

# MINOCYCLINE VASCULAR PROTECTION AFTER ACUTE ISCHEMIC STROKE

By

LIVIA SAMPAIO MACHADO

(Under the Direction of Susan C. Fagan)

## ABSTRACT

Stroke is the third leading cause of death and the leading cause of disability in adults in the United States. The knowledge of the pathophysiology of this disease has increased significantly in the last decades. However, because of population aging and the relative poor impact of stroke primary prevention efforts on certain target populations, it is estimated that the incidence of stroke will continue to rise. Although chronic vascular protection has been traditionally recognized as a strategy to reduce risk and severity of ischemic stroke, there is limited research on the effects of vascular protection acutely after stroke. Because the ischemic insult affects endothelial cells and the components of the blood brain barrier, maintaining the vascular integrity and function after acute stroke may reduce the degree of tissue damage. After cerebral ischemia, matrix metalloproteinases (MMPs) 2 and 9 are involved in the mediation of hemorrhagic transformation, particularly after treatment with tissue plasminogen activator, the only therapy currently available for acute ischemic stroke patients. Minocycline, a drug commonly used in human antimicrobial therapy has been shown to possess anti-inflammatory properties. It can inhibit the degradation of the extracellular matrix in periodontal disease and in disease models of ischemia. However, this MMP inhibition

property of minocycline after stroke and its vascular effects remain unexplored. Minocycline might prevent the MMP- related post-ischemic tissue breakdown. The goal of this study was to understand the mechanisms involved in the neurovascular injury after stroke and evaluate the potential of minocycline as an acute vascular protective therapy in Wistar rats after experimental ischemic stroke. I hypothesized that minocycline treatment after ischemic stroke would be vascular protective acutely through the inhibition of matrix metalloproteinases. I investigated the hemorrhagic transformation, infarct size and overall functional outcome in Wistar rats subjected to three hours of middle cerebral artery occlusion (MCAO) followed by twenty one hours of reperfusion. I also investigated whether components of the blood brain barrier were affected by minocycline treatment. Results presented in this dissertation show that minocycline treatment, particularly with the early intravenous 3 mg/kg dose regimen, is neurovascular protective after stroke in our mechanical model. This neurovascular protection and matrix metalloproteinase inhibition occurs in the same weight-based dose approved for minocycline in humans. Importantly, minocycline remained protective when it was used in combination with tissue plasminogen activator. These results point to a potential for minocycline treatment in acute ischemic stroke patients. However, whether these pre-clinical findings will be translated into human studies remains to be seen.

**INDEX WORDS:** acute ischemic stroke, vascular protection, minocycline, matrix metalloproteinase, tissue plasminogen activator

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LIVIA SAMPAIO MACHADO

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by

LIVIA SAMPAIO MACHADO

Major Professor: Susan Christine Fagan

Committee: Adviy Ergul  
Alvin Terry  
Randall Tackett  
William C. Hill

Electronic Version Approved:

Maureen Grasso  
Dean of The Graduate School  
The University of Georgia  
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## TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS.....	iv
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
CHAPTER	
1. INTRODUCTION AND LITERATURE REVIEW.....	1
2. DELAYED MINOCYCLINE INHIBITS ISCHEMIA-ACTIVATED MATRIX METALLOPROTEINASES 2 AND 9 AFTER EXPERIMENTAL STROKE.....	22
3. MINOCYCLINE AND TISSUE PLASMINOGEN ACTIVATOR: PRECLINICAL EVALUATION PRIOR TO TRANSLATION TO STROKE.....	
PATIENTS.....	46
4. UNPUBLISHED DATA: MINOCYCLINE VASCULAR PROTECTION STUDY.....	76
5. DISCUSSION.....	86
REFERENCES.....	94
APPENDICES	
A. TACTICS FOR VASCULAR PROTECTION AFTER ACUTE ISCHEMIC STROKE.....	99
B. MINOCYCLINE FOR SHORT TERM NEUROPROTECTION.....	111

## LIST OF TABLES

	Page
Tables	
3.1 Effect of minocycline on amidolytic parameters of tPA.....	66
3.2 Mortality rates of Wistar rats after acute ischemic stroke and treatment .....	66

## LIST OF FIGURES

	Page
Figures	
1.1 Schematic figure for the proposed hypothesis .....	2
2.1 MMP activity in the brain of Wistar rats after acute ischemic stroke.....	42
2.2 Impact of minocycline treatment on ischemia-induced MMP activity.....	43
2.3 Impact of minocycline treatment on ischemia-induced MMP protein.....	44
2.4 Effect of multiple minocycline concentrations <i>in vitro</i> on recombinant MMP activity.... .....	45
3.1 Impact of minocycline on fibrinolytic activity of tPA .....	67
3.2.A MMP activity in rat brains after AIS and treatment with tPA and minocycline .....	68
3.2.B MMP protein in rat brains after AIS and treatment with tPA and minocycline .....	69
3.3.A Effect of minocycline treatment on hemorrhagic transformation .....	71
3.3.B Illustration of hemorrhagic transformation in the brain after stroke .....	71
3.3.C Effect of treatments on brain edema after acute ischemic stroke .....	71
3.4.A Effect of treatments on collagen type IV in the rat blood brain barrier after stroke... .....	72
3.4B Effect of treatments on laminin $\alpha$ -1 in the rat blood brain barrier after stroke.....	73
3.5A Impact of treatments on infarct size after acute ischemic stroke.....	74
3.5B Illustration of infarct size with a representative brain of each treatment group.....	74
3.6.A Effect of tPA and minocycline treatments on Bederson Score.....	75
3.6.B Effect of tPA and minocycline treatments on the Paw Grasp Test.....	75

4.1 MMP-9 activity in the Wistar brain after 3 or 24 hours of reperfusion .....	76
4.2.A MMP-2 activity in the plasma of Wistar rats .....	77
4.2.B MMP-9 activity in the plasma of Wistar rats .....	77
4.3.A Brain edema in animals treated with minocycline alone.....	79
4.3.B Brain Tissue hemoglobin in animals treated with minocycline alone.....	79
4.4.A Brain infarct size in animals treated with minocycline alone .....	79
4.4.B Bederson score in animals treated with minocycline alone .....	79
4.5 Minocycline serum concentration at the time of sacrifice .....	80
4.6 Dose-response graph showing the MMP susceptibility to minocycline.....	80
4.7 Tissue hemoglobin content after tPA and minocycline treatment.....	81
4.8.A Elevated body swing test after tPA and minocycline treatment.....	82
4.8.B Beam walk test after tPA and minocycline treatment .....	82
4.9.A MMP-2 activity in the plasma after AIS and treatment with tPA .....	82
4.9.B MMP-9 activity in the plasma after AIS and treatment with tPA .....	82
4.10.A MMP-9 activity of cultured BRECs after OGD damage and treatment.....	83
4.10.B Illustration of a zymogram of cultured cells .....	83
4.11 BREC cell death after OGD and treatment	84
4.12 MMP activity of HBMECs.....	84
4.12 MMP activity of cultured HBMECs after OGD .....	85
5.1 Outline of the project results.....	93



## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

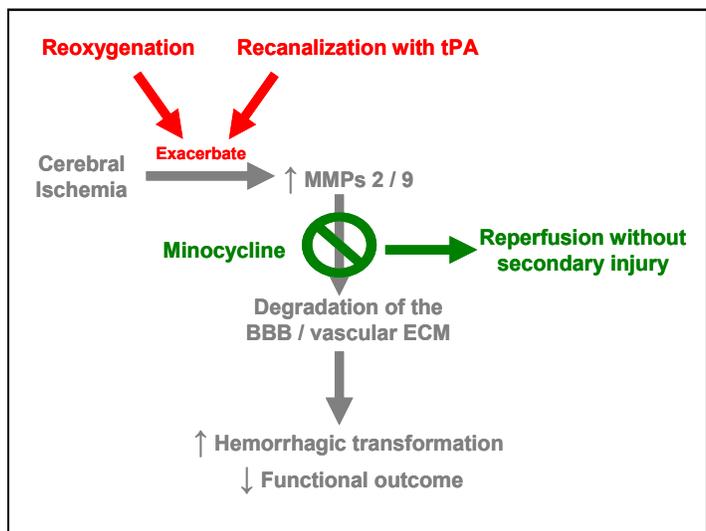
#### A. Statement of the problem and dissertation objectives

Stroke is the third leading cause of death and the leading cause of disability in adults in the United States. The knowledge of the pathophysiology of this disease has increased significantly in the last decades. The relationship between stroke and other cardiovascular conditions has been better defined and the management of specific risk factors has decreased stroke mortality. However, because of the aging population and the relative poor impact of stroke primary prevention efforts on certain target populations, it is estimated that the incidence of stroke will continue to rise. Ischemic stroke of different etiologies comprise over eighty percent of all stroke types. Ischemic stroke damages the brain by severely decreasing the cerebral blood flow and reducing the availability of metabolic sustenance, oxygen and extracellular ions. This imbalance leads to an acute energy failure, excitatory damage and the activation of mechanisms that will lead ultimately to cell death. However, certain areas of the brain, more distal to the occluded artery, are affected in a milder manner due to collateral perfusion. This area is affected only partially and is potentially salvageable by acute therapeutic intervention. The overall acute stroke outcome is dependent ultimately upon the extent

of neuronal cell death, brain edema, vascular health and presence or absence of secondary hemorrhage.

Although chronic vascular protection has been traditionally recognized as a strategy to reduce risk and severity of ischemic stroke, there is more limited research on the effects of vascular protection acutely after stroke. Because the ischemic insult affects endothelial cells and the components of the blood brain barrier, maintaining the vascular integrity and function after acute stroke may reduce the degree of tissue damage.

Matrix metalloproteinases (MMPs) are a family of zinc dependent proteases responsible for the extracellular matrix turnover and degradation of bioactive proteins. In cerebral ischemia, MMPs 2 and 9 have been identified to mediate the degradation of the basal lamina and hemorrhagic transformation. After



**Figure 1.1: Conceptual scheme that outlines the main hypothesis and the objective of this research.**

ischemia, these two enzymes are up-regulated and activated in the sub-acute phase, when other mediators such as proinflammatory proteins are also triggered. The combination of inflammatory factors and MMPs disrupts vascular integrity, increases the permeability of the blood brain barrier and may lead to secondary hemorrhage

formation. These enzymes disrupt the stability of interactions between cells and the supporting extracellular matrix, leading to disturbance of signaling mechanisms.

Minocycline, a second generation tetracycline, is a relatively old drug, commonly used in human antimicrobial therapy. In addition to being antimicrobial, minocycline has been shown to possess anti-apoptotic and anti-inflammatory properties. Due to its lipophilicity, it crosses the blood brain barrier and has been studied for a variety of brain disorders that involve inflammation. Furthermore, minocycline inhibits the degradation of the extracellular matrix in periodontal disease and has been reported to inhibit MMP activity in disease models of ischemia. However, this MMP inhibition property of minocycline and its vascular effects remain unexplored. Minocycline might prevent the MMP- related tissue breakdown.

The overall goal of this study was to understand the mechanisms involved in the neurovascular injury after stroke in order to improve the treatment of acute stroke patients. The main focus of this project was to elucidate the extent of the role of MMPs in the pathophysiology of stroke and to determine if the potential acute MMP inhibition with minocycline would provide neurovascular protection. **The central hypothesis for the proposed research was that minocycline is vascular protective by inhibiting MMPs and consequently the events that compromise the integrity of the vasculature and the blood brain barrier.**

To test this hypothesis, the following specific aims were addressed:

**Specific aim 1: Determine the extent to which acute treatment with minocycline protects the vasculature after acute ischemic stroke and impacts overall functional outcome.**

Our working hypothesis is that minocycline inhibits the deleterious MMPs activated by stroke and decreases vascular breakdown, improving functional outcome after experimental stroke.

**Specific aim 2: Determine if minocycline MMP inhibition occurs in doses that are comparable with doses used in humans.**

Our working hypothesis is that the minimum concentration of minocycline required to inhibit ischemia – activated MMPs is comparable with that proven safe in humans.

**Specific aim 3: Determine the effect of MMP inhibition on the degradation of the blood brain barrier and its components after experimental ischemic stroke.**

Our working hypothesis is that MMP inhibition decreases the degradation of the blood brain barrier basal lamina and endothelial tight junctions.

These aims were addressed by *in vivo* studies with an experimental model of ischemic stroke in rats where hemorrhagic transformation is observed. Furthermore, the studies of specific molecular events occurring in brain endothelial cells after ischemia/reoxygenation were complemented by *in vitro* cellular experiments.

## B. Review of the literature and discussion of the rationale of the project

### **Vascular protection**

The primary goal of experimental therapeutic intervention after cerebral ischemia has been to protect the neuronal cells from irreversible damage and death. This has been the leading approach to research efforts in the acute stroke field. All the experimental agents developed for the treatment of stroke targeted cell pathways that lead to neuronal cell injury and death. Such agents have been called “neuroprotective” because they focused on neurons alone. However, the vasculature is also affected after cerebral ischemia, that can disrupt the close relationship between the microvasculature, their surrounding cells, the extracellular matrix and neurons<sup>1</sup>. This newer understanding of the complexity of the “neurovascular coupling” focuses on the interaction between cell types in the brain and is the new approach to neurovascular protection after stroke.

