# THE EFFECT OF CORM-3 ON THE INFLAMMATORY NATURE OF HAEMORRHAGIC STROKE


by

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Science is facts; just as houses are made of stone, so is science made of facts; but a pile of stones is not a house, and a collection of facts is not necessarily science.

*Jules Henri Poincaré (1854-1912)*

# Abstract

**Objective**– Intracerebral hemorrhage (ICH) is accompanied by a pronounced inflammatory response that mediates brain damage but is also essential for the tissue reparative process. Here we assessed the effect of CORM-3, a water-soluble carbon monoxide-releasing molecule possessing anti-inflammatory properties, on inflammation and brain injury after ICH.

**Design**– *In vivo*, *in vitro* and *ex vivo* laboratory study.

**Setting**– Research laboratory.

**Subjects**– Male Sprague-Dawley rats, 250-350g.

**Interventions and Measurements**– A model of collagenase injection (2 μl) in brain was established to induce ICH. CORM-3 (4 or 8 mg/kg) was administered i.v. at different times as follows: a) 5 min prior to collagenase, b) 3 hours after collagenase and c) 3 days after collagenase challenge. Saline was used as a negative control. Brain damage, brain water content and behavioural assessment were evaluated. The inflammatory response was determined at set intervals after ICH by counting peripheral neutrophils and lymphocytes, neutrophils and activated microglia/macrophages in the ICH area, brain water content and measuring plasma TNF-α levels. BV2 microglia and DI-TNC1 astrocytes were exposed to triton (1%) or CORM-3 (10-100 μM) and cytotoxicity (LDH assay) measured at 24 hours.

**Main Results**– Challenge with collagenase to induce ICH caused marked brain damage and modified the levels of inflammatory markers. Pre-treatment with CORM-3 significantly prevented injury, modulated inflammation and reduced plasma TNF-α. CORM-3 given 3 hours after collagenase significantly increased brain injury and TNF-α production. In contrast, CORM-3 given 3 days after collagenase afforded partial protection, modulated inflammation and decreased TNF-α starting from the day of application. No dose-dependent effects were observed.

**Conclusions**– CORM-3 promotes neuroprotection or neurotoxicity after ICH depending on the time of administration. Beneficial effects are achieved when CORM-3 is given either before or 3 days after ICH, namely, as a prophylactic agent or during the post-acute inflammatory phase.

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# List of abbreviations

| | |
|---|---|
| **WHO** | World Health Organisation |
| **TIA** | Transient ischaemic attack |
| **ICH** | Intracerebral haemorrhage |
| **THF** | Tetrahydrofuran |
| **GAS** | General adaptation syndrome |
| **WBC** | White blood cell count |
| **CRP** | C-reactive protein |
| **ESR** | Erythrocyte sedimentation rate |
| **CO** | Carbon monoxide |
| **HO** | Heme-oxygenase |
| **NMDA** | N-methyl-D-asapartate |
| **AMPA** | Alpha-amino-3-hydroxy-5-methyl-4-isoxanole propionate |
| **MMPs** | Matrix metalloproteinases |
| **ROS** | Reactive oxygen species |
| **SMC** | Smooth muscle cells |
| **MAPK** | Mitogen activated protein kinase |
| **CNS** | Central nervous system |
| **PNS** | Peripheral nervous system |
| **AD** | Alzheimer's disease |
| **PD** | Parkinson's disease |
| **CO-RMs** | Carbon monoxide releasing molecules |
| **NO** | Nitric oxide |
| **LPS** | Lipopolysaccharide |
| **DMEM** | Dulbecco's modified Eagle's medium |
| **APTS** | 8-Aminopyrene-1,3,6-trisulfonic acid trisodium salt |
| **IMS** | Industrial methylated spirit |
| **HCl** | Hydrochloric acid |
| **DPX** | Di-N-Butyl Phthalate in Xylene |

# Chapter I. Introduction

## 1.1 Stroke

### 1.1.1 Overview

Stroke (insult, latin, insultos = jumping), as a clinical diagnosis, was given the definition by the World Health Organisation (WHO) as a syndrome, that is characterized by "rapidly developing clinical signs of focal (global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin" (Anon, 1989). This definition excludes syncopes of cardiac or any other origin and transient ischaemic attacks (TIAs) as syndromes that do not lead to severe brain injury such as an infarction (or to small infarctions) with complete and rapid recovery. As the brain does not store much (in the form of glycogen) glucose, the main energy substrate and is incapable of sustained anaerobic metabolism, ischaemic and haemorrhagic strokes develop rapidly. The rapid nature of onset means that strokes are mainly observed in hospital emergency units.

### 1.1.2 Types of stroke

*Ischaemic stroke*: The most common type of stroke accounting for almost 80% of all strokes is caused by a blood clot or other blockage within an artery leading to the brain.

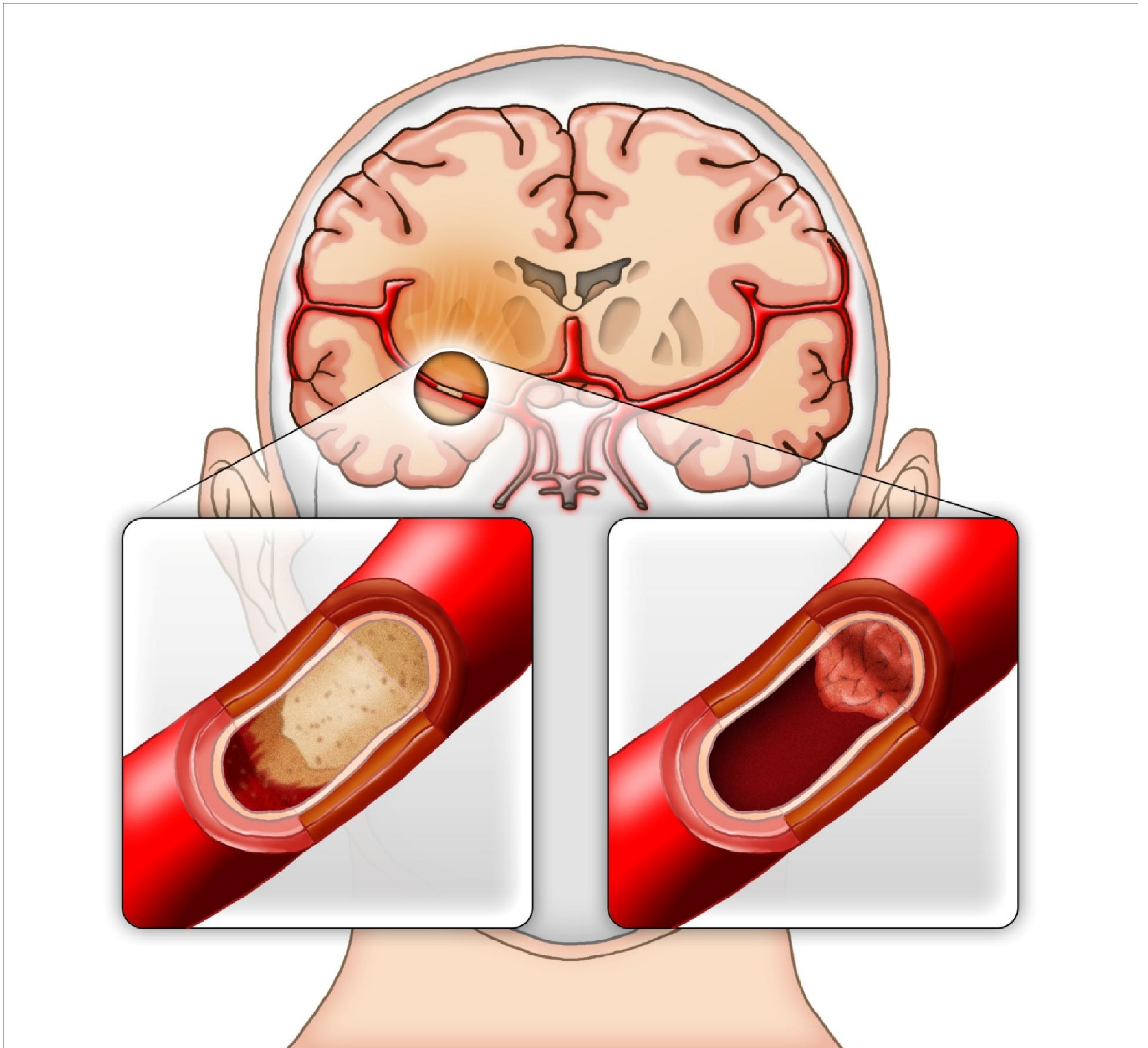

**Figure 1-1 Ischaemic stroke**

*Intracerebral haemorrhage*: An intracerebral haemorrhage is a type of stroke caused by the sudden rupture of an artery within the brain. Blood is then released into the brain tissue, compressing brain structures against the skull. This type of stroke is accountable for almost 20% of cases, and is associated with high mortality and morbidity (Gere et al. 2003).

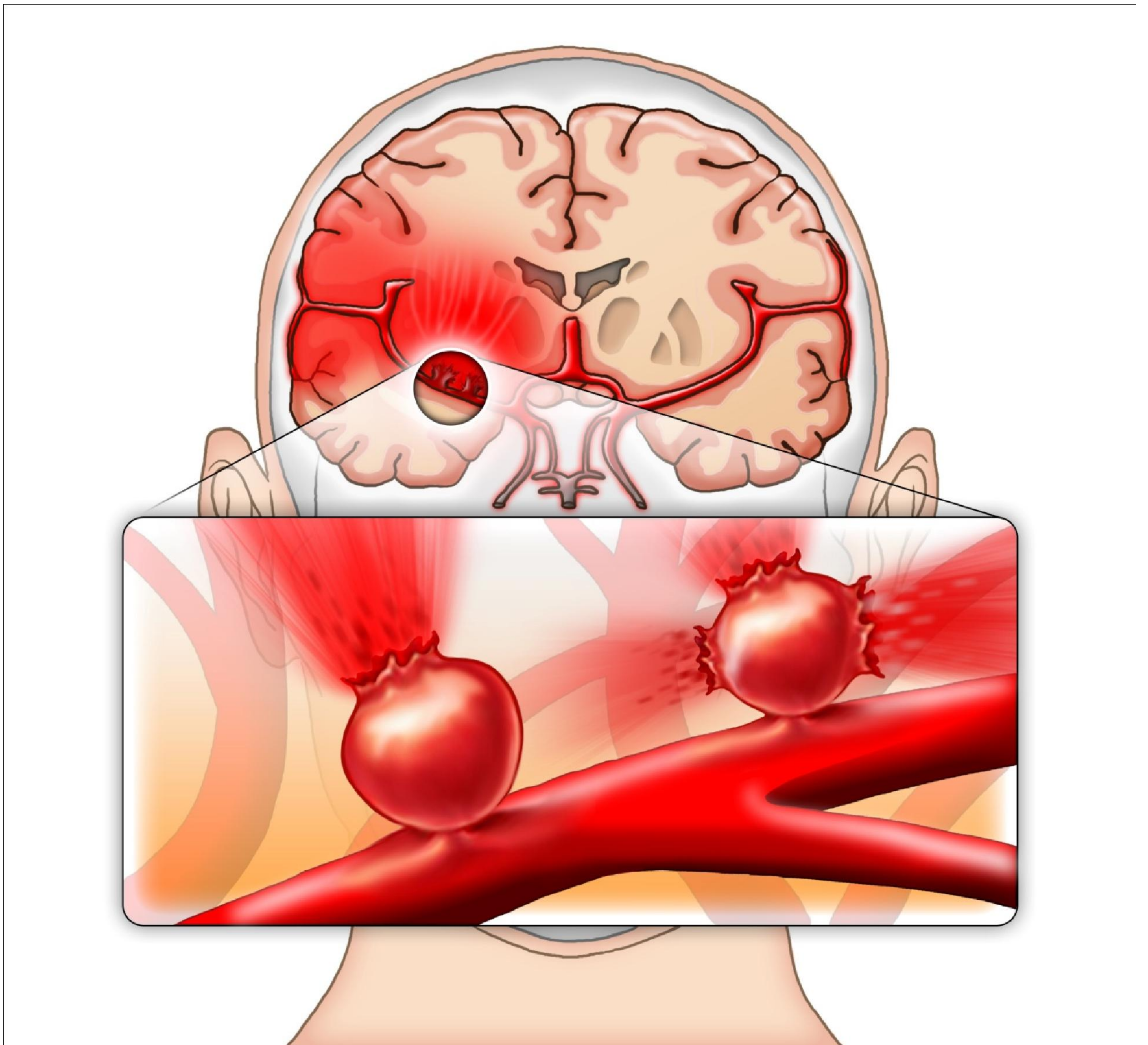

**Figure 1-2 Intracerebral haemorrhage**

*Subarachnoid haemorrhage*: A subarachnoid haemorrhage is also a type of stroke which is caused by the sudden rupture of an artery. A subarachnoid haemorrhage differs from an intracerebral haemorrhage in that the location of the rupture leads to blood filling the space surrounding the brain rather than inside it.

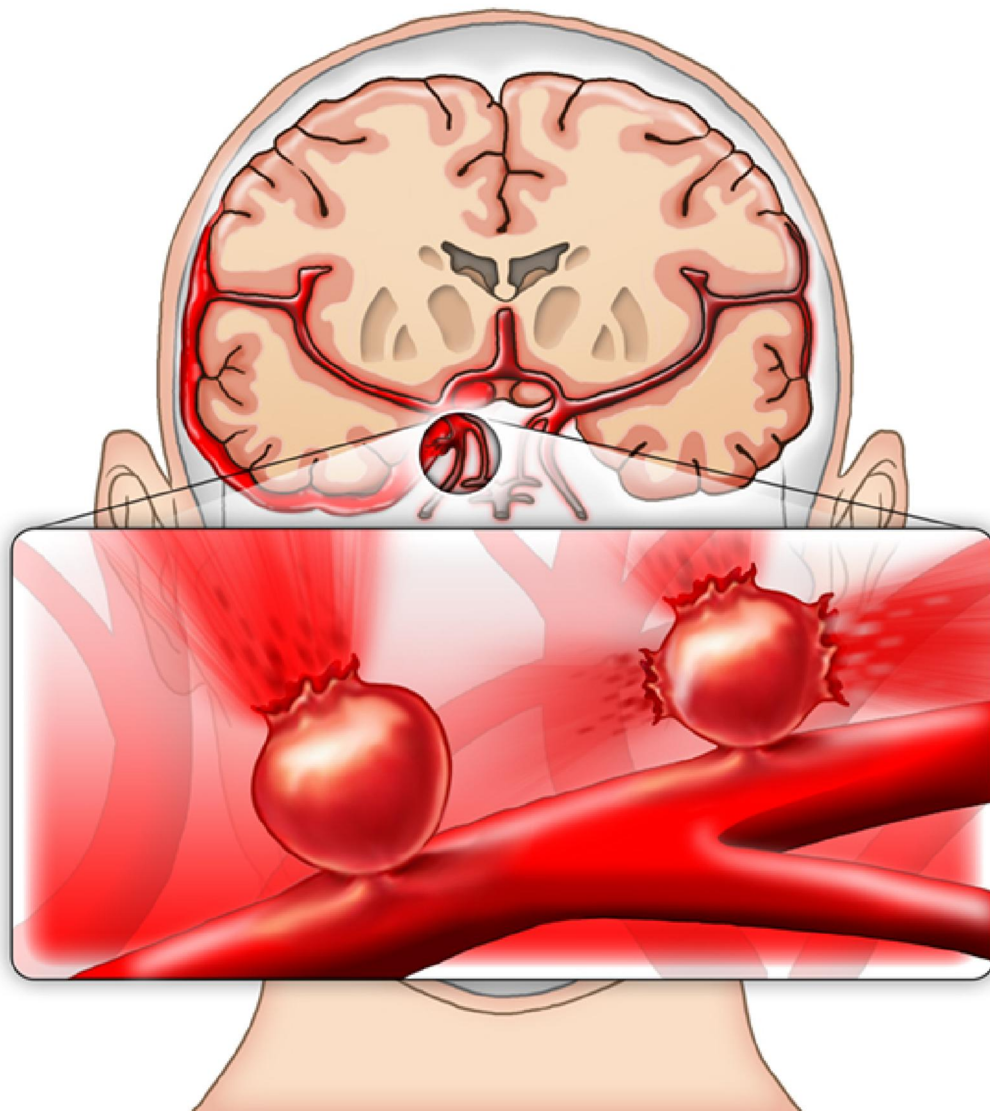

**Figure 1-3 Subarachnoid haemorrhage**

### 1.1.3 Epidemiology

Stroke is the third leading cause of death and is the single biggest cause of disability in the United Kingdom. Ischaemic stroke accounts for 80% of stroke cases, which is why it is the most studied form of stroke, with investigations ranging from aetiology, risk factors and pathogenesis, to clinical picture, treatment and prognosis. In haemorrhagic stroke, intracerebral haemorrhage, accounts for 20% of cases. However, taking into account that intracerebral haemorrhage is accountable for a high proportion of patients who either die or are severely disabled, it has been studied in relatively little detail compared to ischaemic stroke. ICH is responsible for about 50% of all stroke-related death cases and causes disability in 88% of its survivors (Zhang et al. 2006).

The incidence of stroke in the United Kingdom is 2 per 1000 people (Bamford et al. 1988;Warlow 1998). The age and sex indices rise exponentially both in men and women. For example, the incidence for people of 85 years of age is 100 times greater (Williams et al. 2000) than for those aged 35-45 years. The probability of stroke for people who are between 45 and 65 years old is very low, assuming that the individual does not have any other risk factors. But even so, every 1 in 4 men and every 1 in 5 women can be expected to suffer a stroke if they live on into their 80's. In the UK about 130,000 people suffer from a stroke each year, 75 percent of cases occur in individuals over the age of 65 and 50 percent of cases occur in individuals over the age of 75 (Bamford et al. 1988;Goldacre et al. 2008).

Ethnic origin has also been shown to play a role, with a higher incidence among Afro-Corribean and Japanese, and 50 cases per 100.000 which is twice the incidence with Caucasians (Flaherty et al. 2005;Gillum 1988;Suzuki et al. 1987).

30 percent of patients die in the first month after a stroke (Bamford et al. 1990). After a year, only 65 percent of stroke survivors are able to live independently again and the rest remain significantly disabled and may need considerable help with daily tasks. About 5 percent of stroke survivors are eventually admitted to long-term residential care. The fatality rate increases with age. An 85-year-old individual is twice as likely to suffer a fatal stroke than other age groups.

Recent data shows that the incidence of stroke has decreased by 35 percent over recent decades (Rothwell et al. 2004). However, in the UK the mortality rate increases with age from 7 per 100000 people at the age of 35-44 to about 1400 per 100000 people at 75 or over. Worldwide data show that in North America, Western Europe and Japan, stroke mortality rate has decreased during recent decades, but in that time the slope of decline in the UK has now actually stopped decreasing and has plateaued (Goldacre et al. 2008).

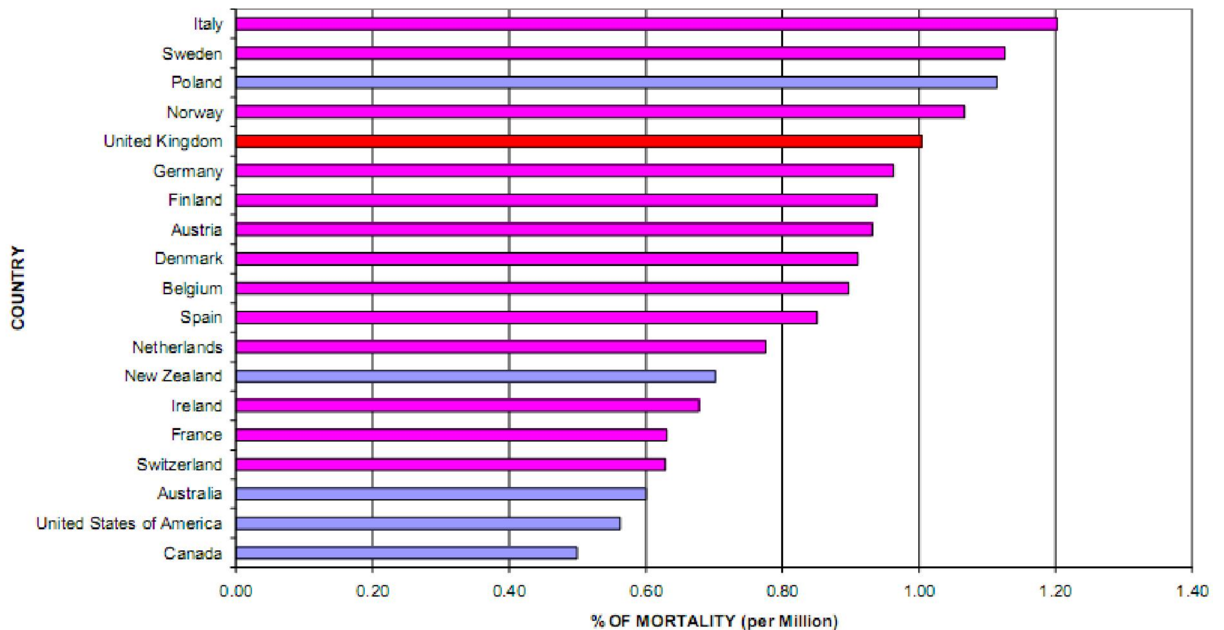


**Table 1 Simple stroke statistics. Table taken from analysis conducted by the World Health Organisation and published in the 'Atlas of Heart Disease and Stroke', 2004, World Health Organisation, p.52-53**

Stroke costs the NHS and the economy about £7 billion a year: £2.8 billion in direct costs to the NHS, £2.4 billion of informal care costs (e.g. the costs of home nursing borne by patients' families) and £1.8 billion in income lost to productivity and disability (Anon, 1989). The total costs of stroke care are predicted to rise in real terms by 30 per cent between 1991 and 2010 (Reducing Brain Damage: Faster access to better stroke care. National Audit Office 2005, http://www.stroke.org.uk). Moreover, most of this money is spent on intracerebral haemorrhagic stroke (ICH) treatment and recovery, even though ICH occurs in only 20% of all stroke cases.

### 1.1.4 Risk factors

Stroke prevention is a primary aim of health programmes, so it is important to identify the risk factors and introduce preventative measures to minimise the probability of stroke occurrence. Though the presence of a risk factor or a combination of risk

factors does not necessarily lead to stroke, and the absence of risk factors does not necessarily mean that a stroke will not occur, the presence of risk factors appears to raise the probability of a stroke occurring. Some of the risk factors can be beneficially influenced, for example by treating arterial hypertension, stopping smoking, and reducing alcohol consumption, but some of them, such as genetic factors, are difficult to influence. A literature review conducted by the WHO showed that some risk factors are “specifically geographic”, but most of them appear to be universal for all peoples. These factors are presented in the table below, summarising these data collected by the WHO (Anon, 1989).

| | Geographical area | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Risk factor** | **North America** | **Europe** | **South America** | **Japan; Oceania** | **China** | **Southeast Asia** | **India** | **Sub-Saharan Africa** |
| Hypertension | + | + | + | + | + | 0 | + | + |
| Systolic | + | + | + | + | + | 0 | + | + |
| Diastolic | + | + | + | + | + | 0 | + | + |
| Diabetes Mellitus | + | + | 0 | - | - | 0 | 0 | + |
| Heart disease | + | + | + | 0 | + | 0 | | 0 |
| Transient ischaemic attack | + | + | 0 | 0 | + | 0 | 0 | 0 |
| Obesity | +/- | +/- | 0 | - | - | 0 | - | + |
| Platelet hyperaggregability | +/- | + | 0 | 0 | 0 | 0 | 0 | 0 |
| Alcoholism | +/- | + | 0 | 0 | + | 0 | 0 | 0 |
| Smoking | + | +/- | 0 | +/- | +/- | 0 | - | 0 |
| Elevated blood lipid levels | | 0 | 0 | +/- | 0 | 0 | + | 0 |
| Cholesterol | +/- | +/- | 0 | - | - | 0 | - | + |
| Triglycerides | +/- | +/- | 0 | - | 0 | 0 | - | 0 |
| Low density lipoproteins | +/- | 0 | 0 | - | +/- | 0 | 0 | 0 |
| Hyperuricemia | +/- | + | 0 | + | 0 | 0 | + | 0 |
| Infections | 0 | 0 | 0 | 0 | + | +/- | + | 0 |
| Genetic or familial factors | +/- | +/- | 0 | 0 | +/- | 0 | 0 | 0 |
| Other | | | | | | | | |
| Migraine | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 |
| Cold temperature | 0 | +/- | 0 | +/- | +/- | 0 | 0 | 0 |
| High-estrogen contraceptives | + | + | 0 | 0 | 0 | 0 | 0 | 0 |
| Socioeconomic status | 0 | +/- | 0 | + | + | 0 | 0 | 0 |
| Haematocrit | | | | | | | | |
| Increased | +/- | 0 | 0 | +/- | 0 | 0 | 0 | 0 |
| Decreased | 0 | 0 | 0 | +/- | 0 | 0 | 0 | 0 |
| Increased fibrinogen | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 |
| Proteinuria | + | 0 | 0 | + | 0 | 0 | 0 | 0 |
| Sodium intake | 0 | 0 | 0 | + | + | 0 | 0 | 0 |

+, yes; +/-, suggestive; -, no; 0, insufficient data

**Table 2 Risk factors for all stroke, 1988, WHO (Special Report From the World Health Organization: Recommendations on Stroke Prevention, Diagnosis, and Therapy, WHO, 1989)**

The most common risk factors that lead to intracerebral haemorrhage include:

- hypertension (Hanggi & Steiger 2008)
- aneurysms
- cerebral amyloid angiopathy, which is characterised by excessive deposition of beta-amyloid, a 30-32 amino acid peptide, in the blood vessels
- diseases of the heart such as atrial fibrillation with presence of thrombi in the atrium
- platelet hyperaggregation
- diabetes mellitus
- infections
- use of alcohol (Klatsky, Armstrong, & Friedman 1989), particularly excessive use in patients with hypertension
- smoking
- advanced age
- male gender
- the influence of low temperatures on the body, which leads to peripheral vasoconstriction and pooling of blood in the body core
- use of high-oestrogen contraceptive drugs
- low quality life index (poor nutrition, etc.)
- high haematocrit levels in males and low haematocrit levels in females
- low intake of potassium (Anon, 1989)
- drug use (heroin, amphetamines, LSD, PCP, "T's and Blues," and marijuana) (Sacco et al. 1997)
- elevated homocysteine levels
- antiphospholipid antibody syndrome

- patent *foramen ovale* (Chong & Sacco 2005)
- race was shown to be a risk factor, as Hispanic men, followed by Afro-corribean men and Japanese and then followed by Caucasian men are more likely to undergo intracerebral haemorrhage (Suzuki et al. 1987)
- thrombolytic treatment for any possible reason

| Risk factor | Geographical area | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **North America** | **Europe** | **South America** | **Japan; Oceania** | **China** | **Southeast Asia** | **India** | **Sub-Saharan Africa** |
| Hypertension | + | + | 0 | + | + | 0 | 0 | 0 |
| Systolic | + | + | 0 | + | + | 0 | + | 0 |
| Diastolic | + | + | 0 | + | + | 0 | + | 0 |
| Diabetes Mellitus | 0 | +/- | 0 | - | - | 0 | 0 | 0 |
| Heart disease | 0 | 0 | 0 | +/- | + | 0 | 0 | 0 |
| Transient ischaemic attack | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Obesity | 0 | 0 | 0 | - | - | 0 | 0 | 0 |
| Platelet hyperaggregability | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Alcoholism | 0 | 0 | 0 | +/- | 0 | 0 | 0 | 0 |
| Smoking | 0 | +/- | 0 | +/- | 0 | 0 | - | 0 |
| Elevated blood lipid levels | 0 | 0 | 0 | 0 | 0 | 0 | +/- | 0 |
| Cholesterol | 0 | - | 0 | 0 | 0 | 0 | - | 0 |
| Triglycerides | 0 | - | 0 | 0 | 0 | 0 | - | 0 |
| Low density lipoproteins | 0 | 0 | 0 | 0 | +/- | 0 | 0 | 0 |
| Hyperuricemia | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Infections | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Genetic or familial factors | +/- | - | 0 | 0 | 0 | 0 | - | 0 |
| Other | | | | | | | | |
| Migraine | 0 | +/- | 0 | 0 | 0 | 0 | 0 | 0 |
| Socioeconomic status | 0 | +/- | 0 | 0 | 0 | 0 | 0 | 0 |
| Haematocrit | 0 | +/- | 0 | 0 | 0 | 0 | 0 | 0 |
| Proteinuria | 0 | +/- | 0 | 0 | 0 | 0 | 0 | 0 |
| Sodium intake | 0 | +/- | 0 | 0 | 0 | 0 | 0 | 0 |
| Hepatic disease | 0 | +/- | 0 | 0 | 0 | 0 | 0 | 0 |

+, yes; +/-, suggestive; -, no; 0, insufficient data

**Table 3 Risk factors for Haemorrhagic stroke, 1988, WHO (Special Report From the World Health Organization: Recommendations on Stroke Prevention, Diagnosis, and Therapy, WHO, 1989)**

The combination of various risk factors has not yet been studied precisely, but in theory a person presenting with multiple risk factors would be expected to be at greater risk of suffering a stroke. This was highlighted by a study showing that high-oestrogen contraceptive drug use together with smoking or hypertension is an important combined stroke risk factor in women, especially of child-bearing age (Special Report From the World Health Organization: Recommendations on Stroke Prevention).

### 1.1.5 Aetiology

Aetiological factors for haemorrhagic stroke are diseases and conditions of the vessels that may lead to malfunction in mechanical and biological properties of vessels with further blood wall disruption and blood leakage. Aetiology factors can be divided into two main groups: primary and secondary.

Primary aetiological factors are: arterial hypertension, cerebral amyloid angiopathy and unknown causes (Ferro 2006).

Hypertension: Despite the fact that arterial hypertension can be controlled and a lot of effort has been made in improving treatment, arterial hypertension remains one of the most common reasons for intracerebral haemorrhage. Many patient studies report that those people who have suffered intracerebral haemorrhage also show signs of suffering from hypertension.

Amyloid angiopathy: In the condition of amyloid angiopathy, usually the most affected are the medium and small sized blood vessels (20-250 μm in diameter) (MacKenzie 1996).

Secondary aetiological factors include:

- traumatic injuries
- aneurysms
- arterio-venous malformations
- cavernomas (Kupersmith, Epstein, & Berenstein 2001)
- a neoplasm with associated haemorrhage
- coagulopathies (Quinones-Hinojosa et al. 2003), which are mainly represented by patients with leukaemia, as disseminated intravascular coagulation
- thrombocytopenia and other platelet disorders
- haemophilia and other hereditary coagulopathies
- hepatic failure
- renal failure
- arterio-venous dural fistulae
- vasculitis
- vasculopathies which can present as dissections, Moya-Moya disease (Kobayashi et al. 2000)
- emboli, atherothrombosis
- surgical procedures and interventions:
    - neurosurgery (arteriovenous malformation surgery, craniotomy (Marquardt et al. 2002), subdural haematoma with burr-hole evacuation, spinal surgery (Friedman, Breeze, & McCormick 2002)
    - neuroradiological interventions
    - carotid endarterectomy
    - carotid angioplasty

    - cardiac transplantation (Ferro et al. 1993)

- brain irradiation (Cheng et al. 2001)
- sonothrombolysis
- medications:
    - anticoagulants
    - thrombolytics
    - antiplatelets
    - sympathomimetics
    - over-the-counter pills and nasal sprays for congestion (e.g. ephedrine, pseudoephedrine and phenylpropanolamine)
- honeybee stings (Remes-Troche et al. 2003)
- alcohol
- drug usage

### 1.1.6 Pathogenesis

An intracerebral haemorrhage (ICH) is bleeding in the brain caused by the rupture of a blood vessel and commonly occurs in the cerebral lobes, basal ganglia, thalamus, brain stem (pons) and cerebellum (Fewel, Thompson, Jr., & Hoff 2003; Mutlu Berry, & Alpers 1963).

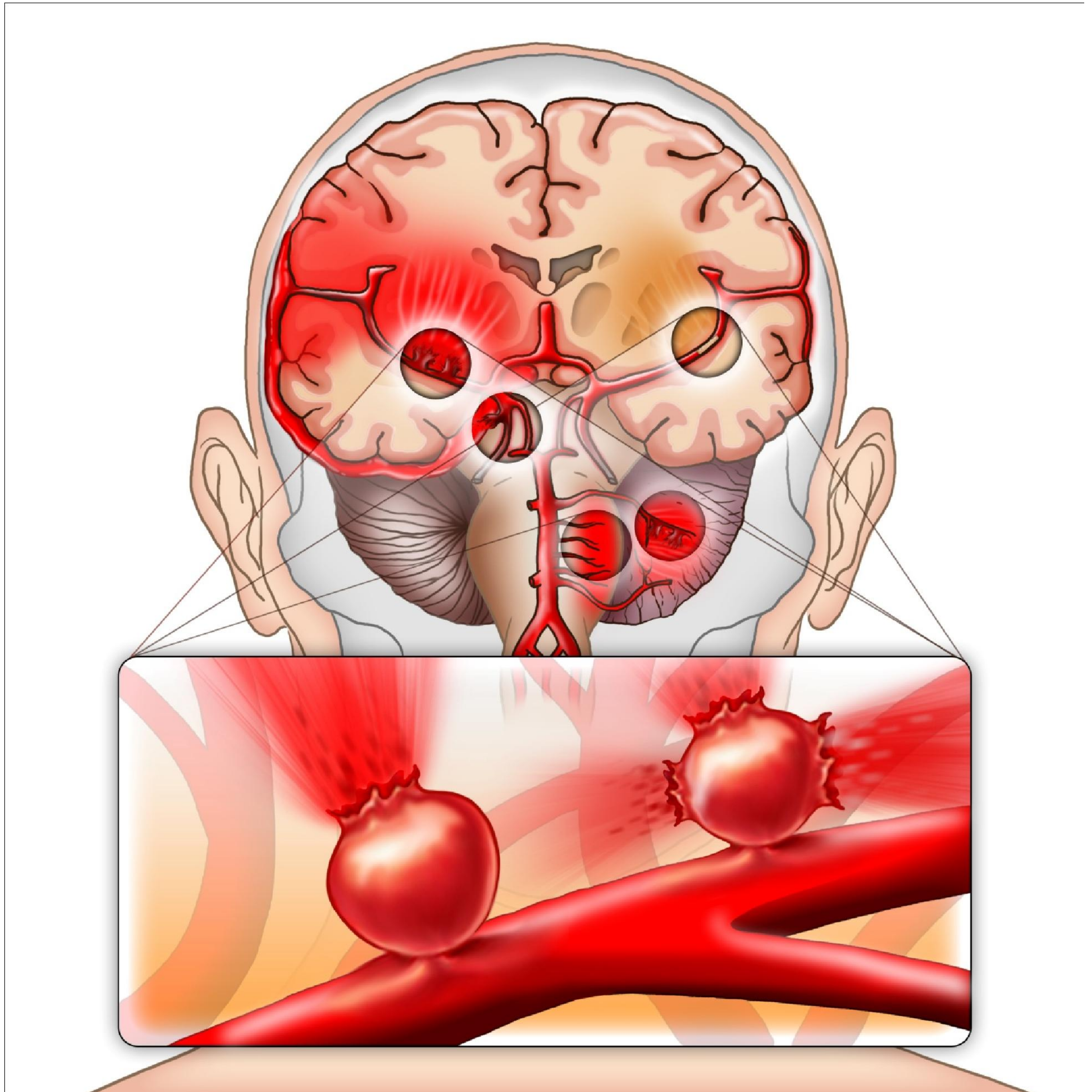

**Figure 1-4 Most Common Sites and Sources of intracerebral haemorrhage**

In most cases intracerebral haemorrhage results from the rupture of small penetrating arteries, which originate from basilar arteries or middle, anterior, or posterior cerebral arteries. Microscopical studies suggest that most ruptures occur at or near the bifurcation of affected arteries (Nahed et al. 2007;Takebayashi & Kaneko 1983).

Haematoma formation was shown not to be a monophasic process, as in some cases the bleeding continues for up to 24 hours (Fujuu et al. 1998; Sahni & Weinberger 2007). Beside the damage from haematoma to the brain tissues, its expansion also results in midline shift, pressing healthy brain tissues against the skull and causing ischemia, thus contributing to neurological deterioration (Zazulia et al. 1999) and brain atrophy (Hutchins et al. 2001).

The distribution of blood into the surrounding brain tissue has a space-occupying effect, moving tissues apart and filling the space in between, and also distributing into extracellular space, thus disturbing the natural conductive pathways resulting in adverse clinical outcome (impairment of neural functions according to the region involved), and pressing healthy tissues against the skull resulting in atrophy like effects and ischaemic degeneration (Hutchins et al. 2001;Liebeskind 2003). The impact of blood leakage is determined by many factors, such as concomitant diseases, location and distribution of the haematoma, state of the body (stressed, distressed) (Badjatia & Rosand 2005; Frizzell 2005).

The post-haemorrhage clot undergoes enzyme destruction and evacuation with further replacement with connective tissue through inflammation (Dabbagh, Chambers, & Laurent 1998;de Fouw et al. 1993;De Fouw, Haverkate, & Bertina 1990;Kalashnyk et al. 2005). During clot destruction a variety of products are released (Sidelmann et al.

2000) modulating inflammation, its quality and outcomes, which we will discuss later in the thesis.

### 1.1.7 Inflammation

Intracerebral haemorrhage, a simple injury in its initial nature triggers an inflammatory response (Becker 1998;Butcher 2006;Emsley & Tyrrell 2002;Perera et al. 2006;Wang & Dore 2007b).

Inflammation is a protective process, which has evolved as an organism's reaction to injury and represents adaptive mechanisms, aimed to recover the integrity and functionality of damaged tissues. Inflammation which have developed is mediated by a stress or general adaptation syndrome at local and systemic levels with the aim of eliminating and replacing necrotizing and dead tissues with connective tissues (Guo et al. 2006;Kowianski et al. 2003;Montaner et al. 2001;Wasserman, Yang, & Schlichter 2008).

Pain, redness, swelling, heat, and loss of function are classical markers and represent local symptoms of inflammation. Systemic symptoms of inflammation are represented mainly by fever, leukocytosis, immunological and other humoral and neural reactions that are specified in the broader picture of general adaptation syndrome (Bennett 2001; Tracy 2006).

### 1.1.8 Systemic level of inflammation

Inflammation is mediated systemically through mechanisms of stress or adaptation syndrome. The definition of general adaptation syndrome (GAS) was proposed by Canadian scientist Dr. Selye in 1936, when it was described as a complex of general defensive mechanisms in the body of living creatures as a reaction to the

impact from strong and prolonged internal and external stimuli (Selye 1937;Selye 1950a;Selye & Fortier 1950). These reactions are to encourage restoration of the disturbed balance and aim to maintain homeostasis. Factors that induce GAS are called stressors, and the condition of the body – stress.

The main signs of GAS are enlargement of the adrenal cortex and amplification of its secretory activity; reduction in spleen size, lymphatic nodes and changes in blood composition (leukocytosis, lymphopenia, eosinopenia); and metabolic disorders with an increase in break down processes. Adaptation to unusual conditions is presented by humoral (that come with blood) stimuli (adrenaline, histamine, serotonin, metabolic products of the tissue breakdown), which lead to the activation of adaptive mechanisms, first of all to the activation of reticular formations and the hypothalamus-hypophysis-adrenal gland axis (Selye 1950; Selye & Horava 1953).

It was reported that catecholamine levels in patients with haemorrhagic stroke increase and that the peak level is reached on day 3-6 with a gradual decrease thereafter (Leow et al. 2007). Glucocorticosteroids, a family of stress hormones, play a role in microcirculation by causing inhibition of vasodilatation and preventing vascular permeability, thus playing an anti-inflammatory role in the recovery process (Peretti & Ahluwalia 2000). Derex et al. showed that primary adrenocortical insufficiency led to the development of the intracerebral haemorrhage and that cortisol has a role in the haemorrhagic stroke recovery process (Derex et al. 1998). Experimentally it was established that cholinergic pathway possesses anti-inflammatory properties and limits damage to the brain itself under/during conditions of haemorrhagic stroke (Lee et al. 2010).

Another systemic hormone, erythropoietin, also participates in haemorrhagic stroke recovery, as it was found that haemorrhaging in erythropoietin treated groups was reduced by 25% in comparison to control groups (Academy of Neurology Annual Meeting in Miami, Florida, USA: 9-16 April, 2005*)*. Erythropoetin reduces oedema and the number of inflammatory cells around haematoma (Lee et al. 2006) and reduces cognitive and motor deficits (Cotena, Piazza, & Tufano 2008).

Free radicals were shown to be a marker of stress processes as they have been found to be highly increased in groups of haemorrhagic stroke patients with lethal outcome (Leow et al. 2007).

High blood glucose levels on admission to hospital is also recognized as a stress-related-response (Fogelholm et al. 2005). Many studies report white blood cell count (WBC), C-reactive protein (CRP) levels and erythrocyte sedimentation rate (ESR) to be increased within 24 hours post-ictus in peripheral blood (Christensen et al. 2002;Christensen & Boysen 2004a; Pedersen et al. 2004;Perera et al. 2006;Suzuki et al. 1995;Zaremba & Losy 2001a). The WBC count within 24 hours of stroke occurrence was shown to be mainly represented by a slightly reduced neutrophil reaction and an increased macrophage infiltration using MRI with contrast enhancing media (Saleh et al. 2004).

Hyperthermia in patients is usually attributed to cytokine-related increase of the hypothalamic temperature set point. There have been a few studies which demonstrated that damaging the hypothalamic region causes hyperthermia, thus suggesting that during haemorrhagic stroke the haematoma itself may influence temperature regulation by compressing the hypothalamus and that hyperthermia may

contribute to secondary brain injury (Deogaonkar et al. 2005). However, a study performed by MacLellan et al. reported that mild and moderate hyperthermia does not worsen outcome after haemorrhagic stroke and is evident even in cases with small intracerebral haemorrhage, suggesting absence of hypothalamic compression (MacLellan & Colbourne 2005). Hypothermia, meanwhile has been shown to be of no benefit in the treatment of haemorrhagic stroke (Fingas et al. 2009).

Since haemorrhagic stroke resolution is realized through mechanisms of inflammation, any increase in body temperature is likely to be due to fever (Fang et al. 2008;Rabinstein & Sandhu 2007).

Schwarz et al. reported fever within 72 hours of 91% of haemorrhagic stroke patient cases. Deogaonkar et al. reported fever in 56% of haemorrhagic stroke patient's within 24 hours. From all the evidence available, it is probably the case that fever development almost invariably accompanies haemorrhagic stroke (Deogaonkar et al. 2005;Schwarz et al. 2000).

Fever that develops in patients with haemorrhagic stroke is represented by leukocytosis (Suzuki et al. 1995), the activity and release of the pyrogens, substances produced by granulocytes which induce rapid and brief fevers (Elfenbein et al. 1979), and mononuclear phagocytes which usually induce prolonged fevers (Atkins & Bodel 1971;Bodel 1976). Pyrogens have also been shown to play a role in central hyperthermia, as Blatteis et al. demonstrated hypothalamic sensitivity to leukocyte pyrogens (Blatteis & Smith 1979).

Fingas et al. found no difference in outcome and functional recovery in rats with induced selective regional hypothermia (Fingas, Clark, & Colbourne 2007), indirectly suggesting that an increase in temperature during ICH is one of the mechanisms of inflammatory response. Hypothermia was also shown to affect the immune system response reducing the number of peripheral lymphocytes and NK cell activity (Saito et al. 2001).

It is obvious that the systemic level of inflammation is very important in the course and outcome of haemorrhagic stroke (Kleinig & Vink 2009;Skinner et al. 2006). However there is still no data on what changes in the systemic level of inflammation provide the best or optimal outcome.

### 1.1.9 Local level of inflammation

Local inflammation develops as a reaction to rapid haematoma formation and blood leakage into the brain tissues and is implemented through a buffer perihaematoma zone, which undergoes certain changes due to its demands.

Early research in understanding the perihaematoma suggested it was the same as the ischaemic penumbra in conditions of ischaemic stroke, (Herweh et al. 2007;Hutchins et al. 2001;MacKenzie 1996), however numerous later studies showed this to be wrong, as there was an absence of ischaemic processes in the perihaematoma zone which indicated hypoperfusion only (Hua et al. 2002;Mayer et al. 1998;Tanaka et al. 1997).

The size of the perihaematoma zone has also been a topic of research and its importance remains controversial to this day (MacKenzie 1996), as sometimes the

perihaematoma found is even bigger than the haematoma itself. From the perspective of the inflammatory response, it is important for the perihaematoma zone to be of just the right size, as has been shown in an example of myocardial infarction the size of the peri-infarction zone plays an important role in outcome (Figueras et al. 1984;Frangogiannis 2006;Malaia, Iabluchanskii, & Sokrut 1990).

Communicative properties of the perihaematoma zone are mainly represented by vascular changes in the region.

**Vascular changes**

Local inflammation includes vascular changes such as vasodilatation, increased permeability of vessels (widened intracellular junctions and contraction of endothelial cells) due to the release of several substances such as histamine, vascular endothelial growth factor (VEGF), bradykinin, nitric oxide and other bioactive molecules (Hohenstein et al. 2005;Hutchins et al. 2001;Lee et al. 2007;Lum & Malik 1996;Tritto & Ambrosio 1999). Most of the vascular changes, as part of the inflammatory response take place in the perihaematoma zone and appear to facilitate the entrance of responsible competent cells. For example, leukocyte-endothelial cell adhesion can be elicited by a number of agents such as superoxide, lactoferrin, histamine, Il-1, hydrogen peroxide, and others (Alabadi et al. 1994;Arndt, Smith, & Granger 1993;Gallia & Tamargo 2006;Juranek & Bezek 2005;Miller, Drummond, & Sobey 2006;Wagner et al. 2003), all produced in the perihaematoma zone.

Nitric oxide, a biologically active gas which is synthesized by a variety of cells, including vascular endothelium, has also been reported as a mediator of vascular

events such as vasodilatation (Dizdarevic 2008;Pluta 2005;Vatter et al. 2007), platelet aggregation and platelet-leukocyte adhesion (Granger & Kubes 1994).

Carbon monoxide (CO) released from the degradation of haem via the haem-oxygenase (HO) pathway was also shown to have vasodilatory properties (Fiumana et al. 2003;Kanu, Whitfield, & Leffler 2006;Ryter, Alam, & Choi 2006), contributing to cell adhesion and migration from the main blood stream into the region of injury (Winestone, Bonner, & Leffler 2003). It was shown that an increase in concentrations of CO in the brain tissue surrounding haematoma was commonly observed in patients (Winestone, Bonner, & Leffler 2003).

**Oedema**

Oedema develops straight after the incidence of the intracerebral haemorrhage (He et al. 2008;Huang et al. 2002;Matsushita et al. 2000;Steiner, Juttler, & Ringleb 2007;Zhang, Li, et al. 2006). In animal models of haemorrhagic stroke, oedema was shown to peak around day 3-4 and to decrease slowly afterwards. In humans, following haemorrhagic stroke, oedema peaks on day 3 and decreases by day 10-20 post-stroke. Whether perihaematomal oedema contributes to the haemorrhagic stroke damage or not still remains unclear. As was shown in several reports (Ropper & King 1984;Zazulia et al. 1999), oedema formation is associated with poor outcome in patients. However, Gebel et al. showed that the presence of oedema in the first few hours after haemorrhagic stroke results in a good clinical outcome (Gebel et al. 2002).

Oedema formation occurs in several phases. The first few hours after intracerebral haemorrhage are characterised by increased hydrostatic pressure and blood clotting with movement of serum from clot into the surrounding tissues (Wagner et

al. 1996). The coagulation cascade and thrombin production are the next to be activated. Besides having a vasogenic nature, oedema also develops as a result of toxicity of certain blood degrading components and cell metabolites, such as thrombin, haem and TNF-α (Bao et al. 2008;Ferro 2006c;Hua et al. 2006;Xi, Keep, & Hoff 2002;Zhang et al. 2006).

Hypoperfusion in the perihaematoma zone may contribute to leukocyte rolling, adhesion and extravasation and it has been suggested that a slow blood flow rate may contribute to neutrophil recruitment (Tritto & Ambrosio 1999), with further necrotized tissues evacuation and replacement with a neuroglial scar. Later studies suggest that oedema is represented mainly by a predominantly cellular component (Hallenbeck et al. 1986;Marmarou 2007), which is essential in inflammatory recovery of post-haemorrhagic brain damage. The quality and composition of the scar tissue is also determined by various factors, including microcirculation and oedema.

The importance of brain oedema during intracerebral haemorrhage was also indirectly shown by Bereczki et al. in their study when mannitol, an osmotic agent and a free radical scavenger, did not lead to improvement or worsening of outcome (Bereczki et al. 2008).

It is obvious that peri-infarction zone formation is determined by the stroke severity, by local and systemic regulatory processes and is reflected in changes in the microcirculation. However the correlation of these events has not been studied as yet.

### Chemotaxis, cell kinetics and dynamics

Blood vessel wall rupture, blood leakage, haematoma formation, and brain tissue soaking with blood, lead to the process called alteration, which is the first stage of the haemorrhagic stroke and the very beginning of the generation of stress signals. These signals are represented by sympathetic activation and increased functional activity of the hypothalamo-pituitary and adrenal system and consequent change of functions of all target organs (Goldstein & Kopin 2007;Harbuz & Lightman 1992;Jezova et al. 2002;Reznikov & Nosenko 2000). Entry of affected metabolic products from the haemorrhagic stroke zone to the circulation system leads to further hormonal activation, migration of leukocytes from the bone marrow to the systemic blood and their further activation and attraction through chemotaxis (Kavelaars et al. 2003;Luster 1998;Renwick et al. 2004). Since the bone marrow deposits mainly contain neutrophils, leukocytosis appears as a shift in cell count; where neutrophils are activated and migrate to the haemorrhagic stroke zone by positive chemotaxis (Furze & Rankin 2008).

Neutrophil infiltration can be observed within 6 hours after ictus and increases gradually at 6-12 hours, peaking at 12-72 hours (Jaremko et al. 2010;Perera, Ma, Arakawa, Howells, Markus, Rowe, & Donnan 2006;Xue & Del Bigio 2000) with a slight decrease at day 7 (Gong, Hoff, & Keep 2000). In a study of early cellular events in the perihaemorrhage zone in 33 fatal cases of spontaneous haemorrhagic stroke, MacKenzie et al. found leukocyte infiltration to be present as early as 5 to 8 h and absent by 72 h (MacKenzie & Clayton 1999;Zhao et al. 2007). Granulocytes were shown to play a role not only in destruction of necrotized tissues, but also in stimulation of subsequent recovery processes in post-haemorrhagic conditions (Park et al. 2005).

Alteration in the haemorrhagic stroke zone triggers further processes, such as structural and immune blood cell migration and proliferation, which are activated by stress.

Immune system cells together with cells involved locally in the area (neurons, astrocytes, damaged cells) release a variety of bioactive substance which play a positive role in chemotaxis, attracting more specific and area-appropriate cells from depots, with the aim of destroying and eliminating the damaged area by phagocytosis (lyzosomal enzymes, free radicals, oxidative burst) (Chuaqui & Tapia 1993;Mena, Cadavid, & Rushing 2004;Perera et al. 2006), and at the same time play the role of chemorepellents, thus regulating the inflammatory response to integrate destructive, eliminative and healing processes.

Activated microglia/macrophages are present in the perihaematoma zone 1-4 hours after haemorrhagic stroke incidence (Gong, Hoff, & Keep 2000;Hickenbottom et al. 1999;Wu et al. 2008;Xue & Del Bigio 2000), reaching their peak on day 7 (Wang et al. 2003;Wang & Tsirka 2005).

Reactive gliosis as a part of the healing process can already be observed at 24-72 hours post-ictus (Guo et al. 2006;Wasserman, Yang, & Schlichter 2008), reaching its peak on day 14-21, leaving numerous resting astrocytes (Kowianski et al. 2003). It was shown that mesynchemal stem cells, that are present in a bone marrow, can differentiate not only into mesodermal, endodermal, ectodermal cells, but also into the neuronal and glial lineages (Andres et al. 2008). Activated neural stem cells were observed on day 2 around a haematoma, increasing at day 4-7, reaching a peak during day 14 followed by a slow decrease (Sgubin et al. 2007;Tang et al. 2004). Observations

on stem cell activation around the haematoma and their migration into the haematoma region suggests the importance of the perihaematoma zone, as stem cells may be activated by local humoral factors (Yokota et al. 2005).

The fact that neural stem cells can migrate into the post-haemorrhagic zone only at a defined time after the disposal of damaged tissues was indirectly shown in the study by Lee et al. when their attempts to restore brain architecture with neural stem cells 2 and 24 hours post-ictus failed (Lee et al. 2008;Masuda et al. 2007;Sun et al. 2008c).

These processes play an important role in both freeing the zone from necrotized tissues and forming in its place primary connective tissue scar. The crucial role in these processes belong to the cooperation and correlation in changes in cellular populations during the different stages of the inflammatory process, kinetics and dynamics of which still demand more research for precise definition.

**Importance of local humoral factors**

*NMDA and Calcium*

At the molecular level, the perihaematoma zone around haematoma is greatly influenced by the levels of glutamate and aspartate, which in the normal state are stored in synaptic terminals, but during overreaction are rapidly ejected into the extracellular space (F.X.Sureda 2008;Germano et al. 2007;Sharp et al. 2008). This process is called excitotoxicity and leads to the opening of calcium channels associated with N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxanole propionate (AMPA) receptors (Sid Shah 2008). Intracellular calcium is responsible for activation of several destructive enzymes such as proteases, lipases, and endonucleases that allow release

of cytokines and other mediators of inflammation (Becker 1998;DeGraba 1998;Hademenos & Massoud 1997).

*Cytokines*

Cytokines are bioactive molecules secreted in regions of stress by activated cells, which play a role in regulation of the immune response at all stages of its progression (Perera et al. 2006;Turrin & Plata-Salaman 2000). Cytokines effect a variety of processes such as proliferation and differentiation of cells, chemotaxis, antigen expression of different markers, activation of immunoglobulin secretion and macrophage cytotoxicity induction (Sid Shah 2008;Turrin & Plata-Salaman 2000). These molecules are usually classified as pro- and anti-inflammatory cytokines.

It has been shown that cytokines are produced by many cells in the brain, such as microglia, astrocytes, neurons and endothelial cells (Kimura et al. 2003;Pantoni, Sarti, & Inzitari 1998;Vila et al. 1999;Wasserman, Zhu, & Schlichter 2007). However, the principal source of cytokines in the brain is activated microglia/macrophages (Emsley & Tyrrell 2002). However, there is also some evidence of involvement of peripherally derived cytokines (Takizawa et al. 2001). After haemorrhagic stroke ictus the permeability of the blood-brain-barrier increases (Lampl et al. 2005) thus leading to migration of mononuclear phagocytes, T-lymphocytes, natural killer cells, and polymorphonuclear neutrophilic leukocytes, which produce and secrete cytokines (Barone & Feuerstein 1999).

It has also been shown that pro- and anti-inflammatory cytokines can induce and potentiate other cytokines and activate positive and negative feedback (Wang & Dore

2007). It should be considered that many cytokines, e.g. TNF-α, play a double role in inflammation, acting as both a pro- and an anti-inflammatory cytokine.

In studying the pathogenetic mechanisms of haemorrhagic stroke the most important cytokines seem to be TNF-α, IL-1b, IL-6 and IL-8 (Perera et al. 2006). It was shown that there was an elevation of IL-1b and TNF-α levels at 3-24 hours post ictus in a double-injection autologous blood rat model of haemorrhagic stroke (Aronowski & Hall 2005). In the collagenase haemorrhagic stroke model an increase in TNF- α levels at 4-8 hours post ictus was observed (Mayne et al. 2001b).  Studies done by Maine et al. reported that intrastriatal infusion of TNF-α-specific antisense oligodeoxynucleotide or adenosine A2A receptor agonists in rats reduced TNF-α mRNA and protein production in brain tissue surrounding a collagenase-induced haematoma (Mayne et al. 2001a;Mayne et al. 2001c). The results showed the reduction in perihaematoma cell death and improvement in neurobehavioral scoring. However the dual role of TNF-α should be taken into account, as TNF-α can potentially repair or damage injured brain tissue (Hallenbeck 2002).

To date, only a few studies have been conducted in patients evaluating cytokine levels after haemorrhagic stroke. In one study of 29 patients IL-6 levels have been reported to be increased significantly at day 1 with a gradual decrease thereafter (Kim et al. 1996). Another study of 124 patients with haemorrhagic stroke showed that elevated plasma concentration levels of TNF-α and IL-6 (Castillo et al. 2002) correlated with the magnitude of subsequent perihaematoma brain oedema.

Studies dedicated to the correlation of cytokines levels to local inflammatory response during haemorrhagic stroke have not yet been conducted (Kadhim, Duchateau, & Sebire 2008).

*Metalloproteases*

Metalloproteases have complex properties in the brain under normal and pathological conditions. Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes involved in the reorganization of the extracellular matrix (Abilleira et al. 2003).

Most of the published data show that extracellular proteases are involved in cell death in many neurological diseases (Nakanishi 2003;Yong et al. 2001). The activity of MMPs can be controlled by many factors among which are free radicals, either acting through latent forms or by mRNA induction through the factor-kappaB site pathway (Kolev et al. 2003;Power et al. 2003;Yong et al. 2001). MMPs increase permeability of capillaries, contributing to brain oedema (Skinner et al. 2006;Wang & Dore 2007b) which probably facilitates entrance of cells into the region.

MMPs are known to be essential for neurogenesis, myelin formation, and axonal growth (Cunningham, Wetzel, & Rosenberg 2005;Kaczmarek, Lapinska-Dzwonek, & Szymczak 2002;Yong 2005). Potential therapies based on MMP inhibition remain controversial. The latest studies suggest that inhibition of MMPs facilitates cell death *in vivo* (Grossetete & Rosenberg 2008).

*Reactive oxygen species (ROS)*

In the injured brain, ROS are released by a variety of cells, including neutrophils, endothelium and activated microglia/macrophages (Facchinetti, Dawson, & Dawson 1998;Wang & Dore 2007b).

Being an important part of oxidative metabolism, high concentrations of ROS can prove lethal (Juranek & Bezek 2005). Reactive oxygen species were shown to contribute to ischaemic brain injury (Crack and Taylor, 2005; Saito et al, 2005) and might also contribute to the outcome of haemorrhagic stroke (Han et al. 2008;Lagowska-Lenard et al. 2008).

As a result of haemorrhage, the extracellular spaces of the brain become exposed to hemoglobin and its breakdown products. Iron and iron-related compounds, including hemoglobin, catalyze hydroxyl radical production and lipid peroxidation (Sadrzadeh et al. 1987;Sadrzadeh & Eaton 1988), in turn, exposing brain cells to increased levels of oxidative stress. Indeed, high levels of oxidative stress, as measured by protein carbonyl formation, have been found within minutes after the onset of autologous blood injection in pig (Hall et al, 2000; Wagner et al, 2002). In addition, intracerebral leakage of lysed erythrocytes into the rat striatum induced marked brain oedema and profound neurological deficits (Wu et al, 2003).

In this setting, increased oxidative stress, measured by protein carbonyl formation, might be associated with reduced Mn-superoxide dismutase and CuZn-superoxide dismutase levels and increased DNA damage.

ROS may also serve as activators for neutrophil chemotaxis (Petrone et al. 1980) suggesting another role of inflammatory response modulation. There is also evidence that ROS may serve in physiological vasodilator mechanisms in cerebral microcirculation (Miller, Drummond, & Sobey 2006). Other studies report failures in using antioxidant therapy, despite the known deleterious role of ROS in homeostasis, suggesting that their overproduction possibly affects recovery processes (Juranek & Bezek 2005). The importance of ROS production for intracerebral haemorrhage was reported by Liu et al. who showed the development of adaptive compensatory mechanisms for free radical production in knockout mice (Liu et al. 2007).

*Thrombin*

Thrombin is a serine protease, one of the main components in the blood coagulation cascade process and is rapidly produced after the occurrence of haemorrhagic stroke (Xi, Reiser, & Keep 2003;Xi, Keep, & Hoff 2006).

High concentrations of thrombin cause inflammation, contributing to brain oedema development and neuronal death (Gingrich & Traynelis 2000;Gong et al. 2001;Xi, Reiser, & Keep 2003). Thrombin affects an opening of the blood brain barrier (Lee et al. 1997;Sansing et al. 2003). Thrombin-induced brain injury was suggested to be mediated by the complement cascade. Injected thrombin caused a 7-fold increase in C9 complement complex and its deposition on neuronal membranes (Gong et al. 2005).

Thrombin was also shown to be one of the stimuli that affect the phosphorylation state of glutamate receptors (Ardizzone et al. 2004), which in turn leads to opening of

the calcium channels of migrated cells and activation of destructive enzymes and further release of cytokines and other mediators of inflammation. Thus thrombin contributed to that stage of the inflammatory response aimed at removing the damaged region.

There are however some beneficial aspects of thrombin activity including haemostasis and the fact that it modulates haematoma enlargement in a certain percentage of the patients during the first day following a haemorrhage. Some studies suggest that low concentrations of thrombin induce protective neuronal effects (Hua et al. 2007). Affecting an opening of the blood brain barrier, thrombin facilitates the crossing of competent cells into the intracerebral haemorrhage zone, thus contributing to recovery via inflammation. Intracerebral injection of thrombin causes gliosis and scar formation (Gingrich & Traynelis 2000;Gong et al. 200;Lee et al. 1995;Yang et al. 2008).

Thrombin is one of many components of the coagulation system of the body that are known to take place in intracerebral haemorrhage. In a study performed by Xi et al. it was shown that formation of a clot results in early oedema formation, suggesting an important role for the whole coagulation system (thrombin particularly) in the inflammatory process and resolution of the problem (Xi et al. 1998). Antovic et al. reported no differences between ischaemic and haemorrhagic stroke in coagulation system activity, suggesting that haemostatic changes are consequences of brain damage rather than primary haemostatic activation only (Antovic et al. 2002).

### 1.1.10 Clinical features and treatment

Intracerebral haemorrhage may be clinically hard to differentiate from ischaemic stroke on a simple physical examination. The physical examination always includes a careful head and neck examination for signs of trauma, infection, and meningeal irritation. An altered level of consciousness or coma is more common with haemorrhagic strokes than with ischaemic strokes. Often, this is due to an increase in intracranial pressure. Meningismus may result from blood in the ventricles.

The clinical signs vary depending on the area of brain affected by haemorrhage and the extent of bleeding. Common symptoms of stroke include abrupt onset of hemiparesis, monoparesis, or quadriparesis; monocular or binocular visual loss; visual field deficits; diplopia; dysarthria; ataxia; vertigo; aphasia; or sudden decrease in the level of consciousness. Haemorrhagic strokes are more likely to elicit symptoms of increased intracranial pressure than other types of stroke: headache, vomiting, nausea. Focal neurologic deficits: The type of deficit depends upon the area of brain involved. If the dominant hemisphere (usually left) is involved, a syndrome consisting of right hemiparesis, right hemisensory loss, left gaze preference, right visual field cut, and aphasia may result. If the nondominant (usually right) hemisphere is involved, a syndrome of left hemiparesis, left hemisensory loss, right gaze preference, and left visual field cut may result. Nondominant hemisphere syndrome also may result in neglect, when the patient has a left-sided hemi-inattention and ignores the left side. If the cerebellum is involved, the patient is at high risk of herniation and brainstem compression. Herniation may cause a rapid decrease in the level of consciousness, apnoea, and death. Other signs of cerebellar or brainstem involvement include the following: gait or limb ataxia, vertigo or tinnitus, nausea and vomiting, hemiparesis or

quadriparesis, hemisensory loss or sensory loss of all 4 limbs, eye movement abnormalities resulting in diplopia or nystagmus, oropharyngeal weakness or dysphagia, crossed signs (ipsilateral face and contralateral body). Many other stroke syndromes are associated with ICH, ranging from mild headache to neurologic devastation. At times, a cerebral haemorrhage may present as a new-onset seizure.

There is no effective medical treatment that can be used in patients with ICH. Patients with suspected subarachnoid haemorrhage should have an urgent neurosurgical consultation for diagnosis and treatment. Patients with cerebellar haemorrhage should have an urgent neurosurgical consultation for consideration of craniotomy and evacuation of the haemorrhage. Patients with supratentorial intracerebral haemorrhage should be cared for on a stroke unit (Canadian Stroke Network and the Heart and Stroke Foundation of Canada: Canadian Stroke Strategy. Canadian Best Practice Recommendations for Stroke Care: 2006. Ottawa, 2006).

Conservative treatment includes: oxygenation, blood pressure management (however it still controversial whether patients should have antipypertensive treatment, as it was also argued that hypertension has a protective mechanism due to preserved cerebral perfusion, especially in patients with increased intracranial pressure (Qureshi et al. 1999); fluid management (the aim is to keep normovolaemia with a central venous pressure between 5 and 12 mmHg and to keep electrolytes in the normal range), seizures (most of the seizures appear within 24 hours after ICH and reflect poor outcome; the recommendations for seizure treatment are anticonvulsants, followed by weaning the patient off of antiepileptic therapy if no seizure is observed after one month); medical therapy (agents that may be beneficial, theoretically, include fresh frozen plasma, factor IX concentrate in human, recombinant factors VIII and IX,

prothrombin concentrate, cryoprecipitate, aminocapronic acid, aprotinin and activated recombinant factor VII (Mayer & Rincon 2006)), intracranial pressure management (the aim of this treatment is to maintain intracranial pressure below 20 mmHg and cerebral perfusion pressure over 70 mmHg (Diringer 1993). However this treatment remains controversial because of lack of evidence. On the other hand there are several studies suggesting the value of mannitol every 4 hrs in patients with large haematoma and high intracranial pressure (Ferro 2006). Corticosteroids should be avoided (Poungvarin et al. 1987), as it has been shown that steroids were of no benefit and can be associated with a higher risk of complication (diabetes, infections) (Ferro 2006).

Surgical treatment remains highly debatable. In the last decade there have been several studies showing both beneficial and non-beneficial effects on early treatment of the intracerebral haemorrhage by clot removal. However recent research shows that there is no beneficial effect from early surgery compared to initial conservative treatment (Tak Fai Cheung & Raymond 2007).

As seen from the treatments available at the moment much recent data has not been taken into account including the role and mechanisms of inflammation, the process of haemorrhage resolution and participation of different cell types at the different stages of the diseases.

### 1.1.11 Haemorrhagic stroke in research

**In vitro and ex vivo models of haemorrhagic stroke**

Prior modelling of neurological disorders *in vivo* and introduction of prospective medications into human patients, generally requires that the research start with simpler models, such as *in vitro* and *ex vivo* ones.

Studies in modelling haemorrhagic stroke include *in vitro* experiments on cell culture lines, and these have been tried and tested on a variety of brain cell lines such as neurons and glial cells (Chen-Roetling, Li, & Regan 2008;Hasegawa et al. 2011;Koeppen, Dickson, & McEvoy 1995;Loftspring, Hansen, & Clark 2010;Smith et al. 2004).

Having established a positive effect using *in vitro* models the next step is to experiment on higher organized structures such as solid tissue cultures. Among the rat brain slice cultures used in experimental models of different brain disorders, the most frequently used are hippocampal, striatal and ventral mesencephalon (containing forebrain and striatum) slices (Anwar et al. 2008).

**Rats as an experimental model**

Rodents, rats in particular, are the laboratory animals that have been studied in the greatest detail, providing numerous behavioural studies, which have shown the similarity in human and rat motor components which are responsible for upper extremity movements in humans and forelimbs in rats during reaching behaviour (Kleim, Boychuk, & Adkins 2007). Thus neostriatum as a site for modelling of haemorrhagic stroke was a logical choise (Canales & Graybiel 2000;Phillips et al. 1993;West et al. 1990). Economy and small size are important factors, along with the fact that these animals have a relatively smooth cerebral cortical mantle, as opposed to the highly convoluted mantle found in many larger species.

There are however, a few major disadvantages to using a rat model. First, the organization of the rat brain is obviously not identical to that of the human brain.

Therefore, the clinical relevance of neuroanatomical information obtained in the rat in principle should be confirmed in humans, which often may not be possible for ethical reasons; and conversely, certain important problems like the neurobiology of language may be difficult if not impossible to study in the rat. Secondarily, the types of genetic analyses that can be carried out in mice will not be possible anytime soon in rats. On the other hand, the mouse brain is often too small for critical analysis with available experimental neuroanatomical techniques (Kleim, Boychuk, & Adkins 2007).

On the other hand, the principle structural organization, functional, behavioural (survival, search for solutions), as well as compensatory mechanisms of lost functions, repair mechanisms (which are realized through inflammation) as well as fundamental biochemical, immune, functional and other processes are common for all the mammalian world, and thus the solutions implemented and found in experimental animals are also applicable in humans.

**Haemorrhagic stroke models**

Under normal conditions blood does not have direct access to neurons in the brain. The blood-brain barrier is formed mainly by astrocytes, endothelial cells and extracellular matrix. When an artery in the brain ruptures, this causes blood leakage into the brain tissues thus causing a non-physiological condition with a cascade of pathological outcomes. This condition is called haemorrhagic stroke.

The volume of blood leaked out, to form haematoma, directly correlates with the clinical signs and outcome. The blood that has leaked out from blood vessels in the brain presses the surrounding tissues against the skull thus forming a zone of ischaemic and haemorrhagic injury.

An ideal experimental haemorrhagic stroke model should have the following characteristics:

- blood deposition in a distribution consistent with the type of haemorrhage desired,
- uniform degree of haemorrhage,
- a mechanism of haemorrhage which closely simulates the human conditions,
- easily performed,
- reasonable cost.

The most common experimental intracerebral haemorrhage stroke models are:

- the blood injection model,
- the bacterial collagenase model.

**Blood injection model**

The model that uses an injection of autologous blood into the brain was firstly proposed by Bullock et al. The blood is collected from the rat's tail vein and stereotaxically injected into the region of interest. The site for injection is based on the common human ICH blood distribution. The basal ganglion is still considered to be the favourite site for blood injection. The volume of the blood injected may vary depending on the how big the haemorrhage is required, but many researchers agree that the volume of the blood should not exceed 100 µl (He et al. 2008;MacLellan, Gyawali, & Colbourne 2006;Strbian, Durukan, & Tatlisumak 2008).

One of the major disadvantages in the use of the blood injection model is that the haematoma size is not easily reproducible in a series of consecutive experiments (Cossu et al. 1991;Kleim, Boychuk, & Adkins 2007;Masuda et al. 1988). The reasons for this lack of reproducibility of haematoma vary but most commonly they are: rupture of ventricle, backflow of the blood infused along the needle track and excess blood being injected.

