This thesis is dedicated to my dear family; my parents (Theresa and Robert Pereira) my sisters (Sheela-Celine & Molly) and my husband (Kevin).

Are treatment strategies designed for acute cerebral ischaemic strokes hazardous or beneficial in cerebral haemorrhage?

Experimental findings from an animal model of intracerebral haemorrhage: Reproducibility of the model & Assessment of Treatment Strategies.

This work is submitted
for the Degree of MPhil

by

Stella Pereira-Jennings

Registered as a candidate of
University College, London
University of London.

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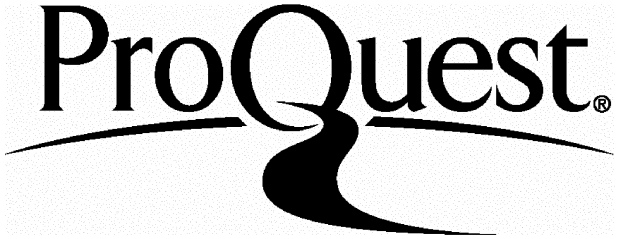

ABSTRACT

Haemorrhagic stroke, although of less frequent occurrence, has the highest mortality rate. In the emergency situation where a patient is suspected of having a stroke, immediate therapeutic strategies have been proposed to stabilise the clinical status and prevent further deterioration. However, these regimes are modelled on ischaemic strokes.

The plan of the project was to determine the possible outcomes of some of these treatments; osmotherapy, inhibition of cyclo-oxygenase, antagonism of the glutamate receptor, induced hypertension, anticoagulation and thrombolysis, when applied to an experimental model of intracerebral haemorrhage. Rosenberg's et al (1990) rat model of collagenase-induced haemorrhage was chosen due its similarity to the clinical situation.

The suitability of Rosenberg's et al (1990) haemorrhagic model was first evaluated. This involved measurements of the haematoma volume, its consequent effect on brain swelling, evidence of a dysfunctional blood brain barrier and any associated ischaemic change. Attention was given to the reliability and reproducibility of these measures.

Results indicate that the cause of cerebral swelling associated with intracerebral haemorrhage is complex. Treatment with either glycerol, dexamethasone or indomethacin were found to be effective in reducing water accumulation, suggesting a role for both cytotoxic and vasogenic oedema respectively.

The haematoma volume was predictably increased significantly by heparin. Clinical outcome was also worse with heparin. Unexpectedly, larger haematomas and worse outcome occurred with the administration of 10mg kg^{-1} indomethacin and 5mg kg^{-1} MK-801.

The possible existence of ischaemic brain damage due to haematoma formation was also addressed in this project. Although no histological proof was evident, there were indirect signs of its likely presence. This was reflected in the improvement of the animals' recovery status seen with induced hypertension, 1 mg kg^{-1} MK-801, and possibly but unexpectedly with tissue plasminogen activator.

It is argued that the collagenase model presents a potentially valuable means of assessing whether new therapies for ischaemic stroke, being investigated in models of cerebral ischaemia, have the potential to improve the outcome of cerebral haemorrhage or cause deterioration.

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CHAPTER I

INTRODUCTION

Section 1: A Historical Perspective

The earliest literature (~ 460BC) on apoplexy (stroke), consists of detailed descriptions of the afflicted patients and their diseased brains. The cause of death of these patients was seen to be an accumulation of 'black bile' in the arteries of the brain (Clarke, 1963). Later in the 19th century the idea of vessels being occluded by material set free in the circulation proximally in the heart or great vessels (embolism) was developed by Virchow (1821-1902), for the lower limb and Kirkes (1852), for the brain. Ischaemic stroke was accepted as being due to thrombosis of diseased cervical and cerebral arteries or embolism particularly from the heart.

In 1658, Johann Wepfer (1620-95), in his postmortem studies of diseased brains of apoplectics, observed the presence of blood in the cerebral tissue. He described four cases of cerebral haemorrhage varying from subarachnoid bleeding at the base of the brain to massive intracerebral haemorrhage (Fields & Lemak, 1989). He proposed that brain haemorrhage could also cause apoplexy. He noted that those most liable to apoplexy were 'the obese, those whose face and hands are livid, and those whose pulse is constantly unequal'. This may be the first observation made, to suggest that hypertension and brain haemorrhage are linked. As to where the bleed originated, Wepfer could not find a ruptured blood vessel. Instead he saw a mesh of very fragile-looking blood vessels, which he did not investigate further.

Leonardo Botallo (1530-1574), observed one postmortem brain, that had showed hematic ventricular invasion due to a rupture of a blood vessel (Fazio, 1983). An autopsy done on Marcello Malpighi (1628-1694) by Baglivi (1669-1706) showed haemorrhage into the left ventricle (Mitchell, 1983) :

" The heart was larger than ordinary, especially the walls of the left ventricle, which were as thick as the breadth of two fingers. When I opened his head I found in the cavity of the right ventricle of the brain, an extravasation of about two pints of black clotted blood which was the cause of his apoplexy and death."

Wepfer was the first to differentiate between the two forms of apoplexy - in one, there is an arterial obstruction preventing the influx of blood (serous apoplexy) and the other, the extravasation of blood into the substance of the brain or the ventricular cavities (sanguinous apoplexy). Later, both Willis (1641-75) and Morgagni (1682-1771) corroborated the existence of these differences between cerebral 'softenings', and cerebral haemorrhage. Morgagni was the first to distinguish between apoplexy charaterised by bleeding into the cerebral parenchyma and the ventricular system as opposed to the general accumulation of blood inside the skull. Hoffman (1660-1742) is accredited for introducing the concept of "cerebral haemorrhage" as a separate anatomo-clinical entity (Fazio, 1983).

The first recorded reference between diseased cerebral vasculature and brain haemorrhage was claimed by Matthew Baille, an English pathologist (1761-1823). The following account is taken from Baille's book, Morbid Anatomy*:*

"It is common in examining the brains of persons who are considerably advanced in life, to find the trunks of the internal carotid artery upon the side of the sella turcica very much diseased, and this disease extends frequently more or less into the small branches. The disease consists in a bony or earthy matter being deposited in the coats of the arteries, by which they lose a part of their contractile and distensile powers, as well as of their tenacity. The same sort of diseased structure is likewise found in their basilar and its branches. The vessels of the brain, under such circumstances of disease, are much more liable to be ruptured than in a healthy state. Whenever blood is

accumulated in unusual quantity, or the circulation is going on in them with unusual vigour, they are liable to this accident, and accordingly in either of these states ruptures frequently happen. Were the internal carotid arteries and the basilar artery not subject to the diseased alteration of structure which we have described, effusions of blood within the cavity of the cranium, where there has been no previous external injury, would be rare".

The source of the bleed in the diseased brain was a contentious issue. Charcot and Bouchard (1868) reported the presence of small grape-like swellings on the cerebral vessels of patients who died from brain haemorrhage. In the absence of diseased vasculature, they inferred that the rupture of these miliary aneurysms, caused the bleed. Osler (1905), Ellis (1909) and Pick (1910) could not confirm Charcot and Bouchard's findings. Ellis argued that these miliary aneurysms were in fact, sites of occlusive thrombi. Even today, there is controversy surrounding these as potential sites of rupture of intracerebral haemorrhage (Challa et al, 1992). They speculated that the injection pressures possibly artifactually distended or caused the rupture of vessel walls. In 1963, Russell, with the aid of X-ray microangiography, demonstrated the presence of aneurysms, particularly in elderly hypertensive patients. He concluded that the frequency of these 'swellings' increased with chronic hypertension and with old age. He wrote:

"The combination of age and hypertension produces a degeneration of muscular and elastic element of small cerebral arteries which goes on to the formation of multiple miliary aneurysms, especially in the basal ganglia"

Cole and Yates (1967) confirmed Russell's observations. They summarised :

".....as hypertension can be adequately controlled in many cases,

such [intracerebral] haemorrhage is, to a large extent, a preventable disease.....We have no evidence as to how long hypertension must be present before aneurysms appear, or of the fate of the lesions if the blood pressure is reduced to normal levels."

Section 2: Current Clinical Situation

Stroke, remains the third highest cause of mortality and a leading cause of physical disability in the Western world. Stroke is described as a clinical syndrome characterised by a rapidly developing episode of focal and at times, global loss of cerebral function with symptoms lasting more than 24 hours, or leading to death, and with no apparent cause other than that of vascular origin (Hatano, 1976). In the United Kingdom, the disease claims about 100,000 victims per annum.

About 70-80% of all strokes are ischaemic (thrombosis and embolism) in nature (Allen et al, 1988). In ischaemic stroke, the blood flow to the brain is compromised through the presence of a thromboembolic occlusion. Strokes due to brain haemorrhage account for only 10-15% (Bamford et al, 1990). In brain haemorrhage, there is a release of blood either into the subarachnoid space (subarachnoid haemorrhage) or into the brain parenchyma (intracerebral haemorrhage) (Broderick et al, 1993b). Haemorrhagic stroke has the higher mortality (Weisberg, 1984). With the development of Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) techniques, it has become easier to distinguish between the two different pathological stroke subtypes before prescribing a treatment regimen.

Section 3: Intracerebral Haemorrhage

Brain haemorrhages are classified:-

- according to the site of the bleeding : extradural, subdural, subarachnoid, parenchymatous, intraventricular
- according to the type of blood vessel ruptured : arterial, venous, capillary
- according to causes : traumatic, degenerative

About 60% of brain haemorrhages are mainly found in the basal ganglia and thalamus and the rest are peripheral lobar haematomas (Caplan, 1993). Hypertension is a leading cause of ischaemic and haemorrhagic stroke (Mohr et al, 1978; Furlan et al, 1979). The commonest brain sites for hypertensive intracerebral haemorrhages (Caplan, 1993) are:-

- putamen, 40%
- thalamus, 12%
- lobar white matter, 15-20%
- caudate nucleus, 8%
- pons, 8%
- cerebellum, 8%

The other non-hypertensive causes of brain haemorrhage and their commonest sites are (Kase, 1986):-

- vascular malformations eg. arteriovenous malformations, angiomas, often subcortical
- intracranial tumours
- cerebral amyloid angiopathy (CAA), these are usually are lobar
- sympathomimetic drugs (amphetamines and cocaine), the sites are similar to hypertensive ones.

- use of anticoagulants, often involving the cerebral white matter and the cerebellum (Kase et al, 1985)
- use of thrombolytic agents

Vascular malformations are primary causes of intracerebral haemorrhage (ICH) in non-hypertensive patients (Kase, 1995). These include AVM's, cavernous angiomas, capillary telangiectasias, and venous angiomas. Toffol et al (1987) stated that ruptured AVM's (tangled mass of blood vessels on CT) are frequently the cause of ICH in patients younger than 45 years of age. Cavernous angiomas have a lower tendency to bleed relative to AVM's (Kase,1995). The choice location of cavernous angiomas is supratentorial (Yamasaki et al, 1986).

About 6-10% of ICH is due to intracranial tumours (Little et al,1979). The main malignant ones are glioblastoma multiforme and metastases. Of the benign type, the pituitary adenoma is the commonest (Wakai et al ,1982). These haemorrhages probably relate to abnormal tumour vessels being more friable.

Cerebral amyloid angiopathy (CAA) is a cerebral vascular lesion. In CAA affected brains, the media and adventitia of small and medium arteries contain amyloid. The actual mechanism of bleeding in (CAA) is not yet settled. Torack (1975) is of the opinion that ICH in CAA patients may in some cases be related to an initial head trauma or a neurosurgical procedure. The incidence of ICH in CAA patients has also been linked to the use of anticoagulants (Pendlebury et al, 1991; Wijdicks and Jack, 1993).

Sympathomimetic drugs implicated in ICH include amphetamines, phenylpropanolamine and cocaine. The onset of ICH is rapid from within minutes to a few hours, after exposure of the powerful drugs. The mechanism of ICH development are listed as follows:

1. the drugs induce acute, transient hypertension

2. angiographic evidence of beading (Margolis and Newton, 1971) - alternate areas of constriction and dilatation of intracranial arteries (not evidenced in cocaine victims).

3. In cocaine cases, the pre-presence of AVM's and aneurysms may be the source of ICH.

Chronic warfarin anticoagulation increases the risk of ICH by eight to eleven fold (Whisnant et al, 1978). Intravenous heparin anticogulation can result in enlargement of the initial haematoma in patients with intracerebral haemorrhage (Kase and Caplan, 1994).

In all of these circumstances leading to haemorrhage, there is either an abnormality of small blood vessels or of haemostasis or blood coagulation. The destructive effect of the rapid egress of a volume of blood into the brain substance is to produce a focal deficit of cerebral function together with the clinical effects of stretch of the meninges and dura, and an elevation of intracranial pressure (headache vomiting, neck stiffness, and reduced conscious level). It is the mechanical effect of the mass that causes death through shifts of brain substance directly or indirectly causing compression of the brain stem and damage to its vital respiratory and cardioregulatory centres.

In recent years it has also been hypothesised that around a cerebral haematoma there may be a zone of brain tissue, that is being at least temporarily damaged by ischaemia, perhaps due to local tissue pressure interfering with tissue perfusion, or through the effects on tissue of the biochemical effects of shed blood. Mendelow (1991, 1993) has, for example produced evidence of ischaemic brain damage due to haematoma formation. He found evidence of reduced blood flow in the zone adjacent but outside the visible clot in a rat model.

Conventional treatment of cerebral haemorrhage depends on surgical evacuation of the haematoma and control of intracranial pressure. Surgical treatment has its limitations (Kanno et al, 1984; Juvela et al, 1989). The size and the location of the haematoma will ultimately determine the success of the operation. The larger the haematoma and its proximity to vital functioning areas will adversely affect the outcome of surgery. The use of osmotherapy e.g. with hypertonic mannitol or glycerol can mitigate against the mass effect of a haematoma by reducing the volume of the brain by causing a degree of tissue dehydration. This 'makes room' for the expanding haematoma. The effect of an infusion of mannitol is temporary with the risk of a rebound as mannitol enters brain substance and reverses the osmotic gradient between the brain and the blood. Controlled hyperventilation by reducing arterial CO_2 levels reduces cerebral blood volume by causing vasoconstriction within the cranium. This again 'makes more room' and lowers pressure. These methods of controlling intracranial pressure 'buys time' for removal or resolution of the haematoma.

Section 4: Cerebral Ischaemia

Although Baillie and others were aware of the association of diseased cerebral arteries and apoplexy, the original connection as we have seen was made with cerebral haemorrhage.

In the modern era interest has focused upon the pathophysiology of cerebral ischaemia and the biochemical mechanism of ischaemic damage to brain parenchyma. Much of modern understanding comes from the study of animal models in which global, or more relevantly, focal ischaemia has been produced.

Initially these experiments were carried out on cats (e.g. Waltz et al,1968) and primates (e.g. Symon et al,1975) with perorbital approaches to the middle cerebral artery which was then clipped or ligated. Waltz showed that ischaemic tissue became

oedematous (increased water content) in a way that paralleled the severe swelling that accounts for early mortality after ischaemic stroke in man. The death of neurons proved to depend on both the depth and duration of ischaemia (Crowell et al, 1970) and Symon et al (1975) showed that adjacent to the densely ischaemic core of the territory of an occluded middle cerebral artery (MCA) was tissue with the metabolic signature of paralysed function e.g. loss of evoked potentials but no leak of cations (K^+) to suggest irreversible disruption of key cellular processes. The idea developed that there might be a window of opportunity during which restoration of flow could rescue tissue, especially that in the penumbra where flow reduction was patchy or more modest.

Normally the level of cerebral blood flow is set by the metabolic demands of the tissue and is protected from fluctuations of blood pressure over a wide range (70-170 mm Hg). Symon and others (1975) showed that this process of autoregulation was impaired or lost in ischaemic tissue where blood flow could become pressure passive. This implied that hypotension would aggravate ischaemia and hypertension might be beneficial. Neurosurgeons employ pressor agents to elevate the blood pressure to reverse ischaemic deficit caused by spasm after subarachnoid haemorrhage once any aneurysm has been clipped. Induced hypertension has been shown to improve the metabolic state of the ischaemic hemisphere in the rat after MCA occlusion (Aspey et al, 1987) but has had little study in man. The reasons for this include the risk of increased oedema once the blood brain barrier is damaged and the fear of haemorrhage.

In a second wave of experiments the chemical sequelae of ischaemia have been dissected out, and this remains an active field (Siesjö, 1992). The initial loss of energy metabolism with falling levels of adenosine triphosphate (ATP) triggers depolarization of neuronal cell membranes. This causes release of neurotransmitters like glutamate

and the opening of voltage gated calcium channels. Glutamate receptors e.g. N-methyl-D-Aspartate (NMDA) and amino-3-hydroxy-5-methyl-4-isoazole propionic acid (AMPA) receptors when activated provide an additional route of entry of calcium ions into the cells. As intracellular calcium levels rise, a series of damaging processes are triggered. Arachidonic acid catabolism leads to the production of thromboxanes, leukotrienes and free radicals. Proteases and deoxyribonucleic acid's (DNA's) are activated which can have direct lytic effects on cell membranes and nuclei, and nitric oxide (NO) synthesis is induced. Acidotic anaerobic metabolism, calcium entry, NO synthesis and free radicals are all capable of cytotoxicity.

The modern treatment of ischaemic stroke is thus actively exploring measures to restore blood flow e.g. by thrombolysis, prevent secondary thrombosis of vessels in ischaemic areas e.g. with heparin, and to block glutamate receptors, calcium flux, or provide free radical scavengers. Treatment of oedema with steroids has been disappointing but osmotherapy and craniotomy are still occasionally utilized to counteract the effect of cerebral swelling.

Thrombolysis, successful in the recanalisation of thrombosed arteries in acute heart attack, is now under intense scrutiny as treatment for ischaemic stroke. The preliminary evidence suggests that it may improve the chances of recovery to an independent life style but at the expense of increased early mortality and morbidity due to haemorrhage into the evolving area of ischaemic brain damage. There is evidence to suggest this risk is least if treatment (with thrombolysis) is instituted early, perhaps less than three hours after the onset of stroke, and only when haemorrhage has been excluded by very early neuro-imaging. Heparin has been widely used in the immediate aftermath of ischaemic stroke to limit extension of the in situ thrombotic occlusion of cerebral vessels in the belief that this would reduce the risk of extension of the volume of infarction on which depends the chance of restoration of function. An international

stroke trial (IST) showed that heparin in this context increases the risk of cerebral haemorrhage (Lancet, 1997).

The use of the anticoagulants, heparin, and warfarin in ischaemic stroke trials have provoked cerebral haemorrhage in some patients. The haemorrhage may be due to blood effusing from damaged vessels on re-perfusion of ischaemic blood vessels. Clearly, the clinical status of ischaemic stroke patients needs to be carefully assessed prior to anticoagulant administration. Elderly ischaemic stroke patients are believed to be more at risk of this secondary complication of haemorrhage.

The frequency of ICH complicating the use of thrombolytic agents, such as streptokinase and recombinant tissue-type plasminogen activator is low (0.4-1.3%) but its development can be fatal (Thrombolysis in myocardial infarction trial, 1989;Kase et al, 1990). Thrombolytic agents are extensively employed in myocardial infarction and are now being used in ischaemic stroke. The mechanism of bleeding is uncertain but it has linked to the prolongation of the thromboplastin time (Kase et al, 1990).

It is assumed therefore that both anticoagulants and thrombolytics (and possibly hypertension) will have adverse and potentially fatal effects if inadvertently given to patients with a haemorrhagic brain lesion but this has received little direct study.

Measures to counteract the metabolic processes thought to be instrumental in neuronal death are being explored intensively. It is assumed from their animal model data that they must be prescribed as soon as possible after the onset of ischaemia, within a very few hours. In view of the logistical difficulties involved in excluding cases of cerebral haemorrhage it would clearly be useful to know what would happen if any of these strategies were applied to cases of cerebral haemorrhage. Some like thrombolysis, heparinsation and hypertension might be expected to aggravate haemorrhage, others like treatment of oedema, and control of the ischaemic process

might be beneficial especially if there proves to be a significant component of ischaemia in the damage created by a haematoma.

Section 5: The Thesis

This thesis describes studies to characterize a recently evolved animal model of cerebral haemorrhage (Rosenberg et al, 1990) to identify the contribution of oedema to the mass effect of a haematoma, to seek evidence of any associated ischaemic neuronal damage and to see whether treatments (existing or innovative) aimed at the ischaemic stroke process have adverse or beneficial effects in cerebral haemorrhage. The belief was that the further exploration of the appropriateness of a cerebral haemorrhage model as well as the initial studies of therapeutic interventions, would help illuminate the debate about the right immediate or emergent management protocol for human stroke victims.

First the animal model will be described (Chapter 3) together with the experiments to document its reproducibility, and the nature of the pathology seen. Experiments to see whether brain swelling is more than can be accounted for by the volume of blood in the haematoma will be combined with a study of the effects of attempts to reduce oedema formation and swelling in Chapter 4. The issue of whether there is ischaemic damage around the haematoma will be addressed by histological observations and a trial of a NMDA antagonist known to be effective in alleviating the extent of ischaemic damage in animal models of ischaemia (Chapter 5). The impact of potentially hazardous treatments such as induced acute hypertension, heparin and tissue plasminogen activator therapy (designed for use for ischaemic stroke) is assessed in Chapters 6 and 7. In Chapter 8, I shall summarize the results and evaluate the relevance of the model and discuss future work.

CHAPTER II

GENERAL METHODOLOGY

Section 1: Animal Preparation

Adult male Sprague Dawley rats (Harlan Olec, UK) weighing between 270-310 grammes (g) were used throughout the project. The rats were fasted overnight, with free access to water, and placed in a temperature and humidity controlled incubator. The animals' body weight were recorded twice; prior to anaesthesia and following 24 hours of survival. The body weight data would indirectly indicate the extent of the animals' survival of the experimental procedures.

Section 2: Surgical Procedures

All procedures were carried out in accordance with The Animals (scientific procedures) Act, 1986 (Home Office, UK).

Section 2.1: Induction of Anaesthesia

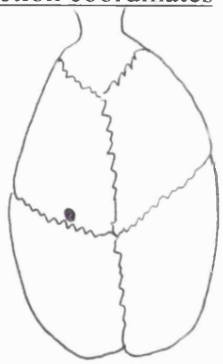
The rats were placed in a Harvard perspex chamber (Harvard Apparatus, Ltd) filled with 4% halothane, delivered in a gas mixture of 70% nitrous oxide and 30% oxygen. They were then anaesthesized with 0.3 ml kg^{-1} Hypnorm, intramuscularly (10 ml kg^{-1}; Janssen Pharmaceutical, Belgium) and 2.5 ml kg^{-1} Diazepam, intraperitoneally (10 ml kg^{-1}; Phoenix Pharmaceuticals, UK). The loss of righting reflex, the lack of response to the tail pinch test and corneal touch was ascertained prior to surgery.Unless otherwise stated all experiments were of 24 hours duration.

Section 2.2: Craniotomy

The rat was placed in a stereotactic head holder (Stoelting, USA). A midline incision was made at the scalp to expose the skull. A bore hole was drilled at 3 millimetres (mm) lateral to the midline and 1mm anterior to bregma (Diagram A). A 26s gauge needle mounted on a Hamilton syringe (25 microlitres) was secured to the needle holder of the Stoelting apparatus. For injection, the needle was lowered 6 mm

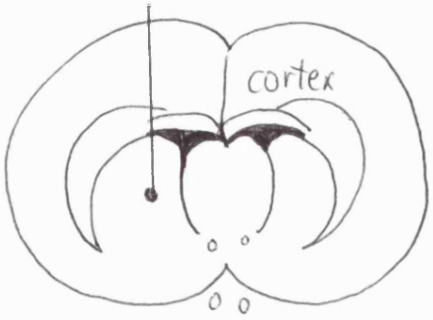
deep into the caudoputamen (Diagram B). Body temperature was maintained by a heating lamp between 37° C-38° C degrees as measured by a rectal thermometer. After the intracerebral injection, the needle was left in situ for a further 5 minutes to stop backflow of the injectate. At the end of the five minutes, the needle was slowly withdrawn over one minute. The craniotomy was sealed with Horsley's bone wax and the skin over the skull sutured (3/0 silk; Mersilk, UK).

Diagram A: Brain injection coordinates



• Site of craniotomy on rats skull.

Diagram B: Site of injection in the coronal brain section



• Site / Target of collagenase injection

Section 2.3: Production of intracerebral haemorrhage

The general method is as described by Rosenberg et al (1990). Intracerebral haemorrhage was effected by a manual injection of 2 microlitres (ul) containing 0.4 units of bacterial collagenase Type IVs (Sigma, UK) over 9 minutes. Control animals either received an equivalent volume of saline or were sham operated. Immediately after the end of the procedures, Vetergesic, an analgesic, (0.04 ml kg^{-1}; Reckitt & Colman, UK) was administered intramuscularly. Dehydration was countered with an intraperitoneal

injection of about 6 millilitres (ml) of normal saline (0.9% sodium chloride). The animals were then returned to the incubator with free access to food and water.

Section 2.4: Assessment of recovery

At the end of the twenty-four hour recovery phase, animals were graded by another member of the laboratory staff (Tarlok Gajree) unaware of the experimental protocol, according to a personally developed simple 5 point scoring system as follows:

0 = awake and moving freely in all directions with no weakness in the fore limbs

1 = either circling when forced with no discernible splaying of fore or hind limbs or moving freely in all directions with slight splaying of fore or hind limbs .

2 = spontaneous circling with accompanying fore or hind paw flexion

3 = alive but paralysed/immobile

4 = died during the 24 hours recovery period

Section 2.5: Mode of Sacrifice - Gravimetric method of perfusion-fixation

The animals were re-anaethesised and subjected to transcardiac perfusion fixation. Heparinised saline (5 units heparin/ml saline) was initially perfused through the heart for 2 minutes or until perfusate ran clear and then finally flushed with 10% saline-buffered formalin for 4 minutes at a constant pressure of 120mmHg. At the end of perfusion, the heads were removed and left overnight in fresh fixative.

Section 2.6: Tissue Preparation

The following day, the brains were removed and placed in fresh 10% saline-buffered formalin for a further two hours. After which, the brains were embedded in gel-albumin mixture (McLean and Singer, 1970- see Appendix 1 for details) prior to sectioning. Ten one millimetre thick slices were cut using a rat brain matrix (department's constructed model). The injection site served as a point of reference for sectioning. Sections 3 (anterior) and 4 (posterior) were located 1 mm away from the site of injection - see Diagram C.

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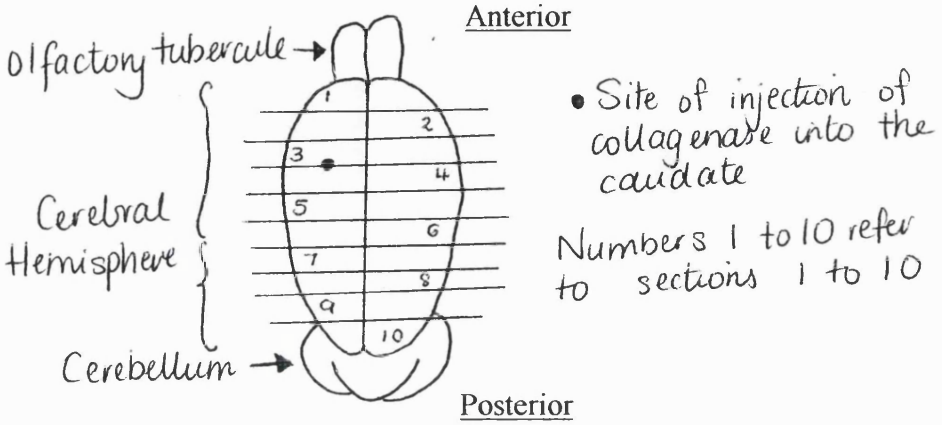
Diagram C: The reference for brain sectioning.



Section 3: Drug Treatment Regimes

These were administered either before the induction or after the induction of cerebral haemorrhage haemorrhage. The details are described in the relevant chapters.

Section 4: Experimental Procedures

Section 4.1:Measurements of cerebral swelling

Two methods were used: Planimetry (Vids software by Synoptics, UK Ltd) and Wet/Dry weight.

Section 4.1a:Planimetry mode of measurement, using VIDS Imaging (Synoptics Ltd).

A Nikon 35 mm camera mounted on a Meiji Techno microscope (objective x1.5) was set up to take complete whole brain sections. The posterior view of all ten brain slices (each 1 mm thick) were photographed from each perfused brain using Kodak Ektachrome colour slide films (100ASA). These photographs of fixed sections were then used to measure the following parameters:-

- hemispherical volume of the ipsilateral and contralateral sides
- haematoma volume. The computer-imposed pseudocolour code is based on the thresholding of 255 grey levels. The computer assigns a colour band to a specific grey level range. There are eight colour bands each with its own grey level range:- black (0-32), magenta (32-63), red (64-95), yellow (96-127), green (128-159), cyan

(160-191), blue (192-223) and white (223-225). The red zone (Plate 1B) corresponds very well to the haematoma core in Plate 1A and the haematoxylin-eosin stained section shown in Plate 1C. This vids colour imaging system was thus used on all brain sections to delinenate measure haematoma volumes.

For each brain, the areas (mm^2) of the ipsilateral and contralateral hemispheres were summed respectively and then multiplied by the thickness of the sections (1 mm) to give brain volume data in mm^3. The % swelling was calculated as follows:

$$= \frac{100 \times [(\Sigma^1_{10} \text{ Volume of ipsilateral hemispheres}) - (\Sigma^1_{10} \text{Volume of contralateral hemispheres})]}{(\Sigma^1_{10} \text{Volume of contralateral hemispheres})}$$

The volume % of the haematoma was calculated as follows:

$$= \frac{100 \times (\Sigma^1_{10} \text{ volume of haematoma})}{(\Sigma^1_{10} \text{volume of ipsilateral hemispheres})}$$

Section 4.1b: Measurement of water content (wet weight/dry weight) .

Perfusion fixation was not necessary. The brains were removed immediately after cervical dislocation. The olfactory tubercules and the cerebellum were trimmed off. The brain was divided into the right and left hemispheres and weighed. The brain tissues

were then left in a vacuum dessicator until dry weight measurements reached a constant value.

$$\% \text{ water content (ipsilateral hemisphere)} = \frac{(\text{wet weight-dry weight}) \times 100}{(\text{wet weight})}$$

Section 4.2: Qualitative assessment of blood brain permeability using Evans blue dye.

1 ml kg^{-1} of 2% ($^w/_v$) Evans blue dye (Sigma, UK) was injected intravenously thirty minutes prior to perfusion fixation. Unbound Evans blue dye was washed out during perfusion fixation.At the end of perfusion, the brains were removed and left in fresh 10% ($^w/_w$) formal saline solution until sectioning.

Section 4.3:Evaluation of 2,3,5-triphenyltetrazolium chloride (TTC) in a rat model of collagenase-induced haemorrhage.

TTC is a relatively colourless water soluble salt which is reduced by mitochondrial dehydrogenase to a deep red coloured lipid soluble product called formazan. Normal living tissue with intact mitochondria will stain red whereas dead/damaged tissue will remain unstained. Prior to fixation, 1ml of heparin (1000 units; Monoparin, CP Pharmaceuticals, Wrexham, UK.) was infused intravenously. Through a thoracotomy, the descending limb of the aorta is clamped for the duration of the perfusion. Four solutions were infused consecutively in at 120mm Hg, through a needle introduced into the left ventricle. Firstly, heparinised saline containing 5 units ml^{-1} was infused for 90 seconds(s) followed by 2M urea (60s), then 2% $(^{w}/_{v})$ TTC (warmed at $37^{0}C$) and finally by 10% formal saline. The head was then removed and left in fresh 10% formal saline overnight and then sectioned immediately.

Section 4.4: Light Microscopy

Once the photographs of the coronal sections were made, the sections were then prepared for wax embedding. The gel-albumin media was removed carefully from the sections so as not to tear the sections. These sections were then put through the 24-hour cycle of a Histokinette tissue processor (Shandon Instruments-UK). At the end of the cycle, the sections were embedded in molten paraffin wax and left overnight to solidify.

Representative eight micron thick coronal sections were cut from each of the brain wax blocks. The dewaxed sections were then stained with either haematoxylin and counterstained with eosin (Appendix 1) or luxol fast blue and counterstained with cresyl violet (Appendix 1). Damaged areas of the brain sections appeared pale (unstained), spongy (vacuolated) and revealed dark, condensed neuronal cells under the light microscope.

15 micron thick sections were also prepared for Glial Acidic Fibrillary Protein (GFAP) immunhistochemistry (Appendix 1). GFAP labelling was performed on coronal sections at the point of needle entry. Sections were stained using primary antibodies against GFAP and diaminobenzidine (DAB). Secondary biotinylated conjugates were demonstrated using an Avidin-biotin-complex (ABC; Vector Ltd.)and diaminobenzidine (DAB). Areas with labelled astroyctes were compared between ipsilateral and contralateral sides. Lesions were localised by loss of staining for GFAP.

Section 4.5: Electron Microscopy

For electron microscopy, Karnovsky's (1965) fixative was used in place of 10% formal saline for perfusion.1 mm^3 blocks of tissue were removed from the haematoma core and the cortical region of the ipsilateral and contralateral hemispheres of the perfused coronal brain section and prepared for electron microscopy (Appendix 2). The status of the cerebral blood vessels (intact or swollen or completely disrupted), mitochondria (intact or swollen or completely disrupted), and the neuropil (intact or vacuolated) were assessed.

Section 5: Statistical Analysis.

