MANIPULATION OF OOCYTES AND EARLY EMBRYOS OF THE COMMON MARMOSET MONKEY *(Callithrix jacchus)*

VIVIENNE SHELLEY MARSHALL

Institute of Zoology,

Regent's Park,

London, NW1 4RY.

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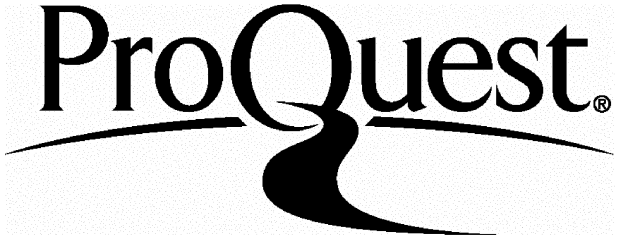

ABSTRACT

Genomic imprinting is a phenomenon whereby some genes are expressed differently depending on whether they are maternally or paternally inherited. In mice, the most notable effects of genomic imprinting appear to be imposed during embryonic development. In humans, it is difficult to study the effects of genomic imprinting on development for obvious ethical reasons, however it is important to discover whether the effects of genomic imprinting on mouse embryonic development are paralleled in primates. The aim of this study was to determine the effects of genomic imprinting on the early embryonic development of a non-human primate, the common marmoset monkey.

To facilitate the investigation, the fertilization rate of marmoset oocytes was increased from 53% to 76% ($p<0.005$) by altering the time between the administration of hCG and laparotomy, and duration of oocyte pre-incubation. The mean maximum cell number (MMCN) of *in vitro* fertilized (IVF) marmoset embryos was increased from 7.7 (± 0.7) when cultured *in vitro* to 15 (± 4.35) when cultured in the oviducts of live mice ($p<0.003$).

The morphological determination of the parental origin of marmoset pronuclei was not possible because both pronuclei formed at the same time after insemination, they were both the same size and both first became visible near the centre of the zygote. Unlike similar studies using mouse zygotes, in marmoset zygotes it was not possible to visualize fluorescent paternal pronuclei after fertilization with marmoset sperm carrying DNA which was stained with a polyspecific fluorochrome.

Pronuclear transfer and electrical fusion of marmoset one-cell embryos was successful in 7/15 (46%) embryos. Marmoset embryos which had undergone sham enucleation and were restored to a normal genetic constitution were able

to develop to an average (± S.E.M) of 3.3 (± 2.3) cells and a maximum of 8 cells.

Parthenogenetic activation of marmoset oocytes was achieved using ethanol (8/47; 17%) and electrical stimulation (68/74; 92%). Marmoset parthenogenones developed to a MMCN (± S.E.M.) of 4.0 ± 0.3 and reached a maximum of 16 cells *in vitro*. There was no significant difference between the percentage of parthenogenetic embryos and IVF embryos reaching each cell stage up to 16 cells.

Three of four IVF embryos, and 2 of 3 marmoset parthenogenones transferred to synchronised recipient marmosets developed to post-implantation stages. To Day 33, when recipient animals were killed, progesterone and inhibin profiles of recipients carrying parthenogenetic embryos (RP) resembled those of recipients carrying normal embryos (RN). However, chorionic gonadotrophin of RP animals remained at non-pregnant levels. Histological analysis of RP animals showed syncytial invasion of the uterine stroma, but only remnants of embryonic membranes.

The development of marmoset parthenogenones to the 16-cell stage is not significantly different from normal IVF embryos. Additionally, implantation of primate embryos can occur without the participation of the paternal genome. By developing the techniques of manipulation of primate embryos and oocytes, this study has provided the basis for further research to elucidate the role of genomic imprinting in primate embryonic development.

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CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Mendel's experiments with garden peas in the mid 19th century led to one of the most important theories of inheritance, the principle of equivalence of reciprocal crosses. That is, it does not matter from which parent the progeny receive their genes, the phenotype will remain the same. This theory has held for over fifty years, with a few exceptions, such as sex-linked traits. However, in recent years, discovery has been made of certain genes which do not adhere to Mendel's theory and whose phenotype depends very much on the parent from which they have been inherited. These genes seem to have some kind of imprint which allows them to "remember" their parental source and changes their phenotypic expression depending on whether they have been maternally or paternally derived.

This differential effect is known as genomic imprinting. The extent to which the genome is imprinted is not yet known, and the mechanism by which imprinting bestows its effect is not yet fully understood, but it is clear that normal prenatal development in mammals cannot proceed without a genetic contribution of both maternal and paternal origin. In some cases, without the contribution of a particular maternal or paternal gene normal development will not proceed.

This chapter summarises normal mammalian fertilization and pronuclear development, the physiological effects of genomic imprinting on early embryonic development in mammals, and the literature regarding the techniques required to

investigate uniparental development in primates.

1.2 FERTILIZATION OF MAMMALIAN OOCYTES

The events surrounding mammalian fertilization include sperm capacitation and penetration of the oocyte vestments, sperm-oocyte fusion, oocyte activation, the incorporation of the sperm into the oocyte cytoplasm and pronuclear development.

Mature, ovulated oocytes have undergone the first meiotic division so that the female gamete consists of an oocyte containing a diploid (2n) set of chromosomes which is arrested at meiotic metaphase II. The vitellus of the oocyte and the first polar body are enclosed within a mucopolysaccharide coat known as the zona pellucida.

During the passage of mammalian sperm through the epididymis, the chromatin in the sperm nucleus is compacted by extensive disulphide cross-linking of nuclear protamines (Calvin 1976). This causes the sperm nucleus to assume a "rigidity" which facilitates the physical penetration of the cumulus mass, corona radiata, and zona pellucida of the oocyte (Bedford 1983).

Before fertilization can take place the sperm must undergo some changes to render it capable of fertilization. These changes are referred to collectively as capacitation. Capacitation involves the removal or change to sperm surface components, which leads to increased permeability and fluidity of the membranes to calcium ions,

resulting in an increased level of intracellular calcium (Plachot and Mendelbaum 1990). Capacitation normally occurs in the female genital tract but can also occur readily in culture medium prior to *in vitro* fertilization. It is unclear exactly what factors are involved in the process of sperm capacitation but a number of enzymes such as neuraminidase (Johnson 1975), β-glucuronidase and β-amylase (Gwatkin 1977) have been suggested.

The acrosome is a membrane bound structure which lies between the anterior region of the sperm nucleus and plasma membrane, and contains hydrolyzing enzymes including acrosin and hyaluronidase. The acrosome reaction is initiated at the surface of the zona pellucida of the oocyte and involves the fusion of the outer acrosomal membrane and the overlying sperm plasma membrane, which releases the acrosomal contents. The acrosome reacted sperm passes through the zona pellucida and enters the peri-vitelline space. Normally one sperm will fuse with the oolemma. Polyspermy is prevented by the "zona reaction" (Wolf 1981). The zona reaction is the term used to describe the refractoriness of the zona pellucida to penetration by more than one sperm. During the penetration of a sperm through the zona pellucida, small membrane-bound organelles located beneath the plasma membrane of the mature oocyte, known as cortical granules, release hydrolytic enzymes which alter the physical and chemical characteristics of the zona pellucida. This cortical granule exocytosis renders the zona impenetrable to more than one sperm.

Sperm-oocyte fusion triggers the activation of the oocyte, i.e. the resumption of meiosis, and subsequent extrusion of the second polar body. It is not known how the sperm activates the oocyte in mammals. It has been proposed that the sperm carries a soluble factor, which has not yet been identified, into the oocyte at sperm-oocyte fusion which causes a release of calcium ions from intracellular stores (Swann 1990) or alternatively, that the sperm binds to an oocyte plasma membrane receptor linked to phosphoinositide turnover, causing an increase in inositol triphophate and release of intracellular calcium (Jaffe 1990). Calcium ions lead to the breakdown of cytostatic factor (Watanabe et al 1989) which, by preventing the degradation of cyclin (Karsenti et al 1987), may maintain high levels of maturation promoting factor, the protein which prevents the cell cycle progressing past the metaphase stage (Murray et al 1989). Therefore it is possible that an increase in the levels of Ca^{2+} may, after a series of changes in the levels of intracellular proteins, lead to the resumption of meiosis.

After extrusion of the second polar body a nuclear membrane forms from cytoplasmic components around the remaining haploid set of maternal chromosomes thus forming the maternal pronucleus.

After sperm-egg fusion the sperm migrates into the oocyte cytoplasm. Soon after the sperm enters the oocyte, the sperm's nuclear envelope disintegrates, allowing the mingling of sperm chromatin with the oocyte cytoplasm (Longo 1985). This

association facilitates the access of cytoplasmic reducing agents to the sperm chromatin, leading to the destruction of the disulphide bonds holding the paternal nuclear chromatin tightly in place. There is some evidence that one of the reducing factors in the oocyte cytoplasm may be a reduced form of glutathione which is present in high quantities in mammalian oocytes and the depletion of which can decrease the decondensation of sperm nuclei (Mahi and Yanagimachi 1975, Calvin and Grosshans 1985). This change in sperm chromatin structure leads to nuclear decondensation. A nuclear membrane forms around the paternal chromatin and the structure can then be recognised as the paternal pronucleus, 6-12 hours after initial sperm penetration in humans (Tesarik and Kopecny 1989, Balakier 1992). Only after the formation of the pronucleus is the decondensed DNA in the paternal pronucleus capable of DNA synthesis (Tesarik and Kopecny 1989).

In most mammalian species, including the human (Palermo et al 1994), but with the exception of the mouse (Schatten et al 1986), the sperm not only carries nuclear material into the oocyte at fertilization but also carries one of the most important organelles involved in mitosis and meiosis, the centrosome. The centrosome is the organelle responsible for the nucleation and organisation of microtubules necessary for the successful progression of mitosis and meiosis (Rappaport 1969, Gould and Borisy 1977, Wheatley 1992). This role includes organising the polarity of the microtubules with the plus end furthest away from the centrosome and the minus end of the microtubule at the centrosome (Schatten 1994). During mitosis, not only

are the chromatin and cytoplasm reproduced and inherited by each new cell, the centrosome is also reproduced and the centrosomes act as the spindle poles during mitosis and meiosis so each cell also inherits a centrosome. Sperm entry initiates the production of a sperm aster which enlarges and moves the paternal pronucleus towards the centre of the zygote (Longo 1987). The migration of the maternal pronucleus begins after contact with the microtubules of the sperm aster. Schatten (1994) proposes that "the surface of the maternal pronucleus is covered with dynein-like, minus-end directed motors". These "motors" would drive the migration of the maternal pronucleus from the periphery towards the centre of the sperm aster. The paternal pronucleus, located at the centre of the sperm aster, and the maternal pronucleus soon become closely apposed. The pronuclear membranes disintegrate, the centrosome splits, the microtubules become bipolar, and the parental chromosomes align along the mitotic spindle. In the mouse, mitosis follows within 24 hours of initial sperm penetration (Hogan et al 1986). The first cleavage division in human embryos occurs slightly later, approximately 36 hours after insemination (Trounson et al 1982).

Cleavage of human embryos to 4 and 8 cells or blastomeres follows approximately 45 and 55 hours after insemination, respectively. Development through morula stages to blastocyst should be expected within five days of fertilization. It has been suggested that blastulation can bew expected between the 4th and 5th cleavage divisions (Hardy et al 1989). These divisions occur approximately every 24 hours in

the human (Hardy et al 1989). The human blastocyst is expected to contain at least 60 nuclei (Trounson and Osborn 1993), although some studies report that after *in vitro* fertilization only 18% of human blastocysts contain this number of nuclei, and the majority (61%) contain less than 29 nuclei (Winston et al 1991). The blastocyst stage is the first stage at which differentiation into two different cell types has occurred (Van Blerkom et al 1976). The blastocyst consists of a trophectoderm (TE) and an inner cell mass (ICM) surrounding a fluid filled cavity, the blastocoel. Approximately 7-9 days after fertilization the human embryo hatches from the zona pellucida and implants in the uterus (Dorkras et al 1991).

Little is known about the immediate post-implantation development of human embryos as these studies cannot be carried out for ethical reasons. However the determination of cells derived from the mouse blastocyst has been investigated in greater detail and a brief outine of these events follows. The TE forms both the mural and polar trophectoderm which, along with the primitive endoderm which is derived from some cells of the ICM, eventually form the parietal yolk sac. The ICM forms both the primitive endoderm and the primitive ectoderm. By day 7 of gestation, the cells of the primitive ectoderm become either the endoderm, (which becomes the intestine, lungs and liver) ectoderm (which becomes skin and nervous system), germ cells or mesoderm. The murine mesoderm divides into extraembryonic mesoderm (which contributes to the visceral yolk sac, the chorioallantoic placenta and the amnion), the genital ridges, and the somites. Pairs of somite blocks form a segmented

pattern along the anterior-posterior axis of the embryo and this process is known as gastrulation. Gastrulation involves the delamination of mesoderm cells to form a primitive streak or groove. At the anterior end of the primitive streak, the Hensen's node appears. The Hensen's node gradually moves posteriorly leaving a trail of notochord cells separating the mesoderm on each side. Next is the formation of the neural folds in the ectoderm and the condensation of the mesoderm cells on either side of the primitive streak into somite blocks. As development continues, the somites are divided into more and more pairs until they eventually become the vertebrae, dermis and muscles. The number of pairs of somite blocks, or somites, is a good indicator of the stage of murine fetal development.

1.3 CONSTRUCTION OF UNIPARENTAL MOUSE EMBRYOS

To study the influence of each parental chromosome set, or genome, on development, mouse embryos with purely maternal or paternal genomes can be constructed.

1.3.1 Creation of parthenogenetic mouse embryos

Mammalian embryos containing only maternal chromosomes can be either parthenogenetic or gynogenetic. Parthenogenetic embryos, or parthenogenones, are derived from unfertilized oocytes that can be stimulated to divide, or activated, by environmental factors such as cold shock (Thibault 1949) or electrical stimulus (Tarkowski et al 1970, Ozil 1990), or by chemical stimulants such as hyaluronidase (Graham 1970), ethanol (Cuthbertson 1983), strontium chloride (O'Neill et al

1991), calcium ionophore A23187 (Winston et al 1991), or media lacking calcium and magnesium (Kaufman et al 1977). Parthenogenetic activation is believed to occur in a similar fashion to the activation of oocytes which occurs at fertilization. Oocytes are held in meiotic arrest by the presence of cytostatic factor (CSF; Murray and Kirschner 1989). CSF prevents degradation of cyclin (Karsenti et al 1987), the protein responsible for maintaining the activity of maturation promoting factor (MPF). Active MPF prevents the cell cycle from progressing past the metaphase stage (Murray et al 1989). Increased concentrations of calcium ions within the oocyte lead to the breakdown of CSF by calpain II (Watanabe et al 1989), allowing cyclin to be broken down, MPF to become inactive and subsequent mitosis of the activated oocyte. An increase in the concentration of calcium ions within the oocyte is believed to be involved in the activation of mammalian oocytes, whether the ions are released from intracellular stores or flow into the oocyte through electroporated membranes. If activation stimulus is applied after extrusion of the first polar body, development usually proceeds in one of four ways, depending on the activation method and the post-ovulatory age of the oocyte (Kaufman 1983). The majority of oocytes undergo the second meiotic division and extrude the second polar body. The remaining haploid set of chromosomes in the oocyte condense into a single pronucleus. DNA replication and cleavage follow, but this homozygous parthenogenone carries only one haploid set of chromosomes. Alternatively, after activation the oocyte may undergo immediate cleavage, without replication of the chromosomes, resulting in two blastomeres each with a haploid set of chromosomes. This mosaic haploid

parthenogenone then continues to cleave at a rate similar to normal embryos. However, in some cases of parthenogenetic activation, the second polar body is not extruded which leaves two sets of chromosomes in the oocyte. These two chromosome sets then either form one or two maternal pronuclei. Replication and division follow, resulting in a heterozygous diploid parthenogenone.

1.3.2 Creation of gynogenetic and androgenetic mouse embryos

Gynogenetic embryos, or gynogenones, also contain only maternal chromosomes but gynogenones are different from parthenogenones in that they contain maternal chromosomes from two different oocytes. Murine gynogenones can be physically constructed by micromanipulation (McGrath and Solter 1983). In murine pronuclear stage embryos, the paternal pronucleus can be distinguished from the maternal pronucleus due to its larger size and greater distance from the second polar body (McGrath and Solter 1983). The paternal pronucleus can be removed from the embryo, and replaced with a maternal pronucleus from another embryo, resulting in a diploid gynogenone (Fig. 1.1). In the same way, mouse embryos containing only paternal chromosomes can be constructed. If the maternal pronucleus is removed and replaced with another paternal pronucleus, a diploid androgenone is formed.

McGrath and Solter (1983) demonstrated that micromanipulation and pronuclear transfer did not affect development when they carried out experiments transferring both male and female pronuclei to previously enucleated zygotes. After this

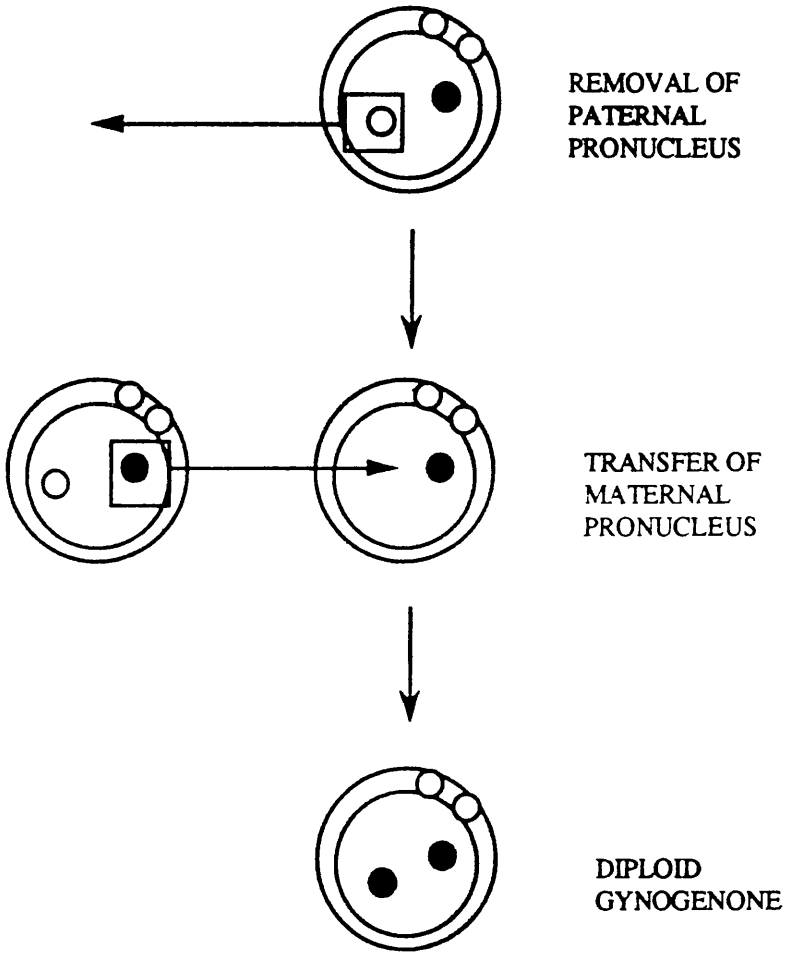

FIGURE 1.1 : Diagram of enucleation and pronuclear transfer to form a diploid gynogenetic embryo. The same principle can be applied to produce a diploid androgenone by removing a maternal pronucleus and replacing it with a second paternal pronucleus.

procedure normal development to term was observed.

1.4 DEVELOPMENT OF MURINE UNIPARENTAL EMBRYOS

1.4.1 Development of murine parthenogenetic embryos

The development of parthenogenetic rabbit embryos was first studied by Thibault (1949) and Chang (1952, 1954). After stimulation by cold shock, parthenogenones developed to blastocyst but no post-implantation development occurred. The first reports of development of parthenogenetic embryos to post-implantation stages was after the *in vivo* activation of mouse oocytes by stimulation of the oviduct with an electric shock (Tarkowski et al 1970). Of 42 embryos which implanted, 45% survived to day 7 of gestation, some of which had reached the early egg cylinder stage. One embryo was recovered on day 10 which had reached the eight-somite stage. Kaufman et al (1977) activated mouse oocytes *in vitro* with media lacking calcium and magnesium. Twenty five percent of these heterozygous diploid parthenogenetic embryos developed to somite stages after transfer to pseudopregnant recipients. The most advanced development was of one embryo to the 25-somite stage, however no development of parthenogenetic embryos to term was achieved.

Several hypotheses were proposed for the failure of parthenogenetic embryos to develop to term. The first was that the presence of homozygous lethal alleles was having a detrimental effect on development. Two experiments were designed to generate embryos with purely maternal chromosomes but with a heterozygous

constitution. After fertilization, extrusion of the second polar body was suppressed by incubating the embryo in cytochalasin B, a microtubule inhibitor (Borsuk 1982, Surani and Barton 1983). This conferred heterozygosity on the embryo because there is some crossing over at the first meiotic division. Subsequent microsurgical removal of the paternal pronucleus (Modlinski 1975, McGrath and Solter 1983) resulted in a diploid, heterozygous, gynogenetic embryo. The second technique used to avoid homozygosity was removal of the paternal pronucleus after fertilization, and replacement with a second maternal pronucleus (McGrath and Solter 1984a). As mentioned previously, McGrath and Solter (1983) demonstrated that micromanipulation and pronuclear transfer *per se* did not affect development. The development of diploid, heterozygous gynogenones was similar to homozygous parthenogenones observed previously, disproving the theory that the homozygosity alone was impairing development.

The second hypothesis proposed for the poor development of parthenogenones was that the cytoplasm of parthenogenetic embryos was abnormal and could not support full-term development. Using microsurgical techniques a paternal pronucleus was transferred into a haploid parthenogenetic embryo (Surani et al 1984). Mann and Lovell-Badge (1984) carried out similar experiments exchanging one maternal pronucleus, from a diploid parthenogenone, with a paternal pronucleus to restore a biparental state within a parthenogenetic cytoplasm. These reconstituted embryos developed normally to term to produce normal, viable offspring, proving that the

parthenogenetic cytoplasm was fully capable of supporting normal development (Surani et al 1984, Mann and Lovell-Badge 1984).

The third hypothesis for the failure of parthenogenones to develop to term was that some extra-nuclear components of the sperm were being carried into the oocyte at fertilization and the absence of these components caused developmental failure. Experiments described above show that even after fertilization, when the embryo would receive any extra-nuclear components of the sperm, if the pronuclei are exchanged so that the embryo contains two maternal genomes instead of a maternal and a paternal genome, the embryo will not develop to term. These experiments demonstrated that a paternal genome is required for normal prenatal development.

In 1977 Hoppe and Illmensee erroneously reported that homozygous diploid parthenogenetic embryos would develop to term. These results have not been repeated (Modlinski 1980, Markert 1982). It has been suggested that the method used by Hoppe and Illmensee to enucleate the embryo may have left a small portion of the paternal pronucleus within the oocyte. This contribution from the paternal genome may have been enough to support development to term (McGrath and Solter 1984a).

1.4.2 Development of murine androgenones

Androgenetic embryos were first constructed by Modlinski in 1975, by

microsurgically removing the maternal pronucleus. Haploid androgenones developed poorly, only cleaving two or three times after 3.5 days in culture. In 1977 Tarkowski prepared haploid androgenones by physically bisecting zygotes. After in vitro culture, haploid androgenones did not cleave more than twice.

Biparental diploid androgenones were first constructed by transfer of a paternal pronucleus to a fertilized embryo after enucleation of the maternal pronucleus (McGrath and Solter 1984a). Sixty-four percent of these androgenones developed to morulae or blastocysts, although more recent work shows that a smaller percentage (10-20%) is usually expected (Howlett 1988, Howlett et al 1989). Barton et al (1984) examined the post-implantation development of androgenetic embryos. After transferring 122 androgenetic embryos, 23% implanted and only 8 embryos were recovered from the uterus on day 10 of pregnancy. These androgenones typically developed to the 5 somite stage but had comparatively large extra-embryonic and yolk sac components compared to the size of fetal tissue. Failure of development of 25% of diploid androgenones can be explained by the YY genotype. YY embryos do not develop beyond two cleavage divisions (Morris 1968). Developmental failure in the remaining 75% of embryos could not be attributed to homozygous lethal alleles since the two male pronuclei were derived from different fathers.

1.4.3 Disparity in the post-implantation development of gynogenetic and androgenetic murine embryos

Reports from Surani and Barton (1983) and Barton et al (1984) showed a surprising phenotypic difference between the development of gynogenetic and androgenetic embryos. Of 228 gynogenetic embryos transferred to pseudopregnant recipients, 18.4% implanted (Surani and Barton 1983). On day 11 of gestation, 77% of control embryos had developed to the 35-somite stage. One gynogenetic embryo was recovered from the contralateral uterine horn on day 11. This gynogenone had developed to the 25-somite stage, however the development of extra-embryonic tissues was extremely sparse. As described in section 1.4.2, androgenones typically developed to the 5 somite stage but had comparatively large extra-embryonic and yolk sac components compared to the size of fetal tissue (Barton et al 1984). In contrast to gynogenones where fetal development is slightly retarded and extra-embryonic tissues are sparse, androgenetic fetuses are extremely retarded with extensive proliferation of the extra-embryonic membranes.

Some parthenogenetic and androgenetic embryos carry a diploid genome. There may be no chromosomal deletions or mutations on these genomes, but the phenotypes of the conceptuses derived from parthenogenetic and androgenetic embryos are markedly different. Clearly, neither the maternal nor the paternal genome alone can support development to term. More important, however, is the observation that each parental genome plays a very different role in early embryogenesis. The maternal genome seems to be required for the development of the fetus and the paternal genome required for extra-embryonic proliferation and differentiation (Barton et al

1984, Surani et al 1984, Howlett et al 1989). Therefore, the parental origin of the genomes must in some way confer differential expression states on the chromosomes. These chromsomes must be able to "remember" their parental origin by carrying some sort of imprint.

1.5 RECONSTRUCTION OF MURINE BLASTOCYSTS

It was postulated that one of the reasons for failure of parthenogenetic/gynogenetic embryos to develop to term was a lack of nutrition at critical stages due to underdeveloped extra-embryonic tissue (Surani et al 1987). This theory was investigated by reconstructing embryos at the blastocyst stage.

Blastocysts can be divided into their ICM and TE components. After removal of the zona pellucida with pronase, the ICM can be cut from the TE with a microneedle. The TE component forms trophoblastic vesicles within approximately 3 hours of this procedure (Barton et al 1985). It was proposed that reconstituting blastocysts by injecting parthenogenetic ICM into normally fertilized TE vesicles may have allowed development of normal extra-embryonic tissues to support the parthenogenetic fetal component. When Barton et al (1985) carried out this procedure, they found that some embryos developed to 30-40 somites. This was the furthest that parthenogenetic fetuses had developed indicating that the normal cells of the TE supported more extensive development of the parthenogenetic fetal component by providing either nutrition or paracrine factors. Since these embryos were only

partially rescued by a normal trophectoderm, it was clear that embryos required both maternal and paternal contributions to support full-term development.

1.6 CONSTRUCTION OF MURINE CHIMERAS

To investigate the ways in which parthenogenetic cells can be supported by cells from normal embryos, chimeras can be constructed. After removal of the zona pellucida with pronase, two different embryos can be joined together during cleavage stages to form a single embryo, or aggregation chimera. Aggregation chimeras develop normally and continue to term. Viable progeny are produced with approximately equal contributions of cells from each embryo to all tissues (Tarkowski 1961, Mintz 1962, McLaren 1976). Chimeras between two different embryos are notated using a double headed arrow. For example, a chimera between a parthenogenetic and a normal embryo is written as a parthenogenetic <-> normal chimera.

1.6.1 Development of parthenogenetic <-> normal chimeras

Parthenogenetic cells can differentiate and contribute to tissues of normal, viable chimeric offspring when aggregated with normally fertilized embryos at cleavage stages (Stevens et al 1977, Surani et al 1977, Anderegg and Markert 1986). After aggregation of 8-cell parthenogenetic embryos with 8-cell fertilized embryos, Anderegg and Markert (1986) observed normal development to blastocyst with rates of chimeric offspring approximately equal to fertilized <-> fertilized chimeras. However, those parthenogenetic <-> fertilized chimeras which did develop to term

were significantly smaller than control chimeras at parturition (Stevens et al 1977, Anderegg and Markert 1986, Thomson and Solter 1989). Parthenogenetic cells are clearly capable of differentiation when they are supported by normal cells, but rates of proliferation may not be normal. Differentiation of parthenogenetic cells has also been demonstrated in extra-uterine sites such as the testis and kidney capsule (Iles et al 1975, Stevens 1978).

Aggregation of embryos with either different isozymes of glucose phosphate isomerase (GPI) (Nagy et al 1987, Surani et al 1988, Fundele et al 1989) or with transgenes incorporated into the genome to act as genetic markers (Thomson and Solter 1989) have been used to follow the fate of parthenogenetic cells during development. These experiments have demonstrated that during post-implantation stages, severe and extensive selective pressure is applied to parthenogenetic cells. Consequently, parthenogenetic cells are virtually eliminated from the extra-embryonic tissues and only survive in the embryonic component (Nagy et al 1987, Surani et al 1988). This selective elimination of parthenogenetic cells from the TE occurs before 6.5 days gestation (Clarke et al 1988, Thomson and Solter 1989) when embryos are undergoing extensive differentiation and proliferation. Not only does elimination occur at these early stages but parthenogenetic cells are specifically eliminated from some tissues of the fetus as development continues and in postnatal chimeric mice, significantly lower numbers of parthenogenetic cells are found in skeletal muscle, liver and pancreas than in brain, heart, kidney and spleen (Fundele et al 1990). Selective

elimination of parthenogenetic cells in aggregation chimeras is both tissue specific and dependent on stage of development. Parthenogenetic cells may be eliminated because they are unable to express genes necessary for participation in events occurring both at particular developmental stages and/or in specific tissues. Further evidence for the stage-specific elimination of parthenogenetic cells is provided by recent work which has shown that parthenogenetic embryos are more likely to die at defined stages in development. Varmuza et al (1993) showed that approximately 50% of murine parthenogenones die during the peri-implantation period, a further 30% die during the pre-gastrulation period and the remaining 20% die around day 7.5, after gastrulation. These workers suggested that, at precise time-points, developmental events were occurring in which parthenogenetic cells were not capable of participating (Varmuza et al 1993).

1.6.2 Development of androgenetic <-> normal chimeras

In contrast to the selective elimination of parthenogenetic cells from the trophectodermal derivatives of chimeras (as discussed above), participation of androgenetic cells in development is specifically confined to the trophectodermal derivatives. Surani et al (1988) found that after transferring androgenetic <-> fertilized chimeras to pseudopregnant recipients, 32% reached parturition but none of the progeny contained androgenetic cells. In a second series of experiments where fetuses were examined at day 10 of gestation, however, androgenetic cells were present but confined to the trophoblast and yolk sac, in some cases contributing up

to 50% of the cells in these tissues. The absence of androgenetic cells in offspring produced from androgenetic <-> fertilized chimeras can be explained if androgenetic cells are selectively eliminated from the embryo proper, and only participate in growth and differentiation of the extra-embryonic derivatives. Just as parthenogenetic cells may not have the necessary genetic information to participate in proliferation of the extra-embryonic derivatives, androgenetic cells may not be able express genes necessary for participation in development of the fetus.

Although full-term development can be achieved when cells with purely maternal or paternal genes are incorporated into chimeras with normally fertilized embryos, the contribution of parthenogenetic or androgenetic cells to the developing fetal and placental tissues is distinctly different. Clearly, the chromosomes in these cells are marked in some way throughout development, and moreover, behave differently because they are derived from different parents.

1.7 THE EFFECTS OF CHROMOSOME DELETION/DUPLICATION ON MURINE EMBRYONIC DEVELOPMENT

Until 1985 the study of differential parental effects had involved the extreme approach of exchanging whole parental genomes and the possibility that chromosomal regions or specific genes were imprinted had not been investigated. Study of parental inheritance of some chromosomal deletion/duplication mutations has revealed interesting phenotypic differences in offspring depending on which

parent contributes the mutation.

The T/t locus which maps to chromosome 17 in the mouse is known to contain 5 lethal mutations (Erickson et al 1978). In 1976, Spiegelman et al had found that inheritance of the t^{w73} mutation, contained within the T/t locus through the paternal line caused unusually high rates of lethality, although maternal transmission had no phenotypic effect. Likewise the hair-pin tail mutation (t^{hp}), also contained within this locus, causes pre- or post-natal death when maternally inherited, but when paternally inherited embryos develop to term and survive to adulthood (Dickie 1965, Johnson 1974). McGrath and Solter (1984b) confirmed that this was a nuclear defect rather than a cytoplasmic one by transferring t^{hp} pronuclei to normal oocytes which resulted in the same phenotypic effect. In 1986 Cattanach also reported lethality due to the presence of two maternal copies without a paternal copy (maternal disomy) of this region of chromosome 17.

Understanding of the effects of the parental contribution of particular chromosomal regions was greatly enhanced by studies in which both copies of a chromosomal region were either maternally or paternally derived (Cattanach and Kirk 1985, Cattanach 1986). Parental duplication (disomy) or deletion (nullisomy) of particular chromosome regions was achieved by crossing animals with different Robertsonian translocations. In mice, Robertsonian translocations are produced when normal separation of chromosomes fails to occur due to centric fusion of pairs of

chromosomes so that offspring receive either two copies or no copies of a chromosome from one parent, but still retain a diploid chromosome constitution. Cattanach (1986) found that parental disomy or nullisomy of most chromosome regions produced normal viable mice. In some cases, however, very different phenotypic effects were evident.

Maternal duplication or paternal deficiency of regions of chromosomes 2, 6, 7, and 8 caused pre- or post-natal death, whereas viable mice were obtained when the duplication was paternally inherited (Cattanach 1986). This pattern of lethality may be caused either by duplication of one chromosome, suggesting a dosage effect, or by the complete absence of one parental chromosome, resulting in a lack of proteins required for embryonic survival. Disomy of the distal region of chromosome 2 exhibited contrasting phenotypes in the progeny depending on parental origin of the translocation (Cattanach 1986). Maternal disomy produced hypokinetic offspring which had arched backs and flat-sided bodies. Paternal disomy, however, resulted in hyperkinetic individuals with short, square and flat bodies.

Cattanach (1986) also found that maternal and paternal duplication of other chromosomal regions produced viable mice but with strikingly different phenotypic variations depending on parental inheritance. Maternal disomy of chromosome 11 produced viable animals that were approximately 30% smaller than their normal littermates. In contrast, those mice inheriting a paternal disomy of chromosome 11

were born approximately 30% larger than their normal littermates. In both types of parental disomy the progeny exhibited normal viability, post-natal growth rates and fertility. The only difference between the genotypes of the individuals exhibiting these anomalous and apparently opposite phenotypes was in the parental derivation of the translocation. Clearly, the parental chromosomes function differently during the development of the embryo.

After finding that differential effects of parental origin were acting on some chromosome regions, the challenge was to examine smaller genetic sequences or specific genes to find out the extent of imprinting in the genome.

1.8 THE EFFECTS OF GENOMIC IMPRINTING ON THE DEVELOPMENT OF TRANSGENIC MOUSE EMBRYOS

When DNA constructs are injected into the pronucleus of a mouse zygote, some of the constructs will be incorporated into the host genome. These constructs are then known as transgenes and serve as genetic markers. Transgenes are usually transcriptionally active but functionally inactive when used as genetic markers. Transgenes have been used to study the inheritance patterns of parts of the genome; to learn more about where imprinted regions are found within the genome; and what mechanisms may be responsible for imprinting.