To minimize possible blood injection into the brain, a double-injection model was introduced (Deinsberger et al. 1996), where the desired volume of blood is injected in two portions at a slow rate over a certain time period with a 7 minute break between the two injections. The pause allows blood to clot along the needle thus preventing backflow, resulting in no leakage into the subarachnoid space.

However, even when limiting the leak, the distribution of the blood injected remains inconsistent and as some researchers report about 25% of haematomas have extensions into the adjacent white matter (MacLellan, Gyawali, & Colbourne 2006;Xue & Del Bigio 2003). Other researchers observe differing amounts of damage after autologous blood injection and an inconsistent positioning of the haematoma (Hua et al. 2002). Irregular morphology and variable location of the haematoma are also problems encountered with the slow infusion technique (Barth et al. 2007).

Recent papers suggest using the curved tip of a metallic microcatheter to create space for the blood to be injected through the cannula and then turning it four full times to the left and right (Barth et al. 2007). However this model is not perfect either, as the cannula used for injection is of a quite a large diameter (23 G) which leaves a stab wound and itself produces some trauma preceding blood injection.

**Bacterial collagenase model**

Collagen IV is the major protein of basal lamina of blood vessels and plays an important role in its integrity. The main principle of this model is that the collagenase disrupts the protein bonds in collagen and thus blood vessel walls are ruptured. Originally the collagenase model was introduced by Rosenberg (Rosenberg et al. 1990) when 0.1-1 units of bacterial collagenase in 2 µl of saline was injected stereotaxically into the brain, and it was reported that 0.5 units gave expected results, while 1 unit of collagenase limited 24-hr survival of the experimental animals. Since then the model has been modified by different authors and different types of collagenase have been used (Strbian, Durukan, & Tatlisumak 2008;Wasserman, Yang, & Schlichter 2008).

The preferred site for injection of collagenase in this injection model remains the striatum. The only other site that has been used with the collagenase injection model is primary somatosensor cortex, which was used in a swine model of intracerebral haemorrhage (Mun-Bryce et al. 2001).

The main disadvantage of the collagenase injection model is thought to be an exaggerated inflammatory response, which was suggested by (Xue & Del Bigio 2003) and later by the National Institute of Neurological Disorders and Stroke (UK) with the idea that collagenase damages tissue at the site of injection and this acts as an artificial chemoattractant.

The collagenase model is most suitable to assess treatments that affect bleeding and haematoma expansion, such as hemostatic therapies (Kawai et al, 2006) or blood pressure therapies. The collagenase model may also help elucidate the side effects of

treatments, such as elevated blood pressure caused by induced hypothermia (MacLellan et al, 2004). The collagenase model was shown to better represent the physiological conditions of haematoma expansion in clinical patients with ICH, as it was suggested that haematoma expansion continues up to 24 hrs (Brott et al, 1997; Fujii et al, 1994; Kazui et al, 1996) after incident.

Most researchers report that this model shows good reproducible results with haemorrhage volumes that correlate well with the amount of collagenase injected (Ferland, Veilleux-Lemieux, & Vachon 2007;Kleim, Boychuk, & Adkins 2007;Terai et al. 2003).

**Haem-oxygenase pathway**

HO is a rate-limiting enzyme in haem degradation. Its activity has been found to be present in almost all animal species and tissues (Maines, 1988). A schematic diagram of the pathway can be seen in Figure 1-5.

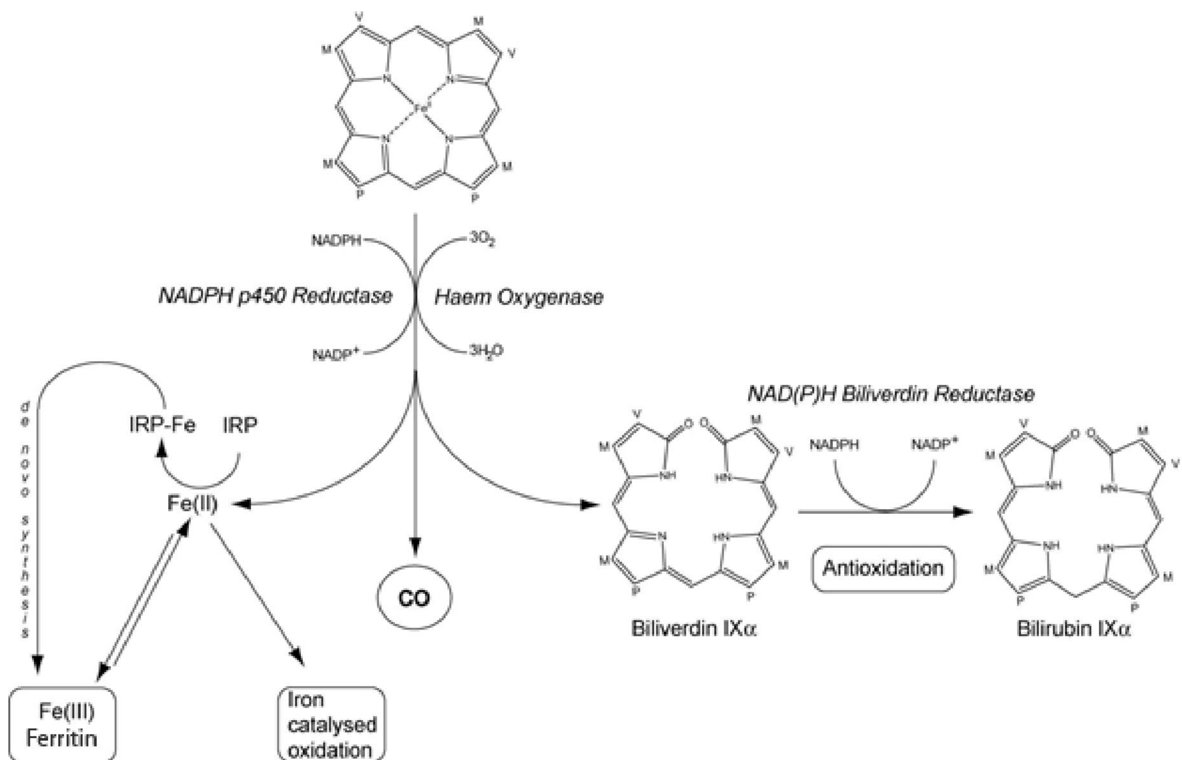


Figure 1-5:- **Schematic representation of the haem oxygenase pathway.**
The diagram shows the major components of the pathway and the various enzymes involved in haem metabolism. Extract from Ryter *et al*, Mol Cell Biochem. 2002 May-Jun;234-235(1-2):249-63.

HO was only identified about 2 decades ago as a distinct microsomal entity that is regulated by metal ions (Maines 1997). It is anchored to the endoplasmic reticulum by a hydrophobic sequence of amino acids at the carboxyl terminus of the protein (Xia et al. 2002). Haem is oxidatively cleaved by HO to biliverdin, carbon monoxide (CO) is then generated as a by-product and ferrous iron is released.

Haem oxygenase has three isoforms, the inducible HO-1 isoform, also known as the heat shock protein, HSP32 (Dwyer et al., 1992; Ewing and Maines, 1991; Maines, 1997), constitutively active HO-2 isoform; and an HO-3 isoform which in comparison with to HO-1 and HO-2 is a poor haem catalyst and has yet to be investigated.

| Properties | HO-1 | HO-2 | HO-3 |
|---|---|---|---|
| **Cellular Localisation** | Microsomes | Mitochondria | ? |
| **Chromosomal Localisation** | 22 q 12 | 16p13.3 | ? |
| **Molecular Weight** | 32 KD | 36 KD | 33 KD |
| **Tissue Distribution** | Liver, kidney, heart, vascular smooth muscle, endothelium, lung and brain | Nervous system, vessels, testes, and intestine | Thymus, spleen, heart, testes, brain |
| **Roles** | Cellular homeostasis, anti inflammatory | Neural signalling and vascular regulation | ? |
| **Regulation** | Haem, oxidative stress, heavy metals, nitric oxide, hypoxia | Glucocorticoids, opiates | ? |
| Modified from (Maines 1997) | | | |

**Table 4 Comparison of all three isoforms of haem oxygenase**

Haem catabolism by HO results in the release of ferrous iron, carbon monoxide (CO) and billiverdin, which is converted into billirubin by billiverdin-reductase. Billirubin (as a result of HO-1 activity) has been found to be present in neurons and astrocytes and may protect them from toxicity (Gennuso et al. 2004;Huang et al. 2005;Nakao et al. 2005). Nanomolar concentrations of bilirubin protect neuronal cells from reactive oxygen species (ROS) activity (Ryter, Alam, & Choi 2006). Bilirubin, released from haem metabolism, has also been suggested as playing a role in vasodilation in the haematoma region after subarachnoid haemorrhage (Suzuki et al. 2003).

The release of ferrous iron, which is toxic to brain tissues in high concentrations, usually goes in parallel with an increase in ferritin levels, the main iron-storage protein in the brain (Wan et al. 2008;Wu et al. 2003).

In one study of haemorrhagic stroke simulation with lysed blood injection, the induction of HO-1 protein was observed in glia surrounding haematoma and immunoreactivity for HO-1 persisted over 4 days (Matz, Weinstein, & Sharp 1997). Later, in vivo experiments suggested that HO-1 activation in the brain during haemorrhagic stroke exacerbates brain injury (Wang & Dore 2007a) in the early stages. However in the later stages, wild type animals showed better improvement compared to HO-1 knock-out animals, suggesting that HO-1 activation might contribute to recovery in the later stages of haemorrhagic stroke. HO-1 was shown to reach its peak on day 3 and then last for a long period and to be mostly from microglial cells (Wu et al. 2003). Activity of HO-1 protein also correlates with activation and migration of polymorphonuclear leukocytes, which is observed at 3-7 days, at the time when the recovery processes begin (Liu et al. 2002;Peake & Suzuki 2004).

CO a diatomic gas that is released from haem catabolism by HO activity, functions as a soluble messenger (Xia et al. 2002). Numerous studies have been conducted studying HO – associated CO properties. The idea of possible CO use in the therapy of haemorrhagic stroke originates from those studies and will be discussed later.

## 1.2 Carbon Monoxide

### Overview

Carbon monoxide, an odourless, colourless, tasteless and corrosive gas, commonly present in our environment (Von Burg 1999), is dubbed the silent killer for its known toxic effects, and has been studied for decades by scientists.

CO does not react with water without significant energy input. Due to its triple bond structure, CO is chemically stable under physiological conditions, it reacts slowly with molecular oxygen ($O_2$), and is involved in redox reactions (Piantadosi 2002). In its free state CO is highly reactive allowing transition metals to effectively promote the reduction of CO. Thus CO easily forms metal carbonyls, which are susceptible to attack on the CO oxygen atoms by electrophiles. Chemical reduction of CO, however, requires temperatures in excess of 100°C which is well above physiological levels. The oxidation or oxygenation of CO to $CO_2$ is also well known in chemistry but again this requires temperatures beyond physiological tolerance for most organisms.

Due to the fact that CO can bind to the iron atoms in haemoglobin, it can significantly reduce the oxygen-carrying capacity of this protein which in turn can lead to tissue hypoxia. At high concentrations (HbCO levels higher than 70%) exposure to CO is lethal.

Apart from exogenous uptake of CO, cells and tissues produce significant amounts of CO as a result of cellular metabolism, from haem degradation in particular via the haem oxygenase pathway (Ryter & Otterbein 2004).

The discovery and thorough investigation of nitric oxide (NO), a gaseous molecule that possesses similar properties to CO, including effects on vascular tone, suggested CO may play a similar role. The very first paradox to consider, bearing in mind that many life scientists consider CO to be a toxic gas, was the omnipresent distribution of the enzyme haem oxygenase in higher living organisms.

**Bioactive properties of CO**

Carbon monoxide has been thoroughly studied over the last few decades. As a biological agent there have been numerous possible functions suggested for this molecule, among which the most pertinent to the current studies are: vascular effects, anti-inflammatory properties, anti-apoptotic and anti-proliferative effects, and neuroprotective effects.

CO released by endothelial cells possesses an important paracrine effect on smooth muscle cells (SMC) and on circulating blood cells (Dulak et al. 2002;Durante & Schafer 1998). CO is capable of maintaining vascular tone in large and small blood vessels and is able to relax vascular smooth muscle (Durante & Schafer 1998;Kaide et al. 2001). Experimentally it was shown that a threshold concentration of 1 μM CO was enough to induce such effects (Maines 1997).

The effects of CO on blood pressure may be realised via the promotion of vasodilation or indirectly via the *nucleus tractus solitarius* in the brain, via alteration of glutamatergic transmission and thus lowering blood pressure (Johnson, Kozma, & Colombari 1999;Maines 1997;Morse & Sethi 2002).

CO release into the vessels may modulate platelet aggregation and adhesion thus affecting the fluidity of the blood (Morita et al. 1995). Furthermore, the induction of HO-1 in endothelial cells indicates the possibility of CO regulating blood pressure *in vivo*, during normal and stressful conditions (Johnson, Kozma, & Colombari 1999;Motterlini et al. 1998a;Motterlini et al. 1998b).

While the local induction of HO-1 and CO production by vascular cells following vascular injury may serve an important protective role in maintaining blood flow and limiting oxidative damage, a more generalised induction of CO synthesis may have deleterious effects. In this respect it was suggested that the widespread induction of HO-1 in the vasculature and the subsequent production of large amounts of CO may contribute to the severe hypotension associated with endotoxin shock (Penney 1988). High levels of CO can directly damage vascular cells by generating reactive oxygen species. CO binds to mitochondrial haem proteins and disrupts mitochondrial electron transport leading to the increased release of superoxide anions, which are produced as a part of normal oxidative phosphorylation. Depending on the amounts generated, CO can exert both beneficial and harmful effects on the vascular system. Pathological excess of CO production results in a range of toxic effects, the earliest detectable difference being an increase in HbCO levels (10-20%), which due to the high affinity of Hb for CO (~240 times greater than oxygen) interferes with oxygen delivery. CO levels of 40-60% generally result in death due to the binding of CO with other haemoproteins e.g., cytochrome oxidase CYP-450, myoglobin.

In the case of vascular damage, where endothelial cells are injured and lost, SMC may be capable of absorbing some of the vasodilatory, anti-proliferative, and anti-thrombotic properties of the endothelium by upregulating CO production via the

inducible HO-1 enzyme (Stanford et al. 2003). Following local injury of the blood vessel wall, circulating monocytes and macrophages infiltrate the site of injury, where they are activated and release various inflammatory cytokines, including Il-1β and TNF-α (Morisaki et al. 2002;Ndisang et al. 2002). These inflammatory mediators may further stimulate the production of cytokines by the blood vessel wall leading to locally increased levels of cytokines and HO-1 induction. The effects of CO on inhibition of platelets and neutrophil migration are likely stimulated by an increasing concentration of cGMP. These characteristics have been suggested as a mechanism by which CO is capable of promoting the survival of transplanted organs as the expression of HO-1 has been shown to protect against chronic rejection (Luckraz et al. 2001;Nakao et al. 2003).

HO-1 was shown to suppress endothelial cell apoptosis via activation of the p38 mitogen activated protein kinase (MAPK) signal transduction pathway (Brouard et al. 2000;Soares et al. 2002). At the site of vascular injury, vascular SMC may be directly affected by arterial levels of fluid shear stress, thus potentially activating HO-1. Based on these findings, CO is likely to be the principle mediator of flow-dependant changes in the vascular tone at sites of blood vessel damage (Wagner et al. 1997). HO-1 induction and CO release by SMC cells at the site of injury may affect vascular homeostasis by preventing blood vessel spasm and SMC proliferation (Otterbein et al. 2003). Moreover, Durante et al demonstrated that SMC can inhibit platelet aggregation via HO-1 catalysed release of CO thus suggesting that CO plays a certain role in thrombus formation in vivo (Durante & Schafer 1998).

CO was shown to suppress cell proliferation in a number of cell types including cancer cells, T-cells and SMCs (Ryter & Otterbein 2004). However, CO was shown to induce proliferation in endothelial cells (Jozkowicz et al. 2003).

CO was suggested to act as a neurotransmitter, intracellular messenger, and modulator of neuroendocrine function (Baranano & Snyder 2001;Dulak & Jozkowicz 2003;Marks et al. 1991;Prabhakar 1998). The discovery of HO-2 throughout the central nervous system (CNS) and peripheral nervous system (PNS) has drawn attention to the possibility that CO acts as an important neural messenger (Morse, Sethi, & Choi 2002).

Several studies investigating certain neurodegenerative disorders, for example Alzheimer's and Parkinson's disease sufferers (AD and PD respectively), demonstrated an increased expression of the HO-1 protein in regions with senile plaques and neurofibrillary tangles, while amyloid precursor protein, which is postulated to be a major cause in the neurotoxicity of AD, leads to inhibition of HO (Baranano & Snyder 2001;Frankel, Mehindate, & Schipper 2000). The exact role and mechanism of HO and HO-associated CO in these and other neurodegenerative disorders is currently unclear.

Haem oxygenase was shown to protect neurons from oxidative stress in cell cultures exposed to hemin (Regan, Chen, & venisti-Zarom 2004). Another study, by Wang et al. demonstrated haem oxygenase as possessing a neuroprotective effect against intracerebral haemorrhage, using an experimental model in HO-2 knock-out mice (Wang, Zhuang, & Dore 2006).

All these studies suggest CO involvement in many processes occurring in the living organism under physiological or both pathological conditions. However the least studied area remains brain damage, in particular intracerebral haemorrhage. Studying CO effects on the brain tissues in haemorrhagic stroke could be facilitated by the use of

carbon monoxide releasing molecules (CO-RMs). The most promising and well studied of these is CORM-3.

**The Development of Carbon Monoxide-Releasing Molecules**

The importance of CO in the biological environment is strongly emerging. Consistent findings have revealed a series of important cellular functions that support a versatile and previously unidentified role for CO gas. It is interesting that many of the novel properties pertaining to CO have strong analogies with the well-established biological activities elicited by nitric oxide (NO). Research in the field of NO has been largely facilitated by the development of a variety of organic compounds that spontaneously release NO and can reproduce a physiological or pathophysiological function of NO. However few attempts had been made to identify or develop similar compounds capable of delivering CO.

The idea that CO could be delivered into the body to target physiological or pathophysiological problems such as the highly successful NO donors is an intriguing one. Such a compound could also be used as a research tool to aid in the elucidation of further functions of CO *in vivo* and *in vitro*. Without the existence of this compound researchers have tried using inhalation of CO gas as an internalisation method. Although the results have been promising, this form of delivery is not targeted and lacks any real control of how much CO is actually being delivered to the site where it is required to act. The systemic effect of CO gas on oxygen transport and delivery via erythrocytes is also potentially dangerous *in vivo*.

During the past few years our laboratory has been working on the characterisation of biological activities associated with transition metal carbonyls as

potential CO-RMs (Boehning et al. 2003;Clark et al. 2003;Foresti et al. 2004a;Johnson et al. 2003;Mann & Motterlini 2002;Motterlini et al. 2001;Motterlini et al. 2002;Motterlini et al. 2003;Motterlini, Foresti, & Green 2002). These complexes are compounds that contain a transition metal e.g., iron, manganese and are surrounded by carbonyl (CO) groups as coordinated ligands. They can be used as catalysts in organic synthesis. Already metal carbonyl complexes are finding novel applications in research in the fields of cell biology, immunology, and pharmacology (Mosi et al. 2002) . Their application in cancer therapy, drug receptor interaction, and malaria is also extremely promising. Bio-organometallic chemistry has now become an emerging discipline that may offer innovative solutions to biological problems. Progress in this field has been possible despite the fact that the majority of biologists and pharmacologists generally and incorrectly view transition metals ions as toxic ‘heavy metals’. The chemical development of anticancer drugs as well as immuno-suppressants based on ruthenium provides evidence of how metal-containing compounds can be used for the development of new pharmaceuticals (Motterlini et al. 2003).

From inorganic chemical studies conducted on these substances *in vitro*, it is known that certain ligands in a metal complex can promote, either sterically or electronically, the dissociation of CO. Furthermore, photodissociation and the consequent elimination of the CO group(s) following exposure to light has been reported in the case of specific metal carbonyls (Motterlini et al. 2002). The discovery that carbonyl complexes possess such interesting and promising features *in vitro* prompted our laboratory to examine the ability of some of these compounds to promote a physiological response in biological systems. The molecules were initially tested to see if they released CO using apparatus as illustrated in Figure 1-6 (Motterlini et al. 2002). The release of CO was stimulated either by the exposure of the carbonyl solution to

cold light in the case of iron pentacarbonyl and dimanganese decacarbonyl ([$Mn_2(CO)_{10}$)]), or by direct addition to the myoglobin in the case of tricarbonyldichlororuthenium (II) dimer ([$Ru(CO)_3Cl_2]_2$). Due to the high affinity of myoglobin for CO any gas released would be captured by the myoglobin and the subsequent conversion of the deoxygenated myoglobin to carboxy myoglobin results in a change in the absorbance curve which gives a measure of the CO released (Motterlini et al. 2002).

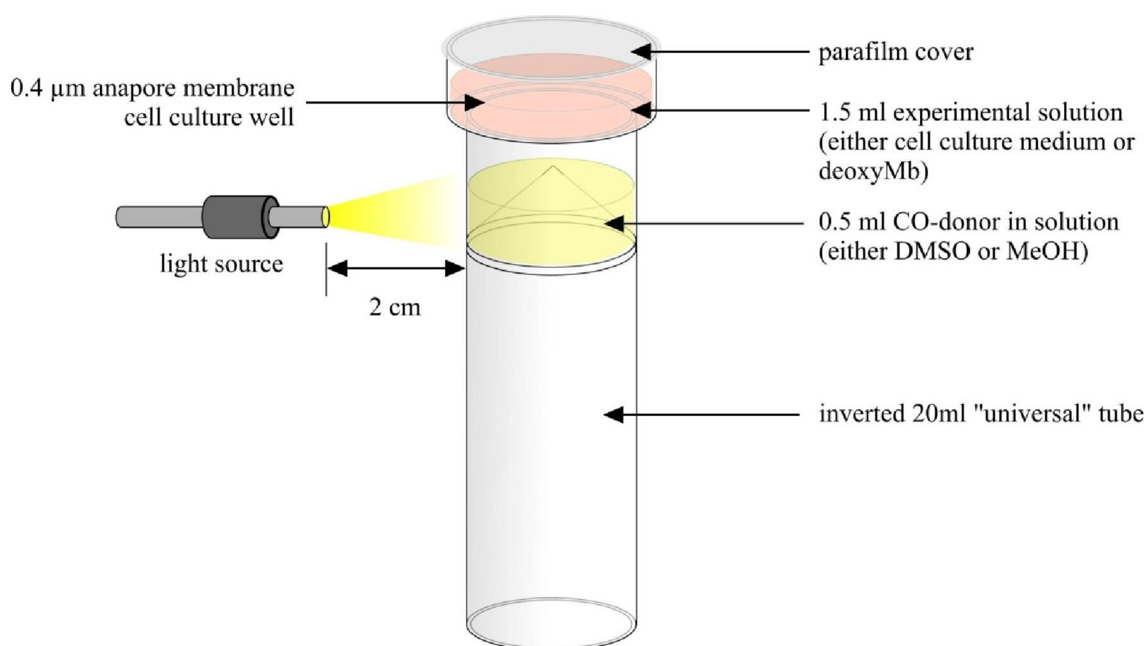


**Figure 1-6 Schematic diagram showing the CO release measurement setup for light stimulated compounds iron pentacarbonyl and dimanganese decacarbonyl.**
CO released by the CO-RM would pass through a 0.4 µM Anapore™ membrane and into the myoglobin solution above. Adapted from Motterlini *et al*, Curr Pharm Des. 2003;9(30):2525-39.

At this point further investigations on iron pentacarbonyl were abandoned due to its deposition of a green brown precipitate. As there were no previous studies on the use of metal carbonyl complexes in biological systems the potential cytotoxic effects of each was evaluated. Neither [$Ru(CO)_3Cl_2]_2$ or [$Mn_2(CO)_{10}$)] caused any appreciable

decrease in cell viability over time at concentrations below 400 μmol/L (Motterlini et al. 2002). The structure of each is shown in Figure 1-7.

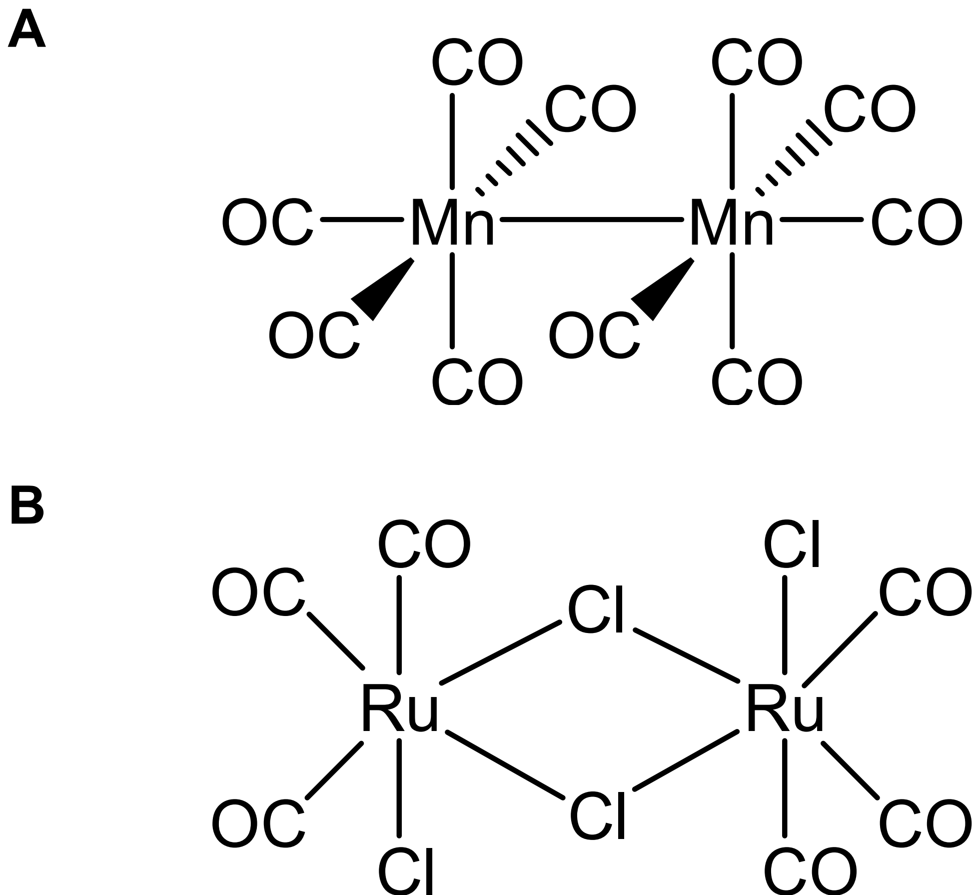


**Figure 1-7 Structure of A) dimanganese decacarbonyl ($[Mn_2(CO)_{10})]$) and B) tricarbonyldichlororuthenium (II) dimmer ($[Ru(CO)_3Cl_2]_2$), CORM-1 and CORM-2 respectively.**

The effect of $[Ru(CO)_3Cl_2]_2$ was then studied in an isolated aortic ring model (Motterlini et al. 2002). The compound exhibited significant vasodilatory effects after the first addition (45% greater than control) and was shown to have a long term effect, as phenylephrine induced contraction was not able to be restored even after extensive washings. This vasodilatory effect was significantly attenuated by the addition of myoglobin to the buffer (which as mentioned earlier avidly binds CO) and ODQ (a selective inhibitor of guanylate cyclase). Interestingly, ODQ only has an observed effect

over the first two additions of $[Ru(CO)_3Cl_2]_2$ but the third addition elicited a substantial vasodilatory effect despite the presence of ODQ. This data shows that CO released from this early identified CO-RM is at least in part cGMP dependent. The failure of ODQ to completely prevent vasodilation may result from CO being in sufficiently high levels to displace ODQ from guanylate cyclase or by circumventing this pathway altogether and exerting relaxation via other independent cellular pathways (Motterlini et al. 2002).

Experiments using an isolated heart model and $[Mn_2(CO)_{10})]$ further confirmed the vasoactive properties of this CO-RM. This metal carbonyl markedly attenuated a L-NAME mediated increase in coronary perfusion pressure; notably, this effect could only be achieved when $[Mn_2(CO)_{10})]$ was stimulated by light to release CO. This corroborates evidence showing that endogenously produced CO can profoundly modulate cardiac vessel function but was achieved using an exogenously applied CO-RM (Motterlini et al. 2002).

Further experiments were carried out investigating the antihypertensive actions of $[Ru(CO)_3Cl_2]_2$ in animals. As seen with previous experiments using hemin to upregulate HO-1, $[Ru(CO)_3Cl_2]_2$ was found to suppress the increase in mean arterial pressure elicited by the intravenous administration of L-NAME further supporting the potential of CO to be use *in vivo* (Motterlini et al. 2002).

These initial experiments opened the door to a host of exciting possibilities in the world of CO-releasing molecules. In addition, with the success of exogenous CO gas delivery in anti-inflammatory and anti-apoptotic models the potential of a therapeutic application for CO-RMs was strongly emerging (Figure 1-8). There was scope to engineer molecules that could release CO with distinct kinetics and have molecules that

were highly specific and thus deliver CO directly to an area of insult. On the back of these first successful experiments our group went on to develop CO-RMs more compatible with biological systems.

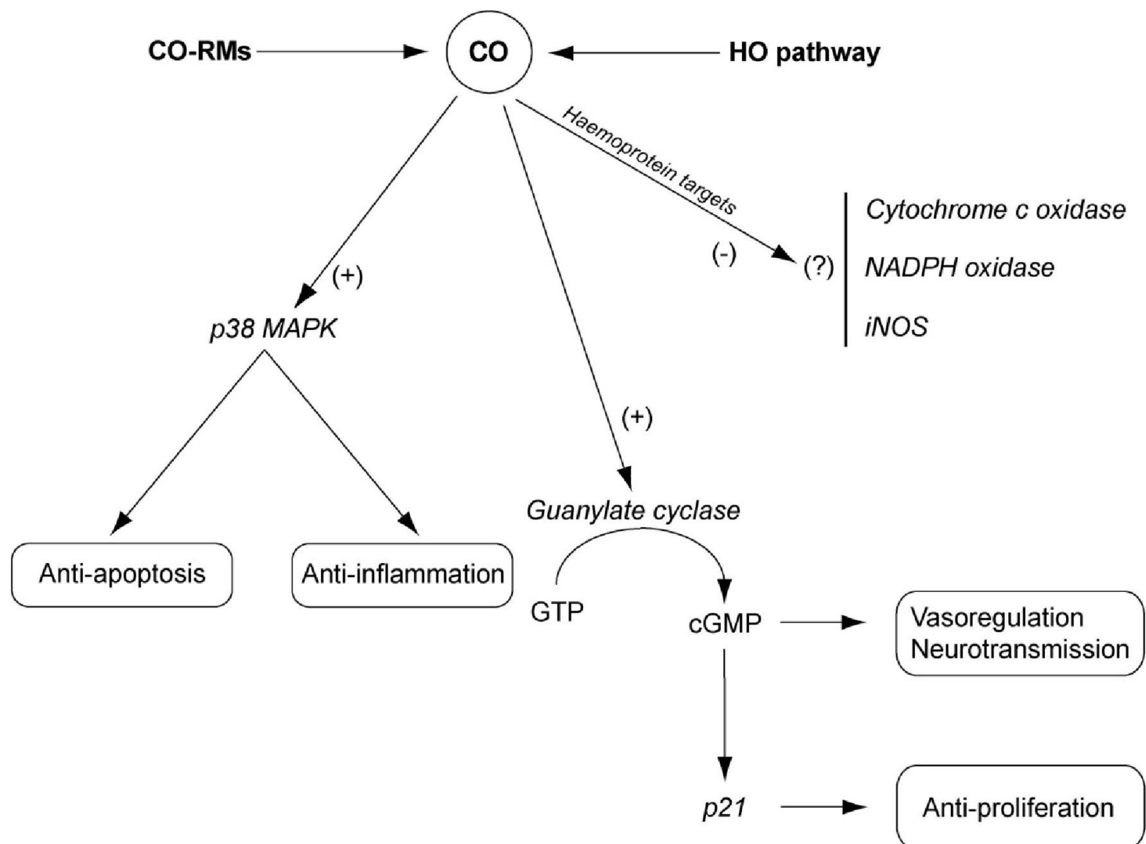


**Figure 1-8 Biological action of CO.**
Endogenously derived CO has been shown to exert many effects. Most endogenously generated CO is derived from the HO pathway but could CO-RMs be used to achieve similar outcomes? Extract from Ryter *et al*, Mol Cell Biochem. 2002 May-Jun;234-235(1-2):249-63.

**CORM-3**

The original CO-RMs were only soluble in organic solvents and required physical (i.e. irradiating light) or chemical (i.e. steric ligands) intervention to favour CO dissociation from these complexes. Interestingly, the versatile chemistry of transition metals allows CO-RMs to be modified by coordinating biological ligands to the metal centre in order to render the molecule less toxic, more water soluble, and to modulate the release of CO. The first such modified molecule was Tricarbonylchloro(glycinato)ruthenium(II) (CORM-3) which released CO and was water soluble (Figure 1-9) (Clark et al. 2003). The development of such a molecule was hugely important as it was biologically 'friendly' and could be used as a prototypic chemical in the development of pharmacologically active compounds capable of delivering CO for therapeutic purposes (Motterlini et al. 2003).

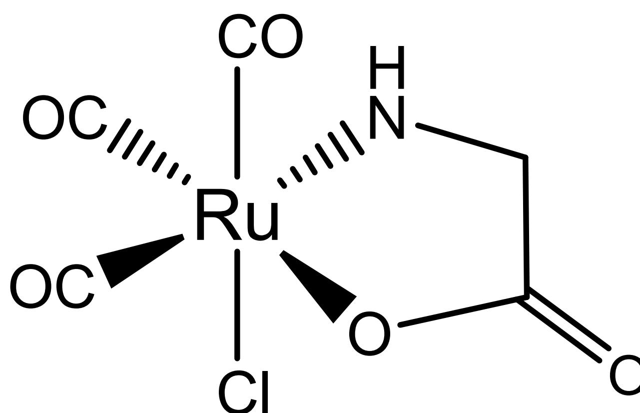


**Figure 1-9 Chemical structure of CORM-3, a water soluble CO-releasing molecule.**

Early investigation of CORM-3 involved testing CO release using the myoglobin assay after preparing the molecule in a number of solutions. The liberation of CO from

CORM-3 in biological systems occurs very rapidly (within 1-5 min) (Motterlini et al.2003).

Interestingly, it was found that CORM-3 remained stable in water ($H_2O$) left over night at room temperature; however, when prepared and left in Krebs buffer (pH = 7.4) in the same conditions it was unable to convert deoxy myoglobin to MbCO and was in effect inactive (iCORM-3) (Motterlini et al. 2003). Infrared spectroscopy suggests that iCORM-3 is a dicarbonyl species, consistent with the loss of one molecule of CO in its formation from CORM-3. This discovery means there is a negative control for the compound which aids in distinguishing between the effects of CO and the compound itself.

CORM-3 stability has been explored further and it has been shown to remain inert in water and acidic pH for over 24 h, whilst, it has been demonstrated to release CO in a number of physiological solutions and biological fluids such as PBS, Kreb's buffer, cell culture medium and human blood plasma (Motterlni et al. 2003). It has also been found that CORM-3 is able to increase HO activity whilst iCORM-3 does not, suggesting that some of the effects witnessed may be partially attributable to this phenomenon (Motterlini et al. 2003).

The development of future CO-RMs which are more biologically suitable and able to release CO in a more targeted fashion continues. There are many aspects of CO-RMs that can be modified to suit the end user. For example, instead of ruthenium other metal ions such as iron commonly found in all animals may elicit less of a toxic effect and aid in biological compatibility (i.e. iron). Another key area of CO-RM development is the kinetics of CO release. By manipulating the ligands, the rate at which CO is

released can be altered, allowing for slow delivery of CO over a period of time or a faster release where needed. CO-RMs that release CO very rapidly (“fast releasers”) in biological systems would be ideal for therapeutic applications where CO acts as a prompt signaling mediator (i.e. neurotransmission, acute hypertension, angina, ischemia-reperfusion). Conversely, chemicals that release CO with a slow kinetic (“slow releasers”) would implement the design of pharmaceuticals that could be more versatile in the treatment of certain chronic diseases (i.e. inflammatory states and chronic hypertension; rejection of transplanted organs) where continuous and long-lasting release of CO may be required.

There are also various issues concerning solubility to consider. Currently water-soluble CO-RMs exist but the need for lipid soluble ones may also be considered. The most intriguing aspect of CO-RM development is specific intra-cellular targeting. The molecules could be synthesised with receptor specific proteins or even some kind of antibody targeting which would allow for the delivery of CO to an exact target thus negating the need for a holistic CO release and any associated side effects of such an approach. The attempt to diversify the multiplicity of CO-RMs that possess a variety of chemical characteristics (i.e. water-soluble vs. lipid-soluble, slow vs. fast releasers) will help to elucidate the biological function of cellular targets that are responsive to CO and will facilitate in due course the design of versatile agents that could be used for the therapeutic delivery of CO in a safe, measurable and controllable fashion (Chatterjee 2004).

### 1.2.1 CO-RMs effects on cell reactivity and inflammation

**Cell cultures**

CO-RMs have been extensively studied over the last decade (Motterlini et al. 2002;Motterlini 2007;Motterlini, Mann, & Foresti 2005). CORM-3, as one of the most promising compounds, has been tested in various cell cultures, among which are microglial cells, macrophages, epithelial cells, cardiac cells, polymorphonuclear granulocytes and vascular endothelial cells (Abdel Aziz et al. 2008;Bani-Hani et al. 2006a;Musameh et al. 2007;Sawle et al. 2005a). Basic studies have indicated CORM-3 to be a non-toxic compound and which has no negative reaction on cells in various likely therapeutic concentrations.

Studies on haemorrhagic stroke, generally use astroglia and microglia cell cultures. The concentration of CO-RMs in these studies varied from 1 μM to 100 μM (Bani-Hani et al. 2006b;Hascalovici et al. 2009;Min et al. 2006). These studies were investigating the role of carbon monoxide on cholesterol synthesis, heme-oxygenase up-regulation and inflammatory markers production in microglial and astrocytes cultures and were not studying the possible toxicity of CO-RMs.

Later studies suggest that CORM-3 is capable of modulating cell reactivity leading to the production of various inflammatory markers (Tranter & Jones 2008;Vannacci et al. 2006). Considering the inflammatory nature of haemorrhagic stroke it is important to understand the effects of CORM-3 on microglia and astroglia cultures. Bani-Hani et al. demonstrated CORM-3 effects on the inflammatory response in BV-2 microglia to lipopolysaccharide (LPS) and INF-gamma showing a significant reduction in nitrite levels and TNF-α production (Bani-Hani et al. 2006b). Papers

investigating the cellular reactivity in astrocytes from the perspective of inflammatory nature of haemorrhagic stroke were not found.

It is important to study CORM-3 toxicity and cell viability in the main cell types of the brain, such as neurons, astrocytes, olygodendrocytes and microglia, and try to define more closely the inflammatory nature of haemorrhagic stroke.

**Brain slices**

As mentioned previously, the results obtained from studies using cell cultures play an important role in investigating CO-RMs and their possible prospective use in clinical practise. However, the brain is a complex structure whose function depends not only on a variety of cell types, but also on their architectural configuration and cellular interactions (Huang 2010;Niggemann, Gebert, & Schulz 2008).

The possible effects of CO-RMs on such complex structure can be investigated to a limited extent using brain slices. A brain slice model allows us to investigate CO-RMs in a simple manner, much like cell culture, with the added benefit of retaining some of the physiological architecture and intercellular relationships of different cell types in the brain.

A literature search on brain slice research revealed only one paper examining the effect of CORM-3, focused on the activation of $Ca^{2+}$ signals in smooth muscle cells of new-born piglet brain slices (Xi et al. 2010).

No publications studying the properties of CO-RMs or CORM-3 in brain slices could be found in the scientific literature.

**Animal work**

Stemming from successful work using CORM-3 in vitro, in vivo experiments have also demonstrated the potential use of CORM-3. CORM-3 has been tested in various animal models, mainly studying its hypotensive, antiarrhythmic properties, organ preservation and possible modulation of inflammatory reactions in experimental animals (Bagul et al. 2008;De et al. 2009;Foresti et al. 2004;Foresti et al. 2005;Guo et al. 2004;Stagni et al. 2009;Varadi et al. 2007).

Many different animal models have been used to determine the effect of CO-RMs and their possible influence on inflammation (Clark et al. 2003;Ryan et al. 2006;Stein et al. 2005a;Vadori et al. 2009). Some of these models include for example ischemia-reperfusion, collagen-induced arthritis, thermal injury and pulmonary injury, and mainly study specific local inflammatory markers (Ferrandiz et al. 2008;Katada et al. 2009c;Sun et al. 2007b;Sun et al. 2008b). As all of these experiments studied local processes and different aspects of inflammation it is difficult to compare and systematize these data. There is no report on inflammatory reactions and the influence of CO-RMs on these systems, nor studies that examine possible CO-RMs effects on inflammation occurring during haemorrhagic stroke.

## 1.3 Hypotheses

- The overall outcome of haemorrhagic stroke after intracerebral haemorrhage is determined by inflammation which by its nature is a compensatory defensive-adaptive reaction of the whole organism in response to haemorrhagic brain damage

- CORM-3 affects haemorrhagic stroke through modulation of inflammation and the general adaptation syndrome

## 1.4 Aim

To examine recovery mechanisms occurring in the intracerebral haemorrhage from the perspective of inflammation theory and to determine the potential role of CORM-3 on the course and outcomes of haemorrhagic stroke and to study their potential for treatment of haemorrhagic stroke.

## 1.5 Objectives

1) To establish a CORM-3 release profile
2) To study CORM-3 toxicity on astrocyte and microglial cell cultures
3) To study CORM-3 toxicity in rat brain tissue slices
4) To develop a standardised model that provides consistency of localisation and evolution of the intracerebral haemorrhage
5) To determine changing parameters in blood tests during intracerebral haemorrhage
6) To determine brain water changes in intracerebral haemorrhage
7) To determine histological brain changes in intracerebral haemorrhage
8) To determine the behavioural profile in intracerebral haemorrhage
9) To show possible CORM-3 effects on the inflammatory process in intracerebral haemorrhage on local, systemic levels and in functional recovery of the brain depending on the time of treatment and dosage
10) To investigate potential CORM-3 properties on the course of haemorrhagic stroke depending on the dose at administration
11) To investigate potential CORM-3 properties on the course of haemorrhagic stroke depending on the time of its administration

# Chapter II. Materials and methods

## 2.1 Cell cultures and experimental design

Rat astrocytes (DI-TNC1) and BV-2 microglial cells were tested for toxicity and viability. The positive control group was given no treatment, while the negative control group was given triton (x100) treatment to reach a 100% cell lysis/death in cell population. CORM-3 effects on toxicity and cell viability were studied using concentrations of 10, 50 and 100 µM.

## 2.2 Brain slices and experimental design

Rat brain striatal slices were studied for toxicity and viability. The positive controls were given no treatment, while the negative control group was measured with triton (x100) in an attempt to achieve a 100% death. CORM-3 effects on toxicity and cell viability were studied using concentrations of 10, 50 and 100 µM.

## 2.3 Animals and experimental design

306 Sprague-Dawley male rats (250-350g) were divided into experimental groups. The development of the haemorrhagic stroke model required 18 Sprague-Dawley male rats having 3 groups of autologous blood (100µl), 2 µl of 0.2 U and 2 µl of 0.5 U collagenase solution injected into the lateral striatum with 6 experimental animals in each group respectively.

The control groups comprised 72 Sprague-Dawley rats consisting of 2 groups of 2 μl of normal saline and 2 μl of 0.2 U collagenase solution injection performed into the lateral striatum of the experimental animals with 36 rats in each group respectively.

To determine the influence of CORM-3 on the development and outcomes of haemorrhagic stroke required 216 Sprague-Dawley rats. The control groups were injected with normal saline into the lateral striatum and CORM-3 administration at a dosage of 8 mg/kg 3 hours post-surgery in 30 Sprague-Dawley rats and CORM-3 administration at the dosage of 8 mg/kg 3 hours post-surgery in experimental animals. CORM-3 effects in haemorrhagic stroke groups required 180 Sprague-Dawley rats. Experimental animals were treated with CORM-3 at two different doses (4 mg/kg and 8 mg/kg) before inducing haemorrhagic stroke, 3 hours after and 3 days post-surgery.

The experimental evaluations were performed 1 day pre-operation and on days 1, 3, 5, 7, 14 and 21 post-operation. The time of evaluation was 11:00 am which was maintained throughout the experimental days. Treatments with CORM-3 for 3 days post-operation administration were performed at 7:00 am. At each time point the set number of the experimental animals (n=6) was used for further behavioral, laboratory, histological, immunohistological examination and brain water content analysis. Each rat from the modeling group was sacrificed by a pentobarbital overdose (60 mg/kg) the following day after operation for histological examination.

## 2.4 Experimental groups

| | |
|---|---|
| Pre-operation group | The pre- operation group was organised in such a way that all animals in each treatment group were observed and evaluated 1 day prior to experimental procedures and these data are represented as day 0 on the figures |
| Group 1 | Injection of 100µl of autologous blood taken from a rat tail vein and administered into the lateral striatum (n=6) |
| Group 2 | Injection of 2 µl of 0.2 U of collagenase IV(S) into the lateral striatum (n=6) |
| Group 3 | Injection of 2 µl of 0.5 U of collagenase IV(S) into the lateral striatum (n=6) |
| Group 4 | Injection of 2 µl of normal saline solution into the lateral striatum of the experimental animals, sacrificed at 6 time points (1, 3, 5, 7, 14 and 21 days post-operation) (n=36) |
| Group 5 | Collagenase injection group (Group 5). Injection of 2 µl of 0.2 U collagenase solution into the lateral striatum of the experimental animals sacrificed at 6 time points (1, 3, 5, 7, 14 and 21 days post-operation) (n=36) |

| | |
|---|---|
| Group 6 | Normal saline injection into the lateral striatum and CORM-3 administration at the dosage of 8 mg/kg 3 hours post-surgery sacrificed at 6 time points (1, 3, 5, 7 and 14 days post-operation) (n=30) |
| Group 7 | CORM-3 administration at a dosage of 8 mg/kg 3 hours post-surgery to intact experimental animals, sacrificed at the day 14 post-operation (n=6) |
| Group 8 | Collagenase injection into the lateral striatum of the experimental animal and CORM-3 administration at a dosage of 4 mg/kg 5 minutes before operation sacrificed at 6 time points (1, 3, 5, 7 and 14 days post-operation) (n=30) |
| Group 9 | Collagenase injection into the lateral striatum of the experimental animal and CORM-3 administration at a dosage of 8 mg/kg 5 minutes before operation sacrificed at 6 time points (1, 3, 5, 7 and 14 days post-operation) (n=30) |
| Group 10 | Collagenase injection into the lateral striatum of the experimental animal and CORM-3 administration at a dosage of 4 mg/kg 3 hours after operation sacrificed at 6 time points (1, 3, 5, 7 and 14 days post-operation) (n=30) |

| | |
|---|---|
| Group 11 | Collagenase injection into the lateral striatum of the experimental animal and CORM-3 administration at a dosage of 8 mg/kg 3 hours after operation sacrificed at 6 time points (1, 3, 5, 7 and 14 days post-operation) (n=30) |
| Group 12 | Collagenase injection into the lateral striatum of the experimental animal and CORM-3 administration at a dosage of 4 mg/kg 3 days after operation sacrificed at 6 time points (1, 3, 5, 7 and 14 days post-operation) (n=30) |
| Group 13 | Collagenase injection into the lateral striatum of the experimental animal and CORM-3 administration at a dosage of 8 mg/kg 3 days after operation sacrificed at 6 time points (1, 3, 5, 7 and 14 days post-operation) (n=30) |

**Table 5 Experimental protocol for animal groups subjected to a different treatment**

## 2.5 Synthesis of Tricarbonylchloro (glycinato) ruthenium (II) (CORM-3)

Tricarbonylchloro(glycinato)ruthenium (II) ([Ru(CO)$_3$Cl(glycinate)] or CORM-3) was synthesised starting from a commercially available compound, tricarbonyldichlororuthenium(II) dimer ([Ru(CO)$_3$Cl$_2$]$_2$) (Sigma Aldrich). Briefly, [Ru(CO)$_3$Cl$_2$]$_2$ (0.129g) and glycine (0.039g) were placed under nitrogen in a round-bottomed flask. Methanol (75ml) and sodium ethoxide (0.034g) were added and the reaction was allowed to continue under stirring for 18 hours at room temperature. The solvent was then removed under pressure and the yellow residue redissolved in

tetrahydrofuran (THF); this was filtered and excess 40 to 60 light petroleum added. The resulting yellow solution was evaporated down to give a pale yellow solid (0.142g, 96% yield). CORM-3 was stored in sealed vials at room temperature and used freshly on the day of the experiment.

### 2.6 CORM-3: Detection of CO release: myoglobin assay

The release of CO from CORM-3 was assessed spectrophotometrically by measuring the conversion of deoxy-myoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO) (Motterlini et al. 2003). The amount of MbCO formed was quantified by measuring the absorbance at 540 nm (extinction coefficient = 15.4 mmol/L$^{-1}$ cm$^{-1}$). Stock solutions of myoglobin (lyophilized horse heart) (Sigma) (66 µmol/L final concentration) were prepared fresh by dissolving the protein in PBS (0.01 M, pH = 7.4) (Sigma). Sodium dithionite (0.1%) (Sigma) was added to convert the myoglobin stock to deoxy-Mb prior to each experiment. A 1 ml quantity of this was measured to obtain a deoxy-Mb curve and then bubbled with CO to measure a MbCO curve. CO-RMs (20, 40 or 60 µM) were added (5 µl) directly to the myoglobin in the cuvette (Sarstedt, Leicester, UK), mixed using a Gilson (1000 µl) and then overlaid with 500 µl mineral oil (Sigma) to prevent CO escaping or the myoglobin becoming oxygenated. Samples were immediately read on a spectrophotometer (Unicam Heλios α) and then read every 5 min for the first 30 min. The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CO to spectra typical of MbCO with two maximal absorption peaks at 540 and 578 nm, respectively (see Figure 2-1).

Subsequent readings were taken every 30 min or when deemed necessary. Normally the readings were continued until the CO release reached a plateau for 2 consecutive readings.

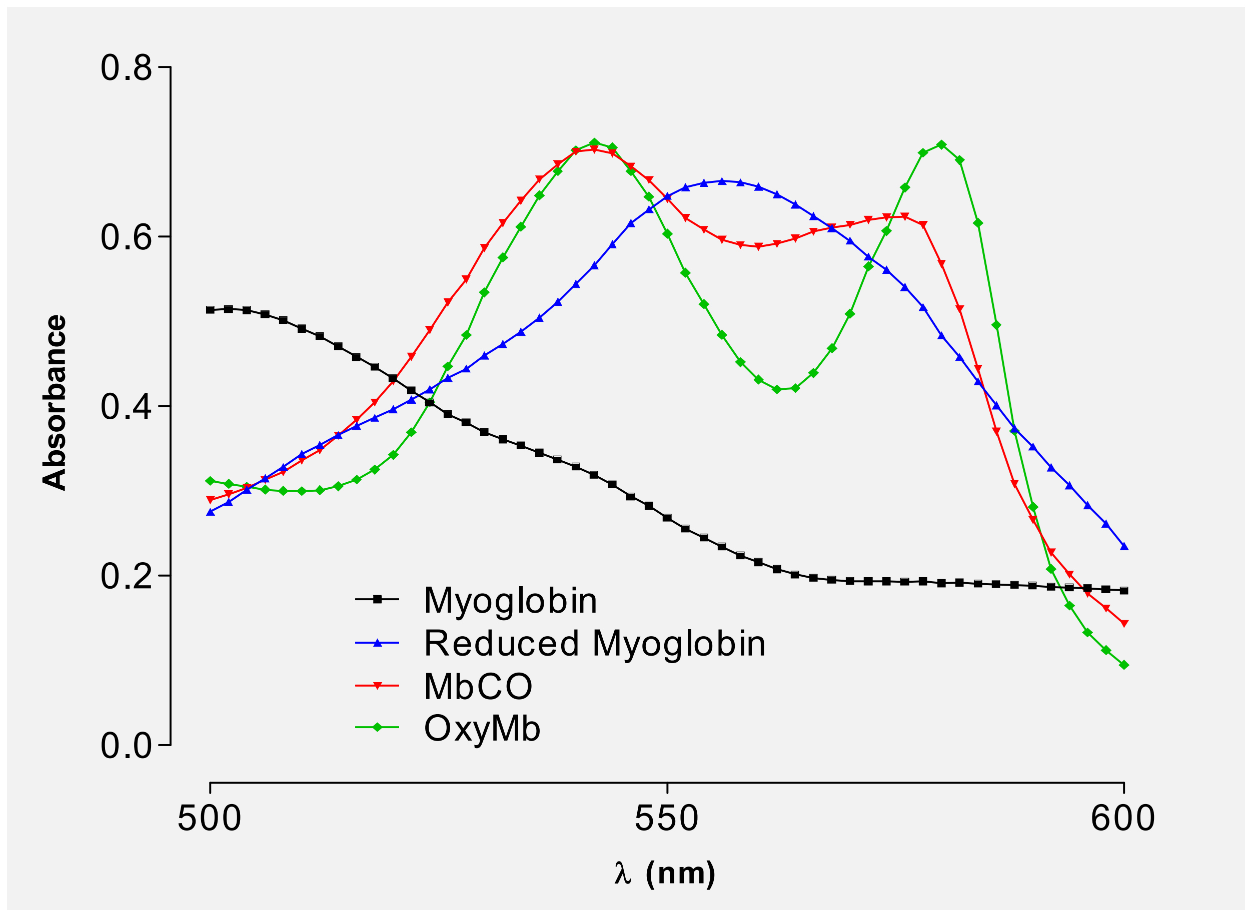


**Figure 2-1 The various spectra of myoglobin.**

This figure shows the alternate spectra that are generated by reducing (sodium dithionite) or exposing myoglobin to carbon monoxide (MbCO) or 95% oxygen (OxyMb).

In order to calculate the total myoglobin in the solution, deoxyMb solution was bubbled for 5 min with 1 % CO gas until the absorbance at 540 nm reached a maximum. This OD value was taken as the maximum MbCO concentration and therefore the total myoglobin concentration of the mixture, although. In theory the solution of myoglobin was converted to ~100 % MbCO but the measured amount was never as high as 66 μM (the initial calculated concentration of Mb) because the myoglobin used was only 90% pure and thus contained impurities such as salts and

denatured myoglobin in the original myoglobin solutions. Total myoglobin concentration was calculated using Equation 2.1.

**Equation 2.1 Equation for calculating total myoglobin concentration in a saturated solution of carmoxy-myoglobin (MbCO). ε = extinction coefficient of MbCO = 15.4 mM$^{-1}$cm$^{-1}$**

$$MbCO_{\max} = \left( \frac{OD_{540}}{\varepsilon} \right) x1000$$

Although the maximum absorbance at 540 nm after saturation with CO gas gives the total MbCO concentration ($MbCO_{max}$), any intermediate values of MbCO (between ~100 % deoxyMb in and ~100 % MbCO) have to be calculated by mathematical iteration from the $OD_{540}$ reading. This is performed as follows.

Firstly, it must be assumed that in the deoxyMb solution there is ~0 % MbCO, so the $OD_{540}$ at this point (always a positive value) indicates 0 μM MbCO. Assuming this, a new extinction coefficient ($\varepsilon_2$) must be calculated to take into account the change in absorbance at 540 nm ($\Delta OD_{540}$). To aid in the accuracy of this calculation, another wavelength is used as a constant reference point. Conveniently, the deoxyMb and MbCO spectra share four isosbestic ($OD_{iso}$) points between 500 and 600 nm (~510, ~550, ~570 and ~585 nm). The value at 510 nm ($OD_{ISO510}$) was taken for these set of the experiments. Thus, the new extinction coefficient was calculated:

**Equation 2.2 Equation needed to calculate an unknown MbCO extinction coefficient.**
**Taking into account the change in absorbance at the isosbestic point ($\Delta Od_{iso510}$) and the change in absorbance at 540 nm ($\Delta OD_{540}$) a new extinction coefficient ($\varepsilon_2$) can be calculated.**

$$\varepsilon_2 = \left( \frac{\Delta \mathrm{OD}_{540} - \Delta \mathrm{OD}_{\mathrm{iso510}} \times 1000}{\mathrm{MbCO}_{\max}} \right)$$

From these calculations, the concentration of MbCO in a solution of myoglobin can be calculated if three things are known about the solution: i) the change in $OD_{540}$ between deoxy- and saturated MbCO (by saturating the Mb solution with CO gas);

ii) the change in the $OD_{iso}$ in the sample from the original deoxyMb solution and; iii) the $OD_{540}$ and $OD_{iso}$ of the sample solution. The following equation is therefore used to calculate the [MbCO] of the unknown sample:

**Equation 2.3 Calculation of unknown MbCO concentrations. Where $\Delta OD_{540}$ is the change in absorbance at 540 nm, $\Delta OD_{iso}$ is the change in absorbance at the isosbestic point, $MbCO_{max}$ is the MbCO concentration calculated after saturation of the Mb with CO gas and $\varepsilon_2$ is the calculated absorption coefficient.**

$$MbCO = \left( \frac{\Delta OD_{540} - \Delta OD_{iso510}}{\varepsilon_2} \right) \times 1000$$

After all the time points have been calculated, These data can be converted from a concentration (µM) to absolute MbCO values (nmoles) by multiplication of the µM value by the volume of myoglobin in the solution (in l). From a graph of MbCO (nmoles) vs. time (min), the incubation time required for any MbCO concentration can be calculated; therefore cells can be exposed to a known concentration of CO using this system.

## 2.7 Cell culture

### 2.7.1 BV-2 microglia and Rat DI-TNC1 astrocytes

Rat microglial BV-2 (European Collection of Cell Cultures) and rat astrocyte DI-TNC1 (Salisbury, Wiltshire, UK) cells were cultured in complete medium consisting of: Dulbecco's modified Eagle's medium (DMEM) or Iscoves modified Dulbecco's medium respectively, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cells were subcultured twice a week and approximately every 3 days respectively when reaching 85% confluence. Initially the existing medium in each flask was removed and discarded.

BV-2 cells were then washed with 5 ml warm (37°C) PBS (Gibco, Paisley, UK) which was replaced with 8 ml warm (37°C) DMEM and the cells scraped using a rubber policeman (Thomas Scientific, Swedesboro, NJ) to remove them from the flask surface. The cells were then pipetted (2 ml) into 4 new T-75 flasks (Sarstedt, Leicester, UK) each containing 8 ml DMEM.

DI-TNC1 cells (>80% confluence in a 75 $cm^2$ flask) were firstly washed with 5 ml of warm (37 °C) PBS to facilitate the action of trypsin (0.25% trypsin-EDTA solution, Sigma). Once the PBS was removed, trypsin (also 37°C) was added to remove cells from the surface of the flask. Cells were then briefly incubated at 37°C for approximately 3 min to allow the trypsin to act. The cell suspension was removed and pipetted into 8 ml of fresh medium to neutralise its action and prevent over exposure of the cells to this agent which could be detrimental. Cells were then centrifuged (1500 x *g*) for 5 min in a bench top centrifuge (SANYO, Harrier 15/80). The supernatant was removed and fresh medium was added to re-suspend the pellet (a 1:3 split would require 6 ml to re-suspend the pellet). Then, 2 ml of the resuspended cells were pipetted into pre-prepared T-75 flasks containing 8 ml fresh medium and incubated. Over an extended period of slow growth where cells failed to reach confluence within 3-4 days, the medium was replaced with fresh medium.

Cultures were maintained at 37°C in a 95% air and 5% $CO_2$ humidified atmosphere and experiments were conducted on cells at approximately 80-90% confluence. All cell culture work was carried out using an ESB JBIO CL II category II laminar flow cabinet.

### 2.7.2 Subculture of cells into 24 wells

Cells were prepared as described above (paragraph 2.7) until the cell pellet was re-suspended (DI-TNC1) or the cells had been scraped (BV-2). At this stage the pellet was resuspended using 2 ml of complete medium whilst the microglia were left as they were. From either cell suspension, 2 ml was then added to a pre-prepared Falcon tube (50 ml, NUNC) containing 49 ml of complete medium. From this mixture 2 ml was added to each well of a 24 well plate, with any remaining cell suspension discarded. The cells were then grown on the plate at 37°C until confluent.

### 2.7.3 Cell viability and toxicity determination

To determine the cellular toxicity of CORM-3, two independent techniques were carried out each quantifying a particular aspect of cell health. The combination of the two gives a good measure of the overall viability of cells subsequent to treatment.

#### 2.7.3.1 Lactate dehydrogenase toxicity assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. The LDH based cytotoxicity detection kit (Roche Diagnostics, UK) is a colorimetric assay for the quantification of cell death and cell lysis based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant. The assay was carried out according to the manufacturer's instructions. Upon completion of treatment the medium of each group was spun (Beckman Avanti™ 30 centrifuge) at 500 g for 5 min at 25°C to remove any cellular debris. Cell free culture supernatant (100 µl) was transferred to a clear flat bottom 96 well plate (NUNC, UK) to which 100 µl of reaction mixture was added. The reaction mixture consisted of a lyophilised catalyst which was reconstituted in 1 ml of distilled water and an INT dye.

The ratio of catalyst to dye was 1:46. The plate was then incubated at 37°C, 95% air and 5% $CO_2$ for 15 min and protected from light. Samples were read on a spectrophotometric plate reader (Molecular Devices VERSAmax tuneable microplate reader) at 490 nm with a reference wavelength of 690 nm and blanked against cell culture medium. The percentage cytotoxicity was determined as in Equation 2.4.

Samples were run in triplicate and a 1 % triton / medium mixture was added to some wells as a positive control, since triton solubilises cell membranes and hence releases maximal LDH protein, resulting in maximal LDH activity.

**Equation 2.4 Calculation of LDH activity associated cytotoxicity, where high control is a triton-induced activity and low control is an activity from a media from a cells with no treatment (blank)**

$$\text{Cytotoxicity (\%)} = \left( \frac{\text{Exp. Value} - \text{Low control}}{\text{High control} - \text{Low Control}} \right) \times 100$$

### 2.7.3.2 Alamar blue viability assay

Cell metabolism was determined using an Alamar Blue assay kit and carried out according to the manufacturer's instructions (Serotec, UK). The medium of the treated cells was removed from all wells on the plate(s) and replaced by 1 ml of a 10% Alamar Blue: 90% complete cell culture medium mixture. The plate(s) was then left to incubate for 4 h after which 200 μl from each well was loaded on to a 96 well plate to be read on a plate reader (Molecular Devices) at 570 nm (subtracting any background absorbance present at 600 nm). The assay is based on the detection of metabolic activity of living cells using a redox indicator which changes from an oxidised form (blue) to a reduced form (red). It has been shown that some ionophores (molecules which carry or transports charged compounds or ions across cell membranes) block the reduction of

Alamar Blue preventing its reduction in cell free extracts. It will, however, accept electrons from the electron transport chain as the Eh (oxidation-reduction potential) of Alamar Blue is similar to that of the cytochromes. The redox dye in Alamar Blue accepts electrons from the electron transport chain in a similar fashion to the terminal reduction of oxygen. The reduced form of Alamar Blue is secreted out of cells and back into the media.

The intensity of the red colour is proportional to the metabolism of the cells, which is calculated as the difference in absorbance between 570 nm and 600 nm and expressed as a percentage of control.

## 2.8 Brain tissue culture

### 2.8.1 Rat brain striatal slices preparation

Prior to brain tissue collection, the medium for maintaining the brain slices was prepared. The medium for brain tissue collection consisted of Gey's balanced salt solution (Calcium Chloride ($CaCl_2$) 0.166 g/L, Potassium Chloride (KCl) 0.97 g/L, Potassium Phosphate Monobasic ($KH_2PO_4$) 0.03 gm/L, Magnesium Chloride ($MgCl^2 \cdot 6H_20$) 0.21 g/L, Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$) 0.07 g/L, Sodium Chloride (NaCl) 8.00 g/L, Sodium Bicarbonate ($NaHCO_3$) 0.227 g/L, Sodium Phosphate Dibasic ($Na_2HPO_4$) 0.12 g/L, Glucose 1.00 g/L) (Sigma) and 45% glucose solution. Pastettes (Alpha Laboratories Ltd), razor blades (Sutherland Helath Ltd), melinex film (Agar Scientific) were stored in 70% alcohol.

Tissue culture plates were prepared by adding the medium and placing the inserts (Millipore) into the wells. The tissue culture plates were placed into incubator (37°C, 95% air and 5% $CO_2$, high humidity). 70% alcohol was used to wipe the hood,

microscope (Leitz, LABOVERT FS), light source (SCHOTT, KL 1500) and working instruments.

Petri dishes containing Geys' balanced salt solution were prepared for brain tissue collection. 6-10 day old Sprague-Dawley rats were sacrificed by decapitation using an approved Schedule I method (scientific procedures). The fur was removed and the skull was peeled of revealing the brain. A pastette was used to remove the brain from the skull and the brain tissue was placed into the Petrie dish. Using a pastette, the olfactorius bulb and the brain tissue behind the chiasma opticus were removed. The cerebral cortex, basal forebrain and septal arc were removed, leaving a cube of striatal tissue.

The cube was placed onto the melinex film and cut with a McIlwain chopper (Mickle Laboratory Engineering) in the frontal pane with a slice thickness of 350-400 µM. Brain slices were separated with pastettes under the microscope (x10) and cultured on the brain tissue plate with 3 striatal slices per well. Brain striatal culture was maintained by changing the medium every 3-4 days. Brain tissue culture was tested for toxicity and viability after 10-14 days of incubation.

### 2.8.2 Brain slices viability/toxicity determination

Rat brain striatal slices were examined using Live/Dead Viability/Cytotoxicity kit (Invitrogen) 24 hours post treatment with CORM-3 and triton. Molecular probes Calcein and Ethidium Homodimer were mixed in equal proportions (2 mM) with the growth medium. Rat brain striatal slices were treated with this molecular probe medium for 30 minutes in an incubator (37°C, 95% air and 5% $CO_2$) then placed in normal growing medium. Rat brain striatal slices were analysed using an Olympus IX51 fluoroscent

microscope at 40 and 100 times magnification via Olympus DP-Soft software. Light microscopy was used to obtain a general picture of the rat brain slice and red and green filters were used for fluorescent microscopy.

## 2.9 Intracerebral haemorrhage models

### 2.9.1 Blood injection model

Rats were placed into the induction box which was connected to an anesthetic machine. Anesthesia was induced using a mixture of oxygen at 1.5 L/min flow together with a gradual increase of the isoflurane concentration up to 4%. After that the rats were placed into the stereotaxic frame and anesthesia was maintained at oxygen 1.5 L/min flow with 2% isoflurane via close-fitting nasal mask. The animals were kept on a heated pad during all surgical procedures.

A midline incision was made in the dorsal region of the scalp, revealing the sagittal suture and location of the bregma. Using a stereotaxic frame (Stoelting) a cranial burr hole was drilled 3 mm lateral to and 0.2 mm anterior to the bregma. Using a 26g needle, ultrasensitive injection pump (Harvard apparatus) and tubing system, 100 µl of autologous tail vein blood was injected into the right basal ganglia (5.5 mm ventral from the skull surface). The infusion of blood was performed over 10 minutes and the needle was kept at the site of injection for 5 minutes to avoid backflow (Rodrigues et al. 2003;Strbian, Durukan, & Tatlisumak 2008). Bone wax (Johnson and Johnson) was placed in the burrhole and the wound was closed with sterile mirsilk sutures (Ethicon).

After the surgical procedure was completed the isoflurane was turned off and rats were exposed to oxygen (2 L/min) via a mask for 5 minutes and then they were placed

into the recovery box. Buprenorphine (subcutaneous dosage of 0.1 U) was used as an analgesic post-operatively (Pfeilschifter et al. 2011).

### 2.9.2 Collagenase injection model

Rats were placed into the induction box which was connected to an anesthetic machine. Anesthesia was induced using a mixture of oxygen at 1.5 L/min flow together with a gradual increase of the isoflurane concentration up to 4%. After that the rats were placed into the stereotaxic frame and anesthesia was maintained at oxygen 1.5 L/min flow with 2% isoflurane via a close-fitting nasal mask. The animals were kept on a heated pad during all surgical procedures.

A midline incision was made in the dorsal region of the scalp, revealing the sagittal suture and location of the bregma. Using a stereotaxic frame (Stoelting) a cranial burr hole was drilled 3 mm lateral to and 0.2 mm anterior to the bregma. Using a 26g needle 0.2U/2ul or 0.5U/2ul Collagenase IV-S (Sigma Aldrich) was injected into the right basal ganglia (5.5 mm ventral from the skull surface). The infusion was performed over 2 minutes and the needle was kept at the site of injection for a further 2 minutes to avoid backflow of the collagenase (Rodrigues et al. 2003;Strbian, Durukan, & Tatlisumak 2008). Bone wax (Johnson and Johnson) was applied to the burrhole and the wound was closed with sterile mirsilk sutures (Ethicon).

After the surgical procedure was completed the isoflurane was turned off and rats were exposed to oxygen (2 L/min) via a mask for 5 minutes and then they were placed into the recovery box. Buprenorphine (subcutaneous dosage of 0.1 U) was used as an analgesic postoperatively (Pfeilschifter et al. 2011).

### 2.9.3 Saline injection model

Rats were placed into the induction box which was connected to an anesthetic machine. Anesthesia was induced using a mixture of oxygen at 1.5 L/min flow together with a gradual increase of the isoflurane concentration up to 4%. After that the rats were placed into the stereotaxic frame and anesthesia was maintained at oxygen 1.5 L/min flow with 2% isoflurane via a close-fitting nasal mask. The animals were kept on a heated pad during all surgical procedures.

A midline incision was made in the dorsal region of the scalp, revealing the sagittal suture and the location of the bregma. Using a stereotaxic frame (Stoelting) a cranial burr hole was drilled 3 mm lateral to and 0.2 mm anterior to the bregma. Using a 26g needle 2µl of sterile saline (Sigma Aldrich) was injected into the right basal ganglia (5.5 mm ventral from the skull surface). The infusion of normal saline was performed over 2 minutes and the needle was kept at the site of injection for 2 minutes to avoid backflow of the saline (Rodrigues et al. 2003;Strbian, Durukan, & Tatlisumak 2008). Bone wax (Johnson and Johnson) was applied to the burrhole and the wound was closed with sterile mirsilk sutures (Ethicon).

After the surgical procedure was completed the isoflurane was turned off and rats were exposed to oxygen (2 L/min) via mask for 5 minutes and then they were placed into the recovery box.