Neurological scores are analysed using non-parametric Chi-square test. Data are presented numerically in tables and graphically as figures. Numerical results are expressed as mean $\pm$ SEM (standard error of the mean) in tables. Coefficient of variability of the data was calculated as a % of standard deviation/mean. In the case of paired samples, student's paired t-test was used to test the significance of the difference between two sets of related data. Analysis of variance (oneway) was used to assess the significance of the differences between groups (unrelated data). In all analyses, a probability of 5% or less was considered to be significant.

Plate 1

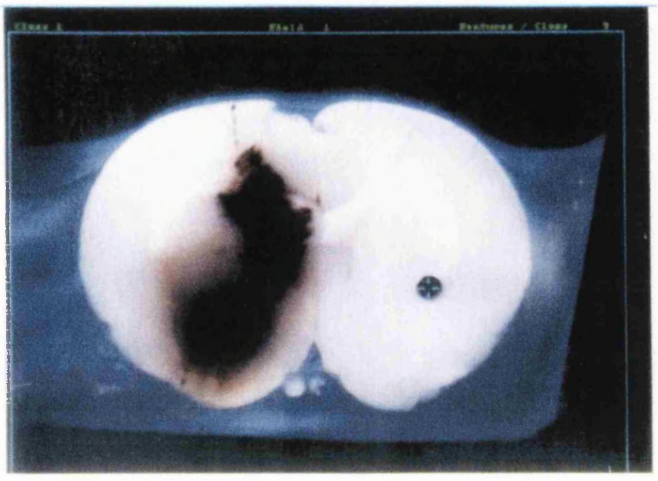
Computer-imposed colour coding used in planimetry measurements of brain sections.

A: Photograph of the coronal brain section of a rat prior to computerised grey level thresholding.

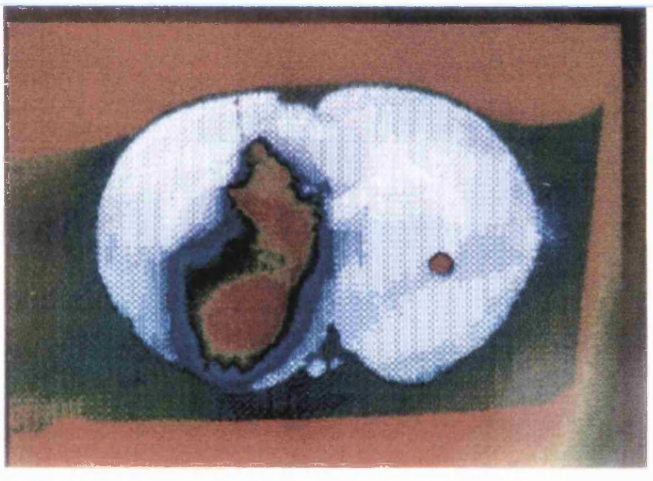
B: The effect of the computerised pseudocolour on the brain section seen in A. The H&E stained pattern (C) is in good agreement with the computerised colour banding.

C: Haematoxylin-Eosin (H&E) stained section taken from the brain section seen in A.

Scale bar = 5 mm



A



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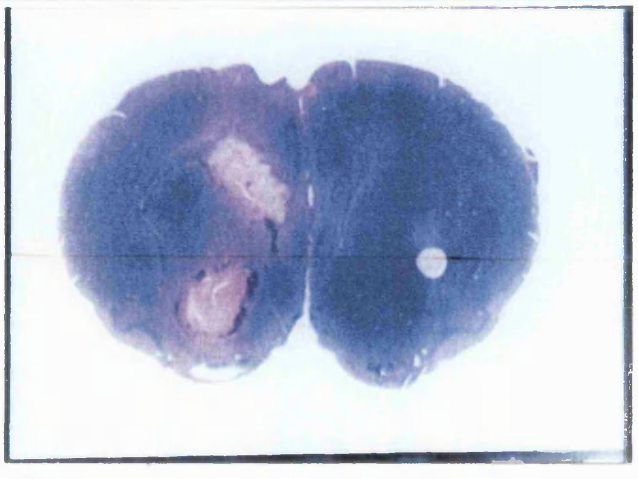


C

CHAPTER III

AN EVALUATION OF A RAT MODEL OF INTRACEREBRAL HAEMORRHAGE

Section 1: Introduction- Animal models of intracerebral haemorrhage

The use of animal models has been fundamental to the understanding of the pathophysiology of many cerebrovascular diseases. Animal models provide the means by which potential/experimental therapies can be tested in a controlled environment. The primary advantage of employing animal models is the ease with which one can manipulate the changes in experimental conditions in order to ascertain the best therapeutic strategy for a given clinical disorder.

The ideal animal model should satisfy the following criteria (Hossmann, 1991):

- must be relevant to the clinical situation
- must be technically easy to perform
- must be highly reproducible
- must avoid extracerebral complicatory side effects

There are several different animal models for intracerebral haemorrhage. The experimental technique used in the production of a focal intracerebral haemorrhage is usually one of three types:

- intracerebral inflation of a microballoon
- intracerebral injection of blood
- intracerebral injection of collagenase

Intracerebral inflation of microballoon

An inert, deflated balloon of known volume (10-50 microlitres) is introduced into the caudate nucleus and then inflated to its full size. The use of a microballoon to simulate an intracerebral haematoma addresses two important issues: (1) the technique permits an in depth study of the effects of introducing an additional inert mass into the

brain within a non-yielding cranium and (2) the versatility of the technique means that one can determine if subsequent removal of the mass improves neurological outcome. The presence of an extraneous mass leads to a build-up of pressure within the brain. Thus, any treatment that is directed at lowering the rise in intracranial pressure, may contribute towards reducing the size of cerebral damage in this context.

Autoradiographic measurement of the local cerebral blood flow in the vicinity of such a 'pseudo-haematoma' produced by a microballoon (Sinar et al, 1987; Nehls et al, 1988, 1990; Mendelow, 1993) revealed that blood flow in the periphery of the mass was reduced but not obliterated. This suggests the possible presence of an area of brain cells that are still viable - an 'ischaemic penumbra' around a haematoma. Consequently, the administration of successful anti-ischaemic treatments may prove to be beneficial in this aspect of intracerebral haemorrhage.

Intracerebral injection of blood

Studies using this form of cerebral haemorrhage model (Nath et al, 1986, 1987; Sinar et al, 1988; Yang et al, 1994; Deinsberg et al, 1996; Matz et al, 1997) have enabled researchers to examine both the biological effect of blood on brain cells and the volume occupancy effect of the haematoma on the brain. The differences between animal models using this form of injection is related to nature and source of blood used. The blood source could be arterial or venous, could be fresh and unheparinised or fresh and heparinised, or in some cases a complete blood clot is injected. The results suggest that both the mass effect of the blood clot and the blood constituents (Koeppen et al, 1995) contribute to brain damage.

The intracerebral injection of collagenase

Rosenberg et al (1990) devised an alternative animal model of intracerebral haemorrhage that closely matches the clinical form. In the clinical condition, intracerebral haemorrhage is very common in patients with chronic hypertension. The cerebral microvessels are fragile and without antihypertensive treatment, may be breached with release of blood into the brain.

In Rosenberg's et al (1990) animal model, to effect the breach of the cerebral microvasculature, bacterial collagenase is injected into the caudate. The enzyme digests the collagen found in the vascular basal lamina. This proteolytic action of collagenase means that there is an efflux of blood, from the circulation, at systemic blood pressure, into the brain.

One most obvious disadvantage that is common to all three techniques is that there is an obligatory, invasive introductory stage. A craniotomy is necessary to facilitate the precise physical placement of the microballoon or needle into the caudate. As we have seen, these different intracerebral haemorrhage models, investigate various aspects of intracerebral haemorrhage. Thus, each of these models can make an important contribution towards understanding the pathogenesis of intracerebral haemorrhage.

Rationale for the choice animal model for intracerebral haemorrhage

In the clinical situation, the presence of blood in the brain is irrefutable and equally so is the disruption of the cerebral microvasculature. Of the three techniques described, the collagenase-induced haemorrhage is thus the closest matched model although the model subjects are young, healthy adult rats and not predisposed to chronic hypertension.

The important feature, of this particular model, is that it allows for the simultaneous examination of the volume/mass effect and the cellular damage caused by the rise in pressure and the biologically active blood.

I therefore chose to employ Rosenberg's et al (1990) intracerebral haemorrhage model for my investigations into intracerebral haemorrhage. Little has been published on its reproducibility, the extent of associated brain swelling and oedema, or on its clinical impact. I addressed these and related issues in the experiments to be reported in this chapter.

Section 2: Materials and Methods

For detailed descriptions of the surgical and experimental procedures and statistical analysis refer to Chapter 2.

A total of one hundred and twenty-eight adult male Sprague Dawley rats weighing between 270 and 300 g were used. The animals were randomly divided into three groups- those that received no intracerebral injection, those that received intracerebral injection of saline, and those that received intracerebral injection of collagenase.

Four parameters were measured following 24 hours of survival: body weight changes, the haematoma volume, the hemispherical volume of the brain and the extent of the breakdown of the blood brain barrier as determined by Evans Blue leakage.

Qualitatively, the neurological recovery status of the experimental animals was determined and the effect of collagenase-induced haemorrhage on the histological appearance of the haemorrhagic lesions was ascertained.

Section 3: Results

There is a clear significant loss ($p < 0.01$) in body weight following haemorrhage when compared to the saline-control and control groups (Table 3A and Figure 3Ai). The coefficient of variability of the pre-haemorrhage and post-haemorrhage body weight within the collagenase-treated cohort is consistent, ~9% and 10% respectively (Figure 3Aii).

A high majority of the animals in the collagenase treated group (63%; chi-square test, $p<0.001$) scored a clinical deficit of 2 (Table 3B and Figure 3B) . Clearly, the production of a haematoma has generated a discernible physical impairment.

The injection of saline (Table 3C and Figure 3Ci) into the caudate has not resulted in any significant contribution to the haematoma volume. Plate 2 shows the development of the haematoma through 10x1 millimetre thick coronal sections from sham operated, saline injected and collagenase-injected rats. The range of haematoma volumes produced within the collagenase treated group is shown in Plate 3. The coefficient of variability is calculated to be 45%.

The haematoma volume was 15.5% $\pm$ 0.96% of the ipsilateral hemisphere (equates to ~ 85 $\pm$ 6 ul). The ipsilateral hemisphere was 13.64% $\pm$ 0.64 % (equates to ~ 64 $\pm$ 3 ul) larger than the volume of the contralateral hemisphere (coefficient of variability of the volume of these hemispheres was 7% in each case- Figure 3Cii). The coefficient of variability in the percent cerebral swelling produced in the ipsilateral was 34%.

The haematoma volume and the associated swelling of the ipsilateral hemisphere (Figure 3Ciii) appears to be closely linked- a correlation coefficient of $r = 0.65$ ($p< 0.01$). There is poor correlation between either the haematoma volume or the volume of brain swelling and the clinical deficit score (correlation coefficient, $r = 0.14$ in both cases ($p > 0.1$)-Figure 3Civ and Figure 3Cv).

Wet and dry weight measurements further confirm an increase in cerebral water content associated with the collagenase-induced intracerebral haemorrhage. There is a significantly higher increase in % water content on the ipsilateral than on the contralateral side (Table 3D and Figure 3Di). The coefficient of variability of the % water content of both ipsilateral and contralateral hemispheres is calculated to be 2% and 1% respectively (Figure 3Dii).

The development of the haematoma and the hemispherical swelling of the ipsilateral beyond the 24 hour survival period, peaks at 24 hours post haemorrhage (Table 3E and Figure 3E). Between 24 and 48 hours, there is a detectable decrease in the volume of both parameters. However, this decrease is not reflected in gain of body weight (Table 3E). At 48 hours of recovery, there is a significant decrease in haematoma size but not in brain swelling. This nevertheless has not improved the clinical deficit score- all the animals (n = 6; 100%) in this recovery group scored a Grade 2.

The haemoglobin assay revealed that the volume of blood produced by the intracerebral injection of collagenase into the rat caudate was 138.39 $\pm$ 13.91 ul (Table 3F and Figure 3Fi). The coefficient of variability in blood volume data for the collagenase-injected group is calculated to be 37% (Figure 3Fii).

The infusion of TTC at time of sacrifice to delineate an ischaemic component did not offer any such evidence -Plate 7A. Visually, TTC reveals two clear zones - the darkly stained haematoma core and the rest of the brain section stained a deep red. The extent of the breakdown of the blood brain barrier by collagenase was demonstrated by Evans blue dye. The dye has diffused from the core of the haematoma sited in the caudate to the more distant cortical regions (Plate 7B).

Light microscopy of the lesion revealed two broad distinct zones; a haematoma core (mainly caudate putamen) that is tightly packed with blood cells with complete destruction of tissue, and an almost intact area beyond the corpus callosum (Plates 5A-B). Plates 4A-D show that saline injection into the caudate effect of intracerebral has not damaged the cellular integrity of either the ipsilateral and contralateral hemispheres.

The coronal section was stained for reactive astrocytes using the glial fibrillary acidic protein immunostain. The expression of this immuno-protein extended well beyond the haematoma core -Plates 6A-D. Areas equivalent to these areas of astrocytic activity, appears normally stained in Haematoxylin/Eosin and Luxol Fast Blue/Cresyl Violet stained sections (Plates 5A and 5B).

At the electron microscopic level, a transverse section taken from normal cortex showed a capillary blood vessel with an intact endothelium and its nuclei, an intact basal lamina, intact mitochondria and intact myelinated and non-myelinated axons complete with ordered microtubules. The neuropil is tightly packed with cellular elements with no vacuolated areas (Plate 8A). A view from the haematoma-bearing hemisphere reveals a totally disrupted region with a structureless neuronal matrix (Plate 8B). Most of the area is filled with large clusters of platelets and red blood cells. There is a certain degree of delamination of the myelinated axons and there are no signs of intact mitochondria. The neuropil of the haematoma is largely vacuolated and is translucent.

Histological analysis was performed on brains in all treatment groups. On examination, no apparent differences were detected in the brain sections between the various treatment groups. The histological evidence presented in this chapter is thus a fair representation of that observed in all treatment groups.

TABLE 3A: The effect of collagenase-induced intracerebral haemorrhage on rat body weight following 24 hours survival.

(Values are presented as mean +/- sem. The number in brackets refer to the number of animals in the group)

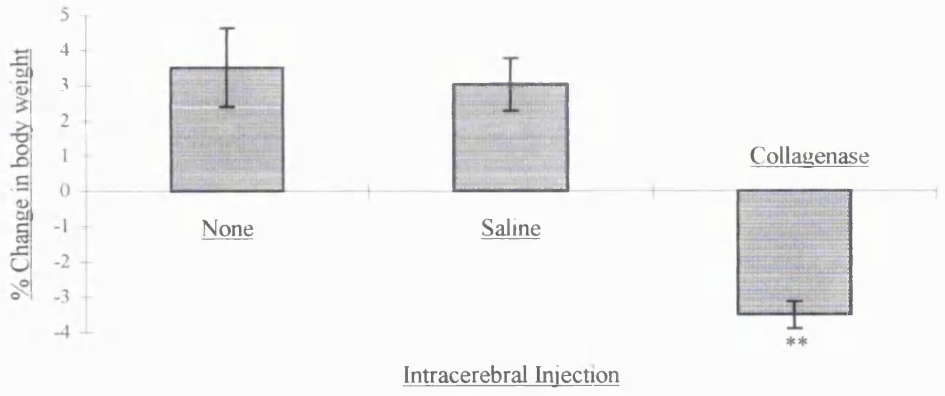
	BODY WEIGHT / g		
Caudate Injection	Pre-Surgery Mean + SEM (n)	Post-Surgery Mean + SEM (n)	% Change in body weight Mean + SEM (n)
None (control)	291.98 ± 5.46 (11)	302.13 ± 6.02 (11)*	(+) 3.52 ± 1.12 (11)
Saline (control)	283.98 ± 3.76 (42)	292.24 + 3.65 (42)*	(+) 3.03 + 0.75 (42)
Collagenase	289.58 ± 2.43 (123)	279.22 ± 2.45 (123)*	(-) 3.53 ± 0.38 (123)**

* paired student's t-test to test differences in body weight before and after haemorrhage within groups; $p < 0.01$

** anova oneway; comparison of collagenase with either saline or normal group; $p < 0.01$.

Figure 3A(i):The effect of collagenase-induced intracerebral haemorrhage on % change in rat body weight after 24 hours of survival.

Data presented as mean +/- sem



** anova oneway; comparison of collagenase with either saline or normal group; $p < 0.01$ (Figure 3A(i)).

Figure 3A (ii): Graph showing the individual body weights before and after intracerebral haemorrhage in the collagenase-treated group.

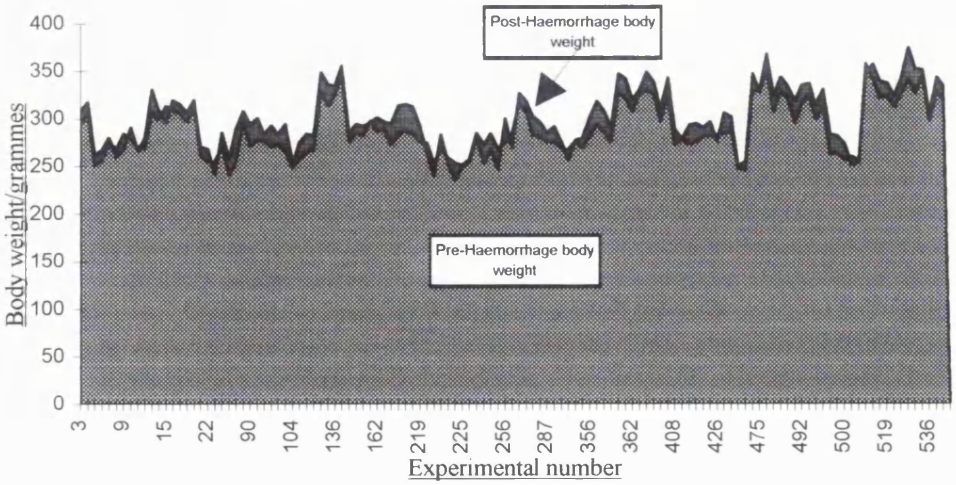


TABLE 3B: The effect of collagenase-induced intracerebral haemorrhage on functional recovery after 24 hours

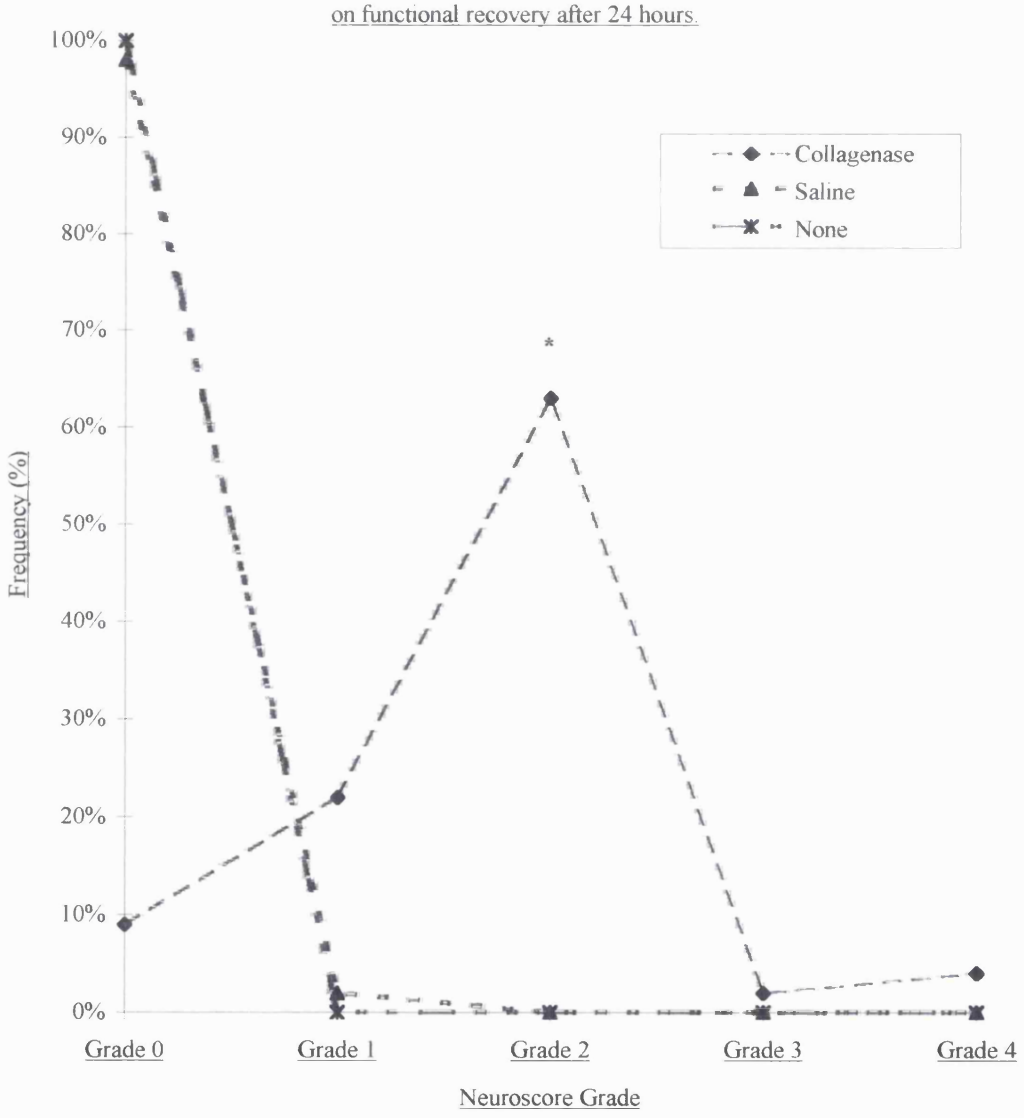
(Values are presented as % of total in the group; n = number of animals used)

Distribution of Neuroscores

Caudate		GRADE					Neurological Score
Injection	n	0	1	2	3	4	Median
None (control)	11	100%	0%	0%	0%	0%	0.0
Saline (control)	42	98%	2%	0%	0%	0%	0.0
Collagenase	128	{9%	22%	63%	2%	4%}*	2.0

* Chi-square test; collagenase vs saline or normal control, $p < 0.01$

Figure 3B: The effect of collagenase-induced intracerebral haemorrhage on functional recovery after 24 hours.



* Chi-square test; collagenase vs saline or normal control, $p < 0.01$

TABLE 3C : The effect of collagenase-induced intracerebral haemorrhage on cerebral volume and haematoma volume following 24 hours of survival.

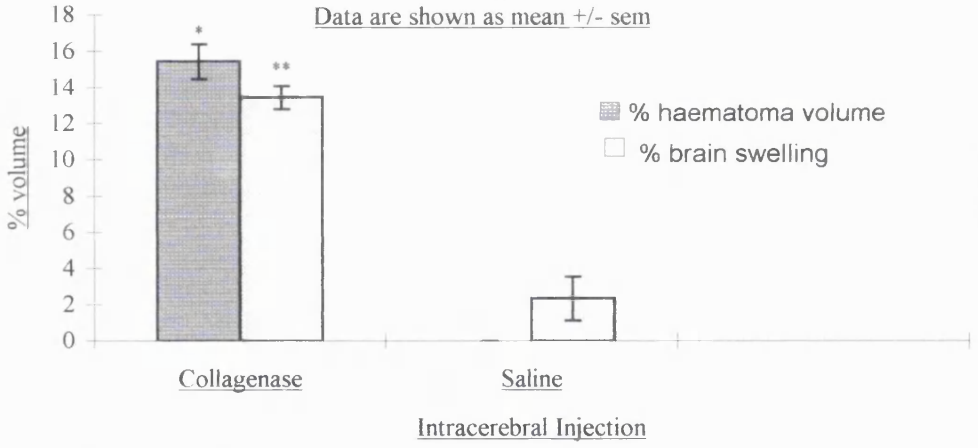
Values are mean ± sem and the number in parentheses refer to number of animals used.

Caudate Injection	% SWELLING Mean + SEM (n)	% HAEMATOMA Mean + SEM (n)
Saline (control)	2.33 ± 1.21 (13)	0.00 ± 0.00 (13)
Collagenase	13.46 ± 0.64 (52)*	15.45 + 0.96 (52)**

*comparison of saline vs collagenase injected (% swelling); anova oneway (unrelated), $p < 0.05$.

** comparison between saline vs collagenase injected (% haematoma); anova oneway (unrelated), $p < 0.05$.

Figure 3C (i): The effect of collagenase-induced haemorrhage on the cerebral volume of both collagenase and saline controls. Data are shown as mean +/- sem



*comparison of saline vs collagenase injected (% swelling); anova oneway (unrelated), $p < 0.05$.

** comparison between saline vs collagenase injected (% haematoma); anova oneway (unrelated), $p < 0.05$.

Figure 3C(ii): Variability of ipsilateral and contralateral hemispherical volumes in all collagenase-injected animals.

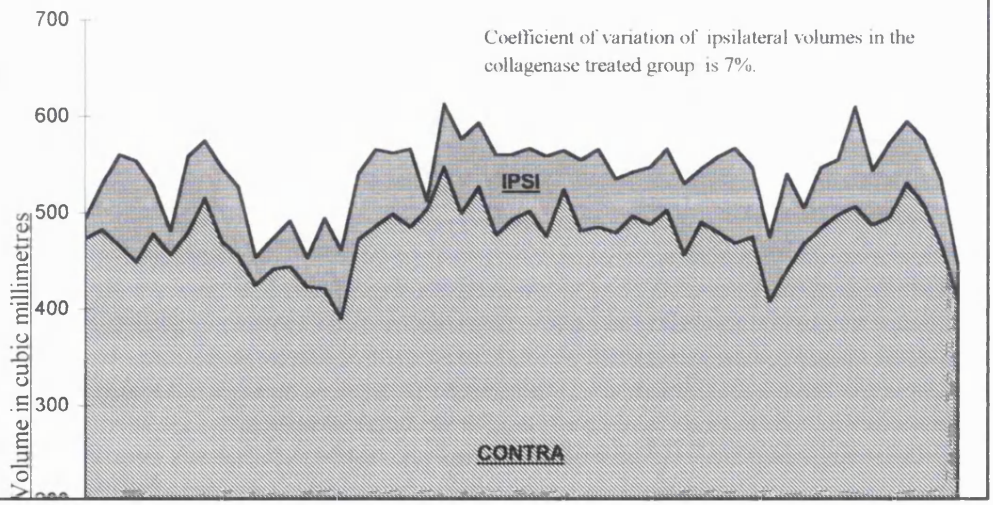


Figure 3C(iii): Regression analysis between haematoma volume data and swelling volume data of all animals in the collagenase-injected group.

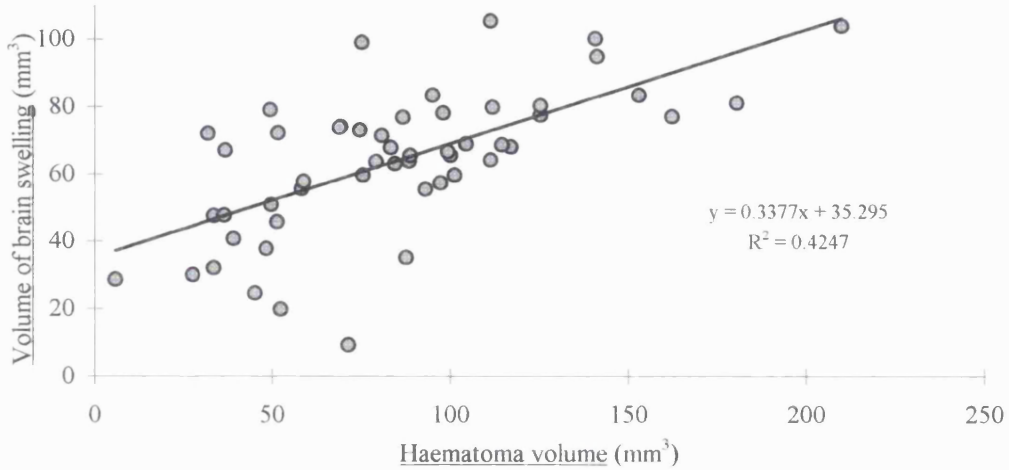


Figure 3C (iv): A scatter plot of % haematoma versus neuroscore deficit.

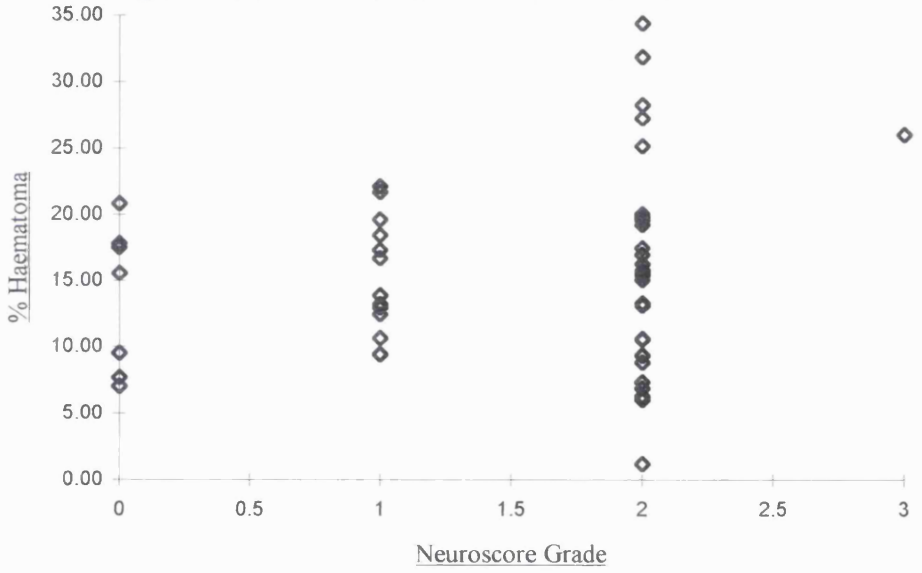


Figure 3C (v): A scatter plot of % cerebral swelling versus neuroscore deficit.

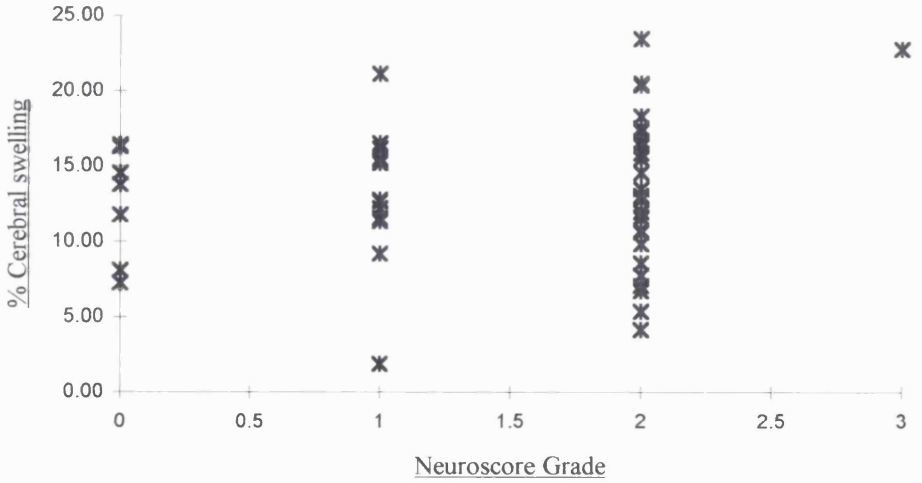


TABLE 3D : The effect of collagenase-induced intracerebral haemorrhage on the percent water content of the ipsilateral and contralateral hemispheres

Values are mean ± sem and the number in parentheses refer to number of animals used

% WATER CONTENT

[(WET WEIGHT-DRY WEIGHT)/WET WEIGHT *100]

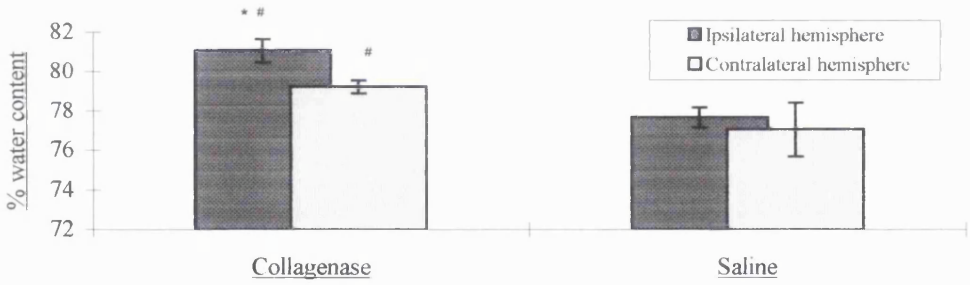
Caudate Injection	IPSILATERAL HEMISPHERE Mean + SEM (n)	CONTRALATERAL HEMISPHERE Mean + SEM (n)
Saline	77.67 ± 0.59(3)	77.07 ± 1.38 (3)
Collagenase	81.08 ± 0.52 (11)*#	79.24 ± 0.32 (11)#

* paired t-test between the ipsilateral and contralateral hemispheres, within group, $p < 0.05$

#comparison between saline and collagenase injected: anova oneway, $p < 0.05$

Figure 3D (i):The effect of collagenase-induced intracerebral haemorrhage on the percent water content of the ipsilateral and contralateral hemispheres

Data presented as mean+/- sem



* paired t-test between the ipsilateral and contralateral hemispheres, within group, $p < 0.05$

#comparison between saline and collagenase injected: anova oneway, $p < 0.05$

Figure 3D(ii): Graph showing the reproducibility of the % water content of both cerebral hemispheres in the collagenase-treated group

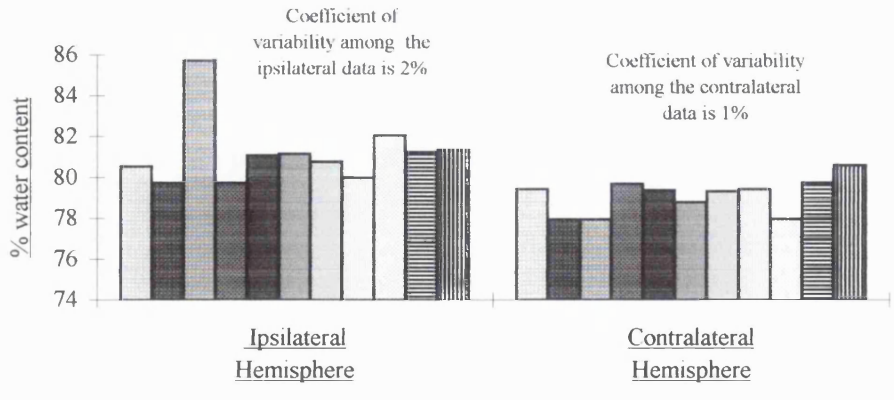


TABLE 3E : The effect of different survival periods on cerebral volume and cerebral haematoma volume in a rat model of collagenase-induced intracerebral haemorrhage.