Swain et al (1987) used a strain of transgenic mice carrying a RSV-S107 autosomal

insert which is a combination of part of the Rous sarcoma virus and a gene from the S107 mouse plasmacytoma cell line. After passage through the male germ line the transgene was expressed. Conversely, when the transgene was passed through the female germ line it was not expressed. De Loia and Solter (1990) also found that the passage of a functional transgene (pL116) through the paternal line, led to expression of the transgene which caused phenotypic abnormalities. Passage of this transgene through the maternal line produced offspring which were phenotypically normal. Although these experiments are consistent with an imprinting effect, there are a number of reasons why they should be interpreted with caution. Since transgenes insert randomly into the genome, the first assumption is that they will behave in the same manner as the endogenous sequences flanking them. This is a major assumption because transgenes may have different patterns of expression merely because they are foreign to the surrounding DNA. Secondly, the introduction of the transgene into the genome may cause some change in expression due to the physical process of incorporation, or it may disturb a gene sequence causing a disruption in the normal pattern of expression. Thirdly, structure of the DNA may affect the insertion of the transgene. For example, the transgene may insert more frequently into heterochromatic or tightly coiled regions of the DNA. It may be interpreted that expression of the transgene represents the whole genome, whereas, if the transgene was preferentially incorporated, the expression of the transgene would only be representative of these heterochromatic regions. This would provide erroneous information about the mechanisms and extent of imprinting in the genome (as

discussed by Reik et al 1990).

To find out more about the effects of genomic imprinting at a single gene level, it was necessary to find examples of endogenous imprinted genes.

1.9 ENDOGENOUS GENES WHICH ARE IMPRINTED IN THE MOUSE GENOME

Recently, four endogenous imprinted genes have been identified. Barlow et al (1991) discovered that the insulin-like growth factor II receptor (IGFIIr) gene, located in the T^{me} region of mouse chromosome 17, was expressed only from the maternal chromosome. Absence of a maternal copy of the IGFIIr gene was lethal by day 15 of gestation. This was the first time an endogenous gene, rather than a chromosomal region, was shown to be imprinted.

DeChiara et al (1991) reported that the insulin-like growth factor II (*IGFII*) gene, located on mouse chromosome 7, was also imprinted. In this case, a targeted disruption of the gene encoding IGFII was employed to block the transcription of the gene. Maternal transmission of the disrupted gene had no phenotypic effect on development. However, transmission of the disrupted gene through the paternal line resulted in progeny that were growth deficient (dwarf phenotype). This gene does not function properly unless a paternally inherited copy is present. *IGFII* is known to have mitogenic properties in the embryo (Czech 1989) so, if the gene does not

function properly when only a maternal copy is present, it is not unexpected that a dwarf phenotype results. The dwarf phenotype may also relate to the size effects observed in parthenogenetic <-> fertilized aggregation chimeras (Stevens et al 1977, Anderegg and Markert 1986, Thomson and Solter 1989). In these chimeras, up to 50% of the cells are of parthenogenetic, or purely maternal origin. If these cells cannot produce functional copies of the *IGFII* ligand due to a lack of paternally inherited *IGFII* genes, this may explain the consistently smaller size of these chimeras.

Passage of the disrupted *IGFII* gene from heterozygous dwarf males mated to normal females results in a 50/50 ratio of normal to dwarf phenotypes. That is, all offspring carrying the disrupted gene have received it through the paternal line and all show the dwarf phenotype. But when heterozygous dwarf females are mated to normal males all the offspring are of normal size. Even though approximately 25% of the offspring carry one disrupted gene and one normally functioning gene for *IGFII*, the disrupted gene no longer has a phenotypic effect after passage through the maternal germ line, and all the offspring are of normal size. This demonstrates one of the most important facets of the phenomenon of genomic imprinting, the ability for the imprint to be completely erased and re-established with transmission through either parental germ line. Failure to erase the imprint would mean the inheritance patterns of the phenotype would resemble those of a mutation.

The mouse *H19* gene which is of unknown function is also imprinted (Bartolomei et al 1991). *H19* maps to chromosome 7 and is closely linked to the *IGFII* gene (Bartolomei et al 1991). The *H19* gene is only expressed from the maternal chromosome (Bartolomei et al 1991). Absence of a maternal copy of *H19* results in late prenatal lethality. Interestingly, although *H19* and *IGFII* are mapped to the same region of chromosome 7, the *IGFII* gene is only expressed from the paternal chromosome, suggesting that the imprinting mechanism can be very precise in its actions.

In 1992, another imprinted gene was discovered. This gene encoding a small nuclear ribonucleoprotein polypeptide N (*Snrpn*), of unknown function, is also located on mouse chromosome 7 and is expressed only from the paternal chromosome (Leff et al 1992). Mice with paternal disomy of the proximal T9H region, which carries the *Snrpn* gene, exhibit decreased post-natal viability, growth retardation, and thin and frail bones (Cattanach et al 1992). Mice with maternal disomy of this region die 3-8 days after birth (Cattanach et al 1992).

1.10 GENOMIC IMPRINTING IN HUMAN DEVELOPMENT

Evidence of a role for genomic imprinting in human development has come from three areas of study. Firstly, the karyotypic analysis of tissues from aborted pregnancies and intrauterine growths, secondly, the study of inheritance patterns of genetic disorders and cancer pre-disposing syndromes, and, thirdly, the molecular

analysis of malignant tumours have been investigated.

1.10.1 The formation and development of hydatidiform moles

The complete hydatidiform mole is the abnormal development of a conceptus with extensive proliferation of extra-embryonic tissue but without any fetal component. The conceptus is usually spontaneously aborted in the second trimester of pregnancy. Karyotypic analysis of complete hydatidiform moles shows that they have the normal complement of 46 chromosomes, usually without any chromosomal abnormalities. The analysis also shows, however, that all of the chromosomes are of paternal origin (Szulman and Surti 1978). Jacobs et al (1980) showed that most complete hydatidiform moles originated from the fertilization of an anucleate oocyte with a single haploid sperm which is then duplicated without cytokinesis, resulting in a diploid androgenetic embryo. It is not unexpected that the development of complete hydatidiform moles strongly resembles the development of experimentally reconstructed androgenetic mouse embryos. This supports the theory that in humans, as in mice, normal development to term cannot proceed without a genomic contribution from both parents.

1.10.2 The development of triploid human fetuses

Human embryos carrying an extra set of parental chromosomes can develop for up to 29 weeks of gestation. Triploid human embryos have either one maternal genome and two paternal genomes (diandric) or one paternal genome and two maternal

genomes (digynic). Diandric triploid embryos result from fertilization of an oocyte either by a diploid sperm or by two sperm. Digynic triploid embryos result from fertilization of a diploid oocyte, which has not undergone either the first or the second meiotic division (McFadden et al 1993).

The development of diandric embryos, not surprisingly, resembles that of complete hydatidiform moles which are androgenetic, and diandric embryos are usually termed partial hydatidiform moles. Partial hydatidiform moles develop relatively normal fetal components but their associated placentas are unusually large and have cystic chorionic villi (McFadden et al 1993). Digynic triploid human embryos develop into retarded fetuses with macrocephaly. The associated placentas are of normal appearance, but are abnormally small (McFadden et al 1993). The phenotypes of diandric and digynic triploid human fetuses and their placentae strongly resemble the phenotypes of androgenetic and gynogenetic mouse embryos, respectively. Clearly, for normal human development not only is a genetic contribution from both parents required, but only one genome from each parent must be contributed. The phenotypes of these triploid fetuses add to the evidence for a role for genomic imprinting in human embryonic development.

The extrapolation of experiments carried out in mice to human embryonic development are not appropriate due to the differences in parental derivation of the centrosome, timing of developmental events, differentiation of tissues of the

developing embryo, and strategies for implantation.

Further experimental elucidation of the role of genomic imprinting on early embryonic development in humans cannot be carried out because the experiments involved would require manipulation of the human embryonic genome and subsequent transfer of those embryos to recipient females. Clearly, these experiments would be totally unacceptable for ethical reasons so for the reasons outlined above it is necessary to use a non-human primate model.

1.11 HUMAN AND NON-HUMAN PRIMATE IN VITRO FERTILIZATION

Although *in vitro* fertilization (IVF) of mammalian oocytes had been achieved in rabbits in the late 1950's (Chang 1959), successful primate IVF was not accomplished until 1969 when Edwards et al reported the *in vitro* fertilization of human oocytes. The first report of non-human primate IVF was some 4 years later, when Gould (1973) achieved fertilization of 11 of 22 squirrel monkey oocytes. Only six of these embryos cleaved and none progressed past the two-cell stage. It was only after the birth of a human infant after IVF and embryo transfer (Steptoe and Edwards 1978), that further progress was reported in IVF of non-human primates. By this time it was considered prudent to develop a non-human primate model for *in vitro* fertilization and development. This would allow investigation of genetic effects of IVF, the requirements for embryo culture, and embryo manipulation which would not be possible using human embryos due to ethical constraints.

The investigation of non-human primate IVF initially centred on the squirrel monkey, which is a relatively small new world primate. Fertilization rates were in the region of 50-60% (Kuehl and Dukelow 1979, Chan et al 1982, Dukelow et al 1983). In 1983, chimpanzee (Gould 1983) and rhesus monkey oocytes (Bavister et al 1983) were successfully fertilized in vitro at rates of 50% and 43%, respectively. Subsequent embryo transfer of rhesus IVF embryos led to the production of live offspring. Bavister et al (1984) transferred 22 rhesus IVF embryos to 11 recipients at the 4-8 cell stage but only one recipient carried the pregnancy to term. In the same year, 17 *in vitro* fertilized cynomolgus macaque embryos were transferred to 7 recipients but again only one embryo developed to term (Balmaceda et al 1984). Despite poor pregnancy rates, these reports demonstrated that non-human primate IVF embryos were also capable of full term development.

IVF in the marmoset monkey was first developed in 1988, with a fertilization rate of 61% (Lopata et al 1988). Embryo transfer following IVF in the marmoset was more successful than in any other primate, including the human. Two of three recipient marmosets became pregnant and three of five marmoset IVF embryos developed to term (Lopata et al 1988). This supported the contention that poor pregnancy rates in other non-human primate species were due to the lack of a reliable system for synchronisation of recipient females. Marmoset monkey recipients can be reliably synchronised using a prostaglandin $F_{2\alpha}$ analogue, cloprostenol, which causes premature luteolysis (Summers et al 1985), and therefore embryo transfers can be carried out when the uterine environment is suitable for embryonic development and

implantation.

The rate of *in vitro* fertilization of human oocytes is now approximately 55% (Trounson and Osborn 1993) but this can vary widely between individual IVF centres. Care must be taken in comparing the rates of IVF between human and non-human primates because human IVF usually involves the use of gametes from at least one potentially infertile individual.

1.11.1 Culture of *in vitro* fertilized primate embryos

The development of primate embryos *in vitro* has always proved difficult (Boatman 1987). Initially a complex medium, TC-199, supplemented with 20% serum, was used to culture squirrel monkey oocytes. These embryos developed poorly, only 52% of fertilized embryos developed beyond two cells in culture and only a few embryos reached 16 cells (Dukelow et al 1983). Ham's F-10, another complex medium which has been shown to support the development of human embryos to the blastocyst stage (Edwards 1972) was used to culture chimpanzee IVF embryos (Gould 1983). However, only five of thirty chimpanzee IVF embryos developed to two cells (Gould 1983). These complex media also failed to support the development of *in vivo* fertilized rhesus and cynomolgus macaque embryos. Eleven of fourteen embryos did not cleave more than once in culture (Kreitmann and Hodgen 1981). In 1983, Bavister et al attempted to culture rhesus monkey IVF embryos in a simple culture medium, TALP supplemented with glutamine, isoleucine, methionine, phenylalanine

and 2% heat inactivated rhesus monkey serum. This proved much more successful for early cleavage stage embryos, 79% cleaved once and 69% of embryos reached the eight-cell stage (Bavister et al 1983). Seventy-one percent of cynomolgus IVF embryos (Balmaceda et al 1984) and 24% of lion-tailed macaque IVF embryos (Cranfield et al 1988) cleaved at least once, and 45% of rhesus monkey IVF embryos reached hatched blastocyst (Wolf et al 1989) after culture in simple medium. Some workers have continued to use complex media for culture of primate IVF embryos but only 14% of chacma baboon IVF embryos (Fourie et al 1987) and 9% of squirrel monkey IVF embryos (Pierce et al 1993) cleaved when cultured in Ham's F-10 and TC-199, respectively.

Bavister et al (1983) noted that 52% of rhesus monkey IVF embryos arrested between the 8 and 16-cell stages whilst cultured in TALP, and suggested that although simple media could support development in early cleavage stages, it was possible that a more complex medium was required for further pre-implantation development. This was borne out by experiments culturing rhesus monkey IVF embryos in CMRL-1066, a complex medium. Although fewer embryos reached the eight cell stage compared to culture in TALP, 68% of 8-cell embryos reached morula when cultured in CMRL-1066 medium but only 13% of 8-cell stage embryos reached blastocyst when cultured in TALP (Boatman 1987).

1.11.2 Development of marmoset embryos *in vitro*

Of nine cleavage stage marmoset embryos, which were fertilized *in vivo* and cultured in M16, a simple medium generally used for mouse embryo culture, only 3 reached the morula stage and 5/9 arrested at or before the eight-cell stage (Harlow 1984). Lopata et al (1988) reported that 3/21 marmoset IVF embryos developed to blastocyst after culture in minimum essential medium (MEM) supplemented with 10% human cord serum. Interestingly, just under 50% of these embryos arrested between the 8 and 16 cell stage (Lopata et al 1988). In a more recent study, marmoset IVF embryos were cultured in MEM and 10% marmoset serum, but only one embryo reached the 32 cell stage and the average development was to 7.7 cells (Wilton et al 1993).

1.11.3 Co-culture of primate embryos

Until the natural physiological environment of the embryo in the oviduct or uterus can be reliably simulated by *in vitro* culture media, embryo development *in vitro* is likely to be compromised. With the aim of mimicking natural conditions there has been much interest in the co-culture of mammalian pre-implantation stage embryos with cell feeder layers.

Culture of marmoset embryos in M16 and BSA or in MEM and marmoset serum on a marmoset oviduct epithelial cell layer had no significantly beneficial effect on the extent of cleavage (Wilton et al 1993). Co-culture in the same media using marmoset skin fibroblast cell layers proved detrimental, marmoset embryos only reached an

average of 3.3 cells (Wilton et al 1993).

1.12 PARTHENOGENETIC ACTIVATION OF PRIMATE OOCYTES

The induction of parthenogenesis in mice has been described in detail in Section 1.3.1. Parthenogenetic activation of primate embryos, however, has proved much more difficult. The only primate in which parthenogenetic activation has been reported is the human.

The stimuli which induce parthenogenetic activation of human oocytes were first studied to prevent the possibility of transfer of parthenogenones to recipient females undergoing IVF treatment. Johnson et al (1990) found that 33% of fresh and 20% of aged human oocytes activated after exposure to acid Tyrode's solution for zona pellucida removal. The pressure used to aspirate oocytes from follicles also caused low rates of activation (Muechler et al 1989). As a check on handling and procedures in clinical IVF programmes, Abramczuk and Lopata (1990) subjected a total of 69 human oocytes to various stimuli to which oocytes are exposed during routine IVF. Exposure to either hyaluronidase, sperm supernatant, ethanol, or cold shock did not induce any activation. These studies showed that it was inherently more difficult to parthenogenetically activate human oocytes than mouse oocytes, and this, for the purposes of routine IVF, was advantageous. However, it became apparent that a method of intentionally activating "spare" oocytes or oocytes that had failed to fertilize would supplement embryonic material for experimental studies of human

pre-implantation development and cytogenetics.

Winston et al (1991) exposed human oocytes to either ethanol or calcium ionophore A23187. Ethanol only activated 16% of oocytes but calcium ionophore activated up to 60% of oocytes. The resulting human parthenogenones underwent a maximum of three cleavage divisions to reach the 8-cell stage *in vitro* (Winston et al 1991). De Sutter et al (1992) and Balakier and Casper (1993) found that puromycin, an inhibitor of protein synthesis, activated up to 91% of human oocytes, but only 16% reached the two-cell stage (De Sutter et al 1992). Clearly, the use of parthenogenones for observations of pre-implantation development requires that embryo viability is not compromised by activation stimulus. At present it appears that calcium ionophore is the most reliable method of activating human oocytes without compromising developmental potential of parthenogenones.

1.13 PRONUCLEAR TRANSFER IN HUMAN ZYGOTES

Another method of creating uniparental embryos is by pronuclear transfer. The literature regarding the manipulation of murine embryos has been discussed in Sections 1.3-1.7. There is little information regarding pronuclear manipulation in human zygotes. Pronuclear transfer has not been reported because, in humans, these types of experiments are ethically unacceptable. Attempts have been made, however, to remove extra pronuclei from triploid embryos to rescue valuable embryonic material (Rawlins et al 1990, Cohen et al 1994, Palermo et al 1994).

The parental origin of pronuclei in one-cell mouse embryos can be determined because the maternal pronucleus is smaller than the paternal pronucleus and is initially located nearer to the second polar body. Determination of the parental origin of human pronuclei has proved much more difficult for a number of reasons. Firstly, the pronuclei are of similar size (Wiker et al 1990). Secondly, the proximity of one pronucleus to the second polar body is not necessarily indicative of its parental origin (Wiker et al 1990). A third possible method of identifying the paternal pronucleus is to look for sperm tail remnants associated with one pronucleus. Wiker et al (1990) found that of 312 pronuclear stage human embryos, only in three (1%) were the sperm tail remnants observed. However, it is possible to successfully remove one of the extra pronuclei from human embryos (Palermo et al 1994, Cohen et al 1994) and development can proceed up to the 16-cell stage (Palermo et al 1994).

If it were possible to reliably identify the parental origin of primate pronuclei this procedure would provide a valuable method of restoring human polyspermic embryos to a diploid state and would also facilitate the production of uniparental non-human primate embryos for research purposes.

1.14 CONCLUSIONS AND AIMS OF THIS STUDY

The majority of information regarding the role of genomic imprinting in mammalian early embryonic development has been derived from studies in the mouse. The effects of genomic imprinting are most strikingly demonstrated by the distinctive phenotypes of mouse embryos carrying only maternal (parthenogenetic) or only paternal (androgenetic) chromosomes and have been described in detail in Section 1.4. Murine parthenogenones develop a relatively normal, if slightly retarded fetus and extremely sparse extra-embryonic tissues. In contrast, murine androgenones develop extensive extra-embryonic tissues associated with very poor fetal development. Uniparental embryos carry the correct number of chromosomes, and these chromosomes do not have any mutations or deletions. Studies using transgenic mice and mice carrying Robertsonian translocations also show that genomic imprinting can have remarkable effects on early embryonic development in mice.

In primates however, the effects of genomic imprinting on early embryonic development are less clear. Winston et al (1991) have parthenogenetically activated human oocytes using calcium ionophore A23187, and demonstrate that human parthenogenones can develop to the eight-cell stage. However, it is of great interest to determine whether uniparental human embryos display similar phenotypic effects to murine uniparental embryos. These studies would involve the genetic manipulation of human embryonic material and subsequent embryo transfer, which is obviously ethically unacceptable. These studies, including pronuclear transfer,

could be carried out using a primate model if gametes and early embryos were readily available.

The common marmoset monkey (*Callithrix jacchus*) is a small new world primate originally from South America. It is polyovular, does not undergo seasonal anoestrus and is easy to breed in captivity. *In vitro* fertilization has been described in this species, so stage-specific early embryos could be made available.

There are, however, a number of prerequisites for the successful accomplishment of these studies, which have never been attempted in any non-human primate. In particular, production of uniparental non-human primates by either pronuclear transfer or by parthenogenetic activation has never been described. The development of techniques required for study of the effects of genomic imprinting on embryonic development in non-human primates include parthenogenetic activation of oocytes, identification of maternal and paternal pronuclei, and protocols for the fusion of pronuclear karyoplasts to one-cell embryos after pronuclear transfer.

The aims of this study were:

1. To modify and extend existing protocols for IVF and marmoset embryo culture. Previous reports of marmoset IVF achieved fertilization rates of 53% and development of 20% of embryos to blastocyst (Lopata et al 1988). However, with the attrition of embryos likely to be high due to the manipulation required to produce uniparental embryos, it was necessary to improve the IVF system to maximise embryonic material.

2. To identify the parental origin of marmoset pronuclei so that successful pronuclear transfer of paternal pronuclei could be carried out to make androgenetic marmoset embryos.

3. To establish a reliable system for the fusion of marmoset one-cell embryos and pronuclear karyoplasts after micromanipulation and pronuclear transfer.

4. To develop a reliable protocol for the parthenogenetic activation of marmoset oocytes and monitor their development *in vitro*.

5. To transfer parthenogenetic embryos to recipient female marmosets and to investigate the development of marmoset parthenogenones *in vivo* using hormonal and histological analysis.

CHAPTER TWO

IN VITRO FERTILIZATION AND EMBRYONIC DEVELOPMENT IN THE MARMOSET MONKEY

2.1 INTRODUCTION

The effects of genomic imprinting on embryonic development can be studied by creating embryos containing only maternal or only paternal chromosomes. This can be achieved by reconstructing one-cell embryos by pronuclear transfer. In order to carry out pronuclear transfer a reliable source of one-cell embryos is required. In some species, it is relatively easy to manipulate and monitor ovulation so that embryos at specific stages of development can be recovered. For instance, studies using the mouse (see Hogan et al 1986) have shown that ovulation can be manipulated using the exogenous hormones pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG). Ovulation usually occurs 10 hours post-hCG injection followed by fertilization approximately 11-13.5 hours after hCG (Hogan et al 1986). This predictable and highly reliable response to hCG, which allows collection of embryos at known developmental stages, is not reproducible in all species.

In the marmoset monkey, the time between hCG administration and ovulation varies quite widely both within and between animals (Harlow 1984) making the collection of embryos at defined developmental stages very difficult. Therefore, when manipulation at pronuclear stages is required, *in vitro* fertilization (IVF) is necessary to determine insemination times and to monitor developmental stage. Lopata et al (1988) developed protocols for IVF

in marmoset monkeys, however on average only 1.7 oocytes were collected from each animal, and only 48% (27/56) of these oocytes fertilized. However, 53% (25/47) of oocytes recovered from larger (>2.5 mm) follicles fertilized (Lopata et al 1988). Although these rates of collection and fertilization were comparable to those of other primate species, the number of animals available for these studies was limited. Therefore, it was in my interest to modify the existing regime to maximise both oocyte collection and fertilization rates to create the largest number of embryos possible. The original protocol, the modifications and changes made to the system, and the results obtained are detailed in this chapter. Some of these findings have been published previously (Wilton et al 1993). However, the results reported in this chapter are more extensive and include the culture of marmoset embryos in the oviducts of live mice.

2.2 MATERIALS AND METHODS

2.2.1 The marmoset colony

The experiments reported in this thesis were carried out on common marmoset monkeys (Callithrix jacchus) which were housed at The Institute of Zoology. The colony is used for both breeding and research. The animals are housed according to Home Office regulations [Animals (Scientific Procedures) Act 1986] and are kept either in breeding pairs or family groups. Marmosets reach maturity at about eighteen months of age (Hearn 1983) and are then removed

from the family group and paired. Each pair is allowed to produce one litter to prove fertility before the pair becomes part of the experimental colony.

2.2.2 The marmoset ovarian cycle

Marmosets have a 27-29 day ovarian cycle (Harlow et al 1983). The follicular phase is 8-10 days, and the luteal phase is 18-20 days (Harlow et al 1983). A prostaglandin $F_{2\alpha}$ analogue, cloprostenol (0.5μg; Estrumate, Coopers Animal Health Ltd., Bristol, UK.) administered between days 10 and 24 of the luteal phase causes premature luteolysis and effectively resets the cycle to the beginning of the follicular phase (Summers et al 1985). Females in the experimental colony are routinely monitored for ovulation. A blood sample (0.3 ml) is taken from the femoral vein and progesterone levels in the peripheral plasma are determined by enzyme-linked immunosorbent assay (ELISA; Hodges et al 1988). The day of ovulation is defined as the day before progesterone rises above 10 ng/ml(Harlow et al 1983).

2.2.3 Synchronisation of marmoset oocyte donors

Oocyte donors received 0.5μg cloprostenol by intramuscular injection at 9am between days 10 and 24 of the luteal phase. The day of cloprostenol administration was designated Day 0. At 1 pm on Day 7 the animals received an intramuscular injection of 75 iu of human chorionic gonadotrophin

(Chorulon, Centaur, Castle Cary, Somerset, U.K.). Laparotomy was carried out between 10 am and 1 pm on Day 8 (i.e. 20-24 hours post hCG injection).

2.2.4 Marmoset oocyte collection

Oocytes were collected by follicular aspiration as described by Lopata et al (1988). Females were anaesthetized with Saffan (~2.5 ml/kg body weight; Centaur, Castle Cary, Somerset, U.K.). The ovaries and uterus were exteriorised by mid-line laparotomy, and the number and size of follicles on each ovary were noted. Follicles less than 2mm in diameter consistently yielded oocytes which had not extruded a second polar body and consequently were too immature to undergo fertilization. For this reason only follicles which were larger than 2mm in diameter were aspirated. Follicles were aspirated with a pulled 1.5 mm diameter glass capillary, broken off at 0.7-0.8 mm diameter, which was attached to a micrometer syringe with rubber tubing. Using the microforge, the tip of the pipette was extended on one side to form a sharp spike so that the follicle could be easily punctured. Once the follicle was punctured, the contents were drawn out. This procedure was repeated two or three times. The follicular contents were expelled into a 35mm sterile petri dish (Merck, Lutterworth, Leics, U.K.) containing alpha modified minimum essential medium (αMEM; Merck, Lutterworth Leics, U.K.) buffered with 25 mM Hepes and supplemented with 0.05 mg/ml streptomycin sulphate, 0.06 mg/ml penicillin (all from Sigma Chemical Co. Ltd., Poole,

Dorset, U.K.), 1 iu/ml heparin (Monoparin; CP Pharmaceuticals Ltd., Wrexham, UK) and 1% heat inactivated marmoset serum.

The reproductive organs of the donor female were coated in 30% w/v Dextran (Pharmacia Fine Chemicals, Milton Keynes, U.K.), to help prevent post-operative adhesions, and replaced in the abdominal cavity. The incision was sutured with 5-0 Dexon (Centaur, Castle Cary, Somerset, U.K.). There were two sets of interrupted sutures, one in the muscle wall and one in the skin. Aureomycin, a broad spectrum antibiotic powder (Centaur, Castle Cary, Somerset, U.K.), was applied subdermally, before the skin was sutured. The animal was given an intramuscular injection of 0.1 ml Clamoxyl LA (Beechams, Crawley, Sussex, U.K.) which is a semi-synthetic penicillin providing antibiotic protection for 48 hours. The animals recovered from anaesthetic within approximately three hours and were returned to their mate or family groups.

2.2.5 Marmoset oocyte grades

As oocytes mature, prior to ovulation, the cells which surround the oocyte (collectively known as the cumulus oophorus) become progressively less compacted. Hence it is possible to estimate the maturity of the aspirated oocyte by the degree of cumulus expansion. The following grades were assigned to the oocytes after assessment of the cumulus : a very expanded

cumulus cell layer (Grade I), an extensive cumulus layer which is not fully expanded (Grade II), 5-6 layers of closely packed cumulus cells (Grade III), and 2-3 layers of very condensed cumulus cells (Grade IV) (Fig. 2.1).

2.2.6 Marmoset sperm preparation

2.2.6 (i) Preparation of sperm collected by electroejaculation

Males marmosets were anaesthetized with Saffan (~2.5 ml/kg body weight; Centaur, Castle Cary, Somerset, U.K.). An electrical probe of approximately 0.9 cm diameter was inserted into the rectum and 5 pulses of 5-10 volts were applied. Ejaculates were collected in a 5 ml plastic tube (Falcon; Marathon Laboratory Supplies, London, U.K.) which contained 500 μl of αMEM supplemented with 10 μM dibutyryl cyclic adenosine monophosphate (dbcAMP), 10 μM caffeine, 6 mg/100ml penicillin, 5 mg/100ml streptomycin sulphate and 15% heat inactivated fetal calf serum (ICN Flow, High Wycombe, Bucks, U.K.) or 15% heat inactivated marmoset serum. The ejaculate was incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 2 hours to allow the ejaculate to disperse. The media containing the ejaculate was overlaid with 500 μl media supplemented with only 10% serum to allow motile sperm to swim up into the less dense upper layer. Both layers of the sperm sample were assessed for motility and number of sperm.

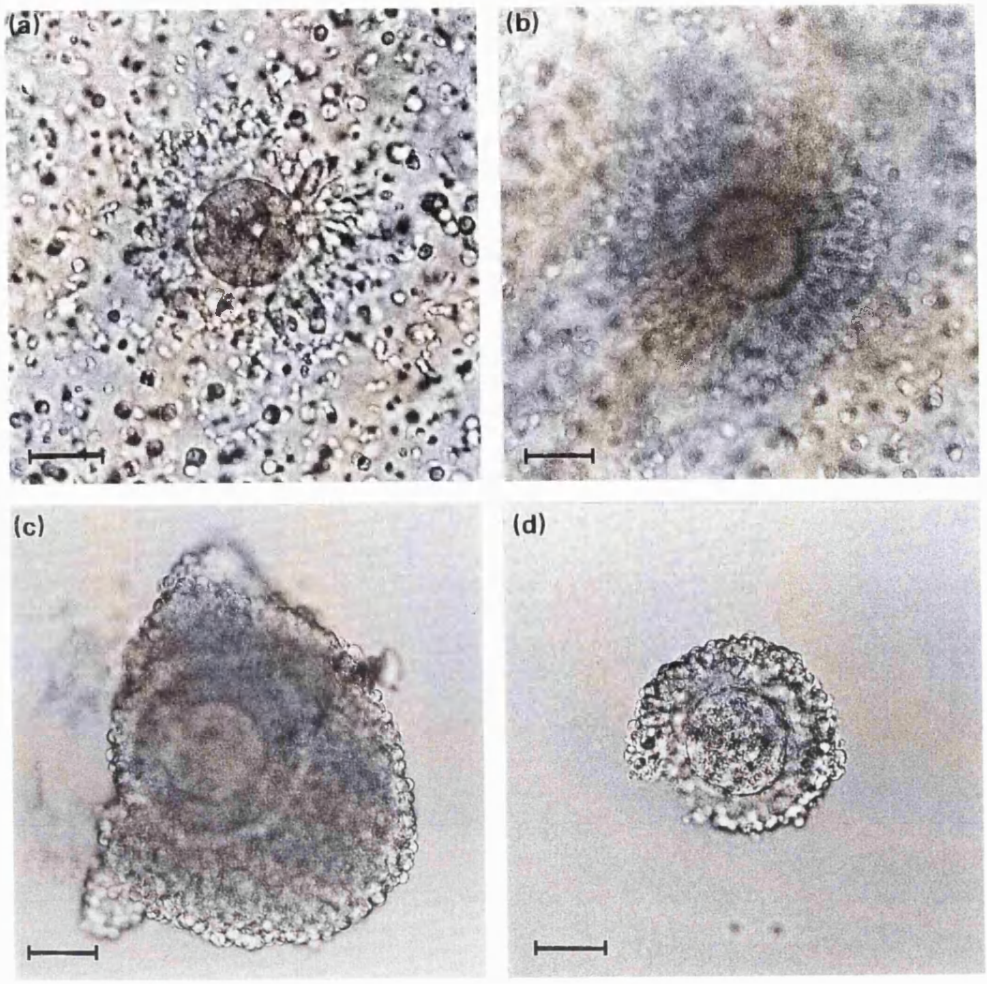

FIGURE 2.1 : Marmoset oocytes of different grades. (a) Grade I - extensive, very expanded cumulus cell layer. (b) Grade II - extensive but less well expanded cumulus than Grade I oocyte. (c) Grade III - a number of layers of closely packed cumulus cells. (d) Grade IV - only a few very densely packed cumulus cell layers.

2.2.6 (ii) Preparation of sperm collected by epididymal dissection

Males were anaesthetized with Saffan (~2.5 ml/kg body weight) and euthanased with 1 ml of 20% w/v Pentobarbitone Sodium (Euthatal; Centaur, Castle Cary, Somerset, U.K.) injected directly into the heart. The epididymides were dissected from the testes and placed in Hepes buffered αMEM. Using a dissecting microscope, blood vessels and excess fatty tissue were removed. Four hundred microlitres of αMEM supplemented with 10 μM dibutyryl cyclic adenosine monophosphate (dbcAMP), 10 μM caffeine, 6 mg/100ml penicillin, 5 mg/100ml streptomycin sulphate and 10% heat inactivated male marmoset serum or fetal calf serum was placed in each well of a 4-well culture plate (Marathon Laboratory Supplies, London, U.K.). In the first well, the epididymis was cut into several pieces with scissors, allowing the sperm to swim out. The epididymis was left in this well for approximately 5 minutes. This process was repeated in the next 3 wells of the plate. In each well the epididymis was cut up further to recover even more sperm. In this way, most of the sperm were collected from each epididymis. The sperm were incubated, in the 4-well plate, at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2.7 Pre-incubation of marmoset oocytes and marmoset sperm

Both oocytes and sperm were incubated separately in αMEM supplemented with 10 μM dibutyryl cyclic adenosine monophosphate (dbcAMP), 10 μM

caffeine, 6 mg/100ml penicillin, 5 mg/100ml streptomycin sulphate and 10% heat inactivated male marmoset serum. This is a slight modification of the media used by Lopata et al (1988) who used human cord serum instead of marmoset serum. Oocytes were incubated for 2-5, 9-11 and 21-29 hours before insemination. Sperm were incubated for at least three hours and up to 7-8 hours before insemination.

2.2.8 Insemination of marmoset oocytes

Insemination was carried out in the wells of a 4-well culture plate. Oocytes were placed into a well containing 400 μl of sperm preparation [see 2.2.6 (ii)]. The concentration of sperm was approximately 10-15 x 10^6 sperm/ml. The insemination time ranged from 12-20 hours. Oocytes were removed from the insemination media, washed and placed in drops of MEM and 10% heat inactivated female marmoset serum and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2.9 Cumulus cell removal and assessment of fertilization

Cumulus cells were easily removed from oocytes after insemination by repeated pipetting with a flame polished pulled pasteur pipette which had an internal diameter very slightly larger than the diameter of the oocyte. Fertilization was confirmed by visualisation of a second polar body and two or more pronuclei.

2.2.10 Culture of marmoset IVF embryos *in vitro*

Embryos were cultured in drops (approximately 50μl) of Minimum Essential Medium (MEM; Life Technologies, Paisley, Scotland, U.K.) supplemented with 6 mg/100ml penicillin, 5 mg/100ml streptomycin sulphate and 10% heat inactivated female marmoset serum. The drops were overlaid with paraffin oil (Merck, Lutterworth, Leics, U.K.). Embryos were observed once or sometimes twice per day using an inverted Olympus OMT-2 microscope fitted with Nomarski optics (Olympus Optical Co. (U.K.) Ltd., London, U.K.). Embryos were assessed by noting the number of cells, the relative cell sizes and their general appearance (eg. granular appearance, particularly dark cells, vacuoles present, etc.). Daily observations were made until there was no further cleavage for at least 48 hours.

2.2.11 Culture of marmoset embryos *in vivo*

2.2.11 (i) Transfer of marmoset embryos to the oviducts of live mice

Immature (3-4 week old) female F_1 (CBA X C57Bl) mice were anaesthetized with 0.7 ml Avertin (Appendix I). The area of the operation was swabbed with 70% alcohol. A dorsal incision approximately 1 cm long was made with scissors. A hole was cut in the muscle wall on either side of the spinal column and the ovary, oviduct and top 0.5 cm of the uterus were exteriorised and held in position with a small serafine clip. A small tear was made in the bursa of the ovary to allow access to the infundibulum. The embryo was loaded into

a pulled glass pasteur pipette attached, by tubing, to a mouth piece. The end of the pipette was then introduced into the infundibulum which was held steady with a pair of fine forceps. The marmoset embryo was expelled into the mouse oviduct. The organs were replaced inside the abdominal cavity and the incision in the skin was closed using 3 or 4 Michel clips (The Holborn Surgical Instrument Co. Ltd., Broadstairs, Kent, U.K.). Each marmoset embryo was left in the recipient mouse for up to 3 days, when the mouse was killed. The oviducts and uterus were dissected and placed in M2 medium. A 30 gauge needle, which had been ground off to remove the sharp point, was threaded through the infundibulum and the oviduct was flushed with approximately 0.25 ml of M2 medium. If the marmoset embryo was not recovered, the uterus was flushed in the same way. There was no question of confusion of a marmoset embryo with a mouse oocyte or parthenogenone due to the large difference in size (marmoset embryo ~120 μm cf. mouse embryo ~85 μm) and the thickness of the zona pellucida (Figure 2.2). After inspection of the embryo, it was transferred into another recipient mouse, as described above, and the process repeated.