Buprenorphine (subcutaneous dosage of 0.1 U) was used as an analgesic postoperatively (Pfeilschifter et al. 2011).

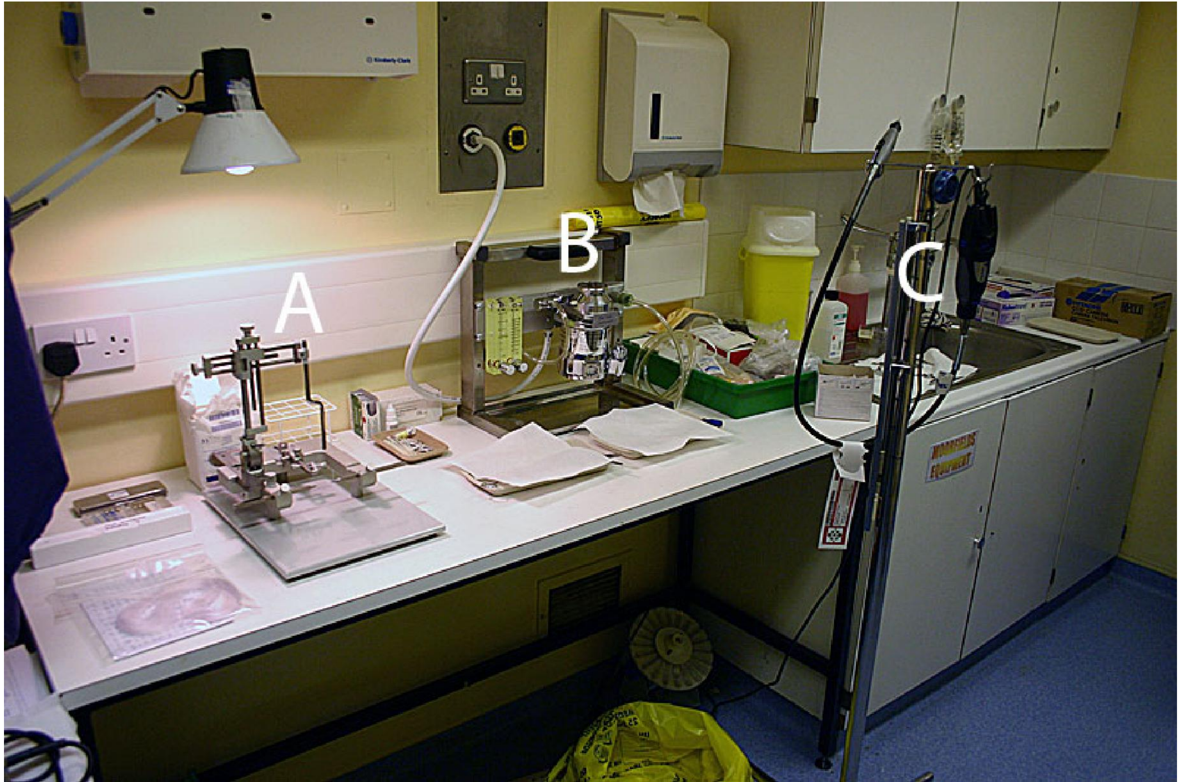


**Figure 2-2 Operation room. A – Stoelting stereotaxic frame, B – Anaestethic machine with isoflurane vaporiser, C – Drill for cranial burr hole drilling**

## 2.10 Blood analysis

### 2.10.1 General blood analysis: White blood cell count

Rat tail vein blood was collected into tubes containing EDTA. The blood was used for a white blood cell count using an ADVIA 2120 hematology system (Bayer Health Care). Leukocyte shift was calculated on the basis of these data collected (Avtandilov et al. 1983;Iabluchanskii, Pilipenko, & Sychev 1982).

**Equation 2-5 Calculation of leukocyte shift (N)**

$$N = \left( \frac{\text{eosinophils} + \text{neutrophils} + \text{basophils}}{\text{Lymphocytes} + \text{monocytes}} \right)$$

### 2.10.2 Enzyme-linked immunosorbent assay for TNF-alpha

The quantification of TNF-α in blood plasma samples was determined using an enzyme-linked immunosorbent assay kit and carried out according to the manufacturer's instructions (R&D systems, UK).

Assay dilutent (50 µl) was added to each well on the plate, pre-coated with the corresponding antibody relating to the cytokine of interest, prior to the addition of the standards, control and samples (50 µl). The samples were gently mixed for 1 min using AM69 Microshaker (Cooke Microtiter System, Dynatech Laboratories, Billinghurst, Sussex, GB), covered with an adhesive plastic cover strip and incubated for 2 h at room temperature. Each well was then aspirated and washed with 400 µl of wash buffer in a process that was repeated a total of 5 times. After the last wash, any remaining wash buffer was aspirated and the plate blotted with clean paper towels. Following washing, an enzyme-linked polyclonal antibody specific to the cytokine of interest was added (100 µl) to each well. The plate was covered with an adhesive strip and incubated at room temperature for 2 h. As before the plate was aspirated and washed five times. Substrate solution (100 µl) was then added to each well and the plate incubated at room temperature for 30 min in the dark.

To halt the reaction, 100 µl of stop solution (provided by manufacturer) was added to each well and the absorbance read at 450 nm (correction wavelength of 570 nm) on a Molecular Devices VERSAmax tuneable spectrophotometric microplate reader. Cytokine concentration was determined by plotting the unknown samples on a standard curve of known cytokine concentrations and is represented on Figure 2-3.

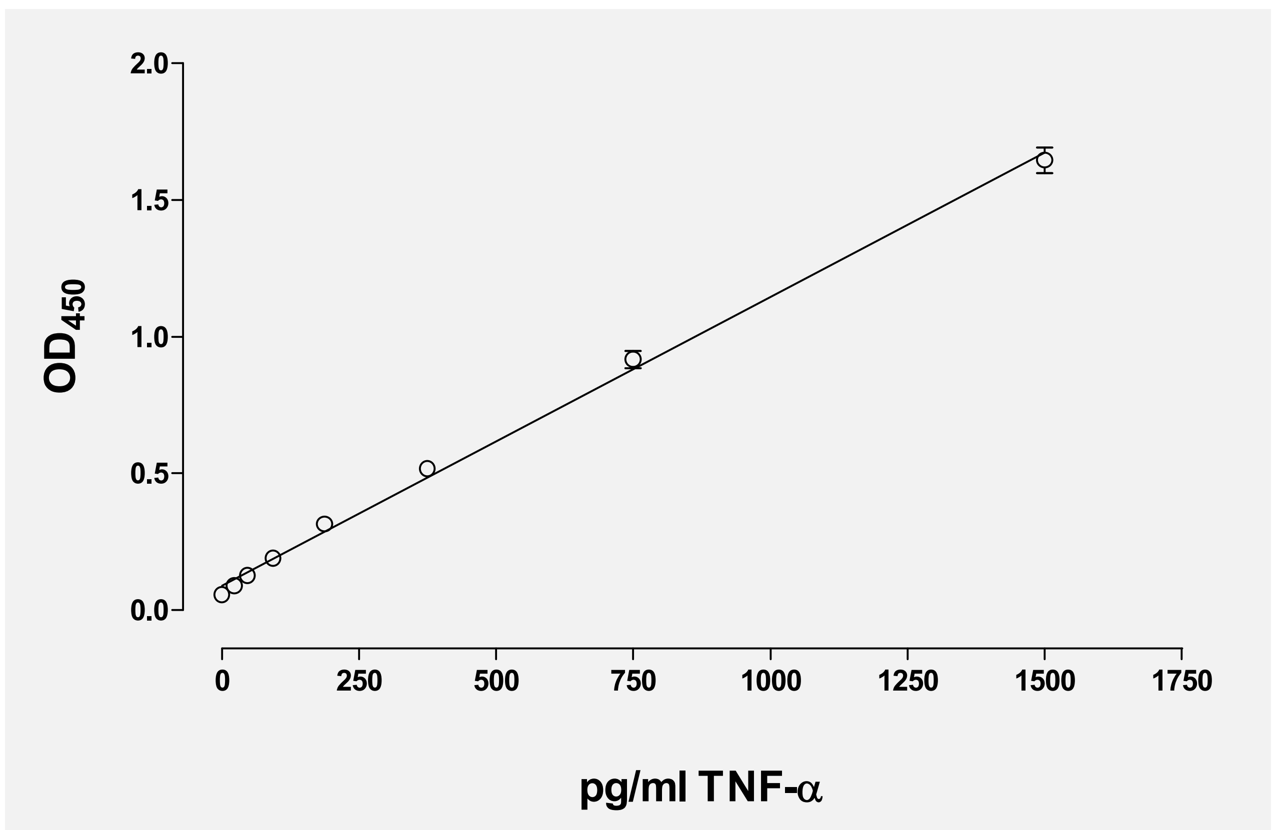


**Figure 2-3:- Standard curve from the TNF-α ELISA**

## 2.11 Brain volumetric, histological and immunohistological procedures

### 2.11.1 Determination of brain intracerebral haemorrhage area

Digital images obtained with a Canon EOS DSLR camera were made for the brain slices performed through the zone of substance injection on day 1 post operation. The damage area was selected using "ImageJ" software by drawing a contour line

around the damaged area. The whole brain slice area was also calculated the same way. Using Microsoft Excel, the percentage of damaged brain area was calculated as the area of the damaged brain tissue compared with the area of the whole hemisphere.

### 2.11.2 Brain oedema measurement

The brain was quickly removed and placed on a cooled surface. The frontal pole (approximately 3 mm thick), the cerebellum and brainstem were removed. The cerebrum was coronally divided into two pieces by sectioning through the needle entry site. The first piece (2 mm thick) was cut ipsilateral and contralateral to the intracerebral haemorrhage. These two ipsilateral and contralateral sections were then used for brain oedema measurement. The other two coronal sections were used for histological analysis (Zhang et al. 2006).

Each section was wrapped in preweighed aluminium foil and weighed to obtain the wet weight (WW) using an electronic analytical balance (Sartorius ultra-sensitive balance), then dried for 24h in an oven at 110°C and weighed again to obtain the dry weight (DW). Brain water content is calculated as the percentage change using the following formula:

**Equation 2-6 Calculation of brain water content**

$$\text{Brain water content} = \left( \frac{WW - DW}{WW} \right) \times 100$$

### 2.11.3 Hematoxylin and eosin staining

Slides were treated with 8-Aminopyrene-1,3,6-trisulfonic acid trisodium salt (APTS) solution and air dried for 24 hours. Sample sections were cut at a thickness of 5 µM (Shandon Retraction AS325 microtome) and placed on APTS slides. Each sample was air dried over night at 60˚C.

Slides were dewaxed with standard procedures using 100% xylene, industrial methylated spirit (IMS) at different concentrations (absolute IMS, 90%, 70%) and running tap water.

Slides were then stained using Gill's hematoxylin (Ethylene glycol 25%, Acetic acid glacial 2%, Aluminum sulfate octadecahydrate 1.8%, Hematoxylin <1%, Sodium iodate 0.004%, Water balanced), differentiated in acid-alcohol, washed with hydrochloric acid (1% HCl in 70% IMS) and then rinsed in running tap water. Slides were then dehydrated using different concentrations of IMS (70%, 90%, absolute IMS) and stored in xylene until ready to mount.

### 2.11.4 Myeloperoxidase staining

Anti-Myeloperoxidase antibody reacts with myeloperoxidase. It stains granules of neutrophils, granulocytes in spleen, bone marrow, tonsil and blood smears (http://www.dako.com/uk/ar45/p110300/prod_products.htm).

Slides were treated with APTS solution and air dried for 24 hours. Sample sections were cut at a thickness of 5 µM (Shandon Retraction AS325 microtome) and placed on APTS slides. Each sample was air dried over night at 60˚C. Slides were

dewaxed with standard procedures using xylene, IMS at various concentrations (absolute IMS, 90%, 70%) and running tap water.

Antigen retrieval was performed using a citrate buffer in a pre-heated water bath for the purpose of breaking the protein cross-links, therefore unmasking the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibodies (Lin & Prichard 2011). After that, sections were then washed in PBS and left to cool.

Liquid blocker pen (Dako UK Ltd) was used to contour the tissue in the slides to prevent the leak of liquids used. Normal goat serum was used to block non-specific-binding in sections. Primary anti-human myeloperoxidase antibody (Dako UK Ltd) in 1:250 dilution was placed in the tissue on the slides and incubated overnight at 4˚C.

Next morning, the slides were washed with PBS, treated with 3% $H_2O_2$ (Sigma) diluted in PBS for 30 minutes and washed with PBS again. ImmPressKit secondary antibody (Vector) was applied to the slides afterwards. Before sections were exposed to 3,3´-diaminobenzidine tetrahychloride (DAB, Research Genetics) at a room temperature, they were washed in PBS and left to air dry.

Sections were counterstained with hematoxylin (Gong, Hoff, & Keep 2000) according to the standard staining procedure. Slides were stained using Harris Hematoxylin, differentiated in acid-alcohol, washed with hydrochloric acid (1% HCl in 70% IMS) and then rinsed in running tap water. Slides were then dehydrated using ascending concentrations of IMS (absolute IMS, 90%, 70%) and stored in xylene until ready to mount.

### 2.11.5 OX42 staining

Anti-CD11b/c equivalent antibody (OX42) recognises a common epitope shared by CD11b/c and precipitates three polypeptides of Mw160kD, 103kD and 95kD, it binds to cells with microglial morphology in brain (http://www.abdserotec.com/catalog/datasheet-MCA275PE.html).

Slides were treated with APTS solution and air dried for 24 hours. Sample section were cut at a thickness of 5 μM and placed on APTS slides. Each sample was air dried overnight at 60˚C. Slides were dewaxed with standard procedures using xylene, IMS in different concentrations (absolute IMS, 90%, 70%) and running tap water.

Antigen retrieval was performed using a citrate buffer in a pre-heated water bath for the purpose of breaking the protein cross-links, therefore unmasking the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibodies. After that, sections were washed in PBS and left to cool (Lin & Prichard 2011).

Liquid blocker pen (Dako UK Ltd) was used to contour the tissue in the slides to prevent any leakage of liquids used. Goat serum was used to block a non-specific-binding in sections. Primary antibody (AbD Serotec) was applied to slides and left overnight at 4˚C.

Next morning slides were washed with PBS, treated with $H_2O_2$ (Sigma) and washed with PBS again. ImmPressKit secondary antibody (Vector) was applied to the slides afterwards. Before sections were exposed to 3,3´-diaminobenzidine tetrahychloride (DAB, Research Genetics) at room temperature, they were then washed in PBS and left to air dry. Sections were counterstained with hematoxylin (Gong, Hoff, & Keep 2000) according to the standard staining procedure. Slides were stained using Harris Hematoxylin, differentiated in acid-alcohol, washed with hydrochloric acid (1% HCl in 70% IMS) and then rinsed in running tap water. Slides were then dehydrated using different concentrations of IMS (absolute IMS, 90%, 70%) and stored in xylene until ready to mount.

### 2.11.6 Densitometric cell count

Densitometric analysis was performed on the brain slices stained for MPO, OX42 and H&E using the freely available software “ImageJ”. Slice images were taken using DPX controller software microscopically at 400 times optical magnification in 7 randomly chosen different fields of view. Images were converted into a 16 bit image. The threshold for red filter for MPO stained neutrophils was set up to 0/100 and calculated by particle analysis with pixel size set from 50 to infinity. Threshold for all granular immune cells was set to 130 and calculated the same way. The number of microglial cells was calculated by substraction of MPO stained cells from the number of total granular immune cells. The number of cells was calculated in all the fields of view and presented as a mean for each slice.

MPO positive stained cells were identified by the positive immunological DAB staining (with nuclear counterstaining) at x400 magnification. Microglial cells were identified by the positive immunological DAB staining at x400 magnification. An example

of positive staining for neutrophils and microglia/macrophages is represented in Figure 2-4.

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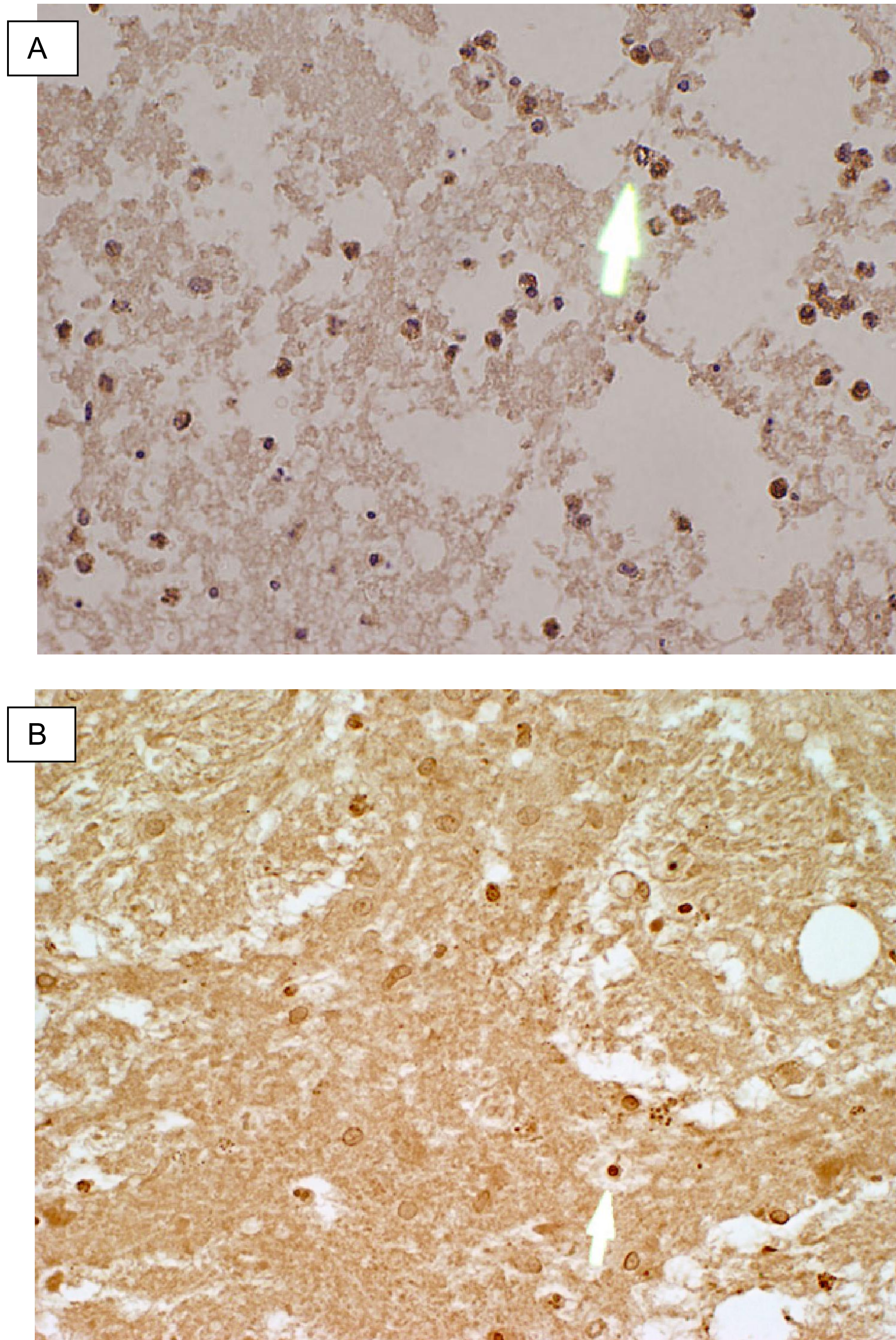


**Figure 2-4 Positive MPO stained neutrophil, counterstained with hematoxylin (A). Positive OX-42 stained microglia cells/macrophages (B). X 400 magnification**

## 2.12 Behavioural testing

Six behavioural tests were used: beam walking, beam balancing, tape removal, forelimb placing, semi-quantitative examination and the forelimb asymmetry test. All animals were trained for 14 days prior to the experimental date. Behavioural testing was performed on the rats at 1, 3, 5, 7, 13 and 21 days post operation. All test scoring was performed by an independent observer blinded to the condition of the animal.

### 2.12.1 Beam walking

Rats were observed crossing the midline segment of a 1 m long horizontal beam. The hind limb use was analysed according to Feeney et al (Feeney, Gonzalez, & Law 1982). Performance was graded as:

0 - rat falls off the beam within 10 sec

1 - rat remains on the beam for more than 19 sec but cannot place the affected limb on the beam

2 - rat is unable to cross but can place affected limb on the beam and maintain balance

3 - rat traverses the beam while dragging the affected limb

4 - rat crosses the beam and places the affected limb on the beam at least once

5 - rat crosses with more than 50% foot slips with the affected limb

6 - rat crosses with fewer than 50% foot slips with the affected limb

7 - rat crosses with 2 or fewer foot slips

Performance of each test day was expressed as an average score of three trials (MacLellan et al. 2006).

### 2.12.2 Beam balancing

The rats were observed by eye, balancing on a beam 2 cm wide and 0.5 m long. The duration of time the animals remained on the beam was recorded for up to 60 sec. Performance of each test day has been expressed as an average score of 10 trials.

### 2.12.3 Tape removal

Sticky dot labels were placed on the contralateral (CLP) and ipsilateral (ILP) paws. The rats were videotaped and the time taken to remove the sticky labels recorded (Figure 2-5).

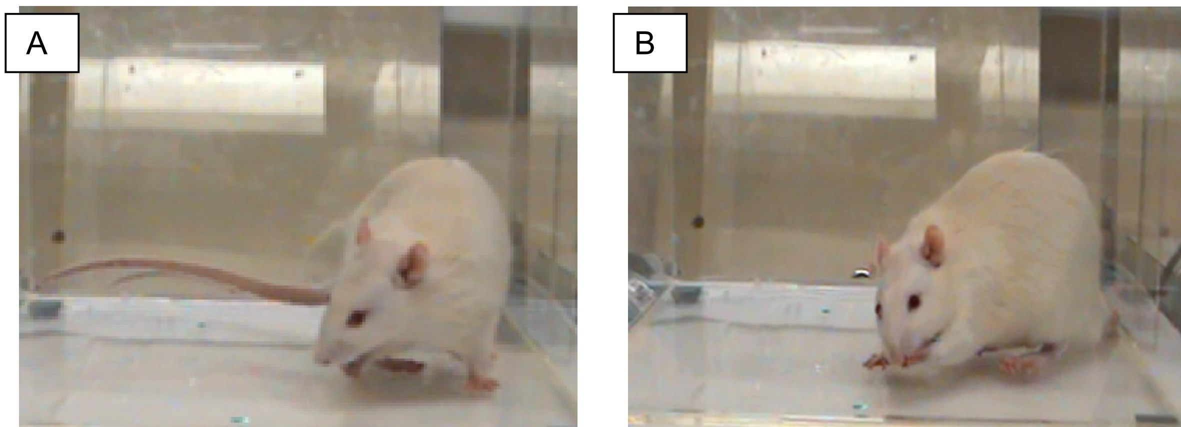


**Figure 2-5 Tape removal test: A - evaluation of time for rat to remove label from right paw, B - evaluation of time for rat to remove label from left paw**

The performance was recorded in seconds and calculated according to the equation below (MacLellan et al. 2006).

**Equation 2-7 Calculation of the score of the tape removal test**

$$\% \text{ deficit} = \left( \frac{\text{Time to remove label from CLP} - \text{Time to remove label from ILP}}{\text{Number of trials}} \right)$$

### 2.12.4 Forelimb placing

Animals were held by their torso, which allowed the forelimbs to hang free. The animal was gently moved up and down before the placing testing to facilitate muscle relaxation and eliminate any struggling movement. Trials during which extreme muscle tension, struggling, or placing of any of the limbs onto the experimenter's hand occurred were not counted. Independent testing of each forelimb was induced by brushing the respective vibrissae on the corner edge of a countertop. Untreated animals place the forelimb ipsilateral to the stimulated vibrissae quickly onto the countertop. Depending on the extent of injury, placing of the forelimb contralateral to the injury in response to contralateral vibrissae contact with the countertop may be impaired. In the experiments each rat was tested 10 times with each forelimb, and the percentage of trials in which the rat placed the appropriate forelimb on the edge of the countertop in response to the vibrissae stimulation was determined (Hua et al. 2002).

### 2.12.5 Semi-quantitative examination

A semi-quantitative neurological examination was used to evaluate the motor performance of the rats prior to, then 1 day, 3, 5, 7, 14 and 21 days after surgery. The behavioural test, scored between 0 (poor or absent) and 4 (normal performance), comprised the following tests: activity, locomotion, positional passivity, visual positioning, climbing, tail rigidity, tremor and hopping, and have been evaluated as:

1 Activity: exploration of the immediate environment when the rat is placed on a novel hard surface (0: no exploration; 1: exploration with head movements only;

2 2: exploration of the immediate environment; 3: normal exploration);

3 Locomotion: the rat is placed on a hard surface and forward progression is observed (0: no displacement; 1: unilateral rotation only; 2: incomplete body movements on both sides, forward progression mainly by rotation; 3: normal linear progression);

4 Positional passivity: during hand restraint, the affected posterior limb (left) is pulled away from the animal and the motor response is observed (0: no flexion of the limb; 1: flexure movements on occasions; 2: normal flexions upon each extension);

5 Visual positioning: the rat is held by the tail above a contact surface and displaced toward a hard surface and the visual positioning of both limbs is evaluated (1: contact with one limb only, other held in a flexed position; 2: partial flexure of the affected limb; 3: both limbs extended to make contact with normal walking movements);

6 Climbing: climbing on a wire grid while skill and symmetry of forelimbs are observed (1: holds wire but cannot let go; 2: tries to climb but has difficulty to do so, little forward movements with important asymmetry; 3: climbs but the movement is moderately asymmetrical; 4: normal climbing, symmetrical movements of both forearms are observed; tail rigidity: the tail is elevated at mid-length and rigidity is observed (1: no flexibility observed, if placed in a curved position remains as such; 2: moderate rigidity; 3: normal flexibility and movements);

7 Tremor: animal is held by the tail in an elevated position so that the front paws remain in contact with a hard surface, stability of hind paws is observed (1: tremor; 2: no tremor);

8 Hopping: the rat is held by hind legs and one forepaw so that the entire weight of the animal is supported by one limb. The animal is moved laterally and hopping movements are evaluated to assess postural adjustments (0: no postural adjustment; 1: delay of the initiation of movement to adjust posture of the affected limb (left) 2: normal postural adjustment) (Ferland, Veilleux-Lemieux, & Vachon 2007).

### 2.12.6 Forelimb asymmetry

Forelimb use during exploratory activity was analysed by videotaping rats in a transparent plastic box for 3 to 10 minutes depending on the degree of activity during the trial. A mirror was placed to one side of the cylinder at an angle to enable the recording of forelimb movements even when the animal was turned away from the camera. Scoring was performed using a video cassette recorder with slow-motion and clear stop-frame capabilities. The behaviour was scored according to the following criteria: (1) independent use of the left or right forelimb for contacting the wall; and (2) simultaneous use of both the left and right forelimbs for contacting the cylinder wall during a full rear and for alternating lateral stepping movements along the wall (Figure 2-6).

**Figure 2-6 Forelimb asymmetry test: The experimental animal was placed into a transparent plastic box. Evaluation of exploratory activity of the rat was by calculation of the number of contacts of the paws with the walls of the box**

Behaviour was quantified by determining the number of occasions when the unimpaired (ipsilateral) forelimb was used as a percentage of the total number of limb use observations on the wall (A); the occasions when the impaired forelimb (contralateral to the injection site) was used as a percentage of the total number of limb use observations on the wall (C); and the occasions when both forelimbs were used simultaneously (or nearly simultaneously during lateral side-stepping movements) as a percentage of the total number of limb use observations on the wall (B). A single overall limb use asymmetry score was calculated as follows: Limb Use Asymmetry (Hua et al. 2002).

**Equation 2-8 Score calculation of the forelimb asymmetry test. A is a percentage of unimpaired limb use on the wall to the total number of forelimb use, C is a percentage of impaired limb use on the wall to the total number of forelimb use, B - is a percentage of both limbs use on the wall to the total number of forelimb use**

$$score = \frac{A}{(A + C + B)} - \frac{C}{(A + C + B)}$$

## 2.13 Statistical evaluation

The results obtained for each test were statistically examined using SPSS Statistics 16.0 software (IBM). Significance between the animal groups differences was evaluated using a non-parametric Mann-Whitney and Kruskal-Wallis test with Bonferoni adjustment. Significance value between the groups was evaluated as <0.05 for Mann-Whitney and for Kruskal-Wallis one-way analysis of variance test respectively.

# Chapter III. The development of the haemorrhagic stroke model

## 3.1 Introduction

An ideal experimental haemorrhagic stroke model should have the following characteristics: 1) blood deposition in a distribution consistent with the type of haemorrhage desired; 2) uniform degree of haemorrhage; 3) a mechanism of haemorrhage which closely simulates the human condition; 4) easily performed; 5) reasonable cost. The most common experimental intracerebral haemorrhage stroke models are: the blood injection model and bacterial collagenase model. It is very important to choose an appropriate model for haemorrhagic stroke modelling as it should provide an opportunity for thorough study of all aspects of the disease. This could eventually help develop new approaches in treatment for the patients suffering haemorrhagic stroke, which is especially important as so few successful treatments are available.

## 3.2 Materials and methods. Experimental protocol.

| | |
|---|---|
| Group 1 | Injection of 100µl of autologous blood taken from a rat tail vein and administered into the lateral striatum (n=6) |
| Group 2 | Injection of 2 µl of 0.2 U of collagenase IV(S) into the lateral striatum (n=6) |
| Group 3 | Injection of 2 µl of 0.5 U of collagenase IV(S) into the lateral striatum (n=6) |

## 3.3 Results

The blood injection group (group 1) proved to be an inconsistent model, as the area of damage varied from random blood distribution and in some instances no blood being injected at all due to slow blood infusion and consequent clotting within the injection tubing system. These data are represented in Figure 3-1.

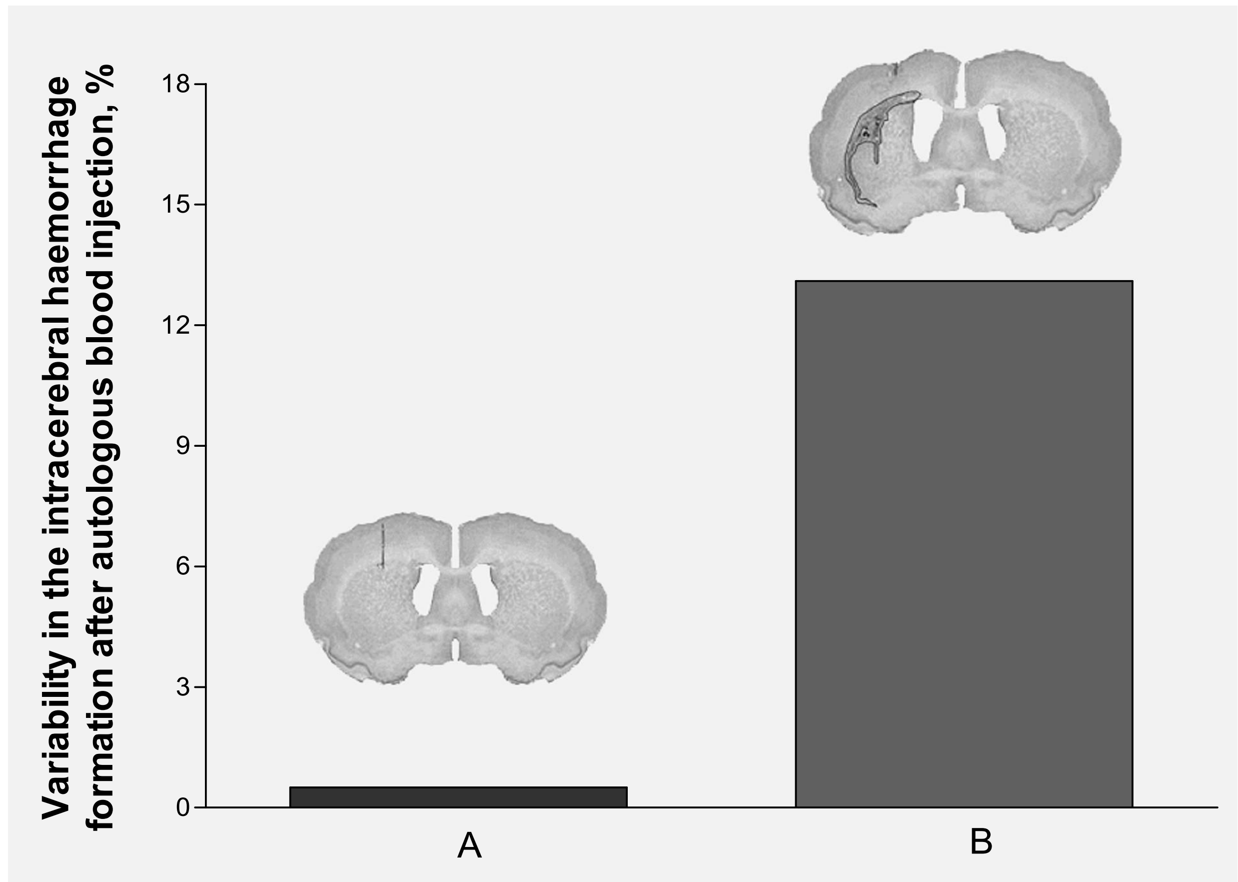


**Figure 3-1 Schematical representation of the intracerebral haemorrhage formation after injection of 100 μl of autologous blood into the right striatum. Blood distribution after the leakage from the injection tract (A) and intracerebral haemorrhage formation (B) after injection of 100 μl of autologous blood.**

The group treated with 0.2 U of collagenase (group 2) showed consistent results in blood distribution in the striatum in which the intracerebral haemorrhage occupied 30% of the hemisphere. The group of animals treated with 0.5 U of collagenase (group 3) led to a formation of the intracerebral haemorrhage which occupied up to 85% of the hemisphere and showed 50% mortality rate 24 hours post treatment. The schematic

intracerebral haemorrhage formation in the 0.2 U (group 2) and the 0.5 U (group 3) collagenase injection groups is presented on the figure below.

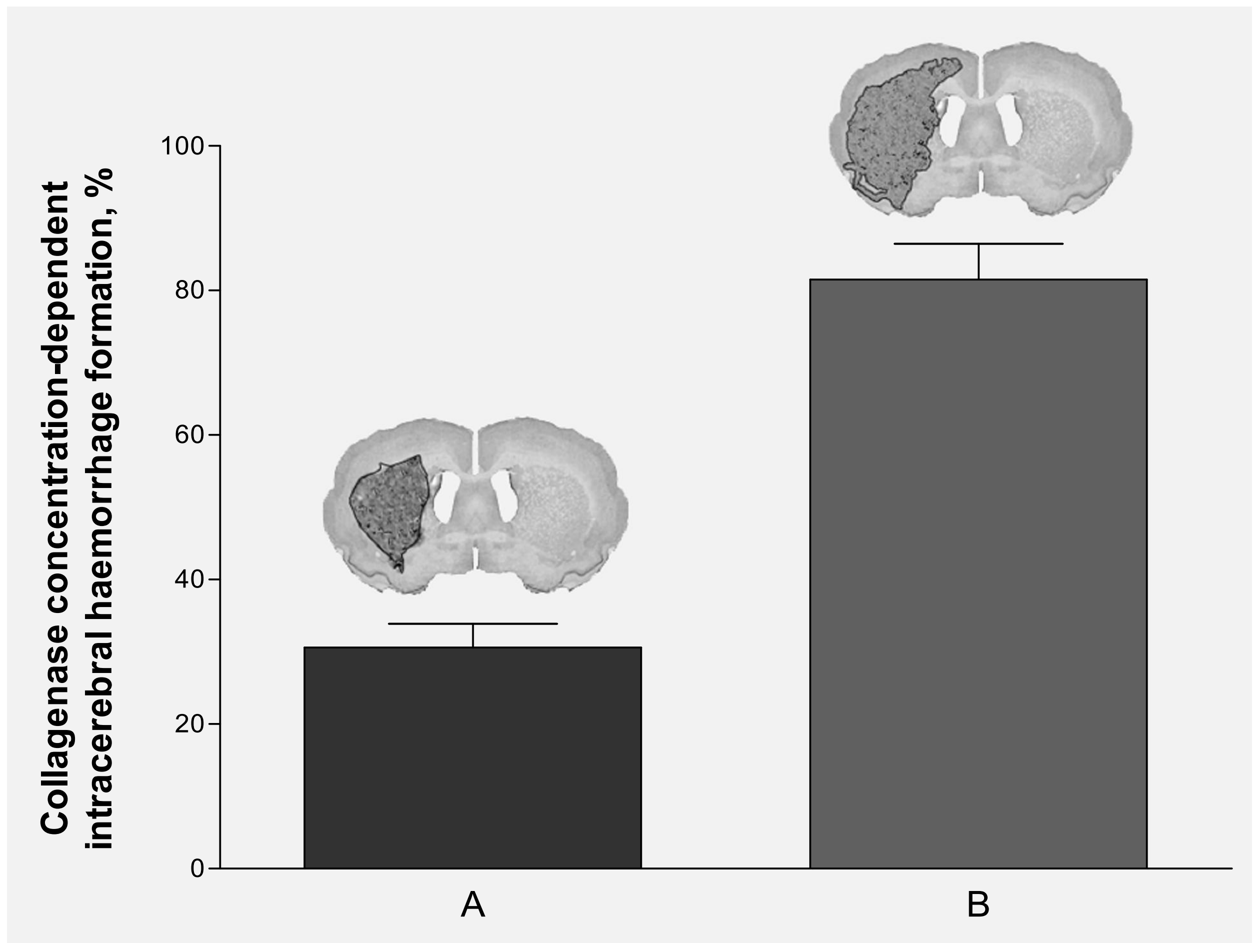


**Figure 3-2 Schematical representation of the intracerebral haemorrhage formation after injection of the collagenase into the right striatum, coronal section performed through the zone of injection. A- 0.2U/2 µl collagenase injection (group 2), B – 0.5U/2ul collagenase injection (Group 3). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively.**

### 3.4 Discussion

Reproducibility in any animal model of disease is of paramount importance in discerning the effects of therapies investigated. This is especially true of an intracerebral haemorrhage model where multiple factors are at work. A comparison of the haemorrhagic stroke zone in the models investigated showed that the collagenase injection model best reflects what is reported to occur in stroke-patients.

Our experiments have shown that the use of the collagenase injection model results in a higher reproducibility in the damage found in the brain when compared to the blood injection model and our data corresponded to the data obtained by other authors (Beray-Berthat et al. 2010).

The inconsistent nature of the blood distribution in the blood injection model can be explained by a number of reasons: large amount of blood injected, partial clotting of the blood in the needle, blood leakage along the needle tract, high pressure due to the volume injected and thus random distribution in the tissues.

As for the collagenase injection model, it was shown previously that injecting 2 µl of collagenase resulted in a consistent and reproducible level of damage (Rosenberg et al. 1990). According to Rosenberg *et al*. the use of 1U of collagenase or above was a lethal dose and only dosages below that level should be considered. In our results, 0.5 U collagenase in 2 µl resulted in a 50% mortality within 24 hours which was too high and thus the model was rejected. Instead we reduced the collagenase concentration to 0.2 U in 2 µl and achieved consistent results in blood distribution in the lateral striatum. The selected concentration was in line with other groups who are also investigating haemorrhagic stroke (MacLellan et al. 2007).

According to data collected on the basis of macroscopical examination of the haematoma formation and blood distribution, 0.2 U collagenase was chosen as the model for intracerebral haemorrhage research by our group.

Standardisation of the approach in experimental haemorrhagic stroke, beginning with the choice of model, is the primary goal for obtaining comparable data. This set of experiments was therefore successful.

# Chapter IV. Release profile, CORM-3 effects on cell and brain tissue culture toxicity and viability.

## 4.1 Introduction

Studying haemorrhagic stroke and the possible effects of CORM-3 on its development requires certain subsidiary data, among which is building up a profile of CO-release from the compound, as the synthesis of such compounds is not standardised yet and CO-RMs have some variations in composition from batch to batch.

CORM compounds have shown a variety of bioactive properties including the ability to modulate cellular reactivity and inflammatory reactions occurring in living organisms (Cepinskas et al. 2008;Desmard et al. 2005;Fiumana et al. 2003;Huang et al. 2002;Katada et al. 2009a;Katada et al. 2009b;Motterlini 2007;Motterlini, Mann, & Foresti 2005;Stefan W.Ryter and Leo E.Otterbein 2004).

The application of CO-RMs in experimental animal models of haemorrhagic stroke for prospective medical use requires investigation of their toxicity on the various cell types of the brain. Among the cells that have a crucial role in local inflammatory reactions in haemorrhagic stroke are astrocytes and microglial cells (Wang & Dore 2007).

Taking into account that the brain is a complex structure which has a variety of cell types, it is important to examine potential CORM-3 toxicity and viability on brain tissue cultures. So far only one paper that examines the function of CO in experimental brain slices in an attempt to analyse glutamate properties in arteriole smooth muscle cells has been published (Xi et al. 2010).

This chapter is focused on studying CORM-3 toxicity and viability on astrocyte, microglial cell and neonatal rat brain striatal tissue cultures.

## 4.2 Results

### 4.2.1 CORM-3 release profile

Having synthesised CORM-3 (a water soluble transition metal carbonyl) the first factor to establish was that it was capable of releasing CO. This was tested using the myoglobin assay technique.

Figure 4-1 (A) shows that CORM-3 converted deoxymyoglobin (deoxyMb) to MbCO. In this instance, there is little change from time zero to 60 min which means that CORM-3 almost instantly saturates the myoglobin solution with CO. The saturated MbCO curve, obtained using CO gas, represents the maximal CO capacity of the myoglobin stock prepared that specific day. Although the stock concentration of myoglobin is 66 µM we can only saturate it to ~51 - 54 µM using CO gas. This is because the myoglobin purchased from Sigma is impure and comes as 90% myoglobin; therefore, the concentration of MbCO we measure having saturated the myoglobin with CO gas is the real concentration of our myoglobin stock (54 µM is indeed ~90% of the expected 66 µM). Importantly, this effect was consistent throughout all myoglobin assays.

Subsequent conversion of the MbCO curve to empirical data (Figure 4-1 (B)) gives us an idea of the CO released in solution. CORM-3 released ~35 μM of CO instantly and then went on to release a maximal of ~40 μM CO after only 5 min. Thus, the kinetics of the reaction are rapid and the half life very short. The overall CO release suggests that one carbonyl group per CORM-3 molecule is liberated which is in agreement with earlier tests (Clark et al. 2003;Foresti et al. 2004a). The release of CO from CORM-3 was concentration dependent. Also, 20 μM and 60 μM CORM-3 released CO in an approximately 1:1 ratio. Addition of CORM-3 to biological buffers results in CO release and leaves an inactive CORM-3 (iCORM-3) not capable of further CO release (Clark et al. 2003;Foresti et al. 2004a) . This was demonstrated by using the myoglobin assay and showed that iCORM-3 was incapable of converting deoxyMb to MbCO.

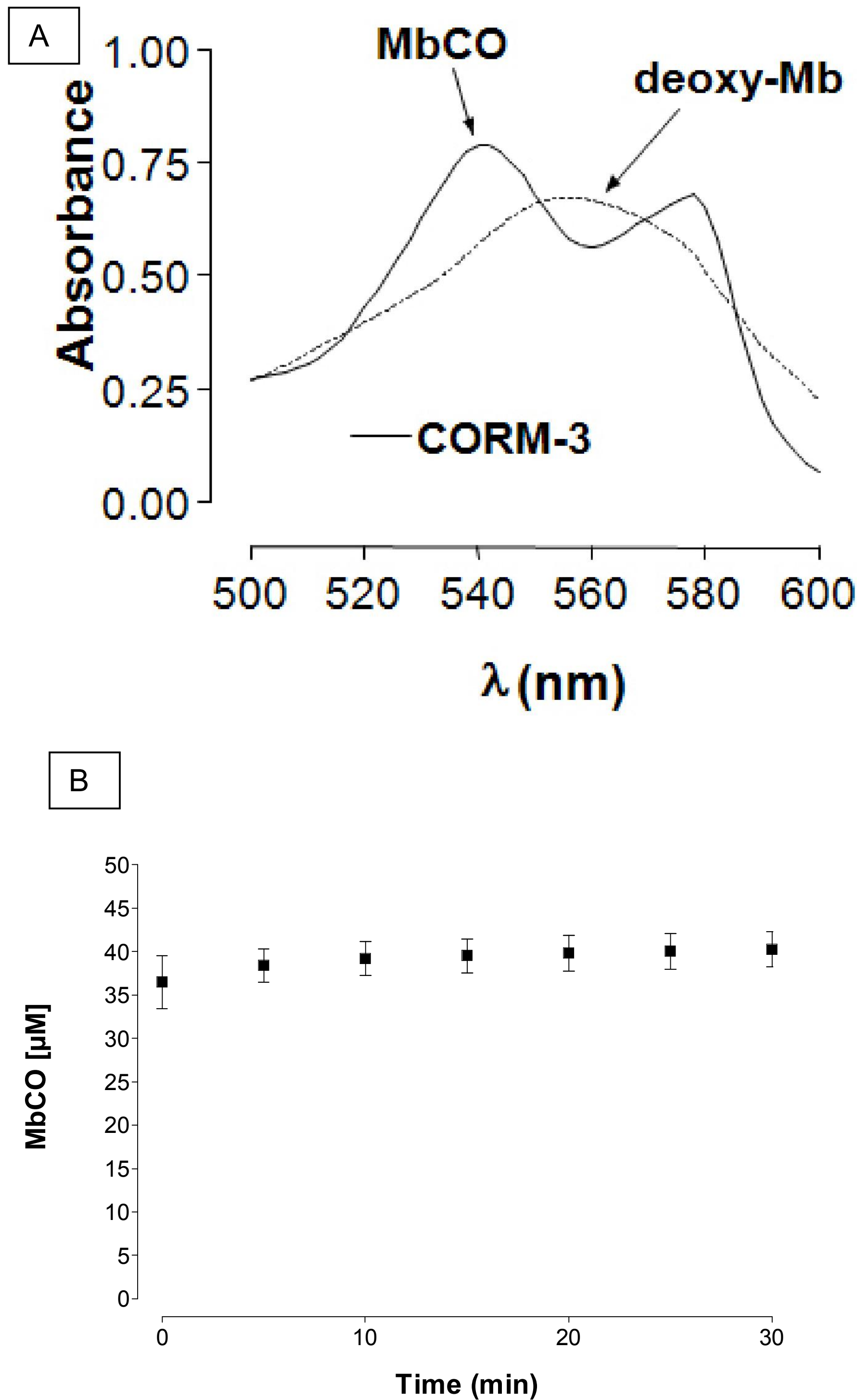


**Figure 4-1 CORM-3 releases a quantifiable amount of CO using the myoglobin assay. A - The maximal absorption peak of myoglobin at 560 nm is gradually converted over time by CORM-3 to spectra typical of MbCO with two maximal absorption peaks at 540nm and 578 nm, respectively. B – the concentration of CO in the solution. Data represent the mean ± SD.**

### 4.2.2 CORM-3 toxicity and viability of cell culture

LDH and alamar blue assays performed on BV-2 cells treated with triton (to achieve high lethality in the whole cell population as a positive control) and CORM-3 (10, 50 and 100 μM) indicated very low statistically non-significant toxicity of CORM-3 on microglial cells (BV-2 cell line) and 100% viability of the cells. These data are represented on Figure 4-2.

LDH and alamar blue assays performed on rat astrocytes (DI-TNC1 cell line) treated with triton (to achieve 100% lethality in cell population) and CORM-3 (10, 50 and 100 μM) indicated very low non-significant toxicity of CORM-3 on astrocytes and 100% viability of the cells. These data are represented on Figure 4-3.

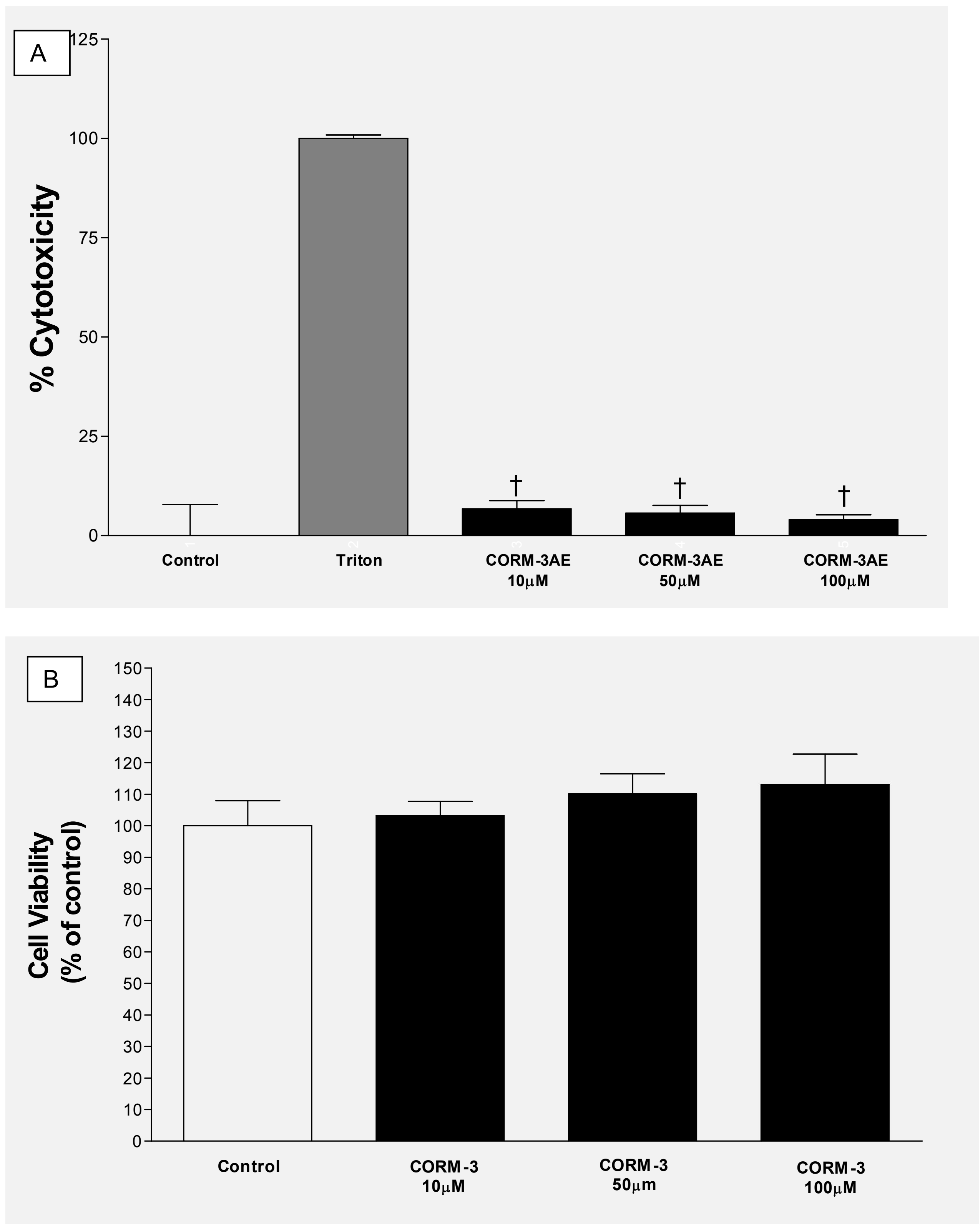


**Figure 4-2 Effects of CORM-3 on cytotoxicity (A) of microglial cells and their viability (B). Data represent the mean ± SD of 6 independent experiments.**

**(A) BV-2 cells were exposed to triton to achieve 100% toxicity and CORM-3 in 3 different concentration (10, 50 and 100 μM). † - $p < 0.05$ vs. triton**

**(B) Cell viability was assessed 24 h after exposure of BV-2 cells to triton and CORM-3 in concentration described above. Viability was expressed as percentage of control. Data represent the mean ± SD.**

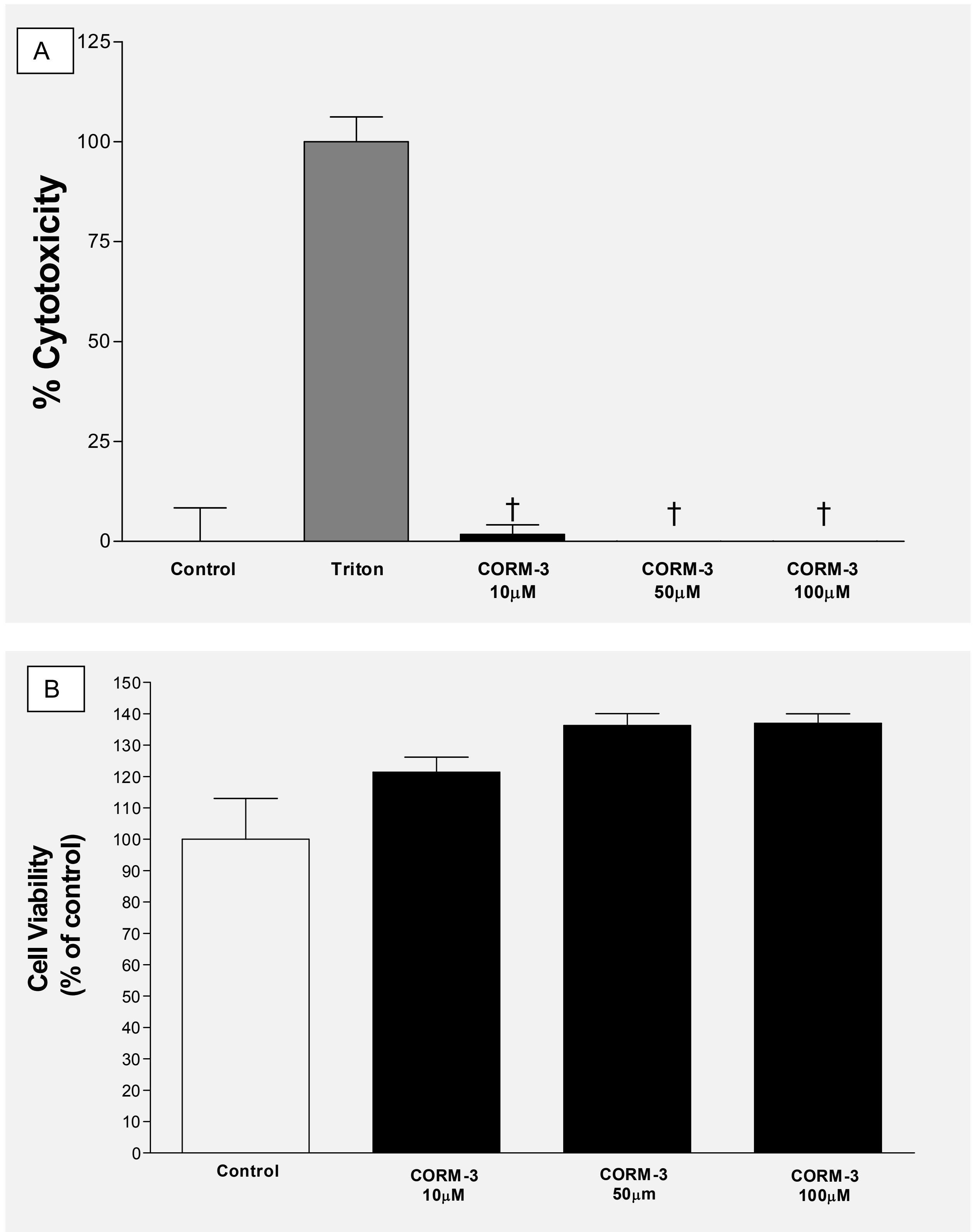


**Figure 4-3 Effects of CORM-3 on cytotoxicity (A) of astrocyte cells and their viability (B). Data represent the mean ± SD of 6 independent experiments.**

**(A) DI-TNC1 cells were exposed to triton to generate what was taken to represent 100% LDH release and CORM-3 in 3 different concentration (10, 50 and 100 µM). † - $p < 0.05$ vs. triton**

**(B) Cell viability was assessed 24 h after exposure of DI-TNC1 cells to triton and CORM-3 in concentration described above. Viability was expressed as percentage of control.**
**Data represent the mean ± SD.**

### 4.2.3 CORM-3 toxicity and viability of brain tissue culture

The control group (with no treatment applied to the brain striatal slices) demonstrated a few dead cells in the slice (Figure 4-4 A-1) and a high viability of the striatal slice (Figure 4-4 A-2). The negative control group treated with 1% triton demonstrated a high toxicity and a large amount of dead cells were observed in the striatal slice s(Figure 4-4 B-1), the viability respectively was very low and no live cells were observed in the areas examined (Figure 4-4 B-2).

Treatment of brain tissue slice cultures with CORM-3, 10, 50 or 100 μM did not cause significant changes in the number of dead cells observed (Figure 4-4 C-1, D-1, E-1) from those in the control group. Rat brain striatal slices remained highly viable after 24 hours treatment with 10, 50 or 100 μM of CORM-3 (Figure 4-4 C-2, D-2, E-2)

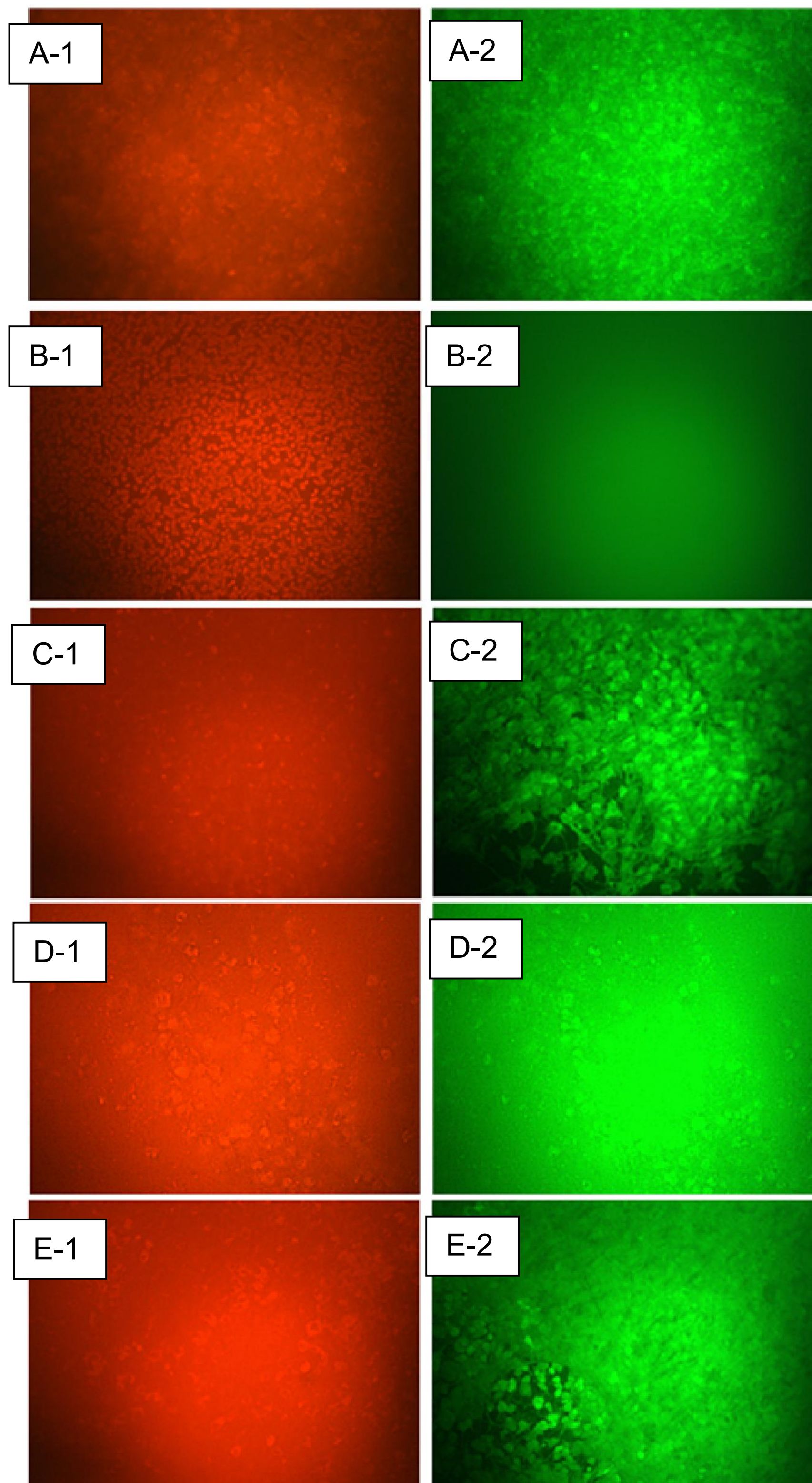


**Figure 4-4 Photographs of the brain striatal slices for qualitative analysis of the number of dead cells (photographs on the left at 100x magnification) and tissue viability (photographs on the right at 100x magnification): control (A-1, A-2), tissue treated with triton 1% (B-1, B-2), tissue treated with CORM-3 10 µM (C-1, C-2), tissue treated with CORM-3 50 µM (D-1, D-2), tissue treated with CORM-3 100 µM (E-1, E-2)**

## 4.3 Discussion

The CO-release profile of the CORM-3 batch used in our experiments indicated that this compound was a fast releasing molecule. CO was released rapidly and the maximum release of ~40 μM CO was observed after 5 minutes *in vitro*.

The high reproducibility of CORM-3's release profile lends itself well to its prospective use as a medication. CORM-3 was tested for toxicity and viability on rat astrocyte DI-TNC1, microglial BV-2 cell cultures and rat brain striatal tissue culture.

CORM-3 was used at 3 different concentrations (10, 50 and 100 μM) and did not show any significant damage to the cells both in BV-2 and DI-TNC1 cell lines that represent some of the main structural elements of the brain and are involved in haemorrhagic stroke. Cells did not suffer any decrease in viability after being exposed to CORM-3 treatment. Our results with BV-2 microglia mirrored the results obtained by our group previously (Bani-Hani, Greenstein, Mann, Green, & Motterlini 2006a), which indicated CORM-3 to be a non-toxic compound. As for the DI-TNC1 astrocyte cultures, we did not find any data that investigated CORM-3 toxicity in this cell type. CORM-3 was demonstrated to be non-toxic in other cell lines, such as murine macrophages (Sawle et al. 2005).

The use of CORM-3 in brain striatal slices also resulted in no toxicity when used at the same concentration as the cell lines, indicating no loss in viability of the slice cultures analysed. We did not find any paper published on the toxicity of CORM-3 in a model of brain tissue culture.

These findings demonstrate that CORM-3 can be used in brain tissue cultures and a brain slice model in pharmacologically active concentrations with no obvious toxicity or effect on viability which highlights the prospective use of the compound *in vivo* in an experimental model of HS.

# Chapter V. The inflammatory nature of haemorrhagic stroke

## 5.1 Introduction

The hypothesis that haemorrhagic stroke post-intracerebral haemorrhage is an inflammatory reparative process demands examination at both systemic and local levels with functional evaluation of the brain. In this respect the systemic responses are assessed in this thesis through blood cell analysis for inflammatory markers. At the local level volumetric brain water content analysis, densitometric analysis of the neutrophilic and microglial infiltration of the intracerebral haemorrhage zone provide indicators of the inflammatory response. Functional recovery was evaluated through behavioural tests. Experimental animals underwent surgical procedures with either saline or collagenase injection into the right striatum.

## 5.2 Materials and methods. Experimental protocol.

| | |
|---|---|
| Group 4<br>(Saline) | Injection of 2 μl of normal saline into the lateral striatum of the experimental animals (n=36) |
| Group 5<br>(Collagenase injection group) | Injection of 2 μl of 0.2 U collagenase solution into the lateral striatum of the experimental animals (n=36) |

## 5.3 Results

### 5.3.1 Systemic level of inflammation

***General blood analysis: White blood cell count***

The saline injection group (Group 4) did not display any significant changes throughout the days of the experiment.

The level of white blood cells in the collagenase injection group (Group 5) decreased on day 1, this was followed by an increase on day 3 and day 7, where the peak formed exceeded pre-operation data. Finally there was a decrease on day 14, reaching the levels of saline injection group (Group 4). These data are represented in Figure 5-1.

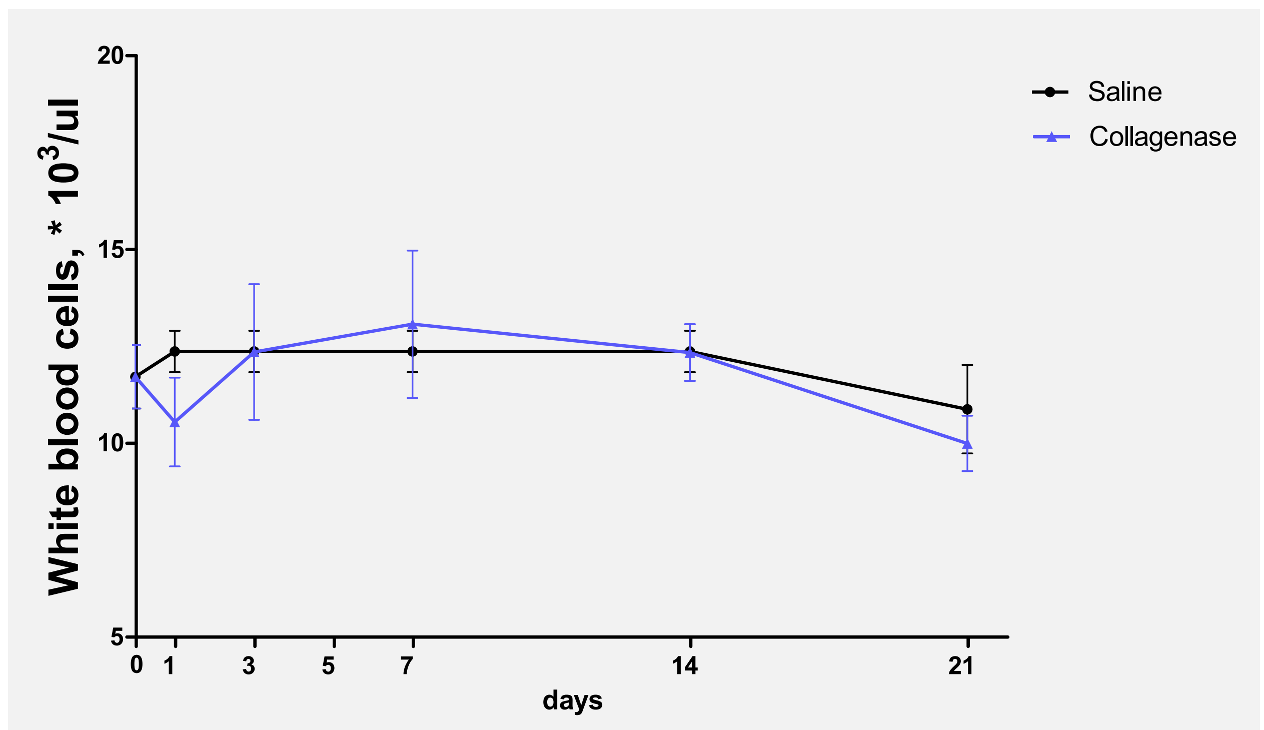


**Figure 5-1 White blood cell count: the saline (Group 4) and the collagenase injection group (Group 5). No statistically significant difference was observed. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively.**

The saline injection group (Group 4) showed a 5% increase on day 1 when compared to pre-operation data, which was lower than the increase seen in the collagenase injection group (Group 5), this increase returned to the level of pre-operation data by day 3 and remained so for the rest of the experiment.

The collagenase injection group (Group 5) showed a rapid statistically significant 10% increase in peripheral blood neutrophil numbers on day 1, a subsequent rapid decrease on day 3 (which was lower than pre-operational level by 5%), another 10% increase on day 7 and subsequent decrease and returning to pre-operation data by day 14 and 21. These data are presented in Figure 5-2.

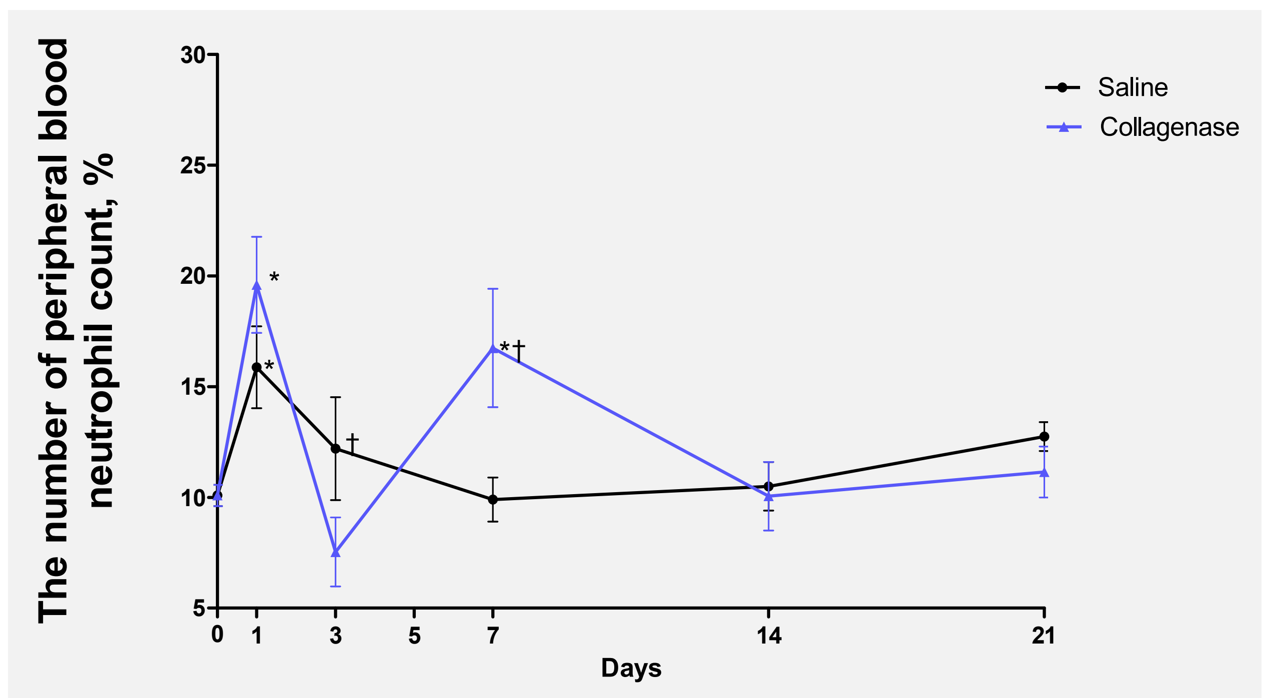


**Figure 5-2 Peripheral blood neutrophil count: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4)**

The saline injection group (Group 4) showed a small non-significant 2% decrease on day 1, a 4% increase on day 3, reaching pre-operation data levels by day 7 where it remained for the rest of the experiment.

