

MINOCYCLINE:
A POTENTIAL DRUG THERAPY FOLLOWING
INTRACEREBRAL HEMORRHAGE?

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ALEKSANDRA SZYMANSKA



MINOCYCLINE: A POTENTIAL DRUG THERAPY FOLLOWING
INTRACEREBRAL HEMORRHAGE?

by

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ABSTRACT

Intracerebral hemorrhage (ICH) is a devastating condition currently lacking a defined line of treatment. An inflammatory response ensues following its onset, and it has been suggested that it contributes to secondary damage following ICH, making inflammation a potential therapeutic target. Minocycline (MC), a commonly used antibiotic, which also has anti-inflammatory properties, has shown promise as a histological protectant in a number of animal stroke models. However, evidence for its ability to meet clinically relevant end-points, such as lessening of functional deficits with a wide therapeutic time window is scarce. Thus the objective of this study was to examine the effects of MC on short-term histopathological changes and long-term functional outcomes in a collagenase induced ICH model in rats. Drug treatment was initiated 3 h following the onset of stroke via intraperitoneal injection.

In accordance with other studies, MC suppressed microglial/macrophage reaction (marker of the inflammatory response) in the perilesion region at 5 days (given for 5 days). However, it did not lead to histological protection as assessed by the size of the infarct volumes at 5 or 28 days. Quantitative sensori-motor tests showed that MC, given for 5 days or 14 days, offered no functional benefit up to 28 days following stroke. A number of factors could account for the lack of therapeutic effect, such as the severity of injury produced and the mode of drug delivery. However, the data suggest that minocycline may have a short therapeutic window and thus limited clinical application after ICH.

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LIST OF ABBREVIATIONS AND SYMBOLS

BBB – blood-brain barrier

BDNF – brain-derived neurotrophic factor

CNS – central nervous system

COX-2 – cyclooxygenase 2

CSF – cerebro-spinal fluid

ECM – extra-cellular matrix

H&E – hematoxylin and eosin

ICH – intracerebral hemorrhage

ip - intraperitoneal

IL-1 β – interleukin 1 β

IL-6 – interleukin 6

MC – minocycline

MC5 – animals receiving minocycline for 5 days

MC14 – animals receiving minocycline for 14 days

MMP – matrix metalloproteinase

NGF – nerve growth factor

NO – nitric oxide

NOS – nitric oxide synthase

p38 MAPK – p38 mitogen-activated protein kinase

PBS – phosphate buffer saline solution

PFA – paraformaldehyde

PGE-2 – prostaglandin 2

PNS – peripheral nervous system

SCI – spinal cord injury

TGF- β – transforming growth factor- β

TNF- α – tumor necrosis factor- α

V – vehicle

V5 – animals receiving vehicle for 5 days

V14 – animals receiving vehicle for 14 days

INTRODUCTION

CLINICAL ASPECTS OF THE INTRACEREBRAL HEMORRHAGE

Intracerebral hemorrhage (ICH) is the most devastating type of stroke. The spontaneous bleeding into the brain parenchyma is usually caused by hypertension-induced blood vessel rupture or aneurysm rupture. Although it accounts for only 10-20% of all strokes occurring in North America, it is associated with the greatest number of deaths. The 30-day mortality rate is 34-50%. Disability is a frequent outcome (Butcher and Laidlaw 2003).

Neurological deterioration usually occurs within the first 24 to 48 h following ICH. Increased mortality and poor outcome are tightly linked to the size of the hematoma and its location. In the past it was thought that hematoma evolution was a monophasic event. It was postulated that bleeding ceased soon after its initial eruption via coagulation and compression by the surrounding tissue. Consequently the early neurological deterioration was attributed to the space-occupying effects (mass effect) of the blood and to the edema. However, it has been shown recently that, in many cases, the hematoma continues to expand up to 24 h following stroke (Mayer 2003). The reasons for the hematoma growth are yet to be clarified. High blood pressure may play a role in prolonged bleeding/re-bleeding from the single site of the original rupture. Secondary bleeding from the vasculature in the surrounding areas may also contribute as the perihematoma tissue suffers damage from mechanic and ischemic injury (resulting from the mass effect of the hematoma), and inflammatory stress following the primary event (Mayer 2003).

The hematoma is a potent instigator of secondary injury following ICH, which further contributes to neurological deterioration. The clot releases osmotically active substances leading to edema. Edema has its greatest increase during the first 24 h (Gebel, Jauch, Brott, Khoury, Sauerbeck, Salisbury, Spilker, Tomsick, Duldner and Broderick 2002). The edema and the mass effect of the hematoma lead to an increase in the intracranial pressure and often result in brain herniation; they may also lead to mechanical and ischemic injury affecting the tissue in the perihematoma region (Mayer 2003). Some substances contained in the clot lead to the breakdown of the blood brain barrier (BBB) further exacerbating edema. Still other clot components (e.g. thrombin) are directly toxic to the surrounding neurons (Lee, Kawai, Kim, Sagher and Hoff 1997).

In the face of substantial primary and delayed cell death, it is not surprising that a robust inflammatory response follows ICH. Histological sections are characterized by the presence of macrophages and neutrophils in the region surrounding the hematoma (Qureshi, Tuhim, Broderick, Batjer, Hondo and Hanley 2001). Inflammation associated with ICH is believed to contribute to secondary injury through exacerbation of edema, weakening of the BBB and cytotoxicity (Mayer 2003). Indeed, in one study the levels of some matrix metalloproteinases (MMPs), which are involved in the breakdown of the extracellular matrix (ECM) and are associated with inflammation and edema, were positively correlated to neurological deterioration in ICH patients (Abilleira, Montaner, Molina, Monasterio, Castillo and Alvarez-Sabin 2003). Another human ICH study showed that the levels of the pro-inflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were correlated with edema (Castillo, Davalos, Alvarez-Sabin, Pumar, Leira, Silva, Montaner and Kase 2002).

As devastating as the processes involved in the pathophysiology of ICH are, what makes this type of stroke even more overwhelming is the fact that there are no effective treatments available. Medical intervention is usually limited to the use of antihypertensive drugs or osmotherapy. In some instances surgical removal of the hematoma is indicated. The role of these treatments in ICH remains controversial (Qureshi, Tuhim et al. 2001). Thus there is a great need to investigate new, viable therapies.

One of the factors limiting the efforts to develop treatments for ICH is the fact that ICH has not been the focus of stroke research to the extent that it has been dubbed the “forgotten stroke” (Castillo et al. 2002). Clinical and experimental studies in ICH have been pursued to a lesser extent. The general view was that ICH was a monophasic phenomenon characterized by irreversible, necrotic cell death. In light of this, tissue seemed unsalvageable. Recent studies, however, point to the prominence of apoptotic-like cell death in the perihematomal regions giving a window of opportunity to counteract the various delayed and detrimental processes (Felberg, Grotta, Shirzadi, Strong, Narayana, Hill-Felberg and Aronowski 2002; Qureshi, Suri, Ostow, Kim, Ali, Shatla, Guterman and Hopkins 2003).

PATHOPHYSIOLOGY OF INFLAMMATION

Inflammation is a natural response to injury and infection. It eliminates foreign substances, toxins, dead cells and leads to repair of damaged tissues (Wyss-Coray and Mucke 2002). However, inflammation may cause collateral damage following the initial insult (Barone and Feuerstein 1999). This latter aspect of inflammation is tolerable in

tissues with high cell turnover, such as skin. It may not be as innocuous in the central nervous system (CNS) where each neuron has a defined place and role, and is part of an intricate network.

Interestingly, the CNS seems to have evolved in order to protect its delicate functions from inflammatory devastation. It has done so by creating an anatomical and physiological shield from the immune system. This phenomenon is called its immune privilege. As part of the immune privilege, the BBB and blood-cerebrospinal fluid (CSF) barrier prevent entrance of plasma proteins and immune cells. Other important components are the lack of conventional lymphatic vessels and dendritic (antigen-presenting) cells (Aloisi 2001). The existence of this privilege supports the notion that there is a need to protect the CNS from the damaging consequences of a full-fledged immune response.

INFLAMMATION FOLLOWING ICH IN RODENT MODELS

Cellular and Molecular Mediators of Inflammation

In the initial stages of an acute inflammatory response the major goal is to destroy and clear the already damaged cells and any foreign substances. Thus it is not surprising that the main mediators of inflammation: microglia/macrophages, neutrophils and astrocytes are capable of releasing toxins and of phagocytosis (Weiss 1989; Aloisi 1999). There is a lot of cross-talk among the cells (i.e. the agents released potentiate each other's actions) (Aschner 1998; Aloisi 1999). The reason why this part of the inflammatory response may become problematic is through its lack of specificity. Most of the

processes mentioned are part of the intrinsic immune response and do not distinguish between injured and intact cells (Aloisi 1999). This tendency to act on (and destroy) both the damaged tissue and the healthy neurons and glia is termed the “bystander effect” (Merrill, Ignarro, Sherman, Melinek and Lane 1993; Aloisi 1999; Van Beek, Elward and Gasque 2003).

The two most commonly used models of rodent ICH are injection of autologous whole blood and collagenase digestion of blood vessels. Both elicit a comparable pattern of inflammatory response (Xue and Del Bigio 2003). And although the inflammatory response in rodent models of ICH has not been described as well as in focal ischemia, they seem to have similar temporal profiles, and they share cellular and molecular mediators (Barone and Feuerstein 1999).

The inflammatory response begins within a few hours after ICH with activation of microglia, the resident macrophages of the CNS. Under normal conditions, these cells are in a state of suppressed immunophenotype. The functions that microglia perform in this instance are still not fully understood, but they seem to contribute to tissue homeostasis as these cells are highly sensitive to the changes in their environment, and there is evidence to show that they are involved in neuroplastic changes (Kreutzberg 1996). However, within hours following a brain injury, such as ICH, microglia start to express various receptors, proliferate and become activated (Gong, Hoff and Keep 2000; Xue and Del Bigio 2003). They seem to be the first cells that respond to CNS injury.