The cerebral microvessels consist mainly of the endothelium, the basal lamina matrix and the end-feet of astrocytes. In physiological circumstances, these elements help regulate and maintain the blood brain barrier whose integrity is essential for the protection of the brain<sup>2</sup>. The functional barrier properties of the blood brain barrier are composed of two main structures: the specialized endothelial cells and the intact basal lamina. The endothelial cell component also comprises the interendothelial cell tight junctions. The proximity of all these cellular components implies ready communication between the endothelial cells and supporting glial cells, while flow alterations with increased neuronal metabolic demand imply also the coupling of neuronal requirements

to microvessel function. During ischemia, however, this relationship among cell types is abruptly changed and the blood brain barrier is disrupted, losing its ability to remain impermeable and that exposes the brain to oxidative and inflammatory stress<sup>3</sup>. The impact of ischemia on the blood brain barrier has been extensively studied. It sets in motion a variety of events that lead to the uncoupling of signals. Pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  as well as vascular endothelial growth factor and nitric oxide have been shown to be involved. Subsequent up regulation of endothelial adhesion molecules lead to transmigration of leukocytes across the endothelium, which furthers the disorganization of the blood brain barrier. Furthermore, reperfusion through damaged cerebral blood vessels exacerbates tissue injury after ischemia and contributes to the degradation of the blood brain barrier ultimately leading to the development of hemorrhage in the brain. This hemorrhagic transformation can occur spontaneously and is mostly associated with large infarcts. Treatment with tissue plasminogen activator, however, has also been shown to increase the risk of developing hemorrhage.

In this context, the vascular health and integrity of the blood brain barrier are critical to help minimize brain injury after ischemia and during reperfusion. "Vascular protection" is a general term that refers to efforts of decreasing the direct damage of ischemia to the cerebral vasculature after acute ischemic stroke. This study explored the effects of the stroke experimental drug minocycline on the vasculature and blood brain barrier after acute ischemic stroke.

## **Minocycline as a potential drug therapy for acute ischemic stroke**

### **Minocycline as a neuroprotective agent**

Minocycline is a commonly used second generation semi-synthetic tetracycline and it is currently used for its bacteriostatic properties. More recent than its development for antimicrobial therapy is its clinical application for inflammatory conditions such as rheumatoid arthritis. A number of studies have been conducted to explore other potential disease states that may respond to minocycline anti-inflammatory effects. Because minocycline is the most lipophilic of all the tetracycline drugs, it has the best tissue absorption into the central nervous system. For that reason it has been studied for inflammatory diseases of the brain, such as Alzheimer's, Parkinson's and Huntington's diseases, multiple sclerosis and acute stroke<sup>4</sup>. Several mechanisms have been proposed to explain its anti-inflammatory effects in the brain<sup>5</sup>. One suggests it involves modulating microglial activation resulting in inhibition of nitric oxide and cytokine release by microglial cells<sup>6</sup>. Another postulated action of minocycline that is relevant to neuroinflammatory diseases is that it protects against cell death. Minocycline acts in the mitochondria, elevating anti-apoptotic regulator Bcl-2 and protecting against cell death *in vitro* and *in vivo* in models of renal ischemia<sup>7</sup>. In the context of cerebral ischemia, there is also compelling evidence that minocycline demonstrates neuroprotective effects. Treatment with minocycline has been shown to decrease neuronal cell death and cerebral infarction after global cerebral ischemia in rats<sup>8</sup>. In addition, minocycline protects against inflammation and decreases cerebral infarction in rats undergoing temporary focal cerebral ischemia<sup>9</sup>. The neuroprotective effect of

minocycline after stroke has been mainly studied in doses higher than that approved for human pharmacotherapy. However, previous studies of our research group have demonstrated that lower minocycline doses given acutely up to 5 hours after the onset of stroke also strongly reduces infarct size and decreases the neurological deficit after acute ischemic stroke in rats<sup>10</sup>. These findings suggest that minocycline – an old, inexpensive and established drug – is a strong candidate for acute therapeutic intervention after stroke.

### **Minocycline and vascular protection**

Yenari et al. explored the association between inflammation generated by activated microglia after stroke and its impact on the blood brain barrier showed that minocycline treatment is able to decrease the disruption and leakage of the blood brain barrier<sup>11</sup>. This study was one of the first to explore the impact of minocycline on the vasculature after brain ischemia. Its findings suggested that minocycline may be a potential drug for vascular protection in the context of stroke.

In *in vitro* study models of angiogenesis, minocycline (as well as its analogue doxycycline) has been shown to attenuate cell migration and also the enzymatic activity of proteolytic enzyme matrix metalloproteinase-9 following stimulation with vascular endothelial growth factor<sup>12</sup>. This effect of minocycline on the matrix metalloproteinase (MMP) enzyme family is believed to be shared by all tetracycline drugs in general and it is not new to the scientific literature. The ability of tetracyclines to decrease the activity of MMPs was first observed in models of periodontal disease. Golub et al. have shown

that tetracyclines protect against collagenolysis and interfere with the MMP activity associated with periodontitis<sup>13 14</sup>. Furthermore, in a model of renal ischemic disease where minocycline treatment was able to decrease MMP-2 and MMP-9 activation, Sutton *et al.* demonstrated that the treatment resulted in a decrease of the microvascular permeability that occurs after an ischemic insult.<sup>15</sup>

Because the enzymatic function of MMPs relies on a specific structural conformation maintained by zinc in their active site, it is speculated that the tetracycline MMP inhibition effect may be due to their ability to chelate divalent ions. All tetracyclines have in their chemical structure an active binding site that confers to them the ability to chelate divalent ions. However, although it is possible that minocycline inhibits MMP activity by disrupting the active conformation of the enzyme, it has been shown that tetracyclines inhibit MMP-1 and MMP-3 by down regulating their gene expression<sup>16</sup>. Furthermore, in a study of neutrophil migration, tetracyclines were able to inhibit this MMP dependent cell function just as seen with EDTA, an MMP inhibitor that acts only directly, via cation chelation.<sup>17</sup> However, when these cells were supplemented with zinc, only the ones treated with EDTA restored their migrating ability. The ones treated with minocycline had only a partial improvement; reinforcing the fact that minocycline may inhibit the MMP enzymes by multiple mechanisms.

Tetracyclines have also been shown to interfere with MMPs in the brain. In studies of cerebral angiogenesis – a process in which MMPs are involved in tissue remodeling and cell migration – doxycycline has been shown to decrease both MMP-9 activity and

mRNA levels as well as angiogenesis in the brain of mice after treatment with vascular endothelial growth factor<sup>18 19</sup>. When compared to minocycline in the aforementioned study, minocycline was more effective in lowering MMP-9 activity than doxycycline. This finding is consistent with the studies of Paemen et al. who determined that minocycline – along with other tetracycline analogs currently not used in humans – is the most effective gelatinase inhibitor *in vitro*<sup>20</sup>.

When Koistinaho et al. studied the role of MMP-9 in stroke and the impact of minocycline treatment on MMP-9 knock out mice in a permanent cerebral ischemia model, they demonstrated that pre-injury treatment was protective only in wild type animals<sup>21</sup>, suggesting a role for MMP-9 in stroke and suggesting minocycline neurovascular protection might be at least partially attributed to its ability to interfere with MMPs. However, this MMP inhibition effect of minocycline after ischemic stroke had not been further elucidated. It remained unclear whether minocycline would retain this MMP inhibition ability when treatment is post-injury and delayed. It was also important to determine if minocycline MMP inhibition would be relevant to reperfusion-induced MMP activation that has been demonstrated after treatment with thrombolytic agent tissue plasminogen activator (tPA). The property of minocycline to inhibit proteolytic enzyme function may explain its ability to prevent increased vascular leakage as seen in the renal ischemia model. It also suggests that this drug may provide vascular protection by preventing proteolysis and the associated degradation of the blood brain barrier after ischemic stroke.

