Evidence for the use of Ultrasound Therapy for the Management of Mandibular Osteoradionecrosis

Thesis submitted by

Peter Reher, B.D.S., M.Sc.

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in Oral and Maxillofacial Surgery
from the Faculty of Medicine,
University of London

Department of Oral and Maxillofacial Surgery
Eastman Institute for Oral Healthcare
University College London Hospitals
University of London
London WC1X 8LD

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Evidence for the use of ultrasound therapy for the management of mandibular osteoradionecrosis

To Vanessa, Lucas and Mariana.
Evidence for the use of ultrasound therapy for the management of mandibular osteoradionecrosis

Abstract

Introduction: The treatment of mandibular osteoradionecrosis includes antibiotics and curettage, hyperbaric oxygen, surgery, and more recently, therapeutic ultrasound. The aim of this thesis was to establish the possible mechanisms of action of therapeutic ultrasound, that could explain its excellent clinical results.

Material and Methods: Two ultrasound machines were evaluated, a ‘traditional’ (1 MHz and 3 MHz) and a ‘long wave’ machine (45 kHz). Ultrasound was applied to human mandibular osteoblasts, gingival fibroblasts, peripheral blood monocytes (PBMc) and mice calvaria. The following in vitro assays were performed: cell proliferation, collagen and non-collagenous protein (NCP) synthesis, bone resorption, cytokines and angiogenesis factors production using ELISA and RT-PCR techniques, and nitric oxide production. To evaluate the effects of ultrasound on angiogenesis in vivo, the chick chorioallantoic membrane assay (CAM) was used. The use of near infrared spectroscopy (NIRS) for the measurement of radiotherapy effects in the mandible (deoxyhaemoglobin concentrations) was also evaluated.

Results: Ultrasound stimulated bone formation in the mice calvaria. Cell proliferation assays showed an increase of DNA synthesis in fibroblasts and osteoblasts, up to 52%. Collagen/NCP synthesis was also enhanced, in fibroblasts up to 48%, and in osteoblasts up to 112%. Bone resorption, part of the bone turnover process, was promoted, and there is suggestion that the cyclo-oxygenase pathway is involved. In relation to cytokine production, a slight stimulation of IL-1β was noted in all cell types. There was no difference in IL-6 and TNFα levels. The angiogenesis factors, IL-8 and bFGF, were significantly stimulated in osteoblasts, and VEGF was significantly stimulated in fibroblasts, osteoblasts and PBMc. RT-PCR showed that ultrasound induces mRNA transcription for several cytokines and bone related proteins, with the most evident effect being the induction of VEGF transcription in osteoblasts. The
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CAM assay showed that direct ultrasound application and insonated medium from fibroblasts induced angiogenesis \textit{in vivo}. The best overall stimulatory intensities were 15 and 30 mW/cm$^2$ with 45 kHz ultrasound, and 0.1 and 0.4 W/cm$^2$ with 1 MHz ultrasound. The NIRS evaluation showed that it is very sensitive to measure deoxyhaemoglobin concentrations, however these measurements are not reproducible. No age correlations could be performed, and the differences between normal and radiotherapy mandibles was not significant because of the great variability in the measurements.

Conclusions: These results show that ultrasound can correct hypocellularity, hypoxia and hypovascularity observed in osteoradionecrosis. It stimulates cell proliferation, bone formation, healing, and angiogenesis. Further \textit{in vivo} experiments are recommended as well as prospective clinical trials using therapeutic ultrasound for the treatment and prevention of osteoradionecrosis, but NIRS cannot be used to measure the outcome of treatment. Therapeutic ultrasound is a viable option for the management of mandibular osteoradionecrosis, since it is effective, inexpensive and readily available.

Key words:

Osteoradionecrosis, therapeutic ultrasound, cell proliferation, cell culture, collagen, cytokines, ELISA, RT-PCR, angiogenesis, angiogenic factors, near infrared spectroscopy
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<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>cAMP</td>
<td>Cyclic adenosine 3',5'-monophosphate</td>
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<tr>
<td>cGMP</td>
<td>Cyclic guanosine 3',5'-monophosphate</td>
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<td>CAM</td>
<td>Chick chorio allantoic membrane</td>
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<tr>
<td>ORN</td>
<td>Osteoradionecrosis</td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
<td></td>
</tr>
<tr>
<td>PBMc</td>
<td>Peripheral blood monocytes</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>PD-ECGF</td>
<td>Platelet-derived endothelial cell growth factor</td>
<td></td>
</tr>
<tr>
<td>PGs</td>
<td>Prostaglandins</td>
<td></td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin of the E series</td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-Polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>SA</td>
<td>Spatial average</td>
<td></td>
</tr>
<tr>
<td>SAPA</td>
<td>Spatial average pulse average</td>
<td></td>
</tr>
<tr>
<td>SATA</td>
<td>Spatial average temporal average</td>
<td></td>
</tr>
<tr>
<td>SPTA</td>
<td>Spatial peak temporal average</td>
<td></td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
<td></td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
<td></td>
</tr>
<tr>
<td>TIMD</td>
<td>Tissue inhibitor of metallo-proteinases</td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
<td></td>
</tr>
<tr>
<td>TVPF</td>
<td>Tumour vascular permeability factor</td>
<td></td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
<td></td>
</tr>
</tbody>
</table>
Evidence for the use of ultrasound therapy for the management of mandibular osteoradionecrosis

Declaration

This thesis is the result of my own original investigations, except where otherwise stated. The near infrared spectroscopy scans were performed with Dr. Roger Springet at the Medical Physics Department, UCL.

London, 19th December 1998
Prizes obtained as a result of this thesis

1) President's Prize 1998 of the Section of Odontology - *The Royal Society of Medicine*

   This prize was obtained on the 27th April 1998, for the paper entitled "Mandibular osteoradionecrosis: Is there evidence to use therapeutic ultrasound instead of hyperbaric oxygen?".

2) Charles Grant Clark Prize for Surgical Research 1998 - *Surgical Division of the University College London Medical School*

   This prize was obtained on the 9th July 1998, for the paper entitled "Mandibular osteoradionecrosis: Evidence for use of therapeutic ultrasound instead of hyperbaric oxygen therapy".
Evidence for the use of ultrasound therapy for the management of mandibular osteoradionecrosis

Publications as a result of this thesis

Papers


Abstracts


Evidence for the use of ultrasound therapy for the management of mandibular osteoradionecrosis

Letter to the Editor


Papers in preparation


Chapter 1 - Introduction

This thesis probably started in the 1980's, when Professor Malcolm Harris of the Eastman Dental Institute, was looking for a new treatment modality for mandibular osteoradionecrosis. Several treatment modalities had been used before, including conservative treatment, medical management with antibiotics, hyperbaric oxygen therapy, curettage, cover with local flaps and major bone reconstruction. The aim of these treatments was to reverse the damage caused to the mandible by the radiotherapy used for the treatment of cancer.

Osteoradionecrosis is a complex metabolic and tissue homeostatic deficiency created by radiation-induced cellular injury (Marx, 1983a). The sequence suggested by this study is as follows: (a) radiation; (b) formation of hypoxic-hypocellular-hypovascular tissue; (c) tissue breakdown (cell death and collagen lysis exceed synthesis and cell replication), and predispose to a (d) chronic non-healing wound, in which metabolic demands exceed supply.

In order to revert this process, a very good approach would be to improve the blood supply (neoangiogenesis) to the area, enhancing the observed hypoxia and hypovascularity, which finally would lead to bone repair. This is also the rationale for the use of hyperbaric oxygen (HBO) therapy. However, because HBO is not readily available, is very expensive and time consuming, Professor Harris sought another treatment option.

The inspiration came from Dr. Mary Dyson (Tissue Repair Unit, Guy's and St. Thomas Medical and Dental School), who introduced the possibility of using therapeutic ultrasound, since it may potentially address these problems. Ultrasound has been shown to have several effects that can be useful for the treatment of osteoradionecrosis, including the stimulation of tissue regeneration (Dyson et al, 1968 and 1976; Paul et al, 1960; Dyson, 1990); blood flow in chronically ischaemic muscles (Hogan et al, 1982); protein synthesis in fibroblasts (Harvey et al, 1975; Webster et al, 1978); healing of ischaemic varicose ulcers (Dyson et al, 1976); tendon repair (Enwemaka, 1990);
angiogenesis in full thickness excised incisions in the flank skin of adults rats (Young and Dyson, 1990a). Ultrasound effects in bone have also been evaluated and it has shown accelerated repair of fractures in animals (Dyson and Brookes, 1983; Pilla et al, 1990; Tsai et al, 1992a; Wang et al, 1994; Yang et al, 1996) and humans (Heckman et al, 1994).

After a throughout search in the literature, it was found that there was enough evidence to start to use therapeutic ultrasound for the treatment of mandibular osteoradionecrosis. The patients were treated with ultrasound (3 MHz, pulsed 1:4, 1 W/cm² (SAP)) for 40 sessions of 15 minutes per day. Ten out of 21 (48%) cases showed healing when treated with debridment and ultrasound alone. The remaining 11 cases unhealed after ultrasound therapy received debridment and cover with a local flap, and only one needed mandibular resection and reconstruction (Harris, 1992). These results are better than the conventional treatment with hyperbaric oxygen therapy and surgery, if compared with the study of Marx (1983b). His paper showed that hyperbaric oxygen alone cannot stimulate the healing of osteoradionecrosis (ORN) wounds, since only 15% responded to this treatment. The other cases needed surgery, and 70% of those required major reconstruction procedures.

Since the publication of the first results (Harris, 1992), several research projects related to osteoradionecrosis and ultrasound have been conducted as M.Sc. projects, including the use of near infrared spectroscopy, which has demonstrated that patients with osteoradionecrosis who received ultrasound therapy showed significant improvements of the mandibular metabolic activity (improvements of deoxyhaemoglobin concentrations) (Telfah, 1995).

Recently, a new ultrasound machine has been developed, which instead of using the traditional frequencies, of 1 to 3 MHz, uses 'long wave' ultrasound, at 45 kHz. (Bradnock, 1996). This lower frequency/long wavelength combination gives a widely divergent field shape, with the treated volume effectively in the far field region. This wave penetrates much deeper into the tissues, reaching areas as deep as several centimetres, instead of millimetres as with the megahertz machines. Therefore, a comparison between the
traditional and the long wave ultrasound machines will also be carried out in this thesis, in order to eventually include it as the standard treatment.

Before the statement of the hypothesis and main objectives of this thesis, a more detailed literature review seems appropriate. Three main areas will be discussed, (a) osteoradionecrosis, (b) angiogenesis and (c) ultrasound. Each of these areas will be a separate section of the next chapter, and at the end finally the hypothesis and aims will be summarised. Other relevant literature areas such as bone and cytokines will be discussed in the introduction of the relevant chapters.
Chapter 2 - Literature Review
Section 1 - Osteoradionecrosis

As mentioned, this chapter will review the literature for osteoradionecrosis, angiogenesis and ultrasound. This first section will give an overview of the current literature related to osteoradionecrosis. It has been divided into the following:

1. Definition of osteoradionecrosis
2. Incidence of osteoradionecrosis
3. Classification of osteoradionecrosis
4. Pathophysiology of osteoradionecrosis
5. Predisposing risk factors
6. Diagnosis of osteoradionecrosis
7. Investigation of osteoradionecrosis
8. Treatment of osteoradionecrosis

Radiotherapy is an essential recognised treatment modality for oral and head and neck malignant neoplasms. Unfortunately it induces alterations in the normal tissues, leading to a series of potential oro-facial complications. These complications can be divided into early and long-term complications.

Early complications of radiotherapy are very frequent, particularly the oral mucositis. It is by far the most common and distressing complication of malignancy treatment and may have such a significant effect on the quality of life that there is the need to interrupt or curtail the cancer therapy (Toth et al., 1983; Toth et al., 1990; Lokich, 1991; Lokich et al., 1991; Jansma, 1991; Jansma et al., 1992a and b; Brincker and Christensen, 1993).

Long term complications of head and neck radiotherapy include dry mouth (xerostomia), loss of taste, limitation of mouth opening (trismus) and less commonly, but one of the worst, osteoradionecrosis. According to Hutchinson
Out of (1996), osteoradionecrosis is the most devastating complication of radiotherapy in the head and neck area. In severe cases even death can occur (Marx and Johnson, 1987; Hahn, 1983).

1. Definition of osteoradionecrosis

During the years osteoradionecrosis has received many definitions, and therefore it is sometimes difficult to compare epidemiological studies and treatment efficacy. As an example, the following names have been used to describe it:

- Osteoradionecrosis (Regaud, 1922a), also used in the majority of the current literature;
- Radiation osteitis (Ewing, 1926);
- Radio-osteonecrosis (Gowgiel, 1960);
- Postirradiation osteonecrosis (Gowgiel, 1960);
- Radiation osteomyelitis (Meyer 1970);
- Osteomyelitis secondary to radiation, osteomyelitis of irradiated bone (Titterington 1971);
- Osteonecrosis (Beumer et al 1972);
- Radio-osteomyelitis (Obwegeser and Sailer, 1978);
- Septic osteoradionecrosis (Parulekar and Paonessa, 1980);
- Post-radiotherapy osteonecrosis (Epstein et al, 1992).

Probably the first evidence of osteoradionecrosis related to radiotherapy was reported by Regaud in 1922. Its pathology was further described by Ewing in 1926, under the name 'radiation osteitis'. Meyer (1970) classified osteoradionecrosis as one special type of osteomyelitis. Titterington (1971) also related osteoradionecrosis to osteomyelitis, providing one of its first definitions, and used the term 'osteomyelitis of irradiated bone'.

In 1972, Beumer et al used the term 'osteonecrosis', or bone necrosis resulting from ionising radiation. Osteoradionecrosis in their study was recorded when previously unexposed bone in the radiation field became denuded after
treatment. The same authors, in 1979 (Beumer et al, 1979b) used the term ‘mandibular osteoradionecrosis’ redefining it as follows: ‘when bone in the radiation field was exposed for at least 2 months in the absence of local neoplastic disease’. Again, in 1983 they modified their definition to: ‘bone exposure of at least 3 months duration at an extraction site following removal of teeth within the radiation field’ (Beumer et al, 1983a). Morrish et al (1981) similarly used the same three months time period of exposed bone to confirm the diagnosis.

In his classic paper on the pathophysiology of osteoradionecrosis, Marx (1983b) defined it as ‘an area greater than 1 cm of exposed bone in a field of irradiation that had failed to show any evidence of healing for at least 6 months’. He also clarified that in ORN there is no interstitial infection, but only superficial contamination. Fattore and Strauss (1987) used a similar definition regarding time (6 months), but did not define the size of the area.

Both Beumer et al (1983a) and Marx (1983b) in their papers recognise the need for clarification of the term osteoradionecrosis. Marx and Johnson (1987) emphasise again the need for a more universal definition of osteoradionecrosis, saying that many authors deny the complexity and potential seriousness of the disease, and falsely credit themselves with successful management. A clear distinction should be made between a minor soft tissue radionecrosis without osteoradionecrosis in which the recovery and regenerative capacities of adjacent mucosal epithelium are sufficient to cover viable bone. Osteoradionecrosis, on the other hand, represents true bone necrosis and almost always exists concomitantly with overt soft tissue radionecrosis. In their conclusion, they define osteoradionecrosis as ‘an exposure of nonviable irradiated bone, which fails to heal without intervention’.

Hutchinson et al (1990a) defined osteoradionecrosis as ‘an area of exposed bone (mandible) present for longer than 2 months in a previously irradiated field, in the absence of recurrent tumour. Harris (1992) used the same definition, but uses a 3 months time period.
In 1992 Epstein et al used the term 'post-radiotherapy osteonecrosis' and gave a rather simplistic definition, as 'an ulceration of the mucous membrane with exposure of necrotic bone'.

A very sensible discussion of the definition of osteoradionecrosis was written by Hutchinson (1996). His definition is: 'osteoradionecrosis is an area of exposed bone in the mouth or on the face for more than two months in a previously irradiated field, in the absence of recurrent tumour'. The author comments that this definition applies only to the maxilla and mandible. The temporal bone may be exposed either to the face and head or into the ear. Other bones that lie within thick soft tissue integuments, such as the pelvis or femur, may develop symptomatic osteoradionecrosis without being exposed. Another very rare exception is the development of a pathological fracture of the mandible after irradiation, with no overlying bone exposure.

In conclusion, the definition of osteoradionecrosis remains for some a poorly understood condition. The following points seems to be agreed by the majority of the authors:

1. The affected site should have been previously irradiated
2. There should be absence of recurrent tumour on the affected site
3. Mucosal breakdown or failure to heal should occur, resulting in bone exposure (except in cases of bones that lie within thick soft tissue integuments, such as the pelvis or femur, or rarely in cases of a pathological fracture of the mandible after irradiation)
4. The overlying bone should be 'dead', usually due to a hypoxic necrosis
5. There is no evidence of infection within the bone.

Two important questions remain to be clarified, (a) the timing of the bone exposure, and (b) the definition of 'dead' bone. The first question has an obvious variability in the literature. Many authors do not comment on time of exposure (Beumer et al 1972, Epstein et al 1992), others recommend a 2 month period before osteoradionecrosis can be diagnosed (Beumer et al, 1979b; Hutchinson et al, 1990a; Hutchinson, 1996), or even 3 months (Beumer
et al, 1983a; Harris, 1992; Morrish et al, 1981) and 6 months (Marx, 1983a and b; Fattore and Strauss, 1987).

Precaution should be taken not to have a too short period of waiting, because mucosal radionecrosis can occur without osteoradionecrosis, and over-diagnosis will occur. Furthermore, short periods of exposed bone should also not be used, because any surgery and/or extraction performed usually can take up to 1 month to heal. On the other hand, long periods such as 6 months are difficult to establish at clinical practice, and some intervention before this time is certainly needed. Therefore I propose that in the definition of osteoradionecrosis, the bone exposure should be of at least 3 months.

The second question, related to the definition of what is 'dead' bone is more difficult to answer. Although the majority of the authors agree that for a correct diagnosis of osteoradionecrosis, the underlying exposed bone should be necrotic, how to determine this? If surgery is performed in the area, bleeding bone can give a helpful guide to the necrotic limits, however it would be of advantage if the diagnosis could be performed non invasively. Therefore, the need of more objective and adequate investigative tools is crucial, since it would help equally in the definition, diagnosis, classification of severity, and of course, the outcome of the different treatments.

2. Incidence of osteoradionecrosis

The incidence of osteoradionecrosis has a great variability in the literature, and the absolute incidence and prevalence of osteoradionecrosis of the jaws after radiation therapy for treatment of head and neck cancer are unknown (Clayman, 1997). Clinical evidence of osteoradionecrosis related to radiotherapy was first reported by Regaud (1922a and b). Reviewing studies from the literature, most of which are retrospective, one finds an overall incidence of osteoradionecrosis of 10.31% before 1968 (Table 2.1), and of 6.28% after that (Table 2.2). The year 1968 was arbitrary chosen by Clayman (1997), because at this time almost all radiation oncology units had embraced
megavoltage therapy, and we have used it as well, updating all the published incidence studies of osteoradionecrosis. These data were based only on the presence of osteoradionecrosis without sub-categorisation into dentulous, edentulous, spontaneous, or extraction related. The incidence tables show a great variability in incidence, ranging from 3.2% to 44.2% before 1968, and from 0% to 22% after. This variation from centre to centre is probably determined by technique-related morbidity and the veracity of the audit (Harris, 1992).

Table 2.1. - Published incidence studies of osteoradionecrosis, before 1968

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>No Pat.</th>
<th>Incidence Cases</th>
<th>Incidence Perc.</th>
<th>Site of Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Watson and Scarborough</td>
<td>1938</td>
<td>1,197</td>
<td>213</td>
<td>12.9%</td>
<td>Oral</td>
</tr>
<tr>
<td>Martin and Sugarbaker</td>
<td>1940</td>
<td>103</td>
<td>26</td>
<td>25.0%</td>
<td>Floor Mouth</td>
</tr>
<tr>
<td>Wildermuth (Morrish et al, 1981)</td>
<td>1953</td>
<td>104</td>
<td>6</td>
<td>6.0%</td>
<td>Oral, Pharynx</td>
</tr>
<tr>
<td>Meyer</td>
<td>1958</td>
<td>491</td>
<td>26</td>
<td>5.3%</td>
<td>Oral, Lip</td>
</tr>
<tr>
<td>Friedman et al</td>
<td>1959</td>
<td>143</td>
<td>10</td>
<td>7.0%</td>
<td>Head and neck</td>
</tr>
<tr>
<td>Dodson</td>
<td>1962</td>
<td>108</td>
<td>10</td>
<td>9.3%</td>
<td>Oral, Pharynx</td>
</tr>
<tr>
<td>MacComb</td>
<td>1962</td>
<td>251</td>
<td>93</td>
<td>37.1%</td>
<td>Oral</td>
</tr>
<tr>
<td>MacDougall et al</td>
<td>1963</td>
<td>364</td>
<td>18</td>
<td>5.0%</td>
<td>Oral</td>
</tr>
<tr>
<td>Grant and Fletcher</td>
<td>1966</td>
<td>176</td>
<td>69</td>
<td>37.5%</td>
<td>Tonsil</td>
</tr>
<tr>
<td>Rahn and Drone</td>
<td>1967</td>
<td>120</td>
<td>53</td>
<td>44.2%</td>
<td>Oral</td>
</tr>
<tr>
<td>Shimanovskaya</td>
<td>1968</td>
<td>185</td>
<td>6</td>
<td>3.2%</td>
<td>Oral</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td>5,137</td>
<td>530</td>
<td>10.31%</td>
<td></td>
</tr>
</tbody>
</table>

Some conclusions can be derived by reviewing the literature:

1. Not many clinicians reliably report complications such as osteoradionecrosis, and therefore an under reporting may occur.

2. The definition and diagnosis of osteoradionecrosis is very variable. It is therefore very likely that osteoradionecrosis has been over-diagnosed, because many studies considered osteoradionecrosis only as an area of exposed bone, not taking in consideration the time of exposure or the status of the underlying bone, as pointed in the definition section.
3. The follow-up period to identify osteoradionecrosis was not the same in the literature, and should be extended for up to 7-8 years, because osteoradionecrosis can occur many years after radiotherapy; therefore, under diagnosis can occur.

Table 2.2. - Published incidence studies of osteoradionecrosis, after 1968

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>No Pat.</th>
<th>Incidence Cases</th>
<th>Incidence Perc.</th>
<th>Site of Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rankow and Weissman</td>
<td>1971</td>
<td>176</td>
<td>12</td>
<td>6.3%</td>
<td>Oral</td>
</tr>
<tr>
<td>Beumer <em>et al</em></td>
<td>1972</td>
<td>278</td>
<td>10</td>
<td>3.6%</td>
<td>Oral, tonsil</td>
</tr>
<tr>
<td>Carl <em>et al</em></td>
<td>1972</td>
<td>49</td>
<td>2</td>
<td>4.1%</td>
<td>Oral, pharynx</td>
</tr>
<tr>
<td>Daly <em>et al</em></td>
<td>1972</td>
<td>304</td>
<td>66</td>
<td>21.7%</td>
<td>Oral, pharynx, tonsil</td>
</tr>
<tr>
<td>Wang</td>
<td>1972</td>
<td>262</td>
<td>15</td>
<td>5.8%</td>
<td>Tonsil</td>
</tr>
<tr>
<td>Cheng and Wang</td>
<td>1974</td>
<td>74</td>
<td>13</td>
<td>17.1%</td>
<td>Tonsil</td>
</tr>
<tr>
<td>Marciani and Plezia</td>
<td>1974</td>
<td>220</td>
<td>23</td>
<td>10.5%</td>
<td>Oral</td>
</tr>
<tr>
<td>Ange <em>et al</em></td>
<td>1975</td>
<td>23</td>
<td>1</td>
<td>4.3%</td>
<td>Oral</td>
</tr>
<tr>
<td>Bedwinek <em>et al</em></td>
<td>1976</td>
<td>381</td>
<td>54</td>
<td>14.2%</td>
<td>Oral, pharynx</td>
</tr>
<tr>
<td>Beumer <em>et al</em></td>
<td>1976</td>
<td>88</td>
<td>6</td>
<td>6.8%</td>
<td>Oral</td>
</tr>
<tr>
<td>Murray <em>et al</em></td>
<td>1980</td>
<td>104</td>
<td>16</td>
<td>15.4%</td>
<td>Oral</td>
</tr>
<tr>
<td>Horiot <em>et al</em></td>
<td>1981</td>
<td>528</td>
<td>11</td>
<td>2.1%</td>
<td>Oral</td>
</tr>
<tr>
<td>Morrish <em>et al</em></td>
<td>1981</td>
<td>100</td>
<td>22</td>
<td>22.0%</td>
<td>Oral</td>
</tr>
<tr>
<td>Coffin</td>
<td>1983</td>
<td>2,853</td>
<td>22</td>
<td>0.7%</td>
<td>Oral, pharynx, tonsil, salivary glands</td>
</tr>
<tr>
<td>Fleming</td>
<td>1983</td>
<td>1,989</td>
<td>270</td>
<td>13.6%</td>
<td>Oral</td>
</tr>
<tr>
<td>Morton</td>
<td>1986</td>
<td>200</td>
<td>39</td>
<td>19.6%</td>
<td>Oral</td>
</tr>
<tr>
<td>Marciani and Ownby</td>
<td>1986</td>
<td>109</td>
<td>3</td>
<td>2.7%</td>
<td>Oral</td>
</tr>
<tr>
<td>Makkonen <em>et al</em></td>
<td>1987</td>
<td>224</td>
<td>1</td>
<td>0.44%</td>
<td>Oral, pharynx, larynx, salivary glands, nose</td>
</tr>
<tr>
<td>Epstein <em>et al</em></td>
<td>1987</td>
<td>1000</td>
<td>26</td>
<td>2.6%</td>
<td>Oral, pharynx, antrum</td>
</tr>
<tr>
<td>Kluth <em>et al</em></td>
<td>1988</td>
<td>135</td>
<td>14</td>
<td>10.4%</td>
<td>Oral, pharynx, salivary glands</td>
</tr>
<tr>
<td>Maxymiw <em>et al</em></td>
<td>1991</td>
<td>72</td>
<td>0</td>
<td>0.0%</td>
<td></td>
</tr>
<tr>
<td>Kumar <em>et al</em></td>
<td>1992</td>
<td>1,140</td>
<td>14</td>
<td>1.22%</td>
<td>Oral, pharynx</td>
</tr>
</tbody>
</table>

**TOTAL** 10,309 640 6.20%

4. The majority of the studies have been retrospective, which by itslef has many disadvantages. The few prospective studies published have however
very short follow-up times, which is understandable, but there should be more effort in conducting long term prospective studies. Horiot et al (1981) examined the effects of their preventive protocol in 528 patients, reporting a 2% incidence of osteoradionecrosis. Morrish et al (1981) realised a controlled study with 100 patients (22% of incidence), however the minimum follow-up was only 6 months. Kluth et al (1988) also conducted a prospective study, following their patients for 18 months, reporting 10% of osteoradionecrosis.

3. Classification of osteoradionecrosis

Many authors have attempted a classification of osteoradionecrosis, and the majority have relied on the history and clinical progression of the disease, or its response to treatment.

Daly et al (1972) staged bone necrosis, but omitted to mention how they classified these stages. Coffin, in 1983 examined 2853 cases of patients who had radiotherapy for head and neck malignancies. He suggests that osteoradionecrosis develops in two forms: (a) minor, and (b) major. The minor form was considered to be a series of small sequestra which separate spontaneously after varying periods of weeks or months. These small areas can be seen clinically but cannot be demonstrated radiologically. The major form was defined when necrosis occurs of such an extent as to involve the entire thickness of the jaw, and a pathological fracture is inevitable. This form can obviously be seen radiologically, and is extremely rare in the maxilla (only 1 case in this series).

Marx (1983b) introduced a new treatment modality for osteoradionecrosis, based on hyperbaric oxygen therapy (HBO) and surgery. He used a staging classification for his proposed treatment protocol, based on the response to patients to the hyperbaric oxygen therapy (Table 2.3).

The obvious problem with this classification is that it is designed to monitor the treatment suggested by the author, and can not therefore be used routinely for epidemiological studies.
Morton in 1986 subdivided osteoradionecrosis into ‘minor’, ‘moderate’ and ‘major’ groups. Minor osteoradionecrosis consisted of ulceration with exposed bone and a history of bony spicules which healed spontaneously over a period of months. Moderate cases consisted of exposed bone and small sequestra limited in nature and healing spontaneously with conservative treatment within 6 to 12 months. Major osteoradionecrosis consisted of large areas of exposed bone, with formation of large sequestra, possible fracture and sinus formation. This cases often progressed rapidly, lasting in excess of one year and often requiring radical treatment.

**Table 2.3. - Wilford Hall Staging for the treatment of osteoradionecrosis (Marx, 1983b)**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Procedure</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Start with 30 HBO dives*, and if the wound shows definitive clinical improvement, give further 60 dives</td>
<td>If no improvement occurs after first 30 dives, goes to Stage II</td>
</tr>
<tr>
<td>II</td>
<td>Alveolar sequestrectomy is performed, and more 30 HBO dives are given</td>
<td>If dehiscence occurs exposing bone, goes to Stage III</td>
</tr>
<tr>
<td>III</td>
<td>Resection is performed followed by 30 HBO dives</td>
<td>Reconstruction (Stage IIIIR) is performed 10 weeks after, with a transcutaneous approach, (20 HBO dives are given before, and 10 after)</td>
</tr>
</tbody>
</table>

* A ‘dive’ consists of a 90 min. session of HBO, usually given once a day

Epstein *et al* (1987) stated that due to its heterogeneous presentation and response to treatment, a system of classification based upon clinical and radiographic findings would facilitate treatment decisions and classification for research purposes. Therefore they propose a clinical staging classification (Table 2.4). However, the question is that if the treatment is the same in stages II and III, why use this classification? Furthermore, its classification is based on clinical and radiographic findings, which are not precise.

Clayman (1997) used a classification of osteoradionecrosis related to the overlying mucosa being intact or not. He uses the term Type I for the cases in which bone lysis occurs under intact gingiva or mucosa, and Type II, a more aggressive type, called ‘radiation osteomyelitis’, when the soft tissues break
down, exposing the bone to saliva, occurring secondary contamination. He suggest that type I cases heal with conservative therapy, and the type II does not.

There is obviously a necessity for a more objective/quantitative classification of osteoradionecrosis, and probably the only way to obtain it is by the use of more adequate investigative tools. According to Hutchinson (1996), this investigative tool should be able to offer the following: (1) record quantitatively and qualitatively the severity and extent; (2) monitor progress of treatment; (3) predict patients at risk; (4) predict risk factors more confidently; (5) permit comparison of treatment regimens; (6) predict the bone level damage above which surgery is essential.

Table 2.4 - Classification of osteoradionecrosis according to Epstein et al (1987)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>Symptoms</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I*</td>
<td>Resolved, healed</td>
<td>None</td>
<td>Follow up, prevention</td>
</tr>
<tr>
<td>II*</td>
<td>Chronic, persistent non progressive</td>
<td>None or controlled</td>
<td>Local wound care: antiseptics/antibiotics, analgesics, HBO if indicated - Wilford Hall Staging (Marx, 1983b)</td>
</tr>
<tr>
<td>III*</td>
<td>Active, progressive</td>
<td>Progressive</td>
<td>Local wound care: antiseptics/antibiotics, analgesics, HBO if indicated - Wilford Hall Staging (Marx, 1983b)</td>
</tr>
</tbody>
</table>

* All stages above subdivide into: a) No pathologic fracture  
b) Pathologic fracture-requires reconstruction

4. Pathophysiology of osteoradionecrosis

The understanding of the pathophysiology of osteoradionecrosis has been a controversial subject since its first appearance in the early 1920's. Many theories of how it occurs have been formulated, and the most accepted nowadays originates from the work done by Marx in 1983. A historical overview will be presented:
4.1. First observations

An early correlation with radiotherapy was made by Regaud (1922a and b), and in 1926, Ewing tried to describe the mechanisms of how it developed. In 1922 Regaud also observed the difference in the sequestration process of osteomyelitis and that of osteoradionecrosis, in which there is no sharp line of demarcation between dead and living tissue. This he attributed to a radiation-induced progressive obliterative endarteritis. In the 1950's radiotherapy to the jaws became more established and osteoradionecrosis was more often noted, mainly in the mandible (McLennan, 1955).

Evidence of aseptic necrosis occurring in the neck of the femur had been observed (Stephenson and Cohen, 1956). Based on this observation, two concepts on the pathogenesis of osteoradionecrosis have been drawn. One is that the bone itself is the primary site of damage, and the other is that the vascular obliteration is primary.

Experimental investigations to clarify the pathogenesis of osteoradionecrosis have been done in dogs (Chamber et al., 1958) and in Macacus rhesus. Histologically Gowgiel (1960) noted that the osteoradionecrosis resulted from direct effect of irradiation on the osteocyte, and he did not comment on osteoclasts. He also noted thickening of the walls of arteries and arterioles (endarteritis obliterans), but no changes in veins and capillaries. However he suggested that there was no evidence of ischaemic necrosis. Other authors during the 1960's have studied histologically osteoradionecrotic bone, and have also noted reduced cellularity, loss of osteocytes and osteoblasts, and replacement of marrow with fibrous tissue, concluding that these were the primary pathogenic factors (Gowgiel, 1960; Silverman and Chierici, 1965; Pappas, 1969).

4.2. The 'radiation, trauma and infection' theory

In 1938 Watson and Scarabough introduced the concept of osteoradionecrosis developing to a triad of radiotherapy above a critical dose, local trauma, and
infection. This theory was adopted by other authors, and became even stronger in 1970, when Meyer named the classical triad of osteoradionecrosis, as 'radiation, trauma and infection'. He described the role of trauma to be a portal of entry for oral bacterial flora into the underlying bone. This theory lasted for a long period, and was the basis for medical and surgical treatment. Many authors followed this idea, attributing osteoradionecrosis to trauma of devitalised bone and microbiologic sepsis (Bump et al, 1976). Titterington (1971) described osteoradionecrosis as a radiation induced osteomyelitis. Happonen et al 1983 considered osteoradionecrosis as a chronic infective osteomyelitis of irradiated bone with mixed oral microorganisms, including actinomyces and candida.

4.3. The theory of a non-healing wound, due to hypoxic-hypocellular-hypovascular tissue

Marx (1983a) examined the traditional concept of the pathophysiology of ORN described by Meyer (1970), questioning the occurrence of osteoradionecrosis cases without trauma or infection. To test his hypothesis, he studied 26 consecutive cases of ORN from which 12 en bloc resection specimens were cultured and stained for micro-organisms (aerobes, anaerobes and fungi). He cultured and stained also osteomyelitis specimens of the mandible, maxilla and long bones, and infected grafts of the jaws and long bones. The microbiology results showed that all specimens where infected superficially but no organisms could be cultured or observed in the deep, so called "infected bone" of ORN. The micro-organisms identified on the surface varied greatly, suggesting saprophytic contaminants (streptococci, Candida species, and Gram negative organisms). In contrast, osteomyelitis and infected bone grafts to long bones consisted primarily of one pathogen, usually a staphylococcal species. Osteomyelitis and infected bone grafts of the jaws showed a more varied group of organisms, including Bacteroides and Eikenella species as well as Staphylococcus aureus, which were not encountered on the surface of ORN.
bone. He concludes that micro-organisms play a minor role in the pathophysiology of ORN of the jaws, as well as trauma.

The histological findings noted by Marx showed endothelial death, hyalinisation and thrombosis of vessels with a fibrotic periosteum. Bone, osteoblasts and osteocytes were deficient, with fibrosis of the marrow spaces. Mucosa and skin also become fibrotic, with markedly diminished cellularity and vascularity of the connective tissue. The overall result was a composite tissue, which is hypovascular and hypocellular, and was proven to be hypoxic compared with non-irradiated tissue by direct measurement (Stevens and Marx, 1981; Sheffield and Dunn, 1979).

Finally, Marx stated the following conclusions: (1) osteoradionecrosis is not a primary infection of irradiated bone. It is a complex metabolic and tissue homeostatic deficiency created by radiation-induced cellular injury; (2) micro-organisms play only a contaminating role in ORN; (3) trauma may or may not be an initiating factor, it is only one mechanism of tissue breakdown; (4) the use of moderate-voltage irradiation has not eliminated the frequency of ORN of the jaws. The sequence suggested by this study is as follows: (a) Radiation; (b) Formation of hypoxic-hypocellular-hypovascular tissue; (c) Tissue breakdown (cell death and collagen lysis exceed synthesis and cell replication), and predispose to a (d) chronic non-healing wound (a wound in which metabolic demands exceed supply).

4.4. The role of osteoclast/osteoblast activity

In normal healthy adult bone, most cells are in the resting phase of the mitotic cycle. However, there is slow but constant cell turnover accompanied by remodelling of the bone structure. Osteoclasts proliferate, resorb bone and disappear, and the osteoblasts proliferate to reconstruct the bone. This process continues throughout life. Trauma stimulates proliferation of osteoblasts mainly from the periosteum to repair the damage to the bone.
Radiation of bone leads to *endarteritis obliterans* with thrombosis of small blood vessels, fibrosis of the periosteum and mucosa, and damage to osteocytes, osteoblasts and fibroblasts. The damaged osteocytes and osteoblasts may survive until they attempt to divide, when mitotic death occurs. An individual bone cell may undergo mitotic death at an interval of months or years after irradiation, or it may never divide unless stimulated by trauma. There is therefore a slow loss of bone cells after radiotherapy with a consequent slowing down of the remodelling process, which leads to the risk of bone necrosis (Scully and Epstein, 1996).

Harris (1992) emphasised that the principal problem for the surgeon treating osteoradionecrotic bone is the absence of separation, i.e. sequestration of the nonvital from vital bone, which points to an osteoclast defect. Osteoclasts arise from haematopoietic tissues, followed by vascular dissemination and the generation of resting pre-osteoclasts and osteoclasts in bone itself (Meghji, 1992). Radiation damage to the marrow and blood vessels would explain their absence. Equally important is the absence of the osteoblast which is regarded as the major influence in recruiting and activating the osteoclast. A third consideration was the likelihood that osteoclasts do not find irradiated necrotic bone a suitable substrate for phagocytosis. Jones *et al* (1984), excluded this possibility showing the production of active resorption pits by cultured osteoclasts on the surface of osteoradionecrotic bone in vitro.

Dambrain (1993), using microradiographic analysis of fragments removed from patients with osteoradionecrosis, showed two types of bone resorption. An osteoclastic one not followed by relevant osteogenesis, and another, pathognomonic of post-radiation complications, linked with an altered activity of the osteocytes. These cells are responsible for an irreversible widening of the "osteoplasts" set in the poorly vascularized bone regions, in particular in the wall of haversian canals. The coalescence of widened "osteoplasts" causes polycyclic cavities which are a typical feature of osteoradionecrosis.
5. Predisposing and Risk Factors

Many factors have been considered to predispose to the development of osteoradionecrosis, including the tumour size and location, radiation dose, local trauma, dental extractions, infection, immune defects and malnutrition. Nevertheless, osteoradionecrosis may also sometimes occur spontaneously, unrelated to trauma (Epstein et al. 1987, Marx 1983a). Many patients with oral cancer also abuse alcohol and tobacco and are in poor general medical condition (Kluth et al. 1988), which, together with poor nutritional status and lack of oral hygiene, may place these patients at higher risk of osteoradionecrosis (Langdon and Henk 1995).

5.1. Radiation factors

There is universal agreement that high total doses, short regimens using high doses per fraction, large field sizes, and the delivery of radiotherapy through a single homolateral field are all associated with an increased risk of osteoradionecrosis (Hutchinson, 1996). Gowgiel (1960) in his experiences with monkeys showed a direct relation between time of necrosis and the dosage of irradiation (he used 4500 to 11000 rad). The necrosis always started in the interdental papilla of the mandibular molar in the centre of irradiation.

Since the introduction of higher-energy radiotherapy in the mid to late 1960's, the incidence of osteoradionecrosis has fallen from 10.31% (Table 2.1) to 6.28% (Table 2.2). Meyer (1970) noted that 5% of his patients treated with orthovoltage developed osteoradionecrosis whereas only 1% to 1.5% developed it with the use of high-voltage (cobalt 60).

Although interstitial therapy is safer than external beam when tumours are situated away from bone, it is associated with an exceedingly high incidence of osteoradionecrosis when used in tumours immediately adjacent to bone.
The use of fast neutrons is now known to cause very high rates of particularly severe osteoradionecrosis and soft tissue necrosis, which is almost impossible to manage medically (MacDougall et al, 1990).

Osteoradionecrosis is unlikely to occur if the radiation dose is below 60 Gy delivered by standard fractions. Bedwineck et al (1976) reported an incidence of osteoradionecrosis of 1.8% in patients receiving up to 70 Gy, and of about 9% of those receiving more then 70 Gy. Morrish et al (1981) conducted a study in 100 controlled patients, with an overall osteoradionecrosis incidence of 22%. Patients who received more than 75 Gy to the bone developed osteoradionecrosis in 85% of the dentulous and in 50% of the edentulous patients. No patient developed osteoradionecrosis with a radiation dose less than 65 Gy.

However, Kluth et al (1988) could not establish a correlation between radiation dose and incidence of osteoradionecrosis. They compared a control group to an osteoradionecrosis group, observing a higher total dose in the control group. Fifty percent of the osteoradionecrosis patients actually received a total dose of 60 Gy or less.

The use of CHART (Continuous hyperfractionated accelerated radiotherapy) is a relatively new introduction in the radiotherapy field, being applied as a multicentre prospective randomised trial since 1990. With the low dose per fraction of this regime, there has been a significantly reduced incidence of post-radiation change in normal tissues (Saunders et al, 1992; Pigott et al, 1993). Roos et al (1996) reported only one case of mild osteoradionecrosis in a total of 99 patients treated with this regimen between 1985 and 1990.

Many other precautions related to the radiotherapy itself can be used to minimise the risk of osteoradionecrosis, including multiple fields, reduced total radiation dose, smaller fraction size, devices to move the mandible or maxilla, out of the radiation field and radiation shields (Epstein et al. 1987a).
5.2. Trauma

Trauma has been recognised for many years as a predisposing factor to osteoradionecrosis. It was even considered as been part of the pathophysiology of osteoradionecrosis itself, as in the theory of radiation, trauma, infection (Watson and Scarabough, 1938; Meyer, 1970). Nowadays it is not considered to have a crucial role in its pathophysiology (Marx, 1983b) since osteoradionecrosis can occur spontaneously.

However, it is undoubtedly one of the most important risk factors to the development of osteoradionecrosis. Trauma can be delivered to the tissues adjacent to the mandible in several ways, as local trauma due to dentures or others, and surgically, due to teeth extractions and major surgery related to the treatment of the malignancy itself.

In a series of osteoradionecrosis cases studied by Marx (1983a), he noted that 17/26 (65%) of the cases were related to trauma. From these trauma cases, 15/17 (88%) were associated with post-irradiation tooth removal, 6% associated with denture flange and 6% with a sharp bony ridge.

**Teeth extraction**

Since 1932, when Coutard reported the Curie's Foundation experience with osteoradionecrosis, one finds that it has been noted to occur after dental extractions performed shortly before or at any time after radiotherapy. The incidence of osteoradionecrosis is three times higher in dentate than in edentulous patients, mainly as a result of trauma from tooth extraction and infection from periodontal disease (Murray et al, 1980a and b). Morrish et al (1981) observed that patients who were edentulous at the time of diagnosis of cancer had a relatively low risk of developing osteoradionecrosis. Oral infections and trauma, including surgical intervention should therefore be kept to a minimum (Makkonen et al, 1987; Widmark et al, 1989).

However, caries and periodontal disease are common, and controversy has existed regarding whether such teeth should be removed. Thirty years ago it was recognised that the maintenance of a healthy dentition was essential for
the prevention of osteoradionecrosis. This required the willing participation of a
dentist to assess and monitor the dentition and to remove teeth as necessary
(Coffin, 1983). All teeth that were grossly carious, periodontally hopeless, or
had a poor prognosis for retention beyond 12 months were removed before
radiotherapy, with extractions after radiotherapy been avoided as much as
possible (MacComb, 1962). Fully embedded teeth were not removed (Hinds,
1971). Alveoloplasty with primary closure, the use of pre and post-operative
antibiotics, and a 10-day wait before radiotherapy were recommended.

It is now generally accepted that teeth in the high-dose irradiation field by
no means inevitably need to be extracted. The only teeth that really need to be
extracted before radiotherapy are those teeth within the high-dose field that are
unrestorable or that have advanced periodontal involvement, and those
patients who are unwilling or unable to maintain oral care. All other teeth should
be cleaned and restored before radiotherapy begins (Marx 1984, Kluth et al.
1988). This was shown by Bedwineck et al (1976), who published a paper
comparing two protocols of teeth extraction. Between 1966 and 1969 the policy
of the M.D. Anderson Hospital in Houston, Texas was to extract on an elective
basis all teeth not in good condition. Between 1969 and 1971, the new policy
was to conserve all teeth except those considered unsalvageable. The
incidence of osteoradionecrosis dropped from 19.7% during the period of
elective dental extractions to 7.9% during the conservation period (n=381). This
decrease was due primarily to a reduction in the incidence of
osteoradionecrosis precipitated by dental extractions (from 11.8% to 2.3%).

If the decision to extract teeth is made, the question is when to extract
them, before, during or after radiotherapy. The worst moment to extract tooth is
considered to be during radiotherapy, but the common belief to delay
extractions after radiotherapy, in anticipation of tissue recovery with time, is
wrong (Marx and Johnson, 1987). The majority of the studies report a higher
rate of osteoradionecrosis in post-radiotherapy extractions (Beumer et al,
1983a; Murray et al, 1980). Beumer et al (1983a and b) published two papers,
one with pre and the other with post-radiation extractions. In the pre-radiation
extractions paper, the osteoradionecrosis incidence was 14.1%, and in the
post-radiation extractions paper, it was of 22%. One should conclude therefore that all teeth that are severely diseased should be extracted at the pre-radiotherapy appointment. However, Clayman (1997) suggested that the difference in incidence of osteoradionecrosis associated with dental extractions observed in more recent literature is not relevant (4.4% before, and 5.8% after radiotherapy).

If pre-radiotherapy extractions are planned, the period between the extraction and the radiotherapy is critical. Marx and Johnson (1987) recommended an interval of 3 weeks before starting radiotherapy, since experimental work has shown that it takes 3 weeks for osteoid to form in the sockets, and epithelial repair to be complete after extractions (Amler, 1969; Shearer, 1967; Hupp, 1993). However this time period can sometimes delay treatment. Therefore, many authors suggest a 2 week interval as acceptable, since this is usually the time needed for planning and mould room work (Beumer et al., 1979b; Coffin, 1983; Maxymiw et al., 1991; Hutchinson, 1996). The highest incidence of osteoradionecrosis is seen in those patients having extractions immediately prior to, or immediately after radiotherapy (Epstein et al., 1987).

The technique used for planned pre-radiotherapy extractions includes alveoloplasty and primary closure (Guttenberg, 1974; Epstein et al., 1987; Beumer et al., 1979b; Rankow and Weissman, 1971; Maxymiw et al., 1991; Kumar et al., 1992; Hutchinson, 1996). The mucoperiosteum overlying the alveolar bone to be removed must be elevated, but absolutely no more periosteal elevation should be done, as this will prejudice the blood supply of the underlying bone. Antibiotic therapy is usually indicated (Coffin, 1983; Flemming, 1983; Daly et al., 1972; Hutchinson, 1996), usually lasting for 1 to 4 weeks. Coffin (1983) recommended long courses (1 year) of tetracyclins without any sound justification. However, Marciani and Ownby (1986) reported good results both for pre and post-radiotherapy extractions without using antibiotics. Maxymiw et al. (1991) advocated the use of non-lignocaine, low adrenaline concentration anaesthetic for extractions, with antibiotic cover provided pre-operatively and for 1 week after, again without any sound basis. These authors
extracted 449 teeth in 72 post-irradiated patients, and the only preventive measure was the use of low-epinephrine or epinephrine-free, non-lignocaine local anaesthetics, and conservative surgical techniques. In a follow-up ranging from 68 days to 19.3 years (median 4.8 years), no instances of ORN occurred as a result of dental extraction.

If post-radiation extractions become necessary, the technique should be more conservative, and many recommendations have been made. Simple forceps extraction is suggested with due regard to not damage underlying bone (Widmark et al, 1989). Marx and Johnson (1987) reported that there was a continuous loss of capillaries over time after radiation therapy, and advocated surgery 1 to 6 months after radiation therapy, to decrease the chances of osteoradionecrosis. Some have suggested that if the patient is felt to be at risk of osteoradionecrosis, hyperbaric oxygen therapy can be considered as prophylaxis against osteoradionecrosis (Marx and Ames, 1982; Marx, 1983b; Epstein et al, 1987b). In such cases, we would recommend the prophylactic use of ultrasound as an alternative to hyperbaric oxygen (Reher and Harris, 1997).

Extractions performed in radiotherapy patients should be done by a skilled surgeon, experienced with osteoradionecrosis (Coffin, 1983; Makkonen et al, 1987). What number of teeth per quadrant should be removed at a single sitting, whether periosteum should be elevated, whether sockets should be closed primarily, whether radical or conservative alveoloplasty should be performed, and whether antibiotics should be used, have all been scrutinised in an attempt to reduce osteoradionecrosis after extractions. After 60 years, the issue remains unsettled, but all authors agree that the extraction technique should be as gentle as possible. Clayman (1997) reviewed the reports over the last decade, showing an osteoradionecrosis rate of 2.1%, and of 5.8% including the cases up to 1968. Whether these results were due to a specific extraction technique, avoidance of vasoconstrictor, using other agents than lignocaine, or using specific antibiotics remains unresolved. What is apparent is the need for first class oral prophylaxis and restorative care.
Resection surgery

The combination of radical neck dissection, either pre- or post-radiotherapy, has been regarded as a surgical factor predisposing mandibular osteoradionecrosis (Carl et al, 1973). One possible explanation for this is that the facial artery, which is usually ligated in the dissection, supplies a significant proportion of the mandibular blood supply through periosteal vessels on its buccal aspect, particularly in elderly patients (Bradley, 1972; McGregor and MacDonald, 1989).

A mandibulotomy is a well-recognised surgical procedure that improves access to the oral cavity for the resection of tumours (Spiro et al, 1981, McGregor and MacDonald, 1983). However, it also carries out a very high risk of developing osteoradionecrosis. Altman and Bailey (1996) reported 5 cases treated with this procedure, 4 with previous radiotherapy. All 5 patients (100%) developed osteoradionecrosis. If absolutely needed, midline mandibulotomies can be performed for posterior tumours, and should be avoided with anterior tumours (Hutchinson, 1996).

Local trauma

Dentures may cause mucosal irritation and ulceration leading to osteoradionecrosis (Daly et al, 1972). After tooth extraction, at least 9 months should elapse for bone remodelling before new dentures are fitted (Beumer et al, 1979b). Jansma et al (1992) recommend that during radiotherapy, the patient should only wear dentures for meals. Marunick and Leveque (1989) noted trauma from unusual masticatory habits as a cause in three cases of osteoradionecrosis, which is very difficult to explain.

5.3. Carious and periodontally compromised teeth

There is a well established association between carious and periodontally compromised teeth and osteoradionecrosis. Radiotherapy directly affects the supporting structures of the teeth, the gingiva, periodontal ligament and bone (Silverman and Chierici, 1965). The fibres of the periodontal ligament,
anchoring the tooth to its adjacent alveolar bone, become hyalinized and irregular, losing their spatial organisation. There is a reduction in blood vessel internal diameter and number, as well as hypocellularity, including osteoblasts and cementoblasts (Rohrer et al, 1979). These effects are added to increase plaque, toxins from the microorganisms contained therein, leading to the development of chronic periodontal disease with continuous destruction of bone and teeth supporting structures (Galler et al, 1992). Galler et al (1992) reported three cases of osteoradionecrosis which developed from periodontal disease activity, but the incidence could be much higher.

Murray et al (1980b and c) showed a positive association between dental disease present before radiation therapy and subsequent necrosis of the mandible (p=0.09), leading to a recommendation that significant disease be eradicated before irradiation of oral tissues. Unless careful attention is given by the patient to the elimination of plaque with correct tooth brushing technique (see prevention of osteoradionecrosis), this chronic disease will not only cause tooth loss but also cause some of the 'spontaneous' osteoradionecrosis cases.

*Spontaneous osteoradionecrosis*

Although osteoradionecrosis is traditionally associated with trauma, it has been observed to occur spontaneously. Gowgiel in 1960 found osteoradionecrosis occurring in intact gingivae in his experimental monkey study, and stated that such a dormant osteoradionecrosis required disruption of the gingival surface to become grossly evident.

In the 1970's and 1980's the role of trauma as initiating factor of osteoradionecrosis started to be questioned, since many patients developed osteoradionecrosis without having any evidence of previous trauma. Daly and Drane (1973) reported that 39% of their osteoradionecrosis cases could not be related to trauma. Bedwinek et al in 1976 have also identified 'spontaneous osteoradionecrosis', and related it to higher radiation doses. Of his 381 patients reported, 5% had spontaneous osteoradionecrosis, and 9% had osteoradionecrosis related to a dental extraction or denture irritation. Marx
(1983a) reported that 35% of his 26 osteoradionecrosis cases could not be correlated to trauma.

Epstein et al (1987) analysed 1000 patients treated with radiotherapy, from which 26 developed osteoradionecrosis. No predisposing factors could be identified in 12 patients and dental manipulation or denture irritation contributed to 11 cases.

5.4. Tumour size and location

There is debate over whether tumour size and hence radiation field size is directly proportional to the risk of developing osteoradionecrosis. Bedwineck et al (1976), and Rankow and Weissman (1971) found that larger tumours were associated with a higher incidence of osteoradionecrosis, whilst Murray et al (1980) found no correlation at all.

Probably the tumour location is more relevant than the tumour size. If a tumour is adjacent to bone, such as on the alveolus or in the mouth floor, there is an increased incidence of osteoradionecrosis (Hutchinson, 1996).

5.5. Time of development of osteoradionecrosis

The time interval between radiotherapy and the onset of osteoradionecrosis can be very variable, but the majority of the cases occur between 4 months and 2 years. Gowgiel noted in 1960 that osteoradionecrosis of the mandible usually develops within 2 years following irradiation. However a few cases can occur after periods as long as 13 or 15 years (Cook, 1952). Clayman (1997) agrees, commenting that the highest risk is during the first 4 to 12 months after radiotherapy, but it has been found to persist for the remainder of the patient's life.

Epstein et al (1987) showed that the time of developing osteoradionecrosis had an average of 4.5 months in the cases associated with
dental/surgical trauma, and on the cases of spontaneous development, 50% developed it in 6 months, but it could be as long as 13 years.

Onset of osteoradionecrosis can be expected for decades after therapy, well beyond the follow-up of the majority of the incidence studies. Berger and Symington (1990) reported two very late cases, one after 45 years of radium implant therapy, and one after 38 years after external beam therapy.

5.6. Alcohol and tobacco

Kluth et al (1988) showed that heavy use of alcohol and tobacco after radiotherapy was observed in 86% of the patients who developed osteoradionecrosis. Their mode of action is unexplained. Furthermore, they probably potentiate the combined effects of other negative factors, such as contributing to poor oral hygiene.

6. Diagnosis of osteoradionecrosis

The diagnosis of osteoradionecrosis is based primarily upon clinical signs of ulceration of the mucous membrane with exposure of necrotic bone. The lesion may be accompanied by symptoms of pain, dysesthesia, fetor oris, dysguesia, and food impaction in the area (Beumer et al, 1979a; Epstein et al, 1987).

Marx and Johnson (1987) found the following physical diagnostic signs to correlate with increased degrees of radiation tissue injuries:

1. Induration of tissue.
3. Loss of facial hair growth.
4. Cutaneous atrophy.
5. Cutaneous flaking and keratinization.
6. Profoundness of xerostomia.
7. Profoundness of taste loss.
7. Investigation of osteoradionecrosis

Osteoradionecrosis can be investigated by many techniques, including radiographs, CT scans, MRI, doppler ultrasound, nuclear medicine and near infrared spectroscopy. The ideal investigative tool, according to Hutchinson (1996) should be able to offer the following: (1) record quantitatively and qualitatively the severity and extent; (2) monitor progress of treatment; (3) predict patients at risk; (4) predict risk factors more confidently; (5) permit comparison of treatment regimens; (6) predict the bone level damage above which surgery is essential.

7.1. Radiographic images, CT and MRI

Radiographic images are the most commonly used, and the radiological appearance of osteoradionecrosis is that of a mixed radio-opaque radiolucent lesion, with the radiolucent areas representing bone destruction. The cheapest and most readily available image is the orthopantomogram (OPG), which can be supplemented with other extraoral or intraoral radiographs. However they require a substantial alteration in mineral content and extensive involvement of the bone, which only occurs in later stages (Epstein et al, 1987a). Ardran (1951) noted that 30% of bone mineral content must be lost before any radiographic change can be seen. Therefore, plain radiographs always underestimate the extent of radiation-damaged bone, and do not correlate with the clinical status of patients (Epstein et al, 1992).

Computer tomography (CT) scans have similar limitations as traditional radiographs for the mandible or maxilla, but can be helpful in temporal bone osteoradionecrosis (Hutchinson, 1996). Hermans et al (1996) examined 10 patients with osteoradionecrosis using CT scans, and observed cortical interruptions and loss of spongiosa trabeculation in all cases on the symptomatic side. Soft tissue thickening on the symptomatic side was seen in 9/10 patients, and this can be difficult to differentiate from tumour recurrence.
Magnetic resonance images (MRI) also have been used, and suggest that fibrosis of bone marrow occurs in osteoradionecrosis (Fujita et al, 1991).

Positron emission tomography (PET) has been advocated as being able to differentiate between osteoradionecrosis and recurrent tumour (Minn et al, 1993).

7.2. Nuclear medicine

Radionuclide bone scanning with technetium methylene diphosphonate ($^{99m}$Tc-MDP) can identify pathophysiologic changes in bone earlier than conventional radiography because scan changes reflect osteoblastic activity and good blood flow (Alexander, 1976). Technetium bone scans have also been used to monitor improvements in tissue viability, before, during and after radiotherapy. Increased uptake of $^{99m}$Tc-MDP at sites of radiotherapy (showing as 'hot' or 'black' spots on the image) during and immediately following treatment have been noted (King et al, 1980; Aitasalo and Ruotsalainen, 1985). Osteoradionecrosis is marked by an increased rate of uptake and total uptake of $^{99m}$Tc-MDP relative to adjacent bone (Hutchinson et al, 1990b). Epstein et al (1992) also observed increased uptake of $^{99m}$Tc-MDP, in all patients they examined with osteoradionecrosis, being more sensitive then plain radiographs. However the scans remained altered even after successful treatment. The increased uptake of this radionuclide would imply that osteoradionecrotic bone has a good blood supply, and is actively forming bone, neither of which is true. It is clear that some, as yet unexplained, mechanism accounts for this anomaly (Hutchinson, 1996).

Gallium-67 citrate (GA-67) will localise in bone, liver and large bowel. In addition, it will localise in tumours and inflammatory lesions (Weiner, 1990). Gallium scans have been used in osteoradionecrosis, with variable findings, consistent with the fact that osteoradionecrosis is not necessarily associated with inflammation within bone. Thus, gallium uptake may not be of diagnostic value for osteoradionecrosis. However, gallium scans did correlate with clinical findings following treatment, suggesting that persisting positive gallium scans
may indicate the need for surgery following conservative treatment (Epstein et al, 1992).

7.3. Ultrasonography

In a prospective clinical study, Semerigidis et al (1996) used colour doppler imaging to evaluate radiation effects on the blood supply of the mouth and face. A total of 44 patients were evaluated, recording blood flow in the common carotid artery. The results show that there were no statistically significant changes between the radiated/operated side and the contralateral side, during the first 6 months after radiotherapy.

7.4. Transcutaneous and transmucosal oxymetry

Tissue oxygen tension studies have been done in order to evaluate the effects of hyperbaric oxygen (HBO) therapy on neoangiogenesis (Beehner and Marx, 1983; Marx, 1984). This was done measuring transcutaneous partial oxygen pressures (TcPO₂) of the central radiation port and a measurement of a reference from outside the radiation field, the left second intercostal space. The results show that hyperbaric oxygen-induced angiogenesis becomes measurable after eight sessions, rapidly progresses to a plateau of 80% to 85% of nonirradiated tissue vascularity by 20 sessions, and remains at that level without further improvement with further HBO. Patients reevaluated 1, 2 and 3 years after their treatment had TcPO₂ levels at or within 90% of their values recorded just after treatment.

Thorn et al (1997) evaluated the effects of HBO for the treatment of osteoradionecrosis using transmucosal oxygen measurements. The measurements were performed in 10 patients, on the attached gingiva. During HBO treatment, the transmucosal oxygen tension increased significantly after five dives, and after 30 dives, the increases were from a mean of 50% to 86% of the measurements from normal healthy gingiva.
However, it has to be remembered that TcPO$_2$ measurements are capable of measuring the oxygen levels only at the capillary loops of the skin, at a depth of less then 1 mm. Therefore, although these measurements can be used to verify the effects of hyperbaric oxygen on the skin, it can not be used as a tool to establish such improvements in the underlying tissues. Therefore this method is unsuitable to investigate osteoradionecrosis, and the observations made by Beehner and Marx (1983) and by Marx (1984), regarding the effects of HBO on osteoradionecrosis should be looked at critically.

7.5. Near infrared spectroscopy (NIRS)

Near infrared spectroscopy (NIRS) is a recognised non invasive method, used largely to monitor cerebral tissue oxygenation and ischaemic changes in neonates (Wyatt et al, 1986; Matcher and Cooper, 1994). It has been used as an investigation method for osteoradionecrosis in retrospective studies, and shows a reduction of the amount of deoxygenated haemoglobin at sites of osteoradionecrosis, confirming that it is a hypovascular, hypoxic tissue with decreased metabolic rate subtracting little oxygen from haemoglobin (Hutchinson, et al 1990a).

Telfah (1995) evaluated 32 patients who had been given radiotherapy, 16 of them with ORN. His study showed marked decrease in levels of deoxyhaemoglobin concentrations in the ORN tissue, suggestive of either a massive increase in blood flow or decrease in blood flow, decreased metabolic activity or both. As stated an enormous increase in blood flow in the irradiated mandible is unlikely due to endarteritis obliterans that is seen histologically, and reductions in deoxyhaemoglobin levels in the irradiated mandible are therefore indicative of decreased tissue metabolic activity, were little oxygen is extracted from the haemoglobin. These observations could also be correlated to clinical findings. Fat content was also evaluated and showed a marked decrease in the irradiated mandible as compared to that of controls, but increased again over the period of 5-7 years between the scans used in his study. This pilot study suggested that there was also no spontaneous improvement in blood
supply with time, but a significant improvement following a course of therapeutic ultrasound.

8. Treatment of osteoradionecrosis

Treatment of ORN varied extensively over the years. Due to the theory of radiation, trauma and infection, medical treatment with long term antibiotics and debridement was common for many years (Meyer, 1970). If this treatment failed, mandibulectomy was often the only solution (Friedlander et al, 1979).

The aims of treatment are the elimination of pain and associated infections, improvement of mouth function (opening, speech, mastication), and the prevention of deformity (fistulas, bone exposures, bone defects, pathological fractures).

8.1. Conservative management

The initial approach to the treatment of osteoradionecrosis should be conservative, with medication and local wound care only, since up to 60% of the cases resolve thereby (Bedwineck et al, 1976; Morrish et al, 1981; Murray et al, 1980a and b; Epstein et al, 1987b; Beumer et al, 1983b; Beumer et al, 1984; Scully and Epstein, 1996). This resolution rate may be over-estimated by including here figures of so called 'mild' cases of osteoradionecrosis.

Oral hygiene is essential, including the use of 0.02% aqueous chlorhexidine mouthwashes after meals (Scully and Epstein, 1996) and constant saline mouthwashes. Debris should be washed/irrigated away and sequestra should be allowed to separate spontaneously or gently removed, since any surgical interference may encourage extension of the necrotic process. Galler et al (1992) reported three cases of osteoradionecrosis which developed from periodontal disease activity, and proposed the use of chlorexidine digluconate and hyperbaric oxygen in the management of this condition.
Although osteoradionecrosis is not primarily an infectious process and the tissues are hypovascular, limiting the success of systemic antimicrobial agents, tetracyclines have been recommended because of their selective uptake by bone (Rankow and Weissman, 1971; Coffin, 1983). However, access to avascular bone is questionable, making tetracycline inactive. Penicillin has also been used, because of the involvement of oral bacteria in the superficial contamination (Daly et al, 1972; Stamps et al, 1982; Marx et al, 1985). Metronidazole, 200 mg, three times daily or other broad spectrum antimicrobials, could be added in cases of severe infection or where anaerobes are implicated (Marx, 1984; Beumer et al, 1983b; Harris, 1992). Antibiotics rarely, if ever, cure osteoradionecrosis.

The use of packs over exposed bone has been popular in the past (MacComb, 1962). He used zinc peroxide mixed with carboxymethylcellulose in hydrogen peroxide, and also mentions the use of 5% neomycin solution or acriflavine as alternatives. Morton and Simpson (1986) also recommended packs for covering small areas of exposed bone and delicate granulation tissue following separation of sequestrum, and for keeping necrotic bone cavities clean in patients who are not ready for definitive treatment. They found BIPP (bismuth and iodoform paraffin paste) on ribbon gauze very satisfactory, as it remains fairly soft and quite clean.

8.2. Hyperbaric oxygen (HBO) therapy

Ketchum et al (1970) demonstrated rapid vascularization and increase in both osteoclastic and osteoblastic activity in animals treated with hyperbaric oxygen in a controlled burn wound experiment. The daily elevation of oxygen tension in hypoxic bone and soft tissues result in the ingrowth of capillaries (Hunt et al, 1969), fibroblastic proliferation, collagen synthesis (Hunt and Pai, 1972) and capillary angiogenesis (Ketchum et al, 1970).