In most pathological settings microglia are aided by systemic macrophages that start infiltrating the hematoma/perihematoma region 2 to 3 days following ICH (Gong et al. 2000; Xue and Del Bigio 2003). Once activated, microglia and macrophages share

many phenotypical markers and have similar functions (Stoll and Jander 1999). The functions include phagocytotic activity, synthesis of cytokines, chemokines and mediators with cytotoxic and cytolytic activity (Aloisi 1999). The substances released include: the inducible form of nitric oxide synthase (NOS), which gives rise to the highly reactive radical nitric oxide (NO), TNF- α and interleukin 1 β (IL-1 β), inflammatory cytokines shown to have toxic properties and MMPs, a family of proteinases involved in the breakdown of various components of the ECM (Kreutzberg 1996; Rosenberg and Navratil 1997; Aloisi 1999). MMPs may be most pertinent to ICH as they are strongly implicated in the breakdown of the BBB and thus to edema formation, which is a concern following ICH. They also may produce bystander damage by severing the cell-cell and cell-ECM communication necessary for normal functioning and survival (Rosenberg 2002).

Neutrophils may be another source of collateral damage following ICH. They start to infiltrate into and around the hematoma within the first 24 h post ICH (Gong et al. 2000; Xue and Del Bigio 2003). When they become activated by specific pro-inflammatory signals they turn into phagocytic cells; they generate and release highly reactive oxygen species (free radicals) and various microbicidal proteins (Weiss 1989). In addition, neutrophils may accumulate in the vessels obstructing blood flow creating ischemic areas (Stoll, Jander and Schroeter 2002).

Also accompanying the inflammatory response is the activation of astrocytes. They become activated during the first week following ICH (Del Bigio, Yan, Buist and Peeling 1996; Kowianski, Karwacki, Dziewiatkowski, Domaradzka-Pytel, Ludkiewicz, Wojcik, Litwinowicz, Narkiewicz and Morys 2003). The influence of these cells has

been thought of as benign in comparison to microglia/macrophages and neutrophils. However, as the resident cells of the CNS they are similar to microglia in the way that they serve as micro-sensors of the injured environment (Little and O'Callaghan 2001). Despite their reputation for providing trophic support to neurons, recent work has demonstrated their ability to produce various pro-inflammatory cytokines, IL-1 β , TNF- α , and the highly cytolytic proteins, perforins (Aschner 1998; Aloisi 1999). Furthermore, astrocytes are capable of phagocytosis (Aloisi 1999).

In summary, many of the substances released by microglia/macrophages, neutrophils, and astrocytes are toxic, as the goal is to destroy and clear the already damaged cells and foreign substances in and around the injury. The direct effect of these diffusible cytotoxins on normal cells, their contribution to the disruption of BBB, and the resulting edema may play an important role in the secondary damage following ICH (Barone and Feuerstein 1999).

In confirmation of the injurious role of inflammation following ICH, a number of studies have shown that suppressing inflammation and its mediators following ICH are beneficial. Pre-treatment of rats with microglia/macrophage inhibitory factor reduced the infarct volume following ICH in rat (Wang, Rogove, Tsirka and Tsirka 2003). In other studies, specific inhibition of TNF- α , MMPs or IL-1 β shortly after the insult was of benefit (Rosenberg and Navratil 1997; Masada, Hua, Xi, Yang, Hoff and Keep 2001; Mayne, Fotheringham, Yan, Power, Del Bigio, Peeling and Geiger 2001; Mayne, Ni, Yan, Xue, Johnston, Del Bigio, Peeling and Power 2001; Power, Henry, Del Bigio, Larsen, Corbett, Imai, Yong and Peeling 2003). Thus blocking the initial, detrimental inflammatory processes following ICH seems a promising target.

All the cellular responses mentioned above peak usually within the first week following ICH. The neutrophil response is short-lived, disappearing within a couple of days. The microglia/macrophage reaction declines after the first week (Xue and Del Bigio 2003). These most turbulent cellular/molecular events in ICH rodent models are associated with delayed cell death and with edema (Yang, Betz, Chenevert, Brunberg and Hoff 1994). However, edema begins to resolve at 5 days and BBB begins to be restored. The hematoma starts to be resolved soon after that (Yang et al. 1994; Del Bigio et al. 1996). This is when the inflammatory response enters a new phase: the emphasis shifts to tissue repair and remodeling (Barone and Feuerstein 1999).

Healing is the main objective of inflammation, especially in an acute situation. Accordingly, some of the inflammatory mediators, microglia/macrophages and astrocytes, whose reactions persist for weeks following ICH, may be involved in the processes that restore tissue homeostasis and promote recovery (Xue and Del Bigio 2003). These processes and the mediators responsible have not been studied and described in detail; however, there is *in vitro* and *in vivo* evidence to show that microglia/macrophages and astrocytes generate various growth factors that may be involved in promotion of neuronal growth and synaptogenesis following a CNS injury. Among them are: brain-derived growth factor (BDNF), nerve growth factor (NGF), and transforming growth factor- β (TGF- β) (Norenberg 1994; Aschner 1998; Nakajima, Honda, Tohyama, Imai, Kohsaka and Kurihara 2001; Streit 2002).

Inflammation as a “Double-Edged Sword”

There is very limited spontaneous recovery after CNS injury as compared to the peripheral nervous system (PNS). This difference has been the topic of many studies, which point to the CNS environment as relatively inhospitable to axonal re-growth due to the presence of various axonal-growth inhibiting factors (Frisen 1997). However, there is also a suggestion that the poor recovery in the CNS is related to the inability of the injured tissue to communicate effectively with the cells of the immune system required for tissue formation/regeneration (Zeev-Brann, Lazarov-Spiegler, Brenner and Schwartz 1998). For example, the PNS has a much more robust macrophage response, which may promote axonal re-growth (Zeev-Brann et al. 1998). Further, *in vitro* and *in vivo* evidence supports the hypothesis that the limited axonal growth and thus recovery in the injured CNS are a result of insufficient microglia/macrophage activation (David, Bouchard, Tsatas and Giftochristos 1990; Rapalino, Lazarovo-Spiegler, Agranov, Velan, Yoles, Fraidakis, Solomon, Geptein, Katz, Belkin, Hadani and Schwartz 1998; Zeev-Brann et al. 1998; Yin, Cui, Li, Irwin, Fisher, Harvey and Benowitz 2003). This implicates the immune system and inflammation as being critical components of recovery after acute CNS injury. Indeed, there are strong opponents of the idea that inflammation and its accompanying gliosis are deleterious processes in the CNS. Instead they argue it is indispensable for tissue homeostasis, repair and regeneration (Streit 2002).

Due to the dichotomy of inflammation after brain injury – the destructive and regenerative forces – it has been dubbed a “double-edged sword” (Barone and Feuerstein 1999). The only consensus which exists right now is that when it comes to inflammation

and its mediators the positive or negative outcome depends on the timing and the circumstances. The challenge is to find ways of interfering with the detrimental effects of inflammation while promoting its beneficial actions.

ANTI-INFLAMMATORY TREATMENTS IN CNS INJURY

Corticosteroid Therapy

Since 1950's, inflammation has been recognized as a major culprit in exacerbating the neurological deterioration following cerebrovascular accidents by effects of cerebral edema, increased intracranial pressure and a disturbed BBB (Russek, Russek and Zohman 1955). Since then, only a handful of clinical trials attempted the use of steroids for the treatment of ICH or cerebral ischemia; they saw either no benefit of the therapy or worsening of condition due to the drug's side effects (e.g. vulnerability to opportunistic infections) (Poungvarin 2004).

Attributing the previous failure of clinical trials to methodological flaws, some argue that steroid therapy for ICH has been abandoned prematurely. For example, the timing of therapy was problematic. That is, the starting time of treatment varied beginning as late as 5 days following the onset of ICH (Rubinstein 1965; Tellez and Bauer 1973; Poungvarin, Bhoopat, Viriyavejakul, Rodprasert, Buranasiri, Sukondhabhant, Hensley and Strom 1987; Desai and Prasad 1998). Considering the fact that a relatively narrow therapeutic window (8h) has already been established for the efficacy of steroid therapy following spinal cord injury (SCI), one would expect the situation to be similar in ICH (Young, Kume-Kick and Constantini 1994).

In favor of corticosteroid therapy are a few animal studies that have shown its benefit in ischemic stroke (Norris 2004). However, it is also possible that corticosteroids are not the optimal choice for neuroprotective therapy following acute CNS injury. Corticosteroids have a narrow scope of actions following injury. They seem to act mainly via inhibition of the production of prostaglandins, leukotrienes and platelet activating factor, which normally contribute to increased vascular permeability (Bennett and Brown 2003). Clinical and basic research experts studying cerebrovascular conditions are slowly realizing the limitations of drugs with overly specific actions. Stroke is a heterogenous condition. It is unlikely that targeting a single process would be beneficial in any treatment plan. Rather, there is a need for multimodal drugs.

Minocycline in CNS injury

Recently, minocycline (MC) – a semi-synthetic tetracycline – has been shown to provide benefit in several animal brain injury models: Parkinson's disease (He, Appel and Le 2001; Wu, Jackso-Lewis, Vila, Tieu, Teismann, Vadseth, Choi, Isciropoulos and Przedborski 2002), Huntington's disease (Chen, Ona, Li, Ferrante, Fink, Szhu, Bian, Guo, Farrell, Hersch, Hobbs, Vonsattel, Cha and Friedlander 2000), traumatic head injury (Sanchez-Meji, Ona, Li and Friedlander 2001), amyotrophic lateral sclerosis (Zhu, Stavrovskaya, Drozda, Kim, Ona, Li, Sarang, Liu, Hartley, Wu, Gullans, Ferrante, Przedborski and Kristal 2002), multiple sclerosis (Brundula, Rewcastle, Metz, Bernard and Yong 2002; Popovic, Schubart, Goetz, Zhang, Linington and Duncan 2002), SCI (Lee, Yune, Kim, Park, Lee, Kim, Oh, Markelonis and Oh 2003; Wells, Hurlbert, Gehlings and Yong 2003; Stirling, Khodarahmi, Liu, McPhail, McBride, Steeves, Ramer

and Tetzlaff 2004; Teng, Choi, Onario, Zhu, Desilets, Lan, Woodard, Snyder, Eichler and Friedlander 2004), ischemic stroke (Yrjanheikki, Keinanen, Pellikka, Hokfelt and Koistinaho 1998; Yrjanheikki, Tikka, Keinanen, Goldsteins, Chan and Koistinaho 1999), and ICH (Power et al. 2003). Tetracyclines are antibiotics. MC has been shown to have both anti-microbial and anti-inflammatory actions, which seem to be independent of each other. In the CNS, the latter have been mainly attributed to the drug's ability to inhibit microglial activation (Tikka, Feibich, Goldsteins, Keinanen and Koistinaho 2001; Tikka and Koistinaho 2001).