The collagenase injection group (Group 5) demonstrated a 6% decrease in the number of peripheral blood lymphocytes on day 1, a rapid significant 12% increase on day 3, a subsequent decrease by day 7 (which was below pre-operational level). On the 14th day of the experiment there was a small increase in the number of peripheral blood lymphocytes in the collagenase injection group (Group 5) by 5% with a subsequent decrease reaching pre-operational levels by day 21. These data are presented in Figure 5-3.

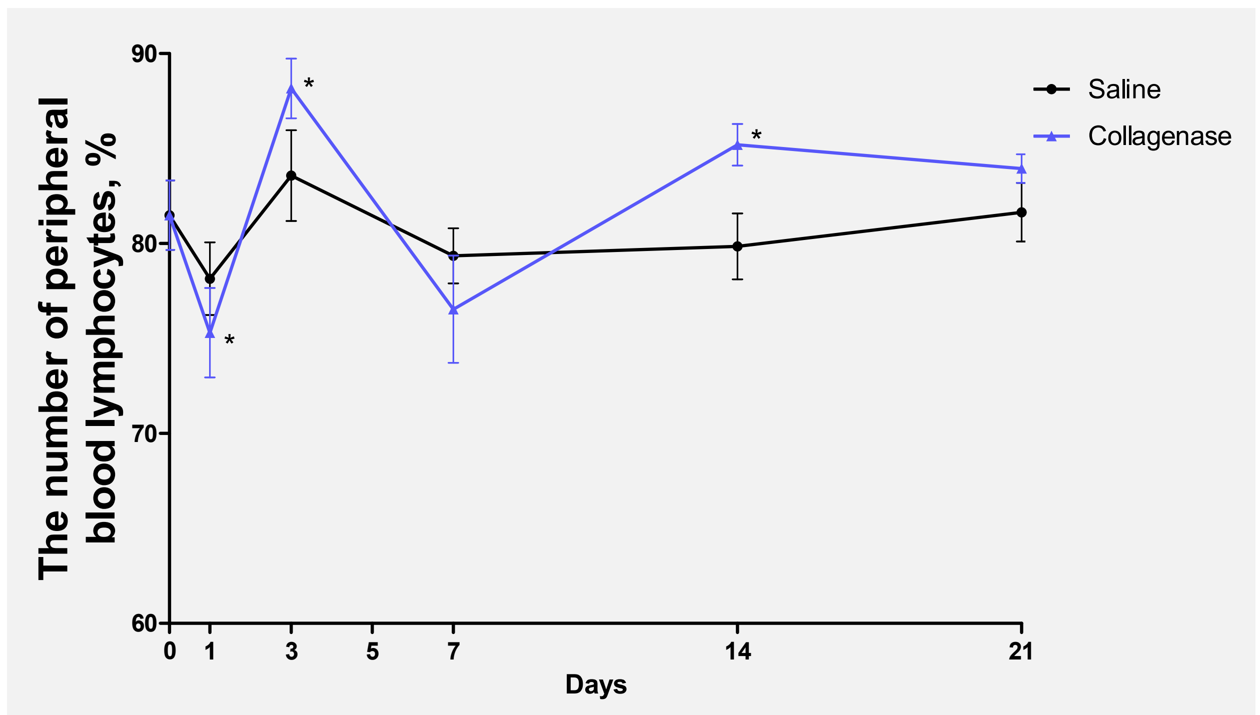


**Figure 5-3 Peripheral blood lymphocyte count: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data**

The leukocyte shift indicated a similar pattern in both the collagenase (group 5) and the saline injection group (Group 4); however the most pronounced variation belonged to the collagenase injection group (Group 5) and suggested the granular stage to be predominant over the first 3 days, with further predominance of an agranular stage around day 14. The rate of changes in leukocyte shift in the collagenase injection group (Group 5) on days 1 and 3 were 2-fold higher than in the saline injection group (Group 4). These data are presented in Figure 5-4.

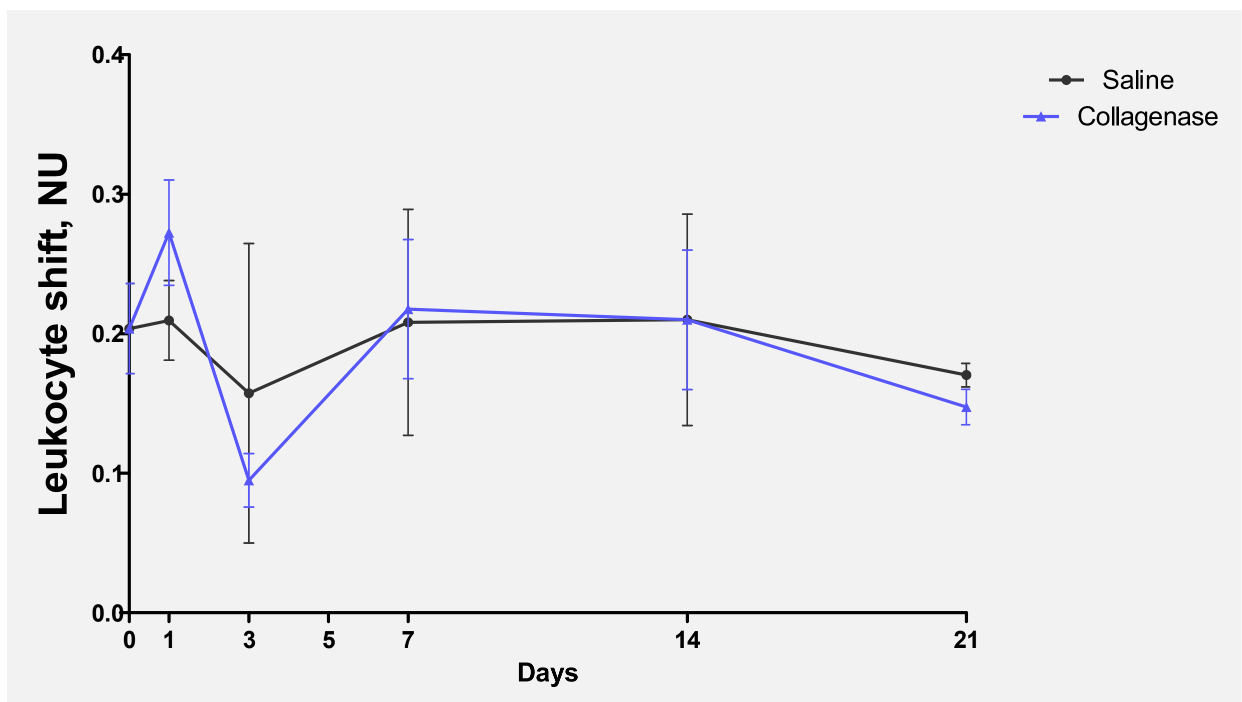


**Figure 5-4 Leukocyte shift: the saline (Group 4) and the collagenase injection group (Group 5) (normalised units). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No significant changes were seen.**

***Enzyme-linked immunosorbent TNF-alpha assay***

The saline injection group (Group 4) did not indicate any increase in the blood plasma TNF-alpha levels throughout the experiment. The collagenase injection group (Group 5) demonstrated a 15-fold increase in TNF-alpha levels in the blood plasma on day 1, compared to the saline injection group (Group 4) and pre-operation data. Further changes in blood plasma TNF-alpha levels in the collagenase injection group (Group 5) indicated a further gradual increase reaching the maximal peak by day 7, which was 23 fold higher than the one observed in the saline injection group (Group 4).

Subsequently in the collagenase injection group (Group 5) blood plasma TNF-alpha levels remained high, with a small decrease on day 14. These data are presented in Figure 5-5.

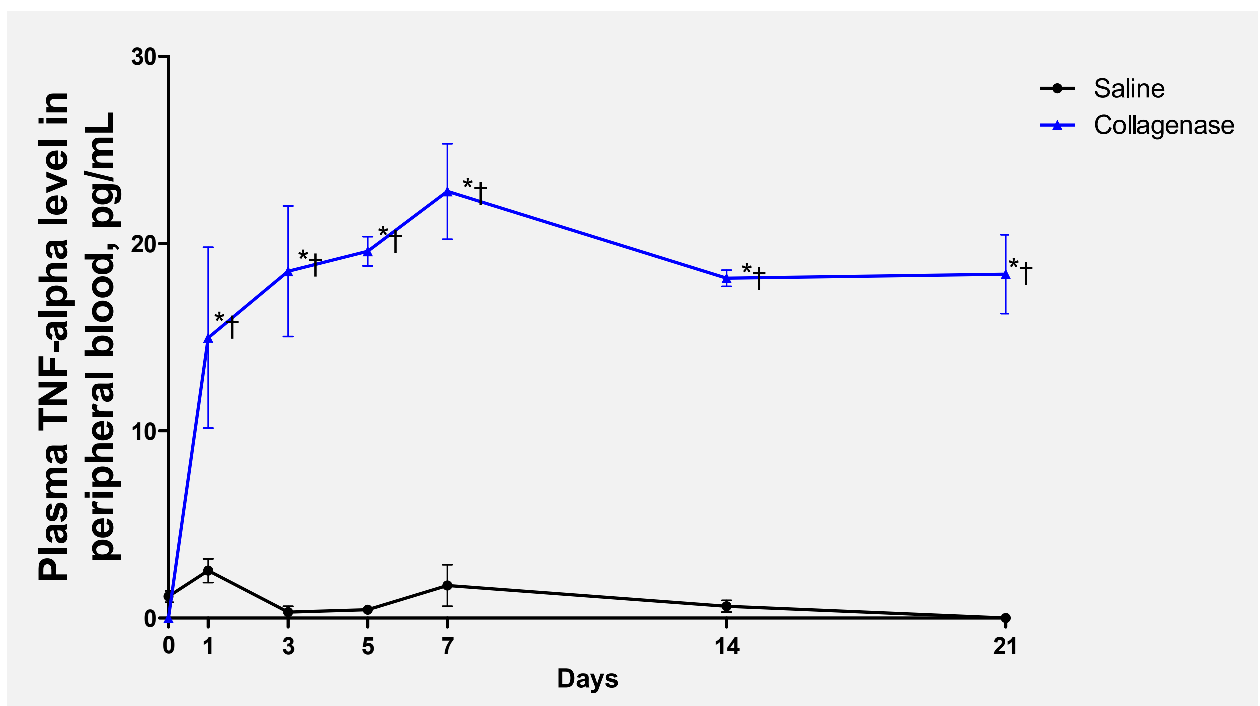


**Figure 5-5 Blood plasma TNF-alpha ELISA assay: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4)**

### 5.3.2 Local level of inflammation

***Determination of brain intracerebral haemorrhage area***

There was no intracerebral haemorrhage formation in the saline injection group (Group 4). In contrast, the injection of collagenase led to a significant intracerebral haemorrhage development. Intracerebral haemorrhage occupied 30% of the affected brain hemisphere. These data are presented in Figure 5-6 and Figure 5-7.

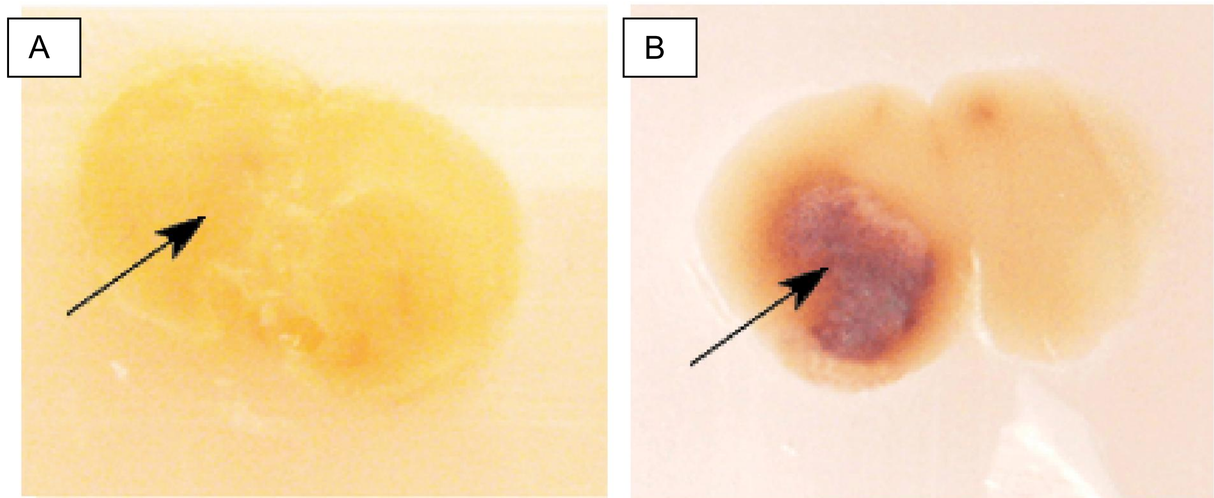


**Figure 5-6 Images of brain slices through the zone of interest obtained on the first day post operation (arrow indicates cross section of area in which either saline or collagenase was injected): A - saline injection – no visible presence of the intracerebral haemorrhage, B - Collagenase injection – visible presence of the intracerebral haemorrhage.**

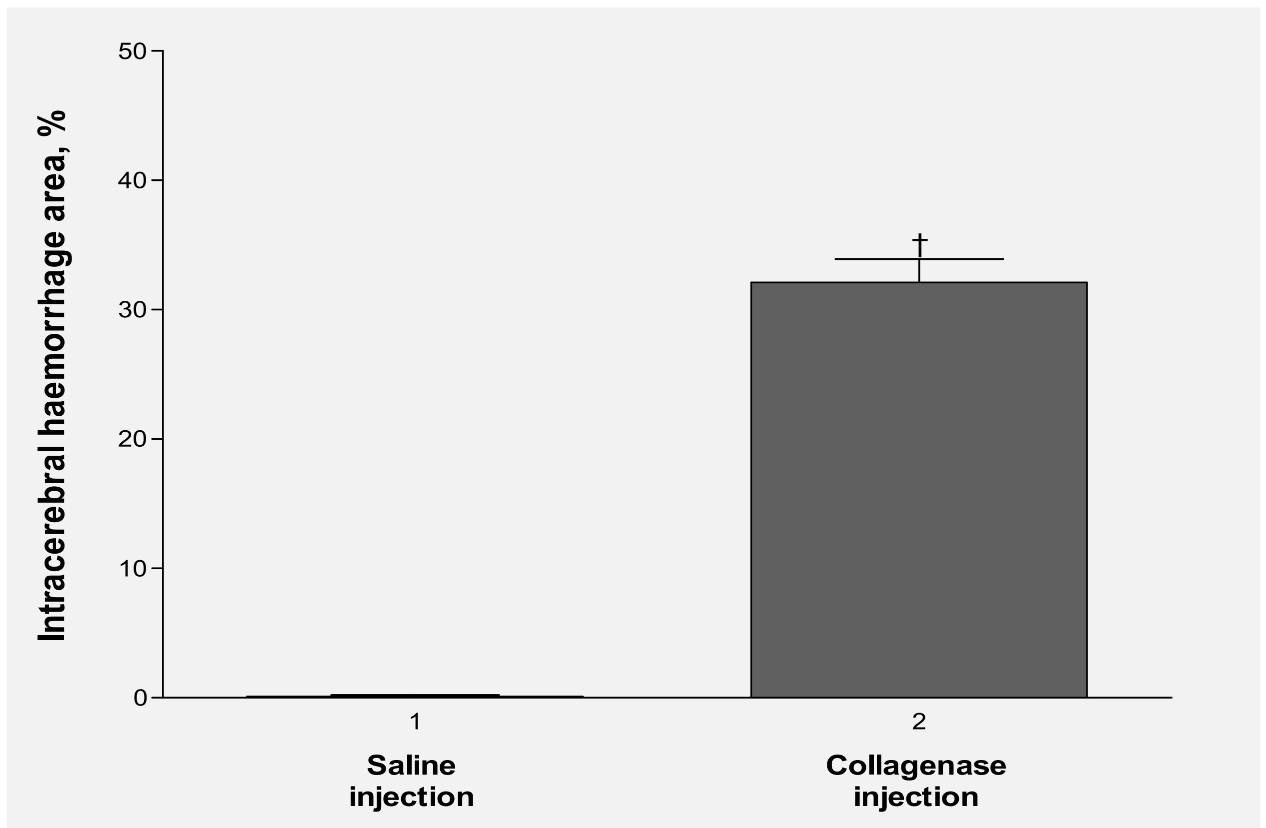


**Figure 5-7 Size of the intracerebral haemorrhage area. Data represent the mean ± SD of n = 6 independent experiments. † - $p < 0.05$ vs. saline**

***Brain water content analysis***

There were no significant changes to brain water content throughout the experiment in the saline injection group (Group 4).

There was a small increase in the brain water content of the unaffected controlateral hemisphere in the collagenase injection group (Group 5), which was significantly higher than in the saline injection group (Group 4). This subsequently decreased by day 5 and reached the levels of the saline injection group (Group 4) and pre-operation data by day 7 and remained stable until day 14. These data are presented in Figure 5-8.

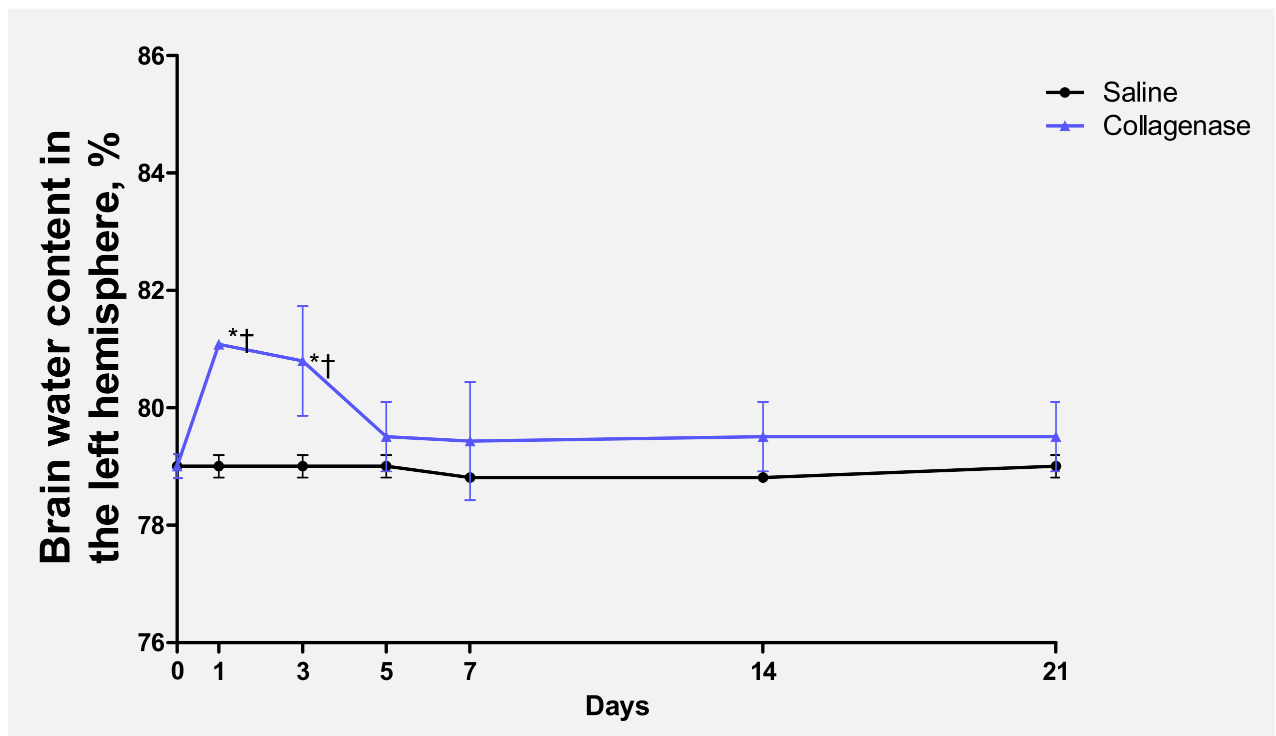


**Figure 5-8 Brain water content: the collagenase (Group 5) and the saline injection group (Group 4), left hemisphere. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4)**

There were no significant changes to brain water content throughout the experiment in the saline injection group (Group 4).

There was a significant increase in brain water content of the affected hemisphere on day 1 and 3 in the collagenase injection group (Group 5) compared to the saline injection group (Group 4). This decreased by day 5 reaching the level of the saline injection group (Group 4) and pre-operation data.

The 7th day of the experiment showed a small increase in the brain water content in the collagenase injection group (Group 5) with further decrease to the saline injection group (Group 4) and pre-operation data levels by day 14 and 21. These data are presented in Figure 5-9.

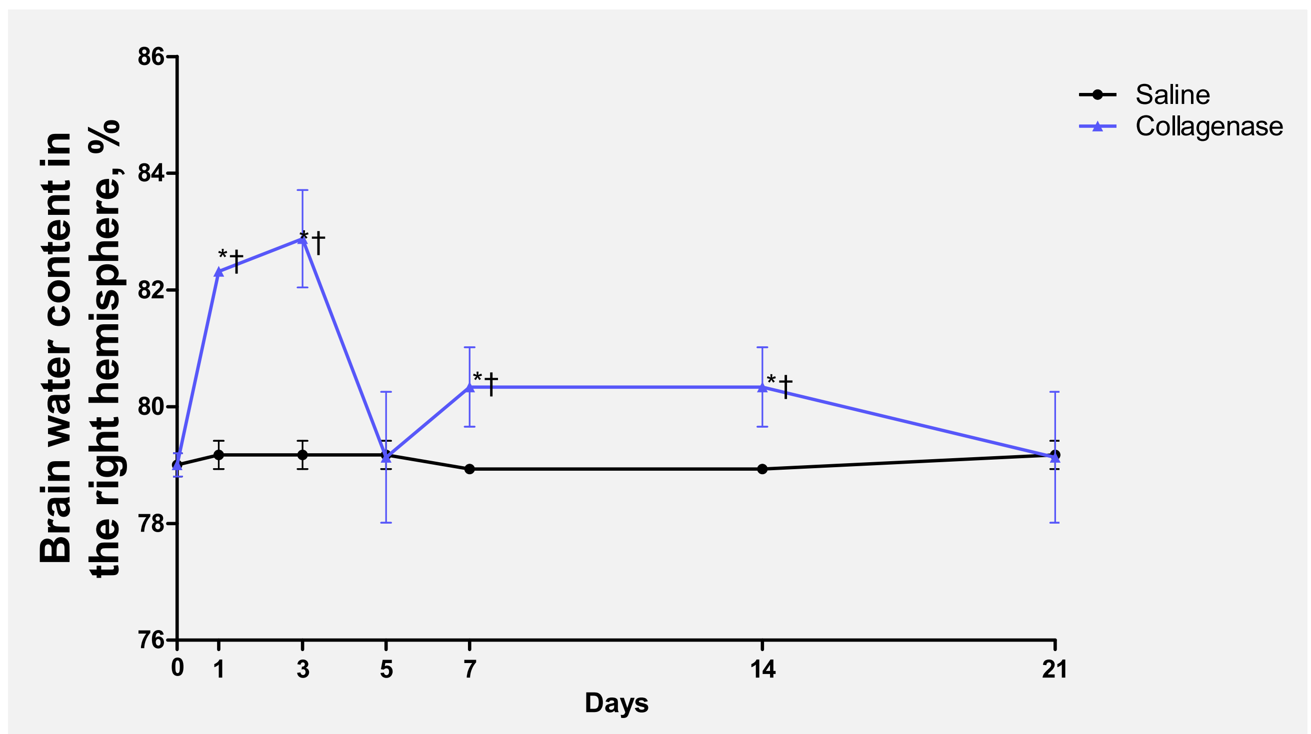


**Figure 5-9 Brain water content: the collagenase (Group 5) and the saline injection group (Group 4), right hemisphere. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4)**

***Densitometric cell count***

There were no significant changes to brain water content throughout the experiment in the saline injection group (Group 4).

There was a significant 18 fold increase in the number of neutrophils in the region of the intracerebral haemorrhage on day 1 in the collagenase injection group (Group 5), which was significantly higher than in the saline injection group (Group 4), this was followed by a gradual decrease on day 3 and 5 reaching the saline injection group (Group 4) and pre-operation data levels by day 14. These data are presented in Figure 5-10.

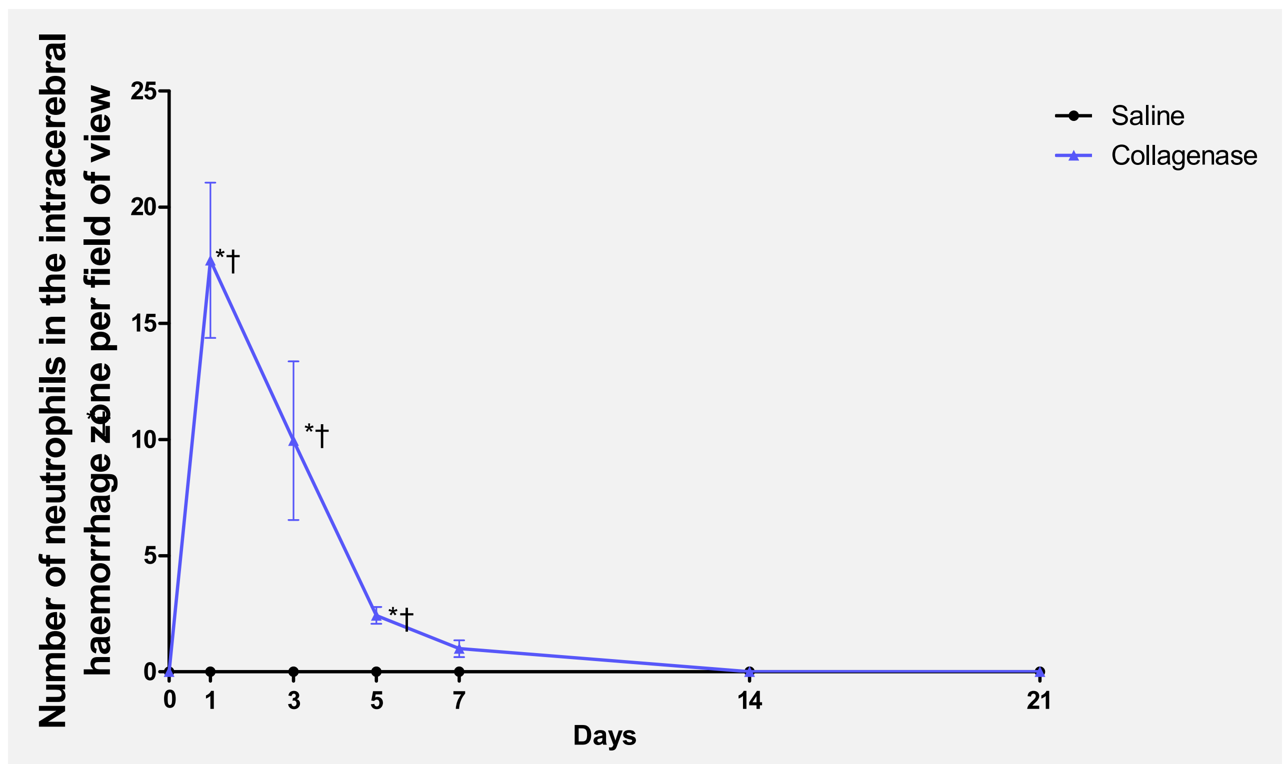


**Figure 5-10 Densitometric cell count analysis: neutrophil cell number (mean) in the intracerebral haemorrhage zone in 7 fields of view. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4)**

There were no significant changes to brain water content throughout the experiment in the saline injection group (Group 4).

There was a 20 fold increase in the number of microglial cells in the region of the intracerebral haemorrhage on day 1 in the collagenase injection group (Group 5), which was significantly higher than in the saline injection group (Group 4).

On the 3rd day of the experiment there was a further 1.3 fold increase in the number of microglial cells in the collagenase injection group (Group 5) with a subsequent gradual 1.75 fold decrease on day 5, falling to near the levels of the saline injection group (Group 4) and pre-operation data by days 7 and 14. These data are presented in Figure 5-11.

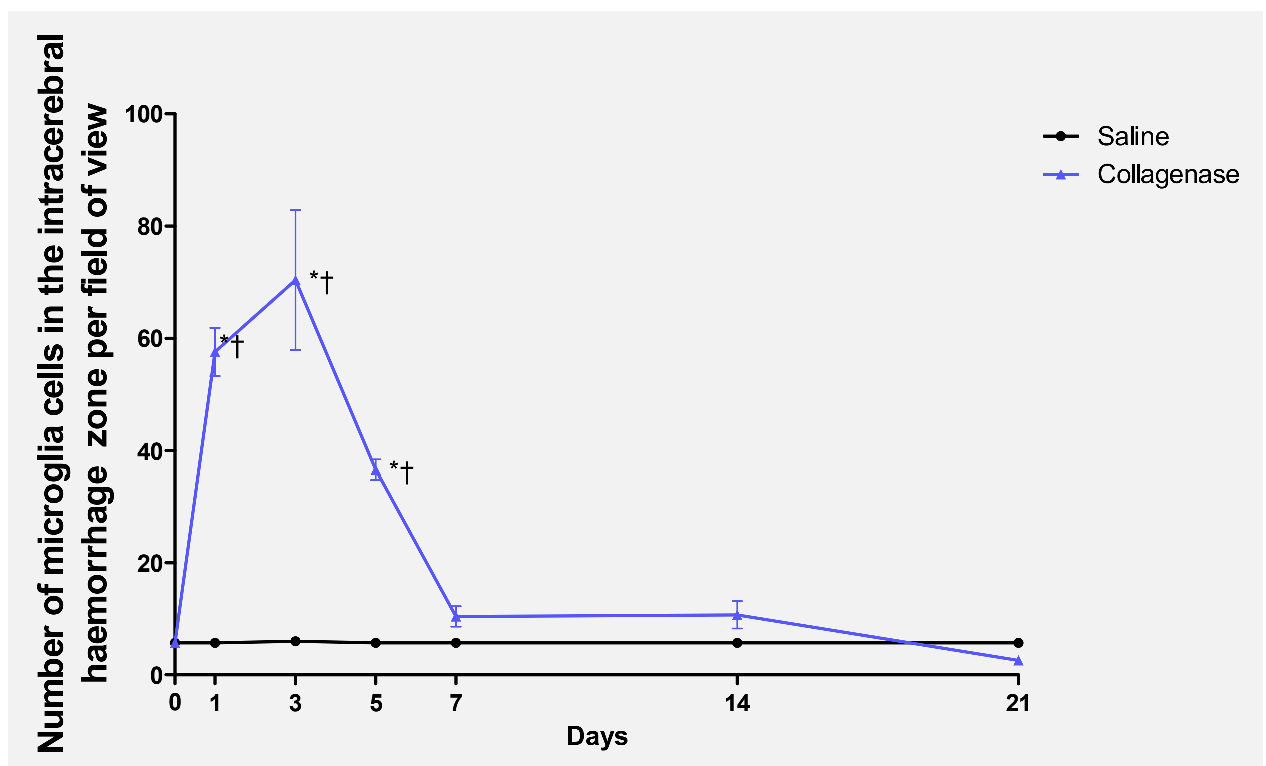


**Figure 5-11 Densitometric cell count analysis: microglial cell number (mean) in the intracerebral haemorrhage zone in 7 fields of view. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4)**

### 5.3.3 Behavioural testing

***Beam walking***

Animals demonstrated maximal performance on the beam walking test throughout the experiment in the saline injection group (Group 4).

There was a statistically significant 1.4 fold decrease in performance on the beam walking test on day 1 in the collagenase injection group (Group 5), compared to pre-operation data, which was followed by a gradual increase on day 3 reaching the levels of pre-operation data and the saline injection group (Group 4) by day 5.

Further changes indicated maximal performance on beam walking test in the collagenase injection group (Group 5) until the end of the experiment on day 21. These data are presented in Figure 5-12.

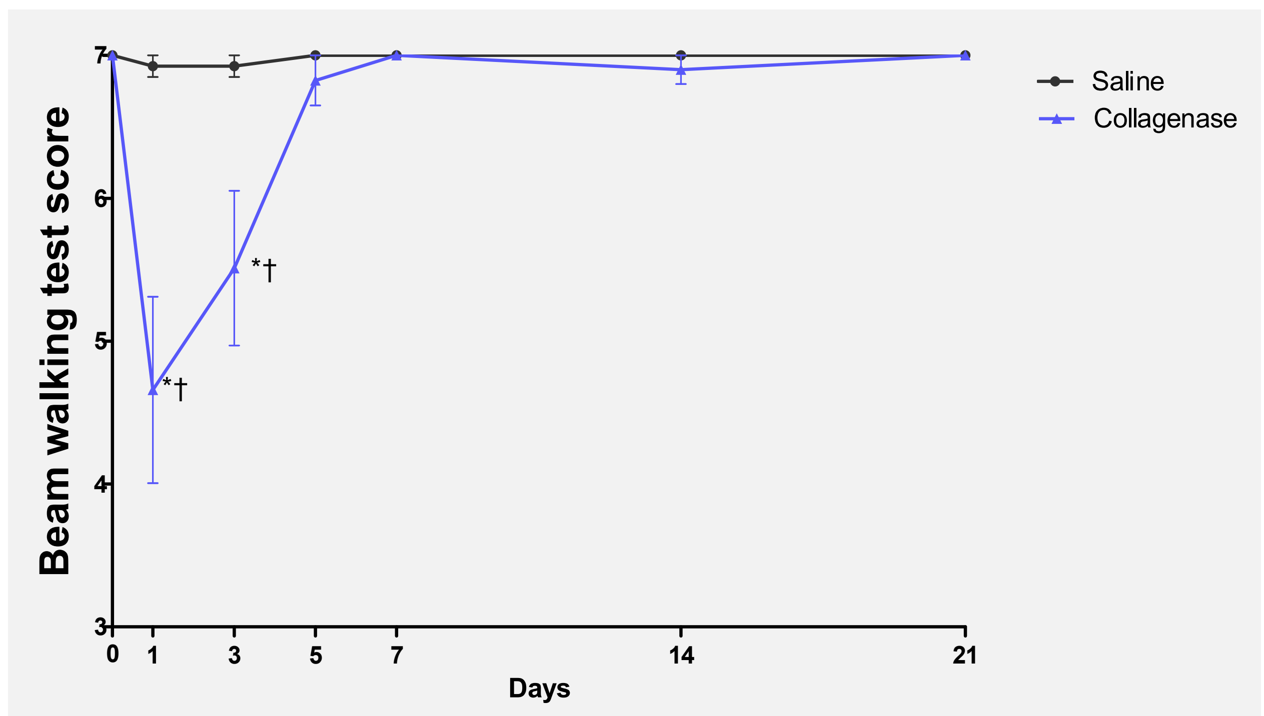


**Figure 5-12 Beam walking test score: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4)**

***Beam balancing***

There was a 1.5 fold decrease in the performance of the beam balancing test on day 1 in the saline injection group (Group 4), with a subsequent 1.3 fold decrease on day 3, when it reached the level of the collagenase injection group (Group 5). Subsequent changes in the beam balancing test in the saline injection group (Group 4) indicated a 2-fold increase in performance by day 5, when it reached the level of pre-operation data and remained at this level until the end of the experiment on day 21.

There was a 3 fold decrease in performance compared to the saline injection group (Group 4) on the beam balancing test in the collagenase injection group (Group 5), which was significantly lower than in the pre-operation data.

Further changes indicated a gradual small improvement in performance on day 3, with a 2.5 fold increase, reaching the level of the saline injection group (Group 4) and pre-operation data by day 5. There were no further changes in the beam walking test in the collagenase injection group (Group 5) throughout the remainder of the experiment. These data are presented in Figure 5-13.

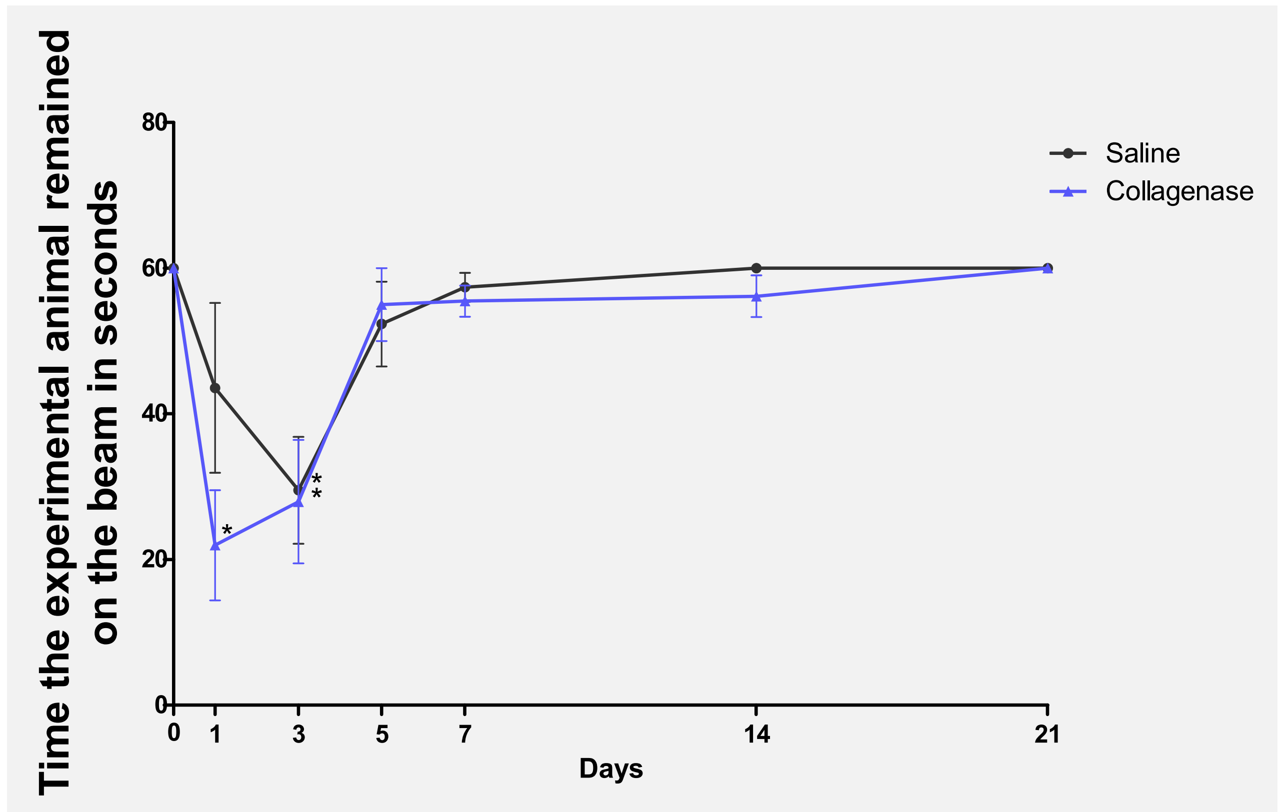


**Figure 5-13 Beam balancing test score: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data**

***Tape removal test***

The saline injection group (Group 4) did not demonstrate any significant difference in tape removal time from the left paw compared to pre-operation data, throughout the experiment.

The collagenase injection group (Group 5) demonstrated a 6 fold increase in tape removal time in the left paw on day 1, with a further 2 fold decrease in removal time over days 3, 5 and 7.

Subsequent changes demonstrated a 2 - fold increase in tape removal time of the left paw in the collagenase injection group (Group 5) on day 14 with a further decrease, reaching the level of pre-operation data by day 21. These data are presented in Figure 5-14.

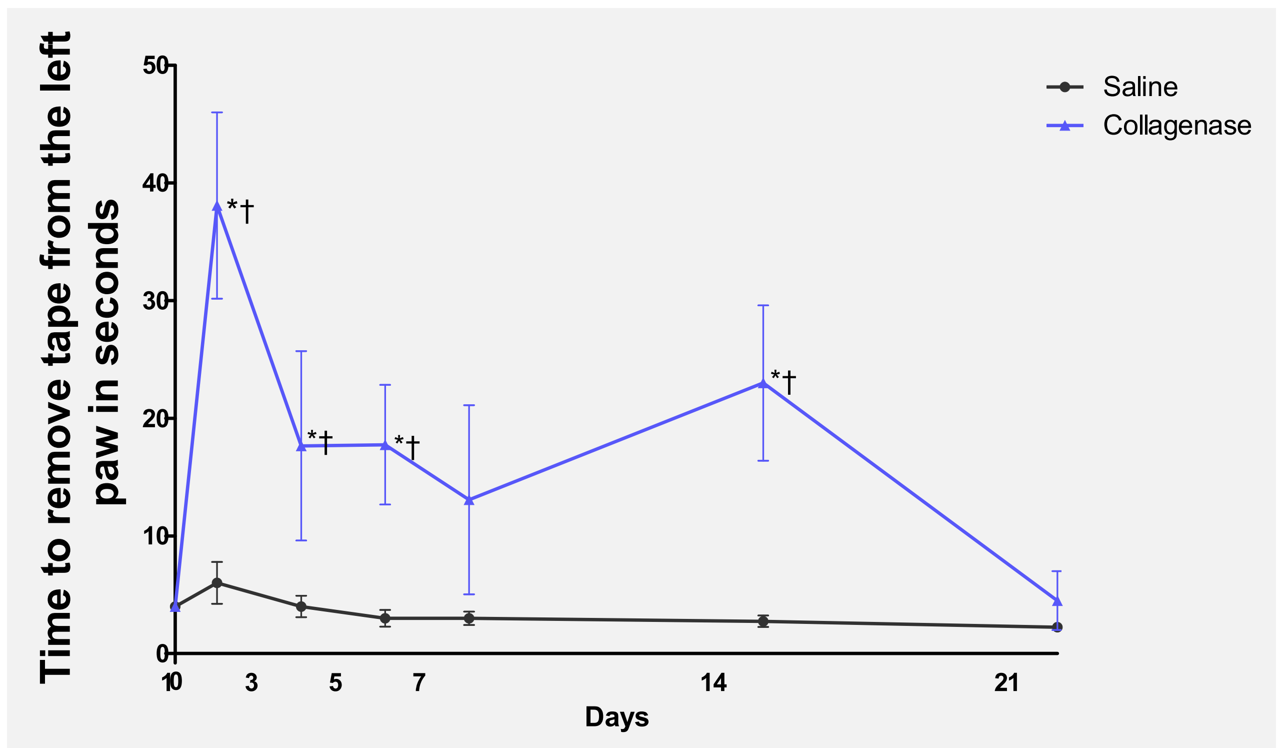


**Figure 5-14 Tape removal test, left paw: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4)**

The collagenase injection group (Group 5) and the saline injection group (Group 4) did not demonstrate any decrease in the performance in tape removal test of the right paw and remained around pre-operation data levels throughout the experiment. These data are presented in Figure 5-15.

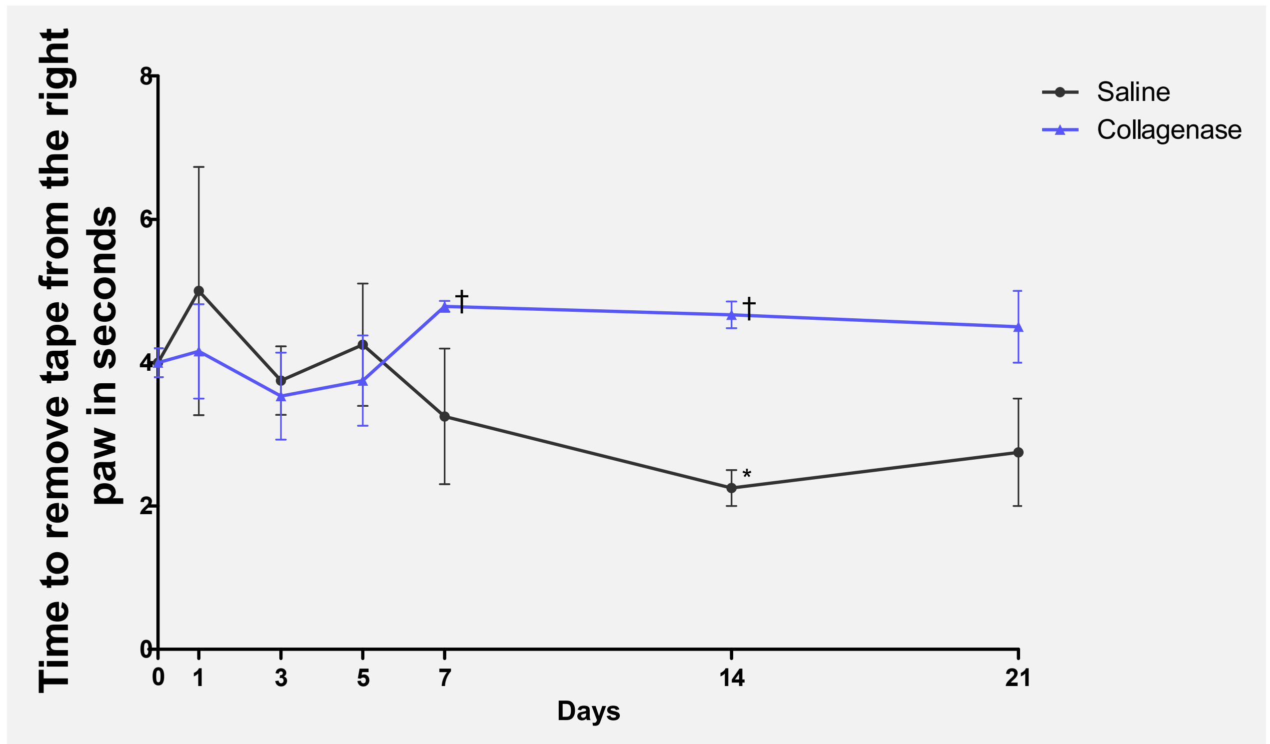


**Figure 5-15 Tape removal test, right paw: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4)**

***Forelimb placing test***

The saline injection group (Group 4) did not demonstrate any changes in the forelimb placing test of the affected paw from pre-operation data throughout the 21 days of the experiment.

The collagenase injection group (Group 5) demonstrated a 1.6 – fold decrease in forelimb placing performance of the affected paw on day 1, comparing to pre-operation data. Further changes indicated a gradual increase in performance of the affected paw on day 3 and 5, reaching pre-operation data levels by day 7 which were maintained through day 14 and 21. These data are presented in Figure 5-16.

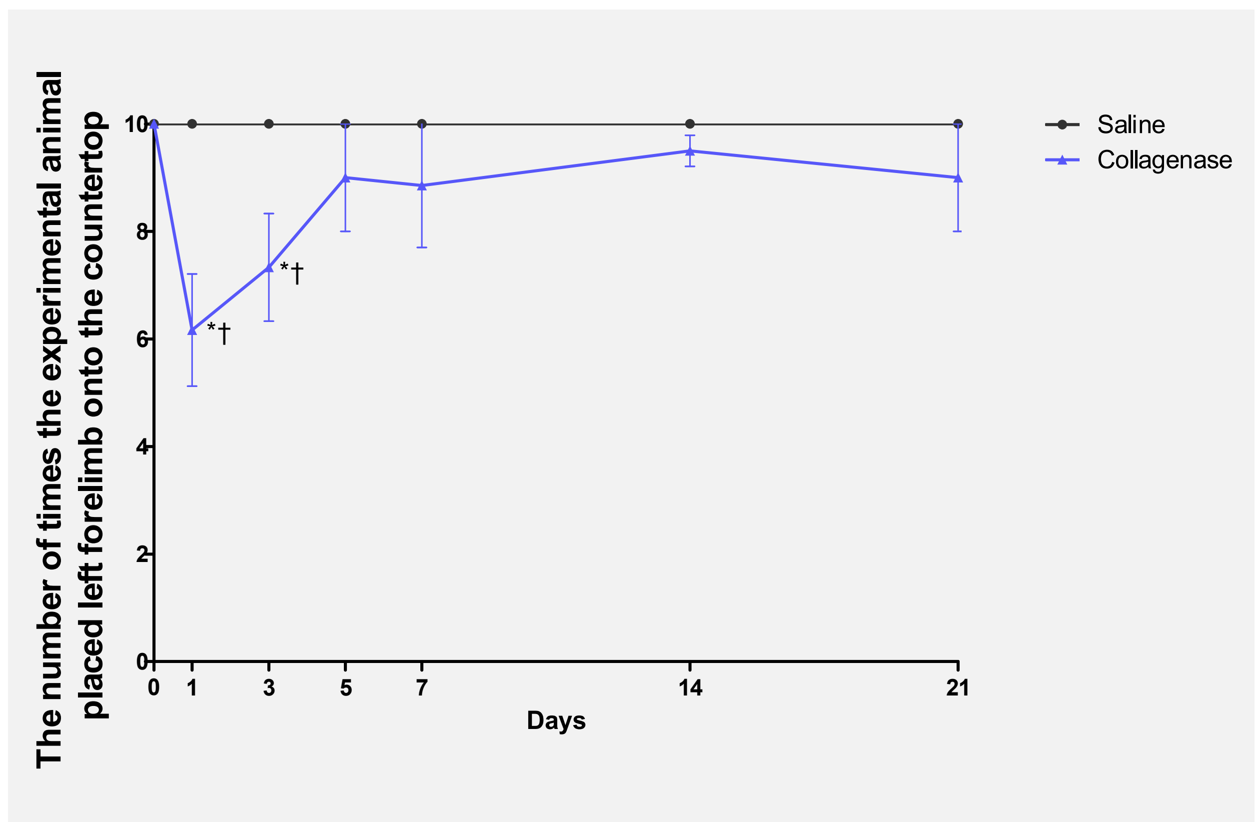


**Figure 5-16 Forelimb placing test, left paw: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data, † - p < 0.05 vs. saline injection group (Group 4)**

The collagenase injection group (Group 5) and the saline injection group (Group 4) did not demonstrate any statistically significant changes in performance in the forelimb placing test and were at the same level as pre-operation data throughout the days of the experiment. These data are presented in Figure 5-17.

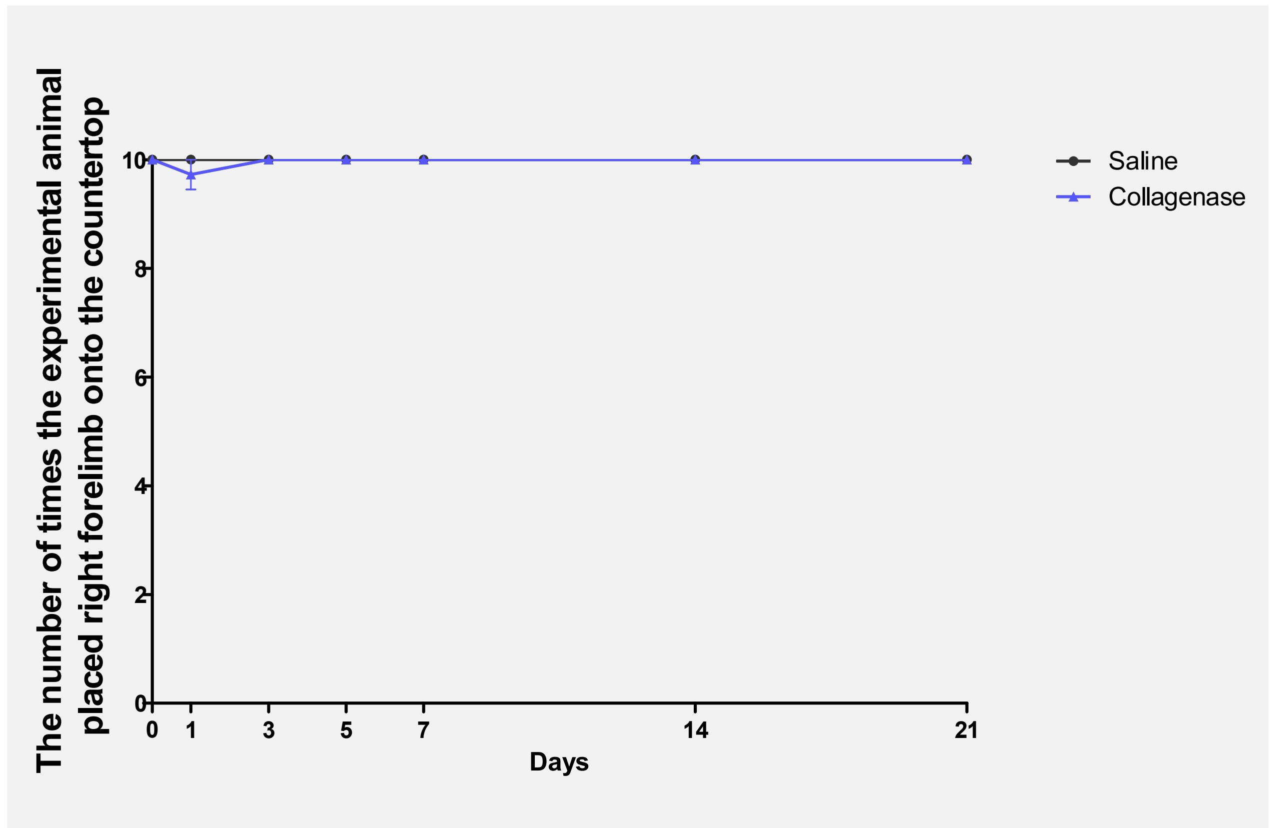


**Figure 5-17 Forelimb placing test, right paw: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

***Semi-quantitative examination***

The saline injection group (Group 4) did not demonstrate any significant changes in performance in semi-quantitative examination from pre-operation data throughout the 21 days of the experiment.

The collagenase injection group (Group 5) demonstrated a significant 1.2 – fold decrease in performance in the semi-quantitative examination on day 1, compared to pre-operation data. Further changes indicated a gradual increase in performance in the collagenase injection group (Group 5) on day 3 and 5 which reached the level of the pre-operation data by day 7. Subsequent changes indicated a small decrease in performance in the semi-quantitative examination in the collagenase injection group (Group 5) at day 14 and remained on the same level until the end of the experiment. These data are presented in Figure 5-18.

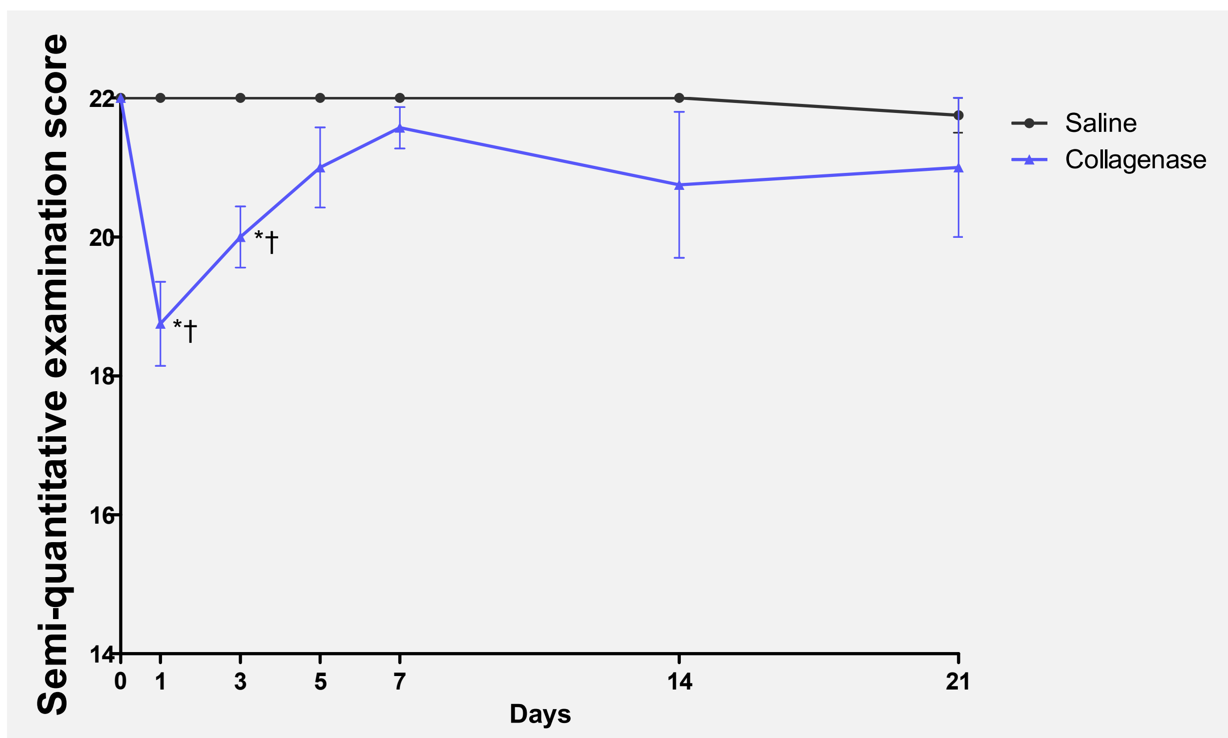


**Figure 5-18 Semi-quantitative examination score: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data, † - p < 0.05 vs. saline injection group (Group 4)**

***Asymmetry test***

The saline injection group (Group 4) performed well over the whole time period of the experiment. The collagenase injection group (Group 5) exhibited poor performance with a decrease in asymmetry test score on days 5 and 7 with subsequent improvement. These data are shown in Figure 5-10.

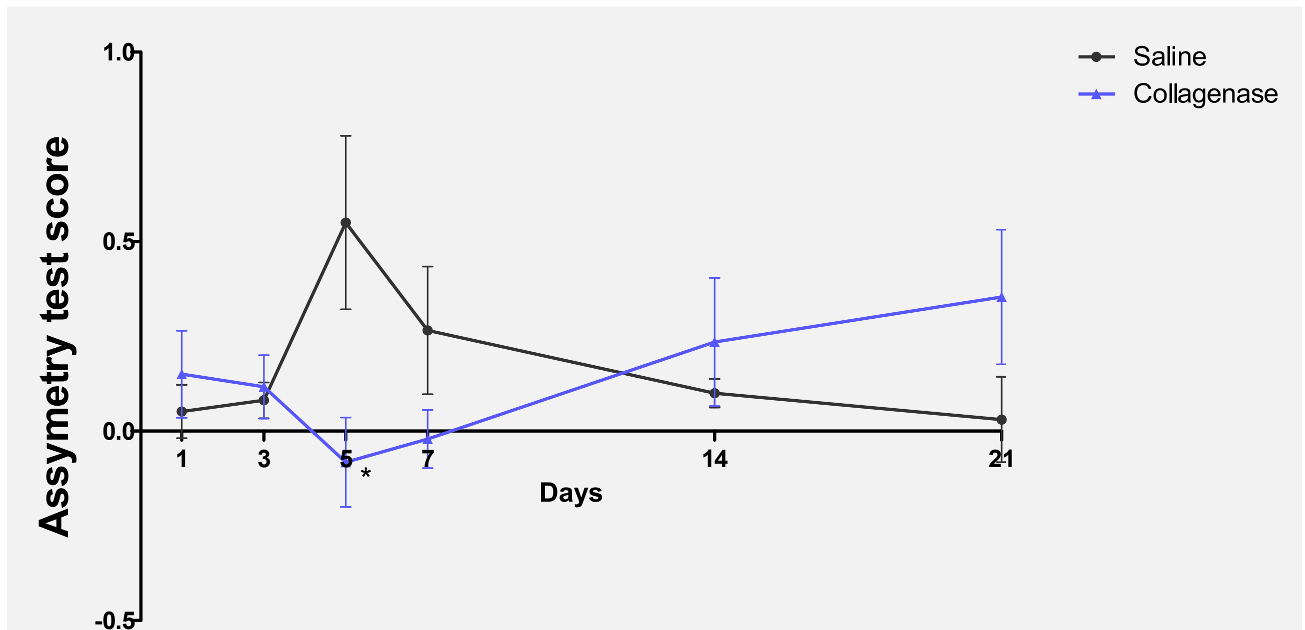


**Figure 5-19 Forelimb use asymmetry test: the saline (Group 4) and the collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ in the collagenase injection group (Group 5) comparing to saline injection group (Group 4)**

### 5.3.4 Correlations between blood and lesion zone leukocytes and behavioural tests

The number of correlations in the saline injection group (Group 4) were small in number and was either moderate or weak in strength.

Moderate positive correlation was observed between:

- the number of peripheral blood lymphocytes and the number of microglia cells in the lesion zone
- the number of peripheral blood neutrophils and plasma TNF-alpha levels.

Moderate negative correlation was found between

- the number of peripheral blood neutrophils and the number of peripheral blood lymphocytes
- the number of peripheral blood lymphocytes and plasma TNF-alpha levels
- the number of microglia and plasma TNF-alpha levels
- the number of peripheral blood neutrophils and the beam walking score
- the number of microglial cells in the lesion zone and the beam walking score
- the beam walking score and the semi-quantitative examination

Weak positive correlations were observed between:

- the number of peripheral blood neutrophils and the number of microglia cells in the lesion zone
- the number of microglia cells in the lesion zone and the forelimb placing test score

Weak negative correlations were observed between

- the number of peripheral blood lymphocytes and the beam walking score
- the number of peripheral blood neutrophils and the semi-quantitative examination
- the number of peripheral blood lymphocytes and the semi-quantitative examination
- the beam walking score and plasma TNF-alpha levels.

These data are presented on Table 6.

**Table 6 Saline injection: matrix of levels pair correlation coefficients between blood and lesion zone leukocytes, TNF alpha and behavioural tests**

| Parameters | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 1 | 0.00 | - 0.30 | 0.12 | 0.00 | - 0.75 | - 0.23 | 0.59 |
| 2 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 3 | | | 0.69 | 0.00 | - 0.04 | - 0.22 | - 0.69 |
| 4 | | | | 0.00 | - 0.65 | 0.17 | - 0.47 |
| 5 | | | | | 0.00 | 0.00 | 0.00 |
| 6 | | | | | | - 0.26 | - 0.27 |
| 7 | | | | | | | 0.00 |

1 – Peripheral blood neutrophil count; 2 – Number of neutrophils in the lesion zone; 3 – Peripheral blood lymphocyte count; 4 – Number of microglia cells in the lesion zone; 5 – Forelimb placing test scores, left paw; 6 – Beam walking test scores; 7 – Semi-quantitative examination scores, 8 – Plasma TNF-alpha levels

In the haemorrhagic stroke group positive strong correlations were found between:

- the number of neutrophils and the number of microglial cells in the intracerebral haemorrhage zone
- forelimb placing test score and plasma TNF-alpha levels
- beam walking test score and plasma TNF-alpha levels
- semi-quantitative examination score and plasma TNF-alpha levels
- forelimb placing of left paw test score and the semi-quantitative examination score

- beam walking test score and semi-quantitative examination score
- forelimb placing of left paw test score and beam walking test score

Negative strong correlation was observed between:

- the number of peripheral blood neutrophils and the number of peripheral blood lymphocytes
- the number of neutrophils in the lesion zone and plasma TNF-alpha levels
- the number of neutrophils in the intracerebral haemorrhage zone and the beam walking test score
- the number of neutrophils in the intracerebral haemorrhage zone and forelimb placing of the left paw test score
- the number of neutrophils in the intracerebral haemorrhage zone and the semi-quantitative examination score
- the number of microglial cells in the intracerebral haemorrhage zone and the beam walking test score
- the number of microglial cells in the intracerebral haemorrhage zone and the forelimb placing of the left paw test score
- the number of microglial cells in the intracerebral haemorrhage zone and the semi-quantitative examination score

Moderate positive correlation was found between:

- the number of peripheral blood neutrophils and the number of neutrophils in the intracerebral haemorrhage zone
- the number of peripheral blood lymphocytes and the forelimb placing of left paw test score

Moderate negative correlation was observed between:

- the number of neutrophils in the intracerebral haemorrhage zone and the number of peripheral blood lymphocytes
- the number of microglia cells and plasma TNF-alpha levels
- the number of peripheral blood neutrophils and the forelimb placing of the left paw test score
- the number of peripheral blood neutrophils and the semi-quantitative examination score

Weak positive correlation was observed between:

- the number of peripheral blood neutrophils and the number of microglia cells in the lesion zone
- the number of peripheral blood lymphocytes and the number of microglial cells in the lesion zone
- the number of peripheral blood lymphocytes and the forelimb placing test score
- the number of peripheral blood lymphocytes and the beam walking score
- the number of peripheral blood lymphocytes and the semi-quantitative examination score

Weak negative correlations were observed between

- the number of neutrophils in the lesion zone and the number of peripheral blood lymphocytes
- the number of peripheral blood neutrophils and plasma TNF-alpha levels

These data are presented in Table 7.

**Table 7 Haemorrhagic stroke group: matrix of levels pair correlation coefficients between blood and lesion zone leukocytes, plasma TNF alpha and behavioural tests**

| Parameters | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 1 | 0.47 | - 0.94 | 0.07 | - 0.48 | - 0.38 | - 0.39 | - 0.14 |
| 2 | | - 0.28 | 0.85 | - 0.97 | - 0.99 | - 0.92 | - 0.77 |
| 3 | | | 0.05 | 0.26 | 0.18 | 0.13 | 0.00 |
| 4 | | | | - 0.84 | - 0.86 | - 0.78 | - 0.58 |
| 5 | | | | | 0.96 | 0.93 | 0.67 |
| 6 | | | | | | 0.93 | 0.79 |
| 7 | | | | | | | 0.93 |

1 – Peripheral blood neutrophil count; 2 – Number of neutrophils in the lesion zone; 3 – Peripheral blood lymphocyte count; 4 – Number of microglia cells in the lesion zone; 5 – Forelimb placing test scores, left paw; 6 – Beam walking test scores; 7 – Semi-quantitative examination scores, 8 – Plasma TNF-alpha levels

## 5.4 Discussion

In this chapter data are presented that strongly supports the hypothesis that haemorrhagic stroke after intracerebral haemorrhage triggers an inflammatory response which by its nature is a compensatory defensive-adaptive reaction of the whole organism in response to the haemorrhagic brain damage. Aseptic inflammation is directed to replace damaged tissue with new connective tissue with partial or complete recovery of lost motor functions.

In our set of experiments the control group for the collagenase injection (that leads to a formation of intracerebral haemorrhage) was represented by an injection of normal saline. In scientific studies of experimental haemorrhagic stroke, apart from the procedure of needle insertion alone, normal saline injection is a widely used technique that serves as a sham group for a number of experiments. The amount of saline injected into the brain tissue varies, but is usually identical to the volume of collagenase solution or autologous blood injected to cause or simulate intracerebral bleeding. Nakamura *et al.*, for example, demonstrated the use of both normal saline (30 μl) and needle insertion as controls in their studies of intracerebral haemorrhage in mice and rats (Nakamura et al. 2004a;Nakamura et al. 2004b). Ardizzone *et al.* also used saline injection of a volume similar to the autologous blood injection group (50 μl) into the lateral striatum to serve as a control in their study of the blockade of glutamate receptors (Ardizzone et al. 2004). Song *et al.* used saline injections as controls in their study of the intracerebral bleeding into the hippocampus (Song et al. 2007). Saline injection has also served as a control in many studies of subarachnoid haemorrhage (Ersahin et al. 2010;Institoris et al. 2011).

Additional rationale for using normal saline as a control in HS models is that collagenase is commonly supplied as a powder and typically is dissolved in normal saline (0.9%) (Ishida et al. 2011) for further experimental use. Thus the effects of saline on brain tissues can be evaluated and the measurements of these effects can be subtracted from the full treatment groups to provide effects due to treatment only.

In studies of experimental HS in which collagenase and normal saline (as a control) are used in very low volumes (up to 2 μl) it has been demonstrated that normal saline does not cause brain injury and does not lead to a physical deficit of the experimental animals (MacLellan et al. 2006). These results were confirmed in our control group for experimental HS.

### 5.4.1 Inflammation at the systemic level

Inflammation has a pivotal role in the systemic response to HS. It mobilises and regulates organism systems as a result of injury and organises a reparative process to attain the best possible outcome. The role of the systemic inflammatory response is part of the general adaptation syndrome or stress-syndrome (Selye & Fortier 1950).

One of the most important factors in systemic inflammation are leukocyte reactions in peripheral blood (Becker 1998;Kleinig & Vink 2009). These reactions form cell pools that generate leukocyte infiltrations in zones of damage, and in a phased way break down and eliminate injured structures followed by their replacement with healthy connective tissue (Frangogiannis 2006).

Most of the biochemical mediators of inflammation, such as transaminases, phosphatases, reactive stage proteins, leukotrienes and cytokines (TNF alpha in

particular) are connected with leukocyte reactions in peripheral blood (Carlson et al. 1999;Otsuka et al. 1990;Zaremba & Losy 2004).

Leukocyte reactions are generally greater in microbial inflammation and smaller in aseptic inflammation (Emsley & Tyrrell 2002;Nakai et al. 2006;Wang & Dore 2007). During microbial inflammation, the leucocytosis factors are represented in the products of microorganisms' catabolism and the lesion focus, in contrast to aseptic inflammation when leucocytosis factors are represented by products of lesion focus only.

In our research, the saline injection group (Group 4) did not demonstrate changes in WBC when compared to pre-operation data. The collagenase injection group (Group 5) demonstrated a decrease in the number of WBC on day 1 with a further increase to reach a peak on day 7 and subsequently reaching the level of the saline injection group (Group 4). These changes in WBC in experimental animals subjected to collagenase injection are typical of leukocyte changes in aseptic inflammation (Christensen & Boysen 2004). Leukocyte reactions during inflammation in experimental animals are mainly characterised with phase changes in quantity and activity of neutrophils and lymphocytes (Kitahara et al. 2002;Rovlias & Kotsou 2001;Xue & Del Bigio 2000). The changes we observed in our study are described in the current chapter.

Saline injection, although it does not lead to visible intracerebral bleeding, does involve some damage to the skull skin and bone integrity, to the brain tissue by the needle insertion and also resulted in pressurising brain tissue as saline is injected. It thus triggers a systemic inflammatory response. In our study, an increase in peripheral blood neutrophils on day 1 with stabilisation by day 5 and a small increase in the

number of peripheral blood lymphocytes on day 3 with stabilisation by day 5 was observed. Initial changes in leukocyte at the systemic level in the saline injection group (Group 4) suggest an adaptive mechanism of inflammation to any injury of the body, characterised by an immediate response at the systemic level (Yoshimoto, Tanaka, & Hoya 2001).