## **Matrix metalloproteinases and their relevance for acute ischemic stroke**

Ischemic stroke activates a complex cascade of events. The first tissue damage occurs as early as a few minutes after the occlusion. Along with mechanisms associated with ion imbalance, inflammation, neuronal excitotoxicity and apoptosis, several processes are activated that target the endothelial cells and the structural components of the blood brain barrier<sup>22</sup>. Within minutes to hours after an ischemic onset, endothelial and inflammatory cells respond to ischemia by releasing pro-inflammatory cytokines, oxidative mediators and extracellular proteases. Following the more acute phase of ischemia, a degradation of the basal lamina is also observed<sup>23</sup> and has been shown to ultimately weaken the blood brain barrier<sup>24</sup>. This extracellular matrix degradation has been associated with a time dependent ischemia-induced protease activation<sup>25</sup>, mainly with two specific matrix metalloproteinases: MMP-2 and MMP-9.

Matrix metalloproteinases are a subfamily of metalloproteinases of which at least 25 members are known. They are classically recognized as matrix-degrading enzymes involved in tissue remodeling during physiologic and pathologic processes. MMP expression and activity are tightly regulated at transcriptional and post translational levels. MMPs 2 and 9, also designated as gelatinases A (72 kDa) and B (92 kDa), are two extracellular MMPs that share similar substrate specificity and require extracellular proteolytic cleavage to become activated. These two enzymes have been extensively studied in the setting of acute ischemic stroke and they are elevated early after experimental stroke in rats<sup>26</sup>. The same trend was observed in the brain of primates

after temporary ischemia<sup>27</sup>. MMP-9 has also been demonstrated to be elevated in infarcted brain tissue collected from postmortem ischemic and hemorrhagic stroke patients<sup>28, 29</sup>. The two gelatinases have also been recognized as mediators of the degradation of the blood brain barrier that occurs after ischemic stroke. A study in mice has established a relationship between time of activation of MMP-9 with increased blood brain barrier permeability and formation of edema<sup>30</sup>. Importantly, the formation of edema and hemorrhagic transformation associated with thrombolysis with tissue plasminogen activator has been linked to MMPs<sup>31</sup>. In this study, rats undergoing temporary cerebral ischemia were treated with tPA. These animals had significantly higher levels of MMP-9 than their control counterparts. Treatment with a broad-spectrum synthetic MMP inhibitor reversed this MMP increase and hemorrhage volume. Although the exact mechanism of tissue damage is not clear, the gelatinases are widely accepted to play a role in the extracellular proteolysis in the brain. One possible pathway involves the ability of MMPs to digest components of the vascular matrix, including collagen, fibronectin and laminin  $\alpha$ -1. Damage to vascular integrity would then lead to disrupted cell-matrix-mediated signaling<sup>32</sup>. Furthermore, the inhibition of these enzymes by synthetic specific class inhibitors has been shown to prevent hemorrhage after thrombolysis also in an embolic model of stroke in rabbits<sup>33</sup>. The breakdown of components of the blood brain barrier, such as laminin and tight junction proteins claudin-5 and occluding have been shown to be reversed with MMP inhibition<sup>34, 35</sup>. Interestingly, studies have shown that the inhibition of MMP-9 with either specific monoclonal antibody targeting MMP-9 or highly specific MMP-9 inhibitor also leads to

reduction of infarct size<sup>36</sup> and rescues neurons from apoptosis<sup>35</sup>. These studies, therefore, reinforce the idea that MMPs do play a role in the overall outcome after stroke and that MMP inhibition may not only decrease the chances of hemorrhagic transformation but also decrease the infarct size.

MMPs are also believed to be involved in the thrombolysis-associated vascular damage after treatment with tissue plasminogen activator (tPA, or alteplase)<sup>37</sup>, which is the only therapeutic agent available for acute ischemic stroke. In a study with endogenous tPA knockout mice, researchers found that infarct size, edema and MMP-9 levels were all decreased compared to their wild-type counterparts<sup>38</sup>. Because tPA is an extracellular protease that is not specific to plasminogen cleavage only, reperfusion with tPA leads to increased chances of hemorrhagic transformation by amplifying the MMP damage in the brain as has been previously speculated<sup>39</sup>. A study with cultured astrocytes showed that tPA can have direct effects on this cell type by upregulating MMP-2 and MMP-9 and this leads to an increase in chemokines and cytokines<sup>40</sup>. Although recanalization with tPA is beneficial, it represents in itself an independent factor for vascular complications and hemorrhage after stroke<sup>41</sup>. In that context, MMP inhibition might also represent a target for decreasing the risks of thrombolysis as shown by Pfefferkorn and collaborators, that showed that MMP inhibition decreased tPA mediated mortality by 30%<sup>42</sup>. Furthermore, it could possibly increase the population who qualifies for tPA treatment since its application is limited to a short therapeutic time window (of 3 to 4.5 hours), after which it is believed the risks outweigh the benefits. The study from the

European cooperative acute stroke study group (ECASS) recently released their trial results that showed that although tPA increased the incidence of bleeding (asymptomatic and symptomatic intracerebral hemorrhage), the mortality rates did not differ and tPA remained beneficial towards an improved outcome when compared to placebo group after the 3 hour time window up to 4.5 hours after the onset of stroke symptoms<sup>43</sup>. However, although this extension in time window will likely benefit more acute ischemic stroke patients, 4.5 hours remains a relatively short period of time.

Due to the broad evidence that MMP-2 and MMP-9 are involved with the degradation of the blood brain barrier and with the increased risk of bleeding after thrombolysis, the inhibition of the proteolytic cascade with minocycline is a logical and appealing approach for vascular protection and therapy of acute ischemic stroke.

## **Summary**

Interference of the MMP cascade has been indeed largely explored as a potential target for vascular protection after stroke. However, there are currently no drugs marketed specifically for their ability to inhibit MMPs. The major clinical obstacle has been that available MMP inhibitors are highly insoluble, not specific within the class and have an unknown human toxicological profile. Because minocycline has demonstrated to inhibit collagenolysis, this drug might inhibit MMPs originated in the brain after ischemia and might represent a potential drug for acute vascular protection.

**In light of this, the results of this study helped assess if minocycline could potentially be useful for acute stroke patients, especially those who are at a higher risk of developing hemorrhagic transformation.**

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## CHAPTER 2

DELAYED MINOCYCLINE INHIBITS ISCHEMIA-ACTIVATED MATRIX  
METALLOPROTEINASES 2 AND 9 AFTER EXPERIMENTAL STROKE<sup>1</sup>

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Published Manuscript: <sup>1</sup> Livia S. Machado, Anna Kozak , Adviye Ergul , David C. Hess,  
Cesario V. Borlongan and Susan C. Fagan

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## **Abstract**

**Background:** Matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) are increased in the brain after experimental ischemic stroke in rats. These two proteases are involved with the degradation of the basal lamina and loss of stability of the blood brain barrier that occurs after ischemia and that is associated with thrombolytic therapy in ischemic stroke. Minocycline is a lipophilic tetracycline and is neuroprotective in several models of brain injury. Minocycline inhibits inflammation, apoptosis and extracellular matrix degradation. In this study we investigated whether delayed minocycline inhibits brain MMPs activated by ischemia in a model of temporary occlusion in Wistar rats.