2.2.11 (ii) Embryo transfer to recipient female marmosets

2.2.11 (ii)a) Synchronisation of recipient marmosets

Recipient females received 0.5 μg of cloprostenol on Day 0 (the same day as the oocyte donors). Females were separated from their male partners on Day

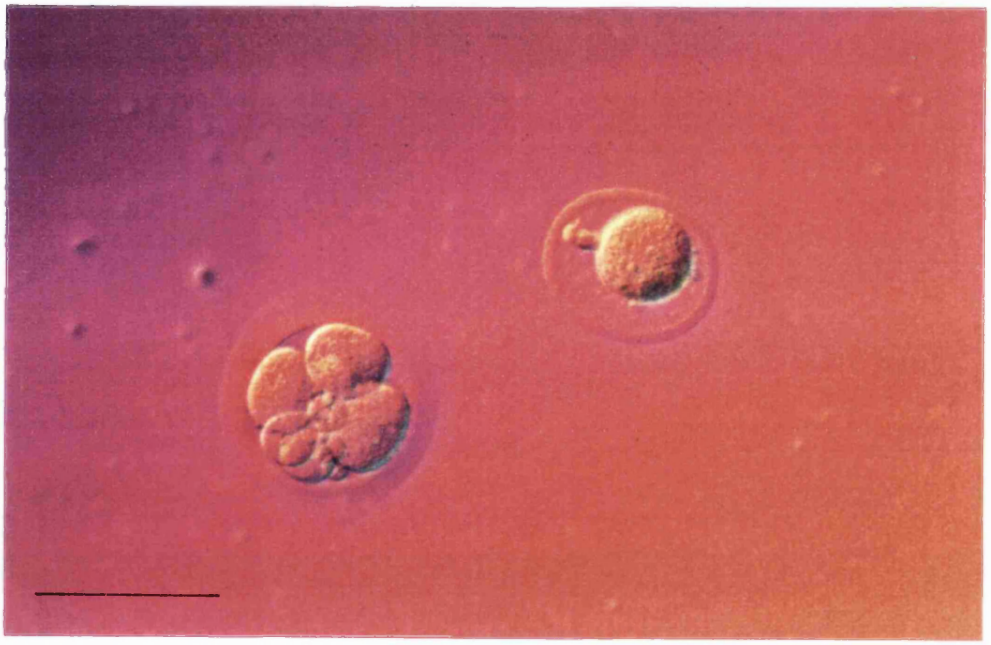

FIGURE 2.2 : 5-cell marmoset embryo retrieved after culture in the oviduct of a live mouse. An unfertilized mouse oocyte, flushed from the same oviduct, is of smaller size and has a thinner zona pellucida. Bar = 100 μm.

5 and housed singly until at least 7 days after embryo transfer. Human chorionic gonadotrophin was administered at 11am on Day 8, and a blood sample was taken. A second blood sample was taken on Day 10 and a progesterone ELISA carried out to confirm ovulation.

2.2.11 (ii)b) Marmoset embryo transfer

Recipient females were anaesthetized with Saffan. A mid-line abdominal incision was made and the uterus and ovaries were exteriorised. The ovaries were checked for the presence of corpora lutea. A hole was punctured in the fundus of the uterus with a 21 gauge (0.8mm x 16 mm) needle (Monoject, Centaur, Castle Cary, Somerset, U.K.). The embryo was loaded, in approximately 2 μl of media, into a pulled glass pasteur pipette attached by tubing to a mouthpiece to permit controlled suction. The pipette was introduced into the uterus of the recipient female through the hole in the fundus. The embryo was expelled by blowing gently into the mouthpiece. Organs were replaced and the animal was sutured as described in Section 2.2.4.

2.2.12 Statistical analyses

Fertilization rates were compared using a x^2 test and a student's *t* test was used to compare the mean cell number of embryos.

2.3 RESULTS

2.3.1 Marmoset oocyte collection

115 laparotomies were performed and in 109 cases pre-ovulatory follicles (> 2mm) were found. Three animals had ovulated prior to laparotomy, 2 animals did not have any pre-ovulatory follicles, and one animal had cystic ovaries with no pre-ovulatory follicles. From 109 laparotomies, 280 pre-ovulatory follicles were aspirated, an average of 2.6 follicles per animal. The oocyte recovery rate was 92% (258/280).

2.3.2 Marmoset oocyte grades

Of the 258 oocytes collected, 19 (7%), 150 (58%), 70 (27%), 15 (6%), and one (1%) were Grade I, II, III, IV, and atretic, respectively. Three oocytes (1%) were at the germinal vesicle stage although they had been aspirated from follicles greater than 2 mm in diameter. Of the 258 oocytes collected, 117 were used for *in vitro* fertilization and the remaining 141 were either not inseminated or were used for other experiments described in this thesis.

2.3.3 Marmoset sperm motility and survival

2.3.3 (i) Electroejaculated marmoset sperm

Ejaculates were successfully collected from 20/31 (65%) males. After dispersal and swim-up procedures motility ranged from 10-90%, however motility was not always progressive and the number of sperm able to swim out of the

ejaculate was very low. All the sperm in these samples were non-motile within 20 hours of collection and there was no difference in longevity depending on whether fetal calf or marmoset serum was used to supplement the media. Only one sample was used to inseminate oocytes (n=4) but no fertilization was obtained.

2.3.3 (ii) Marmoset epididymal sperm

Sperm collected by epididymal dissection survived well in culture and exhibited fast, forward progressive motility. Large numbers of sperm (~25 x 10^6/ml) were obtainable by this method. Sperm remained highly motile for up to 48 hours when incubated in media containing marmoset serum. However, when fetal calf serum was used to supplement the media sperm survival time decreased sometimes to only 6 hours and in all cases all sperm were completely non-motile after 20 hours. Because oocytes were inseminated for up to 16 hours, it is possible that the non-motile sperm, which were probably dead, could have been producing toxic products from cellular breakdown which may have affected embryo viability. For this reason, marmoset serum was used to supplement sperm incubation (and insemination) media.

2.3.4 The timing of marmoset pronuclear formation

A small number of oocytes (n=6) were only incubated with sperm for three hours so that the timing of pronuclear formation could be assessed. Three

hours after insemination, the second polar body had been extruded, but pronuclei were not yet visible. Marmoset pronuclei were first discernible, under phase contrast microscopy, six hours post-insemination. In all embryos observed, both pronuclei became visible at the same time, were positioned very close to each other near the centre of the embryo and were approximately the same size. It was not possible to visibly distinguish between marmoset maternal and paternal pronuclei. Marmoset pronuclei remained visible for 18-22 hours after insemination.

2.3.5 Fertilization rate of marmoset oocytes

Fertilization was determined by the extrusion of a second polar body and the formation of two or more pronuclei. The overall fertilization rate for all oocytes was 76% (89/117). Two were polyspermic fertilization as determined by the presence of three pronuclei.

Fertilization rate was dependent on both oocyte grade and duration of pre-incubation. Fertilization rates were significantly higher for Grade II oocytes (95%) compared to 79% ($p<0.05$), 47% ($p<0.001$) and 55% ($p<0.001$) for grades I, III, and IV, respectively (Table 2.1). Oocytes preincubated for 2-5 hours had an overall fertilization rate of 53% (10/19), which was not significantly increased when oocytes were pre-incubated for 9-11 hours (69%). Overall fertilization rate was significantly higher when oocytes were incubated

for 21-29 hours (92%)($p<0.05$). Within each oocyte grade, the recurring trend was towards an increase in fertilization rate with increased pre-incubation time, as reflected by the overall fertilization rates within each pre-incubation time. It was interesting to note, however, that the less mature the oocyte at collection, the longer the pre-incubation time required before significant increases in fertilization rate were observed. The effect on Grade I oocytes of increasing pre-incubation time from 2-5 to 9-11 hrs was to increase fertilization rate from 40% to 100%. The rate of fertilization of Grade II oocytes also increased from 78% to 100% ($p<0.05$) when incubation time was increased from 2-5 to 9-11 hours and remained high (95%) when oocytes were pre-incubated for 21-29 hours. The fertilization rate of Grade III oocytes significantly increased when incubation time was increased from 9-11 hours (13%) to 21-29 hours (100%; $p<0.001$). There was no significant increase in the fertilization rate of Grade IV oocytes, due to the small numbers in each group, however there was a trend of increasing fertilization rate with increasing pre-incubation time from 0% (2-5 hours) to 30% (9-11 hours) and further to 71% when duration of pre-incubation was increased to 21-29 hours.

2.3.6 Development of marmoset IVF embryos *in vitro*

Of the 87 normally fertilized embryos, 66 were cultured *in vitro.* Sixty embryos (91%) developed to the two-cell stage. One embryo was lost at the

two-cell stage and 53 (82%) of the remaining 65 embryos developed to four cells. Cleavage to eight cells was achieved by 31 (48%) of the embryos, and seven (11%) of these reached 16 cells. Only one embryo divided to 32 cells. The overall mean maximum cell number (± SEM) was 7.7 ± 0.7. There was no significant difference between the maximum cell numbers achieved by embryos resulting from oocytes of different grades (Table 2.2).

Increasing the length of oocyte pre-incubation had a significant effect on mean maximum cell number (Table 2.2). Embryos resulting from oocytes which had been pre-incubated for 2-5 hours reached a maximum of only 6 cells, with a mean maximum cell number of 2.8 ± 0.6, and 40% (4/10) of embryos in this group failed to cleave at all. However, when oocytes were pre-incubated for 9-11 hours, the mean maximum cell number was increased to 9.6 ± 2.5 ($p<0.001$) with only two of 12 embryos (17%) failing to cleave and the maximum development in this group was to 32 cells. Embryos resulting from oocytes pre-incubated for 21-29 hours also developed significantly better than those in the 2-5 hour group ($p< 0.001$). All of these embryos cleaved at least once, developed to a mean maximum cell number of 8.3 ± 0.6, and four embryos (10%) reached sixteen cells.

TABLE 2.1 : Fertilization rates of Grade I, II, III, and IV marmoset oocytes pre-incubated for 2-5, 9-11 or 21-29 hours after collection.

Oocyte Grade	Duration of Preincubation (hours)			
	2-5	9-11	21-29	Total
I	2/5 (40)[a]	2/2 (100)	11/12 (92)[b]	15/19 (79)[h]
II	7/9 (78)	28/28 (100)[c]	19/20 (95)	54/57 (95)[j]
III	1/4 (25)[d]	2/15 (13)[d]	11/11 (100)[e]	14/30 (47)[k]
IV	0/1 (0)	1/3 (30)	5/7 (71)	6/11 (55)[k]
Total	10/19 (53)[f]	33/48 (69)[f]	46/50 (92)[g]	89/117 (76)

b: significantly higher than a ($p<0.05$)
c: significantly higher than d ($p<0.001$)
e: significantly higher than d ($p<0.001$)
g: significantly higher than f ($p<0.05$)
j: significantly higher than k ($p<0.001$)
h: significantly different from j and k ($p<0.05$)

117
cf. 82
35

TABLE 2.2 : Mean maximum cell number achieved by *in vitro* fertilized marmoset embryos resulting from Grade I, II, III, and IV oocytes incubated for 2-5, 9-11 or 21-29 hours before fertilization. Where only one or two embryos were included in a group, the cell number of each embryo is recorded and separated by a comma where necessary.

Oocyte Grade	Duration of Preincubation (hours)			
	2-5	9-11	21-29	Total
I	4,6 n=2	4,5 n=2	7.5±1.4 n=8	6.6±1.0 n=12
II	2.3±0.6 n=7	13.7±3.4 n=7	9.3±1.0 n=18	8.8±1.2 n=32
III	1 n=1	1,8 n=2	7.6±1.1 n=10	6.6±1.1 n=13
IV	-	1 n=1	6.5±1.7 n=4	5.4±1.7 n=5
Total	2.8±0.6[a] n=10	9.6±2.5[b] n=12	8.3±0.6[b] n=40	7.7±0.7 n=62

b : significantly higher than a ($p<0.001$)

2.3.7 Cleavage rates of marmoset embryos fertilized *in vitro*

Marmoset embryos which have been fertilized and have developed *in vivo* reach the 4-cell stage on Day 2 after ovulation (Harlow 1984). Embryos which were fertilized and cultured *in vitro* and had not reached the 4-cell stage by Day 2 after insemination were considered delayed in their development. Increasing the oocyte pre-incubation time from 2-5 to 9-11 hours not only had a beneficial effect on mean maximum cell number it also decreased the number of embryos which were delayed in cleavage. However, increasing the pre-incubation time still further, to 21-29 hours, increased the percentage of delayed cleavage embryos. All embryos resulting from oocytes pre-incubated for 2-5 hours, and 23 (58%) of the embryos resulting from oocytes pre-incubated for 21-29 hours were delayed. Only two (17%) embryos in the 9-11 hour group were delayed in their cleavage time. Delayed embryos only reached a mean maximum cell number of 5.2 ± 0.6 which was significantly lower than that of normally cleaving embryos (10.8 ± 1.1; $p<0.001$). The effects of duration of oocyte preincubation on rate of cleavage were independent of the maturity of the oocyte at fertilization (i.e. oocyte grade).

2.3.8 Development of marmoset embryos *in vivo*

2.3.8 (i) Development of marmoset embryos cultured in live mouse oviducts

A total of 17 embryos were cultured in this system. Six embryos were not recovered from mice, a recovery rate per embryo of 65%. However, of the 39 transfers that were carried out, embryos were recovered on all but six occasions, a recovery rate per transfer of 85%. As described previously, the oviducts and uteri of recipient mice were flushed separately and marmoset embryos were recovered were found in the oviduct in 90% of cases and in the uterus in 10% of cases.

The *in vitro* fertilized marmoset embryos which were recovered in cleavage stages developed to a mean maximum cell number (± SEM) of 15 (± 4.35), which was significantly higher than the mean maximum of 7.7 (± 0.7) cells achieved by embryos which were cultured in MEM and MS ($p<0.003$). Of the embryos recovered, 82% (9/11) developed to the 4-cell stage, 73% (8/11) developed to the eight cell stage, five (45%) reached 16 cells and four (36%) divided to 32 cells. One embryo developed to blastocyst after being cultured *in vitro* from the 40-50 cell stage (estimated number of cells)(Fig 2.3). None of the embryos which reached four cells were delayed in their development, and the embryo which developed to blastocyst reached this stage on Day 10.

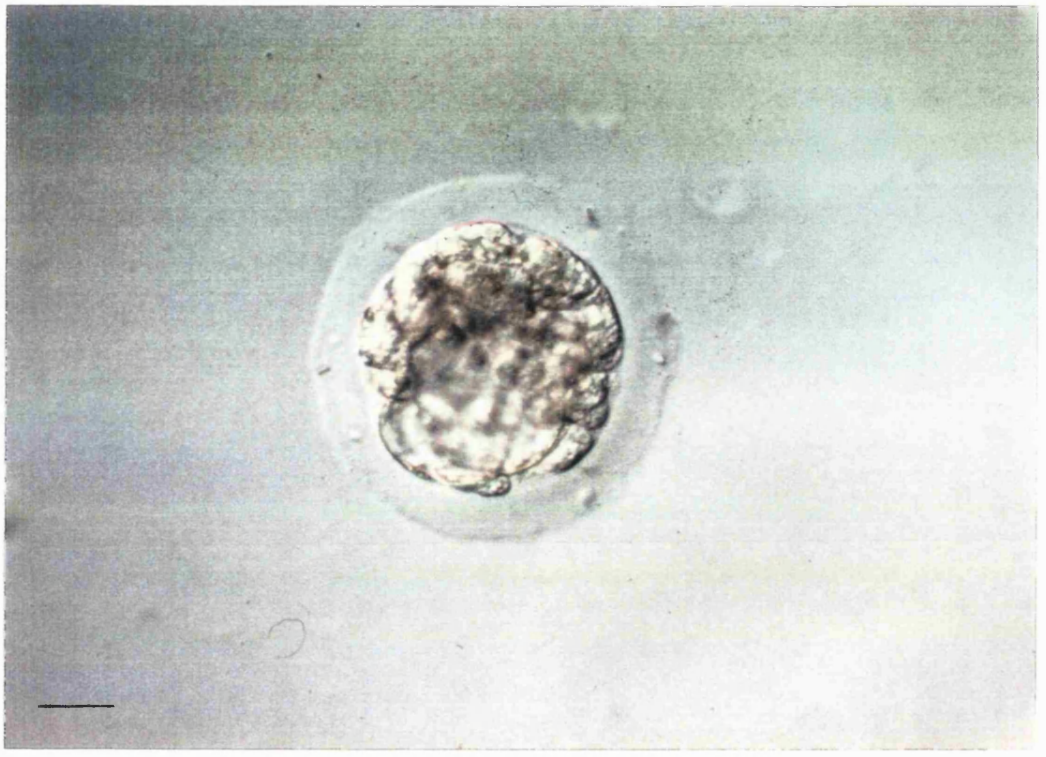

FIGURE 2.3 : *In vitro* fertilized marmoset blastocyst retrieved after culture in the oviducts of live mice. Bar = 25 μm.

2.3.8 (ii) Development of marmoset embryos transferred to recipient marmosets

Four embryos were transferred to synchronised recipients at the four cell stage (Day 3 after fertilization). Three (75%) of these embryos developed to term.

2.4 DISCUSSION

The regime of oocyte collection and IVF described in this chapter was highly successful, demonstrating excellent oocyte recovery rates (92%) and high rates of fertilization (76%). *In vitro* fertilization has been carried out in a number of primate species with fertilization rates ranging from 39% in the baboon (Fourie et al 1987) to 90% in the squirrel monkey (*Saimiri sciureus*; Chan et al 1982). By optimizing the protocol for oocyte donor synchronisation and duration of oocyte *in vitro* maturation, fertilization rates were raised from 53% (Lopata et al 1988) to 76% ($p<0.005$; this study).

The procedures described in this chapter to synchronise females, and to collect marmoset oocytes and sperm, were a modification of those used by Lopata et al (1988) who described three regimes to synchronize females for oocyte collection. The regimes varied in the time between cloprostenol administration and hCG administration, and the time between laparotomy (oocyte collection) and insemination. Lopata et al (1988) found that if the time between cloprostenol

and hCG administration was 192 hours, 45% of animals had ovulated prior to laparotomy, however if this time was shortened to 176 hours, only 14% of animals had ovulated at laparotomy. By decreasing the time between cloprostenol and hCG still further, to 168 hours, no animals had ovulated at laparotomy, but the polyspermy rate at fertilization was 33% (Lopata et al 1988). To minimise both ovulation prior to laparotomy and polyspermy in this study, hCG was administered 172 hours after cloprostenol. This regime meant that only three of 115 animals had ovulated at laparotomy, and the polyspermy rate was kept to a minimum (2%). Clearly, the time between cloprostenol administration and hCG injection is critical. Although increasing the time between cloprostenol administration and hCG yielded larger follicles and more mature oocytes, pre-ovulatory follicles were difficult to aspirate as the contents became very glutinous. This may be a physiological mechanism which helps the oocyte/cumulus complex adhere slightly to the ovary, so that it is not lost before "pick-up" by the fimbria (Dukelow and Vengesa 1986). Kuehl and Dukelow (1979) also described viscous follicular contents in pre-ovulatory squirrel monkey follicles and Lopata et al (1988) obtained lower oocyte recovery rates when aspirating the follicles of marmosets which had been treated with hCG 192 hours after cloprostenol administration. This may have been due to difficulty in aspiration.

Only small numbers of oocytes were available from each animal, so it would be useful to find ways to stimulate the marmoset ovary to produce more oocytes. The administration of pregnant mare serum gonadotrophin (PMSG) or clomiphene citrate followed by hCG stimulates the production of oocytes in primate species such as the cynomolgus macaque (*Macaca fascicularis*; Fujisaki et al 1989, Balmaceda et al 1984, 1988); the chacma baboon (*Papio ursinus*; Fourie et al 1987) and the human (Lopata et al 1978, Edwards et al 1980). Up to 25 oocytes per animal have been collected from the rhesus macaque (*Macaca mulatta*; Lanzendorf et al 1990) and up to 28 oocytes per animal from the pig-tailed macaque (*Macaca nemestrina*; Cranfield et al 1989) after stimulation with these exogenous gonadotrophins. However, human menopausal gonadotrophin (hMG) and hCG administration has no effect on ovulation rate in marmosets (Harlow 1984). It has been shown that the squirrel monkey develops refractoriness to PMSG and therefore becomes resistant to this regime of superovulation, so a combination of follicle stimulating hormone (FSH) and hCG have been used with successful results, and this regime can be repeated at least 16 times without the development of refractoriness (Dukelow and Vengesa 1986). Harlow (1984) demonstrated that marmosets do not develop antibodies to hCG and this was supported by the repeated successful use of hCG in this study. Selection of an appropriate hormone to stimulate follicular development, for instance FSH, and

optimization of its administration time and dose, may result in a reliable superovulation protocol for marmoset monkeys.

Marmoset epididymal sperm retained motility for up to 48 hours in media supplemented with marmoset serum but for only 20 hours in media supplemented with fetal calf serum. The reasons for the difference in survival are not clear but may be due to the presence of particular proteins or growth factors in marmoset serum which are not found in other species, or variations in the structure of these factors which make them compatible with marmoset sperm. Alternatively, there may be toxic factors present in the serum of other species to which marmoset sperm are particularly sensitive, and which compromises their survival. Moore (1981) found that marmoset epididymal sperm survive for only 16 hours in media supplemented with human serum albumin, and survival rates of electroejaculated marmoset sperm in media supplemented with bovine serum albumin were also poor (Harlow 1984).

Epididymal sperm were used in all but one insemination. There was no fertilization of four oocytes using electroejaculated sperm. This lack of fertilizability was reflected by the poor motility and longevity of electroejaculated sperm. We had difficulty in separating sperm from seminal fluid, which

through percoll gradients (unpublished data). Marmoset sperm seem to be particularly susceptible to damage during centrifugation, affecting viability and motility (Harlow 1984, Lopata et al 1988). Lopata et al (1988) found that marmoset sperm prepared by the swim-up method survived longer and had better motility rates than centrifuged sperm, but these authors do not report whether there was a difference in fertilization rate depending on whether electroejaculated or epididymal sperm was used for insemination. Zona-free hamster oocyte penetration tests assess the fertilizing capacity of mammalian spermatozoa *in vitro* (Yanagimachi et al 1976). Harlow (1984) reported that electroejaculated marmoset sperm, which were centrifuged repeatedly during preparation, only penetrated zona-free hamster oocytes on one of 18 (5%) occasions, but epididymal sperm penetrated up to 79% of hamster oocytes (Moore 1981). This would support the contention that marmoset sperm are less capable of *in vitro* fertilization after electroejaculation and centrifugation than epididymal preparation. There is no evidence that electroejaculation *per se* has a damaging effect on sperm. But it is possible that the sequence of events which sperm are exposed to in the course of normal ejaculation might be altered during electroejaculation. For example, sperm may mix with accessory gland secretions either in the wrong order or under the wrong physiological conditions due to inappropriate electrostimulation which may compromise their fertilizing capacity (pers. comm. W.V.Holt). It has been shown that centrifugation of human sperm can result in the release of high

levels of reactive oxygen species which damage sperm membranes and decrease levels of fertilization (Mortimer 1991). During centrifugation, marmoset sperm may also produce high levels of superoxide and hydroxyl radicals, which damage the sperm and impair their fertilizing capacity.

Marmoset embryos extrude the second polar body two to three hours after insemination, and form pronuclei about six hours after insemination. The timing of these events is very similar to that of human *in vitro* fertilized embryos (Lopata et al 1978), however early human pronuclei develop near the oolemma and migrate to the centre of the oocyte (Balakier 1992). I observed that when marmoset pronuclei first became visible they were not situated close to the oolemma, but were nearer to the centre of the oocyte. There is very little information about the pronuclear development of non-human primate species, but this observation indicates that the processes of pronuclear formation, swelling and migration may vary between primates. It would be important to find out whether these differences are reflected in the processes of DNA synthesis and replication in one-cell primate embryos.

Little has been published about the rate of cleavage of marmoset embryos *in vitro*, however Harlow (1984) presented quite extensive findings on the development of *in vivo* fertilized and cultured marmoset embryos which had been collected from the reproductive tract of marmosets at known time periods

after ovulation. Whilst care must be taken in directly correlating the rate of *in vivo* development with that of *in vitro* development, this information does provide some estimate of cleavage rates and some comparison can be made regarding the suitability of the *in vitro* culture system employed. Harlow (1984) found that marmoset embryos which had been fertilized and cultured *in vivo* cleaved once every 24 hours on average, to reach the four-cell stage on day 2 after ovulation, then underwent only one cleavage division to eight cells over the next 48 hours. Summers et al (1988) reported that marmoset blastocysts contain a mean of 120 cells. This equates to one cleavage division per 24 hours from the eight-cell stage on Day 4 to the blastocyst stage on Day 8. However, when fertilized and cultured *in vitro*, the rate of cleavage slowed down and marmoset embryos only reached the eight cell stage by Day 5, the 9-16 cell stage by Day 6.5, and the 16-32 cell stage by Day 8 after fertilization. Moreover, only one embryo in this system reached 32 cells. Undoubtedly, the culture system used did not provide the adequate nutrients or factors required for maximum pre-implantation development at normal rates of cleavage. However, *in vitro* fertilized marmoset embryos cultured in *in vivo* mouse oviducts developed to the blastocyst stage which suggests that the *in vitro* culture system used in these studies compromised viability. It is clear that a reliable and effective culture medium needs to be developed for this species.

Interspecific oviductal or uterine transfer of embryos has been used as an alternative to *in vitro* culture for many species, including mice (Briones and Beatty 1954, Beyer and Zeilmaker 1973), rats (Briones and Beatty 1954, Beyer and Zeilmaker 1973), rabbits (Briones and Beatty 1954, Brinster and Thomson TenBroeck 1969), sheep (Lawson et al 1972), and cattle (Sirard et al 1985). In most cases embryos were transferred to pseudopregnant or synchronised mature recipient females, however, in some cases, the relative development of embryos transferred to immature recipients was studied (Beyer and Zeilmaker 1973, Papaioannou and Ebert 1986). Beyer and Zeilmaker (1973) found that 70% (17/24) of mouse embryos and 44% (47/106) of rat embryos transferred to oviducts of prepuberal mice developed to morulae. Seventy percent (17/24) of mouse zygotes transferred to the oviducts of prepuberal rats also developed to morulae (Beyer and Zeilmaker 1973). These workers concluded that it was not necessary for the oviduct to be synchronised to achieve development to the morula stage. More mouse embryos which were transferred to pseudopregnant recipient mice developed to blastocyst, than mouse embryos which had been transferred to immature mice (84% cf. 69%; Papaioannou and Ebert 1986). However, the number of embryos reaching morula was not significantly different whether the recipient was immature or pseudopregnant. Although this shows that embryonic development is slightly slower in unsynchronised recipients, overall, transferred embryos developed better than those embryos cultured *in vitro* (96% cf. 76% to morula;

Papaioannou and Ebert 1986). Papaioannou and Ebert (1986) also showed that a much higher rate of normal fetal development was achieved, after transfer to synchronised recipients, from embryos which had been cultured in mouse oviducts *in vivo* than from those embryos which had been cultured *in vitro*. I have also shown that marmoset embryos develop further in mouse oviducts *in vivo* than in *in vitro* culture, but have yet to confirm whether culture conditions at pre-implantation stages affect the post-implantation development of marmoset embryos.

It is difficult to address the reasons why culture of embryos in the oviducts of immature females of another species should be superior to *in vitro* culture, without having carried out detailed analysis of the culture media and made comparisons to the oviductal fluid and epithelial components. However, it is possible to make some general assumptions. Firstly, technical/environmental factors must be taken into consideration. Presumably there is little fluctuation in temperature and pH in an *in vivo* system, eliminating some of the environmental variability to which embryos may be subjected in *in vitro* culture conditions. Secondly, and probably more importantly, the oviduct, even in immature females, may produce growth factors such as platelet-derived growth factor (PDGF) or activin (Gandolfi et al 1992), which the embryos require but which are not supplied by culture medium. It is also possible that the physical relationship between the embryo and the oviduct means that the embryo is

constantly being moved or rolled around and this movement may have a beneficial effect, possibly affecting the way in which nutrients are absorbed, rather than relying on passive diffusion which may be the case in *in vitro* conditions.

There are a number of ways in which the culture of marmoset embryos in the oviducts of live mice could be improved or modified. One of the main problems with the procedure is the risk of loss of the embryo. This could be avoided by tying off one or both ends of the oviduct with a suture after embryo transfer (Brinster and Thomson TenBroeck 1969). It would also be beneficial to determine whether there is a critical stage at which the embryo would benefit from *in vivo* culture. If this could be ascertained then it may alleviate the need for successive embryo transfers throughout the pre-implantation period.

Maximum fertilization and developmental rates were obtained from oocytes which had been cultured for 9-11 hours after collection (29-35 hours post-hCG injection). Chan et al (1982) observed that the highest rate of maturation of squirrel monkey oocytes occurred 35 hours post-hCG injection and carried out insemination at this time. These workers obtained fertilization rates of 90% but with limited development post-fertilization. Pre-incubation of marmoset oocytes for 21-29 hours before fertilization decreased the

developmental potential and increased the number of delayed cleavage embryos. This may be due to aging of the oocyte beyond optimum maturity. Webb et al (1986) reported that when mouse oocytes were aged *in vitro*, the meiotic spindle moved from the periphery to the centre of the oocyte and then degenerated causing a disruption in the organisation of the chromosomes. It has been suggested that this disruption could cause aneuploidy (Webb et al 1986), which may explain the delay and/or developmental arrest of marmoset embryos fertilized after 21-29 hours pre-incubation. It is also possible that, although the nuclear maturation of the oocyte increased with longer pre-incubation time (reflected by increased fertilization rate), the cytoplasm may have progressed past optimum maturity and this may be the cause of lower cell number and longer cleavage times in these embryos.

CHAPTER THREE

MANIPULATION AND FUSION OF ONE-CELL MOUSE AND MARMOSET EMBRYOS

3.1 INTRODUCTION

The fusion of cell membranes is important in normal physiological systems such as sperm and oocyte fusion (Moore and Bedford 1978), and can be used to manipulate systems for scientific investigation. Plant protoplasts can be fused to create somatic hybrid plants (Senda et al 1979), possibly with superior growth or production characteristics. B lymphocytes can be immortalised by fusion to myeloma cells for the production of monoclonal antibodies (Kohler and Milstein 1975). Blastomeres from 16-cell sheep embryos can be fused to enucleated oocytes in nuclear transplant experiments (Willadsen 1986). It is also possible to fuse a membrane-bound vesicle to a one-cell embryo (McGrath and Solter 1984) and it is the investigation of this technique which will be discussed in this chapter.

In order to create embryos containing only paternal chromosomes, it is necessary to manipulate the embryo at the one-cell stage and carry out pronuclear transfer. This involves removing the maternal pronucleus from an embryo and replacing it with a second paternal pronucleus from another embryo. The technique used for the removal of a pronucleus from an embryo requires that the pronucleus is contained within a small piece of oolemma. This membrane bound structure containing the pronucleus is termed a karyoplast. When the karyoplast is introduced beneath the zona pellucida of

the recipient embryo, it remains a separate structure unless some sort of fusion stimulus is provided. This fusion of the one-cell embryo (now containing only a paternal pronucleus) and the karyoplast is required to ensure that both pronuclei are enclosed within the same plasma membrane.

The three stimuli most commonly used for the fusion of cell membranes are Sendai virus, polyethylene glycol (PEG), and electrofusion. Because of the small numbers of marmoset embryos which were available for this study it was necessary to find the most reliable and effective method of fusion which would have the least effect on subsequent marmoset embryo viability.

Sendai virus is a paramyxovirus which causes murine pneumonitis and can fuse cells which have viral receptors on their surface (Okada 1993). This includes most mammalian cells and membranes. Inactivated Sendai virus is commonly used to fuse karyoplasts and embryos in pronuclear transfer experiments in mice (McGrath and Solter 1983, Surani et al 1984). However, its ability to fuse membranes in other species is questionable, for instance poor results have been achieved using Sendai virus in cattle embryos (Robl et al 1987). Sendai virus has a number of disadvantages as a fusion system. These are that the production of the virus can be difficult and time-consuming, the virus batches are often variable in their fusogenic activity, and the virus itself does not fuse some types of cells (Pontecorvo 1975). The supply of marmoset embryos is

limited and so it is imperative to use a fusion technique which is efficient and applicable to this species. For these reasons I have not investigated the use of Sendai virus in these studies, but have concentrated on the more defined use of either PEG or electrofusion as a fusogenic system.

PEG is commonly used for the fusion of B lymphocytes with myeloma cells and for the establishment of immortal cell lines for monoclonal antibody production (Kohler and Milstein 1975). The procedures which employ PEG as a fusogenic agent usually involve millions of cells and rates of fusion of up to 50% are acceptable. However, when used for the fusion of karyoplasts to one-cell embryos where numbers are very much smaller, fusion rates of over 80-90% are both desirable and necessary.

The most efficient method of cell fusion currently in use is electrofusion. An electric current is used to disrupt the lipid bilayers of the cell membranes which then form bridges and fuse together. When electrofusion is used for large numbers of cells, the electrofusion protocol generally requires that the cells are subjected to a short series of AC pulses to align the cells in pearl chains, before exposure to the DC (fusion) pulses. This study does not require exposure to AC pulses as the cells are already in contact because of the constraints of the zona pellucida. The cells are subjected to a series of DC pulses which leads to a charge separation in the membrane. Attraction of

opposite electrical charges exerts pressure across the membrane leading to its thinning. When the potential difference across the membrane reaches a critical point, pore formation occurs. Provided the strength of the pulses is not too high, after removal of the electric field the pores will close and the membrane will return to normal. If two cells are in contact during the formation of pores, it is highly likely that the pores from each cell membrane will heal together, leading to fusion. Cytoplasmic bridges will occur where pores have fused together, and one spherical cell will result (Electro Cell Manipulator ECM 200 Operation Manual, 1992).

Electrofusion has been used for various cell fusion and activation systems including the production of monoclonal antibodies, nuclear transfer and oocyte activation in cloning experiments, and for the electroporation of membranes for DNA insertion and transgenesis. Its main advantage is that the number and size of the electrical pulses applied to the membranes can be adjusted to suit the cell type and the species. Electrofusion is highly efficient (80%) between blastomeres in sheep (Willadsen 1986), and cattle (Robl et al 1987), and can be as high as 97% between karyoplasts and one-cell embryos in mice (Kono and Tsunoda 1988).

In the initial studies using PEG, described in this thesis, both zona pellucida-free and zona pellucida-intact 2-cell mouse embryos were used as a model to

determine whether the presence of the zona pellucida had any effect on cell fusion or embryo viability after fusion. Subsequent experiments involved puncturing a hole in the zona pellucida to simulate the status of the embryo after micromanipulation. The aim of this series of experiments was to determine the concentration of PEG and the exposure time which would provide optimum fusion of the two blastomeres within the embryo, without compromising embryo viability. These parameters could then be applied to one-cell embryos, after micromanipulation, to fuse the karyoplast to the embryo.

The feasibility of using electrofusion as a fusogenic agent was tested using mouse and marmoset embryos that had undergone sham pronuclear transfer. These experiments involved restoring embryos to normal status (i.e. one maternal and one paternal pronucleus). After manipulation the one-cell embryo and karyoplast were subjected to an electrical fusion stimulus. The rates of fusion, and the development of fused embryos were monitored. The aim was to determine the effect of the manipulation and fusion process on the viability of both mouse and marmoset embryos with both parental genomes before creating uniparental embryos.

3.2 MATERIALS AND METHODS

3.2.1 Housing of mice

Three to five week old C57/Bl x CBA F_1 mice were housed in 14 hour light:10 hour dark conditions and fed ad libitum on RM 1 (W.M. Lillico and Son, Wonham Mill Ltd., Betchworth, Surrey, U.K.) and had constant access to water.