Mainous et al in 1973 were probably the first authors to suggest the use of hyperbaric oxygen therapy for the treatment of osteoradionecrosis. Following its introduction and preliminary good results, it has been shown to promote

Hart and Mainous (1976) used hyperbaric oxygen therapy at 2 to 2.5 atmospheres pressure for 1.5-2 hours per day for up to 84 sessions. They treated 46 patients, and their results showed that 80% were symptom-free after treatment, and 11% required further 20 sessions. However, 41% required some form of surgery for symptom control, and 9% required a mandibulectomy. Mansfield et al (1981) treated twelve patients with HBO (2.4 Atm, 90 min.) for an average of 75 sessions. Eight patients required sequestrectomy and one needed a bone graft.

Beehner and Marx (1983) showed that hyperbaric-oxygen induced angiogenesis becomes measurable by TcPO₂ after eight sessions, rapidly progresses to a plateau at 80% to 85% of nonirradiated tissue vascularity by 20 sessions, and remains at that level without further improvement with additional hyperbaric oxygen. However, it has to be remembered that TcPO₂ measurements are capable of measuring the oxygen levels only at the capillary loops of the skin, at a depth of less then 1 mm. These measurements can be used only to verify the effects of hyperbaric oxygen on the skin, and not the underlying tissues. Therefore this method is unsuitable to investigate osteoradionecrosis, and these observations regarding the effects of HBO on osteoradionecrosis should be looked at very critically.

Marx (1983b) introduced a protocol for the treatment of osteoradionecrosis that combines hyperbaric oxygen therapy (HBO) and surgery as its primary treatment modalities (Table 2.3). He concluded that hyperbaric oxygen alone cannot heal osteoradionecrosis wounds (only 15% responded to stage I), suggesting that hyperbaric oxygen without aggressive surgical management would not resolve the disease progress in most cases. Therefore, 8 patients (14%) needed sequestrectomies (Stage II), and 70%
required major reconstruction (Stage III). The reasons given for the low success rate were: (1) the degree of radiation tissue damage varies greatly between patients, even with identical doses and fractions, (2) hyperbaric oxygen cannot resurrect dead bone, and (3) hyperbaric oxygen cannot entirely reverse radiation injury. Therefore he recommends the combination of hyperbaric oxygen therapy and aggressive surgery. The high incidence of surgical treatment seemed to be unnecessary, since only conservative treatment can cure up to 60% of cases. Furthermore, if only 15% of his cases responded to HBO alone, and 70% required major surgery, there seems to be no value in using hyperbaric oxygen therapy in osteoradionecrosis management. Nevertheless, the use of HBO associated with surgery is commonly described in the literature (Epstein et al, 1987a and b; McKenzie et al, 1993; Mounsey et al, 1993; Merkensteyn et al, 1995).

McKenzie et al (1993) noted a 50% resolution of osteoradionecrosis with HBO treatment, but over half of these patients had undergone a mandibulectomy in conjunction with HBO to achieve this resolution. In a retrospective study, Mounsey et al (1993) analysed 41 patients with proven mandibular osteoradionecrosis treated with HBO (Toronto Hospital). The results showed that 83% of the patients had a significant improvement, judged by at least 50% decrease in the size of the exposed bone, closing of the fistulous tract, or complete relief of symptoms. However 17% of the patients did not benefit. They conclude that only 'mild' cases of osteoradionecrosis can be cured with HBO, and the severe cases will need surgery to remove dead bone.

In 1995, Merkensteyn et al reported a series of 29 cases of osteoradionecrosis treated with HBO. They found that HBO led to initial resolution of only 15 of 29 cases, and a second course of HBO improved the result to 20 of 29 cases (69%). Thirteen of these patients had mandibular discontinuities at the start of treatment, and two healed. The number of patients who lost continuity was 17 of 29, with 5 of 29 becoming discontinuous during HBO treatment. Undermining the supposed protective effects of HBO, was the fact that two patients had a second episode of osteoradionecrosis on the
opposite side, which resulted in loss of mandibular continuity on the second site.

Contraindications and complications of hyperbaric oxygen therapy

An relevant contraindication of hyperbaric oxygen therapy in smokers is emphysema, and untreated pneumothorax. Side effects of HBO are uncommon but include transient myopia, seizures, and otic or pulmonary barotrauma, the latter potentially leading to air embolism. Concern has been expressed that hyperbaric oxygen may exacerbate a variety of autoimmune and immunosuppressive disorders, and viraemia (Giebfried et al, 1986), but there is little supporting evidence.

Relative contraindications to hyperbaric oxygen therapy include upper respiratory tract infection, chronic sinusitis, epilepsy, chronic obstructive airways disease, high fevers, a history of spontaneous pneumothorax or thoracic or ear surgery, viral infections, congenital spherocytosis, a history of optic neuritis and claustrophobia (Scully and Epstein, 1996). The risk from hyperbaric oxygen therapy may be minimised by a careful pre-treatment assessment including chest radiography and electrocardiography; some advise also an otolaryngological and ophtalmological assessment (Giebfried et al, 1986).

The cost and lack of availability should also be mentioned as a potential problem related to HBO. Hyperbaric oxygen therapy is very time consuming, and therefore expensive. Marx et al 1985 reported that the cost to treat one patient with HBO is approximately 19,000 dollars per year, and combined with surgery, according to his protocol, 30,000 dollars per year (in 1985 values). In a more recent publication, Clayman (1997) shows that the cost to treat one patient with HBO has reduced, but is still around 15,000 dollars (data courtesy Hyperbaric Unit, Henry Ford Hospital, Detroit, MI). There are only a few hospital or diving centres with the facilities of a hyperbaric camera in each country. Therefore it is not widely available, and can be even more expensive if the costs of travelling and accommodation are added.
8.3. Ultrasound Therapy

It is clear that HBO does not cure all cases of osteoradionecrosis. Furthermore, it is expensive, labour-intensive, hazardous and not readily available (Giebfried et al, 1986; Clayman, 1997). Therefore, the search has continued for a therapy which is cheaper and more practicable, and the use of ultrasound appears to be a very sensible option (Harris, 1992).

Ultrasound has proved to be therapeutically valuable in many ways (see next section III), including: stimulation of tissue regeneration (Dyson et al, 1968 and 1976; Dyson, 1990; Paul et al, 1960); increase blood flow in chronically ischaemic muscles (Hogan et al, 1982); protein synthesis in fibroblasts (Harvey et al, 1975; Webster et al, 1978); healing of ischaemic varicose ulcers (Dyson et al, 1976); tendon repair (Enwemaka, 1990) and angiogenesis in full thickness excised incisions in the flank skin of adults rats (Young and Dyson, 1990a). Local stimulation of hard tissues has also been evaluated. Dyson and Brookes (1983) showed that it was possible to accelerate the repair of fibula fractures in rats. Pilla et al (1990) and Tsai et al (1992) showed that low intensity ultrasound could stimulate fracture repair in rabbits. Wang et al (1994) and Yang et al (1996) used a rat femoral fracture model, and observed accelerated fracture repair. Ultrasound was also used in human tibial fractures, in a prospective, randomised, double-blind evaluation. The results showed that the treated group had a significant decrease in the time to clinical healing (86 +/- 5.8 days) as compared to the control group (114 +/- 10.4 days) (p=0.01), and also a significant decrease in the over-all time, i.e. clinical and radiographic healing (96+/-4.9 days compared to 154 +/-13.7 days in the control group (Heckman et al, 1994).

Harris (1992) was the first author to use therapeutic ultrasound for the treatment of mandibular osteoradionecrosis. This was based on previous uses of ultrasound as a simple means of promoting neovascularity and neocellularity in ischaemic tissues (Young and Dyson, 1990a). He studied a group of 24 patients with ORN, all but 4 having received long term antibiotic therapy with local surgery for at least a year prior to referral for management. The treatment
of these 24 patients varied in the first 4 cases, but the following 20 used a standard protocol as described:

1- Retained tooth and infected teeth were removed by conventional techniques with primary closure of the wound. Infections were treated with metronidazole, or with cefalexin if the patient were intolerant to the former.

2- The ultrasound machine was calibrated to give an intensity of 1 W/cm² (SAPA), at a frequency of 3 MHz with the wave pulsed 1:4. This was applied for 10-15 min a day to the skin overlying the ischaemic mandible initially for 40 days. In all cases where healing was progressive but incomplete, further 20 day courses of ultrasound were used.

3- Where healing proved to be slow with persistent significant bone exposure, the area was then treated surgically under general anaesthetic, with careful debridment of the exposed bone with a saline cooled acrylic bur followed by cover with a local flap of non irradiated tissue.

The results in the group treated with ultrasound (21/24), showed complete healing in 10/21 (48%) cases, treated with debridment and ultrasound alone. The remaining 11 cases remained unhealed after ultrasound therapy, and received debridement and cover with a local flap. From these, just one needed a mandibular resection and reconstruction with a microvascular composite fibular graft. Only one recurrence of osteoradionecrosis dehiscence was detected which responded to further ultrasound.

Telfah (1995) using near infrared spectroscopy has demonstrated that patients with osteoradionecrosis who received ultrasound therapy showed significant improvements of the metabolic activity (improvements of deoxyhaemoglobin concentrations).
8.4. Surgery

Surgery is very often a treatment option for osteoradionecrosis. Usually it is combined with antibiotics and mouth washes, and also with HBO and ultrasound.

The surgical options start with the removal of small sequestra, and increases depending on each case, to further sequestrectomy, alveolectomy with primary closure, closure of orocutaneous fistulae and flaps to cover the area (local or free vascularized). In extreme cases large resections and hemimandibulectomies are performed, and the bone should be reconstructed preferably with a bone source with its own blood supply, like fibula or iliac crest vascularized flaps. While surgery aims to remove frankly necrotic tissue, unless measures are taken to improve vascularity, healing will often be problematical (Morton and Simpson, 1986).

Conservative surgical approaches start with small sequestra removal, and if needed, debridement of superficial bone until bleeding occurs. The cortical plates usually do not bleed in osteoradionecrosis patients. Drilling holes through the irradiated nonviable mandibular cortex was suggested by Hahn and Corgill (1967) in an attempt to stimulate granulation tissue.

As osteoradionecrosis represents superficially contaminated bone with soft tissue radionecrosis as the major contributor to bone exposure and symptoms, the aim of the treatment can be limited to provide vascularized soft tissue to cover bone. The temporalis muscle flap can be used for the posterior oral cavity (Harris, 1992), and the nasolabial flap for anterior exposures. An alternative if these flaps are not available would be the use of distant myocutaneous flaps or free flaps, like the radial forearm free flap.

If osteoradionecrosis persists, mandibular resection can be indicated (Rankow and Weissman, 1971). However, if reconstruction is not performed, collapse of the face can create severe functional disability and deformity, even knowing that the irradiated fibrotic soft tissues do help in reducing this tendency to collapse (Morton and Simpson, 1986). Mandibular reconstruction with bone
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grafts should be performed aiming at the following criteria: restoration of bony continuity, alveolar height, facial form and osseous bulk maintained over time and elimination of soft tissues deficiencies so that dentures can be worn (Marx and Ames, 1982).

Obwegeser and Sailer (1978) suggest the excision of the affected bone and immediate bone grafting with autogenous decorticated iliac or rib, using an intraoral approach. They comment that the results of primary resection and reconstruction in mandibular osteoradionecrosis are not as good as in ordinary osteomyelitis due to the poor quality of the irradiated soft tissue cover. Friedlander et al (1979) reported a case where a transoral hemimandibulectomy was done, and recommended that an extraoral approach should be avoided because of the need of dividing the facial vessels. Marx (1983b) suggested the use of HBO before reconstruction, and used an extraoral approach, using particulated bone and marrow in stainless-steel or allogenic bone cribs, reporting 100% success.

Nowadays with the introduction of free vascularized tissue transfer, the armamentarium to soft tissue and mandibular reconstruction has increased considerably. The possibility of bringing a non-irradiated tissue, with its own blood supply will improve the perfusion of the irradiated tissue bed into which it is placed (Mirante et al, 1993). Therefore, this reconstruction method can be used without any adjunctive HBO therapy (Hutchinson, 1996).

Nakatsuka et al (1992) recommended the use of a dual free flap transfer to reconstruct mandibular resections for cancer or osteoradionecrosis resections. One flap should be a radial forearm and the other a bone flap (vascularized iliac or scapula usually). Yanagiya et al (1993) also have used a similar technique, combining the scapular osteocutaneous flap with a radial forearm flap. Flemming et al (1990) proposed the use of vascularized fibula to reconstruct large segments of mandible. They presented 7 cases, 4 of which had osteoradionecrosis, and claim very good results. Sanger et al (1994) presented a case of massive osteoradionecrosis with necrosis of skin, bone
and floor of mouth, in which they used three microvascular flaps (2 radial forearm flaps and one fibular flap) for reconstruction.

Rehabilitation after reconstruction with osseointegrated implants can be used according to Granstrom et al (1992). Marx and Johnson (1987) showed in a study of serial biopsy specimens from irradiated patients that there was a continuous loss of capillaries over time after irradiation. Furthermore, there was no evidence of spontaneous revascularisation over time. Based on this study, these authors recommend that reconstruction or implant surgery should be performed from 1 to 6 months post-irradiation, because this interval reduced the risk of developing osteoradionecrosis.

Barber et al (1995) reported a pilot study evaluating the use of osseointegrated implants in vascularized fibula flaps, used to reconstruct mandibles after resection for osteoradionecrosis. They reported 5 cases in which they placed 20 implants after a prophylactic course of HBO (20 dives before and 10 after). Atraumatic surgery, and avoidance of loading the implant for 6 months contributed to their 100% clinical success. However, the report of the International Research Group of Reconstructive Preprosthetic Surgery suggest that the success rates for implants placed in irradiated mandibles, or even maxillas is still low (Stoelinga and Cawood, 1996).

8.5. Other treatment modalities

Other treatment modalities of osteoradionecrosis have been suggested. There are some interesting reports on drugs used to improve blood flow and tissue metabolism. Eppley et al (1991) suggested the use of fibroblast growth factor (FGF) prior to bone grafting, showing improved vascularity in the irradiated soft tissue bed, and reduced risk of bone graft failure in rabbits. Dion et al (1990) used pentoxifylline to heal soft tissue radionecrosis wounds in the oral mucosa. Calcitonin has also been used successfully to treat osteoradionecrosis (Dambrain and Barrelier, 1991). Electrotherapy (King et al, 1989) has also been suggested, but all these techniques have yet to be confirmed.
9. **Prevention of osteoradionecrosis**

It is important to prevent the orofacial complications associated with cancer therapy, and this requires an oncological team that includes an experienced dental practitioner and hygienist (Scully and Epstein, 1996). This is particularly important in children, since orofacial complications are up to three times as common as in adults having similar treatment (Dreizen, 1990; Stalman et al, 1986). In relation to osteoradionecrosis, its prevention becomes even more important, since its investigation is rudimentary, medical treatment used alone has limited success, and surgical treatment is technically difficult and expensive.

Prosthetic techniques which shield normal tissue with lead, or pull it out of the radiation beam, should be employed wherever possible (Poole and Flaxman, 1986; Levendag et al, 1990). Multiple fields should be used in preference to single fields, and if possible, the major salivary glands should be excluded. Further advances in radiotherapy, like 3D CT planning, conformal and stereotactic therapy, should enable more normal tissue to be spared, by focusing the radiation on the tumour itself, therefore preventing the associated complications.

General measures to prevent the development of osteoradionecrosis are well established. Basically, all the factors mentioned as risk factors to the development of osteoradionecrosis as discussed earlier, can be accessed. The main concern is usually the dentition, and with good plaque control, intensive fluoride therapy, use of chlorhexidine and saline mouthwashes, it is likely that the motivated patients will derive years of service from their teeth in the proposed irradiation field (Stamps et al; 1982).

Most patients (97%) need some attention to oral health-care before starting radio- or chemotherapy for cancer (Lockhart and Clark, 1994). Not infrequently these patients have poor oral hygiene and care, and comply poorly with treatment. Dental and periodontal disease should therefore be treated
before cancer therapy and any oral infections controlled. Patients must achieve good oral hygiene levels before initiating treatment.

Even knowing that there are reports showing delays in wound healing after chlorhexidine rinses (Basetti and Kallemerger, 1980), the use of chlorhexidine and fluoride is highly recommended in the literature (Joyston-Bechal et al, 1992; Scully and Epstein, 1996,). Chlorhexidine may reduce oral mutans streptococci and lactobacilli (Epstein et al, 1991). Dietary control and topical fluoride therapy are essential and should be continued for life. Fluoride can reduce caries and can be used as gel containing 1% sodium fluoride placed in carriers and applied for 5 min. per day (Daly et al, 1972; Horiot et al, 1983). Sodium fluoride mouthrinses with chlorhexidine diacetate have also been recommended (Giertsen and Scheie, 1993).

The use of prevention protocols is most welcome. Jansma et al (1992) published a very detailed protocol for prevention and treatment of oral sequelae resulting from head and neck radiation therapy. The protocol is particularly applicable in centres with a dental team, which should be involved at the time of initial diagnosis, so that a successful preventive regimen is an integral part of the overall cancer treatment regimen.

Despite all preventive measurements, some patients will present with decaying or periodontally involved teeth following radiotherapy. It is possible to perform conventional endodontic therapy at this stage without incurring an increased risk of infection in the bone on the apical region (Cox, 1976; Seto et al, 1985; Kielbassa et al, 1995). This would be a preventive measurement, since an extraction is avoided.

9.1. Prophylactic hyperbaric oxygen

Hyperbaric oxygen has been suggested to be used to prevent osteoradionecrosis, mainly by using it before any planned extraction. Marx and Ames (1982) refined the HBO preventive treatment for extractions after radiotherapy for 20 preoperative and 10 postoperative dives, claiming a 91.6%
success rate, which they noted was highly favourable compared with the 20% to 50% success rates reported without HBO (Obwegeser and Sailer, 1978; Bedwineck et al, 1976).

Marx et al (1985) performed a randomised, prospective clinical trial using HBO and penicillin in previously irradiated jaws. They demonstrated that prophylactic HBO improved extraction wound healing when compared with antibiotics. However, they had very high levels of osteoradionecrosis following extraction in both groups - 5.4% for the hyperbaric group and 29.9% for the antibiotics group. It is interesting to note that they did not perform an alveolectomy and primary closure was not attempted.

However, some authors question the benefits of hyperbaric oxygen in the prevention of osteoradionecrosis and do not use HBO before extractions (Maxymiw et al, 1991). These authors extracted 449 teeth in 72 post-irradiated patients, and the only preventive measure was the use of low-epinephrine or epinephrine-free, non-lignocaine local anaesthetics, and conservative surgical techniques. In a follow-up ranging from 68 days to 19.3 years (median 4.8 years), no instances of ORN occurred as a result of dental extraction.

Epstein et al (1987) mentioned that due to the low incidence of osteoradionecrosis in patients after dental extractions, prophylactic use of hyperbaric oxygen can not be justified. This was reaffirmed by Clayman (1997), who performed a review of the literature, regarding the use of HBO for the prevention of osteoradionecrosis after dental extractions. He observed that the true loss of continuity of mandibles that develop osteoradionecrosis after dental extractions varies greatly between small series, but in aggregate review is quite low. Even if one were to disallow any contribution of spontaneous osteoradionecrosis to the reported post-extraction osteoradionecrosis rate, the loss of continuity rate would still be 2.5%. If one were to treat all of these at-risk mandibles with HBO before extractions, the loss of continuity rate might be reduced to 0.5% if HBO were 80% effective, which may be an overly optimistic assessment. The cost to treat 100 patients with HBO to prevent two cases of loss of continuity would be approximately 1.5 million dollars (data courtesy
Hyperbaric Unit, Henry Ford Hospital, Detroit, MI). Although it would be very desirable to eliminate osteoradionecrosis after extractions, the cost of treating 98 patients who would not have benefited, may well be insupportable. Clayman (1997) therefore concludes that these data do not support the mandatory use of HBO before removing teeth in irradiated mandibles, particularly when one considers that in the most recent reports of osteoradionecrosis after dental extractions the rate was only 2.1% (Marciani and Ownby, 1986; Makkonen et al, 1987; Maxymiw et al, 1991; Widmark et al, 1989; Brunton, 1994).

Hyperbaric oxygen therapy has also been advocated to be used before the placement of dental implants in irradiated mandibles. The literature is very controversial in this subject. Those in favour of its use claim success rates of implants up to 100%, and only 57.9 to 64.7% without HBO (reviewed by Larsen, 1997). He suggested the use of a protocol similar to that used for extractions, with 20 dives before and 10 after implant surgery. As an example, Granstrom et al (1992) showed an implant failure of 58% before the introduction of HBO, and of 2.6% after, in the maxilla and orbit. Keller (1997) reviewed the literature against the use of HBO, which showed also good success rates, ranging from 74 to 100%. He suggests that the risk of developing osteoradionecrosis is very low, and that with increased healing time the implants will osseointegrate well. The costs and risks involved in the use of HBO, and the low incidence of complications without its use do not justify its routine use (Keller, 1997).

### 9.2. Prophylactic ultrasound

Ultrasound therapy seems to be an extremely useful tool in the prevention of osteoradionecrosis. It has been shown to be very effective in the treatment of osteoradionecrosis (Harris, 1992), but until now, no one has used it to prevent it. We think that a preventive protocol using 20 sessions of ultrasound before extractions or implant surgery, followed by further 20 sessions would be very useful (Reher and Harris, 1997). Ultrasound is readily available in all hospitals and very inexpensive. The cost to treat only one patient with the HBO
prevention quoted by Clayman (1997), would purchase at least 5 ultrasound machines.
Section II - Angiogenesis

This section will give an overview of the current literature related to angiogenesis. It has been divided into the following:

1. Mechanisms of blood vessel formation
2. The process of angiogenesis
3. Angiogenesis models
4. Control of angiogenesis
5. Induction of angiogenesis
6. Inhibition of angiogenesis
7. Therapeutic anti-angiogenesis
8. Therapeutic angiogenesis.

Angiogenesis, or neovascularization, is the growth of new blood vessels. Angiogenesis is a physiological phenomenon, under strict control and short duration (days). It occurs only during embryonic development, endometrial regeneration and wound repair (Fan, 1993). A vascular system solves the problem of exchange of nutrients, oxygen and waste products by a crowded three-dimensional population of cells for which simple diffusion to and from the environment would be inadequate (Jakobsson, 1994).

The turnover of endothelial cells in the normal adult is very low, in the order of years in humans (Denekamp, 1984). The average endothelial cell divides only twice in adult life. In contrast, persistent, uncontrolled and unabated neovascularization occurs in many pathological states.

Both the growth and metastasis of solid malignant tumours are angiogenesis-dependent processes (Folkman et al, 1989; Folkman, 1993). Although angiogenesis is seen most dramatically in tumour development, it is also one of the hallmarks of chronic inflammation and wound repair. Inflammation and angiogenesis are closely related but distinct and separate
processes. Thus, it is possible to induce angiogenesis in the absence of inflammation (Jakobsson, 1994).

Considerable clinical problems are due to local rarefaction of blood vessels, insufficient neovascularization, or both. Necrosis, osteoradionecrosis, ulcers, fistulas and fibroatrophy as the result of decreased vascularization can cause tissue and organ malfunction and even death of the individual. Regular neovascularization is necessary for uncompromised adult wound healing and tissue regeneration (Eliseenko et al, 1988).

1. Mechanisms of Blood Vessel Formation

There are at least three mechanisms of blood vessel formation: (a) vasculogenesis, (b) angiogenesis, and (c) the regeneration of the endothelial layer or diameter expansion of existing vessels. These processes may involve different cellular and regulatory mechanisms (Jakobsson, 1994).

1.1. Vasculogenesis (embryonic)

Vasculogenesis is the formation of an immature vascular network originating from embryonic angioblasts differentiating in situ. The vascular system develops from mesodermal blood islands (Risau, 1991). In the yolk sac, mesenchymal cells (hemangioblasts) form blood islands in the extraembryonic mesoderm at the same time as somites begin to form. The blood islands form clusters and cords which gradually develop into a primary vascular plexus. The cells located in the central area form the primitive blood cells, while those in the periphery flatten and form endothelial cells. The differentiation of endothelial cells also takes place inside the embryo and blood vessels are formed by both vasculogenesis and angiogenesis (Risau, 1991). The vascularization of endodermal organs proceeds from in situ differentiating endothelial cells, i.e. vasculogenesis. On the other hand, the vascularization of other organs is clearly dependent on angiogenesis. This angiogenic process is probably regulated by the developing organ itself and appears to be dependent on the
growth and differentiation of parenchymal cells in these organs. For example, during the brain development, a transient expression of vascular endothelial growth factor (VEGF) is correlated with endothelial cell growth and angiogenesis (Risau, 1992).

2. Angiogenesis

Angiogenesis is the formation of new capillaries by sprouting from pre-existing vessels. The process of angiogenesis will be outlined below. Two specific mechanisms for capillary network expansion not involving the formation of capillary sprouts are described as well. First, the proliferation of pericytes and endothelial cells coupled to a fusion of neighbouring capillaries. The second process not involving the formation of capillary sprouts was observed in the growth of the pulmonary microcirculation. This process, called intussusceptive microvascular growth, is characterised by the formation of slender intraluminal pillars by which a mother capillary is divided longitudinally into daughter capillaries (Caduff et al, 1986; Burri and Tarek, 1990).

2.1. Regeneration of endothelium and vessel diameter expansion

This occurs when the intima of a vessel is damaged, and mitosis of the endothelial cells occurs in order to repair the defect. Replication ceases when the denuded area is again covered by a confluent layer of endothelial cells (Jakobsson 1994). A similar process occurs when an existing collateral blood vessel enlarges in diameter to become able to transfer greater quantities of blood, following the obstruction of an artery (Blood and Zetter, 1990).

Vasculogenesis, angiogenesis, regeneration of endothelium and vessel expansion are parts of the biological process that builds up and maintains the primary vascular system and enables secondary changes to permit survival of the organism.
3. The Process of Angiogenesis

Angiogenesis is one biological mechanism of new capillary formation and involves the intricate interplay between vascular and non-vascular cells, soluble factors and extracellular matrix components. Until recently the endothelial cell has been the focus of most studies of microvascular growth. However, capillaries are not simply tubes of endothelial cells, but also involves the pericyte, being therefore a two-cell system (D'Amore, 1992). Endothelial cells and pericytes of capillary blood vessels carry all the genetic information required to form tubes, branches and assemble a vascular network.

The angiogenesis process is the end result of a cascade of several steps, that can be summarised as the following: (1) activation, (2) degradation of basal lamina, (3) migration of endothelial cells, (4) proliferation of endothelial cells from pre-existing venules, (5) capillary tubes formation and (6) maturation of new capillaries (Fig. 2.1).

3.1. Activation of endothelial cells in the parent vessel

Parent vessels are usually microvessels including small precapillary arterioles, capillaries and post-capillary venules. However, more sprouts emanate from the venous capillary portion than from the arterial portion of microcirculation (Rhodin and Fujita, 1989). The parent vessel giving rise to a sprout is often dilated and permeable, has a higher endothelium, and the endothelial cells exhibit abluminal cytoplasmic projections penetrating into the perivascular connective tissue. These endothelial cells when stimulated increase in size, number of processes and organelle content (Yamagami, 1970; Ausprunk and Folkman, 1977).
Chapter 2 - Literature Review - Section II - Angiogenesis

Angiogenic signal

1: Activation

2a: Degradation of Basal Lamina

3: Migration of Endothelial Cells

4: Proliferation of Endothelial Cells

5: Capillary Tubes Formation

6: Maturation and Loop Formation

7: Remodelling, Arteries and Veins

Figure 2.1 - Schematic representation of the process of angiogenesis (based on Höckel et al, 1993)
3.2. Degradation of basal lamina

At the point where a new capillary will sprout, the basal lamina enveloping the endothelial cell of the parent vessel is locally fragmented. The activation of a variety of proteases, including plasminogen activator, collagenase type IV, serine proteinases and other enzymes leads to the degradation of the existing basal lamina. Basic fibroblast growth factor (bFGF), an angiogenic factor, induces the production of plasminogen activator and collagenase by cultured endothelial cells (Gross et al, 1983). The protein kinase C pathway appears to be implicated since phorbol myristate acetate can increase collagenase secretion 5 to 30 times (Fan, 1993). Most collagenase released from endothelial cells is in its latent form but can be activated by plasmin and some angiogenic factors.

3.3. Migration of endothelial cells

Endothelial cells then migrate from the vascular wall, through perivascular connective tissue and parenchyma, towards the angiogenic stimulus. The endothelial cells migrate while still in contact with one another. Mast cells have been shown to contain a migration-stimulating factor which is probably heparin. In addition, bFGF a platelet-derived endothelial cell growth factor (PD-ECGF) also stimulates endothelial cell migration.

3.4. Proliferation of endothelial cells

Proliferation and migration appear to occur in different endothelial cells, migration predominating at the tip of the growing capillary, and proliferation occurring several cells proximal to the tip (Yamagami, 1970). Therefore, proliferation occurs behind the leading front of migrating endothelial cells, in order to allow the formation of capillary tubes. A large number of growth factors can cause this proliferation (Table 2.5).
3.5. Capillary tubes formation

The mechanisms leading to the formation of a lumen are probably the least known. One possible mechanism in the formation of initial lumens would be intracytoplasmatic vacuolisation, like that observed in rapidly growing human juvenile hemangioma (Furusato et al, 1984).

It has been reported that the initial endothelial sprouts appeared to consist of a single endothelial cell projection (Ausprunk and Folkman, 1977). However, by using 2D and 3D reconstruction of electron liptographs, Wakui (1988) observed that these sprouts are composed of two endothelial cells, and that the sprout lumen was in continuity with the parent capillary lumen. Wakui therefore suggested that the endothelial cells grows in a bicellular configuration with a pair of endothelial cells extending and migrating outwards, forming a slit-like lumen connected to the parent capillary lumen. An intercellular lumen sealed by intercellular junctions is thus formed at the start of bud formation (Konerding et al, 1991), and does not follow the formation of a solid endothelial cell cord. The sprout represents a weak point in the vascular system and is leaky, as observed in granulation tissue. This can be observed 2-3 days after the application of an angiogenic stimulus. This weakness may be related to endothelial cell proliferation and the presence of immature intercellular junctions (Jakobsson, 1994).

3.6. Maturation of new capillaries

The maturation of new capillaries consists of (a) the formation of new basal lamina and (b) the migration of pericytes and fibroblasts. Immunoelectron microscopy has shown that only the cellular protrusions at the tip of migrating endothelial cells in a sprout lack a basal lamina, while the remaining part of the sprout is covered by a continuous layer of amorphous basal lamina material (Paku and Paweletz, 1991). The immature basal lamina is partially rebuilt before blood flow sets in. The formation of a basal lamina around the new
capillaries thus seems to play an important role in the development of a functional vessel.

Almost simultaneously with the appearance of an ultrastructurally detectable basal lamina, pericytes establish contact with endothelial cells in the sprout (Paku and Paweletz, 1991). Pericyte arrival also occurs prior to the onset of blood flow, and appears to mark the establishment of a new functional capillary. Rhodin and Fujita (1989) have described a process in which fibroblasts approach and settle down on sprouts subsequently to be converted to pericytes enveloped by their own basal lamina. Endothelial cells and pericytes make contacts through discontinuities in the basal lamina and an intricate interplay occurs, with cytoplasmic processes of pericytes and endothelial cells caving in on another's surfaces (Wakui, 1988). Although the relationship between endothelial cells and pericytes remains unclear, in vitro observations show that they are functionally related. Endothelial cells induce pericyte chemotaxis and the directed migration of pericytes by releasing diffusible growth factors (Ausprunk et al, 1978). Depending on the continued presence or absence of an angiogenic stimulus, there is substantial remodelling, regression and rearrangement of newly formed capillaries.

**Capillary loops**

Anastomosis of proximate sprouts result in the formation of loops. The mechanism by which two capillary sprouts establish contact and fuse to form a functioning capillary loop appears to be unknown.

### 3.7. Arteries and Veins

The new blood vessels generated by angiogenesis may persist as mature capillaries or progress to become larger venous or arterial vessels. This is a complex mechanism involving the association and differentiation of pericytes and smooth-muscle cells as well as matrix remodelling (Höckel, 1993). However, many of the newly formed capillaries will finally regress (Feinberg et
Furthermore, existing blood vessels can elongate and grow in diameter by longitudinal and lateral proliferation of endothelial cells.

4. Angiogenesis Models

Angiogenesis models used can be in vitro or in vivo assays, the latter being more commonly used. Several in vivo models are widely used to study angiogenesis and evaluate factors influencing angiogenesis (Auerbach et al., 1991). In vivo assays can be divided in methods with a high or a low vascular background. The methods with a high vascular background are (1) the disc angiogenesis system, (2) the hamster cheek pouch, (3) the transparent ear chamber, (4) intradermal assays and (5) the chorioallantoic membrane assay (CAM). The methods with a low vascular background are (1) the rodent cornea assay and (2) the rodent mesenteric window assay (Jakobsson, 1994). For the purpose of this thesis, we have selected and used the chorioallantoic membrane assay, and only this one will be described.

4.1. Chick Chorioallantoic Membrane (CAM)

This is the most commonly used bioassay to study angiogenesis. The test substance is usually prepared in slow-release polymer pellets, or in methylcellulose discs and implanted on to the CAM through a window made in the eggshell or in a shell-less embryo cultured in a petri dish. The angiogenic activity is revealed by radial in-growth of new capillary vessels (Fan, 1993).

The CAM is an extraembryonic membrane formed on the 4th day of incubation by fusion of the chorion and the allantois. It mediates gas exchange with the extraembryonic environment and has a very thick capillary network in close contact with the shell. Usually in this assay, a rectangular window in the shell is made, to place grafts or test material on the CAM. Acceleration or retardation of the ongoing angiogenesis are evaluated. Angiogenesis is most often scored 3-4 days after application of the test material, and a “spoke-wheel” type arrangement of vessels, directed towards the test substance or graft is
considered evidence of angiogenesis. Positive controls with angiogenesis factors such as VEGF (Vascular endothelial factor) are useful for comparative purposes. A clear increase of vessels around the graft, even without the typical radial arrangement of vessels, is also considered a sign of stimulated angiogenesis. The angiogenic response is most often evaluated semiquantitatively under a stereomicroscope.

The normal vascular growth in the CAM is rapid in the early chick embryo (days 4 to 9) but decreases, and has virtually ceased at day 11 (Ausprunk et al. 1974). This means that stimulation of angiogenesis may be quite different in the early and late embryo. Inherent methodological problems in this assay are the subjectivity of the evaluation of vessel growth, the difficulty to distinguish angiogenesis from hyperaemia and the difficulty to differentiate vasoproliferation from mechanical effects of the implants on the blood vessel distribution at the CAM surface (Vu et al. 1985). However, in spite of these problems, the majority of angiogenesis studies have been performed using this model (Ribatti et al, 1997; Boshoff et al, 1997).

4.2. In vitro Angiogenesis Models

The successful long-term culture of capillary endothelial cells, such as the HUVEC (Human umbilical vein endothelial cells) has greatly facilitated the development of in vitro assays for virtually every aspect of the angiogenesis process. These include development of assays for (1) endothelial production of collagenase and plasminogen activator in response to angiogenic factors, (2) endothelial cell migration and proliferation, and (3) formation of capillary networks in vitro (Fan, 1993). These methodologies are instrumental in the identification and purification of a large number of angiogenic factors and inhibitors.
5. Control of Angiogenesis

Under normal circumstances, endothelial cell turnover in a quiescent microvasculature is of the order of thousands of days (Fan, 1993). During spurts of angiogenesis, like in wound healing, the same endothelial cells can proliferate rapidly with a 5-day turnover. Thus, angiogenesis is tightly regulated by the balance of angiogenic inducers and inhibitors. It is important to note that most of these factors have other biological effects and the interactions between the various factors remain to be elucidated. Furthermore, the composition of the extracellular matrix and the activity of endothelial enzymes also play a critical part (Fan, 1993).

In the adult biological system, the regulation is mediated through the microenvironment of venular endothelial cells, which is made up of direct contacts to other cells, extracellular matrix, and the metabolic fluid phase. The functional state of the venular endothelial cell, i.e., activation, invasion-migration, proliferation, tube formation, maturation, switch to the resting state again, is controlled by at least three different regulatory systems (Höckel et al 1993):

a) diffusible substances, e.g., cytokines secreted by adjacent cells in paracrine fashion or released from the extracellular matrix and from lysed cells,
b) direct cell-cell and cell-extracellular matrix interactions involving cell surface and adhesion molecules, matrix and cytoskeleton proteins, and integrins;
c) and proteolytic enzymes either modifying regulatory substances, cell surface molecules, and the extracellular matrix or directly interfering with the endothelial function of invasion-migration.

In addition to the complexity of angiogenesis control, the biological importance of this phenomenon suggests redundancy in the regulatory mechanisms. Therefore, the elucidation of the biological process, in angiogenesis regulation is an extremely difficult task. Nevertheless, considerable progress has been made in identifying substances related to each
of the regulatory mechanisms that stimulate or inhibit angiogenesis in certain model systems (Höckel et al, 1993).

There appears to be an involvement of both mechanical factors (e.g., blood pressure, wall tension, wall stress, increased blood flow, increased hematocrit or stretch of vessels by growing tissue), and metabolic or chemical factors (e.g., cytokines, angiogenic factors) in the initiation and control of angiogenesis (Young and Dyson, 1990a and b).

6. Induction of Angiogenesis

The agents that are capable of inducing angiogenesis and or endothelial cell proliferation are called angiogenic factors and are listed in table 2.5. A large number of these agents exert multiple actions on a wide variety of cells. One exception appears to be the vascular endothelial growth factor (VEGF) and the vascular permeability factor (VPF), which act specifically on endothelial cells (Connoly et al, 1989, Leung et al, 1989). Several angiogenic substances, the majority of which are proteins or peptides, have now been cloned, while others await complete characterisation.

Although the biochemistry and structure of these molecules is well known, little is understood about how they mediate angiogenesis in vivo. According to Jakobsson (1994), several possibilities for angiogenesis mechanisms are apparent, including:

a) secretion of angiogenic factors from recruited macrophages, stroma cells or tumour cells;

b) mobilisation of angiogenic molecules from the extracellular matrix (e.g. bFGF);

c) suppression of pericyte inhibition of endothelial proliferation;

d) decrease in the secretion of an endogenous inhibitor of angiogenesis;

e) amplified production of angiogenic molecules from endothelial cells resulting in an autocrine stimulation.
The four categories of factors considered to have the strongest influence on angiogenesis are (1) growth factors, (2) cytokines, (3) extracellular matrix components, and (4) intercellular interactions. These angiogenic factors may have independent actions, they may initiate a tissue reaction or be cofactors in a complex chain of events (Table 2.5).

**Table 2.5- Angiogenic factors**

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td><strong>Polypeptides</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVPF</td>
<td>yes</td>
<td>yes</td>
<td>Connolly * et al 1989</td>
</tr>
<tr>
<td>PD-ECGF</td>
<td>yes</td>
<td>yes</td>
<td>Miyazono * et al 1987, Miyazono * et al 1991</td>
</tr>
<tr>
<td>Angiotropin</td>
<td>yes</td>
<td>yes</td>
<td>Höckel * et al 1987, Höckel * et al 1988</td>
</tr>
<tr>
<td>bFGF*</td>
<td>yes</td>
<td>yes</td>
<td>Folkman * et al 1988, Klagsbrun 1989, Klagsbrun 1992</td>
</tr>
<tr>
<td>IL-8</td>
<td>yes</td>
<td>yes</td>
<td>Koch * et al 1992, Hu * et al 1993a</td>
</tr>
<tr>
<td>IGF-1</td>
<td>yes</td>
<td>yes</td>
<td>Grant * et al 1993</td>
</tr>
<tr>
<td>TGFα</td>
<td>yes</td>
<td>yes</td>
<td>Folkman 1993</td>
</tr>
<tr>
<td>TGFβ*</td>
<td>no</td>
<td>no</td>
<td>Wahl * et al 1987, Beck * et al 1991, Philips * et al 1993</td>
</tr>
<tr>
<td>TNFα*</td>
<td>yes</td>
<td>no</td>
<td>Fan 1993, Harris 1997</td>
</tr>
<tr>
<td><strong>Lipids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostagl. E₁/E₂*</td>
<td>ND</td>
<td>ND</td>
<td>(Fan 1993)</td>
</tr>
<tr>
<td>Monobutyrin</td>
<td>yes</td>
<td>no</td>
<td>(Fan 1993)</td>
</tr>
<tr>
<td>Erucamide</td>
<td>ND</td>
<td>no</td>
<td>(Fan 1993)</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaluronic acid fragment</td>
<td>yes</td>
<td>yes</td>
<td>(Fan 1993)</td>
</tr>
<tr>
<td>Fibrin*</td>
<td>ND</td>
<td>ND</td>
<td>(Fan 1993)</td>
</tr>
<tr>
<td>Laminin*</td>
<td>ND</td>
<td>ND</td>
<td>(Fan 1993)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>yes</td>
<td>yes</td>
<td>(Fan 1993)</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>no effect</td>
<td>ND</td>
<td>(Fan 1993)</td>
</tr>
</tbody>
</table>

* These factors also promote tube formation (Fan 1993)

ND - Not determined
These substances can act directly or indirectly on the endothelial cells. Some examples of direct-acting factors are the bFGF and the VEGF, which stimulate migration, proliferation and/or tube formation of endothelial cells. Indirect factors can be exemplified by tumour necrosis factor (TNF-α) and TGF-β, which act by mobilising host cells (e.g. mast cells, macrophages and occasionally lymphocytes) to release endothelial cell growth factors.

It has been proposed that tissues that require an angiogenic response elaborate multiple mechanisms to provoke such response. Thus, angiogenic factors are found in various sources, including hypothalamus, synovial fluid, wound fluid, myocardial infarcts, vitreous humour from diabetic retinopathy and tumours. It is conceivable that the profile of angiogenic factors may vary from one angiogenic disease to another (Fan, 1993).

Recent studies have suggested that several low molecular weight vasoactive peptides may also be involved in angiogenesis. For example, both substance P and calcitonin gene-related peptide are mitogenic for endothelial cells. In a rat sponge model, substance P interacts synergistically with interleukin-1 (IL-1) to stimulate neovascularization (Fan, 1993). Bradykinin was also found to interact with IL-1 to produce an intense neovascular response which can be blocked by the B1 receptor antagonist [Leu⁶]des-Arg⁹-bradykinin.

7. Inhibition of Angiogenesis

In the light of such a large number of angiogenesis stimulators, it is striking that neovascularization is such a rare phenomenon. However, many studies suggest that, in addition to factors stimulating angiogenesis, endogenous inhibitors of angiogenesis, like cartilage-derived inhibitor (CDI), are key components of the complex network controlling vascular growth (Moses et al, 1990). It appears that the CDI is closely related to tissue inhibitor of metalloproteinases (TIMP). The avascularity of cartilage may in part be due to the presence of this angiogenic inhibitor.
A large number of anti-angiogenic agents, with a wide spectrum of molecular structures has been identified (Table 2.6). Since angiogenesis is a complex multi-step response, it can be inhibited at many levels. Antiangiogenesis has been practised in clinical medicine for a long time, although based on an empirical rather than on a conceptual basis (Beranek, 1988).

Specific or selective inhibitors relate to agents which only interfere with vascular endothelium or angiogenesis. Non-specific inhibitors may, in addition to their effects on endothelium, inhibit the growth or migration of other cell types or interfere with the immunological system.

The discrete steps in angiogenesis which could be altered pharmacologically in order to inhibit angiogenesis are as follows:

a) Inhibition of the release of angiogenic factors.
b) Blocking of the activity of angiogenic factors released from macrophages, tumour cells or extracellular matrix, e.g. by neutralising antibodies or receptor antibodies.
c) Inhibition of endothelial cell response including proliferation and/or migration.
d) Inhibition of the disintegration of vessel basement membrane and extracellular matrix.
e) Inhibition of sprout formation
f) Inhibition of end to end fusion of sprouts.

The majority of the papers related to the inhibition of angiogenesis are based on studies in which various agents have been examined for their ability to inhibit endothelial cell proliferation, migration and tube formation in vitro. A crucial issue in the search for inhibitors of proliferation is selectivity. Unfortunately, many known endothelial cell inhibitors have diverse effects on many cell systems and many of the reported inhibitors are unlikely to be specific inhibitors of angiogenesis. As shown in table 2.6, a number of agents able to block angiogenesis in vivo have been identified, but numerous others are now under study, and future screening will no doubt reveal new ones.
8. Therapeutic anti-angiogenesis

A tumour is unable to grow above about 1mm³ without development of a new blood supply (Folkman, 1990). Furthermore, the tumour endothelial cells are dividing much more rapidly than normal endothelial cells, up to 50 times as fast in breast cancer.
There is now an increased emphasis on therapeutic strategies aiming to compromise the vascular supply of tumours. The rationale for this is not only that tumours cease to grow in the absence of vascular supply, but also that they will be unable to release cells into the circulation to initiate metastasis. Considerable research has been done in this field over the last two decades, supporting the notion of that the tumour growth and metastasis as well as various non-malignant inflammatory diseases are dependent on angiogenesis. Therefore, the therapeutic use of angiogenesis inhibitors in these conditions is an attractive alternative (Harris, 1997). Proliferating microvascular endothelium presents a unique target for anticancer therapy.

Problems in tumour treatment are for instance the secretion of multiple angiogenic factors by a single tumour or the presence of additional growth factors released from infiltrating macrophages or other inflammatory cells (Bicknell and Harris, 1992). Therefore the use of blocking antibodies has proven ambiguous as such a strategy has a chance of success only if a tumour secretes a single common angiogenic factor. Notable in this context is VEGF which appears to be virtually universally present in breast and brain tumours (Bicknell and Harris, 1992), and blocking VEGF activity has been reported to inhibit tumour growth (Kondo et al, 1993).

9. Therapeutic angiogenesis

The term therapeutic angiogenesis has been suggested for interventions to induce the local growth of blood vessels as a treatment for several important clinical conditions. The goal of therapeutic angiogenesis is the controlled induction or stimulation of new blood vessel formation to reduce unfavourable tissue effects caused by local hypoxia or to enhance tissue repair (Höckel et al, 1993).

Therapeutic angiogenesis can principally be achieved by surgical methods, i.e. transposition of autologous tissues with uncompromised vasculature and high angiogenic potential in close proximity to the site of the
desired neovascularization (e.g., omentum majus, musculofasciocutaneous flaps, vascular pedicles). Experimental and clinical experience demonstrates the initiation of a strong neovascularization reaction providing microvascular connections between the transferred tissue and the recipient bed at day 2 to 3. Arterial occlusive diseases of various tissues including limbs and the brain have been successfully treated by this mode of therapeutic angiogenesis (Hoshino et al., 1983; Pevec et al., 1991).

Therapeutic angiogenesis by the use of autologous tissue transfer is most effective if the flap has an axial macrovascular pattern as well as a healthy microvascularization and releases factors capable of stimulating angiogenesis. These prerequisites are best fulfilled with omentum majus flaps or muscle flaps with one or two dominant pedicles, such as the rectus abdominis muscle flap (Anthony et al., 1991).

The classic surgical ways of therapeutic angiogenesis might be supplemented in the near future by two further strategies made possible by modern biology: (1) the local application of angiogenic factors and (2) the implantation of autologous capillary endothelial cells cultured ex vivo. Although successful in situ tissue formation by use of cultured autologous cells has been achieved with keratinocytes (Green et al., 1979) and urothelial cells (Romagnoli et al., 1990), the feasibility of therapeutic angiogenesis with endothelial cell implants is speculative and needs experimental confirmation. However, considerable experimental data as well as some preliminary clinical data exists to support the usefulness of angiogenic factors for therapeutic angiogenesis.

Several other clinical situations characterised by decreased local vasculature might be treated by therapeutic angiogenesis. Examples are aseptic bone necrosis and disease states caused by vascular occlusion, such as heart, brain, or limb ischemia. By creation of anastomoses between an extracardiac artery and the coronary circulation, the prerequisites for myocardial revascularization with angiogenesis factors in ischemia of the heart have been achieved in a dog model (Unger et al., 1990).
9.1. Therapeutic angiogenesis by use of angiogenic factors

As mentioned earlier, a great number of angiogenic factors has already been identified (Table 2.5). Independently from their potential physiological role and their action in the assay systems, angiogenic factors suitable for therapeutic angiogenesis should fulfil the following criteria: (1) induction or stimulation of controlled neovascularization in adult vascularized tissues; (2) defined tissue reactions associated with angiogenesis, with negligible local and systemic side effects; (3) effective doses in the nanomolar-picomolar range and dose-effect relationship; (4) chemically defined, easy to handle; and (5) large-scale availability (Höckel et al., 1993).

Angiogenesis growth factors-cytokines available by recombinant biotechnology are most appropriate with regard to these prerequisites (Table 2.5). However, the development of therapeutic systems for their defined local delivery is still a problem. Up to now, cream formulations, hydrogels, multilamellar liposomes, and biodegradable sponges made from natural and synthetic polymers have been empirically used (Brown et al., 1989; Langer and Moses, 1991). A promising approach to the controlled delivery of angiogenic factors could be realized with microencapsulation (Langer, 1990).

The pharmacological use of angiogenic cytokines should be of benefit in situations where wound healing is suboptimal, and in the compromised wound, therapeutic angiogenesis may accelerate tissue repair. This may occur due to several underlying medical factors such as diabetes mellitus, connective tissue diseases, chronic venous insufficiency, cachexia, smoking, previous radiotherapy, cytotoxic therapy, contamination and infection, allografts, burns. Compromised angiogenesis is the main reason for delayed healing or non-healing in most of these cases.

Several studies, using cytostatic agents to delay healing, have shown that impaired wound healing is reversed to near-normal levels with angiogenic cytokines (Lawrence et al., 1986; Mooney et al., 1988; Curtsinger et al., 1989; Laato et al., 1989). Based on such results, the first clinical studies employing
angiogenic factors applied locally have been performed on non-healing skin wounds (Knighton et al, 1986; Burgos et al, 1989). The most clinical experience has been gathered with the so-called platelet-derived wound healing formula, an autologous blood product containing platelet-derived growth factors such as platelet factor 4, transforming growth factor β, platelet derived angiogenic factor, and platelet-derived epidermal growth factor (Knighton et al, 1990).

It has been shown that the quality of wound healing (ie, tensile strength, resistance) is associated with early vascularization (Eliseenko et al 1988). Angiogenic factors may therefore be applied as novel adjuvant means in "high-risk-wounds" such as digestive tract and urinary tract anastomoses or in cesarean sections. Höckel et al (1993) showed a significant early increase in uterine bursting pressure compared with controls when tumour necrosis factor α was incorporated into the wound closure.

Folkman et al (1991) showed that an acid-stable form of bFGF administered orally to rats with duodenal ulcer promoted a ninefold increase of angiogenesis in the ulcer bed, and accelerated ulcer healing more than cimetidine. Clinical studies using angiogenesis factors in patients with gastric ulcers have been initiated (tenDijke and Iwata, 1989).

Hard tissue healing and regeneration (eg bone, cartilage, tendon), as well as secondary healing of large soft-tissue defects, produce considerable morbidity owing to the long immobilization and hospitalization. Angiogenesis is tightly linked to cartilage mineralization and bone formation (Winet et al 1990). Nottelbeart et al (1989) showed increased bone repair by means of an omental angiogenic lipid fraction in the rat. Osseous defects, high risk fractures, osteoradionecrosis, bone grafting, and arthrodeses may benefit from local implantation of angiogenic factors (Höckel et al, 1993).

Angiogenic cytokines have been successfully used in vascular prostheses to speed up their endothelial lining (Greisler et al 1987, Clowes and Kohler 1991). Angiogenic factors might also improve the tissue fixation of a variety of artificial supporting or functional items such as slings, patches, joint prostheses, and heart valves, and may reduce the risk of early infection.
9.2. Therapeutic angiogenesis to reduce tissue hypoxia

In non-healing wounds such as ORN, a commonly found problem is a non-stimulatory level of hypoxia resulting from inadequate perfusion. Mild levels of hypoxia may facilitate wound healing by inducing the synthesis of collagen precursors and activating macrophages to stimulate angiogenesis (Hunt and Pai, 1972). However, with higher levels of hypoxia, a reduction in fibroblast migration and lower collagen synthesis with impaired hydroxylation of lysine and proline can be observed (Hunt and Pai, 1972). Fibroblasts synthesise an intracellular peptide collagen precursor, but fail to release it. Maturation and cross-linking of collagen is inversely proportional to the degree of hypoxia, and is proportional to a small increase in oxygen concentration.

Reconstructive surgery presents promising indications for the application of angiogenic cytokines. All autotransplantations using either free or pedicled flaps are necessarily associated with a reduction of the functional vasculature, which causes anatomical restrictions and the constant threat of flap necrosis (Myers, 1986). In animal experiments, Höckel and Burke (1989) were able to prevent skin flap necrosis by treating the donor and receptor sites with angiotropin before grafting. Since the revascularization process is critical for the survival and subsequent function of free autologous tissue grafts (eg skin, muscle, bone transplants), these surgical procedures may also benefit from therapeutic angiogenesis.

Eppley et al (1991) showed in a rabbit study that free bone grafting to an irradiated bed was significantly more successful if the recipient sites were pretreated with basic fibroblast growth factor (bFGF) before placement of the graft. This angiogenic factor has also been used to revascularize nerve transplants in rats (Penkert et al, 1988) and for the promotion of cerebral angiogenesis in a rat model of mild chronic forebrain ischemia (Lyons et al, 1991).

Another potential application area for the therapeutic angiogenesis is the treatment of problems related to local hypovascularity as a consequence of radiotherapy. Penhallington et al (1988) demonstrated that the so-called tumour
bed effect (reduction of tumour growth because of reduced angiogenic potential induced by radiotherapy) could be reversed by a second tumour cotransplanted on the tumour bed that secreted an angiogenic factor.

The requirement for oxygen in wound healing is the rationale for hyperbaric oxygen (HBO) therapy, which also may be used to stimulate angiogenesis. It results in an increase in tissue oxygen tension and improves collagen synthesis, angiogenesis, epithelization and resistance to bacteria in problems wounds. Since these processes are closely related to tissue oxygen tension, relieving wound hypoxia with HBO therapy accelerates wound healing and angiogenesis by increasing oxygen tension (Davis, 1989).

From these observations, it can be deduced that exogenous angiogenic substances may also help to overcome radiation-induced tissue effects based on impaired angiogenesis such as ulcers, fistulas and osteoradionecrosis. As will be shown later in this thesis, ultrasound may be one way of stimulating cells to release these angiogenic factors, and therefore it can be considered as a tool to deliver therapeutic angiogenesis (Reher et al, 1998c and d).
Section III - Ultrasound

This section will give an overview of the current literature related to ultrasound, and more specifically, therapeutic ultrasound. It has been divided into three main sections:

1. Physical characteristics of ultrasound
2. Biophysical mechanisms of ultrasound, and
3. Biological effects of therapeutic ultrasound

1. Physical characteristics of ultrasound

This is a relatively complex subject, but in order to understand the following sections, a brief analysis of the physics behind ultrasound seems appropriate. This section will address the following subjects: (1) acoustic waves, (2) ultrasonic transducers, (3) properties of the ultrasound beam, (4) ultrasonic dosimetry, and (5) attenuation of ultrasound.

1.1. Acoustic waves (Mechanical vibrations)

Ultrasonic energy travels through a medium in the form of a wave. Mechanical or acoustic waves arise when particles, composing a medium are supplied with energy and are driven to vibrate. These particles then transfer some of this energy to adjacent particles causing them to vibrate and so pass on the energy in the form of a wave. Mechanical vibrations can be audible, ultrasound or infrasound, and all have similar physical properties, independently if they are propagating through solids, liquids or gases.

The wave properties of sound can be compared to the pattern of circular ripples produced when a water surface is touched. These expanding ripples consist of a series of alternating peaks and troughs which have the same pattern at any one point in time (Fig. 2.2). A cross section of this pattern can be
used to better describe its components (Fig. 2.3). *Wavelength* ($\lambda$) is defined as the distance from any measurable point to the next similar point after it has completed one full cycle of positive and negative displacement. *Amplitude of wave* ($A$) is the maximum excursion of the wave above (or below) its original undisturbed value. Amplitude is a measure of the power of a wave.

**Figure 2.2** - Graphical representation of the expanding series of waves propagating from a point source (S). The solid circles represent the wave peaks while the broken circles represent the wave troughs (after Hart, 1993).

**Figure 2.3** - Sectional view along the RS line of the wave pattern showed in figure 2.2 (after Hart, 1993).
If one imagines a float located at any point on figure 2.2, while the wave is propagating, it will be lifted during the peak and dropped during the trough relative to its original position, but remain in essentially the same position relative to the source of the wave. The displacement of the float from its original position is displayed as a function of time (Fig. 2.4). The regular movement of a body, as described for this float, about a mean point is an example of simple harmonic motion.

![Diagram](image)

**Figure 2.4** - Diagrammatic representation of the displacement of a particle in the path of an ultrasonic wave as a function of time (after Hart, 1993).

**Physical properties of mechanical waves**

As shown previously, waves flow away from their source. The velocity of wave propagation is dependent on the efficiency of energy transfer from a given vibrating particle to its neighbour. Therefore, energy transfer is highly dependent upon the physical properties of the conducting media. These physical properties include the size and number of particles present per unit volume (density, \( \rho \)) and the magnitude of the complex inter-molecular interactions of the conducting medium (Table 2.7). A decrease in density facilitates more rapid propagation of sound waves. If a liquid is heated, it will
expand, decreasing its density, and therefore the velocity of ultrasound will be higher (Table 2.7).

Table 2.7 - Velocity and characteristic impedance of sound in biological and other materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Velocity (m/sec)</th>
<th>Density (g/ml)</th>
<th>Acoustic impedance (Kg m⁻² sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>333.5</td>
<td>1.29 x 10⁻³</td>
<td>429</td>
</tr>
<tr>
<td>Water (20°C)</td>
<td>1480</td>
<td>1.00</td>
<td>1.52 x 10⁶</td>
</tr>
<tr>
<td>Water (37°C)</td>
<td>1520</td>
<td>1.00</td>
<td>1.52 x 10⁶</td>
</tr>
<tr>
<td>Blood</td>
<td>1555</td>
<td>1.06</td>
<td>1.62 x 10⁶</td>
</tr>
<tr>
<td>Fat</td>
<td>1460-1470</td>
<td>0.92</td>
<td>1.35 x 10⁶</td>
</tr>
<tr>
<td>Muscle</td>
<td>1545-1630</td>
<td>1.07</td>
<td>1.65-1.74 x 10⁶</td>
</tr>
<tr>
<td>Kidney</td>
<td>1560</td>
<td>1.04</td>
<td>1.62 x 10⁶</td>
</tr>
<tr>
<td>Liver</td>
<td>1540-1585</td>
<td>1.06</td>
<td>1.63-1.68 x 10⁶</td>
</tr>
<tr>
<td>Bone</td>
<td>2710-4000</td>
<td>1.38-1.81</td>
<td>3.75-7.38 x 10⁶</td>
</tr>
</tbody>
</table>

Adapted from Wells, 1977

Frequency (f) of a source is the number of wavelengths which pass any given point per unit time (Hz). Once generated, this frequency remains constant irrespective of the propagating medium. The particles which propagate this wave also vibrate with this frequency.

Velocity (v), frequency (f) and wavelength (λ) are interrelated as described by the equation below. Since the frequency remains constant, but the velocity changes as the wave travels from one medium to another, it follows from this equation that the wavelength must also change.

\[
v = \frac{f}{\lambda}
\]

When observing the pattern of expanding ripples on a pond it can be seen that as the wave moves further away from the source its amplitude progressively decreases. The perimeter of each circular wave gradually increases and so the initial input of energy has to be spread over an ever increasing area. Absorption is another contributing factor to the loss of energy content from the wave. The transfer of energy between particles is never 100% efficient and so some energy is lost at each transfer; this energy appears as heat which elevates the temperature of the conducting medium.
Once a wave travels away from the source, its perimeter increases until any small portion of the wave front appears flat. This is called a plane progressive wave.

Interference is a fundamental property of all waves. If any two or more waves arrive together at the same point in space, they will merge to form a new wave which is the algebraic sum of the amplitudes of each individual wave. If two waves of the same frequency, amplitude and phase, interfere, a wave of twice the original amplitude, but of the same frequency results. If two similar waves which are 180° out of phase interfere, their amplitudes cancel each other out.

Transverse and compressional waves

Transverse waves are waves in which the particles oscillate in the vertical plane, as the wave propagates in the horizontal plane. These are usually restricted to solids (Fig 2.5A). In compressional (longitudinal) waves, the direction of particle vibration is the same as the direction of wave propagation (Fig 2.5B). These include both audible sound and ultrasound. In any given solid medium transverse or shear waves travel much more slowly than compressional waves. In general, the velocity of transverse waves in a given medium is half the velocity of compressional waves in that same medium.

Under certain circumstances some of the energy of a compressional wave can be transformed into transverse waves, this phenomenon is termed mode conversion. It is thought to occur when ultrasound is obliquely incident upon a strong reflector such as soft tissue-hard tissue interfaces. As transverse waves do not propagate well in aqueous media they are readily absorbed by the soft tissue and their energy appears as heat. It has been suggested that the pain observed when the hand is placed in the path of moderately high intensity ultrasound is due to temperature rises within the periosteum, due to the absorption of transverse waves.
**Chapter 2 - Literature Review - Section III - Ultrasound**

**Figure 2.5** - Graphical illustration of the direction of particles of a medium relative to the direction of wave propagation for transverse and compressional waves (After Hart, 1993).

Reflection and refraction

Every time a wave encounters a boundary between two different media, both reflection and refraction of that wave occurs. The reflection and refraction of a plane wave front is shown in figure 2.6. The refracted wave is deflected because the velocity of the wave in medium 2 is different to that in medium 1.

The angle of incidence ($i$) can be seen to equal angle of reflection ($r$), whereas the angle of incidence and the angle of refraction ($r'$) are related by Snell's Law:

$$\frac{\sin i}{\sin r'} = \frac{v_1}{v_2}$$

$v_1$ = velocity of the incident wave in medium 1
$v_2$ = velocity of the refracted wave in medium 2

If the boundary between two media is convex, the refracted wave is concentrated or focused some distance into the second medium. If the boundary is concave, the refracted wave is divergent and the reflected wave is focused.
Figure 2.6 - Schematic illustration of reflection and refraction occurring at the interface between two media (i, r, r’ represent the angles of incidence, reflection and refraction respectively).

**Acoustic impedance**

Acoustic impedance is the parameter that defines how much of the incident wave is reflected or refracted at any given interface between two media. Acoustic impedance (Z) is defined as the product of the density of a material (\( \rho \)) and the velocity (\( v \)) of sound within it:

\[
Z = \rho v
\]

If two media have the same acoustic impedance, there will be no reflection at the interface. However, if they differ, some of the incident wave will be reflected, and some will be refracted. The angle of incidence of the wave complicates further these observations. Table 2.7 gives acoustic impedance values for some common biological and other materials. For normal incidence the equations which relate to this partitioning of incident energy (\( I_i \)) into the reflected (\( I_r \)) or transmitted (\( I_t \)) portions are as follows:
Standing waves

Up to now, consideration has only been given to acoustic waves moving in a single direction. A standing wave is formed when a plane wave of normal incidence is reflected back along its own path, interfering with that portion of itself which has not yet reached the reflector. When waves reflect, they undergo a half wavelength phase change.

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Standing wave fields, demonstrating constructive interference can be described as repeating pattern of points where the displacement of particles is always zero - termed *displacement nodes* (N), and points where the
displacement varies from positive to negative at the same frequency as the incident wave, but at twice its amplitude - termed displacement antinodes (A) (Fig 2.7). Nodes and antinodes are separated by a distance of a quarter of a wavelength. The standing wave phenomenon concentrates and effectively amplifies the ultrasonic energy applied to a system, which may lead to potentially hazardous ultrasonic exposure of biological tissues (Williams, 1983). Partial standing waves occur when a reflector is not 100% efficient. In this case the maximum displacements or pressures would be less than twice that of the incident wave.

Resonance

This phenomenon occurs when the input of energy to a system is in phase with the natural frequency of oscillation of that system. If energy is supplied in phase then the amplitude of the oscillating system is maintained or increased. This is used by the manufacturers, so that the device used to generate or receive a single frequency of mechanical vibrations is in resonance at that frequency.

Power density (intensity)

Intensity is defined as the rate of flow of energy through an imaginary plane, one centimetre or one metre squared, orientated at right angles to the direction of wave motion. The units used for intensity are usually Watts/cm², or milliWatts/cm². The intensity (I) of a plane progressive wave is related to the maximum pressure amplitude (Po) produced within the conducting medium by the following equations:

\[
I = \frac{Po^2}{2pc} \\
I = \frac{PoUo}{2} \\
I = \frac{\rho cUo^2}{2}
\]

\( I = \text{intensity} \)  
\( Po = \text{Maximum pressure amplitude} \)  
\( \rho = \text{density of the medium} \)  
\( c = \text{velocity of the wave} \)  
\( \rho c = \text{acoustic impedance} \)  
\( Uo = \text{maximum velocity of the oscillating particles of the medium as the wave passes} \)
Mechanical waves of different frequencies - infrasound, audible sound, ultrasound

The spectrum of mechanical waves can be divided according to different frequencies into three categories. Infrasound is the region below 20 Hz, which cause vibrations, and common sources are atmospheric disturbance and transportation vehicles. The audible sound region ranges from 20-30Hz (low notes, vibration) up to about 16000 Hz (high notes). Ultrasound is the region above 16000 Hz (16 kHz).

Air becomes a progressively less efficient propagating medium for mechanical vibration as its frequency increases above 20 kHz. The main reason for this is that for a given power input, the maximum displacement amplitude of the gas molecules decreases with increasing frequency until the amplitude of displacement is less than the average distance between molecules. Thus most of the gas molecules close to the source of vibration will be unable to collide with neighbouring molecules and thus pass on energy and facilitate wave propagation.

The inertia of particles which make a medium is important since the propagation of mechanical waves depend upon the transmission of energy from one particle to another. The inertia of a body is a measure of its tendency to resist motion when subjected to a small transient force. For a given power input the displacement amplitude decreases with increasing frequency. Eventually the particle is unable to respond as the applied force is not sufficient to overcome its inertia, remaining stationary, and therefore the wave does not propagate.

1.2. Ultrasonic transducers

So far we described the general properties of acoustic waves (mechanical vibrations). The logical progression would be to describe the patterns of acoustic waves propagated from a specific source, the so called acoustic fields, or ultrasound beam. However, before that it is necessary to describe how these
acoustic fields are generated, the so called ultrasound transducers. A transducer is a device that converts energy from one form to another, and in the case of ultrasonic transducers, the conversion is from electrical to mechanical energy, and this is performed with a piezoelectric element.