Values are mean ± sem and the number in parentheses refer to number of animals used.

Survival period	% Change in body weight	Neurological score % Grade 2	% Swelling Mean + SEM (n)	% Haematoma Mean + SEM (n)
2 hours	n/a	n/a	1.52 ± 0.49 (10)*	0.04 ± 0.02 (10)**
6 hours	n/a	n/a	10.17 ± 1.45 (6)	14.9 ± 2.47 (6)
24 hours	(-) 3.53 + 0.38 (123)	63 (128)	13.46 ± 0.64 (52)	15.45 + 0.96 (52)
48 hours	(-) 6 ± 1.9 (6)	100 (6)	11.21 ± 0.99 (6)	5.46 ± 1.02 (6)#

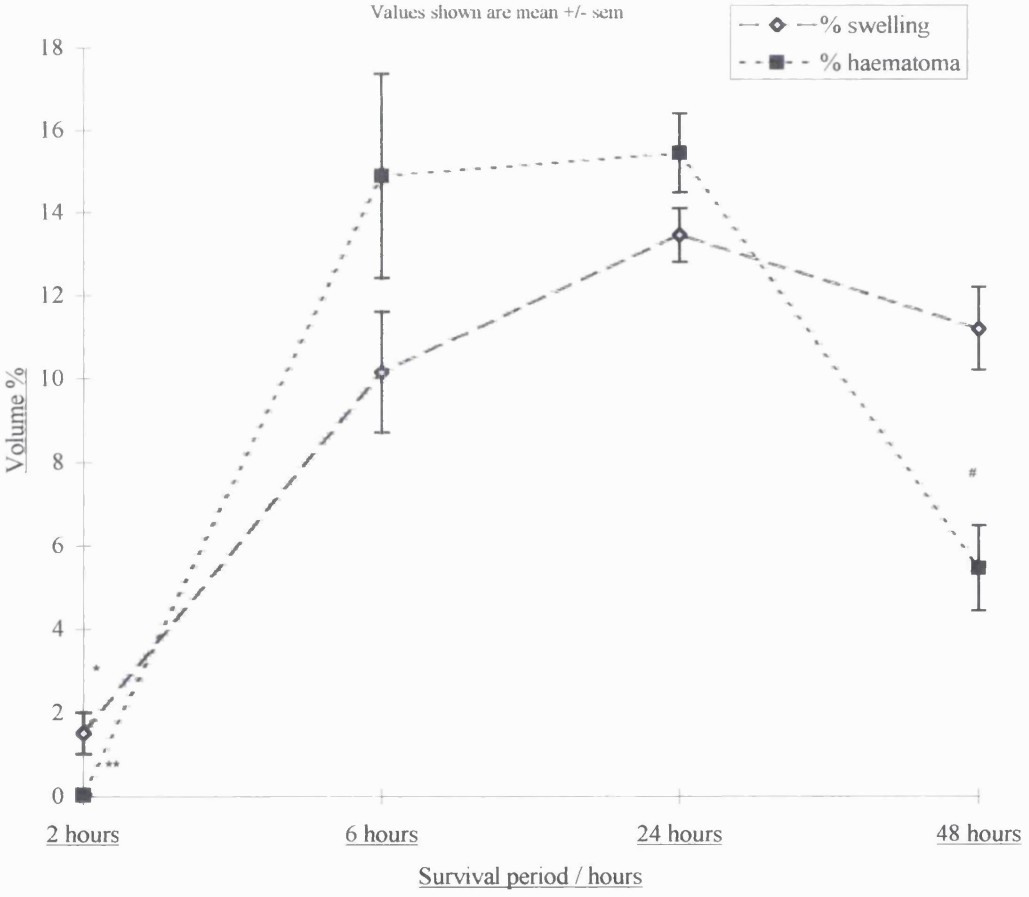
*comparison of % swelling between 2, 6, 24, 48 hrs:anova oneway; $p < 0.0001$.

**comparison of % haematoma between 2, 6, 24, 48 hrs:anova oneway; $p < 0.0001$.

comparison of % haematoma between 6, 24,48 hrs:anova oneway; $p = 0.001$

Figure 3E: The effect of different survival periods on cerebral volume and cerebral haematoma volume in a rat model of collagenase-induced intracerebral haemorrhage.

Values shown are mean +/- sem



*comparison of % swelling between 2, 6, 24, 48 hrs:anova oneway; $p < 0.0001$.

**comparison of % haematoma between 2, 6, 24, 48 hrs:anova oneway; $p < 0.0001$.

comparison of % haematoma between 6, 24,48 hrs:anova oneway; $p = 0.001$

TABLE 3F : Data from the haemoglobin assay on the blood volume contained in the haematoma produced by the collagenase injection.

Values are mean ± sem and the number in parentheses refer to number of animals used

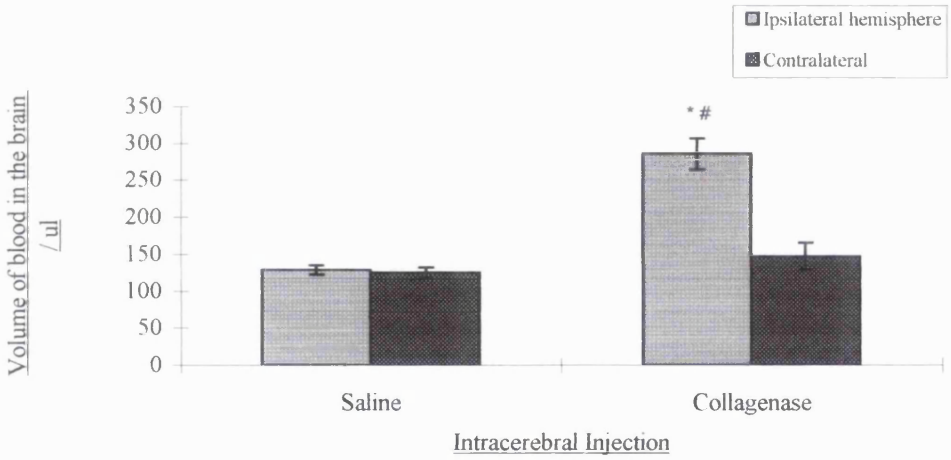
Caudate Injection	VOLUME OF BLOOD (ul) IPSILATERAL HEMISPHERE Mean + SEM (n)	CONTRALATERAL HEMISPHERE Mean + SEM (n)
Saline	129.11 ± 6.16 (3)	125.44 ± 7.30 (3)
Collagenase	285.80 ± 20.71 (26)*#	147.41 ± 18.70 (26)

* comparison between ipsilateral hemispheres of saline and collagenase-injected animals: anova one way, p = 0.02

ipsilateral vs contralateral; paired t-test within group, p < 0.001

Figure 3F(i): The volume of blood contained in both the hemispheres of the saline and collagenase-treated groups.

Values are means +/- sem.



* comparison between ipsilateral hemispheres of saline and collagenase-injected animals: anova one way, p = 0.02

ipsilateral vs contralateral; paired t-test within group, p < 0.001

Figure 3F(ii): The chart shows the range of blood volumes obtained for haematomas in the collagenase treated group..

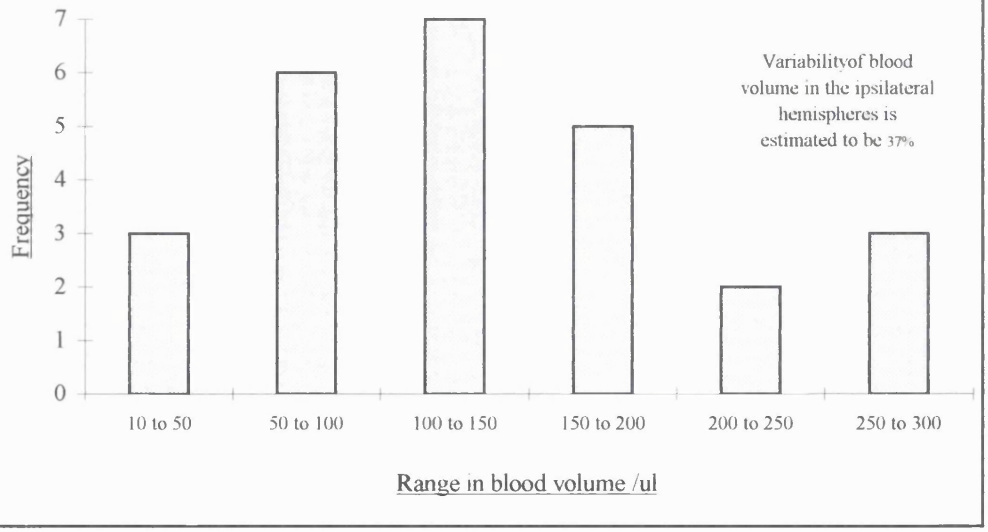


Plate 2

Effect of intracerebral injection of blank, saline and collagenase into the rat caudate.

Ten coronal brain sections were cut using the injection site as the point of reference. The site of injection is always between sections 3 and 4. The contralateral hemispheres were identified by a reference hole.

A: A blank needle was inserted into the left caudate but no intracerebral injection was performed.

B: Intracerebral injection of saline into the right caudate.

C: Intracerebral injection of collagenase into the left caudate.

Scale bar = 5 mm

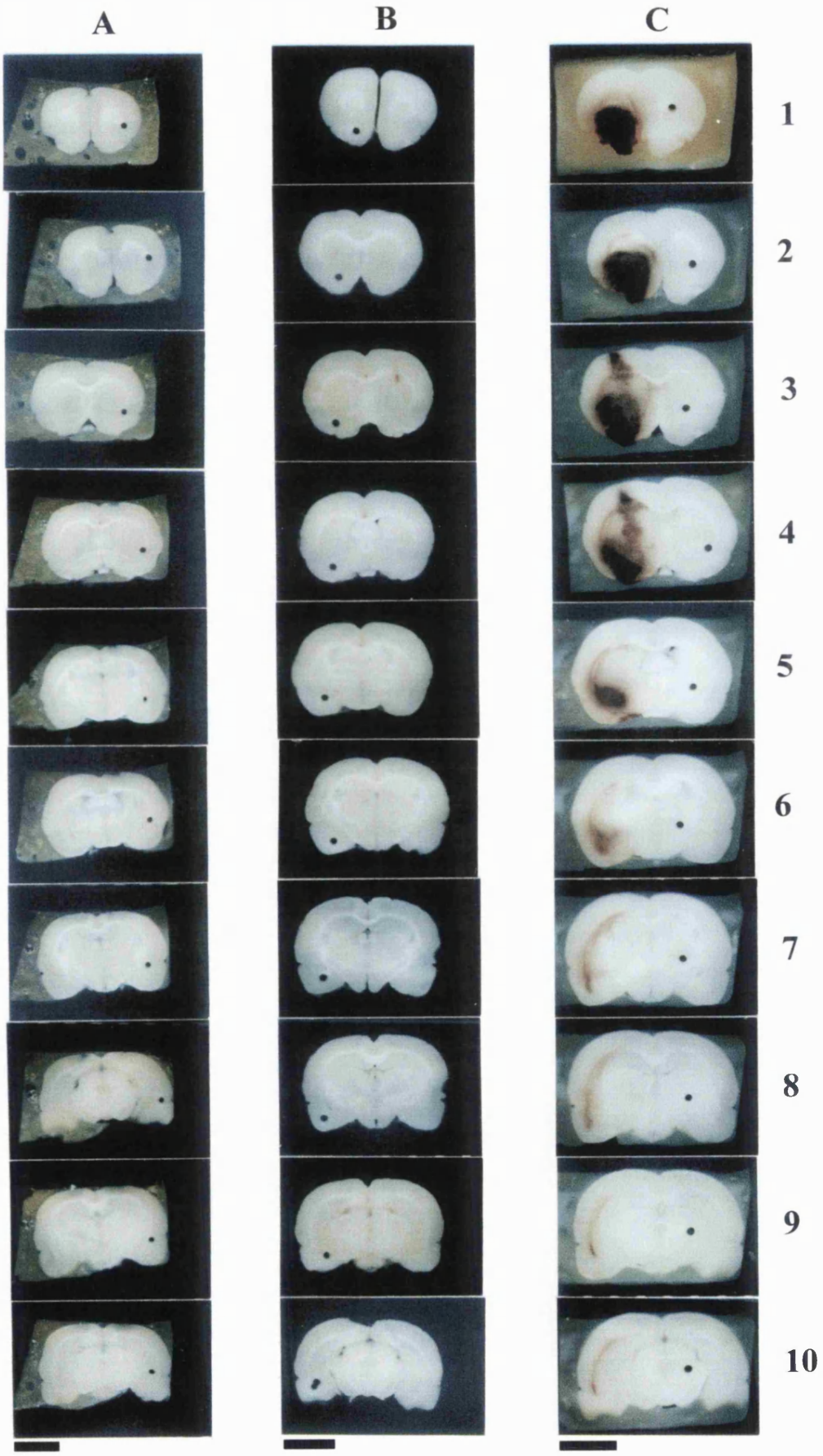

A
B
C
1
2
3
4
5
6
7
8
9
10

Plate 3

Variability in the size of the haematomas produced by intracerebral injection of one dose of collagenase into the rat caudate.

Sections for haematoxylin and eosin staining were cut (8 microns thick) from their respective fixed brains (1 mm thick).

On the left, the appearance of the cut surface and on the right, H & E stained section at the same level.

A: The smallest haematoma volume

B: Representative haematoma volume

C: The largest haematoma volume

Scale bar = 5 mm

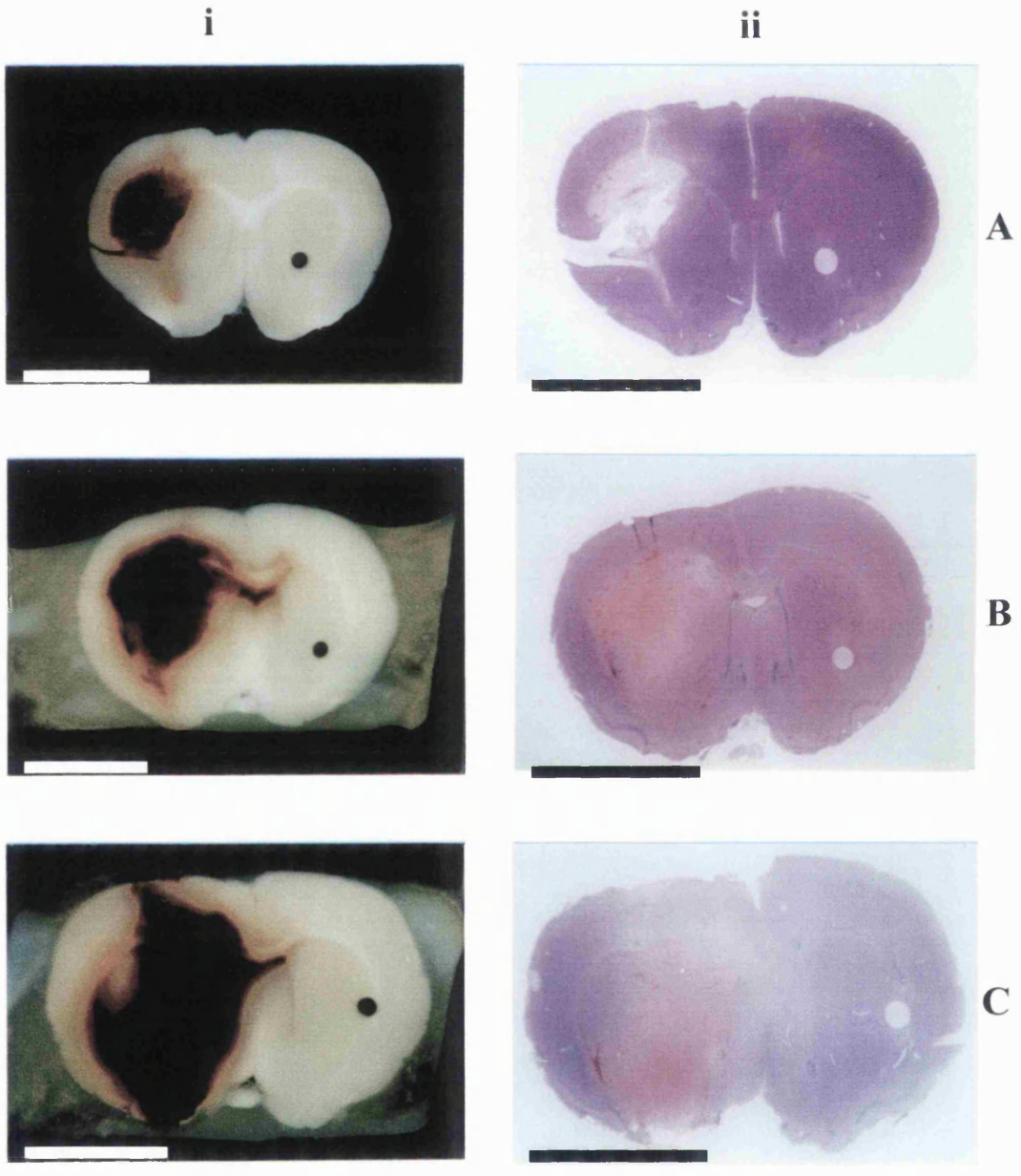

i
ii
A
B
C

Plate 4

Luxol fast blue and Cresyl violet stained coronal paraffin section showing the ipsilateral hemisphere of a saline-injected rat.

A: Photograph shows the point of needle entry (arrow) and the overall 'normal' appearance of the cortex, corpus callosum and caudate.

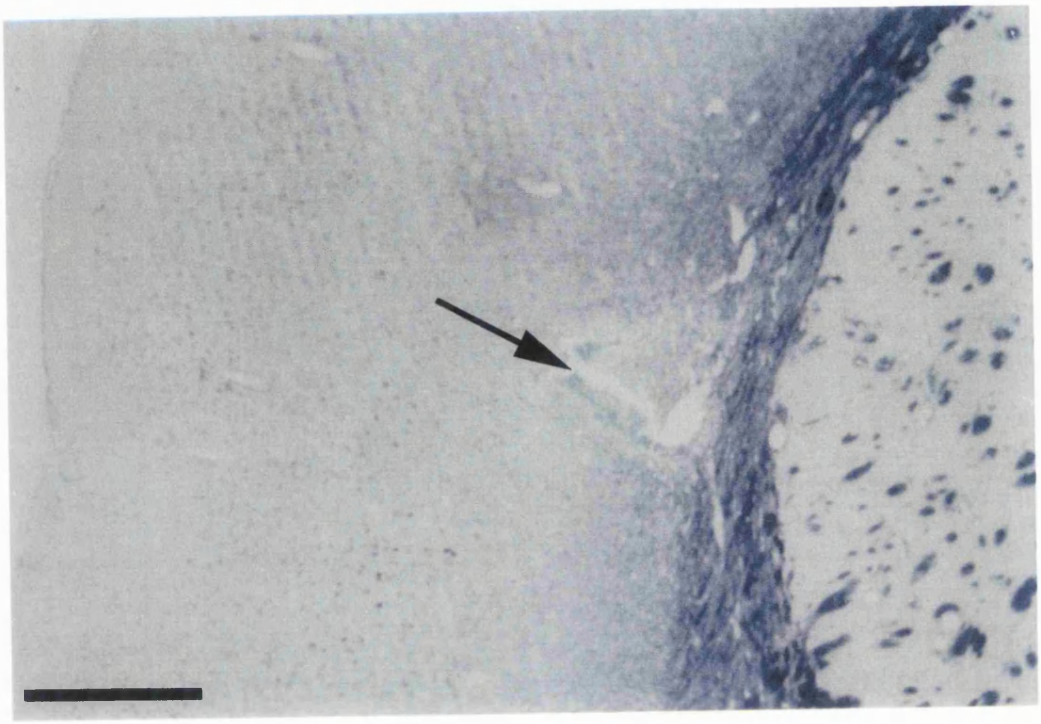
B: A higher magnification of the cortex showing the network of nerve fibres and cells. There is no obvious evidence of damage caused by the saline injection.

Scale bars:

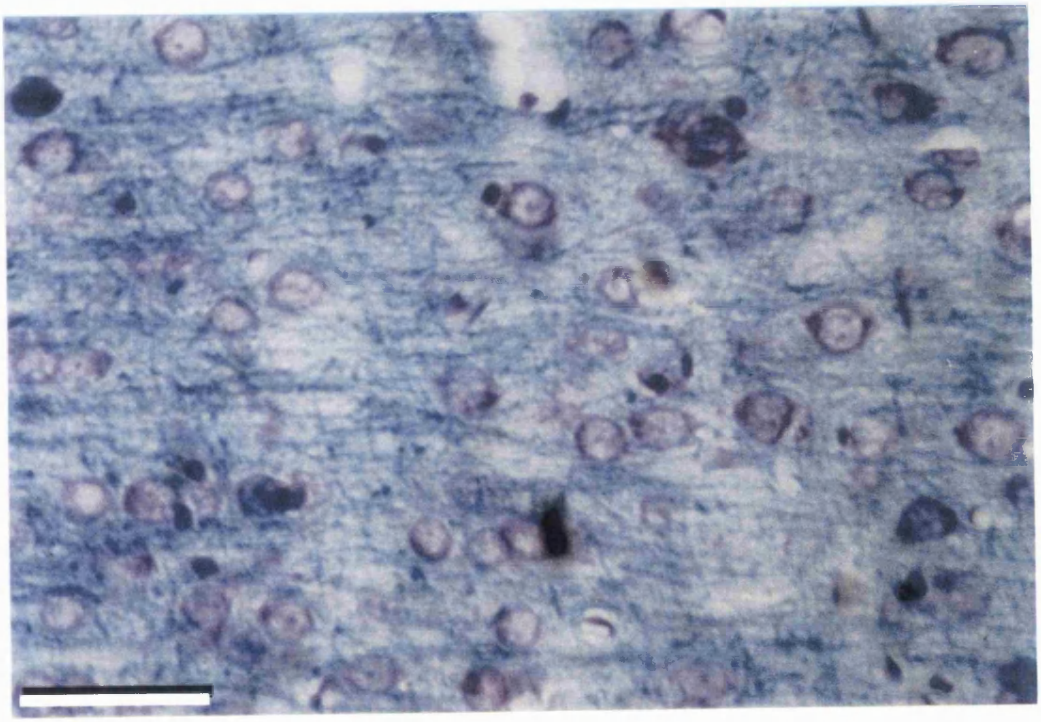
A = 500 um

B = 50 um

Plate 4



A



B

Plate 4

Luxol fast blue and Cresyl violet stained coronal paraffin section showing the ipsilateral hemisphere of a saline-injected rat.

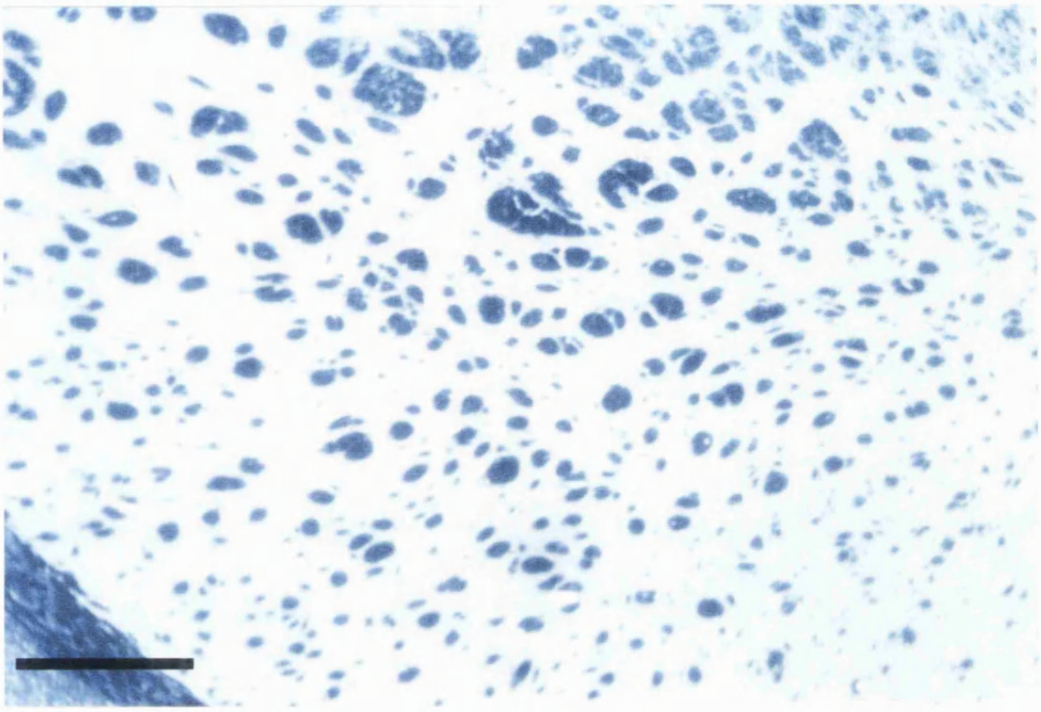
C: Photograph shows the overall 'normal' appearance of the caudate.

D: A higher magnification of the caudate showing the network of nerve fibres, myelin bundles and neuronal cells. No obvious evidence of damage caused by the saline injection is seen.

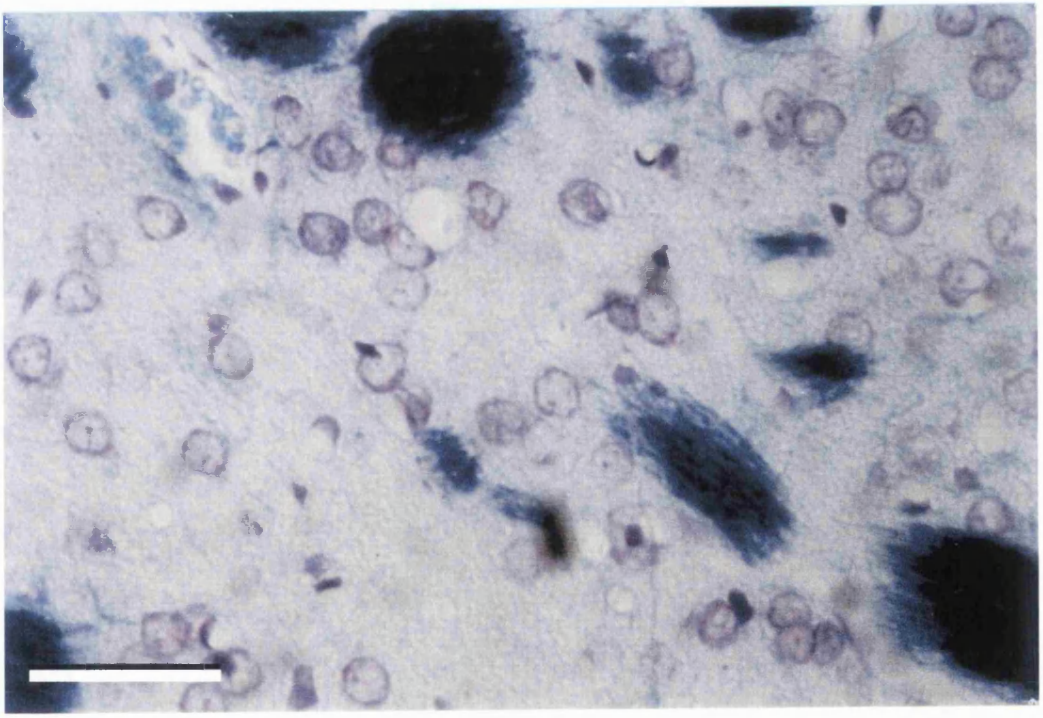
Scale bars:

C = 5 um

D = 50 um



C



D

Plate 5

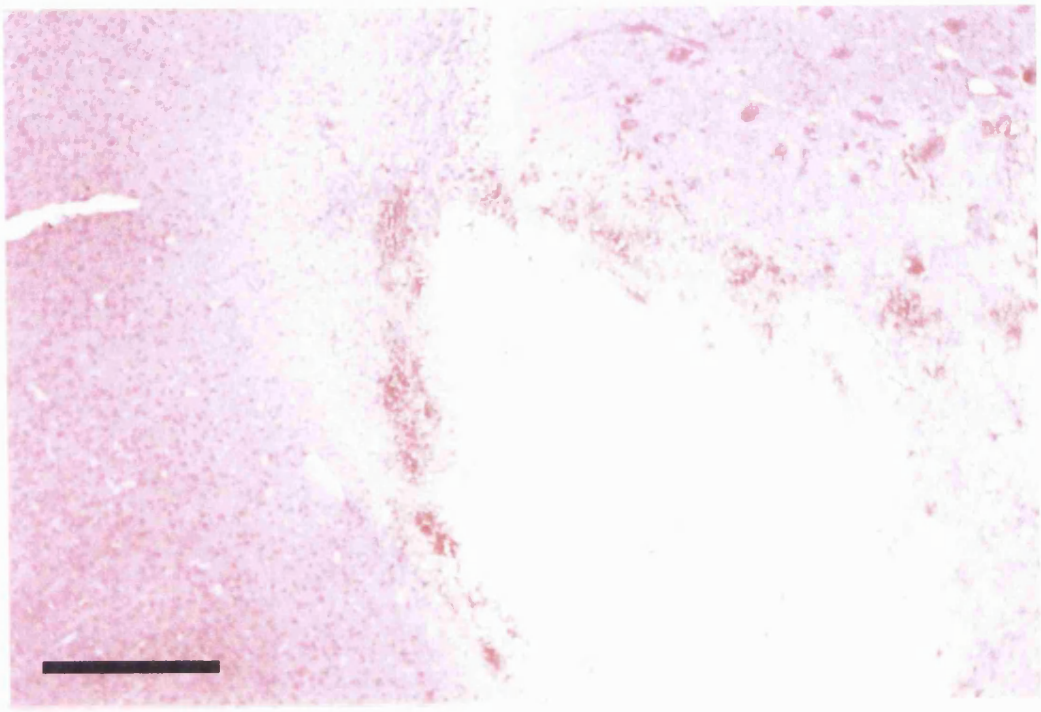
Photomicrograph of a coronal brain section stained with either H&E or Luxol fast blue & Cresyl violet, of the ipsilateral hemisphere from a collagenase-injected rat.

Haematoxylin-Eosin (A) and Luxol fast blue -Cresyl violet (B) stained coronal paraffin section of the ipsilateral hemisphere from a collagenase-injected rat. A and B show the haematoma core bounded by clusters of blood cells.