MC penetrates well into the brain and the CSF. It can be administered orally and is well tolerated by patients (Macdonald, Kelly, Ilen, Noble and Kanegis 1973; Thomas, Le and Jankovic 2003). These characteristics make MC an appealing candidate in clinical trials for ICH.

MC has been shown to be superior to corticosteroid therapy in a mouse model of SCI (Wells et al. 2003). This could be due to the fact that MC is a multimodal drug affecting various processes involved in neurodegeneration. It has been shown to influence inflammation by suppressing microglial/macrophage activation, decreasing the genes for IL-1 β , NOS, cyclooxygenase-2 (COX-2), and prostaglandin E-2 (PGE-2) (Yrjanheikki et al. 1998; Yrjanheikki et al. 1999; Chen et al. 2000; Sanchez-Meji et al. 2001); it suppresses the production and activity of MMPs (Brundula et al. 2002; Power et al. 2003). The drug also has anti-apoptotic properties. It decreases the release of cytochrome c from the mitochondria, attenuates the levels of caspase-3, and it suppresses caspase-independent death pathways (Chen et al. 2000; Zhu et al. 2002; Wang, Zhu, Drozda, Zhang, Stavrovskaya, Cattaneo, Ferrane, Kristal and Friedlander 2003). In

addition, MC reduces free radicals by attenuating the production of NO, and it lessens glutamate-mediated excitotoxic neuronal death seemingly by inhibiting p38 mitogen-activated protein kinase (p38 MAPK) (Amin, Attur, Thakker, Patel, Vyas, Patel, Patel and Abramson 1996; Yrjanheikki et al. 1998; Tikka et al. 2001; Tikka and Koistinaho 2001).

Minocycline in Stroke Models

Previous studies on the role of MC in global and focal ischemic stroke models reported MC neuroprotective effects when the drug was delivered acutely (for 3 to 6 days) (Yrjanheikki et al. 1998; Yrjanheikki et al. 1999). Specifically, the claims were based on short survival times (3 to 6 days), where neuroprotection could not be distinguished from a mere delay of cell death. Infarct volumes or hippocampal cell-counts (in focal and global ischemia, respectively) were the studies' only end-points. Thus the conclusions have been drawn from minimal experimental evidence and poorly designed experiments ominously reminiscent of neuroprotection studies of the past (Hunter, Green and Cross 1995; Corbett and Nurse 1998). The relationship between histopathological changes and clinical outcomes is unclear, and therefore long-term functional assessment is necessary to confirm that MC offers benefits of clinical relevance.

Power et al. (2003) studied the effects of MC in a rat model of collagenase-induced ICH using a survival time of 28 days, and functional recovery was assessed using a combined neurobehavioral score. In that study, MC was delivered for 14 days starting at 1 h post-stroke. It decreased neuronal death at 1 day after stroke, but there was

no difference in infarct volumes at later time points. Minocycline treatment suppressed microglial activation at 4 and 7 days. It also attenuated the levels of the MMP-12 protein at 7 days. Finally, the animals treated with MC achieved a better behavioral score than untreated subjects at 7, 14 and 28 days following stroke.

There is only one study that significantly delays the commencement of MC treatment following injury in order to test its therapeutic window. In that instance, neuroprotection was observed even when the drug delivery was started at 4 h after the onset of focal ischemia (Yrjanheikki et al. 1999). Although the study was based only on short-term histological assessment, it does indicate that MC may have a wide window of opportunity. An important shortcoming of the other MC studies is the fact that the drug was delivered either before or shortly after the onset of stroke (0.5 to 1 h). This is not a clinically relevant end-point since patients are not usually available for medical intervention until hours or days following the onset of stroke (Broderick, Adams, Barsan, Feinberg, Feldmann, Grotta, Kase, Krieger, Mayberg, Tilley, Zabramski and Zuccarello 1999). Inflammation that ensues after acute brain injury has been suggested as a good therapeutic target because it is a prolonged process (the initial, detrimental phase is believed to last up to 7 days post-stroke). Thus theoretically it offers a wide therapeutic window, which should be tested in an experimental setting.

Minocycline as a Potential Therapy Following ICH – Study Hypotheses

The study by Power et al. (2003) is the only one thus far that examined the effects of MC treatment following ICH. It showed that MC offers functional protection when given for a prolonged time (14 days). However, the drug regimen employed may be

problematic. As the detrimental phase of inflammation is believed to occur only within the first week following ICH, it may have encroached on the beneficial phase of inflammation, which contributes to healing, tissue repair and recovery. I hypothesized that MC might be of greater benefit if it were delivered more acutely (5 days) during the initial phase of inflammation, rather than for 14 days.

Another weak point of the Power et al. (2003) study is the fact that it used a relatively crude and subjective neurobehavioral score to test the animals' behavior following ICH. In my studies I decided to use a battery of quantitative behavioral measures to test general as well as skilled sensory-motor behaviors in order to assess the functional benefits of MC. The tests included: staircase reaching (skilled paw reaching to retrieve food pellets), asymmetrical forelimb use (assessment of reliance on a given paw for postural support) and ladder-walking (fore- and hind-limb coordination while crossing a horizontal ladder).

In the Power et al. (2003) study the drug delivery was started at 1 h following ICH and thus did not indicate much about the breadth of the therapeutic window for MC. I attempted to challenge the therapeutic ability of MC by delaying the drug delivery 3 h after stroke. In addition, because the benefits of MC after stroke have been associated with its ability to suppress the inflammatory response by inhibiting microglial/macrophage activation (Yrjanheikki et al. 1998; Yrjanheikki et al. 1999; Power et al. 2003), I used histochemical methods to investigate MC influence on microglia and macrophages.

Therefore, this thesis consists of two main parts: long-term and short-term study. The animals belonging to each of the groups were allowed to survive for 5 or 28 days following ICH, respectively. The goal of the short-term, histological study was to

examine MC effects on microglial/macrophage activation as well as its histologically protective properties. The long-term study was meant to define the effects of MC on the protection of function following ICH using a variety of reliable and quantifiable sensory-motor tests, and to test the hypothesis that shorter exposure to MC (5 days) after ICH is more beneficial than prolonged use of the drug (14 days).

METHODS

ANIMALS

Young adult, male Long-Evans rats were used (280–360 g) supplied by Charles-River. The rationale behind choosing this strain was that they are more out-bred (than the Sprague-Dawley strain, for example) and they have good vision. These factors, we hypothesized, would increase their performance on the behavioral tasks employed in the study. The animals had free access to food and water, except during periods of behavioral training and testing, when their food intake was restricted to approximately 12 g/day. They were housed in groups of 2 to 3 on a reverse 12/12-h light/dark cycle. Behavioral manipulations were performed during the dark cycle. All animals were treated in accordance to the guidelines of the Canadian Council on Animal Care.

INTRACEREBRAL HEMORRHAGE MODEL

Rats were anesthetized with a mixture of 2-3% isoflurane in 30% O₂ and 70% N₂O and placed in a stereotaxic frame. A 26-gauge needle was inserted through a burr hole made in the skull and lowered into the dorso-lateral striatum (coordinates relative to

bregma: 0.7 mm anterior, 3.8 mm lateral, 6.0 mm deep), where it was left for 3 min. ICH was induced by injecting 1.4 μ l sterile saline containing 0.14 U of collagenase (type IV, Sigma, St. Louis, MO) over a 5 min period. The needle was left in place for another 5 min before being slowly removed. Collagenase aliquots of desired concentration were stored at -20° C and thawed immediately before use; the left over after a single injection was discarded.

STUDY DESIGN

To control for selection bias in group designation, the animals were assigned to the drug or vehicle group before surgery on purely random basis. The long-term study animals, however, were first matched for performance on the staircase task.

Short-Term Study Groups

The aim of the short-term study was to examine MC effects on the inflammatory response and histological neuroprotection after ICH. All animals underwent ICH. Starting at 3 h after collagenase injection, one group received either 5 days of MC treatment (MC, n = 11) or 5-day vehicle (sterile water) treatment (V, n = 10). All animals were sacrificed 5 days following ICH.

Long-Term Study Groups

The goal of the long-term study was to assess MC effect on the preservation and/or recovery of function following ICH. All animals were subjected to ICH and then, starting at 3 h post-ICH, they either underwent 5 day MC treatment (MC5, n = 13), 14

day MC treatment (MC14, n = 12), 5 day vehicle treatment (V5, n = 7), or 14 day vehicle treatment (V14, n = 6). The animals were sacrificed at 28 days following ICH.

MINOCYCLINE TREATMENT

MC (Sigma, St. Louis, MO) was stirred in sterile water (15 mg/ml) to produce a clear, yellow solution. Due to some concerns over the drug's stability, MC was prepared fresh every 24 h. It was protected from light and stored at 4° C.

The MC regimen used here emulates those used in previous stroke studies (Yrjanheikki et al. 1998; Yrjanheikki et al. 1999; Power et al. 2003). The animals that were treated with MC for 5 days received an intraperitoneal (ip) injection of 45 mg/kg of MC (starting at 3 h post-ICH) followed by another such injection 12 h later. After that, the MC dose was decreased to 22.5 mg/kg and administered twice daily for another 4 days. In the MC14 group, the animals received identical treatment to MC5 animals for the first 5 days; however, they continued to receive the drug in 22.5 mg/kg dose twice daily for another 2 days after which they received the same dose only once a day for another 7 days. The vehicle groups (V5 and V14) received equivalent volumes of sterile water at equivalent time points. The MC injection causes peritoneal discomfort, therefore both MC and V treated animals were lightly anesthetized with 3% isoflurane in 30% O₂ and 70% N₂O before each injection.

BEHAVIORAL ASSESSMENT

The rats were tested on 3 behavioral tests: staircase reaching, asymmetrical limb use (cylinder test) and ladder-walking to assess their functional deficits over time. The animals were either trained on a given task, or their basal performance was noted before surgery. They were tested starting on the first week post-surgery up to 4 weeks.

Staircase Reaching Test

The staircase reaching test estimates right and left forelimb reaching ability (Montoya, Campbell-Hope, Pemberton and Dunnett 1991). A food-deprived animal (12 g per day) was put on a platform and allowed to retrieve food pellets from a staircase situated on both sides. A staircase consisted of 7 steps where each step contained 3 food pellets. Thus an animal could retrieve a maximum of 21 pellets on each side. The task requires fine digit ability and arm extension, especially with the lower steps of the staircase. Once a pellet is dropped, it is irretrievable. Thus the number of pellets eaten on each side (out of 21) provides an indication of forelimb reaching ability.