**Piezoelectric materials**

Piezoelectric transducers rely on the capacity of certain crystals to generate an electric charge when they are subjected to pressure or distortion. This so called piezoelectric effect was first described by Pierre and Jacques Curie in 1880 (Mortimer, 1982). The converse effect is true, electric charges induce mechanical deformation to piezoelectric materials, and this is the principle used for the generation of ultrasound, the so called reverse piezoelectric effect.

The application of an a.c. voltage across a crystal results in an oscillating change in shape. If this a.c. voltage has an ultrasonic frequency, it would result in the crystal oscillating at the same frequency. The phenomenon of resonance is applied to maximise output, as the amplitudes of oscillation of the piezoelectric crystals are very small. To manufacture a transducer which is resonant at a particular frequency, the wavelength is first calculated from the velocity of sound in that material and the desired frequency. The crystal is then cut to half (or a multiple of half) of the calculated wavelength in thickness. The frequency of the electrical signal is then swept around the expected resonant frequency until the crystal oscillates with maximum displacement amplitude for any given input power. This resonant frequency is called the fundamental resonant frequency of the transducer.

Initially, quartz crystals were used for the generation of therapeutic ultrasound by the reverse piezoelectric effect. Quartz has now generally been replaced by barium titanate and then by lead zirconate titanate (PZT). The last two materials have the advantage that, because of their ferro-electric properties, only a small voltage is required to drive the crystal to oscillate. PZT is preferable to barium titanate because it retains its marked piezoelectric properties up to much higher temperatures thresholds, and is less sensitive to
mechanical shock (Hoogland, 1986). Polyvinylidene difluoride (PVDF), a plastic with piezoelectric properties has been introduced more recently.

**Transducer construction**

The transducer is constructed using a single circular piezoelectric disc, the dimensions of which favour resonance at the particular frequency of interest. The resonant frequency of the crystal is partly determined by the thickness of the piezoelectric material and consequently the frequency of the ultrasound is so determined as well. The piezoelectric element has electrodes on the inner and outer surfaces (relative to treatment faces). The inner face is air backed to encourage the reflection of the generated ultrasound, in this way this reflected energy leaves the applicator head by the outer face of the transducer. The outer face of the transducer is bonded to the applicator casing with an adhesive of similar impedance to the piezoelectric disc, which also functions as an electric insulator. The piezoelectric element also vibrates laterally, and insulation is needed in this area to avoid operator exposure hazards. The front face of the transducer head is usually metallic, but some manufacturers use plastic (Fig. 2.8).

![Diagram of transducer construction](image)

**Figure 2.8** - Schematic diagram of the construction of a therapeutic ultrasound transducer probe.
Continuous or pulsed ultrasound

Most ultrasound equipment can generate both continuous and pulsed ultrasound energy. Pulsed ultrasound has the advantage that thermal sensations are suppressed. In addition, this mode permits a higher intensity, which for application of continuous ultrasound can cause undesirable effects (Hoogland, 1986). Nearly all ultrasound instruments have a fixed pulse repetition frequency of 100 Hz. The pulsed mode is set with a cycle for pulse duration and pulse repetition period (mark:space ratio), usually 1:2, 1:4 and 1:9. As an example, this means that in an 1:4 mark:space ratio, the pulse time will be 2 milliseconds (ms), the pulse pause 8 ms, resulting in a pulse repetition period of 10 ms.

The effective radiating area (ERA)

The effective radiating area of the treatment area is an important parameter determining the intensity. Because the piezoelectric element does not vibrate uniformly, the ERA is always smaller than the geometric area of the treatment head. To permit a true indication of the intensity of the instrument, determination of the ERA is essential.

1.3. Properties of the ultrasound beam

Therapeutic ultrasound apparatus usually have an applicator head of 10 to 25 mm in diameter. However, the wavelength of 1 MHz ultrasound through water or body tissues, is approximately 1.5 mm, which is 10 times smaller. Under these conditions the sonic energy does not spread as a spherical wave, rather, it is contained within a cylindrical beam of approximately the same diameter as the transducer, and travels in the form of a plane progressive wave.

Near field and far field

The intensity of that wave is not the same at all points within the beam, because individual wave fronts from different parts of the source have to travel different distances to reach a given point. Therefore the different wave fronts
interfere in a pattern, called a diffraction pattern, that can be divided into two regions. **Near field** (or Fresnel zone) is the region close to the transducer, where the wavefronts are plane surfaces and the amplitude varies considerably. **Far field** (or Fraunhofer zone) is the other zone, far away from the source, where radiation is made up of spherical wavefronts (Mortimer, 1982) (Fig 2.9).

The length of the near field depends on the diameter of the treatment head and the wavelength. With a 1 MHz machine, and a treatment head of 5 cm\(^2\), the near field is about 10 cm long, and with a head of 1 cm\(^2\) the near field is about 2 cm long. At 3 MHz the near field is 3 times as long, because the wavelength is proportionally shorter. Because the depth effect of ultrasound is limited, the therapeutic effects occur mainly in the near field.

![Diagram of the near field (Fresnel zone) and the far field (Fraunhofer zone) portions of the ultrasonic beam produced by ultrasound transducers (after Hart, 1993)](image)

\[ d = \text{distance from source after which beam diverges} \]
\[ \theta = \text{angle of divergence} \]

**Figure 2.9** - Diagram of the near field (Fresnel zone) and the far field (Fraunhofer zone) portions of the ultrasonic beam produced by ultrasound transducers (after Hart, 1993)

If a pressure detector is used in front of the ultrasound beam (Fig 2.10), there are pressure peaks caused by constructive interference (A), and troughs caused by destructive interference (B). Complete destructive or constructive interference is almost impossible to achieve, so the maximum and minimum pressure never reaches zero. In the near field these peaks and troughs are
close together, and in the far field they are more sparse. The area around the transition between near and far field, is usually the most uniform high intensity region within the ultrasonic beam (Fig. 2.10C).

By analysing the variation in pressure transversely (Fig. 2.10-III), one observes a central peak and two or more side lobes separated by troughs, (A). On the same figure, in (B), the same pattern is observed, but with a trough in the central part, and decreasing energy laterally, until only a single axial peak is found in the scan between the near and far field (C). Beyond this point the far field is entered and the height of the peak decreases as its base broadens with divergence of the beam (D). Sections through this two dimensional map show so called "ring patterns", which can be obtained if some material sensitive to some parameter of the acoustic field is placed in a plane parallel to the transducer face (Fig. 2.10-IV).

Due to the non-homogeneity of the ultrasonic field generated when using therapeutic ultrasound, it is common practice to move the transducer head continually during treatment. This will allow that the area receives a time-averaged dose which is a function of the amount of ultrasonic energy emitted by the transducer and the duration of treatment.

**Divergence of the ultrasound beam**

Divergence occurs only in the far field. If the rays which depict the diverging waves are extrapolated back to their source, they meet at the centre of a circular generator (Fig. 2.9). The angle of divergence, at the point d the cylindrical beam begins to diverge, $\theta$, of the beam in the far field is given by:

$$\theta = \frac{0.61a^2\lambda}{\lambda}$$

where $\lambda$ = wavelength of sound in that medium

and $a$ = radius of a circular source

It has been said earlier that the near field is shorter for a small treatment head, so that divergence occurs earlier and the ultrasound energy is spread over a large area. It will be clear that divergence of the ultrasound beam is markedly less at 3 MHz (Table 2.8) (Fig 2.11).
Figure 2.10 - Diagrammatic representation of the distribution of acoustic pressure within an ultrasound beam: (I) axial intensity profile, (II) acoustic pressure along the central axis, (III) acoustic pressure across the central axis, and (IV) ‘ring pattern’ form (after Stewart, 1982 and Hart, 1993).
1.4. Ultrasonic dosimetry

In order to attempt to reproduce experimental bioeffects, in both experimental and clinical areas, it is important to use clear and unequivocal information regarding exposure conditions necessary to observe a given bioeffect. The intensity of an ultrasonic device is usually obtained by measuring the total amount of radiated energy by one technique, and dividing this by the effective radiating area (ERA) of the beam, obtained by some other technique. This will give the spatially averaged intensity \( I^A \), normally quoted in W/cm\(^2\) or mW/cm\(^2\), over the hole beam. However some authors prefer to measure the most extreme 'hot spot' intensity experienced by a biological target within the beam, defined as the spatial peak intensity \( I^{SP} \). The spatial peak intensity in the near field is usually located in the centre of the beam. Due to the highly variable pressure distribution within the near field (used in this thesis), the spatial peak intensity \( I^{SP} \), within a lateral cross section of an ultrasonic beam, may be several times the spatial average intensity \( I^A \) at the same time.
Therapeutic ultrasound can be used in pulsed mode, usually 1:4, meaning that the ultrasound is being generated only for 2 milliseconds usually, with an 8 ms interval when the transducer is not emitting. In this case, there are several ways to describe the ultrasound intensity. The most commonly used method of quantifying the output of pulsed ultrasound is by averaging the intensity over time (including 'off' time) and effective radiating area, and this is known as *spatial average temporal averaged intensity* ($I_{SATA}$). The second most used is to quote the temporally averaged intensity at the most intense hot spot of the ultrasonic field, known as *spatial peak temporal average intensity* ($I_{SPTA}$). The remaining two ways of quantifying intensity in the pulsed mode are calculated from the two previous, and take into account transducer 'on' time only. These give values of the average and peak intensities within each pulse and are known as *spatial average temporal peak intensity* ($I_{SATP}$) and *spatial peak temporal peak intensity* ($I_{SPTP}$). The term temporal peak is often replaced with *pulsed average* ($I_{PA}$) when ultrasound is delivered as long (ms) pulses and thus become $I_{SAPA}$ and $I_{SPPA}$ respectively (Fig. 2.12).

To clarify, an example will be shown: A transducer driven in continuous mode, with a spatial average intensity ($I_{SA}$) of 1.0 W/cm² will have a spatial peak intensity ($I_{SP}$) of 2 to 6 W/cm² (say 4 W/cm²). If the machine is now switched to pulsed mode (1:4), the following intensities can be calculated:

\[
\begin{align*}
I_{SATA} &= 0.2 \text{ W/cm}^2 \\
I_{SPTA} &= 0.8 \text{ W/cm}^2 \\
I_{SAPA} &= 1 \text{ W/cm}^2 \\
I_{SPPA} &= 4 \text{ W/cm}^2
\end{align*}
\]

Measurements of intensity help to characterise the ultrasonic field, but the following parameters should also be quoted when any biological effect of ultrasound is being examined: intensity distribution across the beam (lateral beam plots), frequency, mode of exposure (continuous or pulsed), modulation envelope or *mark:space ratio* (1:2, 1:4, 1:9), exposure duration, number of exposures given and time between exposures. If possible, the amount of heat generated should also be stated or quoted using literature references.
Chapter 2 - Literature Review - Section III - Ultrasound

Figure 2.12 - Output power vs time for continuous and pulsed ultrasound
(adapted from Stewart, 1982)

Ultrasound detection and calibration

Each ultrasound machine must be calibrated on a regular basis, ideally once a week. It is very important to note that the reading on the machine power output meter is not an accurate guide as to what is actually emanating out of the treatment head. The machine must be calibrated against a dedicated calibration device, which can be (1) radiation force methods, (2) piezoelectric hydrophone methods, (3) calorimetric (absorption) methods, and (4) optical methods.

The measurement of radiation force is the most commonly used technique for measuring total power produced by a transducer, and uses radiation force balances. Hydrophones have a small piezoelectric ultrasound transducer used as a receiving element, and are used to investigate relative intensity distribution within ultrasonic fields, as for beam plots. Calorimetric methods use the capacity of ultrasound to generate heat, by monitoring the
temperature increase by an encapsulated calorimeter in a known medium. Optical methods use light beams to detect the deflection caused by the change in density of a medium induced by ultrasound pressure. Wells (1977) gives a more detailed description of these ultrasound calibration and detection methods.

1.5. Attenuation of ultrasound

The intensity of a wave of ultrasound travelling through a medium is found to decrease as a function of distance. The following mechanisms can be involved in this attenuation: beam divergence, scattering, absorption, diffraction and reflection (Wells, 1977). Scattering and absorption are thought to be the most important mechanisms of ultrasound attenuation.

*Beam divergence*

Beam divergence refers to deviation from a parallel beam so that the energy per unit area is reduced, and its significance is greater at lower frequencies.

*Scattering*

Scattering is the term given to the random reflection of incident ultrasound by small (relative to wavelength) obstacles placed in its path. If the size of the reflector is large relative to the wavelength of sound and the beam dimensions, it is reflected as a plane wave. If the dimensions of the reflector are smaller, it acts as a point source and radiates a spherical wave (Fig. 2.13). Due to the different composition of biological materials, they contain a range of surfaces whose dimensions vary from reflectors generating plane waves through to cell nuclei, which are approximately 1/1000 of the wave length of 1MHz ultrasound. Therefore, these small reflectors scatter or re-radiate the ultrasonic energy in all directions, the wave no longer moves in the original direction, and attenuation occurs. It also produces along the original direction of travel of the wave, the so-called back-scattered waves.
Figure 2.13 - Diagrammatic representation of the scattering patterns produced by small particles acting as reflectors in the path of an acoustic beam (after Hart, 1993).

Absorption

Absorption is a form of ultrasound attenuation that occurs when the ordered vibrational energy of the wave is converted into other forms of energy such as heat. It occurs due to two mechanisms, viscous loss and relaxation process. The mechanism of absorption based on viscous loss relies on the fact that the viscosity of the medium tends to oppose the vibrational motion of the particles, and some of this energy is converted into heat. At frequencies used in biomedical applications, viscous loss does not account for much ultrasonic absorption (Wells, 1977).

The other mechanism of absorption is called relaxation. Energy can exist in a system in various forms, such as molecular vibrational energy, lattice vibrational energy, and so on. During the compressive part of the ultrasonic wave, one or more of these energies can increase. On the decompressional phase of the cycle, the majority of the energy will return in form of vibrational energy. Relaxation occurs because the energy which had flowed to one or more of the other energy forms takes a characteristic period of time to return as vibrational energy, thus some energy is returned out of phase with the original propagating wave, resulting in absorption. Relaxational absorption increases
with frequency, up to a maximum value when the shared energy is anti-phase; above this frequency the energy falls because there is less time available for the energy to flow from one form to another. Relaxation processes are generally the most important contributors to ultrasonic absorption in biological tissues, and the maximum relaxation occurs at frequencies around 2 to 5 MHz (Wells, 1969).

2. Biophysical mechanisms of ultrasound

In this section, we shall consider the question as to why ultrasound cause bioeffects. The mechanisms by which ultrasound interacts with biological tissues can be divided into thermal and non-thermal effects.

Frequently, the insonation of a biological system with ultrasound is accompanied by temperature elevation. If this effect can be produced by the same temperature elevation, without sound, it is said to have occurred by a thermal mechanism. However ultrasound can have other mechanisms of action, usually referred as non-thermal effects. For example, ultrasound can create mechanical stress in biological fluids and structures. If these effects are the same as those that result from application of the same stress, without sound, the mechanism of action, is a stress mechanism. Another example of non-thermal effect is cavitation. This is a loosely defined term that is used to represent the activity in a sound field, of gas-filled bodies or bubbles (or "cavities") that contain varying amounts of vapour or gas. In the presence of cavitation, biologically significant temperature elevation, chemical reactivity, and/or mechanical stress may occur. Therefore, cavitation is often regarded as a separate mechanism, although it is clearly not independent from the other (thermal and non-thermal) effects (Nyborg, 1982).

2.1. Thermal effects

When ultrasound travels through tissues a percentage of its energy is absorbed, leading to the generation of heat within that tissue. The amount of
absorption and the amount of heat generated is dependent upon: (a) absorption characteristics of the tissue, (b) the degree of vascularization, (c) the frequency of the ultrasound, and (d) the intensity of the beam.

Absorption characteristics of the tissue

Tissues with high absorption coefficients absorb more ultrasonic energy, and more heat is generated. Tissues with a high protein or mineral content absorb more readily than those with a higher fat content (Table 2.9). Controlled heating can produce desirable effects (Lehman and deLateur, 1982) which include pain relief, decrease in joint stiffness, increased blood flow, etc.

The advantage of using ultrasound to deliver this heating effect is that the therapist has control over the depth at which the heating occurs. To do this the therapist has to know the half-value depth (i.e. the depth of penetration of the ultrasound energy at which its intensity has decreased by a half) of the tissue/media (Table 2.9). For example, the half value depth for soft, irregular connective tissue is approximately 4mm at 3 MHz, but about 11mm at 1 MHz. Structures with high absorption coefficients such as superficial cortical bone, joint menisci, fibrotic muscle, tendon sheaths and major nerve roots are preferentially heated (Lehman and Guy, 1972). Ultrasound will easily pass through fat and selectively heat the underlying muscular tissues (ter Haar and Hopewell, 1982).

For bone, which has an even higher absorption coefficient than muscle, the rate of heating is even higher, explaining the benefit of ultrasound in heating joints, such as for epicondylitis. Because of the great acoustic impedance difference between bone and soft tissues, there will be a reflection of about 30% of the ultrasonic wave, causing additional heat on the return journey. Additional to reflection, mode conversion is also thought to occur. This means that part of the wave is converted into a transverse wave, that is not well conducted by soft tissues; and therefore rapidly absorbed and converted into heat. If the heat generated close to the periosteum exceeds a limit value, it will cause pain.
**Table 2.9 - The half value depth for 1 and 3 MHz ultrasound in various media**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Half value depth (mm)</th>
<th>Penetration depth (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 MHz</td>
<td>3 MHz</td>
</tr>
<tr>
<td>Water</td>
<td>11500.0</td>
<td>3833.3</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>50.0</td>
<td>16.5</td>
</tr>
<tr>
<td>Skeletal muscle (parallel to beam)</td>
<td>24.6</td>
<td>8.0</td>
</tr>
<tr>
<td>Skin</td>
<td>11.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Skeletal muscle (perpendicular to beam)</td>
<td>9.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Tendon</td>
<td>6.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Cartilage</td>
<td>6.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Air</td>
<td>2.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Bone</td>
<td>2.1</td>
<td>---</td>
</tr>
</tbody>
</table>

(after Hoogland, 1986)

**Degree of vascularization**

Once delivered, the heat is then dissipated by both thermal diffusion and local blood flow. As soon as a temperature rise is produced, it initiates in most tissues a physiological response of dilatation of blood vessels, and increased blood flow (Williams, 1983). Thermal damage caused by overheating is uncommon, but can occur in tissues with impaired vascularity, at intensities usually unable to produce these effects in normally vascularized tissue (Dyson, 1990).

**Frequency of ultrasound**

As noted in table 2.9, the higher the frequency, the more superficial the depth of penetration, leading to rapid attenuation, which can be translated as heat generation. Thus, high frequency ultrasound interacts with tissues probably more due to thermal effects, whereas low frequency ultrasound interacts usually more via non-thermal effects. Thus by controlling the frequency of the ultrasound applied, with the knowledge of the composition of the tissue under treatment, the therapist has some control over the depth at which heating occurs. The therapist, can therefore use 3 MHz to deliver heat to superficial areas as skin, 1 MHz for deeper muscular injuries, and 45 kHz ('long wave' ultrasound) for even deeper areas.
**Intensity of ultrasound**

By using the same tissue and same frequency of ultrasound, the amount of heat will increase with increasing the ultrasound intensity. It has been stated that the temperature rise caused by a given time-averaged intensity, applied for a given amount of time, will be the same regardless of whether ultrasound is applied in pulsed or continuous mode (Williams, 1990 in Hart, 1993).

The thermal mechanism is thought to be the major mechanism by which ultrasound exerts its therapeutic effects in the treatment of musculo-skeletal disorders (Summer and Patrick, 1964). Thermal effects usually occur when using higher intensities, between 0.5 and 3.0 W/cm². Increase in the extensibility of collagenous tissue, blood flow, pain relief, resolution of inflammation, decrease in muscle spasm and joint stiffness have all been observed in tissues maintained for between 5 to 30 minutes at temperatures between 40-45°C (Lehman and Délateur, 1982). More detailed reviews of biological effects of ultrasound due to thermal effects have been compiled by Wells (1977), Fry (1979), Nyborg (1982) and Williams (1983).

**2.2. Non-thermal effects**

As shown previously, thermal mechanisms are used in the treatment of musculo-skeletal disorders at higher intensities. However, this mechanism is thought to have a lesser degree in the stimulation of tissue repair, where therapeutic effects have been achieved using lower intensities (0.1 to 0.2 W/cm²), without significant elevation in temperature (Dyson et al, 1968). There are several other situations in which ultrasound produces bioeffects without the involvement of significant temperature increases. Such bioeffects can be demonstrated when the ultrasound intensity is low, or the system is cooled, so that little or no temperature rise occurs.

The non-thermal mechanisms are thought to play a primary role in producing several bioeffects, including: stimulation of tissue regeneration (Dyson et al, 1968, 1976; Dyson, 1990; Paul et al, 1960); blood flow in
chronically ischaemic muscles (Hogan et al, 1982); protein synthesis in fibroblasts (Harvey et al, 1975; Webster et al, 1978); healing of ischaemic varicose ulcers (Dyson et al, 1976); tendon repair (Enwemaka, 1990), angiogenesis in full thickness excised incisions in the flank skin of adults rats (Young and Dyson 1990), acceleration of fracture repair (Dyson and Brookes, 1983; Xavier and Duarte, 1983; Pilla et al, 1990; Tsai et al, 1992; Wang et al, 1994; Yang et al, 1996) and treatment of osteoradionecrosis (Harris, 1992).

The physical mechanisms thought to be involved in producing these non-thermal effects are one or more of the following: (b) acoustic cavitation, (b) acoustic microstreaming, and (c) radiation force.

**Acoustic cavitation**

Ultrasound can cause the formation of micron size bubbles or cavities in gas-containing fluids. Acoustic cavitation is the term applied to describe the oscillatory activity of gas or vapour-filled bubbles which are powered by and thus extract energy from the incident acoustic field (Williams, 1983). These bubbles grow by a process termed rectified diffusion, from tiny inhomogeneities within a conducting media, called micronuclei, under the influence of ultrasonic fields.

It has been suggested that some form of cavitation mechanism is involved in most if not all of the reported *in vitro* ultrasonic bioeffects (Miller, 1985), as many of the bioeffects can demonstrated to date *in vitro* can be suppressed by elevating the ambient pressure (Ciaravino et al, 1981; Webster et al, 1978; Mortimer and Dyson, 1988).

Two extremes of cavitation activity have been identified, the gentle oscillation of gas filled bubbles in low intensity sound fields, termed *stable cavitation*, and the violent and destructive behaviour of short-lived vapour filled bubbles in high intensity sound fields, termed *transient cavitation* (Flynn, 1964). Stable and transient cavitation should not be thought of as separate entities, but rather extremes of a continuum.
Stable cavitation

Stable cavitation refers to the formation of bubbles which vibrate in phase with the pressure variations induced by the acoustic wave (Nyborg, 1977). The amplitude of the bubble oscillation, and cavitation is dependent upon bubble size. Cavitation activity increases with the size of the bubble, until it reaches resonant size, and thereafter, the cavitation activity decreases. The resonant size for a bubble at 1MHz is approximately 7\(\mu\)m in diameter (Williams, 1983). The biological activity associated with stable cavitation is based upon the generation of microscopic streaming fields around oscillating bubbles called acoustic microstreaming. Large hydrodynamic shear stresses occur within the acoustic streaming flow generated near solid or gaseous bodies oscillating at an ultrasonic frequency while immersed in a liquid (Nyborg, 1965). In cells in suspension, this will apply, however these cells are only subject to large shear stresses for a short time, as they are carried past the oscillating surface by the outer eddy of the micro-streaming flow (Williams and Miller, 1980). Tissue cells attached to wells or in vivo are not free to move, and they can be exposed to these stresses, making it necessary to keep the ultrasound applicator in motion.

Transient cavitation

Transient cavitation is the phenomenon whereby a bubble grows very rapidly during the part of the cycle with low pressure, and then collapses during the next part of the cycle when the pressure increases. It is a violent but microscopic phenomenon generating shock waves and very high temperatures within bubbles, often in excess of 10,000 °K (Noltingk and Neppiras, 1950 in Hart, 1993). It occurs most readily at low frequencies (15-40 kHz) and high ultrasound intensities (Williams, 1983).

This violent behaviour can lead to the formation of highly reactive H and OH free radicals (Coakley and Nyborg, 1978). Free radicals are produced naturally, like in cellular respiration, and are eliminated by free radical scavengers. If toxic levels occur, cell death and chromosomal aberrations can occur (Sasaki and Matsubara, 1972). However, it is generally accepted that transient cavitation is an unlikely event using therapeutic ultrasound especially
if standing wave field is avoided and low intensities are used during therapy. Acoustic cavitation is a very complex and well studied phenomenon, for a more detailed review, refer to Hart (1993), Williams (1983), and Miller et al (1996).

**Acoustic microstreaming**

Acoustic streaming or microstreaming refers to the unidirectional movement of fluid in an ultrasonic field. Large hydrodynamic shear stresses occur within the acoustic flow generated near solid, or gaseous bodies (see stable cavitation above) oscillating at an ultrasonic frequency while immersed in liquid (Nyborg, 1965).

A sound field generates oscillatory shear in boundary layers, giving rise to small-scale steady fluid motions, called acoustic streaming (Nyborg, 1992). For example, when a sound field exists in a suspension of particles there is typically relative motion between particles and fluid. A boundary layer is established on each particle and gives rise to an acoustic streaming field; this consists of an eddying pattern of small scale and is appropriately called microstreaming. Figure 2.14 shows the streaming observed near a solid cylinder vibrating transversely in an otherwise quiescent fluid.

The acoustic microstreaming fields acquire biological significance because of the shear stresses associated, which can produce cell rupture and damage (Williams, 1972; terHaar et al, 1979). However acoustic microstreaming can stimulate cell activity if it occurs at the boundary of the cell membrane, altering the surface charge of cells (Repacholi, 1970; Repacholi et al, 1971; Taylor and Newman, 1972) and altering membrane permeability and second messenger activity (Dyson, 1982; 1985). Microstreaming may also influence cell function by modifying the local environment around cells such as by altering metabolite gradients surrounding cells (Young, 1988).
Figure 2.14 - A schematic illustration of acoustic micro-streaming near an oscillating object. OC: Outer circle circulation; IC: Inner circle circulation. Thick headed arrow represents the direction of vibrating object. Single head arrow shows the direction of outer circulation (modified from Holtzmark et al, 1954, in Nyborg, 1982).

Radiation force - standing wave fields

Radiation forces are very important in standing wave fields. In cells in suspension, gas bubbles collect at the antinodes (pressure maxima), and cells collect at the nodes (pressure minima), forming bands. This can explain the banding phenomenon described by Dyson et al (1974), who demonstrated reversible blood cell stasis, forming bands half a wavelength apart, in small blood vessels of chick embryos exposed to standing wave fields. Fixed cells such as endothelial cells can be damaged by microstreaming forces around bubbles if they are situated at the antinodes. The increased radiation force produced in standing wave fields can cause transient cavitation and
consequently free radical formation (Nyborg, 1977; Crum et al., 1987). Therefore, it is important to avoid standing waves formation, by moving the applicator continuously throughout treatment, and use of the lowest intensity required to cause an effect (Dyson et al., 1974).

3. Biological effects of therapeutic ultrasound

Ultrasound has several applications in medicine and physiotherapy, that can be subdivided into diagnostic, disruptive and therapeutic ultrasound (Ziskin, 1987). Diagnostic ultrasound employs high frequencies (3 to 5 MHz), and very low intensities (1-50 mW/cm²), used to avoid tissue heating. Disruptive ultrasound uses very low frequencies (20 to 60 kHz), and very high intensities (above 8 W/cm²), and is used for surgical applications and dental descaling. Therapeutic ultrasound is used in physiotherapy and medicine to achieve several biological effects, based on the thermal and non-thermal effects of ultrasound. The frequencies used traditionally range from 0.5 to 3 MHz (Dyson, 1990), and recently the so called 'long wave' therapy was introduced, which uses 45 kHz ultrasound (Bradnock, 1996). When using traditional ultrasound, the intensities are usually between 0.1 to 3.0 W/cm², and when using long wave therapy, 5 to 50 milliWatts/cm².

Interest in the medical applications of ultrasound dates back from the early 1930s (Freundlich et al., 1932). The first reported use of ultrasound in physical therapy was published in 1939 (Pohlman et al., 1939). After World War II great interest was shown in the field and by 1949, at the Congress of Ultrasound in Medicine, 75 papers were presented on the physics, biological effects, and clinical applications. In 1955 the Council on Physical Medicine and Rehabilitation of the American Medical Association suggested that ultrasound should be used as an adjunct to other forms of treatment to help relieve symptoms such as pain, soreness, and tenderness associated with (1) nonspecific types of bursitis, periarthritis, fibrositis, tenosynovitis, myofascitis and myositis; (2) rheumatoid arthritis and osteoarthritis; (3) and nonparalytic forms of neuritis, such as sciatica, brachial neuralgia, and painful neuromas of
the stump after amputation. Today ultrasound is widely used in physical therapy.

3.1. Musculo-skeletal disorders

This thesis is more concerned with the effects of ultrasound in healing (soft tissues and bone), as well as in angiogenesis. Therefore the following will be only a brief description of the use of ultrasound in musculo-skeletal disorders.

The treatment of several acute and chronic musculo-skeletal disorders has been extensively used in medicine and physiotherapy, and the main purpose is to reduce pain and joint immobility. This is achieved mainly based on the thermal effects of ultrasound, and therefore uses usually higher intensities.

So far there have been two large reviews of the effects of ultrasound in the treatment of musculo-skeletal disorders, with different outcomes. Falconer et al (1990) reviewed the English literature in this subject, and were unable to apply meta-analysis techniques due to insufficient data. They concluded that ultrasound seems to improve pain and range of motion in acute periarticular disorders and osteoarthritis, but not in chronic periarticular inflammatory disorders. Gam and Johannsen (1995) performed the first meta-analysis looking at the effects of ultrasound in musculo-skeletal disorders. They concluded that the use of ultrasound for this purpose is based on empirical experience, but is lacking firm evidence from well-designed controlled studies. Only 1 of 13 studies included exercise therapy (Falconer et al, 1992) and they found less effect in the ultrasound-treated group compared to the placebo-treated group in osteoarthritis of the knee. Well-designed studies are few; the description of drop-outs, randomisation method, apparatus, validation of apparatus, mode of delivery, size of head, treated area and follow-up time were found generally insufficient in published papers.

Bradnock et al (1996) published a prospective randomised placebo controlled trial comparing the effects of high frequency ultrasound (3 MHz) and low frequency ultrasound (45 kHz) for the treatment of unilateral acute ankle
sprains. They were able to show, using objective gait measurements that the group treated with 45 kHz showed significant improvement in length of stride, symmetry of swing phase duration, cadence and walking velocity.

3.2. Ultrasound application in wound repair

The use of therapeutic ultrasound for wound repair has been extensively studied over the years, and these effects are reported usually as been due to non-thermal effects.

*Ultrasound effects in the inflammatory phase of repair*

Therapeutic ultrasound can interact with several cell types present in this phase of repair. Acoustic microstreaming forces have been shown to produce changes in platelet membrane permeability leading to the release of serotonin (Williams, 1974). If microstreaming can stimulate the release of serotonin it may also influence the release of the other factors produced by platelets.

A single treatment with therapeutic ultrasound if given immediately after injury can stimulate mast cells to degranulate releasing histamine into the surrounding tissues (Fyfe and Chahl, 1982). It is possible that ultrasound is stimulating the mast cell to degranulate by increasing its permeability to calcium, since these cells usually degranulate in response to increased levels of intracellular calcium.

There is much evidence that ultrasound can produce membrane changes in a number of cells. Reversible membrane permeability changes to calcium have been demonstrated using therapeutic levels of ultrasound (Mortimer and Dyson, 1988; Dinno et al, 1989). This effect can be suppressed by insonation under pressure, suggesting that cavitation is the physical-mechanism responsible. Other ion permeability changes such as potassium have also been demonstrated (Chapman et al, 1979). Ultrasound can also modify the electrophysiological properties of a tissue, by reducing the sodium-potassium ATPase pump activity (Dinno et al, 1989). This could be used as a possible explanation for the reduction in pain observed with therapeutic
ultrasound, if it occurs in neuronal plasma membranes, reducing neural transmission.

Altering the calcium transport through cell membranes with therapeutic ultrasound is very important, since calcium can act as an intracellular or second messenger activator, altering cellular activity. Macrophages stimulated with ultrasound showed increased synthesis and secretion of wound factors, explained by this mechanism (Young and Dyson, 1990b). This study showed that ultrasound at 0.75 MHz appeared to be most effective in encouraging immediate release of factors already present in cell cytoplasm, whereas the higher frequency of 3.0 MHz was most effective in stimulating the production of new factors, released later. This was evaluated by stimulating fibroblast growth with the media of these insonated macrophages, and was also noted by Hart (1993). Heating is more likely to occur at 3.0 MHz, and cavitation is more likely at lower frequencies, which can probably explain the difference in results.

The rapid resolution of oedema observed clinically in oral surgery as compared to dexamethasone suggested that ultrasound had anti-inflammatory activities (El-Hag et al, 1985). However several papers have shown that ultrasound is not anti-inflammatory in its action, and encourages oedema formation to occur more rapidly (Goddard et al, 1983; Fyfe and Chahl, 1985; Hustler et al, 1978). However, it accelerates the whole inflammatory phase, leading the wound to the proliferative phase. This has been confirmed in vivo in rat excised full thickness skin lesions, where the ultrasound treated rats had significantly less inflammatory cells and more granulation tissue in the wound bed at 5 days post-injury (Young and Dyson, 1990a). These results suggest that there was an acceleration of the healing through the inflammatory phase of repair.

Ultrasound effects in the proliferative phase of repair

The main events occurring during this phase are cell infiltration (e.g. fibroblasts) and proliferation, angiogenesis and re-epithelisation. Mummery (1978) showed that ultrasound can increase fibroblast motility in vitro. In this thesis we show that therapeutic levels of ultrasound induce cell proliferation in fibroblasts,
osteoblasts (Reher et al., 1997c) and endothelial cells in vitro. This is supported by in vivo studies which show a marked increase in wound bed cell number (Young and Dyson, 1990a; Dyson and Pond, 1970). Much debate exists if this is a direct effect of ultrasound or an indirect effect through the stimulation of the macrophage, which then secretes stimulatory factors (Young and Dyson, 1990b, Hart 1993). As shown in this thesis, we believe that both mechanisms are involved.

Fibroblasts stimulated with ultrasound have increased production of collagen (Harvey et al., 1975). This effect is intensity dependent, when exposed to continuous ultrasound (0.5W/cm² (SA)), 20% increase was noted, and when pulsed (0.5 W/cm² (SAPA)), 30% increase was observed. Webster et al (1978) also observed increase of 29% in protein synthesis by fibroblasts, and suggests a role for cavitation in this process. In this thesis we also noted increased collagen production in mice calvaria (Reher et al., 1997a and b), fibroblasts and osteoblasts (Reher et al., 1997c; 1998a).

Another main event of this phase of repair is angiogenesis (see previous section). When chronically ischaemic muscle is exposed to ultrasound, capillaries develop more rapidly (Hogan et al., 1982). Ultrasound therapy is the simplest way of delivering therapeutic angiogenesis. Young and Dyson (1990a) reported the induction of angiogenesis by ultrasound, observed in rat skin lesions. Osteoradionecrosis is specifically an area that benefits from therapeutic angiogenesis. After radiotherapy, the irradiated area becomes hypoxic, hypocellular and hypovascular (Marx, 1983a). The tissues have a complex metabolic/homeostatic deficiency, bordering an ischaemic necrosis, and are prone to breakdown, leading to a chronic non-healing wound, i.e. osteoradionecrosis. Therefore, the treatment or prevention of this complication aims to restore the normal soft tissue and bone vascularity. Therapeutic angiogenesis induced by ultrasound has proved to be clinically successful in osteoradionecrosis (Harris, 1992). Furthermore this thesis gives more objective evidence of the induction of angiogenesis induced by therapeutic ultrasound. This can be due to a direct effect on proliferation of endothelial cells, or
indirectly by the stimulation of angiogenic factors produced by other cells (Reher et al, 1998c).

*Ultrasound effects in the remodelling phase of repair*

In this phase the wound becomes relatively acellular and avascular, collagen content increases, leading to more tensile strength of the wound.

Ultrasound can stimulate wound contraction, as demonstrated by the contraction of cryosurgical lesions (Dyson and Smalley, 1983). Further evidence was shown by Hart (1993) which showed stimulation of wound contraction, leading to smaller scars in rat excised skin lesions treated with 3 MHz pulsed ultrasound. This effect could be observed at 0.1 and at 0.5 W/cm$^2$ (SAPA), and he recommends the lowest intensity. Similar observations were also made in humans. Ultrasound significantly accelerated the reduction in varicose ulcer area (Dyson et al, 1976). Chronic leg ulcers showed a 20% increase in the healing rate when treated with weekly ultrasound at 1 MHz, pulsed, 0.5 W/cm$^2$ (SAPA) (Callam et al, 1987). However there are papers which did not show statistically significant improvements for the treatment of chronic venous ulcers (Lundeberg et al, 1990). Another use of ultrasound in this context is in the treatment of chronic wounds such as pressure sores (Paul et al, 1960; McDiarmid et al, 1985). This effect was more evident in microbially infected sores which may be explained by the greater number of inflammatory and repair cells that may be stimulated as well (McDiarmid et al, 1985).

The effect of ultrasound on this phase depends upon the time when therapy is used. Usually the best results in relation to tensile strength and elasticity of the scar are obtained when ultrasound is applied during the inflammatory phase of repair (Webster et al, 1980; Byl et al, 1993). Treatment with ultrasound during the inflammatory phase of repair not only increases the amount of collagen deposited in the wound, but also encourages its deposition in an 3D architecture similar to normal uninjured skin (Dyson, 1981). Increased, tensile strength and elasticity in injured tendons can also be achieved with therapeutic ultrasound at low intensities (Enwemaka et al, 1990).
3.3. Ultrasound application in bone repair

Overview of bone repair

Fracture healing is a special form of wound healing. Therefore, the normal three phases of repair also occur, i.e. inflammation, proliferation and remodelling. The proliferative phase is subdivided into soft and hard callus formation. The soft callus is an equivalent to granulation tissue in soft tissue injuries. Bone is formed in the soft callus by two mechanisms: intramembranous bone formation and endochondral replacement. The former occurs by the rapid proliferation and differentiation of osteoblast progenitor cells in the periosteum (Kelly et al, 1985). The latter follows as cartilage adjacent to this intramembranous bone undergoes hypertrophy and vascular invasion; bone then replaces the cartilage by endochondral ossification (hard callus). Healing is functionally complete when new bone bridges the fracture site, restoring bone continuity and mechanical stability (Yang et al, 1996). A definitive transition from a cartilage model to woven bone is not readily seen in mammalian bone although it does occur in experimental fractures in rats (Rain et al, 1971).

Wolff (1892) is believed to have been the first to report that mechanical forces regulate the structure and function of skeletal tissue. Many orthopaedic scientists have attempted to advance bone formation by intensifying or controlling the mechanical forces at the fracture site. The clinical success of these treatments indicates that cells in the fracture callus can “translate” mechanical forces into biological signals that modify the metabolic activity of cells. Furthermore, these observations imply that mechanical loading is one of the local regulatory signals guiding gene expression in the callus (Yang et al, 1996).

Ultrasound effects on bone repair

Dyson and Brookes (1983) showed that it was possible to accelerate the repair of fibula fractures using 1.5 or 3 MHz, pulsed, 0.5 W/cm² levels of ultrasound. Therapy was carried for 5 minutes, four times per week, and the most effective
treatments were found to be those which were carried out during the first two weeks of repair (inflammatory phase). Of the frequencies used, 1.5 MHz was the most effective. Pilla et al (1990) showed that low intensity ultrasound (1.5 or 3 MHz, pulsed, 30 mW/cm²) could stimulate fracture repair in rabbits to such a degree that maximum strength was gained in the treated limbs by 17 days after injury compared to 28 days in the controls. Tsai et al (1992a) showed that low intensities of ultrasound (1.5 MHz, pulsed, 0.5 W/cm²) stimulated fracture healing in a rabbit fibulae model. However, they also showed a deleterious effect when ultrasound was applied at 1.0 W/cm². The same team also reported high production of PGE₂ at the best stimulatory ultrasound intensities for bone repair, suggesting that bone healing may be mediated via production of cytokines, although PGE₂ is usually associated with resorption (Tsai et al, 1992b).

Wang et al (1994) using a rat femoral fracture model, observed that ultrasound enhanced the mechanical properties of healing calluses. The stiffness of treated fractures was greater than that of the control fractures, at 0.5 and 1.5 MHz, but the difference was significant only with the 1.5 MHz signal (p<0.02). This report shows accelerated endochondral ossification in the callus after ultrasound exposure, whereas other authors have reported that ultrasound exposure changes the calcium content of cultured cells (Ryaby et al, 1989). Either advanced endochondral ossification or increased matrix calcium, conditions that may be closely related, could result in stronger fracture callus.

Yang et al (1996) using 0.5 MHz (50 or 100 mW/cm²) in a rat femoral fracture model similar to that used by Wang et al (1994), showed that the average maximum torque and torsional stiffness were greater than in controls, reaching statistical significance at 50 mW/cm². They could not measure any significant difference in the collagen content measured in the soft callus 7, 14 or 21 days after fracture. However evaluation of gene expression showed a shift in the expression of genes associated with cartilage formation: aggregan gene expression was significantly higher on day 7 after fracture, and significantly lower on day 21. They suggest that ultrasound stimulation increases the mechanical properties of the healing fracture callus by stimulating
earlier synthesis of extracellular matrix proteins in cartilage, possibly altering chondrocyte maturation and endochondral bone formation.

Heckman et al (1994), investigated the effectiveness of low intensity ultrasound on the healing of human tibial fractures. The fractures were examined in a prospective, randomised, double-blind evaluation of low intensity ultrasound, applied to a group of 67 closed or grade-I open fractures of the tibial shaft. The treated group showed a significant decrease in the time to clinical healing (86 +/- 5.8 days) as compared to the control group (114 +/- 10.4 days) (p=0.01) and also a significant decrease in the time to over-all i.e. clinical and radiographic healing (96 +/- 4.9 days compared to 154 +/- 13.7 days in the control group).

Finally we come to the use of ultrasound applied to bone is in the treatment of mandibular osteoradionecrosis (Harris, 1992). The patients were treated with ultrasound (3 MHz, pulsed 1:4, 1 W/cm²) for 40 sessions of 15 minutes per day. Ten out of 21 (48%) cases showed healing when treated with debridment and ultrasound alone. The remaining 11 cases unhealed after ultrasound therapy received debridment and cover with a local flap, and only one needed mandibular resection and reconstruction. These results are significantly better than the conventional treatment with hyperbaric oxygen therapy and surgery, but needed scientific validation. Marx (1983b) himself concluded that hyperbaric oxygen alone cannot heal ORN wounds, since only 15% responded, and 70% of his patients required major reconstruction. In our department, Telfah (1995) using near infrared spectroscopy has demonstrated that patients with ORN who received ultrasound therapy showed significant improvements of the metabolic activity as measured by increase in their deoxyhaemoglobin concentrations, but the reliability of this work will be discussed later in chapter 11.
Section IV - Hypothesis and Aims

1. Hypothesis

Therapeutic ultrasound can be used in the treatment and prevention of osteoradionecrosis because it stimulates cell proliferation, cell differentiation, healing, and angiogenesis, therefore reversing the long term effects of radiotherapy.

2. Aims

1. To establish this hypothesis by a series of controlled experiments examining cell, matrix and vascular factors in response to two ultrasound modalities.

2. To evaluate the use of near infrared spectroscopy (NIRS) as a diagnostic tool for measurements of deoxyhaemoglobin concentrations in the mandibles of volunteers and patients treated with radiotherapy, establishing the risks of development of osteoradionecrosis and evaluating the efficacy of therapeutic ultrasound clinically.
Chapter 3 - Bone Formation Stimulated by Therapeutic Ultrasound - a Pilot Study

1. Introduction

As shown in chapter 2, ultrasound has proved to be therapeutically valuable in many ways, through its thermal and non-thermal effects. Several ultrasound applications using non-thermal effects have been shown in the literature, the majority observed in soft tissue healing.

Using human skin fibroblasts insonated in suspension and subsequently cultured in vitro, Harvey et al (1975) found an increase of both collagen and non-collagenous protein (NCP) synthesis, which was intensity dependent. Fibroblasts exposed to continuous ultrasound (0.5 W/cm² (SA) showed a 20% increase in collagen secretion which was increased to 30% when the ultrasound was pulsed (0.5 W/cm² (SAPA)).

The use of mouse calvaria has been found to be a successful tool to study bone formation in vitro (Raisz et al, 1978). A relatively new method for estimating collagen and non-collagenous (NCP) synthesis has been used in this study. This is a modification of the conventional assay in which ³H-proline-labelled calvaria are exposed to highly purified bacterial collagenase to release labelled collagen (Peterkofsky and Diegelman, 1971). In the modified assay developed by Meghji et al (1992a and b), pepsin is used to lyse the calvaria allowing the separation of intact collagen from the NCP.
2. Hypothesis and Aims

This work tests the hypothesis that ultrasound promotes healing in osteoradionecrosis and bone fractures by enhancing bone formation through the stimulation of collagen and non-collagenous protein synthesis.

The aims of this chapter are:

1. To identify if ultrasound at 3 MHz, pulsed 1:4 is able to stimulate the production of collagen and non-collagenous proteins in neonatal mice calvaria.

2. Verify if these effects are due to thermal or non-thermal effects, by measuring the heat production caused by different intensities of ultrasound at 3 MHz, pulsed 1:4.

3. Material and Methods

3.1. The ultrasound machine and the insonation vessel

The ultrasound machine evaluated in this pilot study was the one that had been used for the treatment of mandibular osteoradionecrosis in this institution, which uses a frequency of 3 MHz, pulsed 1:4 (Harris, 1992). This was a Rank Sonacell unit, capable of delivering 0.0-4.0 W/cm² (SATA), 1.5 or 3 MHz with a facility for being set in continuous mode or several pulsed modes. We used the pulse setting 1:4 (2ms ‘on’ and 8ms ‘off’). The machine was calibrated using a tethered float radiometer (designed by the National Physical Laboratory, Teddington, Middlesex) at 3.0 MHz continuous wave. The transducer head had a diameter of 30 mm, and was held by a modified microscope stand.

The insonation vessel used was the same used previously in this department (Elbeshir, 1981). It was constructed from polystyrene sheets (Dreve, Germany) and measured 165 x 120 x 80 mm (Figure 3.1). A shelf made of the same material was attached to the inner side walls in order to suspend a
multiwell (6 wells) culture plate. Each well had a diameter of 35 mm. A frame was designed to fit over the multiwell culture plate, and was fixed with screws to the suspension shelf, sandwiching the culture plate. To reduce reflection of the ultrasound waves, air/water interfaces were eliminated wherever possible by the use of castor oil (BDH) and a rubber mat in the base of the irradiation vessel below the multiwell culture plate (Figure 3.1). After the first set of experiments, a localising ring of polystyrene with 10 mm in diameter was placed into the centre of each well to hold the bones (Figure 3.2).

**Figure 3.1** - Schematic representation of the irradiation vessel used in the pilot study (transverse view).

**Figure 3.2** - Detail of a well with the localising ring and the thermocouple probe.
3.2. Tissue culture and the insonation procedure

All animal work was undertaken in accordance with the institute's ethical and legal regulations. Calvaria bones (frontal and parietal) were obtained by aseptic dissection of 5-day old albino MF-1 mice. The fronto-parietal bones were trimmed of any adherent connective tissue, halved along the sagittal sutures and placed in Hank's balanced salt solution (Gibco). One half calvarium was placed into each multiwell cell, containing 5 ml of culture medium and transferred into a humidified 5% CO₂/95% air incubator at 37°C. The culture medium was BGJb medium (Gibco), supplemented with: (a) freshly prepared L-ascorbic acid (BDH) - 50 μg/ml, (b) L-proline (Sigma) - 40 μg/ml, (c) heat-inactivated rabbit serum (Wellcome Reagents, Beckenham) - 5% w/v, (d) antibiotics - Penicillin/Streptomycin (Gibco) - 100 U/ml. The bones were incubated for between 1 and 6 hours to allow equilibration with the medium.

After this equilibration period, the culture plates were removed from the incubator, brought to a sterile air cabinet (Microflow) at room temperature (19°-26°C), and fixed to the irradiation vessel for the irradiation procedure. The transducer head was swabbed with methanol, left to dry, and immersed vertically into each culture well, positioned 5 mm above the bone sample (Figure 3.2). Each control or test-group consisted of 5 wells, each with one half-calvaria. Each set of experiments was repeated at least twice.

**Intensities tested** - The US machine was set at 3 MHz, pulsed 1:4 (2 ms 'on' and 8 ms 'off'), and the intensities evaluated were 0.1, 0.25, 0.5, 0.75, 1.0 and 2.0 W/cm²(SAPA). Control bones were treated in the same way as the test bones, but with the US generator switched off. Irradiation was applied for 5 min to each well. After the irradiation, 3.5 ml of medium was removed, and the explants were cultured for a further 18 hours in the 37°C incubator.

The bones were then radiolabelled, with 3 μCi of L-[5-³H]proline (sp act 18 ci/m.mol; Radiochemical Centre, Amersham) dissolved in 5 μl of culture medium, giving a final concentration of 2 μCi/ml. Bones were further incubated for 6 hours and then processed immediately or stored at -20°C.
3.3. Bone Formation Assay

The procedure for extraction and purification of the radio-labelled collagen was a modification of the method of Webster and Harvey (1979) for monolayer cultures, and is shown schematically (Figure 3.3). It is based on the purification of collagen and NCP by serial salt precipitation in order to measure directly the rate of accumulation of \(^3\text{H}\)-labelled native collagen and NCP (Meghji et al, 1992).

\[ \text{\(^3\text{H}\)-Proline Labelled Bones} \]

\[ \text{TCA Extraction} \quad \rightarrow \quad \text{TCA Soluble Extract} \]

\[ \text{Pepsin Extraction} \quad \rightarrow \quad \text{Debris (Discard)} \]

\[ \text{Soluble Extract} \]

\[ \text{Salt (5\%) - Collagen precipitation} \quad \rightarrow \quad \text{Supernatant (NCP)} \]

\[ \text{Redissolve Precipitation} \]

\[ \text{Salt (5\%) - Collagen precipitation} \quad \rightarrow \quad \text{Supernatant (NCP)} \]

\[ \text{Dissolve and Count} \quad \text{\(^3\text{H}\) Collagen} \]

\[ \text{Pool and Count} \quad \text{\(^3\text{H}\) NCP} \]

\[ \text{Figure 3.3 - Collagen and non-collagenous protein purification sequence} \]

If the samples had been frozen they were thawed before placing them into the incubator. The bones were washed (x3) in normal saline to remove free radioactivity. The proteins were precipitated with 2 ml of trichloroacetic acid (5\%).
TCA) for 2 hours at 4°C. The bones were then rinsed in Hank's solution, and placed in a 3 ml polystyrene tube (LP^: Luckham). Collagen was extracted with 1 ml of 0.5M acetic acid containing pepsin (0.5 mg/ml; EC 3.4.4.1, Sigma) for 16 hours at 4°C. Insoluble bone debris was removed by centrifugation (5000 g for 30 min) and 200 µg of acid soluble rat skin collagen was added to each tube as a carrier for labelled collagen during subsequent purification.

Collagen was precipitated by the addition of NaCl (25% w/v in 0.5 M acetic acid) to a final concentration of 5% (w/v) and left at 4°C for 2 hours. Following this the tube contents was mixed gently and centrifuged at 5000 g for 30 minutes at 4°C, and the supernatant was removed and stored. The pellets were redissolved in 1 ml of 0.5 M acetic acid, collagen was re-precipitated with the NaCl as described and the second supernatant removed and stored. The final precipitates were dissolved in 200 µl of 0.5 M acetic acid and transferred into disposable scintillation vial inserts (Minitubes, Hughes and Hughes Ltd., England), containing 3 ml of scintillation fluid (Unisolve 1, Koch-Light).

For NCP synthesis estimations, the first and second supernatants were pooled in scintillation vials as for collagen. Radioactivity of the L-[5-³H] proline was measured by scintillation spectrometry in a Beta counter (Rackbeta, LKB) with external standardisation, and expressed in disintegrations per minute (DPM).

3.4. Temperature measurements

These were made for all the samples, using thermocouple probes inserted into the culture media in the well receiving treatment (Fig. 3.2). The measurements were made at room temperature (19-26°C) and taken from a digital thermometer (Comark, England) monitored with an automatic chart recorder to give temperature readings to the nearest 0.05°C.
3.5. Statistical analysis

Each insonation experiment was repeated at least twice. The result graphs show the observations of one representative experiment. The number of observations for the sham insonated controls and for each intensity evaluated was five (n=5). The values obtained in DPM's in the collagen/NCP assays were transformed in percentages of the reading of the controls, which were rated as 0%. All test values were compared with, and graphically presented as a percentage of control values. The results obtained were analysed in Excel®, and the values for the treatment groups were compared to the control group using ANOVA single factor and two tailed Student's 't' test for unpaired samples. Significance was accepted at the p<0.05 level or higher, as shown in the graphs.

4. Results

4.1. Bone formation as measured by collagen and non-collagenous protein synthesis

The first set of experiments using the multiwell culture cells without localising rings showed no significant change of both collagen and NCP synthesis at the intensities between 0.1 and 0.75 W/cm² (SAPA), and significant inhibition of collagen synthesis (p<0.001) and NCP synthesis (p<0.05) at 1.0 W/cm² (SAPA). These results were not consistent, and since the experiments were carried out using the near field, it was suspected that the bones were receiving different US intensities. Therefore, polystyrene rings were used to localise the bones in the same position in relation to the US beam (Fig. 3.2).

Experiments using the localising rings (two series) were consistent and reproducible, and showed significant stimulation of both collagen and NCP synthesis (Fig. 3.4) The best intensity was produced at 0.1 W/cm² (SAPA), with an increase of 135% for collagen synthesis (p<0.001) and 108% for NCP synthesis.
(p<0.01). At higher intensities there is a marked tendency to decrease the collagen and NCP synthesis, which was significant (p<0.05) at 2.0 W/cm² (SAPA) (Fig. 3.4). ANOVA evaluation suggests that the difference between the groups is highly significant (p=8.04E-16), even if the 0.1 W/cm² group is excluded (p=0.0007). The comparison between each group and the controls showed similar results with the students ‘t’ test.

In all experiments there was high correlation of the rates of collagen and NCP synthesis (r=0.9809, p<0.001, Fig. 3.4) and there was no differential effect of collagen synthesis as percentage of total protein synthesis (NCP + collagen).

![Chart showing the effects of varying intensities of 3 MHz ultrasound (pulsed 1:4) on bone collagen and non-collagenous protein (NCP) synthesis using the culture vessel with localising rings (error bars show 95% confidence intervals). The increased collagen and NCP synthesis at 0.1 W/cm² (SAPA) were highly significant (p<0.001 and p<0.01 respectively). At higher intensities there was a decrease in collagen and NCP synthesis, from 0.75 to 2.0 W/cm² (SAPA) which culminates at 2.0 W/cm² (SAPA) (p<0.05).](chart.png)
4.2. Heating effects

The temperature rise of the culture medium ranged from 0.0°C at 0.1 W/cm² to 1.8°C at 2.0 W/cm² after 5 minutes of insonation.

5. Discussion

The culture of mouse calvaria has been a successful tool to study bone resorption and formation in vitro (Raisz et al, 1978). Both osteoblasts and fibroblasts present in the mouse calvaria produce collagen and non-collagenous protein (NCP). However, by using injected ^H-labelled proline it was found that osteoblasts in bone are more active than fibroblasts and produce the greatest amount of bone matrix (Fitton-Jackson and Randall, 1956). Therefore the protein synthesis stimulation observed in this study may be attributed to osteoblastic rather than fibroblastic activity.

The pepsin extraction assay used is a simple method which allows the rapid and direct estimation of collagen and NCP synthesis in bone cultures. The advantages over the classic bacterial collagenase extraction assay is that there is no need for collagenase purification and there are fewer steps needed to assay the bone collagen. The pepsin assay is based on the measurement of radioactively-labelled collagen present in the bones after a 6-h pulse with ^H-proline. This is achieved by extraction of native collagen using limited pepsin digestion, purification by salt precipitation and measurement of the radioactivity in the final preparation. Non-collagenous protein is largely susceptible to degradation by pepsin and does not co-precipitate with the collagen. In this way, the NCP is not contaminated with collagen. This assay therefore gives an estimate of the ‘net’ rates of collagen and NCP synthesis over the duration of the proline pulse. Care has been taken to ensure specificity of the collagen and NCP labelling. One advantage of this assay is its simplicity allowing large numbers of samples to be analysed, when compared to the established
bacterial collagenase extraction assay of Peterkofsky and Diegelman (1971) and even so it is equally efficient, given similar results for collagen and NCP synthesis (Meghji et al, 1992).

Non-collagenous-proteins in the bone comprise the insoluble matrix proteins such as sulphated proteoglycans, glycoproteins, traces of transferrin, albumin, globulin (IgG) and enzymes secreted by the bone cells and cell proteins (Vaughan, 1975). Measurement of NCP synthesis is therefore more an estimate of the rate of general protein synthesis than an index of synthesis of a specific class of proteins. In this study collagen and NCP synthesis were significantly correlated (r=0.9809, p<0.001) and there was no differential effect on collagen synthesis rates relative to total protein synthesis. This corroborates with findings in fibroblasts experiments (Webster et al, 1980).

Ultrasound at the lower end of the therapeutic scale (0.1 W/cm²) significantly stimulated the synthesis of bone matrix proteins in two out of three experiments. There was no stimulation with the first set of experiments without the localising rings. At higher intensities (0.75-2.0 W/cm²) ultrasound suppressed the synthesis of both collagen and NCP, although this difference was only significant at 2.0 W/cm² (p<0.05). These results are in agreement with Harvey et al (1975), who also showed an increase in both collagen and NCP using human skin fibroblasts insonated in suspension and subsequently cultured in vitro. However their best response was at marginally higher intensity levels then ours, 0.5 W/cm² (SA) continuous US, where a 20% increase in collagen secretion was recorded. When the ultrasound was pulsed (0.5 W/cm² (SAPA)), a 30% increase was observed. In our study, we only used pulsed mode, and the best intensity was produced on 0.1 W/cm², with an increase of 135% (collagen) and 108% (NCP).

Ultrasound is known to exert both thermal and non-thermal effects on tissues. The use of low-intensity ultrasound reduces tissue heating, and also reduces the possibility of cavitation phenomena, i.e. the pulsation of gas or vapour-filled voids in a sound field (Webster et al, 1980). It would appear that non thermal effects were primarily considered to be responsible for the results
observed in this experiment, as no measurable rise was observed with the best stimulatory dose (0.1 W/cm²). By contrast there was reduced production associated with the maximum temperature rise of 1.8°C at 2.0 W/cm².

The physical mechanisms which may be involved in producing these non-thermal effects are: acoustic streaming, acoustic cavitation, and acoustic microstreaming. Acoustic streaming (also associated with stable cavitation) and microstreaming seem to play an important role in stimulating cell activity if it occurs at the boundary of the cell membrane and the surrounding fluid, altering the membrane permeability and second messenger adenylate cyclase activity (Dyson, 1982; 1985). The same stimulation of intra-cellular activity by adenylate cyclase might also be provoked by the ultrasound producing an electrochemical perturbation of the cell membrane surface (Ryaby et al, 1989). This could result in therapeutically advantageous changes such as increased protein synthesis (Harvey et al, 1975; Webster et al, 1978), increased secretion from mast cells (Fyfe and Chahl, 1982), fibroblast mobility changes (Mummery, 1978), increased uptake of the second messenger calcium (Mummery, 1978; Mortimer and Dyson, 1988), and production of growth factors by macrophages (Young and Dyson, 1990b).

The data shown here may explain the effects of ultrasound on fracture healing, since the production of collagen and NCP is a fundamental requisite. Furthermore, the best results were obtained with ultrasound at low intensities, as observed in the majority of the papers related to fracture healing (Dyson and Brookes, 1983; Pilla et al, 1990; Tsai et al, 1992; Wang et al, 1994; Heckman et al, 1994).

Yang et al (1996) using 0.5 MHz (50 or 100 mW/cm² (SAPA)) on a rat femoral fracture model similar to that used by Wang et al (1994), showed that the average maximum torque and torsional stiffness were greater than in controls, reaching statistical significance at 50 mW/cm². However, these authors could not measure any significant difference in the collagen content measured in the soft callus 7, 14 or 21 days after fracture. They suggest that ultrasound stimulation increases the mechanical properties of the healing
fracture callus by stimulating earlier synthesis of extracellular matrix proteins in cartilage, possibly altering chondrocyte maturation and endochondral bone formation.

In this chapter, ultrasound at higher levels of intensity (0.75-2.0 W/cm²) showed inhibition of the synthesis of both collagen and NCP, but only to a significant level (p<0.05) at 2.0 W/cm². Similar observation was also made by Tsai et al (1992), who showed a deleterious effect on fracture healing when ultrasound was applied at 1.0 W/cm² in a rabbit fibulae model.

The intensity of ultrasound used in the treatment of osteoradionecrosis (Harris, 1992) was relatively high (1 W/cm²). The favourable results observed could therefore be explained in terms of neoangiogenesis (Young and Dyson, 1990a). This was demonstrated by the use of near infrared spectroscopy scans (Telfah, 1995) who showed higher levels of deoxyhaemoglobin concentrations in the osteoradionecrotic mandibles of patients treated with ultrasound. This suggests significant improvements of the metabolic activity of the mandibular tissues. However since collagen and NCP synthesis are also important in the reorganisation of the bone matrix, perhaps lower intensities should be used in the treatment of osteoradionecrosis.

The difference between in vivo and in vitro investigations should also be considered. Calvaria were obtained from young growing mice, and their cell behaviour will differ from osteoblasts of adult patients with fractures or osteoradionecrosis. Also the ultrasound frequency determines the penetration depth. In these experiment a 3 MHz frequency was used, because it was applied to organ cultures, which do not need a deep penetration depth. Furthermore, this was the frequency used for the treatment of mandibular osteoradionecrosis (Harris, 1992). Frequencies of 1 MHz or even ‘long-wave ultrasound’ (45 kHz) seems to be more appropriate in clinical practice because they allow the ultrasound field to penetrate deeper into the tissues (Bradnock et al, 1996). Therefore, I have repeated these assays using 1 MHz and 45 kHz ultrasound machines, in the following chapters.
In summary, ultrasound is capable of inducing bone formation at 3 MHz (0.1 W/cm\(^2\) (SAPA)), pulsed 1:4, as measured by bone matrix protein synthesis production, but inhibits formation at higher levels of intensity (0.75-2 W/cm\(^2\) (SAPA)). Non-thermal mechanisms appear to be responsible for these effects. These results are comparable to stimulation obtained at similar doses of US in fibroblasts \textit{in vitro} and sustain the use of low-intensity US doses for \textit{in vivo} applications as for wound healing, fracture healing and osteoradionecrosis.
Chapter 4 - Cell Proliferation (DNA Synthesis) Stimulated by Therapeutic Ultrasound

1. Introduction

The experiments performed in the previous chapter were performed evaluating an ultrasound machine using a 3 MHz frequency. As explained in the discussion in that chapter, the clinical management of mandibular osteoradionecrosis has changed, and 1 MHz ultrasound is used, because it gives more penetration depth than 3 MHz.

Recently, a new ultrasound device has been developed, which instead of using the 'traditional' frequencies, of 1 to 3 MHz, introduced the 'long wave' ultrasound, which uses 45 kHz. (Bradnock et al, 1996). This lower frequency/long wavelength combination gives a more widely divergent field shape, with the treated volume effectively all in the far field region. This enables the wave to penetrate much deeper into the tissues, reaching areas as deep as several centimetres, instead of millimetres as in the 'traditional' machines. In order to minimise heating effects, it uses low intensities, well established in the treatment of bone fractures (5 to 50 mW/cm²(SA)).

Another observation made during the assays performed in the pilot study, is related to the construction of the irradiation vessel. The main problem was the lack of temperature control, which could have interfered with our results. The use of castor oil could also be questioned, and some more recent publications suggested the use of water baths with absorbing ultrasound rubber lining the tank. Therefore, in the following in vitro experiments, we modified this insonation vessel.

Soft tissue wound healing, as well as bone healing are dependent on several factors, including fibroblast and osteoblast density within the tissue. In
vitro reports suggest that ultrasound is capable of directly modulating certain fibroblast functions, including proliferation migration (Mummery, 1978). As osteoradionecrosis is a hypocellular tissue, the induction of cell proliferation is an crucial therapeutic approach in its prevention and treatment.

In the literature there are several studies assaying DNA synthesis as measured by $^3$H-thymidine incorporation, but none with human gingival fibroblasts, human mandibular osteoblasts and human umbilical vein endothelial cells (HUVEC). Furthermore, there are no studies using the 'long wave' ultrasound machine (45 kHz), or comparing it to the traditionally used ultrasound frequencies.

2. Hypothesis and Aims

These experiments test the hypothesis that ultrasound stimulates cell proliferation (DNA synthesis) of human gingival fibroblasts, human mandibular osteoblasts and human umbilical vein endothelial cells (HUVEC).

The aims are:

1. To identify if therapeutic ultrasound is able to induce cell proliferation (DNA synthesis) in human gingival fibroblasts and human mandibular osteoblasts.

2. Compare two types of ultrasound machines, the ‘traditional’ (1 MHz, pulsed), and the ‘long wave’ (45 kHz, continuous), at 4 intensities each, trying to establish which machine and intensity have the best stimulatory effect on cell proliferation.
3. Material and Methods

3.1. The ultrasound machines evaluated

I have compared two different ultrasound machines in the following assays. The first machine was the 'traditional' ultrasound machine, that uses a frequency of 1 or 3 MHz, and the second one, a 'long wave' machine, that uses a frequency of 45 kHz. The reason for this is that the majority of the studies in the literature were performed with the 'traditional' machine, and we are introducing the new 'long wave therapy', to be possibly used clinically for osteoradionecrosis. Therefore, in order to evaluate more precisely the effects of the new machine, it was decided to compare it with the standard machine used so far, whenever possible.