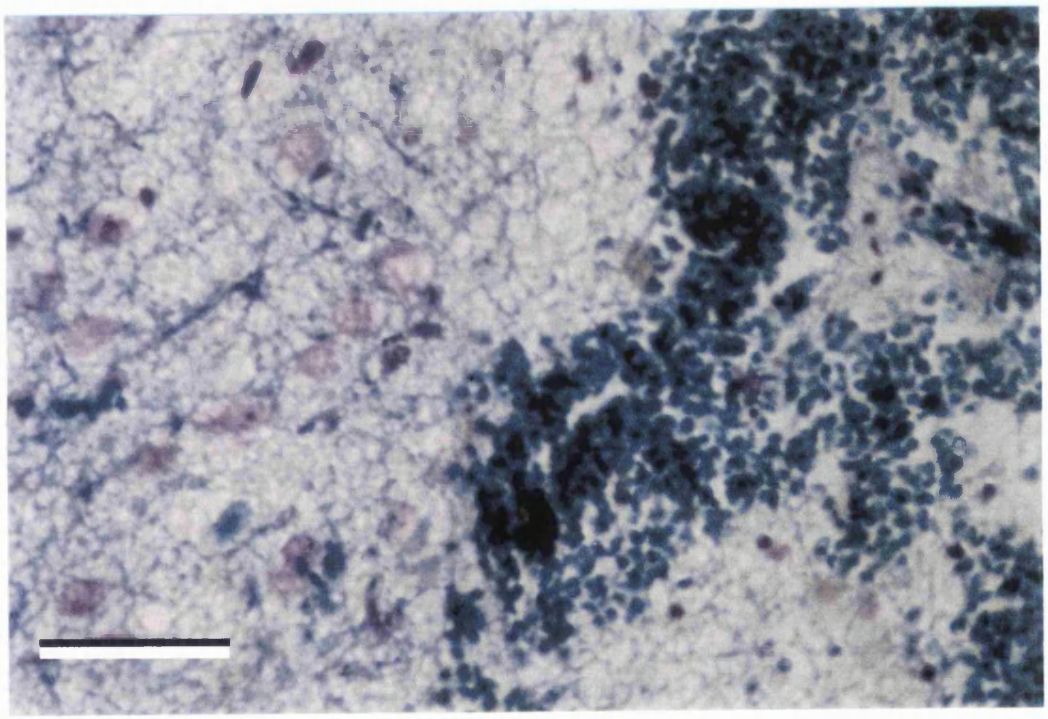
Scale bars:

A = 500 um

B = 100 um



A



B

Plate 6

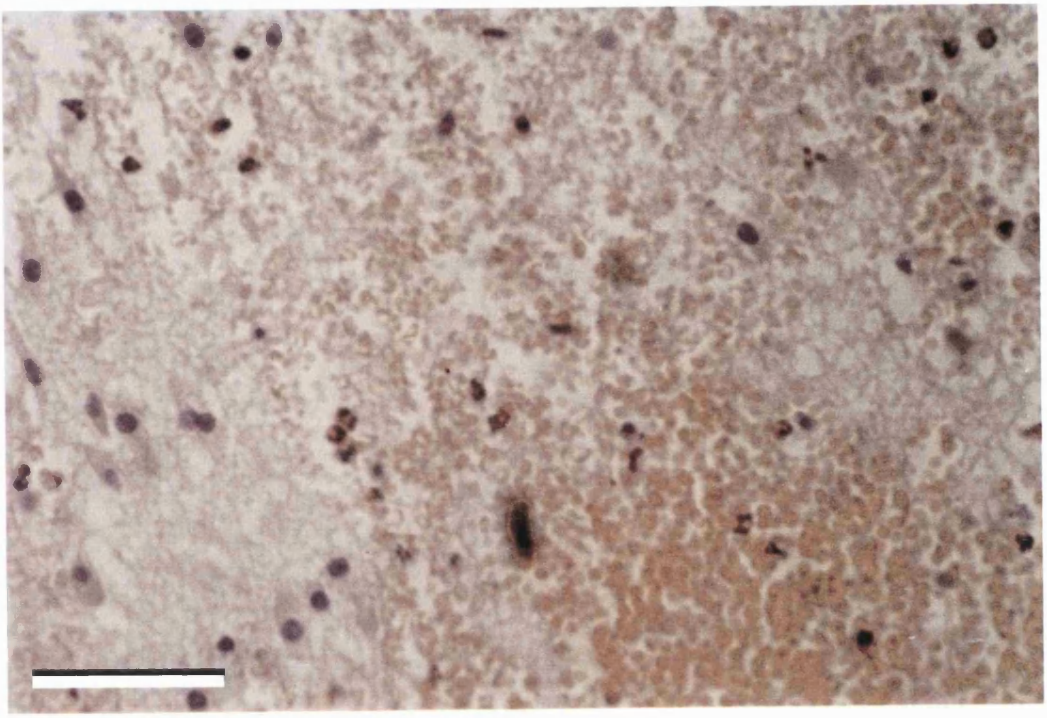
GFAP immunohistochemistry in the cortex and caudate of both the ipsilateral and contralateral hemispheres from collagenase-injected rat.

Formal-saline fixed, paraffin-embedded sections (15 microns thick) stained for astrocytic activity using rabbit anti-cow GFAP (primary antibody).

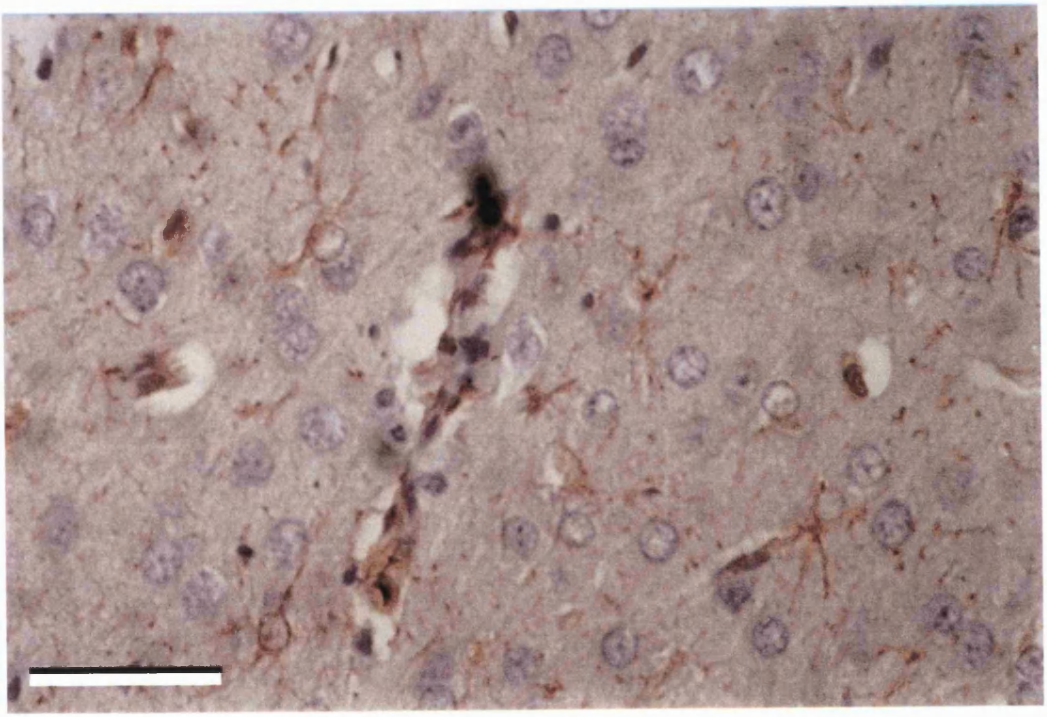
A: Ipsilateral caudate shows total absence of astrocytic activity. The haematoma core is filled with clusters of blood cells.

B: Some astrocytic activity seen in the ipsilateral cortical tissue.

Scale bar = 50 um



A



B

Plate 6

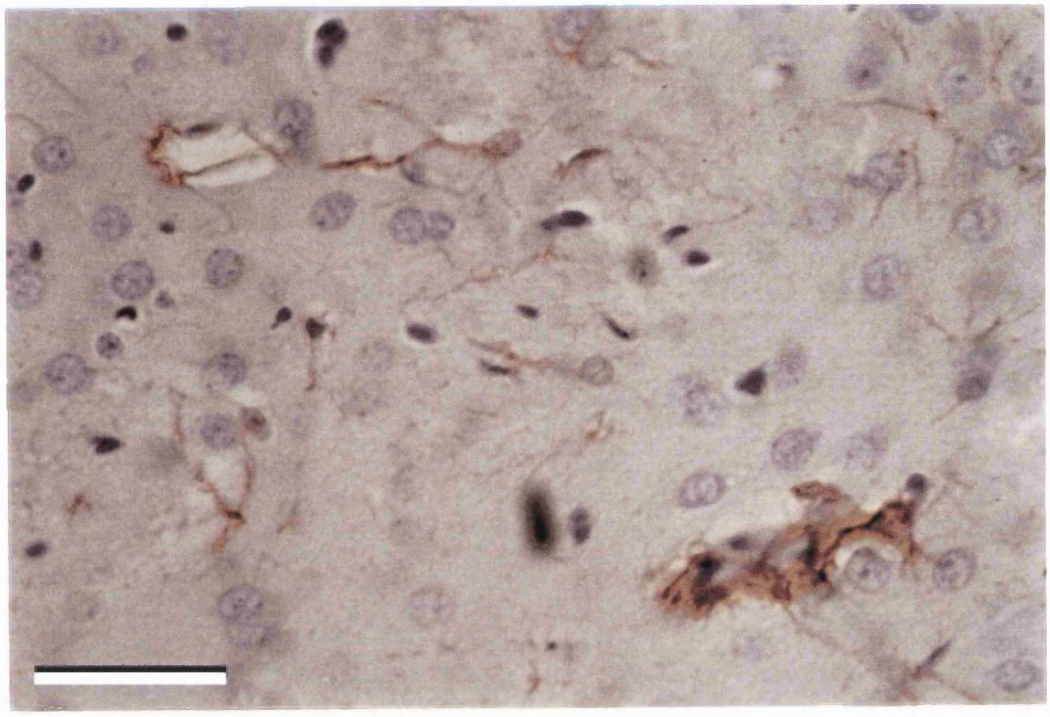
GFAP immunohistochemistry in the cortex and caudate of both the ipsilateral and contralateral hemispheres from collagenase-injected rat.

Formal-saline fixed, paraffin-embedded sections (15 microns thick) stained for astrocytic activity using rabbit anti-cow GFAP (primary antibody).

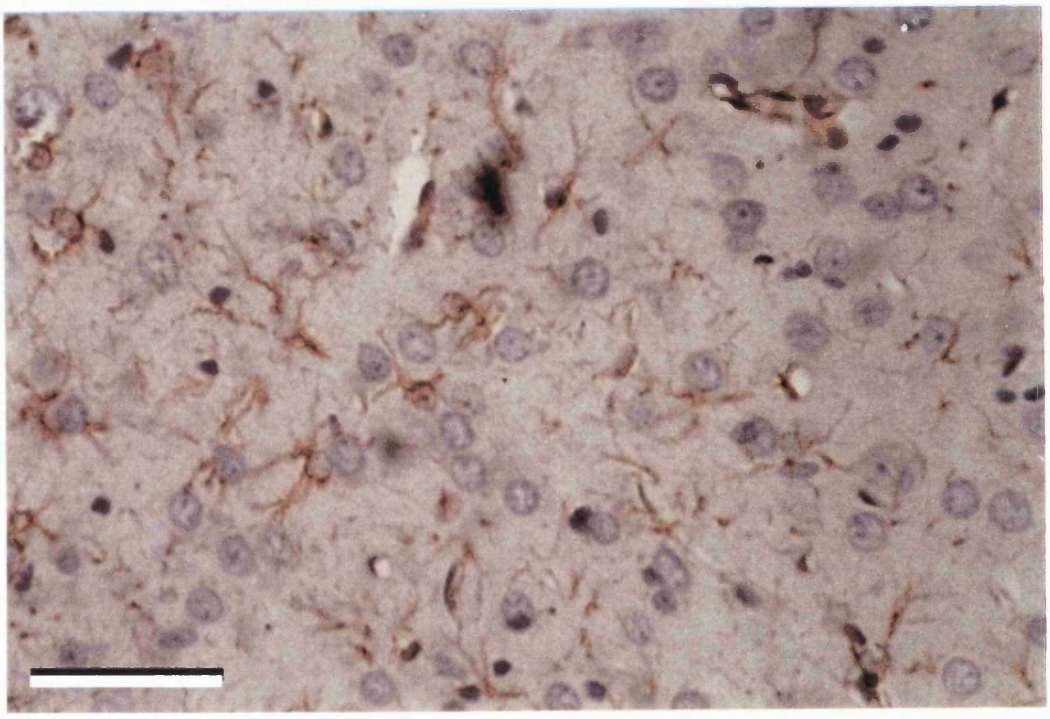
C: Contralateral caudate shows some astrocytic activity

D: Increased astrocytic activity seen in the contralateral cortical tissue.

Scale bar = 50 um



C



D

Plate 7

Transcardiac perfusion of 2,3,5-triphenyltetrazolium chloride (TTC) and intravenous infusion of Evans blue dye following 24 hours after cerebral haemorrhage.

Ten 1 mm coronal sections were cut from formal-saline fixed collagenase-injected rats.

A: Transcardiac perfusion of 2,3,5-triphenyltetrazolium chloride (TTC) 24 hours after cerebral haemorrhage. Only two zones were visualised; (1) the haematoma core and (2) unaffected region. TTC staining did not reveal any areas in either of the two cerebral hemispheres approximating to an ischaemic region.

B: 1 ml/kg of a 2% solution of Evans blue dye was given intravenously an hour before perfusion by fixation. The dye has extrasavated to an area larger than the haematoma core. There is some leakage into the contralateral hemisphere through the corpus callosum.

Scale bar = 5 mm

Plate 7

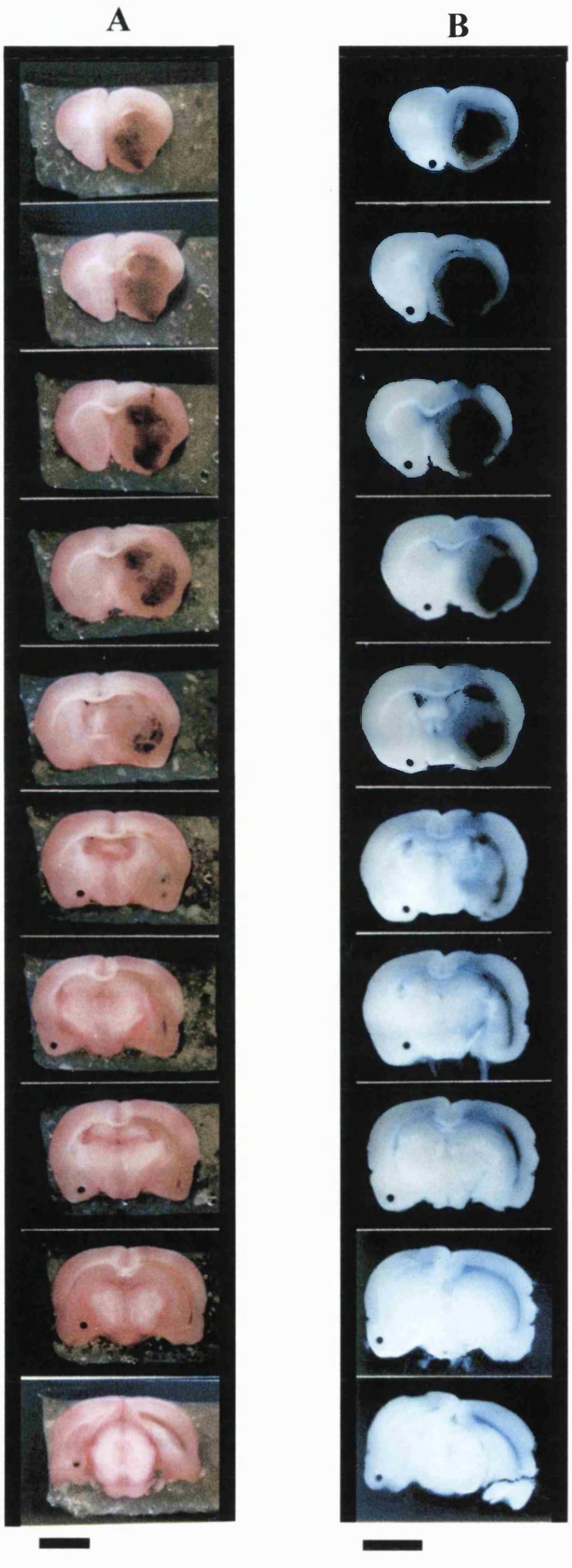


Plate 8

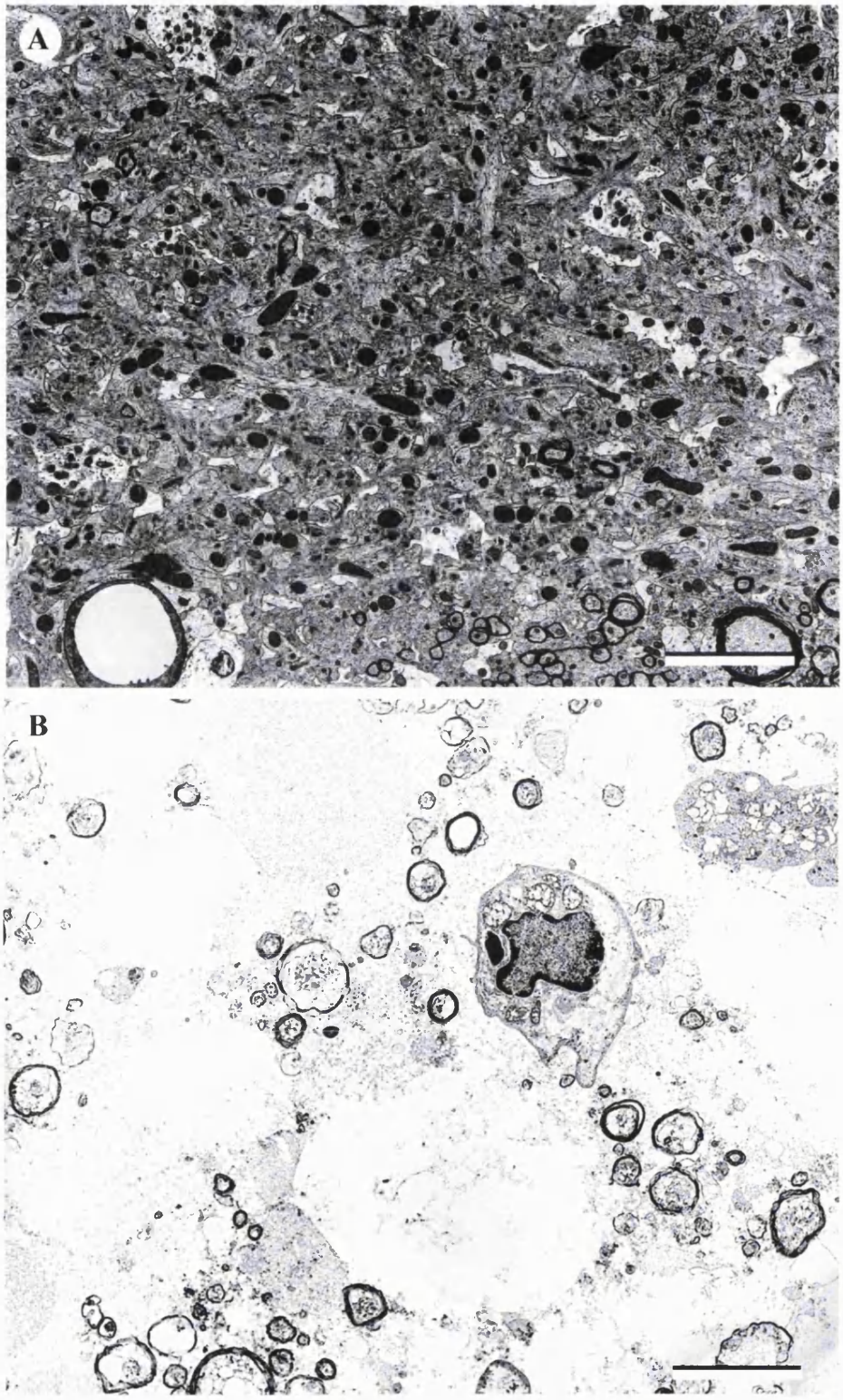
Electron micrographs of the ipsilateral hemisphere from a collagenase-injected rat.

A: An ultrathin section from the ipsilateral cortex. The cellular elements are densely packed with little/no damage to the neuropil matrix.

B: Ipsilateral caudate showing the damaging effects of cerebral haemorrhage. The core is largely vacuolated and translucent.

Scale bar = 2.5 um

Plate 8



Section 4: Discussion

Technically the procedure employed to simulate an intracerebral haemorrhage through the direct injection of collagenase into the caudate is relatively easy to perform. However, the method demands great care so as to avoid artificially aggravating the brain injury following haemorrhage. Data from the saline-injected group reveal that with care the effects due to the mechanical insertion of the needle and the manual injection of saline (0.9% sodium chloride) into the brain are negligible (Tables 3A-D).

The dose of collagenase (0.4 units) chosen for this study was adequate in that it resulted in a large majority of animals with some form of neurological deficit (91%). Dose response curves were not done primarily because the focus of the project was to characterise the model. Further increases or decreases in the dosage would have either raised the mortality rate or reduced the numbers scoring a deficit of 2 or 3. Sixty-three percent of collagenase-treated animals treated exhibited a clinical scoring of Grade 2 (Table 3B). Interestingly, there were instances (9% of animals) where an identical injection of collagenase resulted in a clinical score of 0 (Table 3B). The mortality rate in this model was less than 5% (Table 3B). These characteristics offered a good background for the primary focus of the further studies on interventions.

Statistically, the small values cited for the standard error of the mean for the haematoma volume, cerebral oedema and % water content indicates that there is little deviation from the population mean (Tables 3C-D) and thus suggests that the reproducibility of the model is reasonably high. In each case, the location of the haematoma was always found to be in and around the same area (caudate; Plates 2, 3 and 7). The margin of error in carrying out repeated planimetry measurements was always never more than 10% (data not presented).

The data obtained for the haematoma volume (85 $\pm$ 6 ul) and the volume of blood (138 $\pm$ 14 ul) contained in the haematoma are not matched. Possible reason for the variance in these measurements may include a higher density of packing of red blood cells in the clot compared to free haemoglobin measurement in unclotted blood. Also there may be an underestimation in haematoma volume from planimetry measurements based on brain slices.

Infusion of Evans Blue dye prior to perfusion fixation revealed that dye leakage extended well beyond the haematoma (Plate 7A). This would seem to imply that brain regions remote from the core but within the ipsilateral hemisphere are affected by vasogenic oedema. This is in agreement with the data on the increased volume of cerebral swelling and % water content of the ipsilateral hemispheres.

TTC staining did not reveal an intermediate region between the haematoma core and unaffected areas. The haematoma core was stained black/dark brown and the rest of the tissue in the ipsilateral hemisphere and the whole of the contralateral area stained red (Plate 7B) indicating undamaged tissue with active mitochondrial dehydrogenase.

At both the light (Plates 4 and 5) and electron microscopic (Plate 8) levels, the three areas of interest in the haematoma-bearing hemisphere are:

(a) the core of the haematoma

(b) the appearance of the perihaematoma boundary if clearly delineated

(c) the region beyond the perihaematomal boundary

The haematoma core proved to be composed of blood cells. The haemorrhage has caused the destruction of the brain tissue in the haematoma of the ipsilateral hemisphere but not of the contralateral hemisphere. There was no evidence of an

ischaemic injury zone between the haematoma core and untraumatised tissue. This supports the TTC findings.

Immunohistochemistry with GFAP revealed that in response to the haemorrhagic insult in the ipsilateral hemisphere, astrocytes in the cortex of the ipsilateral hemisphere and in the contralateral hemisphere responded by the production of GFAP (Plate 6). There is a complete destruction of astrocytes associated with the haematoma core and this is in agreement with Koeppen et al findings (1995) who found that the haemorrhagic insults caused a destruction of astrocytes in the perifocal zone.

Data from the collagenase-induced haemorrhage time course experiments (Table 3E) showed that both the haematoma and the associated cerebral swelling increased from 2 to 24 hours. Afterwhich, there was a fall in both measures. However, this reduction was not reflected as improvement in clinical scores. This seems to suggest that any brain injury sustained at 24 hours post-haemorrhage is irreversible thereafter.

An obvious weakness of this model is the use of perfectly healthy animals, which contrasts with the vascular pathology in human victims for example of hypertensive cerebral haemorrhage. Otherwise, the intracerebral haemorrhage model appears simple, reproducible, versatile and should therefore lend itself well to the exploration of potential therapies for intracerebral haemorrhage.

CHAPTER IV

THE EFFECT OF MANNITOL, GLYCEROL, DEXAMETHASONE AND INDOMETHACIN TREATMENT ON CEREBRAL SWELLING IN A RAT MODEL OF INTRACEREBRAL HEMORRHAGE.

Section 1: Introduction

Brain swelling is a life threatening, clinically important secondary complication caused by a variety of brain injuries. Brain swelling may be defined as an increase in brain volume. This increase in brain volume may be due to an increase in cerebral blood volume (congestive brain swelling) or an increase in the water content of the cerebral tissue (cerebral oedema). In cerebral oedema, the water accumulation can either be intracellular (cytotoxic) or extracellular (vasogenic) or a combination of both (Klatzo, 1967).

Cytotoxic Oedema

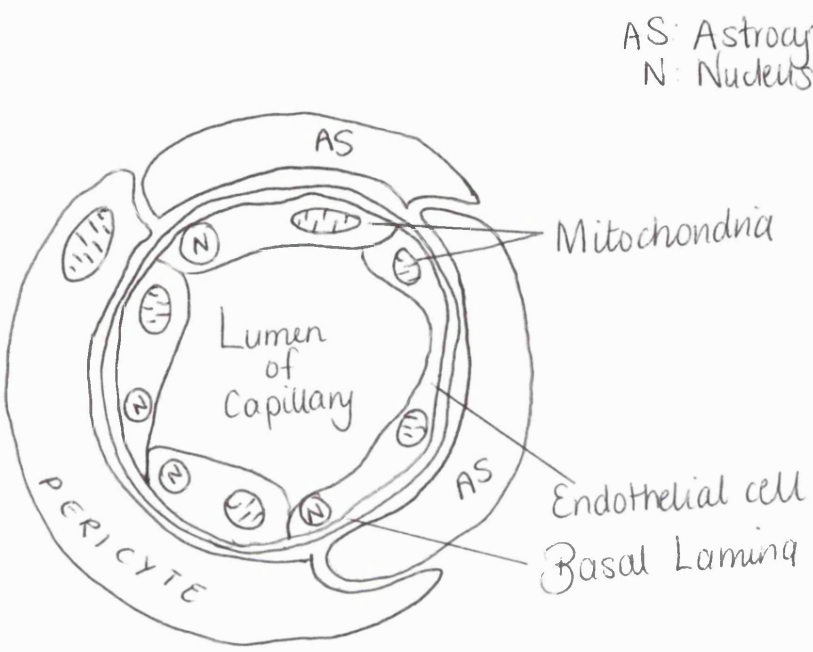
When energy failure occurs, there is an eventual swelling of all cellular elements because of a disturbance of the ionic pump and transport mechanisms for sodium, chloride, bicarbonate, potassium and calcium, leading to an accumulation of water intracellularly. This form of oedema is seen in ischaemic-hypoxia brain damage (Siesjo, 1984). The blood brain barrier, in this type of oedema, remains intact.

Vasogenic oedema.

The brain is normally protected from variations in the contents of the blood perfusing its capillary bed, by the blood brain barrier (BBB) which limits the egress of solutes across the capillary endothelium. The morphological substrate of the blood brain barrier in cerebral vessels is composed of the continuous layer of collagen-based basal lamina, endothelial cells with tight junctions and with little or no vesicular transport, the

astrocytes, the pericytes and the perivascular microglia (Diagram D). Lipophilic compounds can easily cross the blood brain barrier by simple osmotic diffusion, and the transcellular transport of hydrophilic solutes is made possible by facilitated diffusion and active transport.

Diagram D: The interaction between the neuron, glial cell, and the cerebral capillary.



Vasogenic oedema occurs when there is ;

1. an increase in intravascular pressure
2. damage and increased permeability of the cerebral vascular wall
3. a decrease in plasma colloid osmotic pressure

Any one of these conditions would encourage a net outflow of water from the vascular bed into the brain's extracellular space. Under hydrostatic pressure, water, sodium, chloride and protein molecules extrasavate from blood vessels that are located principally in grey matter, and the oedema fluid then moves on to accumulate in the extracellular space of white matter. This type of oedema is easily produced experimentally, for example by application of intense cold to the surface of the brain

(Clasen et al, 1962; Klatzo, 1967).

In intracerebral haemorrhage, the breach in the cerebral vessels facilitates the entry of whole blood and its components into the brain parenchyma, at arterial/venous pressure. This increases the extracellular space volume. As the tissue pressure increases, the blood vessels become compressed, leading to decreased blood flow. The reduced blood supply of oxygen and glucose to tissues distant from the core of the haematoma may thus cause ischaemia. Since the cranium is inflexible, the intracranial pressure rises as the brain expands, which leads to brain herniation, culminating if unchecked, in brain stem damage and death of the patient.

Patently, the cerebral swelling induced by cerebral haemorrhage, consists of the haematoma mass, and the ensuing processes suggest that both vasogenic and cytotoxic oedema may also develop with the former as the dominant event.

The amelioration of cerebral oedema (cytotoxic and vasogenic types) has been researched in several forms of brain injury clinically as well as experimentally, utilizing hyperosmotherapy, dexamethasone and indomethacin.

Osmotherapy is frequently the preferred clinical approach with the aim of reducing intracranial pressure. Glycerol and mannitol produce an osmotic effect by drawing water from the oedematous tissue into the intravascular compartment of the brain, but only in regions where the blood brain barrier is intact. However, when the blood brain barrier is damaged, the osmolar agent(s) enter the damaged brain and can actually promote the influx of more water into the brain (Chen et al, 1990). One of the side effects of long term osmotherapy, more especially with mannitol than with glycerol (Garcia et al, 1991; Node et al, 1990), is this 'rebound' phenomenon as the hyperosmotic compound enters the brain. The cause of this has been linked to the

metabolic stability of the compound and depends on the dose, concentration , duration and the infusion method of the treatment .

Results from different animal models of cold injury (von Berenberg et al, 1994; Kamezawa et al, 1994; Wang et al, 1994; Garcia et al, 1991; Chen et al, 1990; Guisado et al, 1976) and acute ischaemia (Paczynski et al, 1997; Otsubo et al, 1994; Kashiwagi et al, 1990; Ohta et al, 1990; Mujsce et al, 1990) seem to suggest that in the main, at a certain dose and with the appropriate route of administration, both glycerol and mannitol are effective in reducing brain swelling. Plotnikov (1981) reported that glycerol, given 30 minutes and even 1 to 2 days post cerebral haemorrhage, suppressed the development of cerebral oedema in cats.

Small clinical controlled trials of intravenous glycerol have claimed benefits for patients with acute ischaemic stroke (Azzinibdu et al, 1994; Hansen & Boysen, 1992; Yu et al, 1992;Bayer et al, 1987; Gilsanz et al, 1975). However, Yu et al (1993) found glycerol administration did not benefit patients with intracerebral haemorrhage. There is very little evidence of clinical trials involving mannitol. Despite this lack of information, mannitol has become the main stay of therapy for both ischaemic and haemorrhagic stroke patients, where lowering of the ensuing high intracranial pressure is urgent.

Corticosteroids, for example dexamethasone, reduce peritumoural vasogenic oedema (Heiss et al, 1996; Molnar et al, 1995; Chumas et al, 1997). They appear to reduce oedema formation by reducing the abnormally high vascular permeability - this may occur by the endothelium reacquiring the blood brain barrier characteristics which are lacking in the microvasculature of brain tumours.

There have been fourteen randomised clinical trials in acute ischaemic stroke, using corticosteroids (Qizilbash & Murphy, 1993). Overall these clinical trials did not

show any significant differences between treated and control groups. This implies that the oedema in ischaemic stroke differs from that around tumours, the latter with more severe damage to the cerebral capillary endothelium, the former perhaps with a more mixed aetiology of oedema. As with Yu's et al study with glycerol (1993), Poungvarin et al (1987) found that dexamethasone did not show any beneficial effects in patients with supratentorial intracerebral haemorrhage .

The release of arachidonic acid from the membrane phospholipids is associated with various pathological insults , such as ischaemia, hypoxia and trauma. Leukotrienes and prostanoids are the products from enzymic oxygenation of arachidonic acid by lipo-oxygenase and cyclo-oxygenase respectively (Wolfe, 1982). The leukotrienes and prostanoids influence the permeability of the microvasculature and have been shown to be active mediators of brain oedema (Baethmann et al, 1997; Chan et al, 1978). Indomethacin reduces brain oedema by inhibiting the enzyme, cyclo-oxygenase (Gaudet et al, 1980, 1979; Moncada et al, 1978).

Studies utilising cerebral ischaemic animal models have shown that indomethacin reduced oedema (Yamamoto et al, 1996; Olsson et al, 1992; Deluga et al, 1991; Sasaki et al, 1988; Sutherland et al, 1988), and improved cerebral perfusion (Shigeno et al, 1985; Hallenback et al, 1979 ; Furlow et al, 1978).

In 1994, a multicentre randomised trial of indomethacin therapy in neonatal intraventricular haemorrhage was reported (Ment et al, 1994). The results show that indomethacin was effective in limiting extension of intraventricular haemorrhage. No other types of clinical trials of indomethacin appear to have been reported in either ischaemic or haemorrhagic stroke.

In this part of the study, these three approaches to ameliorate the brain swelling in a rat model of intracerebral haemorrhage, are investigated:

1. Osmotherapy, using glycerol and mannitol
2. A corticosteroid, dexamethasone
3. A cyclooxygenase inhibitor, indomethacin

Section 2: Materials And Methods

The preparation of the animals for surgery, the production of intracerebral haemorrhage, and experimental procedures is as detailed in Chapter 2.

One hundred and forty-three adult male Sprague-Dawley rats, weighing between 280-300 g body weight were used in this study. All experiments involved a twenty-four recovery period.

The protocol involved the measurement of body weight (before surgery and before sacrifice), an assessment of the recovery status, volume of cerebral swelling and haematoma and the water content of the brain following potentially therapeutic administration.

Animals were randomly assigned into four main treatment groups: glycerol, mannitol, indomethacin and dexamethasone. All drug solutions were prepared freshly on the day of the experiment.

Mannitol

Six male adult Sprague Dawley rats were used. Mannitol was purchased ready made as a 20% (w/v) sterile solution (Middlesex Hospital Pharmacy). A dose of 6.5 ml kg^{-1} was administered as a single bolus intraperitoneally thirty minutes prior to the induction of cerebral haemorrhage.