The animals were pre-trained twice a day until they reached the performance specified by a set of criteria: 1) trained for a minimum of 16 trials (8 days); 2) the average of the last 8 trials was to be ≥ 12 pellet; 3) the standard deviation of the last 8 trials was to be ± 2 pellets; 4) if the left paw did not meet the criteria but the right one did then the left hemisphere was lesioned; 5) the left hemisphere was lesioned when an animal showed a strong preference for the right paw (defined as follows: average of last 8 trials for left paw was to be ≤ 15 pellets and for the right paw 4 pellets more, or the difference between

the left and right averages of last 8 trials was ≥ 5 pellets); 6) if an animal was showing a potential for improvement (i.e. presence of a high outlier) then training was continued; 7) animals that did not meet the criteria and showed no potential for improvement after 14 days were excluded.

The average of the last 4 trials (2 days) was considered the animals' pre-stroke ability. The animals were then tested for 2 days starting at 7 days post-stroke and then again at 2 and 4 weeks. At each time point, the average of the 4 trials was used to establish the animals' ability. Post-stroke performance was expressed in the following terms: (post-stroke ability/ pre-stroke ability) x 100%.

This test does not provide detailed information on reaching ability to the same extent as the Whishaw reaching task (e.g. precision of retrieval, compensation vs. recovery) (Whishaw, O'Connor and Dunnett 1986). However, it is much less laborious than the Whishaw task, and it sensitively measures the extent of sensori-motor deficit of the reaching paw as compared to pre-training and as compared to the uninjured limb.

Asymmetrical Forelimb Use

In this test, the extent to which an animal relies on a given forelimb for weight-bearing postural support is assessed (Jones and Schallert 1994). The test employs a transparent 25 cm diameter cylinder. A food-deprived animal was placed in the cylinder, and its behavior was video-recorded. The number of bilateral and single wall contacts used for postural support during a 3 min period (or until 20 contacts were made) were counted from the tape by an observed blinded to the rats' treatment. Reliance on the ipsilateral paw was assessed using the following formula: (ipsilateral contacts/(ipsilateral

+ contralateral + bilateral contacts)) x 100%. The animals were tested before surgery to determine the baseline scores, and then the test was repeated at 1, 2 and 4 weeks after ICH.

Ladder-Rung Walking Test

This test was adapted from a previous study (Metz and Whishaw 2002). It examines an animal's ability to cross a 1 m horizontal ladder whose rungs are irregularly spaced (from 1 cm to 5 cm apart); it requires coordinated, skilled limb-paw placement. The spacing between the rungs of the ladder was changed for each testing day to prevent the animal from learning rung location. Rats were acquainted with the task for 3 days before surgery by crossing the ladder whose rungs were regularly spaced 2 cm apart (2 runs across for the first 2 days and then 4 runs across on the 3rd day), and then they were tested at 1, 2 and 4 weeks after ICH (one training run plus 3 test runs at each time point). The food-deprived animals were videotaped as they walked across the ladder. The foot faults: deep slips/misses, minor slips, placement errors (correction, replacement) on the contralateral and ipsilateral side were counted from a video-tape by an observer blinded to the rats' identities. The deficits were represented as a sum of all foot faults committed.

HISTOLOGY

Animals were sacrificed either at 5 or 28 days following stroke. They were transcardially perfused using heparinized saline for 2 min followed by 4 % paraformaldehyde (PFA) for 6 min. The brains were post-fixed in 4 % PFA for 24 h, after which they were placed in a 20 % sucrose-phosphate buffer solution for 4 days.

Frozen brain tissue was cut on a cryostat into 40 μm sections and stained with hematoxylin and eosin (H&E) to assess the infarct volumes. For the short-term survival animals, a set of free-floating sections was taken. The tissue was stored in cryoprotectant at -20°C until it was used for histochemistry. Peroxidase labeled isolectin B₄ (Sigma; L5391, St. Louis, MO) was used to assess microglial/macrophage activation. This lectin has a high affinity for terminal-D-galactosyl residues. Although it stains various structures in the PNS and CNS, no other glial cells except for microglia exhibit binding (Streit 1990). The protocol used has been described before (Streit 1990). Briefly, endogenous peroxidase activity was inhibited with hydrogen peroxide. Sections were then washed in 0.1 M phosphate buffer saline solution (PBS) containing 0.1% Triton X-100 and cations added as salts (0.1 mM CaCl₂, 0.1 mM MgCl₂). They were then incubated in peroxidase labeled isolectin B₄ (20 mg/ml in 0.1 M PBS containing 0.1% Triton X-100 and cations) for 2 h at room temperature. Lectin binding sites were localized with 3,3'-diaminobenzidine-H₂O₂ as peroxidase substrate. Every 16th section was mounted on slides. Negative controls were done whereby the protocol was followed except that the tissue was incubated without the lectin.

INFARCT VOLUME MEASUREMENTS

Infarct volume estimations were performed by an observer, who was blind to the identity of the experimental groups. Measurements were taken for all animals from the H&E stained sections using the NIH Image Software (Bethesda, MD). Starting randomly (a coin was thrown to decide whether to commence analysis with the section where the lesion first became visible or with the following section), every 16th section was analyzed

until the posterior end of the lesion was reached. The cross-sectional area of the infarct was calculated using the following formula: area of intact tissue in the contralateral hemisphere – area of intact tissue in the ipsilateral hemisphere. Infarct volume per section was calculated by multiplying the infarct area by the section thickness and the distance between sections. Total infarct volume was calculated by summing the volumes of all sections considered for a given animal.

ASSESSMENT OF MICROGLIAL/MACROPHAGE ACTIVATION

Microglial/macrophage activation was assessed at 5 days post-ICH based on isolectin B₄ histochemistry using a semi-quantitative method. Rating scores were designed to estimate the striatal microglia/macrophage response. Cortical response was not looked at as not all of the sections considered had cortical damage. Rating was performed by a person who was blind to the experimental groups. For each animal, two to three sections were selected in which there was sub-total injury to the striatum; this allowed an assessment of the microglial/macrophage response in the peri-hematoma region of striatum. The final score was expressed as a mean rating of those sections. Striatal response was rated using a 4 point scale, the score of 3 marking the greatest microglial/macrophage activation: 3 – pronounced, uniform band of activated cells around the hematoma; 2 – continuous but uneven band of activated cells; 1 – discontinuous distribution of activated cells; 0 – lack of activated cells (Figure 1).

STATISTICAL ANALYSIS

Statistical analysis for long-term survival infarct volumes was done using analysis of variance (ANOVA). Behavior (staircase reaching, asymmetrical forelimb use, and ladder walk) was analyzed using repeated measures ANOVA. Over-time behavioral differences within a group were assessed using a paired t-test. The size of the infarct was correlated with the resulting behavioral deficits by a line of linear regression. The differences in short-term survival infarct volumes and weight were measured with an unpaired t-test. Differences in microglial/macrophage activation based on the semi-quantitative rating score were determined using the Kruskal-Wallis analysis. All values are represented as mean \pm standard error of the mean.

RESULTS

ICH MODEL

The model produced large (although variable) lesions, encompassing the striatum, white matter, as well as the cortex (Fig. 2). These histopathological changes were accompanied by significant sensori-motor deficits, and these deficits were correlated with the size of the infarct (Fig 3). The model was associated with low mortality (3 out of 62 animals operated on died).

Animals tended to drop in weight as a result of ICH (V groups dropped 7-8 % of their pre-surgery weight) (Fig. 4). It took approximately 2 weeks for the animals to get back to their pre-surgery levels, after which they began to gain weight rapidly (data not shown). There was low morbidity (other than sensori-motor deficits) associated with the

ICH model. Two out of 59 surviving animals required supplementation with Ringer's lactate solution starting on second day following ICH because they were not drinking and/or eating sufficiently (one received 5 injections over 3 days and another received a single bolus injection). Interestingly, both of the animals were receiving MC treatment.

SIDE EFFECTS OF MC TREATMENT

Most MC treated animals had diarrhea for the duration of the drug intake. In order to assess the effect of MC on body weight, animal body weights following ICH were represented as a percentage of the pre-surgery values (Fig. 4). Short and long term animals (59 subjects) were pooled into 2 groups: animals receiving MC treatment (MC) or receiving V injections (V) (Fig. 4). The depicted weights are only of those animals that were receiving the drug at the given time point or the surviving V animals. And so on Days 1-5, the MC group consists of 36 animals and the V group consists of 23, whereas on Day13 the numbers are 12 and 13, respectively. On Days 1 and 2, weight loss was similar between the two groups, and it was most likely resultant of surgery and ICH. Whereas the V groups began to regain weight after that, the MC subjects failed to do so. The difference between the MC and V animals reached significance 5 days post-ICH ($p < 0.05$). And although the MC animals were not statistically different from V groups on the last day of MC treatment (Day 13), their body weights were reduced in comparison and remained so up to 28 days following ICH (data not shown).

The MC animals sacrificed at 5 days had yellow, pasty deposits on their liver and stomach/intestine (animals were sacrificed 3-6 h following last MC injection). The amount of the deposit varied from animal to animal as judged by visual inspection. As

MC is a yellow powder and has poor water solubility, it was assumed that it was the drug that was accumulating within the peritoneal cavity. The observation was confirmed by the authors of another study that used IP injections of MC (Fagan, Edwards, Borlongan, Xu, Arora, Feuerstein and Hess 2004). Two of MC-treated animals had bleeding of the stomach/intestine (possibly a result of MC effect on the gut flora). There were no obvious signs of peritonitis as a result of the numerous injections in any of the subjects.

MC TREATMENT IS NOT NEUROPROTECTIVE

The majority of the infarct was localized to the striatum. However, all animals had damage to the corpus callosum. Also, all animals showed some cortical damage along the damaged corpus callosum. The internal capsule of many animals was affected (42 animals out of 62). Figure 2 shows representative images of what the damage looked like at 5 and 28 days following ICH.

In the short-term study, MC treatment had no effect on the infarct volumes. Table 1 summarizes the results. The infarct volumes for the V treated and MC treated animals were virtually identical: $39.3 \text{ mm}^3 \pm 4.6$ and $38.5 \text{ mm}^3 \pm 5.7$, respectively. It should be noted that these numbers could be overestimates of the actual amount of abnormal tissue present. At 5 days following ICH, healthy tissue might be obscured by the hematoma and edema, which are then at their peak (Yang et al. 1994; Del Bigio et al. 1996).