**Results:** Both MMP-2 and MMP-9 were elevated in the ischemic tissue as compared to the contra-lateral hemisphere after 3 hours occlusion and 21 hours survival ( $p < 0.0001$  for MMP-9). Intraperitoneal minocycline at 45mg/kg concentration twice a day (first dose immediately after the onset of reperfusion) significantly reduced gelatinolytic activity of ischemia-elevated MMP-2 and MMP-9 ( $p < 0.0003$ ). Treatment also reduced protein concentration of both enzymes ( $p < 0.038$  for MMP-9 and  $p < 0.018$  for MMP-2). *In vitro* incubation of minocycline in concentrations as low as 0.1  $\mu\text{g/ml}$  with recombinant MMP-2 and MMP-9 impaired enzymatic activity and MMP-9 was more sensitive at lower minocycline concentrations ( $p < 0.05$ ).

**Conclusion:** Minocycline inhibits enzymatic activity of gelatin proteases activated by ischemia after experimental stroke and is likely to be selective for MMP-9 at low doses. Minocycline is a potential new therapeutic agent to acute treatment of ischemic stroke.

## Background

Matrix metalloproteases (MMPs) are a family of zinc dependent proteases responsible for the extracellular matrix turnover and degradation of bioactive proteins. In cerebral ischemia, MMPs 2 and 9, also designated as gelatinases A (72 kDa) and B (92 kDa) have been identified to mediate the degradation of the basal lamina<sup>1,2</sup> and hemorrhagic transformation<sup>3</sup>. MMPs 2 and 9 have been shown to be elevated a few hours after ischemia<sup>4</sup> and to maintain increased activity for days after the onset<sup>2,5</sup>. The inhibition of these enzymes by specific class inhibitors reverts breakdown of laminin<sup>6</sup> and prevents increased barrier permeability, edema, and hemorrhage after ischemic stroke<sup>7-9</sup>. The development of MMP inhibitors as therapeutic agents has been limited by their poor solubility.

Minocycline is a commonly used semi-synthetic tetracycline with anti-inflammatory and anti-apoptotic properties<sup>10,11</sup>. Minocycline interferes with MMP activity<sup>12-13</sup> and has been shown to be neuroprotective in cerebral ischemia<sup>14</sup> and in other models of brain injury<sup>15,16</sup>.

In this study we investigated whether minocycline prevents ischemia-induced MMP activation in a temporary model of temporary focal cerebral ischemia in rats, and explored the dose-response relationship and possible specificity of *in vitro* MMP-inhibition by this drug. Because MMP-2 and MMP-9 have distinct mechanisms of activation and different roles after stroke, the potential selectivity of minocycline for either enzyme should be determined. Furthermore, MMP inhibition may be the central

mechanism through which minocycline provides neurovascular protection <sup>17</sup>. There are currently no drugs marketed specifically for their ability to inhibit systemic MMPs. Minocycline could represent a new clinical approach to inhibiting acute MMP activation, reducing the risk of development of hemorrhage and protecting the patient from further damage after acute ischemic stroke.

## **Results**

### **MMP activity after ischemic stroke**

The 3 hour ischemia followed by 21 hour survival after reperfusion caused increased activity of MMP-2 and MMP-9 in the ischemic hemisphere (Fig. 2.1) as compared to the contra-lateral hemisphere (n=8, p<0.0001 for MMP-9, MMP-2 activity was increased, but not significantly). The densitometric analysis revealed also that intra-peritoneal minocycline 45 mg/kg treatment twice a day (first dose immediately after reperfusion), significantly reduced the ischemia-induced increments in both MMP-2 and MMP-9 active gelatinolytic forms (n= 6 for control and 8 for treated animals) (p<0.0003) (Fig. 2.2). The same treatment regimen with intra-peritoneal minocycline also significantly reduced the ischemia-induced increase in MMP total protein concentration (Fig. 2.3) (n= 6 for PBS and 8 for minocycline treated groups, p<0.038 for MMP-9 and p<0.018 for MMP-2).

### **Neurological evaluation**

All animals had a significant deficit (scored 3 points in the Bederson Scale) prior to reperfusion, demonstrating successful MCAO. The neurological scores did not show a

significant difference between the treatment and control groups. The scale shows a high variability between animals and a much higher sample size is required to detect a significant difference (data not shown).

### ***In vitro* MMP inhibition by minocycline**

The densitometric analysis revealed that minocycline at 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml, 500 µg/ml and 1000 µg/ml concentrations significantly inhibited the activity of recombinant MMP-2 and recombinant MMP-9 as compared to the control enzymatic activity. The inhibitory effect of minocycline in both enzymes was similar or slightly greater than the positive control EDTA in 1 µg/ml concentration (not shown). There was a significant interaction between the two proteases and minocycline drug concentration such that, at concentrations of 100 µg/ml and below, the percent inhibition is significantly higher for MMP-9 ( $p < 0.05$ ) (Fig 2.4).

### **Discussion**

This study demonstrated for the first time that delayed treatment with minocycline inhibits MMPs that are elevated after temporary experimental cerebral ischemia. In addition, this inhibitory effect is present over a wide range of clinically relevant and safe concentrations of minocycline. In fact, the minocycline concentration expected in the brain after a normal dose of 200 mg in humans (3 mg/kg in rodents), is equivalent to 0.5-1 µg/ml<sup>18</sup> and is higher than the lowest *in vitro* doses tested. Furthermore, our results reinforce previous findings that minocycline is a potent inhibitor of MMP-9 *in vivo*<sup>19</sup> and *in vitro*<sup>13</sup> and suggests a sensitivity of this enzyme for the drug when compared

to MMP-2. Our drug dosing regimen was based on previous studies demonstrating therapeutic efficacy of intra-peritoneal minocycline 45 mg/ kg in experimental stroke <sup>14</sup>, <sup>18</sup> and to achieve a constant plasma concentration since the timing of MMP activation spike has not yet been identified either in humans or in rodents. Intra-peritoneal delivery of minocycline in our model of stroke also insures that regardless of differences in the pharmacokinetics of this drug in rodents, the drug will be present whenever the activation of the MMP enzymes occurs, as the delivery of the drug is slow and constant.