3.2.2 Superovulation of mice

Three to four week old female C57/Bl x CBA F_1 mice were superovulated with an intraperitoneal injection of 5 iu pregnant mare serum gonadotrophin (PMSG; Folligon, Centaur, Castle Cary, Somerset, U.K.) followed by an intraperitoneal injection of 5 iu human chorionic gonadotrophin (hCG; Chorulon, Centaur, Castle Cary, U.K.) 48 hours later.

3.2.3 Mating of mice

Female mice which had undergone the superovulation treatment were housed overnight with C57/Bl x CBA F_1 males, and mating was confirmed by the presence of a vaginal plug the following morning (Day 1). Fertilization was assumed to have taken place in the middle of the dark period.

3.2.4 Mouse embryo recovery

Female F_1 mice were killed by cervical dislocation at 9 am on the day after observing the cervical plug (Day 2). The abdomen was opened and the oviducts were dissected from the uterus and ovarian bursa. The oviducts were then placed in a petri dish containing approximately 2 ml of Medium M2 (Quinn et al 1982; Appendix II). Embryos were flushed from the oviducts using a blunt ended 30 gauge needle attached to a 1 ml syringe. The syringe was filled with M2 and the needle was inserted into the infundibulum of the oviduct. Approximately 0.1 ml of M2 medium was flushed through the oviduct expelling the embryos into the dish of M2.

3.2.5 Removal of the zona pellucida from mouse embryos

Two-cell mouse embryos were transferred in groups of 3-4 to a petri dish containing approximately 2 ml of acid tyrodes (Hogan et al 1986 : Appendix III). Embryos were briefly incubated in acid tyrodes until the zona pellucida had completely disappeared. The embryos were transferred back into M2 medium and thoroughly washed.

3.2.6 Puncture of the zona pellucida of mouse embryos

Approximately 5 μl of M2 medium was placed in the centre of the cavity of a glass cavity slide, which had previously been coated in silicon, and was covered with light paraffin oil to prevent evaporation. Ten to twenty 2-cell embryos

were placed in the M2 medium and the whole slide was transferred to an inverted Olympus OMT-2 microscope fitted with Nomarski optics and micromanipulators. A hole was punctured in the zona pellucida of the 2-cell embryos with a pulled glass enucleation pipette of approximately 20 μm diameter. Care was taken to hold each embryo with its cleavage furrow parallel to the enucleation pipette to minimise lysis during manipulation (Fig 3.1).

3.2.7 Preparation of Polyethylene Glycol

Polyethylene glycol 1000 (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) was mixed with Medium M2 at various weight/volume concentrations in 10 ml plastic tubes. It was necessary to heat the mixture of PEG and M2 to dissolve the PEG. This was achieved by surrounding the falcon tubes with warm water (approximately 40-50 °C). Once in solution, the pH was adjusted with 1M NaOH to 7.2.

3.2.8 Exposure of two-cell mouse embryos to polyethylene glycol

Two-cell mouse embryos were exposed to various concentrations of PEG:M2 for times ranging from 10 to 300 seconds to determine the most appropriate concentration and exposure to fuse the blastomeres without lysis. The highest concentration of PEG was 50:50 w/v PEG:M2 medium. After exposure to the initial concentration of PEG, the embryos were passed sequentially through

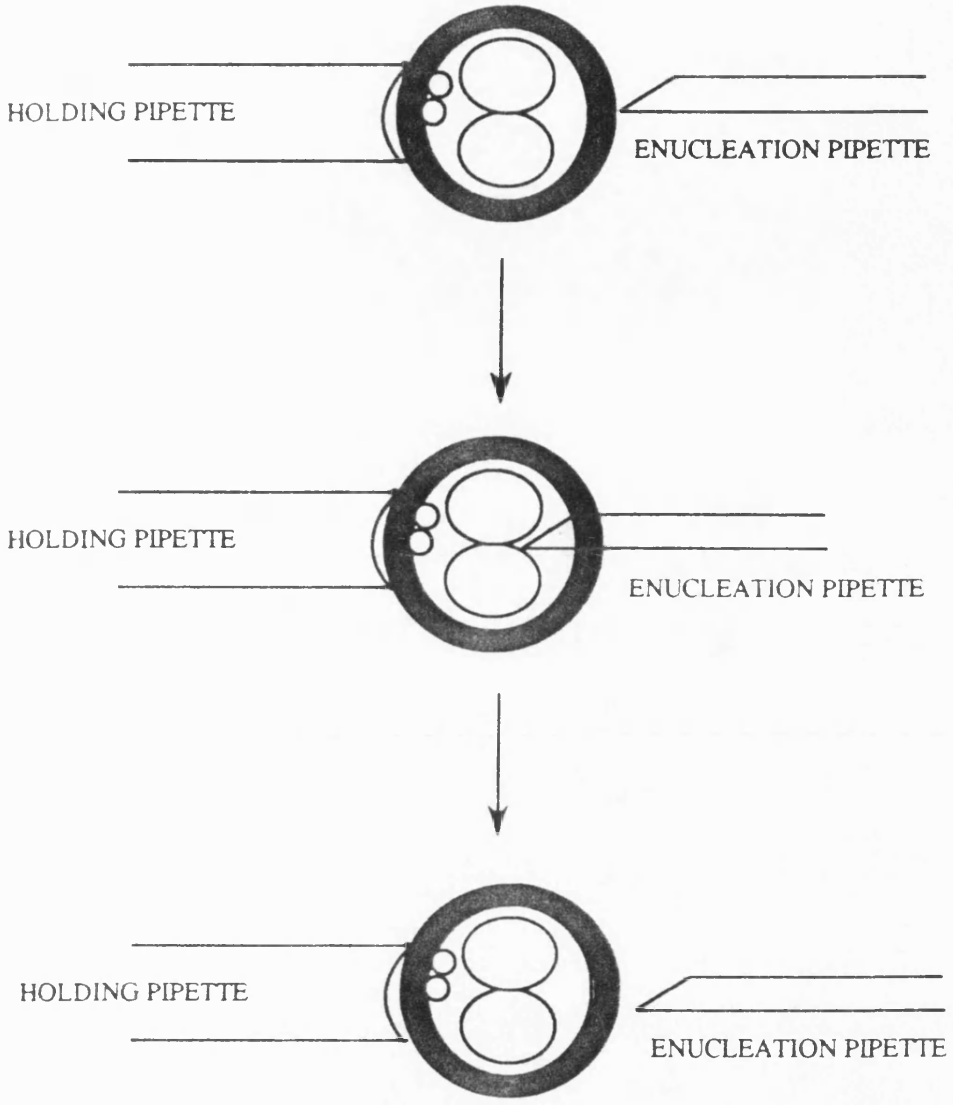

FIGURE 3.1 : Diagram showing the orientation of 2-cell mouse embryos during zona pellucida puncture. In this case, the enucleation pipette was only used to puncture the zona pellucida.

concentrations of 40%, 30%, 20%, and 10% w/v PEG to minimise detrimental effects of rapid changes in osmolarity. After treatment the embryos were washed thoroughly in M2 and cultured in drops of M16 (approx 50 μl) under paraffin oil in a humidified atmosphere of 5% CO_2 in air, at 37 °C.

3.2.9 Preparation of Phytohemagglutinin

Phytohemagglutinin (PHA; Sigma Chemical Co. Ltd., Poole Dorset, U.K.) was prepared as a 5 mg/ml solution in PBS. This was stored frozen at 20 °C in 40 μl aliquots. The stock solution of phytohemagglutinin was diluted with M2 to a working concentration of 400 μg/ml. Embryos were exposed to this concentration of phytohemagglutinin for 30 seconds before exposure to PEG.

3.2.10 Micromanipulation of mouse embryos

A 10 μl drop of M2 medium supplemented with 5 μg/ml cytochalasin D (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) and 0.1 μg/ml colcemid (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) was placed in the well of a siliconised cavity slide and covered with a layer of paraffin oil to prevent evaporation. One-cell mouse embryos were transferred to the medium on the slide which was placed on the heated stage of an Olympus IMT-2 inverted microscope (Olympus Optical Co. (U.K.) Ltd., London, U.K.) fitted with micromanipulators (Zeiss and Leitz), and Nomarski optics (Olympus Optical Co. (U.K.) Ltd., London, U.K.) which allow pronuclei to be viewed clearly.

Micromanipulation pipettes were prepared from 1.0 or 1.5 mm diameter glass capillary tubes (Clark Electromedical Instruments Ltd., Pangbourne, Reading, UK). Glass capillaries were pulled using a Kopf vertical pipette puller (Model 720, David Kopf Instruments, Tujunga, California, USA). Pipettes were broken at the appropriate diameter, heat-softened and bent to a 30° angle to the horizontal using a De Fonbrune microforge (Vacuum Instruments and Products Ltd., Middlesex, UK). Enucleation pipettes were bevelled to make a pointed end on the pipette using a grinder (Research Instruments Ltd., Cornwall, UK). Final diameters of the two pipettes were as follows:

	Internal diameter	External diameter
Holding pipette:	17 μm	85 μm
Enucleation pipette :	15 μm	19 μm

The embryos were placed in the micromanipulation drop and manipulated one at a time before being washed thoroughly and returned to culture media. Manipulation involved holding the embryo stationary by gentle suction through the holding pipette and raising it slightly from the bottom of the glass slide. The enucleation pipette was used to puncture the zona pellucida, but not the oolemma. One pronucleus was gently aspirated into the enucleation pipette without rupturing the oolemma. The enucleation pipette, still

containing the pronucleus surrounded by the oolemma, was removed from the embryo (Fig. 3.2). When the pipette was withdrawn, the oolemma pinched off, leaving the karyoplast within the enucleation pipette and separate from the embryo. In these control experiments, the karyoplast was returned to the embryo from which it had just been removed and placed under the zona pellucida. The karyoplast remained separate from the embryo but was held adjacent to it by the confines of the zona pellucida. The embryo was washed and returned to culture media for at least one hour before fusion stimulus was applied.

3.2.11 Collection and fertilization of marmoset oocytes

Collection and fertilization of marmoset oocytes was carried out as described in Sections 2.2.4 to 2.2.8.

3.2.12 Micromanipulation of marmoset embryos

Marmoset embryos were manipulated in the same way as mouse embryos (Section 3.2.10; Fig. 3.3) except that the manipulation pipettes were larger bore. When used for marmoset embryos, the pipettes were the following sizes:

	Internal diameter	External diameter
Holding pipette:	40 μm	100 μm
Enucleation pipette:	25 μm	30 μm

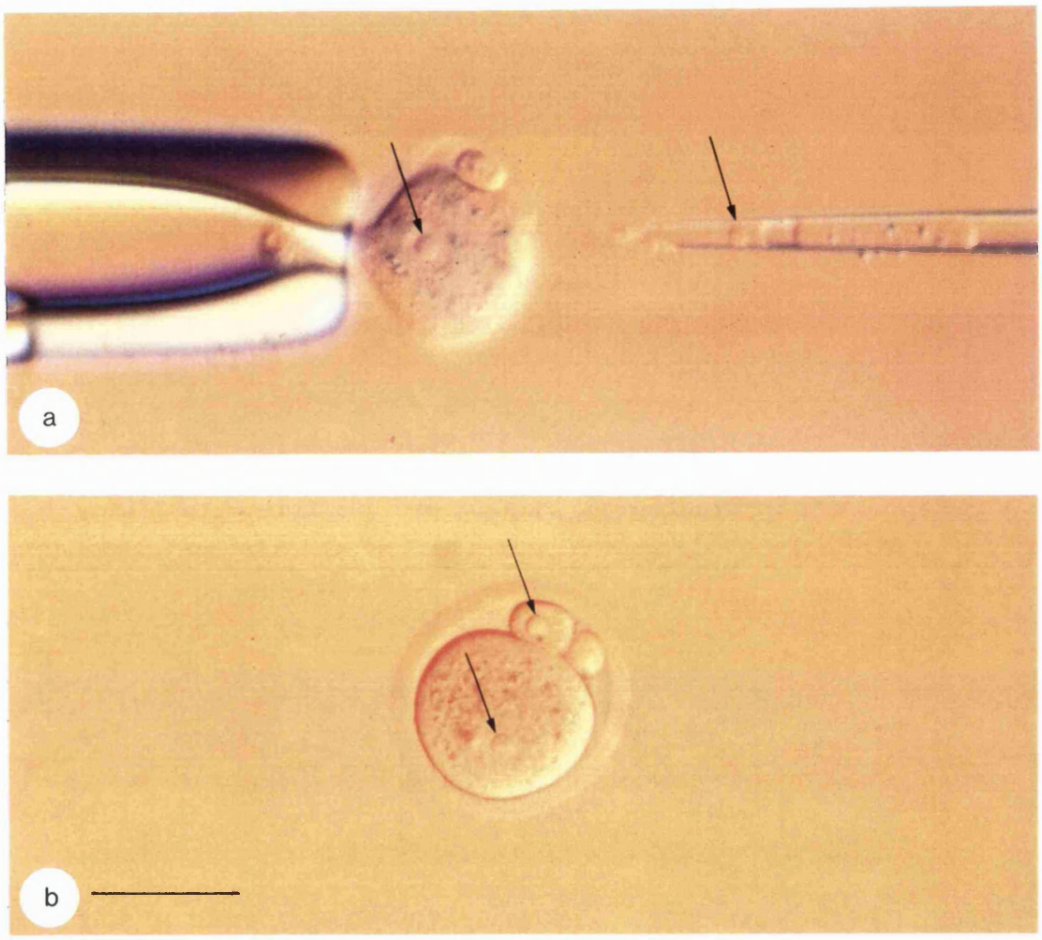

FIGURE 3.2 : Sham enucleation and pronuclear transfer of a one-cell mouse embryo. Pronuclei are shown by arrows. (a) One pronucleus is removed from the embryo. (b) The pronuclear karyoplast is replaced within the zona pellucida but has not yet been exposed to fusogenic stimulus. Bar = 50 μm.

3.2.13 Electrofusion of embryos

The electrofusion system incorporates a pulse generator (BTX Electro Cell Manipulator 200, Skatron Instruments Ltd., Newmarket, UK) attached to an electrofusion chamber (Part No. 450, Skatron Instruments Ltd., Newmarket, UK). The electrofusion chamber consists of two parallel, stainless steel, 0.5 mm diameter electrodes attached to a glass slide, 0.5 mm apart. The chamber was filled with electrofusion medium (Appendix IV). The embryos were washed in electrofusion medium, placed in the chamber, and subjected to one pulse of either 2 kV/cm (mouse embryos) or 1.5 kV/cm (marmoset embryos) for 70 μsec. The embryos were washed with M2 and returned to culture media. Fusion had usually occurred within 30 minutes of treatment (Fig. 3.4). If fusion had not occurred, the electrofusion protocol was repeated up to three times.

3.2.14 Transfer of mouse embryos to recipient female mice

Mouse embryo transfer was carried out as described in Section 2.2.11 (i).

3.2.15 Statistical analyses

Analysis of the difference between the proportions of embryos which fused, lysed or failed to cleave was carried out using a proportional *t*-test (CSS: Statistica; Statsoft UK, Letchworth, U.K.).

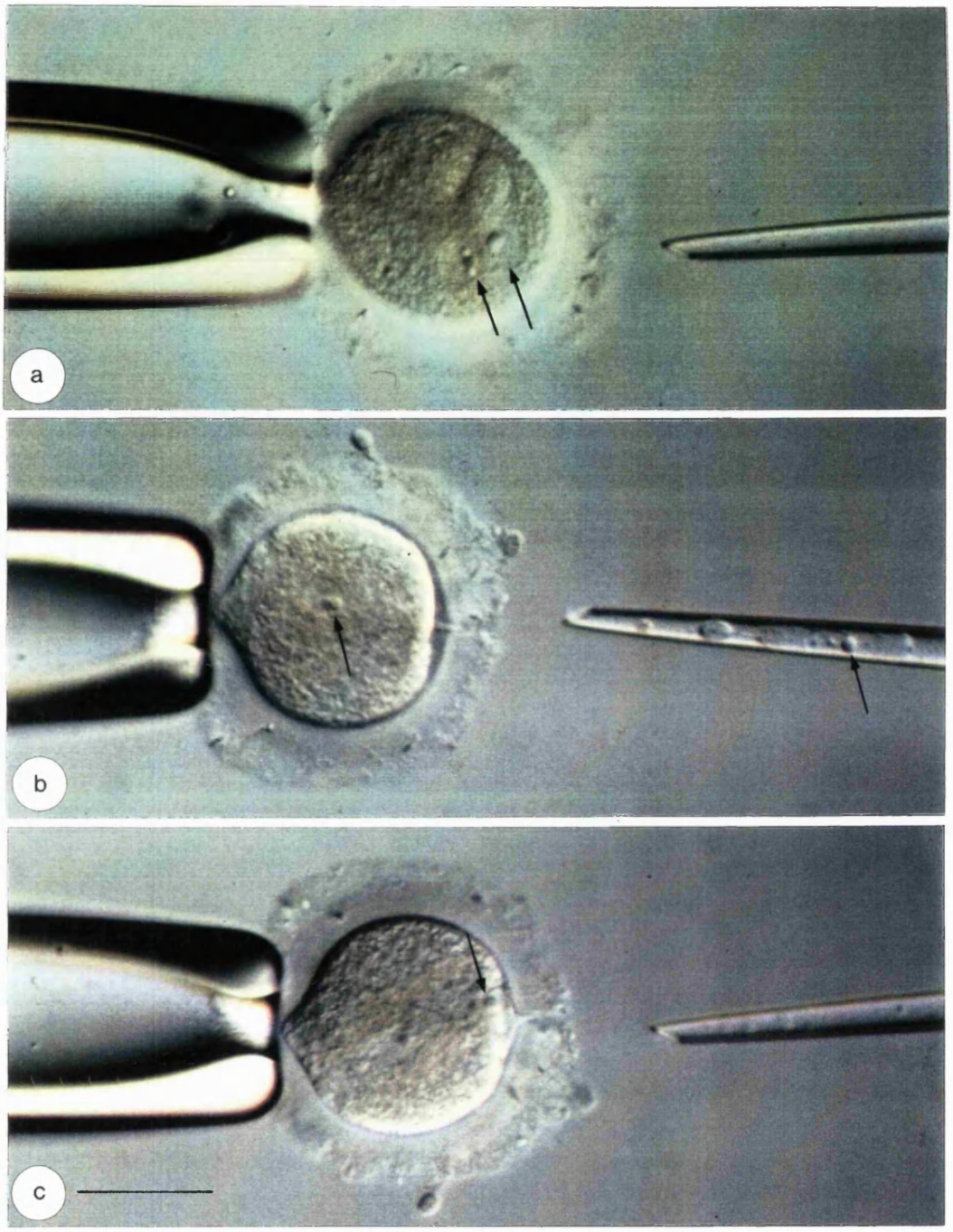

FIGURE 3.3 : Sham enucleation and pronuclear transfer of a one-cell marmoset embryo. Pronuclei are shown by arrows. (a) Before enucleation. (b) One pronucleus is removed from the embryo and is membrane enclosed in the enucleation pipette. (c) The pronuclear karyoplast is replaced within the zona pellucida but has not yet been exposed to any fusogenic stimulus (the pronucleus within the embryo is no longer in focus). Bar = 50 μm.

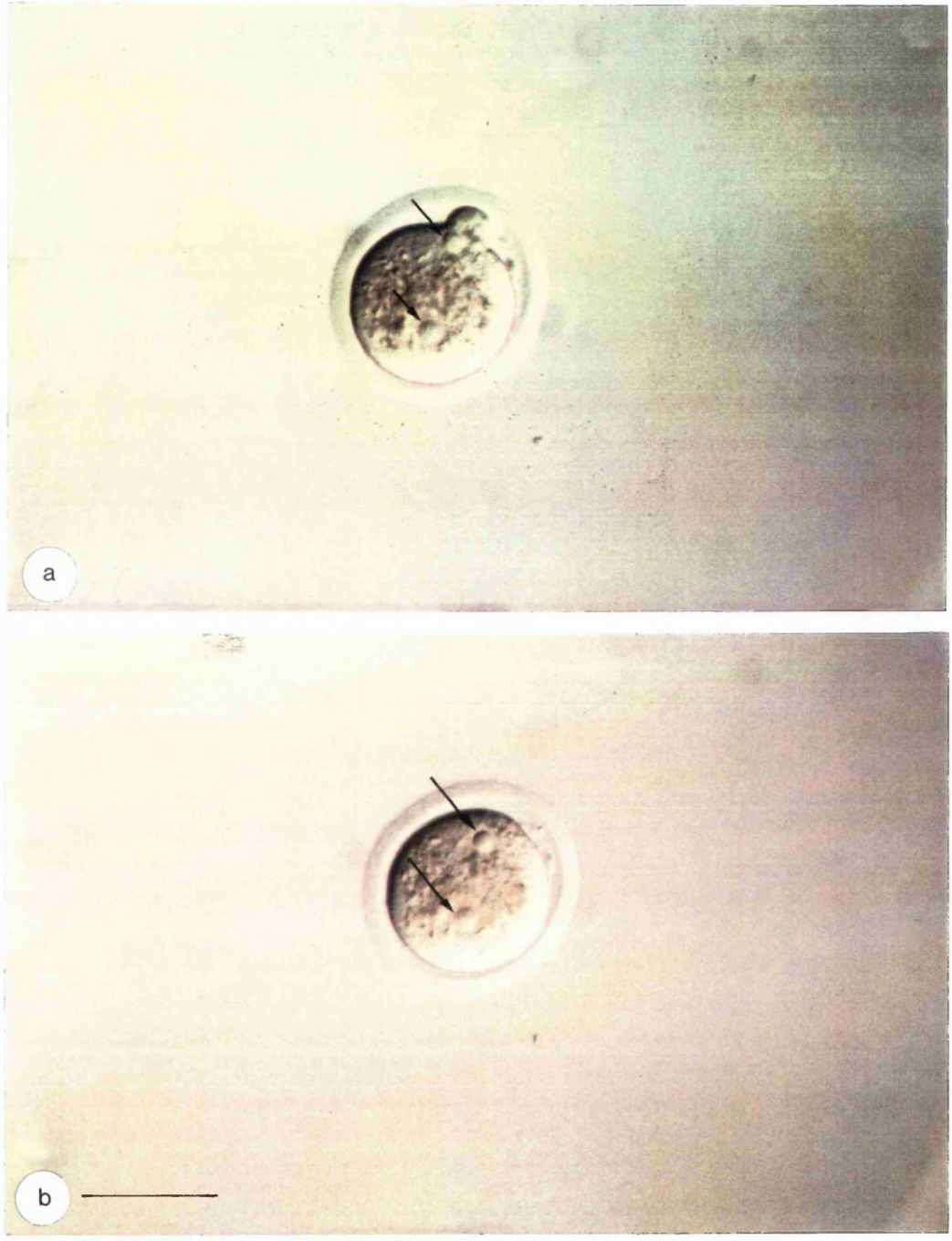

FIGURE 3.4 : Electrofusion of a one-cell mouse embryo and pronuclear karyoplast shown in Fig. 3.2. Pronuclei are shown by arrows. (a) The membranes have fused and the pronucleus is migrating into the embryo proper. (b) The same embryo after the completion of fusion. Bar = 50 μm.

3.3 RESULTS

3.3.1 Treatment of zona pellucida-free 2-cell mouse embryos with PEG

Zona pellucida-free 2-cell mouse embryos were exposed to 25% and 50% w/v PEG for 90 seconds. There was no fusion of blastomeres within embryos. There was no cleavage of any embryos exposed to PEG. All embryos exposed to 50% w/v PEG lysed within one hour of treatment. Sixty-three percent (15/24) of zona-free 2-cell mouse embryos not exposed to PEG (controls) developed to the blastocyst stage after five days (data not shown).

3.3.2 Treatment of zona-intact 2-cell mouse embryos with PEG

Zona-intact 2-cell mouse embryos were exposed to 50% w/v PEG for 30, 90, 180 or 300 seconds (Table 3.1). There was no significant difference between the fusion rates of embryos exposed to PEG for up to 300 seconds. However, rates of lysis were significantly higher in zona-intact 2-cell mouse embryos exposed to PEG for 180 and 300 seconds ($p<0.01$) than in embryos exposed to PEG for shorter time periods. The viability of embryos did not appear to be compromised by exposure to 50% PEG for up to 90 seconds, with 8/9 embryos reaching the blastocyst stage (Table 3.1).

TABLE 3.1 : Development of zona-intact 2-cell mouse embryos exposed to 50% w/v PEG for 30, 90, 180 or 300 seconds

Number ofembryos treated	Time of exposure (seconds)	Embryos lysed (%)	Embryos fused (%)	Development of fused embryos	Development of intact unfused embryos
10	0	0[a]	n.d.	n.a.	1 x 4-cell 1 x morula 8 x blastocyst
9	30	3 (33)[a]	0	n.a.	1 x 5-cell 1 x 12-cell 1 x morula 3 x blastocyst
9	90	0[a]	3 (33)	3 x blastocyst	1 x 4-cell 5 x blastocyst
10	180	10 (100)[b]	0	n.a.	n.a.
9	300	9 (100)[b]	0	n.a.	n.a.

n.d. - not done ; n.a. - not applicable
a: significantly lower than b ($p<0.01$)

3.3.3 Fusion and development of 2-cell mouse embryos with a breached zona pellucida exposed to PEG

Two-cell embryos with a hole punctured in the zona pellucida were exposed to 50% w/v PEG for 10, 30, or 60 seconds (Table 3.2). There was no significant difference between the fusion or lysis rates observed after exposure to PEG for up to 30 seconds, however the rate of lysis was significantly higher ($p<0.01$) when embryos were exposed to PEG for 60 seconds. Embryo viability was affected by longer exposure to PEG. The number of embryos which either lysed or failed to cleave after treatment rose from 0/18 in control embryos to 3/10 when embryos were exposed to PEG for 10 seconds ($p<0.05$). This trend was also evident when exposure times were increased to 30 and 60 seconds with combined rates of lysis and cleavage failure of 85% (17/20; $p<0.001$) and 100% (10/10; $p<0.001$), respectively. Only one of the treated embryos reached the morula stage. None of the other 39 embryos exposed to 50% PEG developed beyond the 8-cell stage. This contrasts with the development of 78% (14/18) of control embryos which reached the blastocyst stage.

Two-cell embryos with a hole punctured in the zona pellucida were exposed to 40% w/v PEG for 45, 75, or 120 seconds (Table 3.3). Although 30% of embryos exposed to PEG for 120 seconds fused, this was not significantly different from embryos treated with 40% PEG for 45 or 75 seconds, or from

TABLE 3.2 : Development of 2-cell mouse embryos with a hole punctured in the zona pellucida exposed to 50% w/v PEG for 10, 30 or 60 seconds

Number of embryos treated	Time of exposure (seconds)	Embryos lysed (%)	Embryos fused (%)	Development of fused embryos	Development of intact unfused embryos	Total number of embryos lysed or failed to cleave (%)
18	0	0[a]	n.d.	n.a.	3 x 4-cell 1 x 8-cell 14 x blastocyst	0[c]
10	10	2 (20)[a]	3 (30)	2 x 2-cell 1 x morula	1 x 2-cell (cf) 3 x 4-cell 1 x 8-cell	3 (30)[d]
20	30	9 (45)[a]	5 (25)	5 x 1-cell (cf)	3 x 2-cell (cf) 2 x 4-cell 1 x 8-cell	17 (85)[e]
10	60	10 (100)[b]	0	n.a.	n.a.	10 (100)[e]

n.d. - not done ; n.a. - not applicable ; cf - cleavage failure

b: significantly higher than a ($p<0.01$) ; d: significantly higher than c ($p<0.05$) ; e: significantly higher than d ($p<0.001$)

TABLE 3.3 : Development of 2-cell mouse embryos with a hole punctured in the zona pellucida exposed to 40% w/v PEG for 45, 75 or 120 seconds

Number of embryos treated	Time of exposure (seconds)	Embryos lysed (%)	Embryos fused (%)	Development of fused embryos	Development of intact unfused embryos	Total number of embryos lysed or failed to cleave (%)
10	0	0	n.d.	n.a.	3 x 4-cell 7 x blastocyst	0
8	45	0	0	n.a.	7 x 4-cell 1 x 8-cell	0
8	75	0	0	n.a.	1 x 2-cell (cf) 5 x 4-cell 1 x 8-cell 1 x morula	1 (13)
10	120	1 (10)	3 (30)	3 x 2-cell	2 x 4-cell 3 x morula 1 x blastocyst	1 (10)

n.d. - not done ; n.a. - not applicable ; cf - cleavage failure

control embryos. The number of those embryos which either did not cleave or lysed after treatment did not significantly increase from control embryos at this concentration of PEG. However, the development of fused embryos was poor, each embryo only undergoing one cleavage division. It is also worth noting that although 7/10 control embryos reached the blastocyst stage, only one of 26 (4%) intact but unfused embryos exposed to PEG formed a blastocyst.

3.3.4 Fusion and development of 2-cell mouse embryos with a breached zona pellucida exposed to phytohemagglutinin and PEG

Two-cell mouse embryos with a breached zona pellucida were exposed to 40% PEG for 120 seconds after 30 seconds phytohemagglutinin exposure (Table 3.4). Control embryos were also exposed to PHA but not to PEG. Twenty percent (2/10) of embryos fused and one embryo lysed. Both embryos which fused developed to the blastocyst stage. Overall 6/10 treated embryos developed to blastocyst which was not significantly different from the development of 9/10 control embryos to the blastocyst stage. Two-cell mouse embryos with a breached zona-pellucida were exposed to 45% PEG for 60, 90 or 120 seconds (Table 3.5). The lowest fusion rate was of those embryos exposed to PEG for 60 seconds (6%). There was a significant increase in fusion as exposure times were increased ($p<0.001$) with a maximum fusion rate of 70% in those embryos exposed to PEG for 120 seconds. However, lysis

TABLE 3.4 : Development of 2-cell mouse embryos with a hole punctured in the zona pellucida exposed to 400 μg/ml phytohemagglutinin for 30 seconds followed by exposure to 40% w/v PEG for 120 seconds

Number of embryos treated	Time of exposure (seconds)	Embryos lysed (%)	Embryos fused (%)	Development of fused embryos	Development of intact unfused embryos
10	0	0	n.d.	n.a.	1 x 8-cell 9 x blastocyst
10	120	1 (10)	2 (20)	2 x blastocyst	1 x 4-cell 1 x 8-cell 1 x morula 4 x blastocyst

n.d. - not done ; n.a. - not applicable

TABLE 3.5 : Development of 2-cell mouse embryos with a hole punctured in the zona pellucida exposed to 400 μg/ml phytohemagglutinin for 30 seconds followed by exposure to 45% w/v PEG for 60, 90 or 120 seconds

Number of embryos treated	Time of exposure (seconds)	Embryos lysed (%)	Embryos fused (%)	Development of fused embryos	Development of intact unfused embryos	Total number of embryos lysed or failed to cleave (%)
40	0	0[a]	n.d.	n.a.	2 x 2-cell (cf) 5 x 4-cell 2 x morula 31 x blastocyst	2 (5)[g]
47	60	0[a]	3 (6)[d]	1 x 2-cell 2 x blastocyst	3 x 2-cell (cf) 14 x 4-cell 7 x 8-cell 4 x morula 16 x blastocyst	3 (6)[g]
55	90	23 (42)[b]	18 (33)[e]	13 x 1-cell (cf) 2 x 8-cell 3 x blastocyst	3 x 2-cell (cf) 5 x 4-cell 4 x 8-cell 1 x 12-cell 1 x blastocyst	39 (71)[h]
77	120	11 (14)[c]	54 (70)[f]	43 x 1-cell (cf) 5 x 2-cell 2 x 4-cell 4 x blastocyst	9 x 2-cell (cf) 2 x 4-cell 1 x 8-cell	63 (82)[h]

n.d. - not done; n.a. - not applicable; c.f. - cleavage failure

All numbers in the same column with different superscripts are significantly different ($p<0.001$).

rates were also significantly increased to 42% when exposure time was raised to 60 seconds ($p<0.001$). Surprisingly, lysis decreased significantly when exposure to PEG was increased to 120 seconds ($p<0.001$). Only 6% (3/47) of embryos exposed to PHA followed by PEG for 60 seconds failed to cleave after treatment. A total of 18 (38%) embryos exposed to PEG for 60 seconds, including two embryos which had undergone fusion, developed to the blastocyst stage. This was significantly less than the number of control embryos reaching the blastocyst stage (78%; $p<0.001$). When exposure times were increased to 90 and 120 seconds only 7% and 5% of embryos, respectively, reached the blastocyst stage ($p<0.001$). Correspondingly, the number of embryos which either failed to cleave or lysed after treatment increased from 3/47 (6%) to 39/55 (71%) when exposure times were increased from 60 to 90 seconds ($p<0.001$) and rose to 82% (63/77) when the exposure time was increased to 120 seconds.

A similar pattern was observed when two-cell mouse embryos, with a punctured zona pellucida, were exposed to 50% PEG for 30, 60 or 90 seconds following PHA exposure (Table 3.6). Increasing the exposure time from 30 to 60 seconds and then further, to 90 seconds, had no significant effect on fusion rate. There was no significant difference in the number of lysed embryos when exposure time was increased from 30 to 60 seconds, however there was a

TABLE 3.6 : Development of 2-cell mouse embryos with a hole punctured in the zona pellucida exposed to 400 μg/ml phytohemagglutinin for 30 seconds followed by exposure to 50% w/v PEG for 30, 60, or 90 seconds

Number of embryos treated	Time of exposure (seconds)	Embryos lysed (%)	Embryos fused (%)	Development of fused embryos	Development of intact unfused embryos	Total number of embryos lysed or failed to cleave (%)
40	0	0	n.d.	n.a.	2 x 2-cell (cf) 5 x 4-cell 2 x morula 31 x blastocyst	2 (5)[c]
29	30	9 (31)[a]	5 (17)	2 x 1-cell (cf) 1 x 2-cell 1 x 4-cell 1 x 8-cell	2 x 2-cell (cf) 9 x 4-cell 4 x 8-cell	13 (45)[d]
19	60	4 (21)[a]	6 (32)	6 x 1-cell (cf)	6 x 2-cell (cf) 3 x 4-cell	16 (84)[e]
19	90	12 (63)[b]	6 (32)	6 x 1-cell (cf)	1 x 2-cell (cf)	19 (100)[e]

n.d. - not done; n.a. - not applicable; cf - cleavage failure

b: significantly higher than a ($p<0.05$) ; d: significantly higher than c ($p<0.001$) ; e: significantly higher than d ($p<0.001$)

significant increase in the number of embryos which did not cleave after treatment, as reflected by the increase from 45% to 84% ($p<0.01$) in the total number of embryos which lysed or failed to cleave. A further increase in time of exposure from 60 to 90 seconds caused 100% of embryos to lyse or fail to undergo cleavage. Only 16/29 (55%) of embryos exposed to PEG for 30 seconds cleaved and the maximum development was to eight cells. Just three embryos exposed to PEG for 60 seconds cleaved and reached four cells.

3.3.5 Micromanipulation and electrofusion of one-cell mouse embryos

A total of 75 mouse embryos were manipulated in 9 experiments (Table 3.7). A pronuclear karyoplast was removed, replaced beneath the zona pellucida and electrofusion was used to restore the mouse embryo to its biparental status. Sixty-nine (92%) embryos were successfully manipulated. Of these, 52 (75%) fused after electrical stimulus was applied. One embryo (2%) lysed during electrofusion. The overall efficiency of the system, when combining the results from manipulation and electrofusion was 69% (52/75; Table 3.7).

TABLE 3.7 : Development of one-cell mouse embryos after removal, replacement and fusion of a pronuclear karyoplast.

Experiment Number	Number of embryos manipulated	Number of embryos successfully manipulated[a]	Fusion of Karyoplast[b]	Development of fused embryos
1	5	4	3	1 x 1-cell (cf), 2 x morula
2	10	10	4	1 x morula, 3 x blastocyst
3	6	6	3	1 x 8-cell, 2 x blastocyst
4	14	12	12	7 x 2-cell, 2 x 4-cell, 3 x morula
5	4	3	2	TRANSFERRED (see Table 3.8)
6	6	6	3	TRANSFERRED (see Table 3.8)
7	4	4	4	4 x blastocyst
8	13	12	11	4 x 2-cell, 3 x 4-cell 1 x 8-cell, 3 x morula
9	13	12	10	2 x 1-cell (cf), 2 x 2-cell, 2 x 8-cell, 2 x morula, 2 x blastocyst
TOTAL	75	69 (92)	52 (75)	

[a] : (% of embryos manipulated)
[b] : (% of embryos successfully manipulated)
f: cleavage failure

3.3.6 Development of manipulated and electrically fused mouse embryos

3.3.6 (i) Development *in vitro*

Forty-seven manipulated and fused embryos were cultured *in vitro*. Ninety-four percent (44/47) underwent at least one cleavage division. Thirty-one embryos (66%) reached the four cell stage, and 26 (55%) continued to cleave to eight cells. Forty-seven percent (22/47) of embryos formed compacted morulae and eleven embryos (23%) reached the blastocyst stage after five days (Table 3.7).