Similarly, in the long-term study there was no effect of MC treatment, 5 or 14 day long, on infarct size ($F_{(35,2)} = 0.12$, n.s.)(see Table 1). The infarct volumes were as follows: $27.8 \text{ mm}^3 \pm 3.1$ for the V animals (V5 and V14 pooled together), $27.1 \text{ mm}^3 \pm 3.8$

for the MC5 animals, and $27.3 \text{ mm}^3 \pm 2.5$ for the MC14 group. The infarct volumes appear smaller at 28 days than at 5 days because by the later time point the hematoma had resolved, the tissue had undergone reorganization and shrinkage (Jenkins, Maxwell and Graham 1989).

MC TREATMENT DECREASES THE MICROGLIAL/MACROPHAGE REACTION

At 5 days post-ICH, the microglial/macrophage response was most pronounced inside the core of the hematoma and in the peri-hematoma region in the striatum. Minocycline treatment visibly suppressed that response (Fig. 5). The semi-qualitative 4-point rating scale confirmed that the V treated animals had a higher microglial/macrophage response (2.5 ± 0.08), than the MC animals (1.6 ± 0.23 ; $p < 0.001$) (Fig. 6).

MC TREATMENT DOES NOT PROVIDE ANY FUNCTIONAL BENEFIT

Staircase Reaching Test

Before stroke, trained animals were normally able to retrieve around 17 pellets out of the maximum of 21. There were no differences across the groups in the animals' ability to perform the task either pre- (data not shown) or post-ICH ($F_{(35,2)} = 0.188$, n.s.). Only data from day 7 and 28 are reported (Fig. 7). V5 and V14 animals did not differ, and so the groups were pooled together into a single V group. Staircase performance at each time point post-stroke was represented as percent of the animal's pre-stroke performance (i.e. if an animal was able to retrieve 10 pellets pre-stroke and only 5 post-

stroke, then the post-stroke staircase performance was 50%). There was a lot of variability among the animals in terms of impairment, such that following ICH some were unable to pick up a single pellet (0% of their pre-surgery performance) while others were at an almost normal performance (100%). Seven days after ICH, animals across all groups were strongly but comparably impaired: V = 41.1% ± 9.0, MC5 = 36.2% ± 11.4, and MC14 = 37.4% ± 7.2. There was no significant recovery over time (spontaneous or due to drug treatment), and the groups remained impaired up to 28 days: V = 49.1% ± 7.8, MC5 = 39.4% ± 9.7, and MC14 = 43.9% ± 7.7

Asymmetrical Forelimb Use

There were no differences among the groups at any time point tested ($F_{(35,2)} = 1.308$, n.s.) (Fig. 8). The animals had a normal pre-surgery reliance on their paws: approximately 30% for each right, left and bilateral contacts. Specifically, V subjects (V5 and V14 were pooled together) used the (future) ipsilateral paw 35.8% ± 3.1; the numbers were 32.8% ± 2.5 and 34.7% ± 2.8 for the MC5 and MC14 groups, respectively. Following ICH, all groups showed a significant impairment when compared to their pre-ICH performance as they exhibited a strong shift in reliance on ipsilateral paw placements: V = 64.1% ± 6.0, MC5 = 66.9% ± 6.2, MC14 = 70.0% ± 6.7. There was a slight trend for recovery at 28 days as the animals resumed using bilateral touch. However, they still had significant impairment: V = 59.8% ± 4.8, MC5 = 57.1% ± 3.5, MC14 = 56.74% ± 2.9. Only the data from day 7 and day 28 post-ICH are reported.

Ladder-Rung Walking Test

There were no differences among the groups in their performance at any of the time-points tested ($F_{(35,2)} = 1.188$, n.s.) (Figure 9). The average numbers of foot faults out of 3 trials performed are presented. Pre-ICH, all groups made some mistakes: V animals had 2.1 ± 0.3 foot faults, and MC5 and MC14 animals had respectively 2.4 ± 0.2 and 2.4 ± 0.1 foot faults. Across all groups, the animals made significantly more foot faults 7 days after ICH when compared to their pre-surgery scores: V = 3.6 ± 0.3 , MC5 = 3.3 ± 0.2 , MC14 = 3.9 ± 0.4 . At 28 days, the relative impairment was statistically significant for the MC14 group: 3.8 ± 0.4 , whereas the MC5 and V animals showed a strong trend: 3.5 ± 0.3 , 3.1 ± 0.3 , respectively.

DISCUSSION

Minocycline has shown promise as a neuroprotectant in a number of models of acute and chronic CNS disease (Yrjanheikki et al. 1998; Yrjanheikki et al. 1999; Chen et al. 2000; He et al. 2001; Sanchez-Meji et al. 2001; Brundula et al. 2002; Popovic et al. 2002; Wu et al. 2002; Zhu et al. 2002; Lee et al. 2003; Power et al. 2003; Wells et al. 2003; Stirling et al. 2004; Teng et al. 2004). It has been hailed for the heterogeneity of its potentially beneficial actions: anti-apoptotic, anti-inflammatory, antioxidant, as well as its paucity of negative side effects (Thomas et al. 2003). In this study, MC was tested as a potential therapy in a collagenase-induced rodent model of ICH. It was premised that various processes, such as the release of toxic clot components (Lee et al. 1997), ischemia and inflammation ensue for hours and days following ICH and contribute to secondary neurodegeneration and neurological worsening (Mayer 2003). The goal of

MC treatment was to block the inflammatory response and salvage enough tissue in the penumbral regions in order to improve functional outcomes. To assess the effect of MC treatment on long-term protection of function, sensitive and quantifiable sensori-motor tests were used. It was also hypothesized that more acute MC treatment (5 days) might provide greater benefit than a longer exposure to the drug (14 days), as the latter may interfere with inflammation-mediated tissue reorganization and repair essential to recovery.

No histological protection was observed in this study. The animals treated with MC (for 5 days) had infarcts of similar volume to vehicle (V) treated subjects at both 5 and 28 days post-ICH. Also, there was no difference in infarct volumes between the two long-term survival MC groups, MC5 and MC14, pointing to the fact that, at least at the histological level, there was no benefit of the shorter MC treatment over the more prolonged dosing regimen. The lack of histological protection translated into a lack of functional benefit of MC up to 28 days post-ICH. The behavioral tests used: staircase reaching, asymmetrical forelimb use and ladder walk showed all groups to be comparably impaired 7 days following stroke, and there were no differences among the groups at any later time point. Neither the more acute nor the prolonged MC treatment had any effect on protection of function. Thus MC provided no therapeutic effect.

An interesting finding of the study was that despite significant inhibition of microglia/macrophages there was no neuroprotection with MC. This poses an important question. Are microglia/macrophages really key in mediating the positive actions of MC reported in some studies (Yrjanheikki et al. 1998; Yrjanheikki et al. 1999; He et al. 2001; Tikka and Koistinaho 2001; Wu et al. 2002; Power et al. 2003)? My findings suggest

that a delayed microglial/macrophage suppression is inconsequential to the neurologic outcomes following ICH, yet it is possible that there exists some early, critical period when these cells contribute significantly to neurodegeneration. In support of this view are results from a study where pre-treatment of rats with a macrophage inhibiting factor, tuftsin fragment 1-3, led to an impressive reduction in the infarct volume following collagenase-induced ICH (Wang et al. 2003).

The above results are consistent with those of Power et al. (2003) in terms of the histological findings but stand in contrast with respect to the functional protection reported. There are a number of points that must be kept in mind when trying to reconcile these results: the differences in the time of commencement of MC treatment, in the severity of injury, in the mode of functional assessment employed as well as the disparity in the rat strains used.

The first important difference between the two studies is that MC was given at 3 h rather than 1 h following ICH as was done in the Power et al. (2003) study. In both studies, MC was administered by ip injection. Recently, however, this mode of MC delivery has been criticized due to less than optimal and reliable bioavailability of the drug in the CNS (Fagan et al. 2004). Consistent with the findings of this study, I noticed an accumulation of the drug on the surface of the liver, stomach and intestine in the short-term survival MC animals. The accumulation did not lead to any obvious morbidity, but it may have been associated with a delayed absorption into the blood (Fagan et al. 2004). Indeed, the Fagan et al. (2004) study reported incomplete and inconsistent absorption of MC into the bloodstream with peak serum concentrations attained at 2.5 h following the injection. Such delays in the effective drug concentration are problematic especially

following acute CNS injury when prompt delivery is necessary. It is possible that the 3 h delay of MC administration presumed in my study was, in effect, closer to 6 h, potentially missing the window of therapeutic opportunity. In the Power et al. (2003) study peak absorption would have occurred earlier. This could account for the differences in the drug efficacy between the two studies. A more consistent and prompt delivery of MC, such as via intravenous injection, might make MC more effective following ICH.

If, in fact, MC has a short therapeutic window, it does not bode well for viability of MC treatment in acute CNS injury in humans where significant delays exist between the onset of injury and hospital arrival. In an attempt to investigate whether the lack of benefit noted in the present study was due to the drug's narrow therapeutic window, an additional group of animals received MC immediately following ICH (n = 10). Immediate MC significantly reduced injury at 5 days after ICH (data not shown). Unfortunately, interpretation of those results is complex as MC has been shown to interact directly with collagenases and inhibit their action (Greenwald, Moak, Ramamurthy and Golub 1992). Therefore, the neuroprotection seen here cannot be attributed to MC actions countering secondary processes following ICH as the drug may have simply reduced collagenase activity. Such MC-collagenase interaction may also have contributed to the protection in the Power et al. (2003) study where MC was administered 1 h after the collagenase injection. In future studies, the ICH model employing injection of autologous blood should be used. It will allow to determine conclusively if MC has selective, protective actions independent of possible collagenase inhibiting action.

Another point of disparity between the Power et al. (2003) study and mine is the severity of injury. The amount of collagenase used here almost tripled that used in the Power et al. (2003) study (1.4 μ l containing 1.4 units vs. 0.5 μ l containing 0.05 units). Although the authors did not report infarct volumes, injury size correlates with the amount of collagenase used (Terai, Suzuki, Sasamata and Miyata 2003). Thus the striatal damage in the Power et al. (2003) study would have been considerably less than in this experiment. It is possible that a threshold of ICH damage exists past which neuroprotective therapy is useless, although two studies argue against this notion. The first reported that striatal damage caused by comparable collagenase amounts to that used in this study (0.7 μ l containing 1.4 units) was amenable to treatment (Mayne et al. 2001). In that study, direct injection of TNF- α antisense oligodeoxynucleotide into the ipsilateral striatum did not ameliorate the volumes of infarcts but led to improved functional outcomes. The weakness of the study lies in the fact that function was assessed using a subjective, neurobehavioral rating score. In a more recent study, ICH was induced by injecting 1 μ l of saline containing 0.2 units of collagenase into the striatum (MacLellan, Girgis and Colbourne 2004). The infarct volumes at 4 weeks exceeded those in the present study (\sim 35 mm³ vs. \sim 27 mm³). Despite the severity of the injury, treatment with hypothermia (delayed to 12 h post-ICH) led to significant histological and functional protection. Functional outcomes were measured using sensitive sensori-motor tests such as the ones used in my study. Thus there is some evidence to support the notion that the amount of injury produced in this study can be attenuated. Still, it does not rule out the possibility that MC may not be as effective as hypothermia or TNF- α antisense oligodeoxynucleotide infusion.