Neutrophilic reactions in peripheral blood possess phase characteristics. Although changes in peripheral blood neutrophil levels in the collagenase injection group (Group 5) are similar to the ones in saline injection group (Group 4) on day 1, the rate in first group is much more pronounced. In contrast to the saline injection group (Group 4), we observed a decrease in the number of peripheral blood neutrophils towards the level of pre-operation data from day 1. The level of neutrophils in the collagenase injection group (Group 5) demonstrated a significant decrease, lower than in the pre-operation data on day 3 with a subsequent pronounced increase on day 7, which returned to pre-operation data level only on day 14. A pronounced increase in the number of neutrophils in the peripheral blood on the first day after experimental haemorrhagic stroke may be explained by the system reaction of the body to injury and stress and follows the processes that take place in aseptic inflammation (Papassotiriou et al. 2008). A rapid decrease in the number of blood neutrophils on day 3 in the collagenase injection group (Group 5) can be explained by their phagocytosis in the zone of the intracerebral haemorrhage, where the processes of inflammation are under way (Butterfield, Best, & Merrick 2006), and by the release of lymphocytes into the systemic blood main stream (Furze & Rankin 2008). The increase in the number of peripheral blood neutrophils on day 7 may be explained by the large number of neutrophils phagocytised with the zone of the intracerebral haemorrhage and thus the

need for the restoration of its number as their role is in initiating the reparative processes (Butterfield, Best, & Merrick 2006).

Lymphocytic reactions in peripheral blood possess phase characteristics as well. A rapid increase in their number on day 3 might be elicited by stimuli that come from a destruction zone and their decrease on day 5 might be explained by their phagocytosis in the zone of the intracerebral haemorrhage. Further increased levels of peripheral blood lymphocytes, until the end of the experiment correlate with patients' haemorrhagic stroke data and might be explained by a change in their role from microglial cells to the ones that provide a range of cytokines for the proliferative stage of inflammation of tissue replacement (Boyce et al. 2000;Farini et al. 2007;Huaux et al. 2003;Martin & Muir 1990).

A gradual increase in TNF-alpha levels over the first 3 days following in the haemorrhagic stroke, maintaining the plateau until the end of the experiment in the collagenase injection group (Group 5) in contrast to saline injection group (Group 4) where no TNF-alpha was observed throughout the experiment, also suggests a correspondence of cytokine involvement in inflammation in the experimental animals (Carlson et al. 1999;Zaremba & Losy 2004). Our data supports other authors' studies of TNF alpha levels, which suggest an overproduction of several pro-inflammatory cytokines in the patients with stroke (Nazar & Szymanska 2000;Zaremba & Losy 2001).

Changes observed in the number of blood leukocytes, the phase changes of neutrophils and lymphocytes number in particular, in general correspond to the changes observed in human patients (Christensen et al. 2002;Christensen & Boysen 2004b;Christensen & Boysen 2004a;Pedersen et al. 2004;Perera et al. 2006;Suzuki et

al. 1995;Zaremba & Losy 2001a). Conformance of increase in blood plasma TNF-alpha levels to leukocyte reactions in the blood may be explained with its cellular origin (its synthesis by cells and increase in the number of neutrophils and lymphocytes) and proves the crucial role of these cells in system mechanisms of inflammation (Zaremba, Skrobanski, & Losy 2001b).

### 5.4.2 Local inflammation

Local inflammation is characterised by blood flow disturbances, oedema, destruction of damaged tissue and its elimination, cell proliferation and replacement of damaged tissue with connective tissue (Frangogiannis 2006).

Being a physiological substance, saline injection did not elicit any intracerebral haemorrhage in the hemisphere subjected to treatment. In contrast, collagenase injection indicated haematoma which occupied 30% of the affected hemisphere. This is due to collagenase disrupting the basal lamina of the cerebral blood vessels, thus causing a blood leak into the surrounding non-vascular tissue (Rosenberg & Navratil 1997).

Brain water content did not change significantly in the saline injection group (Group 4), indicating no local inflammatory processes occurred. In contrast, the collagenase injection group (Group 5) demonstrated an increase in the brain water content in both hemispheres, with oedema levels significantly higher in the injected hemisphere. An increased level of brain water content of the affected hemisphere, which had a tendency to decrease and reach the level of pre-operation data by day 5

and 7, was similar to the data obtained by other studies of experimental haemorrhagic stroke (Huang et al. 2002;Sansing et al. 2003;Zhang et al. 2006).

Saline injection did not result in any neutrophils being present in the zone of needle insertion, nor any changes in the number of microglial cells. In contrast, collagenase injection caused a rapid increase in the number of neutrophils in the zone of the intracerebral haemorrhage, which peaked on day 1 and gradually decreased to the level of pre-operation data by day 5 and 7. As for microglial cells, the collagenase injection group (Group 5) showed an increase in the number of neutrophils in the region of the intracerebral haemorrhage with a maximal peak on day 3 with a subsequent gradual decrease reaching the levels of the pre-operation data.

An increased number of neutrophils in the zone of the intracerebral haemorrhage was in agreement with data from other authors, who demonstrated neutrophil infiltration in other experimental models of haemorrhagic stroke beginning to be present in the zone of the intracerebral haemorrhage within 12 hours *post ictus* (Del Bigio et al. 1996;Gong, Hoff, & Keep 2000). An infiltration by neutrophils of the zone of the intracerebral haemorrhage in our experimental model correlated with the basis of aseptic inflammation and neutrophil attraction into the zone of injury with the purpose of destroying damaged tissues (Xue & Del Bigio 2000), and with other inflammatory models of haemorrhagic stroke (Frangogiannis 2006;Gong, Hoff, & Keep 2000) and human patients' data with haemorrhagic stroke (MacKenzie & Clayton 1999;Mena, Cadavid, & Rushing 2004). The destruction of damaged tissue in the early stage of inflammation occurs via a variety of bioactive enzymes released from neutrophils in the zone of injury, such as gelatinase, collagenase, glucoronidase, elastase,

myeloperoxidase, phosphatase, etc. (Castle, Ling, & Chibber 1984;Chevrier et al. 2006;Kettle & Winterbourn 2001;Sopata et al. 1989;Turner et al. 1979).

Neutrophil activity and their attraction to other brain regions is regulated via production of various cytokines (Takizawa et al. 2001;Wang & Dore 2007), e.g. TNF-alpha was shown to mediate apoptosis (Otsuka et al. 1990), produced by macrophages and various antibodies produced in the body to control neutrophil life span (Chitnis et al. 1996). Barone and Feuerstein showed that peripherally derived cytokines can cross the blood-brain barrier (Barone & Feuerstein 1999), whose permeability increases after intracerebral haemorrhage (Lampl et al. 2005). A strong link between neutrophils in the early stage of inflammation to the next stage of microglial infiltration was described by Atzeni et al., when demonstrated that neutrophils are responsible for the early activation of T- and B-lymphocytes (Atzeni et al. 2002).

An increase in the number of microglial cells that was observed in our experimental model of haemorrhagic stroke (which peaked on day 3) is explained by the demands of the lesion focus, in which cells are already undergoing the processes of destruction, during the elimination of damaged tissue, as a consequence of aseptic inflammation. It was shown that microglial cells are controlled by a number of cytokines, which are widely produced by neutrophils and as products of cellular catabolism in the injured zone, thus providing a signal for their further activation and infiltration (Hanisch 2002;Wang & Dore 2007). Microglial cell changes we observed correlated well with other authors using experimental models of haemorrhagic stroke (Kowianski et al. 2003;Power et al. 2003) which indicated numbers peaking around 48 hours *post ictus* (Kowianski et al. 2003;Zhao et al. 2007) and also in data obtained from human patients (Skinner et al. 2006;Wang & Dore 2007b). When they have finished their role in cellular

damage, microglial cells undergo apoptosis as a part of the next stage of inflammation. Bzowka et al. demonstrated that macrophages undergoing apoptosic change in their cytokine production profile, indicating a downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory ones, leading to a proliferative stimulation (Bzowska et al. 2002). Apart from cytokines produced by microglial cells, a variety of proteases are also released, such as cathepsins which also contribute to tissue repair (Wang & Tsirka 2005).

A further decrease in the number of neutrophils and microglial cells in the zone of the intracerebral haemorrhage and their increase in numbers in peripheral blood that was observed on day 7 for neutrophils and the prolonged increase in the number of peripheral blood lymphocytes( starting from day 14) suggest their involvement in the signalling and engagement process of recovery. Butterfield et al. demonstrated these cells to be responsible and strongly connected to the reparative processes occurring in inflammation (Butterfield, Best, & Merrick 2006). A strong correlation between cell numbers in the zone of the intracerebral haemorrhage and in peripheral blood suggests a strong link between systemic level of these cells with local levels of inflammation.

A decrease of neutrophil and microglial cell numbers in the zone of the intracerebral haemorrhage correlated with brain water content, indicating a cellular component of oedema. Hence, medical reduction of brain water content should be considered to be an important stage in treatment of haemorrhagic stroke, as it's a reduction may lead to reduction of competent cellular components in the zone of the intracerebral haemorhage and thus to changes in the inflammatory response. The importance of brain oedema in the intracerebral haemorrhage was also inderictly shown by Bereczki et al. in their study when mannitol, an osmotic agent and a free radical

scavenger, did not lead to an improvement or worsening of outcome (Bereczki et al. 2008).

### 5.4.3 Behavioural tests

Loss of motor function is one of the clinical signs of inflammation (Lucignani 2007) in the early stage of inflammation is associated with alterative processes and is gradually improved with the progression of reparative processes.

As a representation of functional recovery of the brain in the haemorrhagic stroke model used in our experiments, behavioural tests demonstrated changes in their performance over the 21 days of the experiments.

Saline injection did not lead to any functional loss in the experimental animals as no significant structural damage was caused to the brain that would evoke inflammatory response on the local level. However, some of behavioural tests, such as the beam balancing test indicated a decrease in the performance over first 3 days as was observed in the collagenase injection group (Group 5). This was, we believe, stress-related and mood-dependent in the experimental animal that have undergone a surgical procedure the day before.

The collagenase injection group (Group 5) showed poor performance in most of the tests observed on the first day after stroke with further gradual improvement which reached the level of the saline injection group (Group 4) and pre-operational levels by day 7. These findings mirrored the data obtained by other authors in their experimental models of haemorrhagic stroke (Ferland, Veilleux-Lemieux, & Vachon 2007;MacLellan et al. 2006;Metz & Whishaw 2000). The loss of functions in our experimental model

followed the observations in human patients with haemorrhagic stroke, indicating functional disability after stroke with subsequent gradual improvement (Miyai et al. 2000;Paolucci et al. 2003).

Some of the tests such as the tape-removal test and the asymmetry test were not support the conclusions found by other authors (MacLellan et al. 2006; Hua et al. 2002). A poor performance in the tape-removal test over the days of the experiment and a decrease in the performance in the asymmetry test on day 5 only, suggest the data obtained depend on the preference of the experimental animals. Being a non-forced-type of functional test, the results obtained from these two tests depended on the mood of the experimental animals despite there being a previous training programme. Thus we made a decision to use forced-type-tests for our further experiments.

A negative correlation between peripheral blood and intracerebral haemorrhage zone neutrophils with performance in the three schosenbehavioural tests, and positive correlation between these tests and peripheral blood lymphocytes and microglial cells was observed. Taking into account the facts that microglial cells and peripheral blood lymphocytes, are capable of stimulating reparative processes in the lesion focus (Butterfield, Best, & Merrick 2006;Wang & Tsirka 2005) and the fact that they were measure in the blood at a significantly higher level at the end of the experiment, suggest that functional recovery and behavioural improvement of the experimental animals run alongside the processes of regeneration and reparation of damaged tissues, in parallel.

A correlation between functional improvement and peripheral blood and local cell kinetics suggest a connection between systemic levels of cells as well as local levels of

inflammation and functional recovery of the brain, and mirrors the phased processes that take place in resolution of haemorrhagic stroke damage.

# Chapter VI. CORM-3 effects on the inflammatory nature of the experimental haemorrhagic stroke

## 6.1 Introduction

Previously we have described that CORM-3, which is a chemical compound that is capable of rapidly liberating CO molecules, does no harm to brain glial cells at the doses/concentrations we used in our experiments. These findings agreed with the results obtained by other authors who have studied the properties of CO-RMs in other various cell lines (Bani-Hani, Greenstein, Mann, Green, & Motterlini 2006a;Sawle et al. 2005).

Earlier we have also demonstrated that brain striatal slices incubated with CORM-3 did not show any signs of toxicity either, resulting in 100% viability of the slices analysed, at the doses/concentrations we used.

In chapter 5 we have shown that a compensatory reparative mechanism occurs in our model of a haemorrhagic stroke after intracerebral haemorrhage which appears to occur through the processes of inflammation which are represented at a both the local and systemic levels of the organism.

Based on these data obtained from various inflammatory models and the ability of CO-RMs to modulate an inflammatory reaction, it is logical to predict that these compounds may have an impact on the inflammation generated during and after a haemorrhagic stroke and could influence its outcome.

Since we did not find any data currently in the scientific literature investigating the potential role of CORM compounds in the compensatory reparative mechanisms in haemorrhagic stroke, we hypothesised that CO-RMs, CORM-3 in particular, might affect haemorrhagic stroke through inflammation and modulate the general adaptation syndrome.

Considering that inflammation is determined by the general adaptation syndrome and might progress in different ways, and that inflammation is a process organised temporarily in stages which are fundamentally different (i.e. destruction of tissues, removal of damaged tissues and its replacing with connective tissue), we propose another hypothesis, concerning the impact of CO-RMs on haemorrhagic stroke, CORM-3 in particular is likely to be influenced by the time of administration, because of the temporal/rapid nature of its release.

Therefore it was decided to evaluate CORM-3 administered at these different times. Firstly, CORM-3 was given prior to haemorrhagic stroke as it might influence the general adaptive syndrome and positively affect haemorrhagic stroke outcomes. Secondly, it was administered 3 hours post stroke, which represents a potential therapeutic window for the early intervention into stroke in men, when the destructive

stage of inflammation is under way. Finally, it was given 3 days post stroke, when the destructive stage of inflammation is substituted by the reparative stage.

However, despite the time of CORM-3 administration, it is logical to predict that being a bioactive compound, CORM-3 might also have a dose-dependent effect. Based on a literature review, the concentrations of CO-RMs used in various animal models varied from 4 to 15 mg per kg (Caumartin et al. 2011;De et al. 2009;Lancel et al. 2009). Therefore the groups o f 4 mg/kg and 8 mg/kg of CORM-3 treatment were selected to be used for the selected time points of administration.

Evaluation of the influence of CORM-3on haemorrhagic stroke outcomes also requires control groups for comparison, which are represented by the group of CORM-3 administration to intact animals and CORM-3 administration to animals with saline being injected into the lateral striatum, as opposed to collagenase.

## 6.2 The effect of CORM-3 on the saline-treated group

### 6.2.1 Materials and methods. Experimental protocol

| Group | Protocol |
|---|---|
| Group 4 (Saline) | Injection of 2 μl of 0.2 U saline solution into the lateral striatum of the experimental animals (n=30) |
| Group 6 (Saline + CORM-3) | Saline injection into the lateral striatum and CORM-3 administration at a dosage of 8 mg/kg, 3 hours post-surgery (n=30) |
| Group 7 (Intact animals + CORM-3) | CORM-3 administration at a dosage of 8 mg/kg, 3 hours post-surgery (n=6) |

### 6.2.2 Results

#### 6.2.2.1 Systemic level of inflammation

**General blood analysis: white blood cell count**

The saline + CORM-3 group (Group 6) demonstrated a small non-significant increase in the number of white blood cells on days 3, 5 and 7 with further drop and reaching the levels of saline injection group (Group 4) on days 14 and 21.

The intact animals + CORM-3 group (Group 7) compared to the saline + CORM-3 and the saline injection group (Group 4) did not display any significant changes throughout the 14 days of the experiment, although there was fluctuation around pre-operation data levels and a small non-significant increase on day 3. These data are presented on Figure 6-1.

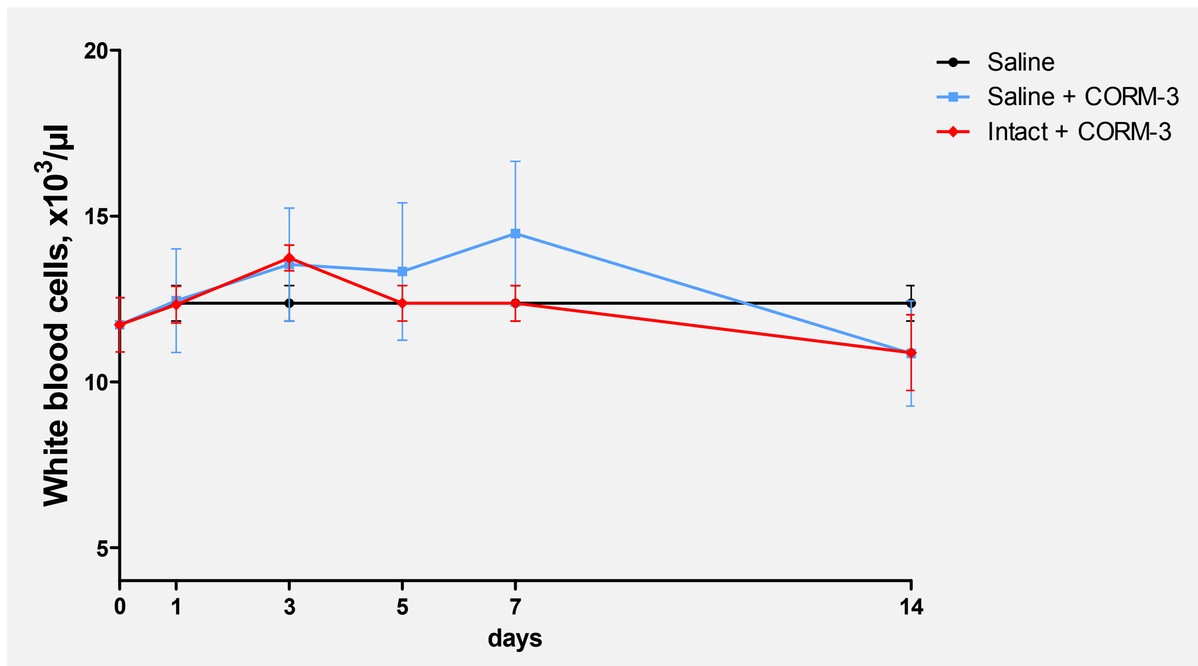


**Figure 6-1 White blood cell count: the saline (Group 4), saline + CORM-3 (Group 6), intact + CORM-3 (Group 7) groups data are represented. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No significant changes were seen.**

The saline + CORM-3 group (Group 6) demonstrated a 15% increase in the number of peripheral blood neutrophils on day 1, when compared to pre-operation data, which was significantly higher than the number in the saline injection group (Group 4). A further significant 10% decrease in the number of peripheral blood neutrophils in the saline + CORM-3 group (Group 6) was observed on days 3 and 5; however it was higher than in saline injection group (Group 4) and pre-operation data. The saline + CORM-3 group (Group 6) demonstrated subsequent significant 3% increase on day 7 in the number of peripheral blood neutrophils and a further decrease, reaching the levels of saline injection group (Group 4) and pre-operation data on days 14 and 21. The intact animals + CORM-3 group (Group 7) remained at the levels of pre-operation data throughout the days of the experiment, indicating no significant changes from pre-operational levels in levels of peripheral blood neutrophils. These data are presented in Figure 6-2.

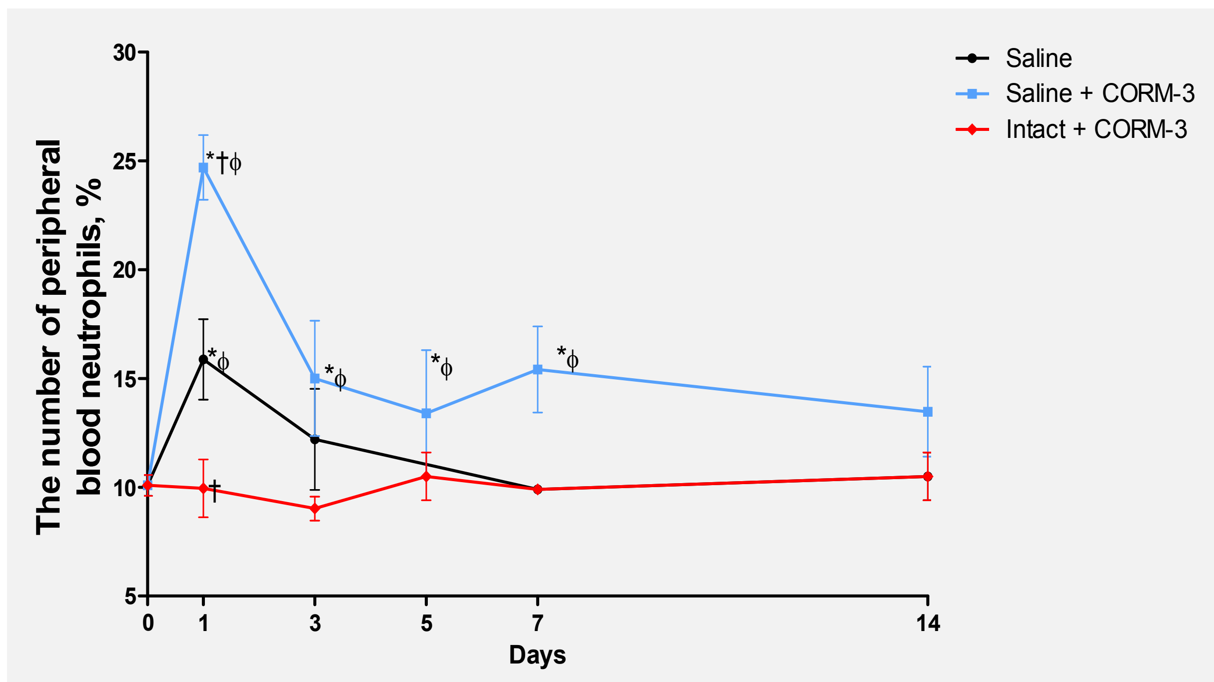


**Figure 6-2 Peripheral blood neutrophil count: the saline (Group 4), saline + CORM-3 (Group 6), intact + CORM-3 (Group 7) groups. Data represent the mean ± SD of n = 6 independent**

**experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4), Φ - $p < 0.05$ vs. intact + CORM-3 group (Group 7)**

The saline + CORM-3 group (Group 6) demonstrated a significant 15% decrease in the number of peripheral blood lymphocytes on day 1, when compared to pre-operation data, which was significantly lower than the one observed in the saline injection group (Group 4).

A further gradual 10% and 12% increase in the number of peripheral blood lymphocytes in the saline + CORM-3 group (Group 6) was observed on day 3 and 5 respectively, when it reached the levels of the saline injection group (Group 4) and pre-operation data.

By the 7th day of the experiment, it was seen that there was a 2% decrease in the number of peripheral blood lymphocytes in the saline + CORM-3 group (Group 6), which was non-significantly lower than the one in pre-operation data and the saline injection group (Group 4).

The intact + CORM-3 group (Group 7) demonstrated a small increase in the number of peripheral blood lymphocytes on day 1, which was somewhat higher when compared to pre-operation data and significantly higher than in saline injection group (Group 4).

A further gradual decrease in the number of peripheral blood lymphocytes was observed on days 3 and 5, which then reached the levels of pre-operation data and the saline injection group (Group 4). These data are presented in Figure 6-3.

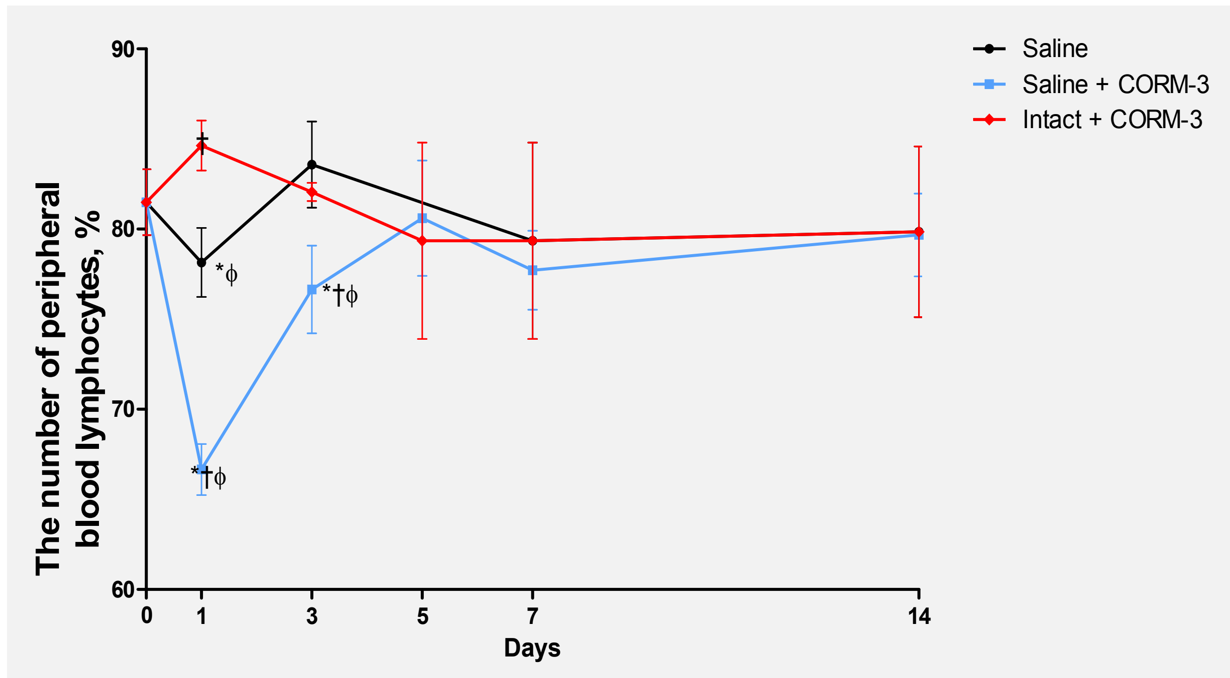


**Figure 6-3 Peripheral blood lymphocyte count: the saline (Group 4), saline + CORM-3 (Group 6), intact + CORM-3 (Group 7) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, † - $p < 0.05$ vs. saline injection group (Group 4), Φ - $p < 0.05$ vs. intact + CORM-3 group (Group 7)**

### Enzyme-linked immunosorbent TNF-alpha assay

The saline + CORM-3 (Group 6) and Intact + CORM-3 (Group 7) groups demonstrated very similar plasma TNF-alpha level to the saline injection group (Group 4) throughout the 14 days of the experiment, which was similar to pre-operation data level. These data are presented in Figure 6-4.

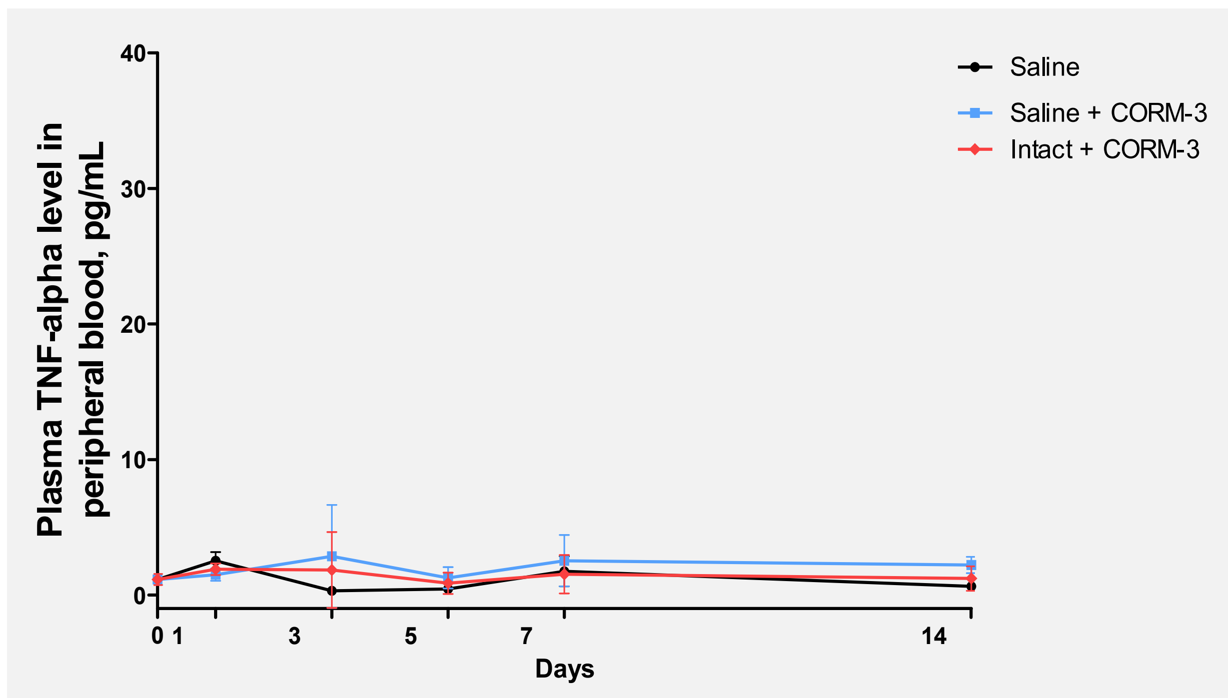


**Figure 6-4 Blood plasma TNF-alpha ELISA assay: the saline (Group 4), saline + CORM-3 (Group 6), intact + CORM-3 (Group 7) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

#### 6.2.2.2 Local level of inflammation

**Determination of brain intracerebral haemorrhage area determination**

The saline + CORM-3 (Group 6) and the saline injection groups (Group 4) demonstrated no visible intracerebral haemorrhage formation. The results are represented in Figure 6-5.

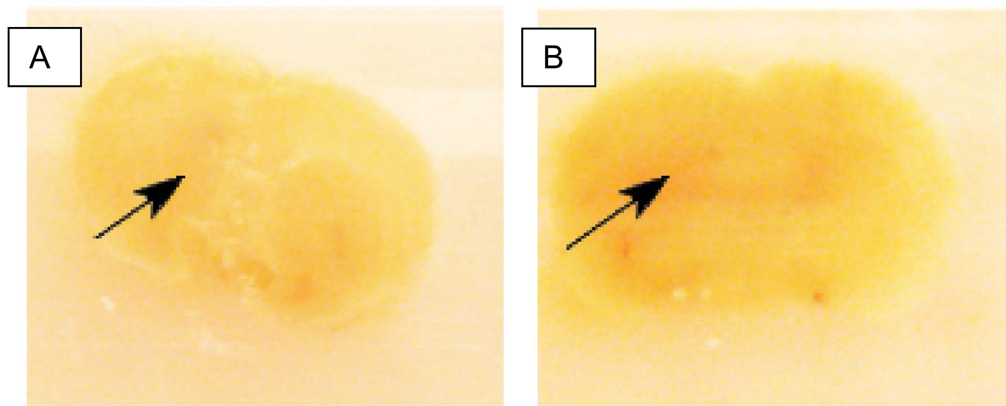


**Figure 6-5 Images of the brain slices through the zone of interest obtained on the first day post op (arrow indicates an absence of the intracerebral haemorrhage): A - saline injection group (Group 4), B - saline + CORM-3 injection group (Group 6)**

**Brain water content analysis**

The saline + CORM-3 group (Group 6) demonstrated very similar brain water content in the unaffected hemisphere compared to the saline injection group (Group 4) throughout the 14 days of the experiment. These data are presented in Figure 6-6.

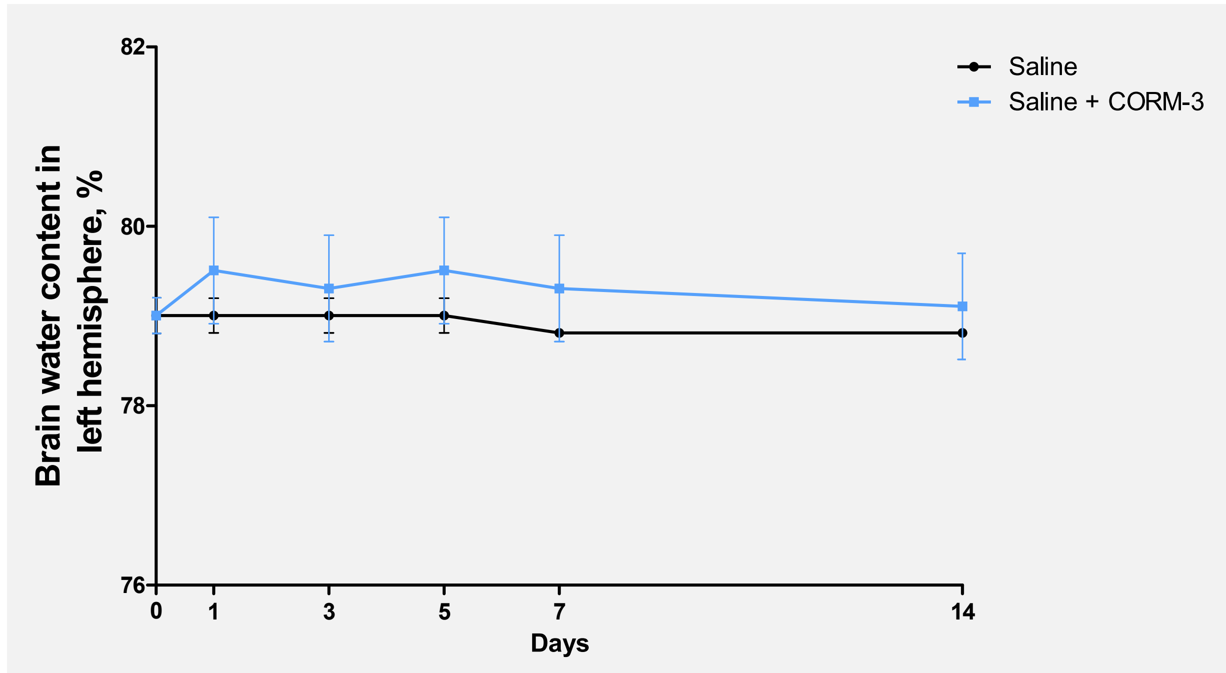


**Figure 6-6 Brain water content: the saline (Group 4), saline + CORM-3 (Group 6) groups, left hemisphere. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

The saline + CORM-3 group (Group 6) did not demonstrate any significant differences from the saline injection group (Group 4) in the affected hemisphere throughout the 14 days of the experiment. These data are presented in Figure 6-7.

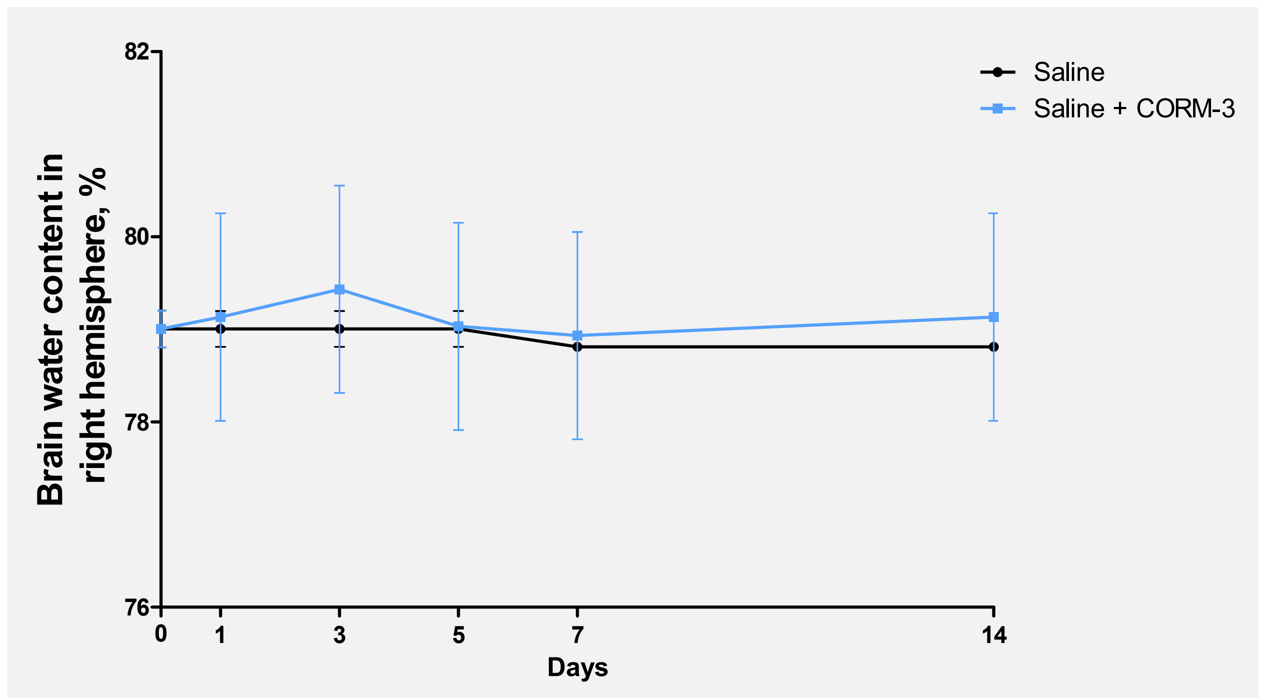


**Figure 6-7 Brain water content: the saline (Group 4), saline + CORM-3 (Group 6) data represented for right hemisphere. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

**Densitometric cell count**

The saline + CORM-3 group (Group 6) demonstrated no presence of neutrophils in the region of injection, which was similar to the levels of the saline injection group (Group 4) and the pre-operation data.

The saline + CORM-3 group (Group 6) demonstrated the number of microglial cells in the region of injection to be at the same level as was measure in the saline injection group (Group 4) and pre-operation data. These data are presented in Figure 6-8.

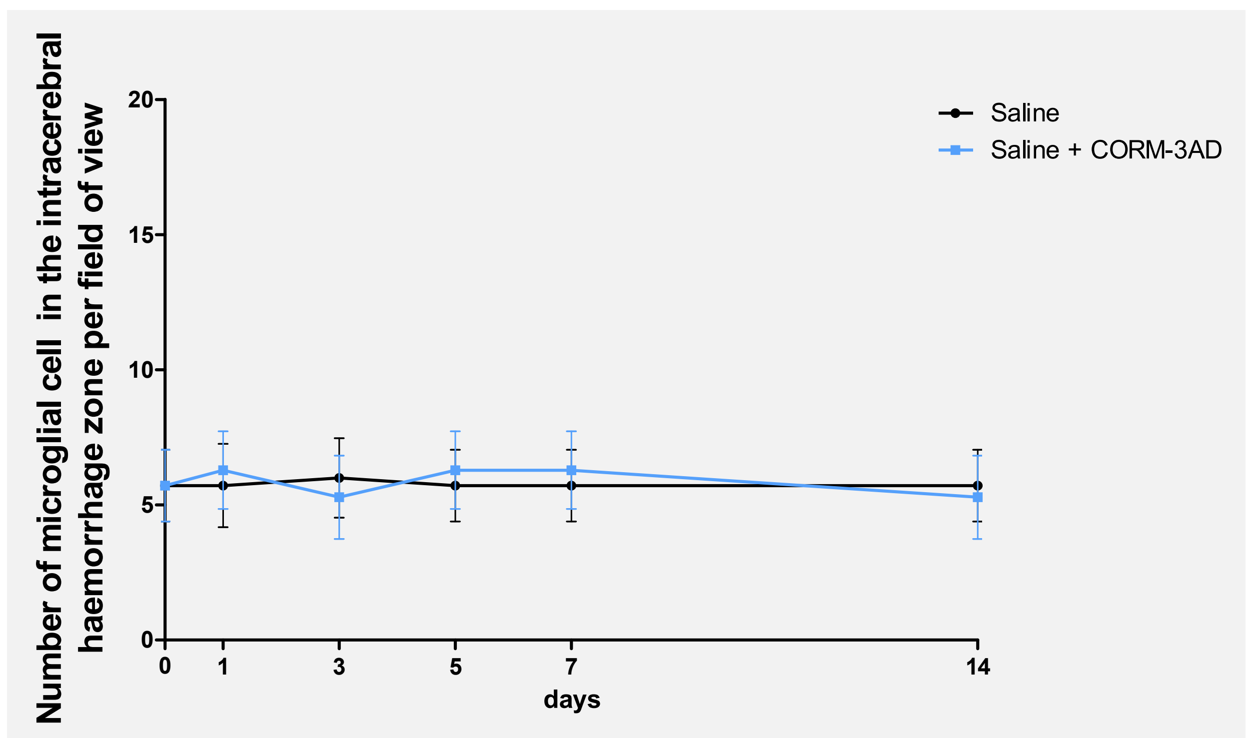


**Figure 6-8 Densitometric cell count analysis: microglial cell number (mean) in the intracerebral haemorrhage zone in 7 fields of view. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

### 6.2.2.3 Behavioural testing

***Beam walking***

The saline + CORM-3 group (Group 6) and the saline injection group (Group 4) demonstrated the some performance in the beam walking test throughout the 14 days of the experiment without any statistically significant difference. These data are presented in Figure 6-11.

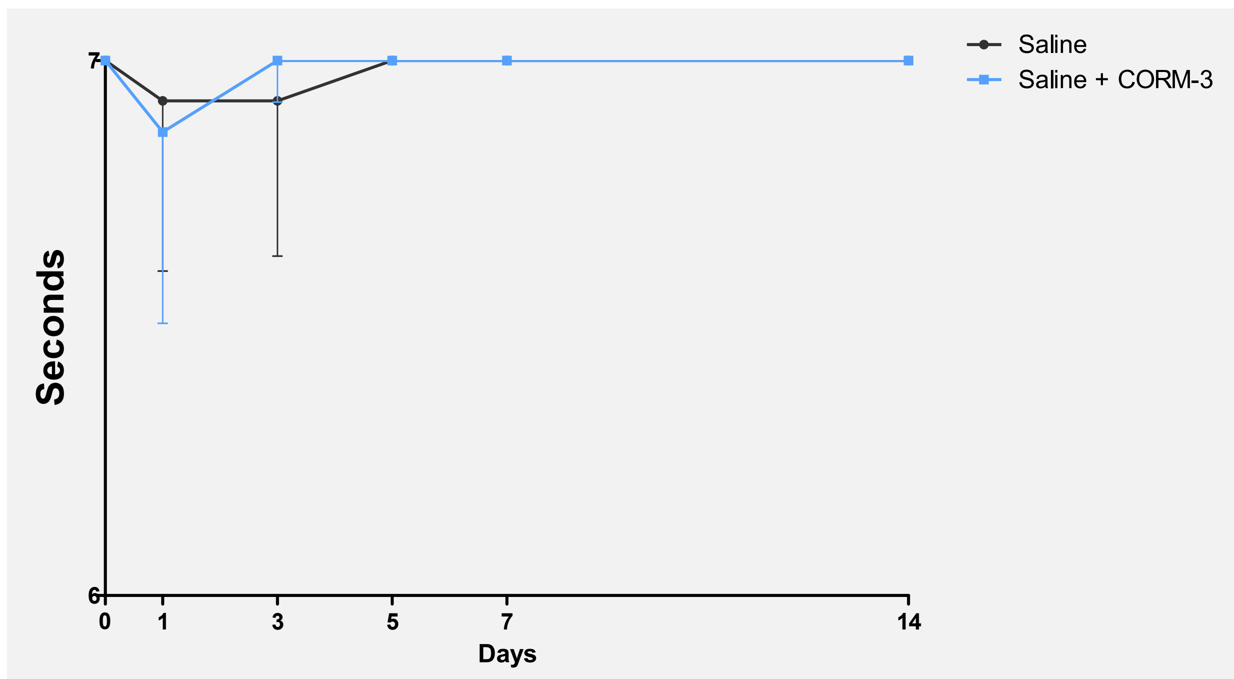


**Figure 6-9 Beam walking test score: saline (Group 4), saline + CORM-3 (Group 6) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

***Forelimb placing test***

The saline + CORM-3 group (Group 6) and the saline injection group (Group 4) demonstrated the same performance in the forelimb placing test of the affected paw throughout the 14 days of the experiment. These data are presented on Figure 6-10.

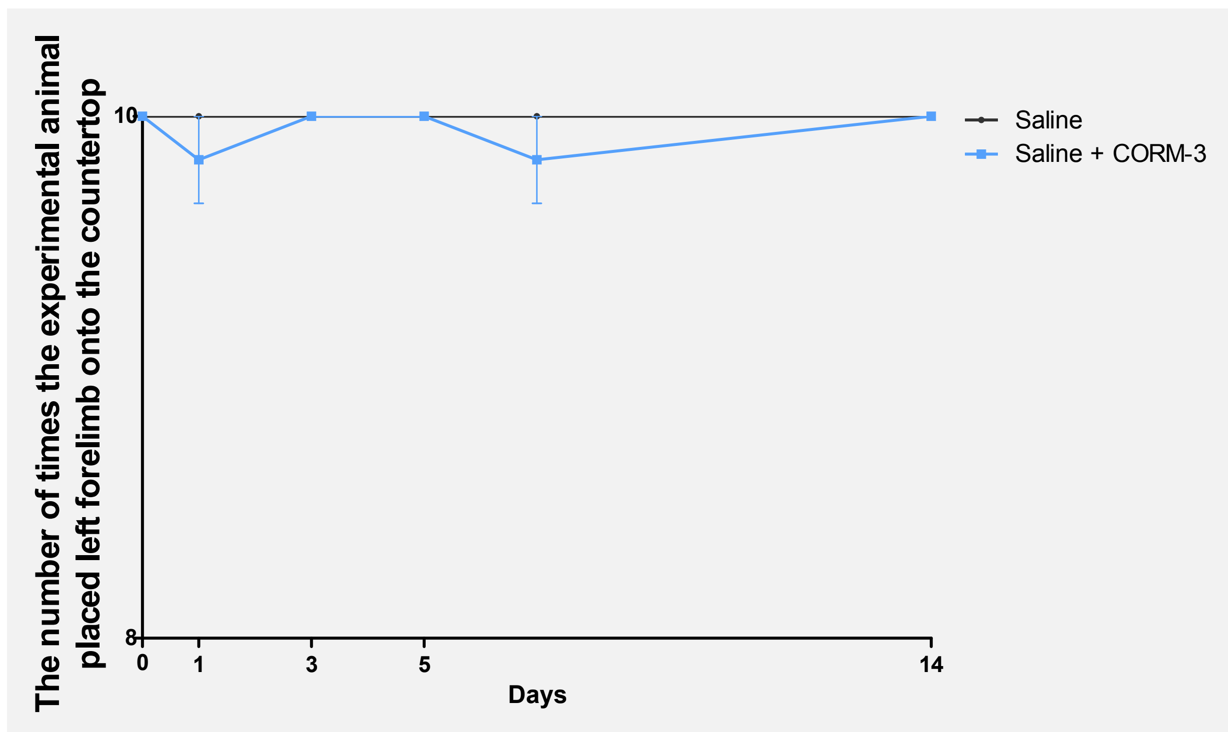


**Figure 6-10 Forelimb placing test, left paw: saline (Group 4), saline + CORM-3 (Group 6) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

The saline + CORM-3 group (Group 6) and the saline injection group (Group 4) demonstrated the same performance in the forelimb placing test of the unaffected paw throughout the 14 days of the experiment. These data are presented in Figure 6-11.

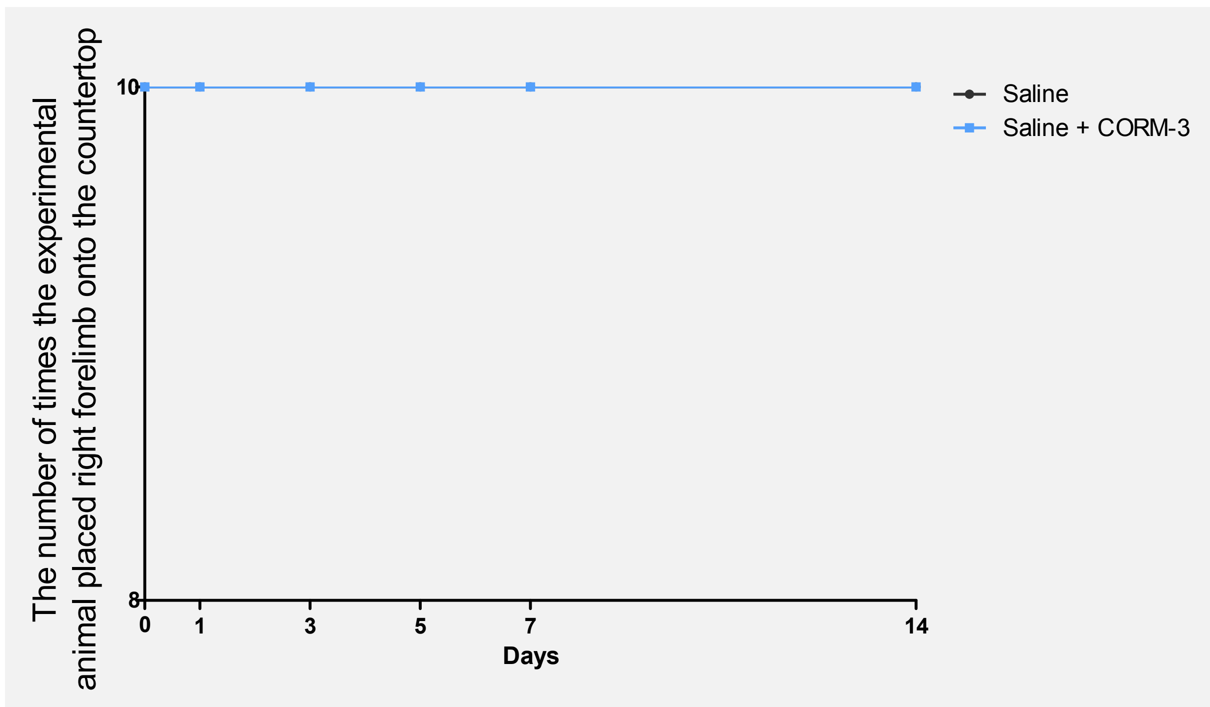


**Figure 6-11 Forelimb placing test, right paw: the saline (Group 4), saline + CORM-3 (Group 6) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

***Semi-quantitative examination***

The saline + CORM-3 group (Group 6) and the saline injection group (Group 4) demonstrated similar performance in semi-quantitative test with only a non-significant small variation of the saline + CROM-3 group (Group 6) compared to pre-operation data throughout the 14 days of the experiment. These data are presented in Figure 6-12.

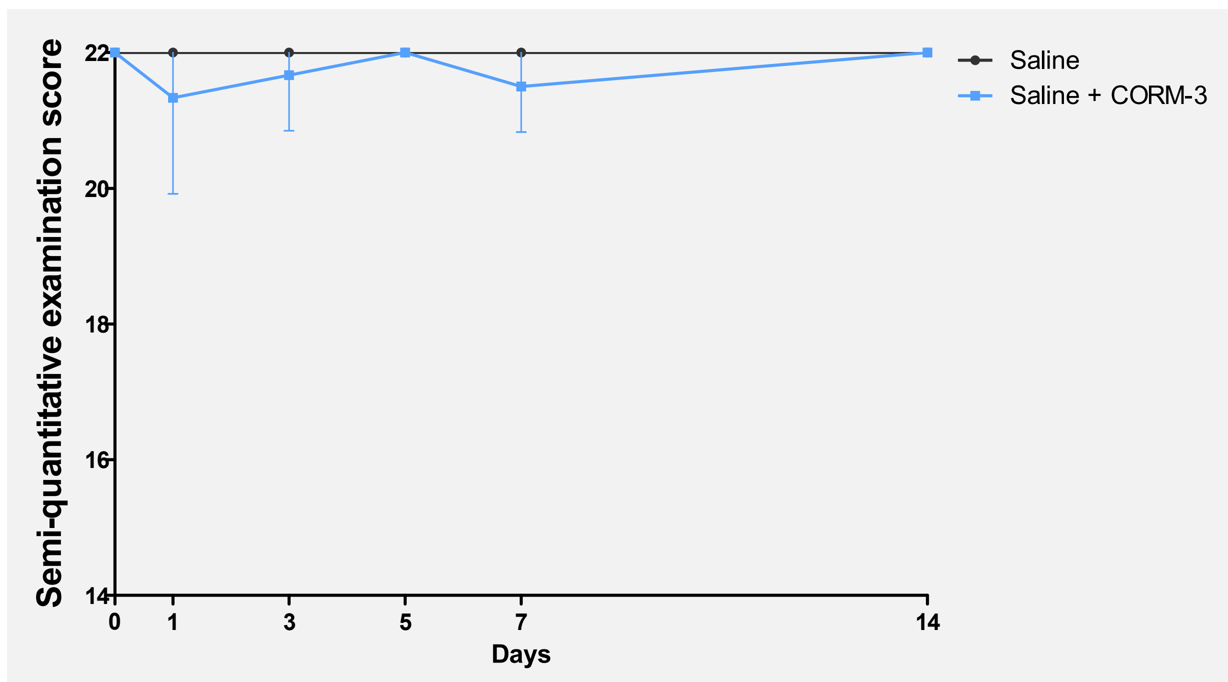


**Figure 6-12 Semi-quantitative examination score: saline (Group 4), saline + CORM-3 (Group 6) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No significant changes were seen.**

#### 6.2.2.4 Correlations between blood and lesion zone leukocytes and behavioural tests

A strong positive correlation was observed between:

- the number of peripheral blood lymphocytes and the beam walking test score
- the number of peripheral blood lymphocytes and the semi-quantitative examination test score
- the forelimb placing test and the semi-quantitative examination test score

A strong negative correlation was observed between:

- the number of peripheral blood neutrophils and the number of peripheral blood lymphocytes
- the number of peripheral blood neutrophils and the forelimb placing test score
- the number of peripheral blood neutrophils and the beam walking test score
- the number of peripheral blood neutrophils and the semi-quantitative examination test score

A moderate positive correlation was observed between:

- the number of peripheral blood neutrophils and the number of microglia cells in the lesion zone
- the number of peripheral blood lymphocytes and forelimb placing test score
- the beam walking test and plasma TNF-alpha levels
- the forelimb placing test and the beam walking test score
- the beam walking test and the semi-quantitative examination test score

A moderate negative correlation was observed between:

- the number of peripheral blood lymphocytes and the number of microglia cells in the lesion zone
- the number of peripheral blood neutrophils and plasma TNF-alpha levels
- the number of microglia cells in the lesion zone and plasma TNF-alpha levels
- the number of microglia cells in the lesion zone and the forelimb placing test score
- the number of microglia cells in th lesion zone and the beam walking test score
- the number of microglia cells in the lesion zone and the semi-quantitative examination test score

A weak positive correlation was observed between:

- the number of peripheral blood lymphocytes and plasma TNF-alpha levels
- the forelimb placing test and plasma TNF-alpha levels.

A weak negative correlation was observed between:

- the semi-quantitative examination test and plasma TNF-alpha levels.

**Table 8 Saline + CORM-3: matrix of levels pair correlation coefficients between blood and lesion zone leukocytes, plasma TNF alpha and behavioural tests**

| Parameters | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 1 | 0.00 | - 0.99 | 0.42 | - 0.71 | - 0.98 | - 0.81 | - 0.34 |
| 2 | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 3 | | | - 0.31 | 0.67 | 0.96 | 0.83 | 0.23 |
| 4 | | | | - 0.67 | - 0.41 | - 0.41 | - 0.62 |
| 5 | | | | | 0.61 | 0.87 | 0.08 |
| 6 | | | | | | 0.69 | 0.47 |
| 7 | | | | | | | - 0.14 |

1 – Peripheral blood neutrophil count; 2 – Number of neutrophils in the lesion zone; 3 – Peripheral blood lymphocyte count; 4 – Number of microglia cells in the lesion zone; 5 – Forelimb placing test scores, left paw; 6 – Beam walking test scores; 7 – Semi-quantitative examination scores, 8 – Plasma TNF-alpha levels

## 6.3 The effects of CORM-3 administered 5 minutes pre-operation treatment in an animal model of HS

### 6.3.1 Materials and methods. Experimental protocol.

| | |
|---|---|
| Group 5<br>(Collagenase injection) | Collagenase injection group (Group 5). Injection of 2 µl of 0.2 U collagenase solution into the lateral striatum of the experimental animals (n=30) |
| Group 8<br>(CORM-3 4 mg/kg group) | Collagenase injection into the lateral striatum of the experimental animal and CORM-3 administration at a dosage of 4 mg/kg 5 minutes i/v prior to inducing HS (n=30) |
| Group 9<br>(CORM-3 8 mg/kg group) | Collagenase injection into the lateral striatum of the experimental animal and CORM-3 administration at a dosage of 8 mg/kg 5 minutes i/v before operation 5 minutes prior to inducing HS (n=30) |

## 6.3.2 Results

### 6.3.2.1 Systemic level of inflammation

***General blood analysis: White blood cell count***

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg (Group 9) groups demonstrated a similar small increase in the number of white blood cells during days 1 and 3, which was higher than the one observed in the collagenase injection (Group 5) and in the pre-operation data. The CORM-3 4 mg/kg group (Group 8) demonstrated a decline in white cell count on day 5 and reached the levels of the collagenase group (Group 5) by day 7. The CORM-3 8 mg/kg group (Group 9), as compared to the CORM-3 4 mg/kg group (Group 8), showed a further increase on day 5 and a decrease by day 7, reaching the levels of CORM-3 4mg/kg (Group 8), collagenase (Group 5) and pre-operation data. These data are presented in Figure 6-13.

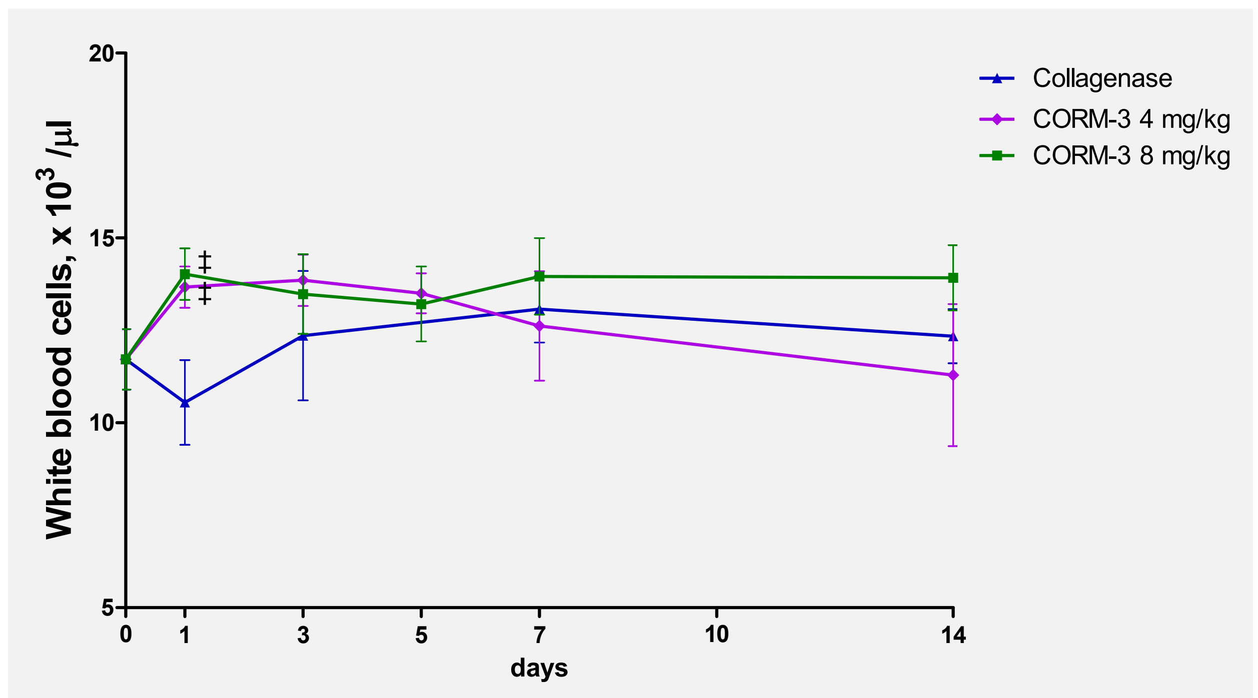


**Figure 6-13 White blood cell count: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg (Group 8), collagenase + CORM-3 8 mg/kg (Group 9) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) demonstrated a similar 3% increase in the number of peripheral blood neutrophils on day 1, compared to pre-operation data, which was significantly lower than in the collagenase injection group (Group 5).

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) showed a further decrease in the number of peripheral blood neutrophils on day 3. In the CORM-3 4 mg/kg group peripheral blood neutrophils reached the level of pre-operation data and the CORM-3 8 mg/kg reached the level of the collagenase injection group (Group 5), which was lower than in the CORM-3 4 mg/kg group by 2%, which was not statistically significant. Further changes in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) were similar to pre-operation data until the end of the experiment. These data are presented in Figure 6-14.

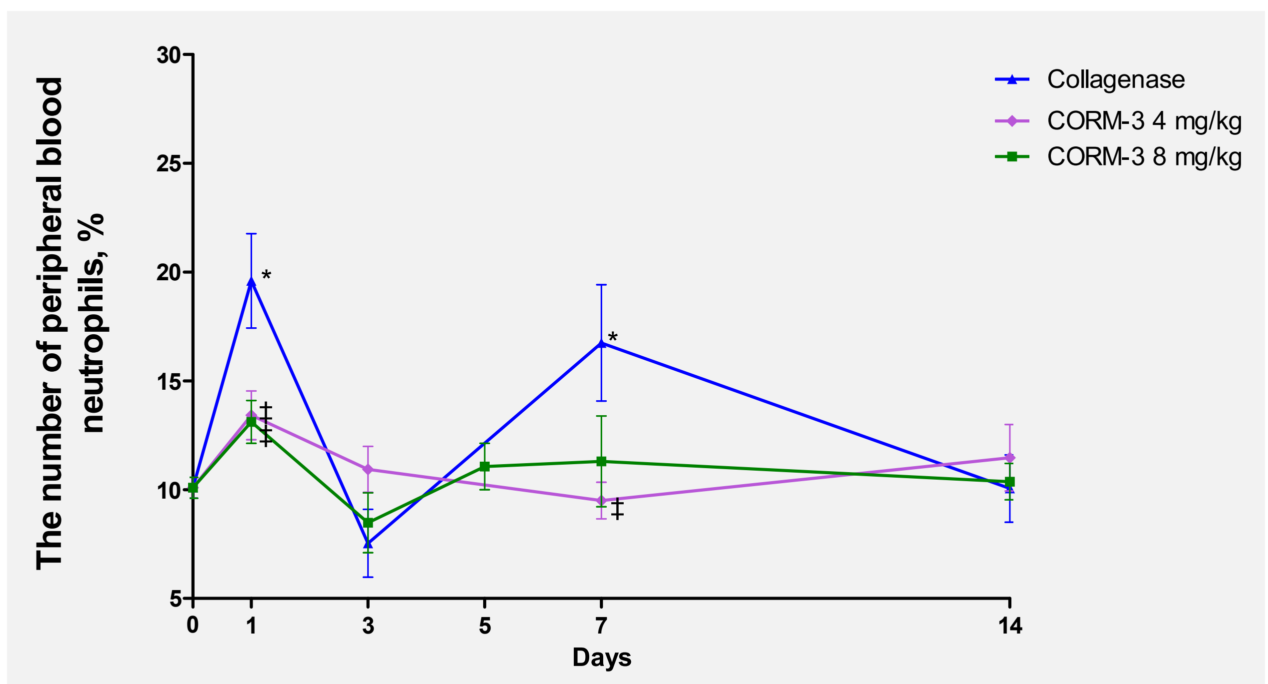


**Figure 6-14 Peripheral blood neutrophil count: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg (Group 8), collagenase + CORM-3 8 mg/kg (Group 9) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) demonstrated similar behaviour in peripheral blood lymphocyte number on day 1, which was at the same level as pre-operation data and significantly higher than what was measured in the collagenase injection group (Group 5).

The 3rd day of the experiment demonstrated an increase in the number of peripheral blood lymphocytes in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) by 8%, compared to pre-operation data, which was non-significantly lower than in the collagenase injection group (Group 5).

The trends in the number of peripheral blood lymphocytes in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) were similar, remaining above pre-operational levels by 6%, indicating a small but non-significant decrease in CORM-3 8 mg/kg group (Group 9) on day 5. These data is represented in Figure 6-15.

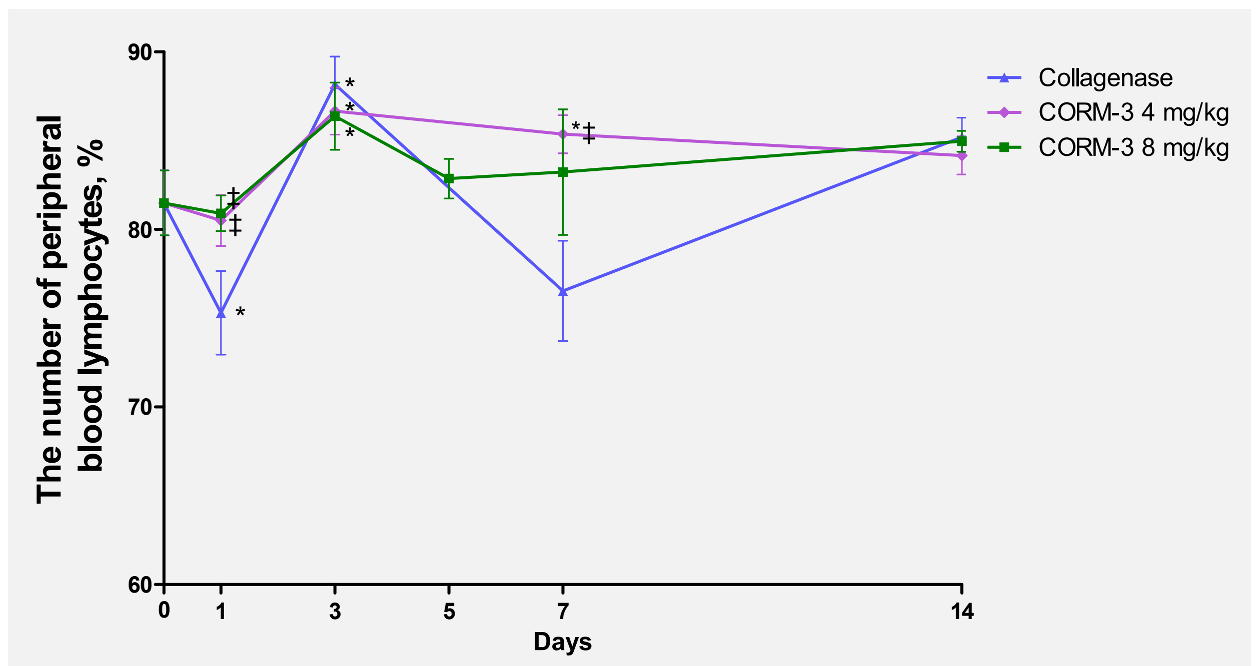


**Figure 6-15 Peripheral blood lymphocyte count: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg (Group 8), collagenase + CORM-3 8 mg/kg (Group 9) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data, ‡ - p < 0.05 vs. collagenase injection group (Group 5)**

***Enzyme-linked immunosorbent TNF-alpha assay***

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) demonstrated similar behaviour in changes of plasma TNF-alpha levels throughout the 14 days of the experiment.

There was a 4-fold increase in plasma TNF-alpha level on day 1 in the CORM-3 treatment groups, which was significantly higher than in pre-operation data and lower than in the collagenase injection group (Group 5). The level of plasma TNF-alpha in the group of the CORM-3 4 mg/kg (Group 8) was non-significantly higher than the one observed in the CORM-3 8 mg/kg (Group 9) treatment group.

Further changes in plasma TNF-alpha levels indicated a small decrease in the CORM-3 4 mg/kg group (Group 8) on day 3, while the CORM-3 8 mg/kg group (Group 9) remained at the same level.

Subsequent changes indicated plasma TNF-alpha levels remained at the same level in both CORM-3 treatment groups until the end of the experiment, which was significantly higher than in pre-operation data and significantly lower than in the collagenase injection group (Group 5). These data are presented in Figure 6-16.

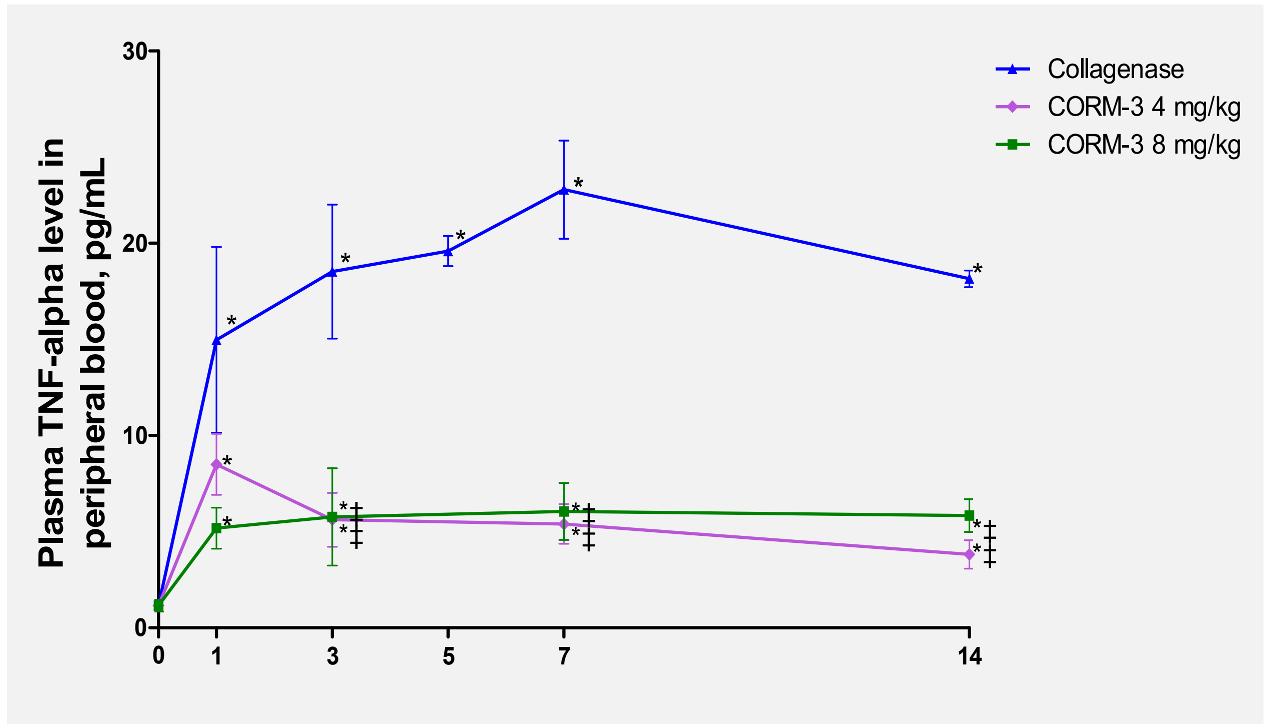


**Figure 6-16 Blood plasma TNF-alpha ELISA assay: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg (Group 8), collagenase + CORM-3 8 mg/kg (Group 9) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

#### 6.3.2.2 Local level of inflammation

***Brain intracerebral haemorrhage area determination***

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9), comparing to the collagenase injection group (Group 5), demonstrated a statistically significant decrease in the size of the intracerebral haemorrhage by 20% and 14% respectively.