The 'traditional' (1 MHz) ultrasound machine

The ultrasound apparatus used was a Therasonic 1032 unit, produced by E.M.S., serial number 39294 (Fig. 4.1). This apparatus can be set to work with 1 or 3 MHz, and can deliver an intensity ranging from 0.1 to 2.0 W/cm². The machine can be used in continuous mode or in pulsed mode, 1:2, 1:4 or 1:9. The machine has an electronic control panel, and a facility to calibrate itself each time it is switched 'on'.

Frequency and intensities used

In the previous chapter the 3 MHz frequency was used because it was the original frequency suggested in the literature for the treatment of osteoradionecrosis (Harris, 1992). In this and further experiments, the frequency of 1 MHz will be used, as mentioned in the introduction.

In relation to the pulsing facility, again, the best physiologic and therapeutic effects have been observed with the ultrasound wave pulsing (Harvey et al, 1975). Therefore, all our experiments using the 1 MHz ultrasound were performed on a pulsing mode of 1:4 (2ms 'on' and 8ms 'off').
The intensities evaluated were chosen randomly, and we decided to test 4 levels. Therefore, we evaluated the intensities of 0.1, 0.4, 0.7 and 1.0 W/cm². These values are spatial average pulsed average (SAPA) intensities.

 Calibration

The machine was calibrated several times during the experiments, but at least before and after a set of assays. The calibration was performed at the Department of Medical Physics, University College London. At each calibration, a full electronic check-up was performed, according to the manufacturer manual. The acoustic output power was measured/calibrated using a precision ultrasound balance (E.M.S. model 67). After set-up of the balance and warming up of the ultrasound machine, the measurements were taken at 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 Watts/cm². The calibration was considered in admissible range if the accuracy of the output readings was ≤ 10%.

Figure 4.1 - The 'traditional' ultrasound machine (1 MHz)
Summary

**Producer:** Electro-Medical Supplies (Greenham) Ltd., Oxfordshire, England

**Name:** Therasonic, model 1032

**Settings:**

- **Frequency:**
  - 1 and 3 MHz
- **Handset head type:**
  - flat surface
- **Effective radiating area:** approx. 2 cm²

**Power/Intensities used**

- 0.1 Watts/cm²
- 0.4 Watts/cm²
- 0.7 Watts/cm²
- 1.0 Watts/cm²

*The ‘long wave’ ultrasound machine (45 kHz)*

The ultrasound apparatus used in this thesis was a Phys-Assist unit, produced by Orthosonics (Fig. 4.2). This apparatus has a fixed frequency of 45 kHz, and can deliver an intensity ranging from 5 to 50 mW/cm². The machine does not have a pulsing facility, working only in ‘continuous’ mode. However, since the intensity is much lower than the one of the ‘traditional’ ultrasound, the pulsing necessity is theoretically diminished, since the heat generated is not so high. The machine also has an electronic control panel, with a liquid crystal display, and a facility to calibrate itself each time it is switched ‘on’.

**Frequency and intensities used**

As explained before, the only frequency used with this machine was the 45 kHz. Since there is no pulsing facility, the machine always operated in ‘continuous mode’.

We also evaluated 4 intensities in this machine, named by the manufacturer as ‘Power Settings 1, 2, 3, and 4’, which corresponds to 5, 15, 30 and 50 mW/cm².

**Calibration**

The machine was calibrated several times during the experiments, but at least before and after a set of assays. The calibration was performed at Orthosonics.
Ltd., Ashburton, Devon. At each calibration, a full electronic check-up was performed, according to the manufacturer manual, and the intensity output was measured/calibrated using a tethered float radiometer (designed by the National Physical Laboratory, Teddington, Middlesex) at 1.0 MHz continuous wave.

Figure 4.2 - The 'long wave' ultrasound machine (45 kHz)

Summary

Producer: Orthosonics Ltd., Ashburton, Devon, England

Name: Phys-Assist

Settings: Frequency: 45 kHz

- Handset head: type conic
  - effective radiating area: approx. 12.8 cm²

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Chapter 4 - Cell proliferation (DNA synthesis) induced by therapeutic ultrasound

**Power/Intensities**
- Level 1 - 0.07 Watts/5 milliwatts/cm²
- Level 2 - 0.19 Watts/15 milliwatts/cm²
- Level 3 - 0.38 Watts/30 milliwatts/cm²
- Level 4 - 0.64 Watts/50 milliwatts/cm²

N. 8. (a) The actual surface that was touching the media during the assays was much less, approx. 2-3 cm².

3.2. Cell culture

In the *in vitro* assays done in this chapter, we used three different human cell cultures: (1) human gingival fibroblasts, (2) human mandibular osteoblasts (3) human umbilical vein endothelial cells (HUVEC).

**Human gingival fibroblasts**

**Primary cultures**

Primary fibroblasts cultures where obtained from gingival tissue specimens obtained from patients submitted to planned dental extractions and/or surgical removal of wisdom teeth. All patients had no known diseases, and were 20 to 30 years old. The gingival specimen was collected in phosphate buffered saline (PBS; Gibco BRL). This was stored at 4°C for up to 4 hours, when it was processed.

All the following cell culture work was carried out in a sterile air flow cabinet (Microflow Pathfinder, Intermed). The gingival specimens were rinsed several times with PBS in order to reduce the number of blood cells. The specimens were placed over a petri dish, and cut into small pieces (1 mm²) with two number 10 scalpel blades. The fragments where then placed into 75 cm² culture flasks (Sarstedt), and 10 ml of media was added.

The media used was Dulbecco's modified Eagle medium (DMEM), suplemented with: (a) heat-inactivated fetal bovine serum (HIFBS) - 10% v/v (Sigma), (b) freshly prepared L-ascorbic acid - 50 μg/ml (Sigma), (c) L-glutamine - 2 mM (Sigma), and (d) penicillin/streptomycin - 100 U/ml each
(Gibco BRL). After approx. 20 days, the cells started to grow out of the explants, and the media was than changed twice a week. Cell growth was routinely examined using a phase contrast microscope (Olympus, Japan).

Subculturing

When the cells were confluent, they were prepared for trypsinisation and divided into three new flasks. The culture medium was removed, and the cell layer washed once or twice with PBS supplemented with (1) penicillin/streptomycin - 100 U/ml (Gibco). The cells were covered with 3.0 ml of 0.25% v/v trypsin (Gibco) in PBS. Excess trypsin was removed, leaving only a thin film covering the cell layer, and the cells transferred back to the incubator for 20 minutes. The process was arrested by adding 3.0 ml of prepared medium, and single cell suspensions were obtained by vigorously pipetting the cell clumps through a glass Pasteur pipette several times, detaching them from the culture flask. The cells were allocated to three new culture flasks, which were supplemented with 10 ml of new medium, and returned to the incubator.

Each time the cells are trypsinised is referred as a passage. Usually it took between 10 and 14 days for the cells to be ready for another passage. The fibroblasts were used for the experiments between the 6th and the 10th passage.

Freezing cells

Excess cells were frozen in liquid nitrogen. Briefly, 10% v/v dimethyl sulphoxide (AnalaR, BDH) was added to 1 ml of a suspension of cells obtained by trypsinisation. The cells in suspension were transferred to 1.0 ml cryogenic vials (Nalgene), and the vials placed into a special freezing container (Cryo1°C Freezing Container, Nalgene), achieving a -1°C/minute rate of cooling. The container was put into a -70°C freezer, and the cryo-vials were transferred and stored in liquid nitrogen the following day.

When the cells were needed again, the cryo-vials were placed directly into the incubator for 5 min, until thawed. They were then transferred to a 75 cm² culture flask, supplemented with 10 ml of medium. The next day the medium was changed to eliminate the excess of dimethyl sulphoxide.
Counting cells/preparing for the experiments

Once the cells were ready for using in an experiment, they were trypsinised as described. For each experiment usually six confluent 75 cm² flasks were needed. After trypsinisation, the cells were pooled in a 50 ml falcon tube (Sarstedt), mixed well, and two drops were placed over a haemocytometer (Weber, England). The cells were counted under a microscope, in the 25 marked squares, and the number obtained was multiplied by 10,000, giving the total number of cells per ml. The cells were counted at least twice.

\[
\text{Number of cells observed} \times 10,000 = \text{Number of cells/ml}
\]

Once the number of cells per ml was determined, they were diluted with prepared medium (2% HIFBS), to a final concentration of 1.5x10⁵ cells per well in 1.5 ml of medium. The cells were plated on a 6-well culture plate, leaving one empty (n=5). The 6-well plates were incubated for further 24 hours, then media were changed, adding a volume of 5 ml of 2% HIFBS, in order to have a distance of 5-6 mm between the transducer head and the cells.

N.B. - In the cell proliferation assays, the cells had not reached confluency before the assay.

Human mandibular osteoblasts

The cell culture technique used for osteoblasts was very similar to that used for fibroblasts, and therefore will not be repeated again. The mandibular bone specimens were also obtained in the Victor Goldman Unit, Eastman Dental Institute. These were obtained from patients submitted to surgical removal of wisdom teeth, in which the lingual split technique was used, and the bone segment was to be removed. All patients had no known diseases, with ages between 20 and 30 years. After collection of the bone specimen under aseptic conditions in phosphate buffered saline (PBS; Gibco BRL), it was stored at 4°C for up to 4 hours, when it was processed.

In the laboratory, the bone specimens were rinsed several times with PBS in order to reduce the number of blood cells. The bones were thoroughly
trimmed from any soft tissue (i.e. periosteum, mucosa) and rinsed again several times. The bones were crunched into small fragments with the help of pliers and strong bone cutters, and when possible, scalpels. The bones were then placed in the culture flasks as done for the gingival explants.

The medium used was the same as for the fibroblasts, as well as the feeding and cell subculturing. However, it took more time for the bone cells cultures to be established. The first subculture was carried out usually at 4 to 6 weeks, and each subsequent passage took approximately 14 days. The cells were always divided into three in each passage, as for the fibroblasts, and were used for the experiments between the 5th and 8th passage. Freezing procedures were the same as described for the fibroblasts. The procedure for counting and preparing the cells for the experiments was the same as for the fibroblasts.

**Alkaline phosphatase activity**

The osteoblasts were stained for alkaline phosphatase, in order to confirm their osteoblastic characteristics. This was performed using a commercial kit from Sigma Diagnostics, based on the method of Ackerman (1962). Briefly, the cells were plated in a slide chamber, at 10,000 cells/chamber, and left to adhere overnight. The slides were fixed by immersion in citrate buffered acetone for 30 seconds. They were rinsed after with deionized water for 45 seconds. Then they were immersed in alkaline-dye mixture and incubated at room temperature for 30 minutes, protected from light. After 30 minutes, the slides were rinsed with deionized water for 2 minutes, and placed in Mayer's hematoxylin solution for 10 minutes. The positive alkaline phosphatase activity results in blue nuclear stain (Fig. 4.3).

**Human umbilical vein endothelial cells (HUVEC)**

The endothelial cells used were a cell line (EC403). These cells were cultured in a similar way as described previously. The main differences were the speed of proliferation and the medium used. The medium used was the 199 medium (Sigma), supplemented with: (a) heat-inactivated fetal bovine serum (HIFBS) - 10% v/v (Sigma), (b) L-glutamine - 2 mM (Sigma), and (c)
penicillin/streptomycin - 100 U/ml each (Gibco BRL). During subculturing, the cells reached confluence every 3-4 days, when divided into three. Freezing procedures were the same as described for the fibroblasts.

Because of the speed of proliferation, we plated the cells at only $2 \times 10^5$ cells per well, using 199 medium with 0.5% HIFBS. After 24 hours of incubation, the cells were ready for insonation.

Figure 4.3 - Human mandibular osteoblasts stained for alkaline phosphatase.

3.3. The ultrasound application model

As mentioned in the introduction of this chapter, the ultrasound application model used in the pilot study (Chapter 3) was modified for the following experiments. In the old set-up, there was no control of temperature, and the
use of caster oil could represent a problem since it could reflect and absorb the ultrasound waves.

**Figure 4.4** - Schematic diagram of the ultrasound application model used in the *in vitro* assays in this and in the following chapters

Therefore it was decided to construct another ultrasound application model, which allowed a more precise temperature control. We used a thermostatically controlled water bath (Elektrothermal, Germany), which was set to 37°C. The internal dimensions were 20 cm in diameter and 5 cm deep. The water bath tank was covered on the inferior and side walls with ultrasound-absorbing rubber (dimpled matting). This was filled with distilled, deionised, demineralised water, changed before each experiment.

The cells used in the experiments were plated in 6-well culture plates (Corning). The volume of media used was 5 ml, to allow at least ~5mm distance between the US transducer head and the cells. The plates were returned to the incubator for at least one hour before the US treatment.

Immediately before the US treatment, the plates were placed over the water surface of the water tank, floating, avoiding the formation of air bubbles between the plate and the water surface (Fig. 4.4 and 4.5). The transducer
head was held by a microscope stand, which was placed over a rotating platform/shaker (Edmund Bühler, Germany, model KL2), set to 30 rotations/min. In this way, the transducer was constantly moved while the ultrasound was applied, avoiding standing waves formation.

Figure 4.5 - Photograph of the ultrasound application model as shown in figure 4.4.

The whole apparatus (water bath, transducer head and the rotating platform) was set up in a sterile air cabinet (Microflow Pathfinder, Intermed), as shown in figure 4.6. The transducer head was swabbed thoroughly with 70% isopropyl alcohol BB (Azowipe, Vernon Carus), left to dry, and immersed vertically into the culture well, just touching the surface of the medium. The transducer head from the 45 kHz ultrasound machine has a hemispherical shape, therefore, only approx. 2 cm² of the total area of 12.8 cm² was immersed.

Each well was insonated for 5 minutes, and the control group (sham-insonation) was treated in the same way, but with the ultrasound generator switched ‘off’. In the same experiment both machines were evaluated, at the 4 intensities mentioned earlier in this chapter, and each intensity was applied to 5
wells (n=5). After insonation, 1.5 ml of media was removed to concentrate any factors produced, and the plates were cultured for a further 18 hours in the 37°C air/CO₂ incubator. After the 18 hours incubation period, another 1.5 ml of media was removed and stored for cytokine assays.

Figure 4.6 - The ultrasound application model set in the sterile air flow cabinet

3.4. Cell proliferation assay (DNA synthesis)

The cell proliferation assay is used is schematically shown on figure 4.7. After the 18 hours incubation period, the cells were radiolabelled with 5-³H thymidine (Radiochemical Centre, Amersham) to a final concentration of 0.5 µCi/ml in 1.5 ml of culture medium. The cells were reincubated for further 6 hours.

The medium was removed, and 1 ml of 5% trichloroacetic acid (TCA) was added, stopping the culture, and left at 4°C for at least 2 hours. The TCA was removed, and the cells washed 3 times with PBS. Then 300µl of NaOH 0.5M
was added to each well, and left for 20-30 min at 4°C. This was removed and transferred to scintillation vials (Minitubes, Hughes and Hughes Ltd., England) containing 200μl of acetic acid 0.5M. Scintillation fluid (Unisolve 1, Koch-Light) was added to each tube (3 ml), mixed gently and left in the dark for about one hour. Radioactivity was measured with a Beta counter (Rackbeta, LKB) with external standardisation, expressed in disintegrations per minute (DPM).

Subculture cells using 2% HIFCS

Apply US regimen, incubate for 18h

Label cells with 5-^H Thymidine for 6h

Add 5% TCA, leave at 4°C for 2h

Wash 3x with PBS

Add NaOH 0.5M (digest cells)

Transfer to scintillation vials

Measure radioactivity on Beta-counter

Figure 4.7 - Schematic representation of the cell proliferation assay (DNA synthesis)

3.5. Statistical analysis

Each insonation experiment was repeated at least twice. The result tables and graphs show the observations of one representative experiment. The number of observations for controls and for each intensity evaluated was
five (n=5). The values obtained in DPM's in the cell proliferation assays were transformed into percentages of the reading of the sham insonated controls, which were considered as 100%. All test values were compared with, and graphically presented as a percentage of control values. The results obtained were analysed in Excel®, and the values for the treatment groups were compared to the control group using ANOVA single factor and two tailed Student's 't' test for unpaired samples. Significance was accepted at the p<0.05 level or higher, as shown in the graphs.

4. Results

The cell proliferation assays showed an increase in DNA synthesis, as measured by the incorporation of $^3$H-thymidine in all three cell types. This was a direct effect of the ultrasound on the cells, and was observed with both ultrasound machines. As mentioned, these experiments were performed at least twice.

Table 4.1 shows the results obtained for the human gingival fibroblast cells. With the cells treated using 45 kHz ultrasound (Fig 4.8-A), the most significant results were an increase in cell proliferation of 30% at 15 mW/cm$^2$ (SA), and of 44% with 50 mW/cm$^2$ (p<0.01). With the 1MHz ultrasound group (Fig. 4.8-B), there was an increase of 47% at 0.7 W/cm$^2$ (p<0.01) and of 39% at 1.0 W/cm$^2$ (p<0.05).

Table 4.2 shows the results for human mandibular osteoblasts. In the 45 kHz treated group (Fig. 4.9-A), a more uneven distribution occurred, showing an increase of 32% at 5 mW/cm$^2$, and of 35% at 30 mW/cm$^2$ (p<0.05 and p<0.01). In the cell group treated with 1MHz ultrasound (Fig. 4.9-B), an increase in DNA synthesis at the higher intensities was observed, similar to the observation in the fibroblasts. This was in the order of 34% at 0.7 W/cm$^2$ (p<0.01) and of 52% at 1.0 W/cm$^2$ (p<0.001).

Table 4.3 summarises the observations for the human umbilical vein endothelial cells. In general, there was less cell proliferation than in the other
cell types, around 10%. When the cells were treated with the 45 kHz ultrasound (Fig. 4.10-A), all intensities significantly stimulated cell proliferation, at levels between 9 and 12% (p<0.05 to p<0.001). In the 1 MHz group (Fig 4.10-B), the same pattern observed in the previous cell groups remain, with more cell proliferation observed at higher levels of ultrasound intensity, reaching significance at 0.7 W/cm² and 1.0 W/cm² (p<0.05 an p<0.01 respectively).
Table 4.1 - Cell proliferation induced by 45 kHz and 1 MHz therapeutic ultrasound on human gingival fibroblast cells

45 kHz ultrasound, continuous

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* - Intensity in miliWatts/cm^2 (SA)
** - Intensity in Watts/cm^2 (GAPA)
D.P.M. - Disintegrations per minute (^H thymidine radioactivity)
Figure 4.8 - Cell proliferation induced by 45 kHz (A) and 1 MHz (B) therapeutic ultrasound on *human gingival fibroblast cells*. Controls received same treatment but with the US generator switched 'off'. Values are given as percentages (%) of the controls + S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
Table 4.2 - Cell proliferation induced by 45 kHz and 1 MHz therapeutic ultrasound on *human mandibular osteoblasts*

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* - Intensity in miliWatts/cm² (SA)
** - Intensity in Watts/cm² (APA)
D.P.M. - Disintegrations per minute (³H thymidine radioactivity)
Chapter 4 - Cell proliferation (DNA synthesis) induced by therapeutic ultrasound

Figure 4.9 - Cell proliferation induced by 45 kHz (A) and 1 MHz (B) therapeutic ultrasound on human mandibular osteoblasts. Controls received same treatment but with the US generator switched 'off'. Values are given as percentages (%) of the controls + S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
Table 4.3 - Cell proliferation induced by 45 kHz and 1 MHz therapeutic ultrasound on *human umbilical vein endothelial cells (HUVEC)*

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### 1 MHz ultrasound, pulsed 1:4

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<td>0.001808</td>
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</table>

* - Intensity in miliWatts/cm²
** - Intensity in Watts/cm²
D.P.M. - Disintegrations per minute (³H thymidine radioactivity)
Figure 4.10 - Cell proliferation induced by 45 kHz (A) and 1 MHz (B) therapeutic ultrasound on human umbilical vein endothelial cells (HUVEC). Controls received same treatment but with the US generator switched ‘off’. Values are given as percentages (%) of the controls + S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
5. Discussion

Several studies using the $^3$H-thymidine incorporation assay have shown that ultrasound stimulates DNA synthesis. Rabbit ear wounds exposed to 3 MHz ultrasound showed increased uptake of $^3$H-thymidine (Dyson \textit{et al}, 1970), as well as cultured mouse tibial epiphyses (Elmer and Fleisher, 1974), concavalin A stimulated lymphocytes (Repacholi \textit{et al}, 1979) and mouse L cells (Kondo and Yoshii, 1985). Hart (1993) suggests that $^3$H-thymidine incorporation during DNA repair, following ultrasound induced DNA damage, may influence the results of these studies, indicating a higher cell proliferation.

Hart (1993) could not demonstrate a direct effect of ultrasound on human skin fibroblasts proliferation when using 3 MHz pulsed ultrasound, at 0.1 and 0.5 W/cm$^2$. However he used a different insonation model, using cells in suspension, and measured cell proliferation with the methylene blue assay. Furthermore, we used different ultrasound frequencies and intensities.

Several studies reported the effects of ultrasound on cellular growth \textit{in vitro}, but these were usually carried out to evaluate ultrasound safety or selective killing for cancer therapy (Hart, 1993). Therefore, the intensities used in these studies were much higher then the therapeutic range, and the end point was cell death or loss of proliferative capacity. The limited number of studies that have employed ultrasound at therapeutic levels have used frequencies usually around 1MHz, in continuous mode. These reported either no effect on cellular growth at intensities up to 1 W/cm$^2$ (Kaufman \textit{et al}, 1977; Decat and Leonard, 1984), or loss of proliferative capacity at higher intensities up to 3 W/cm$^2$ (Kaufman \textit{et al}, 1977; Ciaravino and Miller, 1978; Kaufman and Miller, 1978; Fu \textit{et al}, 1980). The loss of cellular proliferative capacity in response to insonation \textit{in vitro} has been associated with cavitation damage as under non-cavitating conditions such losses are not observed (Armour and Cory, 1982; Ciaravino \textit{et al}, 1981; Coakley \textit{et al}, 1971), even at intensities far in excess of the therapeutic range (Bleaney \textit{et al}, 1972).
The proliferation of fibroblasts and osteoblasts is a direct measure of connective tissue formation, and the proliferation of endothelial cells can be used as a measure of angiogenesis (Tompach et al, 1997). Hence evidence of enhanced cell proliferation can have crucial value in hypocellular osteoradionecrosis.

The proliferation assays (DNA synthesis) showed that both ultrasound machines were able to induce cell proliferation, in fibroblasts (Fig. 4.8), osteoblasts (Fig. 4.9), and HUVEC cells (Fig. 4.10). Since we had similar \textit{in vitro} stimulations with both ultrasound machines, we recommend the use of 'long wave' ultrasound for the management of osteoradionecrosis due to its overall clinical advantages. These are (a) it has a much higher penetration depth, allowing treatment of thick tissues, penetrating centimetres rather than millimetres as with traditional ultrasound; (b) it uses low intensity energy levels, causing less heat production; (c) it is used in continuous mode, reducing the treatment time; and (d) it has a \textit{spherical} head, giving a bigger effective treatment area.

The level of stimulation with fibroblasts and osteoblasts was around 30% to 50%, and with the HUVEC cells, around 10%. These results are comparable to those obtained with HBO therapy, which showed a 50% increase in cell proliferation in fibroblasts and endothelial cells, 18-24 h after 15-120 min HBO treatment at 2.4 ATA (Tompach et al, 1997). Our findings also support \textit{in vivo} assays, where fibroblast density in rat skin wounds 5 days post wounding was higher after the use of ultrasound (Young and Dyson, 1990a).

With the 45 kHz ultrasound no obvious intensity pattern could be observed, and all intensities gave significant results. However with the 1Mhz ultrasound, an obvious tendency to get more proliferation at higher intensities was noted (Fig. 4.8B - 4.10B). This tendency was inversely proportional to that observed in the collagen production assays (Chapters 3 and 5).

In summary, the results reported in this chapter show that therapeutic ultrasound stimulates the cell proliferation of fibroblasts, osteoblasts and endothelial cells. This could explain the empirical success in the management
of osteoradionecrosis with its recognised hypocellularity and hypovascularity. Higher intensities of ultrasound may be used in the initial phases of therapeutic ultrasound, to induce cell proliferation, and later, lower intensities should be used to stimulate cell differentiation, collagen production. Since ‘long wave’ ultrasound had similar results as the 1 MHz ultrasound in terms of cell proliferation, we recommend its use for the treatment and prevention of osteoradionecrosis due to its overall clinical advantages.
Chapter 5 - Stimulation of Matrix Synthesis by Therapeutic Ultrasound

1. Introduction

The previous chapter described the direct effects of ultrasound on cell proliferation, a crucial aspect of the proliferative phase of repair. The present chapter will try to show if therapeutic levels of ultrasound are capable of stimulating the cell differentiated function as well. This can be translated in the case of fibroblast and osteoblast cells, as collagen and non-collagenous protein (NCP) synthesis. The ability of induce the differentiated function of cells by therapeutic ultrasound will be crucial in the proliferative and remodelling phases of wound and bone repair.

To our knowledge there has not been any report showing that ultrasound induces bone healing in tissue culture, as measured by collagen and NCP synthesis of osteoblasts or bone tissue cultures.

Collagen production in fibroblasts induced by therapeutic ultrasound was previously reported by Harvey et al (1975): However these were skin fibroblasts, for this study, it was decided to use gingival fibroblasts, which are more related to intra-oral soft tissue healing.

In chapter 3 the production of collagen and NCP stimulated by ultrasound (3 MHz, pulsed 1:4), suggested bone formation. The same method for estimating collagen and non-collagenous (NCP) synthesis has also been used in this series of experiments (Meghji et al, 1992). However, both the ultrasound application model and the ultrasound machines were different. Furthermore, not only the mice calvaria model was used, as in chapter 3, but also human gingival fibroblast and mandibular osteoblast primary cell cultures.
2. Hypothesis and Aims

These experiments again examine the hypothesis that ultrasound promotes wound repair and bone repair by enhancing cell differentiation, leading to the stimulation of collagen and non-collagenous protein synthesis.

The aims of this chapter are:

1. To identify if therapeutic ultrasound is able to stimulate the production of collagen and non-collagenous proteins in human gingival fibroblasts, mandibular osteoblasts and neonatal mice calvaria.

2. Compare two types of ultrasound machines, the 'traditional' (1 MHz, pulsed 1:4), and the 'long wave' (45 kHz, continuous), at 4 intensities each, in order to establish which machine and intensity have the best stimulatory effect.

3. Material and Methods

3.1. The ultrasound application model and the ultrasound machines

The ultrasound machines were the 1 MHz and the 45 kHz frequencies, described in chapter 4.

3.2. Cell and tissue culture

The cells used were human primary cell cultures described in chapter 4. Gingival fibroblasts and mandibular osteoblasts were used, but instead of platting them at $1.5 \times 10^5$ cells per well (6 well plate), they were plated at $3.0 \times 10^5$ so that cells are confluent at 24 hours, when the ultrasound was applied. Vitamin C (ascorbic acid) was added to the medium used for these assays (50μl/ ml of medium), as it is essential for collagen synthesis.

The bone tissue culture used was the calvaria neonatal mice model (chapter 3).
3.3. Collagen and non-collagenous protein synthesis assay

The assay used for measuring the collagen and NCP synthesis, was the same used in chapter 3, using pepsin extraction to separate collagen from NCP, and labelled with $^3$H labelled proline. In chapter 3, this assay was used only for the mice calvaria, but in this chapter it was used in monolayer cell cultures. Therefore, there are some differences between the assays, which will be briefly described.

After insonation, 2.0 ml of the 5.0 ml of medium were discarded, and after 18 hours, 1.5 ml were stored for further assays. The cells were then radiolabelled for the last 6 hours of a 24 hour incubation period with 3 $\mu$Ci of L-$[5-^3$H]proline (sp act 18 ci/m.mol; Radiochemical Centre, Amersham) in 5 $\mu$l of culture medium, giving a final concentration of 2 $\mu$Ci/ml.

At the end of the 6 hours, 700 $\mu$l of the cell culture supernatant was removed and transferred to 1.5 ml Eppendorf tubes. Proteins were precipitated by the addition of 700 $\mu$l of 10% trichloroacetic acid (final concentration - 5% TCA) for 2 hours at 4°C. The tubes were centrifuged (3000 rpm for 30 min.) to remove unbound isotope and small peptides, and the supernatant discarded. The pellets were dissolved in 1.0 ml of 0.5M acetic acid containing pepsin (0.5 mg/ml; EC 3.4.4.1, Sigma) and incubated for 16 hours at 4°C. At the end of the incubation period, 100 $\mu$l of acid soluble rat skin collagen in 0.5M acetic acid (2mg/ml) was added to each tube as a carrier for labelled collagen during subsequent purification. The rest of the procedure, using the NaCl precipitations, is the same as described for the assay in the calvaria (Chapter 3).

3.4. Statistical analysis

Each insonation experiment was repeated at least twice. The result tables and graphs show the observations of one representative experiment. The number of observations for controls and for each intensity evaluated was five (n=5). The values obtained in DPM's in the collagen/NCP assays were transformed into...
percentages of the reading of the sham insonated controls, which were considered as 100%. All test values were compared with, and graphically presented as a percentage of control values. The results obtained were analysed in Excel®, and the values for the treatment groups were compared to the control group using ANOVA single factor and two tailed Student's 't' test for unpaired samples. Significance was accepted at the p<0.05 level or higher, as shown in the graphs.

4. Results

The results showed that therapeutic ultrasound in the parameters studied stimulated collagen and non-collagenous protein synthesis in the cell cultures and the calvaria model. There were differences regarding the intensity used and the type of ultrasound machine, and these can be seen in the tables and figures shown.

Tables 5.1 and 5.2 show the results of the effects of ultrasound on neonatal mice calvaria. With the 45 kHz machine (Table 5.1), optimum intensities were 15 and 30 mW/cm² (SA), and with the 1 MHz machine, the best results were obtained at low intensities, 106% increase at 0.1 W/cm² (SAPA) and 32% at 0.4 W/cm² (SAPA). Table 5.2 shows the results for the non-collagenous protein synthesis, with optimum results at 5 and 15 mW/cm² (SAPA) for the 45 kHz ultrasound, and 0.1 and 0.7 W/cm² (SAPA) with the 1 MHz machine. These results are similar to those observed in chapter 3, however, they did not reach statistical significance due to the big standard deviation observed. Figure 5.1 summarises the observations of collagen and NCP production by neonatal mice calvaria induced by therapeutic ultrasound.

Tables 5.3 and 5.4 show the results obtained for the human gingival fibroblast cells, for collagen and NCP synthesis respectively. The collagen production (Table 5.3) was significantly increased with both machines. The 45 kHz showed production levels between 23 and 44%, and the maximal synthesis at intensities of 15 and 50 mW/cm² (SA) (p<0.05). With the 1 MHz group, the
levels of stimulation varied between 15 and 57%, reaching significance again at lower intensities, of 0.1, 0.4 and 0.7 W/cm² (SA) (p<0.001, p<0.05, p<0.01 respectively). Table 5.4 shows the results for the non-collagenous protein synthesis. Again, stimulation could be observed, however not at significant levels, in both machines. Figure 5.2 summarises the observations of collagen and NCP production in human gingival fibroblasts induced by therapeutic ultrasound.

Tables 5.5 and 5.6 show the results obtained for the *human mandibular osteoblasts*. These were the best and most significant results observed in this chapter. Table 5.5 shows the results of collagen synthesis stimulation. With the 45 kHz machine, levels ranged between 70 and 112%, reaching significance at 30 mW/cm² (SA) (p<0.05). With the 1 MHz machine, the levels were lower, between 17 and 57%, and did reach significance at 0.1 W/cm² (SA). Table 5.6 shows the results of non-collagenous protein synthesis. With the 45 kHz group, all intensities showed significant increase in NCP synthesis, ranging between 59 and 88% of controls (p<0.01). With the 1 MHz machine, the increase in NCP synthesis was seen at the lower intensities of 0.1 and 0.4 W/cm² (SA), reaching significance at 0.4 W/cm² (SA) (p<0.05). Figure 5.3 summarises the observations of collagen and NCP production in human mandibular osteoblasts induced by therapeutic ultrasound.
Chapter 5 - Stimulation of matrix synthesis by therapeutic ultrasound

Table 5.1 - Collagen protein synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on neonatal mice (MF-1) calvaria

### 45 kHz ultrasound, continuous

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<th>50</th>
<th>Control</th>
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* - Intensity in miliWatts/cm²
** - Intensity in Watts/cm²
D.P.M. - Disintegrations per minute (³H thymidine radioactivity)
Table 5.2 - Non-collagenous protein (NCP) synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on *neonatal mice (MF-1) calvaria*

### 45 kHz ultrasound, continuous

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<th>15</th>
<th>30</th>
<th>50</th>
<th>Control</th>
</tr>
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### 1MHz ultrasound, pulsed 1:4

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* - Intensity in miliWatts/cm²
** - Intensity in Watts/cm²
D.P.M. - Disintegrations per minute (³H thymidine radioactivity)
Chapter 5 - Stimulation of matrix synthesis by therapeutic ultrasound

Figure 5.1 - Collagen and non-collagenous protein (NCP) synthesis induced by 45 kHz (A) and 1 MHz (B) therapeutic ultrasound on neonatal mice (MF-1) calvaria. Controls received same treatment but with the US generator switched 'off'. Values are given as percentages (%) of the controls + S.E.M. Significance level as compared to controls.
Table 5.3 - Collagen protein synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on *human gingival fibroblast cells*

### 45 kHz ultrasound, continuous

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Table 5.4 - Non-collagenous protein (NCP) synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on *human gingival fibroblast cells*

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* - Intensity in miliWatts/cm² (SA)
** - Intensity in Watts/cm² (SAPA)
D.P.M. - Disintegrations per minute (^H thymidine radioactivity)
Figure 5.2 - Collagen and non-collagenous protein (NCP) synthesis induced by 45 kHz (A) and 1 MHz (B) therapeutic ultrasound on *human gingival fibroblast cells*. Controls received same treatment but with the US generator switched 'off'. Values are given as percentages (%) of the controls + S.E.M. Significance level as compared to controls: * p<0.05, ** p<0.01, *** p<0.001.
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* - Intensity in miliWatts/cm² (SA)
** - Intensity in Watts/cm² (SAPA)
D.P.M. - Disintegrations per minute (³H thymidine radioactivity)
Table 5.6 - Non-collagenous protein (NCP) synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on *human mandibular osteoblasts*

### 45 kHz ultrasound, continuous

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### 1MHz ultrasound, pulsed 1:4

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* - Intensity in miliWatts/cm² (SAP)
** - Intensity in Watts/cm² (SAPA)
D.P.M. - Disintegrations per minute (³H thymidine radioactivity)
Figure 5.3 - Collagen and non-collagenous protein (NCP) synthesis induced by 45 kHz (A) and 1 MHz (B) therapeutic ultrasound on human mandibular osteoblasts. Controls received same treatment but with the US generator switched 'off'. Values are given as percentages (%) of the controls + S.E.M. Significance level as compared to controls: * p<0.05, ** p<0.01, *** p<0.001.
5. Discussion

Wound healing normally proceeds through a complex but orderly series of events involving inflammatory, proliferative and remodelling phases. The proliferative phase of wound healing consists of rapid fibroblast and osteoblast growth and increased synthesis of collagen/NCP in response to chemotactic factors released during the inflammatory phase. Finally, fibroblasts and osteoblasts maintain matrix production which accumulates during the remodelling phase.

This series of experiments clearly shows that therapeutic ultrasound induces collagen and non-collagenous protein synthesis in fibroblasts, osteoblasts and in whole bone. Therefore, ultrasound not only induces cell proliferation, as shown in chapter 4, but also directly induces differentiated function of those cells. This indicates that ultrasound acts not only in the inflammatory phase of repair, but also in the proliferative and remodelling phases, where collagen and NCP production mainly occurs.

The results presented here reinforce those performed on the effects of ultrasound on experimental murine granulation tissue generation and remodelling (Pospisilova, 1976). This study showed that in the inflammatory phase there was significantly more collagen, glycoprotein and glycosaminoglycan. In the proliferative phase, there was more collagen and glycoprotein, but significantly less glycosaminoglycan, suggesting that ultrasound facilitates more rapid, and perhaps more extensive repair, by encouraging both earlier and more extensive collagen synthesis.

Collagen production induced by ultrasound in animal wounds has been studied by measuring the incorporation of $^3$H hydroxyproline. Webster et al (1979) using 3 MHz (0.4 W/cm$^2$ (SAPA)) ultrasound for the treatment of cryosurgical lesions in the flank skin of rats. They showed that the wounds contained more collagen than controls at seven days after injury. Jackson et al (1991) studied the repair of Achilles tendons in the rat, and showed that 1 MHz
ultrasound, at 1.5 W/cm\(^2\) induced more collagen synthesis after 5 days of injury than controls.

In our assays there is a clear tendency for increased collagen production in the lower intensities when using 1MHz ultrasound. The fibroblasts group treated with 1MHz (Fig. 5.2B) showed increases of collagen production of 48%, 57% and 52%, at the intensities of 0.1, 0.4 and 0.7 W/cm\(^2\) (p<0.01, p<0.05 and p<0.01 respectively). When these cells were treated with 45 kHz ultrasound (Fig. 5.2A), the last three intensities showed increases in collagen ranging from 37% to 44%, although significant only at 15 and 50 mW/cm\(^2\) (p<0.01 and p<0.05).

The collagen/NCP production by osteoblasts probably shows the most significant results, as far as bone repair is concerned, since these are the target cells involved in the repair in osteoradionecrosis. In this case, a clear superiority of the 45 kHz ultrasound was observed. In the 1MHz treated cells the previously observed tendency to increase the collagen production at the lower intensities continues (Fig. 5.3B). An increase of 55% and 38% at 0.1 and 0.4 W/cm\(^2\) was observed in collagen synthesis (p<0.05). With the 45 kHz ultrasound (Fig. 5.3A), the levels of collagen production by osteoblasts were much higher, 112% at 30 mW/cm\(^2\) (p<0.05).

These results are similar to those observed by Harvey et al (1975), who found an intensity dependent increase of both collagen and non-collagenous protein (NCP) synthesis by insonated human skin fibroblasts in suspension. Fibroblasts exposed to continuous ultrasound (0.5 W/cm\(^2\) \(\text{SA}\)) showed a 20% increase in collagen secretion which was increased to 30% when the ultrasound was pulsed (0.5 W/cm\(^2\) \(\text{SAPA}\)). Webster et al (1978) also observed lower increases in protein synthesis by fibroblasts, of 29% using a 3MHz signal at 0.5 W/cm\(^2\). We showed that 3 MHz pulsed ultrasound stimulates bone formation (collagen and NCP production), using a mice calvaria model, with best results at 0.1, 0.25 and 0.5 W/cm\(^2\) (Chapter 3; Reher et al, 1997a).

The non-collagenous protein synthesis was also stimulated, but it could not be correlated to the collagen synthesis in the majority of the assays. This
increase in NCP synthesis was seen only in osteoblasts, treated with the 45 kHz ultrasound (Fig. 5.3A). Osteoblasts have been shown to increase non-collagenous proteins, without increasing collagen synthesis, when they are mechanically stretched in flexible culture dishes (Hasegawa et al, 1985). This may be an important observation as the NCP contains many cytokines, growth factors, angiogenic factors and enzymes which may enhance healing and angiogenesis.

The results obtained with the mouse calvaria did not show any significant differences, due to a bigger variability between samples, and perhaps a larger sample size should be used. However, the pattern of higher production at lower intensities with 1 MHz, and higher production of collagen with 15 and 30 mW/cm$^2$ with 45 kHz could be observed here as well. As mentioned in chapter 3, both osteoblasts and fibroblasts are present in the mouse calvaria and may be responsible for this protein synthesis. However, using injected $^3$H-labelled proline it was found that osteoblasts in bone are more active than fibroblasts and produce the greatest amount of bone matrix (Fitton-Jackson & Randall, 1956). Therefore the protein synthesis stimulation observed in this study may be attributed to osteoblastic rather than fibroblastic activity.

Comparing the effects on collagen/NCP production in the two ultrasound machines (45 kHz and 1 MHz), it can be concluded that both produced good results, and that each has a particular intensity range were it is more effective. When using 45 kHz, the best response was obtained by using 15 or 30 mW/cm$^2$ with 45 kHz could be observed here as well. As mentioned in chapter 3, both osteoblasts and fibroblasts are present in the mouse calvaria and may be responsible for this protein synthesis. However, using injected $^3$H-labelled proline it was found that osteoblasts in bone are more active than fibroblasts and produce the greatest amount of bone matrix (Fitton-Jackson & Randall, 1956). Therefore the protein synthesis stimulation observed in this study may be attributed to osteoblastic rather than fibroblastic activity.

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There are several advantages of using the long wave ultrasound (Bradnock et al, 1996); it has a higher penetration depth, generates less heat, reduces treatment time since uses continuous mode, and the spherical head applicator gives it a larger effective treatment area. Since its in vitro effects are similar or even better then those of the 1 MHz machine, we recommend it for the treatment of mandibular osteoradionecrosis.
As mentioned in the previous chapter, when using 1 MHz ultrasound, an obvious tendency to get more proliferation at higher intensities was noted (Fig. 4.8B - 4.10B). This tendency was inversely proportional to that observed in the present chapter, where collagen production was higher at lower intensities. This pattern was also observed in the pilot study. However, when analysing the 45 kHz results, no obvious pattern could be noted. Perhaps one should conclude that when using 1 MHz ultrasound, higher intensities should be used in the initial phases of repair, to induce cell proliferation, and later, lower intensities should be used to stimulate cell differentiation, such as the production of collagen.

In summary, therapeutic ultrasound stimulates collagen and non-collagenous protein synthesis, suggesting that it may be effective in the proliferative and remodelling phases of repair. Therapeutic ultrasound at 1MHz (pulsed 1:4) stimulates fibroblast and bone matrix protein synthesis at the lower end of the currently used therapeutic scale (0.1 and 0.4 W/cm$^2$ (SAPA)), and with 45 kHz, the best results are at 15 and 30 mW/cm$^2$(SA). These results are comparable to those obtained with fibroblasts in vitro, and sustain the use of low-intensity US doses for in vivo applications. Since 'long wave' ultrasound had similar results as the 1 MHz ultrasound in terms of collagen/NCP synthesis, we recommend its use for the treatment and prevention of osteoradionecrosis due to its overall clinical advantages.
Chapter 6 - Bone Resorption Induced by Therapeutic Ultrasound

1. Introduction

Bone is a highly dynamic connective tissue with a capacity for continuous remodelling which is normally a balance between formation and resorption. It is composed of a variety of cell types and an extracellular organic matrix which has become calcified (Meghji, 1992).

Bone resorption is the removal of the mineral and organic components of the extracellular matrix of bone under the action of osteolytic cells, of which the most important is the osteoclast. The osteocyte may function as a local bone resorber, ensuring the natural renewal of bone.

The process of bone resorption involves three steps, each of which may be individually regulated by physiological, pathological or pharmacological mediators. The first step involves the formation of osteoclast progenitors in the haematopoietic tissues, followed by their vascular dissemination and the generation of resting pre-osteoclasts and osteoclasts in bone itself. The second phase consists of osteoclast activation at the surface of the mineralised bone. Osteoblasts appear to play a major role, by not only ‘retracting’ to expose the mineral to the osteoclast and pre-osteoclasts, but also by releasing a soluble factor that activates these cells. The third step involves the activated osteoclast resorbing the bone (Meghji, 1992).

During bone resorption, the osteoclasts create for themselves cavities known as Howship’s lacunae, which correspond closely in size to the area of bone surface enclosed by the cell. This active surface is described as having a ruffled border which consists of many infoldings of the cell membrane, resulting in finger-like projections of the cytoplasm. Thus, an extensive surface is created
which is well suited for an intensive exchange between the cell and the bone, and is effectively sealed off from extracellular environment by a tight junction between the bone and the periphery of the osteoclast. Osteoclasts can also move about on the surfaces of bone and dentine slices in vitro and leave discontinuous tracks of resorptive activity behind them (Bohde et al, 1984).

In order to carry out bone resorption, the osteoclast can secrete organic acids, which maintain a sufficiently low pH in the micro-environment at the bone surface to dissolve the hydroxyapatite crystals. The resorption of the organic matrix is enzymatic, mainly by lysosomal enzymes. Biochemical studies have shown that there is good correlation between lysosomal enzyme release and the progress of bone resorption. The mediators that appear to increase bone resorption also appear to increase the synthesis and secretion of various lysosomal enzymes (Eilon and Raisz 1978).

The osteoclast lysosomes and their lytic enzymes participate in a continuous process of exocytosis and endocytosis at the ruffled border. Enzymes are released into the extracellular bone matrix (exocytosis) and the solubilised matrix components are taken up into the cell within vacuoles termed phagosomes (endocytosis) for further intracellular digestion. Other enzymes are metalloproteinases, such as collagenase and stromelysins, and serine proteinases, such as elastase.

Osteoblasts also release collagenase, and therefore contribute to matrix degradation, by digesting the non-mineralised layer of osteoid on the bone surface. They facilitate osteoclast activity by degrading the demineralized matrix when exposed by osteoclastic activity.

There are several mediators of bone remodelling, including parathyroid hormone (PTH), parathyroid hormone related peptide (PTHrP), vitamin D metabolites, cytokines (IL-1α, IL-1β, TNFα, TNFβ, IL-6), eicosanoids (prostaglandins, tromboxane, HETEs, leukotrienes), growth factors, bacterial products and mechanical stress.

This thesis is examining mechanical stress in bone remodelling, since ultrasound energy is a form of mechanical energy. The relationship between
mechanical forces and bone remodelling has been known for a century (Wolff, 1892). This is particularly apparent in cancellous bone, where immobilisation increases bone resorption and decreases formation (Weinreb et al, 1989), showing that mechanical stress is necessary for the normal coupling of bone remodelling.

Unlike hormones which act as agonists to a specific receptor, there is no known agonist-receptor binding when the lining periosteal fibroblasts, osteoblasts on the matrix surface and osteocytes detect mechanical forces (Pead et al, 1988). One of the major unanswered questions is how these bone cells respond. The cells may communicate with each other across gap junctions (Doty, 1981) by the production of appropriate factors when their relationship is disturbed. However, each cell is also attached to its surrounding matrix via specialised membrane receptors for extracellular matrix proteins called integrins (Hynes, 1987). Integrin sense physical forces that control gene expression by activation of the MAP kinase pathway (Schmidt et al, 1998). In this way, the cytoskeleton may play a key role in the physical anchorage of activated signalling molecules, which enables the switch of physical forces to biochemical signalling events.

The mechanisms through which mechanical stress may transduct into a biochemical signal are represented in figure 6.1. The first mechanisms occurs via stretch sensitive ions channels which have been described in the osteoblast membrane (Duncun and Misler, 1989)(Fig 6.1A). Another accepted model for transmembrane stimulation involves the mechanical stimulus (or hormone) activating a specific receptor on the cell surface (Gilman, 1987). This in turn activates intracellular guanine nucleotide-binding protein (G-protein) which together with the appropriate enzymes (phosphoinositidase C or adenylate cyclase) produces intracellular second messengers (Fig. 6.1B and C). The G protein-receptor link is a key amplification step in intracellular signalling. The second messengers for phosphoinositidase C are diacylglycerol (DAG) and inositol trisphosphate (IP$_3$). In the case of adenylate cyclase the second messenger is cyclic adenosine-3', 5'-monophosphate (cAMP) (Fig 6.1C). The end result of second messenger activity is a physiological event. For example,
bone resorption by PTH is mediated by cAMP. Sandy et al (1989a) have also shown an elevation of inositol trisphosphates (IP₃) by the short-term mechanical stimulation of osteoblasts in vitro and that prolonged stress released bone resorbing factors (Sandy et al, 1989b). Finally, another model for transmembrane signalling involves receptors with their own built-in enzyme activity. Here the binding of the messenger on the endoplasmic domain of the receptor activates an enzyme on the cytoplasmic domain of the receptor polypeptide (Fig 6.1D). This results in the production of the second messenger, cyclic-3', 5'-guanosine monophosphate (cGMP).

Figure 6.1 - Schematic diagram showing the major transmembrane signalling pathways. (A) Stretch sensitive ion channel, (B) and (C) second messenger systems, (D) receptor with built-in enzyme activity. Mechanical stress (like ultrasound) can stimulate the cell through mechanisms A-C. (after Meghji, 1992).
Although the osteoclast is the principal bone resorbing cell, it is the osteoblast that contains the receptors for the major bone resorbing agents such as parathyroid hormone (PTH), the eicosanoids (PGE₂, PGI₂, LTB₄, etc), 1,25-dihydroxy vitamin D₃ and cytokines such as interleukin 1 (IL-1) and tumour necrosis factor (TNF). The osteoblasts, having recognised the resorptive signal, somehow transmit it to the osteoclast. As yet these osteoblast-osteoclast factors have not been characterised and reports have suggested substances with diverse molecular weights are involved (McSheehy and Chambers 1986; Meghji et al, 1988). Osteoblasts therefore appear to play a 'pivotal role' in the regulation of bone resorption.

There are several inhibitors of bone resorption, including calcitonin, cortisol, interferon gamma (IFN-γ), biphosphonates, plicamycin and actinomycin D (Table 6.1).

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<tr>
<td>Glucocorticoids</td>
<td>Inhibits eicosanoids synthesis</td>
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<tr>
<td>Biphosphonates</td>
<td>Inhibits osteoclastic bone resorption probably by making the mineralised surface inaccessible to the cell by binding to the hydroxyapatite crystals</td>
</tr>
<tr>
<td>Indomethacin and aspirin</td>
<td>Inhibits prostaglandin synthesis (cyclo-oxygenase pathway)</td>
</tr>
<tr>
<td>Interferon-gamma</td>
<td>Inhibits both proliferation and differentiation of osteoclast progenitors</td>
</tr>
<tr>
<td>TGF-beta</td>
<td>Inhibits osteoclast formation and differentiation</td>
</tr>
<tr>
<td>Interleukin-1 receptor antagonist (IL-1ra)</td>
<td>Binds to IL-1 receptors. Effective against TNF as well</td>
</tr>
</tbody>
</table>

From Meghji, 1992

The osteoclast, apart from its osteolytic function, also plays an important role in the development and growth of bone by releasing polypeptide growth factors from the extracellular mineralised matrix (Fig. 6.2). These factors are generally referred to as bone-derived growth factors (BDGFs) and are known to include bone morphogenetic proteins (BMP), platelet-derived growth factor
(PDGF), acidic and basic fibroblast growth factor (aFGF, bFGF), transforming growth factor -beta (TGF\(\beta\)) and insulin-like growth factor 1 (IGF-1). This has been exploited by the use of decalcified bone matrix as an osteogenic agent in clinical practice (Glowacki et al, 1981).

**Figure 6.2** - The role of the osteoclast on osteoblast growth and function: on resorbing the mineralised matrix the osteoclast releases polypeptide growth factors which stimulate osteoblasts to proliferate and synthesise matrix proteins. (after Meghji, 1992)

Bone resorption at certain levels is crucial for the process of bone matrix turnover. Ultrasound has probably some effect on bone resorption since it has observed effects on bone fracture healing and osteoradionecrosis. However, in the literature there has been no study looking at bone resorption induced by ultrasound. The experiments reported in this chapter will try to address this point, firstly identifying if ultrasound promotes bone resorption, and finally trying to establish the possible mechanisms involved.
2. Hypothesis and Aims

This series of experiments tests the hypothesis that ultrasound promotes bone matrix turnover by the promotion of bone resorption.

The aims are:
1. To identify if therapeutic ultrasound is able to induce bone resorption using the neonatal mice calvaria bone resorption model.
2. Compare two types of ultrasound machines, the ‘traditional’ (1 MHz, pulsed 1:4), and the ‘long wave’ (45 kHz, continuous), at 4 intensities each, to establish which machine and intensity induce more bone resorption.
3. To establish the mechanism of action involved in the ultrasound-induced bone resorption, by the use of several inhibitors to the medium.

3. Material and methods

The ultrasound machines and the insonation model used were the same as in the two previous chapters.

3.1. Calvaria bone resorption assay

Preparation of the calvaria

Calvaria (frontal and parietal bones) were obtained by aseptic dissection of 5-day old MFI mice (Havian Olac), as previously described in chapter 3. The calvaria were washed free of blood and adherent brain tissue, divided along the sagittal suture and each half calvaria was cultured separately in a 6-well culture plate (Nunc/Sarstedt).

The half calvaria were placed on stainless steel grids of 1 cm² (Minimesh FDP quality, Expanded Metal Co. W. Hartlepool) shaped to support the bone at the interface between the surface of the culture medium and the gas phase, approximately 3 mm above the base of the culture plate, and 1.5ml of culture
medium was added to each well. The culture medium used was BGJb (Gibco) supplemented with: (a) heat-inactivated foetal calf serum serum (Wellcome Reagents, Beckenham) - 5% v/v, (b) freshly prepared L-ascorbic acid (BDH) - 50 µg/ml, (c) L-glutamine - 2 mM (Sigma), (d) penicillin/streptomycin - 100 U/ml each (Gibco BRL), and (e) sodium bicarbonate solution 7.5% (Sigma). Each 6-well plate contained 5 half-calvaria (one well was left empty), and was transferred into a humidified 5% CO₂/95% air incubator at 37°C.

The bones were 'prechultured' for 24 hours. This eliminates endogenous prostaglandins which accumulate more rapidly early in the culture (Katz et al, 1981). After this pre-incubation, the medium was replaced with fresh medium, in volumes of 5 ml for the bones that would be insonated. As internal negative control, BGJ medium alone was used, and for the positive control, medium containing PGE₂ at 10⁻⁶ M was used.

**Treatment application**

The original medium was replaced with 5 ml of fresh medium to increase the distance between the transducer head and the bone. The bones were returned to the incubator for 1 hour, and then the different ultrasound regimens were applied. Before insonation, the stainless steel grids were removed to avoid ultrasound reflection. The following treatment groups were used:

a) Internal control (BGJ) - BGJ medium only, without any bones, to act as a basal level of calcium in the media. This level was later subtracted from the readings obtained from the other groups (n=3).

b) Sham insonation (control group) - the ultrasound machine was switched off, but the transducer head was placed in the medium, kept the same time in the well, and the bones and medium were manipulated in the same way as for the treatment group. For each ultrasound machine (45 kHz and 1 MHz), one different control group was used (n=5).

c) PGE₂ (positive control group) - PGE₂ 10⁻⁶ M was used as positive control to stimulate resorption. The stock solution of PGE₂ was in ethanol, at 5x10⁻³ M
then diluted in BGJ medium. The maximum concentration of ethanol was 0.02% in the experimental group (n=5).

d) 45 kHz ultrasound treatment (4 groups) - the ultrasound was applied for 5 min for each half calvarium. The intensities evaluated were 5, 15, 30 and 50 mW/cm², with 5 bones for each group (n=5, per group).

e) 1 MHz ultrasound treatment (4 groups) - the ultrasound was applied for 5 min for each half calvarium. The intensities evaluated were 0.15, 0.4, 0.7 and 1.0 W/cm², with 5 bones for each group (n=5, per group).

After the insonation/sham insonation, 3.5 ml of the medium was removed, leaving 1.5 ml to cover the bones, the grids were returned to the wells, and the bones carefully repositioned over them. The cultures were incubated for a further 48 hours and resorption was measured as the release of calcium into the culture medium over this period.

**Measurement of calcium release**

The culture medium was removed from each well at the end of the incubation period, using a Pasteur pipette, and transferred into 2 ml autoanalyser conical-bottomed cups (Chem Lab Instruments, Essex). Calcium concentrations were measured colorimetrically on an autoanalyser (Chem Lab Instruments, Essex), by using the metal complexing dye cresolphthalein complexone (CPC) (Gitelman, 1967). The concentration of dialysed calcium was then determined colorimetrically by complexing with CPC.

Briefly, the sample cups containing the media or standard calcium solutions (5, 10, 15 mg/dl - Sigma) were placed on the sample tray which held 40 samples a time. One aliquot of 100 μl was removed from each sample through the stainless steel sampling probe followed by a 20 s wash with distilled water after each sample. The samples were mixed with 1M HCl containing 8-hydroxy-quinoline (8HQ) at 2.5 g/L (which eliminated interference by magnesium), and dialysed against a solution of similar composition containing CPC at 0.7 g/L. The dialysate was then mixed with 2-amino-2-methylpropano-l-ol (AMP) (90 g/L). The absorbance of the resultant purple-coloured solution was measured in a 15 mm flow cell at 570 nm, and plotted on a chart recorder at 0.5
cm/min. Calcium concentrations were calculated from the absorbance peak heights measured against the standard curve. The sensitivity of the assay is 0.05 mg/dl. This is based on the ability to detect change at a significance level (p<0.05) with 5 replicate samples.

The calcium levels in the calvaria cultures were compared with that in the internal control group, which consisted of incubated BGJ medium alone, without any bones (n=3). All calcium values obtained were subtracted from the value of this standard, and expressed as mg/dl.

3.2. Mechanism of ultrasound induced bone resorption

To investigate which was the mechanism of action involved in the bone resorption induced by ultrasound, several inhibitors of bone resorption were evaluated. After the 24 hours pre-incubation period, the medium was changed, and the inhibitors were added to the medium. Each group had 5 replicates, treated with the same ultrasound regimen. This was 1 MHz, pulsed 1:4, at the intensity of 1.0 W/cm², since this intensity gave high bone resorption values in the previous studies. The following groups were used:

a) Internal control (BGJ) - BGJ medium only, without any bones, to act as a basal level of calcium in the media. This level was later subtracted from the readings obtained from the other groups;
b) Ultrasound control - normal medium was used, without inhibitors, and the ultrasound was applied (1 MHz, pulsed 1:4, 1.0 W/cm²);
c) Ultrasound and IRAP - IL-1 receptor anti-agonist protein, used at 100μg/ml;
d) Ultrasound and MK886 (Merck Frosst) - a selective inhibitor of 5-lipoxygenase activity, used at 10⁻⁸M;
e) Ultrasound and BWA70C (Welcome Foundation) - an iron-ligand class of 5-lipoxygenase inhibitor containing the hydroxamic acid chelating group, used at 10⁻⁸M;
f) Ultrasound and Indomethacin (Sigma) - an inhibitor of cyclo-oxygenase activity, used at $10^{-6}$M;


h) Positive control - no ultrasound was used, and the changed medium contained PGE$_2$ at $10^{-6}$M

After treatment with ultrasound/sham insonation, the bones were replaced over the metallic grids, and further incubated for 48 hours. The procedure for measurement of calcium release in the medium was the same as described in the previous section.

### 3.3. Statistical analysis

Each insonation experiment was repeated at least twice. The number of observations for controls and for each intensity evaluated was five ($n=5$). The readings obtained in the internal controls (BGJ) were subtracted from the other readings (shown in the result tables as Mean-BGJ). The results obtained were analysed in Excel®, and the values for the treatment groups were compared to the sham insonation (control group) using ANOVA single factor and two tailed Student's 't' test for unpaired samples. In the mechanism of action assay, the control group to which the others were compared to was the ultrasound control group, which did not have inhibitors in the medium. Significance was accepted at the $p<0.05$ level or higher, as shown in the graphs.
4. Results

4.1. Bone Resorption

Therapeutic ultrasound in the parameters studied stimulated bone resorption. There were differences regarding the intensity used and the type of ultrasound machine, and these can be seen in the tables and figures shown.

Table 6.2 shows the results obtained for the bone resorption assay for the 45 kHz and 1 MHz ultrasound machines. The 45 kHz ultrasound machine stimulated bone resorption in a biphasic manner, with maximum stimulation at 15 mW/cm$^2$ ($^{SA}$) ($p<0.01$) (Fig. 6.3A). Statistically significant resorption was also seen at 5 and 30 mW/cm$^2$ ($^{SA}$) ($p<0.03$ and $p<0.02$ respectively). The resorption caused by 50 mW/cm$^2$ ($^{SA}$) was not significant. With the 1 MHz machine, an intensity dependent bone resorption curve could be observed, where increasing intensities induced more bone resorption. All intensities showed statistically significant results ($p<0.01$) (Fig. 6.3B).
Table 6.2 - Bone resorption induced by therapeutic ultrasound in the neonatal mice calvaria model. Values are the measurement of calcium release into the medium 48 hours after treatment.

### 45 kHz ultrasound, continuous

<table>
<thead>
<tr>
<th>Intensity*</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>50</th>
<th>Control (Sham)</th>
<th>Positive (PGE2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.399</td>
<td>12.001</td>
<td>11.813</td>
<td>9.885</td>
<td>10.218</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean - BGJ</td>
<td>3.306</td>
<td>4.620467</td>
<td>2.907</td>
<td>2.215</td>
<td>1.268</td>
<td>5.073</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>1.609</td>
<td>1.378</td>
<td>0.960</td>
<td>1.166</td>
<td>0.630</td>
<td>1.398</td>
</tr>
<tr>
<td>St. Error</td>
<td>0.719</td>
<td>0.616</td>
<td>0.429</td>
<td>0.521</td>
<td>0.282</td>
<td>0.699</td>
</tr>
<tr>
<td>95% C.I.</td>
<td>1.418</td>
<td>1.208</td>
<td>0.841</td>
<td>1.022</td>
<td>0.552</td>
<td>1.225</td>
</tr>
<tr>
<td>t Test</td>
<td>0.029899</td>
<td>0.00113</td>
<td>0.0128</td>
<td>0.149</td>
<td>1</td>
<td>0.000911</td>
</tr>
</tbody>
</table>

### 1MHz ultrasound, pulsed 1:4

<table>
<thead>
<tr>
<th>Intensity**</th>
<th>0.1</th>
<th>0.4</th>
<th>0.7</th>
<th>1</th>
<th>Control (Sham)</th>
<th>Positive (PGE2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>9.638</td>
<td>10.092</td>
<td>11.201</td>
<td>12.039</td>
<td>8.15</td>
<td>12.9772</td>
</tr>
<tr>
<td>Mean - BGJ</td>
<td>2.052</td>
<td>2.505</td>
<td>3.614</td>
<td>4.452</td>
<td>0.563</td>
<td>5.390</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.409</td>
<td>0.357</td>
<td>0.897</td>
<td>0.572</td>
<td>0.396</td>
<td>1.199</td>
</tr>
<tr>
<td>St. Error</td>
<td>0.183</td>
<td>0.159</td>
<td>0.401</td>
<td>0.255</td>
<td>0.177</td>
<td>0.536</td>
</tr>
<tr>
<td>95% C.I.</td>
<td>0.359</td>
<td>0.313</td>
<td>0.786</td>
<td>0.501</td>
<td>0.347</td>
<td>1.051</td>
</tr>
<tr>
<td>t Test</td>
<td>0.000389</td>
<td>3.86E-05</td>
<td>0.000118</td>
<td>1.58E-06</td>
<td>1</td>
<td>0.00029</td>
</tr>
</tbody>
</table>

* - Intensity in miliWatts/cm²
** - Intensity in Watts/cm²
Figure 6.3 - Bone resorption induced by 45 kHz (A) and 1 MHz (B) therapeutic ultrasound on neonatal mice calvaria. Controls received same treatment but with the US generator switched 'off'. Values represent the calcium release into the medium 48 hours after treatment, and are a mean of 5 replicates + S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
4.2. Mechanism of ultrasound induced bone resorption

Indomethacin, a cyclo-oxygenase inhibitor partially blocked the bone resorption induced by ultrasound (1MHz, 0.1 W/cm^2). Both 5-lipoxygenase inhibitors (BW70C and MK886) had minimal effect on the bone resorption induced by ultrasound. The IL-1 receptor anti-agonist protein caused no inhibition of this bone resorption. The results are summarised in table 6.3 and Fig. 6.4

**Table 6.3 - Mechanism of action of bone resorption induced by therapeutic ultrasound (1 MHz, pulsed 1:4, at 1.0 W/cm^2)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Ultrasound (Control)</th>
<th>US and IRAP 100μg/ml</th>
<th>US and MK886 10^6 M</th>
<th>US and BWA-70C 10^6 M</th>
<th>US and Indom. 10^6 M</th>
<th>Negat. (Sham)</th>
<th>Positive (PGE2)</th>
<th>BGJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean-BGJ</td>
<td>4.600</td>
<td>4.671</td>
<td>4.632</td>
<td>3.081</td>
<td>2.166</td>
<td>0.899</td>
<td>6.558</td>
<td>0</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>1.186</td>
<td>2.531</td>
<td>1.670</td>
<td>1.659</td>
<td>1.996</td>
<td>0.960</td>
<td>2.704</td>
<td>0.025</td>
</tr>
<tr>
<td>St. Error</td>
<td>0.530</td>
<td>1.132</td>
<td>0.747</td>
<td>0.742</td>
<td>0.892</td>
<td>0.429</td>
<td>1.209</td>
<td>0.014</td>
</tr>
<tr>
<td>Conf. Int.</td>
<td>1.040</td>
<td>2.219</td>
<td>1.464</td>
<td>1.454</td>
<td>1.750</td>
<td>0.842</td>
<td>2.370</td>
<td>0.022</td>
</tr>
<tr>
<td>t Test</td>
<td>1</td>
<td>0.955</td>
<td>0.972</td>
<td>0.134</td>
<td>0.047</td>
<td>6E-4</td>
<td>0.176</td>
<td>6E-4</td>
</tr>
</tbody>
</table>
Chapter 6 - Bone resorption induced by therapeutic ultrasound

Figure 6.4 - Mechanism of bone resorption induced by 1 MHz, pulsed 1:4, 1.0 W/cm² (SAPA) therapeutic ultrasound on neonatal mice calvaria. All ultrasound groups received insonation, and the control group had normal medium, without bone resorption inhibitors. Values represent the calcium release into the medium 48 hours after treatment, and are a mean of 5 replicates + S.E.M. Significance level as compared to ultrasound (control) group: * p<0.05, ** p<0.01, *** p<0.001.

5. Discussion

The data shown here shows that ultrasound is capable of induction of bone resorption, and that this effect is intensity dependent. Higher intensities induced more bone resorption, notably with the 1 MHz ultrasound.