A third important difference between the two MC studies was the quality of behavioral assessment used. The mode of behavioral assessment employed in the Power et al. (2003) study may have overestimated the therapeutic efficacy of MC treatment following ICH. The graded neurobehavioral score used consisted of three parts that tested spontaneous circling, forelimb flexion, and beam walking. Each part was scored separately, and then a composite score out of 10 was reported. However, there were no detailed descriptions of the behavior attributed to a given score. For example, beam walking was graded from 0 for normal movement across the beam to 4 for falling off the beam. No explanation was provided for what constituted a “normal” crossing or the scores in between the two extremes, which is crucial for an objective assessment of impairment. There are also problems with regards to spontaneous circling as employed here. Spontaneous circling usually disappears within the first few days following striatal injury as the animals begin to compensate (Glick and Cox 1978). In fact, asymmetrical rotation following striatal lesion is rarely studied as a spontaneous phenomenon. Rather, dopaminergic agonists (e.g. amphetamine) are routinely used to unmask lesion-induced asymmetries and to re-induce circling (Grabowski, Brundkin and Johansson 1993). Based on this, it is surprising that spontaneous circling would have been sensitive enough to detect impairment up to 4 weeks following injury as reported by Power et al. (2003). An additional weakness of the functional evaluation used in the Power et al. (2003) study is the lack of any controls. There was no pre-surgery assessment/training or sham operated group. Baseline scores would have normalized the results and controlled for any innate differences among the animals. Indeed, normal animals do exhibit directionally biased circling behavior (Glick and Cox 1978). However, the Power et al.

(2003) study examined only post-ICH behavior and only in two groups of animals: animals receiving ICH plus saline treatment and ICH plus MC treatment. At 7 days post-ICH, the saline treated animals had an average score of ~ 4 (4/10), which was significantly worse than the score ~ 3 earned by the MC group. At 28 days, the scores were ~3 and ~2, respectively. The differences, although statistically significant, are small and of questionable validity.

A final disparity between my study and the one by Power et al. (2003) is the fact that different rat strains were used (Long-Evans vs. Sprague-Dawley, respectively). Despite that MC suppressed the microglial/macrophage response and caused weight loss in both of the strains (Power et al. 2003), MC may still have been less effective as a neuroprotectant in the Long-Evans rats. One study suggests that the animal strain used can influence the immune response following CNS trauma (Popovich, Wei and Stokes 1997). The authors compared the inflammatory response of Sprague-Dawley and Lewis rats following SCI contusion. It was found that the Lewis strain sustained greater and longer macrophage activation. Differences in terms of antigenic expression and T-cell infiltration were noted as well. The implication of those findings is such that, due to variations in inflammatory response, different strains could be characterized by different responses to anti-inflammatory treatment, such as MC.

This study is among a handful of studies that report negative findings regarding MC efficacy in neurological disorders. They include a rat model of SCI (Zang da and Cheema 2003), the R6/2 mouse model of Huntington's (Smith, Woodman, Mahal, Sathisvam, Ghazi-Noori, Lowden, Bates and Hockly 2003), and the mouse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease in which MC actually

exacerbated neuronal death (even though it was associated with a suppressed microglial/macrophage response) (Yang, Sugama, Chirichigno, Gregorio, Lorenzl, Shin, Browne, Shimizu, Joh, Beal and Albers 2003) . Although, all of the above cast a shadow on the drug's therapeutic value in CNS disease, there are a number of potential experimental variants, which could account for the negative results. Among them are issues of drug dosing regimen variations, injury severity, and MC stability. For example, Smith et al. (2003) prepared MC fresh weekly, and there are suggestions that MC is unstable and should be prepared daily (Hersch, Fink, Vonsattel and Friedlander 2003). Another factor that could account for the negative results of the MC studies is the possibility that MC has some detrimental actions that obscure its positive, neuroprotective effects. The MC doses used in the present study were comparable to those used in other studies (Yrjanheikki et al. 1999; Power et al. 2003). The treatment lead to diarrhea and weight loss in MC treated animals, but the latter was statistically significant only on day 5 following ICH. There were no differences between the MC5 and MC14 animals, which would be expected had MC caused morbidity. Finally, the drug did not cause any obvious functional or histological disturbances in normal subjects (data not shown). Although MC did not seem to have major negative side effects, it is possible that it had some adverse actions at the molecular level. One possibility is its effect on growth factors. MC is capable of suppressing vascular endothelial growth factor and basic fibroblast growth factor in non-nervous tissue (Sasamura, Takahashi, Miyao, Yanase, Masumori, Kitamura, Itoh and Tsukamoto 2002). Both of these factors have been shown to have important roles as neuroprotectants and in angiogenesis following acute CNS injury (Liang, Kanthan, Shuaib and Wishart 1997; Sun, Jin, Xie,

Childs, Mao, Logvinova and Greenberg 2003). Thus MC may have actions that contribute to both favorable and deleterious outcomes following CNS injury. Accordingly, studies report toxic (Yang et al. 2003), none (Smith et al. 2003), and neuroprotective (Zhu et al. 2002) MC effects in CNS. It is possible that the therapeutic potential of MC depends on a proper balance between the “positive” and “negative” properties of the drug.

In summary, ip MC treatment commenced 3 h after ICH is neither histologically nor functionally protective, even though it suppresses the microglial/macrophage response in the perihematoma region. The contrasting report by the authors of the Power et al. (2003) study may be due to differences in timing of drug delivery, which could be indicative of MC having a narrow therapeutic window. Other factors that could account for the disparity of results are severity of injury and the nature of behavioral tests used. As there are other studies that report negative findings with respect to MC therapy in CNS disease (Smith et al. 2003; Yang et al. 2003; Zang da and Cheema 2003), more research is needed in order to elucidate the drug’s therapeutic potential and mechanisms of action.

One other caveat that makes it difficult to gauge the usefulness of MC as a treatment in clinical CNS disease: the extrapolative value of rodent models to the human situation. For example, animal models of ICH do not perfectly emulate the complex and dynamic nature of clinical hemorrhage. In humans the hematoma expansion is a prolonged process lasting up to 24 h following the initial insult, whereas in the collagenase ICH model, the hemorrhaging stops within the first 4 h (Del Bigio et al. 1996; Mayer 2003). Thus there is a clear difference in the time course (and possibly in

the extent) of the processes contributing to the secondary neurodegeneration following ICH. As MC is believed to counteract many of these processes, it is still possible that the drug could prove efficacious in the clinical setting where the therapeutic window may be longer than in rodent models.

With respect to ICH, the effects of MC treatment in rodents should be studied in the autologous blood injection model where MC is delivered intravenously. If future studies, both in animals and humans, determine an impractically short therapeutic time window, it will limit the drug's clinical application in ICH therapy. However, MC may still be found useful as a prophylactic intervention for ICH. As the drug is well tolerated in patients, individuals identified as running high risks of ICH (e.g. persons with ominously high blood pressure or ICH survivors, who are generally liable to future re-bleeding) could be put on chronic MC treatment (Labovitz and Sacco 2001; Koistinaho 2002). Future research involving MC prophylaxis and/or treatment in ICH and other types of acute CNS injury should proceed with caution.

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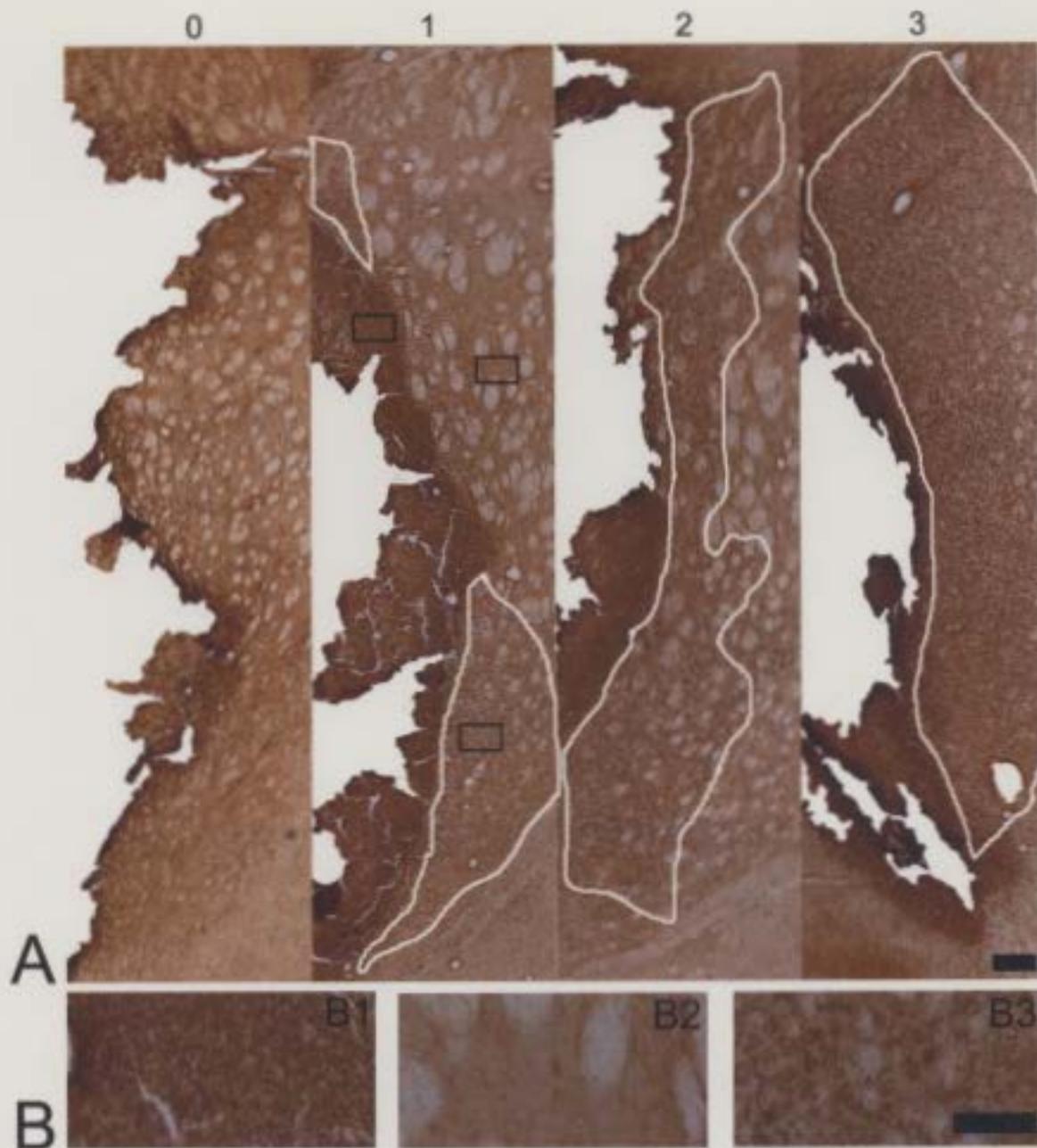