Glycerol

A total of forty male adult Sprague Dawley rats were employed. Two doses of glycerol (Sigma Chemical Co., UK) concentrations were tested; 10% and 20%. Dilutions

were made up in 0.9% (w/v) sterile sodium chloride. Animals received either one of the two doses at 6.5 ml kg^{-1} thirty minutes after the end of the collagenase injection.

Dexamethasone

Thirteen male Sprague Dawley rats were used. Dexamethasone was supplied as 4 mg ml^{-1} (Middlesex Hospital Pharmacy). Animals received a single bolus of 4 mg kg^{-1} dexamethasone intraperitoneally thirty minutes prior to the production of the cerebral haemorrhage.

Indomethacin

A total of seventy six male Sprague Dawley rats were used. Two doses were tried; 4 mg kg^{-1} and 10mg kg^{-1}. Indomethacin was given as a single bolus intraperitoneally thirty minutes before cerebral haemorrhage was initiated.

Section 3: Results

Loss in body weight ranged from 2.3 ± 2.4% to 4.54 ± 0.95% (Table 4A and Figure 4A). There was no clear evidence of a therapeutic effect in mannitol and dexamethasone treated groups ($p = 0.62$, Anova oneway). The greatest variability in weight loss was seen in these two groups whose sample sizes were also the smallest.

The effect of the treatments on the clinical scores is summarised in Table 4B and depicted in Figure 4B. In all groups there is a shift of the scores towards the left, indicating an amelioration of clinical damage. The results reached statistical significance in two treatment cases; 20% glycerol and 10 mg/kg indomethacin. Mannitol did not reach statistically significance perhaps once again due to the small sample size.

With the exception of dexamethasone, haematoma size when compared with controls was either unchanged or insignificantly smaller in all treated groups studied (Table 4C and Figure 4C). Dexamethasone treatment was associated with reduction in the size of the haematoma from 15.45 ± 0.96% to 9.8 ± 0.85% ($p = 0.04$; Anova oneway). It should be noted that the data were obtained from a group of seven animals.

In each group, the haematoma bearing hemisphere was significantly larger than the contralateral (Table 4C and Figure 4C). The extent of the swelling in all treated groups, apart from the 4 mg/kg indomethacin group, was not significantly different from controls. The percentage swelling associated with the lower indomethacin dose was 10.49 ± 0.94% compared to 15.45 ± 0.96% ($p = 0.02$; Anova oneway).

Measurement of the hemispheric water content is reported in Table 4D and shown graphically in Figure 4D. In all groups, the water content of the haematoma bearing hemisphere significantly exceeded that of the contralateral hemisphere. All treated groups show evidence of water reduction in both hemispheres when compared with controls ($p \leq 0.01$; Anova oneway).

TABLE 4A: The effect of mannitol, glycerol, dexamethasone and indomethacin treatment on body weight in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

(Values are presented as mean +/- sem. The number in brackets refer to the number of animals in the group).

Caudate Injection	Drug Dose	Treatment Time	% Change in body weight Mean + SEM (n)
Collagenase	None	None	(-) 3.52 ± 0.38 (123)
	Mannitol		
Collagenase	20%	Pre 30 mins	(-) 2.52 ± 1.89 (6)
	Glycerol		
Collagenase	10%	Post 30 mins	(-) 3.97 ± 0.72(20)
Collagenase	20%	Post 30 mins	(-) 4.54 ± 0.95 (20)
	Dexamethasone		
Collagenase	4 mg/kg	Pre 30 mins	(-) 2.3 ± 2.4 (13)
	Indomethacin		
Collagenase	4 mg/kg	Pre 30 mins	(-) 3.18 ± 0.56 (44)
Collagenase	10 mg/kg	Pre 30 mins	(-) 4.45 ± 0.65 (32)

Figure 4A: The effect of mannitol, glycerol, dexamethasone and indomethacin treatment on body weight in a rat model of collagenase-induced intracerebral haemorrhage in the rat.

Data shown with their means and sem.

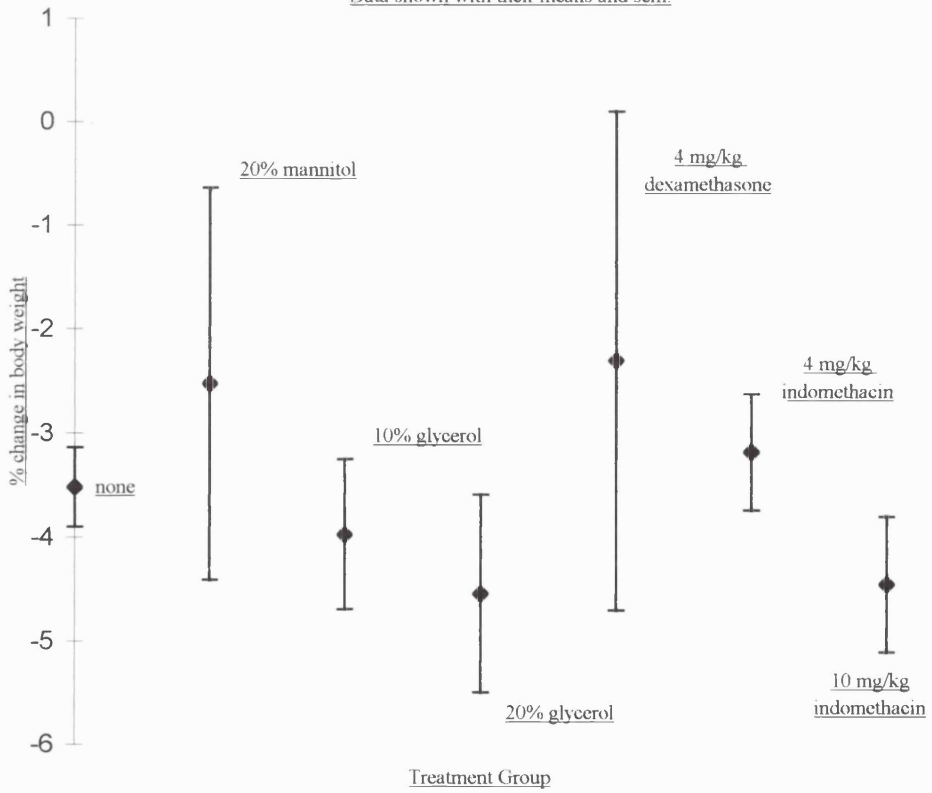


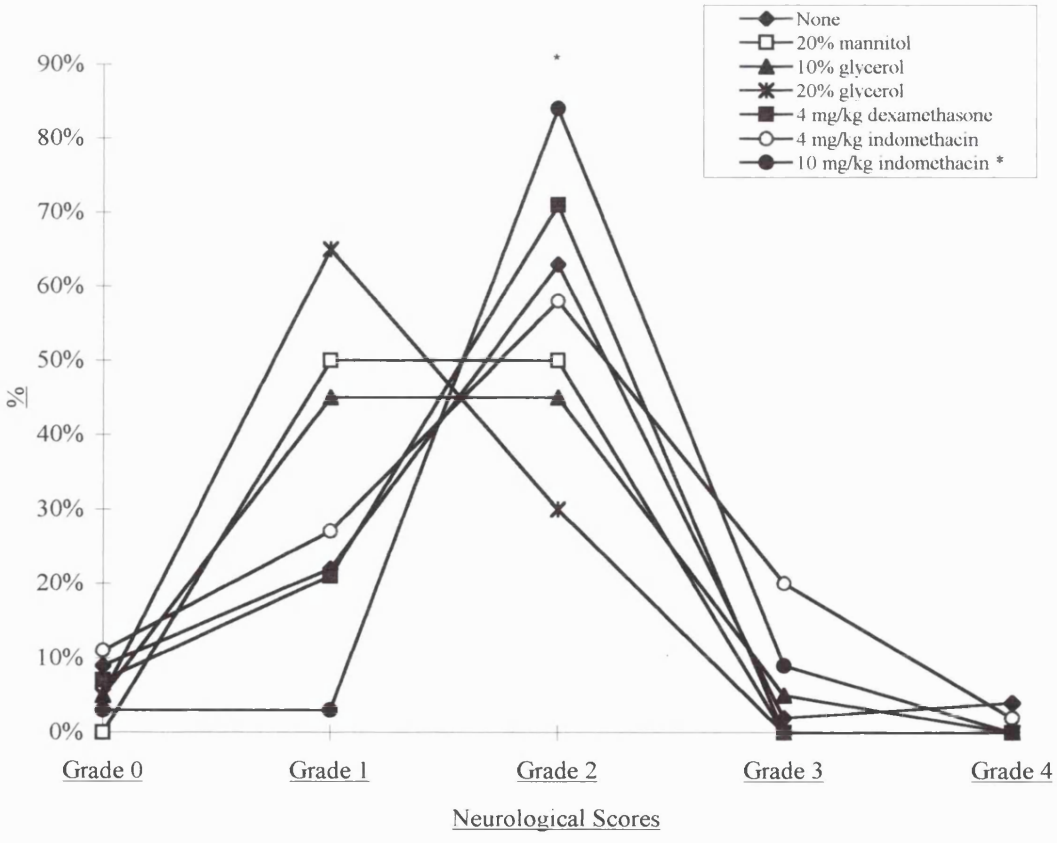
TABLE 4B: The effect of mannitol, glycerol, dexamethasone and indomethacin treatment on neurological scores in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

Numbers in brackets refer to the number of animals.

Caudate Injection	Drug Dose	Distribution of Neurological scores 0	1	2	3	4	Neurological Score Median
Collagenase	None	10%	22%	63%	2%	4%	2.0 (128)
	Mannitol						
Collagenase	20%	0%	50%	50%	0%	0%	1.5 (6)
	Glycerol						
Collagenase	10%	5%	45%	45%	5%	0%	1.5 (20)
Collagenase	20%	{5%	65%	30%	0%	0 %}*	1.5 (20)
	Dexamethasone						
Collagenase	4 mg/kg	7%	21%	71%	0%	0%	2.0 (13)
	Indomethacin						
Collagenase	4 mg/kg	11%	22%	62%	2%	2%	2.0 (45)
Collagenase	10 mg/kg	{3%	3%	84%	9%	0%}*	2.0 (32)

* Chi-squared test; collagenase vs treated, $p < 0.01$

Figure 4B: The effect of mannitol, glycerol, dexamethasone and indomethacin treatment on neurological scores in a rat model of collagenase-induced intracerebral haemorrhage.



* Chi-squared test; treated vs nontreated control, $p < 0.01$

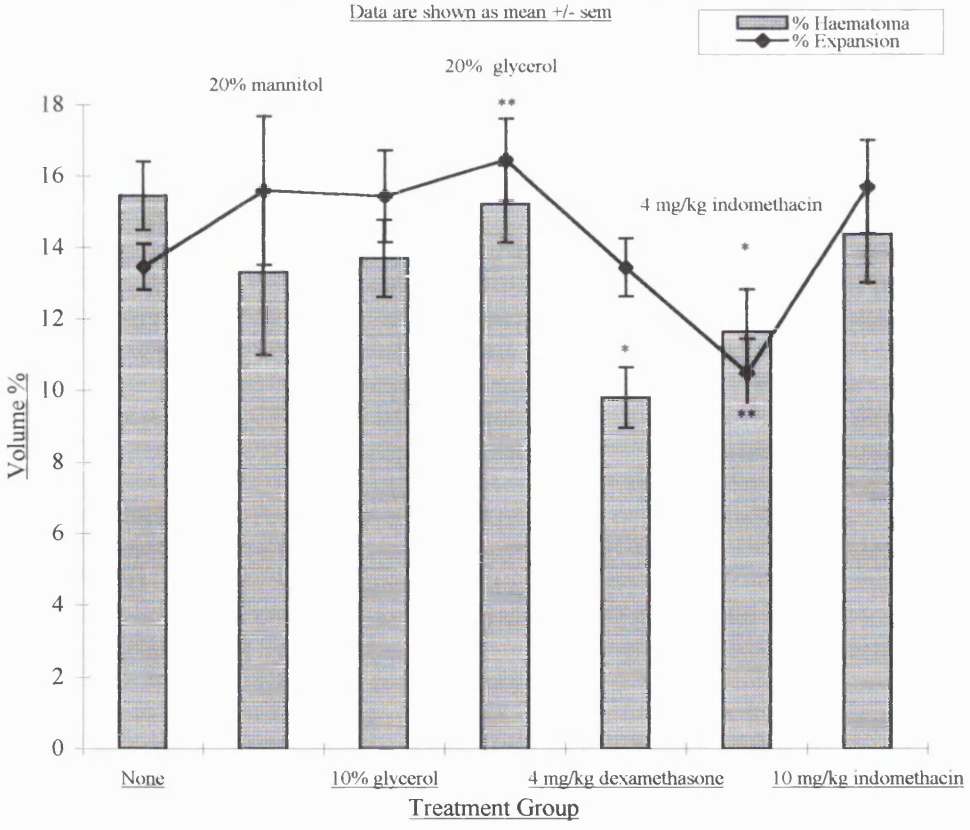
TABLE 4C: The effect of mannitol, glycerol, dexamethasone and indomethacin treatment on % cerebral swelling and haematoma size in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival (planimetry data)

Values are mean ± sem and the number in parentheses refer to number of animals used.

Caudate Injection	Drug Dose	Treatment Time	% Haematoma Mean + SEM (n)	% Cerebral swelling Mean + SEM (n)
Collagenase	None	None	13.46 ± 0.64 (52)	15.45 + 0.96 (52)
	Mannitol			
Collagenase	20%	Pre 30 mins	15.60 ± 2.08 (6)	13.29 ± 2.30 (6)
	Glycerol			
Collagenase	10%	Post 30 mins	13.69 ± 1.08 (20)	15.43 ± 1.29 (20)
Collagenase	20%	Post 30 mins	15.21 ± 1.08 (20)	16.46 ± 1.15 (20)**
	Dexamethasone			
Collagenase	4 mg/kg	Pre 30 mins	9.80 ± 0.85 (7)*	13.43 ± 0.81 (7)
	Indomethacin			
Collagenase	4 mg/kg	Pre 30 mins	11.63 ± 1.18 (18)*	10.49 ± 0.94 (20)**
Collagenase	10 mg/kg	Pre 30 mins	14.36 ± 1.36 (21)	15.68± 1.31 (21)

* collagenase vs treated (% haematoma); anova oneway, p = 0.04
**collagenase vs treated (% cerebral swelling); anova oneway, p = 0.02

Figure 4C: The effect of mannitol, glycerol, dexamethasone and indomethacin treatment on % cerebral swelling and haematoma size in a rat model of collagenase-induced intracerebral haemorrhage (planimetry data).
Data are shown as mean +/- sem



* collagenase vs treated (% haematoma); anova oneway, p = 0.04
**collagenase vs treated (% cerebral swelling); anova oneway, p = 0.02

TABLE 4D : The effect of glycerol, dexamethasone and indomethacin treatment on % water content of both ipsilateral and contralateral hemispheres in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

Values are mean ± sem and the number in parentheses refer to number of animals used

		% WATER CONTENT [(WET WEIGHT-DRY WEIGHT)/WET WEIGHT *100]	
Caudate Injection	Drug Dose	Ipsilateral hemisphere Mean + SEM (n)	Contralateral hemisphere Mean + SEM (n)
Collagenase	None	81.08 ± 0.52 (11)*	79.24 ± 0.32 (11)
	Glycerol		
Collagenase	10%	78.88 ± 0.56 (6)*#	77.63 ± 0.04 (6)+
	Dexamethasone		
Collagenase	4 mg/kg	79.73 ± 0.05 (5)*#	78.39 ± 0.11 (5)+
	Indomethacin		
Collagenase	4 mg/kg	79.36 ± 0.35 (15)*#	77.99 ± 0.23 (15)+
Collagenase	10 mg/kg	79.54 ± 0.16 (5)*#	78.29± 0.15 (5)+

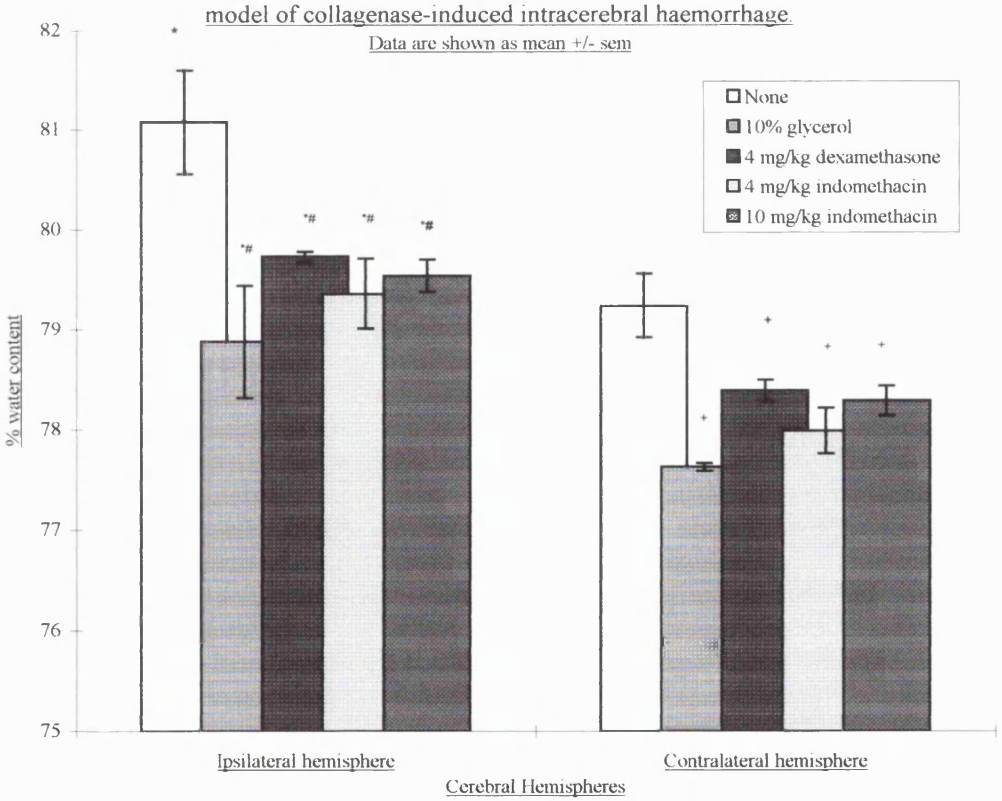
* paired t-test between ipsilateral and contralateral hemispheres (within groups), $p<0.01$

comparison between collagenase (untreated) and treated animals (ipsilateral): anova one way, $p<0.001$

+ comparison between collagenase (untreated) and treated animals (contralateral): anova one way, $p < 0.001$

Figure 4D: The effect of glycerol, dexamethasone and indomethacin treatment on % water content of both ipsilateral and contralateral hemispheres in a rat model of collagenase-induced intracerebral haemorrhage.

Data are shown as mean +/- sem



* paired t-test between ipsilateral and contralateral hemispheres (within groups), $p<0.01$

comparison between collagenase (untreated) and treated animals (ipsilateral): anova one way, $p<0.001$

+ comparison between collagenase (untreated) and treated animals (contralateral): anova one way, $p < 0.001$

Section 4: Discussion

The interventions studied in these experiments were not expected to have any effect on the size of the haematoma produced by the intracerebral injection of collagenase because of their anti-oedemic function. Surprisingly, a significantly smaller haematoma was measured in the steroid-treated group. This may be spurious and due to the small group size, though the possibility that steroid stabilisation of membranes might affect the action of collagenase remains, and would need separate study.

All these treatments aimed at limiting cerebral swelling due to oedema showed a reduction in brain water content though this was only reflected in a reduced volume expansion in one group. This was the group given 4 mg kg^{-1} of indomethacin. The small size of the group and the lack of effect with 10 mg kg^{-1} prompts caution in attributing this to a drug specific effect.

That the small effect on water content has a biological impact is suggested by the 'clinical' assessments. Weight loss was least in the steroid treated group which might be due to an effect on the stress response rather than reflecting any change due to a reduction in oedema however. The neurological scores however suggest a trend to improved status in all treated groups.

In the absence of clear differences in volume expansion of the haematoma bearing hemisphere, one might speculate that reduction in oedema may have improved cerebral perfusion and caused the better clinical outcome. Alternatively, the method of volume measurement may be too crude to reflect subtle changes in intracranial pressure (ICP). Direct measurement of ICP and cerebral blood flow would be needed to answer this question.

CHAPTER V

THE EFFECT OF A NON-COMPETITIVE N-METHYL-D-ASPARTATE ANTAGONIST (MK-801) ON COLLAGENASE-INDUCED INTRACEREBRAL HAEMORRHAGE.

Section 1: Introduction

The N-Methyl-D-Aspartate (NMDA) receptor is one of three pharmacological distinct postsynaptic membrane glutamate receptors identified in the brain (Greenmeyre et al, 1985). The other two being Quisqualate, and Kainate-named after their agonists. The NMDA is the most abundant excitatory amino acid receptor in the mammalian central nervous system (Monaghan & Cotman, 1987) and has the highest affinity for endogenous glutamate (Olverman et al, 1984).

Structurally, the NMDA receptor (Diagram E) has four different binding sites (Watkins et al, 1987);

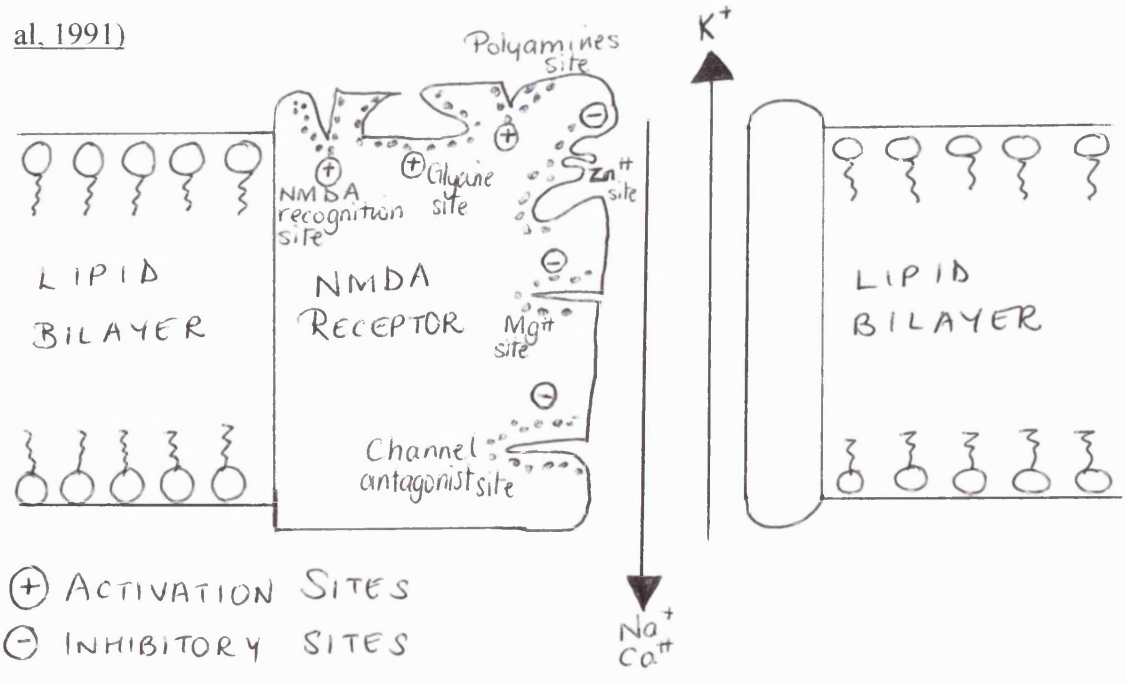
(1) phenycyclidine (PCP) binding site

(2) agonist recognition site

(3) modulation sites recognised by glycine, polyamines and zinc ions

(4) cationic magnesium binding site

Diagram E: A schematic representation of the NMDA receptor (taken from Scatton et al, 1991)



The action of the NMDA receptor is dictated by which of the sites are occupied. In its inactive state, the magnesium ions are bound to the receptor. Activation of the receptor, as in depolarisation, loosens the bound magnesium ions (Scatton et al, 1991). The binding of glycine (co-agonist), and polyamines attenuates the stimulation of the NMDA receptor whereas that of zinc ions results in an inhibitory effect (Scatton et al, 1991). The PCP site of the NMDA receptor, is a ligand-gated ionic channel for sodium and calcium ions. These receptor-operated ion channels have a relatively high permeability to calcium (MacDermott et al, 1986; Rothman et al, 1987). Continuous activation of the NMDA receptor therefore, will result in increased concentration of intracellular calcium. If left unchecked, excessive calcium will lead to an abnormal activation of enzymes, such as, proteases and phopholipases with damaging consequences (Siesjo, 1981).

Section 5.1.1: Glutamate as a Neurotoxin

The neurotoxicity of glutamate on tissue was shown by several investigators (Lucas & Newhouse, 1957; Curtis et al, 1959; Harreveld & Fifkova, 1971; Beneviste et al, 1984; Choi et al, 1987 and Olney et al, 1989). Lucas and Newhouse (1959) found that the glutamate destroyed the inner neural layer of the retina. Curtis et al (1959) were the first to demonstrate the toxic action of glutamate on the central nervous tissue, on cat spinal cord. Choi et al (1987) showed that the exogenous glutamate (five minute exposure, 100uM) to a cortical neural culture killed the cells. It was Olney (1989) who coined the term excitotoxin, to describe the damaging effect of high glutamate concentration.

The exact mechanism by which glutamate and other excitatory amino acids effect cell damage is still unclear. From in vitro neuronal cell culture studies, Choi et al, (1987) and Rothman (1985) put forward two mechanisms:

(1) the early influx of sodium (Chan et al, 1979) and chloride lead to acute swelling of neurons, described by Albers et al (1989) as acute excitotoxic neuronal swelling

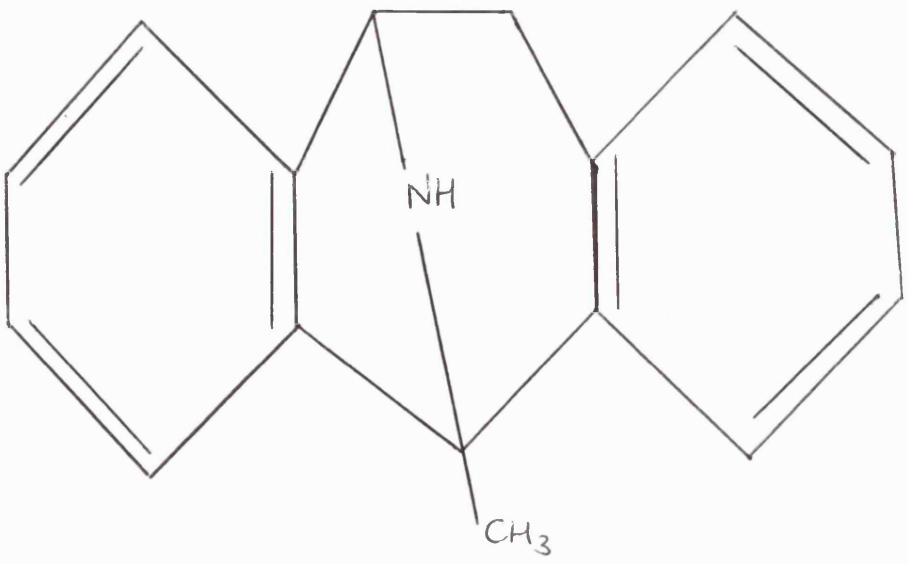
(2) followed by the influx of excessive calcium which leads to delayed tissue death.

Section 5.1.2: MK-801

The compound MK-801(Figure 5.1.2) {(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepte -5,10-imine maleate} was originally discovered in the early 1980's as a potent, orally active anticonvulsant (Clineschmidt et al, 1982). Wong et al (1986) revealed that MK-801 is a powerful , non-competitive antagonist of glutamate receptors, particularly for the N-methyl-D-aspartate (NMDA) receptor subtype.

The lipophillic nature of MK-801 (Diagram F) is a significant feature of the drug, for it indicates that the drug can easily cross the tightly regulated blood brain barrier (Wong et al, 1986). Wong et al (1986) observed from radio-ligand studies that in the rat brain, the greatest number of MK-801 binding sites (Wong et al, 1986) were seen in the hippocampus followed by cerebral cortex, and then the corpus striatum.

Diagram F: Structure of MK-801(taken from Kemp et al, 1987)



Foster et al (1987) found that a single dose of MK-801 (10 mg kg^{-1}, intraperitoneally) was sufficient to prevent neuronal degeneration in the hippocampus and striatum caused by local injection of NMDA. Kemp et al (1987) showed that the neuroprotective effect of MK-801 was communicated through the occupancy of the PCP site. The binding of MK-801 rendered the sodium and calcium channel impervious to entry of sodium, chloride and calcium ions into the neuronal cells (Wong et al, 1986).

Beneviste et al (1984) observed an elevation in glutamate concentration in different animal models of focal cerebral ischaemia. The administration of MK-801 either pre- or post - ischaemia was found to be neuroprotective (Kemp et al, 1987; Gill et al, 1987; Gill et al, 1988; Boast et al, 1988; Duverger 1987; Ozyurt 1988; Park 1988a, b; Bielenburg 1989; Shearman, 1989) - the treatment reduced the volume of the ischaemic infarct in some cases by 50%.

However, these beneficial effects of MK-801 have not been echoed in some studies of global cerebral ischaemia. Albers (1992) suggested that this variance in MK-801's neuroprotective action is perhaps linked to the presence of an ischaemic penumbra, which is clearly detected in models of focal cerebral ischaemia.

MK-801 has been tested in one animal model of subdural haematoma (Kuroda et al, 1994) and was found to be neuroprotective. However, the idea of investigating the effect of MK-801 has not been fully considered in intracerebral haemorrhage.

The rationale of studying the effect of MK-801 on intracerebral haemorrhage was prompted by the cerebral blood flow data reported by Mendelow (1991) using a microballoon to simulate the mass effect of a haematoma. The group found a zone of reduced blood flow between the periphery of the haematoma and normal non-infarcted tissue, hence hinting at an ischaemic component. Consequently, this warranted an

investigation into the effectiveness of cerebral ischaemic therapies in intracerebral haemorrhage. It was hoped that the results obtained on testing MK-801 on Rosenberg's et al (1990) model of intracerebral haemorrhage would elucidate the contribution of glutamate toxicity to brain damage induced by cerebral haemorrhage and thereby verify the existence of any ischaemic penumbra, around a haematoma.

Section 2: Materials and Methods

In this study, a total of 86 adult male Sprague-Dawley rats, weighing between 285-290 g, were employed. The animals were initially anaesthetised with Halothane followed by Hypnorm and Diazepam, as described in Chapter II.

Section 2.1: Treatment Protocol

The animals were divided into three groups and were administered one of three doses of MK-801 (a gift from Merck Sharpe & Dohme); 1mg kg^{-1}, or 5 mg kg^{-1} or 10 mg kg^{-1} interperitoneally thirty minutes prior to intracerebral injection of collagenase.
The 24-hour survival weight loss and mortality of the animals were recorded. Recovery animals were graded with the aid of the simple neurological scale (Chapter II). Measurements were made of hemispherical expansion, haematoma volume using fixed rat brains, and percent water content was determined using fresh unfixed brains.

Section 2.2: Statistical Analysis

Paired t-tests were used to analyse the significance of the differences in hemispherical expansion. Unpaired t-tests were performed on data emanating from different samples. The effects of the different pre-dosing regimens on body weight loss, functional recovery, planimetry measured hemispherical expansion, size of the haematoma and the water content were evaluated via application of one way analysis of variance (ANOVA).

Section 3: Results

Body weight fell in all groups with evidence of a dose related increase in loss in those given MK-801 (Table 5A and Figure 5A). The effect of 1 mg/kg was to produce a small non-significant increase in weight loss from 3.52 $\pm$ 0.38% to 4.21 $\pm$ 0.63%. The loss with 5 mg/kg and 10 mg/kg doses was significantly greater (6.87 $\pm$ 0.48% and 7.36 $\pm$ 0.43% respectively; anova oneway, $p < 0.001$).

The neurological assessment is shown in Table 5B and Figure 5B. The group receiving 1 mg/kg MK-801 had the lowest median score and the greatest proportion of animals showing little or no adverse effect (69% were scored between 0 and 1 compared with 31% of controls; $p < 0.01$). There was no evidence of such a therapeutic effect with the higher dose regimes.

The haematoma measured in the group receiving 1 mg/kg MK-801 was, presumably by chance, the smallest though the difference was not formally significant (Table 5C and Figure 5C; anova oneway, $p = 0.43$). Similarly, this group had an insignificantly smaller degree of hemispheric swelling measured by planimetry (anova oneway, $p = 0.056$). The group receiving 5 mg/kg had the largest haematomas (anova oneway, $p = 0.11$).

The wet weight dry weight method of calculating hemispheric water content however revealed a significant reduction in the water content of the ipsilateral hemisphere, compared to untreated animals, in the group receiving the lowest dose of MK-801 (Table 5D and Figure 5D). A significant reduction in water content in the contralateral hemisphere was seen for both 1 mg/kg and 10 mg/kg doses.