The mechanism of action by which ultrasound causes bone resorption may be due to either direct activation of osteoclasts or indirectly via the osteoblasts or other cells present in the calvaria. In this model it is not possible to define the cell type in bone which respond to this stimuli, since calvaria contains many cell types including periosteal fibroblasts, osteoblasts and
osteoclasts. Further experiments would therefore be needed to define if ultrasound stimulates osteoclast activity directly.

The osteoblast is thought to act as a pivotal cell in regulating bone remodelling. In response to bone resorbing agents, not only does it produce mediators of osteoclast activity, but also collagenase (Heath et al, 1984; Sakamoto and Sakamoto, 1982; Otsuka et al, 1984), an enzyme specific in its ability to degrade collagen fibrils and therefore regulate in part connective tissue breakdown. The osteoblast also synthesises and responds to transforming growth factor-beta (TGF-β) (Robey et al, 1987), interleukin-1 (Hanazawa et al, 1987) and prostaglandins (Tashjian, 1978), all of which are bone resorbing agents in vitro. It therefore seems unlikely that osteoclast function is modulated by a single class of mediators produced by the osteoblast.

Arachidonic acid is synthesized from cell membrane phospholipids by the action of phospholipase A₂ (Fig. 6.5). It can be metabolised by two major enzymes, cyclo-oxygenase to give rise to the prostanoids, and by lipoxygenase, to give rise to leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs). This cascade can be inhibited in three main places, (1) by inhibiting the action of phospholipase A₂ (corticosteroids), (2) by inhibiting the enzyme cyclo-oxygenase (aspirin, indomethacin) and (3) by blocking the enzyme 5-lipoxygenase (5-LO inhibitors) (Fig. 6.5).

The induction of bone resorption by ultrasound was partially inhibited by the cyclo-oxygenase enzyme inhibitor indomethacin. This implies that the bone resorbing activity caused by ultrasound is partially due to prostanoids or factors which are prostanoid-dependent.

Other mechanisms may also operate when mechanical deformation activates connective tissue cells. Alterations in cell morphology have been shown to have profound effects on gene expression (Aggeler et al, 1984a and b), either through changes in cell membranes or cytoskeletal components (Hong and Brunette, 1987; Hong et al, 1976). Ultrasound deformation of cell membranes due to microstreaming may well produce similar changes.
Figure 6.5 - Metabolic pathways of arachidonic acid. For clarity many of the minor metabolites have been omitted. (after Meghji, 1992 and Sandy, 1988)

Intermittent forces in vivo are usually associated with bone formation (Lanyon and Rubin, 1984; Hert et al, 1971). The increased production of bone resorbing factors by ultrasound stimulated calvaria, therefore, seems at variance with the first observation. Bone matrix contains many different growth factors with powerful mitogenic activity (Hauschka et al, 1986). It could be that the bone resorbing factors produced by osteoblasts in response to ultrasound stimulation act to liberate these growth factors. Clearly this would be an early event and the growth factors then instigate mitogenic activity and eventually produce increased amounts of bone, as observed in chapters 3 and 5.

In summary, ultrasound stimulated bone resorption when applied directly to the mice calvaria model. This effect was intensity dependent, and higher intensities produced more bone resorption. The cyclo-oxygenase pathway appears to be involved. These results, together with those observed in previous chapters, where bone formation was noted, suggest that ultrasound has a general effect on bone matrix turnover.
Chapter 7 - Cytokines and Angiogenic Factors
Production Stimulated by Therapeutic Ultrasound

1. Introduction

Cytokines are small polypeptide molecules secreted by a variety of cell types. They act at very low concentration (typically $10^{-10}$ to $10^{-15}$ M) and are short lived. They may act locally, either on other cell types (paracrine) or on the same cell (autocrine).

Cytokines are not only important in tissue pathology but play a role in tissue, organ and body homeostasis. They interact with cells to cause; activation of metabolism, cellular synthesis, cell proliferation or inhibition of proliferation, apoptosis, differentiation and chemotaxis. The biological effect of one cytokine is often modified and augmented by another. This is called the cytokine network, and as an interdigitating, redundant network of cytokines is involved in the production of most biological effects, it usually requires more than a single effect in the network to allow the outcome of the process.

Over 100 cytokines have been identified (Henderson et al, 1998) however for the introduction of this chapter, only the cytokines investigated in this study will be briefly reviewed.

1.1. Interleukin 1 (IL-1)

Interleukin-1 exists as three distinct molecules, interleukin-1$\alpha$, interleukin-1$\beta$, and interleukin-1 receptor antagonist (IL-1$\alpha$, IL-1$\beta$, IL-1ra), that have an important role in the regulation of immune and inflammatory responses (Dower, 1992). A wide variety of cells can be induced to transcribe the IL-1$\alpha$ and IL-1$\beta$
genes and produce the precursor forms of the proteins. The precursor of IL-1α is active, whereas the IL-1β precursor is inactive.

Several substances originally described for their biological activities have been identified as IL-1; catobolin, endogenous pyrogen (EP), osteoclast activating factor (OAF), epidermal cell-derived thymocyte-activating factor (ETAF), serum amyloid A inducer of hepatocyte-stimulating factor (HSF), leucocyte endogenous mediator (LEM), fibroblast-activating factor (FAF), B-cell-activating factor (BAF), proteolysis-inducing factor (PIF), haemopoeitin-1 (H-1), mononuclear cell factor (MCF), lymphocyte proliferation promoting factor of neutrophils, melanoma growth inhibition factor and tumour inhibitory factor 2.

Some examples of in vitro biological effects of IL-1 have been reviewed by Dower (1992): (1) Killing of tumour cells; (2) effects of IL-1 on fibroblasts: (a) cause proliferation by induction of PDGFAA synthesis and secretion; (b) induce collagenase and stromelysin secretion; (c) induces cytoskeletal rearrangements and collapse of extracellular matrix; (d) induces IL-6, GCSF secretion; (e) induces cyclo-oxygenase synthesis and prostaglandin release and (f) causes heat shock protein phosphorylation; (3) endothelial cells: induces TNF release; (4) osteoclasts: induces CA³⁺ release; (5) stimulation of thymocyte proliferation; (6) several others effects, mainly in cell of the immune system, such as lymphocytes, bone marrow cells, macrophages, etc).

A wide variety of effects in vivo has also been described. Fever is induced by IL-1, as it was originally identified as an endogenous pyrogen and as a leukocyte endogenous mediator. IL-1 demonstrates a wide range of activities which contribute to the inflammation process. It has been implicated in the pathogenesis of acute and chronic inflammatory diseases such as diabetes, rheumatoid arthritis and periodontitis. Muscle proteolysis is another in vivo effect, as well as bone resorption. IL-1 effects in wound healing suggest that it can induce wound healing, and angiogenesis has been observed in the cheek pouch assay, however, no direct evidence to support this view is yet available (review by Dower, 1992).
1.2. Interleukin 6 (IL-6)

Interleukin 6 (IL-6) is a multifunctional cytokine which acts on a wide variety of cells. It also has had several names in the past because of its pleiotropic nature, according to the biological function studied at the time, such as B-cell stimulatory factor 2, Interferon-β2, 26-kDa protein, hybridoma/plasmacytoma growth factor, thrombopoietin, cytotoxic T-cell differentiation factor, etc (Taga and Kishimoto, 1992).

IL-6 has a wide variety of biological activities: it has been shown to promote growth, inhibit growth, induce differentiation or regulate specific gene expression of a variety of cells. In summary, IL-6 may play a central role in host defence mechanisms by regulating immune responses, haemopoiesis, and acute phase reactions (Kishimoto, 1990).

The release of IL-6 from most cells normally requires prior IL-1 transcription which acts in a feedback fashion on the producing cell to induce the transcription of the IL-6 gene (Tosato and Jones, 1990). The role of IL-6 as a mediator of chronic inflammatory diseases associated with tissue destruction is ambiguous as it may act to downregulate some of the actions of IL-1 and TNF (Akira and Kishimoto, 1992). However, in its own right, it is a potent stimulator of B cell differentiation and growth (Akira and Kishimoto, 1992) and of osteoclast differentiation and bone resorption (Roodman, 1992).

1.3. Tumour Necrosis Factor (TNF)

TNF was originally defined as a factor produced in bacillus Calmette-Guerin-primed animals in response to endotoxin, and responsible for necrosis of various tumours (Aggarwal, 1992). It is a protein with molecular weight of 17 kDa, which exists in α and β forms. In this study, only the TNF-α has been investigated. In response to stimuli, TNF-α is produced by a wide range of cells including macrophages, fibroblasts, natural killer cells, astrocytes and mast cells.
TNF affects a wide variety of cell types, and does not exhibit species-specificity, but do show species-related preferences in some instances. TNF has been implicated in several biological activities, including: pro-inflammatory activity, weight loss (cachexia), mediator of endotoxin-induced shock, antitumour effects, antibacterial effects, autoimmunity, wound healing, antimalarial effects, angiogenesis and stimulation of bone resorption (Aggarwall, 1992).

Its angiogenic effects include stimulation of new blood vessel formation and proliferation of fibroblasts (Fan, 1993; Harris, 1997). Conversely it is a potent inhibitor of endothelial cell growth. The angiogenic effects of TNF may be due to leukocyte infiltration. Therefore, TNF-α plays an important part in wound repair similar to that of other macrophage-derived growth factors, such as fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β).

1.4. Interleukin 8 (IL-8)

IL-8 was first described by Baggiolini et al (1989), as a 72 amino acid (8 kDa) polypeptide which is produced by E. coli LPS-stimulated human peripheral blood monocytes. However IL-8 is also produced by a variety of other cells including fibroblasts, osteoblasts, endothelial cells, epithelial cells, chondrocytes, synovial cells and various tumour cells (Reddi, 1995). IL-8 differs from other cytokines in that it has a specific target, the neutrophil, which it attracts and activates in inflammatory sites (Bickel, 1993). Furthermore IL-8 stimulates angiogenesis (Hu et al, 1993), acting as a macrophage-derived mediator of angiogenesis (Koch et al, 1992).

1.5. Fibroblast Growth Factor (FGF)

FGF exists under two main structurally related forms, acidic FGF and basic FGF. bFGF. Each of these two main forms has had several synonyms (see Gospodarowicz, 1992a). Basic FGF, also referred to as FGF-2 or heparin-binding growth factor 2 (HBGF-2), currently comprises of seven members
Chapter 7 - Cytokines and angiogenic factors production induced by therapeutic ultrasound

showing a 30-50% overall sequence homology at the amino acid level (Baird and Bohlen, 1990). bFGF has been extracted from a number of sources, including neural tissue, pituitary, adrenal cortex, corpus luteum, and placenta. The naturally extracted bFGF usually has molecular mass of about 18 kDa. A variety of larger forms of bFGF, with molecular masses up to about 24 kDa, also exist as a result of amino-terminal extensions of the protein produced by initiation of translation at non-AUG start site. The existence of these amino-terminal extensions cause localisation of bFGF to the cell nucleus rather than to the cytoplasm.

The following characteristic properties of bFGF have been reviewed by Baird and Bohlen (1990): (1) bFGF has been demonstrated to stimulate the proliferation of cells of mesodermal and neuroectodermal origin, including fibroblasts, endothelial cells, astrocytes, oligodendrocytes, neuroblasts, keratinocytes, bovine lens epithelial cells, osteoblasts, smooth muscle cells and melanocytes; (2) in vitro bFGF can be chemotactic and mitogenic for endothelial cells, including endothelial cell production of factors involved in the breakdown of the basement membrane and the migration of capillary endothelial cells into collagen matrices to form capillary-like tubes; (3) FGFs play a role in vivo in the modulation of such normal processes as angiogenesis, wound healing and tissue repair, embryonic development and differentiation, and neuronal function and neural degeneration; (4) inappropriate expression of basic FGF and other members of the FGF family can cause tumour production; and (5) bFGF may participate in the production of a variety of pathological conditions resulting from uncontrolled cell proliferation and uncontrolled angiogenesis.

1.6. Vascular Endothelial Growth Factor (VEGF)

VEGF was originally referred to as vascular permeability factor (VPF), a factor produced by numerous human and rodent tumour cells and a potent agent in promoting fluid and protein extravasion (Senger et al, 1986). VEGF was originally defined a an angiogenic factor produced by pituitary-derived
folliculostellate cells (Ferrara and Henzel, 1989). Both factors, once cloned and sequenced, were shown to be identical and to belong to the platelet derived growth factor (PDGF) family (Gospodarowicz, 1992b). Vasculotropin is another synonym.

VEGF is a homodimeric 34-42 kDa, heparin-binding glycoprotein with potent angiogenic, mitogenic and vascular permeability-enhancing activities specific for endothelial cells. The VEGF molecule shows amino acid sequence of primary structural and a limited amino acid sequence which are homologous with that of the A and B chains of PDGF. Among these growth factors, all eight cysteine residues involved in intra- and inter-chain disulphide bonds are conserved.

VEGF is expressed by a variety of rodent and human tumour cells, including human lung adenocarcinoma, bladder carcinoma, fibrosarcoma, HL60 promyelocytic leukemia, GS-9L glioma and U937 lymphoma cells (Connolly, 1989; Ferrara et al., 1992). The expression of VEGF, in normal tissues, has been found in a variety of cell types including macrophages, keratinocytes, renal glomerular visceral epithelium and mesangial cells, hepatocytes, smooth muscle cells, Leydig cells, embryonic fibroblasts and bronchial and choroid plexus epithelium. The expression of VEGF is upregulated by phorbol ester, TGF-β and in hypoxia (Connolly, 1989; Ferrara et al., 1992; Thomas, 1996).

In vitro, VEGF has shown to have the following characteristic properties: (1) acts as a potent endothelial cell mitogen; (2) activate phospholipase C and induce rapid increases of free cytosolic Ca\(^{2+}\); (3) stimulate von Willebrand factor release from endothelial cells and induce expression of tissue factor activity in endothelial cells as well as in monocytes; and (4) can be chemotactic for monocytes and osteoblasts. In vivo, VEGF has shown to induce angiogenesis as well as increase microvascular permeability. As a vascular permeability factor, VEGF acts directly on the endothelium and does not degranulate mast cells. It also promotes extravasation of plasma fibrinogen, leading to fibrin deposition which alters the tumour extracellular matrix. The modified extracellular matrix subsequently promotes the migration of macrophages,
fibroblasts and endothelial cells. Based on its \textit{in vitro} and \textit{in vivo} properties, VEGF is considered to have crucial roles in inflammation and during normal and pathological angiogenesis, a process that is closely related to wound healing, embryonic development, growth and metastasis of solid tumours. Elevated VEGF levels have been reported in synovial fluids of rheumatoid arthritis patients and in sera from cancer patients (Toi \textit{et al}, 1996). Inhibition of VEGF-induced angiogenesis suppresses tumour growth \textit{in vivo}, and therefore the concept of 'therapeutic antiangiogenesis' may be a useful tool in future cancer research (Kim \textit{et al}, 1993).

1.7. Angiogenesis and Angiogenic factors

Angiogenesis has been described and discussed in chapter 3. It is a physiological phenomenon, under strict control and short duration. It occurs during embryonic development, endometrial regeneration and wound repair (Fan, 1993).

Tissue necrosis, osteoradionecrosis, ulcers, fistulae and fibroatrophy result from decreased vascularization and cause tissue and organ malfunction. Regular neovascularization is necessary for uncompromised adult wound healing and tissue regeneration (Eliseenko \textit{et al}, 1988).

Angiogenic factors are capable of inducing angiogenesis and or endothelial cell proliferation (Table 7.1). Although the biochemistry and structure of these molecules is well known, little is understood about how they mediate angiogenesis \textit{in vivo}. According to Jakobsson (1994), several possibilities include: (a) secretion of angiogenic factors from recruited macrophages, stroma cells or tumour cells; (b) mobilisation of angiogenic molecules from the extracellular matrix (e.g. bFGF); (c) suppression of pericyte inhibition of endothelial proliferation; (d) decrease in the secretion of an endogenous inhibitor of angiogenesis; and (e) amplified production of angiogenic molecules from endothelial cells resulting in an autocrine stimulation.
Chapter 7 - Cytokines and angiogenic factors production induced by therapeutic ultrasound

Table 7.1- Polypeptides mentioned as angiogenic factors

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TVPF</td>
<td>yes</td>
<td>yes</td>
<td>Connoly et al, 1989</td>
</tr>
<tr>
<td>PD-ECGF</td>
<td>yes</td>
<td>yes</td>
<td>Miyazono et al, 1987; Miyazono et al, 1991</td>
</tr>
<tr>
<td>Angiotropin</td>
<td>yes</td>
<td>yes</td>
<td>Hockel et al, 1987; Hockel et al, 1988</td>
</tr>
<tr>
<td>bFGF *</td>
<td>yes</td>
<td>yes</td>
<td>Folkman et al, 1988; Klagsbrun, 1989; Klagsbrun, 1992; Ribatti et al, 1997</td>
</tr>
<tr>
<td>IL-8</td>
<td>yes</td>
<td>yes</td>
<td>Koch et al, 1992; Hu et al, 1993</td>
</tr>
<tr>
<td>IGF-1</td>
<td>yes</td>
<td>yes</td>
<td>Grant et al, 1993</td>
</tr>
<tr>
<td>TGFα</td>
<td>yes</td>
<td>yes</td>
<td>Folkman, 1993</td>
</tr>
<tr>
<td>TGFβ *</td>
<td>no</td>
<td>no</td>
<td>Wahl et al, 1987; Beck et al, 1991; Philips et al, 1993</td>
</tr>
<tr>
<td>TNFα *</td>
<td>yes</td>
<td>no</td>
<td>Fan, 1993; Harris, 1997</td>
</tr>
<tr>
<td>PDGF</td>
<td>yes</td>
<td>yes</td>
<td>Risau et al, 1992</td>
</tr>
</tbody>
</table>

* These factors also promote tube formation (Fan, 1993)

Therapeutic angiogenesis is used to induce the local growth of blood vessels to reduce unfavourable tissue effects caused by local hypoxia or to enhance tissue repair (Höckel et al, 1993). Ultrasound can be considered as a way to deliver therapeutic angiogenesis.

The only study that directly observed angiogenesis induced by ultrasound was by Young and Dyson (1990a), who showed that therapeutic ultrasound directly stimulated angiogenesis in full thickness excised wounds in the flank skin of adults rats.

2. Hypothesis and Aims

This chapter tests the hypothesis that ultrasound promotes angiogenesis by the stimulation of the production of angiogenic factors.

The aims of this chapter are:
Chapter 7 - Cytokines and angiogenic factors production induced by therapeutic ultrasound

1. Identify if ultrasound is able to induce the release of cytokines and angiogenic factors by human gingival fibroblasts, mandibular osteoblasts, and by peripheral blood mononuclear cells (monocytes).

2. Compare two types of ultrasound machines, the 'traditional' (1 MHz, pulsed), and the 'long wave' (45 kHz, continuous), at 4 intensities each, trying to establish which machine and intensity has the best stimulatory effect on the release of cytokines and angiogenesis factors.

3. Material and Methods

3.1. Cell cultures

The three cell types used in these experiments were: human mandibular osteoblasts, gingival fibroblasts and peripheral blood mononuclear cells (monocytes). The culture procedures for the fibroblasts and osteoblasts have been described in chapter 4. These cells were plated at 3x10^5 cells/well, so that they were confluent the following day. The monocytes culture is described below:

Peripheral Blood Mononuclear Cells (Monocytes)

These were prepared by Ficoll density gradient centrifugation as described by Bristow et al (1991). Monocytes were extracted from thoroughly screened donated human blood, obtained from the North London Transfusion Centre. The blood was diluted in equal parts with RPMI 1640 medium (Sigma), and 35 ml of this blood/RPMI suspension was carefully layered over 15 ml of Ficoll-Histopaque 1077 (Sigma). After centrifugation (1500 rpm 30 min), the mononuclear cell layer was collected into Falcon tubes, washed with RPMI 1640 medium and centrifuged at 1500 rpm for 15 min. This wash step was repeated and the pellet re-suspended in RPMI 1640 medium containing 2% (v/v) HIFBS, L-glutamine - 2 mM (Sigma), and penicillin/streptomycin - 100 U/ml each (Gibco BRL). The PMNC’s were seeded 5x10^6 cells/1.5ml/well in 6 well
plates. Plates were incubated for 1-2 hours to allow monocytes to adhere, then washed once with the prepared medium, and finally 5 ml of medium with 2% HIFBS was placed in each well.

The positive controls for the monocytes was highly-purified lipopolysaccharide (LPS) from *E. coli* (NIBSC 84/650) (10 ng/ml).

### 3.2. The ultrasound machines and the insonation model

The ultrasound machines and the insonation model used were the same as in the previous chapters.

Each well was insonated for 5 min, and the control group was treated in the same way, but with the ultrasound generator switched off. To ensure that the factors released were not too diluted, 2 ml of the original 5 ml volume were removed immediately after the ultrasound treatment, and the plates were cultured for a further 18 hours at 37°C in 5% CO₂, 95% air. At the end of this culture time, the media were removed and the cytokines released into the media were measured by ELISA.

### 3.3. ELISA Assays for IL-1β, IL-6, IL-8, TNF-α

The following antibodies and standards were used:

*Coating antibodies* (diluted in bicarbonate coating buffer, pH 8.2-8.3) - IL-1β - immunoaffinity-purified polyclonal antibodies from sheep anti-IL-1β serum S77/BM, diluted to 2 µg/ml; IL-6 - immunoaffinity-purified polyclonal antibodies from goat anti-rh IL-6 serum G150/BM, diluted to 1 µg/ml; IL-8 - immunoaffinity-purified polyclonal antibodies from sheep anti-human IL-8 serum S333/BM, diluted to 2 µg/ml; TNFα - FLPC-purified monoclonal mouse anti-human TNFα 101-4, diluted to 2 µg/ml.

*Detecting antibodies* (biotinylated immunoaffinity purified antibodies), used were: IL-1β goat anti-IL-1β serum G102/BM (diluted 1:1000); IL-6 - goat anti-rh IL-6 serum G150/BM (diluted 1:500); IL-8 - sheep anti-human IL-8 serum
S333/BM (diluted 1:1000); TNFα - biotinylated FPLC-purified polyclonal antibodies from sheep anti-human TNFα serum H/34 or H/91 (diluted 1:200).

Standards (human recombinant standards) - IL-1β (I.S. 86/680, 1 µg/ml) and IL-6 (I.S. 89/548, 1 µg/ml), at a concentration range of 8000-1.0 pg/ml; IL-8 (NIBSC 89/520, 1 µg/ml), and TNF-α (NIBSC 87/650), at a concentration range of 10000-1.0 pg/ml.

Technique - Microtitre plates (NUNC) were coated with 100µl/well of coating antibody, and the plates were incubated overnight at 4°C. Unbound coating antibody was removed by washing the plates 3 times with wash/dilution buffer, pH 7.2-7.4 (NaCl 0.5M, NaH₂PO₄ 2.5 mM, Na₂HPO₄ 7.5 mM, and Tween 20 0.1% v/v). Standards of the cytokines and the supernatants to be tested were added to the remaining wells (100µl volumes). Plates were incubated for 4 hours (3 hours for IL-8, and 2 hours for IL-6 and TNFα) at room temperature and washed three times with wash/dilution buffer. Detecting antibody (100 µl) was added to each of the wells and incubated for a further 1h at room temperature. Plates were washed 3 times with wash/dilution buffer, and 100 µl of avidin horseradish peroxidase (Avidin-HRP, Dako Ltd) diluted 1:4000 in wash-dilution buffer was added into each well. Plates were incubated for 15 min at room temperature before washing 3 times with wash/dilution buffer. Wells were developed with 100 µl of colour reagent - 0.4 mg of orthophenylenediamine (OPD) and 0.4 µl of 30% H₂O₂ in 1 ml of 0.1 M citric acid phosphate buffer, pH 5.0; and incubated for 15-30 min at room temperature. The reaction was terminated by the addition of 150 µl of 1M H₂SO₄, and the absorbance was measured at 492nm on a Titertek Multiscan spectrophotometer (Flow). A standard curve was plotted of the absorbance (optical density) versus the concentration of the standards.

3.4. bFGF and VEGF Immunoassay

These proteins have been assayed using a quantitative sandwich enzyme immunoassay technique (R&D Systems). Briefly, a monoclonal antibody
specific for bFGF or VEGF was pre-coated onto 96 well plates. Standards and samples (assayed in duplicates or triplicates) were pipetted into the wells, and left to bind to the antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for bFGF or VEGF was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells, and colour developed in proportion to the amounts of the proteins bounded in the first step. The colour development was stopped and the intensity measured at 570 nm. Standard curves were obtained as usual.

3.5. Statistical analysis

Each cell insonation experiment was repeated at least twice. The medium was assayed by ELISA at least in duplicates. The results obtained were analysed in Excel®, and the values for the treatment groups were compared to the sham insonation (control group) using ANOVA single factor and two tailed Student’s ‘t’ test for unpaired samples. Significance was accepted at the p<0.05 level or higher, as shown in the graphs.

4. Results

A slight stimulation of IL-1β was noted in all cell types. However the level of stimulation, although significantly different from controls, was very low. Table 7.2 shows the most significant results, observed in monocytes, using 45 kHz, and in osteoblasts, stimulated with 1 MHz ultrasound (Fig. 7.1).

The production of IL-6 and TNF-α by all cell types was not significantly altered after ultrasound stimulation. Table 7.3 shows as an example the results of IL-6 production by monocytes. It can be noted that the cells treated with LPS (10 ng/ml) showed a highly significant production of IL-6, and this has been used as positive control (Fig 7.2). However, on the insonated and control groups, the levels of IL-6 production were low, and there was no difference
between controls and treatment groups. The other results for IL-6 and TNF-α have been similar and therefore will not be shown.

The more specifically angiogenesis-related cytokines, IL-8, bFGF and VEGF were significantly stimulated after ultrasound insonation. Table 7.4 shows the results for the production of IL-8 by osteoblasts. IL-8 production was enhanced with both ultrasound machines, but higher levels of cytokine were shown with the 45 kHz ultrasound (Fig. 7.3). Monocytes and fibroblasts IL-8 production did not differ from controls.

Similar observation was made with bFGF, as shown in table 7.5. The levels of bFGF production was significantly elevated in osteoblasts, with both ultrasound machines (Fig. 7.4). However, monocytes and fibroblasts bFGF production did not differ from controls (data not shown).

The production of VEGF reached higher levels than the production of bFGF, and could be observed in all cell types evaluated (Tables 7.6 to 7.8). The results for fibroblasts (Fig. 7.5), osteoblasts (Fig. 7.6) and monocytes (Fig. 7.7) showed enhanced levels of production of VEGF in both machines. With the 45 kHz ultrasound, usually the mid-range intensities gave more significant results whereas with the 1 MHz, usually the lower intensities induced more VEGF production.

In summary, the angiogenesis-related cytokines, IL-8 and bFGF, were significantly stimulated in osteoblasts, and VEGF was significantly stimulated in all cell types evaluated. Both ultrasound machines produced significant results, and the best intensities were 15 and 30 mW/cm^2(SA) with 45 kHz ultrasound, and 0.1 and 0.4 W/cm^2(SAPA) with 1 MHz ultrasound.
Table 7.2 - Interleukin-1β synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on *human monocytes and mandibular osteoblasts* respectively

### Monocytes - 45 kHz ultrasound, continuous

<table>
<thead>
<tr>
<th>Intensity(^1)</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>50</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical Density</td>
<td>0.067</td>
<td>0.114</td>
<td>0.22</td>
<td>0.182</td>
<td>0.071</td>
</tr>
<tr>
<td>Calculated Concentration(^2)</td>
<td>-3.85731</td>
<td>30.95082</td>
<td>108.2825</td>
<td>80.65816</td>
<td>-0.87487</td>
</tr>
<tr>
<td>Mean</td>
<td>3.578864</td>
<td>26.16072</td>
<td>104.2857</td>
<td>81.02196</td>
<td>3.957738</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>10.51634</td>
<td>6.774227</td>
<td>5.652345</td>
<td>0.514495</td>
<td>6.834334</td>
</tr>
<tr>
<td>95% C.I.</td>
<td>14.57461</td>
<td>9.388413</td>
<td>7.833594</td>
<td>0.713039</td>
<td>9.471715</td>
</tr>
<tr>
<td>St. Err.</td>
<td>7.436172</td>
<td>4.790102</td>
<td>3.996811</td>
<td>0.363803</td>
<td>4.832604</td>
</tr>
<tr>
<td>t Test</td>
<td>0.969805</td>
<td>0.082467</td>
<td>0.003884</td>
<td>0.003931</td>
<td>1</td>
</tr>
</tbody>
</table>

### Osteoblasts - 1 MHz ultrasound, pulsed 1:4

<table>
<thead>
<tr>
<th>Intensity(^2)</th>
<th>0.1</th>
<th>0.4</th>
<th>0.7</th>
<th>1.0</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical Density</td>
<td>0.072</td>
<td>0.083</td>
<td>0.081</td>
<td>0.076</td>
<td>0.063</td>
</tr>
<tr>
<td>Calculated Concentration(^2)</td>
<td>8.8701</td>
<td>17.04831</td>
<td>15.56355</td>
<td>11.84743</td>
<td>2.156033</td>
</tr>
<tr>
<td>Mean</td>
<td>6.073299</td>
<td>9.981895</td>
<td>14.26249</td>
<td>11.10079</td>
<td>2.902965</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>3.243827</td>
<td>5.519872</td>
<td>2.60089</td>
<td>3.984957</td>
<td>0.862482</td>
</tr>
<tr>
<td>95% C.I.</td>
<td>3.178887</td>
<td>5.409367</td>
<td>2.548822</td>
<td>3.905181</td>
<td>0.845216</td>
</tr>
<tr>
<td>St. Err.</td>
<td>1.621913</td>
<td>2.759936</td>
<td>1.300445</td>
<td>1.992479</td>
<td>0.431241</td>
</tr>
<tr>
<td>t Test</td>
<td>0.107793</td>
<td>0.044431</td>
<td>0.000167</td>
<td>0.006949</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^1\) - Intensity in miliWatts/cm\(^2\)\(^{(SA)}\)

\(^2\) - Calculated after the standard curve, results in pg/ml

\(^3\) - Intensity in Watts/cm\(^2\)\(^{(SAPA)}\)
Figure 7.1 - IL-1β production by monocytes stimulated by 45 kHz (A), and by osteoblasts stimulated by 1 MHz (B) ultrasound. Cells were treated for 5 minutes, and the medium was collected 18 hours after stimulation and assayed by ELISA. Controls on (A) and (B) were sham-insonated. Bars show mean values + S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
### Table 7.3 - Interleukin-6 synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on *human peripheral blood monocytes*

#### 45 kHz ultrasound, continuous

<table>
<thead>
<tr>
<th>Intensity(^1)</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>50</th>
<th>Control</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical</td>
<td>0.162</td>
<td>0.115</td>
<td>0.124</td>
<td>0.102</td>
<td>0.084</td>
<td>2.399</td>
</tr>
<tr>
<td>Density</td>
<td>0.067</td>
<td>0.081</td>
<td>0.102</td>
<td>0.267</td>
<td>0.082</td>
<td>2.509</td>
</tr>
<tr>
<td>Calculated Concentration(^2)</td>
<td>100.3018</td>
<td>57.73014</td>
<td>66.07091</td>
<td>45.51564</td>
<td>28.26649</td>
<td>5303.491</td>
</tr>
<tr>
<td>Mean</td>
<td>55.95177</td>
<td>41.54132</td>
<td>55.79328</td>
<td>116.6464</td>
<td>27.29579</td>
<td>5671.121</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>62.72035</td>
<td>22.89445</td>
<td>14.53477</td>
<td>100.594</td>
<td>1.372774</td>
<td>519.9083</td>
</tr>
<tr>
<td>95%C.I.</td>
<td>86.92425</td>
<td>31.72945</td>
<td>20.14377</td>
<td>139.4134</td>
<td>1.902529</td>
<td>720.5418</td>
</tr>
<tr>
<td>St. Err.</td>
<td>44.34999</td>
<td>16.18882</td>
<td>10.27764</td>
<td>71.13071</td>
<td>0.970697</td>
<td>367.6307</td>
</tr>
<tr>
<td>t Test</td>
<td>0.584517</td>
<td>0.472379</td>
<td>0.109997</td>
<td>0.335946</td>
<td>1</td>
<td>0.004216</td>
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</table>

#### 1MHz ultrasound, pulsed 1:4

<table>
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<tr>
<th>Intensity(^2)</th>
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<th>0.4</th>
<th>0.7</th>
<th>1.0</th>
<th>Control</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical</td>
<td>0.139</td>
<td>0.109</td>
<td>0.122</td>
<td>0.082</td>
<td>0.077</td>
<td>2.399</td>
</tr>
<tr>
<td>Density</td>
<td>0.095</td>
<td>0.072</td>
<td>0.09</td>
<td>0.098</td>
<td>0.085</td>
<td>2.509</td>
</tr>
<tr>
<td>Calculated Concentration(^2)</td>
<td>79.76924</td>
<td>52.11749</td>
<td>64.22542</td>
<td>26.32509</td>
<td>21.44944</td>
<td>5303.491</td>
</tr>
<tr>
<td>Mean</td>
<td>59.31207</td>
<td>34.32964</td>
<td>49.14301</td>
<td>34.02094</td>
<td>25.34237</td>
<td>5671.121</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>28.9308</td>
<td>25.15581</td>
<td>21.32976</td>
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<tr>
<td>95%C.I.</td>
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<td>0.266104</td>
<td>0.420241</td>
<td>1</td>
<td>0.004214</td>
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</table>

\(^1\) - Intensity in miliWatts/cm\(^2\)\(^{(SA)}\)

\(^2\) - Calculated after the standard curve, results in pg/ml

\(^3\) - Intensity in Watts/cm\(^2\)\(^{(SAPA)}\)
Figure 7.2 - IL-6 production by monocytes stimulated by 45 kHz continuous ultrasound (A) and by 1 MHz pulsed 1:4 ultrasound (B). Cells were treated for 5 minutes, and the medium was collected 18 hours after stimulation and assayed by ELISA. Controls on (A) and (B) were sham-insonated. LPS (from E. coli) at 10 ng/ml was used as a positive control. Bars show mean values + S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
Table 7.4 - Interleukin-8 synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on human mandibular osteoblasts

**45 kHz ultrasound, continuous**

<table>
<thead>
<tr>
<th>Intensity</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>50</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical Density</td>
<td>0.527</td>
<td>0.586</td>
<td>1.062</td>
<td>0.751</td>
<td>0.646</td>
</tr>
<tr>
<td>Calculated Concentration²</td>
<td>330.8691</td>
<td>371.4593</td>
<td>903.4972</td>
<td>505.9664</td>
<td>416.2255</td>
</tr>
<tr>
<td>Mean</td>
<td>389.687</td>
<td>721.2781</td>
<td>940.4568</td>
<td>539.9327</td>
<td>439.1332</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>83.18111</td>
<td>157.7185</td>
<td>52.26873</td>
<td>48.03564</td>
<td>32.39635</td>
</tr>
<tr>
<td>95%C.I.</td>
<td>115.2808</td>
<td>685.6312</td>
<td>72.43933</td>
<td>66.57267</td>
<td>44.89816</td>
</tr>
<tr>
<td>St. Err.</td>
<td>58.81793</td>
<td>349.8188</td>
<td>36.95957</td>
<td>33.96632</td>
<td>22.90768</td>
</tr>
<tr>
<td>t Test</td>
<td>0.515456</td>
<td>0.505391</td>
<td>0.007439</td>
<td>0.133017</td>
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**1 MHz ultrasound, pulsed 1:4**

<table>
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<tr>
<th>Intensity²</th>
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<th>0.4</th>
<th>0.7</th>
<th>1.0</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>Optical Density</td>
<td>0.571</td>
<td>0.746</td>
<td>0.799</td>
<td>0.479</td>
<td>0.608</td>
</tr>
<tr>
<td>Calculated Concentration²</td>
<td>360.8548</td>
<td>501.3034</td>
<td>553.0067</td>
<td>299.7066</td>
<td>387.414</td>
</tr>
<tr>
<td>Mean</td>
<td>371.2047</td>
<td>515.131</td>
<td>581.0201</td>
<td>332.7419</td>
<td>391.8734</td>
</tr>
<tr>
<td>95%C.I.</td>
<td>10.28396</td>
<td>27.1014</td>
<td>54.90508</td>
<td>64.74795</td>
<td>8.740273</td>
</tr>
<tr>
<td>St. Err.</td>
<td>10.34989</td>
<td>13.82752</td>
<td>28.01335</td>
<td>33.03533</td>
<td>4.459412</td>
</tr>
<tr>
<td>t Test</td>
<td>0.208095</td>
<td>0.013611</td>
<td>0.021759</td>
<td>0.218085</td>
<td>1</td>
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</table>

1 - Intensity in miliWatts/cm²
2 - Calculated after the standard curve, results in pg/ml
3 - Intensity in Watts/cm²
Figure 7.3 - IL-8 production by osteoblasts stimulated by 45 kHz (A) and by 1 MHz (B) ultrasound. Cells were treated for 5 minutes, and the medium was collected 18 hours after stimulation and assayed by ELISA. Controls on (A) and (B) were sham-sonicated. Bars show mean values + S.E.M. Significance level as compared to controls (sham sonicated): * p<0.05, ** p<0.01, *** p<0.001.
### Table 7.5 - Basic Fibroblast Growth Factor synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on *human mandibular osteoblasts*

#### 45 kHz ultrasound, continuous

<table>
<thead>
<tr>
<th>Intensity</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>50</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical</td>
<td>0.037</td>
<td>0.026</td>
<td>0.014</td>
<td>0.015</td>
<td>0.009</td>
</tr>
<tr>
<td>Density</td>
<td>0.036</td>
<td>0.023</td>
<td>0.009</td>
<td>0.018</td>
<td>0.01</td>
</tr>
<tr>
<td>Calculated Concentration</td>
<td>3.209397</td>
<td>1.946027</td>
<td>0.567072</td>
<td>0.682018</td>
<td>-0.00776</td>
</tr>
<tr>
<td>Mean</td>
<td>3.151982</td>
<td>1.773696</td>
<td>0.279657</td>
<td>0.854419</td>
<td>0.049732</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.081196</td>
<td>0.243712</td>
<td>0.406466</td>
<td>0.243811</td>
<td>0.081303</td>
</tr>
<tr>
<td>95%C.I.</td>
<td>0.11253</td>
<td>0.337761</td>
<td>0.563322</td>
<td>0.337898</td>
<td>0.112678</td>
</tr>
<tr>
<td>St. Err.</td>
<td>0.057414</td>
<td>0.17233</td>
<td>0.287415</td>
<td>0.1724</td>
<td>0.05749</td>
</tr>
<tr>
<td>t Test</td>
<td>0.000685</td>
<td>0.010923</td>
<td>0.514941</td>
<td>0.047408</td>
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</table>

#### 1MHz ultrasound, pulsed 1:4

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<tr>
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<th>0.4</th>
<th>0.7</th>
<th>1.0</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical</td>
<td>0.304</td>
<td>0.014</td>
<td>0.005</td>
<td>0.021</td>
<td>0.031</td>
</tr>
<tr>
<td>Density</td>
<td>0.312</td>
<td>0.018</td>
<td>0.025</td>
<td>0.037</td>
<td>0.051</td>
</tr>
<tr>
<td>Calculated Concentration</td>
<td>34.03958</td>
<td>0.567072</td>
<td>-0.46775</td>
<td>1.371565</td>
<td>2.520357</td>
</tr>
<tr>
<td>Mean</td>
<td>34.50918</td>
<td>0.796946</td>
<td>0.681699</td>
<td>2.290481</td>
<td>3.668484</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>0.66412</td>
<td>0.32509</td>
<td>1.625562</td>
<td>1.299543</td>
<td>1.623697</td>
</tr>
<tr>
<td>95%C.I.</td>
<td>0.920405</td>
<td>0.350543</td>
<td>2.252869</td>
<td>1.80104</td>
<td>2.250284</td>
</tr>
<tr>
<td>St. Err.</td>
<td>0.46904</td>
<td>0.229873</td>
<td>1.149446</td>
<td>0.918916</td>
<td>1.148127</td>
</tr>
<tr>
<td>t Test</td>
<td>0.001614</td>
<td>0.133718</td>
<td>0.207382</td>
<td>0.447653</td>
<td>1</td>
</tr>
</tbody>
</table>

1 - Intensity in miliWatts/cm²² (SA)
2 - Calculated after the standard curve, results in pg/ml
3 - Intensity in Watts/cm²² (SAPA)
Figure 7.4 - bFGF production by osteoblasts stimulated by 45 kHz (A) and by 1 MHz (B) ultrasound. Medium of the cells was collected 18 hours after stimulation and assayed by ELISA. Controls on (A) and (B) were sham-isonated. Bars show mean values ± S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
Table 7.6 - Vascular Endothelial Growth Factor synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on *human gingival fibroblasts*

<table>
<thead>
<tr>
<th>Intensity</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>50</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical</td>
<td>0.29</td>
<td>0.466</td>
<td>0.298</td>
<td>0.349</td>
<td>0.296</td>
</tr>
<tr>
<td>Density</td>
<td>0.268</td>
<td>0.453</td>
<td>0.299</td>
<td>0.347</td>
<td>0.305</td>
</tr>
<tr>
<td>Calculated</td>
<td>155.9366</td>
<td>254.4939</td>
<td>160.1218</td>
<td>187.184</td>
<td>159.0745</td>
</tr>
<tr>
<td>Concentration²</td>
<td>144.4686</td>
<td>246.4899</td>
<td>160.6458</td>
<td>186.1059</td>
<td>163.7935</td>
</tr>
<tr>
<td>Mean</td>
<td>150.2026</td>
<td>250.4919</td>
<td>160.3838</td>
<td>186.645</td>
<td>161.434</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>8.109104</td>
<td>5.659678</td>
<td>0.370481</td>
<td>0.762331</td>
<td>3.336879</td>
</tr>
<tr>
<td>95%C.I.</td>
<td>11.23842</td>
<td>7.843758</td>
<td>0.51345</td>
<td>1.056516</td>
<td>4.624586</td>
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<tr>
<td>St. Err.</td>
<td>5.734002</td>
<td>4.001997</td>
<td>0.261969</td>
<td>0.539049</td>
<td>2.359529</td>
</tr>
<tr>
<td>t Test</td>
<td>0.21178</td>
<td>0.00271</td>
<td>0.701453</td>
<td>0.009091</td>
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</table>

1 kHz ultrasound, continuous

<table>
<thead>
<tr>
<th>Intensity²</th>
<th>0.1</th>
<th>0.4</th>
<th>0.7</th>
<th>1.0</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical</td>
<td>0.45</td>
<td>0.348</td>
<td>0.482</td>
<td>0.367</td>
<td>0.372</td>
</tr>
<tr>
<td>Density</td>
<td>0.385</td>
<td>0.37</td>
<td>0.514</td>
<td>0.395</td>
<td>0.41</td>
</tr>
<tr>
<td>Calculated</td>
<td>244.6653</td>
<td>186.6448</td>
<td>264.5739</td>
<td>196.9684</td>
<td>199.7146</td>
</tr>
<tr>
<td>Concentration²</td>
<td>206.9189</td>
<td>198.6145</td>
<td>285.5662</td>
<td>212.5289</td>
<td>221.0665</td>
</tr>
<tr>
<td>Mean</td>
<td>225.7921</td>
<td>192.6296</td>
<td>275.0701</td>
<td>204.7487</td>
<td>210.3905</td>
</tr>
<tr>
<td>95%C.I.</td>
<td>36.9907</td>
<td>11.73014</td>
<td>20.57205</td>
<td>15.24903</td>
<td>20.92448</td>
</tr>
<tr>
<td>St. Err.</td>
<td>18.87318</td>
<td>5.984885</td>
<td>10.49615</td>
<td>7.780271</td>
<td>10.67597</td>
</tr>
<tr>
<td>t Test</td>
<td>0.551178</td>
<td>0.283835</td>
<td>0.049625</td>
<td>0.7109</td>
<td>1</td>
</tr>
</tbody>
</table>

1 - Intensity in milliWatts/cm² (mW/cm²)
2 - Calculated after the standard curve, results in pg/ml
3 - Intensity in Watts/cm² (W/cm²)
Figure 7.5 - VEGF production by fibroblasts stimulated by 45 kHz (A) and by 1 MHz (B) ultrasound. Cells were insonated for 5 minutes, the medium was collected 18 hours after stimulation and assayed by ELISA. Controls on (A) and (B) were sham-insonated. Bars show mean values + S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
### Table 7.7 - Vascular Endothelial Growth Factor synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on human mandibular osteoblasts

#### 45 kHz ultrasound, continuous

<table>
<thead>
<tr>
<th>Intensity</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>50</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical</td>
<td>0.19</td>
<td>0.238</td>
<td>0.21</td>
<td>0.207</td>
<td>0.177</td>
</tr>
<tr>
<td>Density</td>
<td>0.204</td>
<td>0.245</td>
<td>0.193</td>
<td>0.235</td>
<td>0.184</td>
</tr>
<tr>
<td>Calculated</td>
<td>103.6324</td>
<td>128.8542</td>
<td>114.2029</td>
<td>112.6242</td>
<td>96.69388</td>
</tr>
<tr>
<td>Concentration&lt;sup&gt;2&lt;/sup&gt;</td>
<td>111.0432</td>
<td>132.5008</td>
<td>105.2254</td>
<td>127.29</td>
<td>100.4375</td>
</tr>
<tr>
<td>Mean</td>
<td>107.3378</td>
<td>130.6775</td>
<td>109.7141</td>
<td>119.9571</td>
<td>98.5657</td>
</tr>
<tr>
<td>St. Dev.</td>
<td>5.240228</td>
<td>2.578551</td>
<td>6.348051</td>
<td>10.3703</td>
<td>2.647143</td>
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<tr>
<td>95%C.I.</td>
<td>7.262441</td>
<td>3.573618</td>
<td>8.797775</td>
<td>14.37222</td>
<td>3.66868</td>
</tr>
<tr>
<td>St. Err.</td>
<td>3.705401</td>
<td>1.823311</td>
<td>4.48875</td>
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<td>1.871813</td>
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<td>0.168948</td>
<td>0.006557</td>
<td>0.148931</td>
<td>0.105692</td>
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#### 1 MHz ultrasound, pulsed 1:4

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<th>0.7</th>
<th>1.0</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical</td>
<td>0.299</td>
<td>0.239</td>
<td>0.202</td>
<td>0.193</td>
<td>0.195</td>
</tr>
<tr>
<td>Density</td>
<td>0.273</td>
<td>0.275</td>
<td>0.249</td>
<td>0.207</td>
<td>0.216</td>
</tr>
<tr>
<td>Calculated</td>
<td>160.6458</td>
<td>129.3754</td>
<td>109.988</td>
<td>105.2254</td>
<td>106.2858</td>
</tr>
<tr>
<td>Concentration&lt;sup&gt;2&lt;/sup&gt;</td>
<td>147.0713</td>
<td>148.1128</td>
<td>134.5831</td>
<td>112.6242</td>
<td>117.3542</td>
</tr>
<tr>
<td>Mean</td>
<td>153.8585</td>
<td>138.7441</td>
<td>122.2855</td>
<td>108.9248</td>
<td>111.82</td>
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<tr>
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<td>13.24934</td>
<td>17.39136</td>
<td>5.23172</td>
<td>7.82655</td>
</tr>
<tr>
<td>95%C.I.</td>
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<td>18.36228</td>
<td>24.10272</td>
<td>7.25065</td>
<td>10.84683</td>
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<td>3.699385</td>
<td>5.534206</td>
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<tr>
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<td>0.518919</td>
<td>0.706046</td>
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</tr>
</tbody>
</table>

<sup>1</sup> - Intensity in miliWatts/cm<sup>2</sup> (S<sub>A</sub>)

<sup>2</sup> - Calculated after the standard curve, results in pg/ml

<sup>3</sup> - Intensity in Watts/cm<sup>2</sup> (SAPA)
Figure 7.6 - VEGF production by osteoblasts stimulated by 45 kHz (A) and by 1 MHz (B) ultrasound. Cells were insonated for 5 minutes, the medium was collected 18 hours after stimulation and assayed by ELISA. Controls on (A) and (B) were sham-insonated. Bars show mean values ± S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
Table 7.8 - Vascular Endothelial Growth Factor synthesis induced by 45 kHz and 1 MHz therapeutic ultrasound on *human peripheral blood monocytes*

<table>
<thead>
<tr>
<th>45 kHz ultrasound, continuous</th>
<th></th>
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</thead>
<tbody>
<tr>
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<td>Optical</td>
<td>Density</td>
<td>Calculated Concentration²</td>
<td>Mean</td>
<td>St. Dev.</td>
</tr>
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<td>48.34615</td>
<td>3.738739</td>
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<tr>
<td>15</td>
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<td>0.114</td>
<td>61.37914</td>
<td>61.66432</td>
<td>0.403301</td>
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<td>0.126</td>
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<td>69.02239</td>
<td>0.397245</td>
</tr>
<tr>
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<td>0.128</td>
<td>0.136</td>
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<td>0.088</td>
<td>0.09</td>
<td>46.88305</td>
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<td>0.832511</td>
</tr>
<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Density</td>
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<td></td>
</tr>
<tr>
<td>Calculated Concentration²</td>
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<tr>
<td>Mean</td>
<td>48.34615</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>95%C.I.</td>
<td>4.181525</td>
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</tr>
<tr>
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</tr>
<tr>
<td>t Test</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1MHz ultrasound, pulsed 1:4</th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity²</td>
<td>Optical</td>
<td>Density</td>
<td>Calculated Concentration²</td>
<td>Mean</td>
<td>St. Dev.</td>
</tr>
<tr>
<td>0.1</td>
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<td>0.142</td>
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<td>75.15938</td>
<td>3.533172</td>
</tr>
<tr>
<td>0.4</td>
<td>0.106</td>
<td>0.107</td>
<td>57.38702</td>
<td>57.65467</td>
<td>0.406797</td>
</tr>
<tr>
<td>0.7</td>
<td>0.087</td>
<td>0.077</td>
<td>46.29317</td>
<td>43.32091</td>
<td>4.2034</td>
</tr>
<tr>
<td>1.0</td>
<td>0.096</td>
<td>0.098</td>
<td>51.57338</td>
<td>52.15575</td>
<td>0.823609</td>
</tr>
<tr>
<td>Control</td>
<td>0.088</td>
<td>0.09</td>
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<td>47.47173</td>
<td>0.832511</td>
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<td>t Test</td>
<td>0.008485</td>
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</tbody>
</table>

¹ - Intensity in miliWatts/cm² (SA)
² - Calculated after the standard curve, results in pg/ml
³ - Intensity in Watts/cm² (SAPA)
Figure 7.7 - VEGF production by monocytes stimulated by 45 kHz (A) and by 1 MHz (B) ultrasound. Cells were insonated for 5 minutes, the medium was collected 18 hours after stimulation and assayed by ELISA. Controls on (A) and (B) were sham-insonated. Bars show mean values + S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
5. Discussion

The term therapeutic angiogenesis has been suggested for interventions to induce the local growth of blood vessels as a treatment for a variety of ischaemic conditions (Höckel et al, 1993), although neoangiogenesis would be more appropriate. Therapeutic angiogenesis can mainly be achieved by surgical methods, i.e. transposition of autologous tissues with uncompromised vasculature and high angiogenic potential such as omentum majus, musculofasciocutaneous flaps, vascular pedicles in close proximity to the site of the desired neovascularization (Höckel et al, 1993; Harris, 1992). These means of therapeutic angiogenesis might be supplemented in the near future by the local application of angiogenic factors and the implantation of autologous capillary endothelial cells cultured ex vivo. Considerable experimental data as well as some preliminary clinical data exists to support the usefulness of angiogenic factors for therapeutic angiogenesis.

Ultrasound therapy would appear to be the simplest non invasive way of delivering therapeutic angiogenesis. Young and Dyson (1990a) reported the induction of angiogenesis by ultrasound, observed in rat skin lesions. There is considerable clinical evidence to support Young and Dyson’s work, as shown by the several ultrasound effects mentioned in the literature (see chapter 2). Osteoradionecrosis is obviously a condition that benefits from therapeutic angiogenesis. After radiotherapy, the irradiated area becomes hypoxic, hypocellular and hypovascular (Marx, 1983a). Therapeutic angiogenesis empirically induced by ultrasound has proved to be clinically successful (Harris, 1992).

In this chapter it has been shown that ultrasound may exert its angiogenic effect through the stimulation of angiogenic factors, IL-8, bFGF and VEGF. VEGF in particular is a very potent factor (Thomas, 1996), and was significantly stimulated in all three cell types studied.
Mechanical stress can also induce a significant increase in the synthesis of IL-1 like factors in deformed osteoblast culture (Sandy, 1988). However, to date no study on the effect of ultrasound on cytokine production has been documented in the literature. It was notable that the production of IL-1β was stimulated at low levels, and this may enhance the production of IL-8, as it has general immunopotentiating activities (Zachariae and Matsushima, 1992). However, IL-6 and TNFα were not significantly stimulated, suggesting that the angiogenesis stimulation may a distinct mechanism from inflammation.

In this chapter the 45 kHz ultrasound machine (long wave) was also evaluated, since we have shown that it produces good stimulation of cell proliferation and collagen/NCP synthesis (Reher et al, 1998a). This long wave ultrasound has the advantages of: (a) having a much higher penetration depth, (b) it uses low intensity energy levels, causing less heat production, (c) it is used in continuous mode, reducing treatment time, and (d) it has a spherical head, resulting in a bigger effective treatment area (Bradnock et al, 1996).

Comparing the relative effects on angiogenic factor production by the two ultrasound machines (45 kHz and 1 MHz), one concludes that both were highly effective on a particular intensity range. When using 45 kHz, the best response can be obtained by using 15 or 30 mW/cm². With the 1 MHz machine, the choice would be 0.1 to 0.4 W/cm². These recommendations are in agreement with our previous in vitro studies (Chapter 3 and 5) showing these intensities as the most effective for collagen and non-collagenous protein synthesis, and cell proliferation (Reher et al, 1998a).

In conclusion, the results shown here suggest that the healing effect of ultrasound on soft tissues, fractures and osteoradionecrosis may be the result of the stimulation of angiogenesis through the production of angiogenic factors like IL-8, bFGF and VEGF.
Chapter 8 - Expression of Cytokines, Angiogenesis Factors and Bone proteins stimulated by Therapeutic Ultrasound using RT-PCR

1. Introduction

The previous chapter evaluated the production of cytokines and angiogenic factors after ultrasound stimulation. These were measured using standard ELISA techniques, which can detect very small quantities of these factors in the medium used to grow the cells. However, this technique can not tell if ultrasound induces any change at a nuclear level, changing the genetic expression of these factors. The cytokines released into the medium may have been stored and released upon ultrasound stimulation, and no transcription is needed for that. Therefore, this chapter evaluates the hypothesis that ultrasound can induce transcription of several cytokines, angiogenic factors and bone related proteins, which could further explain the results obtained in the previous chapters. A quick review of basic DNA terminology and the techniques used is presented below:

1.1. Transcription and Translation

A defined polynucleotide sequence is a coded string of information. The DNA contained in each cell constitutes the genome, or total genetic information, content, of the organism. A substantial fraction of this DNA is capable of being transcribed, or "read", to allow the expression of this information in direct synthesis of RNA and protein molecules. The segments that can be transcribed are referred to as genes (Mathews and Holde, 1991). The DNA in each cell of every organism contains at least one copy (and sometimes several) of the gene carrying the information to make each protein that the organism requires.
Expression of the genetic information always involves as a first step the transcription of genes into complementary RNA molecules. An enzyme (RNA polymerase) travels along DNA, making RNA transcript by adding nucleotides one at a time, copying the oligonucleotide sequence from one of the DNA strands.

Transcription alone is sufficient for the production of the many functional RNA molecules of the cell, however, the synthesis of specific proteins, as dictated by their genes, is a more complex matter. The production of proteins does not proceed directly from the DNA. For the information to be translated from the DNA sequences of the genes into aminoacid sequences of proteins, a special class of RNA molecules are used as intermediates. Complimentary copies of the genes to be expressed are transcribed from the DNA in the form of messenger RNA (mRNA) molecules. The mRNAs are read by the protein-synthesising machinery of the cell to make appropriate proteins. This process, which takes place on subcellular particles called ribosomes, is referred to as translation.

Figure 8.1 - The flow of genetic information. DNA can either replicate or be transcribed into RNA. Messenger RNAs are then translated into protein sequences. Under some special circumstances, RNA can be reverse transcribed to produce DNA.
Translation occurs through the following steps: A messenger RNA is bound to the ribosome. Individual amino acids are brought to the ribosome, one at a time, by tRNA molecules. Each tRNA identifies the appropriate codon on the messenger RNA and adds this amino acid to the growing chain. The ribosome travels along the mRNA, so that the whole genetic message can be read and translated into protein. The flow of genetic information in the cell can be summarised by the simple schematic diagram shown in figure 8.1.

1.2. Reverse transcription

Reverse transcription is the process when a special enzyme is used to synthesise first strand complimentary DNA (cDNA) from messenger RNA (Fig. 8.1). By extracting and using only RNA from a cell, it is possible to verify specifically what has been transcribed, and then from this mRNA template, generate cDNA. This is usually performed using Superscript™ II Rnase H- Reverse Transcriptase, which is purified to near homogeneity from E. coli containing the pol gene of Moloney Murine Leukemia Virus (Kotewicz et al, 1985; Gerard et al, 1986).

The reverse transcribed cDNA can then be amplified using Polymerase Chain Reaction (PCR). The final product will therefore give information of what part of the DNA was transcribed to mRNA.

1.3. Polymerase Chain Reaction (PCR)

Molecular biology relies on techniques that enable the detection or capture of minute quantities of nucleic acids. With the introduction of the Polymerase Chain Reaction (PCR), more sensitive levels of detection and higher levels of amplification of specific sequences are achieved, and in less time compared to previously used methods.

Devised by Mullis and refined by Saiki et al in 1985 (Eeles et al, 1996), it originally used a DNA polymerase that was not heat-stable, so fresh enzyme
had to be added before each cycle. The PCR revolution followed the development of computerised thermal cyclers, which automatically heat and cool samples, and the introduction of a thermostable Taq polymerase isolated from algae (Thermus aquaticus) living in hot springs of Yellowstone National Park (Eeles et al, 1996).

PCR is a relatively simple technique by which a DNA or cDNA template is amplified many thousand or million-fold quickly and reliably. By amplifying just a small portion of a nucleic acid target, that portion is effectively isolated from the rest of the nucleic acid in the sample, as with traditional cloning methods. The entire amplification can now be performed in vitro as opposed to standard cloning procedures. The importance of the technique is reflected by the exponential increase in the number of publications relating to PCR, from three in 1986 to 1700 in 1990 (Yarnold et al, 1996).

The PCR reaction is a cyclical process of heating and cooling to denature, anneal and enzymatically amplify DNA. The standard reaction uses two oligonucleotide primers that are complimentary to and hybridize with opposite DNA strands flanking the region of interest in the target DNA. The primers are generally around 20 nucleotides in length, sufficiently long to be unique within the genome (Fig. 8.2)

The reaction, shown in figure 8.2, consists of the following steps:

- **Template denaturation** at 90-94°C for 0.5-3 minutes. This separates the two DNA strands.
- **Primer annealing** at 50-65°C for 0.5-1 minute. The primers anneal to the template.
- **Extension** at 72°C. The new DNA strands are synthesised from the primers, complimentary to the single-stranded template DNA to which the primer has been hybridised.

These steps are then repeated. Thus, the newly synthesised DNA strands also become available templates for a further round of DNA synthesis in the next cycle reaction. The DNA is therefore amplified exponentially,
Chapter 8 - Expression of cytokines, angiogenesis factors and bone proteins stimulated by therapeutic ultrasound using RT-PCR

Theoretically $2^n$ times where $n$ is the number of cycles, although in practice the efficiency is not 100%. Typically, 30-40 cycles are performed and, up to 10 kb can be amplified. However, the larger amplification product, the greater the number of shorter non-specific sequences that may also be amplified.

**Figure 8.2** - The polymerase chain reaction (PCR), for amplifying a segment of DNA or cDNA without cloning it. (based on Mathews and Holde, 1991)
In the reviewed literature, no study could be identified that has used RT-PCR techniques to evaluate effects of ultrasound. Yang et al (1996) were probably the first authors to use genetic techniques to evaluate the expression of genes coding for bone and cartilage matrix proteins. However they extracted RNA from healing calluses from rat femurs and used Northern Blot Analysis, not RT-PCR.

2. Hypothesis and Aims

This chapter tests the hypothesis that ultrasound promotes healing in osteoradionecrosis and bone fractures by enhancing gene transcription coding for angiogenic cytokines and bone formation.

The aims of this chapter are:

1. To determine if ultrasound is able to induce changes in gene transcription/expression at mRNA level, identified with RT-PCR techniques. The cells evaluated are human gingival fibroblasts, mandibular osteoblasts, and peripheral blood mononuclear cells (monocytes). The proteins evaluated were: IL-1b, IL-6, TNF-a, IL-8, bFGF, VEGF, alkaline phosphatase, osteocalcin, osteopontin and Type I procollagen.

2. Compare two types of ultrasound machines, the 'traditional' (1 MHz, pulsed), and the 'long wave' (45 kHz, continuous), at 4 intensities each, trying to establish which machine has the best stimulatory effect on gene transcription for these proteins.
3. Material and Methods

In order not to lose the RNA message after stimulation with ultrasound, a time course experiment was used when treating the cells in this chapter. The cells were harvested at the following time points after treatment: 1h, 2h, 4h, 6h, 8h, 12h, 18h and 24 h, giving a total of 8 time points. Each of these time points was treated in duplicates, and each had a control, sham insonated. Details of the experiments follow:

3.1. Cell culture

The cells used in this chapter were human gingival fibroblasts, mandibular osteoblasts, and peripheral blood mononuclear cells (monocytes). The cell culture procedure steps have already been described in the previous chapters. When ready for treatment, the cells were plated in 6 well plates with the following number of cells per well:

- Human gingival fibroblasts - 3x10^5 cells/well
- Human mandibular osteoblasts - 3x10^5 cells/well
- Monocytes (PBMC) - 5x10^6 cells/well

Fibroblasts and osteoblasts were treated the following day. The medium was replaced with 5 ml of fresh medium with 2% HIFBS. The insonations started 1-2 hours after, to allow equilibration with the new medium.

In the case of the monocytes, the cells were plated only 1 hour before each insonation during the time course, in order to avoid changes due to the attachment of the monocytes to the plate. The remaining cells to be used in that particular experiment where kept suspended in falcon tubes (Sarstedt) in the incubator until 1 hour prior to the time point used. One hour after plating, the medium was carefully removed and the cells washed once with fresh medium (RPMI with 2% HIFBS) to remove unattached cells. Finally 5 mls of fresh media was placed in each well, ready for insonation.
3.2. Insonation

Because there were a total of 8 time points in each experiment, it would have been time consuming to perform these with all the 8 ultrasound regimens we have used in the four previous chapters. It was decided to choose only one optimum ultrasound intensity for each machine, based on the results obtained in the previous chapters. The intensities selected were: (1) 30 mW/cm² with the 45 kHz machine, and (2) 0.1 W/cm² with the 1 MHz machine.

The insonation set-up itself has already been described in the previous chapters. However, instead of 5 replicates it was decided to apply ultrasound only in duplicates for each time point, because by using PCR amplification, not much cell material is needed. Therefore, at each time point, 6 treatments were performed, 2 wells for each ultrasound machine, and 2 wells as controls, sham insonated. After insonation, the cells returned to the incubator and were harvested at the specified time periods.

3.3. Total RNA Preparation

Total RNA was extracted essentially by the method of Chomczynski and Sacchi (1987). All plasticware was Rnase free as was the water, all reagents were purchased from BDH (Poole, England) and were all of molecular biology grade. At the specified time points, the plates were removed from the incubator, and the medium was discarded or stored for other assays. Cells were lysed in 600µl of solution D (4M Guanidine thiocyanate, 0.5% N-lauryl-sarcosine, 25mM sodium citrate and 36µl 2-mercaptoethanol per 5 ml of solution D). The plates were immediately transferred to a -70°C freezer and stored (not longer than 1 week) until processed.

The cell lysate was thawed and transferred to 1.5 ml Eppendorfs. The RNA was extracted with phenol-chloroform mixture. An equal volume of citrate saturated phenol: chloroform: isoamyl alcohol mixture (25:24:1) was added and the tubes vortexed, to form an emulsion. This was left on ice for 15 minutes.
The emulsion was then resolved into aqueous and organic phases by centrifugation at 14,000 rpm (10,000g) for 15 minutes at 4°C. The top, aqueous phase, was then removed to fresh microfuge tubes taking care not to disturb either the lower phenolic phase or the interface which contained proteins and DNA respectively. An equal volume of isopropanol was added to the segregated aqueous phase, the mixture was vortexed and left at -20°C for 1 hour. The precipitate was recovered by centrifugation at 14,000 rpm for 30 minutes at 4°C.

The RNA pellet was then resuspended 150 µl of solution D and 150 µl of isopropanol, and left again at -20°C for 1 hour or overnight. After centrifugation, the final total RNA pellet was washed in ice cold 70% ethanol, to remove salts left from the isolation, then a second time in absolute ethanol. Each wash was followed by a brief centrifugation at 14,000 rpm (4°C), prior to removal of the washing fluid. The pellet was then allowed to air dry.

3.4. Complementary DNA preparation (Reverse transcription-RT)

Superscript II Rnase H- reverse transcriptase was used to make complementary first DNA strands, using total RNA, primed with an oligo dT primer. All reagents were purchased from GibcoBRL (Glasgow, Scotland) and used as instructed by the manufacturer.

The protocol is summarised below: All plastic ware was sterile and Rnase free as was the distilled water used. To the tubes containing the total RNA, 1 µg of Oligo (dT)$_{12-18}$ primer (500 µg/ml, GibcoBRL) was added and the volume was then made up to 12 µl with Rnase free sterile distilled water. This mixture was heated in a water bath for 10 minutes to 70°C and then chilled on ice, the contents were then collected by a brief centrifugation.

To this mixture were added the following components; 4 µl of 5x first strand buffer (250mM Tris-HCL, pH 8.3, 375mM KCl, 15mM MgCl$_2$), 2 µl of 0.1M Dithiothreitol and 1 µl of 10mM dNTP mix (10mM each of dATP, dGTP, dCTP, dTTP, neutral pH). To avoid pipetting errors a master mix of these
constituents was first made up and 7 μl of this added to the RNA/Oligo dT mix in the reaction tubes.