Figure 1. Representative images denoting the four-point rating scale used in the assessment of microglia/macrophage activation in the peri-hematoma striatum as marked by isolectin B₄ histochemistry (A). The darkest areas correspond to the hematoma itself and were not considered in the assessment (upper box in A and enlarged in B1). The areas containing activated cells are enclosed by white lines (lower box in A and enlarged in B3). The lowest response was marked as 0, and it was characterized by a lack of activated cells in the perihematoma region (middle box in A and enlarged in B2). A score of 1 was given to a discontinuous distribution of activated cells. A score of 2 was given to a continuous but uneven border of activated cells. The highest response was marked as 3, and it was characterized by a pronounced, uniform band of activated cells. Scale bars = 100 μ m.

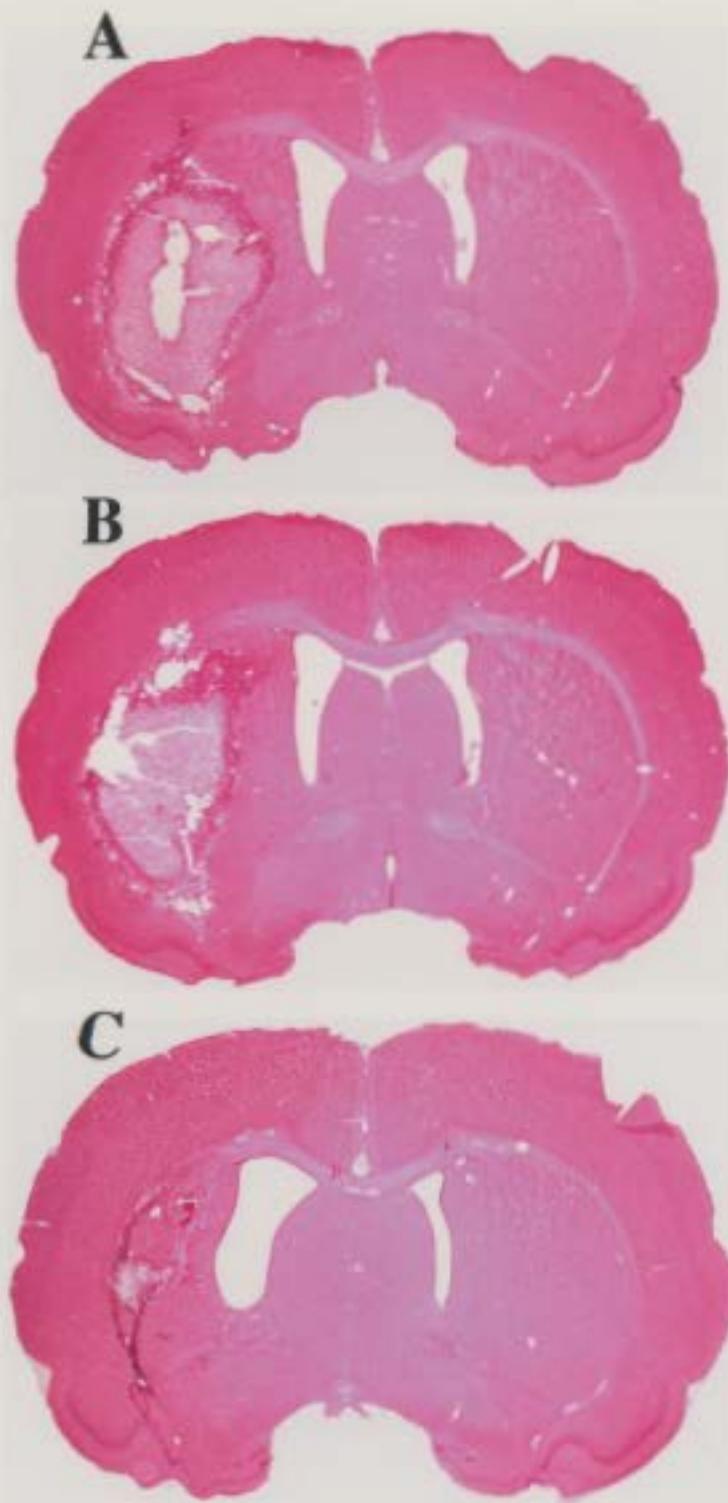


Figure 2. Representative areas of injury. The H&E stained tissue shows the injury at 5 (A-B) and 28 days (C) post-ICH. Short term infarct volumes were of comparable size between the MC (A) and V (B) treated groups. The majority of injury was localized to the striatum; however, the overlying cortex and the white matter were affected as well. There were no differences among the V, MC5 and MC14 groups at 28 days (C). Note the striatum has collapsed, and the ventricle is enlarged.

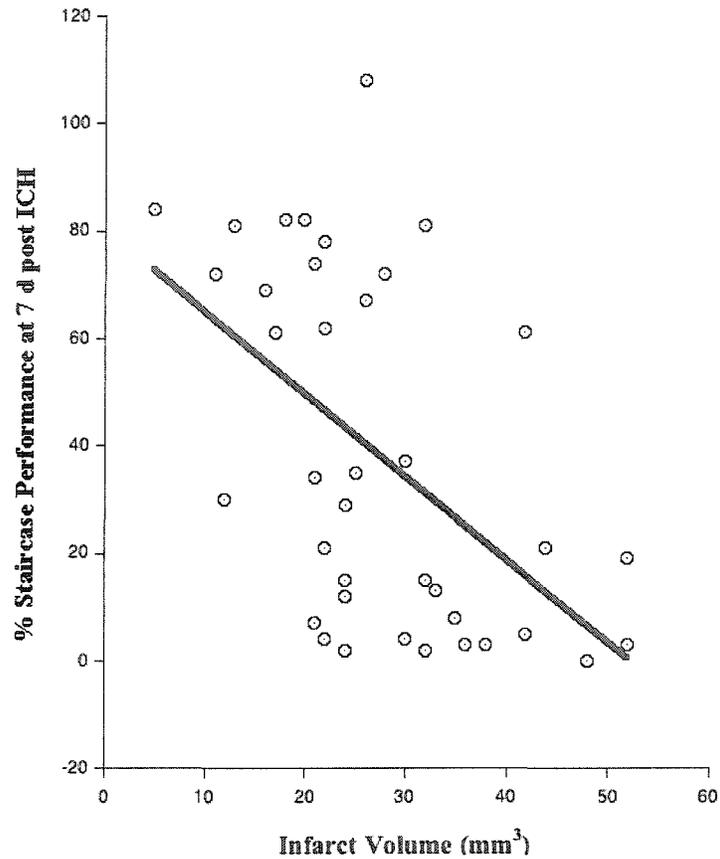


Figure 3. Infarct volumes and sensori-motor deficits correlated by a line of linear regression. Since there were no histological or functional differences among the groups, all the animals were combined. There is a significant correlation ($p < 0.001$) between the infarct volumes of the long-term groups (V, MC5 and MC14) and their staircase reaching performance at 7 d post-ICH (represented as the percent of the pre-ICH score where 100 % indicates no impairment); $R = 0.52$.

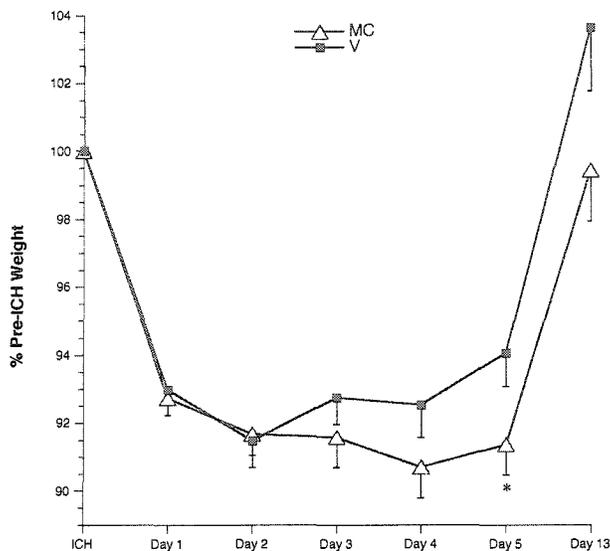


Figure 4. Average body weights following ICH represented as a percentage of the pre-surgery values. All experimental animals – short and long-term – were divided into two groups: MC and V. MC animals presented here are only those that were receiving the drug at the given time points. All animals lose weight as a result of the surgery as indicated by the ~7-8% drop on Day 1 and 2 post-ICH. MC treatment impedes weight gain in the following days. The difference between the groups reaches significance on Day 5 (* $p < 0.05$). All the values in this and subsequent graphs represent the mean \pm SEM.