The size of the intracerebral haemorrhage in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9), similar to the collagenase injection group (Group 5), was significantly different from the saline injection group (Group 4), where no intracerebral haemorrhage was observed. These data are presented in Figure 6-17 and Figure 6-18 respectively.

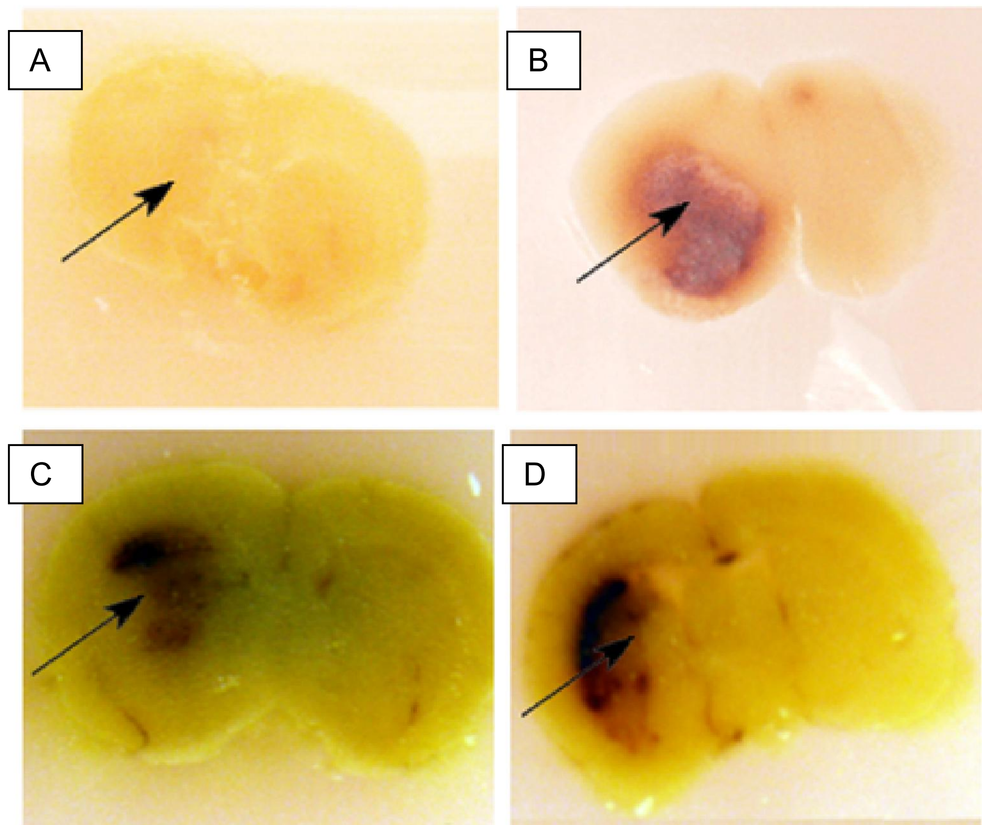


**Figure 6-17 Images of the brain slices through zone of interest obtained on the first day post operation (arrow indicates presence or absence of the intracerebral haemorrhage): A - Saline injection group (Group 4), B - Collagenase injection group (Group 5), C - Collagenase + CORM-3 4 mg/kg injection group (Group 8), D - Collagenase + CORM-3 8 mg/kg injection group (Group 9)**

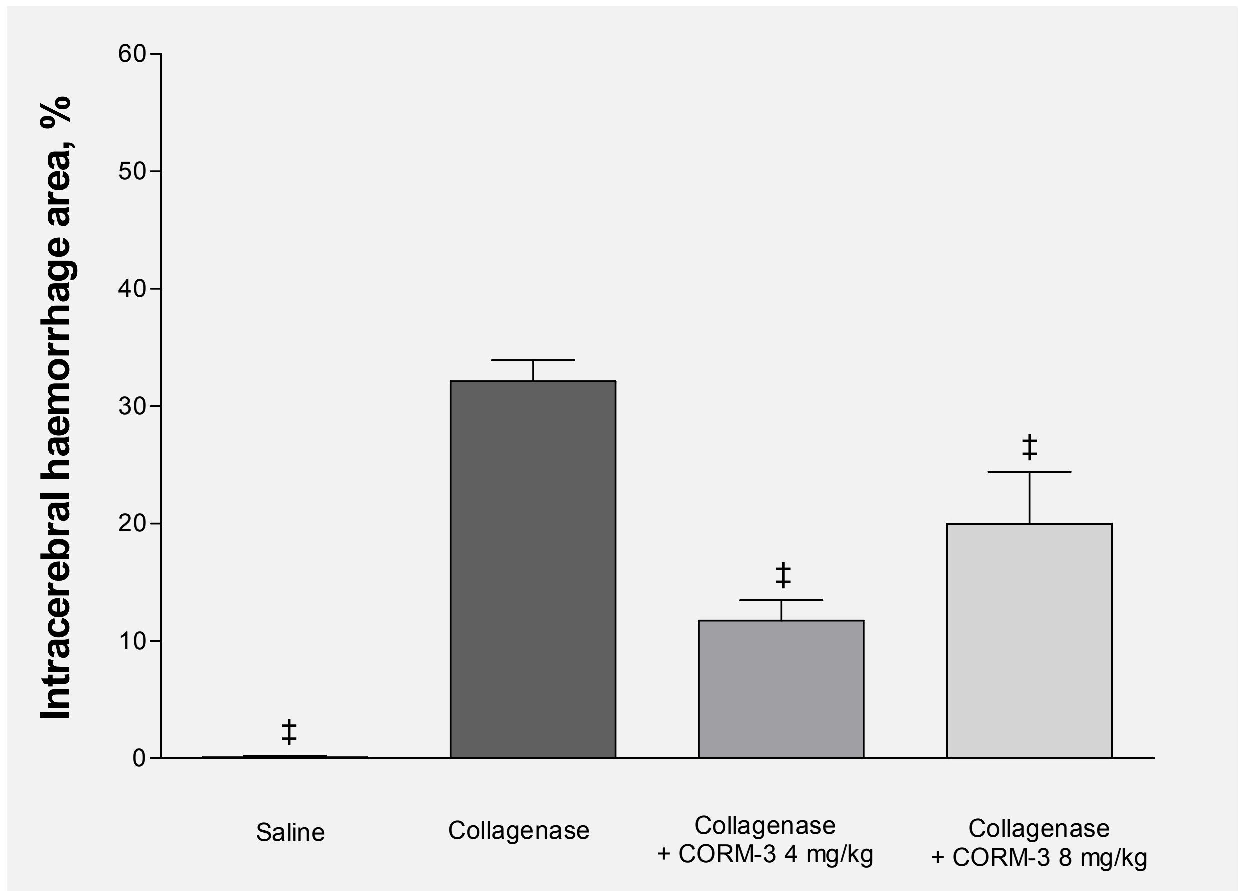


**Figure 6-18 Size of the intracerebral haemorrhage area. Data represent the mean ± SD of n = 6 independent experiments. ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

***Brain water content analysis***

The CORM-3 4 mg/kg group (Group 8) demonstrated a small increase in brain water content in the unaffected hemisphere on day 1, which was smaller than the one observed in the collagenase injection group (Group 5) but greater than in the pre-operation data. The CORM-3 8 mg/kg group (Group 9) demonstrated a brain water content level (on day 1) which was significantly greater than in the pre-operation data.

Further changes in the brain water content level in the CORM-3 4 mg/kg group (Group 8) indicated a non-significant decrease and reached the level of the collagenase injection group (Group 5) and pre-operation data by day 5 and 7 after a small non-significant increase on day 3, and remained at the level of the saline injection group (Group 4) until the end of the experiment.

Subsequent changes in the brain water content level in the CORM-3 8 mg/kg group (Group 9) demonstrated a small decrease on day 3, a small increase on day 5 with a further decrease reaching the level of pre-operation data by day 7 and 14. These data are presented in Figure 6-19.

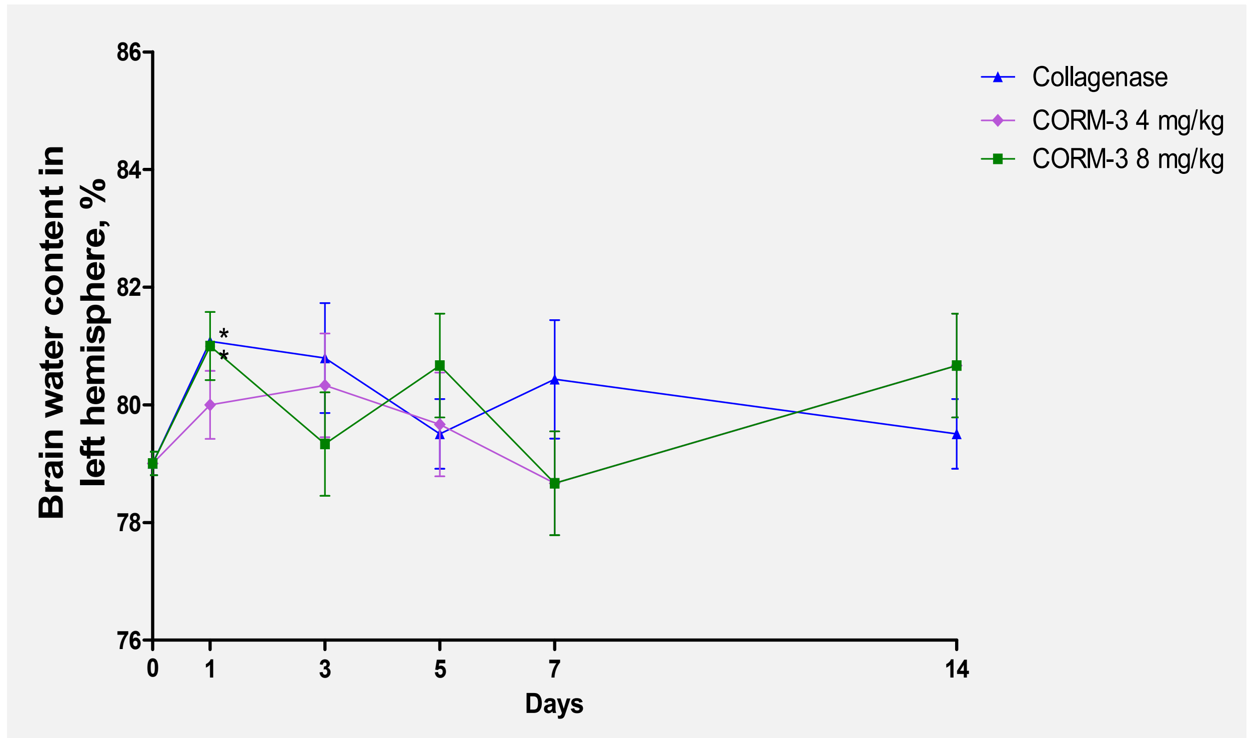


**Figure 6-19 Brain water content for the left hemisphere. Collagenase (Group 5), collagenase + CORM-3 4 mg/kg (Group 8), collagenase + CORM-3 8 mg/kg (Group 9) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) indicated a similar increase in the brain water content of the affected hemisphere, which was lower than in the collagenase injection group (Group 5) and significantly higher than the pre-operation data.

Data from the 3rd and 5th days of the experiment demonstrated a gradual decrease in the brain water content of the affected hemisphere in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) and reached the level of the collagenase injection group (Group 5) and pre-operation data on day 5. Further changes in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) indicated the brain water content to be at the same level of the pre-operation data until the end of the experiment. These data are presented in Figure 6-20.

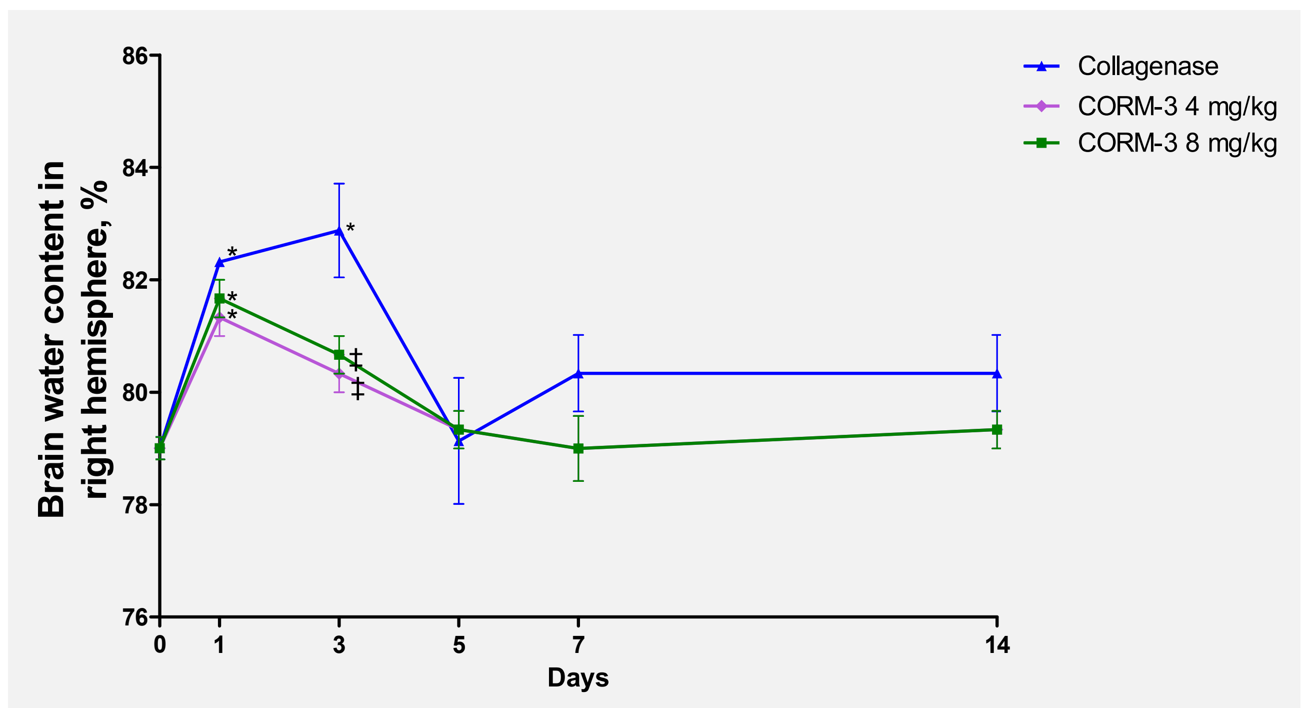


**Figure 6-20 Brain water content for the right hemisphere: collagenase (Group 5), collagenase + CORM-3 4mg/kg (Group 8), collagenase + CORM-3 8 mg/kg (Group 9) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

***Densitometric cell count***

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) demonstrated a rapid 40-fold increase in the number of activated neutrophils on day 1, which was significantly higher than in the collagenase injection group (Group 5) and pre-operation data.

On day 3 of the experiment there was a decrease in the number of activated neutrophils in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9); however the levels remained significantly higher than in the collagenase injection group (Group 5) and pre-operation data.

Further changes in the number of activated neutrophils in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) showed a decrease and reached the levels of the collagenase injection group (Group 5) and pre-operation data by day 5 and 7 and remained at the same level until the end of the experiment. These data are presented in Figure 6-21.

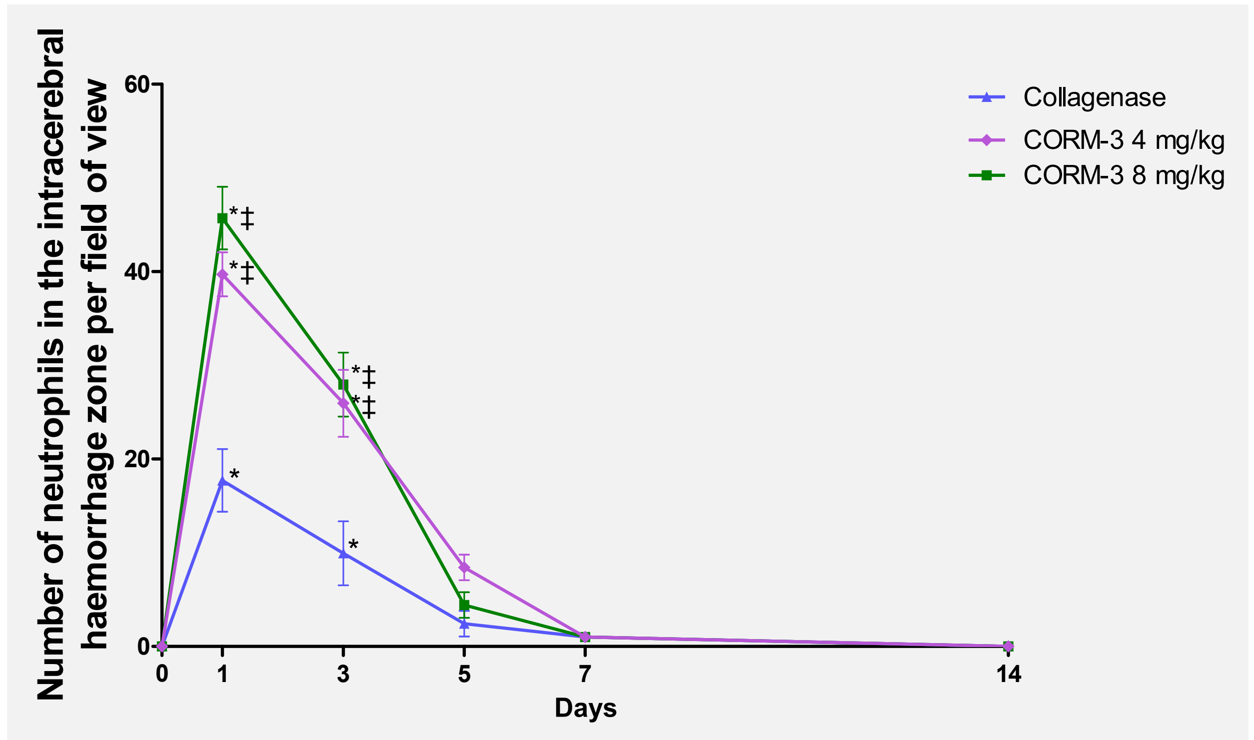


**Figure 6-21 Densitometric cell count analysis: neutrophil cell number (mean) in the intracerebral haemorrhage zone in 7 fields of view. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) demonstrated a similar 8-fold increase in the number of microglial cells on day 1, which was lower than in the collagenase injection group (Group 5) and significantly higher than the one observed in pre-operation data.

The 3rd day of the experiment indicated a decrease in the number of activated microglia in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9), which remained lower than in the collagenase injection group (Group 5) and significantly higher than in pre-operation data. Further changes in the number of microglial cells in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) showed a decrease, reaching the level of pre-operation data by day 5, then remaining at the same level until the end of the experiment. These data are presented in Figure 6-22.

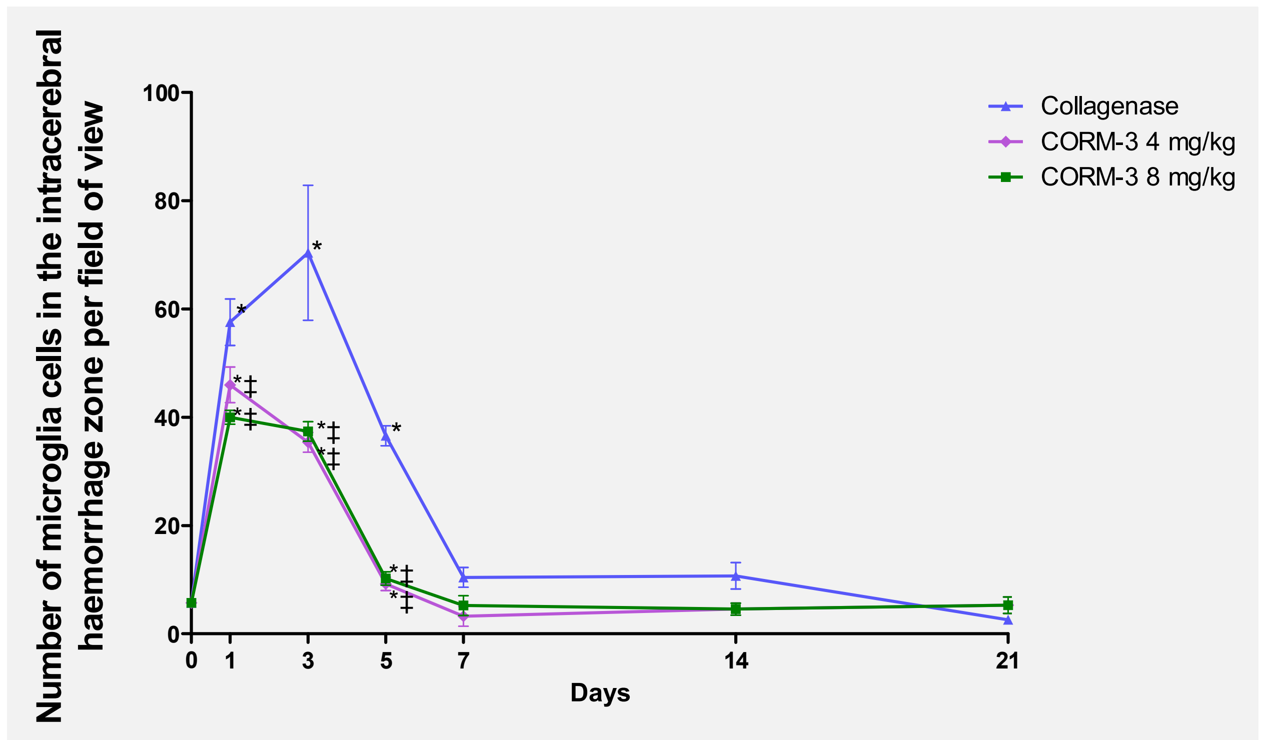


**Figure 6-22 Densitometric cell count analysis: microglial cell number (mean) in the intracerebral haemorrhage zone in 7 fields of view. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

### 6.3.2.3 Behavioural testing

***Beam walking***

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg (Group 9) groups indicated (similar to the collagenase injection group (Group 5)) a 1.5 – fold decrease in performance in the beam walking test on day 1, with the CORM-3 4 mg/kg group performance being slightly higher than the collagenase injection group (Group 5) and the CORM-3 8 mg/kg (Group 9) group being slightly lower than in the collagenase injection group (Group 5).

Further changes indicated a gradual improvement in the performance in the beam walking test in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) on day 3, which was somewhat higher than in the collagenase injection group (Group 5), and reached the level of the collagenase injection group (Group 5) and pre-operation data on day 7.

Further changes in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) in the beam walking test indicated maximal performance until the end of the experiment. These data are presented in Figure 6-23.

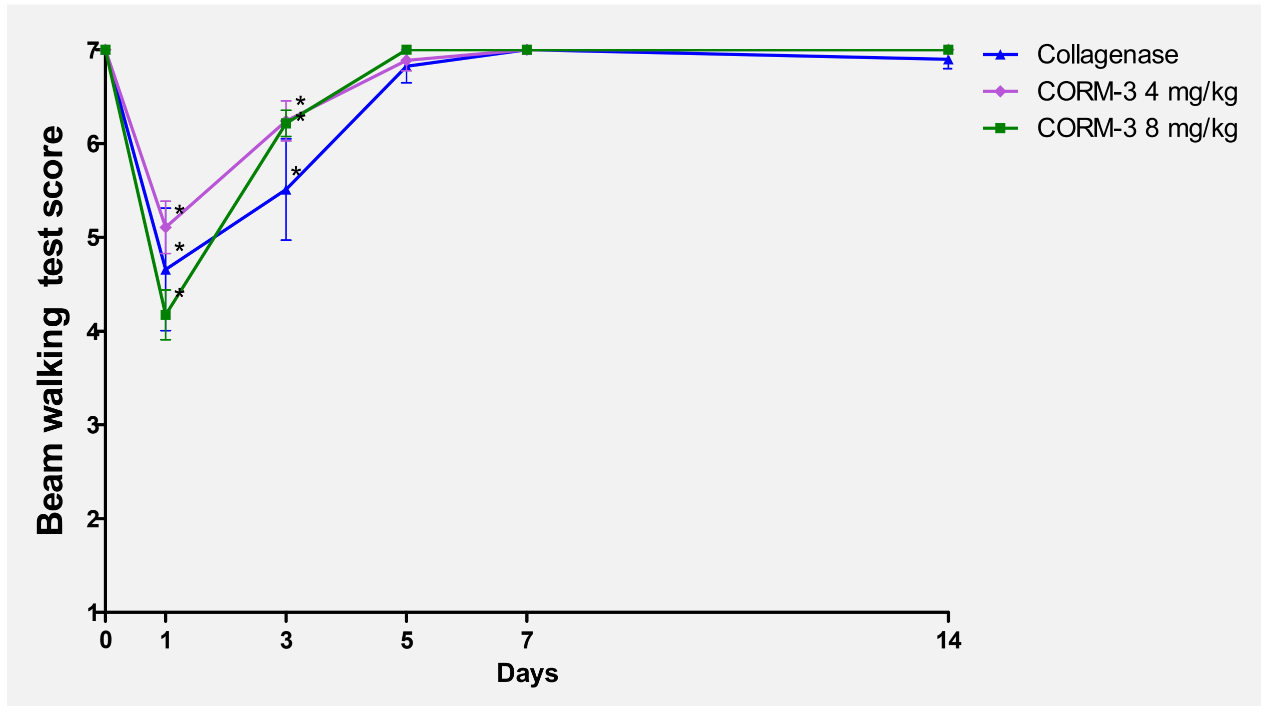


**Figure 6-23 Beam walking test score: the collagenase, collagenase + CORM-3 4mg/kg, collagenase + CORM-3 8 mg/kg groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data**

***Forelimb placing test***

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) demonstrated 2 – and 2.5 – fold decreases in performance in the forelimb placing test on day 1, compared to pre-operation data being 1.2 – and 1.5 – fold lower than in the collagenase injection group (Group 5) respectively.

Further changes in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) indicated a gradual increase in performance in the forelimb placing test on day 3, when CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) showed slightly better performance than in the collagenase injection group (Group 5).

The 5$^{th}$ day of the experiment demonstrated an improvement in the forelimb placing test in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9), when they have reached the pre-operation test scores.

Further changes indicated maximal performance in the forelimb placing test in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) until the end of the experiment, in contrast to the collagenase injection group (Group 5), when maximal performance was reached by day 14. These data are presented in Figure 6-24.

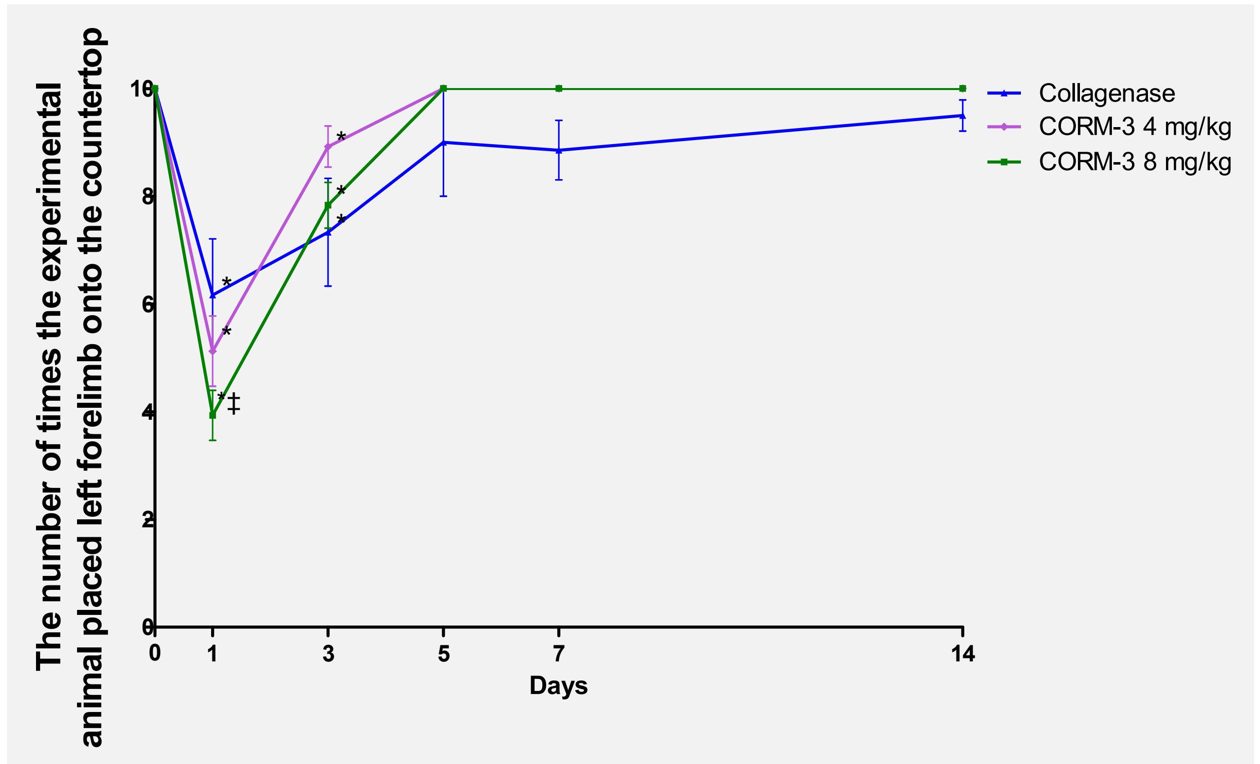


**Figure 6-24 Forelimb placing, left paw: the collagenase, collagenase + CORM-3 4mg/kg, collagenase + CORM-3 8 mg/kg groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) demonstrated (similar to the collagenase injection group (Group 5)) maximal performance in the forelimb placing test of the unaffected paw throughout the days of the experiment, which was at the level of pre-operation data. These data are presented in Figure 6-25.

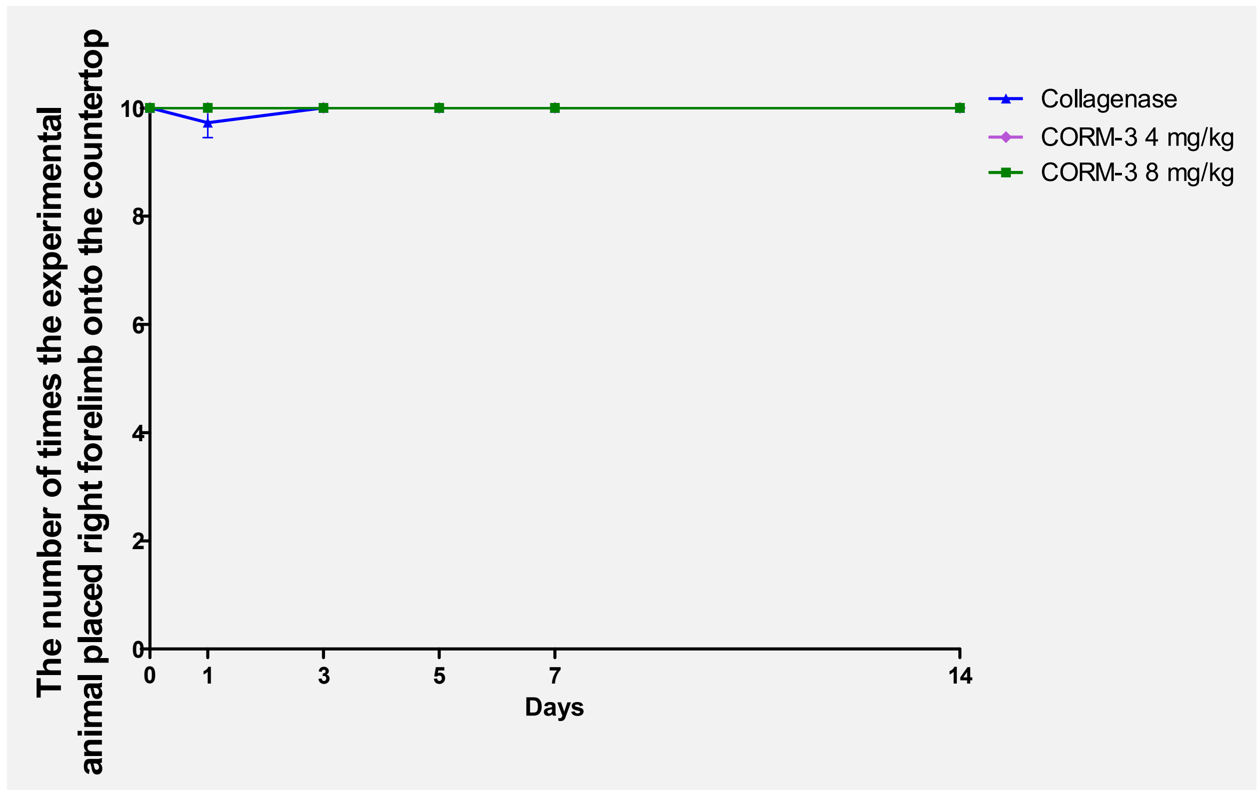


**Figure 6-25 Forelimb placing, right paw: the collagenase, collagenase + CORM-3 4mg/kg, collagenase + CORM-3 8 mg/kg groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

***Semi-quantitative examination***

The CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) demonstrated (similar to the collagenase injection group (Group 5)) a 1.15 – fold decrease in performance in semi-quantitative examination on day 1, with CORM-3 8 mg/kg demonstrating slightly lower performance than in the collagenase injection group (Group 5).

Further changes indicated a gradual improvement in the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) in the semi-quantitative examination, which was higher than in the collagenase injection group (Group 5) and reached the pre-operation data level by day 5 in the CORM-3 8 mg/kg group and by day 7 in the CORM-3 4 mg/kg group.

Further changes showed the CORM-3 4 mg/kg (Group 8) and the CORM-3 8 mg/kg groups (Group 9) performance in the semi-quantitative examination remained at the level of pre-operation data until the end of the experiment, while the collagenase injection group (Group 5) demonstrated a slightly lower performance with a small decrease on day 14. These data are presented in Figure 6-26.

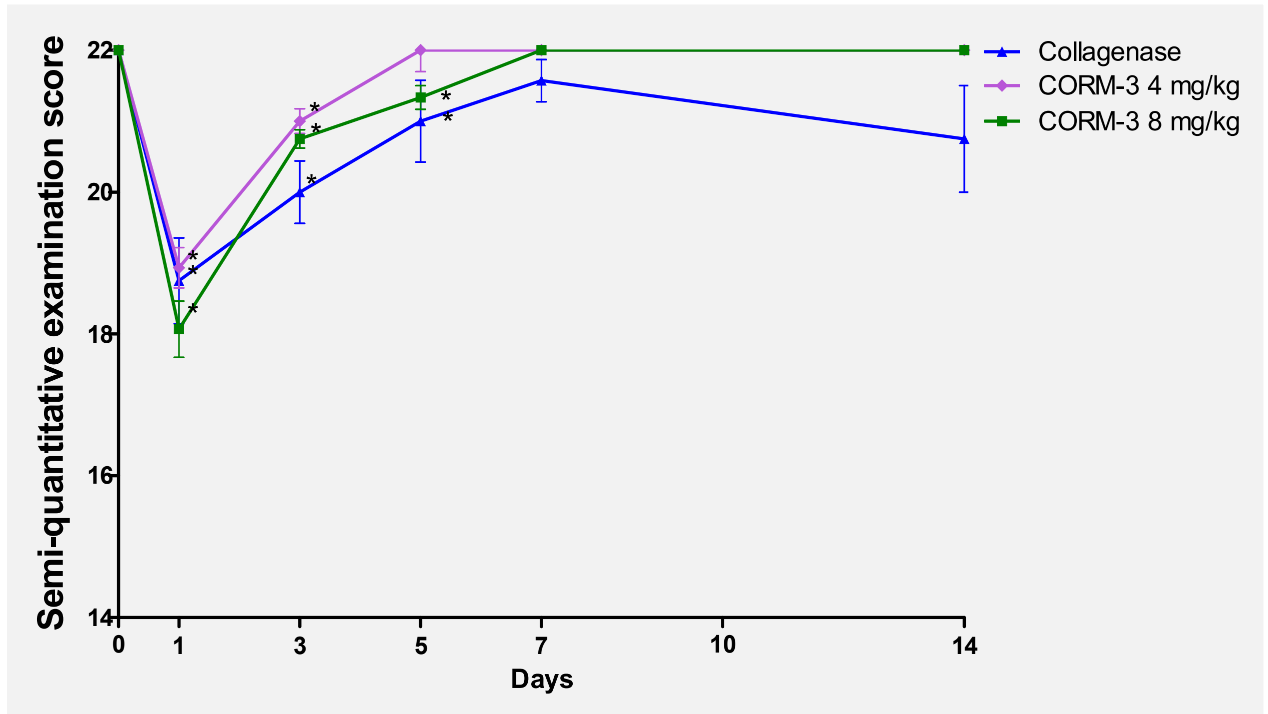


**Figure 6-26** Semi-quantitative examination score: the collagenase, collagenase + CORM-3 4mg/kg, collagenase + CORM-3 8 mg/kg groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data

#### 6.3.2.4 Correlations between blood and lesion zone leukocytes and behavioural tests

A strong positive correlation was observed between:

- the number of peripheral blood neutrophils and the number of neutrophils in the lesion zone
- the number of peripheral blood neutrophils and the number of microglia cells in the lesion zone
- the number of neutrophils in the lesion zone and the number of microglia cells in the lesion zone
- the number of neutrophils in the lesion zone and plasma TNF-alpha levels
- the number of microglia cells in the lesion zone and plasma TNF-alpha levels
- the number of peripheral blood lymphocytes and the forelimb placing test score
- the number of peripheral blood lymphocytes and the beam walking test score
- the forelimb placing test score and the beam walking test score
- peripheral blood lymphocytes and semi-quantitative examination score
- the forelimb placing test score and semi-quantitative examination score
- the beam walking test score and semi-quantitative examination score

A strong negative correlation was observed between:

- the number of peripheral blood neutrophils and the number of peripheral blood lymphocytes
- the number of peripheral blood neutrophils and the forelimb placing test score
- the number of microglia cells in the lesion zone and the forelimb placing test score
- the number of peripheral blood neutrophils and the beam walking test score

- the number of neutrophils in the lesion zone and beam walking test
- the number of microglia cells in the lesion zone and the beam walking test score
- the number of peripheral blood neutrophils and the semi-quantitative examination score
- the number of neutrophils in the lesion zone and the semi-quantitative examination score
- the number of microglia cells and the semi-quantitative examination score
- the forelimb placing test score and plasma TNF-alpha levels
- the beam walking test score and plasma TNF-alpha levels
- the semi-quantitative examination score and plasma TNF-alpha levels.

A moderate positive correlation was observed between:

- the number of peripheral blood neutrophils and plasma TNF-alpha levels.

A moderate negative correlation was observed between:

- the number of neutrophils in the lesion zone and the number of peripheral blood lymphocytes
- the number of peripheral blood lymphocytes and the number of microglia cells in the lesion zone
- the number of peripheral blood lymphocytes and plasma TNF-alpha levels.

**Table 9 HS + CORM-3 4 mg/kg treatment: matrix of levels pair correlation coefficients between blood and lesion zone leukocytes, plasma TNF alpha and behavioural tests**

| Parameters | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 1 | 0.74 | - 0.82 | 0.75 | - 0.87 | - 0.84 | - 0.87 | 0.59 |
| 2 | | - 0.54 | 0.99 | - 0.91 | - 0.97 | - 0.94 | 0.87 |
| 3 | | | - 0.48 | 0.84 | 0.73 | 0.78 | - 0.69 |
| 4 | | | | - 0.87 | - 0.95 | - 0.92 | 0.80 |
| 5 | | | | | 0.98 | 0.99 | - 0.91 |
| 6 | | | | | | 0.99 | - 0.90 |
| 7 | | | | | | | - 0.90 |

1 – Peripheral blood neutrophil count; 2 – Number of neutrophils in the lesion zone; 3 – Peripheral blood lymphocyte count; 4 – Number of microglia cells in the lesion zone; 5 – Forelimb placing test scores, left paw; 6 – Beam walking test scores; 7 – Semi-quantitative examination scores, 8 – Plasma TNF-alpha levels

A strong positive correlation was observed between:

- the number of neutrophils in the lesion zone and the number of microglia cells in the lesion zone
- the forelimb placing test score and the beam walking test score
- the forelimb placing test score and the semi-quantitative examinations core
- the beam walking test score and the semi-quantitative examination score
- the number of neutrophils in the lesion zone and plasma TNF-alpha levels
- the number of microglia cells in in the lesion zone and plasma TNF-alpha levels.

A strong negative correlation was observed between:

- the number of peripheral blood neutrophils and the number of peripheral blood lymphocytes
- the number of neutrophils in the lesion zone and the forelimb placing test score
- the number of microglia cells in the lesion zone and the forelimb placing test score
- the number of neutrophils in the lesion zone and the beam walking test score

- the number of microglia cells in the lesion zone and the beam walking test score
- the number of neutrophils in the lesion zone and the semi-quantitative examination score
- the number of microglia cells in the lesion zone and the semi-quantitative examination score
- the forelimb placing score and plasma TNF-alpha levels
- the beam walking test score and plasma TNF-alpha levels
- the semi-quantitative examination score and plasma TNF-alpha levels.

A moderate positive correlation was observed between:

- the number of peripheral blood neutrophils and the number of neutrophils in the lesion zone
- the number of peripheral blood neutrophils and plasma TNF-alpha levels
- the number of peripheral blood lymphocytes and the forelimb placing test score
- the number of peripheral blood lymphocytes and the beam walking test score
- the number of peripheral blood lymphocytes and the semi-quantitative examination score

A moderate negative correlation was observed between:

- the number of neutrophils in the lesion zone and the number of peripheral blood lymphocytes
- the number of peripheral blood lymphocytes and plasma TNF-alpha levels
- the number of peripheral blood neutrophils and the forelimb placing test score
- the number of peripheral blood neutrophils and the beam walking test score

- the number of peripheral blood neutrophils and the semi-quantitative examination score

A weak positive correlation was observed between:

- the number of peripheral blood neutrophils and the number of microglia cells in the lesion zone.

A weak negative correlation was observed between:

- the number of peripheral blood lymphocytes and the number of microglia cells in the lesion zone.

**Table 10 HS + CORM-3 8 mg/kg treatment: matrix of levels pair correlation coefficients between blood and lesion zone leukocytes, plasma TNF alpha and behavioural tests**

| Parameters | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 1 | 0.28 | - 0.98 | 0.02 | - 0.48 | -0,55 | - 0.55 | 0.56 |
| 2 | | - 0.32 | 0.96 | - 0.97 | -0,95 | - 0.95 | 0.87 |
| 3 | | | - 0.09 | 0,49 | 0,55 | 0.59 | - 0.58 |
| 4 | | | | - 0.87 | - 0.83 | - 0.84 | 0.75 |
| 5 | | | | | 0.99 | 0.98 | - 0.93 |
| 6 | | | | | | 0.99 | - 0.94 |
| 7 | | | | | | | - 0.96 |

1 – Peripheral blood neutrophil count; 2 – Number of neutrophils in the lesion zone; 3 – Peripheral blood lymphocyte count; 4 – Number of microglia cells in the lesion zone; 5 – Forelimb placing test scores, left paw; 6 – Beam walking test scores; 7 – Semi-quantitative examination scores, 8 – Plasma TNF-alpha levels

## 6.4 Effects of CORM-3 administered 3 hours post-operation on HS

### 6.4.1 Materials and methods. Experimental protocol.

| | |
|---|---|
| Group 5 (Collagenase injection group) | Collagenase injection group (Group 5). Injection of 2 µl of 0.2 U collagenase solution into the lateral striatum of the experimental animals (n=30) |
| Group 10 (CORM-3 4 mg/kg group) | Collagenase injection into the lateral striatum of the experimental animals and CORM-3 administration at the dosage of 4 mg/kg 3 hours after inducing HS (n=30) |
| Group 11 (CORM-3 8 mg/kg group) | Collagenase injection into the lateral striatum of the experimental animals and CORM-3 administration at the dosage of 8 mg/kg 3 hours after inducing HS (n=30) |

### 6.4.2 Results

#### 6.4.2.1 Systemic level of inflammation

***General blood analysis: White blood cell count***

The CORM-3 4 mg/kg group (Group 10) showed a small increase in the number of white blood cells on day 1, which was similar to pre-operation data and higher than in the collagenase injection group (Group 5).

The CORM-3 8 mg/kg group (Group 11) demonstrated a small increase in the number of white blood cells on day 1, which was higher than in the CORM-3 4 mg/kg group (Group 10) and the collagenase injection group (Group 5).

On the 3rd day of the experiment the levels of white blood cells in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) reached the level of pre-operation data, which was similar to the collagenase injection group (Group 5).

Further changes in the number of white blood cells indicated a small decrease in the CORM-3 4 mg/kg group (Group 10) on day 7, which was lower than in the collagenase injection group (Group 5) and pre-operation data; and an increase in the CORM-3 8 mg/kg group (Group 11) on day 7, which was higher than in the collagenase injection group (Group 5) and pre-operation data.

Subsequent changes in the number of white blood cells in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) reaching the levels of the collagenase injection group (Group 5) and pre-operation data occurred by day 14. These data are presented in Figure 6-27.

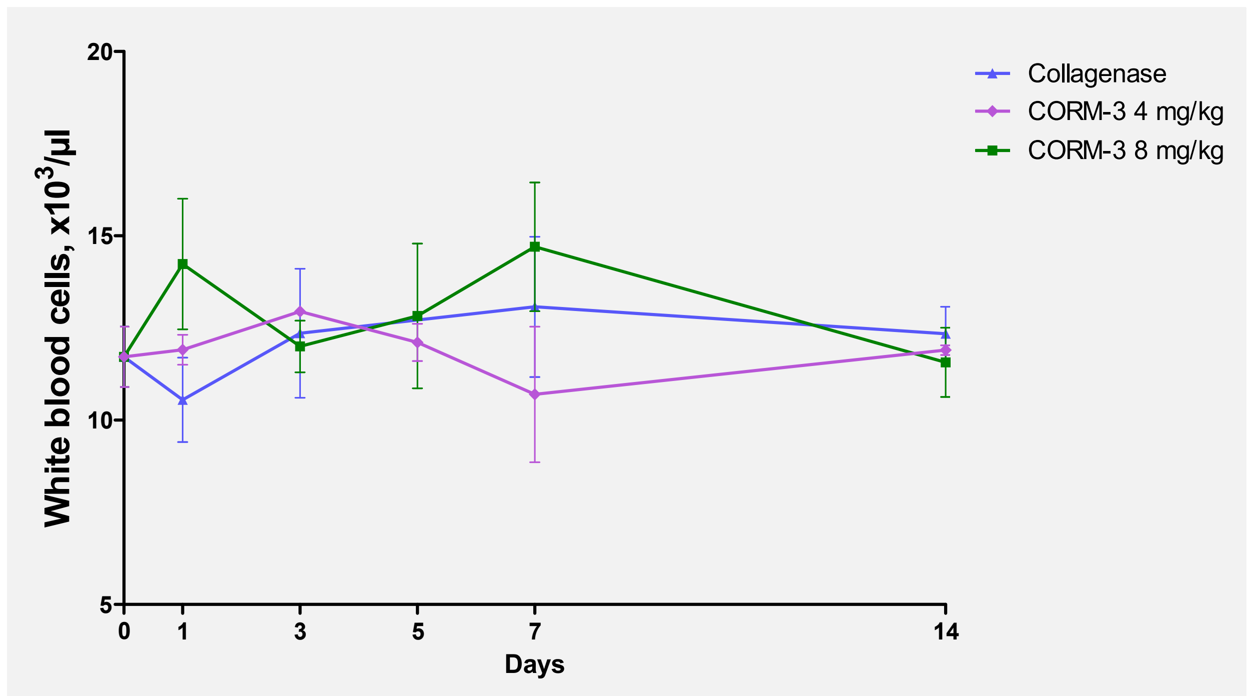


**Figure 6-27 White blood cell count: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 10), collagenase + CORM-3 8 mg/kg (Group 11) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated a similar 22% and 23% increase in the number of peripheral blood neutrophils on day 1, which was similar to that observed in the collagenase injection group (Group 5) and significantly higher than the one in pre-operation data.

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) showed a further decrease in the number of peripheral blood neutrophils on day 3, indicating the CORM-3 4 mg/kg group (Group 10) reaching the level of the collagenase injection group (Group 5) and the CORM-3 8 mg/kg group (Group 11), being somewhat higher than in the collagenase injection group (Group 5) and the level of pre-operation data.

Further changes in the CORM-3 4 mg/kg group (Group 10) showed a 7% - increase in the number of peripheral blood neutrophils on day 5 with a subsequent decrease reaching the levels of pre-operation data by days 7 and 14. In contrast to the CORM-3 4 mg/kg group (Group 10), further changes in the CORM-3 8 mg/kg group (Group 11) indicated an 11% increase in the number blood neutrophils on day 7, was also observed in the collagenase injection group (Group 5) but was significantly higher, with a subsequent decrease reaching the levels of pre-operation data by day 14. These data are presented in Figure 6-28.

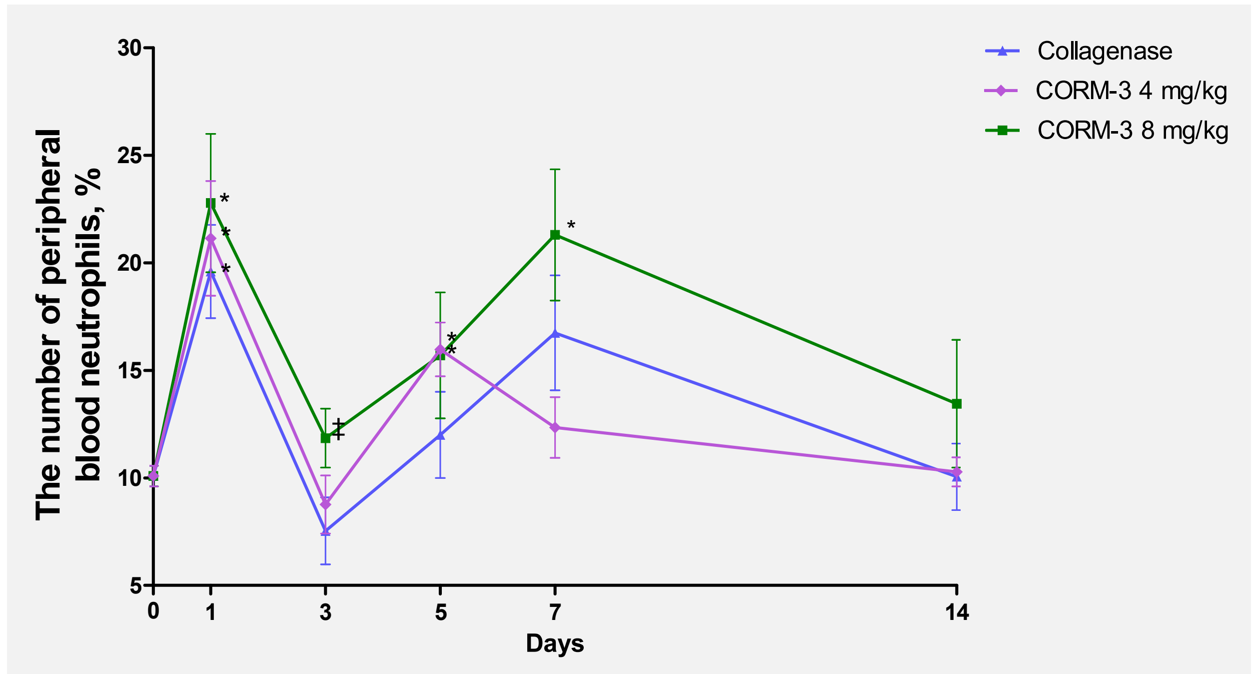


**Figure 6-28 Peripheral blood neutrophil count: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 10), collagenase + CORM-3 8 mg/kg (Group 11) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated a similar 9-10% decrease in the number of peripheral blood lymphocytes on day 1, when compared to pre-operation data, which were somewhat lower than the change observed in the collagenase injection group (Group 5).

On the 3rd day of the experiment an increase in the number of peripheral blood lymphocytes in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) was observed, being lower by 6% than in the collagenase injection group (Group 5) and also lower than the level of pre-operation data. Further changes in peripheral blood lymphocytes in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) indicated a small decrease on day 5 with subsequent restoration to pre-operational levels on day 7 and 14. These data are presented in Figure 6-29.

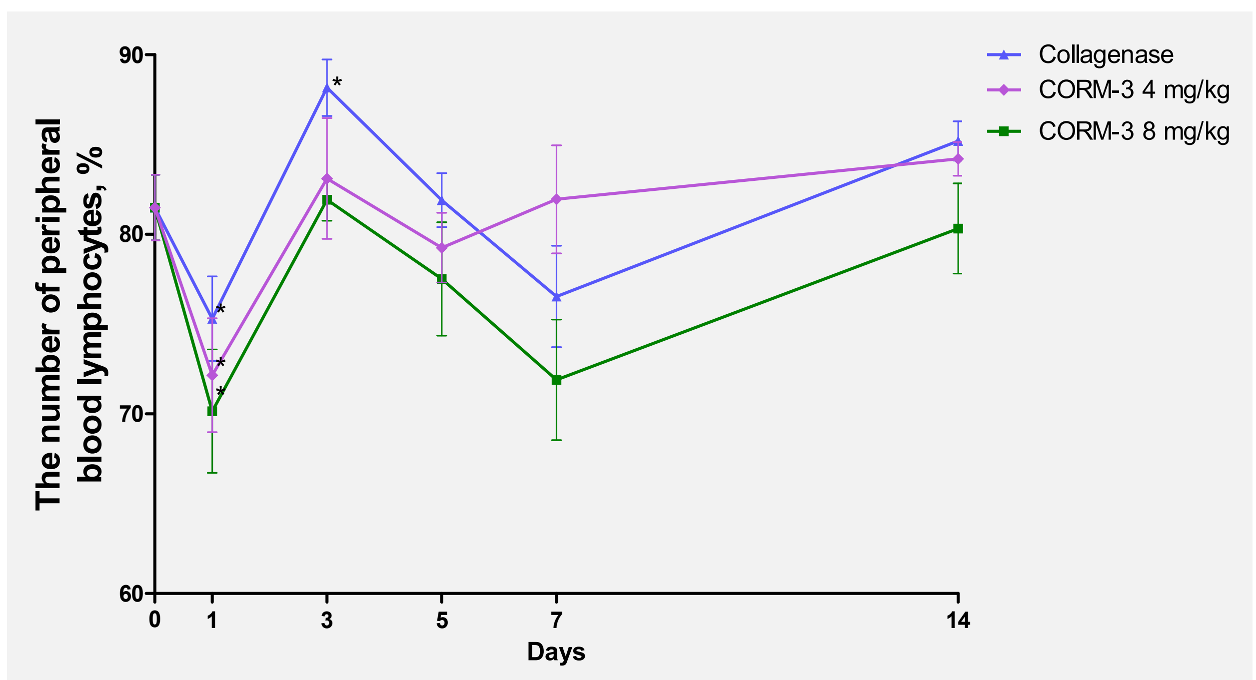


**Figure 6-29 Peripheral blood lymphocytes count: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 10), collagenase + CORM-3 8 mg/kg (Group 11) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data**

***Enzyme-linked immunosorbent TNF-alpha assay***

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated a similar 16-fold increase in plasma TNF-alpha levels on day 1, which was significantly higher than pre-operation data and the collagenase injection group (Group 5).

Data from later time points indicated that the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) remained at the same level until the end of the experiment, which was significantly higher than pre-operation data, and had a similar level to the collagenase injection group (Group 5) on day 5 and 7. These data are presented in Figure 6-30.

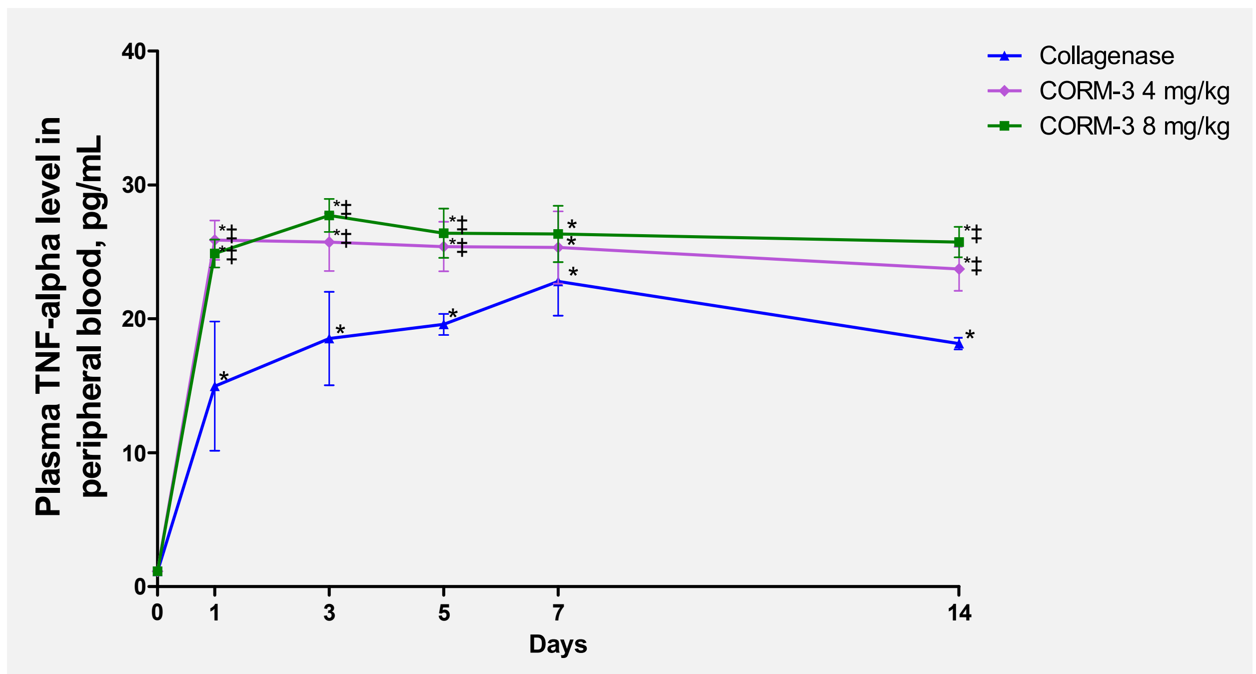


**Figure 6-30 Blood plasma TNF-alpha assay: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 10), collagenase + CORM-3 8 mg/kg (Group 11) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

#### 6.4.2.2 Local level of inflammation

***Brain intracerebral haemorrhage area determination***

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg (Group 11) groups, compared to the collagenase injection group (Group 5), demonstrated an increase in the size of the intracerebral haemorrhage by 15% and 14% respectively. The size of the intracerebral haemorrhage in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) was significantly different from the saline injection group (Group 4), where no intracerebral haemorrhage was observed. These data are presented in Figure 6-31 and Figure 6-32.

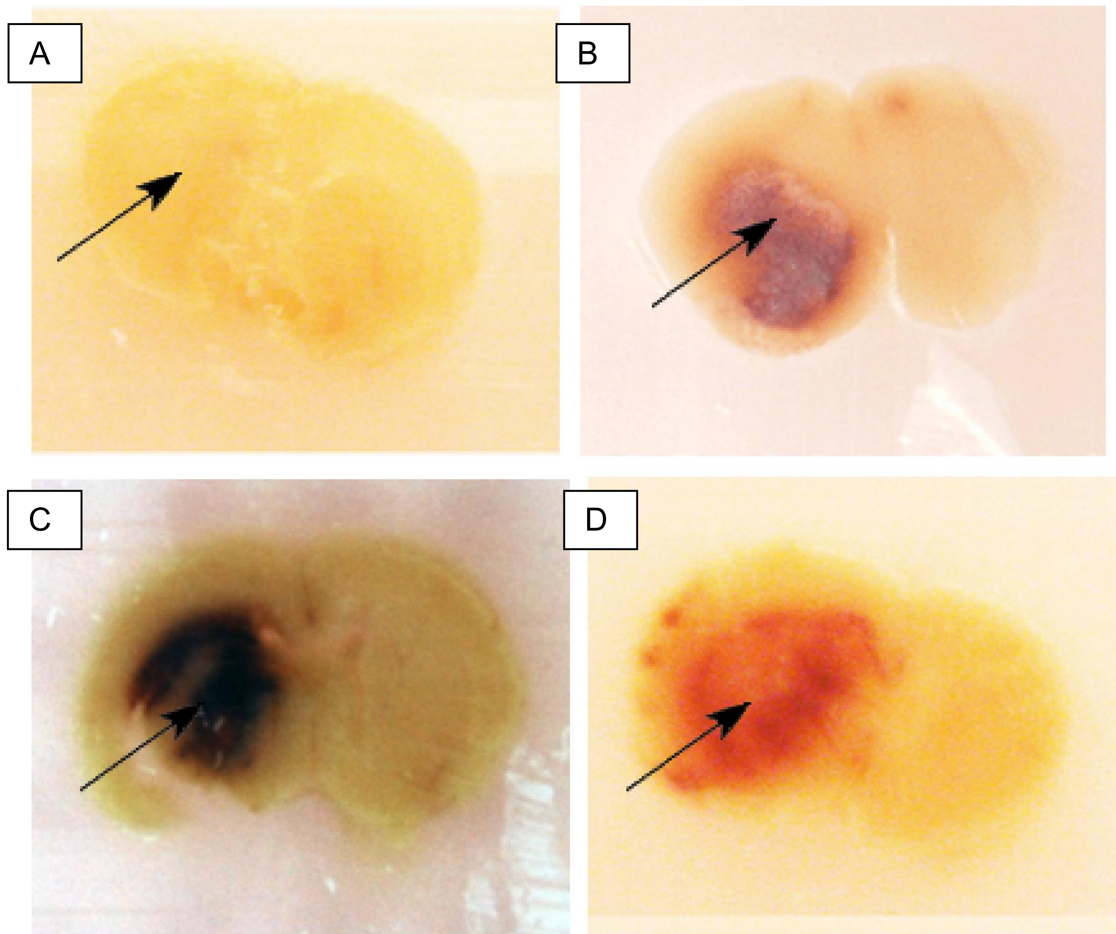


**Figure 6-31 Images of brain slices through the zone of interest, obtained on the first day post operation (arrow indicates presence or absence of the intracerebral haemorrhage): A - Saline injection group (Group 4), B - Collagenase injection group (Group 5), C - Collagenase + CORM-3 4 mg/kg injection group, D - Collagenase + CORM-3 8 mg/kg injection group**

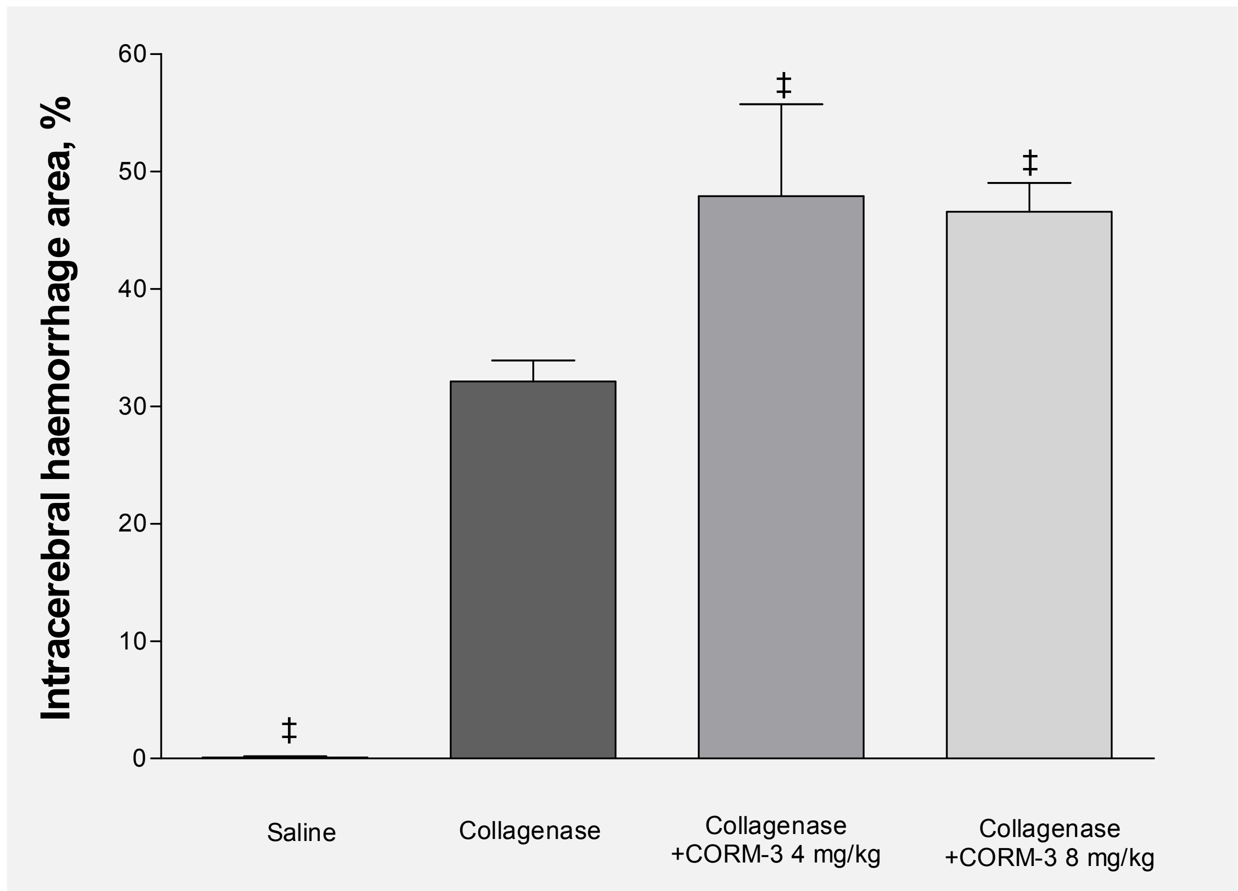


**Figure 6-32 Size of the intracerebral haemorrhage area: ‡ - $p < 0.05$ vs. collagenase injection group (Group 5). Data represent the mean ± SD of n = 6 independent experiments.**

***Brain water content analysis***

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated a small increase in the brain water content of the unaffected hemisphere on day 1, which was lower than in the collagenase injection group (Group 5) and higher than pre-operation data.

The 3$^{rd}$ and 5$^{th}$ day of the experiment indicated a gradual decrease in brain water content in the unaffected hemisphere in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) reaching the level of the collagenase injection group (Group 5) and pre-operation data by day 5.

Further changes in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) indicated a similar small decrease in brain water content on day 7, compared to the collagenase injection group (Group 5) and pre-operation data levels. These data are presented in Figure 6-33.

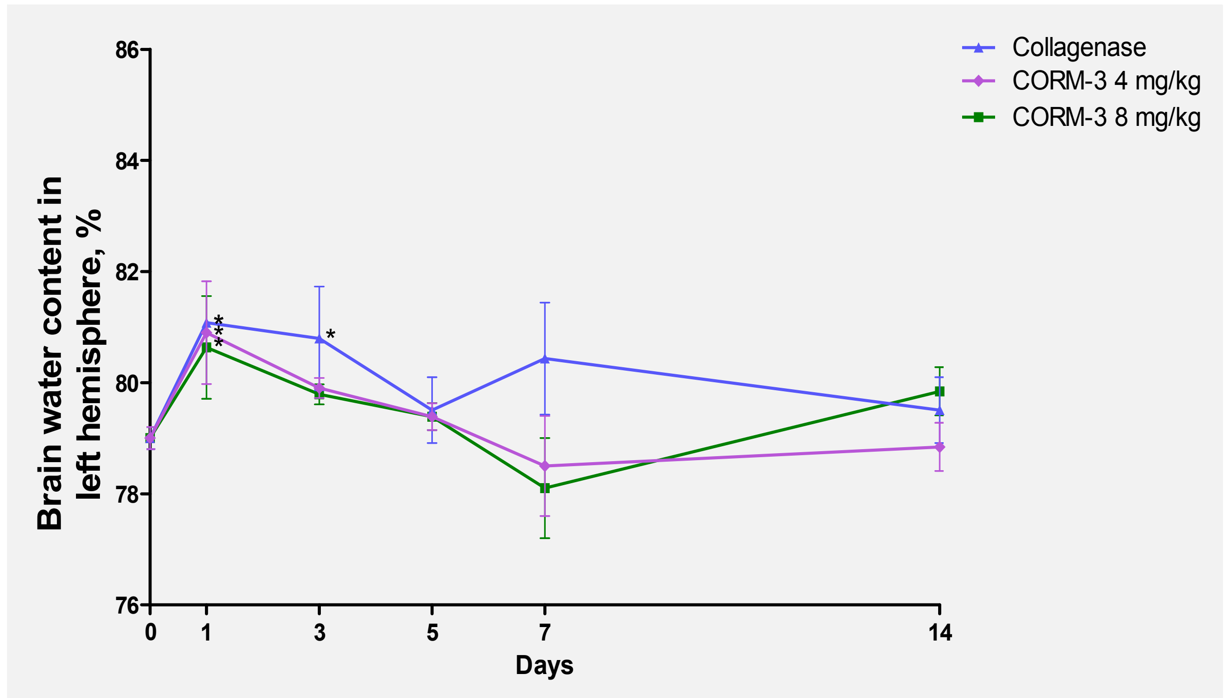


**Figure 6-33 Brain water content for the left hemisphere: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 10), collagenase + CORM-3 8 mg/kg (Group 11) groups data are represented. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data**

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated an increase in brain water content in the affected hemisphere on day 1, which was similar to the level of the collagenase injection group (Group 5) and significantly higher than pre-operation data.

On the 3rd day of the experiment there was a small decrease in the brain water content of the affected hemisphere in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11), which was somewhat lower than in the collagenase injection group (Group 5) and significantly higher than pre-operation data.

Further changes in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) showed the brain water content remained on the same level at day 5, which was significantly higher than in the collagenase injection group (Group 5) and pre-operation data, with a further decrease reaching the level of pre-operation data by day 7.

On the 14th day of the experiment a small increase in the brain water content of the affected hemisphere in CORM-3 8 mg/kg group (Group 11) was observed, which was similar to the collagenase injection group (Group 5) and higher than in the CORM-3 4 mg/kg group (Group 10) and pre-operation data. These data are presented in Figure 6-34.