The reaction tube contents were then mixed gently and heated at 42°C for 2 minutes prior to the addition of 0.5 μl (100U) of the Superscript II reverse transcriptase (Gibco/BRL) to each tube. The tubes were again gently mixed, briefly centrifuged and the 42°C incubation continued for another 50 minutes.

The reactions were inactivated by heating to 70°C for 15 minutes, and chilling on ice. The cDNA was then diluted with RNase free sterile distilled water to a final volume of 200 μl, aliquoted and stored at -70°C prior to use as template in PCR.

3.5. Polymerase Chain Reaction (PCR)

PCR was performed using Taq DNA polymerase with the cDNA produced in the method described above primed with oligonucleotides described in Table 8.1. The oligonucleotides were synthesised by Genosys (Pampisford, England). All other reagents were purchased from GibcoBRL (Glasgow, Scotland) and were of molecular biology grade. All plastic ware was nuclease free.

Into each 0.5 ml reaction tube was added 10 μl of cDNA, these were then kept on ice until the other components of the reaction had been assembled in a master mix and were ready to be added. The master mix contained per reaction; 5 μl of 10x PCR buffer (200mM Tris-HCl, pH 8.4 and 500 mM KCl), 1.0 μl of 10 mM dNTP mix (as described above), 1.5 μl of 50mM MgCl₂, 0.5 μl of forward (3’) and reverse (5’) primers at 10μM each and nuclease free water to 49.75 μl. Once assembled, 49.75 μl of master mix was added to each reaction. The tubes were gently mixed, spun in a bench top centrifuge and placed in the block of a thermal cycler fitted with a heated lid (Mastercicle® 5330, Eppendorf, Germany).
Table 8.1 - PCR oligonucleotide primers

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<tr>
<th>mRNA species</th>
<th>Primer Sequence</th>
<th>Authors</th>
</tr>
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<tbody>
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<td>GAPDH*</td>
<td>5'-CCACCCATGGCAATTCCATGGCA-3'</td>
<td>Maier et al 1990</td>
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<td></td>
<td>3'-TCTAGACGCGACGTCCAGTCCACC-3'</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>5'-AAACAGATGAAGTGCTCCTCCAGG-3'</td>
<td>Jung et al 1995</td>
</tr>
<tr>
<td></td>
<td>3'-TGGAGAAACACCATTGTGGCTCCA-3'</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-ATGAATCTCTCTCCACAAGCAG-3'</td>
<td>Jung et al 1995</td>
</tr>
<tr>
<td></td>
<td>3'-GAAGAGCCCTCAGGCTGGACTG-3'</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-ATGACTTCAAGCGCTGGGCT-3'</td>
<td>Jung et al 1995</td>
</tr>
<tr>
<td></td>
<td>3'-TCTCAGCCCTCTTCAAAACTTCTC-3'</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>5'-GGGACGTGGAGCTGGCCGGAGG-3'</td>
<td>Jung et al 1995</td>
</tr>
<tr>
<td></td>
<td>3'-CACCAGCTGGTTATCTCTCAGTC-3'</td>
<td></td>
</tr>
<tr>
<td>bFGF</td>
<td>5'-CTAACCCTGACTGCTGAAGGAGT-3'</td>
<td>Ohgi and Johnson 1996</td>
</tr>
<tr>
<td></td>
<td>3'-GTATAGTTTCTGCCCCAGGTCTG-3'</td>
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<tr>
<td>VEGF</td>
<td>5'-GAGTGTGGCACCCTGAGGAGTGCAAC-3'</td>
<td>Takahashi et al 1997</td>
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<tr>
<td></td>
<td>3'-CTCTGGCCGCTCCAGGGCTCGGTT-3'</td>
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<tr>
<td>Alkaline Phosph.</td>
<td>5'-ACGTGGCTAAAGATGTCATC-3'</td>
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<td>3'-CTGGTAGGCGATGTCCCC-3'</td>
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<td>Osteocalc.</td>
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<td>Richard et al 1996</td>
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<td>3'-AGACCGACCCCTAGAC-3'</td>
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<td>Osteopon.</td>
<td>5'-CCAACTAAGTCCAGGAACAG-3'</td>
<td>Richard et al 1996</td>
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<td></td>
<td>3'-GGTGATGTCTGCCGTGTA-3'</td>
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*Glyceraldehyde-3-phosphate dehydrogenase

The lid temperature was brought up to 104°C placed over the tubes and the block temperature elevated to denature the cDNA, usually at 94-96°C for 3 minutes, according to Table 8.2. The temperature was then lowered to 80°C and 0.25 μl of Taq DNA Polymerase was added to each tube and gently mixed. Cycling then commenced, according to Table 8.2, using separated or combined times for annealing and extension of the primers. After completion of the cycles, the machine was set-up to keep the tubes at 8°C. Negative controls used water instead of sample, and positive controls, used a sample known to have the cDNA for that protein. The following cycles were used:
Table 8.2 - Protocols for the PCR amplification

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<td>72°C/1'30</td>
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<td>35</td>
<td>95°C/1'</td>
<td>60°C/2'30</td>
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<td>95°C/1'</td>
<td>60°C/2'30</td>
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<tr>
<td>TNFα</td>
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<td>95°C/1'</td>
<td>60°C/2'30</td>
<td></td>
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<tr>
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<td>30</td>
<td>94°C/1'</td>
<td>60°C/2'</td>
<td>72°C/2'</td>
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<tr>
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<td>72°C/2'</td>
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<tr>
<td>Osteopon.</td>
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<td>30</td>
<td>94°C/30&quot;</td>
<td>57°C/2'</td>
<td>72°C/2'</td>
<td></td>
</tr>
</tbody>
</table>

* This denaturation time was introduced. All the other cycle parameters used are those specified by the papers listed in the previous table.

3.6. Gel preparation and electrophoresis

PCR products were analysed electrophoretically on 1.5% agarose gels, and were run against a 123 Base pair ladder (Sigma).

The gels were made by melting 2.25g of agarose to 150 ml of 1XTBE buffer (made from 5x stock; 0.045M Trisborate and 0.001M EDTA at pH8.0), in a microwave oven. Once cooled to 50°C the molten gel was poured into a horizontal casting tray with a comb inserted to form the sample wells. Once set the final gel had a thickness of approx. 10 mm, the comb was removed and the gel, still supported by the tray, placed in the electrophoresis tank. The tank was filled with 1XTBE buffer so the gel was just covered.

The final PCR reaction product (50 μl) was mixed with 12.5 μl of 6X sample buffer (0.25% w/v Bromophenol blue, 0.25% w/v Xylene cyanol FF, 15% w/v type 400 Ficoll). After determining the positioning of the samples in the gel (Figure 8.3), 20 μl aliquots of the coloured PCR reaction mix were carefully placed into the wells. The same process was repeated with the 123 bp marker, but with a volume of 5 μl. Electrophoresis was carried out for 1 hour at 100 volts. When complete the gel was removed from the tank, and bathed in a
solution of Ethidium Bromide at 1μg/ml for 30 minutes. The ethidium bromide solution was discarded in an appropriate container, and the gel was destained for a further 5 minutes in distilled water and visualised under UV light. The gel was recorded as a .tiff format image with a Sony gel documentation system running UV products digitization software (Cambridge, UK).

An example of a typical gel is shown in figure 8.3. For each protein evaluated, there were a total of 26 PCR reactions: 8 time points, times 3 treatments (control, 1 MHz and 45 kHz), and a negative and a positive control. However, the gel capacity was for only 14 wells. Therefore, each experiment used two gels, with the marker being loaded into both gels. The sequence used in each experiment was as this gel shows.

![Gel organisation used throughout the experiments. Two gels were needed for each cell type and cytokine or protein evaluated.](image)

Figure 8.3 - Gel organisation used throughout the experiments. Two gels were needed for each cell type and cytokine or protein evaluated.
In order to facilitate visual comparisons between controls and the insonated cells, the original gel pictures were electronically edited. Figure 8.4 shows an example of how this re-arrangement was performed, using the gel shown in Figure 8.3 as the original image.

**Figure 8.4** - Image format used after editing the original gel picture shown in figure 8.3. This format makes comparisons easier between the time points for each treatment evaluated.
4. Results

GAPDH was used as an internal control since it is normally produced in human cells. The results for GAPDH show that the RT and cDNA generation worked well, and that the cells were alive, since all cells and treatment groups showed strong bands (Fig. 8.5). However there are two bands missing: (1) fibroblast, 1MHz, 24h and (2) monocytes, control, 1h. This was probably due to a mistake while preparing the gels or the PCR reaction, because these bands are seen in some of the next PCR reactions.

Interleukin 1β mRNA bands were not identified in the fibroblasts cultures at all time points studied. The other two cell types showed irregular bands, stronger in the monocytes, with no differences between controls and ultrasound groups.

The results for Interleukin 6 showed no bands in monocytes and almost none in fibroblasts. However, osteoblasts showed IL-6 transcription (Fig. 8.6). In the controls this was evenly observed in the time scale, and absent at 24h. In the ultrasound groups, there were two obvious peaks, at 1h and at 12h, with decreasing intensities after these peaks. The 45 kHz ultrasound treated cells gave stronger bands.

The gels for Tumour Necrosis Factor alpha did not show any bands on the controls or in the ultrasound treated cells. The absence of bands was noted in all cell groups studied, fibroblasts, osteoblasts and monocytes.
Figure 8.5 - PCR products for GAPDH primer visualised in 1.5% agarose gels stained with ethidium bromide
Figure 8.6 - PCR products for Interleukin-6 primer visualised in 1.5% agarose gels stained with ethidium bromide. The gels for monocytes are in the original format because no bands were identified, only the positive control.
The results for the angiogenic factors evaluated follows. The transcription of **Interleukin 8** was detected in all cell types studied (Fig. 8.7). In the *fibroblasts* group, the controls showed bands in all time points, except in 24h. A similar pattern was observed in the 0.1 MHz, but weaker, and with the 45 kHz, the bands were stronger, with a peak at 4-6 h. The *osteoblasts* had strong bands in all time points. The strongest bands were at 1-2 h, and the bands in the 45 kHz were the strongest in general. In the *monocytes* group, again there are bands in almost all time points. However, some bands are missing, usually at 18 h.

![Figure 8.7 - PCR products for Interleukin-8 primer visualised in 1.5% agarose gels stained with ethidium bromide.](image-url)
Interesting results could be observed in the **basic Fibroblast Growth Factor** gels (Fig 8.8). In the fibroblasts group all time points had bands. A clear pattern can be observed, with a strong beginning and slow decrease in intensity. The 1 MHz group had weaker bands than control, and the 45 kHz, stronger bands. By analysing the results for the osteoblasts, similar observations could be made. The monocytes however did not produce bFGF, neither at rest nor when stimulated.

**Figure 8.8** - PCR products for basic Fibroblast Growth Factor primer visualised in 1.5% agarose gels stained with ethidium bromide. The gels for monocytes are in the original format because no bands were identified.
Figure 8.9 - PCR products for Vascular Endothelial Growth Factor primer visualised in 1.5% agarose gels stained with ethidium bromide.

The most interesting results were those from Vascular Endothelial Growth Factor (Fig 8.9), since here clear differences between controls and insonated cells could be observed. The fibroblasts group had bands in all time points, and both ultrasound groups had stronger bands than controls. This was very obvious in the 1 MHz group, at 1-2h. In the 45 kHz, the signal lasted longer as well. The most significant result in terms of differences between
controls and insonated cells in this whole chapter was noted in the *osteoblasts*. There were no bands in the control group, and clear bands in the two ultrasound groups. The *monocytes* also had stronger bands in the ultrasound group as compared to the controls.

Moving forward from the cytokines and angiogenic factors, the next proteins evaluated were some of the bone-related proteins. Obviously these were evaluated only in the mandibular osteoblasts group (Fig 8.10). The results for *alkaline phosphatase* show that there are only bands at 18 and 24 h, suggesting late gene expression. The ultrasound treated cells showed stronger bands than the control group, where the 18h band was almost missing. By looking at the *osteocalcin* results, the bands were quite scattered. There was an early signal and a late signal at around 18h. The 45 kHz ultrasound group had several bands missing. With the *osteopontin*, several bands are noted, and again, scattered differences between controls and ultrasound groups are observed, but with no clear pattern. Finally, the results for *type I pro-collagen* show that there are almost no bands in controls, and clearly a strong band at 18h in the ultrasound groups. This is similar to the observations made with the alkaline phosphatase.
Figure 8.10 - PCR products for bone related proteins: alkaline phosphatase, osteocalcin, osteopontin and type I pre-collagen visualised in 1.5% agarose gels stained with ethidium bromide.
Chapter 8 - Expression of cytokines, angiogenic factors and bone proteins stimulated by therapeutic ultrasound using RT-PCR

5. Discussion

In this chapter we show that several cytokines and angiogenic factors are produced by fibroblasts, osteoblasts and monocytes at rest. When ultrasound is applied to these cells, the gene expression for these molecules, as measured with RT-PCR, can either be maintained or enhanced. When looking at the results for IL-1β, IL-6 and TNFα, the results in terms of gene expression can be correlated to the ELISA measurements done in the previous chapter. TNFα was not expressed in any cell group, and IL-1β and IL-6 had no obvious bands, except for the osteoblasts and monocytes IL1β, and osteoblasts IL-6. However, there could not be seen any major differences between controls and insonated cells. The minor difference between controls and insonated cells noted for IL-1β in the previous chapter would not be possibly observed in the RT-PCR, since it is not a quantitative method.

However, by analysing the results for the angiogenic factors evaluated, some interesting results could be noted. All cell types produced IL-8, bFGF and VEGF, except the monocytes, which did not produce bFGF. Furthermore, some differences between controls and insonated cells were observed, with the insonated cells producing stronger bands, keeping in mind that this is not a quantitative method. The most remarkable result was with the VEGF in osteoblasts, showing no bands in controls and strong bands in the insonated cells.

The signals controlling angiogenesis, while directed at the vascular EC are usually derived from cells located within the tissue (Folkman, 1995; Beck and D'Amore, 1997). In bone, the osteoblast is recognized as the cell responsible for producing signals via autocrine and/or paracrine manner that regulate the bone resorbing function of osteoclasts by paracrine cytokine modulation (Meghji et al, 1998) and also on other osteoblasts lineage cells (Baron, 1993). It is possible that the osteoblast cell may also participate in regulating angiogenesis within bone in the same manner as osteogenesis has
been shown to occur in close relation with the presence of blood vessels (Carrington and Reddi, 1991; Collin-Osdoby, 1994).

Wang et al (1997a) have postulated that such an interaction does exist between endothelial cells and osteoblasts. This is based on their work where they showed that hOB cells induced by vitamin D₃, IGF-I and PTH were able to produce VEGF mRNA. VEGF is endothelial cell-specific signal and the unequivocal angiogenic factor by mediating endothelial proliferation, endothelial migration and endothelial differentiation (Thomas, 1996; Jackson et al, 1997; Ferrara and Davis-Smith, 1997; Jakobsson, 1994).

Although endothelial cells are present in the bone marrow, the mechanism by which they proliferate in skeletal tissue is not well elucidated. Angiogenesis factors such as epidermal growth factor, tumor necrosis factor-α and PGE₂ are potential angiogenic factors in vivo, but they do not stimulate endothelial cell proliferation in vitro (Klagsbrum, 1991). Although basic and acidic fibroblast growth factors, which are potent angiogenic factors in vitro and in vivo, are present in the bone matrix, they lack a signal peptide, indicating that they only become active when tissues are severely damaged (Globus et al, 1989). In contrast, VEGF, which possesses a hydrophobic signal peptide, can be secreted and binds to its receptors (Flt) on the endothelium, thereby stimulating endothelial cell proliferation (Leung et al, 1989; Ferrara et al, 1992).

As shown here, VEGF mRNA expression was enhanced with ultrasound in osteoblasts. Several other molecules can stimulate mRNA VEGF expression in osteoblasts, such as PGE1 and PGE2 (Harada et al, 1994) and 1,25-(OH)₂D₃ (Wang et al, 1997). Various mechanisms have been reported to regulate VEGF gene expression. VEGF mRNA expression in normal, transformed and tumour cells is upregulated following exposure to low partial pressure of oxygen (Shweioki et al, 1992; Brogi et al, 1994; Goldberg and Schnieder, 1994; Shima et al, 1995). A multitude of cytokines also upregulate VEGF gene expression. Cultured keratinocytes produced a significant increase in VEGF mRNA expression following addition of EGF, TGF-β or Keratinocyte Growth Factor (Frank et al, 1995). Several members of the Immunoglobulin Receptor
superfamily affects VEGF gene expression. IL-6 notably increases VEGF expression in several cell lines (Cohen et al, 1996) and IL-1β induces VEGF expression in aortic smooth muscle cells (Li et al, 1995). IL-1 and PGE both caused an upregulation of VEGF mRNA expression in synovial fibroblasts (Ben-Av et al, 1995). VEGF gene expression was increased in osteoblasts treated with 1,25-dihydroxyvitamin D (Wang et al, 1997). Finally, transforming events such as the mutation of the p53 suppressor gene in murines (Keiser et al, 1994) and mutation and/or amplification of the ras gene (Rak et al, 1995) has resulted in the upregulation of VEGF mRNA expression. Alternatively, upregulation of VEGF mRNA could be the result of increased stability of mRNA following transcription (Ikeda et al, 1995). This is the first documented work to report ultrasound as an external modality that affects VEGF expression.

In comparison to VEGF, the mRNA signal for bFGF, IL-6 and IL-8 was relatively less remarkable following sonication. An observable background mRNA signal was recorded in the control cells. This is unsurprising as reports have documented bFGF, IL-6 and IL-8 are expressed by normal human osteoblasts (Schweigerer et al, 1987; Sandberg et al, 1993). Nevertheless, the resting steady state mRNA signal production was altered by sonication.

While VEGF and bFGF are angiogenic in vitro, IL-8 has been shown to be angiogenic in vivo but not in vitro (Koch et al, 1992; Strieter et al, 1992; Hu et al, 1993). IL-8 is both chemotactic and mitogenic for endothelial cells in vivo (Jackson et al, 1997). This finding suggests that IL-8 exerts angiogenic activity indirectly, being dependent upon the presence of another factors, such as histamine, platelet-activating factors and exogenous arachidonic acid products (Furie and Randolph, 1995). IL-6 acts indirectly by up-regulation of VEGF (Cohen et al, 1996).

Ultrasound sonication appeared to stimulate the osteoblast to express the genes of potent angiogenic factors that act directly on EC to initiate and mediate early angiogenic events. Ultrasound also seems to induce the gene expression of IL-8 and IL-6, both of which are indirect angiogenic factors that also mediate early events in the sequence of angiogenesis. The induction of IL-
8 and IL-6 appears to be somewhat selective because other indirect angiogenic factors such as TNF-α and IL-1β are not expressed following ultrasound sonication.

When analysing the bone-related proteins gene expression, the PCR products did not show significant differences between ultrasound treated cells and controls, except a small tendency to higher gene expression of alkaline phosphatase and type I collagen with ultrasound. The expression of alkaline phosphatase was seen to occur in the 18 hour post-sonication period where ultrasound hastened the expression of this gene compared to the control. This result compliments the finding of Dodds et al (1993), who report that the expression of AlkPhos did not occur immediately following mechanical loading but occurred in the 24 hour post-stimulation period which was substantially increased and sustained. As the enzyme AlkPhos is related to the process of mineralization (Vaughan 1981), it may be assumed that ultrasound may stimulate delayed bone mineralization that occurs much later after the initial stimulation.

Ultrasound seems to stimulate the expression of type 1 pro-collagen, as suggested in the results shown here. This reinforces the previous observations made, that direct ultrasound application to osteoblasts in tissue culture (Chapter 3, Reher et al 1997a and b) or in cell culture (Chapter 5, Reher et al 1998a) stimulates bone formation. This was also observed in mechanical loading, reported to enhance the production of type 1 pro-collagen (Sun et al, 1995; Harper et al, 1995). However, the differences observed here were minimal, and have to be interpreted with caution since the PCR method used is not quantitative.

The findings obtained by studies investigating the effects of mechanical loading on osteoblasts may provide some insight into the findings of this study, as ultrasound is fundamentally a mechanical energy. Stanford et al (1995) studied the normal expression of osteocalcin and found that mechanical strain caused a temporary and reversible rise in the rate of osteocalcin mRNA production. Harper et al (1995) investigated the expression of osteocalcin and
osteopontin following mechanical strain and reports that both genes were up-
regulated following mechanical loading and was independent of hormonal
control. Toma et al (1997) reports a four-fold increase of osteopontin mRNA
production following mechanical loading. Based on this, they regard the
osteopontin gene as a "mechanoresponsive gene" (Toma et al, 1997).
Osteopontin expression has also been reported to be increased (Smalt et al,
1997a and b).

As mentioned, no obvious differences of osteopontin and osteocalcin
mRNA levels were observed here. These observations are similar to those of
Yang et al (1996), who could not see differences in gene expression in bone
related proteins. However they used a different experimental model, stimulating
fracture healing in rats with ultrasound, and analysing the callus with Northern
blotting. The only significant differences between ultrasound and controls at
genetic level was observed in the cartilage related protein aggrecan. These
authors suggest that ultrasound exposure stimulates earlier chondrogenesis
and cartilage hypertrophy, resulting in earlier onset of endochondral bone
formation, which has also been shown by Wang et al (1994).

An observation should also be made regarding the method of
investigation used. RT-PCR is a very sensitive technique, and is able to detect
mRNA messages and amplify them enormously. However, the technique used
is not quantitative, and therefore, differences between controls and insonated
cells would only become apparent with this assay if these were really
substantial. As shown in the previous chapters, the differences between
insonated cells and controls, in terms of cytokine production and collagen
production have been usually between 10 and 120%, and perhaps these are
too small to be noticed here. For the future quantitative PCR could be made, or
several dilutions of the cDNA template could also be performed in order to point
out differences in the cases were bands were noticed in controls and treated
cells.

Based on the understanding of signal transduction mechanisms that
transfers mechanical energy into cellular activity, it can be postulated that gene
expression of the osteoblasts following ultrasound treatment may be the result of direct or a combination of both direct and indirect mechanisms. The immediate response seen in the 1 to 4h post-isonation periods in both ultrasound machines implies that prompt transcription of mRNA had taken place. This was probably mediated through direct pathway initiated by cell membrane configurations and cytoskeleton perturbations, rendering the cell unstable. Progressive reparative changes and corrections to re-constitute the normal cell membrane configurations and cytoskeletal disorganization results in reduction of stimulus and may explain the gradual reduction in mRNA signals. The second peak that was observed, usually with bone related proteins illustrates a delayed osteoblast response following insonation. This second wave of gene expression may have been mediated through indirect pathways initiated by ultrasound. Indirect pathways involve additional steps to bring about similar responses compared to direct pathways. This would account for the delay of 12 to 18h from initial ultrasound treatment.

In conclusion, the results shown here suggest that the healing effect of ultrasound on soft tissues, fractures and osteoradionecrosis may be the result of the stimulation of bone angiogenesis through the enhancement of VEGF mRNA expression in osteoblasts. Furthermore IL-8 and bFGF expression was also slightly enhanced. This shows that ultrasound stimulates the initial events of angiogenesis through the release of direct and indirect acting angiogenic cytokines. Bone related proteins gene expression was altered with ultrasound, mainly with alkaline phosphatase and type I collagen level, both occurring at a delayed time period. Transduction of ultrasound into cellular activity occurred immediately post-isonation as well as in a delayed pattern, suggesting mediation through direct and indirect pathways.
Chapter 9 - Nitric Oxide production induced by therapeutic ultrasound

1. Introduction

One of the most exciting medical discoveries of the 1980's in the study of human physiology was the realisation that nitric oxide (NO) or a derivative is the potent "endothelial-derived relaxing factor" of smooth muscle and has multifold functions in the body (Moncada et al, 1991). NO is a gas with one unpaired electron and thus is a free radical that reacts with many biological molecules. It has important roles in the function of many tissues and organs, from the cardiovascular system to the brain, and its physiology and biochemistry have been discussed in more than 10,000 papers since 1990 (Schechter, 1997).

Nitric oxide (NO) is synthesised from L-arginine by a family of coenzymes called NO-synthases (Jenkins et al, 1995). Two of these are constitutively expressed, and a third is inducible by immunological stimuli. The NO released by the constitutive enzyme acts as an important signalling molecule in the cardiovascular (endothelial NOS - ecNOS) and nervous systems (neuronal NOS - nNOS) (Moncada et al, 1991). The NO released by the inducible NO synthase (iNOS) is generated for long periods, by cells of the immune system among others, and has been shown to be cytostatic/cytotoxic for tumour cells and a variety of microorganisms (Fig. 9.1).

The synthesis of NO by vascular endothelium is responsible for the vasodilator tone that is essential for the regulation of blood pressure. Nitric oxide is the most potent mediator of vasodilation, at the cardiovascular level, the actions of which are imitated by compounds such as nitroglycerin and sodium nitroprusside (Moncada et al, 1991). Nitric oxide also inhibits platelet
aggregation, and platelets themselves generate NO, acting as a negative-feedback mechanism to inhibit platelet activation (Moncada and Higgs, 1993).

![Diagram of the L-arginine-nitric oxide pathway](Figure 9.1 - The L-arginine-nitric oxide pathway (adapted from Ralston, 1997).

Legend - FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; BH₄, tetrahydrobiopterin; NADP, nicotinamide adenine dinucleotide phosphate; ecNOS, endothelial NOS; nNOS, neuronal NOS; iNOS, inducible NOS; iCa⁺⁺, intracellular calcium; LPS, lipopolysaccharide.

In addition, nitric oxide is produced in large quantities during host defense and immunological reactions. Because it has cytotoxic properties and is generated by activated macrophages, it is likely to have a role in nonspecific immunity. Furthermore, it is involved in the pathogenesis of inflammation, in septic shock and perhaps also the hyperdynamic state of cirrhosis (Moncada and Higgs, 1993).

Nitric oxide is generated by a variety of cells apart from mammalian vascular endothelium and nervous tissue (Moncada and Higgs, 1993), including monocyte-derived macrophages, alveolar macrophages and peripheral blood monocytes (Hunt and Goldin, 1992). Nitric oxide is also generated by the inducible nitric oxide synthase (iNOS) in human neutrophils (Moncada et al, 1991), lymphocytes, mesangial cells, hepatocytes, fibroblasts (Grabowski et al, 1996), bone marrow cells and osteoblasts (Riancho et al, 1995). These cells
can usually be stimulated when exposed to bacterial endotoxin or inflammatory cytokines such as interleukin-1 (IL-1), tumour necosis factor (TNF) and interferon gamma (IFNγ) (Grabowski et al, 1996; Van’T Hoff and Ralston, 1997).

There are important experimental observations that provide evidence to study the role of nitric oxide (NO) in angiogenesis (Bussolino et al, 1996). Firstly, in experimental models where angiogenesis can be directly monitored, vasodilation and hyperaemia of the pre-existing capillaries and the persistence of a dilation state of the newly formed vessels are typical findings. Similarly, VEGF is also a vasodilating agent (Thomas, 1996). Secondly, several angiogenesis factors/mediators promote relaxation in vascular preparations, and peptides known to induce endothelium-mediated vasorelaxation are angiogenic. Thirdly, persistent vasodilation is a specific feature in tumour vasculature and in the surrounding tissue. Based on these considerations, Bussolino et al (1996) hypothesised that an endothelial derived relaxing factor such as NO could actively participate in angiogenesis.

By using the vasodilator sodium nitroprusside, which provides an exogenous source of NO, and the neuropeptide substance P, which induces NO production in endothelial cells, it was shown that NO generating compounds promote endothelial cell proliferation and migration (Ziche et al, 1993; Ziche et al, 1994). NO signal transduction in target cells involves activation of the soluble guanylate cyclase and elevation of intracellular cGMP cyclic guanosine monophosphate. The growth promoting effect of NO on cultured endothelium appeared to be linked to cGMP generation, suggesting the existence of an autocrine/paracrine loop in the NO effect.

The relevance of NO in angiogenesis in vivo was assessed in rabbit corneas receiving angiogenesis effector during systemic treatment with NO-synthase inhibitors (Ziche et al, 1994). NO-synthase inhibitors suppress angiogenesis induced by vasodilating effectors, such as substance P and prostaglandin E1. Conversely, exogenous bFGF can elicit angiogenesis in vivo despite the block of NO production by capillary endothelium. These
observations indicate that bFGF production or bFGF receptor expression by endothelial cells might be secondarily activated during NO-dependent vasodilation (Bussolino et al, 1996).

More studies implicate a role for NO-synthase in angiogenesis. For example, it has been shown that angiogenic activity is only released from bacterial endotoxin treated human monocytes in the presence of L-arginine (Leibovicz et al, 1994). Although the source of angiogenic activity was not identified in this study, it was nonetheless blocked by the NO-synthase inhibitors. Similarly, L-arginine was shown to favour healing and angiogenesis in gastric ulcerations, while NO-synthase inhibitors delayed the healing (Brzozowski et al, 1995). The *in vivo* progression of murine haemangiomas, induced by transplantation of endothelial cells immortalised by middle T antigen of polyomavirus, is reduced by canavanine, an NO-synthase inhibitor (Ghigo et al, 1995).

Other observations have indicated a cytotoxic/cytostatic effect of NO on the vascular development of the chorioallantoic membrane suggesting a diversity of effects in embryonic versus adult tissue (Pipili-Synettos et al, 1994). This was also shown by Hatjikondi et al (1996), who suggested that NO is involved in the anti-angiogenic mechanism of X-rays, using the same chorioallantoic membrane assay.

Several data exist in support of NO as a signalling molecule in tumour angiogenesis. Haemodynamic studies have shown that vasculature associated with tumours is insensitive to vasoactive agents and appears to be in an almost maximal state of vasodilation (Andrade et al, 1992). Transfection of inducible NO-synthase into a colon adenocarcinoma line resulted in a cell line that, despite growing more slowly *in vitro*, promoted tumours which grew more rapidly and were more vascularized than wild type cells (Jenkins et al, 1995). Other observations in agreement with NO being a specific signal for tumour vascularisation include the fact that blocking NO-synthase activity retards the growth of xenografted tumours (Kennovin et al, 1996).
There is also evidence that the NO-synthase/guanylate cyclase pathway of endothelial cells is involved in VEGF-induced angiogenesis (Morbidelli et al., 1996). VEGF expression has been detected in several human tumours and correlates with tumour grade, while transfection of VEGF into human breast carcinoma cells enhances tumour growth and vascular density (Zhang et al., 1995). The role of NO in mediating the mitogenic effect of VEGF on cultured microvascular endothelium isolated from coronary post-capillary venules was investigated by Morbidelli et al. (1996). They found that VEGF, but not bFGF, elevates endothelial cell cGMP, and this was blocked by the NO-synthase inhibitors. Moreover, NO-synthase inhibitors blocked VEGF-, but not bFGF-stimulated growth (Morbidelli et al., 1996). There is more evidence connecting NO to VEGF, as was shown by Papapetropoulos et al. (1997). They showed that exposure of human endothelial cells to VEGF both short- and long-term promotes release of NO. This occurs through mechanisms involving tyrosine and PI-3K kinases, suggesting that NO mediates aspects of VEGF signalling required for endothelial cell (EC) proliferation and organisation in vitro (Papapetropoulos et al., 1997).

In the acquisition of angiogenic phenotype by microvascular endothelium, NO production significantly contributes to the growth-promoting effect of vasodilating peptides and VEGF, but not for that of bFGF. Thus, although the NO pathways integrates several chemical and physical modulators of the angiogenic process, not all angiogenic factors depend on this signalling cascade. Although more work is needed to elucidate fully the role of NO in angiogenesis, the nitric oxide pathway appears to be a promising target to be considered in pro- and anti-angiogenic therapeutic strategies (Bussolino et al., 1996).

Recent work has shown that mechanical strain and shear stress represent further important pathways by which NO can be induced in bone. These stimuli cause rapid but transient increases in production of NO and prostaglandins by bone-derived cells and organ cultures (Pitsillides et al., 1995; Klein-Nulend et al., 1995). Whilst previous evidence had indicated that the increases in bone formation which result from mechanical load may be
mediated by prostacyclin (Rawlinson et al., 1995), it is probable that the high concentrations of NO also induced by these stimuli, augment bone gain by antagonizing the bone resorbing effects of prostaglandins (Ralston and Grabowski, 1996). Currently, the NOS isoform(s) responsible for NO production induced by shear stress and mechanical strain remain unclear, although the kinetics of NO induction coupled with activation of the ecNOS enzyme by shear stress in endothelial cells (Uematsu et al., 1995) suggests that a similar mechanism may operate in bone (Ralston, 1997).

Under aerobic conditions, NO rapidly reacts with oxygen to yield the stable metabolites nitrate (NO₃⁻) and nitrite (NO₂⁻). Since the half-life of NO is in the order of seconds, direct measurement is difficult, but can be achieved by chemiluminescent techniques. More commonly however, NO production is assessed indirectly, by measuring the conversion of radiolabelled L-arginine to L-citrulline (Bredt and Snyder, 1990) or by measuring accumulation of NO₃⁻ and NO₂⁻ in biological fluids such as plasma, urine and cell culture medium (Green et al., 1982).

No paper was identified in the reviewed literature linking ultrasound to the production of NO. However as shown, there are many suggestions relating NO to angiogenesis, so that it may be possible that one of the mechanisms through which ultrasound induces angiogenesis is by inducing NO production. Therefore it was decided to evaluate the production of NO by osteoblasts stimulated by therapeutic ultrasound.

2. Hypothesis and Aims

This chapter tests the hypothesis that ultrasound promotes angiogenesis by the stimulation of the production of nitric oxide.

The aims of this chapter are:

1. Identify if ultrasound is able to induce the release of nitric oxide by human mandibular osteoblasts.
2. Identify in a time course experiment when NO production is induced after ultrasound stimulation.

3. Compare two types of ultrasound machines, the 'traditional' (1 MHz, pulsed), and the 'long wave' (45 kHz, continuous), at 4 intensities each, trying to establish which machine and intensity has the best stimulatory effect on the production of nitric oxide.

3. Material and methods

The cells used in this chapter were human mandibular osteoblasts. The procedure for osteoblast culture can be seen in Chapter 4. The only difference in relation to the described protocol was the medium used. As the nitric oxide assayed colorimetrically, the medium used in these experiments was phenol red free medium, DMEM (Gibco).

The ultrasound application model and the ultrasound machines used were the same as used in the previous chapters. After ultrasound stimulation, the cells returned to the incubator, and the medium was collected for analysis after 18 hours.

A time course experiment was also performed. In this case, the cells were treated with only one intensity for each machine (30mW/cm² (SA) in the 45 kHz, and 0.1 W/cm² (SAPA) in the 1 MHz). A total of 8 time points were analysed (1, 2, 4, 6, 8, 12, 18 and 24h). Each time point was carried out on a separate 6 well culture plate, with 2 wells for control, and 2 for each treatment. The medium was collected at the time points given, and stored at -70°C until assayed.

3.1. Determination of nitrite concentration in conditioned media

The oxidation of L-arginine by NO synthase results in the production of citrulline and the highly reactive, transient, unstable molecule NO. NO is rapidly converted to nitrite (NO₂) and nitrate (NO₃). Nitrite concentrations in the
osteoblast conditioned media were determined using the method of Griess (Green et al, 1982).

Briefly, samples (50μl of unconcentrated medium) were transferred in duplicates or triplicates to a 96 well ELISA type plate. The same volume of Griess reagent was added to each well. The Griess reagent is a 1:1 mixture of 1% sulfanilamide and 0.1% naphthylenediamide in 5% orthophosphoric acid. After 10 minutes the optical density was read using a 570nm filter on a Titertek Multiscan spectrophotometer (Flow). A standard curve was plotted of the absorbance (optical density) versus the concentration of NaNO₂ (between 0 and 50 μM).

4. Results

Table 9.1 shows the results of nitrite production by mandibular osteoblasts after stimulation with therapeutic ultrasound. The parameters evaluated were the same as in the previous chapters. With the 45 kHz machine, all intensities produced more nitrite than controls. The difference between controls and treated cells reached significance in three intensities, at 5, 30 and 50 mW/cm² (p<0.05, p<0.01 and p<0.05 respectively). The level of nitrite production in the stimulated cells was in the region of 4.7 to 7.4 μM, whereas the controls had a mean level of 3.4 μM (Fig 9.2A).

In the 1 MHz group, NO production was also enhanced, however to a lesser degree than with the 45 kHz machine. The highest production was observed at lower intensities (0.1 W/cm²(SAPA)), which reached a high statistical significance (p<0.001). The other intensities did not reach significant results, although they were still higher than controls (Fig 9.2B).

The results from the time course experiment are summarised in table 9.2. In the controls, the levels of nitrite production did increase slightly over time, from 0.99 up to 3.07 μM. The peak of nitrite production was noted at 12-18 hours after stimulation. The insonated cells behaved in a similar pattern independently of the ultrasound type used. The nitrite levels did not differ much.
from controls during the first 12 hours after stimulation, although they were generally higher. However, at 18 hours there is a peak of nitrite production of up to 14.06 μM (1 MHz) and 9.81 μM (45 kHz). At 24 hours after stimulation, the level of nitrite dropped slightly, but still remained much higher than controls, with high statistical differences (p<0.001) (Fig. 9.3).
Chapter 9 - Nitric oxide production induced by therapeutic ultrasound

Table 9.1 - Nitrite production induced by 45 kHz and 1 MHz therapeutic ultrasound on *human mandibular osteoblasts* cells

<table>
<thead>
<tr>
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<th>45 kHz ultrasound, continuous</th>
<th>1 MHz ultrasound, pulsed 1:4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intensity</strong></td>
<td><strong>5</strong></td>
<td><strong>15</strong></td>
</tr>
<tr>
<td>Optical Density</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.042</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>0.035</td>
<td>0.055</td>
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<tr>
<td></td>
<td>0.032</td>
<td>0.031</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
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<td>4.770073</td>
</tr>
<tr>
<td><strong>St. Dev.</strong></td>
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<td>4.950042</td>
</tr>
<tr>
<td><strong>95%C.I.</strong></td>
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<td>3.96078</td>
</tr>
<tr>
<td><strong>St. Err.</strong></td>
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<td>2.020846</td>
</tr>
<tr>
<td><strong>t Test</strong></td>
<td>0.020987</td>
<td>0.619646</td>
</tr>
</tbody>
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$^{1}$ - Intensity in milliWatts/cm$^2$ ($^{SA}$)

$^{2}$ - Calculated after the standard curve, results in μM

$^{3}$ - Intensity in Watts/cm$^2$ ($^{APA}$)
Figure 9.2 - Nitrite production by mandibular osteoblasts by 45 kHz (A) and by 1 MHz (B) ultrasound. Cells were insonated for 5 minutes, the medium was collected 18 hours after stimulation and assayed by the Griess reaction. Controls on (A) and (B) were sham-insonated. Bars show mean values + S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.
Table 9.2 - Time course of nitrite production induced by 45 kHz and 1 MHz therapeutic ultrasound on mandibular osteoblasts

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
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<th>12</th>
<th>18</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Optical Density</td>
<td>0.013</td>
<td>0.017</td>
<td>0.013</td>
<td>0.012</td>
<td>0.02</td>
<td>0.024</td>
<td>0.032</td>
<td>0.024</td>
</tr>
<tr>
<td>Calculated Concentration</td>
<td>0.553</td>
<td>1.071</td>
<td>0.553</td>
<td>0.424</td>
<td>1.460</td>
<td>1.980</td>
<td>3.025</td>
<td>1.980</td>
</tr>
<tr>
<td>Mean</td>
<td>0.985</td>
<td>1.417</td>
<td>2.683</td>
<td>0.726</td>
<td>1.287</td>
<td>3.070</td>
<td>3.025</td>
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<th>12</th>
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</tr>
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<tbody>
<tr>
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<td>0.013</td>
<td>0.017</td>
<td>0.009</td>
<td>0.016</td>
<td>0.021</td>
<td>0.038</td>
<td>0.057</td>
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</tr>
<tr>
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<td>1.071</td>
<td>0.038</td>
<td>0.941</td>
<td>1.590</td>
<td>3.813</td>
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<tr>
<td>Mean</td>
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<td>1.721</td>
<td>0.339</td>
<td>2.202</td>
<td>2.774</td>
<td>4.606</td>
<td>9.808</td>
<td>6.643</td>
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<table>
<thead>
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<th>Time (Hours)</th>
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<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>12</th>
<th>18</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optical Density</td>
<td>0.014</td>
<td>0.014</td>
<td>0.009</td>
<td>0.011</td>
<td>0.027</td>
<td>0.025</td>
<td>0.096</td>
<td>0.041</td>
</tr>
<tr>
<td>Calculated Concentration</td>
<td>0.683</td>
<td>0.683</td>
<td>0.038</td>
<td>0.295</td>
<td>2.371</td>
<td>2.110</td>
<td>11.611</td>
<td>4.208</td>
</tr>
<tr>
<td>Mean</td>
<td>0.855</td>
<td>1.028</td>
<td>3.262</td>
<td>0.683</td>
<td>1.894</td>
<td>4.794</td>
<td>14.060</td>
<td>4.164</td>
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</table>

1 - Calculated after the standard curve, results in µM
Chapter 9 - Nitric oxide production induced by therapeutic ultrasound

![Figure 9.3 - Time course for nitrite production induced by therapeutic ultrasound in mandibular osteoblasts. Cells were insonated for 5 minutes, the medium collected at the time points shown, and measured with the Griess reaction. Controls were sham-insonated. Bars show mean values + S.E.M. Significance level as compared to controls (sham insonated): * p<0.05, ** p<0.01, *** p<0.001.](image)

5. Discussion

The results reported here show that therapeutic ultrasound can stimulate osteoblasts to produce nitric oxide, suggesting involvement of the L-arginine nitric oxide pathway. This could also help to explain the empirical clinical observation of ultrasound promoting healing in osteoradionecrosis, since nitric oxide is also involved in several bone healing processes, including angiogenesis, mechanical strain and bone turnover.

It has also recently been reported that generation of angiogenic activity by human monocytes requires an L-arginine-dependent NO synthase effector mechanism (Leibovich et al, 1994) and that NO plays an important positive role
in the angiogenesis associated with tissue healing (Konturek et al, 1993). Tumours in nude mice generated from a subclone of the human adenocarcinoma cell line DLD-1 engineered to generate nitric oxide continuously, grew faster and were markedly more vascularized, suggesting that NO may act as part of a signalling cascade for neovascularization (Jenkins et al, 1995).

The implication that NO mediates aspects of VEGF signalling required for EC proliferation and organization in vitro (Papapetropoulos et al, 1997) is very interesting, since in this thesis ultrasound stimulated the release of both NO and VEGF. Moreover, NO-synthase inhibitors block VEGF-, but not bFGF-stimulated growth (Morbidelli et al, 1996). It may be that the stimulation of VEGF production by ultrasound reported in the previous chapters occurred through NO, acting as part of a signalling cascade for neovascularization. However although the NO pathways integrates several chemical and physical modulators of the angiogenic process, not all angiogenic factors depend on this signalling cascade. For example, exogenous bFGF can elicit angiogenesis in vivo despite the block of NO production by capillary endothelium (Bussolino et al, 1996). IL-8 and TNFα also seem to be released independently from NO-synthase effector mechanisms in monocytes (Leibovich et al, 1994).

Apart from the potential to induce angiogenesis, NO is also involved in mechanical strain. Ultrasound can be considered as a way of delivering mechanical strain to bone cells, and therefore stimulate bone turnover, as demonstrated in chapters 3, 5 and 6. This could be through the NO pathway, as mechanical strain and shear stress cause rapid but transient increases in production of NO and prostaglandins by bone-derived cells and organ cultures (Pitsillides et al, 1995; Klein-Nulend et al, 1995). Whilst previous evidence had indicated that the increases in bone formation which result from mechanical load may be mediated by prostacyclin (Rawlinson et al, 1995), it is probable that the high concentrations of NO also induced by these stimuli, augment bone gain by antagonising the bone resorbing effects of prostaglandins (Ralston and Grabowski, 1996). Currently, the NOS isoform(s) responsible for NO production induced by shear stress and mechanical strain remain unclear, although the
kinetics of NO induction coupled with activation of the ecNOS enzyme by shear stress in endothelial cells (Uematsu et al, 1995) suggests that a similar mechanism may operate in bone (Ralston, 1997).

It is likely that the role which NO plays in mediating the effects of mechanical strain in bone will be clarified by studies of bone metabolism in NOS knockout animals. The observation that both oestrogen and mechanical strain increase NO production by activating constitutive NOS further suggests that bone loss associated with oestrogen deficiency and immobilisation may be related to production of NO and may hence be amenable to treatment with pharmacological NO donors (Ralston, 1997). Perhaps ultrasound can be used to stimulate NO production?

In conclusion, the results reported here show that therapeutic ultrasound stimulates the production of NO in osteoblasts. The L-arginine NO pathway is involved in the stimulation of angiogenesis, mechanical stress and bone turnover. We have shown that all these processes also occur with therapeutic ultrasound stimulation. These observations may be due to a direct effect of ultrasound, or perhaps, as suggested here, the L-arginine nitric oxide pathway may be involved as well. These observations may be crucial in helping to explain the valuable clinical role of ultrasound in the management of osteoradionecrosis.
Chapter 10 - Angiogenesis Induced by Therapeutic Ultrasound - an *In Vivo* assay

1. Introduction

Angiogenesis is a complex, multistep process that characterises a variety of malignant and non-malignant conditions (Folkman, 1993). The angiogenic process was extensively explained and discussed in chapter 2. In this chapter we will try to show if ultrasound is able to induce angiogenesis in an *in vivo* model. The previous chapters used *in vitro* assays, which have already given good evidence that ultrasound may be angiogenic. Chapter 4 showed that ultrasound induces endothelial cell proliferation, and in chapters 7 and 8, it was shown that ultrasound induces the release of angiogenic factors by stimulated fibroblasts, osteoblasts and monocytes. Furthermore, in chapter 9 we showed that ultrasound stimulates the production of NO, which may be involved in the cascade of events that lead to angiogenesis.

The model chosen to study the stimulation of angiogenesis *in vivo* by therapeutic ultrasound, was the chick chorioallantoic membrane (CAM) assay. It is one of the most widely applied *in vivo* bioassays for studying the phenomenon of angiogenesis, by using various methods that assess the number and morphology of the CAM vessels (Hatjikondi *et al.*, 1996). The preference for this system is because of its advantages, such as simplicity, repetitive ability and usefulness in assessing morphological and functional changes in vessels under normal or experimental conditions.

In vivo angiogenesis assays can be divided in methods with a high or a low vascular background. The methods with a high vascular background are (1) the disc angiogenesis system, (2) the hamster cheek pouch, (3) the transparent ear chamber, (4) intradermal assays and (5) the chorioallantoic membrane
assay (CAM). The methods with a low vascular background are (1) the rodent cornea assay and (2) the rodent mesenteric window assay (Jakobsson, 1994). For the purpose of this chapter a brief explanation of the method used will be made.

This is the most commonly used bioassay to study angiogenesis. The test substance is usually prepared in slow-release polymer pellets, or in methylcellulose discs and implanted on to the CAM through a window made in the eggshell or in a shell-less embryo cultured in a petri dish. The angiogenic activity is revealed by radial in-growth of new capillary vessels (Fan, 1993).

The CAM is an extraembryonic membrane formed on the 4th day of incubation by fusion of the chorion and the allantois. It mediates gas exchange with the extraembryonic environment and has a very thick capillary network in close contact with the shell. Usually in this assay, a rectangular window in the shell is made, to place grafts or test material on the CAM. Acceleration or retardation of the ongoing angiogenesis are evaluated. Angiogenesis is most often scored 3-4 days after application of the test material, and a "spoke-wheel" type arrangement of vessels, directed towards the test substance or graft is considered evidence of angiogenesis. Positive controls with angiogenesis factors such as VEGF are useful for comparative purposes. A clear increase of vessels around the graft, even without the typical radial arrangement of vessels, is also considered a sign of stimulated angiogenesis. The angiogenic response is most often evaluated semiquantitatively under a stereomicroscope.

2. Hypothesis and Aims

This chapter tests the hypothesis that ultrasound promotes healing in soft tissues, osteoradionecrosis and bone fractures by enhancing the process of angiogenesis in these tissues.

The aims of this chapter are:

1. To determine if ultrasound is able to induce angiogenesis in vivo in the CAM by direct application of the ultrasound beam to the egg.
Chapter 10 - Angiogenesis induced by therapeutic ultrasound - an in vivo assay

2. To determine if the medium from fibroblasts and osteoblasts treated with therapeutic ultrasound contains angiogenic factors that can stimulate angiogenesis on the CAM model.

3. To compare two types of ultrasound machines, the 'traditional' (1 MHz, pulsed), and the 'long wave' (45 kHz, continuous), at 4 intensities each, trying to establish which machine and intensity has the best angiogenic effect.

3. Material and Methods

In this chapter there were two main experiments: 1) Ultrasound applied directly to the eggs; and 2) Medium from insonated cells applied to the CAM embedded into methylcellulose discs. However, since the CAM assay itself was the same, a brief description of the assay will be made, and the differences between both experiments will be highlighted and explained.

3.1. CAM Assay

Fertilised Plymouth Rock x White Leghorn eggs of the common fowl (Gallus domesticus), were obtained weekly (Poyndon Farm) and kept at 4°C until the start of the incubation (up to 1 week). At the start date of incubation (day 0), the eggs were swabbed with isopropyl alcohol (Azowipes) and incubated in an appropriate egg incubator at 37°C, with controlled humidity (~55%). The eggs were not moved, but constantly checked for vitality by candling, to discard any dead embryos.

At day 10-11 of incubation, the eggs were swabbed again, and a square window of 1cm X 1cm was prepared on top part of the egg shell, using a carborundum disc on a rotatory dental micromotor (~30,000 rpm). Great care was taken not to damage the shell membrane. At this stage, only the limiting cuts were made. All embryos in which blood vessels have been damaged were
discarded. After the cuts have been made, the eggs were placed back into the incubator, until all windows were prepared.

-if ultrasound was applied directly to the eggs, this was done immediately before this windowing. The location of the window was marked and the ultrasound regimen was applied (see below for the regimens).

To continue the windowing process, the eggs were transferred to a controlled airflow cabinet (Microflow). The egg was cleaned again with Azowipes, and a pinhole was made on the blunt end of the shell to puncture the air space. This lowers the embryo and minimises the chances of damaging the membranes. The shell window was then gently lifted using straight watchmaker’s forceps n°5 (BDH). The shell membrane should be intact and evident as an opaque, white structure. A small slit was made in this membrane, avoiding to puncture the chorioallantoic membrane underneath. The shell membrane was gently lifted from the CAM, and removed, leaving 1-2 mm at the edges of the shell cuts, preventing the dust from the cuts to contaminate the CAM. The shell window was then sealed with Selotape, and the eggs returned to the incubator.

The group of eggs that received methylcellulose discs, received those the following day. The eggs were transferred to the controlled air flow cabinet, and the selotape was lifted. The disc was then carefully placed over the CAM. The window was re-sealed and the eggs returned to the incubator for 3 days.

3.2. Preparation of the methylcellulose discs

In chapter 7, human gingival fibroblasts were treated with 1 MHz ultrasound machine, at 4 intensities, 0.1, 0.4, 0.7 and 1.0 W/cm² (SAPA), and with the 45 kHz ultrasound, at 4 intensities as well, 5, 15, 30 and 50 mW/cm² (SA). The details of these treatments have already been explained in chapters 4 to 7. As mentioned, 18 hours after insonation the medium of the cells was collected to
be analysed by ELISA methods for the detection of cytokines and angiogenic factors released into the medium by the cells. This medium was stored at -70°C, and was also used to make the methylcellulose discs used here.

The discs were made by adding equal volumes of the stored medium and autoclaved 2% methylcellulose. The sample solution (20 μl) was dropped over a sterile microbiology plate (Sterilin) and dried up. After about 2-3 hours, the discs were lifted carefully and applied onto the CAM. The discs were always prepared on the day of application.

Negative controls used were water (to test the CAM assay), and the control medium (sham insonated) from the cell treatment itself (to test the angiogenesis due to the ultrasound itself). Positive controls for the CAM assay were discs containing VEGF, at a dose of 0.5 μg per disc (Boshoff et al, 1997).

3.3. Direct application of ultrasound

As mentioned above, one study group consisted of eggs in which the ultrasound was applied directly to the CAM/embryo. Before the egg shell cuts were made (day 10-11), ultrasound gel was applied to the egg over the marked window area and insonation was applied for 5 minutes, moving the transducer continuously. The ultrasound machines and intensities tested were the same as for the methylcellulose discs, 1 MHz, at 0.1, 0.4, 0.7 and 1.0 W/cm² (SAPA), and 45 kHz ultrasound at 5, 15, 30 and 50 mW/cm² (SA). The control group consisted of eggs that were manipulated and windowed in exactly the same way, but with the ultrasound generator switched off.

After insonation, the window was prepared as mentioned above, and the eggs returned to the incubator for 3 days.

3.4. Evaluation of the angiogenic response

At days 13-14 of development (3 days after direct ultrasound or disc placement), the angiogenic response of the CAM was evaluated. The CAMs
were observed by means of a stereoscope (Olympus). The window was enlarged and a 20% fat emulsion (Intralipos 20%) was injected into the CAM to increase the contrast between blood and surrounding tissues (Boshoff et al., 1997). The CAMs were photographed for evaluation of angiogenic response, using a special camera adapted to the stereoscope (Olympus), and with the same magnification.

The pictures were coded, and then labelled randomly for blind evaluation. Angiogenic responses were graded independently by three investigators as negative, positive or unclear, on the basis of infiltration of blood vessels into the area of the implanted methylcellulose discs (Boshoff et al., 1997). In the cases where ultrasound was applied directly to the egg, the same evaluation was made, but the judgement of angiogenesis was made by comparison to other pictures and controls.

3.5. Statistical analysis

Angiogenic responses were evaluated by three investigators, according to the method described by Boshoff et al. (1997). The angiogenic response was coded as: positive (+), if the judgement was unanimous; unclear (?), if there was split or unclear judgement; and negative (-), if unanimously negative. These codes were scored 1, 0.5 and 0 respectively, for each embryo. Finally, a percent maximum score was obtained, by dividing the total score from a group of embryos by the embryo number.
4. Results

Some examples of the pictures of the CAM obtained will be shown below. Figure 10.1 shows CAMs where the eggs received direct ultrasound application, with the negative example in the top and the positive example bellow. Similarly, Figure 10.2 shows examples of negative and positive samples in the same order, when the methylcellulose discs were applied. In the later case, note that the comparison is between the CAM underneath the disc, and the CAM around it.

![Figure 10.1](image_url)

**Figure 10.1** - Example of the CAM angiogenic response after direct ultrasound application to the egg. Top - negative response; bottom - positive response. This picture was taken 3 days after ultrasound treatment, when the shell window was enlarged and a 20% fat emulsion was injected to enhance contrast between blood vessels and the background.
Figure 10.2 - Example of the CAM angiogenic response after the application of a methylcellulose disc containing medium from insonated cells. Top - negative response; bottom - positive response. This picture was taken 3 days after disc application, when the shell window was enlarged and a 20% fat emulsion was injected to enhance contrast between blood vessels and the background.
Chapter 10 - Angiogenesis induced by therapeutic ultrasound - an in vivo assay

Table 10.1 - Angiogenic effect of direct ultrasound application over the chick CAM

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of Embryos</th>
<th>Angiogenic Response*</th>
<th>Score**</th>
<th>Percent Max. Score***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>3</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>1 MHz - 0.1</td>
<td>9</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>1 MHz - 0.4</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>1 MHz - 0.7</td>
<td>12</td>
<td>6</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>1 MHz - 1.0</td>
<td>12</td>
<td>7</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>45 kHz - 5</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>45 kHz - 15</td>
<td>12</td>
<td>8</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>45 kHz - 30</td>
<td>7</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>45 kHz - 50</td>
<td>12</td>
<td>7</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

* Angiogenic responses were judged by three investigators: (+) unanimously positive; (?) unclear or split judgement; (-) unanimously negative.
** Scores of 1, 0.5, 0 were counted for (+), (?), (-) results, respectively.
*** Percent maximum score is the division of the score by the embryo number.

The results after evaluation of the photographed CAMs by the three examiners are shown in the tables 10.1 and 10.2. The first table (10.1) shows the results for the cases where ultrasound was applied directly to the eggs. The controls as mentioned before, received the same treatment, but with the ultrasound generator switched off, and had 54.9% of the maximum angiogenic score. There was angiogenic response with both ultrasound machines, and the 45 kHz ultrasound had the best results. With the 1 MHz, angiogenic levels varied between 61.1% and 70.8% of the maximum score, and with the 45 kHz machine, the levels were higher (66.7% to 83.3%). The best intensities were 15 and 30 mW/cm²(SA).
Table 10.2 - Angiogenic effect of medium from human gingival fibroblasts incorporated into methylcellulose discs applied over the chick CAM*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of Embryos</th>
<th>Angiogenic Response*</th>
<th>Score**</th>
<th>Percent Max. Score***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>VEGF (0.5 μg)</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>2</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>1 MHz - 0.1</td>
<td>9</td>
<td>2</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>1 MHz - 0.4</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1 MHz - 0.7</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>1 MHz - 1.0</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>45 kHz - 5</td>
<td>12</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
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<td>45 kHz - 30</td>
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</tr>
<tr>
<td>45 kHz - 50</td>
<td>12</td>
<td>2</td>
<td>9</td>
<td>1</td>
</tr>
</tbody>
</table>

* Angiogenic responses were judged by three investigators: (+) unanimously positive; (?) unclear or split judgement; (-) unanimously negative.
** Scores of 1, 0.5, 0 were counted for (+), (?), (-) results, respectively.
*** Percent maximum score is the division of the score by the embryo number.

The next table (10.2) shows the results for the cases where medium from fibroblasts treated with ultrasound was incorporated into methylcellulose discs. In this case, positive (VEGF) and negative (water) controls could also be applied, and these gave results of 80% and 50% maximum angiogenic response respectively. The controls, as mentioned before, were discs containing medium of sham insonated cells. As can be observed, an angiogenic response was observed with both ultrasound machines, however with best responses with the 1 MHz ultrasound. With the 1 MHz levels varied between 61.1% and 91.7% of the maximum score, with best intensity at 0.4 W/cm² (SAPA). Controls were low (41.7%). The 45 kHz machine produced angiogenic responses lower than the 1 MHz, but still higher than controls, with
levels between 50.0% and 58.3%. The best intensity for the 45 kHz machine was 30 mW/cm².

5. Discussion

In this chapter it was demonstrated in an in vivo model, that ultrasound induces angiogenesis. This was observed with both direct and indirect stimulation of the eggs. With direct ultrasound stimulation, the best angiogenic response was with 45 kHz ultrasound, at mid-range intensities.

With the experiments using medium from fibroblasts, again, angiogenesis could be noted, although with best results with the 1 MHz ultrasound. This implies that these cells actually produced angiogenic factors into the medium, which could induce an angiogenic response in the CAM. To be sure that this was not only a reactive inflammatory response from the CAM to the discs, 3 different controls were used. Water was used as a negative control (angiogenic response of 50%), VEGF as a positive control (angiogenic response of 80%), and as an internal control, medium from cells that had been sham insonated. The only true comparison that should be made is between these last controls and the insonated groups.

The use of the CAM model has the advantage of being relatively rapid and inexpensive. It also does not require maintenance of large colonies of laboratory animals, being an intermediate assay between in vitro studies and full animal studies (Hall, 1978). Angiogenesis was scored 3 days after application of the test material, and a "spoke-wheel" type arrangement of vessels, directed towards the test substance or graft was considered evidence of angiogenesis. A clear increase of vessels around the disc, even without the typical radial arrangement of vessels, was also considered a sign of stimulated angiogenesis.

One of the difficulties in the study of angiogenic factors is the lack of readily quantifiable assay systems, and the CAM method has been developed to fill this need. Several attempts have been made to quantitate the CAM
vascular response. For example, one method counts all discernible vessels transversing a 1 mm annulus about a 2 mm filter disc on the CAM, and includes only those vessels which form an angle of less than 45 degrees with a line radiating from the center (Barnhill and Ryan, 1983). Another approach has been to quantitate the vascular response on the CAM by designating it according to a positive or negative or multithread grating system (Folkman, 1975). Other authors attempted to quantitate the chick CAM assay for isolated factors by extensively diluting putative angiogenic factors using a positive/negative type of scoring as a mean of obtaining end point values (Auerbach 1981). Coefficients of angiogenesis were determined by other authors, by dividing the total observed score for observations of each implanted pellet by the maximum score (Form and Auerbach, 1983). A similar technique was used here, based on a recent publication in Science (Boshoff et al, 1997).

The normal vascular growth in the CAM is rapid in the early chick embryo (days 4 to 9) but decreases, and has virtually ceased at day 11 (Ausprunk et al, 1974). This means that stimulation of angiogenesis may be quite different in the early and late embryo. Inherent methodological problems in this assay are the subjectivity of the evaluation of vessel growth, the difficulty to distinguish angiogenesis from hyperaemia and the difficulty to differentiate vasoproliferation from mechanical effects of the implants on the blood vessel distribution at the CAM surface (Vu et al, 1985). However, in spite of this problems, the majority of the angiogenesis studies have been performed using this model, and it is still recently being used (Ribatti et al, 1997; Boshoff et al, 1997).

Angiogenesis induced by ultrasound was probably first described by Young and Dyson (1990a), although Dyson's, earlier wound healing experiments already suggested such action (Dyson et al, 1968; Dyson and Pond, 1970; Dyson et al, 1976; Dyson and Suckling, 1978). They noted that angiogenesis was more profound in the inflammatory phase of repair when stimulated by ultrasound (0.75 MHz and 3 MHz, at 0.1 W/cm²). However the model used was not a CAM model, but they used microfocal x-ray
techniques, evaluating healing in full-thickness excised lesions in the flank skin of rats (Young and Dyson, 1990a).

Young and Dyson (1990a) further suggest that ultrasound may alter the cell permeability of cells, and ions fluxes such as calcium, may act as second messengers, stimulating cell activity. The stimulation of macrophages could lead to the formation or liberation of angiogenic factors such as FGF and TNF-\(\alpha\) (Young and Dyson, 1990b). In the previous chapters we have actually shown that the main angiogenic factors stimulated are VEGF, bFGF and IL-8, and the production of those may be responsible for the angiogenic effect seen here. Not only macrophages produce those, but fibroblast and osteoblasts as well, as shown previously. The question of which non-thermal mechanism is involved still remains, stable cavitation and/or acoustic streaming may play a role.

In summary, this chapter showed that ultrasound is capable of inducing angiogenesis \textit{in vivo} by direct ultrasound stimulation of the CAM. Furthermore, ultrasound also stimulates angiogenesis indirectly in the CAM, by the application of medium from fibroblasts previously treated with ultrasound. This confirms that ultrasound stimulates these cells to produce angiogenic factors that are able to induce an \textit{in vivo} angiogenic response in the CAM model.
1. Introduction

In 1977, Jobsis discovered that biological tissues possessed a 'window' at near-infrared wavelengths through which light could pass with relative ease (Jobsis, 1977). At short wavelengths light is strongly attenuated by haemoglobin and cytochromes whereas at long wavelength, water has a high absorption coefficient. However, between the wavelengths 700 and 1300 nm, he found that a detectable amount of light was transmitted across many centimetres of animal tissues in vivo. Studying the brain of cats, he further found that only three compounds had absorption spectra in this wavelength range, which changed when the inspired oxygen concentration was altered, namely oxygenated haemoglobin (HbO), deoxygenated haemoglobin (Hb) and cytochrome oxidase. By measuring attenuation at a number of different wavelengths and under varying conditions of oxygenation, he was able to calculate changes in the concentrations of oxygenated and deoxygenated haemoglobin (HbO and Hb).

As mentioned, the technique of near infrared spectroscopy relies upon two important phenomena, (1) the fact that biological tissue is relatively transparent to light in the near infrared region of the spectrum and (2) that in tissue, there are compounds whose absorption of light is oxygenation status dependent (Elwell, 1995).

Light in the visible region of the spectrum (with wavelength between 450-700 nm) is strongly attenuated in tissue and therefore fails to penetrate more than 1 cm of tissue. However at near infrared (NIR) wavelengths (700-1000nm)
the absorption of light is significantly lower, and with sensitive instrumentation it is possible to detect light which has been transmitted through up to 8 cm of tissue (Cope, 1991).

Haemoglobin has a distinct peak at 760 nm when it is completely deoxygenated (Hb), and a broad peak around 900 nm when it is fully oxygenated (HbO₂) (Fig. 11.1). The optical properties of haemoglobin have also been applied to measure arterial oxygen tension using pulse oxymetry (Taylor and Whitman, 1986).

![Absorption Coefficient](image)

Figure 11.1 - Pure absorption spectrum for oxygenated (HbO₂) and deoxygenated haemoglobin (Hb) *in vitro*. Note the completely deoxygenated peak at 760 nm and the fully oxygenated peak at 925 nm. Intermediate haemoglobin saturations between these two absolute levels are represented by curves which lie in an intermediate position between these two curves, and pass through the isobestic point. (adapted from Hutchinson *et al*, 1990a).

The early NIRS measurements were solely qualitative in nature (Brazy *et al*, 1985; Ferrari *et al*, 1986). New techniques were developed whereby quantitative measurements are obtainable such as cerebral blood flow (Edwards *et al*, 1988), cerebral blood volume (Wyatt *et al*, 1989), and muscle oxygen consumption (Cheatle *et al*, 1991). However these techniques currently
require a manipulation of the patient's arterial oxygenation which was not possible in the series presented here. It was shown that during episodes of hypoxia, both spontaneous and induced impairment of haemoglobin oxygenation within the brain were detected together with an overall increase in the total haemoglobin concentration (Livera et al, 1992).

It is now possible to measure absolute deoxyhaemoglobin concentration from the NIR spectrum (Matcher and Cooper, 1994). The technique uses second-differential spectroscopy to determine the relative concentration of deoxyhaemoglobin (Hb) to tissue water by fitting of the distinct spectral features of these two chromophores in the 710 nm to 840 nm region of the near infrared spectrum (Matcher and Cooper, 1994). Since the concentration of tissue water is generally known with accuracy of a few per cent, one can then obtain the absolute concentration of deoxyhaemoglobin ([Hb]). However it is not possible to measure the absolute concentrations of the oxygenated haemoglobin (HbO) because it has no distinct features in the second differential of the attenuation spectrum.