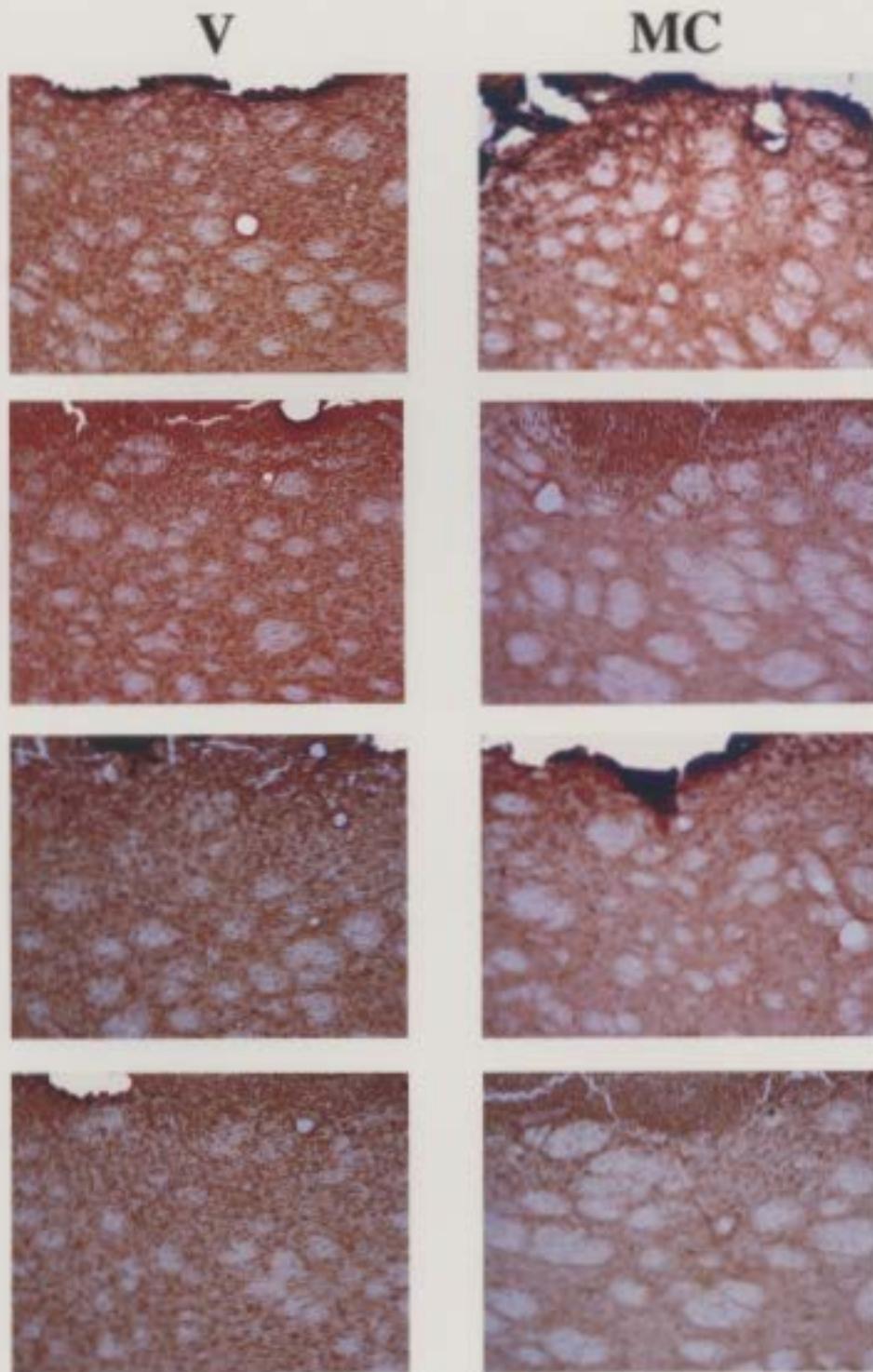


Figure 5. Microglial/macrophage activation in the peri-hematoma striatum as detected by isolectin B4 histochemistry at 5 days post-ICH. Top of each image corresponds to the hematoma border. Four representative animals from the V and MC short-term (5 day) groups are shown. MC treatment reduced the microglial/macrophage response.

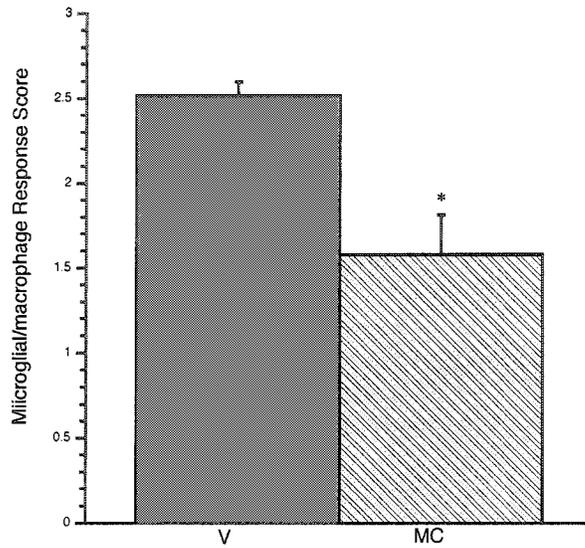


Figure 6. Semi-quantitative score of the microglial/macrophage response in the peri-hematoma striatum based on the isolectin B₄ histochemistry indicates that MC suppresses the microglia/macrophages. The vehicle treated animals (V, n=14) had a significantly higher microglial/macrophage reaction than the groups receiving MC (MC, n = 11). * indicates different from V $p < 0.001$.

TABLE 1. Infarct Volumes*.

Treatment	5 Days Post-ICH	28 Days Post-ICH
V**	39.5 ± 6.2 (n = 10)	27.8 ± 3.1 (n = 13)
MC5	38.3 ± 5.7 (n = 11)	27.1 ± 3.8 (n = 13)
MC14	n/a	27.3 ± 2.5 (n = 12)

* Values in mm³ represent mean ± SEM

** Both V5 and V14 combined

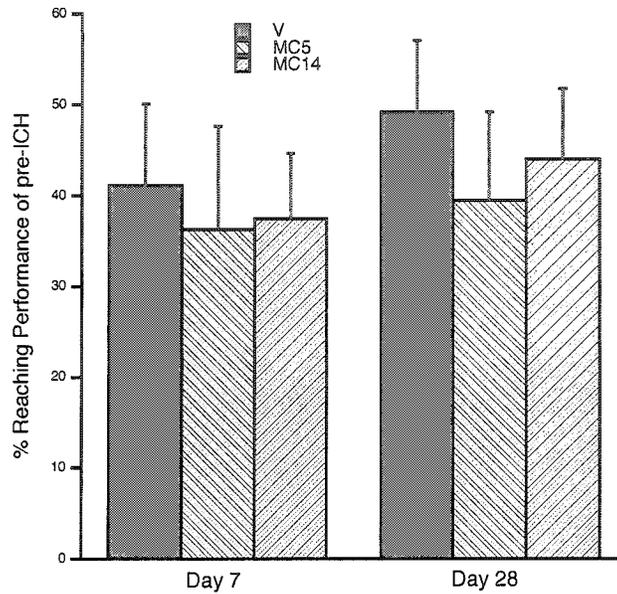


Figure 7. Staircase reaching test. Individual skilled reaching with the limb contralateral to the lesion is expressed as a percentage of pre-ICH performance level. ICH resulted in significant impairments in all groups. There was no drug-induced or spontaneous recovery. The vehicle groups, V5 and V14, were not significantly different at any time point and therefore were pooled for statistical analyses. There were no differences among the experimental groups at any time point. V, n=13; MC5, n=13; MC14, n=12.

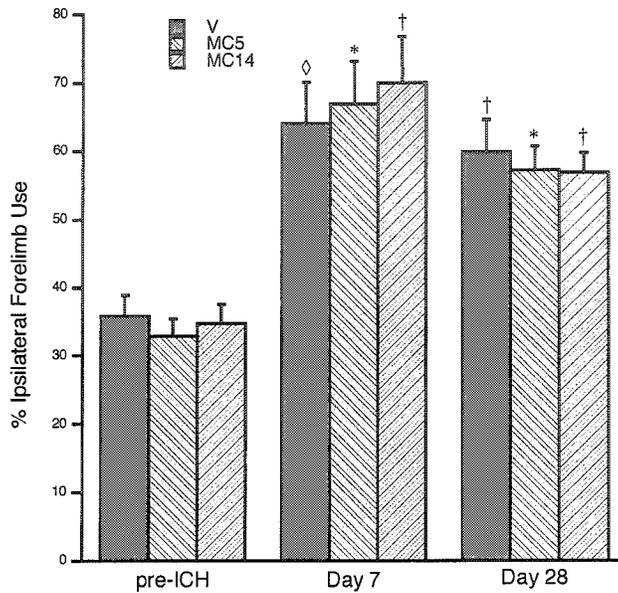


Figure 8. Asymmetrical forelimb use. Preferential forelimb use was examined before and post-ICH in a cylinder test, and it was expressed as the number of contacts by the paw ipsilateral to the lesion over the total number of contacts (ipsilateral + contralateral + bilateral). There were no differences among the experimental groups at any time point. V5 and V14 groups were pooled. All groups remained significantly impaired up to 28 days following ICH as compared to their pre-surgery performance. V, n=13; MC5, n=13; MC14, n=12. *, †, † indicates different from pre-surgery performance $p < 0.0001$, $p < 0.001$, $p < 0.01$, respectively.

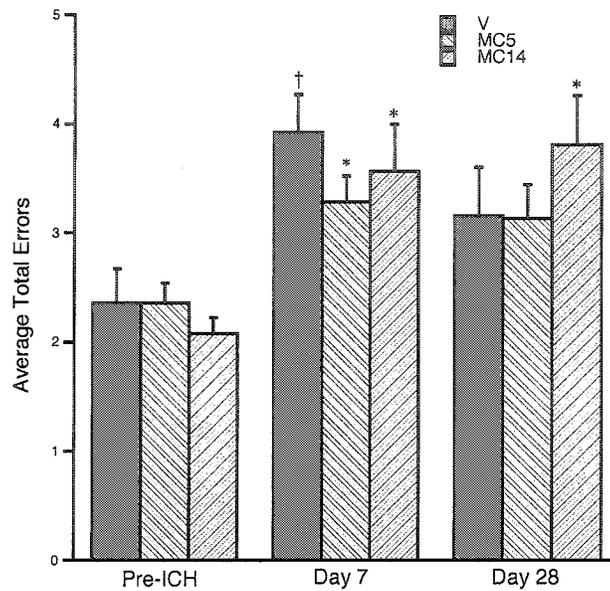


Figure 9. Ladder walk test. Individual number of total foot errors (including slips and placement errors) was calculated by averaging the performances of 3 trials at each time point. There were no differences among the experimental groups at any time. V5 and V14 were pooled. All groups were significantly impaired at 7 days following stroke when compared to their pre-surgery performance. At 28 days, only the MC14 was still statistically different from its pre-ICH score. V, n=13; MC5, n=13; MC14, n=12. *, †, indicates different from pre-surgery performance $p < 0.01$, $p < 0.05$, respectively.