Minocycline has been shown to be neuroprotective <sup>14</sup>, <sup>20-21</sup>. One common pathophysiological mechanism of models of brain ischemic damage is the dysregulation of the proteolytic cascade at the level of the endothelial and microglial cells. Interference of this cascade by minocycline might be the central pathway for these neurovascular protective properties of decreasing tissue injury and also providing functional recovery. Ischemic stroke activates a complex cascade of events <sup>22</sup>. The first tissue damage occurs as early as a few minutes after the occlusion. Within hours after stroke onset, endothelial and inflammatory cells respond to ischemia releasing proteases MMP-2 and MMP-9 that culminate in degradation of the basal lamina and ultimate weakening of the blood brain barrier <sup>23</sup>. The subsequent events are leakage of leukocytes into the parenchyma, and brain edema followed by neuronal death that occurs in the later hours of ischemia. Since MMP-2 and MMP-9 are believed to be largely responsible for the degradation of the blood brain barrier and also involved in the signaling of neuronal cell death <sup>24-25</sup>, the inhibition of the proteolytic cascade is a logical target for several models of brain injury that involve matrix degradation and vascular instability <sup>26</sup>.

Our studies point to an apparent selectivity of minocycline for MMP-9 with low dose minocycline. This could reflect either a time profile difference between the activation of the two enzymes, the pathway minocycline interferes, or a chemical selectivity for MMP-9. Among the main differences between their molecular pathways, MMP-2 is constitutively expressed, while MMP-9 can be stimulated at the level of gene activation by multiple stimuli including ischemia. Both enzymes are expressed as pro-forms and after they are secreted they require chemical or proteolytic activation to become functional<sup>27</sup>. In endothelial brain cells, little is known about the presence and regulation of the different MMPs<sup>28-29</sup>. Further studies are necessary to determine the mechanisms of activation of MMPs in the brain after ischemia and the mechanism by which minocycline decreases MMP activity.

Minocycline has in its chemical structure an active binding site that confers the ability to chelate divalent ions. Our *in vitro* studies confirm that minocycline is able to inhibit gelatin digestion by MMPs by interacting with these enzymes. MMPs require zinc in their active site for functional activity and removal of the zinc results in change of conformation and inactivation of the enzyme. Tetracyclines have been shown to inhibit collagenolysis<sup>30, 31</sup> and inhibit MMP-9 activity<sup>14</sup>. Previous studies, however, have shown that multiple tetracyclines can inhibit collagenase MMPs via down-regulation of gene expression in a cellular model of rheumatoid arthritis using cultured chondrocytes<sup>32</sup> and that pre-treatment with minocycline down-regulates pro-MMP expression after permanent cerebral ischemia<sup>17</sup>. Furthermore, because minocycline has been shown to be neuroprotective in different models of brain injury, it is likely to act by multiple

mechanisms, with MMP inhibition being a central link for its anti-inflammatory and anti-apoptotic properties in the ischemic cascade and reperfusion. Our studies are pioneer in investigating the ability of minocycline to decrease MMP activity in the setting of a temporary model of stroke, and with treatment occurring post reperfusion. Increase in MMP activity and its consequences are relevant in stroke from the standpoint of ischemic damage itself and also from the standpoint of delayed reperfusion damage alone.

In order to determine the clinical relevance of these findings for acute stroke treatment, the next step is to determine if the MMP inhibition by minocycline in a model of temporary occlusion will result in decreased blood brain barrier degradation and decreased hemorrhage formation. In addition, the vascular protection properties of minocycline should also be studied for ischemic stroke after treatment with tissue plasminogen activator (tPA). tPA is currently the only available acute treatment for ischemic stroke patients but its application is limited due to the increased risk of serious intracerebral bleeding <sup>33</sup>. Because tPA is an extracellular protease and is not highly specific for plasminogen cleavage, reperfusion with tPA might amplify the MMP damage in the brain as has been previously speculated <sup>34, 35</sup> and concurrent administration of minocycline with tPA or other reperfusion agents might be a potential approach for reestablishing blood flow without compromising the vasculature. These studies are currently ongoing.

## **Conclusions**

Our studies provide evidence that the inhibition of MMP enzymatic activity can be achieved with intraperitoneal treatment of minocycline after experimental ischemic stroke. MMP inhibition is likely a mechanism through which minocycline is neuroprotective in stroke. Furthermore, the *in vitro* inhibitory effect of minocycline is observed at very low therapeutic concentrations with MMP-9 being more. Because thrombolytic agents exacerbate the proteolytic cascade, minocycline raises optimism for the acute treatment of ischemic stroke patients. Minocycline is a candidate neuroprotective agent to be used in combination with tPA.

## **Methods**

### **Drug preparation and regimen**

Minocycline powder was purchased from Sigma Aldrich CO., Saint Louis, MO. The drug was prepared fresh one hour prior to administration by dissolution in phosphate buffered saline (PBS) pH 7.4 (Fisher Scientific, Pittsburgh, PA). The preparation tube was covered with aluminum foil and protected from light. Minocycline was administered intraperitoneally to animals in two doses of 45 mg/kg, the first one being 5 minutes after the onset of reperfusion and the second one 12 hours later. This dosing regimen was based on previous studies<sup>14, 18</sup>. The injection was 9 ml/kg. Animals were randomized between the minocycline treated group and the PBS treated group and received equivalent injection volumes.

### **Animal procedures and experimental stroke**

The Institutional Animal Care and Use Committee (IACUC) of the Veterans Affairs Medical Center approved the protocol. Male Wistar rats purchased from Charles River Laboratory (Wilmington, MA) were used. Animals used were within a range of 270-300g of body weight. Cerebral ischemia was induced by intraluminal suture occlusion of the MCA<sup>36</sup>. A 20 mm 3.0 nylon monofilament with round tip was inserted through the right internal carotid artery up to the origin of the MCA. After 3 hours of ischemia the animals were reperfused by removing the suture. The animals were anesthetized with isoflurane inhalation in a glass chamber prior to stroke procedure and reperfusion. The anesthesia was kept by 2% isoflurane during surgery. Body temperature was maintained with heating pad during surgery and reperfusion. The animals were evaluated neurologically and for motor ability using the Bederson Scale<sup>37</sup> prior to reperfusion and sacrifice. Prior to sacrifice, which occurred 24 hours after stroke, the animals were anesthetized with a cocktail of ketamine (45 mg/kg) and xylazine (15 mg/kg) via intramuscular injection. The animals were then perfused with ice cold PBS, sacrificed and the brains were extracted.

### **Tissue processing, immunoblotting and gelatin zymography**

After extraction, the brain was washed with PBS and placed in a coronal matrix. The olfactory bulb and the first 2 mm-thick slice of the anterior brain were discarded. Four consecutive 2 mm slices corresponding to the major infarcted area and the contralateral hemisphere were separated as a block. The ischemic and non ischemic hemispheres were then separated, placed in cryotubes and flash frozen in liquid

nitrogen. The tubes were stored in a -80°C freezer until homogenization. The samples were homogenized in 450 µL of cold working buffer containing 50 mM Tris-HCl (pH 7.5), 75 mM NaCl, and 1 mM PMSF as described by Heo and collaborators<sup>3</sup>. The tissue was processed with a homogenizer for 10 minutes and centrifuged at 4°C for 20 minutes at 13000 rpm. The supernatants were separated, frozen and kept at -80°C until use. The total protein concentration was assessed by the Bradford method.