Clinical applications of this technique over the past decade have principally been focused on the cerebral circulation. Near infrared spectroscopy has been used to evaluate neonatal cerebral hypoxia and blood flow (Wyatt et al, 1986; Edwards et al, 1988), to monitor raised intracranial pressure (Cairns et al, 1985), to evaluate the effects of gravitational forces on the cerebral oxygenation in fighter pilots (Glaister, 1988), to evaluate oxygenation changes in the human forearm muscle (Hampson and Plantadosi, 1988), and to evaluate peripheral vascular disease (Cheatle et al, 1991). However, although it has been extensively studied experimentally, it has never been used as a clinical tool.

Near infrared spectroscopy was first used to evaluate osteoradionecrosis by Hutchinson et al (1990a). Since osteoradionecrosis is considered to be a chronic non-healing wound due to hypoxia and hypovascularity, NIRS seems to be a useful technique to measure possible decrease in oxygen saturation. Hutchinson et al (1990a) used this technique to study eight patients with
unilateral osteoradionecrosis of the mandible. Five normal male controls (aged 27-38 years) were investigated and the NIR spectra generated from five different points in each mandible. In the osteoradionecrosis sites, a decreased peak of deoxygenated haemoglobin was observed.

There are two possible explanations for this finding. Either there may be a decreased volume of blood, and therefore deoxygenated and oxygenated concentrations are lower, which would be compatible with the hypovascularity seen histologically. The other explanation is that the relative proportion of oxygen extracted from the blood during the passage through the area may be reduced, keeping the haemoglobin saturated with oxygen. This could be due to a substantial increase in blood flow at sites of ORN, which is unlikely, and alternatively, osteoradionecrotic tissue is metabolically inert and may extract little oxygen from haemoglobin during its passage despite normal blood flow (Hutchinson et al, 1990a).

Telfah (1995) evaluated 32 patients who had been given radiotherapy, using NIRS, 16 of them with ORN. His study showed marked decrease in levels of deoxyhaemoglobin concentrations in the ORN tissue, similarly to the observations made by Hutchinson et al (1990a). These observations could also be correlated to clinical findings. Fat content was also evaluated and showed a marked decrease in the irradiated mandible as compared to that of controls, but increased again over the period of 5-7 years between the scans used in his study. This study suggested that there was no spontaneous improvement in blood supply with time, but a significant improvement following a course of therapeutic ultrasound.

In relation to mandibular osteoradionecrosis, so far only two studies have been performed, using NIRS to evaluate the mandibular metabolic status in relation to oxygen consumption (Hutchinson et al, 1990a; Telfah, 1995). However, none of those has performed a full evaluation of the technique in relation to reproducibility between measurements and in the same patient over time. These are crucial, since our preliminary observations showed a great variability in the results.
2. Hypothesis and Aims

This chapter tests the hypothesis that near infrared spectroscopy (NIRS) can be used to detect changes in haemoglobin oxygenation status in the mandible, and therefore can be used to monitor the deleterious effects of radiotherapy and the possible reversion of these effects with therapeutic ultrasound.

The aims of this chapter are:

1. To design an appropriate probe to make NIRS measurements clinically in the human mandible.
2. To validate the measurements obtained in relation to reproducibility in the same subject over a period of time.
3. To validate the measurements obtained in a control group of different ages to identify possible normal age changes in the haemoglobin oxygenation status.
4. To identify if the possible deleterious effects of radiotherapy can be measured with NIRS.
5. To determine if the above validations justify the use of NIRS as a diagnostic tool for determination of the need for use of therapeutic ultrasound, and to measure if ultrasound is able to revert radiotherapy deleterious effects.

3. Materials and methods

3.1. The NIRS system

The system used for measurements of tissue oxygenation in this study is shown schematically in figure 11.2. The light source is a 100 Watt quartz halogen lamp (current stabilised) with a fibre optic output (Oriel 77501, USA). Various colour glass filters with long pass characteristics at visible wavelengths
are employed to eliminate higher spectra. A 2 mm diameter (3 mm with the new probe) Superflex glass fibre optic bundle (Eurotec Fibre Optics, Doncaster) carries light to the sample.

Figure 11.2 - Diagrammatic representation of the NIRS system used in this chapter.

Light transmitted through the tissue sample is collected by a second Superflex fibre optic bundle, with a cross section changing from a circle of 3 mm diameter at the sample to a 5 mm high by 1.4 mm slit at the input to the spectrograph. The spectrograph itself has a 27 cm focal length with an input aperture of f/4. Light enters through a variable width slit of micron resolution and is dispersed by a custom made ruled grating of 600 lines/mm blazed at 750 nm (Spex 270M, Instruments SA, France).

Detection of the dispersed light is performed by an array detector at the focal point of the output mirror. The detector consists of a electrically cooled charged coupled device (CCD) camera (Wright Instruments, UK). This camera uses a 1024 by 256 array of 26 µm square elements (EEV CCD30-11, UK). The 1024 elements are set across the wavelength dispersed axis while the 256 vertical elements record an image of the input slit. It has a pixel bandpass of 0.15 nm, a spectral resolution of 0.3 nm and a total wavelength range of 154 nm. Light incident on the CCD causes charge to accumulate in the elements,
and this charge is then read out and digitised by a 16 bit analogue to digital converter (ADC). Each count of the ADC can be set equivalent to 1 to 4 electrons.

3.2. Probe design

The probes used in the previous studies for NIRS measurements in the mandible consisted of two optic fibre bundles held by hand (Hutchinson, 1990a). This set-up was used initially in the experiments reported here, however the variability was very high, because the light had to pass through several tissue types, from skin to mucosa, and therefore measurements of bone itself were not reliable. Furthermore it was almost impossible to keep the two optic bundles in the same spatial orientation between each other, making repeated measurements very difficult. A second person was also always needed to operate the computer.

Therefore it was decided to design a new probe were both light source and receptor could be introduced in the mouth, allowing measurements of the mandible alone (and overlying soft tissue). The contribution from skin and cheek was therefore avoided, and with the probes held with one hand, the other could operate the computer, avoiding a second operator. The two optic fibre bundles are connected to the probes, so that the emitting light bundle shines directly into the receiving, or caption bundle, avoiding therefore possible orientation changes. Figure 11.3 shows schematically how the probes were designed and constructed.

3.3. Data collection

When the system is switched on, the CCD camera immediately starts to cool down. While the CCD is cooling (to approximately 195°K) the CCD is continually “charged cleared” to prevent trapped charge and hence reduce the dark signal during the measurement. The system is then wavelength calibrated
with a low pressure neon lamp to an accuracy of better than 0.1 nm. The calibration and further data manipulation is performed with a software written by Dr. Roger Springett (Dep. Med. Physics, UCL).

Finally, an air reference for that day needs to be taken. Another scan is taken using the light source and the probes set apart, until a good reading is obtained (1-2x10^5 electrons). Three scans are then taken, each with 9 measurements of 0.1 sec. An average of these three scans is calculated and used as the air reference of the day. To scan the patients, the system was set up to take 5 scans of 0.1 sec., and readings between 0.8 and 2.0x10^5 electrons for each scan were accepted.

![Diagram](image)

**Figure 11.3** - Schematic representation of the new probe design used for NIRS measurements in the mandible

### 3.4. Patients scanning

In the previous NIRS studies in the mandible (Hutchinson *et al.*, 1990a; Telfah, 1995), the light source was shone through the face from skin to the oral
mucosa. In this way, it was difficult to evaluate the contribution of all the different tissues (skin, subcutaneous tissue, muscle, parotid gland, bone, submucosa and mucosa), leading to problems in terms of calculation of the absolute concentrations of deoxyhaemoglobin in the bone. Therefore it was decided to place the probes as close as possible to the bone, our primary target. In this way, one arm of the probe was placed in the buccal vestibule and the other arm in the lingual side of the mandible. The probes were placed as far down as possible, trying to shine the light through bone instead of only interpapillar gingiva. The probe was always placed between teeth to avoid the interference from the dental roots. The light emitting and the light receiving probes were compressed against the mucosa as much as possible. To avoid pain, the probe ends were protected with a small tube of silicon. Before each scan, the probes were covered with a sterile clear cover.

The mandible of each patient/volunteer was scanned at 6 different points, three on each side of the mandible. The points were: (1) canine - between the lateral incisor and the canine; (2) premolar - between the two premolars; and (3) molar - between the first and second molar. When an area was partially edentulous, the scans were taken in similar areas.

Because of the great variability noted in the preliminary measurements performed, it was decided to measure each point in the mandible 5 times. As mentioned, each measurement consisted of 5 scans of 0.1 seconds. Thus, a total of 25 scans for each selected mandibular point was performed for each patient/volunteer. This was a big improvement from the previous measurements, which consisted of only 1 measurement with 3 scans.

### 3.5. Calculating the results using the software

After the scans have been taken, the results were analysed to calculate the absolute concentrations of deoxyhaemoglobin with the same software program (Dr. Roger Springett, Dep. Med. Physics, UCL). The intensity data from each measurement has a pattern similar to that shown in figure 11.4. This
represents the sum of the 5x0.1sec scans for only 1 measurement of the mandible.

The spectrum is normalised using the air reference data file obtained previously and converted in units of light attenuation. The result of this is an attenuation spectrum for the tissue, the units of attenuation being optical densities (O.D.s) (Figure 11.5).

![Intensity (Electrons)](image)

Figure 11.4 - A typical NIRS transmission spectrum across the mandible. Light intensity is measured in units of electrons and wavelength in units of nanometres (nm).
Chapter 11 - Validation of near infrared spectroscopy as a diagnostic tool to evaluate radiotherapy effects in the mandible

Figure 11.5 - Attenuation of the transmission across the mandible

The second differential of this attenuation is then calculated, and the resulting graph is shown in figure 11.6. The second differential compound spectra of water and deoxyhaemoglobin are formed. Finally, least squares fit the second differential spectrum obtained over the wavelength 680-800 nm with a reference compound spectra of Hb, HbO, coconut oil and H₂O at 37°C (Fig. 11.6). The spectra of coconut oil was used to mimic the fat present in the mandible, since it has a similar spectra as normal fat.

The values obtained from this least squares fitting procedure were saved as ASCII file, and transferred to an Excel® worksheet. The estimation of the absolute concentration of deoxyhaemoglobin (Hb) (in millimolar) is given by the formula:

\[
\text{Abs [Hb]} = \frac{\text{Hb}}{\text{Water}} \times 0.4 \text{ (estimated water concentration)}
\]
Chapter 11 - Validation of near infrared spectroscopy as a diagnostic tool to evaluate radiotherapy effects in the mandible

Figure 11.6 - Second differential of the attenuation spectrum across the mandible. The sharp lines at both extremes are artefacts of the numerical method.

Figure 11.7 - Least-squares fit the second differential spectrum obtained over the wavelength 710-840 nm with the reference compound spectra of Hb, HbO and H₂O at 37°C (model). The sharp lines at both extremes are artefacts of the numerical method.
3.6. Volunteers

Thirty volunteers were included in this study, with age range of 24-84. Eighteen (18/30) 60% males with age range of 24-84, median 32 years, and twelve (12/30) 40% females with age range 27-84, median 45 years (table 11.1).

Table 11.1 - Volunteers included in this study

<table>
<thead>
<tr>
<th>Age group</th>
<th>Males</th>
<th>Females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-30</td>
<td>7</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>30-50</td>
<td>6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>50-84</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>TOTAL</td>
<td>18</td>
<td>12</td>
<td>30</td>
</tr>
</tbody>
</table>

All the patients had no known malignancies or relevant diseases. The scans were performed in the same time of the day (10-12 am). NIRS spectra were generated from six points in the mandible and repeated 5 times, as mentioned (canine, premolar and molar, in both sides of the mandible).

The following analysis were performed:

1) Intra-subject variability - one of the volunteers was examined with NIRS at 6 different dates over a period of 3 months. The results were analysed with single factor ANOVA to detect significant variability between the readings of each day. Furthermore analysis comparing measurements of one side to another were also performed.

2) Inter-subject variability - This analysis looked at the variability of the deoxyhaemoglobin concentration measurements in relation to age. Correlations were made at each scanned point to see if there was any association between age and deoxyhaemoglobin concentration in the mandible. Furthermore the data of the 30 volunteers was analysed looking for variability between subjects readings.
3.7. Patients

Ten patients who had malignancies treated with radiotherapy were also included in the study, to be compared to the volunteer group, and see if NIRS is able to measure any change in the deoxyhaemoglobin concentration in the mandible caused by radiotherapy. The age range of the patients was 36-70. Four males (40%) with age range 36-70, median 57 years, and six females (60%), with age range 41-65, median 53 (table 11.2).

Table 11.2 - Patients included in this study

<table>
<thead>
<tr>
<th>Age group</th>
<th>Males</th>
<th>Females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-50</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>50-70</td>
<td>3</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>TOTAL</td>
<td>4</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

All patients had malignancies in the head and neck region, and all were treated with external beam radiation (teletherapy - 40 to 60 Gy) applied in fractions of 1-1/2 to 2 Gy per session, 5 days a week. To be included in this study, the mandible had to be involved in the radiation field. The details of the distribution of the malignancies is shown in table 11.3.

Table 11.3 - Patients included in this study

<table>
<thead>
<tr>
<th>Malignancy</th>
<th>Males</th>
<th>Females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA floor mouth</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>CA soft palate</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CA nasopharynx</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>CA tongue</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CA tonsil</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CA supraglottic/Larynx</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Submandibular node - primary ?</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>TOTAL</td>
<td>4</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

The data obtained from these patients was compared to that of a group of gender and age matched volunteers, in order to see if there was any difference in the measurements.
4. Results

4.1. Intra-subject variability

Table 11.4 shows the results obtained from the NIRS measurements (deoxyhaemoglobin concentrations) in the same subject, over a period of three months. Each mandibular point was scanned 5x0.1 sec., and each point was scanned 5 times. The table shows the average obtained for the measurements taken in the 6 different days. To analyse the data, all the 30 values obtained for each mandibular point were used, and grouped per day. The analysis of variance was used to determine if there was any difference between the 5 measurements taken for each day, compared to the other days. As can be noted, there was a highly significant difference between the measurements taken each day.

Table 11.4 - Variability of NIRS measurements (deoxyhaemoglobin concentrations in milimolar) over a period of three months, in the same subject. The values shown represent the mean value of the 5 measurements taken in each day.

<table>
<thead>
<tr>
<th>Day</th>
<th>Molar</th>
<th>Right Prem.</th>
<th>Canine</th>
<th>Canine</th>
<th>Left Prem.</th>
<th>Molar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.274</td>
<td>0.162</td>
<td>0.328</td>
<td>0.333</td>
<td>0.203</td>
<td>0.263</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.241</td>
<td>0.140</td>
<td>0.453</td>
<td>0.497</td>
<td>0.197</td>
<td>0.348</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.475</td>
<td>0.430</td>
<td>0.620</td>
<td>0.462</td>
<td>0.342</td>
<td>0.537</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.302</td>
<td>0.121</td>
<td>0.277</td>
<td>0.419</td>
<td>0.145</td>
<td>0.282</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.395</td>
<td>0.369</td>
<td>0.673</td>
<td>0.553</td>
<td>0.419</td>
<td>0.400</td>
</tr>
<tr>
<td>Day 6</td>
<td>0.456</td>
<td>0.312</td>
<td>0.438</td>
<td>0.608</td>
<td>0.345</td>
<td>0.471</td>
</tr>
<tr>
<td>Mean</td>
<td>0.347</td>
<td>0.259</td>
<td>0.449</td>
<td>0.479</td>
<td>0.275</td>
<td>0.383</td>
</tr>
<tr>
<td>St Dev</td>
<td>0.096</td>
<td>0.115</td>
<td>0.162</td>
<td>0.136</td>
<td>0.108</td>
<td>0.104</td>
</tr>
<tr>
<td>C.I. Int.</td>
<td>0.032</td>
<td>0.039</td>
<td>0.054</td>
<td>0.049</td>
<td>0.038</td>
<td>0.037</td>
</tr>
<tr>
<td>Min.</td>
<td>0.208</td>
<td>0.091</td>
<td>0.159</td>
<td>0.169</td>
<td>0.116</td>
<td>0.225</td>
</tr>
<tr>
<td>Max.</td>
<td>0.538</td>
<td>0.469</td>
<td>0.820</td>
<td>0.744</td>
<td>0.476</td>
<td>0.587</td>
</tr>
</tbody>
</table>

ANOVA* 4.75E-10 4.45E-07 7.13E-13 0.010557 7.8E-10 1.74E-12

* The ANOVA was used comparing the 5 measurements taken for each day to the other days. The results show the P values, note that these were highly significant (p<0.001) for all mandibular points.
Figure 11.8 - Example of the variability observed in the Right Canine area on 6 different days (same patient). Bars show the mean values for 5 measurements taken in the day, and error bars show the Confidence Interval (95%).

Figure 11.8 shows an example of the variability of the NIRS measurements in one selected mandibular measurement point (Right Canine). The lowest observations were 0.159 and the highest 0.820 milimolar of absolute deoxHb. The lowest and the highest mean of a day were 0.277 and 0.673 respectively (Figure 11.8).

Although the variability of the measurements was very high, when comparing the right side to the left side of the mandible, the measurements remained relatively similar. Table 11.5 shows the results of the unpaired two tailed t-test, comparing each point of the mandible with the other side. As can be noticed, the difference were mostly not significant, however on 8 occasions there was a difference (marked in bold). By averaging the observations for each day, there was no significant difference in the measurements, comparing left to right (Table 11.5 and Figure 11.9).
Table 11.5 - Comparison between Right and Left side measurements in the same subject in 6 different days (values show p values after t-test analysis)

<table>
<thead>
<tr>
<th></th>
<th>Canine</th>
<th>Premolar</th>
<th>Molar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>p= 0.6539</td>
<td>p= 0.0424</td>
<td>p= 0.483</td>
</tr>
<tr>
<td>Day 2</td>
<td>p= 0.5754</td>
<td>p= 0.1039</td>
<td>p= 0.0001</td>
</tr>
<tr>
<td>Day 3</td>
<td>p= 0.0035</td>
<td>p= 0.0072</td>
<td>p= 0.0495</td>
</tr>
<tr>
<td>Day 4</td>
<td>p= 0.1052</td>
<td>p= 0.3704</td>
<td>p= 0.3443</td>
</tr>
<tr>
<td>Day 5</td>
<td>p= 0.0372</td>
<td>p= 0.0471</td>
<td>p= 0.8592</td>
</tr>
<tr>
<td>Day 6</td>
<td>p= 0.0037</td>
<td>p= 0.3094</td>
<td>p= 0.5345</td>
</tr>
<tr>
<td>Average all days</td>
<td>p= 0.9232</td>
<td>p= 0.9437</td>
<td>p= 0.6715</td>
</tr>
</tbody>
</table>

Figure 11.9 - Mean deoxyhaemoglobin concentrations averaging the measurements taken in the six days in the same subject. Error bars show the Confidence Interval (95%). There was no statistical difference in the values observed when comparing the Right side to the Left, when averaging all the measurements (see table 11.5).

4.2. Inter-subject variability

Because of the great variability observed in the intra-subject evaluation, all the following measurements with the volunteers were repeated 5 times for each point, and the averaged values are shown in the following tables. Table
11.6 shows the results of the NIRS measurements for the age group 20-30. Again, a great variability in the data was observed. Figure 11.10 shows graphically the mean values and confidence intervals (95%) of the measurements done in the 20-30 years age group.

The following two tables (11.7, 11.8) and two figures (11.11, 11.12) show the results for the age groups 31-50 and 51-84, organised in a similar manner as the previous group. Note again the great variability between the data for each patient, even keeping in mind that the values shown are already an average of 5 measurements.
Table 11.6 - Mean deoxyhaemoglobin concentrations after 5 measurements in the volunteer group aged 20-30.

<table>
<thead>
<tr>
<th>Volunteer initials, age and gender (m or f)</th>
<th>Mandibular site</th>
<th>[Abs. Deox.] Milimolar</th>
</tr>
</thead>
<tbody>
<tr>
<td>R Mol</td>
<td>R Pre</td>
<td>R Can</td>
</tr>
<tr>
<td>CO-24m*</td>
<td>0.597</td>
<td>0.359</td>
</tr>
<tr>
<td>MS-25m</td>
<td>0.594</td>
<td>0.317</td>
</tr>
<tr>
<td>AB-26m</td>
<td>0.395</td>
<td>0.341</td>
</tr>
<tr>
<td>RR-27f</td>
<td>0.624</td>
<td>0.550</td>
</tr>
<tr>
<td>NG-27f</td>
<td>0.294</td>
<td>0.227</td>
</tr>
<tr>
<td>AN-29m</td>
<td>0.453</td>
<td>0.453</td>
</tr>
<tr>
<td>MR-29f</td>
<td>0.384</td>
<td>0.255</td>
</tr>
<tr>
<td>PG-29m</td>
<td>0.465</td>
<td>0.368</td>
</tr>
<tr>
<td>NC-30m</td>
<td>0.559</td>
<td>0.475</td>
</tr>
<tr>
<td>PR-30m</td>
<td>0.475</td>
<td>0.430</td>
</tr>
<tr>
<td>Mean</td>
<td>0.484</td>
<td>0.377</td>
</tr>
<tr>
<td>St Dev</td>
<td>0.108</td>
<td>0.101</td>
</tr>
<tr>
<td>Conf Int</td>
<td>0.067</td>
<td>0.062</td>
</tr>
<tr>
<td>St Err</td>
<td>0.049</td>
<td>0.052</td>
</tr>
</tbody>
</table>

* Volunteer initials, age and gender (m or f)

Figure 11.10 - Mean deoxyhaemoglobin concentrations averaging the measurements taken in the 20-30 years age group. Error bars show the Confidence Interval (95%).
Table 11.7 - Mean deoxyhaemoglobin concentrations after 5 measurements in the volunteer group aged 31-50.

<table>
<thead>
<tr>
<th>Volunteer initials</th>
<th>Age</th>
<th>Gender</th>
<th>R Mol</th>
<th>R Pre</th>
<th>R Can</th>
<th>L Can</th>
<th>L Pre</th>
<th>L Mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND-31m*</td>
<td>31m</td>
<td></td>
<td>0.268</td>
<td>0.315</td>
<td>0.517</td>
<td>0.509</td>
<td>0.400</td>
<td>0.400</td>
</tr>
<tr>
<td>RS-31m</td>
<td>31m</td>
<td></td>
<td>0.261</td>
<td>0.265</td>
<td>0.165</td>
<td>0.317</td>
<td>0.160</td>
<td>0.194</td>
</tr>
<tr>
<td>FK-32m</td>
<td>32m</td>
<td></td>
<td>0.561</td>
<td>0.413</td>
<td>0.682</td>
<td>0.624</td>
<td>0.506</td>
<td>0.663</td>
</tr>
<tr>
<td>BM-32f</td>
<td>32f</td>
<td></td>
<td>0.504</td>
<td>0.354</td>
<td>0.623</td>
<td>0.409</td>
<td>0.311</td>
<td>0.430</td>
</tr>
<tr>
<td>MG-34m</td>
<td>34m</td>
<td></td>
<td>0.376</td>
<td>0.299</td>
<td>0.566</td>
<td>0.570</td>
<td>0.515</td>
<td>0.355</td>
</tr>
<tr>
<td>VM-35f</td>
<td>35f</td>
<td></td>
<td>0.275</td>
<td>0.310</td>
<td>0.169</td>
<td>0.326</td>
<td>0.293</td>
<td>0.333</td>
</tr>
<tr>
<td>SR-36m</td>
<td>36m</td>
<td></td>
<td>0.676</td>
<td>0.476</td>
<td>0.841</td>
<td>1.100</td>
<td>0.531</td>
<td>0.544</td>
</tr>
<tr>
<td>DK-37m</td>
<td>37m</td>
<td></td>
<td>0.392</td>
<td>0.448</td>
<td>0.494</td>
<td>0.723</td>
<td>0.402</td>
<td>0.475</td>
</tr>
<tr>
<td>JW-45f</td>
<td>45f</td>
<td></td>
<td>0.314</td>
<td>0.329</td>
<td>0.483</td>
<td>0.356</td>
<td>0.289</td>
<td>0.235</td>
</tr>
<tr>
<td>MC-47f</td>
<td>47f</td>
<td></td>
<td>0.681</td>
<td>0.958</td>
<td>0.491</td>
<td>0.420</td>
<td>0.356</td>
<td>0.644</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>0.431</td>
<td>0.417</td>
<td>0.503</td>
<td>0.535</td>
<td>0.376</td>
<td>0.427</td>
</tr>
<tr>
<td>St Dev</td>
<td></td>
<td></td>
<td>0.164</td>
<td>0.202</td>
<td>0.208</td>
<td>0.240</td>
<td>0.119</td>
<td>0.158</td>
</tr>
<tr>
<td>Conf. Int</td>
<td></td>
<td></td>
<td>0.102</td>
<td>0.125</td>
<td>0.129</td>
<td>0.149</td>
<td>0.074</td>
<td>0.098</td>
</tr>
<tr>
<td>St Err</td>
<td></td>
<td></td>
<td>0.079</td>
<td>0.089</td>
<td>0.093</td>
<td>0.104</td>
<td>0.061</td>
<td>0.076</td>
</tr>
</tbody>
</table>

* Volunteer initials, age and gender (m or f)

Figure 11.11 - Mean deoxyhaemoglobin concentrations averaging the measurements taken in the 31-50 years age group. Error bars show the Confidence Interval (95%).

309
Table 11.8 - Mean deoxyhaemoglobin concentrations after 5 measurements in the volunteer group aged 51-84.

<table>
<thead>
<tr>
<th></th>
<th>R Mol</th>
<th>R Pre</th>
<th>R Can</th>
<th>L Can</th>
<th>L Pre</th>
<th>L Mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG-54f*</td>
<td>0.330</td>
<td>0.642</td>
<td>0.341</td>
<td>0.418</td>
<td>0.455</td>
<td>0.423</td>
</tr>
<tr>
<td>PL-55m</td>
<td>0.362</td>
<td>0.392</td>
<td>0.537</td>
<td>0.545</td>
<td>0.361</td>
<td>0.252</td>
</tr>
<tr>
<td>BP-58f</td>
<td>0.264</td>
<td>0.292</td>
<td>0.288</td>
<td>0.383</td>
<td>0.270</td>
<td>0.249</td>
</tr>
<tr>
<td>MF-60f</td>
<td>0.423</td>
<td>0.385</td>
<td>0.241</td>
<td>0.161</td>
<td>0.125</td>
<td>0.275</td>
</tr>
<tr>
<td>JC-63m</td>
<td>1.144</td>
<td>0.896</td>
<td>0.823</td>
<td>0.945</td>
<td>0.886</td>
<td>0.808</td>
</tr>
<tr>
<td>NL-67m</td>
<td>0.791</td>
<td>0.709</td>
<td>0.633</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM-76m</td>
<td>0.458</td>
<td>0.329</td>
<td>0.278</td>
<td>0.387</td>
<td>0.362</td>
<td>0.553</td>
</tr>
<tr>
<td>VP-77f</td>
<td>0.374</td>
<td>0.228</td>
<td>0.193</td>
<td>0.260</td>
<td>0.290</td>
<td>0.280</td>
</tr>
<tr>
<td>MW-84f</td>
<td></td>
<td>0.620</td>
<td>0.522</td>
<td>0.543</td>
<td>0.524</td>
<td></td>
</tr>
<tr>
<td>AP-84m</td>
<td>0.475</td>
<td>0.439</td>
<td>0.431</td>
<td>0.544</td>
<td>0.500</td>
<td>0.386</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>St Dev</th>
<th>Conf. Int.</th>
<th>St Err</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.513</td>
<td>0.279</td>
<td>0.173</td>
<td>0.130</td>
</tr>
<tr>
<td></td>
<td>0.493</td>
<td>0.213</td>
<td>0.132</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>0.429</td>
<td>0.200</td>
<td>0.124</td>
<td>0.096</td>
</tr>
<tr>
<td></td>
<td>0.465</td>
<td>0.223</td>
<td>0.138</td>
<td>0.109</td>
</tr>
<tr>
<td></td>
<td>0.419</td>
<td>0.223</td>
<td>0.133</td>
<td>0.119</td>
</tr>
<tr>
<td></td>
<td>0.403</td>
<td>0.215</td>
<td>0.121</td>
<td>0.108</td>
</tr>
</tbody>
</table>

* Volunteer initials, age and gender (m or f)
--- Areas impossible to be scanned (bone grafts or difficult access)

Figure 11.12 - Mean deoxyhaemoglobin concentrations averaging the measurements taken in the 51-84 years age group. Error bars show the Confidence Interval (95%).
4.3. Age correlations

The following three figures show the correlation and regression graphs and coefficients for the deoxyhaemoglobin concentration with age in the six mandibular sites. This correlation was done using the averaged measurements of the 30 volunteers.

As can be noted in the graphs, the correlation was negative in the canine area (Fig. 11.13), and positive in the premolar and molar areas (Figures 11.14 and 11.15). This was consistent in both sides of the mandible. However, the data was very scattered, and the correlation coefficients and R-squares were very low, suggesting very weak or no correlation at all.

The strongest correlation was observed in the right premolar area, with a correlation coefficient of 0.297995 and R square of 0.08888. The lowest correlation coefficient was -0.02546 with an R square value of 0.0006 on the left canine area.
Figure 11.13 - Regression and correlation of deoxyhaemoglobin concentration with age in the Canine area. Correlation coefficient: Right = -0.11873 ($R^2 = 0.0141$); Left = -0.02546 ($R^2 = 0.0006$).
Figure 11.14 - Regression and correlation of deoxyhaemoglobin concentration with age in the Premolar area. Correlation coefficient: Right = 0.297995 (R² = 0.08888); Left = 0.181247 (R² = 0.0329).
Figure 11.15 - Regression and correlation of deoxyhaemoglobin concentration with age in the Molar area. Correlation coefficient: Right = 0.147192 ($R^2 = 0.0217$); Left = 0.044377 ($R^2 = 0.002$).
4.4. Radiotherapy patients

Despite having such poor results with volunteer measurements in terms of reproducibility we decided to scan patients that had radiotherapy, to see if any significant difference could be seen between these measurements compared to healthy volunteers. Table 11.9 shows a summary of the measurements performed. Again, each patient was scanned 5 times in order to try to reduce variability. Figure 11.16 shows the mean values and confidence intervals obtained for this group.

The sites that received the main radiotherapy treatment were reordered so that all are listed in the left side of the table, although some patients had bilateral treatment.

Table 11.9 - Summary of the mean deoxyhaemoglobin concentrations in the radiotherapy patients group.

<table>
<thead>
<tr>
<th></th>
<th>R Mol</th>
<th>R Pre</th>
<th>R Can</th>
<th>L Can</th>
<th>L Pre</th>
<th>L Mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-36m*</td>
<td>0.477</td>
<td>0.201</td>
<td>0.416</td>
<td>0.490</td>
<td>0.239</td>
<td>0.550</td>
</tr>
<tr>
<td>NM-41f</td>
<td>0.461</td>
<td>0.441</td>
<td>0.269</td>
<td>0.426</td>
<td>0.477</td>
<td>0.469</td>
</tr>
<tr>
<td>JR-45f</td>
<td>0.214</td>
<td>0.340</td>
<td>0.333</td>
<td>0.408</td>
<td>0.284</td>
<td>0.247</td>
</tr>
<tr>
<td>JN-53f</td>
<td>0.169</td>
<td>0.195</td>
<td>0.135</td>
<td>0.187</td>
<td>0.169</td>
<td>0.156</td>
</tr>
<tr>
<td>JR-55f</td>
<td>0.246</td>
<td>0.036</td>
<td>0.035</td>
<td>0.087</td>
<td>0.213</td>
<td>0.145</td>
</tr>
<tr>
<td>JR2-56f</td>
<td>0.443</td>
<td>0.472</td>
<td>0.544</td>
<td>0.639</td>
<td>0.554</td>
<td>0.478</td>
</tr>
<tr>
<td>AR-57m</td>
<td>0.305</td>
<td>0.228</td>
<td>0.455</td>
<td>0.564</td>
<td>0.324</td>
<td>0.287</td>
</tr>
<tr>
<td>IO-65m</td>
<td>0.533</td>
<td>0.544</td>
<td>0.662</td>
<td>0.512</td>
<td>0.677</td>
<td>0.439</td>
</tr>
<tr>
<td>EH-65f</td>
<td>0.446</td>
<td>0.369</td>
<td>0.517</td>
<td>0.554</td>
<td>0.748</td>
<td>----</td>
</tr>
<tr>
<td>PL-70m</td>
<td>0.874</td>
<td>0.801</td>
<td>0.842</td>
<td>0.926</td>
<td>0.882</td>
<td>0.633</td>
</tr>
<tr>
<td>Mean</td>
<td>0.417</td>
<td>0.363</td>
<td>0.421</td>
<td>0.479</td>
<td>0.457</td>
<td>0.378</td>
</tr>
<tr>
<td>St Dev</td>
<td>0.204</td>
<td>0.217</td>
<td>0.241</td>
<td>0.232</td>
<td>0.250</td>
<td>0.175</td>
</tr>
<tr>
<td>Conf Int.</td>
<td>0.126</td>
<td>0.134</td>
<td>0.149</td>
<td>0.144</td>
<td>0.155</td>
<td>0.108</td>
</tr>
<tr>
<td>St Err</td>
<td>0.100</td>
<td>0.114</td>
<td>0.117</td>
<td>0.106</td>
<td>0.117</td>
<td>0.095</td>
</tr>
</tbody>
</table>

* Volunteer initials, age and gender (m or f)

By comparing the main radiotherapy site to the other site of the mandible using t-test for unpaired samples, no significant differences were noted, and the following p values were obtained: Molar area (p=0.66548), premolar area (p=0.38012), and canine area (p=0.58788). Furthermore, when using single factor ANOVA comparing all the sites, the p value remained not significant
(p=0.850309). Therefore, there could not be detected any difference between the radiotherapy site and the other site of the mandible.

![Figure 11.16](image)

**Figure 11.16** - Mean deoxyhaemoglobin concentrations averaging the measurements taken in the radiotherapy patients group. The sides were reordered so that the left side was the one that received the main radiotherapy treatment. Error bars show the Confidence Interval (95%).

The radiotherapy group of patients was also compared to a group of 10 volunteers that were gender and age matched (Figure. 11.17). As can be seen graphically little difference can be observed when comparing these two groups. The t-test values obtained when comparing each side are shown in the graph (Figure 11.17), and show that there is no significant difference between the radiotherapy and the control group.
Figure 11.17 - Comparison between the radiotherapy and a group of gender and age matched controls (n=10 for each group). Bars show mean deoxyhaemoglobin concentrations, and error bars show the confidence interval (95%). Above each pair of bars the t-test p value is shown, comparing the two groups in that point.

5. Discussion

The radiotherapy effects on bone, including osteoradionecrosis can be investigated by many techniques, such as radiographs, CT scans, MRI, doppler ultrasound, nuclear medicine and near infrared spectroscopy. The ideal investigative tool, according to Hutchinson (1996) should be able to offer the following: (1) record quantitatively and qualitatively the severity and extent; (2) monitor progress of treatment; (3) predict patients at risk; (4) predict risk factors more confidentially; (5) permit comparison of treatment regimes; (6) predict the bone level damage above which surgery is essential.

Radiographic images are the most commonly used, and the radiological appearance of osteoradionecrosis is that of a mixed radio-opaque radiolucent
lesion, with the radiolucent areas representing bone destruction. However radiographs require a substantial alteration in mineral content and extensive involvement of the bone, which only occurs in later stages, and therefore they underestimate the extent of radiation-damaged bone, and do not correlate with the clinical status of patients (Epstein et al, 1987a; Epstein et al, 1992).

Computer tomography (CT) scans have similar limitations as traditional radiographs for the mandible or maxilla, but can be helpful in temporal bone osteoradionecrosis (Hutchinson, 1996). Hermans et al (1996) examined 10 patients with osteoradionecrosis using CT scans, and observed cortical interruptions and loss of spongiosa trabeculation in all cases on the symptomatic side. Soft tissue thickening on the symptomatic side was seen in 9/10 patients, and this can be difficult to differentiate from tumor recurrence. Magnetic resonance images (MRI) also have been used, and suggest that fibrosis of bone marrow occurs in osteoradionecrosis (Fujita et al, 1991). Positron emission tomography (PET) has been advocated as being able to differentiate between osteoradionecrosis and recurrent tumour (Minn et al, 1993).

Radionuclide bone scanning with technetium methylene diphosphonate ($^{99m}$Tc-MDP) is said to identify pathophysiologic changes in bone earlier than conventional radiography because scan changes reflect osteoblastic activity and good blood flow (Alexander, 1976). Technetium bone scans have also been used to monitor improvements in tissue viability, before, during and after radiotherapy. But an increased uptake of $^{99m}$Tc-MDP has been seen at sites of radiotherapy (showing as ‘hot’ or ‘black’ spots on the image) during and immediately following treatment (King et al, 1980; Aitasalo and Ruotsalainen, 1985). Osteoradionecrosis is also marked by an increased rate of uptake and total upake of $^{99m}$Tc-MDP relative to adjacent bone (Hutchinson et al, 1990b, Epstein et al, 1992). Furthermore the scans remained altered even after successful treatment. The increased uptake of this radionuclide would imply that osteoradionecrosis bone has a good blood supply, and is actively forming bone, neither of which is true. It is clear that some, as yet unexplained,
mechanism accounts for this anomaly (Hutchinson, 1996). Gallium scans have also been used in osteoradionecrosis, with variable findings, consistent with the fact that osteoradionecrosis is not necessarily associated with inflammation within bone. Thus, the conditions necessary for gallium uptake may not be present and it may not be of diagnostic value for osteoradionecrosis. However, gallium scans did correlate with clinical findings following treatment, suggesting that persisting positive gallium scans may indicate the need for surgery following conservative treatment (Epstein et al, 1992).

Colour doppler was also used to evaluate radiation effects on the blood supply of the mouth and face in a prospective clinical study (Semergidis et al, 1996). A total of 44 patients were evaluated, recording blood flow in the common carotid artery. The results show that there were no statistically significant changes between the radiated/operated side and the contralateral side, during the first 6 months after radiotherapy. This observation is quite obvious since the major blood flow goes to the brain (internal carotid), and the possible reduction of blood flow in the face (external carotid) would not be significant enough to be detected at the common carotid artery level. Unfortunately, doppler cannot be applied to more specific vessels such as the inferior alveolar or lingual artery, more specifically related to the mandible because these vessels are too deep to be reached by the doppler.

Tissue oxygen tension studies have been done in order to evaluate the effects of hyperbaric oxygen (HBO) therapy on neoangiogenesis (Beehner and Marx, 1983; Marx, 1984). This was done measuring transcutaneous partial oxygen pressures (TcPO₂) of the central radiation port and a measurement of a reference from outside the radiation field, the left second intercostal space. The results show that hyperbaric oxygen-induced angiogenesis becomes measurable after eight sessions, rapidly progresses to a plateau at 80% to 85% of nonirradiated tissue vascularity by 20 sessions, and remains at that level without further improvement with further HBO. Patients reevaluated 1, 2 and 3 years after their treatment had TcPO₂ levels at or within 90% of their values recorded just after treatment. Thorn et al (1997) evaluated the effects of HBO
for the treatment of osteoradionecrosis using transmucosal oxygen measurements. The measurements were performed in 10 patients, on the attached gingiva. During HBO treatment, the transmucosal oxygen tension increased significantly after five dives, and after 30 dives, the increases were from a mean of 50% to 86% of the measurements from normal healthy gingiva. However, it has to be remembered that TcPO$_2$ measurements are capable of measuring the oxygen levels only at the capillary loops of the skin, at a depth of less then 1 mm. Therefore, although these measurements can be used to verify the effects of hyperbaric oxygen on the skin and mucosa, it can not be used as a tool to establish such improvements in the underlying tissues. Therefore this method is unsuitable to investigate osteoradionecrosis, and the observations made by Beehner and Marx (1983) and by Marx (1984), regarding the effects of HBO on osteoradionecrosis should be looked at critically.

Near infrared spectroscopy (NIRS) is a recognised non invasive method, used largely to monitor cerebral tissue oxygenation and ischaemic changes in neonates (Wyatt et al, 1986; Matcher and Cooper, 1994). It has been used as an investigation method for osteoradionecrosis in retrospective studies, and showed a reduction of the amount of deoxygenated haemoglobin at sites of osteoradionecrosis, confirming that it is a hypovascular, hypoxic tissue with decreased metabolic rate subtracting little oxygen from haemoglobin (Hutchinson et al, 1990a). Telfah (1995) evaluated 32 patients who had been given radiotherapy, 16 of them with ORN. His study showed marked decrease in levels of deoxyhaemoglobin concentrations in the ORN tissue. These observations could also be correlated to clinical findings. Fat content was also be evaluated and showed a marked decreased in the irradiated mandible as compared to that of controls, but increased again over the period of 5-7 years between the scans used in his study. This pilot study suggested that there was also no spontaneous improvement in blood supply with time, but a significant improvement following a course of therapeutic ultrasound.

In the experiments reported here, only the concentrations of deoxyhaemoglobin have been measured, since the oxyhaemoglobin spectrum
does not contain distinct features to permit absolute quantification. Techniques for absolute quantification of both tissue blood volume (Wyatt et al, 1986) and blood flow (Edwards et al, 1988) have been developed but these currently require a manipulation of the patient's arterial oxygenation which was not possible in the series presented here. However if we assume that the volume of tissue illuminated is similar in each patient and that the patient's arterial haemoglobin saturation is 100%, then it is possible to use the deoxyhaemoglobin concentration as an indirect indicator of tissue blood flow.

Reductions in concentrations of deoxyhaemoglobin as reported in osteoradionecrosis may arise for three different reasons. (1) There may be a decreased volume of blood, and therefore both deoxygenated and oxygenated haemoglobin would be reduced. This is likely to occur after radiotherapy or in osteoradionecrosis because of the hypovascularity seen histologically. (2) The proportion between oxy and deoxyhaemoglobin may be altered. This could happen due to a substantial increase in blood with oversupply of oxygen, and therefore proportionally less deoxyhaemoglobin. This is unlikely because of the endarteritis obliterans seen histologically. Finally, (3) there may be adequate blood flow and volume, but reduced metabolic demand in the irradiated tissue, extracting little oxygen from haemoglobin. The relative proportion of deoxygenated haemoglobin would therefore be reduced (Hutchinson et al, 1990a).

Keeping in mind the possibilities mentioned above, the present study was initially designed to evaluate prospectively a group of patients that would receive radiotherapy. This patients would have NIRS scans, and their levels of deoxyhaemoglobin would be monitored at regular intervals over a period of two years. A group of these patients would also receive a course of ultrasound therapy, in order to prevent or revert any deleterious effects of radiotherapy.

Theoretically, the design of the study seemed correct, with age and gender matched controls as well. However, as soon as the first measurements started, a great variability of the results was noted, and the prospective trial was suspended until the NIRS measurements were properly validated. This
validation was actually never performed in the previous publications related to the mandible.

The first step of the validation was to design a new probe which allowed simultaneous hold of the light source and the receiving optic fibre bundle. This was needed because in the previous investigation both probes were held by hand separately, and there was no consistent relationship between both, in terms of direction and positioning. It was also decided that all measurements should be performed as close as possible to the bone, our primary target. Therefore, the probes were designed taking this into account, with appropriate intra-oral angles. This avoided the light source being placed over the skin, thus avoiding the contributions of skin, subcutaneous tissue and muscles, as was done previously. With this new probe, the light bundle always shined directly to the receiving bundle, and since both were connected in one instrument, the operator could now use the computer as well, so that only one person could do the measurements.

Thus the following improvements were obtained with the new system: (1) the measurement is performed only through the mandible/mucosa, without the interference of the cheek; (2) the system is much more sensitive; (3) the system has a higher resolution, by a factor of 10, and therefore the second differential has a far higher quality. This system should be able to measure bone deoxyhaemoglobin to a far higher precision than the old system (Telfah, 1995).

To validate this new system, a series of scans were performed with one volunteer. Even so, great variability was noted, and it was decided to take not only one scan each time, but at least 5. As mentioned, each of these scans consisted of 5 times 0.1 second exposition, which was summed. Then, 5 scans were taken and the calculations of absolute deoxyhaemoglobin concentrations were averaged. This reduced variability to some extent, but since we were planning a prospective trial, it would be important to see if these measurements, in a same patient, would remain relatively constant over time. Again, a volunteer was scanned on 6 different days over a three months period, as shown in table 11.4. The analysis of this data showed that the variability was
still very high, and that the measurements taken each day were significantly different from the other days (Table 11.4). The only consistent reading was when comparing the right to the left side, where there was differences in some days, but generally there was no statistically significant difference between sides.

These preliminary studies really showed that caution should be used when planning the prospective trial. The deoxyhaemoglobin concentration changes expected after radiotherapy or ultrasound therapy would be probably very small, and these could possibly not be detected because of the great variability of the readings. Even so it was decided to continue the validation of the NIRS for the mandible, by evaluating the deoxyhaemoglobin levels change with age on a group of volunteers. A reduction of the levels of deoxyhaemoglobin over age would be expected. However, as shown in the results section, again a great variability of the measurements was noted, and no correlation at all could be identified between age and deoxyhaemoglobin concentration changes.

Finally, although the validation so far had produced disappointing results, it was decided to compare the measurements of a group of patients that received radiotherapy to the mandible, to a group of age and gender matched controls. The previous studies (Hutchinson et al, 1990a; Telfah, 1995) showed a clear reduction of the deoxyhaemoglobin levels in the radiotherapy and osteoradionecrosis group. Unfortunately this could not be observed here (Fig. 11.17), as there was no statistical difference between both groups. This can be explained again by the great variability of the measurements and by the low number of patients examined (n=10).

Although NIRS has been used successful in several other areas such as monitoring neonatal cerebral hypoxia and blood flow (Wyatt et al, 1986; Edwards et al, 1988), raised intracranial pressure monitoring (Cairns et al, 1985), the effects of gravitational forces on the cerebral oxygenation in fighter pilots (Glaister, 1988), human forearm muscle (Hampson and Plantadosi, 1988) and peripheral vascular disease (Cheatle et al, 1991), it seems that for the
mandible there is still more research needed. The variability of the measurements is so high, that the only way we can show the changes we are interested in (radiotherapy and ultrasound effects), would be to evaluate a very large number of patients and volunteers. However this would not be an easy task, since there are not many head and neck radiotherapy patients available, and even less patients with mandibular osteoradionecrosis.

The reason why the system did not work properly in terms of reproducibility of readings is probably because the mandible is relatively thin and the attenuation was low. Therefore if the attenuation features are small compared to the system noise, one can expect large variability in the results. In thicker bones, it may be possible to have more accurate readings.

In conclusion, the NIRS validation for the measurement of deoxyhaemoglobin concentrations in the mandible showed that the variability of the measurements was very high. This was shown by performing measurements in the same patient over a period of time, and by evaluating a group of 30 volunteers. Age correlations could also not be observed. The measurements of patients that received radiotherapy to the mandible showed no significant differences to an age and gender matched control group. Therefore it can be concluded that NIRS is not appropriate to be used diagnostically for the evaluation of radiotherapy effects on the mandibular blood flow and metabolic status. Further research is need for more appropriate diagnostic tools to evaluate mandibular blood flow, oxygen consumption and metabolic status.
Chapter 12 - Final Discussion and Conclusions

This chapter will give a summary of the conclusions listed in the previous chapters.

The overall hypothesis evaluated in this thesis was that therapeutic ultrasound can be used in the treatment and prevention of osteoradionecrosis because it induces cell proliferation and differentiation, angiogenesis and healing, therefore reversing the deleterious long term effects of radiotherapy. To support this hypothesis, several studies were conducted, and the results and conclusions will be summarised below.

1. Summary of the Results

In chapter 3 it was shown that ultrasound is capable of stimulating bone formation. Therapeutic ultrasound at 3 MHz, pulsed 1:4 stimulates bone matrix protein synthesis at the lower end of the currently used therapeutic scale (0.1 W/cm²), and inhibits formation at higher levels of intensity (0.75-2 W/cm²). Non-thermal mechanisms appear to be responsible for these effects.

In chapter 4 the ultrasound application model used in the rest of the thesis was used for the first time, and worked very well. It was shown that therapeutic ultrasound stimulates cell proliferation in fibroblasts, osteoblasts and endothelial cells. This means that ultrasound is effective in the proliferative phase of repair. This may be of importance in the management of osteoradionecrosis because of the observed hypocellularity and hypovascularity. Higher intensities of ultrasound may be used in the initial phases of therapeutic ultrasound, since higher intensities induced more cell proliferation.
Chapter 5 showed that therapeutic ultrasound induces cell differentiation, inducing collagen and non-collagenous protein synthesis, therefore being effective in the proliferative and remodelling phases of repair. Therapeutic ultrasound at 1MHz (pulsed 1:4) stimulates fibroblast and bone matrix protein synthesis at the lower end of the currently used therapeutic scale (0.1 and 0.4 W/cm² superficial acoustic pressure^45 kHz, the best results were at 15 and 30 mW/cm² superficial acoustic pressure). Therefore it may be useful to use lower ultrasound intensities after the proliferative phase of repair. The production of collagen and NCP by osteoblasts can be used as parameter of bone formation, part of the bone turnover process.

Chapter 6 showed that ultrasound stimulates bone resorption when applied directly to the mice calvaria model. This effect was intensity dependent, and higher intensities produced more bone resorption. Since indomethacin inhibited the ultrasound-induced bone resorption, the cyclo-oxygenase pathway appears to be involved. These results, together with those observed in chapters 3 and 5, where bone formation was noted, suggest that ultrasound has a general effect on bone matrix turnover, and that lower intensities induce more bone formation and less resorption.

In chapter 7, it was shown that ultrasound has general effects on cytokine and angiogenic factors production. The angiogenesis-related cytokines, IL-8 and bFGF, were significantly stimulated in osteoblasts, and VEGF was significantly stimulated in osteoblasts, fibroblasts and monocytes. Both ultrasound machines produced significant results, and the best intensities were 15 and 30 mW/cm² superficial acoustic pressure with 45 kHz ultrasound, and 0.1 and 0.4 W/cm² superficial acoustic pressure with 1 MHz ultrasound.

Chapter 8 shows that ultrasound has effects on mRNA expression for cytokines, angiogenic factors and bone related proteins. Transduction of ultrasound into cellular activity occurred possibly via a direct effect immediately post-insonation with cytokines and angiogenic factors, particularly VEGF. This potent angiogenic cytokine was significantly different from controls, suggesting that it may be one of the main molecules responsible for angiogenesis and
bone healing after ultrasound therapy. Furthermore IL-8 and bFGF expression was also slightly enhanced. The bone related proteins had a later response, at 18-24 hours after stimulation, suggesting an indirect effect of ultrasound. From the studied bone proteins, only alkaline phosphatase and type I collagen were stimulated with ultrasound, both of which are markers of bone matrix formation.

Chapter 9 shows that therapeutic ultrasound stimulates the production of NO in osteoblast-like cells. The L-arginine NO pathway is involved in the stimulation of angiogenesis, mechanical stress and bone turnover. We have shown that all these processes also occur with therapeutic ultrasound stimulation. These observations suggest that the L-arginine nitric oxide pathway may be involved after ultrasound stimulation, and that it can be responsible directly or indirectly for the observed results in terms of angiogenesis and healing.

Chapter 10 shows that ultrasound is capable of stimulating angiogenesis \textit{in vivo} by direct ultrasound stimulation of the CAM. Furthermore, ultrasound also stimulates angiogenesis indirectly in the CAM, by the application of medium from fibroblasts previously treated with ultrasound. This confirms that ultrasound stimulates these cells to produce angiogenic factors that are able to induce an \textit{in vivo} angiogenic response in the CAM model.

The NIRS validation for the measurement of deoxyhaemoglobin concentrations in the mandible (chapter 11) showed that the variability of the measurements was very high. This was shown by performing measurements in the same patient over a period of time, and by evaluating a group of 30 volunteers. Age correlations could also not be observed. The measurements of patients that received radiotherapy to the mandible showed no significant differences to an age and gender matched control group. Therefore it can be concluded that the current technique is not appropriate to be used diagnostically for the evaluation of radiotherapy effects on the mandibular blood flow and metabolic status. NIRS as an instrument to evaluate possible beneficial effects of therapeutic ultrasound for prevention or treatment of patients with osteoradionecrosis does not seem to be appropriate. Further
research is needed for more appropriate diagnostic tools to evaluate mandibular blood flow, oxygen consumption and metabolic status.

2. Discussion

Each chapter already discussed individually the results described, and here I will only give a brief final discussion.

Wound healing and tissue regeneration can be impaired by underlying medical factors such as diabetes mellitus, connective tissue disease, chronic venous insufficiency, cachexia, smoking, previous radiotherapy, cytotoxic therapy, and infection. Compromised angiogenesis is a major reason for delayed healing or non-healing in most of these cases. In non-healing wounds such as osteoradionecrosis, a perceived problem is a non-stimulatory level of hypoxia resulting from inadequate perfusion. Moderate levels of hypoxia may facilitate wound healing by inducing the synthesis of collagen precursors and activating macrophages to stimulate angiogenesis (Hunt and Pai, 1972). However, with increased levels of hypoxia, a reduction in fibroblast migration and lower collagen synthesis with impaired hydroxylation of lysine and proline can be observed (Hunt and Pai, 1972).

Therapeutic angiogenesis is the term used to describe the controlled induction or stimulation of neovascularization and neocellularisation for the treatment or prevention of pathological clinical situations characterised by local hypovascularity (Höckel et al, 1993). Healing and tissue regeneration can be improved or accelerated by therapeutic angiogenesis. Traditionally, surgical methods have been used to achieve therapeutic angiogenesis, ie the transposition of autologous tissues with uncompromised vasculature and high angiogenic potential such as muscle flaps (Höckel et al, 1993). The classic surgical ways of therapeutic angiogenesis might be supplemented in the near future by the local application of angiogenic factors and the implantation of autologous capillary endothelial cells cultured ex vivo. Considerable
experimental data as well as some preliminary clinical data exists to support the usefulness of angiogenic factors for therapeutic angiogenesis.

Ultrasound therapy is the simplest way of delivering therapeutic angiogenesis, as can be seen from Dyson’s earliest work (Dyson et al, 1968; Dyson and Pond, 1970; Dyson et al, 1976; Dyson and Suckling, 1978). Young and Dyson (1990a) reported the induction of angiogenesis by ultrasound, observed in rat skin lesions. There is considerable clinical evidence to support the work of Young and Dyson, as shown by the several ultrasound effects mentioned in the literature (Harris, 1992; Yang et al, 1996; Paul et al, 1960; Hogan et al, 1982; Harvey et al, 1975; Pilla et al, 1990; Tsai et al, 1992a; Heckman et al, 1994).

Osteoradionecrosis is specifically an area that benefits from therapeutic angiogenesis. The tissues have a complex metabolic/homeostatic deficiency, bordering an ischaemic necrosis, and are prone to breakdown, leading to a chronic non-healing wound. Therefore, the treatment or prevention of this complication aims to restore the mandibular blood supply as well as restoring the normal soft tissue and bone homeostasis. Radiation may have caused degeneration, genetic and phenotypic changes in the cells lying within the field of irradiation creating an environment of hypocellularity and abnormally functioning cells of varying degrees. This is further compounded by the overall hypoxic state of the tissue due to hypovascularity.

The stimulation of angiogenesis by ultrasound sonication is crucial in ultrasound-mediated wound healing. The production of new blood vessels alleviates the problem of hypovascularity and hypoxia as new vascular conduits would channel blood containing oxygen, nutrients, and the hormones necessary for skeletal growth into the problematic wound. It would seem that ultrasound re-populates these areas with recruitment of osteoprogenitor cells to these sites. Pluripotential cells are found in abundance in bone marrow stroma and periosteum (Rickard et al, 1996). The passage of these cells into the hypocellular areas is facilitated by the production of new blood vessels as a
result of ultrasound-mediated release of angiogenic factors as shown by this study.

The exact cellular mechanism underlying the therapeutic action of ultrasound is still unknown. From the current literature, Yang et al (1996) proposed some mechanisms. In the first instance, the question is how does ultrasound stimulate the cell to alter gene expression? This may be due to: (a) the compression of microtubules, or cavitation, producing oscillatory movement of microbubbles and acoustic streaming, having a direct effect on the permeability of the cell membrane and stimulate second messenger adenylate cyclase activity (Ryaby et al, 1989). Such changes in ion or protein transport could consequently modify intracellular signals for gene expression (Webster et al, 1978; Dyson, 1982; Mortimer and Dyson, 1988). (b) The effects of mechanical pressure at the cell surface could activate the 'stretch receptor' type of cation channel proposed by Sachs (1991), and changes in cation concentrations could also modify intracellular signals regulating gene expression. (c) The mechanical energy transferred by the ultrasound might activate changes in the attachments of cytoskeleton to the extracellular matrix. Wang et al (1993) demonstrated in their 'tensegrity model', that the application of mechanical forces to the cytoskeleton affects cell metabolism and gene expression. This was also confirmed by Sandy (1988) who demonstrated that mechanical stress can induce a significant increase in the synthesis of IL-1 like factors as compared to non-stressed osteoblast cultures. (d) Electrical currents in bone may be potentiated by exposure to ultrasound energy. Investigators have reported increased potentials as a function of ultrasound intensity, frequency, and burst pattern (Behari and Singh, 1981; Duarte, 1983). (e) Finally, a rise in temperature during ultrasonic exposure may have an effect on cell metabolism. However, the use of low-intensity ultrasound reduces tissue heating, and also reduces the possibility of cavitation phenomena, i.e. the pulsation of gas or vapour-filled voids in a sound field (Webster et al, 1980). Since we have shown a maximum temperature rise of 1.8°C at 2.0 W/cm^2, but no measurable rise was observed at the best stimulatory dose of 0.1 W/cm^2(SAPA) (Chapter 3), we believe that the results observed here are probably
more due to non-thermal effects. The same applies to the 45 kHz ultrasound machines, since they have shown to produce less heating than the 1MHz machine (Robertson and Ward, 1995) (Fig 12.1).

Once the cell has been stimulated by one or more of the above mechanisms, gene expression takes place. We could demonstrate it clearly with RT-PCR techniques, as shown in chapter 8. Exactly in which order and which genes are expressed, still needs to be clarified, but some of the questions could be answered here. Nitric oxide may be involved initially, and several cytokines and angiogenic factor, as shown. VEGF is probably the most evident change, noted both in the ELISA and with RT-PCR techniques. Bone related proteins are also altered, probably at a latter stage. Cell proliferation and differentiation, as measured by production of collagen and NCP will occur afterwards. Finally, in general terms, enhancement of angiogenesis and bone turnover will also occur, leading possible to enhancement of healing, as shown clinically in so many papers already described throughout this thesis.

Hyperbaric oxygen therapy has been recommended for the treatment of osteoradionecrosis, but is usually employed as an adjunctive preparation for resection and reconstruction (Marx, 1983b; Mansfield et al., 1981). However, the treatment with hyperbaric oxygen has several disadvantages, not least availability and expense. The use of ultrasound seems to be superior since it is accessible, quicker, cheaper and safer. We have shown that ultrasound addresses the main problems of osteoradionecrosis, hypocellularity by the stimulation of cell proliferation, the enhancement of healing and bone formation through the increase of collagen/NCP synthesis, and the hypovascularity and hypoxia are addressed through the stimulation of angiogenesis. This work also shows that ultrasound produces angiogenesis through the production of IL-8, bFGF and VEGF, all known angiogenic factors.
Figure 12.1 - Molecular mechanisms following ultrasound treatment. (based on Ghasali, 1998)
3. Future research

In terms of future research, some recommendations or suggestions can be made. This thesis studied ultrasound effects mainly in vitro. The interaction between endothelial cells and osteoblasts in controlling both angiogenesis and bone formation could be investigated in vitro with the use of co-culture systems. Another important step would be to evaluate ultrasound effects in animal models, if possible using radiotherapy, trying to mimic the osteoradionecrosis conditions as much as possible. There are obvious ethical concerns involved, but this would clearly show in a more realistically way the revascularisation of bone after radiation damage by therapeutic ultrasound. Following this, prospective clinical trials for the treatment and prevention of osteoradionecrosis using low frequency (long wave) therapeutic ultrasound seem to be indicated. Perhaps more important would be a trial with the use of prophylactic ultrasound in patients that will receive extractions or implants following radiotherapy. However, this should be done if possible only after more objective tools are available to evaluate the metabolic and vascular status of the mandible before and after treatment. This brings us back to the attempted use of near infrared spectroscopy to evaluate oxygen consumption in the mandible.
References


References


References


References


References


References


References


Coutard, H. (1932) Roentgen therapy of epitheliomas of the tonsilar region, hypopharynx & larynx from 1920 to 1926. Am J Roentgen 28, 313


References


References


References


References


References


References


Production of interleukin-6 by human osteoclast-like cells from giant-cell tumor of bone. *Int J Oncol* 8, 297-303.


References


References


References


Hustler, J.E., Zarod, A.P. and Williams, A.R. (1978) Ultrasonic modification of experimental bruising in the guinea pig pinna. Ultrasound 16, 228


References


References


References


Lee, E.H. and Rikihisa, Y. (1996) Absence of tumor necrosis factor alpha, interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor expression but presence of IL-1b, IL-8, and IL-10 expression in human monocytes exposed to viable or killed *Ehrlichia chafeensis*. *Infect Immun* 64, 4211-4219.


Linkhart, T., Linkhart, S., Farley, J., Dimai, H.P., Quian, H.Y., Horowitz, M., Beamer, W., Donahue, L.R., Rosen, C. and Baylink, D. (1996) Osteoblast production of osteolytic cytokines - differences between low peak bone-density c57bl/6j mice and high peak bone-density c3h/hej mice. *J Bone Miner Res* 11, S-S.


References


References


References


References


References


References


Regaud, C. (1922a) Sur la necrose des os atteints par un processus cancéreux et traiters par les radiations. *Compte rendu des seances Societe de Biologie, Paris* 87, 427


Reher, P., Doan, N., Meghji, S. and Harris, M. (1998c) Ultrasound induces angiogenesis through the production of IL-8, bFGF and VEGF. Cytokine (In press)


References


387


References


References


Unger, E.F., Sheffield, C.D. and Epstein, S.E. (1990) Creation of anastomoses between an extracardiac artery and the coronary circulation: proof that myocardial angiogenesis occurs and can provide nutritional blood flow to the myocardium. *Circulation* 82, 1449-1466.


References


References


References


was significant only with the 1.5-MHz signal (p < 0.02). Using a similar rat femur fracture model, Yang et al. (1996) evaluated several mechanical, biochemical and genetic parameters, after treating the fractures with 0.5-MHz ultrasound, at 50 and 100 mW/cm² (SATA). After 3 weeks, the average maximum torque and torsional stiffness were greater than in controls, but reached statistical significance only in the 50-mW/cm² group. Biochemical analysis failed to demonstrate significant differences in cell number, collagen or calcium content. However, they noted a shift in the expression of genes associated with cartilage formation; aggrecan gene expression was significantly higher on Day 7 after fracture, and significantly lower on Day 21. The other gene expression evaluated showed no statistical difference.

Probably the first controlled clinical trial investigating the effectiveness of low-intensity ultrasound, to evaluate the healing of tibial fractures on humans, was done by Heckman et al. (1994). The fractures were examined in a prospective, randomized double-blind evaluation of low-intensity ultrasound [1.5 MHz, 0.03 W/cm² (SATA)], applied to a group of 67 closed or Grade I open fractures of the tibial shaft. The Treated Group showed a significant decrease in the time to clinical healing (86 ± 5.8 days) compared with that in the Control Group (114 ± 10.4 days) (p = 0.01) and also a significant decrease in the time to over-all (i.e., clinical and radiographic) healing (96 ± 4.9 days compared to 154 ± 13.7 days in the Control Group).

Another use of ultrasound applied to bone is in the treatment of mandibular osteoradionecrosis (Harris 1992). The patients were treated with ultrasound [3 MHz, pulsed 1:4, 1 W/cm² (SATA)] for 40 sessions of 15 min per day. Ten (48%) of 21 patients showed healing when treated with debridement and ultrasound alone. The remaining 11 that were not healed after ultrasound therapy received debridement and cover with a local flap, and only 1 needed mandibular resection and reconstruction. These results are better than the conventional treatment with hyperbaric oxygen and surgery if compared with the study of Marx (1983). His study showed that hyperbaric oxygen alone cannot stimulate the healing of osteoradionecrosis (ORN) wounds, because only 15% responded to this treatment. The other patients needed surgery, and 70% of those required major reconstruction procedures. Telfah (1995), using near infrared spectroscopy, has demonstrated that patients with ORN who received ultrasound therapy showed significant improvements of the metabolic activity as measured by an increase in their deoxyhaemoglobin concentrations.

Using human skin fibroblasts isonated in suspension and subsequently cultured in vitro, Harvey et al. (1975a, 1975b) found an increase of both collagen and noncollagenous protein (NCP) synthesis, which was intensity dependent. Fibroblasts exposed to continuous ultrasound [0.5 W/cm² (SATA)] showed a 20% increase in collagen secretion that was increased to 30% when the ultrasound was pulsed [0.5 W/cm² (SATA)].

The use of mouse calvaria has been found to be a successful tool to study bone formation in vitro (Raisz et al. 1978). A relatively new method for estimating collagen and noncollagenous (NCP) synthesis has been used in this study. This is a modification of the conventional assay in which ³H-proline-labelled calvaria are exposed to highly purified bacterial collagenase to release labelled collagen (Peterkoefsky and Diegelmann 1971). In the modified assay (Meghji et al. 1992), pepsin is used to lyse the calvaria, allowing the separation of intact collagen from the NCP. This system was used to investigate the effect of ultrasound on bone matrix synthesis under controlled conditions, and to identify the most effective ultrasound intensity capable of inducing both types of protein synthesis.

**MATERIALS AND METHODS**

*The ultrasound machine and irradiation vessel*

The ultrasound apparatus used in this study was a Rank Sonacell unit, capable of delivering 0.0-4.0 W/cm² (SATA), 1.5 or 3.0 MHz with a facility for pulsing at 1:1 (continuous) or 1:4 (pulsed: 2 ms on and 8 ms off). The machine was calibrated using a tethered float radiometer (designed by the National Physical Laboratory, Teddington, Middlesex, UK) at 3.0-MHz continuous wave. The transducer head had a diameter of 30 mm, and was held by a modified microscope stand.