TABLE 5A: The effect of MK-801 pretreatment (1, 5 and 10 mg/kg) on body weight in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

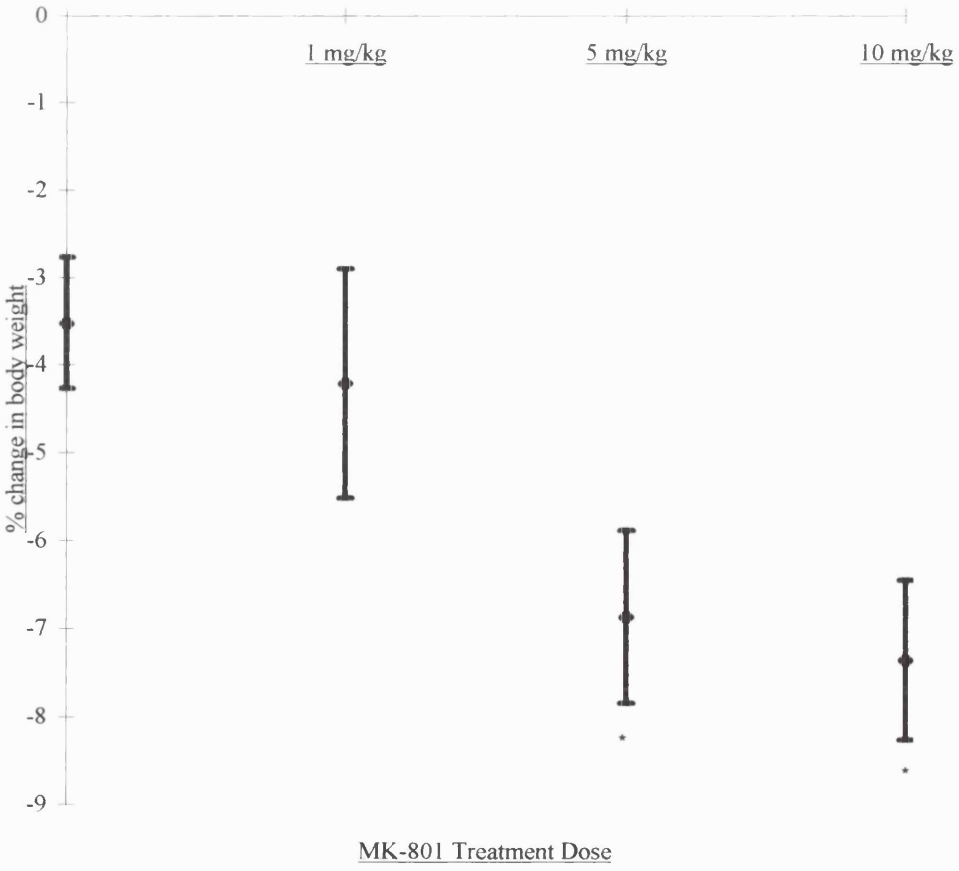
(Values are presented as mean +/-sem. The number in brackets refer to the number of animals in the group)

Caudate Injection	MK-801 Dose	% Change in body weight Mean + SEM (n)
Collagenase	None	(-)3.52 ± 0.38 (123)
Collagenase	1 mg/kg	(-)4.21 ± 0.63 (23)
Collagenase	5 mg/kg	(-)6.87 ± 0.48 (42)*
Collagenase	10 mg/kg	(-)7.36 + 0.43 (20)*

*Comparison of treated (MK-801) with non-treated (collagenase only); Anova oneway,p <0.001

Figure 5A: The effect of MK-801 pretreatment (1, 5 and 10 mg/kg) on body weight in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

Data shown as mean with the 95% confidence intervals.



*Comparison of treated (MK-801) with non-treated (collagenase only); Anova oneway,p <0.001

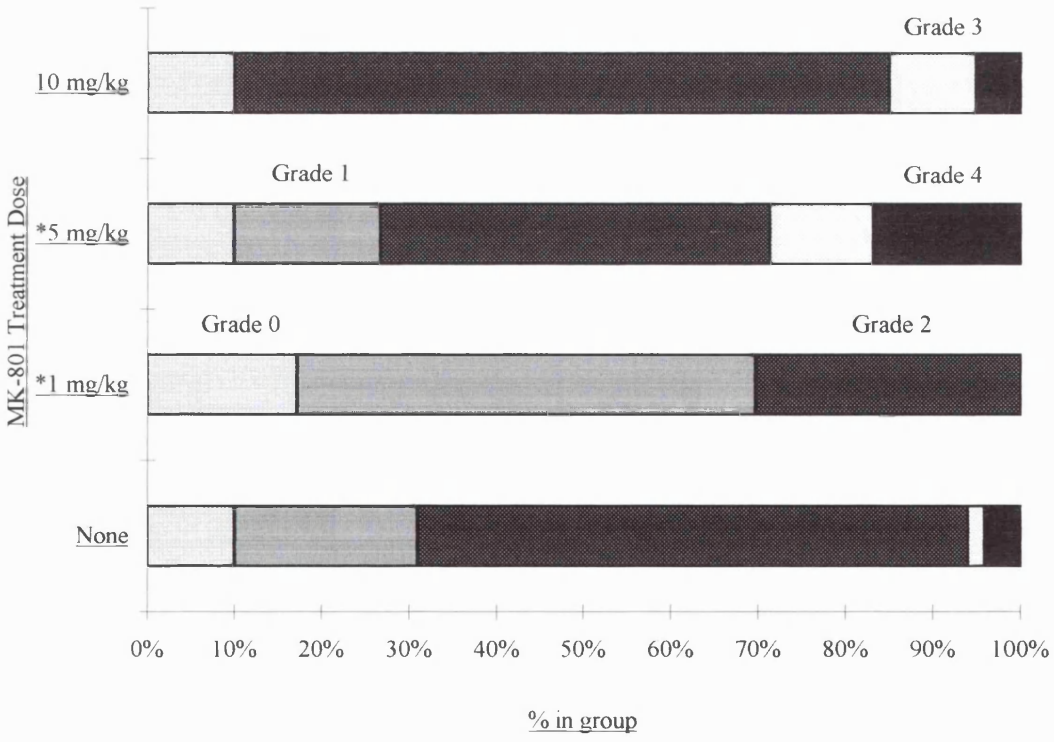
TABLE 5B The effect of MK-801 pretreatment (1, 5 and 10 mg/kg) on neurological scores in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival .

(Values are presented as % of total in the group. The number in brackets refer to the number of animals).

Caudate Injection	MK-801 Dose	Distribution of Neuroscores 0	1	2	3	4	Neurological Score Median
Collagenase	None	10%	21%	63%	2%	4%	2.0 (128)
Collagenase	1 mg/kg	{17%	52%	30%	0%	0%}*	1.0 (23)
Collagenase	5 mg/kg	{10%	17%	45%	12%	17%}*	2.0 (42)
Collagenase	10 mg/kg	10%	0%	75%	10%	5%	2.0 (20)

* Comparison between MK-801 treated vs nontreated (collagenase only); Chi-square test, p <0.01

Figure 5B:The effect of MK-801 pretreatment (1, 5 and 10 mg/kg) on neurological scores in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival .



* Comparison between MK-801 treated vs nontreated (collagenase only); Chi-square test, p <0.01

TABLE 5C : The effect of MK-801 pretreatment (1, 5 and 10mg/kg) on cerebral swelling and haematoma size in a rat model of intracerebral haemorrhage after 24 hours of survival (planimetry data)

Values are mean ± sem and the number in parentheses refer to number of animals used.

Caudate Injection	MK-801 Dose	% Haematoma Mean + SEM (n)	% Cerebral swelling Mean + SEM (n)
Collagenase	None	15.45 ± 0.96 (52)	13.46 ± 0.64 (52)
Collagenase	1 mg/kg	13.91 ± 1.68 (17)	10.98 ± 1.09 (17)
Collagenase	5 mg/kg	18.56 ± 1.80 (19)	12.06 ± 1.17 (19)
Collagenase	10 mg/kg	15.58 ± 1.08 (13)	13.25 ± 0.88 (13)

Figure 5C: The effect of MK-801 pretreatment (1, 5 and 10mg/kg) on cerebral swelling and haematoma size in a rat model of intracerebral haemorrhage after 24 hours of survival (planimetry data)

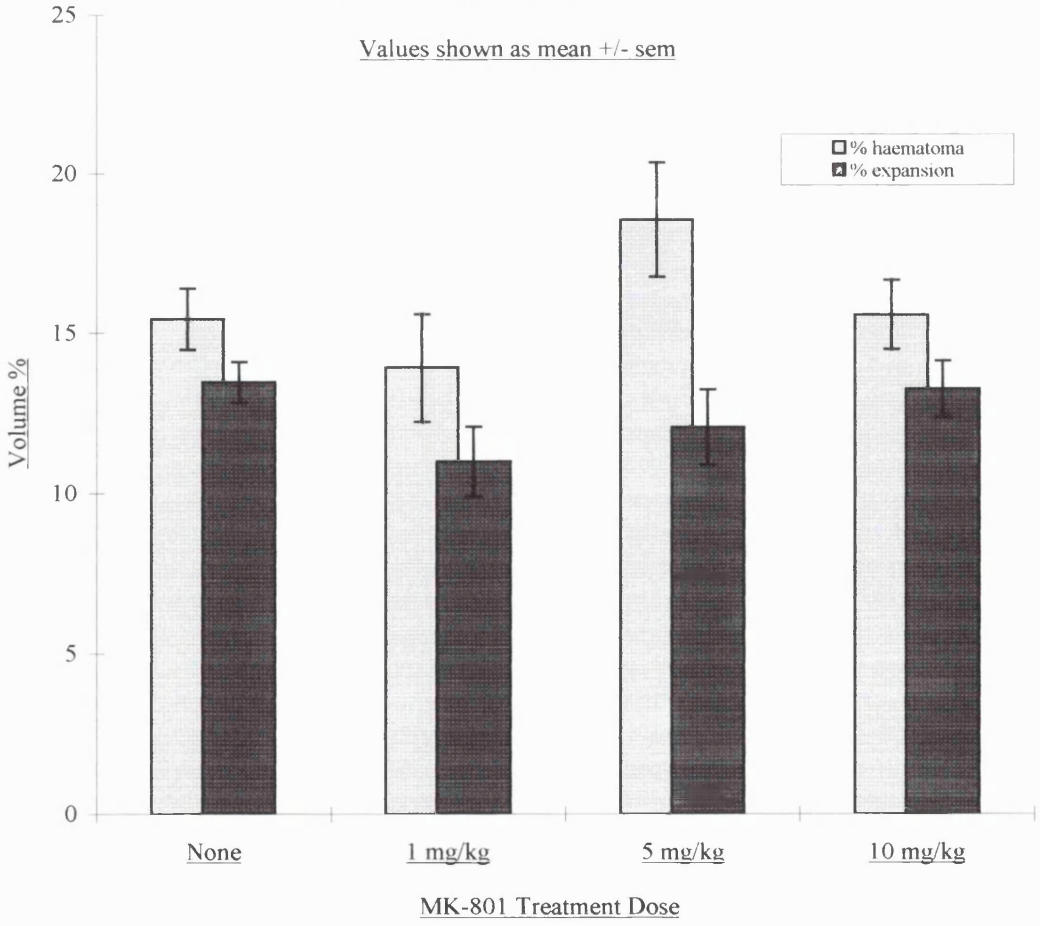


TABLE 5D: The effect of MK-801 pretreatment (1, 5 and 10 mg/kg) on % water content of both ipsilateral and contralateral hemispheres in a rat model of intracerebral haemorrhage after 24 hours of survival.

Values are mean ± sem and the number in parentheses refer to number of animals used

% WATER CONTENT

[(WET WEIGHT-DRY WEIGHT)/WET WEIGHT *100]

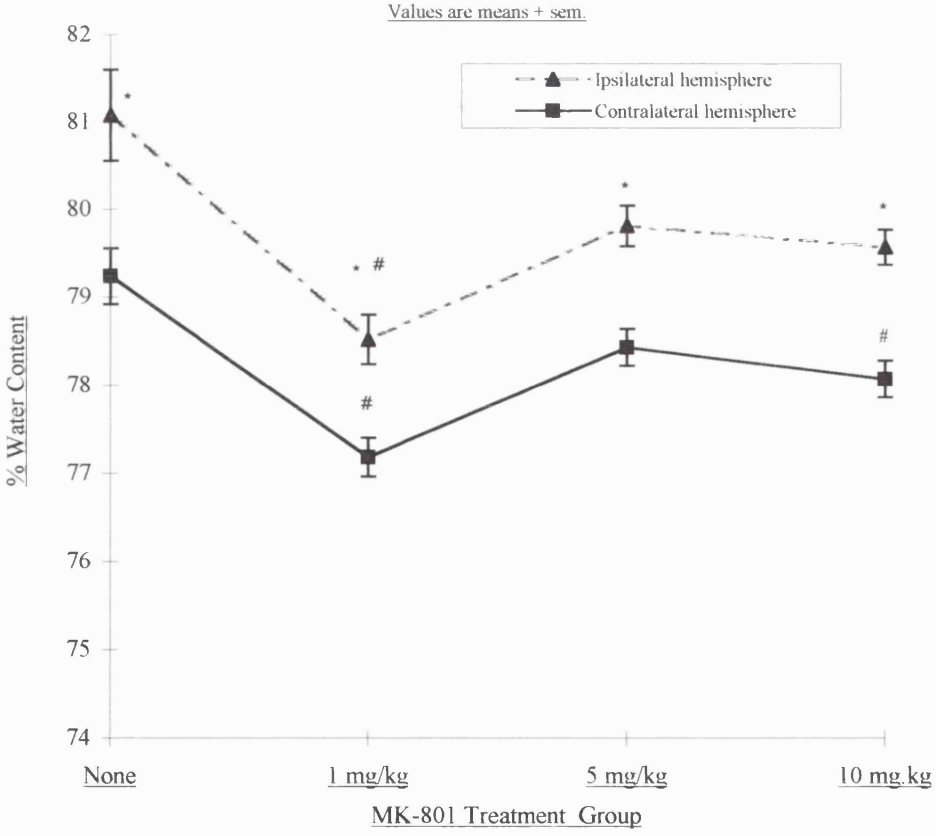
Caudate Injection	MK-801 Dose	Ipsilateral hemisphere Mean + SEM (n)	Contralateral hemisphere Mean + SEM (n)
Collagenase	None	81.08 ± 0.52 (11)*	79.24 ± 0.32 (11)
Collagenase	1 mg/kg	78.52 ± 0.28 (5)*#	77.18 ± 0.22 (5)#
Collagenase	5 mg/kg	79.81 ± 0.23 (7)*	78.43 ± 0.21 (7)
Collagenase	10 mg/kg	79.57 ± 0.20 (7)*	78.07 ± 0.20 (7)#

* comparison between ipsilateral and contralateral hemispheres within group; paired t-test , $p < 0.03$

comparison of ipsilateral /contralateral hemispheres between MK-801 treated and untreated rats; Anova oneway, $p < 0.01$

ure 5D: The effect of MK-801 pretreatment (1, 5 and 10 mg/kg) on % water content of both ipsilateral and contralateral hemispheres in a rat model of intracerebral haemorrhage after 24 hours of survival.

Values are means + sem.



* comparison between ipsilateral and contralateral hemispheres within group; paired t-test , $p < 0.03$

comparison of ipsilateral /contralateral hemispheres between MK-801 treated and untreated rats; Anova oneway, $p < 0.01$

Section 4: Discussion

There is not much documentation relating to the role of glutamate and calcium as possible major contributors to brain damage in cerebral haemorrhage. One of the many reasons to investigate MK-801 in cerebral haemorrhage was provided by Mendelow et al (1991). He presented evidence of an ischaemic element associated with the primary haemorrhagic event. If this ischaemic component was a substantial feature in the collagenase-haemorrhage model, then the application of MK-801 at the neuroprotective doses should be seen to be beneficial.

The doses of MK-801 tested in this study were those reported to be neuroprotective in global and focal cerebral ischaemic animal models (Gill et al, 1987; Kemp et al, 1987; Albers et al, 1992; Park et al, 1994). MK-801 has been shown to reduce cerebral infarction by as much as 50% via non-competitive antagonism of the NMDA receptor, when administered pre-insult (Gill et al, 1987).

It is tempting to interpret the results in the haemorrhage model as showing a dose dependent effect. At the lowest dose chosen, weight loss was insignificantly aggravated, clinical scoring suggested improved function and this was paralleled as in the last chapter by reduced oedema. This could be due to amelioration of ischaemic oedema around the haematoma and even in the contralateral hemisphere where diaschesis may produce some glutamate driven 'ischaemic' oedema. The higher doses of MK-801 had no such effects on function, and aggravated weight loss despite the 10 mg/kg group showing reduced water content. Kane et al (1994) using a microballoon to simulate the mass effect of a haematoma found that MK-801 in a dose of 3 mg/kg did not confer protection against cerebral oedema.

One possible reason for an adverse effect of higher doses may relate to hypotension. MK-801 can cause hypotension more particularly in anaesthetised

animals (Park et al 1988a, b) and Osborne et al (1987) showed that hypotension could increase ischaemic brain damage by as much as 50%. Blood pressure was not monitored in these animals so this must remain speculative.

The fact that haematoma volumes were lowest in the 1mg/kg group and highest in the 5 mg kg^{-1} may have had a complicating influence on the results, but the haematoma size of 10 mg kg^{-1} group was the same as for untreated controls. These differences were insignificant, and they do not account in a simple way for the drug effects on water content.

There is in this data therefore a suggestion that MK-801 may ameliorate the functional impact of a haematoma perhaps through ischaemic oedema. Therefore despite the lack of histological proof, there may indeed be an ischaemic component in the effects of haematomas.

CHAPTER VI

THE EFFECT OF EARLY, ACUTE INDUCED HYPERTENSION ON AN ANIMAL MODEL OF EXPERIMENTAL INTRACEREBRAL HAEMORRHAGE.

Section 1: Introduction

Hypertension is a leading cause of primary intracerebral haemorrhage and infarction (Bronner et al, 1995; Broderick et al, 1993; Harmsen et al, 1990; Brott et al, 1986; Kannel et al, 1983). Long term treatment of hypertension reduces the risk of strokes (Collins et al, 1990). The control of blood pressure, in the immediate period after the onset of stroke whether ischaemic or haemorrhagic, is however controversial and plagued with theoretical difficulties.

Cerebral blood flow is autoregulated between mean blood pressure of 65-145mm Hg, such that no change in flow results from pressure variation within that range. Above this, a breakthrough leads to increased blood flow and below it to a fall. In damaged tissue for example after stroke, autoregulation is lost and blood flow becomes pressure passive (Adams and Powers, 1997). The overaggressive lowering of blood pressure may therefore increase the likelihood of extending the area of infarction in ischaemic stroke, and hence may ultimately lead to a worse neurologic outcome (Britton et al, 1980). In such patients, hypotensive treatment will produce poor cerebral perfusion of areas distant from the lesion core (Graham, 1989). An initial increase in arterial blood pressure will theoretically be beneficial by tending to increase blood flow to the ischaemic penumbra (Allen et al, 1988). Thus, in acute ischaemic stroke patients, very early hypertension might improve metabolic consequences of acute cerebral ischaemia through improving perfusion to ischaemic tissue (Rordorf et al, 1997; Fagan, 1997) and in turn reduce cytototoxic cerebral oedema.

However, a sustained raised arterial pressure after the first day or so, may tend to increase the volume of irrepairably damaged tissue because of the evolving damage to the blood brain barrier and the risk of vasogenic oedema and secondary haemorrhage.

In patients with aneurysmal subarachnoid haemorrhage, the ensuing vasospasm may lead to ischaemic focal changes with, for example, hemiparesis. Phenylephrine-induced hypertension, following clipping of the aneursym, increased cerebral blood flow, improved vasospasm-related ischemic neurologic deficits, and decreased brain injury after subarachnoid haemorrhage (Rordorf et al, 1997; Hayashi et al, 1984; Fagan, 1997; Wise et al, 1972). The use of phenylephrine was associated with minimal morbidity (Miller et al, 1995), when the risk of haemorrhage had been eliminated by control of the aneurysm.

The data from these focal cerebral ischaemic studies is very relevant in the context of cerebral haemorrhage, as several experimental studies of intracerebral haemorrhage have implied the presence of an ischaemic component around a haematoma (see General Introduction). This theoretically might be alleviated by increasing the blood flow through induced hypertension, though the risks of aggravating haemorrhage would appear obvious.

Despite the evidence of the benefits of early hypertension in acute cerebral ischaemia associated with vasospasm, there is still no agreed consensus, as to whether early induced hypertension should be tried in other stroke patients. If very early treatment was required, there would be a real risk that patients with cerebral haemorrhage might receive pressure treatment before the nature of their lesion was adequately evaluated.

In light of this information, I have investigated the effect of induced, acute hypertension on Rosenberg's et al (1990) model of intracerebral haemorrhage. The

question being posed is whether early and short term hypertension, reduces the volume of any secondary ischaemic injury and cerebral swelling, or exacerbates haemorrhage and vasogenic brain swelling, and whether such changes have an impact on the neurological outcome.

Section 2: Materials And Methods

Twenty-one adult male Sprague-Dawley rats weighing between 270-300g were used in this study. Animals were anaesthetised initially with halothane and then with diazepam and hypnorm (as described in Chapter 2: General methodology). The right femoral artery was cannulated for arterial blood sampling and blood pressure recording and the left femoral vein cannulated for infusions. Body temperature was monitored by a rectal probe and maintained by a heating lamp.

Section 2.1: Treatment Protocol

After physiological baseline values were established for arterial blood pressure, pCO2, pO2, pH and glucose, a craniotomy was performed and either collagenase or saline was injected into the caudate nucleus of one of the hemispheres. An infusion of Metaraminol bitartrate (Aramine, Sigma Chem Co.) was started 30 minutes following induction of haemorrhage. Animals received a continuous intravenous infusion of metaraminol bitartrate to maintain the mean arterial blood pressure 30-40 mm Hg above pre-haemorrhage level for a further 30 minutes. At the end of the thirty minutes, the infusion was stopped but the blood pressure recording continued for a further 30-60 minutes. Animals were allowed to recover for 24 hours.

The brains were used for planimetry measurement, and wet weight dry weight determinations (see Chapter 2 for experimental details).

Section 2.2: Statistical treatment of data is as described previously (Chapter 2).

Section 3: Results

Following infusion of Aramine, there was an immediate rise of arterial blood pressure from 70 mm Hg to 120 mm Hg (an average increase of 30-40 mm Hg above baseline). This elevation was maintained for thirty minutes until infusion was stopped (Blood Pressure Traces 1 to 3). The blood pressure began to decline slowly after infusion stopped but remained above baseline until end of recording (1 hour). Prior to sacrifice baseline values were again found.

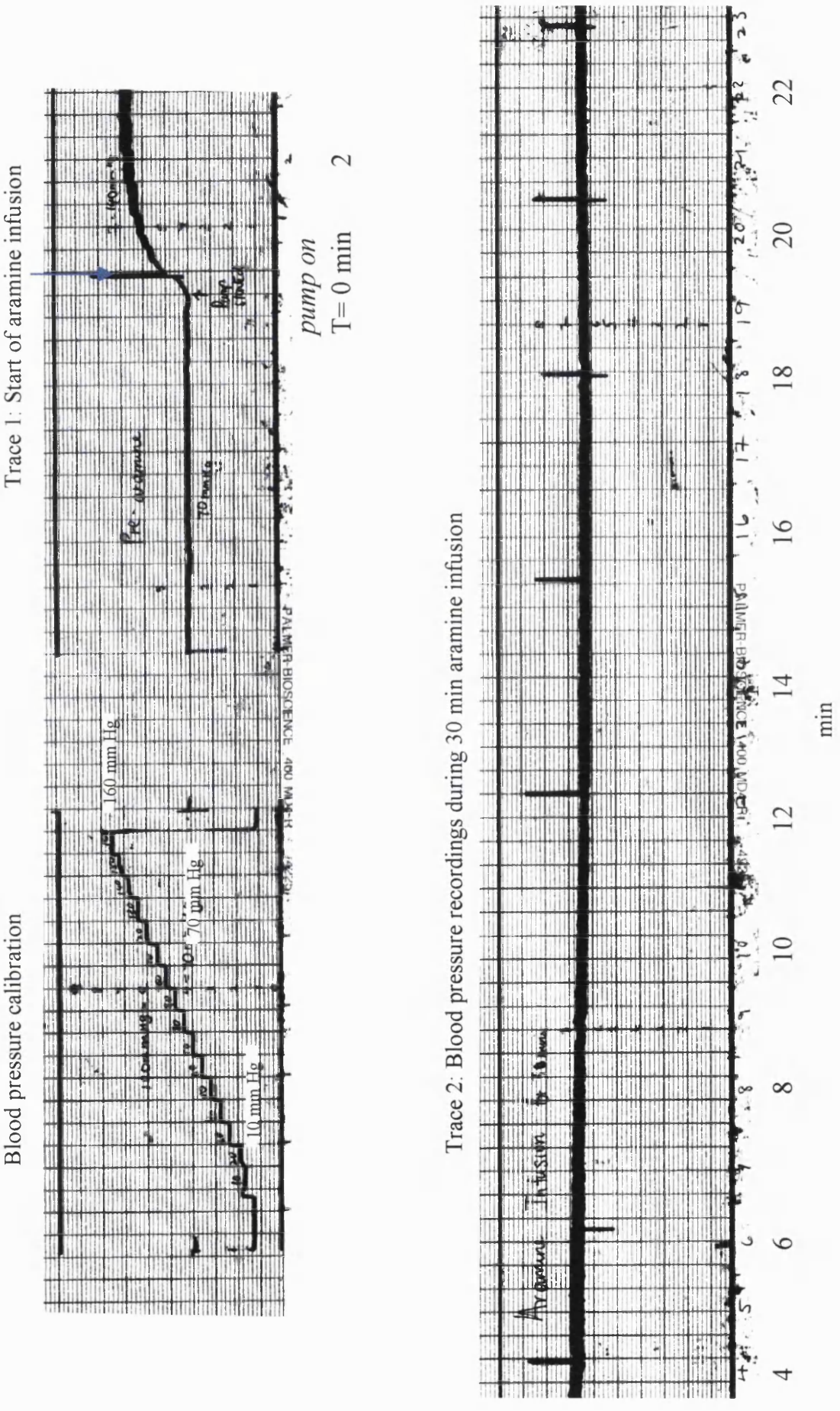
No significant differences (Anova oneway; $p = 0.03$) were seen in post recovery weight loss between those given Aramine and the untreated collagenase animals (Table 6A and Figure 6A).

The functional assessment is set out in Table 6B and depicted in Figure 6B. More animals scored in the best categories (score of 0 and 1) after receiving the pressor agent (58% compared to 31%; chi-squared $p = 0.03$) and they had a lower median score.

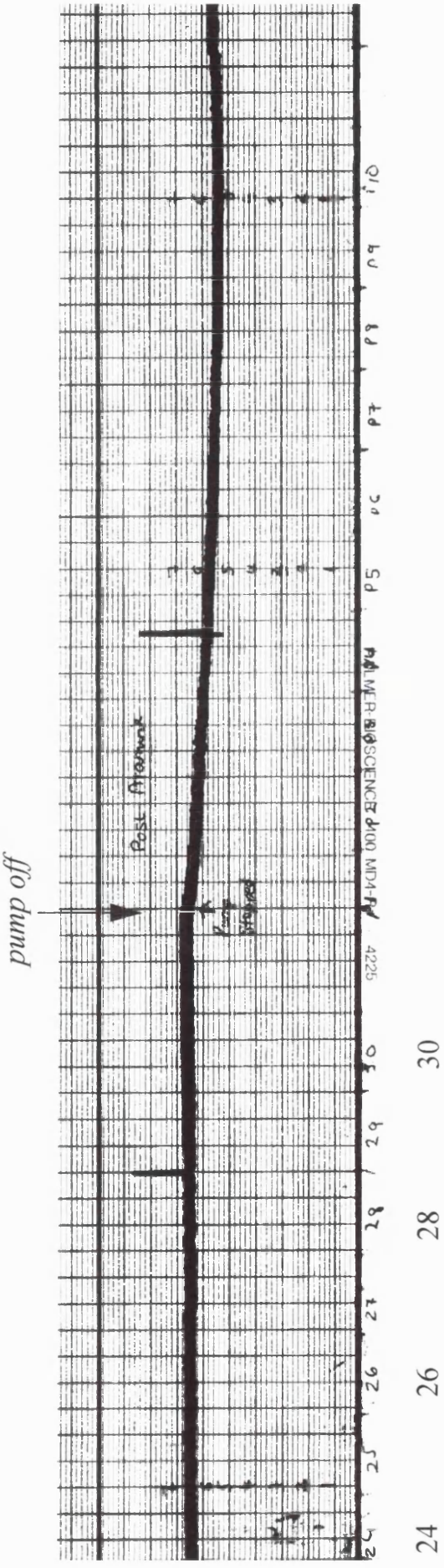
There was no evidence that Aramine influenced the size of the haematoma (Table 6C and Figure 6C), or that of the swollen hemisphere in the planimetry data (Anova oneway, $p>0.1$).

Although the water content of both the ipsilateral and contralateral hemispheres were lower in the treated group (Table 6D and Figure 6D) the differences failed to reach statistical significance (Anova oneway, $p = 0.06$ and $p = 0.08$ respectively).

Blood pressure recordings (Traces 1-3): Elavation of blood pressure induced by infusion of aramine post collagenase injection



Blood pressure recordings (Traces 1-3): Elavation of blood pressure induced by infusion of aramine post collagenase injection (cont.)



Trace 3: Blood pressure recordings post aramine infusion

TABLE 6A: The effect of post-induced hypertension on body weight in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

(Values are presented as mean +/- sem. The number in brackets refer to the number of animals in the group)

Caudate Injection	Aramine Infusion	Treatment Time	Treatment Duration	% Change in bw Mean + SEM (n)
Collagenase	None	None	None	(-) 3.53 ± 0.38 (123)
Collagenase	Yes	Post 30 mins	30 mins	(-) 2.93 ± 1.21 (18)

Figure 6A: The effect of post-induced hypertension on body weight in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

Data are shown as mean +/- sem

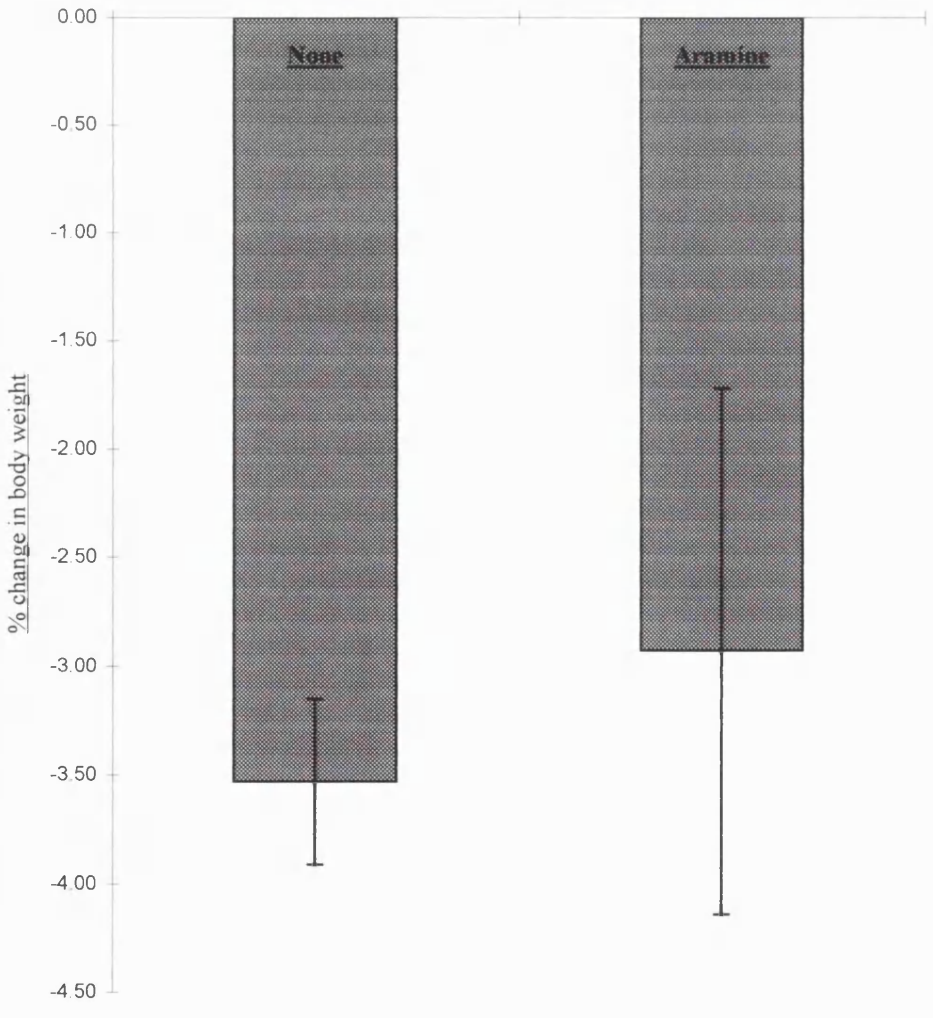


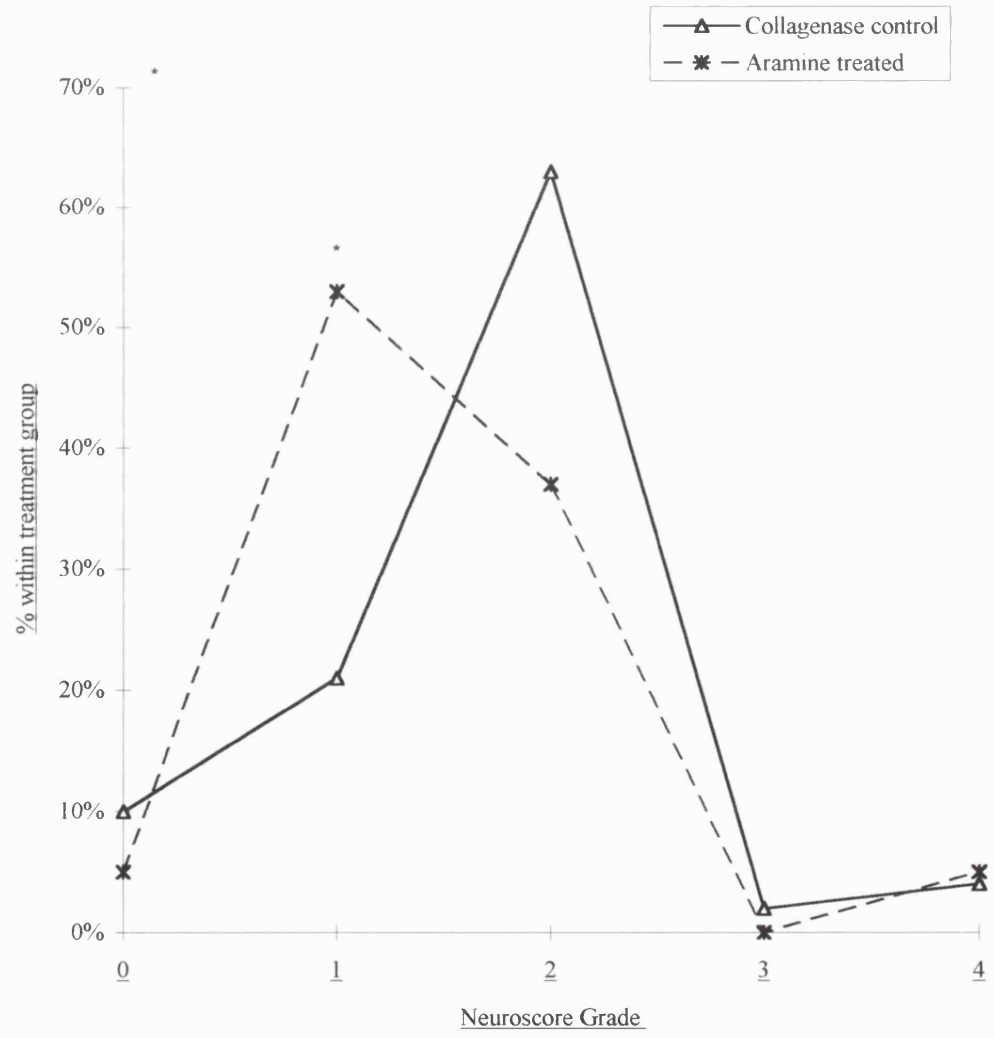
TABLE 6B: The effect of post-induced hypertension on neurological scores in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

(Values are presented as % of total in the group. The number of animals used are shown in brackets)

Caudate Injection	Aramine Infusion	Distribution of Neuroscores Grade 0	1	2	3	4	Neurological Score Median
Collagenase	None	10%	21%	63%	2%	4%	2.0 (128)
Collagenase	Yes	{0.05	53%	37%	0%	0.05}*	1.0 (19)

*Chi-square test between untreated and treated groups groups, p <0.05

Figure 6B:The effect of post-induced hypertension on neurological scores in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.