3.3.6 (ii) Development *in vivo*

Five manipulated and fused mouse embryos were transferred, at the two-cell stage, to two recipient female mice (Table 3.8). All five embryos (100%) had implanted, and three (60%) had formed fetuses on Day 15 of gestation. A total of 10 control embryos (non-manipulated two-cell embryos) were transferred to the contralateral oviducts. All control embryos implanted and six (60%) formed fetuses (Table 3.8). Clearly, there was no difference between the *in vivo* development of manipulated and non-manipulated mouse embryos.

TABLE 3.8 : Development of mouse embryos after sham pronuclear transfer and electrofusion (I) or control embryos (II) and transfer to pseudopregnant recipient mice

I. Sham pronuclear transfer and electrofusion

Experiment Number	Number transferred	Number transferred to pseudopregnant recipients	Implantations[a]		Fetuses	
			No.	%	No.	%
1	2	2	2	100	2	100
2	3	3	3	100	1	33
TOTAL	5	5	5	100	3	60

II. Controls

Experiment Number	Number transferred	Number transferred to pseudopregnant recipients	Implantations[a]		Fetuses	
			No.	%	No.	%
1	4	4	4	100	3	75
2	6	6	6	100	3	50
TOTAL	10	10	10	100	6	60

[a]: Includes embryos which were resorbed and embryos which developed into fetuses

TABLE 3.9 : Development of one-cell marmoset embryos after removal, replacement and fusion of a pronuclear karyoplast.

Experiment Number	Number of embryos manipulated	Number of embryos successfully manipulated[a]	Fusion of Karyoplast[b]	Development of fused embryos
1	6	0	-	-
2	5	4 (80)	3 (75)	1 x 2-cell 1 x 4-cell 1 x 8-cell
3	3	2 (33)	1 (50)	1 x 2-cell
4	1	1 (100)	0 (0)	-
5	3	2 (33)	1 (50)	1 x 2-cell
6	3	2 (33)	2 (100)	1 x 4-cell 1 x 1-cell
TOTAL	21	11 (52)	7 (64)	

[a] : (% of embryos manipulated); [b] : (% of embryos successfully manipulated)

3.3.7 Micromanipulation and electrofusion of one-cell marmoset embryos

A pronuclear karyoplast was removed, replaced beneath the zona pellucida and electrofusion was used to restore the marmoset embryo to its biparental status. Of the 21 embryos which were manipulated, eleven (52%) had a pronucleus removed and successfully replaced beneath the zona pellucida (Table 3.9). However, in the first experiment, which involved six embryos, all six lysed during the manipulation process. This was due to the use of manipulation pipettes which were too small in diameter. In subsequent experiments, when the diameter of the enucleation pipette had been increased to 25 μm, 11/15 (73%) of marmoset embryos were successfully manipulated. Eleven embryos were subjected to the fusion process. Seven (64%) fused and were restored to biparental status (Table 3.9). Three marmoset embryos fused after a single electrical pulse and four embryos required two or more electrical pulses to facilitate fusion. Of the five embryos which did not fuse, two karyoplasts lysed after the fusion pulse was applied. The remainder of the unsuccessfully fused embryos remained intact. The overall success rate of this system, combining the results from the manipulation and fusion was 30% (7/21) or, disregarding the first experiment for the reasons outlined above, the overall success rate was 46% (7/15).

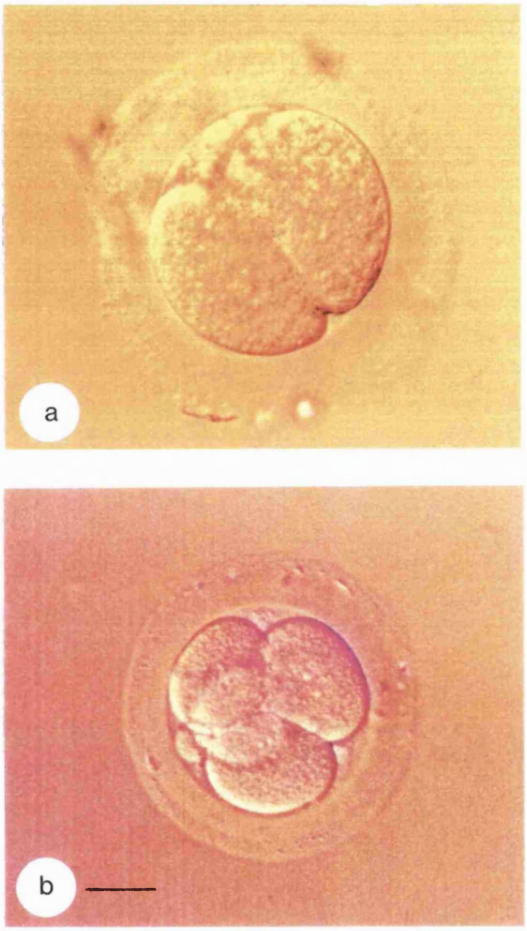

FIGURE 3.5 : (a) 2-cell and (b) 4-cell marmoset embryo after sham enucleation and pronuclear transfer, and electrofusion. Bar = 25 μm.

3.3.8 Development of marmoset embryos after manipulation and electrofusion

Only one of the seven successfully electrically fused marmoset embryos failed to cleave after treatment (Table 3.9). Six embryos reached the 2-cell stage, three embryos continued to cleave to four cells, and one embryo reached 8 cells (Fig. 3.5). The average development (± SEM) was to 3.3 (± 2.3) cells, which is significantly less than the development of in vitro fertilized, non-manipulated, marmoset embryos which developed to 7.7 (± 0.7) cells ($p<0.04$; Section 2.3.6).

3.4 DISCUSSION

These studies show that blastomeres from two-cell mouse embryos can be fused using PEG, and that karyoplasts and one-cell embryos from mice and marmosets can be fused using electrical stimulation.

The successful fusion of cell membranes using PEG requires a sequence of events to occur. Firstly, juxtaposition of the two cell membranes is necessary. Two-cell mouse embryos used in this study were physically constrained by the zona pellucida and the membranes agglutinated by exposure to phytohemagglutinin (PHA; Mintz et al 1973). The next events in the fusion sequence are modifications to the structure and organisation of the lipids and/or the proteins in the membrane to allow fusion to occur. PEG appears

to increase the permeability of the membranes to Ca^{2+} ions, decrease the amount of free water in solution, and change the surface potential of the membranes (reviewed by Lucy 1980). All of these changes in the membrane allow fusion to occur. The third event in the process of cell fusion is stabilisation of the membranes after fusion (Lucy 1982).

Polyethylene glycol has mainly been used for the fusion of somatic cells and maximum rates of fusion of 40-50% have been achieved using a 60 second exposure to 50% w/v ratio of PEG/water. PEG is not commonly used for the fusion of mammalian blastomeres, although the protocol described above has been used to fuse porcine and mouse zona-free oocytes (Fulka et al 1986). Eglitis (1980) used PEG to fuse 4-cell mouse blastomeres to create tetraploid mouse embryos. The highest rates of fusion (57%) were obtained after exposure of zona pellucida-free 4-cell mouse blastomeres to a 45% w/v solution of PEG for 120 seconds, after PHA exposure. In this study, the highest rate of fusion using polyethylene glycol was 70%, achieved by exposing 2-cell mouse embryos, with a hole punctured in the zona pellucida, to the same treatment. Eglitis (1980) found that ninety percent of fused blastomeres cleaved at least once and 30-40% of tetraploid embryos cavitated, whereas I found that this particular treatment led to significant decreases in the extent of cleavage and the number of embryos reaching the blastocyst stage compared to control embryos. The cavitation of these embryos should not be affected by

decreased cell number because compaction and blastocyst formation are temporal events and are not related to cell number. Mouse embryos go through a transition from control by the maternal genome to the embryonic genome at the two-cell stage (Flach et al 1982). Therefore, two-cell mouse embryos may be more susceptible to the toxic effects of PEG than four-cell mouse embryos. The influx of Ca^{2+} may also affect the cell cycle control at the two-cell stage. Eglitis (1980) concluded that the reason for decreased cavitation efficiency in fused embryos compared to control embryos was due to the toxic effects of PEG exposure.

To achieve high rates of fusion it was necessary to increase exposure time and concentration of PEG. The recurring trend observed in these experiments was that increased exposure to PEG led to a concomitant increase in both lysis rate and the number of embryos which did not cleave after treatment. The exposure times and concentrations of PEG which sustained embryo viability were unable to induce fusion.

The toxicity of PEG can be partially alleviated by the pre-treatment of embryos with phytohemagglutinin (Eglitis 1980). PHA also has agglutination characteristics which help in the fusion process (Mintz et al 1973). Its protective qualities are demonstrated by comparing embryos which were exposed to the same treatment regime with or without PHA. For example,

100% of two-cell mouse embryos, with a punctured zona pellucida, exposed to 50% w/v PEG for 60 seconds, without PHA exposure, lysed after treatment (Table 3.2). However, when two-cell mouse embryos, with a punctured zona pellucida, were pre-treated with PHA the lysis rate dropped to 21% (Table 3.6). In spite of the decrease in lysis rate, subsequent development of these embryos was poor and only three of nineteen embryos cleaved (Table 3.6). A similar comparison can be made between two-cell mouse embryos, with a hole punctured in the zona pellucida, exposed to 40% w/v PEG for 120 seconds, again with or without PHA exposure. In this instance, there was no significant difference in lysis or fusion rates. However, only 1/10 of the embryos which were not pre-treated with PHA developed to blastocyst (Table 3.3), compared to 6/10 blastocysts derived from embryos which had been exposed to PHA (Table 3.4). These results suggest that PEG has a bimodal effect on embryo viability which is time dependent. Firstly, it may inflict a toxic effect which affects developmental capacity by reducing embryo cleavage and, secondly, with longer exposure, the cell membranes may be unable to complete the stabilisation process required after fusion and consequently the cells lyse.

Removal of the zona pellucida from 2-cell mouse embryos diminished the likelihood that the third and final event in the process of PEG fusion, the stabilisation of the membranes, would occur. When zona-free 2-cell mouse embryos were exposed to 50 % w/v PEG for 90 seconds, 100% lysed. In

contrast, none of the zona-intact mouse embryos which underwent the same treatment lysed and 89% developed to blastocyst. This suggests that there may be a time lag, during which the PEG is passing through the zona pellucida, before it affects the cell membranes. This would effectively decrease the length of exposure of the membranes to PEG and hence decrease the lysis rate.

Fused two-cell mouse embryos are tetraploid and this may compromise the extent of development. However, it has been shown by Eglitis (1980), that up to 73% of tetraploid mouse embryos fused by PEG can develop to the blastocyst stage. Kaufman and Webb (1990) also found that up to 95% of tetraploid mouse embryos produced by electrofusion at the two-cell stage and transferred to synchronised recipients, implanted and 69.6% of tetraploid embryos formed fetuses with beating hearts after 10 days of gestation.

Fusion of blastomeres of two-cell mouse embryos was carried out to investigate the feasibility of using PEG as a fusion system. The ultimate aim was to apply this technique to marmoset embryos. It has been demonstrated that fusion of mouse blastomeres with PEG can be achieved and, in some cases, development will continue after fusion. But this system was not efficient enough for use in a species where the number of embryos available was limited. For this reason, the use of electrical stimulation as a fusion system was investigated.

Although there was no difference between the rates of fusion when comparing PEG to electrical stimulation, the advantages of electrofusion over PEG fusion are demonstrated in the developmental capacity of the fused embryos. At the highest rates of fusion, only 5% of PEG treated two-cell mouse embryos developed to blastocyst, and 82% either lysed or failed to cleave, but 29% of electrically fused one-cell mouse embryos developed to blastocyst and only 8% failed to cleave. Embryo viability is less likely to be compromised by using electrofusion as there is no exposure of embryos to potentially toxic chemicals.

Fusion of marmoset karyoplasts to one-cell embryos was successful in 64% of cases. Pronuclear stage marmoset embryos developed to an average of 3 cells, and a maximum of 8 cells, after micromanipulation and electrofusion. This demonstrates the potential of this system and if it had been possible to gain access to a greater number of marmoset embryos for this study, it is likely that an even more efficient electrofusion protocol could have been developed which would have increased the fusion efficiency. This is the first successful demonstration of this procedure on the embryos of any primate, including the human. Attempts have been made to remove the extra pronucleus from human tripronuclear embryos and two of three embryos survived the process (Rawlins et al 1988). However, there was no cleavage of either of the two successfully manipulated embryos. Palermo et al (1994) successfully enucleated dispermic and digynic human zygotes and cleavage continued after

this process, but for ethical reasons it has not been shown whether fusion of a pronuclear karyoplast to a one-cell human embryo is possible.

Table 3.10 shows how the results obtained for mouse and marmoset embryos in this study compare with the rates of electrofusion obtained by other workers for other species. Depending upon the species under investigation, pulse parameters required to obtain high rates of fusion vary. There appears to be an association between pulse number and pulse strength. Fewer pulses are required to achieve fusion if the pulse strength is increased and vice versa. The only exception being the electrofusion of rabbit blastomeres to oocytes which required three very strong pulses. By altering pulse size, pulse duration, and the number of pulses applied to embryos it is possible to customize the electrofusion system to suit the species under investigation.

The creation of embryos carrying only paternal chromosomes requires the success of two fundamental techniques. Firstly, the determination of the parental origin of the pronuclei, and secondly, pronuclear transfer and fusion. This chapter has described the development of an efficient fusion system. The following chapter (Chapter 4) describes the techniques applied to determine the parental origin of marmoset pronuclei.

TABLE 3.10 : Pulse parameters required to obtain electrofusion of cell types in various species.

Species	Cell types fused	Strength of pulse (kV/cm)	Pulse duration (μs)	Number of pulses	Maximum fusion (%)	Source
Marmoset	PN/1-cell	1.5	70	1-3	64	This study
Mouse	PN/1-cell	2.0	70	1	72	This study
Rat	PN/1-cell	1.5	200	2	81-92	Kono et al (1988)
Rabbit	8-cell/oocyte	3.6	60	3	94	Collas and Robl (1990)
Pig	2-8-cell/oocyte	1.2	30	1	~80	Prather et al (1989)
Sheep	16-cell/oocyte	0.75	100	3	~90	Willadsen (1986)
Cow	32-64-cell/oocyte	0.75	50	3	~70	Bondioli et al (1990)

PN - pronuclear karyoplast

CHAPTER 4

DETERMINATION OF THE PARENTAL ORIGIN OF MARMOSET PRONUCLEI

4.1 INTRODUCTION

The fundamental requirement when creating uniparental embryos by enucleation and pronuclear transfer, is to distinguish between the maternal and paternal pronuclei. In mouse embryos, the maternal pronucleus is larger and is initially situated closer to the second polar body than the paternal pronucleus (McGrath and Solter 1983). There is some indication that these parameters can also be used to distinguish between maternal and paternal pronuclei in human embryos (Rawlins et al 1988). Unfortunately, in marmoset embryos there appears to be no size difference between the pronuclei, and it is difficult to differentiate between the first and second polar bodies (Fig. 4.1). Both pronuclei of marmoset embryos form at the same time after fertilization (Chapter 2), unlike hamster embryos in which the maternal pronucleus forms before the paternal pronucleus (Perreault et al 1987). Clearly, a system of pronuclear identification is required for marmoset embryos.

The DNA of mouse gametes and pronuclei can be stained with a polyspecific nuclear fluorochrome, Hoechst 33342, with little effect on subsequent fertilization or development (Tone and Kato 1986). This enables the pronuclei to be visualised without fixing the embryo. DNA staining has been used to determine the interaction of the two parental genomes with the egg

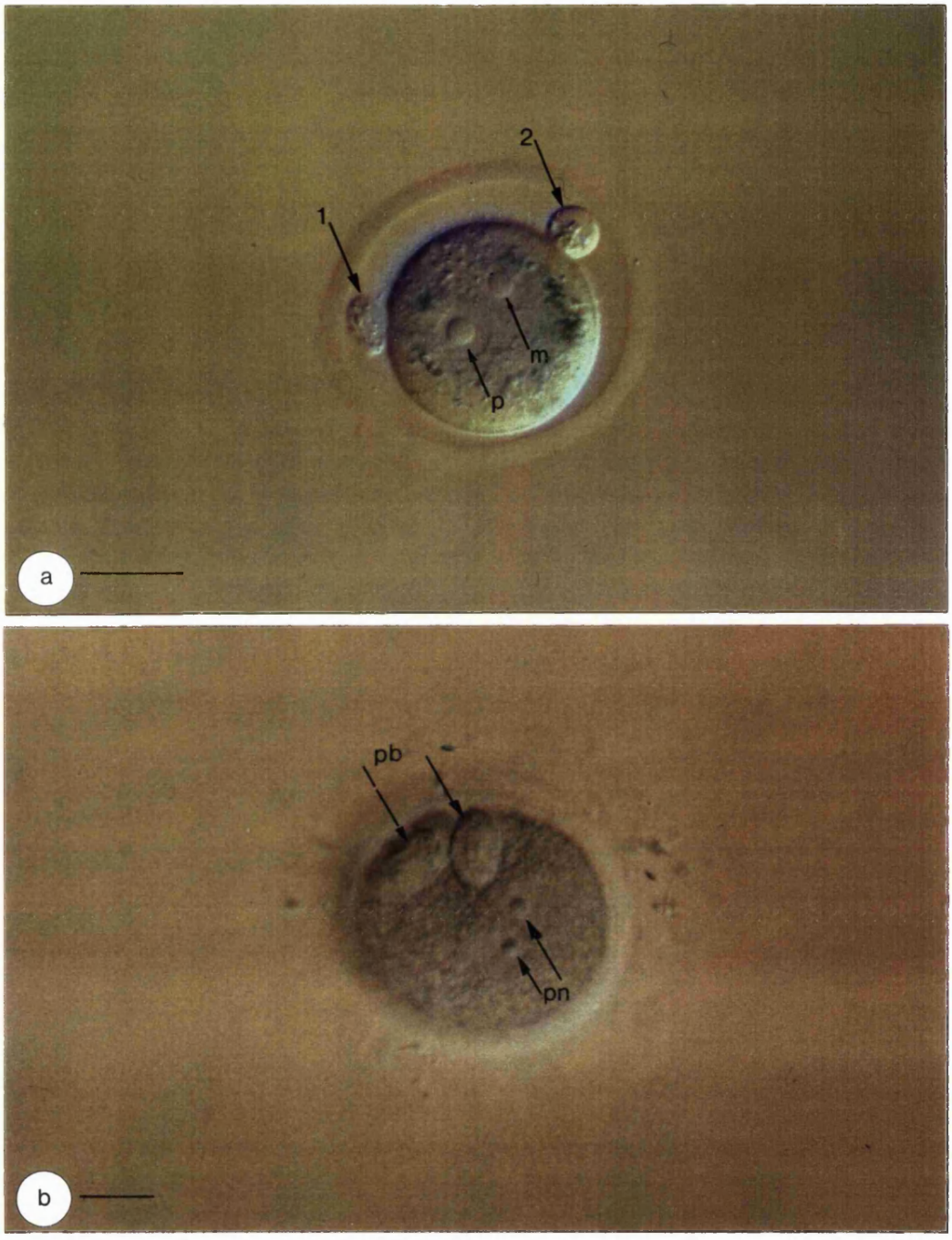

FIGURE 4.1 : (a) One-cell mouse embryo showing maternal pronucleus (m) and larger paternal (p) pronucleus, and first (1) and second (2) polar bodies. (b) One-cell marmoset embryo. There is no size difference between pronuclei (PN), and it is not possible to differentiate between the first and second polar bodies (PB). Bar = 25 μm.

cytoplasm, and organisation of chromatin during pronuclear formation and cell cleavage (Eichenlaub-Ritter et al 1988, Mori et al 1988, Adenot et al 1991).

Identification of the parental origin of marmoset pronuclei might be successful if fertilization was obtained from stained gametes. Fluorescence of either the paternal or the maternal pronucleus would result from staining of either the sperm or the oocyte, respectively. Prolonged exposure to ultra-violet light in conjunction with staining does, however, cause a loss of viability (Tone and Kato 1986). For this reason, it would be desirable to stain only gametes from the parent whose pronucleus is to be removed from the embryo. Production of gynogenetic embryos would be preceded by fertilization of oocytes with stained sperm. At the pronuclear stage the stained paternal pronucleus would be removed to create a gynogenote, thus eliminating the possibility that abnormal DNA would contribute to the embryonic genome. Alternatively, if the study involved production of androgenotes, the oocyte would be stained and, after fertilization, removal of the maternal pronucleus would leave the unstained paternal pronucleus within the embryo. The initial requirements of this procedure are that the gametes will take up fluorochrome, that normal fertilization will take place, that the pronuclei will fluoresce, and that the exposure to ultra-violet light will be short enough that it will not affect the viability of the embryo. All these requirements can be fulfilled in mouse embryos, with fertilization rates of up to 90% using stained oocytes and sperm

(Tone and Kato 1986). Less than 10 seconds exposure to ultra-violet light has no effect on the development of mouse embryos to blastocyst (Tone and Kato 1986).

In this chapter I describe experiments using mouse embryos and gametes to verify the fluorescent staining and detection of DNA using Hoechst 33342. Subsequently, I have exposed marmoset gametes to fluorochrome and carried out IVF using stained gametes. Effects of exposure to stain on marmoset sperm motility, IVF, and marmoset embryo development are presented.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Hoechst 33342

A 2 mg/ml stock solution of Hoechst 33342 (Bisbenzimide; Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) was made using purified water. This stock solution was snap frozen in aliquots of 20 μl and stored at -20 °C. A working concentration of 2 μg/ml was prepared by diluting 10 μl of stock solution with 10 ml of M16 medium (Whittingham 1971).

4.2.2 Staining of pronuclear stage mouse embryos

Pronuclear stage mouse embryos were collected as described in Section 3.2.4. Those embryos with two pronuclei were incubated in M16 and 2 μg/ml

Hoechst 33342 for 2 hours before being visualised for fluorescence under ultra-violet light (Section 4.2.12).

4.2.3 Collection of mouse sperm

Epididymal sperm was collected according to the method described by Fraser and Drury (1975). Ten-twelve week old C57/Bl x CBA male mice were killed by cervical dislocation. One epididymis was dissected and placed in a 100 μl drop of equilibrated T6 (Fraser and Drury 1975; Appendix V) under paraffin oil in a 35 mm diameter culture dish. The epididymis was teased apart, to allow sperm to swim out into the medium, and the dish was placed in a humidified atmosphere of 5% CO_2 in air at 37 °C, for approximately 2 hours.

4.2.4 Staining of mouse sperm with Hoechst 33342

The sperm from one epididymis was released into T6 medium with 15 mg/ml bovine serum albumin (BSA; Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) and 2 μg/ml Hoechst 33342. The sperm was incubated in this media for 30 minutes then centrifuged for 4 minutes, resuspended in T6 with BSA, recentrifuged for 4 minutes, and again resuspended in T6 and 15 mg/ml BSA. The sperm was incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 2 hours and adjusted to a concentration of 1 x 10^6 /ml.

4.2.5 Collection of mouse oocytes

Female CBA x C57Bl/6 F_1 mice were superovulated using the same protocol as described in Section 3.2.2. Twelve hours post-hCG injection, the mice were killed by cervical dislocation. The oviducts were dissected, and placed in M2 medium. The oviducts were torn apart with sharp forceps, releasing the cumulus/oocyte masses. Cumulus enclosed oocytes were immediately placed into drops of T6 with sperm (Section 4.2.3) for insemination.

4.2.6 Insemination of mouse oocytes

Cumulus enclosed eggs were incubated with the sperm for 2 hours, washed 5 times in T6 before transferring to M16 medium, and incubated under oil in an atmosphere of 5% CO_2 in air. Embryos were examined for the presence of two pronuclei and fluorescence of the paternal pronucleus 12 hours post-insemination.

There were three controls:

(1) Some oocytes (n=11) were not incubated with sperm as a control for parthenogenetic activation.

(2) Oocytes were incubated with sperm which was not stained with Hoechst 33342, to compare the fertilizing potential of stained and unstained sperm.

(3) Oocytes were incubated with stained sperm but were not exposed to fluorescent light to compare with the effects of combining sperm staining and ultra-violet exposure.

4.2.7 Collection of marmoset sperm

Marmoset sperm was collected as described in Section 2.2.6 (ii).

4.2.8 Exposure of marmoset sperm to Hoechst 33342

Motile marmoset epididymal sperm was incubated for 30 minutes in Alpha Modification of Minimum Essential Medium Eagle (αMEM) supplemented with 10 μM caffeine, 10 μM dibutyryl cyclic AMP, 10% marmoset serum and 2μg/ml Hoechst 33342. The stain was washed out of the sperm by centrifuging the sperm for 5 minutes, resuspending, centrifuging again for 5 minutes and resuspending in the same media without Hoechst 33342. Marmoset sperm was examined for motility and fluorescence.

4.2.9 Collection of marmoset oocytes

Marmoset oocytes were collected as descibed in Section 2.2.4.

4.2.10 Exposure of marmoset oocytes to Hoechst 33342

Marmoset oocytes were incubated in Alpha Modification of Minimum Essential Medium Eagle (αMEM) supplemented with 10 μM caffeine, 10 μM dibutyryl cyclic AMP, 10% marmoset serum and 2μg/ml Hoechst 33342 for 2 hours. Oocytes were subsequently washed thoroughly and placed with sperm for insemination.

4.2.11 Insemination of marmoset oocytes and assessment of fertilization

Insemination and assessment of fertilization was carried out as described in Sections 2.2.8 and 2.2.9.

4.2.12 Visualisation of fluorescence

Gametes and embryos were examined with a Zeiss Axioskop microscope (Zeiss, Welwyn Garden City, Herts, U.K.) fitted with epifluorescence. Hoechst 33342 is visible under ultra-violet light with a wavelength of 395-440 nm, and was viewed using Filter set No. 5 (Zeiss, Welwyn Garden City, Herts, U.K.).

4.3 RESULTS

4.3.1 Staining of pronuclear stage mouse embryos

Mouse pronuclear stage embryos were stained after fertilization to confirm that the parameters for this method, used by Tone and Kato (1986) would be successful under conditions in our laboratory. In the 23 one-cell embryos exposed to Hoechst 33342 and examined under ultra-violet light, both pronuclei exhibited fluorescence (Fig 4.2).

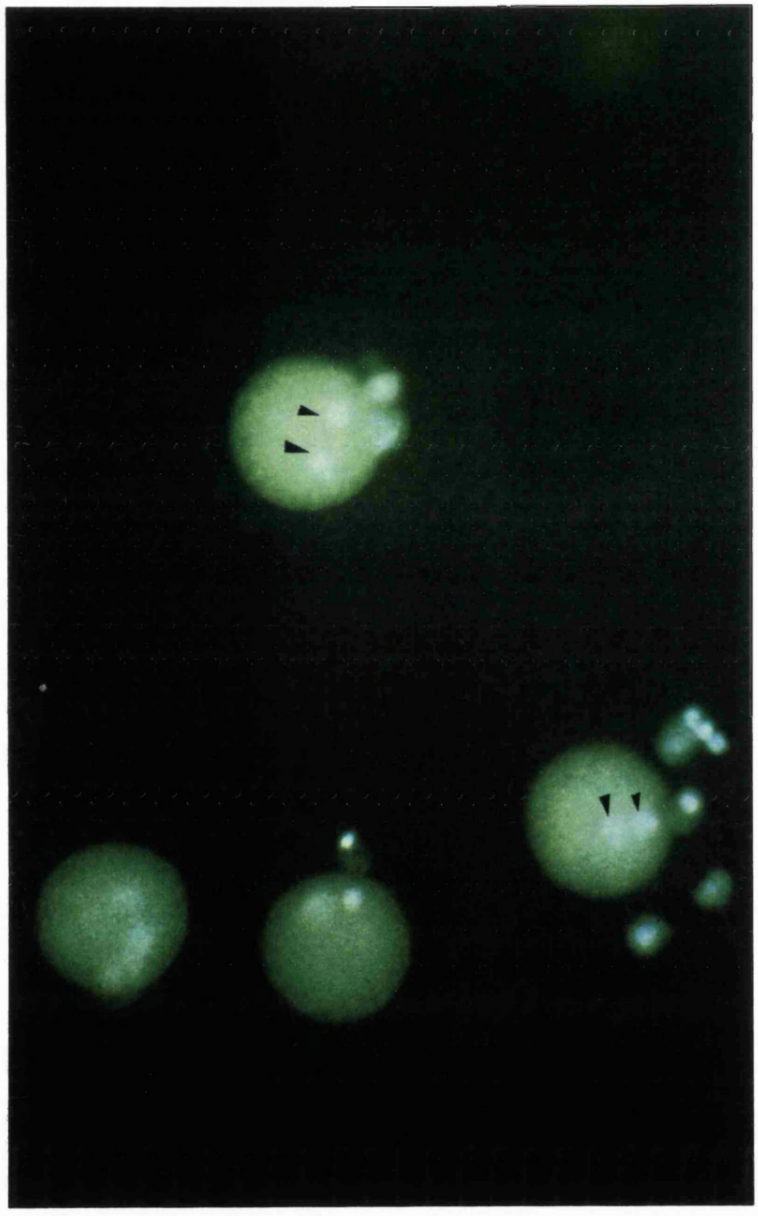

FIGURE 4.2 : One-cell mouse embryos after exposure to Hoechst 33342, showing fluorescent pronuclei (arrow heads). Polar bodies and some cumulus cells are also fluorescent.

4.3.2 Fluorescent staining of mouse sperm and IVF

This experiment was designed to confirm that stained mouse sperm could fertilize mouse oocytes and that the paternal pronucleus would fluoresce, and to record the subsequent development of these embryos compared to controls. Sixteen (50%) mouse oocytes were fertilized by stained mouse sperm. This was not significantly different from the number of mouse oocytes fertilized by unstained sperm (30/45; 66%)(Figure 4.3). Embryos fertilized by stained sperm were exposed to ultra-violet light at the pronuclear stage. This showed that the male pronucleus was fluorescent, but this exposure had a detrimental effect on development as none of the exposed and stained embryos underwent more than one cleavage division (n=21)(Figure 4.3). Nineteen percent (6/32) of the embryos fertilized with stained sperm, but not exposed to ultra-violet light, cleaved to the 8-cell stage but none developed further. Of control embryos, which had been fertilized with unstained sperm and were not exposed to ultra-violet light, 49% (22/45) developed to the 8-cell stage, 36% (16/45) to the morula stage, and 29% (13/45) developed to blastocyst (Figure 4.3). No parthenogenetic activation occurred in control oocytes.

4.3.3 Staining of marmoset epidydimal sperm

A preliminary experiment was carried out to determine whether the same concentration of Hoechst 33342 used for mouse sperm (2 μg/ml) would also

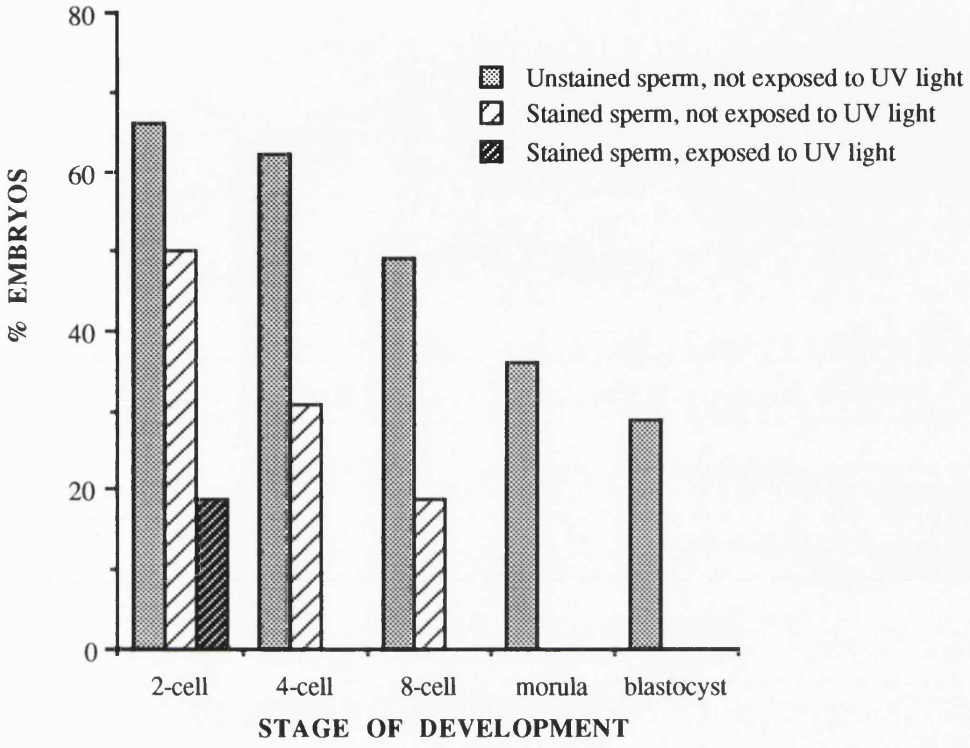

FIGURE 4.3 : The development of mouse embryos produced after in vitro fertilization with stained or unstained sperm and with or without exposure to UV light at the pronuclear stage.

cause fluorescence of marmoset sperm. Marmoset sperm did fluoresce as shown in Figure 4.4.

4.3.4 Fertilization of marmoset oocytes with stained sperm

Three of nine (33%) marmoset oocytes which were incubated with stained sperm fertilized. Although numbers are very small in this study, the fertilization rate decreased from 76% with unstained marmoset sperm ($p<0.005$; see Section 2.3.5) to 33% with stained sperm. The decrease in fertilizing capacity could be due to the centrifugation which is required to recover the sperm from the stain solution before insemination.

4.3.5 Fertilization of stained marmoset oocytes

Sixty-five percent (11/17) of marmoset oocytes exposed to 2 μg/ml Hoechst 33342 fertilized, not significantly different from that of untreated marmoset oocytes (Chapter 2). This indicates that this concentration of stain has no detrimental effect on the capability of marmoset oocytes to be fertilized.