The irradiation vessel was constructed from polystyrene sheets (Dreve, Dusseldorf, Germany) and measured 165 × 120 × 80 mm (Fig. 1). A shelf made of the same material was attached to the inner side walls to suspend a multiwell (6 wells) culture plate. Each well had a diameter of 35 mm. A frame was designed to fit over the multiwell culture plate, and was fixed with screws to the suspension shelf, sandwiching the culture plate. To reduce reflection of the ultrasound waves, air/water interfaces were eliminated wherever possible by the use of castor oil (BDH) and a rubber mat in the base of the irradiation vessel below the multiwell culture plate (Fig. 1). After the first set of experiments, a localising ring of polystyrene 10 mm in diameter was placed into the centre of each well to hold the bones (Fig. 2).

*The irradiation procedure/tissue culture*

All animal work was undertaken in accordance with the institutional and legal regulations. Calvaria
(frontal and parietal) bones were obtained by aseptic dissection of 5-day-old albino mice. The fronto-parietal bones were trimmed of any adherent connective tissue, halved along the sagittal sutures and placed in Hanks balanced salt solution (Gibco, Paisley, UK). One half calvarium was placed into each multiwell cell, containing 5 mL of culture medium, and transferred into a humidified 5% CO₂/95% air incubator at 37°C. The culture medium was the BGJb (Gibco) medium, supplemented with: 1. Freshly prepared L-ascorbic acid (BDH), 50 μg/mL; 2. L-proline (Sigma, Poole, UK), 40 μg/mL; 3. heat-inactivated rabbit serum (Wellcome Reagents, Beckenham, UK), 5% w/v; and 4. antibiotics, penicillin/streptomycin (Gibco), 100 U/mL. The bones were incubated for between 1 and 6 h to allow equilibration with the medium.

After this equilibration period, the culture plates were removed from the incubator, brought to a sterile air cabinet (Microflow, Andover, UK) at room temperature (19–26°C), and fixed to the irradiation vessel for the irradiation procedure. The transducer head was swabbed with methanol, left to dry, and immersed vertically into each culture well, positioned 5 mm above the bone sample (Fig. 2). Each Control or Test Group consisted of at least 5 wells, each with 1 half calvaria. Each set of experiments was repeated at least twice.

**Intensities tested**

The US machine was set at 3 MHz, pulsed 1:4 (2 ms on and 8 ms off), and the intensities evaluated were 0.1, 0.25, 0.5, 0.75, 1.0 and 2.0 W/cm² (SATA). Control bones were treated in the same way as the test bones, but with the US generator switched off. Irradiation was applied for 5 min to each well. After the irradiation, the explants were cultured for a further 20 h in the 37°C incubator.

The medium was then changed, using 1.5 mL, and 3 μCi of L-[5-3H]proline (sp. act. 18 Ci/mmol; Radiochemical Centre, Amersham, UK) dissolved in 30 μL of culture medium was added, giving a final

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**Fig. 1. Schematic representation of the irradiation vessel (transverse view).**

**Fig. 2. Detail of a well with the localising ring and the thermocouple probe.**
Fig. 3. Collagen and noncollagenous protein purification sequence.

Bone formation assay

The procedure for extraction and purification of the radiolabelled collagen was a modification of the method of Webster and Harvey (1979) for monolayer cultures, and is shown schematically in Fig. 3. It is based on the purification of collagen and NCP by serial salt precipitation to measure directly the rate of accumulation of $^3$H-labelled native collagen and NCP (Meghji et al. 1992).

If the samples had been frozen, they were first thawed by placing them into the incubator. The bones were washed (3 times) in normal saline to remove free radioactivity. The proteins were precipitated with 2 mL of trichloroacetic acid (5% TCA) for 2 h at 4°C. The bones were then rinsed in Hanks solution, and placed in a 3-mL polystyrene tube (LP3; Luckham). Collagen was extracted with 1 mL of 0.5 M acetic acid containing pepsin (0.5 mg/mL; EC 3.4.4.1, Sigma) for 16 h at 4°C. Insoluble bone debris was removed by centrifugation (5000 g for 30 min) and 200 μg of acid-soluble rat skin collagen was added to each tube as a carrier for labelled collagen during subsequent purification.

Collagen was precipitated by the addition of NaCl [25% (w/v) in 0.5 M acetic acid] to a final concentration of 5% (w/v) and left at 4°C for 2 h. Following this, the tube contents were mixed gently and centrifuged at 5000 g for 30 min at 4°C, and the supernatant was removed and stored. The pellets were redissolved in 1 mL of 0.5 M acetic acid, collagen was re-precipitated with the NaCl as described and the second supernatant removed and stored. The final precipitates were dissolved in 200 μL of 0.5 M acetic acid and transferred into disposable scintillation-vial inserts (Minitubes, Hughes and Hughes Ltd., London, UK), containing 3 mL of scintillation fluid (Unisolve 1, Koch-Light, Haverhill, UK).

For NCP synthesis estimations, the first and second supernatants were pooled in scintillation vials as for collagen. Radioactivity of the L-[5-$^3$H]proline was measured by scintillation spectrometry (Rackbeta, LKB) with external standardisation, and expressed in disintegrations per minute (DPM).

The results obtained were analysed using single-factor ANOVA and Student’s t-test for unpaired samples. All test values were compared with, and graphically presented as, a percentage of control values.

Temperature measurements

Temperature measurements were made for all the samples, using thermocouple probes inserted into the culture media in the well receiving treatment (Fig. 2). The measurements were made at room temperature (19–26°C) and taken from a digital thermometer (Comark, London, UK), monitored with an automatic chart recorder to give temperature readings to the nearest 0.05°C.

RESULTS

Bone formation (collagen/noncollagenous protein synthesis)

The first set of experiments using the multiwell culture cells without localising rings showed no significant change of either collagen or NCP synthesis at the intensities between 0.1 and 0.75 W/cm² (SATA), and significant inhibition of collagen synthesis ($p < 0.001$) and NCP synthesis ($p < 0.05$) at 1.0 W/cm² (SATA). These results were not consistent and, because the experiments were carried out using the near field, it was suspected that the bones were receiving different US intensities. Therefore, polystyrene rings were used to hold the bones always in the same position in relation to the US beam (Fig. 2).

Experiments using the localizing rings (2 series) were more consistent and reproducible, and showed significant stimulation of both collagen and NCP syn-
Fig. 4. The effects of varying intensities of 3-MHz (pulsed 1:4) ultrasound on bone collagen and noncollagenous protein (NCP) synthesis using the culture vessel with localising rings (error bars show 95% confidence intervals). Collagen and NCP synthesis increase at 0.1 W/cm² (SATA) were highly significant (p < 0.001 and p < 0.01, respectively). At higher intensities, there is a decrease in collagen and NCP synthesis from 0.75 to 2.0 W/cm² (SATA) that culminates at 2.0 (p < 0.05).

The best intensity was produced at 0.1 W/cm² (SATA), with an increase of 135% for collagen synthesis (p < 0.001) and 108% for NCP synthesis (p < 0.01). At higher intensities, there was a marked tendency to decrease the collagen and NCP synthesis, which was significant (p < 0.05) at 2.0 W/cm² (SATA) (Fig. 4). ANOVA evaluation suggests that the difference between the groups is highly significant (p = 8.04e−16), even if the 0.1 W/cm² group is excluded (p = 0.0007). The comparison between each group and the control showed results similar to Student’s t-test.

In all experiments, the rates of collagen and NCP synthesis were highly correlated (r = 0.9809, p < 0.001; Fig. 4), and there was no differential effect of collagen synthesis as percentage of total protein synthesis (NCP + collagen).

**Heating effects**

The temperature rise of the culture medium ranged from 0.0°C at 0.1 W/cm² to 1.8°C at 2.0 W/cm² after 5 min.

**DISCUSSION**

The culture of mouse calvaria has been a successful tool to study bone resorption and formation in vitro (Raisz et al. 1978). Both osteoblasts and fibroblasts present in the mouse calvaria produce collagen and noncollagenous protein (NCP). However, using injected ¹H-labelled proline, it was found that osteoblasts in bone are more active than fibroblasts and produce the greatest amount of bone matrix (Fitton-Jackson and Randall 1956). Therefore, the protein synthesis stimulation observed in this study may be attributed to osteoblastic, rather then fibroblastic, activity.

The pepsin extraction assay used is a simple method that allows the rapid and direct estimation of collagen and NCP synthesis in bone cultures. The advantages over the classic bacterial collagenase extraction assay is that there is no need for enzyme (i.e., collagenase) purification and there are fewer steps needed to assay the bone collagen. The pepsin assay is based on the measurement of radioactively labelled collagen present in bones after a 6-h pulse with ¹H-proline. This is achieved by extraction of native collagen using limited pepsin digestion, purification by salt precipitation and measurement of the radioactivity in the final preparation. Noncollagenous protein is largely susceptible to degradation by pepsin and does not co-precipitate with the collagen. In this way, the NCP is not contaminated with collagen. This assay, therefore,
gives an estimate of the 'net' rates of collagen and NCP synthesis over the duration of the proline pulse. Care has been taken to ensure specificity of the collagen and NCP labelling. One advantage of this assay is its simplicity, allowing large numbers of samples to be analysed, when compared with the established bacterial collagenase extraction assay of Peterkofsky and Diegelmann (1971) and, even so, it is equally efficient, given similar results for collagen and NCP synthesis (Meghji et al. 1992).

Noncollagenous proteins in the bone comprise the insoluble matrix proteins, such as sulphated proteoglycans, glycoproteins, traces of transferrin, albumin, globulin (IgG) and enzymes secreted by the bone cells and cell proteins (Vaughan 1975). Measurement of NCP synthesis is, therefore, more an estimate of the rate of general protein synthesis than an index of synthesis of a specific class of proteins.

In this study, collagen and NCP synthesis were significantly correlated ($r = 0.9809$, $p < 0.001$) and there was no differential effect on collagen synthesis rates relative to total protein synthesis. This corroborates the findings in fibroblasts experiments (Webster et al. 1980). However, these effects on collagen synthesis were different from those obtained using insulin (Canalis et al. 1977), which suggests that ultrasound may act through a generalized nonspecific stimulation of cell growth and activity.

Ultrasound at the lower end of the therapeutic scale (0.1 W/cm$^2$) was found to stimulate significantly the synthesis of bone matrix proteins in 2 of 3 experiments (no stimulation with the first set of experiments without the localizing rings). At higher intensities (0.75–2.0 W/cm$^2$), ultrasound suppressed the synthesis of both collagen and NCP, although this difference was only significant at 2.0 W/cm$^2$ ($p < 0.05$). These results are in agreement with Harvey et al. (1975a, 1975b), who also showed an increase in both collagen and NCP using human skin fibroblasts isonated in suspension and subsequently cultured in vitro. However, their best response was at higher intensity levels than ours, 0.5 W/cm$^2$ (SATA) continuous US, where a 20% increase in collagen secretion was recorded and, when the ultrasound was pulsed [0.5 W/cm$^2$ (SATA)], a 30% increase was observed. In our study, we only used pulsed mode, and the best intensity was produced on 0.1 W/cm$^2$, with an increase of 135% (collagen) and 108% (NCP).

Ultrasound is known to exert both thermal and nonthermal effects on tissues. The use of low-intensity ultrasound reduces tissue heating, and also reduces the possibility of cavitation phenomena (i.e., the pulsation of gas or vapour-filled voids in a sound field) (Webster et al. 1980). In our study, nonthermal effects were primarily considered to be responsible for the results, because the maximum temperature rise was of 1.8°C at 2.0 W/cm$^2$, and no measurable rise was observed with the best stimulatory dose (0.1 W/cm$^2$).

The physical mechanisms that may be involved in producing these nonthermal effects are acoustic streaming, acoustic cavitation and acoustic microstreaming. Acoustic streaming (also associated with stable cavitation) and microstreaming seem to play an important role and can stimulate cell activity if it occurs at the boundary of the cell membrane and the surrounding fluid, altering the membrane permeability and second messenger activity (Dyson 1982, 1985). This could result in therapeutically advantageous changes, such as increased protein synthesis (Harvey et al. 1975a, 1975b; Webster et al. 1978), increased secretion from mast cells (Fyfe and Chahl 1982), fibroblast mobility changes (Mummery 1978), increased uptake of the second messenger calcium (Mortimer and Dyson 1988; Mummery 1978) and production of growth factors by macrophages (Young and Dyson 1990).

It is speculated that the ultrasound pressure wave may mechanically deform connective tissue cell membranes, altering their ionic permeability and thereby activating the intracellular second messenger adenylate cyclase. The same stimulation of intracellular activity by adenylate cyclase might also be provoked by the ultrasound producing an electrochemical perturbation of the cell membrane surface (Ryaby et al. 1989).

The results observed in our study can explain the results of fracture healing, in which the best results were obtained with ultrasound at low intensities. Dyson and Brookes (1983) showed that it was possible to accelerate the repair of fibular fractures using therapeutic levels of ultrasound [1.5 or 3 MHz, pulsed, 0.5 W/cm$^2$ (SATA)]. Pilla et al. (1990) showed that low-intensity ultrasound [1.5 MHz, pulsed, 30 mW/cm$^2$(SATA)] could stimulate fracture repair in rabbits, so that maximum strength was gained in the treated limbs by 17 days after injury compared to 28 days in the controls. Tsai et al. (1992) showed that low intensities of ultrasound (1.5 MHz, pulsed, 0.5 W/cm$^2$) stimulate fracture healing in a rabbit fibulae model. Wang et al. (1994), using a rat femoral fracture model, observed accelerated fracture repair at 21 days, using 0.5 and 1.5 MHz, but the difference was significant only with the 1.5 MHz signal ($p < 0.02$). Our results also confirm the studies performed in human tibial fracture healing (Heckman et al. 1994), which also used low ultrasound intensities.

Yang et al. (1996) using 0.5 MHz [50 or 100 mW/cm$^2$ (SATA)] on a rat femoral fracture model similar to that used by Wang et al. (1994), showed
that the average maximum torque and torsional stiffness were greater than in controls, reaching statistical significance at 50 mW/cm². However, these authors could not measure any significant difference in the collagen content measured in the soft callus 7, 14 or 21 days after fracture. They suggest that ultrasound stimulation increases the mechanical properties of the healing fracture callus by stimulating earlier synthesis of extracellular matrix proteins in cartilage, possibly altering chondrocyte maturation and endochondral bone formation.

In our study, ultrasound at higher levels of intensity (0.75–2.0 W/cm²) showed inhibition of the synthesis of both collagen and NCP, but only to a significant level (p < 0.05) at 2.0 W/cm². A similar observation was also made by Tsai et al. (1992), who showed a deleterious effect on fracture healing when ultrasound was applied at 1.0 W/cm² in a rabbit fibula model.

The intensity of ultrasound used in the treatment of osteoradionecrosis (Harris 1992) was relatively high (1 W/cm²). The favorable results observed could therefore be explained in terms of ultrasound promoting angiogenesis (Young and Dyson 1990), rather than due to effects on protein synthesis. This was well demonstrated by the use of near infrared spectroscopy scans (Telfah 1995), which showed higher levels of deoxyhaemoglobin concentrations in the osteoradionecrotic mandibles of patients treated with ultrasound. This suggests significant improvements of the metabolic activity of the mandibular tissue, probably due to neoangiogenesis. However, because collagen and NCP synthesis are also important in the reorganisation of the bone matrix, perhaps lower intensities should be used in the treatment of osteoradionecrosis.

Differences between in vivo and in vitro investigations should also be considered. Calvaria were obtained from young growing mice, and their cell behaviour would vary from osteoblasts of patients with fractures or osteoradionecrosis.

The ultrasound frequency determines the penetration depth. In our study, we have used a 3-MHz frequency because we have applied it to superficial tissues, which do not need a deep penetration depth. However, lower frequencies of 1 MHz or even ‘long-wave ultrasound’ (45 kHz) seems to be more appropriate in clinical practice for the penetration of deeper tissues (Bradnock et al. 1996).

SUMMARY

1. Therapeutic US stimulates bone matrix protein synthesis at the lower end of the currently used therapeutic scale [0.1 W/cm² (SATA), and inhibits formation at higher levels of intensity [0.75–2 W/cm² (SATA)].

2. Nonthermal mechanisms appear to be responsible for these effects.

3. Our results are comparable to stimulation obtained at similar doses of US in fibroblasts in vitro and sustain the use of low-intensity US doses for in vivo applications, such as for wound healing, fracture healing and osteoradionecrosis.

4. Future research should be done to identify which noncollagenous proteins are released by bone tissue after stimulation by US, such as growth and angiogenesis factors.

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REFERENCES


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Original Paper

Therapeutic Ultrasound for Osteoradionecrosis: an In Vitro Comparison Between 1 MHz and 45 kHz Machines

P. Rcher,¹,² N. Doan,¹ B. Bradnock,³ S. Meghji¹ and M. Harris¹

¹Department of Oral and Maxillofacial Surgery, Eastman Dental Institute for Oral Health Care Sciences, University College London, 256 Gray's Inn Road, London WC1X 8LD, U.K.; ²Department of Morphology, UFMG, Belo Horizonte, Brazil; and ³Institute of Orthopaedics, Royal National Orthopaedics Hospital, London, U.K.

Therapeutic Ultrasound for Osteoradionecrosis: an In Vitro Comparison Between 1 MHz and 45 kHz Machines

P. Reher,¹,² N. Doan,¹ B. Bradnock,³ S. Meghji¹ and M. Harris¹

¹Department of Oral and Maxillofacial Surgery, Eastman Dental Institute for Oral Health Care Sciences, University College London, 256 Gray's Inn Road, London WC1X 8LD, U.K.; ²Department of Morphology, UFMG, Belo Horizonte, Brazil; and ³Institute of Orthopaedics, Royal National Orthopaedics Hospital, London, U.K.

Mandibular osteoradionecrosis is a serious chronic complication which may follow radiotherapy. Therapeutic ultrasound is a highly effective, inexpensive and readily available means of promoting revascularisation and healing. 'Long wave' ultrasound increases penetration depth and, therefore, seems to be more appropriate than traditional high frequency ultrasound. The aim of this study was to compare a new treatment using 45 kHz with the current standard 1 MHz machine. A traditional 1 MHz machine, pulsed 1:4, at intensities of 0.1, 0.4, 0.7 and 1.0 W/cm² (SAPA) was compared with a long wave machine, 45 kHz, at intensities of 5, 15, 30 and 50 mW/cm² (SAPA). The ultrasound was applied to human gingival fibroblasts and mandibular osteoblasts in vitro. Cell proliferation (DNA synthesis) and collagen and non-collagenous protein synthesis assays were performed, using radiolabelled thymidine and proline, respectively. Controls were sham-sonated and all readings were given as a percentage of controls. Fibroblast proliferation increased by 47% at 0.7 W/cm² (1 MHz) and by 43% at 50 mW/cm² (45 kHz), and osteoblast proliferation increased by 52% at 1.0 W/cm² (1 MHz), and by 35% at 30 mW/cm² (45 kHz). Fibroblast collagen production increased by 48% at 0.1 W/cm² (1 MHz), and by 44% at 15 mW/cm² (45 kHz) and osteoblast collagen production increased by 55% at 0.1 W/cm² (1 MHz) and by 112% at 30 mW/cm² (45 kHz). Long wave ultrasound was, therefore, capable of inducing a comparable or even higher enhancement of bone formation compared with traditional ultrasound, which, with its greater penetration, may accelerate the healing effect of ultrasound on osteoradionecrosis. The suggested intensity for 45 kHz ultrasound is 30 mW/cm². © 1998 Published by Elsevier Science Ltd. All rights reserved.

Key words: bone healing, cancer, cell proliferation, collagen synthesis, osteoradionecrosis, radiotherapy, radiotherapy effects, therapeutic ultrasound


INTRODUCTION

Radiotherapy is an essential treatment modality for oral and head and neck malignant neoplasms. Unfortunately, it induces alterations in the normal tissues, resulting in early and long-term complications. Mandibular osteoradionecrosis is the most serious long-term complication of radiotherapy, with a variable incidence, ranging from 2 to 44.2%. With adequate prevention, the incidence is still around 2-5%. Treatment modalities include curettage and antibiotics [1, 2], hyperbaric oxygen therapy [3, 4], resection and reconstruction and, more recently, revascularisation with therapeutic ultrasound, which was successfully introduced by Harris in 1992 [5].

Ultrasound is the term applied to sound waves, the frequency of which is above the limit of human audibility, which is approximately 20 kHz. Ultrasound is a propagating pressure wave that can transfer mechanical energy into the tissues. Its applications can be divided into diagnostic, surgical and therapeutic [6]. Diagnostic ultrasound employs a
frequency between 3 and 5 MHz, and very low intensities (1–50 mW/cm²) are used to avoid tissue heating. Surgical ultrasound (disruptive) uses very low frequencies (20–60 kHz) and very high intensities (above 8 W/cm²) although other surgical non-disruptive applications use high frequencies (HIFU operates at 0.5–3 MHz and at kW/cm²). As the term suggests, therapeutic ultrasound is used principally in physiotherapy. This can be through its ability to generate heat, and also the ‘activation’ of tissues, the so-called non-thermal effects. Several non-thermal applications have been described in the literature, and some soft tissue applications include the stimulation of tissue regeneration [7–10], enhanced blood flow in chronically ischaemic muscles [11], protein synthesis in fibroblasts [12,13], the healing of ischaemic varicose ulcers [8], tendon repair [14] and angiogenesis in full thickness excised incisions in the flank skin of adult rats [15].

Ultrasound effects in bone have also been evaluated. Accelerated repair in fibula fractures has been reported, with the best results when treated with 1.5 MHz, using 0.5 W/cm² [16]. Similarly, Tsai and colleagues [17] showed that low intensities of ultrasound (1.5 MHz, pulsed, 0.5 W/cm²) stimulate fracture healing in a rabbit fibula model. However, they also showed a deleterious effect when ultrasound was applied at 1.0 W/cm². For clarity, the intensity measurements used here are spatially averaged intensity (SA), used for continuous ultrasound, and spatial average pulsed averaged (SAPA), for pulsed ultrasound.

The use of much lower intensities (diagnostic range) has been recommended for the acceleration of the normal fracture repair process (1.5 MHz, pulsed, 30 mW/cm²) [18–20]. Using a rat femur fracture model, Yang and associates [21] observed greater torque and torsional stiffness after treating the fractures with 0.5 MHz ultrasound, which reached significance at 50 mW/cm². They also noted a shift in the expression of genes associated with cartilage formation: aggrecan gene expression was significantly higher on day 7 after fracture, and significantly lower on day 21. The first controlled clinical trial investigating the effectiveness of low intensity pulsed ultrasound (1.5 MHz, 30 mW/cm²), to evaluate the healing of tibial fractures on humans was reported in 1994 [22]. The treated group showed a significant decrease in the time to clinical healing (86 ± 5.8 days) as compared with the control group (114 ± 10.4 days).

As mentioned above, we have established the use of ultrasound as an important means of revascularisation of mandibular osteoradionecrosis [5]. Patients were treated with ultrasound (3 MHz, pulsed 1:4, 1 W/cm²) for 40 sessions of 15 min/day. 10 out of 21 (48%) cases showed healing when treated with debridment and ultrasound alone. 11 cases showed lesser healing after ultrasound therapy, but healed completely after debridment and cover with a local flap, and only 1 needed mandibular resection and reconstruction. These results are superior to the conventional treatment with hyperbaric oxygen therapy and surgery [23], where hyperbaric oxygen alone only achieved complete healing of osteoradionecrosis in 15% of cases, and 70% required resection and major reconstruction. Using near infrared spectroscopy, it has been demonstrated that patients with osteoradionecrosis who received ultrasound therapy showed significant improvements in metabolic activity, as measured by an increase in their deoxyhaemoglobin concentrations [24]. Bone formation stimulation was also observed in vitro, using 3 MHz pulsed 1:4 ultrasound, with the best results at low intensities (0.1 W/cm²) [25].

Recently, a new ultrasound device has been developed, which instead of using the traditional frequencies of 1–3 MHz, uses ‘long wave’ ultrasound, at 45 kHz [26]. This lower frequency/long wavelength combination gives a widely divergent field shape, with the treated volume effectively in the far field region. This wave penetrates much deeper into the tissues, reaching areas as deep as several centimetres, instead of millimetres as with the megahertz machines. In order to minimise heating effects, it uses low intensities (5–50 mW/cm²).

The purpose of this study was to evaluate in vitro this long wave machine, comparing it with a traditional 1 MHz ultrasound machine. The parameters evaluated were cell proliferation (DNA synthesis), and collagen/non-collagenous protein synthesis, in fibroblasts and osteoblasts.

**MATERIALS AND METHODS**

**Cell cultures**

The cell types used in the experiments were human gingival fibroblasts and mandibular osteoblasts. The fibroblasts were cultured from gingival tissue specimens obtained from patients admitted for planned dental extractions and/or surgical removal of wisdom teeth. The osteoblasts were cultured from bone obtained from mandibular osteotomies performed for surgical removal of wisdom teeth. All patients had no known diseases, and were 20–30 years of age. The specimens were rinsed several times with phosphate buffered saline (PBS, Gibco, Paisley, U.K.), minced and cultured in 75 cm² culture flasks using Dulbecco’s modified Eagle medium (DMEM), complemented with heat-inactivated fetal bovine serum (HIFBS) 10% v/v (Sigma, Dorset, U.K.), freshly prepared l-ascorbic acid 50 µg/ml (Sigma), L-glutamine 2 mM (Sigma), and penicillin/streptomycin 100 U/ml each (Gibco). The cells were insonated into a humidified 5% CO₂/95% air incubator at 37°C. After approximately 10 days, the cells started to grow out of the explants, and the media was changed twice a week. When the cells were confluent, they were trypsinised (0.025% w/v trypsin in PBS) and divided 1 in 3. The cells were used between the sixth and 10th passage for the fibroblasts and between the fourth and eighth passage for the osteoblasts. For the proliferation assays, they were plated in 6 well plates (Corning, New York, U.S.A.) at 1.5×10⁵ cells/well and for the collagen assays at 3×10⁵ cells/well. Each well was filled with 5 ml of the media used for the cell cultures, but with different concentrations of HIFBS. For the cell proliferation assays (DNA synthesis), the media used contained 1% HIFBS, the positive control group had 10% HIFBS. In the collagen assays, the media used contained 10% HIFBS. The cells were insonated the following day.

**The ultrasound machines evaluated**

A ‘traditional’ ultrasound machine, that uses a frequency of 1 or 3 MHz, and a ‘long wave’ machine, that uses a frequency of 45 kHz, were used. The ‘traditional’ ultrasound machine was a Therasonic 1032 unit, produced by E.M.S., Oxfordshire, U.K. This apparatus can be set to work with 1 or 3 MHz, and can deliver an intensity ranging from 0.1 to 2.0 W/cm². It also has a pulsing facility, and can be set to continuous or pulsed mode, pulsing 1:2, 1:4 or 1:9. The machine has an electronic control panel, a facility to do an electronic check each time it is switched on, and an alert signal...
if there is no coupling gel or liquid. The handset head has a flat surface and an effective radiating area of approximately 2.0 cm². The apparatus was set to 1 MHz, pulsed 1:4 (2 msec ‘on’ and 8 msec ‘off’) and the intensities evaluated were 0.1, 0.4, 0.7 and 1.0 W/cm². Several calibrations were performed during the experiments, but at least once before and after each set of assays. The calibration was performed at the Department of Medical Physics, University College London. At each calibration, a full electronic check-up was performed, according to the manufacturer’s manual. The acoustic output power was measured/calibrated using a precision ultrasound balance (E.M.S. model 67). After setting up the balance and warming up the ultrasound machine, the measurements were taken at 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 W/cm². The calibration was considered in the admissible range if the error in accuracy of the output readings was ≤ 10%.

The ‘long wave’ ultrasound machine (45 kHz) used in this study was a Phys-Assist unit, produced by Orthosonic (Ashburton, Devon, U.K.). This apparatus has a fixed frequency of 45 kHz, and can deliver an intensity ranging from 5 to 50 mW/cm². It does not have a pulsing facility, working in the ‘continuous’ mode, which at low intensities does not produce harmful tissue damage. The machine also has an electronic control panel, with a liquid crystal display, and a facility to calibrate itself each time it is switched on. The handset head type is conic and has an effective radiating area of approximately 12.8 cm². The intensities evaluated were 5, 15, 30 and 50 mW/cm². Calibration was performed several times during the experiments, but at least once before and after each set of assays. The calibration was performed at Orthosonic Ltd, and was considered satisfactory if the acoustic intensity ranged from 45 to 55 mW/cm², at power 4, and corresponding values at the other settings.

The ultrasound application model

A thermostatically controlled water bath (Electrothermal, London, U.K.) was used to maintain a constant temperature of 37°C during the assays. The tank had an internal diameter of 20 cm and a depth of 5 cm and was covered on the inferior and side walls with ultrasound-absorbing rubber. The water-bath was filled with distilled, de-ionised, demineralised water which was changed before each experiment. The cells used in the experiments were prepared in six well culture plates, which had a diameter of 35 mm, and a plate thickness of 1 mm. The plates were placed floating directly over the water surface, taking care not to let any air bubbles form between the plate and the water surface (Figure 1). The transducer was held by a microscope stand, which was placed over a rotating platform/shaker (Edmund Bühler, Tübingen, Germany, model KL2), set to 30 rotations/min. In this way, the transducer was constantly moved while the ultrasound was applied, avoiding the production of standing waves. The whole apparatus (water bath, transducer head and the rotating platform) was set up in a sterile air flow cabinet (Microflow Pathfinder, Intermed, Andover, U.K.). The transducer head was swabbed with 70% isopropyl alcohol BB (Azowipe, Vernon Carus, Lancashire, U.K.), left to dry, and immersed vertically into the culture well, just touching the surface of the medium. Each well of the culture plate had 5 ml of medium, and in this way, the distance between the transducer head and the cells/bones was approximately 5-6 mm. The transducer head from the 45 kHz ultrasound machine has a conic shape, therefore, only approximately 2–3 cm² of the total area of 12.8 cm² was immersed. However, since most of the energy comes through the centre of the head, we believe that there was not much energy loss. The ultrasound was applied to five wells (n = 5) for each evaluated intensity. Each well was sonicated for 5 min, and the control group was treated in the same way, but with the ultrasound generator switched off. After sonication, the plates were cultured for a further 18 h in the 37°C incubator.

Cell proliferation assay (DNA synthesis)

After 18 h in the incubator, 3.5 ml of the media was removed, the cells were radiolabelled with 5-³H thymidine (specific activity 14.3 Ci/mmol, 1 ml/1 mCi, Amersham, Buckinghamshire, U.K.) to a final concentration of 0.5 μCi/ml in 1.5 ml of culture medium. The cells were re-incubated for a further 6 h, when the incubation was terminated. The media was removed, 1 ml of 5% trichloroacetic acid (TCA) was added to the wells, and the plates were kept in the fridge (4°C) for 2 h. The TCA was then removed, and the cells washed three times with PBS. Then, 300 μl of 0.5 M NaOH was added to each well, and left for 20–30 min at 4°C. This was removed and transferred to scintillation vials (Minitubes, Hughes and Hughes Ltd, Lancashire, U.K.) containing 200 μl of 0.5 M acetic acid. Scintillation fluid (NBS Biologicals, Cambridge, U.K.) was added to each tube, and radioactivity measured with a beta counter (Wallac 1409, Turku, Finland) with external standardisation, expressed in disintegrations per minute (DPM).

Collagen-non-collagenous protein synthesis assay

After 18 h in the incubator, 3.5 ml of the media was removed, the cells were radiolabelled with 5-³H proline (specific activity 31.0 Ci/mmol, 1 ml/1 mCi, Amersham) to a final concentration of 2 μCi/ml in 1.5 ml of culture medium. The cells were re-incubated for a further 6 h, and thereafter 700 μl of the media was transferred to Eppendorf tubes containing 700 μl of 10% TCA (final concentration 5% TCA), and left at 4°C for at least 2 h. The tubes were centrifuged at 4°C (2500 rpm for 30 min) to remove unbound isotope and small peptides from the cells, and the supernatant discarded. The pellets were suspended in 1 ml 0.5 M acetic acid containing pepsin (0.5 mg/ml, EC 3.4.4.1, Sigma) and left at 4°C. Collagen was extracted using limited peptic digestion (16 h). Rat acid soluble collagen was added (100 μl of collagen in 5 mg/ml in 0.5 M acetic acid) to act as a carrier for the newly

![Figure 1. Schematic representation of the ultrasound insonation model used during the assays. The transducer was kept in motion to avoid the formation of standing waves, and inserted into the culture medium above the cells. These were plated into 6 well plates that were placed floating over the water tank (37°C). The whole system was placed in a sterile air flow cabinet, and insonation was applied for 5 min for each well.](image-url)
formed collagen. Collagen was precipitated by the addition of NaCl to a final concentration of 5% (w/v) in 0.5 M acetic acid, for 3 h at 4°C. The tubes were mixed gently and centrifuged at 4,000 rpm for 30 min at 4°C. The supernatant containing the non-collagenous protein was stored in scintillation vial inserts (Minutubes, Hughes and Hughes Ltd). The pellets were resuspended in 1 ml of 0.5 M acetic acid, and the collagen reprecipitated with NaCl as described, for 2–3 h. The tubes were centrifuged again, at 5,000–8,000 rpm for 30 min at 4°C, and the second supernatant (non-collagenous protein) added to the first supernatant. The final precipitates with the purified collagen were resuspended in 400 μl of 0.5 M acetic acid and transferred into another disposable scintillation vial insert. Each scintillation vial was filled with 3 ml of scintillation fluid (NBS Biologicals) and radioactivity was measured as for the proliferation assays.

Statistical analysis

Each experiment was repeated at least twice. The number of observations for controls and for each intensity evaluated was five (n = 5). The values obtained in DPMs were transformed into percentages of the controls (sham-insonated), which were considered as 0%. All test values were compared with, and graphically presented as a percentage of control values. The results obtained were analysed using ANOVA single factor and Student’s t test for unpaired samples.

RESULTS

Cell proliferation (DNA synthesis)

The cell proliferation assays showed an increase in DNA synthesis with both ultrasound machines. In the fibroblasts group treated with 1 MHz ultrasound (Figure 2a), the most significant results were an increase of 47% at 0.7 W/cm² (P < 0.01) and of 37% at 1.0 W/cm² (P < 0.05). In the group treated with 45 kHz (Figure 2b), increases of 30 and of 43% were observed, with 15 and 50 mW/cm², respectively (P < 0.01). When osteoblasts were treated with 1 MHz ultrasound (Figure 3a), again an increase in DNA synthesis at the higher intensities was observed. This was in the order of 34% at 0.7 W/cm² (P < 0.01) and of 52% at 1.0 W/cm² (P < 0.001). In the 45 kHz treated group (Figure 3b), a more uneven distribution occurred, showing an increase of 32% at 5 mW/cm², and of 35% at 30 mW/cm² (P < 0.05 and P < 0.01).

Collagen/non-collagenous protein synthesis

In these assays, there was a clear tendency for increased collagen production in the lower intensities when using 1 MHz ultrasound. The fibroblasts group treated with 1 MHz (Figure 4a) showed increases of collagen production of 48, 57 and 52%, at intensities of 0.1, 0.4 and 0.7 W/cm², respectively (P < 0.01, 0.05 and 0.01, respectively). When these cells were treated with 45 kHz ultrasound (Figure 4b), the last three intensities showed increases in collagen ranging from 37 to 44%, although significant only at 15 and 50 mW/cm² (P < 0.01 and P < 0.05). The collagen/non-collagenous protein production by osteoblasts were probably the most significant results, since these are the target cells involved in repair in osteoradionecrosis. In this case, a clear superiority of the 45 kHz ultrasound was observed. In the 1 MHz treated cells, the previously observed tendency to increase collagen production at the lower intensities continued (Figure 5a).
were able to induce proliferation, in fibroblasts and osteoblasts

Figures 4 and 3). This is a crucial effect, since osteoradionecrosis is a hypocellular tissue. However, this can also be interpreted as having a deleterious affect, since the cells may be involved in cellular division, and not in the production of collagen and other physiological proteins. This was observed when the cells were stimulated with the 1 MHz machine, when increased proliferation was observed for the same intensities that caused reduced collagen production (Figures 3a, 5a). The 45 kHz ultrasound revealed similar observations in fibroblasts, but on osteoblasts the proliferation was high at the same intensities which increased collagen synthesis.

The procedure for extraction and purification of the radio-labelled collagen was a modification of the method of Webster and Harvey for monolayer cultures [28]. The pepsin assay was based on the measurement of radioactively labelled collagen present in cells and culture medium after a 6 h pulse with 

\[ \text{H} \]-proline. This is achieved by extraction of native collagen using limited pepsin digestion, purification by salt precipitation and measurement of the radioactivity in the final preparation [29]. Non-collagenous protein is largely susceptible to degradation by pepsin and does not coprecipitate with the collagen. This assay, therefore, gives an estimate of the ‘net’ rates of collagen and non-collagenous protein synthesis over the duration of the proline pulse. One advantage of this assay is its simplicity, allowing large numbers of samples to be analysed, when compared with the established bacterial collagenase extraction assay of Peterkofsky and Diegelman [30], and is equally efficient [29].

In the current collagen/non-collagenous protein synthesis assays, both ultrasound machines induced collagen production.

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In the current collagen/non-collagenous protein synthesis assays, both ultrasound machines induced collagen production.

![Figure 4. Fibroblast collagen and non-collagenous protein (NCP) synthesis induced by 1 MHz (a) and 45 kHz (b) ultrasound. Controls received the same treatment but with the ultrasound generator switched 'off'. Values are given as percentages of the controls. Standard error bars are shown. Significance of results: *P<0.05, |P<0.01, |P<0.001.](image)

![Figure 5. Osteoblast collagen and non-collagenous protein (NCP) synthesis induced by 1 MHz (a) and 45 kHz (b) ultrasound. Controls received the same treatment but with the ultrasound generator switched 'off'. Values are given as percentages of the controls. Standard error bars are shown. Significance of results: *P<0.05, |P<0.01, |P<0.001.](image)
With the 1 MHz machine, this was more evident with lower intensities, both in fibroblasts and in osteoblasts (Figures 4a, 5a). However, in osteoblasts, the 45 kHz ultrasound machine produced much higher increases in collagen synthesis (up to 112%) than those for the 1 MHz machine (maximum of 59%) (Figure 5). These results are higher than those observed by Harvey and colleagues [12], who found an increase of both collagen and non-collagenous protein synthesis, which was intensity dependent, using human skin fibroblasts sonicated in suspension and subsequently cultured in vitro. Fibroblasts exposed to continuous ultrasound (0.5 W/cm²) showed a 20% increase in collagen secretion, which was increased to 30% when the ultrasound was pulsed (0.5 W/cm²). Webster and associates [13] also observed lower increases in protein synthesis by fibroblasts of 29% using a 3 MHz signal at 0.5 W/cm². Sebek and colleagues [25] using a mice calvaria model showed that 3 MHz pulsed ultrasound stimulates bone formation (collagen and non-collagenous protein production), with the best results at 0.1, 0.25 and 0.5 W/cm².

Non-collagenous protein synthesis was also stimulated, but it could not be correlated to the collagen synthesis in the majority of the assays. Furthermore, the non-collagenous protein synthesis increase reached significance only in osteoblasts, and mainly with the 45 kHz ultrasound (Figure 5b), where all intensities significantly stimulated the cells. This may be an important observation, as the non-collagenous protein contains many cytokines, growth and angiogenic factors and enzymes which may enhance healing and angiogenesis.

The molecular mechanisms by which ultrasound alters cell function or protein synthesis are still not known, although some mechanisms have been mentioned [21]. The compression of microtubules, or cavitation, producing oscillatory movement of microbubbles and acoustic streaming, could have a direct effect on the permeability of the cell membrane and enhance second messenger activity. Such changes in ion or protein transport could consequently modify intracellular signals for gene expression [13,31–33]. The effects of mechanical pressure at the cell surface could also activate the 'stretch receptor' type of cation channel [34], and changes in cation concentrations could also modify intracellular signals regulating gene expression. Alternatively, mechanical energy transferred by the ultrasound might activate changes in the attachments of cytoskeleton to the extracellular matrix, affecting cell metabolism and gene expression [21]. Electrical currents in bone may be potentiated by exposure to ultrasound energy. Investigators have reported increased potentials as a function of ultrasound intensity, frequency and burst pattern [35–37]. Finally, a rise in temperature during ultrasonic exposure may have an effect on cell metabolism. The use of low intensity ultrasound reduces tissue heating, and also reduces the possibility of cavitation phenomena, i.e. the pulsation of gas or vapour filled voids in a sound field [38]. We have shown a maximum temperature rise of 1.8°C at 2.0 W/cm² (SAPA), but no measurable rise was observed at the best stimulatory dose of 0.1 W/cm² (SAPA) [25]. The use of 45 kHz ultrasound machines has been shown to produce less heating than the 1 MHz machine [39].

The intensity of ultrasound used in the clinical treatment of osteoradionecrosis [5] was relatively high (3 MHz, pulsed 1:4, 1 W/cm²(SAPA)). The favourable results observed could, therefore, be explained in terms of ultrasound promoting heating or angiogenesis [15], rather than due to the effects on collagen protein synthesis, which is higher at lower intensities. This was supported by the use of near infrared spectroscopy scans of irradiated mandibles [24], which showed higher levels of deoxyhaemoglobin concentrations in the osteoradionecrotic mandibles of patients after treatment with this ultrasound regimen. This suggests significant improvements of the metabolic activity of the mandibular tissue, probably due to neoangiogenesis. However, since collagen and non-collagenous protein synthesis are also important in the reorganisation of the bone matrix, the lower intensities should be used in the treatment of osteoradionecrosis. With 45 kHz ultrasound, intensities between 15 and 50 mW/cm² seem appropriate as the best results in our series were achieved with 30 mW/cm².

The in vitro assays reported here demonstrated comparable effects on cell proliferation and collagen/non-collagenous protein synthesis for 1 MHz and 45 kHz. The best effects are achieved by the 45 kHz ultrasound, mainly in collagen/non-collagenous protein synthesis by osteoblasts. These results support the use of low intensity regimens. Our recommended ultrasound regimens are 0.1 W/cm² pulsed 1:4 if using 1 MHz, and 30 mW/cm² continuous insonation if using 45 kHz. However, we recommend the use of 'long wave' ultrasound for the treatment and prevention of osteoradionecrosis, due to its overall clinical and in vitro advantages.

In conclusion, 'long wave' ultrasound appears to have significant therapeutic advantages, which are that it has a much higher penetration depth, allowing treatment of thick tissues, penetrating centimetres rather than millimetres as with traditional ultrasound. It uses low intensity energy levels, causing less heat production, and is used in continuous mode, reducing the treatment time, with a spherical head, giving a large effective treatment area.


**Acknowledgements**—We would like to thank Dr M. Dyson (Tissue Repair Unit, Guy’s and St Thomas Medical and Dental School) for her kind help and advice on the set-up of our preliminary experiments with ultrasound. This research was supported by P. Reher’s scholarship from CAPES, Ministry of Education, Brazil.
Dear Dr Meghji

Re: MS# G0661 - “Effect of ultrasound on the production of IL-8...”

Acceptance date: 30 September 1998

I am pleased to inform you that your revised manuscript received good reviews and has been accepted for publication in CYTOKINE. You will receive galley proofs from the publisher in due course.

Kind regards,

Yours sincerely

Professor G W Duff
Editor

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Effect of ultrasound on the production
of IL-8, basic FGF and VEGF

Authors: Peter REHER $^{1,2}$, Nghiem DOAN$^1$ Brian BRADNOCK$^3$, Sajeda MEGHJI$^{1,4}$, Malcolm HARRIS$^1$

Institution: Department of Oral & Maxillofacial Surgery
Eastman Dental Institute for Oral Health Care Science
and University College London Hospitals
256 Gray's Inn Road
London WC1X 8LD
United Kingdom

Reprints-Request: Dr. Sajeda Meghji
Address - above
Tel: 00 44 171 915 1271
FAX 00 44 171 915 1259
Email: s.meghji@eastman.ucl.ac.uk

Short Title: Ultrasound stimulates IL-8, bFGF and VEGF production

Key words: Angiogenic factors - Cytokines - ELISA - Osteoblasts - Therapeutic ultrasound.

$^1$ Department of Oral & Maxillofacial Surgery, Eastman Dental Institute/UCL, London, U.K.
$^2$ Federal University of Minas Gerais, Belo Horizonte, Brazil
$^3$ Institute of Orthopaedics, Royal National Orthopaedics Hospital, London, U.K.
$^4$ To whom correspondence and reprint requests should be addressed
Mandibular Osteoradionecrosis: Evidence for the use of therapeutic ultrasound instead of hyperbaric oxygen

Authors:
Mr. Nghiem DOAN, BDS, MPH - MSc Student Oral & Maxillofacial Surgery
Mr. Peter REHER, DDS, MSc - Assistant Professor
Dr. Sajeda MEGHJI, BSc, MPhil, PhD - Senior Lecturer
Professor Malcolm HARRIS, BDS, MD, FDSRCS, FFDRCSI - Head of Department

Institution: Department of Oral & Maxillofacial Surgery
Eastman Dental Institute for Oral Health Care Science
University College London
256 Gray's Inn Road
London WC1X 8LD
England, U.K.

Key words: Angiogenesis, Bone healing, Cytokine, Cell proliferation, Collagen synthesis, ELISA, Fibroblasts, Monocytes, Osteoblasts, Osteoradionecrosis, Therapeutic ultrasound, Hyperbaric Oxygen Therapy.

Short Title: Ultrasound or HBO for Osteradionecrosis?

1 Department of Oral & Maxillofacial Surgery, Eastman Dental Institute/UCL, London, U.K.
2 Federal University of Minas Gerais, Belo Horizonte, Brazil
RE-EXTRACTION HBO: EMPIRICISM OR SCIENCE?

To the Editor:—I read with great interest the clinical controversies in the March 1997 issue. Dr Lewis Clayman has presented many interesting statistics regarding osteoradionecrosis (ORN) and has concluded from these statistics that mandatory hyperbaric oxygen (HBO) treatment before extractions in irradiated patients is not justified. I agree, that with proper clinical judgement and extensive experience, one can sometimes avoid HBO. However, as we all have seen, this is usually not the case. Most of post-extraction (ORN) cases have been caused by inexperienced general dentists and even oral maxillofacial surgeons with limited to no experience regarding the severe, devastating, disfiguring results of mandibular discontinuity and soft tissue loss secondary to ORN. Even in the most experienced hands, reconstruction is at best acceptable, but occasionally impossible.

In my opinion, statistics and articles like Dr Clayman's are a large reason for the severe ORN cases I continue to see, even in private practice. Dentists, oral surgeons, ENT surgeons, and radiation oncologists have become cavalier about ORN and use such statistics to justify their lack of proper precautions, and also in my opinion, blatant malpractice. Most of these practitioners do not see the consequences of their actions, because these patients come to us expecting an easy resolution to their problem. There is no such solution.

I ask Dr Clayman, if he or someone close to him was at risk of being one of those statistically insignificant patients who develop severe ORN, with all of its sequela, would he then opt for prophylactic HBO? I know I would.

JEFFREY M. BROWN
Port St. Lucie, FL

In Reply:—I thank Dr Brown for his passion and concern about my article recommending against the use of mandatory hyperbaric oxygen (HBO) before dental extractions in irradiated patients. However, I find his suggestions to be disquieting because he refutes the validity of my conclusions drawn from direct analysis of primary source data on the basis of his personal observations unsupported by data. This is an example of the conflict between strongly held beliefs based “on my clinical experience” and clinical decision-making based on data and conclusions subject to controls and peer review. It is a manifestation of the conflict between empiricism and science.

I have reviewed all of the available literature since the turn of the century on the subject of osteoradionecrosis (ORN) and extractions related to ORN. Only the data directly relating to extractions, ORN, and HBO have been presented in my article. The introductory section clearly places great emphasis on the importance of careful extraction techniques performed by senior surgeons. Tables 2 and 3 note that the incidence of ORN is relatively low since 1968 (5.4% for post-extraction ORN and 4.4% for pre-radiation extractions) and since 1986 (Table 5: note) it has dropped to 2.1% for post-radiation therapy extractions. This represents progress without the addition of HBO. Perhaps this has occurred because of improved radiotherapy delivery techniques attributable to the multiple leaf collimator in use since the early 1980s, as well as from a generally improved awareness of the consequences of ORN coupled with improved surgical techniques. These lower incidence rates do not encourage a “cavalier” attitude toward the management of the irradiated patient requiring extractions. Furthermore, I fail to understand how statistics and articles like Dr Clayman’s are a large reason for the severe ORN cases I continue to see, even in private practice. Somewhere he also brings “blatant malpractice” into the same sentence. Since my article is the only one in our literature that has delved into the data presented in all of the studies reviewed and actually re-analyzed the raw data in regard to the subject of the report, I wonder how he can attribute the occurrence of severe ORN cases seen in his practice to my article. The recommendation for or against a technique also does not speak to the issue of malpractice.

Regarding the last paragraph, I believe my entire article makes the case that HBO has been oversold as a sure cure or preventive measure for ORN. HBO used prophylactically might save 2% of the jaws at risk for developing ORN from losing continuity. Is this an acceptably high gain measured against the unnecessary delivery of HBO, which is both expensive and not without risk for the other 98%? Who is willing to pay for the unnecessary 98% of treatments? HBO as a treatment modality for ORN is well established as a suitable therapeutic choice, but it is certainly not the only choice.

Dr Brown mistakenly ascribes to patients the possibility of being “statistically insignificant.” No patient is insignificant. However, the occasional disastrous loss of mandibular continuity cannot mandate HBO for all patients.

In a world of limitless resources, perhaps HBO would be available to all. However, even under these ideal economic conditions, hyperbaric chambers are not located in close proximity to patients in many areas of the country, and patients with intercurrent illnesses and/or claustrophobia could not avail themselves of this treatment. In a world of escalating costs and limited resources, a therapy that prophylactically might save mandibular continuity in 2% of cases to which it is applied would be most unlikely to be funded.

Finally, Dr Brown’s implication that it is “blatant malpractice” to manage dental extractions in the irradiated patient in a way contrary to his beliefs is inflammatory, unsupported, and does not belong in a scientific discourse.

LEWIS CLAYMAN
Detroit, MI

ULTRASOUND FOR THE TREATMENT OF OSTEORADIONECROSIS

To the Editor:—We would like to contribute to the fascinating debate on osteoradionecrosis. ORN following den-
Almus: The intent of our study was to develop an external fixation device for experimental mandibular distraction around an arc and to demonstrate the ability to use distraction osteogenesis to reconstruct defects in the anterior mandible around an arc. To demonstrate outgrowth of the alveolar artery and to show bone regeneration. To transfer to humans, the knowledge and experience obtained from and techniques developed in a previously animal study. To reconstruct the front of the mandible and the floor of the oral cavity with distraction osteogenesis in patients operated upon because of oral cancer. Methods: A six cm defect was created in the mandible in four healthy adult dogs. Distraction osteogenesis was used to lengthen the mandible. An external apparatus was designed to allow distraction around an arc. The bony defect was regenerated using bifocal distraction osteogenesis at a rate of 1 mm a day. Two patients with oral cancer, who had been declared operable and in whom part of the mandible was to be removed, was operation upon with excision of the tumour and a suprahyoid neck dissection. The bony defect and soft tissue was in both cases regenerated using trifocal distraction osteogenesis at a rate of 1 mm a day. Results: The bony defect was regenerated in three of the four dogs using bifocal distraction osteogenesis. The inferior alveolar artery and nerve were found to recanalize through the regenerate segments in three of the four animals. The apparatus developed proved to be very stable. The animals showed normal occlusal function after removal of the external apparatus. Nine centimetres of the front of the mandible and the floor of the oral cavity have been reconstructed in two patients being treated for oral cancer. They have obtained normal sensation in the reconstructed soft tissue in the mouth. Conclusions: We have a theoretically well-founded hope that distraction osteogenesis is a better alternative in reconstructing bone in patients operated upon for oral cancer. It is our hope that reconstruction time will be reduced and the post-operative period less painful, and that cosmetic appearance will improve and psychological disadvantages be diminished. Further studies are necessary. A new distraction device is being developed in cooperation with Leibinger®.

Abstracts
1145
Agarose (Targel) and Vitamin K 4. Preparations Reduce Tumors in Tumor-bearing mice.
B. CARIN* 1, S. MEGHIN*, S. HODGES† and M. HARRIS† (The Easter Day Institute UK)
Background: Agarose an anionic, soluble, gelatin-like polymer, is used to reduce tumor growth.
An agarose film treatment has also been reported [Hu et al. 1997. Cancer Research 57:4398-4403]. On the other hand, Vitamin K 4 (Phelochromic Coenzyme), which is used in the treatment of osteoporosis, has been reported to decrease the size of tumors in mice [Manolescu et al. 1993. Int J Cancer. 52:1-7].

Aim: The aim of this study was to determine the effects of agarose and Vitamin K 4 on tumor growth in tumor-bearing mice.

Methods: Agarose film was prepared by dissolving the polymer in water and casting it on glass slides. Vitamin K 4 was administered orally to mice. The tumor model used was the MCF-7 breast carcinoma model, which is commonly used in studies of tumor growth.

Results: Mice treated with agarose film showed a significant decrease in tumor size compared to the control group. Mice treated with Vitamin K 4 showed a slight decrease in tumor size, but this was not statistically significant.

Conclusions: Agarose film and Vitamin K 4 may have potential anti-tumor effects. Further studies are needed to confirm these findings.

1146
In vivo study of metal release from magnets and screws. J.R. MATTHEW, I.W. FRAME, (University of Birmingham, School of Dentistry, St. Chads Quay, Birmingham, B1 3NU, UK)

Aims: The aim of this study was to investigate the metal release from magnets and screws used in orthopedic surgery.

Materials and Methods: The study involved 50 magnets and 50 screws of different types and sizes. The magnets were made of different materials, including samarium-cobalt and iron-nickel-cobalt. The screws were made of stainless steel and titanium.

Results: The study showed that magnets and screws release various metals, including iron, nickel, cobalt, and copper. The metals released were detected in the surrounding tissue and were found to be within the acceptable limits for human health.

Conclusions: The release of metals from magnets and screws is within the acceptable limits for human health. Further studies are needed to investigate the long-term effects of metal release on the surrounding tissue.

1147
Nitric oxide (NO) has been suggested to be an important mediator in degenerative and inflammatory musculoskeletal disorders. Since interleukin-1 (IL-1) is implicated in these diseases, we evaluated the effects of mechanical strain on NO production in chondrocytes in the presence of recombinant human IL-1β and IL-1α. The study was conducted in a rat model with the following groups:

- Control group (unstrained chondrocytes in the absence of rhlL-10 treatment (1.1 μM), when compared to unstrained chondrocytes in the presence of rhlL-10 treatment (1.1 μM), when compared to unstrained chondrocytes in the absence of rhlL-10 treatment (1.1 μM), when compared to unstrained chondrocytes in the presence of rhlL-10 treatment (1.1 μM).

Methods: Bone-anchored transplants were treated with DBM alone or DBM combined with GTM (P < 0.05).

Results: The data suggest that mechanical strain may be an important mediator in the degeneration of NO production in chondrocytes.

Conclusions: The findings support the use of mechanical strain as a potential therapeutic strategy for degenerative and inflammatory musculoskeletal disorders.

1148
Ultrasound induced angiogenesis through the production of IL-8, BFGF & VEGF
J. KREHER, M. DOAN*, S. MEGHIN†, M. HARRIS† (Dep. of Morphology, UPHNO, Brussels, Belgium)

Introduction: Angiogenesis is a term given to the biological process leading to the formation of new blood vessels by sprouting from the existing blood vessels. The good results of therapeutic ultrasound on wound healing, fracture healing and osteoporosis could be explained through the enhancement of angiogenesis: part of the angiogenic stimulus is the mechanical stress generated by the vibration of a piezoelectric transducer. The study was conducted in a rat model with the following groups:

- Control group (unstrained chondrocytes in the absence of rhlL-10 treatment (1.1 μM), when compared to unstrained chondrocytes in the presence of rhlL-10 treatment (1.1 μM), when compared to unstrained chondrocytes in the absence of rhlL-10 treatment (1.1 μM), when compared to unstrained chondrocytes in the presence of rhlL-10 treatment (1.1 μM).

Methods: Bone-anchored transplants were treated with DBM alone or DBM combined with GTM (P < 0.05).

Results: The study showed that mechanical strain may be an important mediator in the degeneration of NO production in chondrocytes.

Conclusions: The findings support the use of mechanical strain as a potential therapeutic strategy for degenerative and inflammatory musculoskeletal disorders.

1149
B-scan ultrasonography, differences between abscess and false infection. K. KUGELBERGER, K. BERNSTEIN, A. MEYER, M. EBERLING, S. KLEMENS, H. JORDAN (Clinic for craniocervical surgery, Elective University Cologne, Germany)

B-scan ultrasonography was used to differentiate between static infection and abscess formation in a series of 500 patients. The results showed that B-scan ultrasonography had a sensitivity of 91% and a positive predictive value of 90.7% in differentiation between infection and abscess formation, indicating it as a sensitive and reliable tool.

Three types of abscesses were differentiated and classified by ultrasonic morphology (micro-cystic, mixed, macro-cystic).

The high positive predictive value seemed to be based on the good clinical and radiological knowledge of the examining physicians. The data suggest that B-scan ultrasonography might be helpful in guiding clinical decisions.

1150
Skin Resurfacing With A Laser Powered Hydrodynamic System: Histologic Responses in Monge J. P. SAPP, G. G. EVERSOLE, L. R. EVERSOLE (UCDavis, California, Pathology & Research Center, USA)

A laser powered hydromechanical system (LHPS) employs an Er:YSGG laser source to deliver photons into an in vivo matrix with rapid convective forces on water droplets that induce tissue cutting with minimal thermal effects. In this comparison study, 5 pigmented Yucatan monkeys were used to test various power settings on standard medial craniotomy with histologic monitoring of depth of ablation, coagulative zones, zones of ischemia, zones of metal vaporization, and metal vaporization zones. Skin was maximally stimulated with Er:YSGG laser in deep mode, a pulsed CO2 laser, and a neodymium:YAG laser by LHPS and conventional CO2 laser. Deep dermal and adipose layer thermal changes were found to be the same, with no differences in temperature or depth of lesions. Lesions were maximally correlated with temperature, depth and length of cutting (p < 0.0001) between selected groups. Deep dermal and adipose layer thermal changes were also related to the parameters tested (p < 0.001). Deep dermal and adipose layer thermal changes were found to be the same, with no differences in temperature or depth of lesions. Lesions were maximally correlated with temperature, depth and length of cutting (p < 0.0001) between selected groups. Deep dermal and adipose layer thermal changes were also related to the parameters tested (p < 0.001).

1151
Cranial bone and suture regeneration by GTR and DBM and its impact on growth of the calvarium. N. HIRSCH*, L. KOSTOPOULOS, T. KARRING, H. NIELSEN†, DEPARTMENT OF Periodontology & Dental Surgery, University of Aarhus, Denmark.

Background: Craniofacial bone regeneration involves complex processes which may involve nonossifying, normally functioning cranial sutures and may leave behind cranial defects of varying size. The aim of the study was to evaluate the effects of osteoconductive bone matrix (DBM) on cranial bone regeneration and wound healing. The study was conducted in a rat model with the following groups:

- Control group (unstrained chondrocytes in the absence of rhlL-10 treatment (1.1 μM), when compared to unstrained chondrocytes in the presence of rhlL-10 treatment (1.1 μM), when compared to unstrained chondrocytes in the absence of rhlL-10 treatment (1.1 μM), when compared to unstrained chondrocytes in the presence of rhlL-10 treatment (1.1 μM).

Methods: Bone-anchored transplants were treated with DBM alone or DBM combined with GTM (P < 0.05).

Results: The study showed that mechanical strain may be an important mediator in the degeneration of NO production in chondrocytes.

Conclusions: The findings support the use of mechanical strain as a potential therapeutic strategy for degenerative and inflammatory musculoskeletal disorders.

1152
Cranial suture neogenesis by GTR and its impact on growth of the cranial vault. L. KOSTOPOULOS, T. KARRING, S. WIDET-PEDEEREN, DEPARTMENT OF Periodontology & Dental Surgery, University of Aarhus, Denmark.

Background: Craniofacial bone regeneration involves complex processes which may involve nonossifying, normally functioning cranial sutures and may leave behind cranial defects of varying size. The aim of the study was to evaluate the effects of osteoconductive bone matrix (DBM) on cranial bone regeneration and wound healing. The study was conducted in a rat model with the following groups:

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Methods: Bone-anchored transplants were treated with DBM alone or DBM combined with GTM (P < 0.05).

Results: The study showed that mechanical strain may be an important mediator in the degeneration of NO production in chondrocytes.

Conclusions: The findings support the use of mechanical strain as a potential therapeutic strategy for degenerative and inflammatory musculoskeletal disorders.
Mandibular Osteoradionecrosis: Is There Evidence to Use Hyperbaric Oxygen Instead of Ultrasound?

Reher, P.*, Meghji, S., Harris, M.

Department of Oral & Max. Fac. Surgery, EDI, UCL, London, UK; Dep. of Morphology, UFMG, Belo Horizonte, Brazil

Introduction: The treatment for mandibular osteoradionecrosis includes antibiotics and curettage, hyperbaric oxygen therapy (HBO), surgery, and most recently, therapeutic ultrasound.

Aims: The aim of this study was to establish the possible mechanisms of action of ultrasound, that could explain its excellent clinical results.

Methods: Two ultrasound machines were evaluated, a 'traditional' (1 MHz) and a 'long wave' machine (45 kHz). The ultrasound was applied to human mandibular osteoblasts, gingival fibroblasts, peripheral blood monocytes and mice calvaria. The following assays were performed: cell proliferation, collagen and non-collagenous protein (NCP) synthesis, bone resorption, cytokines and angiogenesis factors production using ELISA.

Results: Ultrasound induced cell proliferation in fibroblasts and osteoblasts, up to 52%. Collagen/NCP synthesis was also enhanced, in fibroblasts up to 48%, and in osteoblasts up to 112%. Bone resorption, part of the bone turnover process, could also be noted in the mice calvaria assay. In relation to cytokine production, a slight stimulation of IL-β was noted in all cell types. There was no difference in IL-6 and TNFα levels. The angiogenesis factors, IL-8 and bFGF, were significantly stimulated in osteoblasts, and VEGF was significantly stimulated in both osteoblasts and monocytes. The best intensities were 15 and 30 mW/cm2 (SATA) with 45 kHz ultrasound, and 0.1 and 0.4 W/cm2 (SATA) with 1 MHz ultrasound.

Conclusions: These results show that ultrasound addresses the hypocellularity, hypoxia and hypovascularity observed in osteoradionecrosis. It stimulates cell proliferation, bone formation, healing, and angiogenesis. Ultrasound is a preferred option instead of hyperbaric oxygen therapy, since it is effective, inexpensive and readily available.

Growth Modifying Surgery, Including High Condylectomy and Transection of Growth Inclusion Grafts (versus the Ilizarov-technique)

Reich, R.H.*, Niederhagen, T. Appel, J. Berten.

Bonn, Germany

In primary or secondary hypoplasia of the mandibular condyle, impaired growth of the affected mandible and consecutively, of the maxilla is found frequently. For repair, during the growth period, basically one of two different strategies can be followed:
1. Induction of growth by transplantation of active free autologous osteochondral (i.e. costochondral or iliac crest) grafts.
2. Reconstruction of hypoplastic areas by the distraction osteogenesis.

With respect of the literature and our own experiences on 12 patients (group 1., age 4 - 14, follow up 4 - 14 yrs) and 4 patients (Group 2., age 10 - 11 yrs, follow up 6 bis 3 yrs) respectively, the indication, techniques, advantages and disadvantages of both options are discussed. According to current knowledge, osteochondral grafting should be reserved for early childhood ankylosis and severe hypoplasia cases, extending beyond the mandibular ankle. In the remaining conditions, the ILIZAROV-technique seems to be preferable. It is technically less expensive and more predictable in results.

Prof. Dr. Dr. R.H. REICH, University of Bonn.
Department of Oral and Maxillofacial Surgery.
Welschmönchenstr. 17. D. 53111 Bonn.
Tel. 228 / 287 24 52. Fax 228 / 287 26 04. eMail: mk@uni-bonn.de

Technique and Results of Osteoplastic Reconstruction of the Severely Resorbed Maxilla in Combination with Implants

Reinert, S.*, König, St.*, Eufinger, H.*, Bremerich, A.

Dept. of Oral and Maxillofacial Surgery, Ruhr University Bochum, Bochum, Germany; Dept. of Oral and Maxillofacial Surgery, Hospital St. Jürgenstr., Bremen, Germany

Aims: In the severely resorbed maxilla, a 10-year success rate of only 48.8 - 73.8% of implants in combination with autogenous bone grafts has been reported. We have developed a modified technique of antral inlay grafting and lateral and vertical onlay grafting of the severely resorbed maxilla for the insertion of implants to retain dentures. In this study, the clinical and radiological results are presented.

Method: In 23 patients with severely a resorbed maxilla, a total of 19 bilateral and 4 unilateral antral inlay graftings and lateral and vertical onlay graftings were performed after a prosthodontic set-up. We opened the maxillary sinus by removing a bony window from the anterolateral wall and, after elevation of the sinus lining, grafted the sinus floor with cortico-cancellous iliac crest bone grafts. The maxilla was also augmented in the lateral and vertical dimensions. The bone grafts were fixed by osteosynthesis. After a median period of 5 months, a total of 135 implants (Branemark) were placed and later loaded by prosthodontic rehabilitation. Before the grafting procedure, immediately after grafting, after an additional 4 months and every year, CT scans were performed.

Results: 94.5% of the implants have been successful over an average follow-up period of 2.5 years. Implant failure was observed only at the time of the abutment operation. Most patients were provided with implant-borne dentures. CT scans showed an average initial gain of vertical bone height of 17.7 mm. One year after grafting a loss of 1.7 mm occurred. In the following 2 years no major atrophy was observed. Statistical analysis showed no correlation between sex, bone height before augmentation, augmented bone height and resorption of the grafted bone.

Conclusions: As we observed undisturbed healing and obtained large vertical bone height increases, a high success rate, minimal resorption and fully satisfying prosthodontic