*Chi-square test between untreated and treated groups groups, p <0.05

TABLE 6C : The effect of post-induced hypertension on cerebral swelling and haematoma volume in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival (Planimetry data).

(Values are mean +/- sem and the number in parentheses refer to number of animals used)

Caudate Injection	Aramine Infusion	% Haematoma Mean + SEM (n)	% Cerebral swelling Mean + SEM (n)
Collagenase	None	15.45 ± 0.96 (52)	13.46 ± 0.64 (52)
Collagenase	Yes	15.23 ± 1.59 (13)	13.09 ± 1.89 (13)

Figure 6C: The effect of post-induced hypertension on cerebral swelling and haematoma volume in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival (planimetry data).

Data shown are mean +/- sem

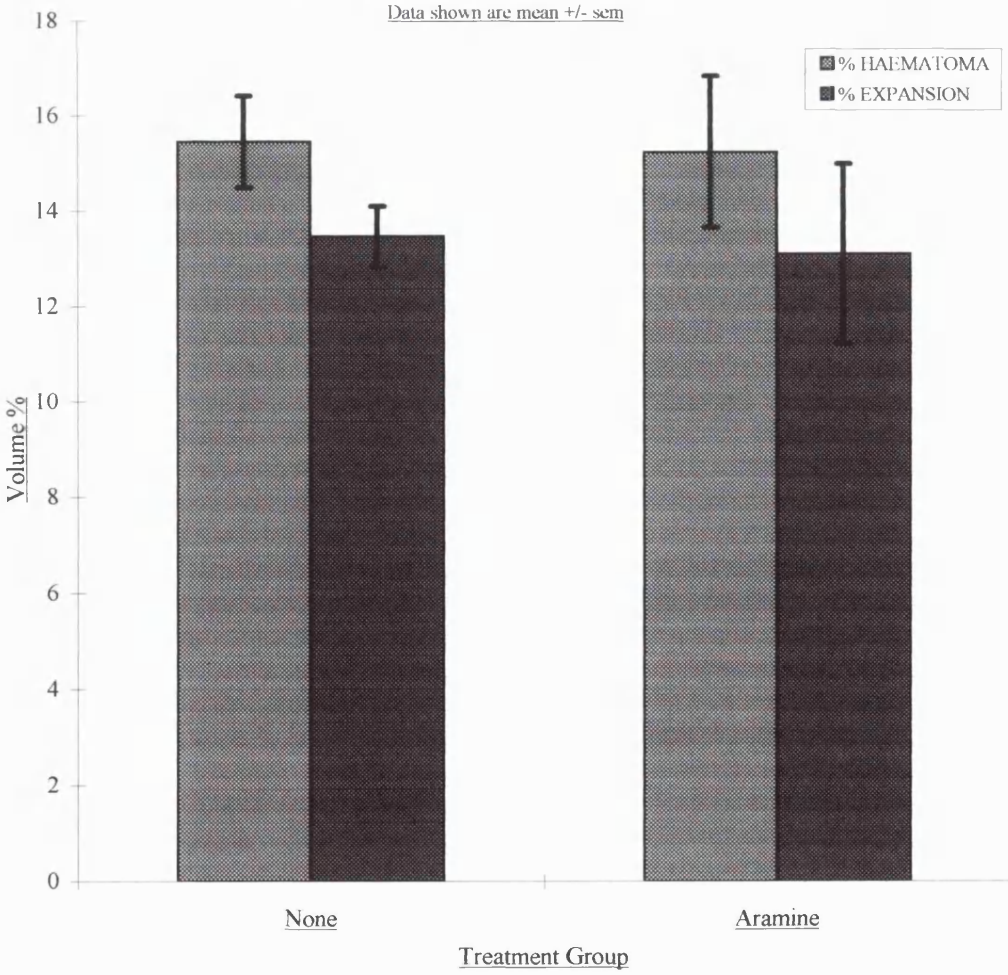


TABLE 6D: The effect of post-induced hypertension on % water content of both hemispheres in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

(Values are mean +/- and the number in parentheses refer to number of animals used)

% WATER CONTENT

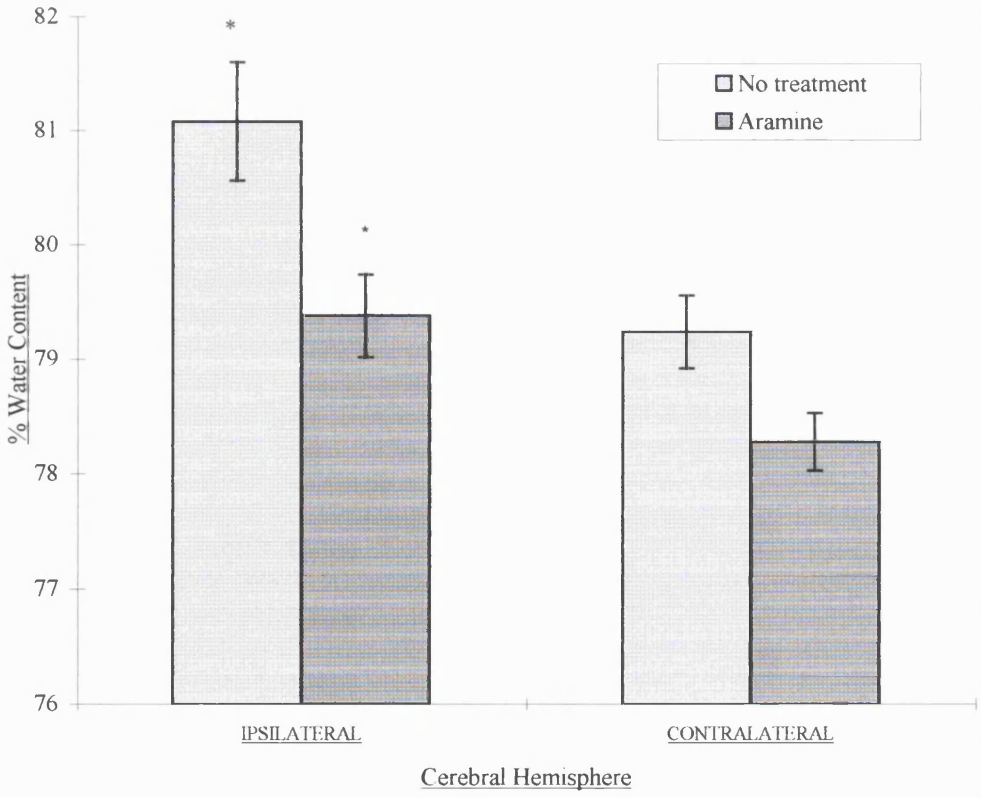
[(WET WEIGHT-DRY WEIGHT)/WET WEIGHT *100]

Caudate Injection	Aramine Infusion	Ipsilateral hemisphere Mean + SEM (n)	Contralateral hemisphere Mean + SEM (n)
Collagenase	None	81.08 ± 0.52 (11)*	79.24 ± 0.32 (11)
Collagenase	Yes	79.38 ± 0.36 (5)*	78.28 ± 0.25 (5)

* comparison between ipsilateral & contralateral hemispheres within groups, student's t-test (paired);
Collagenase (untreated control) p = 0.03
Collagenase (Aramine-treated) p = 0.005

Figure 6D: The effect of post-induced hypertension on % water content of both hemispheres in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

Values shown are mean +/- sem.



* comparison between ipsilateral & contralateral hemispheres within groups, student's t-test (paired);
Collagenase (untreated control) p = 0.03
Collagenase (Aramine-treated) p = 0.005

Section 4: Discussion

Aspey et al (1987) showed that early induced hypertension reduced the metabolic impact of focal cerebral ischaemia presumably due to improved cerebral perfusion in the presence of disrupted autoregulation. Delayed treatment led to an increase in vasogenic oedema.

The present experiments were designed to see if there was any such beneficial effect of a brief early period of induced hypertension in a model of cerebral haemorrhage. It was assumed an adverse effect on haematoma size would result from prolonged elevation of pressure, particularly as the Evans Blue experiment had confirmed that by 24 hours the blood barrier is breached widely (see Chapter 3).

There is a suggestion in the current results of improved functional outcome associated with the brief, early use of a pressor agent, in that more animals behaved normally or showed the mildest neurological sequelae associated with the induction of a haematoma. With this protocol, haematoma size was not increased and there was a tendency to reduced oedema formation rather than to aggravation.

Clearly a more detailed study would be needed to discover the dose and time parameters within which any such possible ameliorating effect might be extended. The results do again support to a small degree the idea that an ischaemic component is part of the mechanism of disturbed brain function due to a cerebral haematoma.

CHAPTER VII

THE EFFECTS OF HEPARIN AND TISSUE PLASMINOGEN ACTIVATOR ON COLLAGENASE -INDUCED INTRACEREBRAL HAEMORRHAGE

Section 1: Introduction

This chapter focuses on the impact of anticoagulant/thrombolytic therapy, currently used in thrombo-embolic ischaemic strokes, on intracerebral haemorrhage. Three categories of compounds affecting haemostasis and coagulation have been evaluated in clinical trials for ischaemic strokes :

(1) anticoagulants, e.g., heparin, which inhibits the formation of fibrin,

(2) fibrinolytic / thrombolytic agents e.g., streptokinase, urokinase and tissue-plasminogen activator, which digest fibrin in blood clots.

(3) antiplatelet drugs, e.g., aspirin, which reduce platelet adhesion/aggregation

Although their rationale is obvious in thromboembolic stroke, they all carry the theoretical risk of exacerbating cerebral haemorrhage. I therefore decided to study the effect of heparin and of thrombolysis in the collagenase induced haemorrhage model to (a) quantify any increase in the volume of the haematoma due to the induced 'coagulopathy' and to (b) explore the usefulness of the model as a parallel for clinical studies.

Section 1.1: Heparin

Conventional treatment has involved the use of heparin in the acute aftermath of cerebral ischaemia, and has been used for 40 years. Heparin is a glycosaminoglycan, consisting of repeated sequences of sulphated glucosamine and glucuronic or iduronic acid, linked to form a strongly negatively charged organic acid and is located mainly in mast cells.

The anticoagulant action of heparin relies upon the presence of the natural coagulation inhibitors heparin co-factor II and antithrombin III in plasma. Thus, careful

monitoring of the coagulation tests (whole blood clotting time, thrombin time, or activated partial thromboplastin time) is called for whenever heparin is used, to prevent bleeding complications. Consequently, the use of heparin is contraindicated in the presence of clotting disorders. Heparin interferes with platelet function and this in itself could also lead to unwanted episodes of bleeding. The clinical rationale for the use of heparin in ischaemic stroke relate to its ability to prevent new clot formation and limit the propagation of existing thrombi.

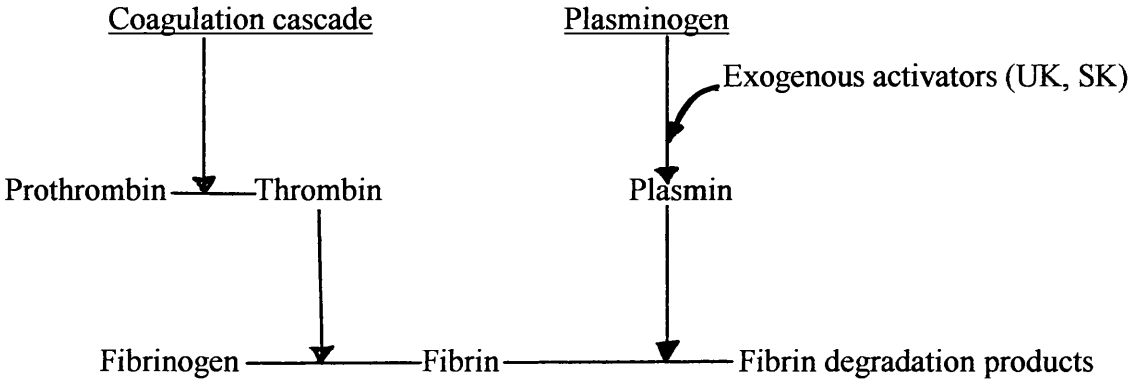
Recently a large multicentre trial assessed its safety and efficacy. The International Stroke Trial on heparin (The Lancet, 1997) has highlighted the difficulty in the use of heparin; it demonstrated a decrease in the absolute number of recurrent ischaemic strokes. There was however an increased risk of severe intracranial bleeding, on treatment especially in combination with aspirin. The ability to treat the ischaemic phase, within the first few hours is thus limited by the perceived need to guarantee that no patient with cerebral haemorrhage receives anticoagulant treatment.

Section 1.2: Thrombolysis

The possibility of improving patient outlook, by systemic administration of thrombolytics, has also led to a series of new trials (National institute of neurological disorders and stroke, NINDS and European acute stroke trial, ECASS II) , but again a risk of haemorrhage has been demonstrated.

Blood has a inherent fibrinolytic system that effectively manages blood clots. This is helped by the presence of a number of enzyme precursors, activators and inhibitors. The lysis of blood clots, within the circulatory system, is initiated when inactive plasminogen is converted to plasmin (Diagram G). Plasmin digests the fibrin component of the blood clot thereby liquefying the solid mass.

Diagram G: A simplified scheme of Fibrinolysis and Coagulation cascades (Taken from Narayan et al, 1985)



Streptokinase and Urokinase

Streptokinase (SK) and urokinase (UK) activate the plasminogen-plasmin system. Streptokinase is a purified exotoxin from C beta-hemolytic streptococci. It forms a complex in plasma with plasminogen which develops activator activity. The activator complex then forms plasmin largely from unbound plasminogen. Urokinase, synthesized by the renal parenchymal cells, acts by direct proteolytic cleavage of plasminogen (an inactive precursor of plasmin) to form plasmin.

These fibrinolytic/thrombolytic enzymes activate plasminogen both at the thrombus surface and in the plasma and this leads to more plasmin being released. However, the systemic release of plasmin can paralyze the clotting process by destroying many of its components and thereby causing a lytic state. Thus treatment with these agents can bring about bleeding complications (Sakurama et al, 1994).

There have been five large randomized trials of intravenous thrombolytic therapy for acute stroke (Brott et al, 1997). Three of these trials that assessed the effects of streptokinase in ischaemic stroke, had to be stopped because of excess

mortality and excess intracranial haemorrhage in the treated group (Donnan et al, 1995; Multicentre acute stroke trial -Italy, MAST-I, 1995).

Section 1.3: Tissue Plasminogen Activator

Tissue plasminogen activator (t-PA) is a synthetic fibrinolytic protein that converts plasminogen to its active form, plasmin specifically in the presence of fibrin. t-PA has a half-life of about 5 minutes compared to 23 minutes for SK. Unlike SK or UK, t-PA does not affect the circulating plasminogen levels and therefore produces less systemic fibrinogenolysis. Tissue-type plasminogen activator has high affinity for fibrin and requires fibrin as a stimulator for plasminogen activation (Sakurama et al, 1994). Thus, intravenous administration of t-PA should induce thrombolysis with reduced haemorrhagic complication.

At the experimental level, Zivin (1985, 1988) and Lyden (1989) have reported that t-PA treatment reduced neurologic damage in experimental embolic stroke, but not without a risk of cerebral haemorrhage. Carlson (1988) and Loscalzo (1988) also reported that there may be a particular risk (low) of brain haemorrhage in patients treated with a higher doses of t-PA.

The low risk of associated haemorrhage and a significant reduction in brain infarction was sufficient to initiate t-PA clinical trials (NINDS 1995; ECASS-I 1995; ECASS-II 1998). The National Institute of Neurological Disorders and Stroke trial (NINDS, 1995) found that when t-PA at a dose of 0.9 mg kg^{-1} was given within 3 hours after ischaemic stroke onset, the treated patients fared better than those on placebo. The ECASS I trial (1995), used a higher dose of t-PA (1.1 mg kg^{-1}) and administered six hours after onset of symptoms, found no significant differences between treated and placebo groups. The findings of the second ECASS (1998) study, designed using the same t-PA dose as the NINDS trial and the same time point as

ECASS I, were inconclusive. The t-PA trials demonstrated that when the drug is given within 3 hours of symptom onset and at a low dose the likelihood of total or near recovery is increased by 30% (Brott et al, 1997). The accompanying risk of cerebral haemorrhage varied between 6.4 and 8.8%. The dosage, the severity of the stroke and also the time of administration have proved critical to avoid this complication.

In this study I used the rat model of intracerebral haemorrhage to establish whether an intravenous infusion of heparin or t-PA would increase the size of a cerebral haematoma and thereby enhance the severity of its clinical sequelae, or conceivably ameliorate any ischaemic component associated with haematoma and improve outcome.

Section 2: Materials and Methods

The preparation of the animals for surgery and the induction of haemorrhage is as described in detail in Chapter 2 (General Methodology). All drug solutions were made fresh on the day of the experiment.

Section 2.1: Treatment Protocol

Heparin

Twenty five male Sprague Dawley rats were used. The minimum dose that affected the clotting time of the blood (personal communication with College's veterinarian) was employed. The heparin dose of 325 IU kg^{-1} was made up in sterile saline (0.9% sodium chloride). Thirty minutes after the haemorrhage was produced, heparin was administered intravenously at a constant rate over thirty minutes.

Tissue-type Plasminogen Activator (t-PA)

t-PA (Alteplase ^{TM}) was supplied by Boehringer Mannheim, UK. The compound was dissolved in sterile saline (0.9% sodium chloride) at a concentration of 1 mg ml^{-1}. The specific activity of t-PA was 580,000 IU mg^{1}.

Twenty male Sprague Dawley rats were used. The animals were fasted overnight with tap water provided ad libitum. Two t-PA dosing regimes were examined: 1 mg kg^{-1} was administered thirty minutes post haemorrhage and 3 mg kg^{-1} was given after a delay of ninety minutes. The intravenous injection of t-PA, commenced with a 10% bolus followed by a constant infusion over thirty minutes.

Section 2.2: Tissue preparation and Data Analysis

Tissue preparation and statistical treatment of the data obtained were carried out as described in detail in Chapter 2 (General Methodology).

Section 3: Results

Body weight fell in heparin treated animals more than in untreated controls (4.77 $\pm$ 0.54% compared to 3.52 $\pm$ 0.38%) though the difference was not significant. The group of animals receiving the low dose of t-PA (1 mg kg^{-1}) showed a small weight gain (+) 1.18 $\pm$ 1.12% which was significantly different from the untreated control data (Anova oneway, $p < 0.001$). The higher dose of t-PA (3 mg kg^{-1}) was associated with a insignificant increase in weight loss (-) 5.1 $\pm$ 0.19% (Table 7A and Figure 7A).

As shown in Table 7B and Figure 7B, the median score for functional status was unchanged by heparin and the higher dose of t-PA but improved by 1 mg kg^{-1} t-PA. Thus 93% of animals given this lower dose of t-PA scored in the best two categories (scores of 0 and 1) compared with 31% of untreated controls (chi-square test, $p < 0.01$).

All treated groups had larger haematomas though the difference was only significant for heparin treated animals and those given 1 mg kg^{-1} (Table 7C and Figure 7C). Heparin and 1 mg kg^{-1} t-PA administration was associated with a haematoma occupying 23.29 $\pm$ 2.44 % (anova oneway, $p < 0.001$) and 19.71 $\pm$ 0.99% (anova oneway, $p = 0.03$) of the hemisphere respectively compared to 15.45 $\pm$ 0.96% in untreated animals.

Despite this, the hemispheric swelling recorded with the 1 mg kg^{-1} dose of t-PA was smaller than controls albeit not significantly. The higher dose of t-PA and heparin both produced more hemispheric swelling, that with 3 mg kg^{-1} t-PA reaching statistical significance. Wet weight dry weight measurements in a small number of animals suggested that hemispheric water content was reduced bilaterally by both heparin and t-PA (Table 7D and Figure 7D).

TABLE 7A: The effect of Heparin and t-PA treatment on body weight in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

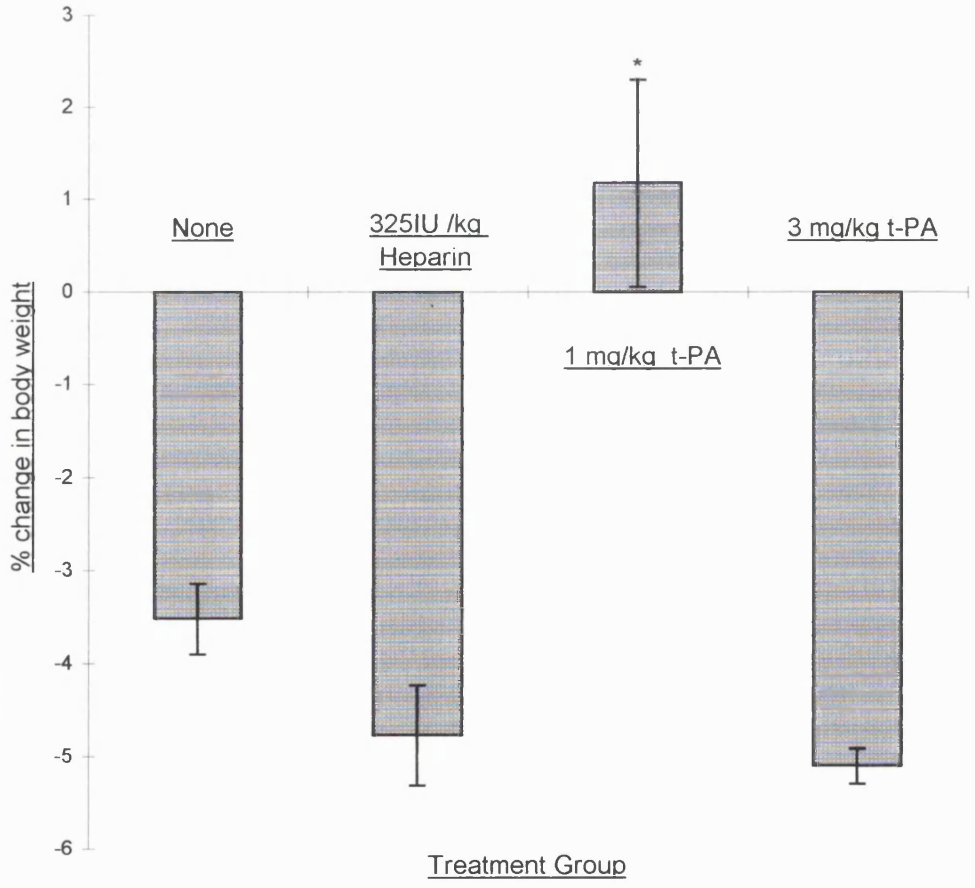
(Values are presented as mean +/- sem. The number in brackets refer to the number (of animals in the group)

Caudate Injection	Drug Treatment	Drug Dose	Treatment Time	% Change in body weight Mean + SEM (n)
Collagenase	None	None	None	(-)3.52 ± 0.38 (123)
Collagenase	Heparin	325 IU/kg	Post 30 mins	(-)4.77 ± 0.54 (25)
Collagenase	t-PA	1 mg/kg	Post 30 mins	(+) 1.18 ± 1.12 (14)*
Collagenase	t-PA	3 mg/kg	Post 90 mins	(-) 5.10 ± 0.19 (6)

*comparison of treated group with untreated collagenase group; anova oneway, p<0.001

Figure 7A: The effect of Heparin and t-PA on body weight in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

Values shown are mean +/- sem.



*comparison of treated group with untreated collagenase group; anova oneway, p<0.001

TABLE 7B: The effect of Heparin and t-PA treatment on neurological scores in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

Numbers in brackets refer to number of animals in the group

Caudate	Drug	Distribution of Neuroscores					Neurological Score
Injection	Dose	0	1	2	3	4	Median
Collagenase	None	10%	21%	63%	2%	4%	2.0 (128)
	Heparin						
Collagenase	325 IU/kg	{4%	31%	54%	8%	4%}*	2.0 (26)
	t-PA						
Collagenase	1 mg/kg	{53%	40%	0%	0%	7%}*	0.0 (15)
Collagenase	3 mg/kg	0%	0%	83%	17%	0%	2.0 (6)

*comparison between nontreated collagenase vs treated groups;Chi-square test; , $p<0.01$.

Figure 7B:The effect of Heparin and t-PA treatment on neurological scores in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

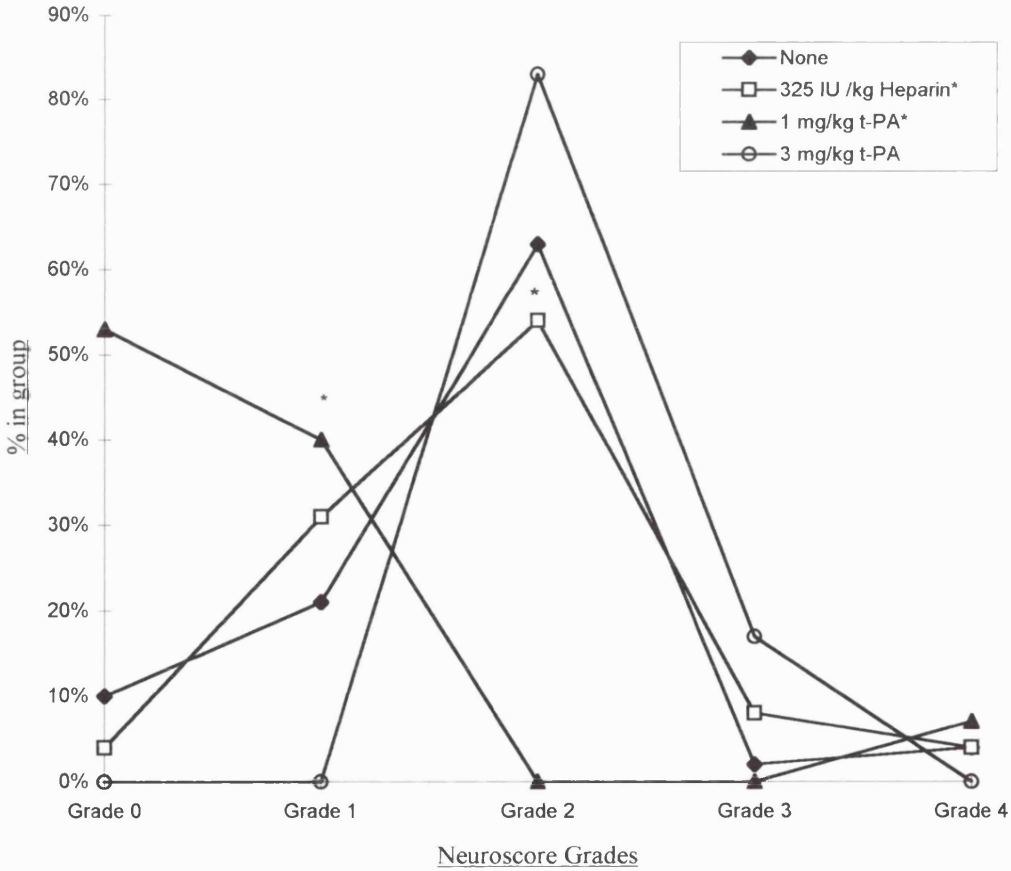


TABLE 7C : The effect of Heparin or t-PA treatment on cerebral swelling and haematoma size in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

(Values are mean ± sem and the number in parentheses refer to number of animals used).

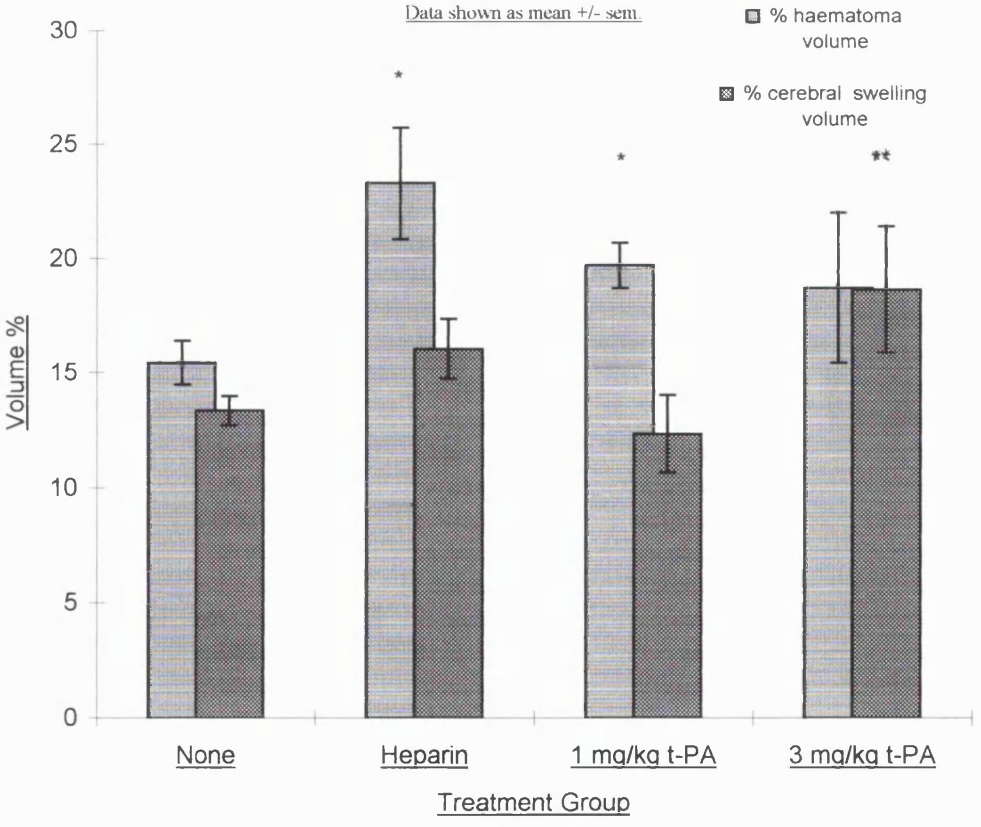
			HEMISPHERICAL VOLUME (mm^3)	
Caudate	Drug	Treatment	% Haematoma	% Cerebral swelling
Injection	Dose	Time	Mean + SEM (n)	Mean + SEM (n)
Collagenase	None	None	15.45 ± 0.96 (52)	13.46 ± 0.64 (52)
	Heparin			
Collagenase	325 IU/kg	Post 30 mins	23.29 ± 2.44 (18)*	16.06 ± 1.31 (18)
	t-PA			
Collagenase	1 mg/kg	Post 30 mins	19.71 ± 0.99 (14)*	12.3 ± 1.69 (14)
Collagenase	3 mg/kg	Post 90 mins	18.72 ± 3.28 (6)	18.65 ± 2.76 (6)**

* comparison between treated group and untreated collagenase group; Anova oneway: $p < 0.001$ (Heparin) and $p = 0.03$ (1 mg/kg tPA)

** comparison of treated group vs collagenase untreated group; Anova oneway, $p = 0.02$

Figure 7C: The effect of Heparin or t-PA treatment on cerebral swelling and haematoma size in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

Data shown as mean +/- sem.