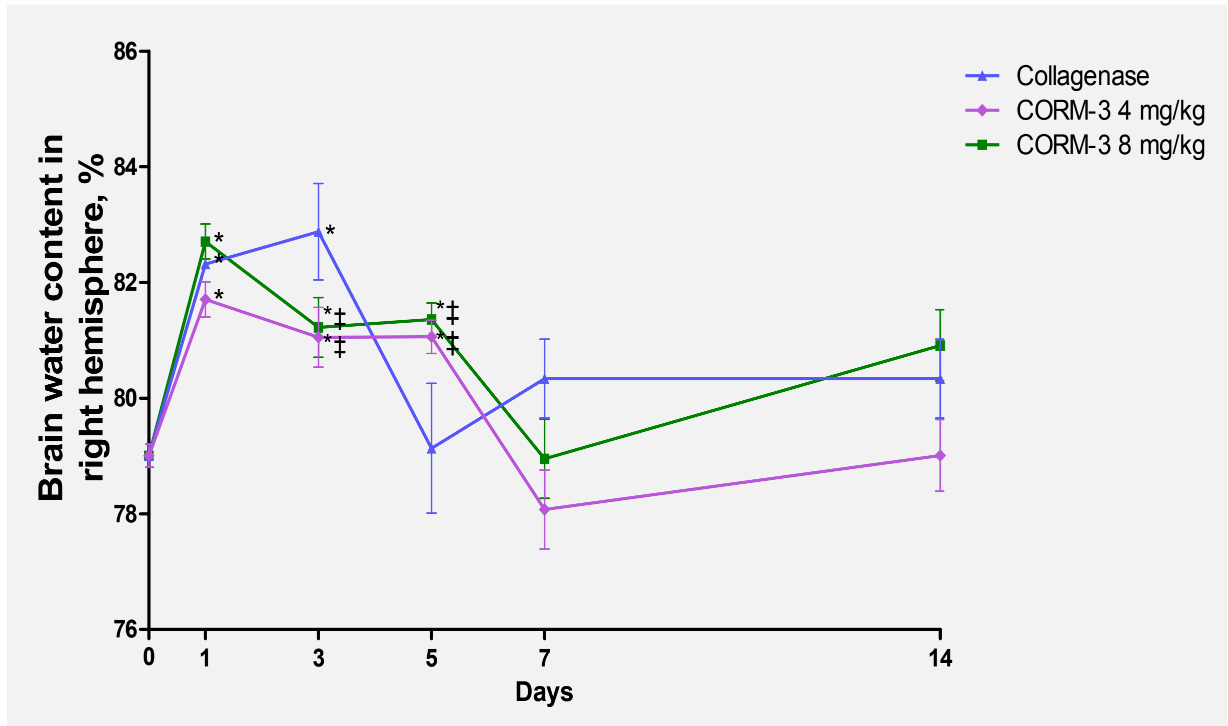


**Figure 6-34 Brain water content for right hemisphere: collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 10), collagenase + CORM-3 8 mg/kg (Group 11) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

***Densitometric cell count***

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated an increase in the number of neutrophils in the region of the intracerebral haemorrhage on day 1, which was similar to the level of the collagenase injection group (Group 5) and significantly higher than in pre-operation data.

On the 3rd day of the experiment a further rapid 3-fold increase in the number of neutrophils occurred in the region of the intracerebral haemorrhage in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) with a further increase on day 5, which was significantly higher than in the collagenase control and pre-operation data. In contrast to the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11), the number of neutrophils in the region of the intracerebral haemorrhage in the collagenase injection group (Group 5) demonstrated a decrease on day 3 and reached the level of pre-operation data by day 5.

Further changes in the number of neutrophils in the region of the intracerebral haemorrhage in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated a decrease on day 5, with the levels of neutrophils remaining significantly higher than the level in the collagenase injection group and pre-operation data.

The levels of neutrophils in the region of the intracerebral haemorrhage, in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11), reached the level of the collagenase injection group (Group 5) and pre-operation data

by day 7, and remained at the same level until the end of the experiment. These data are presented in Figure 6-35.

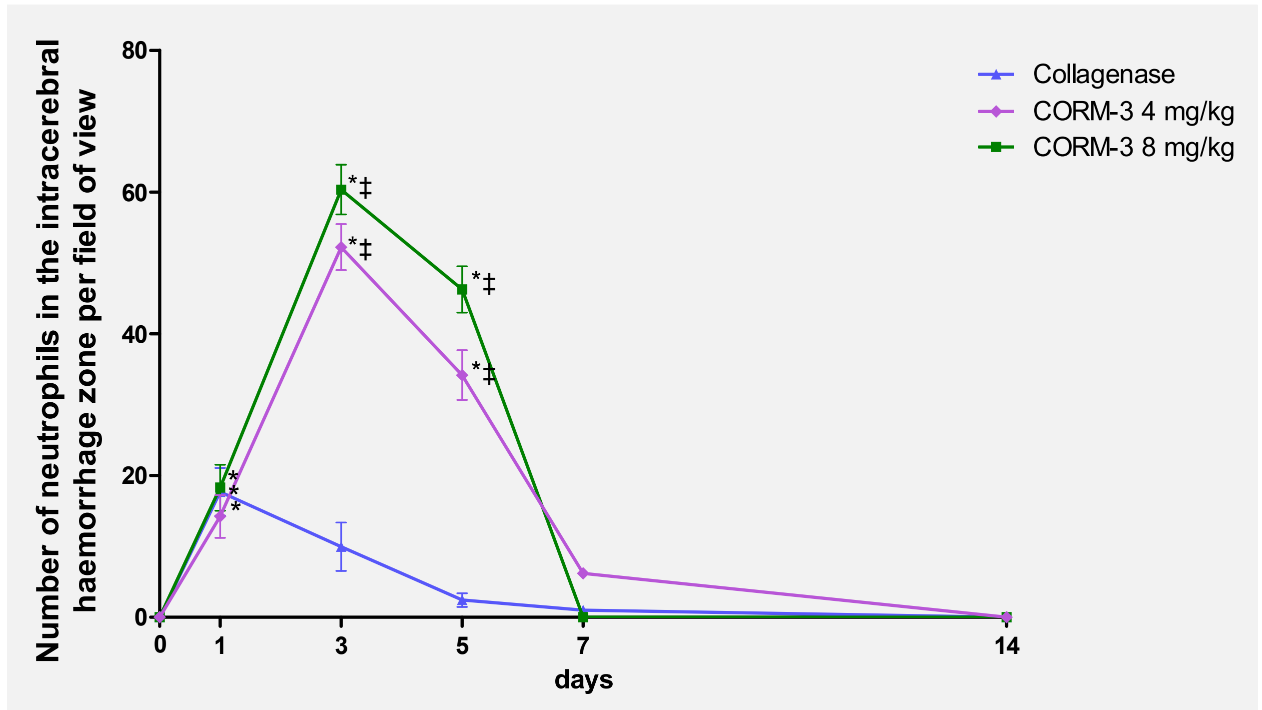


**Figure 6-35 Densitometric cell count analysis: neutrophil cell number (mean) in the intracerebral haemorrhage zone in 7 fields of view. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated a 4-fold increase in the number of microglial cells in the region of the intracerebral haemorrhage on day 1, which was significantly lower than in the collagenase injection group (Group 5) and significantly higher than pre-operation data.

On the 3rd day of the experiment a further increase occurred in the number of microglial cells in the region of the intracerebral haemorrhage in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11), where they reached the levels of the collagenase injection group (Group 5).

Further changes in the number of microglial cells in the region of the intracerebral haemorrhage in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) showed a further additional small increase on day 5, exceeding the levels of the collagenase injection group (Group 5) and remaining significantly higher than in pre-operation data. In contrast to the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) the number of microglial cells in the region of the intracerebral haemorrhage in the collagenase injection group (Group 5) showed a 1.75 – fold decrease on day 5.

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated a subsequent decrease in the number of microglial cells in the region of the intracerebral haemorrhage, reaching the levels of the collagenase injection group (Group 5) injection group and pre-operation data on day 7. These data are presented in Figure 6-36.

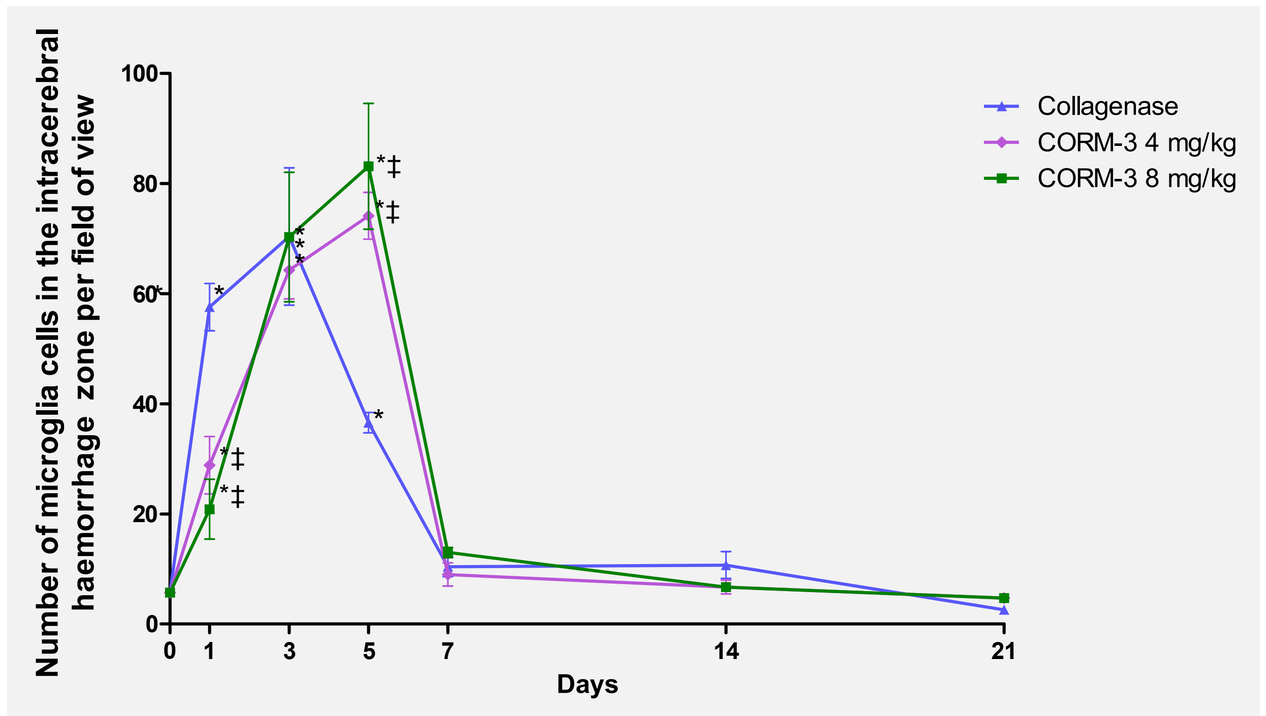


**Figure 6-36 Densitometric cell count analysis: microglial cell number (mean) in the intracerebral haemorrhage zone in 7 fields of view. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

#### 6.4.2.3 Behavioural testing

***Beam walking***

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated a 2.3 – fold decrease in performance in the beam walking test on day 1, compared to pre-operation data, which was 1.4 – fold lower than in the collagenase injection group (Group 5).

Further changes in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) indicated a gradual improvement in performance in the beam walking test on day 3, when it reached the level of the collagenase injection group (Group 5).

On the 5$^{th}$ day of the experiment a further improvement in performance in the beam walking test in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) was observed. However the mean score in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) was lower than in the collagenase injection group (Group 5) and pre-operation data.

Further changes in the beam walking scores showed an improvement in the CORM-3 4 mg/kg group (Group 10) on day 7, which reached the level of the collagenase injection group (Group 5) and pre-operation data. The CORM-3 8 mg/kg group (Group 11) demonstrated a further decrease in performance in the beam walking test on day 7, which was 1.3 – fold lower than in pre-operation data, with a further improvement, reaching the levels of the collagenase injection group (Group 5) and pre-operation data by day 14. These data are presented in Figure 6-37.

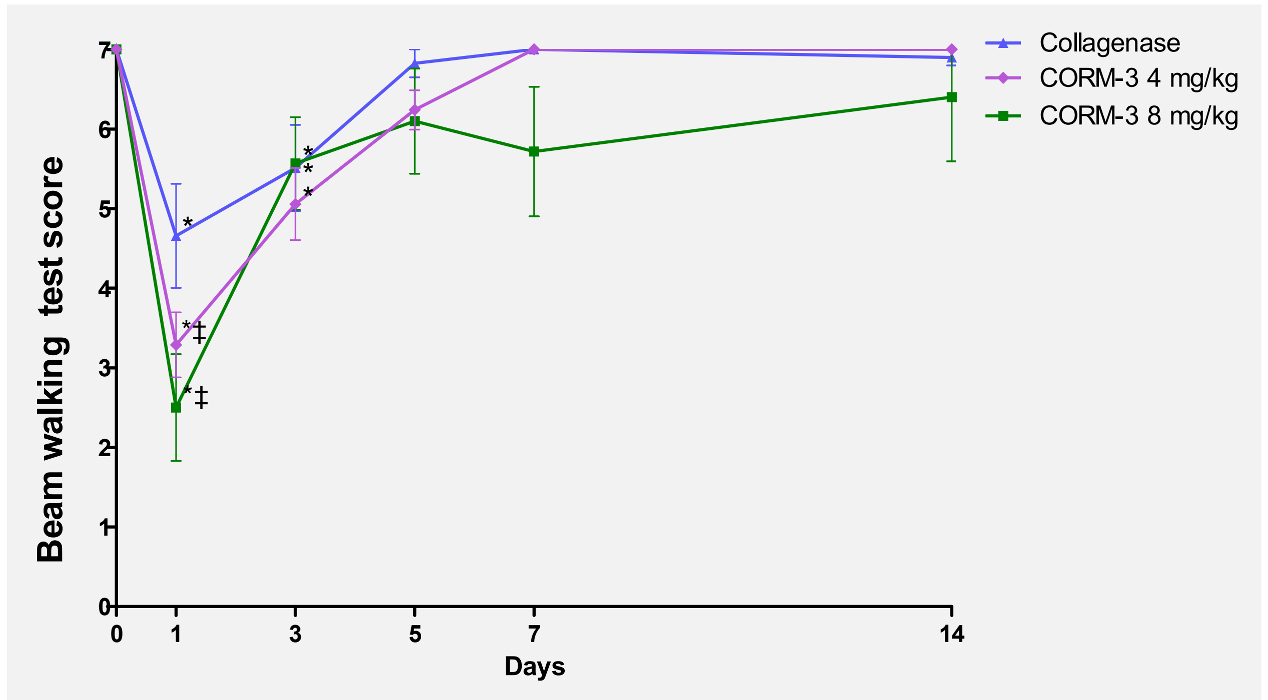


**Figure 6-37 Beam walking test score: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 10), collagenase + CORM-3 8 mg/kg (Group 11) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data, ‡ - p < 0.05 vs. collagenase injection group (Group 5)**

***Forelimb placing test***

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated a similar significant 5-fold decrease in performance in the forelimb placing test of the affected paw on day 1, which was 3 – fold worse than the one observed in the collagenase injection group (Group 5).

Further changes indicated an improvement in performance in the forelimb placing test of the affected paw on day 3, with the levels of the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) being slightly lower than in the collagenase injection group (Group 5).

The 5$^{th}$ day of the experiment showed a further increase in performance in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11), when it reached the level of the collagenase injection group (Group 5). Further changes indicated an improvement, reaching pre-operational performance in the CORM-3 4 mg/kg group (Group 10) on day 7.

The CORM-3 8 mg/kg group (Group 11) showed a slightly lower performance than in pre-operation data, and slightly higher than in the collagenase injection group (Group 5) until the end of the experiment. These data are presented in Figure 6-38.

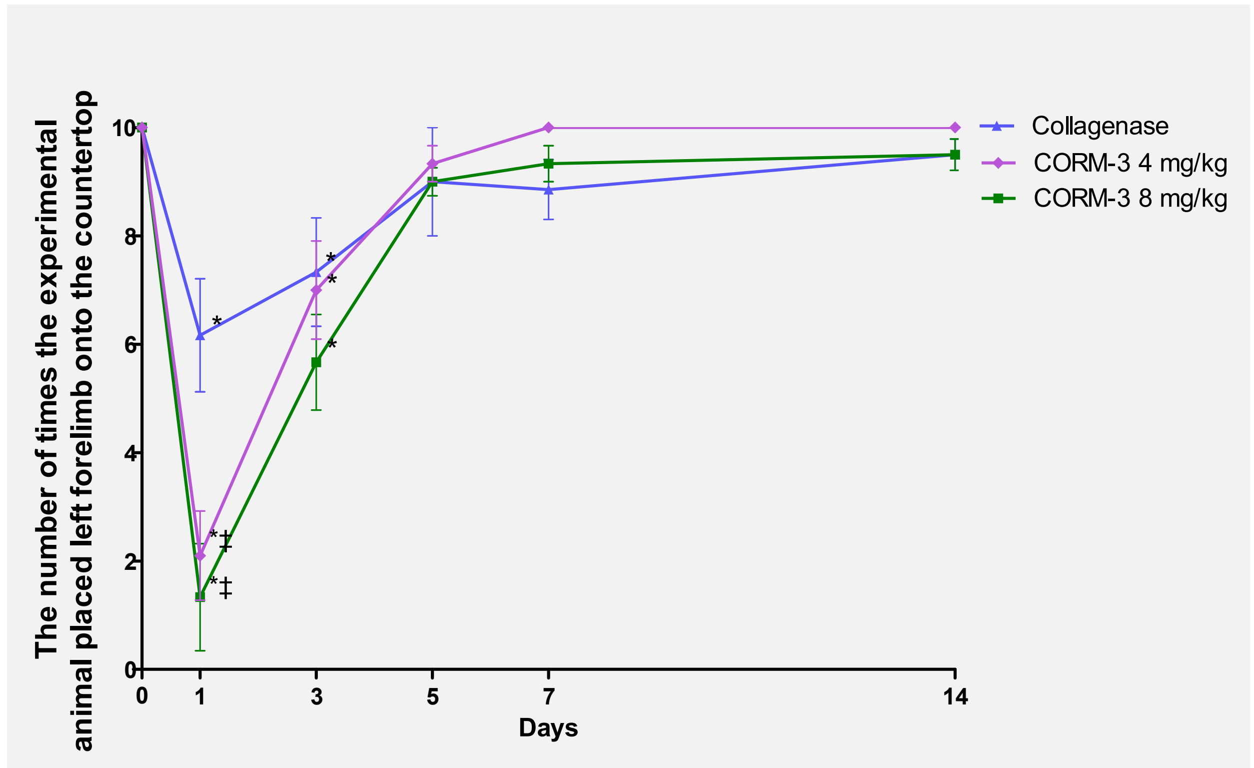


**Figure 6-38 Forelimb placing, left paw: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 10), collagenase + CORM-3 8 mg/kg (Group 11). Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated (similar to the collagenase injection group (Group 5)) a maximal performance in the forelimb placing test of the unaffected paw throughout the days of the experiment, which was at the level of pre-operation data. These data are presented in Figure 6-39.

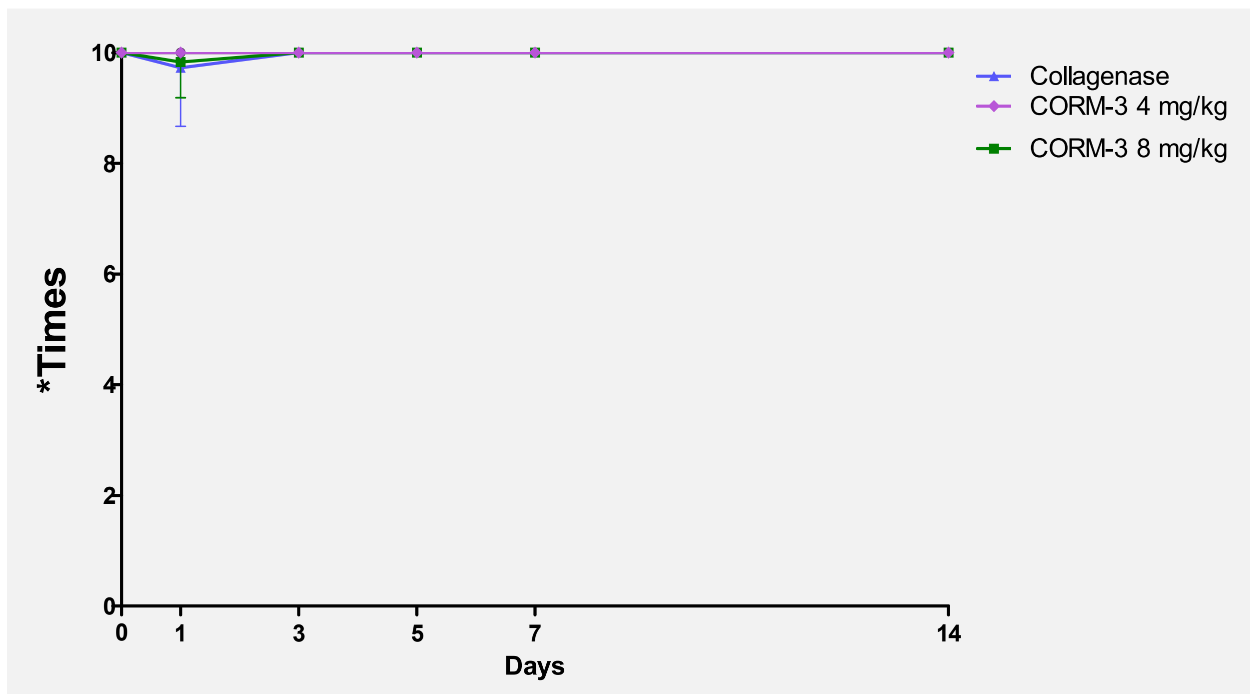


**Figure 6-39 Forelimb placing, right paw: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 10), collagenase + CORM-3 8 mg/kg (Group 11) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No significant difference was observed.**

***Semi-quantitative examination***

The CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) demonstrated a 1.3 – fold decrease in performance in the semi-quantitative examination on day 1, compared to pre-operation data, which was 1.1 – fold lower than in the collagenase injection group (Group 5).

Further changes in performance in the semi-quantitative examination in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) indicated a gradual decrease on day 3, when CORM-3 4 mg/kg group (Group 10) reached the level of the collagenase injection group (Group 5) and the CORM-3 8 mg/kg group (Group 11) and remained somewhat lower than in the collagenase injection group (Group 5).

On the 5th day of the experiment, the data indicated a similar increase in performance in the semi-quantitative examination in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) to the collagenase injection group (Group 5).

Further changes indicated an improvement in performance in the semi-quantitative examination in the CORM-3 4 mg/kg group (Group 10) on day 7, when it reached the level of the collagenase injection group (Group 5) and the pre-operation data. The CORM-3 8 mg/kg group (Group 11) demonstrated no improvement in the semi-quantitative examination on day 7 and remained at the same level as it was observed on day 5.

On the 7th day of the experiment there was an increase in the CORM-3 4 mg/kg group (Group 10) and the CORM-3 8 mg/kg group (Group 11) reaching the level of pre-operation data, despite the collagenase injection group (Group 5) which demonstrated a small decrease in performance in the semi-quantitative examination on day 14. These data are presented in Figure 6-40.

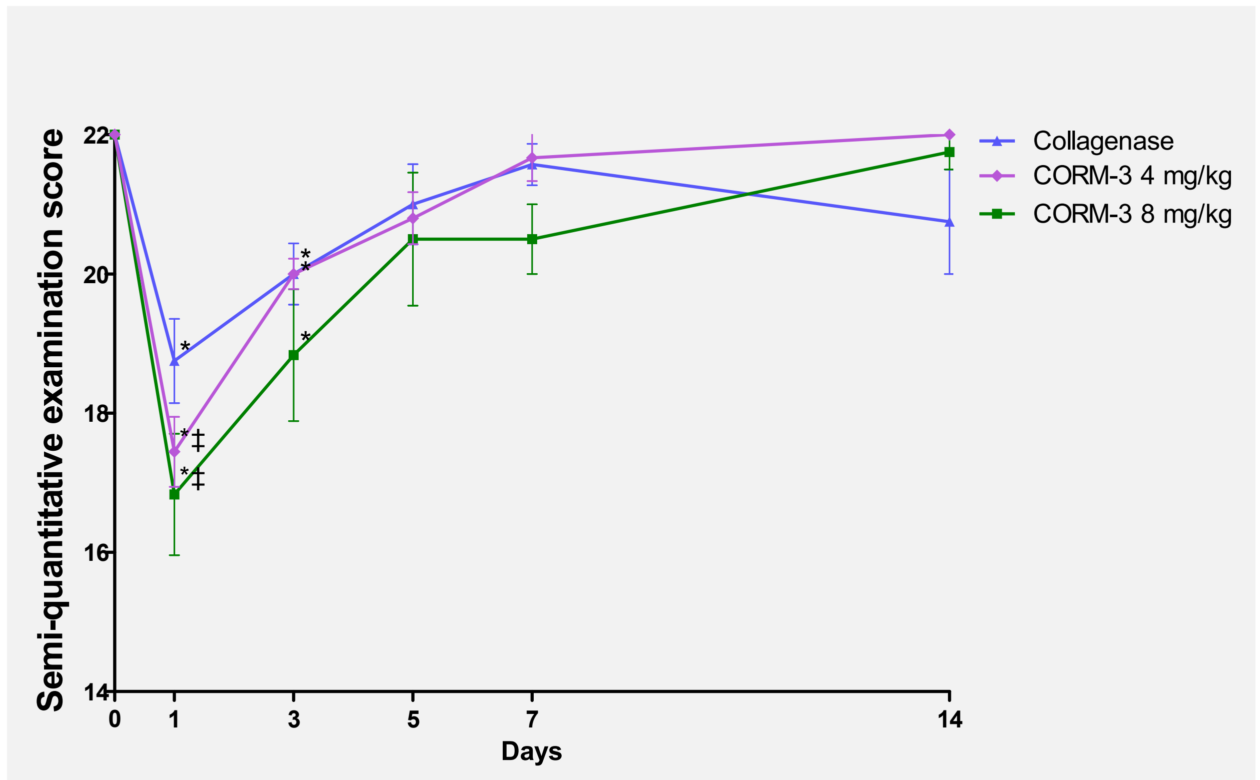


**Figure 6-40 Semi-quantitative examination score: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 10), collagenase + CORM-3 8 mg/kg (Group 11) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

#### 6.4.2.4 Correlations between blood and lesion zone leukocytes and behavioural tests

A strong positive correlation was observed between:

- the number of neutrophils in the lesion zone and the number of microglia cells in the lesion zone
- the number of peripheral blood lymphocytes and the forelimb placing test score
- the forelimb placing test score and the beam walking test score
- the number of peripheral blood lymphocytes and the semi-quantitative examination score
- the forelimb placing test score and the semi-quantitative examination score
- the beam walking test score and the semi-quantitative examination score

A strong negative correlation was observed between:

- the number of peripheral blood neutrophils and the number of peripheral blood lymphocytes
- the number of peripheral blood neutrophils and the semi-quantitative examination score

A moderate positive correlation was observed between:

- the number of peripheral blood neutrophils and plasma TNF-alpha levels
- the number of neutrophils in the lesion zone and plasma TNF-alpha levels
- the number of microglia cells in the lesion zone and plasma TNF-alpha levels
- the number of peripheral blood lymphocytes and the beam walking test score

A moderate negative correlation was observed between:

- the number of peripheral blood lymphocytes and plasma TNF-alpha levels
- the number of peripheral blood neutrophils and the forelimb placing test score
- the number of peripheral blood neutrophils and the beam walking test score
- the number of neutrophils in the lesion zone and the beam walking test score
- the forelimb placing test score and plasma TNF-alpha levels
- the beam walking test score and plasma TNF-alpha levels
- the semi-quantitative examination score and plasma TNF-alpha levels.

A weak positive correlation was observed between:

- the number of neutrophils in the lesion zone and the number of peripheral blood lymphocytes
- the number of peripheral blood lymphocytes and the number of microglia cells in the lesion zone.

A weak negative correlation was observed between:

- the number of peripheral blood neutrophils and the number of neutrophils in the lesion zone
- the number of peripheral blood lymphocytes and the number of microglia cells in the lesion zone
- the number of neutrophils in the lesion zone and the forelimb placing test score
- the number of microglia cells in the lesion zone and the forelimb placing test score
- the number of microglia cells in the lesion zone and the beam walking test score
- the number of neutrophils in the lesion zone and the semi-quantitative examination score

- the number of microglia cells in the lesion zone and the semi-quantitative examination score

**Table 11 HS + CORM-3 4 mg/kg treatment: matrix of levels pair correlation coefficients between blood and lesion zone leukocytes, plasma TNF alpha and behavioural tests**

| Parameters | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 1 | - 0.19 | - 0.97 | 0.05 | - 0.68 | - 0.49 | - 0.73 | 0.45 |
| 2 | | 0.06 | 0.91 | - 0.15 | - 0.33 | - 0.25 | 0.58 |
| 3 | | | - 0.10 | 0.84 | 0.69 | 0.88 | - 0.59 |
| 4 | | | | - 0.11 | - 0.20 | - 0.25 | 0.53 |
| 5 | | | | | 0.97 | 0.99 | - 0.60 |
| 6 | | | | | | 0.95 | - 0.61 |
| 7 | | | | | | | - 0.69 |

1 – Peripheral blood neutrophil count; 2 – Number of neutrophils in the lesion zone; 3 – Peripheral blood lymphocyte count; 4 – Number of microglia cells in the lesion zone; 5 – Forelimb placing test scores, left paw; 6 – Beam walking test scores; 7 – Semi-quantitative examination scores, 8 – Plasma TNF-alpha levels

A strong positive correlation was observed between:

- the number of neutrophils in the lesion zone and the number of microglia cells in the lesion zone
- the forelimb placing test score and the beam walking test score
- the forelimb placing test score and the semi-quantitative examination score
- the beam walking test and the semi-quantitative examination score

A strong negative correlation was observed between:

- the number of peripheral blood neutrophils and the number of peripheral blood lymphocytes.

A moderate positive correlation was observed between:

- the number neutrophils in the lesion zone and the number of peripheral blood lymphocytes
- the number of peripheral blood lymphocytes and the number of microglia cells in the lesion zone
- the number of neutrophils in the lesion zone and plasma TNF-alpha levels
- the number of peripheral blood lymphocytes and plasma TNF-alpha levels
- the number of microglia cells in the lesion zone and plasma TNF-alpha levels
- the number of peripheral blood lymphocytes and the forelimb placing test score
- the number of peripheral blood lymphocytes and the beam walking test score
- the number of peripheral blood lymphocytes and the semi-quantitative examination score
- the forelimb placing test score and plasma TNF-alpha levels
- the beam walking test score and plasma TNF-alpha levels

A moderate negative correlation was observed between:

- the number of peripheral blood neutrophils and the number of neutrophils in the lesion zone
- the number of peripheral blood neutrophils and the number of microglia cells in the lesion zone
- the number of peripheral blood neutrophils and plasma TNF-alpha levels
- the number of peripheral blood neutrophils and the forelimb placing test score
- the number of peripheral blood neutrophils and the beam walking test score
- the number of peripheral blood neutrophils and the semi-quantitative examination score

A weak positive correlation was observed between:

- the number of microglia cells in the lesion zone and the forelimb placing test score
- the number of neutrophils in the lesion zone and the beam walking test score
- the number of microglia cells in the lesion zone and the beam walking test score
- the semi-quantitative examination score and plasma TNF-alpha levels.

A weak negative correlation was observed between:

- the number of neutrophils in the lesion zone and the forelimb placing test score
- the number of microglia cells in the lesion zone and the semi-quantitative examination score

**Table 12 HS + CORM-3 8 mg/kg treatment: matrix of levels pair correlation coefficients between blood and lesion zone leukocytes, plasma TNF alpha and behavioural tests**

| Parameters | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 1 | - 0.51 | - 0.99 | - 0.47 | - 0.40 | - 0.68 | - 0.46 | - 0.67 |
| 2 | | 0.49 | 0.94 | - 0.23 | 0.04 | - 0.32 | 0.66 |
| 3 | | | 0.45 | 0.41 | 0.69 | 0.47 | 0.67 |
| 4 | | | | 0.02 | 0.22 | - 0.10 | 0.63 |
| 5 | | | | | 0.94 | 0.97 | 0.34 |
| 6 | | | | | | 0.92 | 0.58 |
| 7 | | | | | | | 0.22 |

1 – Peripheral blood neutrophil count; 2 – Number of neutrophils in the lesion zone; 3 – Peripheral blood lymphocyte count; 4 – Number of microglia cells in the lesion zone; 5 – Forelimb placing test scores, left paw; 6 – Beam walking test scores; 7 – Semi-quantitative examination scores, 8 – Plasma TNF-alpha levels

## 6.5 Effects of CORM-3 administered 3 days post HS

### 6.5.1 Materials and methods. Experimental protocol.

| | |
|---|---|
| Group 5 (Collagenase injection group) | Collagenase injection group (Group 5). Injection of 2 μl of 0.2 U collagenase solution into the lateral striatum of the experimental animals (n=30) |
| Group 12 (CORM-3 4 mg/kg group) | Collagenase injection into the lateral striatum of the experimental animals and CORM-3 administration at a dosage of 4 mg/kg 3 days after operation inducing HS (n=30) |
| Group 13 (CORM-3 8 mg/kg group) | Collagenase injection into the lateral striatum of the experimental animals and CORM-3 administration at a dosage of 8 mg/kg 3 days after operation inducing HS (n=30) |

### 6.5.2 Results

#### 6.5.2.1 Systemic level of inflammation

***General blood analysis: White blood cell count***

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13), qualitively similar to the collagenase injection group (Group 5), showed a decrease in the number of white blood cells on day 1, with a further increase by day 3 and day 5, reaching the level of pre-operation data. The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13), compared to the collagenase control and pre-operation data, demonstrated a greater increase in the number of white blood cells on day 7 with further a decrease, reaching the levels of the collagenase group and pre-operation data by day 14. These data are presented in Figure 6-41.

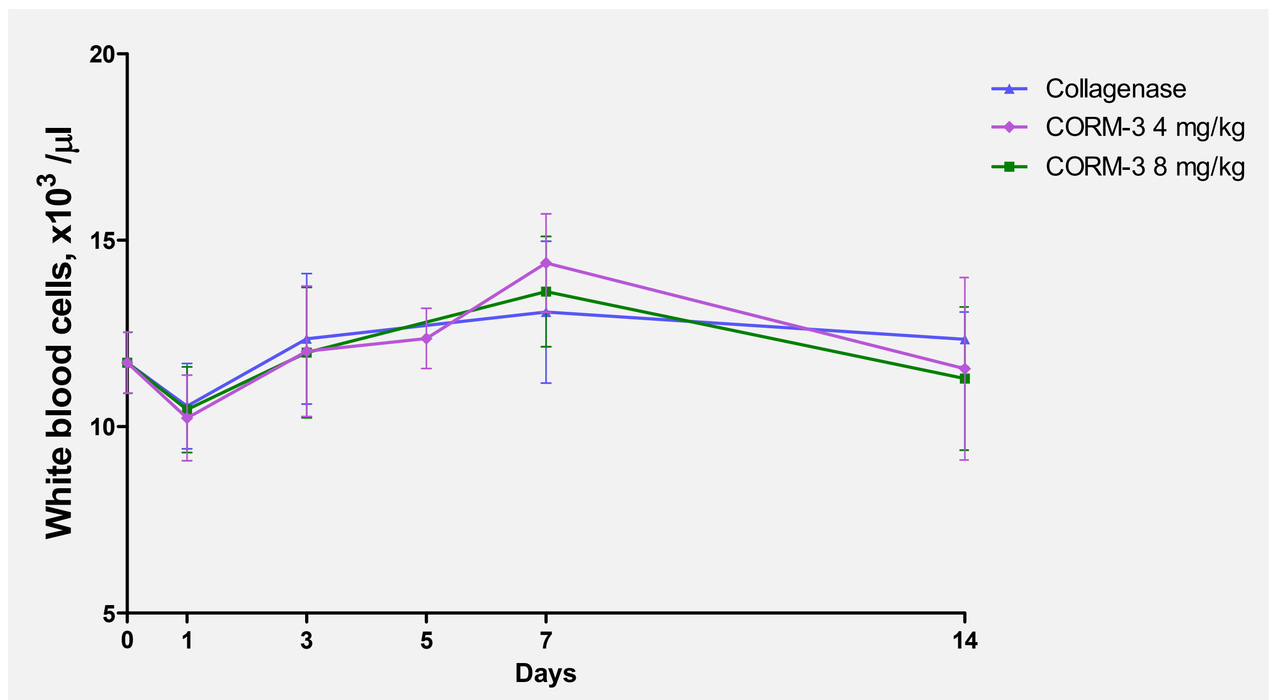


**Figure 6-41 White blood cell count: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 12), collagenase + CORM-3 8 mg/kg group (Group 13) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. † - < 0.05 for all groups comparing to the collagenase injection group (Group 5)**

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) demonstrated an 18% increase in the number of peripheral blood neutrophils on day 1, which was similar to the collagenase injection group (Group 5).

On the 3$^{rd}$ day of the experiment an 8% decrease in the number of peripheral blood neutrophils in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) was observed, indicating that levels were higher than in the collagenase injection group (Group 5) and lower than in pre-operation data.

Further changes in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) in the number of peripheral blood neutrophils remained at the level of pre-operation data throughout the 14 days of the experiment, compared to the collagenase injection group (Group 5), where the number of peripheral blood neutrophils increased on day 7 and reached the level of pre-operation data by day 14. These data are presented in Figure 6-42.

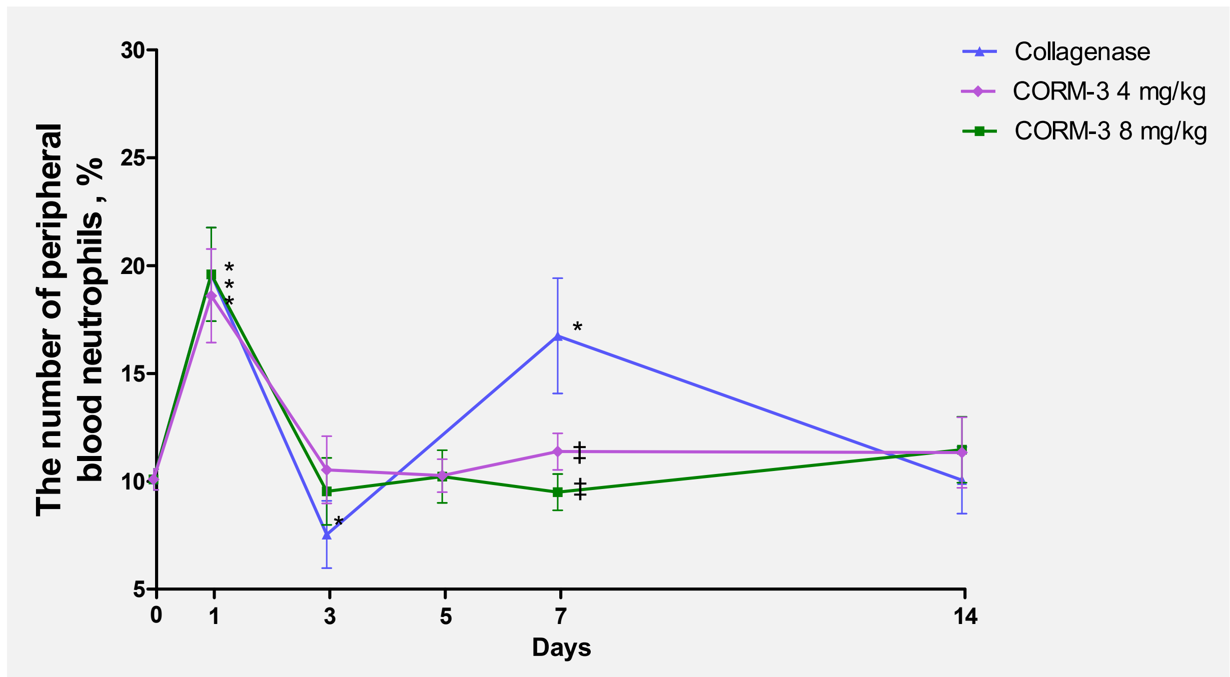


**Figure 6-42 Peripheral blood neutrophil count: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 12), collagenase + CORM-3 8 mg/kg group (Group 13) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) demonstrated a similar 4% decrease in the number of peripheral blood lymphocytes, which was similar to the collagenase injection group (Group 5) and pre-operation data.

On the 3rd day of the experiment, an increase in the number of peripheral blood lymphocytes (8-10%) was noted, which was somewhat lower than in the collagenase injection group (Group 5) and somewhat higher than pre-operation data. Further measurements of the number of peripheral blood lymphocytes indicated that the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) remained at the same level until the end of the experiment, which was higher than the levels measured observed in the collagenase control group and in pre-operation data by 5%. These data are presented in Figure 6-43.

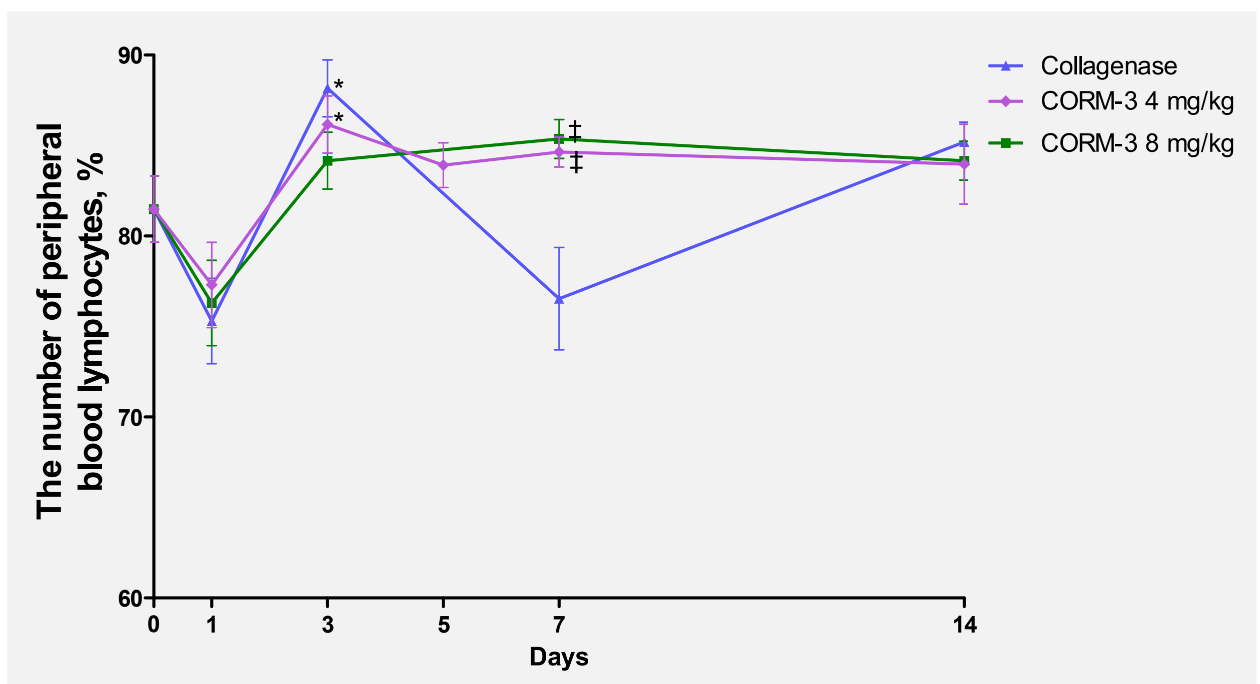


**Figure 6-43 Peripheral blood lymphocytes count: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 12), collagenase + CORM-3 8 mg/kg group (Group 13) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

***Enzyme-linked immunosorbent TNF-alpha assay***

CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) demonstrated similar changes to the collagenase injection group (Group 5), i.e. a 10-fold increase in plasma NTF-alpha levels on day 1, which was significantly higher than in pre-operation data, with a further 1.3 - fold increase on day 3.

Further changes indicated a decrease in plasma TNF-alpha levels in both CORM-3 treatment groups on day 5, which was significantly higher than in pre-operation data and significantly lower than in the collagenase injection group (Group 5).

Subsequent measurements indicated CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) maintained the same level of plasma TNF-alpha until the end of the experiment. These data are presented in Figure 6-44.

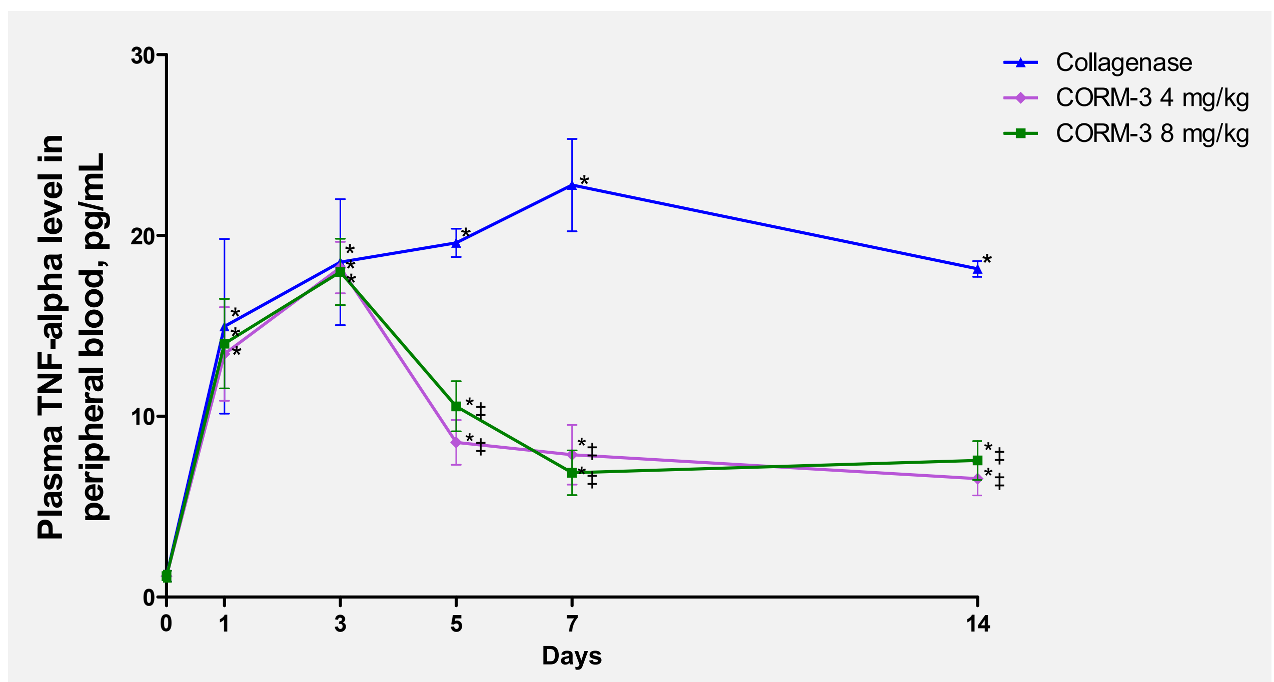


**Figure 6-44 Blood plasma TNF-alpha assay: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 12), collagenase + CORM-3 8 mg/kg group (Group 13) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data, ‡ - p < 0.05 vs. collagenase injection group (Group 5)**

#### 6.5.2.2 Local level of inflammation

***Brain intracerebral haemorrhage area determination***

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13), compared to the collagenase injection group (Group 5), demonstrated a decrease in the size of the intracerebral haemorrhage by 6% and 7% respectively.

The size of the intracerebral haemorrhage in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13), as in the collagenase injection group (Group 5), was significantly different from the saline injection group (Group 4), where no intracerebral haemorrhage was observed. These data are presented in Figure 6-45 and Figure 6-46 respectively.

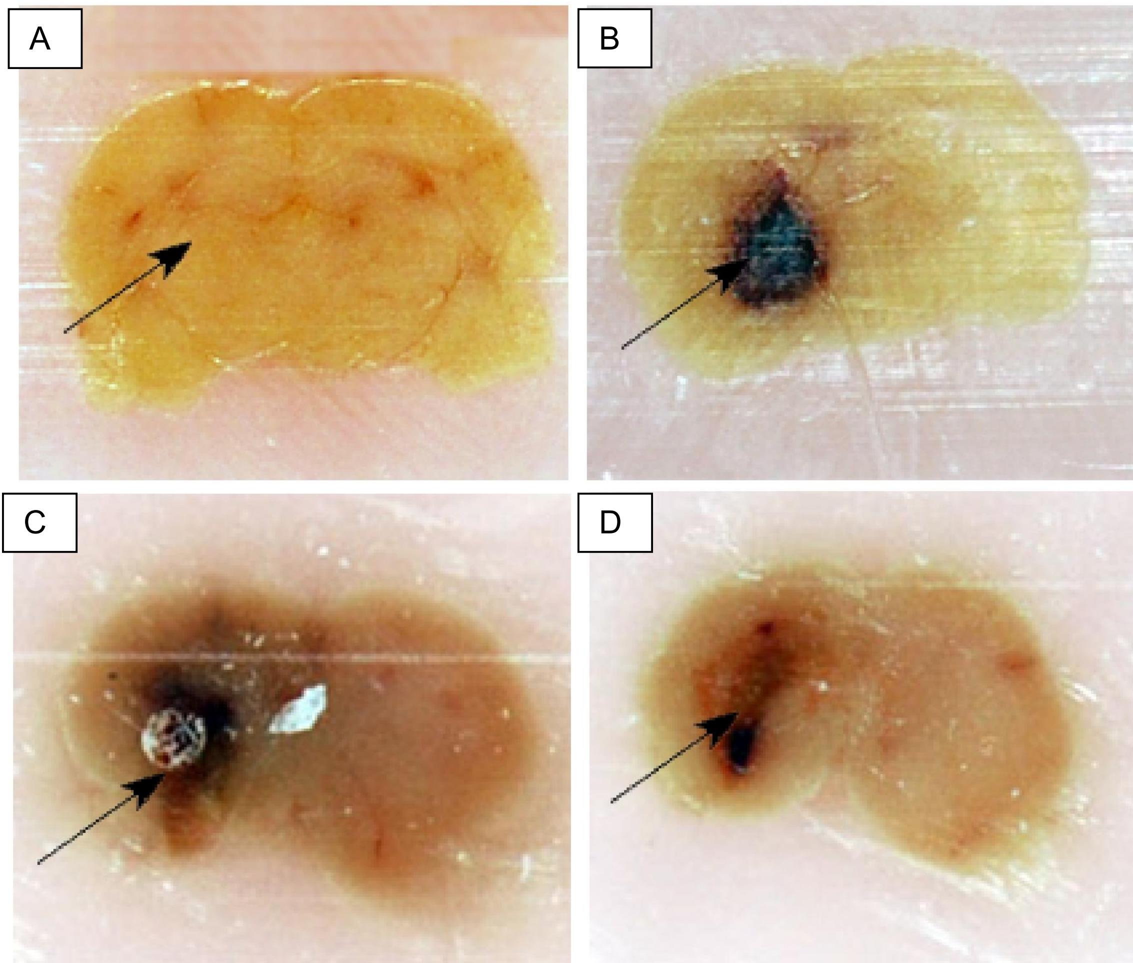


**Figure 6-45 Images of the brain slices through zone of interest obtained on the fifth day post op (arrow indicates presence or absence of the intracerebral haemorrhage): A - Saline injection group (Group 4), B - Collagenase injection group (Group 5), C - Collagenase + CORM-3 4 mg/kg injection group (Group 12), D - Collagenase + CORM-3 8 mg/kg injection group (Group 13)**

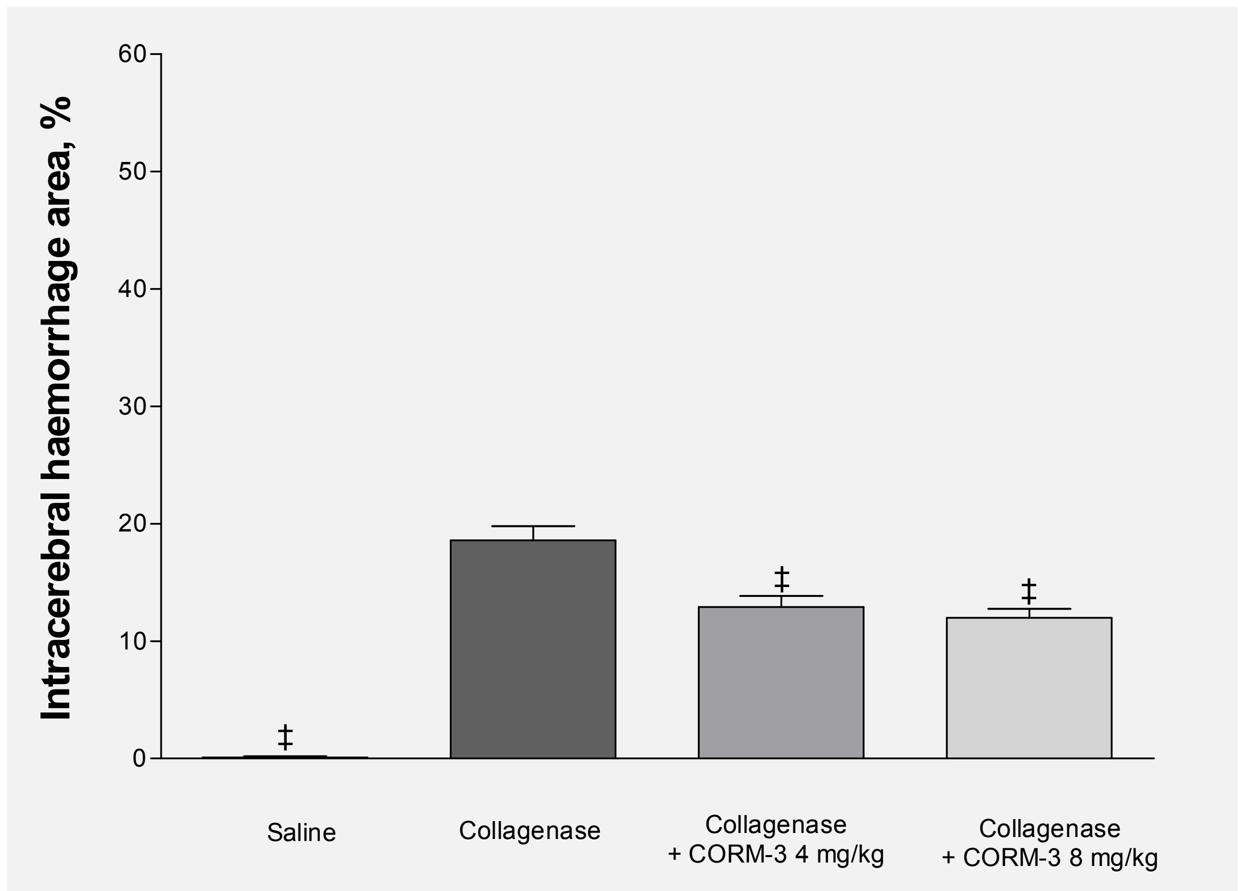


**Figure 6-46 Size of the intracerebral haemorrhage area. Data represent the mean ± SD of n = 6 independent experiments. ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

***Brain water content analysis***

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) showed an increase in the brain water content of unaffected hemisphere on day 1, which was similar to the collagenase injection group (Group 5) and significantly higher than in pre-operation data.

Further changes in brain water content of the unaffected hemisphere in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) were similar to that observed in the collagenase injection group (Group 5), indicating gradual decrease and reaching the level of pre-operation data on day 3 and day 5.

On the 7$^{th}$ day of the experiment, a lower brain water content was measured in the unaffected hemisphere in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) than in the collagenase injection group (Group 5).

Subsequent changes in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) showed non-significant variation of brain water content levels around pre-operation data. These data are presented in Figure 6-47.

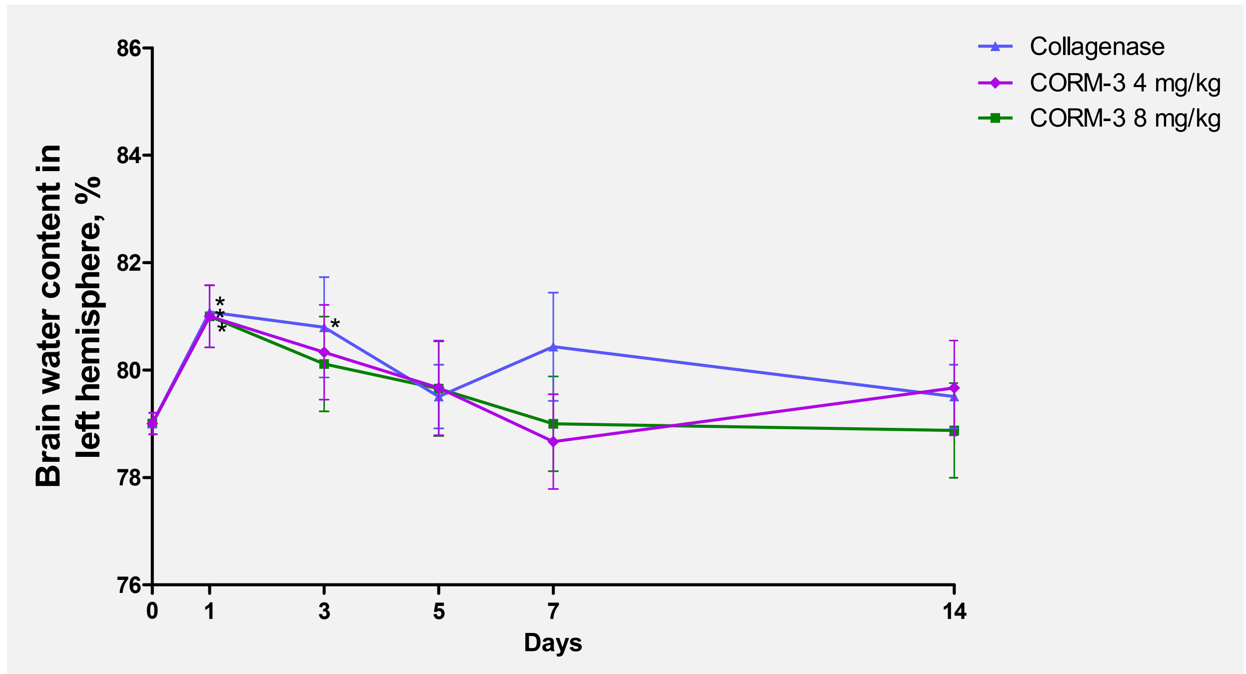


**Figure 6-47 Brain water content: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 12), collagenase + CORM-3 8 mg/kg (Group 13) groups, left hemisphere. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data**

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) demonstrated an increase in the brain water content of the affected hemisphere on day 1, which was similar to the one observed in the collagenase injection group (Group 5) and significantly higher than in pre-operation data.

On the 3$^{rd}$ day of the experiment, a gradual decrease in the level of brain water content of the affected hemisphere was observed in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13), which was lower than in the collagenase injection group (Group 5) and higher than in pre-operation data, and reached the levels of the collagenase injection group (Group 5) and pre-operation data by day 5.

On the 7$^{th}$ day of the experiment, a lower brain water content was observed in the affected hemisphere in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) than in the collagenase injection group (Group 5).

The brain water content levels in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) remained at the level of pre-operation data until the end of the experiment. These data are presented in Figure 6-48.

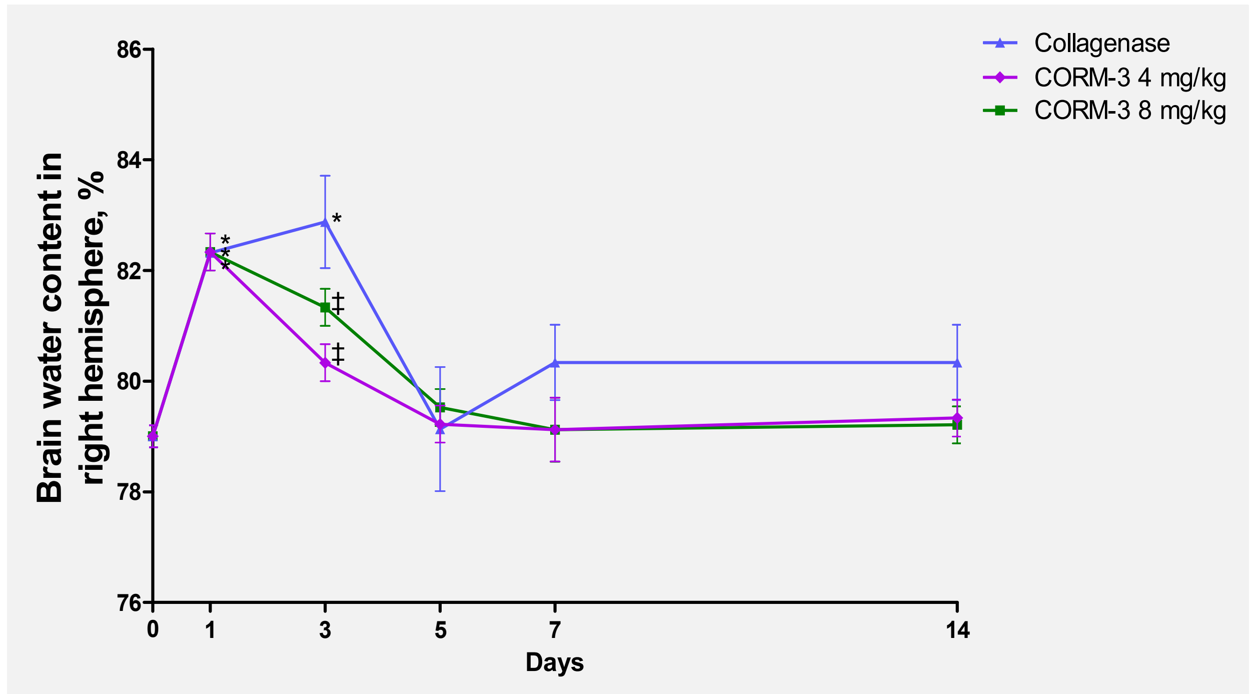


**Figure 6-48 Brain water content: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg (Group 12), collagenase + CORM-3 8 mg/kg (Group 13) groups, right hemisphere. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

***Densitometric cell count***

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) demonstrated a similar 15% increase in the number of neutrophils on day 1, which was similar to the collagenase injection group (Group 5).

Further changes in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) demonstrated similar data to the collagenase injection group (Group 5), which was exemplified by a gradual decrease in the number of activated neutrophils on day 3, reaching the levels of pre-operation data by day 5 and 7. These data are presented in Figure 6-49.

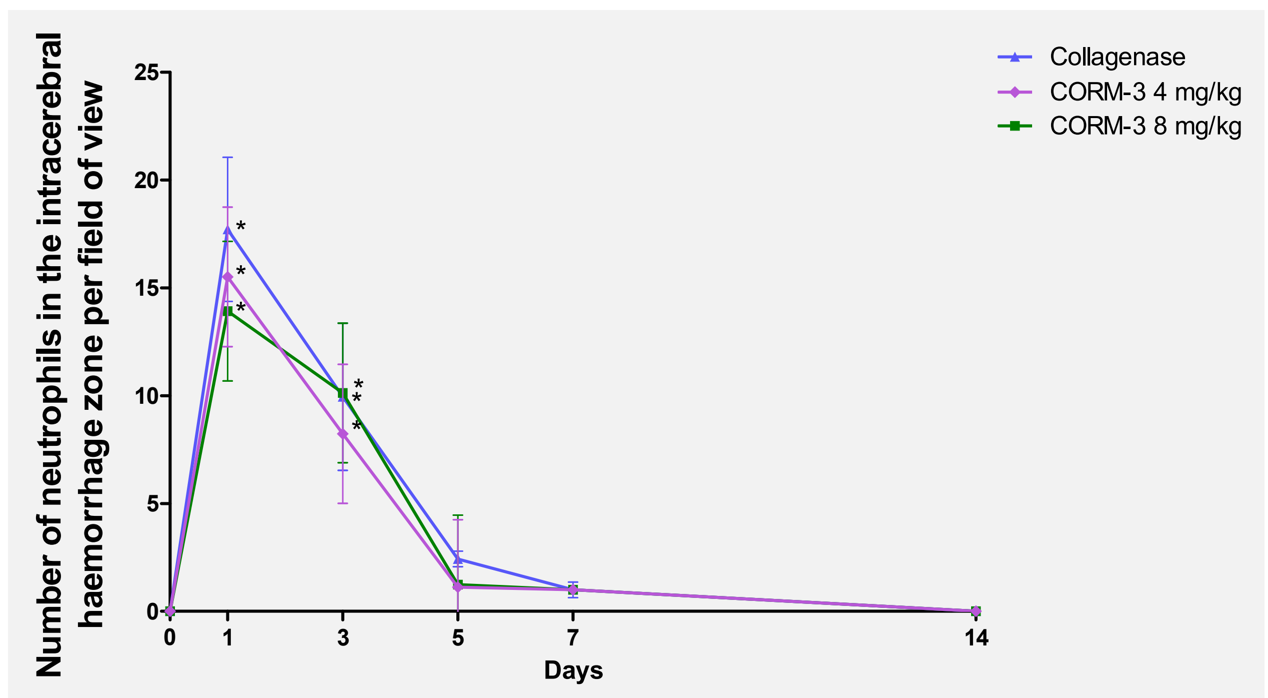


**Figure 6-49 Densitometric cell count analysis: neutrophil cell number (mean) in the intracerebral haemorrhage zone in 7 fields of view. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data**

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) demonstrated a similar 30-fold increase in the number of microglial cells, which was similar to the collagenase injection group (Group 5) and significantly higher than in pre-operation data.

On the 3rd day of the experiment a further small increase in the number of microglial cells in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) was observed, which was similar to the collagenase injection group (Group 5) and significantly higher than in pre-operation data.

Further changes in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) microglial cell numbers showed a rapid decrease reaching the levels of pre-operation data by day 5, being 2-fold lower than in the collagenase injection group (Group 5), which was statistically significant.

Subsequent changes in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) showed the number of microglial cells remained at the same level as in pre-operation data from day 7 until the end of the experiment. These data are presented in Figure 6-50.

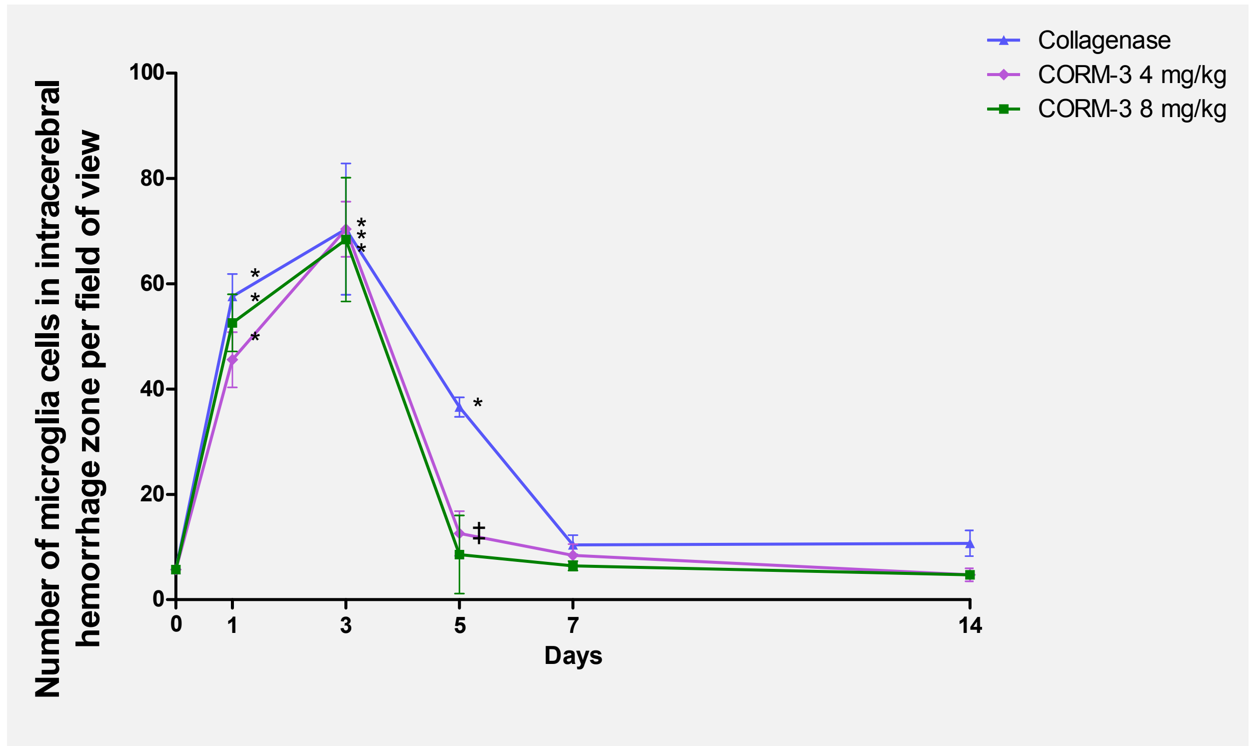


**Figure 6-50 Densitometric cell count analysis: microglial cell number (mean) in the intracerebral haemorrhage zone in 7 fields of view. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data, ‡ - $p < 0.05$ vs. collagenase injection group (Group 5)**

### 6.5.2.3 Behavioural testing

***Beam walking***

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) demonstrated a similar 1.4 – fold decrease in the performance in the beam walking test on day 1, comparing to pre-operation data, which was similar to the one observed in the collagenase injection group (Group 5).

On the 3$^{rd}$ day of the experiment the data showed a 1.16 – fold improvement in the performance of semi-quantitative examination in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13), which was a better performance than the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) compared to the collagenase injection group (Group 5).

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) showed a further improvement in performance in the beam walking test and reached the level of the collagenase injection group (Group 5) and pre-operation data by day 5.

Further changes in performance in the beam walking test showed the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) remained at the same level until the end of the experiment. These data are presented in Figure 6-51.