4.3.6 Observation of fluorescence

None of the pronuclei in marmoset embryos derived from either stained marmoset oocytes or from stained marmoset sperm were visible when exposed to ultra-violet light at the pronuclear stage. The first polar body of stained marmoset oocytes was fluorescent, indicating that the stain did penetrate the

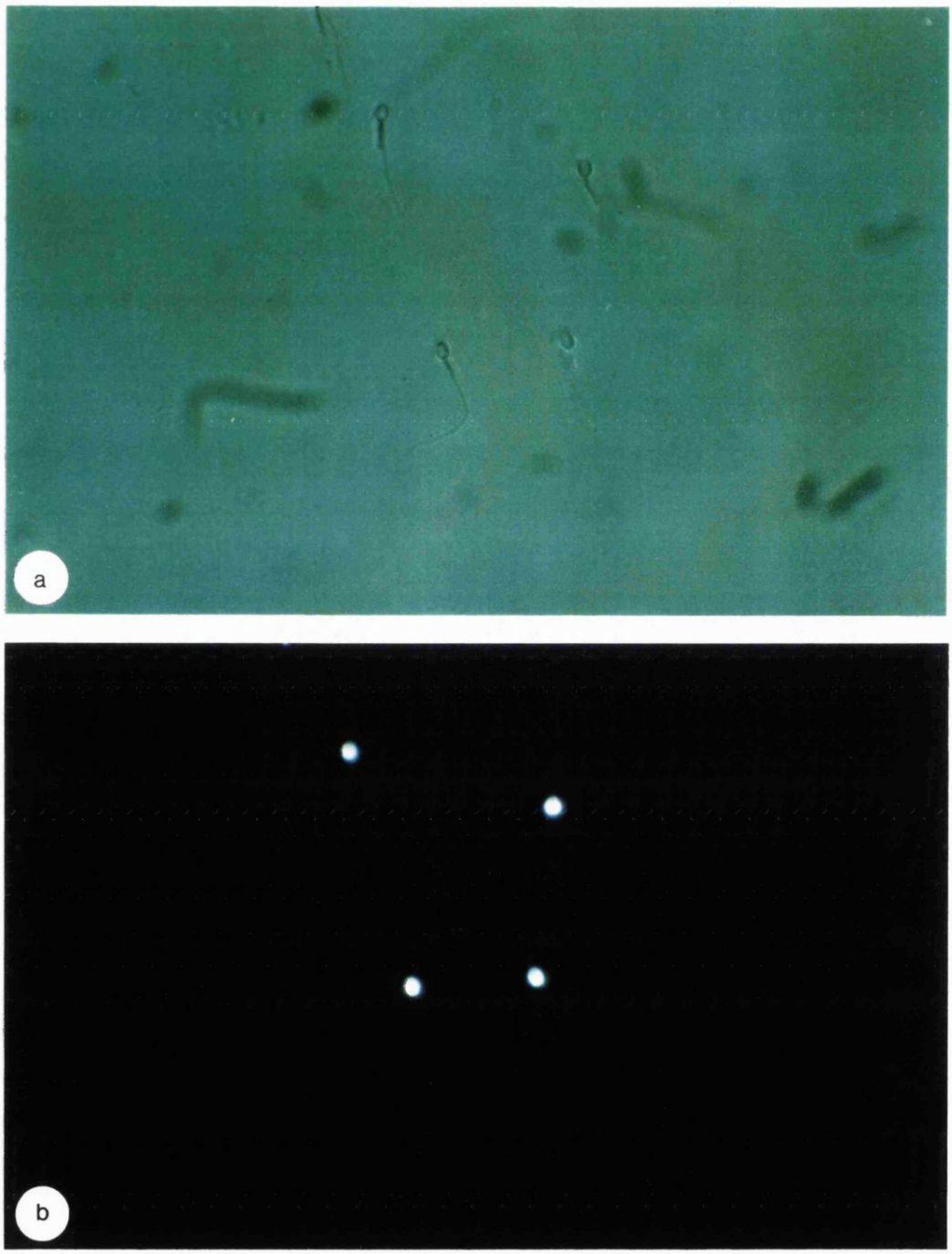

FIGURE 4.4 : (a) Marmoset sperm stained with Hoechst 33342 and (b) exposed to ultra-violet light. Immotile sperm were intentionally photographed to show that the stain is taken up.

cumulus and zona pellucida, and that the concentration of stain used was high enough to obtain visible fluorescence of marmoset DNA.

4.4 DISCUSSION

Hoechst 33342 staining is most commonly used as a pre-requisite for flow cytofluorometric sorting of X- and Y-chromosome bearing sperm in domestic species. Offspring have been produced from pigs (Johnson 1991), rabbits (Johnson 1992), and cattle (Cran et al 1993) after artificial insemination with stained sperm. This confirms that the viability of the DNA in the sperm of these species is not compromised by exposure to Hoechst 33342.

The DNA of marmoset oocytes and sperm can be stained with a polyspecific nuclear fluorochrome, Hoechst 33342. Marmoset sperm motility was not detrimentally affected by exposure to Hoechst 33342. Luttmer and Longo (1986) reported that there were no effects of exposure to Hoechst 33342 on the motility of hamster, surf clam and sea urchin sperm.

Fertilization rates using stained mouse sperm were not significantly decreased compared to unstained sperm. Luttmer and Longo (1986) observed that staining with Hoechst 33342 had a detrimental effect on fertilizing capacity of hamster sperm, as they were not able to fertilize hamster oocytes with intact zona pellucidae. This defect of fertilization is presumably associated with

sperm penetration of the zona pellucida since stained sperm were able to fertilize zona-free hamster oocytes (Luttmer and Longo 1986). Stained marmoset sperm also fertilized significantly less marmoset oocytes than unstained marmoset sperm. A marked decrease in the fertilizing capacity of marmoset sperm may have been associated with the processing of the sperm to remove it from media containing the stain before insemination. Marmoset sperm are particularly susceptible to damage during centrifugation (Chapter 2, this thesis; Harlow 1984). Centrifugation could be avoided by using highly technical methods of achieving fertilization with stained sperm, such as intra-cytoplasmic sperm injection (ICSI; Palermo et al 1992). However, for the purposes of this study, the zona pellucida would need to be punctured twice, firstly during sperm injection and again during enucleation. It would be extremely difficult to find the same hole in the zona pellucida to use for enucleation. The force required to puncture the zona pellucida during the enucleation process may force part of the oolemma and cytoplasm through the first hole in the zona pellucida. This may decrease the efficiency of creation of uniparental marmoset embryos and is likely to compromise embryo viability.

Staining did not affect the ability of marmoset oocytes to undergo fertilization. Neither was the fertilization rate of mouse oocytes, stained with similar concentrations of Hoechst 33342, significantly decreased (Tone and Kato

1986). This further supports the contention that the decreased fertilization rate of stained marmoset sperm was not due to effects of the stain on DNA, but because stained sperm had difficulty in physically penetrating the oocyte vestments.

Hoechst 33342 and 4',6'-diamidino-2-phenylindole (DAPI) have been used to observe fluorescent DNA in mouse (Tone and Kato 1986), pig (Critser and First 1986) and cattle embryos (Critser and First 1986). Embryos are usually fixed soon after exposure to stain so subsequent embryo viability need not be taken into consideration. To use fluorescent staining as an identification system for pronuclei in this study, the marmoset embryos must remain viable after staining and the stain must not effect changes to the epigenetic structure of the DNA. If the DNA structure was affected the technique would become inappropriate because it may alter the genomic imprinting of the DNA. Consequently, the intention was to remove the stained pronucleus from the embryo, leaving the unstained pronucleus to contribute to development. Unfortunately, no fluorescent pronuclei were visible after fertilization of marmoset oocytes exposed to Hoechst 33342. The reason for this is unclear. Critser and First (1986) have shown that the pronuclei of both bovine and porcine embryos, which were stained with Hoechst 33342 after fertilization, could be observed under ultra-violet light. It has also been demonstrated that mouse pronuclei will fluoresce when exposed to Hoechst 33342 (Tone and

Kato 1986, Critser and First 1986, This Study). However, it is also difficult to observe fluorescence of human pronuclei (K. Hardy, pers. comm.). This would suggest that there are distinct species differences in either the pronuclear structure, the ability of the DNA to incorporate the fluorescent dye, or the consistency of the cytoplasm which could mask fluorescence. The latter is unlikely since pronuclei are visible under normal phase contrast microscopy. Also, it has been demonstrated here that marmoset DNA can incorporate Hoechst 33342 as shown by fluorescence of sperm (Section 4.3.3; Fig 4.4). Therefore there may be differences between the pronuclear structure of primate embryos and other species. This contention is supported by Tesarik and Kopecny (1989) who found, during experiments to assess the nucleic acid synthesis of the human paternal pronucleus, that DNA synthesis started later in human embryos than in other species. Their conclusion was that there are marked interspecies differences in the way in which the egg cytoplasm affects pronuclear structure and development, and nucleic acid synthesis. If the patterns of human embryonic DNA synthesis are different from other species it is possible that human pronuclear DNA structure is different also, and this difference may apply to other primates such as the marmoset monkey. The pronuclear DNA structure may affect exhibition of fluorescence.

To facilitate fluorescence of marmoset pronuclei, the incubation time or the concentration of stain could be increased. Previous experiments have shown that marmoset DNA/chromatin will fluoresce, it is simply not observable at the pronuclear stage. The benefit gained by changing these parameters may be eclipsed by detrimental toxic effects on fertilization rate and embryo viability. Tone and Kato (1986) demonstrated that an increase in the concentration of Hoechst 33342 used for staining mouse sperm from 2.5 μg/ml to 10 μg/ml decreased fertilization rate from 76% to 44%, and regardless of concentration, staining of mouse and porcine embryos led to a decreased rate of development to blastocyst (Critser and First 1986). Critser and First (1986) noted that "longer incubation times or altered incubation conditions may increase the frequency of staining but are likely to interfere with development". These detrimental effects could be caused by a combination of exposure to the stain and exposure to ultra-violet light, which may induce the production of free radicals within the embryo (Critser and First 1986). Luttmer and Longo (1986) reported that within a few minutes of ultra-violet exposure the development of stained sea urchin eggs was arrested. More than 10 seconds ultra-violet exposure also decreased the percentage of stained mouse embryos which developed to blastocyst from 62% to 47% (Tone and Kato 1986).

The results presented in this chapter led to the consideration of alternative techniques to identify marmoset pronuclei. These are discussed in detail in Chapter 7.

CHAPTER 5

PARTHENOGENETIC ACTIVATION OF MARMOSET OOCYTES AND THE DEVELOPMENT OF MARMOSET PARTHENOGENONES *IN VITRO*

5.1 INTRODUCTION

Parthenogenetic activation is the induction of oocytes to resume meiosis without fertilization, and is a simple and effective method of producing embryos containing only maternal chromosomes. Various chemical and environmental stimuli have been used to activate murine oocytes. These include ethanol (Cuthbertson 1983), strontium chloride (O'Neill et al 1991) and electrical stimulation (Whittingham 1980). Calcium ionophore A23187 has recently been used to parthenogenetically activate human oocytes that had failed to fertilize (Winston et al 1991).

Parthenogenetically activated mouse oocytes, which contain only maternal chromosomes, develop to post-implantation stages (Tarkowski 1970, Kaufman et al 1977). One explanation for the failure of parthenogenetic embryos to develop to term has been the lack of nutrition at critical stages due to a very poorly developed extra-embryonic component (Surani and Barton 1983). In mouse embryos it appears that without a paternal genome, development of the extra-embryonic tissues will not proceed normally (Surani et al 1984). This phenomenon, whereby both parental genomes are required for complete and normal embryonic development to term, has been described as genomic imprinting (Section 1.4). Only in the mouse have the effects of genomic imprinting on mammalian early embryonic development been extensively studied (reviewed by Surani et al 1987).

A reliable method of activating non-human primate oocytes would enable studies to determine whether the maternal genome alone can support development of primate embryos at pre-implantation stages. After transfer of parthenogenetic embryos it may be possible to determine the role of the maternal genome in primate post-implantation development.

In this chapter I describe investigations of the rates of activation of marmoset oocytes using ethanol, electrical stimulation, calcium ionophore A23187, and strontium, and the *in vitro* development of marmoset parthenogenones.

5.2 MATERIALS AND METHODS

5.2.1 Marmoset oocyte collection

The collection of marmoset oocytes was carried out as described in Section 2.2.4. Some oocytes were exposed to sperm as part of the IVF study (Chapter 2) and the sperm preparation and insemination procedures are described in Sections 2.2.6 (ii) and 2.2.8, respectively.

5.2.2 The removal of cumulus cells from marmoset oocytes

Oocytes which had been exposed to sperm were gently pipetted with a flame polished pasteur pipette which dispersed the cumulus cells from the zona

pellucida. Oocytes which had not been exposed to sperm were incubated briefly in 0.1% hyaluronidase (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) in Medium 2 (Quinn et al 1982) and gently pipetted with a flame polished pasteur pipette to remove cumulus cells. After cumulus cell removal it was possible to visualise the first polar body. When a second polar body and pronuclei could not be seen and there was no cleavage for at least 48 hours after insemination, oocytes were considered to be unfertilized. Only unfertilized oocytes which had extruded a first polar body and so were presumably in meiotic metaphase II were used in this study. Cumulus free marmoset oocytes were incubated in MEM supplemented with 10% heat inactivated marmoset serum in a humidified atmosphere of 5% CO_2 in air, at 37 °C, until activation stimulus was applied.

5.2.3 Exposure of marmoset oocytes to ethanol

Marmoset oocytes were incubated in 7% ethanol (Absolute alcohol 100, Hayman Ltd., Witham, U.K.) in phosphate buffered saline (PBS; Life Technologies, Paisley, Scotland, U.K.) for 5 minutes at room temperature (Cuthbertson 1983). Oocytes were washed in and returned to MEM supplemented with 10% heat inactivated marmoset serum and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Nineteen oocytes in this treatment group had not been exposed to sperm. The remaining oocytes (28/47) had been exposed to sperm as part of another study but had failed to

fertilize as confirmed by absence of a second polar body and pronuclei and lack of cleavage. Forty oocytes were not exposed to ethanol until at least 3 days after collection (4 days post-hCG). This delay was partly due to the time taken to be certain that fertilization had not occurred i.e. confirmation that no cleavage had taken place for at least 48 hours after insemination. Seven oocytes were exposed to ethanol 10 hours after collection (34 hours post-hCG) to determine whether post-meiotic aging affected the rate of activation.

5.2.4 Exposure of marmoset oocytes to electrical stimulation

Electrical pulses were generated by an Electro Cell Manipulator 200 (BTX Inc., San Diego, California, U.S.A.). The pulses were applied to the oocytes in a chamber consisting of 2 parallel, stainless steel, 0.5 mm diameter electrodes attached to a glass slide, 0.5 mm apart (Part No. 450, BTX Inc., San Diego, California, U.S.A.). The chamber was filled with electrofusion medium (Appendix IV). The oocytes were subjected to either one series of 6 electrical pulses (DC) or two series of six electrical pulses, 30 minutes apart. Each pulse was of 2kV/cm and 70 μsec duration.

5.2.5 Exposure of marmoset oocytes to strontium chloride

Oocytes were incubated in 1.7 mM strontium chloride (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) in Medium 16 (Whittingham 1971) for 1 hr at 37°C in an atmosphere of 5% CO_2 in air (O'Neill et al 1991). All six oocytes

in this group had been exposed to sperm and had failed to fertilize. These oocytes were all exposed to strontium chloride five days after collection (6 days post-hCG).

5.2.6 Exposure of marmoset oocytes to calcium ionophore A23187

Calcium ionophore A23187 (Sigma Chemical Co. Ltd., Poole, U.K.) was prepared as a stock solution at a concentration of 500 μM in dimethylsulphoxide. A working dilution of 5 μM was prepared in M2. Winston et al (1991) showed that higher rates of activation of human oocytes were achieved if oocytes were cultured in serum-free medium before exposure to calcium ionophore. For this reason, marmoset oocytes were transferred to M2 for one hour before incubation with 5μM calcium ionophore A23187 in M2 for 5 minutes at room temperature. All oocytes in this group had failed to fertilize when exposed to marmoset sperm. These oocytes were exposed to calcium ionophore A23187 five days after collection (6 days post-hCG).

5.2.7 Culture of marmoset oocytes

After activation stimulus was applied all oocytes were washed thoroughly and cultured in ~100 μl drops of MEM supplemented with 10% heat inactivated female marmoset serum. The drops were overlaid with paraffin oil (Merck, Lutterworth, Leics, U.K.) to prevent evaporation. Oocytes were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

5.2.8 Assessment of parthenogenetic activation of marmoset oocytes

Oocytes were examined 18-20 hours after treatment when oocytes had either cleaved, formed pronuclei, or failed to activate. Observations of development *in vitro* were made daily. Oocytes were left in culture till developmental arrest, determined by lack of cleavage for at least 24 hours.

5.2.9 Statistical analyses

The differences in the proportions of oocytes undergoing each type of activation after either ethanol or electrical exposure were analysed by ANOVA (CSS: Statistica; Statsoft UK, Letchworth, U.K.).

5.3 RESULTS

5.3.1 Assessment of activation and development of marmoset oocytes after exposure to ethanol

Of 47 oocytes exposed to ethanol, eight (17%) activated. Three types of activation were observed. Five (63%) activated oocytes underwent immediate cleavage (IC) and had divided to 2 cells within 18 hours of treatment; two oocytes (25%) extruded a second polar body and formed one pronucleus (2PB,1PN); one oocyte formed two pronuclei without extruding a second polar body (1PB,2PN).

The only oocytes which divided in culture were those which underwent immediate cleavage. One of these embryos developed to 16 cells, one to 6 cells, one to 3 cells and two embryos developed to 2 cells *in vitro* (Table 5.1). Overall, the average cell number (± S.E) of ethanol activated parthenogenones was 4.0 ± 1.7.

Oocytes which did activate had undergone treatment at least 4 days post-hCG injection. None of the oocytes which were exposed to ethanol 34 hours post-hCG activated.

Of the oocytes which activated when exposed to ethanol, seven had previously been exposed to sperm but had failed to fertilize. All of these oocytes had been incubated with sperm which fertilized other oocytes.

5.3.2 Assessment of activation and development of marmoset oocytes exposed to electrical stimulation

Two of four oocytes (50%) activated when exposed to a single series of six electrical pulses. One oocyte cleaved immediately (IC) and developed to 6 cells in culture while the other oocyte formed two pronuclei (2PN, 1PB) and developed to 4 cells in culture.

TABLE 5.1. The activation and development of marmoset oocytes exposed to 7% ethanol in phosphate buffered saline for 5 minutes.

Age at treatment (post-hCG)	Number of oocytes treated (n)	Number of oocytes activated (% of n)	Type of activation		
			IC (haploid)	1PN,2PB (haploid)	2PN,1PB (diploid)
34 hours	7	0 (0)	-	-	-
6 days	40	8 (20)	2 x 2-cell 1 x 3-cell 1 x 6-cell 1 x 16-cell	2 x 1-cell	1 x 1-cell
TOTAL	47	8 (17)	5	2	1

Ninety-two percent (68/74) of marmoset oocytes activated after exposure to two series of six electrical pulses (Table 5.2). Thirty (44%) activated oocytes extruded a second polar body and formed one pronucleus (1PN,2PB). The embryos which activated in this way developed to an average (± S.E.) of 3.9 (± 0.3) cells. Thirty-two percent (22/68) of oocytes which activated formed two pronuclei without extruding a second polar body (2PN,1PB) and developed to an average of 4.5 (± 0.5) cells. Nine (13%) oocytes underwent immediate cleavage and developed to an average of 3.9 (± 0.8) cells in culture. The 7 remaining marmoset oocytes (10%) formed a single pronucleus but did not extrude a second polar body (1PN,1PB) and developed to an average of 3.4 (± 0.6) cells (Figs. 5.1 and 5.2). Two oocytes failed to activate and four oocytes were degenerate 24 hours after treatment. Overall, the average cell number (± S.E.) of electrically stimulated parthenogenones was 4.0 ± 0.3.

The type of activation (1PN,2PB; 2PN,1PB; 1PN,1PB or IC) made no significant difference to the extent of development *in vitro*. There was also no significant difference between the *in vitro* development of haploid and diploid parthenogenones in this treatment group.

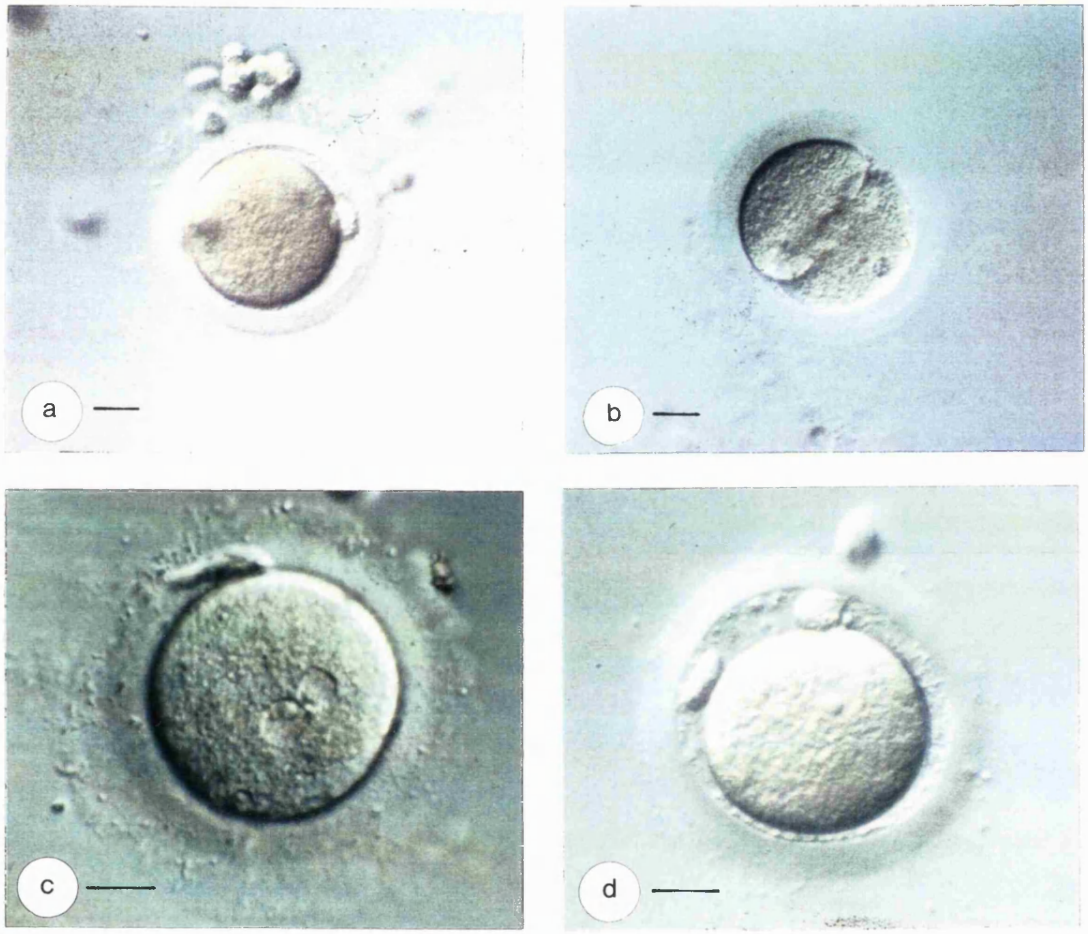

FIGURE 5.1 : Marmoset oocyte before (a) and after parthenogenetic activation. (b) Immediate cleavage. (c) 2PN, 1PB. (d) 1PN, 2PB. Bar = 25 μm.

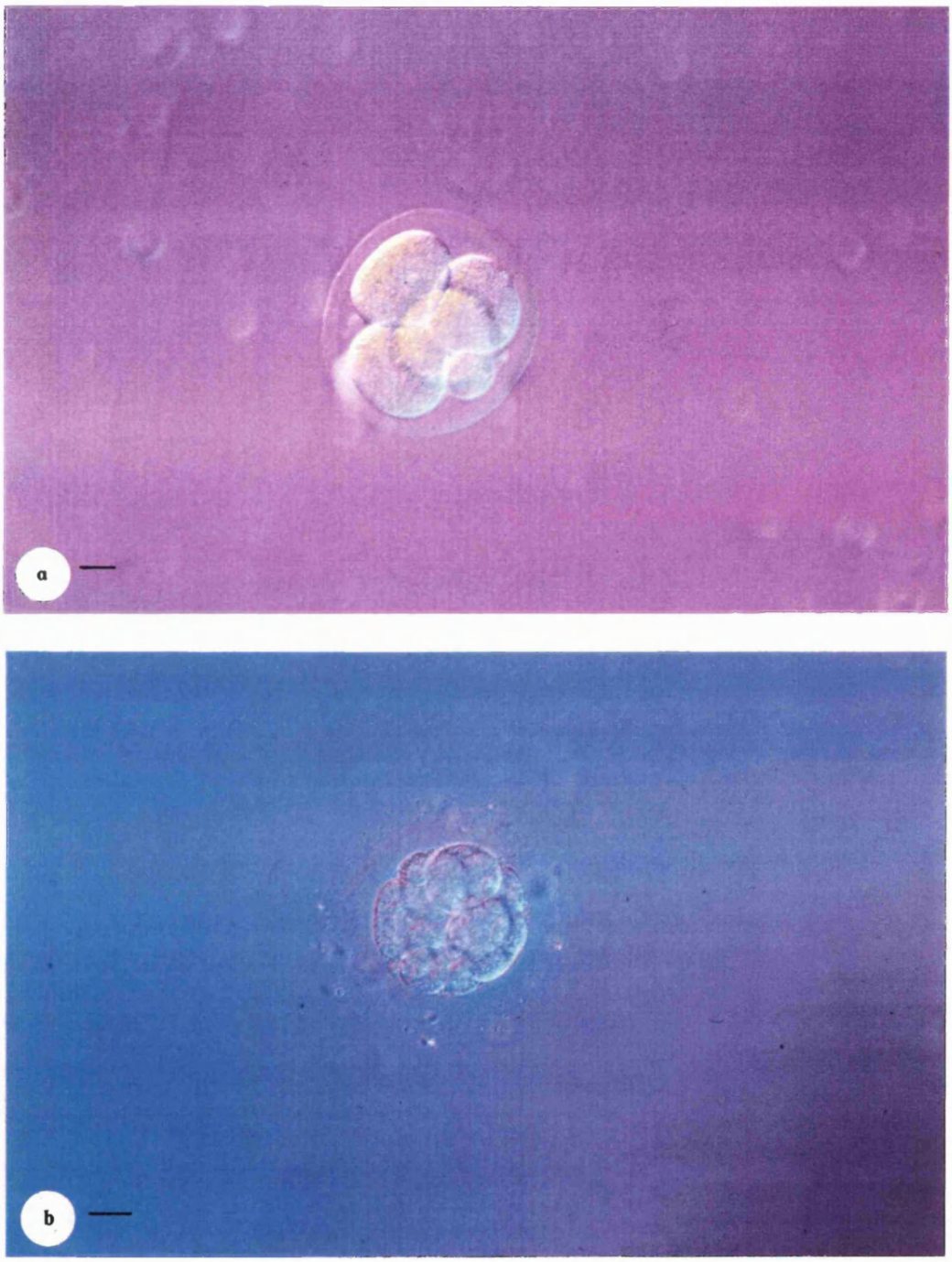

FIGURE 5.2 : Marmoset parthenogenones at the (a) 4-cell and (b) 12-cell stage. Bar = 25 μm.

TABLE 5.2 The activation and development of marmoset oocytes exposed to two series of six electrical pulses.

Number of oocytes treated (n)	Number of oocytes activated (% of n)	Type of activation			
		IC (haploid)	1PN,2PB (haploid)	2PN,1PB (diploid)	1PN,1PB (diploid)
74	68 (92)	4 x 2-cell 1 x 3-cell 2 x 4-cell 2 x 8-cell	2 x 1-cell 6 x 2-cell 5 x 3-cell 9 x 4-cell 1 x 5-cell 4 x 6-cell 1 x 7-cell 2 x 8-cell	5 x 2-cell 6 x 3-cell 3 x 4-cell 1 x 5-cell 1 x 6-cell 6 x 8-cell	3 x 2-cell 3 x 4-cell 1 x 6-cell
NUMBER OF OOCYTES ACTIVATED (% of n)		9 (12)	30 (41)	22 (30)	7 (9)
AVERAGE CELL NUMBER (± SEM)		3.9 (±0.8)	3.9 (±0.3)	4.5 (±0.5)	3.4 (±0.6)

TABLE 5.3 Parthenogenetic activation rates of marmoset oocytes using five different treatments.

Treatment	Number of oocytes treated	Number of oocytes activated	Activation rate %
Strontium Chloride (1.7 mM)	6	0	0
Calcium Ionophore (5 μM)	7	0	0
Ethanol (7 %)	47	8	17[a]
Electrical current (6 pulses)	4	2	50[b]
Electrical current (2 x 6 pulses)	74	68	92[c]

c: significantly higher than a ($p<0.001$) and b ($p<0.01$)

5.3.3 Assessment of marmoset oocytes following strontium chloride exposure

No marmoset oocytes exposed to strontium chloride 6 days post-hCG activated (Table 5.3).

5.3.4 Assessment of marmoset oocytes following exposure to calcium ionophore A23187

No marmoset oocytes exposed to calcium ionophore A23187 6 days post-hCG activated (Table 5.3).

5.3.5 Comparison of the activation and development of ethanol and electrically stimulated marmoset parthenogenones

Most ethanol activated oocytes underwent immediate cleavage (5/8, 63%). However, most of the oocytes which activated after electrical stimulation extruded a second polar body and formed one pronucleus (30/68, 44%). There was no significant difference between the *in vitro* development of ethanol stimulated and electrically stimulated parthenogenones which reached an average (± S.E.) of 4.0 ± 1.7 and 4.0 ± 0.3 cells, respectively.

5.4 DISCUSSION

This study has shown that marmoset oocytes can be parthenogenetically activated by electrical stimulation or ethanol treatment. Ninety-two percent

of marmoset oocytes were activated by exposure to multiple electrical pulses. Oocytes from other species have been activated by electrical pulses and 100% of rabbit oocytes have been activated after exposure to repetitive electrical pulses (Ozil 1990). The mechanism by which electrical stimulation causes parthenogenetic activation is not known. Electrical pulses may cause calcium channels in the plasma membrane to open which would allow an influx of Ca^{2+} ions into the oocyte (reviewed by Rasmussen, 1989). It has been shown that increases in the level of intracellular free calcium are associated with mouse oocyte activation (Cuthbertson et al 1981). Ozil (1990) suggests that activation is caused by an association of calcium ions with inositol 1,4,5-triphosphate which leads to a release of Ca^{2+} ions from intracellular stores. Oocytes are held in meiotic arrest by the presence of cytostatic factor (CSF; Murray and Kirschner 1989). CSF prevents degradation of cyclin (Karsenti et al 1987), the protein responsible for maintaining the activity of maturation promoting factor (MPF). Active MPF prevents the cell cycle from progressing past the metaphase stage (Murray et al 1989). Increased concentrations of calcium ions within the oocyte lead to the breakdown of CSF by calpain II (Watanabe et al 1989), allowing cyclin to be broken down, MPF to become inactive and subsequent mitosis of the activated oocyte. An increase in the concentration of calcium ions within the oocyte is presumably involved in the activation of mammalian oocytes, whether the ions are released from intracellular stores or flow into the oocyte through electroporated membranes.

Human oocytes can be activated by ethanol and calcium ionophore A23187 at rates of 16% and 50-60%, respectively (Winston et al 1991). I have shown that ethanol can activate marmoset oocytes at similar rates (17%) to human oocytes but only when marmoset oocytes are aged for at least 4 days post-hCG injection. No activation, however, was achieved by treating aged marmoset oocytes with calcium ionophore A23187 or strontium chloride. Winston et al (1991) found that, to achieve high rates of activation, human oocytes required a period of incubation in serum-free medium before exposure to calcium ionophore. Marmoset oocytes may require longer pre-incubation in serum-free medium before calcium ionophore exposure. It is difficult to explain why strontium exposure did not activate marmoset oocytes as strontium, like ethanol, is believed to induce activation by increasing intracellular free calcium levels (O'Neill et al 1991). Only seven marmoset oocytes were exposed to strontium and, had more oocytes been available for this part of the study, a response may have become evident. More experimental material would also have provided the opportunity to expose oocytes to strontium chloride at various times after hCG.

Human oocytes activated with calcium ionophore developed to an average of 4 cells and reached a maximum of 8 cells *in vitro* (Winston et al). Marmoset parthenogenones activated with either ethanol or electrical stimulation also

reached an average of 4 cells in culture and one parthenogenone developed to 16 cells. This would suggest that the preimplantation developmental potential of human and marmoset parthenogenones *in vitro* is similar and does not depend solely on the type of activation stimulus used.

Marmoset oocytes underwent four different types of activation. These were the formation of two pronuclei without extrusion of a second polar body (2PN, 1PB); extrusion of a second polar body and formation of one pronucleus (1PN, 2PB); formation of one pronucleus without the extrusion of a second polar body (1PN, 1PB); and immediate cleavage (IC). The types of activation observed are similar to those reported for the mouse (Kaufman 1983). The difference in the proportion of oocytes which underwent each type of activation after ethanol exposure or electrical stimulation may have depended on the ages of the oocytes as well as the treatment. Ethanol activated oocytes were at least 2 days older than those treated with electrical current. Webb et al (1986) reported differences in the types of activation for mouse oocytes of different ages. When treated with ethanol, fresher oocytes (16-20 hours post-hCG) more often underwent 1PN,2PB activation and as the oocytes aged (25 hours post-hCG) the incidence of IC increased, until 32-36 hours post-hCG when activated oocytes formed one pronucleus without extrusion of a second polar body (Webb et al 1986). The high incidence of immediate cleavage in aged oocytes has been explained by Szollosi (1971) who observed that the

meiotic spindle migrates to the centre of mouse oocytes during the aging process. Although the marmoset oocytes used in this study had been aged for at least 48 hours post-hCG and for up to six days before treatment there was no overt sign of degeneration or fragmentation. It may be that marmoset oocytes tolerate long term *in vitro* culture, and that the changes in the type of parthenogenetic activation that are seen in *in vivo* mouse oocytes over 36 hours (Webb et al 1986) also occur in marmoset oocytes but over a longer time span. This effect may be related to the length of the cell cycle in pre-implantation stages; marmoset embryos take four days to reach the eight cell stage (Harlow 1984), the same length of time taken for mouse embryos to reach blastocyst (McLaren 1982).

Aging of oocytes *in vitro* may also explain why some oocytes had failed to fertilize when exposed to sperm but were capable of activation. Sperm were not incapable of fertilization in all cases as some oocytes in the same cohorts fertilized. It has now been demonstrated in mouse (Collas et al 1989) and rabbit (Collas and Robl 1990) oocytes that extended aging *in vitro* increases the rate of activation when electrical stimulus is used. Therefore, although these marmoset oocytes were incapable of fertilization 10 hours after collection, the extra time in culture may have promoted nuclear or cytoplasmic events which facilitated parthenogenetic activation.

There was no significant difference between the *in vitro* development of electrically stimulated haploid and diploid parthenogenones which develop to an average of 4 and 5 cells, respectively. This differs from the mouse where half of the haploid parthenogenones only undergo one cleavage division but about 90% of diploid parthenogenones reach the blastocyst stage (Howlett 1988). However, in marmosets, even normal embryos with both parental genomes develop poorly *in vitro* (Wilton et al 1993, Chapter 2, this Thesis). *In vitro* fertilized marmoset embryos only develop to an average of 8 cells in culture, probably due to a culture system which lacks the correct nutritional requirements for this species (Wilton et al 1993, Chapter 2). Any differences in the development of haploid and diploid marmoset parthenogenones might not manifest themselves until later in development.

This study has shown that marmoset oocytes can be parthenogenetically activated by electrical stimulation and by ethanol treatment. Activation of 92% of marmoset oocytes can be achieved using multiple electrical pulses. This is the highest reported rate of activation of primate oocytes. The development of a reliable method of parthenogenetic activation of primate oocytes lays the foundation for embryo transfer to determine the extent of parthenogenetic development *in vivo*, and assessment of the role of the parental genomes in primate embryonic development.

CHAPTER 6

THE DEVELOPMENT OF MARMOSET PARTHENOGENONES *IN VIVO*

6.1 INTRODUCTION

The type of implantation observed in primate species varies between interstitial, or highly invasive penetration of the embryo into the uterine stroma (as seen in humans, reviewed by Johnson and Everitt 1980) and superficial implantation seen in many other non-human primate species including baboons, rhesus monkeys and marmoset monkeys (Moore et al 1985). Timing of implantation also varies widely from 6-7 days after ovulation in humans (Hertig et al 1956), to 11-12 days after ovulation in marmoset monkeys (Moore et al 1985). A common feature in primate placentation is the formation of a haemo-chorial placental interface, irrespective of the degree of trophoblast invasion. This type of placentation is not observed in common laboratory species and therefore it is difficult to extrapolate findings related to mouse implantation, for example, to those in the human.