On the day of the experiment, the volume equivalent to 50 µg of total protein was loaded into fresh made 10% polyacrylamide gels (Immunoblotting) or gelatin zymography gels. For the zymography experiments, the gels were electrophoretically separated under non reducing conditions and 100 V. After electrophoresis the zymogram gels were washed in 125 ml 2.5% Triton twice for 20 minutes. The gels were then incubated in activation buffer (Zymogram Development Buffer, Bio-Rad, Hercules, CA) for 20 hours at 37°C. The next day, the gels were stained with Coomassie Blue R-250 Staining Solution (Bio-Rad) for 3 hours and destained for 25 minutes with Destain Solution (Bio-Rad). The gelatinolytic activity of the samples was assessed by densitometric analysis (Gel-Pro v 3.1, Media Cybernetics, Carlsbad, CA) of the bands as a relative comparison to a standard band of recombinant enzyme. To minimize inter-gel variability, all gels had a control lane loaded with 0.5 ng recombinant enzyme, which was used as a standard optical density and enzyme amount (in ng). The lytic bands identified in the zymogram gels were subjected to molecular weight identification with the use of pre-stained standard protein marker (Bio-Rad). For the immunoblotting experiments, the gels were transferred into nitrocellulose membranes for one hour.

Membranes were blocked with blotting grade blocker non-fat dry milk (Bio-Rad). After washing with 0.1% tween 20- tris- buffered saline (TTBS), the membranes were incubated with either anti MMP-2 human (mouse) monoclonal antibody (Oncogene Research Products) or anti MMP-9 monoclonal mouse antibody overnight at 4°C. Membranes were washed again in TTBS, incubated with secondary antibody (goat anti-mouse IgG, horseradish peroxidase conjugated antibody, Calbiochem) for one hour and finally developed with horseradish peroxidase development solution (ECL advance detection kit, Amersham). The membranes were exposed to autoradiography films (Hyblot CL, Denville Scientific Inc.). The density of the sample bands for the immunoblots and zymograms were expressed as maximal optical density relative to the standard band.

### ***In vitro* MMP inhibition**

The ability of minocycline to inhibit MMPs was tested by a direct, cell free *in vitro* system, in which minocycline was allowed to incubate at room temperature with recombinant active MMP-2 and MMP-9 (Oncogene, EMD Biosciences, La Jolla, CA). Each enzyme was incubated separately. 0.05 nanogram of recombinant enzyme (1:10,000 dilution from the 5 µg/ 50 µl commercial stock) was incubated with a range of clinically relevant concentrations: 0.1, 0.5, 1, 10, 50, 100, 500 and 1000 µg/ml of minocycline prepared in deionized purified water. Two control samples were used: the negative control in which the enzyme was incubated with no drug, and the positive control in which the enzyme was incubated with 10 µg/ml EDTA. The samples were incubated for 2 hours and separated on 10% zymogram gels as described above. The

gels were allowed to run in 100V under non-reducing conditions. After electrophoresis, the gels were washed twice with 2.5 % Triton for 20 minutes each cycle. They were then incubated for 20 hours with Zymogram Development Buffer at 37°C. The gels were stained with Coomassie Blue R-250 (Bio-Rad) for 3 hours and destained with Destain Solution (Bio-Rad) for 25 minutes. The gelatinolytic bands were analyzed with densitometry (GelPro).

### **Statistics**

A 2 MMP type (MMP-2 and MMP-9) by 2 group, (saline or minocycline), analysis of variance (ANOVA) on the ranks of the enzyme activity was used to determine the differences in MMP activity, treatment group and their interaction. A 2 MMP type (MMP-2 or MMP-9) by 6 concentrations (minocycline 0.1 µg/ml, minocycline 0.5 µg/ml, minocycline 1 µg/ml, minocycline 100 µg/ml, minocycline 500 µg/ml, and minocycline 1000 µg/ml) ANOVA was used to determine differences in percent inhibition for MMP type, minocycline concentration, and their interaction. A Tukey's test was used to adjust for post-hoc multiple comparisons. Statistical significance was determined at  $p < 0.05$  and SAS 9.1.3 was used for all analyses.

### **Authors' contributions**

LSM assisted with surgeries, prepared and administered the treatments, performed zymography and prepared the manuscript. AK performed the stroke surgeries and contributed to the preparation of the manuscript. AE contributed to the optimization of zymograms, data interpretation and preparation of the manuscript. DCH and CVB

contributed to the design, interpretation and preparation of the manuscript. SCF coordinated all aspects of this work and the manuscript. All authors approved the final version of the manuscript.

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**Legends:**

Figure 2.1: The ischemic hemisphere has increased MMP-9 (significant increase) and MMP-2 activity as compared to the contra-lateral non ischemic control hemisphere in non-treated animals. Sample sizes: n=8 for both PBS and minocycline treated groups ( $p < 0.0001$  for MMP-9 increase). Each band represents one different replicate sample. Molecular weights were determined with the use of pre-stained protein standards.

Figure 2.2: Intra-peritoneal minocycline 45 mg/kg treatment twice a day (first dose immediately after reperfusion), significantly reduced the ischemia-induced increase in MMP-2 and MMP-9 gelatinolytic activities (represented by the active form bands; 67 and 86 kDa respectively). Sample sizes: PBS n=6; Minocycline n=8 ( $p < 0.0003$ ). Each band represents one different replicate sample.

Figure 2.3: Intra-peritoneal minocycline 45 mg/kg treatment twice a day (first dose immediately after reperfusion), significantly reduced the ischemia-induced increase in MMP-2 and MMP-9 total protein concentrations as compared to PBS treated control animals. Sample sizes: PBS n=6; Minocycline n=6 ( $p < 0.038$  for MMP-9 and  $p < 0.018$  for MMP-2). Each band represents one different replicate sample.

Figure 2.4: Minocycline concentrations ranging within 0.1 and 1000  $\mu\text{g/ml}$  inhibits the activity of 0.05 ng recombinant human MMP-2 and MMP-9 as compared to the control enzymatic activity. At low concentrations, MMP-9 inhibition is greater than that of MMP-2 ( $p < 0.05$ ).