* comparison between treated group and untreated collagenase group; Anova oneway: $p < 0.001$ (Heparin) and $p = 0.03$ (1 mg/kg tPA)

** comparison of treated group vs collagenase untreated group; Anova oneway, $p = 0.02$

TABLE 7D : The effect of Heparin and t-PA treatment on % water content of both Ipsilateral and Contralateral hemispheres in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

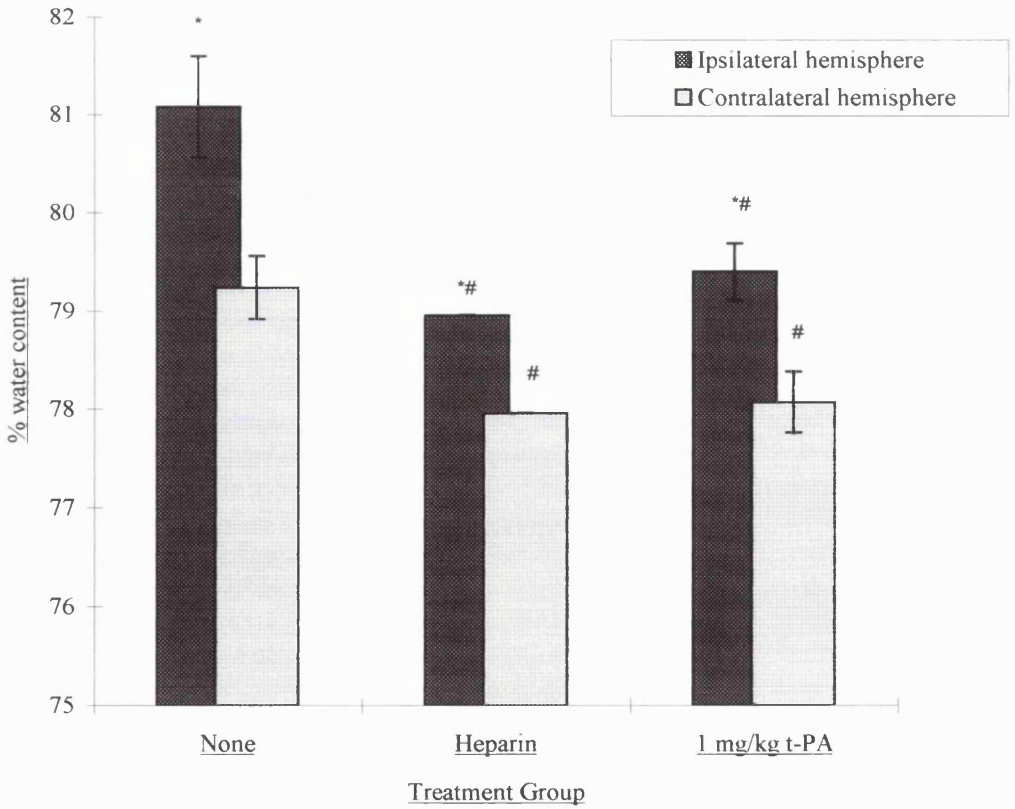
(Values are mean ± sem and the number in parentheses refer to number of animals used).

		% WATER CONTENT [(WET WEIGHT-DRY WEIGHT)/WET WEIGHT *100]	
Caudate	Drug	Ipsilateral hemisphere	Contralateral hemisphere
Injection	Dose	Mean + SEM (n)	Mean + SEM (n)
Collagenase	None	81.08 ± 0.52 (11)*	79.24 ± 0.32 (11)
	Heparin		
Collagenase	325IU /kg	79.43 ± 0.005 (6)*#	78.13 ± 0.001 (6)#
	t-PA		
Collagenase	1 mg / kg	79.40 ± 0.29 (8)*#	78.07 ± 0.31 (8)#

*comparison between ipsilateral and contralateral hemispheres within groups,paired t-test;
p (collagenase untreated)=0.03; p (Heparin)<0.0001; p (tPA)=0.002
comparison between treated groups and untreated collagenase group; Anova oneway, p <0.05

Figure 7D: The effect of Heparin and t-PA treatment on % water content of both Ipsilateral and Contralateral hemispheres in a rat model of collagenase-induced intracerebral haemorrhage after 24 hours of survival.

Data are shown as mean +/- sem



*comparison between ipsilateral and contralateral hemispheres within groups, paired t-test;
p (collagenase untreated)=0.03; p (Heparin)<0.0001; p (tPA)=0.002
comparison between treated groups and untreated collagenase group; Anova oneway, p <0.05

Section 4: Discussion

The conventional view that heparin treatment is contraindicated in cerebral haemorrhage (despite the desire to use it for deep vein thrombosis prophylaxis in stroke patients for example) is supported in this model. Heparin use even for a brief period was associated with larger haematomas, a more swollen hemisphere, greater body weight loss, and the greatest number of animals in adverse grades 3 and 4 (Table 7B and

Figure 7B).

The results with 3 mg kg^{-1} t-PA were also as predicted. Haematomas were larger, brain swelling greater, weight loss increased and this group had no animals in the best two functional categories.

The results of 1 mg kg^{-1} t-PA were unexpected. Despite the larger haematoma, brain swelling was reduced though not significantly, body weight loss was prevented and the greatest number of animals (68%) were normal or only mildly affected on the functional scale.

The reason for this result is not immediately obvious. If there is an ischaemic component in the haematoma bearing hemisphere and this is due to microvascular occlusion by thrombotic plugging then it is conceivable that t-PA might improve perfusion. Higher doses would be expected to disrupt haemostasis to such an extent that the direct effects on a haematoma outweigh any influence on associated ischaemia. The actual volumes of haematoma do not make this argument secure however as the larger dose of t-PA was not associated with the largest haematomas.

It would be interesting to see if lower doses of t-PA would also show a therapeutic effect with even less impact on haematoma size. A detailed study of dose

CHAPTER VIII

SYNOPSIS

Section 1. Summary of experimental findings

As was discussed in some detail in Chapter 1, stroke is a major cause of death and disability. Although ischaemic stroke predominates (~80%) the development of strategies for its treatment such as the inhibition of thrombosis, the institution of thrombolysis, manipulation of blood pressure and pharmacological attempts to affect ischaemic neuronal death have been complicated by the need to treat early and in most cases to know before treatment that the patient does not have a cerebral haemorrhage.

Animal models have played an important role in the search for treatment in ischaemic stroke but less has been done to model cerebral haemorrhage to highlight difficulties or to reveal similar responsiveness to new treatments. I therefore began by assessing the available models.

The first experimental haemorrhage models included the direct injection of blood into the brain. These either involved hand injection, the use of an infusion pump or to approximate arterial blood pressure, a catheter from the femoral artery. Alternatively a microballoon was inflated to simulate the mass effect of a cerebral haematoma.

Blood used in the blood injection model however, does not issue from the cerebral vasculature and the microballoon model is potentially limited in that it only parallels a rapidly expanding mass. Nevertheless, the data from these models together (Kane et al, 1994; Kingman et al, 1987; Nehls et al, 1990; Mendelow, 1993; Sinar et al, 1987) have highlighted some of the reactions that potentially contribute to the

The collagenase model (Rosenberg et al, 1990) is potentially closer to clinical haemorrhage as it involves disruption of blood vessels within the brain substance. As the basal ganglia are the commonest site for hypertensive cerebral haemorrhages

(Caplan, 1993), this is applied in animal models set up to mimic human cerebral haemorrhage. The two main aims of the studies reported in this thesis therefore were to characterise the collagenase model of cerebral haemorrhage and to assess its suitability for therapeutic studies by piloting some of the latter.

The collagenase model evaluated in Chapter III has in my hands, proved technically more reliable in that backflow of the injectate was a rarity compared with that associated with blood injections (Yang et al, 1991). Each intracerebral injection of collagenase elicited a haemorrhagic event - a 100% success rate. Also, the haematoma always affected the target caudate nucleus (100%).

However, the clinical sequelae, the size of the haematoma and the degree of associated brain swelling, all remained somewhat variable. These features were studied in detail and reported in Chapter III. The presence of a haematoma in the caudate caused a reduction in the rat's body weight of 3.53 $\pm$ 0.38% (mean $\pm$ sem) on average. The clinical sequelae were variable with 9% showing no physical abnormality, 25% of the sample displaying either a splayed limb or some unilateral circling, 63% showing definite unilateral circling with splayed limbs. Mortality rate was low at 4%. The haematoma volume of 15.5 + 0.96 % (mean $\pm$ sem) with a coefficient of variability 48% was associated with an estimated volume of shed blood of 138.4 +/- 13.9 microlitres (mean $\pm$ sem) with a coefficient of variation 51%. The mass effect of the haematoma and associated oedema was revealed by the measured increase in hemispheric volume on the haematoma bearing side. This amounted to a volume increase of 13.5 + 0.64% (mean $\pm$ sem) with a coefficient of variability of 33% suggesting that the model would be suitable for studies on treatment of mass effect. The component of volume change due to oedema was also calculated from wet weight dry weight measurements and revealed volumes of swelling of 20 $\pm$ 5.3%

(mean ± sem) with a calculated coefficient of variability of 1-2 %, once again suggesting that the model would be suitable for studies on treatment of mass effect. This still represents a composite of haematoma and oedema in so far as the shed blood contributes directly to the measurements. The extravasation of Evans blue dye beyond the haematoma margin testifies to the vasogenic component of the oedema seen in this haemorrhagic insult. This has also been visualised by Rosenberg's group in nuclear magnetic resonance spectroscopic studies (Mun-Bryce et al, 1993).

In Chapters IV -VII, I explored the usefulness of the collagenase model in the assessment of therapeutic interventions (other than surgical aspiration). These addressed the safety in the presence of haemorrhage, of therapy aimed at ischaemic brain injury, and sought indirect evidence of the presence of ischaemic change around a haematoma that might be revealed by a positive treatment effect.

Osmotherapy (Chapter 4) with glycerol and the inhibition of cyclo-oxygenase by indomethacin resulted in some reduction in the volume of cerebral swelling. Dexamethasone, although reported to be effective in reducing peri-tumoural oedema, was not convincing in this model. Rosenberg et al (1995) reported that mannitol had no effect on brain oedema but found that atrial natriuretic peptide (ANP) was effective in reducing haemorrhagic oedema even when given as late as 4 hours after the insult. The size of the haematoma remained unaffected through these anti-oedemic treatments. Throughout these studies, evidence was sought for the presence of a reversible ischaemic component to the adverse effects of the haematoma. It has been hypothesised, that around a cerebral haematoma there may be a zone of brain tissue that is being at least temporarily damaged by ischaemia perhaps due to local tissue pressure interfering with tissue perfusion, or through the effects on tissue of the biochemical effects of shed blood. Neither the Haematoxylin and Eosin stain nor the

Luxol Fast blue and Cresyl Violet stain revealed a likely zone of ischaemic damage. Although no clear histological evidence of ischaemic damage was thus seen, the 'invasion' of tissue immediately adjacent to the haematoma by the blood cells makes such visualisation difficult, a problem also reported by Sinar et al (1988). The use of TTC (triphenyltetrazolium chloride), a marker stain for mitochondrial activity, also failed to reveal a zone outside the haematoma that might reflect ischaemic damage. This is in agreement with Patel's findings of minimal brain damage outside the haematoma, as shown by good TTC staining, following intracerebral haemorrhage (Patel et al, 1999).

The most useful method of detecting an ischaemic zone has been the direct measurement of regional cerebral blood flow (CBF). With injection of blood into the cerebral substance, regional CBF falls though not all studies have shown severe reductions to ischaemic levels, and levels have often been reported to return to normal as early as 3 hours after the original insult (Ropper et al,1982; Nath et al, 1987; Jenkins et al, 1990). The clearest evidence of a zone of low CBF to ischaemic levels have been found with the bloodless balloon (Mendelow, 1991).

Most recently, Yonezawa et al (1999) using a collagenase-induced haemorrhage model, found that regional cerebral blood flow in the ipsilateral regions decreased in the first four hours, returned to baseline values by 24 hours and then fell again and remained low after the first month (presumably due to tissue loss).

Much interest has been aroused concerning the mechanism of these flow changes. Mendelow reported a greater reduction in cerebral blood flow following uncontained (for example, subarachnoid intraventricular haemorrhage) than contained intracerebral haemorrhage, suggesting a role for pressure effects. However flow fell without pressure rise in the latter situation. This raised the possibility that compressed

brain released vasoactive materials though, no direct tissue pressure or dialysis experiments have been reported to evaluate this.

Ropper et al (1982) found that 'treated' blood, e.g., centrifuged blood, had a more immediate effect on CBF than whole blood. The injection of an inert plastic did not affect the CBF. Ropper et al (1982) also observed a transient hyperperfusion of either or both cortical regions. Matz (1997) found that lysed blood caused more activation of heat shock proteins (HSP 70) than whole blood. Koeppen et al (1995) found that washed red blood cells recruited less phagocytes than whole blood. These findings suggest that the CBF changes may relate to blood products released at the edge of the haematoma, one of these may be iron.

Lee et al (1996, 1997) reported thrombin to be the most damaging blood component and to be one of the leading vasoactive components that contributes to brain oedema in response to haemorrhage. This response is blocked through the use of a thrombin inhibitor. Whole body irradiation to remove the platelets and the white blood cells also led to a reduction in associated cerebral oedema (Kane et al, 1992).

MK801, shown to ameliorate ischaemic brain damage in other contexts by inhibiting excitotocixity, at 1 mg kg^{-1} improved outcome in this haemorrhage model (Chapter V). The security of this conclusion is compromised by the fact that this group of animals had a smaller haematoma though the difference in volume was not statistically significant, and the correlation of volume and outcome as well.

A brief pulse of induced hypertension far from increasing haematoma size, produced a trend towards reduced oedema and produced a significant improvement in functional outcome (Chapter VI).

Heparin (Chapter VII), traditionally considered hazardous in the presence of haemorrhage (and putting time constraints on treatment until haemorrhage can be

excluded by scanning), increased haematoma size and with it brain swelling and adverse clinical sequelae. Elger et al (1994) also found heparin doubled the size of a collagenase-induced haematoma in the rat. The clinical consensus and the collagenase model results suggest heparin is more likely to be hazardous than beneficial in patients with haemorrhage. In recent trials in ischaemic stroke in man, heparin has been given early to some 375 patients with haemorrhagic stroke with fewer than predicted adverse effects with an insignificant trend to increased mortality (Keir et al, 2000).
Saxena et al (2000) found that intravenous infusion of diaspirin cross-linked hemoglobin delayed the fall in blood pressure in patients with acute ischaemic stroke without haemorrhagic transformation, oedema or hypertensive encephalopathy.

The results with t-PA were intriguing. Thrombolysis with t-PA (Chapter VII) appeared, when given early at 1 mg kg^{-1} to improve secondary swelling and outcome without affecting haematoma size. Rosenberg et al (1990) described microvascular plugging in the collagenase haematoma bed, so t-PA could conceivably favour reperfusion. Larger doses given after 90 minutes had no such beneficial effects. Whether this beneficial effect at low dose was through an amelioration of transient ischaemia as discussed above or to some other mechanism is not understood. Clinical experience has established that t-pa in ischaemic stroke in man given 1-6 hours after the ictus is associated with an increased tendency to haemorrhage so much more would need to be done to explore this and the time dependency of such an effect. The model would also lend itself to further studies of the use of craniotomy (Altumbacic et al, 1998) to decompress cerebral haematomas and the use of t-PA injected into the haematoma to accelerate its dissolution.

Summary

My overall conclusion about the collagenase model is that, it is currently the best of the small animal models of cerebral haemorrhage. The collagenase model has the definite theoretical advantage of involving bleeding into the cerebral substance, that the bleeding is well contained, reliably produced in a clinically relevant site and is reproducible in volume with a predictable effect on brain swelling. It involves as in human pathology rupture of blood vessel and the haematoma can be detected by MRI (Del Bigio et al, 1996) so offering the possibility of monitoring.

If a model of cerebral haematoma was to be used in the screening of new treatments for acute stroke, it should highlight the risks associated with any intervention that might prove deleterious in the presence of haemorrhage even if beneficial in the context of ischaemia. The collagenase model in the current studies appropriately highlighted the risk with heparin administration with increase in haematoma volumes and deficit.

The disadvantages of the model include the need for craniotomy, the obscuring of subtle histological change by spreading haemorrhage and the absence of hypertension-the commonest background cause in man . It might be of interest to induce collagenase in spontaneously or renal hypertensive rats to mimic that aspect of human cerebral haemorrhage.

The pilot studies of these interventions have suggested that very early hypertension may be safe to evaluate further, and that early t-PA treatment may even have something to offer the patient with haemorrhage but this controversial suggestion would clearly need more detailed work. Iron chelating agents might be effective if free radical release due to shed blood proves to have a role in the cellular damage. The

results of some of the 'therapeutic' interventions could be interpreted as favouring an ischaemic component.

Criticism of the therapeutic intervention studies reported in this thesis might include the lack of direct measurements of regional blood flow to further pursue the question of an ischaemic zone. It was however felt that at a practical level, a functional definition of reversibility of deficit or of pathophysiological changes like tissue swelling would be a relevant way to explore the impact of any ischaemic change. Also interventions were restricted to the immediate aftermath of the induction of haemorrhage. This strategy was taken to parallel the clinical situation and avoid the later stages when the adverse effects of heparin, t-PA, and hypertension had all been documented, but this may have led to an underestimation of the effect of the interventions.

Finally, I would suggest that when treatments for ischaemic stroke are at the animal model stage as well as being tested in middle cerebral artery occlusion models of cerebral ischaemia, it would be valuable to run in parallel studies in this or an improved model of cerebral haemorrhage.

Section 2: Scope for further research

This study has sought to determine the feasibility of employing a rat model of collagenase-induced intracerebral haemorrhage as a test bed for interventional treatment as planned in man for acute stroke. Although the results suggest the model is appropriate and has reasonable reproducibility, further studies would be valuable in several areas:

- choice of animal model
- experimental protocol

Choice of animal model

It would be interesting to repeat the study in spontaneously hypertensive animals, perhaps to 'model' the role of hypertension in human cerebral haemorrhage and to explore other ways of causing major compromise of small vessels in the brain parenchyma (via different enzymatic systems) or combining vessel damage with anticoagulation preferably in a closed skull preparation.

Experimental protocol

In assessing the safety and possibly the efficacy of new ischaemic stroke treatments in the presence of haemorrhage varying dosages, times of administration and short and long term outcomes all need consideration. The length of the recovery period is another experimental feature that requires further work. 24 hours is the longest recovery time tested in these studies. The results from longer term experiments would shed more light on the potential length of any treatment time window.

Thrombin (Lee et al, 1997, 1996) and free radicals (Peeling et al, 1998) have been identified as mediators of neuronal damage in a haemorrhage model. Further studies of the compounds released in a haematoma could be pursued with microdialysis techniques followed by trials of specific antagonists or 'blockers', including free radical scavengers and iron chelating agents.

The treatments in my studies have been administered as single injections, either prior to the creation of the lesion or after the lesion (post 30 minutes). Multiple and combination treatments and later timings will provide important information on treatment windows. Post stroke treatment is the approach in clinical practice and this should be mimicked with any regime showing efficacy in animal models. Sustained treatment, i.e., multiple infusions rather than single bolus injections may be necessary to arrest late deterioration as seen in clinical stroke, or maximise therapeutic effect.

This project has been successful in establishing the practicality of using the collagenase model to screen stroke treatments in cerebral haemorrhage. More needs to be done to refine the protocols. Although the evidence of a major role for ischaemia in the pathophysiology of haemorrhagic stroke remains equivocal, attempts to blockade the ischaemic metabolic pathways are still worth assessing alongside more direct attempts to reduce the volume of the haematoma.

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APPENDIX

APPENDIX 1

Light microscopy techniques

(i) Albumin and Gelatin Mix

Solutions

Albumin Solution: 45 grammes albuminn is added in small amounts to 100 ml 0.2M Sorensen's phosphate buffer pH 7.0

Gelatin solution : Add 0.75 grammes gelatin to 50 ml 0.2M Sorensen's phosphate buffer pH 7.0 and heat. Once cool, add to (1). Add a few granules (0.005%) sodium azide to prevent fungal/bacterial growth.

1. Trim off olfactory tubercules and spinal cord-just posterior to cerebellum.
2. Oil plastic mould with mineral oil and position perfused brain in with anterior face and right lateral face next to walls of mould.
3. Add 0.3 ml 4%(v/v) glutaraldehyde to 3 ml of albumin/gelatin mix and pour over the brain.
4. Add 0.2ml 4% (v/v) glutaraldehyde to 2 ml albumin/gelatin mix and add to first layer of (3) and leave to set.
5. Once set, remove brain from mould and store in 8% (v/v)glutaraldehyde overnight at 4° C.
6. Place albumin/gelatin embedded brain on brain matrix and cut 10 x1 mm thick coronal sections.

(ii)Histokinette: Shandon Citadel 2000 Tissue Processor Schedule

1. 70% (v/v) Industrial Methylated Spirit (IMS): 2 hours
2. 70% (v/v) IMS: 2 hours

3. 90% (v/v) IMS: 2 hours
4. 90% (v/v) IMS: 2 hours
5. 100% (v/v) IMS: 1 hour
6. 100% (v/v) IMS: 2 hours
7. 100% (v/v) IMS: 2 hours
8. 50:50 (v/v) mixture of 100% IMS:Chloroform: 1 hour
9. Cedar Wood oil: 3 hours
10. Toulene: 1 hour
11. Fibrowax: 2 hours
12. Fibrowax: 2 hours

Total Processing time: 22 hours

(iii) Haematoxylin and Eosin Staining Protoccol

1. Dewax sections in 2 changes of Xylene each for 3 minutes
2. Rehydrate in 2 changes 100% (v/v) absolute alcohol each for 90 seconds
3. Rehydrate in 90% (v/v) absolute alcohol for 2 minutes
4. Rehydrate in 70% (v/v) absolute alcohol for 2 minutes
5. Wash under running tap water for 2 minutes
6. Immerse in Harris' Haematoxylin for 10 minutes
7. Wash under running tap water for 10 minutes
8. Rinse in acid alcohol (1% (v/v) concentrated Hydrochloric acid in 70% (v/v) absolute alcohol)
9. Wash under running tap water for 10 minutes
10. Counterstain in 1% (w/v)Eosin for 5 minutes

11.Dehydrate in ascending series of absolute alcohol ; 70% (v/v) , 90%(v/v) and 100 (v/v) at 2 minutes each

12. Immerse in 2 changes of fresh Xylene for 3 minutes each

13.Mount in Diastrene Plasticiser Xylene (DPX)

(iv) Luxol Fast Blue and Cresyl Violet Staining Protoccol

Solutions:

Luxol Fast Blue stain: 1g Luxol Fast Blue in 1000 ml 95% (v/v) absolute alcohol . Acidified with 5 ml of with 10% (v/v) acteic acid .

0.05% (w/v) Lithium Carbonate: 0.5 g in 1000 ml of double distilled water

0.01 % Cresyl Violet:: 0.1g in 1000 ml in double distilled water. Acidified with 5 drops of 10% acetic acid per 30 ml of stain.

1. Dewax sections in 2 changes of Xylene each for 3 minutes
2. Rehydrate in 95% (v/v) absolute alcohol for 2 minutes
3. Stain overnight in 0.1% (w/v) Luxol Fast Blue (made up in 95% absolute alcohol) at 60° C
4. Wash in 95% (v/v)absolute alcohol
5. Wash in distilled water
6. Differentiate in 0.05% (w/v) Lithium Carbonate (made up in 70% absolute alcohol)
7. Continue differentiation in 70% (v/v) absolute alcohol
8. Wash in distilled water
9. Counterstain in acidified 0.01% (w/v) Cresyl Violet for 10 minutes at 60° C
10. Wash briefly in distilled water
11. Dehydrate in 95% (v/v) absolute alcohol

12. Clear in 2 changes of fresh Xylene for 3 minutes each

13.Mount in DPX

(v) Immunohistochemistry with Glial Fibrillary Acidic Stain

Solutions

Vecta Stain ® Elite ABC Kit (Rabbit IgG) (Vector Ltd, Peterborough, UK))

DAB substrate (Vector)

Avidin/Biotin Blocking Kit (Vector)

Control Antibodies (Rabbit IgG) (Vector)

Polyclonal Rabbit Anti-cow GFAP (DAKO): Diluted 1 in 200 in TBS

Polyclonal Rabbit Laminin (DAKO): Diluted 1 in 100 in TBS

Horse radish Peroxidase (HRP) blocking solution: 0.03% (v/v) Hydrogen Peroxide in Methanol

Trypsin Tablets (Sigma)

Tris Buffer buffered Saline (TBS): 100 ml Tris buffer and 900 ml saline

1. Dewax sections in 2 changes of Xylene : 2 x3 minutes
2. Dehydrate through ascending series of alcohols - 100% (v/v) (2x90 seconds), 90% (v/v) (2 minute) and 70% (v/v) (2 minutes)
3. Wash in double distilled water : 3 x 5 minutes
4. Rinse in TBS : 3 x 5 minutes
5. Block endogenous peroxidase with HRP blocking solution for 20 minutes
6. Wash in running tap water for 10 minutes
7. Warm slides to 60° C by placing slides in warmed 60° C double distilled water
8. Add trypsin solution (warmed at 37° C) and leave at 37° C for 10 minutes

9. Wash in running water for 10 minutes

10. Wash in TBS : 3 x 5 minutes

11. Apply normal swine serum and leave for 10 minutes

12. Tip off serum, apply primary antibody (rabbit anti-cow GFAP). Negative controls in normal rabbit serum. Leave overnight at 4° C

13. Wash in TBS : 3 x 5 minutes

14. Apply biotinylated secondary antibody: 30 minutes at room temperature

15. Wash in TBS: 3 x 5minutes

16. Apply Avidin-Peroxidase conjugate to sections: 30 minutes at room temperature

17. Wash in TBS: 3 x 5 minutes

18. Treat sections with activated DAB:10 minutes

19. Wash in running tap water: 10 minutes

20. Counterstain in Harris' Haematoxylin: 30s econds

21. Differentiate in acid alcohol if necessary

22. Wash in running tap water : 10 minutes

23. Dehydrate in ascending series of absolute alcohol ; 70% (v/v) , 90%(v/v) and 100 (v/v) at 2 minutes each

24. Clear in 2 changes of fresh Xylene for 3 minutes each

25. Mount in DPX

APPENDIX 2

Electron microscopy techniques

(i) Transmission Electron Microscopy: Sample Preparation

Karnovsky's Fixative (J Cell Biol (1963);17:19)

25% glutataraldehyde: 80 ml

40% Formaldehyde: 50 ml

0.2M Sodium Cacodylate buffer: 490 ml

Anyhydrous Calcium chloride: 5 mg

Double distilled water: 280 ml

Adjust to pH 7.4

1. Place tissue (1 millimetre cube brain tissue) in fresh Karnovsky's fixative and leave overnight at 4° C
2. Place in 0.1M sodium cacodylate buffer pH 7.4
3. Osmicate (1% osmium tetraoxide in 0.1 M sodium cacodylate buffer) for 2 hours
4. Wash in buffer: 3x15minutes
5. Wash in saline : 3x15 minutes
6. Place in 50% (v/v) absolute alcohol: 15 minutes
   70%(v/v) absolute alcohol: 15 minutes
   90%(v/v) absolute alcohol: 15 minute
7. Place in 100 % (v/v) absolute alcohol: 2 x 30 minutes
8. Place in propylene oxide: 30 minutes
9. Place in 50:50 mixture of propylene oxide and Araldite overnight on a rotator at room temperature
10. Place in fresh resin the following morning
11. Place in 60° C oven 10 minutes (to evaporate propylene oxide)
12. Place tissue in rubber moulds. Leave at room temperature for 30 minutes.
13. Place in 37° C oven overnight to polymerise
14. Place in 60° C oven overnight to polymerise

APPENDIX 3: The estimation of blood volume in a haematoma - Hb Assay.

Standard curve 1.

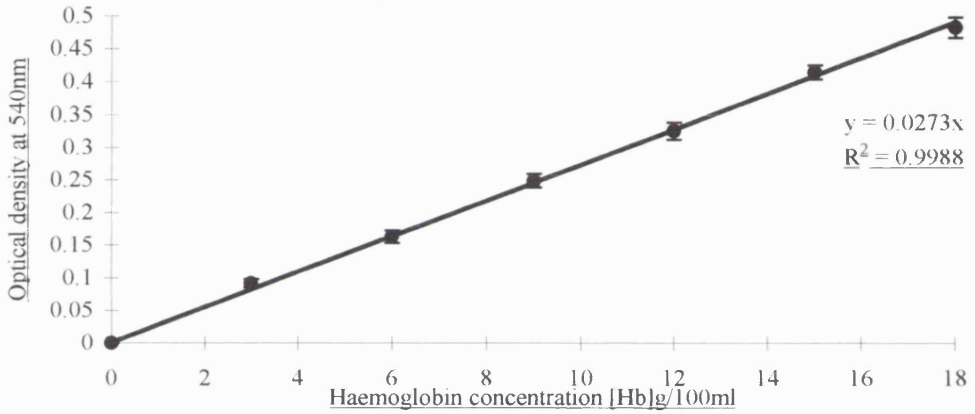
The Sigma Hb Kit was used. From a stock solution of 18 g/100 ml haemagoblin, dilutions (3, 6, 9, 12, 15, 18) were made up in Drabkins Reagent. The solutions were left to stand for 30 minutes at room temperature before the readings were taken at 540nm.

CALIBRATION CURVES

[Hb]; g/100ml	0	3	6	9	12	15	18
CAL 1	0	0.096	0.14	0.24	0.32	0.395	0.48
CAL 2	0	0.105	0.1865	0.27	0.345	0.44	0.5025
CAL 3	0	0.075	0.155	0.224	0.29	0.4	0.44
CAL 4	0	0.0925	0.1725	0.2625	0.3475	0.425	0.51
Mean	0	0.0921	0.1635	0.2491	0.3256	0.4150	0.4831
Standard Error	0	0.0063	0.0101	0.0105	0.0134	0.0106	0.0157

Standard Curve 1 :Haemoglobin Assay

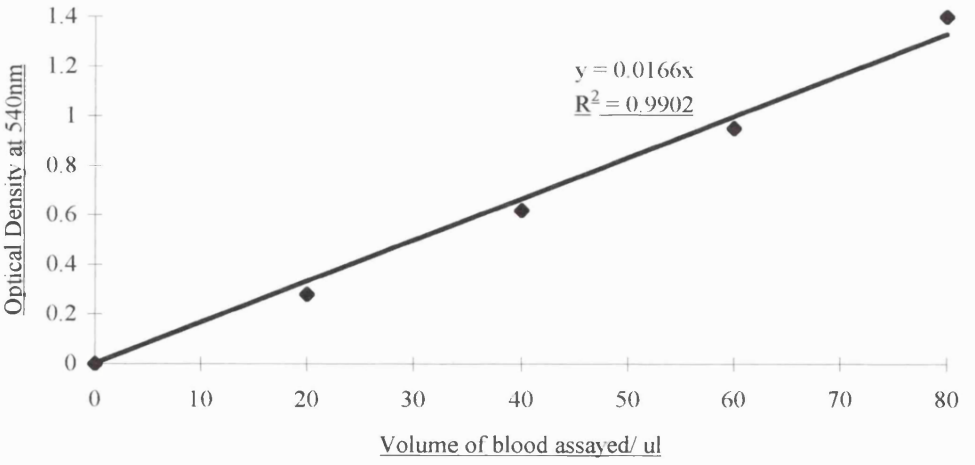
Data presented as mean +/- sem



Standard curve 2

blood ul	OD (540 nm)	Hb/100ml
0	0	0
20	0.28	10.26
40	0.62	22.71
60	0.95	34.80
80	1.4	51.28

Standard curve 2: Blood volume estimations



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Thrombin (Lee et al, 1997, 1996) and free radicals (Peeling et al, 1998) have been identified as mediators of neuronal damage in a haemorrhage model. Further studies of the compounds released in a haematoma could be pursued with microdialysis techniques followed by trials of specific antagonists or 'blockers', including free radical scavengers and iron chelating agents.

The treatments in my studies have been administered as single injections, either prior to the creation of the lesion or after the lesion (post 30 minutes). Multiple and combination treatments and later timings will provide important information on treatment windows. Post stroke treatment is the approach in clinical practice and this should be mimicked with any regime showing efficacy in animal models. Sustained treatment, i.e., multiple infusions rather than single bolus injections may be necessary to arrest late deterioration as seen in clinical stroke, or maximise therapeutic effect.

This project has been successful in establishing the practicality of using the collagenase model to screen stroke treatments in cerebral haemorrhage. More needs to be done to refine the protocols. Although the evidence of a major role for ischaemia in the pathophysiology of haemorrhagic stroke remains equivocal, attempts to blockade the ischaemic metabolic pathways are still worth assessing alongside more direct attempts to reduce the volume of the haematoma.

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