Early stages of implantation in the marmoset monkey have been described by Moore et al (1985). Syncytial trophoblast adjacent to the embryonic pole begins to penetrate the uterine endometrium 13-16 days after ovulation, 1-3 days after initial attachment. By 16 days after ovulation, there is lateral invasion of the trophoblast into the endometrial epithelium, and syncytiotrophoblast is adjacent to the maternal capillaries. Syncytium also starts migrating into the stromal tissue. Embryonic development by day 16

includes the formation of the amniotic cavity and proliferation of the extraembryonic ectoderm, but the parietal yolk sac has not yet formed. Nineteen days after ovulation, there is a decidual response in the stromal cells. The trophoblast of the embryos covers the dorsal and ventral surfaces of the endometrium, and the embryonic disc and yolk sac has formed. Twenty-three days after ovulation, embryos are only at a slightly more advanced stage than embryos observed after 19 days. The amnion is considerably larger and there is more extensive proliferation of the trophoblast. The syncytiotrophoblast is still quite superficial, cytotrophoblast cells are obvious and there is much more interaction with the maternal blood system as well as formation of new capillaries. At this stage, a decidual reaction can still be seen in the stroma. By day 29 Moore et al (1985) observed that the embryos had a well developed yolk sac and a trilaminar embryonic disc. There was also a ridge of "redundant trophoblast tissue" on the surface of the endometrium (Moore et al 1985). Thirty-one days after ovulation the maternal blood vessels are surrounded by syncytiotrophoblast and the cytotrophoblast has extended further into the syncytiotrophoblast. A discontinuous basal lamina surrounds the primitive fetal blood vessels which have now differentiated. No information is available regarding the period between Days 31 and 45 of gestation in the marmoset. By Day 45, the mesoderm furhter extends into the cytotrophoblast and nucleated erythroblast can be found in the fetal blood vessels. The maternal

blood vessels are still intact and the basal laminae are disorganised and starting to degrade.

Since normal marmoset embryos fertilized *in vitro* often show poor development in culture (Chapter 2, Wilton et al 1993), it is difficult to determine whether the poor development of marmoset parthenogenones is due to the absence of the paternal genome or simply reflects inappropriate culture conditions. In contrast, normal *in vitro* fertilized marmoset embryos develop well after transfer and up to 100% of embryos reach term (Chapter 2, Summers et al 1987). This suggests that manipulation of marmoset oocytes *in vitro* need not compromise subsequent development if optimal conditions are provided. For this reason, useful information about the role of the parental genomes in early marmoset development is more likely to be provided by monitoring the development of parthenogenetic embryos after transfer to recipient females.

The investigation of mammalian parthenogenetic development beyond implantation is limited to only a few species; mice (Tarkowski 1970, Kaufman et al 1977, Surani and Barton 1983), rabbits (Ozil 1990) and cattle (Boediono and Suzuki 1994).

Murine parthenogenones develop poorly after transfer to synchronised recipients, and generally die at about 10 days of gestation, before reaching the 25-somite stage (Tarkowski 1970, Kaufman et al 1977, Surani and Barton 1983). These parthenogenones are retarded in their development compared to control embryos and develop very sparse extra-embryonic tissues (Surani and Barton 1983). It has been proposed that one of the reasons for the failure of murine parthenogenones to reach term was because of the poor development of extra-embryonic tissues, which diminished the supply of nutrients to the developing embryo (Surani et al 1987). Similarly, rabbit parthenogenones transferred to recipient females did not develop beyond the 30-somite stage and all parthenogenetic fetuses died by the twelfth day of gestation (Ozil 1990). Interestingly however, although rabbit parthenogenones were severely retarded compared to control embryos, the development of the extra-embryonic tissues was proportional to the development of the fetal component (Ozil 1990). It appears that the failure of rabbit parthenogenones was not due to an under-developed extra-embryonic component. Three bovine parthenogenones survived to days 57, 62 and 67 of gestation, but the morphology of the conceptuses was not determined (Boediono and Suzuki 1994).

CG has long been accepted as the major luteotrophic factor produced by the embryo in the early stages of pregnancy in primates (reviewed by Webley and

Hearn 1994). CG can first be detected in the peripheral plasma of pregnant marmosets within 14-18 days after ovulation which is about 3-4 days after implantation (Chambers and Hearn 1979, Hearn et al 1988). CG is produced by the trophoblast cells of the developing embryo at the blastocyst stage, and subsequently by the syncytiotrophoblast after attachment and implantation (Johnson and Everitt 1980). CG has two sub-units, α and β. Biological activity is dependent on the β sub-unit, but the dimer (or combination of the two sub-units) is needed for maximum biological response (Webley and Hearn 1994). Both active and passive immunization against the β sub-unit of CG during pregnancy in the marmoset will induce abortion within 6 days and 4 weeks, respectively (reviewed by Hearn 1979). This would suggest that CG is required for maintenance of the corpus luteum and hence maintenance of pregnancy.

Immunoreactive inhibin (ir-inhibin) is produced by the luteal cells of the marmoset ovary (Webley et al 1991b). Levels of ir-inhibin in the peripheral plasma of marmoset monkeys have been shown to rise significantly higher in conception cycles than non-conception cycles by day 8 after ovulation (Webley et al 1991b). This is before the detection of CG and 3-4 days before implantation.

The aim of this study was to transfer marmoset parthenogenetic embryos to synchronised recipient females and to determine the morphology of parthenogenetic marmoset embryos *in vivo*. Pregnancy was monitored using levels of progesterone, chorionic gonadotrophin (CG) and immunoreactive inhibin (ir-inhibin) in the peripheral plasma. Progesterone is produced by the corpus luteum (CL) during the luteal stage of the cycle of all primates and continues to be produced by the CL throughout the early stages of pregnancy in the marmoset (Hearn 1983).

It would be important to determine how long a parthenogenetic pregnancy might be maintained in a primate, but, assuming that the conceptus would be lost at some stage, no morphological observations about the developing parthenogenetic conceptus would be obtained. For this reason, animals which were considered pregnant 33 days after ovulation were sacrificed. The uteri were collected for sectioning and histological examination.

6.2 MATERIALS AND METHODS

6.2.1 Parthenogenetic activation of marmoset oocytes

Parthenogenetic activation of marmoset oocytes was carried out as described in Section 5.2.4 and marmoset parthenogenones were cultured to the 4-cell stage as described in Section 2.2.10. Only diploid parthenogenetic embryos,

either 2PN,1PB or 1PN,1PB, were transferred to the uteri of synchronised recipient marmosets.

6.2.2 Transfer of parthenogenetic marmoset embryos to recipient marmosets

Parthenogenetic embryos were transferred at the four-cell stage to synchronised recipient female marmosets (n=3). Recipients were synchronised as described in Section 2.2.11 (ii)a), and embryo transfers were carried out as described in Section 2.2.11 (ii)b). Two normal *in vitro* fertilized embryos were transferred to recipient females (n=2) as controls. Due to the small number of animals available, it was not possible to carry out sham transfers to control for the effects of the transfer procedure without the presence of an embryo.

6.2.3 Monitoring of recipient female marmosets after embryo transfer

6.2.3 (i) Blood sampling of recipient marmosets

Approximately 0.5 ml of blood was taken from the femoral vein of recipient female marmosets 2-3 times per week. The animal was held in a specially designed restraining device, and blood was withdrawn, from the femoral vein, with a 25 gauge needle attached to a heparinised 1 ml syringe. After blood sampling, the animals received 0.5 ml of iron syrup (Fersamal; Centaur, Castle Cary, Somerset, U.K.) orally, as a reward. The blood sample was centrifuged in the syringe casing at ~2500 r.p.m. for 10 minutes. Plasma was aspirated

using a glass pasteur pipette and placed in plastic tubes (Philip Harris Scientific, London, U.K). The plasma samples were stored frozen at -20 °C.

6.2.3 (ii) Progesterone assay

Progesterone levels in the peripheral plasma of recipient females were monitored by enzyme-linked immunosorbent assay (ELISA), developed for marmoset monkeys (Hodges et al 1988). Progesterone ELISAs were carried out weekly. The day of ovulation (Day 0) was determined as the day before progesterone levels rose above 10 ng/ml (Harlow et al 1983). Progesterone levels could be expected to remain high (above 50 ng/ml) throughout the luteal phase (18-19 days post-ovulation; Hearn 1983). High progesterone levels after Day 18-19 would normally indicate the establishment of pregnancy (Hearn 1983).

6.2.3 (iii) Luteinizing hormone/chorionic gonadotrophin bioassay

Chorionic gonadotrophin in the peripheral plasma was measured by mouse Leydig cell bioassay (Van Damme et al 1974, Hearn et al 1988). Briefly, marmoset plasma is incubated with mouse Leydig cells. Chorionic gonadotrophin (CG) and/or luteinizing hormone (LH) stimulates Leydig cells to produce a quantity of testosterone proportional to the amount of CG/LH in the sample. By measuring the level of testosterone by radioimmunoassay,

after co-incubation, the level of LH/CG in the peripheral plasma can be deduced.

The level of CG was expected to rise sharply around the day of ovulation due to the administration of exogenous human CG (hCG), used to synchronise recipient animals. Levels of CG would then drop to baseline levels (< 20 mIU/ml), and remain low if a pregnancy was not established (Hearn et al 1988). If a pregnancy was established CG should be detected in the peripheral plasma from around Day 13-14 (30 mIU/ml) and would continue to rise to levels of 1000 mIU/ml by day 60 of gestation (Hearn et al 1988). After day 60, CG levels begin to fall (Hearn et al 1988).

6.2.3 (iv) Inhibin assay

The concentration of ir-inhibin in the peripheral plasma of recipient marmosets was measured by Dr. P. G. Knight, Department of Biochemistry and Physiology, University of Reading. The assay was carried out as described by Webley et al (1991b), with some modifications. Briefly, levels of ir-inhibin were measured by radioimmunoassay (RIA), using antiserum to the N-terminal sequence of the α subunit of human inhibin, raised in sheep. In the original protocol, the tracer was monomeric inhibin α subunit. For this study the 32 kilodalton dimer of inhibin was used as the tracer.

6.2.4 Sacrifice of recipient female marmosets

Recipients which had high (> 50 ng/ml) progesterone until at least Day 26 (controls) or Day 33 (recipients of parthenogenetic embryos) were considered to be pregnant and these animals were sacrificed on Day 26 or 33 of pregnancy, respectively. The females were anaesthetized with 1 ml of Saffan (~ 2.5 ml/kg body weight; Centaur, Castle Cary, Somerset, U.K.) administered by intramuscular injection. Once under general anaesthesia, the peritoneum and thoracic cavity were opened. The ovaries were inspected for the presence of corpora lutea (CL). A 21 gauge butterfly catheter (Centaur, Castle Cary, Somerset, U.K.), which was connected to a 50 ml syringe, was inserted into the left ventricle of the heart and held in place with artery forceps. The vena cava was cut posterior to the renal veins. The animal was perfused with heparinised (1,000 iu/ml) phosphate buffered saline (200 ml; PBS; Merck, Lutterworth, Leics, U.K.), delivered through the catheter. When most of the blood had been replaced by heparinised PBS, 200 ml 2.5 % glutaraldehyde was delivered through the same catheter. After fixation of the animal, the uterus was dissected and stored in 2.5 % glutaraldehyde until it could be sectioned.

6.2.5 Sectioning and staining of marmoset uteri

Sectioning of marmoset uteri was carried out by the Department of Histopathology at the Royal Free Hospital School of Medicine (London). Three micron thick paraffin wax sections were cut (Fig. 6.1) and every fifth

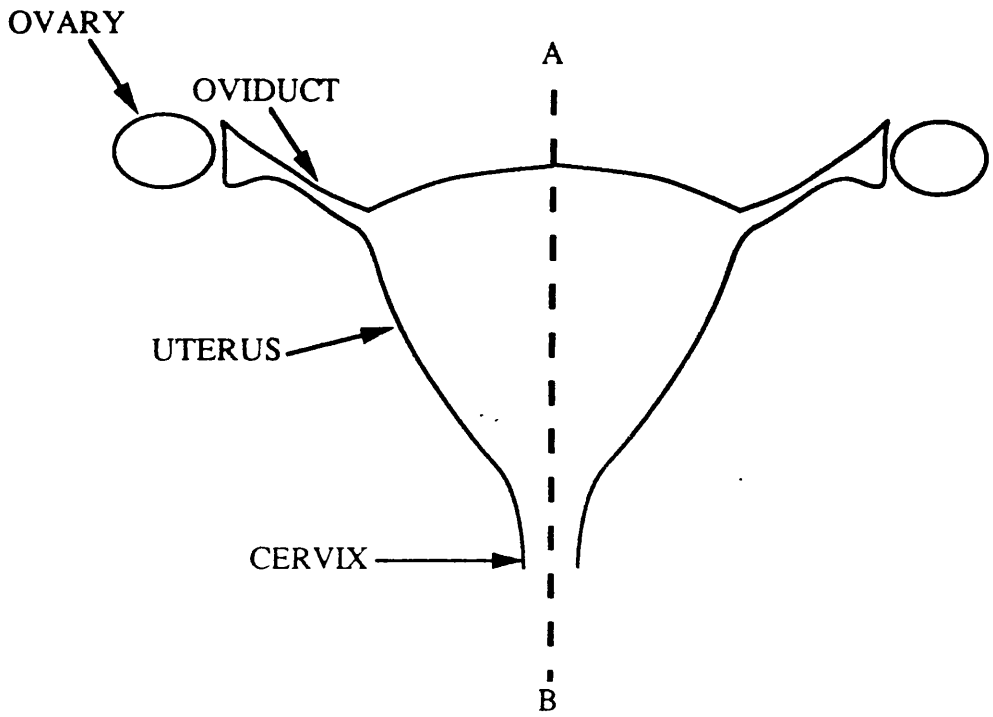

Figure 6.1 : Diagram of marmoset uterus and the direction of sections cut for histological analysis (A-B).

section was mounted on a glass slide and stained with haematoxylin and eosin (H & E). Sections were inspected under bright field microscopy and relevant sections were photographed.

6.3 RESULTS

6.3.1 Endocrine profiles of recipient marmosets after transfer of biparental *in vitro* fertilized marmoset embryos

6.3.1 (i) Progesterone

The levels of progesterone in the peripheral plasma of both recipient animals (345W and 457W) rose steadily until about 10-12 days after ovulation when levels started to plateau at about 100-120 ng/ml (Fig. 6.2 and 6.3). Progesterone levels remained high until day 26 when the animals were sacrificed.

6.3.1 (ii) Chorionic gonadotrophin

Levels of CG rose around the day of ovulation (Day 0), due to the administration of exogenous hCG used to synchronise the recipient animals (Fig. 6.2 and 6.3). CG levels then dropped to baseline levels (<40 miu/ml) by Day 10-12. By Day 16-18 CG levels were beginning to rise and reached levels of at least 100 miu/ml before dropping again to about 80 miu/ml when the animals were sacrificed (Fig. 6.2 and 6.3).

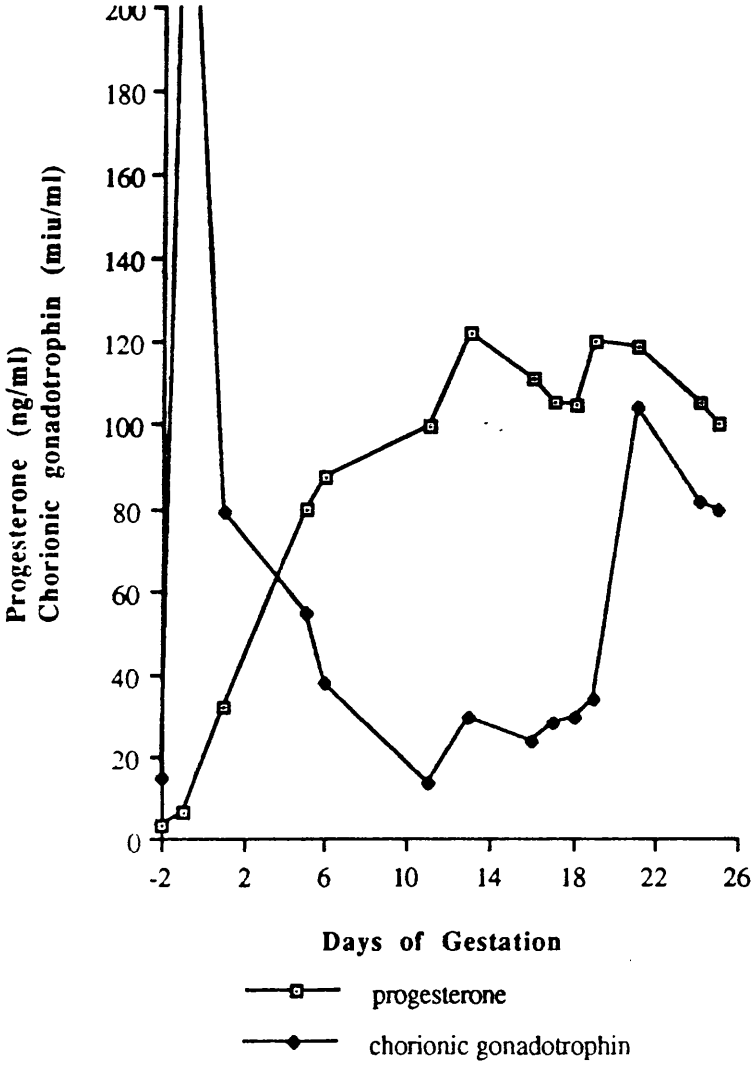

FIGURE 6.2 : Progesterone and chorionic gonadotrophin profile of marmoset (345W) after transfer of a normal embryo at the 4-cell stage.

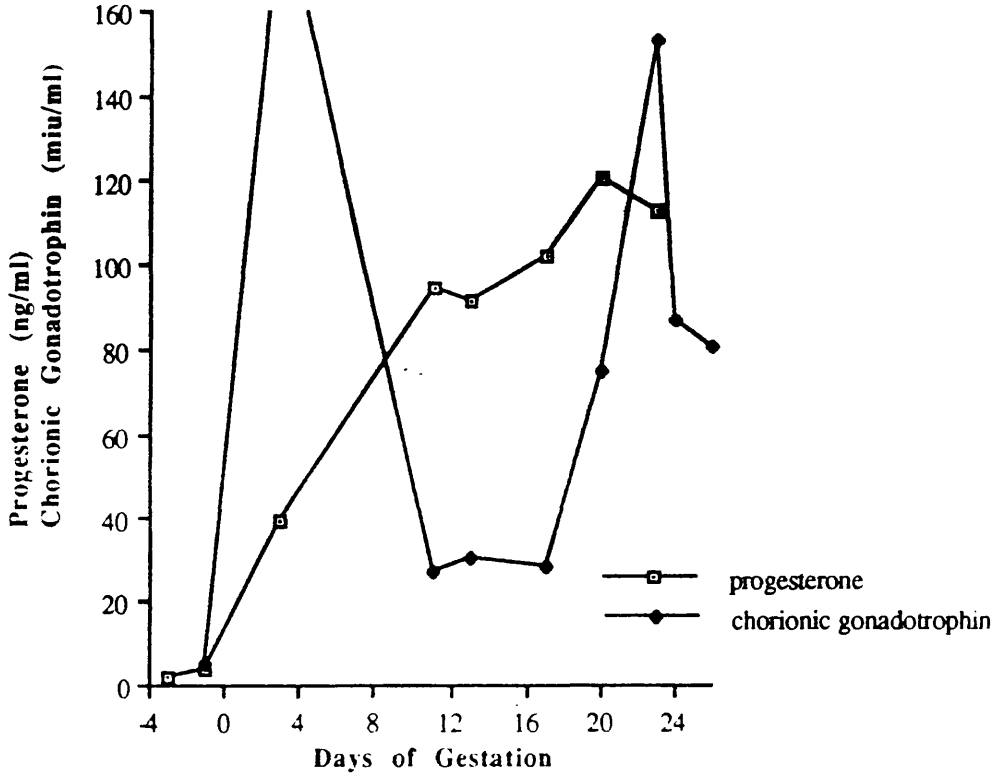

FIGURE 6.3 : Progesterone and chorionic gonadotrophin profile of marmoset (457W) after transfer of a normal embryo at the 4-cell stage.

6.3.2 Endocrine profiles of recipient marmosets after transfer of parthenogenetic embryos

6.3.2 (i) Progesterone

Progesterone levels rose to over 10 ng/ml on Day 1 after ovulation. In one recipient animal (323W), levels of progesterone in the peripheral plasma rose steadily until Day 6 after ovulation (Fig. 6.4). Progesterone levels plateaued at 70-100 ng/ml until about Day 15 after ovulation, when they started to drop, and were back to baseline (follicular phase levels) by Day 22 after ovulation. On the basis of the progesterone profile, this animal (323W) was considered non-pregnant. In the other two recipient animals (469W and 491W), a quite different profile occurred (Fig 6.5 and 6.6). Progesterone levels rose to about 120 ng/ml by Day 13-16, and remained above 60 ng/ml until Day 33. These two animals were considered pregnant due to the maintenance of high levels of progesterone for almost twice the length of a normal luteal phase and were killed on Day 33. The maintenance of high progesterone levels for 33 days after ovulation would suggest that the corpus luteum (CL) was maintained up to Day 33. This was confirmed visually when the recipient marmosets were killed.

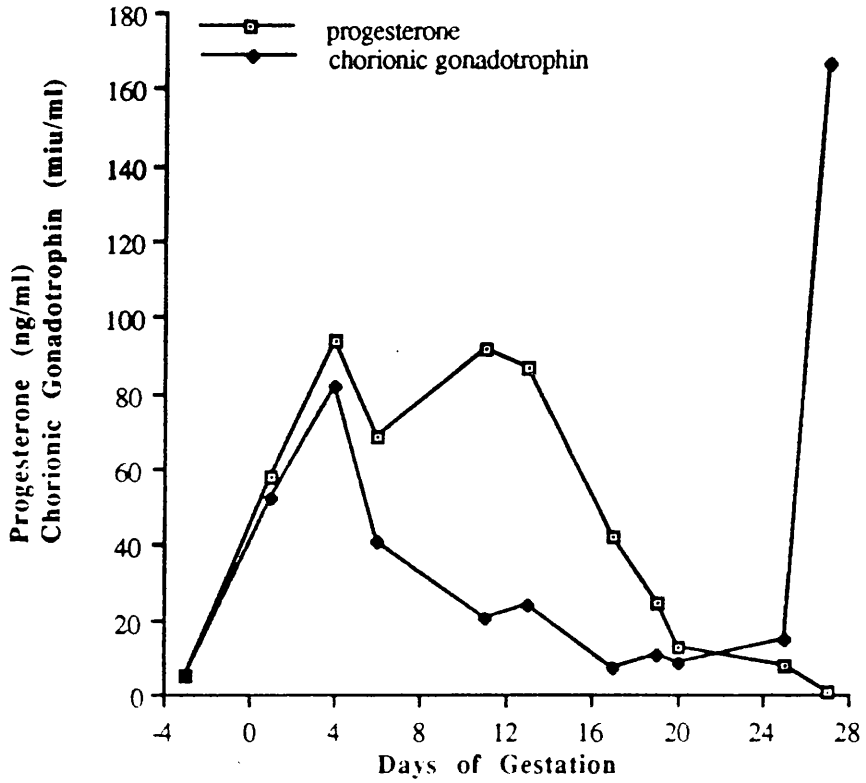

FIGURE 6.4 : Progesterone and chorionic gonadotrophin profile of marmoset (323W) after transfer of a parthenogenetic embryo at the 4-cell stage.

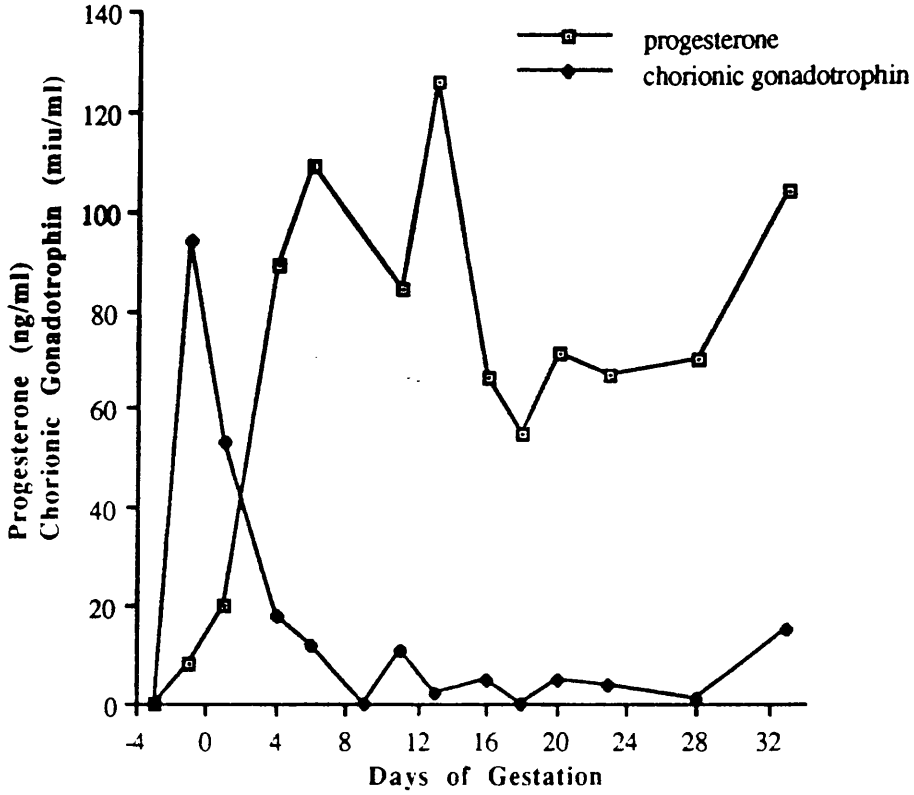

FIGURE 6.5 : Progesterone and chorionic gonadotrophin profile of marmoset (469W) after transfer of a parthenogenetic embryo at the 4-cell stage.

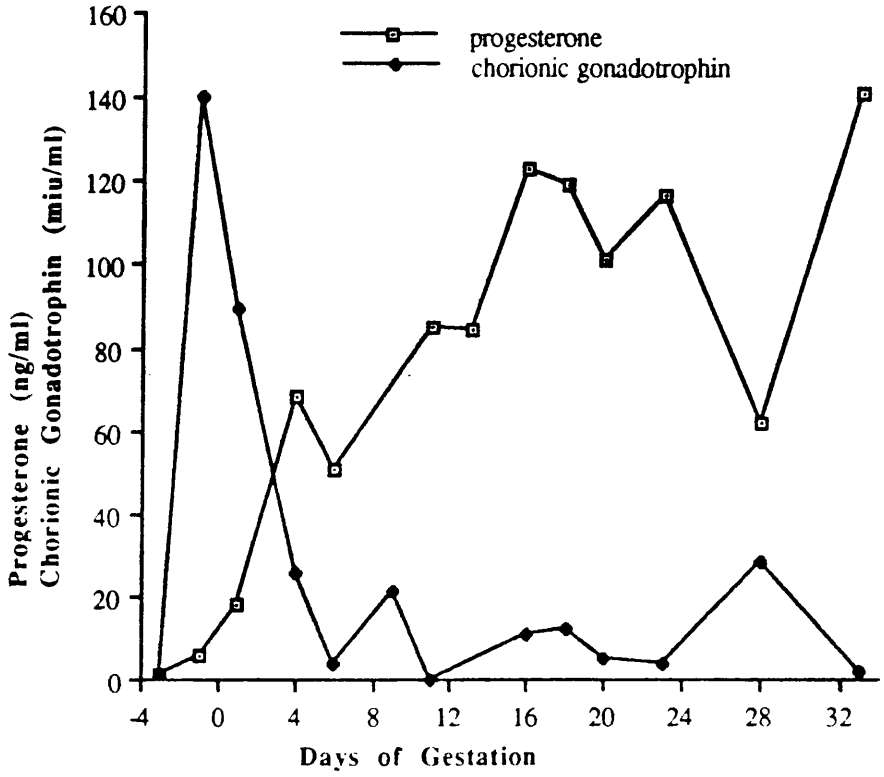

FIGURE 6.6 : Progesterone and chorionic gonadotrophin profile of marmoset (491W) after transfer of a parthenogenetic embryo at the 4-cell stage.

6.3.2 (ii) Chorionic gonadotrophin

CG levels in the animal considered non-pregnant by the progesterone profile (323W) dropped to baseline levels (below 30 mIU/ml) by Day 12 and remained low until a second peak was recorded on Day 30 (Fig. 6.4). As the bioassay measures both CG and LH, the second peak coincided with the expected endogenous LH surge before the next ovulation. The animals considered pregnant by progesterone levels (469W and 491W), showed a peak of CG/LH around the day of ovulation, which corresponded to the administration of exogenous hCG (Fig. 6.5 and 6.6). However, by Day 4 after ovulation, CG/LH levels dropped to baseline and remained low until Day 33 (Fig. 6.5 and 6.6). Baseline levels of CG would normally indicate a non-pregnant animal, since CG is initially produced by the embryo (reviewed by Webley and Hearn 1994) and can be used as an early indicator of pregnancy. Because the progesterone profiles and CG profiles for these two animals did not seem to correlate ir-inhibin was measured in addition to determine the status of recipient animals (Section 6.1).

6.3.3 Inhibin levels in animals receiving either normal or parthenogenetic embryos

Previous studies, using multiple students' *t* tests to compare between ir-inhibin levels at specific time points, have shown that a significant difference between conception and non-conception cycles can be detected from Day 8/9 after

ovulation (Webley et al 1991b). Another method of analysing changes in data levels over time is to measure the area under the curve (AUC). The AUC provides a single measure for each set of data and is more appropriate for the analysis of this data because of the small number of animals involved. A comparison was made of the AUC for one non-pregnant animal (323W), two normal pregnancies (345W and 467W), and the two animals which received parthenogenetic embryos (469W and 491W) (Fig. 6.8). Ir-inhibin levels during the first 26 days after transfer were 52 % and 120 % higher in the peripheral plasma of animals which received parthenogenetic embryos than both the normal pregnancies and the non-pregnant animal, respectively (Fig. 6.8). Ir-inhibin levels in the peripheral plasma of normal pregnant animals was ~45 % higher than the non-pregnant animal (Fig. 6.8).

6.3.4 Histological evidence for implantation of normal and parthenogenetic embryos

6.3.4 (i) Histology of uteri of recipient female marmosets which recieved normal embryos

The morphology of the normal embryos at early implantation stages after transfer to recipient females was similar to that described previously (Moore et al 1985). Placental membrane (cytotrophoblast) displayed extensive but superficial attachment to the luminal surface of the endometrial epithelium, covering almost the entire uterine lumen. A typical cross-section of the uterus

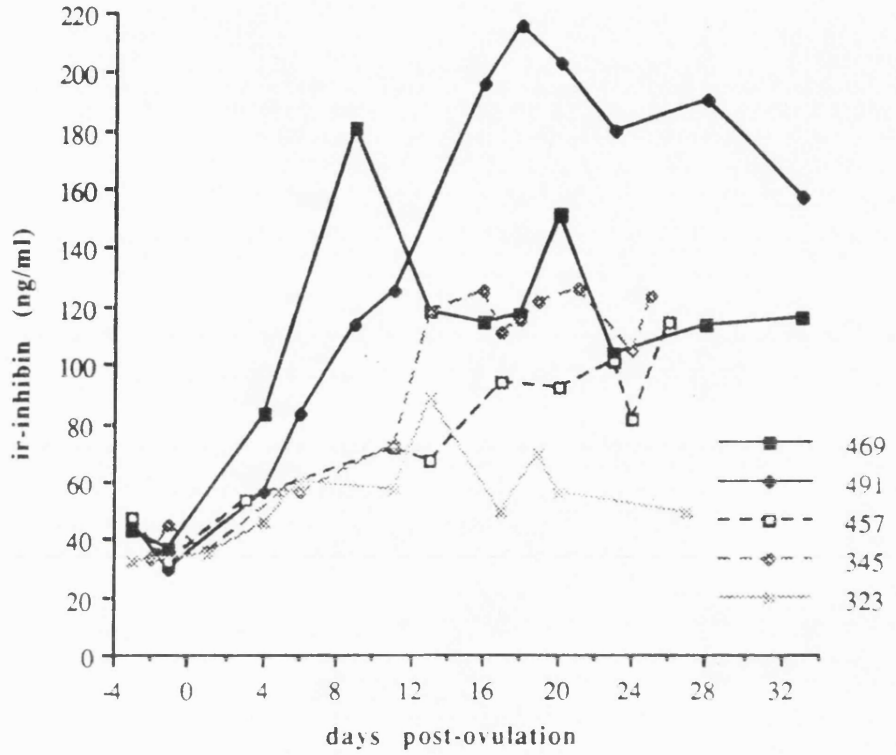

FIGURE 6.7 : Ir-inhibin profiles of recipient marmosets after transfer of normal (345W and 457W) and parthenogenetic (323W, 469W and 491W) embryos at the 4-cell stage.
All recipients except 323W were believed to be pregnant.

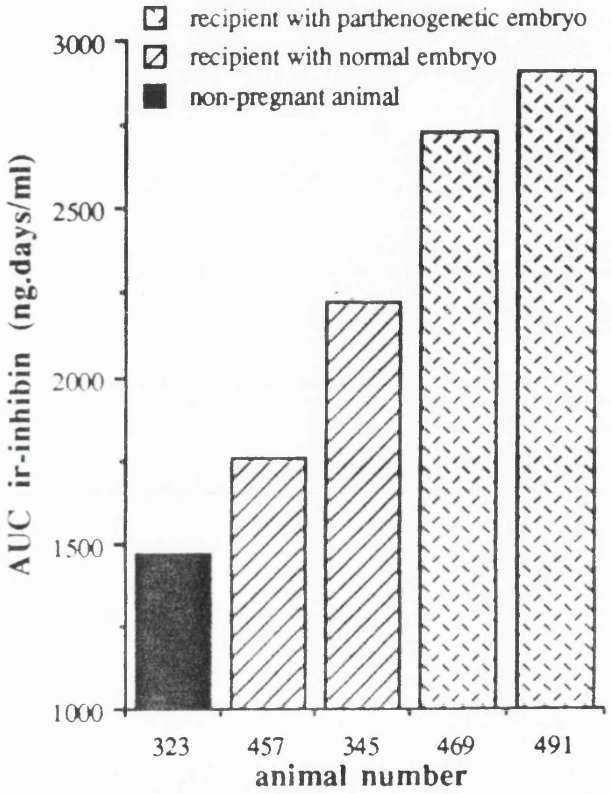

FIGURE 6.8 : Area under the curve (Fig. 6.7) of ir-inhibin in recipient marmosets from 2 days pre-ovulation to 26 days post-ovulation.

of the pregnant females 26 days after ovulation is shown in Figure 6.9. The chorionic membrane is attached to the luminal endometrium and the developing fetus (with amniotic sac) is clearly visible. For comparison, the uterine lumen of a non-pregnant female in the luteal stage of the cycle is shown in Figure 6.10. At higher magnification, the invasion of the syncytiotrophoblast into the stromal tissue of the endometrium can be seen as 'finger-like' projections of tissue displaying multinucleated cells typical of syncytium. This cell layer surrounds the blood vessels underlying the apical endometrial epithelium which has undergone a typical hypertrophy and proliferative response (Fig. 6.11). By comparison, the apical endometrium of a non-pregnant marmoset shows no hypertrophy of blood vessels and the endometrium is of regular appearance (Fig. 6.12). The decidual reaction in the marmoset monkey is minor compared to that of other primates (Moore et al 1985), so the lack of a decidual reaction seen in Figure 6.11 is not necessarily an indication of a non-pregnant animal.