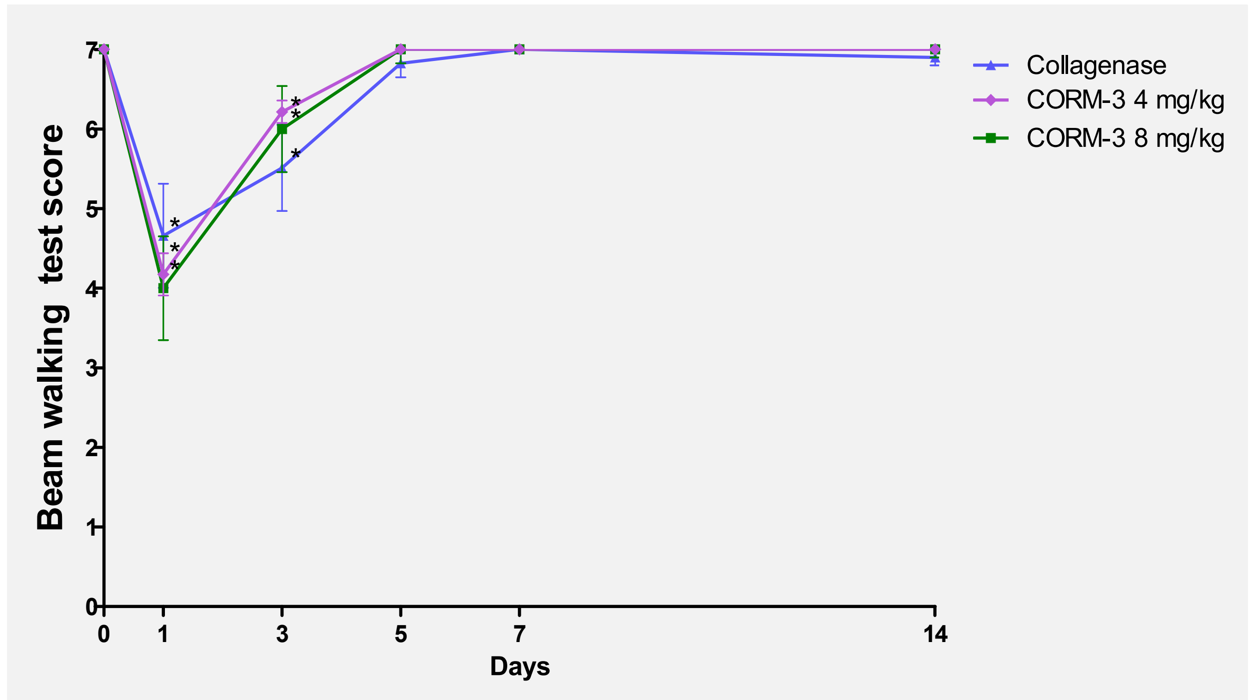


**Figure 6-51 Beam walking test score: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 12), collagenase + CORM-3 8 mg/kg group (Group 13) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data**

***Forelimb placing test***

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) showed similar 1.6 – fold decreases in performance in the forelimb placing test of the affected paw on day 1, compared to pre-operation data, which was similar to the collagenase injection group (Group 5). The CORM-3 4 mg/kg group (Group 12) performance was somewhat lower and the CORM-3 8 mg/kg group (Group 13) performance was somewhat higher than in the collagenase injection group (Group 5).

On the 3rd day of the experiment a small improvement in performance was observed in the forelimb placing test of the affected paw in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13), when it reached the level of the collagenase injection group (Group 5).

In contrast to the collagenase injection group (Group 5), subsequent changes indicated a further improvement in performance in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) in the forelimb placing test, when it reached the level of pre-operation data.

Further measurements indicated the performance of the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) remained at the same level as pre-operation data until the end of the experiment. These data are presented in Figure 6-52.

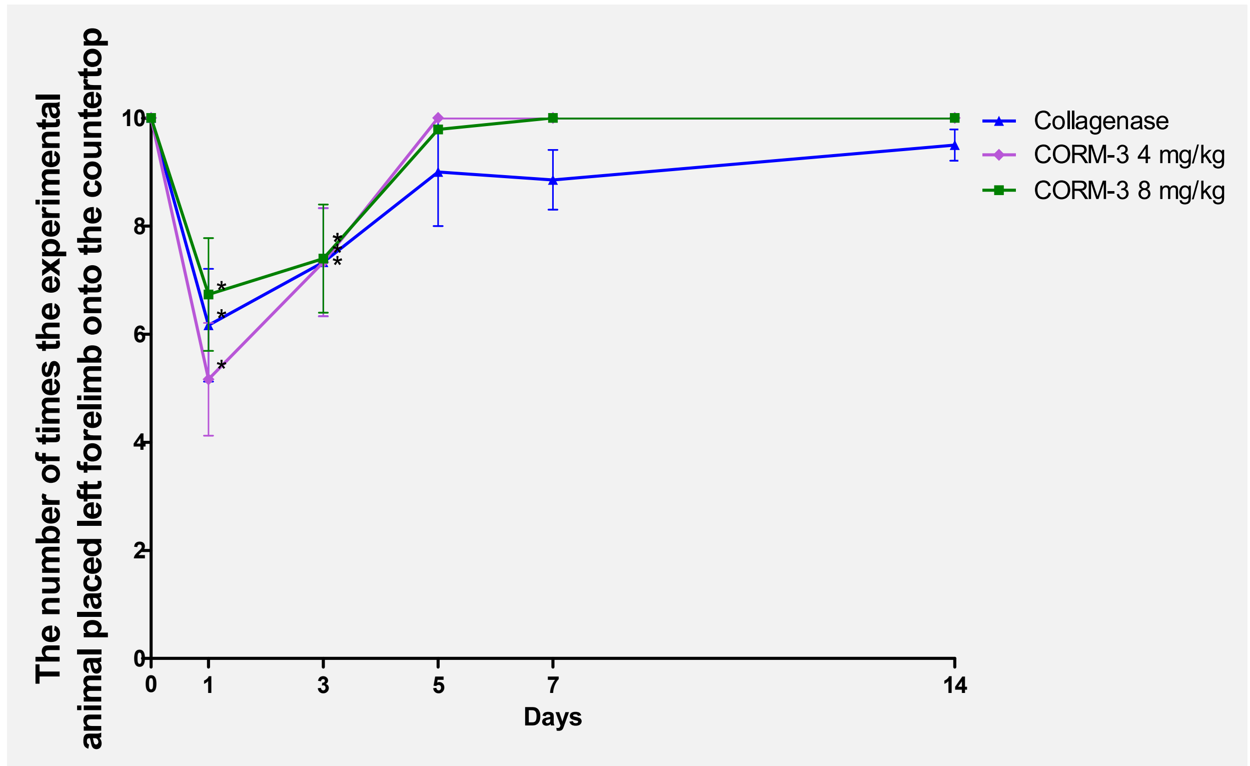


**Figure 6-52 Forelimb placing, left paw: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 12), collagenase + CORM-3 8 mg/kg (Group 13) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - $p < 0.05$ vs. pre-operation data**

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) demonstrated similar to the collagenase injection group (Group 5), maximal performance in the forelimb placing test of the unaffected paw throughout the 14 days of the experiment, which was also at level of pre-operation data. These data are presented in Figure 6-53.

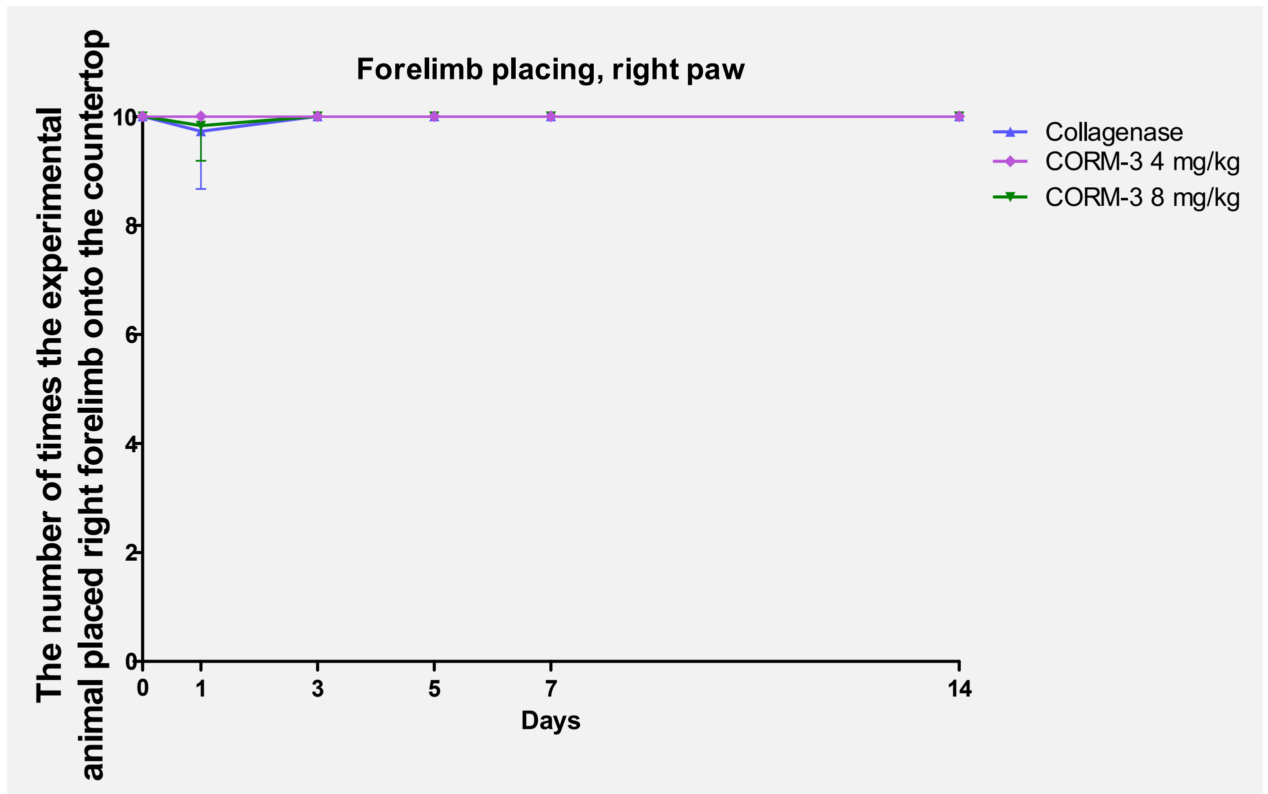


**Figure 6-53 Forelimb placing, right paw: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 12), collagenase + CORM-3 8 mg/kg (Group 13) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. No statistically significant changes were seen.**

***Semi-quantitative examination***

The CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) demonstrated a similar 1.2 – fold decrease in performance in the semi-quantitative examination on day 1, compared to pre-operation data, which was similar to the collagenase injection group (Group 5).

On the 3rd day of the experiment an improvement in performance was noted in the semi-quantitative examination in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13), when they reached the level of the collagenase injection group (Group 5).

Subsequent changes indicated a further improvement in the CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) on day 5, being somewhat higher than in the collagenase injection group (Group 5), and reached the level of pre-operation data by day 7.

Further measurements indicated the performance of CORM-3 4 mg/kg group (Group 12) and the CORM-3 8 mg/kg group (Group 13) remained at the same level as pre-operation data until the end of the experiment. These data are presented in Figure 6-54.

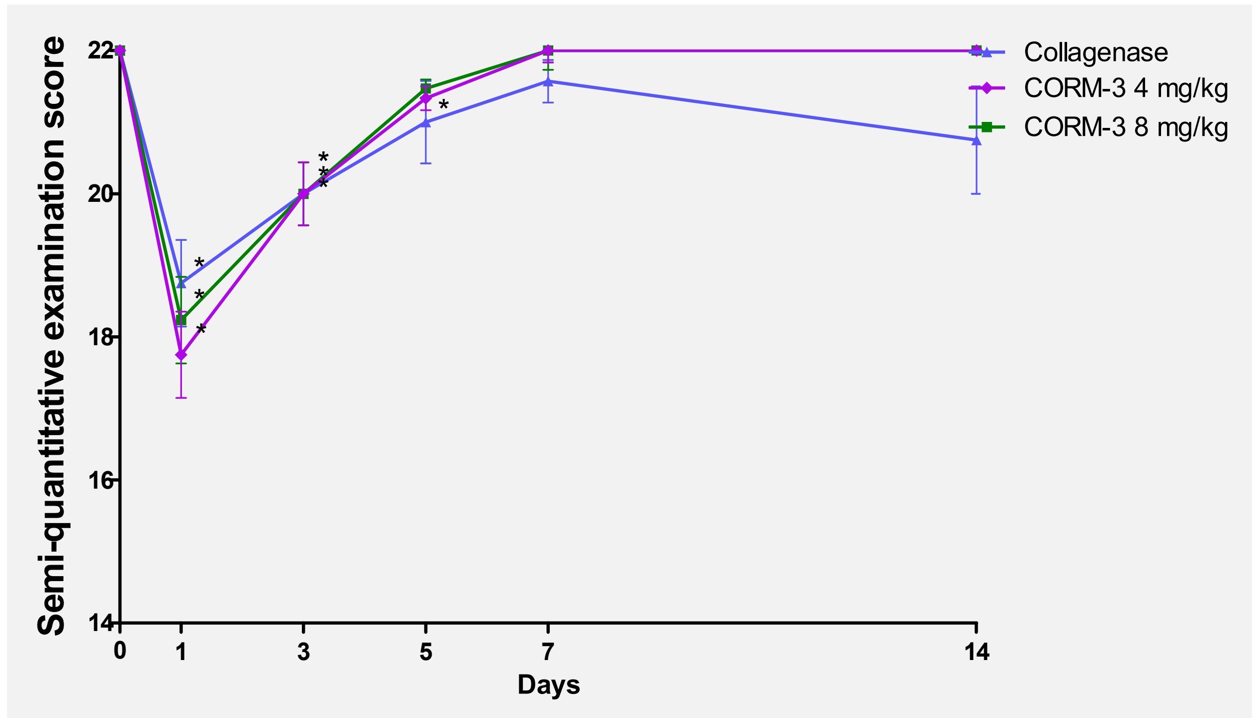


**Figure 6-54 Semi-quantitative examination score: the collagenase (Group 5), collagenase + CORM-3 4 mg/kg group (Group 12), collagenase + CORM-3 8 mg/kg (Group 13) groups. Data represent the mean ± SD of n = 6 independent experiments for each time point respectively. * - p < 0.05 vs. pre-operation data**

#### 6.5.2.4 Correlations between blood and lesion zone leukocytes and behavioural tests

A strong positive correlation was observed between:

- The number of peripheral blood neutrophils and the number of neutrophils in the lesion zone
- the number of neutrophils in the lesion zone and the number of microglia cells in the lesion zone
- the number of neutrophils in the lesion zone and plasma TNF-alpha levels
- the number of microglia cells in the lesion zone and plasma TNF-alpha levels
- the number of peripheral blood lymphocytes and the beam walking test score
- the forelimb placing test score and the beam walking test score
- the number of peripheral blood lymphocytes and the semi-quantitative examination score
- the forelimb placing test score and the semi-quantitative examination score
- the beam walking test score and the semi-quantitative examination score

A strong negative correlation was observed between:

- the number of peripheral blood neutrophils and the number of peripheral blood lymphocytes
- the number of neutrophils in the lesion zone and the number of peripheral blood lymphocytes
- the number of peripheral blood neutrophils and the forelimb placing test score
- the number of microglia cells in the lesion zone and the forelimb placing test score

- the number of peripheral blood neutrophils and the beam walking test score
- the number of neutrophils in the lesion zone and the beam walking test score
- the number of microglia cells in the lesion zone and the beam walking test score
- the number of peripheral blood neutrophils and the semi-quantitative examination score
- the number of neutrophils in the lesion zone and the semi-quantitative examination score
- the number of microglia cells in the lesion zone and the semi-quantitative examination score
- the forelimb placing test score and plasma TNF-alpha levels
- the semi-quantitative examination score and plasma TNF-alpha levels.

A moderate positive correlation was observed between:

- the number of peripheral blood lymphocytes and the forelimb placing test score

A moderate negative correlation was observed between:

- the number of peripheral blood neutrophils and the forelimb placing test score
- the number of microglia cells in the lesion zone and the beam walking test score
- the beam walking test score and plasma TNF-alpha levels.

A weak positive correlation was observed between:

- the number of peripheral blood neutrophils and the number of microglia cells in the lesion zone
- the number of peripheral blood neutrophils and plasma TNF-alpha levels

A weak negative correlation was observed between:

- the number of peripheral blood lymphocytes and the number of microglia cells in the lesion zone
- the number of peripheral blood lymphocytes and plasma TNF-alpha levels

**Table 13 HS + CORM-3 4 mg/kg treatment: matrix of levels pair correlation coefficients between blood and lesion zone leukocytes, plasma TNF alpha and behavioural tests**

| Parameters | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 1 | 0.83 | - 0.96 | 0.27 | - 0.67 | - 0.94 | - 0.81 | 0.22 |
| 2 | | - 0.71 | 0.76 | - 0.97 | - 0.97 | - 0.99 | 0.73 |
| 3 | | | - 0.09 | 0.53 | 0.86 | 0.72 | - 0.04 |
| 4 | | | | - 0.89 | - 0.58 | - 0.76 | 0.99 |
| 5 | | | | | 0.88 | 0.97 | - 0.87 |
| 6 | | | | | | 0.96 | - 0.54 |
| 7 | | | | | | | - 0.73 |

1 – Peripheral blood neutrophil count; 2 – Number of neutrophils in the lesion zone; 3 – Peripheral blood lymphocyte count; 4 – Number of microglia cells in the lesion zone; 5 – Forelimb placing test scores, left paw; 6 – Beam walking test scores; 7 – Semi-quantitative examination scores, 8 – Plasma TNF-alpha levels

A strong positive correlation was observed between:

- the number of neutrophils in the lesion zone and the number of microglia cells in the lesion zone
- the number of peripheral blood lymphocytes and the number of microglia cells in the lesion zone
- the number of peripheral blood lymphocytes and the forelimb placing test score
- the number of peripheral blood lymphocytes and the beam walking test score
- the forelimb placing test score and the beam walking test score
- the number of peripheral blood lymphocytes and the semi-quantitative examination score

- the forelimb placing test score and the semi-quantitative examination score
- the beam walking test score and semi-quantitative examination score
- the number of neutrophils in the lesion zone and plasma TNF-alpha levels
- the number of microglia cells and plasma TNF-alpha levels

A strong negative correlation was observed between:

- the number of peripheral blood neutrophils and the number of peripheral blood lymphocytes
- the number of neutrophils in the lesion zone and the number of peripheral blood lymphocytes
- the number of peripheral blood neutrophils and the forelimb placing test score
- the number of neutrophils in the lesion zone and the forelimb placing test score
- the number of microglia cells in the lesion zone and the forelimb placing test score
- the number of peripheral blood neutrophils and the beam walking test score
- the number of neutrophils in the lesion zone and the beam walking test score
- the number of microglia cells in the lesion zone and the beam walking test score
- the number of peripheral blood neutrophils and the semi-quantitative examination score
- the number of neutrophils in the lesion zone and the semi-quantitative examination score
- the number of microglia cells and the semi-quantitative examination score
- the forelimb placing test score and plasma TNF-alpha levels
- the semi-quantitative examination score and plasma TNF-alpha levels.

A moderate positive correlation was observed between:

- the number of peripheral blood neutrophils and the number of neutrophils in the lesion zone
- the number of peripheral blood neutrophils and the number of microglia cells in the lesion zone

A moderate negative correlation was observed between:

- the number of peripheral blood lymphocytes and the number of lymphocytes in the lesion zone
- the number of peripheral blood lymphocytes and plasma TNF-alpha levels
- the beam walking test score and plasma TNF-alpha levels

A weak positive correlation was observed between:

- the number of peripheral blood neutrophils and plasma TNF-alpha levels

**Table 14 HS + CORM-3 8 mg/kg treatment: matrix of levels pair correlation coefficients between blood and lesion zone leukocytes, plasma TNF alpha and behavioural tests**

| Parameters | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| 1 | 0.68 | - 0.99 | 0.36 | - 0.79 | - 0.90 | - 0.83 | 0.23 |
| 2 | | - 0.78 | 0.92 | - 0.99 | - 0.93 | - 0.96 | 0.83 |
| 3 | | | - 0.49 | 0.87 | 0.95 | 0.91 | - 0.38 |
| 4 | | | | - 0.85 | - 0.72 | - 0.79 | 0.96 |
| 5 | | | | | 0.98 | 0.98 | - 0.74 |
| 6 | | | | | | 0.98 | - 0.60 |
| 7 | | | | | | | - 0.71 |

1 – Peripheral blood neutrophil count; 2 – Number of neutrophils in the lesion zone; 3 – Peripheral blood lymphocyte count; 4 – Number of microglia cells in the lesion zone; 5 – Forelimb placing test scores, left paw; 6 – Beam walking test scores; 7 – Semi-quantitative examination scores, 8 – Plasma TNF-alpha levels

## 6.6 Inter-chapter results comparison

### 6.6.1 Systemic level of inflammation

Early pre-treatment with CORM-3, 5 minutes prior to intracerebral haemorrhage, led to a small increase in WBC on day 1 which subsequently then returned to pre-operational levels, compared to the collagenase injection group, where WBC levels decreased slightly over 1 day but then returned to the level of pre-operation data. CORM-3 administered 3 hours post intracerebral haemorrhage elicited a non-significant variation of WBC around the pre-operational level. When injected 3 days post intracerebral haemorrhage, WBC levels were not significantly different to the collagenase injection group over the first 3 days, but slightly increased compared to the collagenase injection group level on day 7. This was in contrast to the early pre-treatment group when the number of WBC were slightly increased on day 1 followed by a further decrease to pre-operation data and to the 3 hours post intracerebral haemorrhage treatment group, where the number of WBC were similar to the level of pre-operation data.

CORM-3 administered 5 min prior intracerebral haemorrhage resulted in an increase in the number of peripheral blood neutrophils which was lower than in the collagenase injection group on day 1, ultimately reaching pre-operational level on days 3 and 5. In contrast to early pre-treatment, 3 hours post intracerebral haemorrhage treatment resulted in an increase in the number of peripheral blood neutrophils on day 1 with the levels higher than in the collagenase injection group, which then decreased reaching pre-operational level but subsequently rapidly increased by day 5. On the $7^{th}$ day of the experiment in the 3 hours post intracerebral haemorrhage group, the levels of

peripheral blood neutrophils were similar to the level in the collagenase injection group, decreasing to the level of pre-operation data by day 14. When CORM-3 was given 3 days post intracerebral haemorrhage, the peripheral blood neutrophil level remained the same as in the collagenase injection group over the first three days, which was significantly higher than in the early pre-treatment group and slightly lower than in the 3 hours post intracerebral haemorrhage group. Subsequently in the 3 days post intracerebral haemorrhage treatment group the level of peripheral blood neutrophils remained at the pre-operation data level until the end of the experiment.

The lymphocytic reaction in peripheral blood in the early pre-treatment group showed a levels similar to pre-operation data on day 1, which was higher than in the collagenase injection group, this increased until the levels of peripheral blood lymphocytes reached the level of the collagenase injection group. Peripheral blood lymphocyte levels in the group which had 3 hours post intracerebral haemorrhage CORM-3 administration displayed similar changes to the collagenase injection group. The lymphocyte level was lower than in the collagenase injection group over the first 5 days, ultimately reaching the level of pre-operation data. The peripheral blood lymphocytosis in the 3 hours post intracerebral haemorrhage group peripheral blood was significantly lower over the course of the experiment compared to the early pre-treatment group. In contrast, CORM-3 administered 3 days post intracerebral haemorrhage, appeared to stabilise the level of lymphocytes that were observed on day 3 with no change observed over the remainder of the experiment. The level of peripheral blood lymphocytes in the 3 days post intracerebral haemorrhage group was similar to the level in the early pre-treatment group and higher than in the 3 hours post intracerebral haemorrhage treatment group.

Changes were also observed in plasma TNF-alpha levels between the groups. CORM-3 in the early pre-treatment group increased the level of TNF-alpha in plasma samples starting from day 1, which was lower than in the collagenase injection group. The level of TNF-alpha remained raised until the end of the experiment. CORM-3 administered 3 hours post intracerebral haemorrhage led to increased plasma TNF-alpha levels which were higher than the ones observed in the collagenase injection and early pre-treatment group. In contrast to the collagenase injection groups, when TNF-alpha levels peaked on day 7 with further decrease, the 3 hours post intracerebral haemorrhage treatment group demonstrated stable high levels until the end of the experiment. In the 3 days post intracerebral haemorrhage group, levels of plasma TNF-alpha over the first 3 days were similar to the collagenase injection group. The addition of CORM-3 reduced the TNF-alpha level on day 5, but these were still higher than in the early pre-treatment group and significantly lower than in the collagenase injection and 3 hours post intracerebral haemorrhage treatment groups.

### 6.6.2 Local level of inflammation

Locally, early pre-treatment with CORM-3 led to attenuation of intracerebral haematoma size in contrast to the collagenase injection group, in which haematoma occupied 30% of the affected hemisphere, and the 3 hours post intracerebral haemorrhage treatment, where haematoma size was significantly enlarged, exceeding the size of the collagenase injection group. On day 5 the haematoma in the group which was treated with CORM-3 on the 3$^{rd}$ day indicated a small decrease in its size compared to the data in the collagenase injection group.

Brain water content in the unaffected hemisphere in all three CORM-3 treatment groups indicated a similar increase to the collagenase injection group on day 1 with a subsequent decrease reaching pre-operation data levels by day 5.

As for the affected hemisphere, the early CORM-3 pre-treatment group demonstrated a lower brain water content level on day 1 than in the collagenase injection group, with a reduction, reaching the levels of pre-operation data on day 5.

In contrast, 3 hours post intracerebral haemorrhage treatment, led to a high stable brain water content over first three days, similar to the collagenase injection group, which remained at the same level over the next two days, exceeding the levels of the early pre-treatment group, which eventually decreased to the level of pre-operation data by day 5.

As for 3 days post intracerebral haemorrhage treatment, the brain water content level was similarly high on day 1 compared to the collagenase injection group and the 3 hours post intracerebral haemorrhage treatment group, there was a subsequent decrease in its levels on day 3 reaching the pre-operation data level on day 5.

The densitometric cell count of the early pre-treatment group indicated a significant increase in the number of neutrophils compared to the collagenase injection group and pre-operation data on day 1, with a gradual decrease reaching the level of pre-operation data by day 5.

There was an increase in the number of neutrophils in the zone of the intracerebral haemorrhage in the 3 hours post intracerebral haemorrhage CORM-3 treatment group, which reached the level of the collagenase injection group on day 1 and was significantly lower than the one in early pre-treatment group. However, a further increase in the number of neutrophils reached the maximal peak on day 3, compared to the early pre-treatment and the collagenase injection groups where a peak was reached on day 1. Further changes in the 3 hours post intracerebral haemorrhage treatment group indicated a decrease in the number of neutrophils which reached the levels of pre-operation data by day 7.

The number of neutrophils in the region of the intracerebral haemorrhage in the group receiving CORM-3 treatment 3 days post intracerebral haemorrhage was similar to the level in the collagenase injection group, indicating a peak on day 1, which was significantly lower than in the early pre-treatment group and similar to the level of the 3 hours post intracerebral haemorrhage treatment group, returning to the level of pre-operation data by day 5.

The densitometric cell count in the early pre-treatment group showed an increase in the number of microglial cells in the region of the intracerebral haemorrhage on day 1, with the levels of microglial cells being lower than measured in the collagenase injection group. Also, on day 1, a peak in the number of microglial cells in the region of the intracerebral haemorrhage of the early pre-treatment group was noted, in contrast to the collagenase injection group, when the maximal peak occurred on day 3. There was a decrease in the number of microglial cells in the region of the intracerebral

haemorrhage which reached the level of pre-operation data by day 7 in the pre-treatment group, as was also observed with collagenase control group.

There was an increase in the number of microglial cells in the intracerebral haemorrhage zone on day 1 in the 3 hours post intracerebral haemorrhage CORM-3 treatment group, which was lower than in the collagenase injection group and the early pre-treatment group. A further increase in the number of microglial cells in zone of the intracerebral haemorrhage led to a peak on day 5, exceeding the level of the collagenase injection group and early pre-treatment group. Subsequent changes indicated a rapid decrease in the number of microglial cells in the intracerebral haemorrhage zone which reached the level of pre-operation data by day 7, as was observed in the collagenase injection group, but which was slightly delayed in time when compared to the early CORM-3 pre-treatment group.

The number of microglial cells in the region of the intracerebral haemorrhage over the first three days was very similar in the collagenase injection group to the 3 days post intracerebral haemorrhage CORM-3 treatment group, indicating the level of microglial cells were higher than in the early pre-treatment group and lower than in the 3 hours post intracerebral haemorrhage group. Further changes indicated an earlier decrease in the number of microglial cells in the region of the intracerebral haemorrhage, reaching the level of pre-operation data, compared to the collagenase injection group and the 3 hours post intracerebral haemorrhage treatment group, similar to the one observed in the early CORM-3 pre-treatment group.

### 6.6.3 Behavioural testing

There was no observed difference between saline and saline plus CORM-3 injection groups in the behavioural tests throughout the experiment, indicating maximal performance in both groups. Thus, CORM-3 administered after a small HS injury does not lead to brain damage in this particular animal model and resultant structural changes that would result in behavioural performance loss, over the 14 days period of study

CORM-3 treatment in the early pre-treatment group resulted in a similar decrease in performance in all the behavioural tests to the collagenase injection group on day 1. The CORM-3 treatment group was found to possess faster improvement indicating and better performance than in the collagenase injection group on day 3. Maximal performance in the CORM-3 treatment group was observed on the 5$^{th}$ day of the experiment.

CORM-3 administered 3 hours post haemorrhagic stroke resulted in a significant decrease in functional performance on day 1, compared to the collagenase injection group and early pre-treatment group. There was an improvement in functional recovery in the CORM-3 treatment group, however the level of improvement was lower than in the collagenase injection group and the early pre-treatment group on day 3. There was a similar level of functional recovery in the 3 hours post haemorrhagic stroke CORM-3 treatment group compared to the collagenase injection group on the 5$^{th}$ day of the experiment, which was lower than the one observed in the early pre-treatment group. The maximal stable performance was not reached until the end of the experiment

indicating a small variation around pre-operation data in the 3 hours post intracerebral haemorrhage CORM-3 treatment group.

Behavioural tests in the 3 days post haemorrhagic stroke CORM-3 treatment group indicated similar performance to the collagenase injection group over the first three days, which was somewhat lower than in the early pre-treatment group and significantly higher than in the 3 hours post haemorrhagic stroke treatment group. CORM-3 administration at 3 days post haemorrhagic stroke resulted in an improvement in functional recovery, demonstrating that the level of behavioural performance on day 5 was slightly higher than in the collagenase injection group and similar to the early pre-treatment group. The maximal performance in the CORM-3 treatment group at 3 days post haemorrhagic stroke was reached on day 5 and 7.

## 6.7 Discussion

In this chapter we discuss the hypothesis that CORM-3 affects haemorrhagic stroke through inflammation and modulates the general adaptation syndrome, and that the impact of CORM-3 on haemorrhagic stroke is determined by the time of its administration post HS.

### 6.7.1 The effect of CORM-3 on intact animals

Our results showed that CORM-3 administered without any surgical intervention, did not lead to any changes in observed tests in the kinetics of blood leukocyte numbers and plasma TNF alpha levels, tests we used to determine systemic levels of inflammation.

However, when administered at the same time as the surgical intervention of saline injection into the lateral striatum, CORM-3 contributed to a neutrophilic prevalence in WBC over the first three days, but without increasing the level of plasma TNF-alpha, with the local level of inflammation and functional recovery of the brain remaining unaffected. In the literature there is no data on the effects of CORM-3 in intact experimental animals in HS.

The difference that we observed between data obtained from CORM-3 treated intact animals, CORM-3 plus saline injection and saline injection only groups could be explained by a property of CORM-3 which triggers inflammation in stressed experimental animals subjected to an insult or injury (Bagul et al. 2008;Katada et al. 2009;Lancel et al. 2009).

### 6.7.2 CORM-3 effects on HS inflammation

Bearing in mind that CORM-3 is capable of modulating inflammation occurring in a living organism with little damage, we compared the effects of CORM-3 on systemic and local levels of inflammation and on functional recovery of the brain using different concentrations and different times of administration in an experimental model of haemorrhagic stroke.

#### 6.7.2.1 Systemic level of inflammation

These data suggest that the effect of CORM-3 on the systemic level of inflammation during haemorrhagic stroke is determined by the time of its administration according to the phases of inflammatory response that develop as a result of intracerebral haemorrhage.

We did not find any published data investigating the properties of CORM-3 on the WBC in any experimental model of haemorrhagic stroke and any other aseptic inflammation models.

However, changes observed in our experiments regarding peripheral blood leukocyte kinetics in the early pre-treatment group, suggest that CORM-3 may cause a general adaptation syndrome in experimental animals (Selye & Fortier 1950) and leads to the pre-organization of the systemic inflammation response (Kleinig & Vink 2009), indicating a decrease in neutrophilic reaction during the very first hours and days following HS and a more pronounced lymphocytic reaction starting from day 3, which could possibly have led to the attenuation of brain damage.

CORM-3 administered 3 hours post intracerebral haemorrhage exacerbated an alterative stage of inflammation via significant changes in neutrophilic and lymphocytic reactions in blood, which led to a delayed neutrophilic and depressed lymphocytic reaction in blood. Taking into account that in some medical cases, non-intracerebral blood leak in haemorrhagic stroke patients can continue for up to 24 hours (Fujii et al. 1998;Miyai et al. 2000;Sahni & Weinberger 2007), another CO-RM effect may have taken place. Vasodilation, one of the principal properties of CO-RMs (Motterlini et al. 2002;Motterlini 2007), could have contributed to a longer intracerebral blood leak and thus the enlargement of haematoma with subsequent changes in leukocyte kinetics.

Changes observed in the peripheral blood of the experimental animals subjected to 3 days post intracerebral haemorrhage treatment with CORM-3, indicated a normalisation of neutrophil levels and more pronounced lymphocytic reaction, which is normally responsible for the reparative stage of inflammation and usually remains elevated in patients with a favourable prognosis following haemorrhagic stroke (Kitahara et al. 2002).

A smaller increase in the plasma levels of TNF-alpha in the early-pre-treatment group compared to the collagenase injection group correlated with changes observed in peripheral blood leukocytes, suggesting its cellular origin was from this cell type (Zaremba, Skrobanski, & Losy 2001b). Increased levels of plasma TNF-alpha throughout the experiment suggest its involvement in the systemic response and reparative stages of inflammation running in experimental animals (Carlson et al. 1999b;Zaremba & Losy 2004).

Pronounced changes in leukocyte kinetics in the 3 hours post intracerebral haemorrhage group correlated with overproduction of plasma levels of TNF-alpha. In this instance, considering that TNF-alpha, acting as a pro-inflammatory cytokine is capable of exacerbating brain damage (Hallenbeck 2002), overproduction of this cytokine in the acute stage of inflammation compared to the collagenase injection group, could have contributed to stimulation of the alterative stage. Liu et al. demonstrated that suppression of TNF-alpha levels led to attenuation of ROS production in their model of thermally-injured mice and thus to lesser damage observed (Liu et al. 2008). High levels of TNF-alpha throughout the experiment suggest its involvement in reparative processes and its anti-inflammatory properties that take place in the inflammatory response to tissue injury (Hallenbeck 2002).

A positive correlation with leukocyte kinetics in the group of animals 3 days post intracerebral haemorrhage treated with CORM-3 once again suggests the cellular origin of TNF-alpha produced in plasma of the experimental animals and its crucial role in mediating an inflammatory response to stroke injury and its involvement into alterative and reparative stages of inflammation (Carlson et al. 1999;Hallenbeck 2002).

Contradictory data concerning serum and CSF levels of TNF-alpha associates with brain damage and leukocyte kinetics (Christensen et al. 2002;Intiso et al. 2004;Zaremba & Losy 2001a;Zaremba, Skrobanski, & Losy 2001b) suggest the importance of this cytokine and its systemic response occurring in stroke (Feuerstein, Goldman, & Feuerstein 2002).

#### 6.7.2.2 Local level of inflammation

CORM-3 administered 5 minutes prior to intracerebral haemorrhage led to higher WBC levels compared to the collagenase injection group, thus more neutrophils entered the intracerebral haemorrhage area. The smaller percentage of neutrophils in the blood compared to the collagenase injection group could be explained by their initial higher levels. Saturating the zone of the intracerebral haemorrhage with neutrophils, and in addition probably a faster response in the pre-treated rat, led to faster destruction of cells together with better scavenging processes thus decreasing the haematoma size.

The lower levels of microglial cells observed in the densitometric analysis could possibly be explained by the demands of the organism, dictated by the haemorrhage size formed. Increased levels of blood lymphocytes and decreased numbers of microglial cells by day 5, suggest further lymphocyte activity was shifted towards tissue replacement processes, as it was shown that lymphocytes, a precursor of microglial cells (Martin & Muir 1990), are capable of regulating proliferation, differentiation and migration of stem cells in the zone of intracerebral haemorrhage (Butterfield, Best, & Merrick 2006;Huaux et al. 2003). A faster decrease in the number of observed cells in the region of the intracerebral haemorrhage, compared to the collagenase injection group, correlated with the brain water content levels in the affected hemisphere. The brain water content decreased on day 3, and was lower than in the collagenase injection group, thus suggesting the predominance of a cellular component.

CORM-3, administered 3 hours post intracerebral haemorrhage triggered an alterative stage of inflammation. A decrease in the neutrophil levels in general blood analysis on day 3 correlated with a dramatic increase in neutrophils in the zone of the

intracerebral haemorrhage on the same day, thus indicating shifting and elongation of the alterative stage of inflammation. It may be that late oversaturation of the intracerebral haemorrhage zone with neutrophils in a zone where the reparative processes should have started could lead to a more intensive destruction of tissues in the intracerebral haemorrhage zone, with delayed processes associated with new connective tissue organisation thus contributing to the blood leakage. It is known that damaged tissue releases a variety of bioactive compounds that are capable of interacting with nearby tissues and contributing to the overall damage, as it can be observed indirectly in ischemia-reperfusion models (Bora & Sharma 2010;Kinross et al. 2009). Despite the fact that some authors indicated that CO-RMs are capable of attenuating a neutrophilic infiltration (Mizuguchi et al. 2009;Sun et al. 2007;Sun et al. 2008), our data did not suggest this. We believe this happened due to overriding CORM-3 effects attenuating leukocyte infiltration with its effects at a systemic level and the more pronounced organism response to a larger injury.

An increasing number of microglial cells in the zone of the intracerebral haemorrhage may be a reaction to damage enlargement. The larger damage to cerebral tissue demands a larger response of immunocompetent cells capable of scavenging damaged tissue.

Changes observed on day 3 with the CORM-3 treatment group suggest that CORM-3 has influenced both systemic and (brain) local environments. Although there were no differences found in the intracerebral haemorrhage zone neutrophil numbers in the collagenase injection group over the first 3 days, systemically their numbers were reduced and stabilised by day 5. Taking into account the fact that CORM-3 was

administered at the post-acute stage of the inflammatory response to the intracerebral haemorrhage we believe that CORM-3 via its effects attenuated neutrophil activity (Masini et al. 200;Mizuguchi et al. 2009). Thus, despite the fact that the number of neutrophils in the zone of the intracerebral haemorrhage in the 3 days *post ictus* treatment group was similar to the one observed in the collagenase injection group, neutrophil activity was not as high as in the 3 hours *post ictus* treatment group, which led to a smaller damage area in the affected hemisphere.

Initially similar levels of microglial cells in the 3 days CORM-3 treatment group as compared with the collagenase injection group indicated that CORM-3 reduced their number earlier. A decrease in the level of microglial cells and lymphocyte levels remaining high in peripheral blood suggests lymphocytes shifted their activity from a scavenging role to a tissue replacement (Farini et al. 2007). According to post intracerebral haemorrhage neutrophils and microglial cell dynamics and their earlier reduction compared to the collagenase injection group, a decrease in brain water content starting from day 3 is consistent, and once again suggests a cellular component to the subsequent brain oedema and pivotal role in its development.

We associate the oedema level with a predominant cellular component, as a correlation was observed between the decreaseing numbers of immunocompetent cells in the brain with reduction of the oedema.

We did not find published data investigating the effects of CORM-3 on haematoma size and the cellular kinetics of alterative and reparative processes in an experimental model of haemorrhagic stroke. The results observed in size of haematoma

and cellular kinetics of the alterative and reparative nature following CORM-administration correspond to other studies of mechanisms run of aseptic inflammation (Dolgina & Kakhetelidze 1972;Ertas et al. 2005;Kotilainen et al. 1996). The phase progression at the local level has also been described in the pathomorphology of haemorrhagic stroke in humans (Bykovnikov 1991;Vlasiuk 1986;Zulch 1974).

The times of CORM-3 administration in our experimental haemorrhagic stroke model are reflected not only in general adaptation syndrome in blood leukocyte kinetics and plasma TNF-alpha levels, but also reflect a specific pattern of local processes in the brain, in particular in the damaged area, cellular reactions of alterative and reparative stages of inflammation, which in the end influence the outcome of haemorrhagic stroke.

#### 6.7.2.3 Behavioural testing

We did not find published data investigating the effect of CO-RMs on the functional recovery of the brain. Indirectly, CO-RMs have demonstrated positive effects on myocardium functional properties in the study by Musameh et al., where it was shown that systolic and diastolic functions after cold storage of rat hearts were significantly improved after CORM-3 treatment (Musameh et al. 2007). Varadi et al. also demonstrated an improvement in post-ischaemic myocardial recovery after treatment with CORM-3 (Varadi et al. 2007). Recognising that post injury myocardium heals through the mechanisms of inflammation, CO-RMs effects, should be considered alongside their time of administration. Other investigators that demonstrated positive effects of CO-RMs on injured myocardium used these compounds in a pre-treatment manner, however, if CO-RMs were used in early post-injury time or 3 days post injury, the results, we believe, may be similar to ours.

The loss of functions of the organ involved in the pathological process is one of the major manifestation of inflammation (Lucignani 2007). A dependence of these changes and restoration of functions is consistent with the effects of CORM-3 on the functional recovery of the brain in our animal model. Thus, our findings suggest, that apart from the crucial role of CORM-3 modulating a dynamic process of inflammation, the time of its intervention in the dynamics of inflammation should be considered equally importance.

The results obtained from the behavioural examination of the experimental animals with haemorrhagic stroke in correlation with the cellular kinetics of immunocompetent cells and the systemic and local levels of cytokines produced by these cells, e.g. TNF-alpha, at different times and concentrations of CO-RMs treatment suggest, that this approach may be useful in drugs that could be aministered systemically.

#### 6.7.2.4 Dose-dependent effects of CORM-3

CORM-3 possesses a variety of bioactive properties and is capable of modulating an inflammatory response to intracerebral haemorrhage in experimental animals. CORM-3 showed an ability to differently modulate inflammation depending on its time of administration according to the phase of inflammation occurring in the living organism, thus suggesting a prospective use for haemorrhagic stroke treatment. As with any prospective medication it was important to study its dose-dependent and time-dependent effect on the course of haemorrhagic stroke.

Reading the literature, there are only a few specific doses of CO-RMs used in in vivo models. Guo et al. used a 3.54 mg/kg doses in their model of myocardial infarction, Vadori et al. used 4 mg/kg odse in their models of allo- and xenografted organs, Chen et al. used a 8 mg/kg dose in their model of pancreatitis in rats (Chen et al. 2010;Guo et al. 2004;Maicas et al. 2010;Stein et al. 2005;Vadori et al. 2009). All of these studies demonstrated a potential beneficial use of CO-RMs in various models, suggesting CO-RMs possess a variety of protective properties.

Based on these data we decided to investigate two concentrations of CORM-3 in our experiments, 4 mg/kg and 8 mg/kg respectively. The results obtained from our experiments did not indicate a significant difference and in some cases showed no difference between the two concentrations of CORM-3 used.

However, despite the fact that we did not observe a dose-dependent effect of CORM-3 in a wide dose spectrum, our results suggest that these two concentrations possess a very similar effect on inflammation in experimental animals subjected to haemorrhagic stroke. However, taking into account that the difference between our concentrations was 2-fold, similar positive effects in the early pre-treatment group and the 3 days post intracerebral haemorrhage treatment group suggests its beneficial use for attenuation of brain injury during haemorrhagic stroke and neuroprotection.

# Chapter VII. General discussion and conclusion

We confirmed the properties of CORM-3 as a bioactive compound with the ability to liberate CO molecules in 1 to 1 manner. Apart from its stable release profile, CORM-3 was found to be non-toxic when testing it on the viability of two the main cellular compounds of the brain (microglia and astroglia cells). Brain tissue slices also suggest CORM-3 to be non-toxic at the doses and times used, indicating a 100% viability of tissue culture when subjected to high concentrations of compound (10, 50, 100 μM).

In the search for a representative model of haemorrhagic stroke we selected a collagenase injection model due to its reproducibility and stability. The optimum concentration of collagenase and the volume of injection were experimentally determined to be best at 0.2U/2μl.

Previous experimental investigations suggest the hypothesis that haemorrhagic stroke is resolved through inflammation, which is represented at a systemic level by changes in peripheral blood white cell level and brain level with changes in brain water content and histological structure with typical cellular reactions, which are followed by a partial functional recovery in behavioural test performance. Thus the importance of inflammation as one of the main processes during the course of haemorrhagic stroke was highlighted.

Taking into account the various phases of inflammation which occur in living organisms in various inflammatory models and in haemorrhagic stroke, the crucial role of the time of administration of CORM-3 post HS was highlighted.

CORM-3 administered 5 minutes prior to intracerebral haemorrhage led to an attenuation of brain damage with a lowering of systemic and local responses and better functional recovery. The positive effects of CORM-3 on the course of haemorrhagic stroke in the early pre-treatment group suggest its prospective use as a prophylaxis in the clinic in men but this still needs more experimental work.

CORM-3 given 3 hours post intracerebral hemorrhage facilitated an inflammatory response and evolution of haemorrhagic stroke pathology contributing to a hyperreactive cellular environment. Whether its effects on vasodilation contributed to blood leakage and thus the more pronounced reaction of the organism remains unclear, however previous experiments with CORM-3 given pre-treatment and evidence of its ability to modulate an inflammatory response suggest a crucial role. Despite the fact that CORM-3 led to larger damage in the early treatment group post stroke in our experimental model, the prospective use of this compound *in vivo* should be investigated further due to the time dependent effects observed.

Late CORM-3 administration, at 3 days post intracerebral haemorrhage led to attenuation of brain damage with correspondent systemic and local levels of organismal reactions and with better functional recovery. The positive effects of CORM-3 in the reparative stage of haemorrhagic stroke suggest its prospective use as an additional medication for patients recovering from HS.

CORM-3 demonstrated an important property in its ability to modulate a general adaptation reaction at a systemic and a local (brain) level and thus increase functional recovery of the brain, but these effects are determined by the time of its administration, which may reflect the phase of inflammation.

The use of two different concentrations, one of which was 2-fold higher than the other, showed no statistically significant difference between CORM-3's effects in all the time-dependant groups.

The positive effects of CORM-3 administration in the early pre-treatment group and 3 days post intracerebral haemorrhage treatment group suggests its beneficial use for attenuation of brain injury during haemorrhagic stroke and neuroprotection. Thus it will be critical to determine the precise time and concentration dependent effects of CORM-3 and any of its analogues with slower CO releasing properties, before they are used in the clinic in man.

# Chapter VIII. Future perspectives

The data obtained from our experiments suggested an inflammatory nature of haemorrhagic stroke and demonstrated that CORM-3 affects the course and resolution of haemorrhagic stroke via a general adaptation syndrome and inflammatory mechanisms and that CORM-3 is capable of modulating a resolution of haemorrhagic stroke damage depending on the time of its administration.

Current investigations into CORM-3 have studied it in particular models and systems (i.e. *in vitro, ex vivo* and *in vivo* model of inflammation). These early studies allow us to define the biological suitability, toxicity, concentration range of use, mechanism of action and the overall therapeutic potential. The role of CO has been investigated in many models and its effect on various biological functions have been reported. CO-RMs should be investigated in as many such systems as possible to corroborate or dispute the existing findings of CO gas and HO upregulation based experiments.

There are many short and long terms goals to be achieved and many ways for furthering this study which include:

1) The development of a models of haemorrhagic stroke with variation in adaptation syndrome in experimental animals, e.g. models of suppressed immune response

with anti-inflammatory medications, examples of which can be found in clinical practise

2) Examination of possible CORM-3 effects on the necrotic phase of inflammation in experimental haemorrhagic stroke with initial low adaptation syndrome level, that modulates haemorrhagic stroke in the elderly

3) Examination of possible CORM-3 effects on the necrotic phase of inflammation in experimental haemorrhagic stroke with initial hyperreactive adaptation syndrome level, that modulates haemorrhagic stroke in young people and babies

4) Evaluation of currently used medications for haemorrhagic stroke treatment for their effects on its resolution depending on the time of their administration and the current phase of the inflammatory reaction occurring after intracerebral haemorrhage

5) A development of the systemic control methods of aseptic inflammation and inflammation-associated restoration of functions with the aim of increasing accuracy in diagnostics and treatment of haemorrhagic stroke in patients

6) Examination of the possibility of applying data obtained in ischaemic stroke, to other brain tissue injuries in pathological conditions with the purpose of developing innovative methods of diagnostics and treatment in clinical practise.

# Chapter IX. Appendices

## Appendix 1: CORM-3: Detection of CO release: myoglobin assay

The following section comprises a step by step guide to performing a myoglobin assay to test the CO release from CO-RMs.

1) Prepare the spectrophotometer making sure the cuvettes (1.5 ml plastic) (Sarstedt) are in place and that they are at 37 °C (heated carousel).

2) Zero the baseline using 1 ml PBS (0.01M, pH = 7.4) (Sigma)

3) Prepare a 66 µmol/l (final concentration) stock of myoglobin (lysophilised horse heart) (Sigma) by adding 50 ml PBS (0.01M, pH = 7.4) (Sigma) to 56 mg myoglobin.

4) Warm the myoglobin in a water bath at 37 °C.

5) Once warm, add sodium dithionite (0.1 % or ~ 2 heaped spatulas) (Sigma) to convert the myoglobin stock to deoxymyoglobin (deoxy-Mb).

6) Add 1 ml deo

7) xy-Mb to a cuvette and read the absorbance between 500 and 600 nm. These should be a single peak around 560 nm. This absorbance value is taken as your zero MbCO value.

8) With the same cuvette, bubble the deoxy-Mb with CO gas for 1 min to saturate the myoglobin. Read the sample between 500 and 600 nm again. The curve should now have two peaks with the second peak smaller than the first. The first peak should be around 540 nm and the second 578 nm. This is your 100 % MbCO value.

9) Using the deoxy-Mb stock add 1 ml to a cuvette.

10) Prepare the CO-RM you wish to test. The final concentrations used in this study were 20, 40 and 60 µM. These were prepared as 4, 8 and 12 mM stocks respectively. The final volume added, therefore, was 5 µl.

11) Add your compound to the myoglobin.

12) Mix the myoglobin-CO-RM solution gently using a Gilson pipette (1000 µl) so as not to oxygenate the solution. This would result in two peaks of equal size.

13) Layer the myoglobin with 500 µl of mineral oil (Sigma) to prevent CO escaping and the myoglobin becoming oxygenated.

14) Read the sample. For the purpose of this study samples were read every 5 min for the first 30 min and every 30 min thereafter. Depending on the CO releasing characteristics of the CO-RM there will either be no change, a gradual conversion from deoxy-Mb to MbCO or a near instant conversion.

## Appendix 2: Rat brain striatal slice preparation

### Preparation of medium

1. Using a sterile pipette, prepare 2 ml aliquots of a 45% glucose solution (Sigma) in sterile tubes. Store aliquots at room temperature until required
2. Using a sterile pipette prepare 1 ml aliquots of 1 mM L-glutamine (Sigma) in sterile tubes. Store aliquots at -20°C and thaw immediately prior to use

### Preparation of 200 ml standard growth medium

1. Defrost heat-inactivated horse serum (Gibco) and gently agitate to ensure is fully mixed
2. In a laminar flow hood, add the following reagents into a 75 cm$^2$ vented flask:
    i. 100 ml MEM + Earle's salts (Gibco)
    ii. 50 ml Hank's Balanced Salt Solution (MP Biomedicals)
    iii. 50 ml Heat-inactiavated horse serum
    iv. 2 ml 45% glucose solution
    v. 1 mM L-glutamine
    vi. Gently invert flask 2-3 times to ensure solutions are well mixed (do not shake as serum has a high protein content and the solution will form froth very easily)
3. Prepare Gey's balanced salt solution. In a laminar flow hood add the following reagents to a 75 cm$^2$ vented flask:

a. 200 ml of Gey's balanced salt solution

b. 2 ml 45% glucose solution.

c. Invert flask 2-3 times to ensure solutions are well mixed.

4. Store at 4°C until required.

**Preparation of materials**

1. Pastettes (Alpha Laboratories Ltd), the chopping disk, blade guard and nut from the McIlwain tissue chopper should be stored in 70% alcohol solution at all times until required.
2. Double-edged razor blades (Sutherland Helath Ltd) should be stored in separate dish containing 70% alcohol. It is important that blades are in alcohol for at least 60 minutes prior to use as there is a fine oil-coating on them that need to be dissolved.
3. Melinex sheeting (Agar Scientific) should be cut to size and sterilised in 70% alcohol (can go in with the pastettes, etc.).
4. Prepare tissue culture plates by adding 1.2 ml of complete medium into each well of a 6-well plate.
5. Remove Millicell inserts (Millipore) from packaging one at a time and place into wells slowly using a pair of sterile forceps.
6. It is essential to ensure that the entire surface of the insert is in contact with the medium (insert will turn transparent when wet) and that there are no bubbles under the inserts. 3-6 wells per rat will be required if preparing rat striatal slice cultures.
7. Clearly label plates with date and place in an incubator (37°C, 95% air and 5% $CO_2$, high humidity) until required.

**Immediately prior to culturing**

1. Remove the pastettes and double-edged razor blades from alcohol and place on paper towel in the hood. Remove excess alcohol with paper towel and leave until required. Tear filter paper into 1/4 or 1/6 and keep with other items until required.

2. Prepare pairs of 60 mm Petri dishes containing Gey's balanced salt solution. Using a 5 ml pipette transfer 0.2 - 0.3 ml solution into one dish and 6 ml into a second. Replace the lids and store at 4°C until required.

3. Ensure that the tissue chopper, dissecting microscope (Leitz, LABOVERT FS), dissecting area and light source (SCHOTT, KL 1500) are wiped down with 70% alcohol

4. Place dissecting instruments into 70% alcohol.

5. Insure the presence of a further (dry) 60 mm Petrie dish for collecting the brain into.

6. Set the McIlwain tissue chopper as follows:

a. Set the micrometer to the required slice thickness (300 μM).

b. Slide in the white plastic chopping disk and one piece of melinex onto the circular platform.

c. Place a razor blade onto the screw at the end of the chopping arm. Fix it with blade guard and nut.

**Preparation of slice cultures**

1. Remove one pair of Petri dishes containing Gey's balanced salt solution from the fridge and place in the laminar flow hood.

2. Sacrifice 6-10 day old Sprague-Dawley rat pup using an approved Schedule I method and decapitate using large scissors.

3. Remove fur and skin from the surface of the skull using a large pair of scissors.

4. Insert a small pair of dissecting scissors into the back of the skull and cut carefully along the midline until just past the bregma. Ensure that the points of the scissors are pointing upwards to avoid damaging brain tissue.

5. Using small dissecting scissors, make a small incision perpendicular to the midline at the level of bregma. Using a pair of small forceps peel back the skull tissue

until the whole hemisphere of the brain and cerebellum can be seen clearly. Repeat on the contralateral side.

6. With the head held horizontally, insert a pastette vertically into the front of the brain and scoop the pastette under the brain, lift the whole brain out of the skull. Collect the brain into a 60 mm Petri dish for further dissection.

7. In the laminar flow hood, hemisect the brain using a pastette and then dissect out the region of interest using an appropriate dissection protocol.

8. For experiments with the striatum, collect striatal slices as soon as the striatum is dissected out and, using a pastette, transfer it to the Petri dish containing 0.2 - 0.3 ml of Gey's balanced salt solution

9. Using a pastette, cut out the cerebral cortex, basal forebrain and septal area, leaving the cube of striatal tissue, and then transfer to the melinex strip so that the blade is set to cut the frontal plane of the tissue.

10. Press "reset" on the tissue chopper and then turn on the power switch. The chopper should automatically start cutting. Make sure it does not cut through the melinex film. Once complete, turn off the power switch on the chopper, raise the chopping arm and slide out the melinex film.

11. In the laminar flow hood, remove the lid from the Petri dish containing 6 ml of Gey's balanced salt solution, invert the melinex film and slightly bend it so that the ends are raised relative to the centre. Gently touch the striatum to the surface of the Gey's balanced salt solution and agitate until the tissue is transferred into the Petri dish.

12. Using a dissecting microscope and two pastettes, gently separate the individual slices.

13. Transfer 3 - 4 slices into each well of a 6-well plate.

**Preparation of slice cultures**

1. Striatal brain slice culture require medium changes every 3-4 days
2. To change the medium:
   i. Pre-warm serum-containing medium in the incubator at 37°C for 30 minutes.
   ii. Remove plates from incubator and transfer then to the laminar flow hood
   iii. Take the lids off two plates, and then rest the six well plates on the edge of the lids at an angle so that the medium pools in the lower portion of each well.
   iv. Lift out each well with a pair of sterile forceps and using 1 ml sterile pipette, remove 1 ml of medium from each well. Take care not to disturb medium in the wells. Discard the medium removed into a beaker.
   v. Using a 10 ml sterile pipette, transfer 1 ml of fresh medium back into each well.
   vi. Replace the lids onto plates and return to the incubator.

**Appendix 3: Enzyme-linked immunosorbent TNF-alpha assay**

Blood plasma samples were collected. Rat tail vein blood was collected in an Eppendorf tube and spun down at 10 000 g (Beckman Avanti 30). Collected plasma was frozen at -80˚C.

*Plate Preparation*

1. Dilute the capture antibody to the working concentration in PBS without any carrier protein. Immediately coat a 96-well microplate with 100 µL per well of the diluted capture antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with wash buffer, repeating the process two times for a total of three washes. Wash by filling each well with wash buffer (400 µL) using a manifold dispense autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining wash buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of reagent diluent to each well. Incubate at room temperature for a minimum of 1 hour.
4. The aspiration/wash was repeated as in step 2. The plates are now ready for the addition of sample.

*Assay Procedure*

1. Add 100 µL of sample or standards in reagent diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate for 2 hours at room temperature.
2. Repeat the aspiration/wash as in step 2 of plate preparation.

3. Add 100 μL of the detection antibody, diluted in reagent diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.

4. Repeat the aspiration/wash as in step 2.

*Preparation*

1. Add 100 μL of the working dilution of the Streptavidin-HRP conjugate to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

2. Repeat the aspiration/wash as in step 2.

3. Add 100 μL of substrate solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

4. Add 50 μL of stop solution to each well. Gently tap the plate to ensure thorough mixing.

5. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. Set wavelength correction to 540 nm.

## Appendix 4: Hematoxylin and Eosin staining

*Slide preparation*

1. Prepare 3-aminopropyltriethoxysilane (APTS) treated slides:
2. Soak slides in a 2% APTS solution (diluted IMS) for 2 minutes.
3. Rinse slides twice in double distilled $H_20$.
4. Air dry the slides.
5. Cut 5 µM thickness sections from the brain samples. Place sections in the APTS-slides and dry over night at 60˚C. For each sample you need 2 sections, one to test and the other to act as a negative control (the negative control in this was section without the primary antibody).

*De-waxing and hydration of the slides:*

1. Place slide in Xylene 1 for de-waxing for 5 minutes.
2. Place slide in Xylene 2 for de-waxing for 2 minutes.
3. Place slide in Absolute IMS for hydrating for 2 minutes.
4. Place slide in 95% IMS for hydrating for 2 minutes.
5. Place slide in 70% IMS for hydrating for 2 minutes.
6. Rinse slide in running tap water for 2 minutes.

*Staining the slides*

1. Stain the slides in Gill's Hematoxylin 1.5 minutes or Harris Hematoxylin 10 minutes.
2. Rinse the slides in running tap water 2 minutes.

3. Diffirentiate in acid-alcohol.

4. For Gills Hematoxylin (1%HCl in 70% IMS) for 3 seconds.

5. For Harris Hematoxylin – 10 seconds.

6. Rinse the slides in running tap water 4 minutes.

7. Stain the slides in 0.5% aqueous Eosin 5 minutes.

8. Rinse the slides in the tap water 20 seconds.

9. Dehydrate the slides in 70% IMS 30 sec.

10. Dehydrate the slides in 95% IMS 30 sec.

11. Place the slides in absolute IMS 2 minutes.

12. Place the slides in Xylene 1 2 minutes.

13. Place the slides in Xylene 2 - until readyto mount.

14. Mount in Di-N-Butyl Phthalate in Xylene (DPX) mountant.

## Appendix 5: Myeloperoxidase staining

*Slide preparation*

1. Prepare 3-aminopropyltriethoxysilane (APTS) treated slides:
2. Soak slides in a 2% APTS solution (diluted IMS) for 2 minutes.
3. Rinse slides twice in double distilled $H_20$.
4. Air dry the slides.
5. Cut 5 μM thickness sections from the brain samples. Place sections in the APTS-slides and dry over night at 60˚C. For each sample you need 2 sections, one to test and the other to act as a negative control (the negative control in this was section without the primary antibody).

*De-waxing and hydration of the slides:*

1. Place slide in Xylene 1 for de-waxing for 5 minutes.
2. Place slide in Xylene 2 for de-waxing for 2 minutes.
3. Place slide in Absolute IMS for hydrating for 2 minutes.
4. Place slide in 95% IMS for hydrating for 2 minutes.
5. Place slide in 70% IMS for hydrating for 2 minutes.
6. Rinse slide in running tap water for 2 minutes.

***Antigen Retrieval***

Device: Steamer

Buffer/pH value: Citrate Buffer/pH 6.0

Citrate Buffer Antigen Retrieval Protocol

Description: Formalin or other aldehyde fixation forms protein cross-links that mask the antigenic sites in tissue specimens, thereby giving weak or false negative staining for immunohistochemical detection of certain proteins. The citrate based solution is designed to break the protein cross-links, therefore unmasking the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibodies.

*Solutions and Reagents*:

Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0):

Tri-sodium citrate (dihydrate) --------- 2.94 g

Distilled water -------------------------- 1000 ml

Mix to dissolve. Adjust pH to 6.0 with 1N HCl and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4°C for longer storage.

*Procedure*:

- Pre-heat the steamer or water bath with staining dish containing sodium citrate buffer or citrate buffer until the temperature reaches 95-100°C.
- Immerse the slides in the COPLIN jar. Incubate for 10 minutes. (put the slides into the COPLIN jars in pairs).
- Turn off the steamer or water bath and remove the COPLIN jar, leave at room temperature and allow the slides to cool for 20 minutes.

- Rinse the sections twice in PBS for 2 min. Leave a few drops of PBS on the slide, it is ready to put the serum on.

Note: Microwave or pressure cooker can be used as alternative heating source to a steamer or water bath.

1. Use a liquid blocker pen to contour the tissue in the slide.
2. Non-specific-binding is blocked using 10% normal goat serum or horse normal serum in PBS for 60 minutes at room temperature.
3. Take the excess serum by gentle tapping the slide.
4. The sections are then incubated in 1:250 dilution of rabbit anti-human myeloperoxidase (DAKO Corporation) in PBS overnight at 4˚C.
5. The slides are then washed 3 times in PBS.
6. The slides are then treated with 3% $H_2O_2$ in PBS for 30 minutes.
7. Wash 3 times in PBS.
8. Incubate the sections with ImpressKit anti-Rabbit for 40 minutes .
9. Wash the slides 3 times in PBS and then wash 3 times with double distilled $H_2O$.
10. Expose the sections to stable 3,3'-diaminobenzidine tetrahychloride (DAB, Research Genetics) until a slight brown colour appears. Wash with PBS (for 2.5 mL water there is 1 drop of buffer, 2 drops of DAB, 1 drop of hydrogen peroxide).
11. Some sections are counterstained with hematoxylin (Gong, Hoff, & Keep 2000).
12. Wash the slides in running tap water.
13. Stain the slides in Harris Hematoxylin for 10 sec.
14. Wash the slides in running tap water for 2 minutes.
15. Differentiate in 1 % hydrochloric acid for 3 sec (1%HCl in 70% IMS) (3 dips).

16. Wash the slides in running tap water for 2 minutes.
17. Dehydrate the slides in 70% alcohol for 30 sec.
18. Dehydrate the slides in 95% alcohol for 30 sec.
19. Dehydrate the slides in absolute alcohol for 2 minutes.
20. Clear the slides in xylene (1 minute in xylene1 and 1 minute in xylene2).
21. Mount using DPX mounting reagent .

**Appendix 6: OX42 staining**

*Slide preparation*

1. Prepare 3-aminopropyltriethoxysilane (APTS) treated slides:
2. Soak slides in a 2% APTS solution (diluted IMS) for 2 minutes.
3. Rinse slides twice in double distilled $H_20$.
4. Air dry the slides.
5. Cut 5 μM thickness sections from the brain samples. Place sections in the APTS-slides and dry over night at 60˚C. For each sample you need 2 sections, one to test and the other to act as a negative control (the negative control in this was section without the primary antibody).

*De-waxing and hydration of the slides:*

7. Place slide in Xylene 1 for de-waxing for 5 minutes.
8. Place slide in Xylene 2 for de-waxing for 2 minutes.
9. Place slide in Absolute IMS for hydrating for 2 minutes.
10. Place slide in 95% IMS for hydrating for 2 minutes.
11. Place slide in 70% IMS for hydrating for 2 minutes.
12. Rinse slide in running tap water for 2 minutes.

***Antigen Retrieval***

Device: Steamer

Buffer/pH value: Citrate Buffer/pH 6.0

Citrate Buffer Antigen Retrieval Protocol

Description: Formalin or other aldehyde fixation forms protein cross-links that mask the antigenic sites in tissue specimens, thereby giving weak or false negative staining for immunohistochemical detection of certain proteins. The citrate based solution is designed to break the protein cross-links, therefore unmasking the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibodies.

*Solutions and Reagents*:

Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0):

Tri-sodium citrate (dihydrate) --------- 2.94 g

Distilled water -------------------------- 1000 ml

Mix to dissolve. Adjust pH to 6.0 with 1N HCl and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4°C for longer storage.

*Procedure*:

- Pre-heat the steamer or water bath with staining dish containing sodium citrate buffer or citrate buffer until the temperature reaches 95-100°C.
- Immerse the slides in the COPLIN jar. Incubate for 10 minutes. (put the slides into the COPLIN jars in pairs).
- Turn off the steamer or water bath and remove the COPLIN jar, leave at room temperature and allow the slides to cool for 20 minutes.

- Rinse the sections twice in PBS for 2 min. Leave a few drops of PBS on the slide, it is ready to put the serum on.

Note: Microwave or pressure cooker can be used as alternative heating source to a steamer or water bath.

1. Use a liquid blocker pen to contour the tissue in the slide.
2. Non-specific-binding is blocked using 10% normal goat serum or horse normal serum in PBS for 60 minutes at room temperature.
3. Take the excess serum by gentle tapping the slide.
4. The sections are then incubated in 1:100 dilution of mouse anti-rat CD11b (OX42 clone) (AbD Serotec) in PBS overnight at 4˚C.
5. The slides are then washed 3 times in PBS.
6. The slides are then treated with 3% $H_2O_2$ in PBS for 30 minutes.
7. Wash 3 times in PBS.
8. Incubate the sections with ImpressKit anti-Mouse for 40 minutes.
9. Wash the slides 3 times in PBS and then wash 3 times with double distilled $H_2O$
10. Expose the sections to stable 3,3'-diaminobenzidine tetrahychloride (DAB, Research Genetics) until a slight brown colour appears. Wash with PBS (for 2.5 mL water there is 1 drop of buffer, 2 drops of DAB, 1 drop of hydrogen peroxide).
11. *Some sections are counterstained with hematoxylin (Gong, Hoff, & Keep 2000).
12. Wash the slides in running tap water.
13. Stain the slides in Harris Hematoxylin for 10 sec.
14. Wash the slides in running tap water for 2 minutes.
15. Differentiate in 1 % hydrochloric acid for 3 sec (1%HCl in 70% IMS) (3 dips).

16. Wash the slides in running tap water for 2 minutes.
17. Dehydrate the slides in 70% alcohol for 30 sec.
18. Dehydrate the slides in 95% alcohol for 30 sec.
19. Dehydrate the slides in absolute alcohol for 2 minutes.
20. Clear the slides in xylene (1 minute in xylene1 and 1 minute in xylene2).
21. Mount using DPX mounting reagent.

## Appendix 7: Publications and participation in conferences

### PUBLICATIONS:

1. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. CORM-3, a carbon monoxide-releasing molecule, alters the inflammatory response and reduces brain damage in a rat model of haemorrhagic stroke. The paper accepted for publication on 17.07.2011, Critical Care Medicine (doi: 10.1097/CCM.0b013e31822f0d64)

2. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Relationship between leukocyte kinetics and behavioural tests changes in the inflammatory process of haemorrhagic stroke recovery. International Journal of Neuroscience, December 2010, Vol. 120, No. 12 , Pages 765-773 (doi:10.3109/00207454.2010.523129)

3. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Experimental haemorrhagic stroke: search for a better model. Visnik, Karazin’s Kharkiv National University, Kharkiv, Ukraine, 2010

**PUBLISHED ABSTRACTS:**

1. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Effects of a carbon monoxide-releasing molecule (corm-3) on haemorrhagic stroke zone dynamics. International Journal of Stroke (2010), 5 (Suppl.2); ISSN: 1747-1747

2. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Effects of corm-3 on the dynamic of white blood cells in haemorrhagic stroke. International Journal of Stroke (2010), 5 (Suppl.2); ISSN: 1748-1748

3. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Inflammatory nature of haemorrhagic stroke and dynamic of functional recovery. EFNS European Journal of Neurology 17 (Suppl. 3), 120-120, 2010

4. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Effect of CORM-3 on restoration of movement functions following haemorrhagic stroke. EFNS European Journal of Neurology 17 (Suppl. 3), 109-109, 2010

5. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Effects of a carbon monoxide-releasing molecule (CORM-3) on behavioural recovery in an experimental model of haemorrhagic stroke. Proceedings of the 15$^{th}$ World Congress of Psychophysiology (I.O.P.). Budapest,

Hungary, September 1-4, 2010. International Journal of Psychophysiology 77 (2010) 250-251

**MEETINGS PRESENTED AT:**

1. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Effects of CORM-3 on dynamics of white blood cells in haemorrhagic stroke. 7th World Stroke Congress, Seoul, South Korea. October 2010.

2. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Effects of a carbon monoxide-releasing molecule (CORM-3) on haemorrhagic stroke zone dynamics. 7th World Stroke Congress, Seoul, South Korea. October 2010.

3. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Effects of CORM-3 on restoration of movement functions following haemorrhagic stroke. 14th Congress of the European Federation of Neurological Societies, Geneva, Switzerland. September 2010

4. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Inflammatory nature of haemorrhagic stroke and dynamic of functions recovery. 14th Congress of the European Federation of Neurological Societies, Geneva, Switzerland. September 2010

5. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green and Roberto Motterlini. Effects of a carbon monoxide-releasing molecule (CORM-3) on behavioural recovery in an experimental model of haemorrhagic stroke. 15$^{th}$ World Congress of Psychophysiology, Budapest, Hungary, September 2010

6. Andriy Yabluchanskiy, Philip Sawle, Shervanthi Homer-Vanniasinkam, Colin J. Green, Barry Fuller and Roberto Motterlini. Effect of CORM-3 on inflammation induced by haemorrhagic stroke. Stroke@UCL - Scientific Meeting, UCL, London, UK, October 2009

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