6.3.4 (ii) Histology of uteri of recipient female marmosets which received parthenogenetic embryos

In the two females which displayed biochemical evidence of pregnancy (469W and 491W; Section 6.3.2), there was also histological evidence of implantation although in both cases the morphology was distinct from that of pregnant females which received normal embryos. In cross-sections of the uterus of

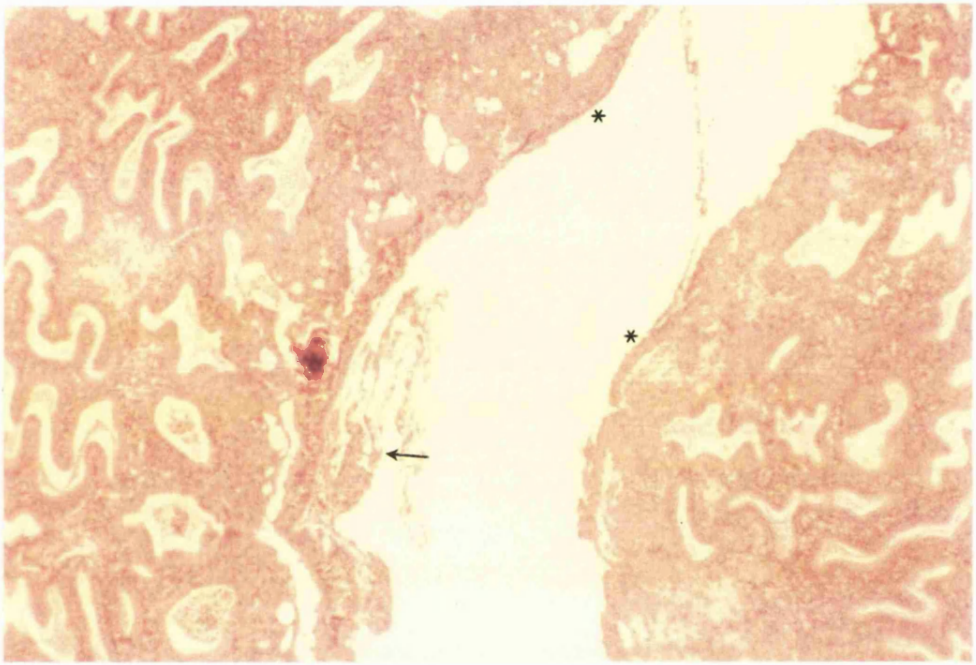

FIGURE 6.9 : Cross section of the uterine lumen and epithelium of a marmoset on Day 26 of pregnancy (normal embryo). The fetal disc and yolk sac is present (arrowed). The chorionic membrane is attached to the endometrium on the embryonic and ad-embryonic face (*). Syncytiotrophoblast has invaded the apical region of the endometrium and new blood vessels have formed. Haematoxylin and eosin, x 140.

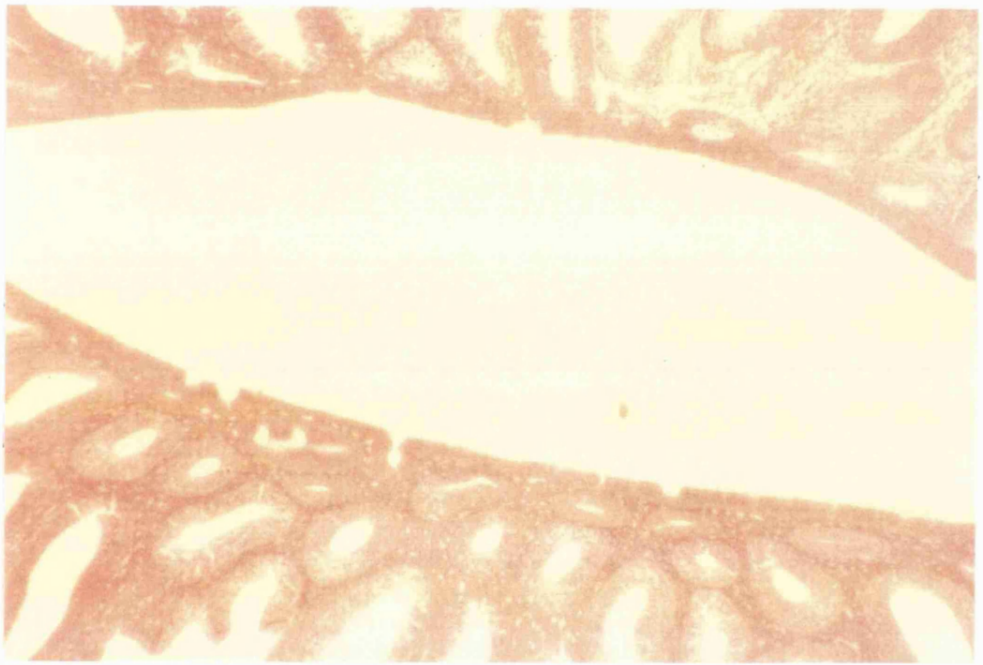

FIGURE 6.10 : Cross section of the uterine lumen of a non-pregnant marmoset. Note the regular appearance of the endometrial epithelium. Haematoxylin and eosin, x 140.

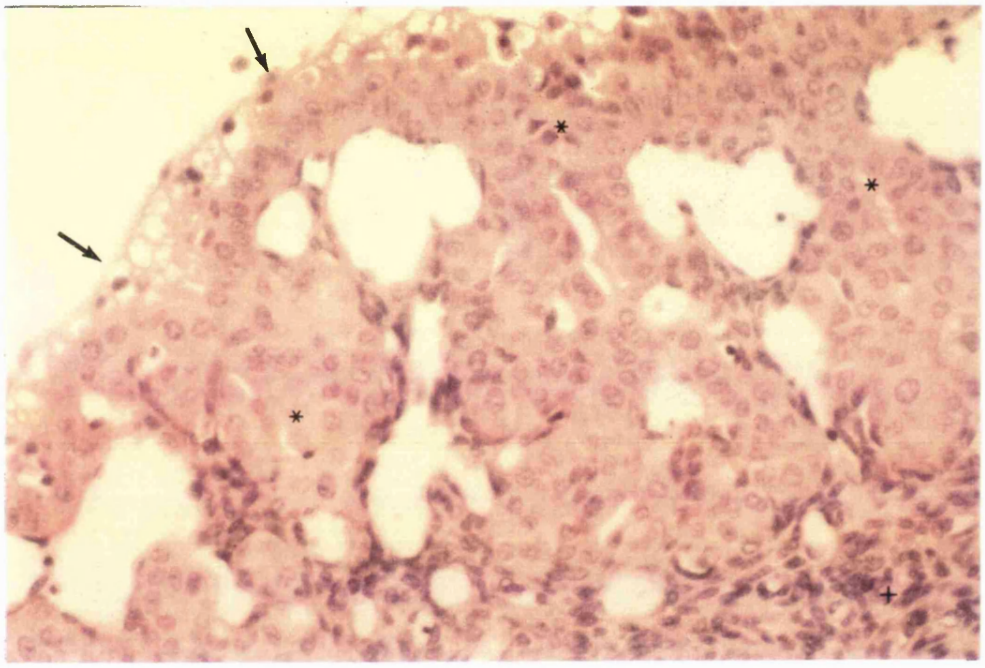

FIGURE 6.11 : Cross section of the apical implantation site of a normal embryo on Day 26 of pregnancy. The characteristic chorionic membrane (arrowed) is present on the apical surface of the endometrium while "fingers" of syncytiotrophoblast (*) have invaded the endometrial stroma and surrounded the underlying blood vessels. The latter have undergone a typical hypertrophy and proliferative response. Interstitial stromal cells are present basally(+). Haematoxylin and eosin, x 420.

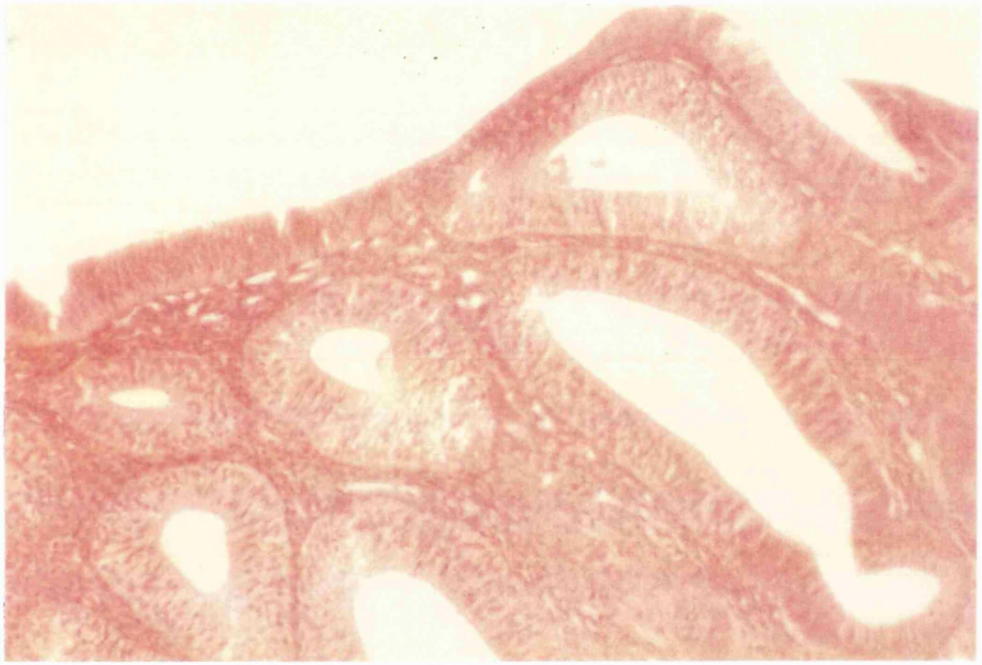

FIGURE 6.12 : Cross section of the apical endometrium of a non-pregnant marmoset. Note the relative lack of blood vessels and the regular appearance of the epithelium. Haematoxylin and eosin, x 420.

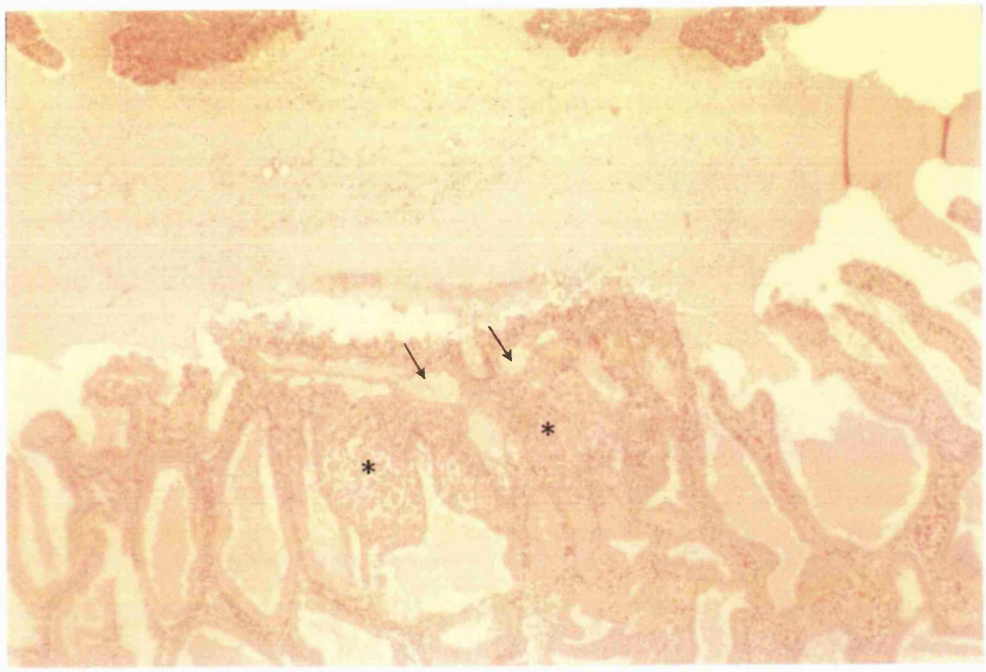

FIGURE 6.13 : Cross section of the uterine epithelium of a marmoset (491W) on Day 33 post-ovulation after receiving a parthenogenetic embryo. An implantation site is present on one face of the endometrium although there is no fetus present and only remnants of placental membranes within the lumen. Presumptive syncytiotrophoblast has invaded the stroma (*) and blood vessels have proliferated at the implantation site (arrow). Haematoxylin and eosin, x 240.

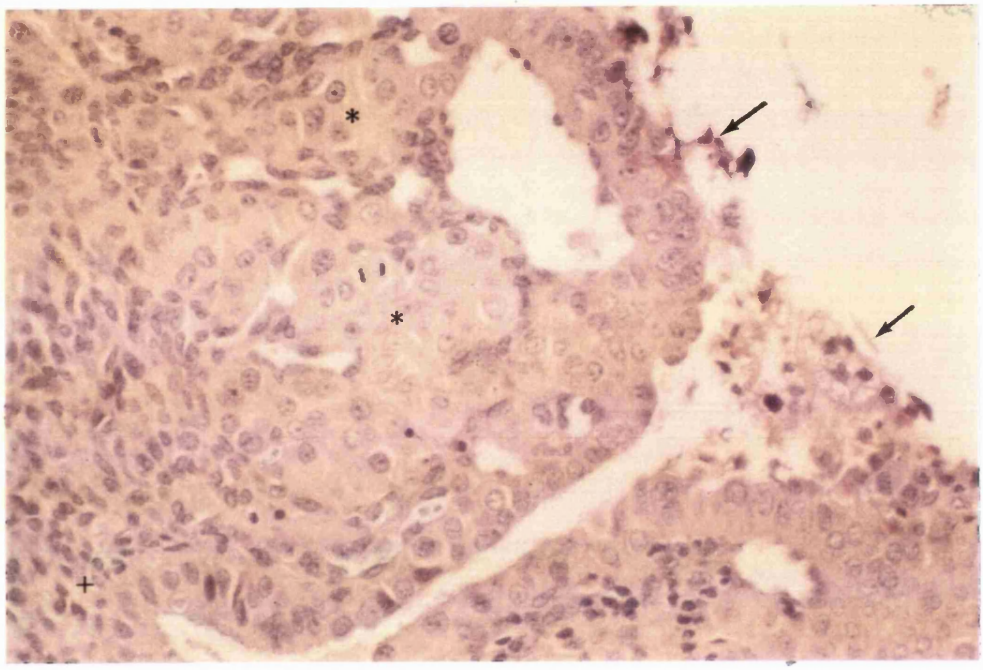

FIGURE 6.14 : Cross section of the implantation site (Fig 6.13) at higher magnification. Degenerative membrane is present at the luminal surface of the endometrium (arrowed). "Fingers" of syncytiotrophoblast (*) have invaded the stroma and surrounded the blood vessels underlying the epithelium. Interstitial stromal tissue is present basally (+). Note the similarity to Figure 6.11. Haematoxylin and eosin, x 420.

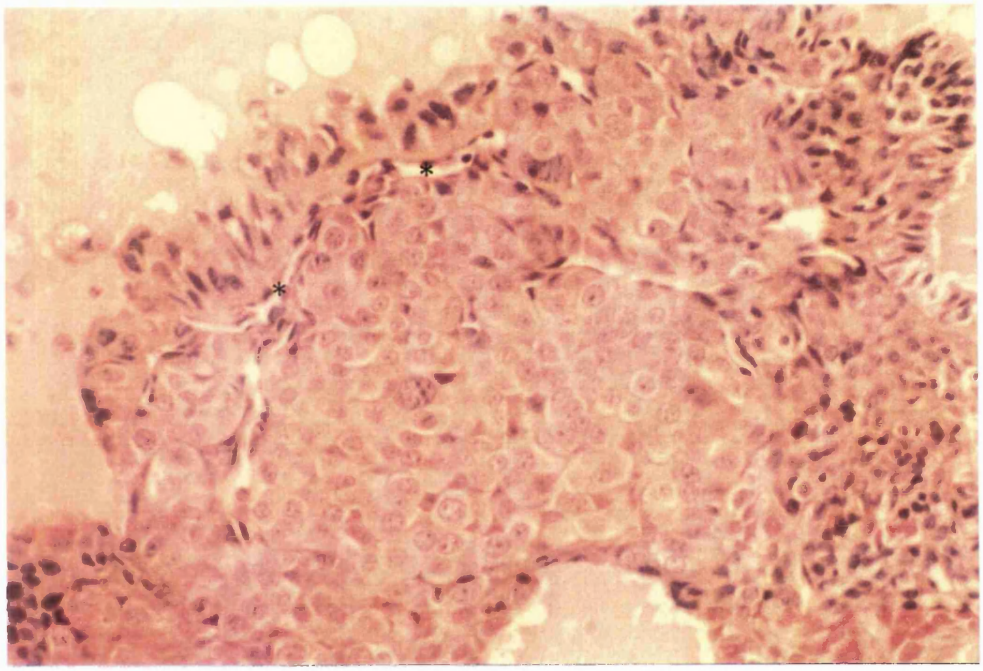

FIGURE 6.15 : Cross section of part of the implantation site of marmoset 469W on Day 33 post-ovulation after receiving a parthenogenetic embryo, at high magnification. Sloughed cells, possibly of placental tissue, are present in the lumen and on the apical endometrial endothelium which has a disorganised appearance. Syncytial cells are present in the stroma. Blood vessels are present but have collapsed (*). Haematoxylin and eosin, x 420.

female 491W a plaque of tissue was observed near the apical surface of the endometrium and presumptive syncio trophoblast is surrounding blood vessels underlying the epithelium (Fig. 6.13). At higher magnification, syncytial tissue in the stroma, similar to that seen in the normal pregnant animals, was observed. Although remnants of membranes were present on the surface of the endometrium (Fig. 6.14, arrow) a definite membrane could not be determined. There was no discernible fetal tissue. Cross-sections of the uterus of female 469W also exhibited a plaque of putative trophectoderm (Fig. 6.15) and a more distinct decidual reaction. Epithelium in the vicinity of the plaque was disorganised, but neither placental membranes nor fetal tissue were observed. Syncytium could be seen underlying the endometrial epithelium and this also had very similar histology to that seen in normal pregnant animals.

6.4 DISCUSSION

This chapter describes the endocrine response of recipient females to the transfer of either normal or parthenogenetic marmoset embryos, and the subsequent development of these embryos *in vivo*.

Histological analysis of recipient uteri showing invasion of syncytium into the stromal tissue of the endothelium indicated that implantation of parthenogenetic marmoset embryos occurred. Two of three parthenogenetic embryos transferred to recipient females developed beyond pre-implantation

stages. Post-implantation development of parthenogenetic marmoset fetuses appeared to be minimal. Only remnants of membranous material were present in the uterine lumen 33 days after ovulation. However, these remnants and the extent of syncytiotrophoblast invasion into the uterine stroma indicate that a developing conceptus was present for at least some time after implantation. At what stage embryonic loss occurred is difficult to determine purely from the extent of syncytial invasion because it is possible that this invasion continued after loss of the embryo proper. To find out more about the extent and morphology of parthenogenetic development in marmosets after implantation, it would be necessary to sacrifice recipient animals at a series of earlier stages of gestation.

For marmoset parthenogenetic embryos transferred to recipient animals, the endocrine and histological results described in this chapter appear to be contradictory. In marmosets pregnant with normal embryos, CG from the embryo provides a luteotrophic action, inducing increased levels of luteal progesterone. Inhibin levels rise significantly higher than in non-pregnant animals by day 8 of gestation, and histological investigation shows development of a fetus and extensive proliferation of the extra-embryonic membranes. However, the results accumulated for the endocrine response of recipient marmosets receiving parthenogenetic embryos do not strictly follow this pattern. Although progesterone and inhibin levels were maintained at high

levels, suggesting continued luteotrophic support, the major luteotrophin provided by the embryo, CG, remained at baseline (non-pregnant) levels. Despite these low levels of CG, parthenogenetic embryos still implanted as determined by the histological evidence.

It is possible that enough CG was produced by marmoset parthenogenones to reach the ovary and cause a luteotrophic effect, but that CG was not produced in sufficiently high quantities to be measurable in the peripheral circulation. Webley et al (1989) have shown that marmoset luteal cells are exposed to an unknown luteotrophin 2 days after implantation. This is before CG is measurable in the peripheral circulation. These workers propose that the CL may be able to respond to extremely small amounts of CG, but do not rule out the possiblity that the CL has been maintained by some other luteotrophic or anti-luteolytic factor whilst CG builds up to high enough concentrations to provide luteotrophic support (Webley et al 1989). The latter possibility is supported by evidence from inhibin studies in marmosets. In contrast to the production of inhibin by the embryo in humans, inhibin is thought to be produced by the CL in marmosets (Webley and Hearn 1994). In marmoset monkeys there is a significant increase in inhibin production by day 8 after ovulation in conception cycles compared to non-conception cycles. The production of CG by marmoset embryos is undetectable both *in vitro* and *in vivo* at this early stage of development (Webley et al 1991b). It is possible that

another unknown factor is produced by the embryo at pre-implantation stages which causes this significant rise in inhibin levels.

Alternatively, CG may not be produced by parthenogenetic embryos at all. In the face of very strong evidence that CG is required to prevent luteolysis to enable implantation and post-implantation embryonic development in primates (reviewed by Webley and Hearn 1994), could the CL be maintained in the apparent absence of chorionic gonadotrophin from the embryo? If so, then there must then be some other factor produced by the embryo which causes a luteotrophic effect. There have been some suggestions for other "maternal recognition" factors such as early pregnancy factor (Morton et al 1977), platelet-activating factor (O'Neill 1985) and histamine releasing factor (Cocchiara et al 1987). Further investigation would be necessary to determine whether any of these factors are playing a role in the luteotrophic support of marmoset embryos.

Why wouldn't marmoset parthenogenetic embryos produce CG? In humans the β-subunit of CG is situated on chromosome 19 (Solomon and Rawlings 1991), an area syntenic to the distal part of mouse chromosome 7 (Hall 1990), which has some areas known to be imprinted (Cattanach 1986). De Groot et al (1993) have shown that the abundance of total β-subunit of CG is proportional to the number of paternal genomes carried by hydatidiform moles

(2 paternal), partial moles (2 paternal, 1 maternal), triploid conceptuses (2 maternal, 1 paternal) and normal conceptuses (1 paternal, 1 maternal). Therefore, there is some circumstantial evidence that the gene for the β-subunit of CG may be imprinted and only expressed from the paternal chromosome (Haig 1993, De Groot et al 1993). If this is the case then parthenogenetic embryos would not be expected to produce CG as they do not carry a paternal genome.

The implantation of parthenogenetic marmoset embryos supports the contention, made earlier in this chapter (Section 6.1), that the poor development of marmoset parthenogenones *in vitro* may be due to suboptimal culture conditions rather than inherent genetic effects. A reliable method of culturing marmoset embryos to blastocyst would provide a means to study both the production of CG and the expression of the gene for the β-subunit of CG by peri-implantation stage marmoset parthenogenones. Additionally, the expression of other factors liable to act as embryonic signals for maternal recognition could be examined.

Only very small numbers of animals were available to provide information about the development of marmoset parthenogenetic embryos *in vivo*, and the response of recipient animals. However, to validify the results several parameters of development were measured. Not only were the responses of

marmosets which received parthenogenetic embryos different from animals receiving normal embryos, both animals which received parthenogenetic embryos had a similar response.

Further investigation must be carried out to demonstrate whether the interesting preliminary findings reported in this chapter are statistically valid. If marmoset parthenogenetic embryos do not produce CG they may provide an important model system for further research into the maternal recognition of pregnancy in primates.

CHAPTER SEVEN

CONCLUSIONS

7.1 CONCLUSIONS

Genomic imprinting is a phenomenon whereby some genes are expressed differently depending on whether they are maternally or paternally inherited. For the past ten years, genomic imprinting has been extensively studied in the mouse and striking morphological differences can be found between embryos containing only maternal or only paternal chromosomes (Chapter 1). Imprinting also bestows effects on specific chromosome regions and, to date, four genes have been identified which have different expression states depending on which parent they have been inherited from. In the mouse, the most notable effects of genomic imprinting appear to be imposed during embryonic development, in some cases causing prenatal lethality.

In humans, it is difficult to study the effects of genomic imprinting on development for indisputable ethical reasons. However, a role for genomic imprinting in human early embryonic development can be deduced from the analysis of hydatidiform moles, and the development of diandric and digynic triploid fetuses. Clearly, in humans, studies involving the deliberate manipulation of the genome and subsequent embryo transfer are totally unacceptable. But it is important to discover whether the remarkable effects of genomic imprinting on embryonic development in the mouse are paralleled in primates.

The aim of this study was to determine the effects of genomic imprinting on the early embryonic development of a non-human primate, the common marmoset monkey. To achieve this aim a number of techniques were required, some of which had never been developed in any other primate species. The limited availability of marmoset gametes and embryos necessitated maximum efficiency of all procedures.

In vitro fertilization of marmoset embryos had been developed and fertilization rates of 53% had been achieved (Lopata et al 1988). However, by altering the time between the administration of hCG and laparotomy, and duration of oocyte pre-incubation, it was possible to increase the fertilization rate to 76% ($p<0.005$; Chapter 2, Wilton et al 1993). Constraints due to the small numbers of marmosets available for oocyte collection and the limited time available for this study curtailed the development of an appropriate *in vitro* culture medium. However, the transfer of marmoset embryos into the oviducts of live mice doubled the mean maximum cell number from 7.7 (± 0.7) to 15 (± 4.35; $p<0.003$). Bavister et al (1983) reported that complex media (TC-199 and Ham's F-10) retarded the development of rhesus monkey embryos after IVF. It is possible that the development of marmoset embryos was compromised by culture in another complex media, MEM. It has also been recently reported that the presence of glucose in media for culture of early human IVF embryos may have a detrimental effect on development to the

blastocyst stage (Conaghan et al 1993). These are the type of changes which may lead to the development of a suitable culture medium for marmoset embryos.

To produce uniparental embryos by pronuclear transfer, it is necessary to identify the parental origin of the pronuclei. In mouse embryos the maternal pronucleus is identifiable due to its smaller size and proximity to the second polar body. I found that the parental origin of marmoset pronuclei could not be determined visually (Chapter 4). Both marmoset pronuclei formed approximately 6 hours after insemination, they were both the same size, and they first became visible near the centre of the zygote. After fertilization using gametes stained with a polyspecific fluorochrome, it was not possible to visualize fluorescent pronuclei.

Pronuclear identification might be possible using the following techniques. Firstly, it may be possible to irradiate marmoset oocytes, making the maternal genome functionally inactive before fertilization. It is not known whether this procedure would compromise fertilization of primate oocytes, but if fertilization did take place, an androgenetic embryo would result. For experiments concerning genomic imprinting, this procedure would require extensive validation to ensure that the maternal chromosomes had been made completely functionally inactive. Additionally, it would be necessary to ensure

that the subsequent development of these androgenetic embryos was not modified by the initial irradiation process, rather than the genetic constitution of the embryo.

A second method of determining the parental origin of marmoset pronuclei would be to ascertain this information in retrospect. It may be possible to remove a single pronucleus from the embryo and, using polymerase chain reaction (PCR), accumulate enough DNA to identify the parental source by microsatellite analysis. If existing primers for the human genome were not appropriate, it would be necessary to design primers specifically for the common marmoset. This would involve making a DNA library, scanning the library for positive clones, sequencing the clones, and using the sequences to make primers for the flanking regions of microsatellites. Primers for highly polymorphic microsatellites could then be used for PCR. Subsequent comparison of the size of the microsatellites in the pronuclear DNA of the embryo and the parental DNA may reveal the parental origin of the pronucleus. Clearly, this method requires extensive groundwork and was beyond the scope of this thesis.

Micromanipulation and electrical fusion of marmoset one-cell embryos was successful in 46% of embryos. Marmoset embryos which had undergone sham enucleation and were restored to a normal genetic constitution were able to

develop to the 8-cell stage. The results were encouraging as these procedures were only carried out on 15 marmoset embryos. It is likely that further experience to improve pronuclear transfer techniques and modification of the protocol for fusion will produce a system which is more efficient.

The approaches discussed above will provide a means to create primate embryos carrying only paternal chromosomes. However, the same extreme approach is not required for the production of primate embryos carrying only maternal chromosomes, as these embryos can be produced by parthenogenetic activation. I have developed a highly efficient protocol for electrical stimulation which can reliably activate 92% of marmoset oocytes.

The maternal genome alone can support development to the 16-cell stage in marmoset embryos. Marmoset parthenogenones developed to an average of 4 cells *in vitro*. There was no significant difference between the percentage of parthenogenetic embryos and normal IVF embryos reaching each cell stage up to 16 cells, although one IVF embryo reached 32 cells in culture (Fig. 7.1). On this basis the conclusion must be that the absence of a paternal genome has no effect on the extent of marmoset embryonic development in this *in vitro* system. It would be interesting to find out whether other factors such as gene expression are altered by the absence of the paternal genome at these early stages.

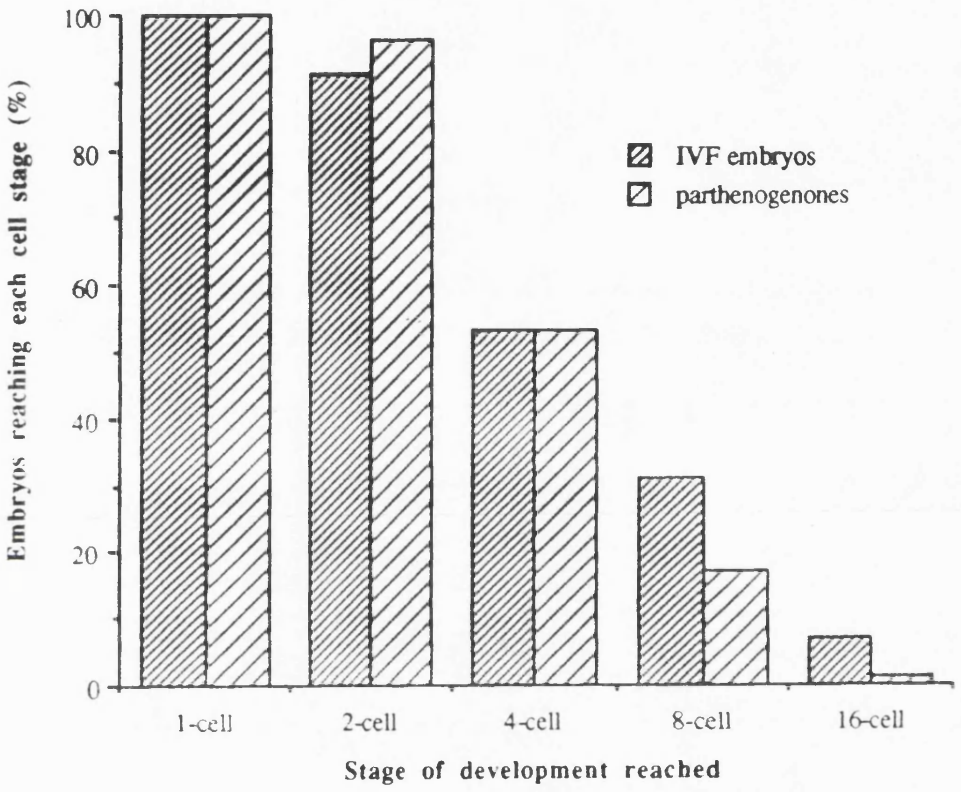

Figure 7.1 : The development of IVF marmoset embryos and marmoset parthenogenones in culture

The similarity between the extent of development of marmoset IVF embryos and marmoset parthenogenones indicate that the culture system may be affecting the extent of both normal and parthenogenetic development *in vitro*. This premise is also supported by evidence that 3 of 4 IVF embryos (Chapter 2), and 2 of 3 marmoset parthenogenones (Chapter 6) transferred to recipient marmosets developed to post-implantation stages. Again, an improved culture system designed for early marmoset embryos would significantly enhance the possibilities for further research using marmoset parthenogenones.

The endocrine response of recipient marmoset females to the transfer of parthenogenetic embryos is intriguing and invites further investigation. Did a parthenogenetic fetus develop at all? If so, was it lost at an earlier stage in gestation? Were the cellular remains, found in the uterine lumen, of fetal origin? These questions could be answered by collecting marmoset uteri carrying parthenogenetic conceptuses at a series of earlier stages in gestation to determine the presence or absence of a developing fetal component.

This study has shown that primate embryos carrying only maternal chromosomes can develop to post-implantation stages. It has been shown previously that mouse and rabbit parthenogenones can implant, and post-implantation development can progress to the 25 and 30 somite stage, respectively.

The mechanism for maternal recognition of pregnancy varies widely between species. In mice, the corpora lutea are maintained by prolactin secreted during coitus. The CL will be maintained for 10-11 days after coitus whether the female is pregnant or not. This is known as pseudopregnancy. The CL of pseudopregnancy will only be converted to the CL of pregnancy if lactogen is produced by the placenta of the developing conceptus. It is possible that the failure of murine parthenogenones to develop beyond 10 days of gestation is, in part, due to diminished placental lactogen, produced by extremely underdeveloped placental membranes, leading to luteal regression. This would also explain why parthenogenetic cells can be rescued in chimeras with normal embryos. That is, the normal cells provide a more extensive extra-embryonic component which is capable of producing enough lactogen for luteal maintenance.

It is possible that the abnormal development of marmoset parthenogenones *in vivo* was due to a lack of CG production. Production of an unknown embryonic signal prior to and around the time of attachment and implantation may have facilitated the implantation of marmoset parthenogenones. This early signal may have had a luteotrophic effect on the CL which, in turn, caused an increase in luteal inhibin production. Subsequent failure of parthenogenetic development may have been due to the lack of CG produced by parthenogenetic embryos. To test this theory it would be possible to

administer exogenous hCG to marmosets which had received parthenogenetic embryos, thus artificially mimicking the production of this hormone in normal pregnancy. Webley et al (1991a) have performed a similar experiment to show that exogenous CG can rescue the CL and hence stimulate increased progesterone production. This approach might promote further development of marmoset parthenogenetic embryos and the morphology of early primate parthenogenones could be analysed.

This study has shown that the development of marmoset parthenogenones to the 16-cell stage is not significantly different from normal IVF embryos. Additionally, implantation of primate embryos can occur without the participation of the paternal genome. By developing the techniques of pronuclear transfer, karyoplast fusion of one-cell primate embryos and parthenogenetic activation of primate oocytes, this study has provided the basis for further research to determine the role of CG in maternal recognition of pregnancy in primates and to elucidate the role of genomic imprinting in primate embryonic development.

APPENDICES

APPENDIX I : Avertin

1. Dissolve 1 mg of 2,2,2-tribromoethanol in 1 ml of butan-2-ol to make Stock solution.

2. Dilute 1.2 μl of Stock in 10 ml of sterile saline. The mixture may need to be warmed under a hot tap for the Stock solution to dissolve.

3. Inject ~0.04ml/g body weight intraperitoneally into mouse.

4. Full anaesthesia should take effect within 5-10 minutes.

APPENDIX II : M2 medium

	g/l
NaCl	5.533
$NaHCO_3$	0.349
KCl	0.356
KH_2PO_4	0.162
$MgSO_4.7H_2O$	0.293
$CaCl_2.2H_2O$	0.252
HEPES	4.969
Sodium lactate	4.349 g of 60% syrup
Sodium pyruvate	0.036
BSA	4.000
Penicillin (Potassium salt)	0.060
Streptomycin sulphate	0.050
D-Glucose	1.000
Phenol Red	0.010
H_2O	up to 1 litre

Weigh out Hepes and dissolve in 50 ml of water. Weigh out penicillin and streptomycin and dissolve in 50 ml water. Weigh out $CaCl_2$ and dissolve in 50 ml of water. Weigh out the remaining substances, apart from BSA and sodium lactate, into a one litre volumetric flask, and add 500 ml of water. Dissolve. Add the dissolved antibiotics, HEPES and calcium chloride to the volumetric flask. Add the sodium lactate, and add sufficient water to bring the volume up to one litre. Pour the contents of the voulmetric flask into a beaker and

sprinkle the BSA over the surface of the media. Allow to dissolve. If necessary, adjust the pH to 7.2-7.4. Check that the osmolarity is 285-287 mOsm.

APPENDIX III : Acid Tyrode's solution

	g/100 ml
NaCl	0.800
KCl	0.020
$MgCl_2.6H_2O$	0.010
$CaCl_2.2H_2O$	0.024
D-Glucose	0.100
PVP*	0.400
H_2O	up to 100 ml

* : Polyvinylpyrrolidone

Weigh out all the compounds into a 100 ml voulmetric flask and dissolve in water. Adjust the pH to 2.5 with HCl.

APPENDIX IV : Electrofusion medium

	g/l
Mannitol	54.66
$MgCl_2.6H_2O$	0.020
$CaCl_2.2H_2O$	0.014
H_2O	up to 1 litre

Weigh out all the compounds into a volumetric flask and dissolve in the water.

APPENDIX V : T6 Medium

	g/100 ml
NaCl	0.726
$NaHCO_3$	0.21
KCl	0.02
NaH_2PO_4	0.0056
Penicillin (Na)	0.006
$MgCl_2$	0.01
$CaCl_2$	0.0264
D-Glucose	0.1
Phenol Red	0.1
H_2O	up to 100 ml

Weigh out $CaCl_2$ and dissolve in 10 ml of H_2O. Weigh out remaining compounds into a 100 ml volumetric flask and dissolve in 50 ml of the H_2O. Add dissolved $CaCl_2$, and make the volume up to 100ml.

Check that the osmolarity is 288-290 mOsm.

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