Figure 2.1

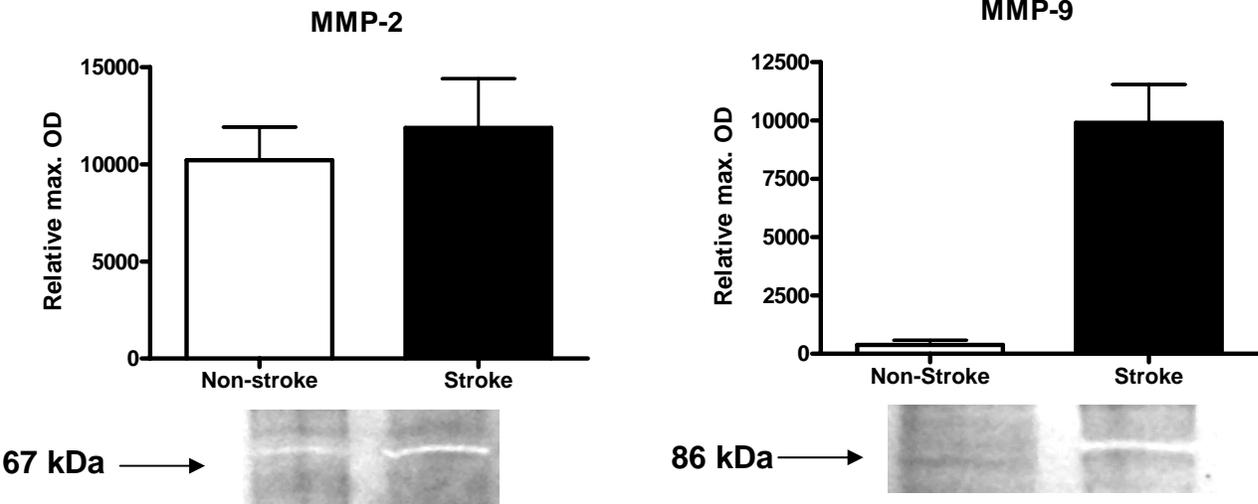


Figure 2.2

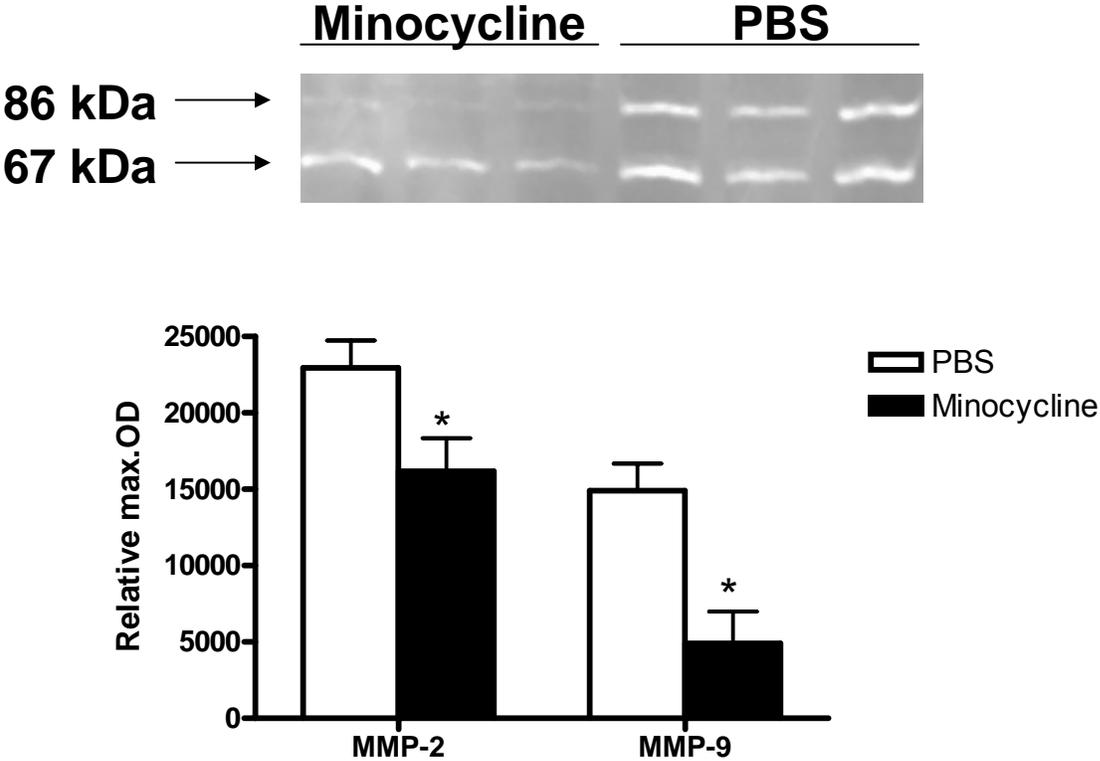


Figure 2.3

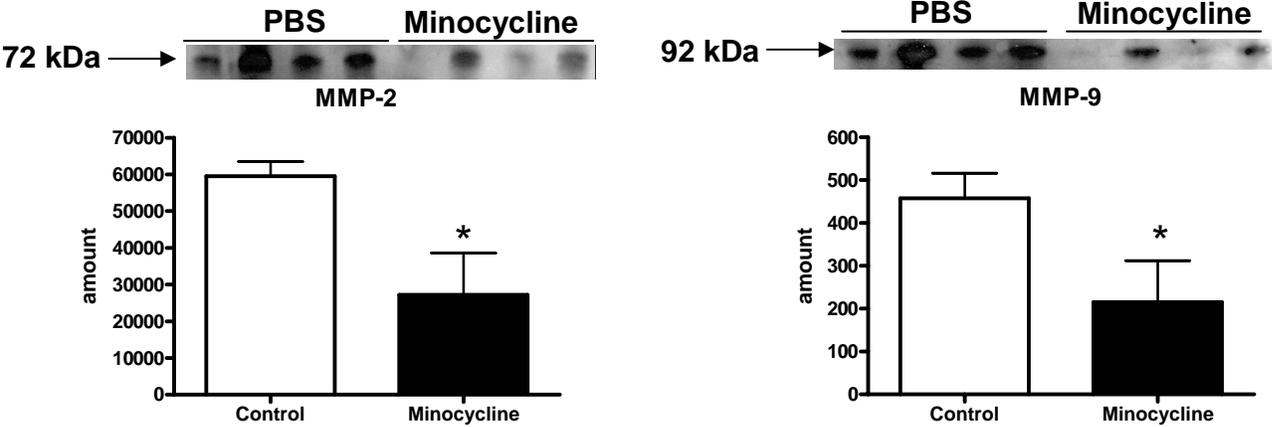
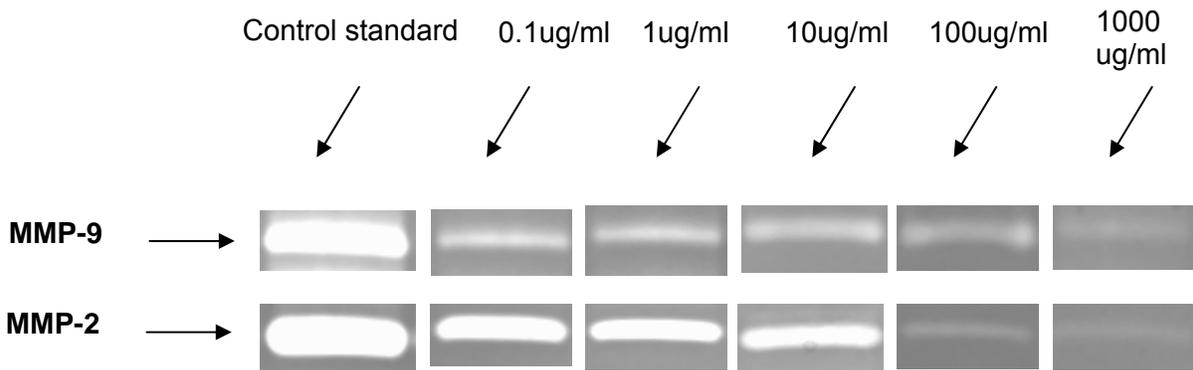
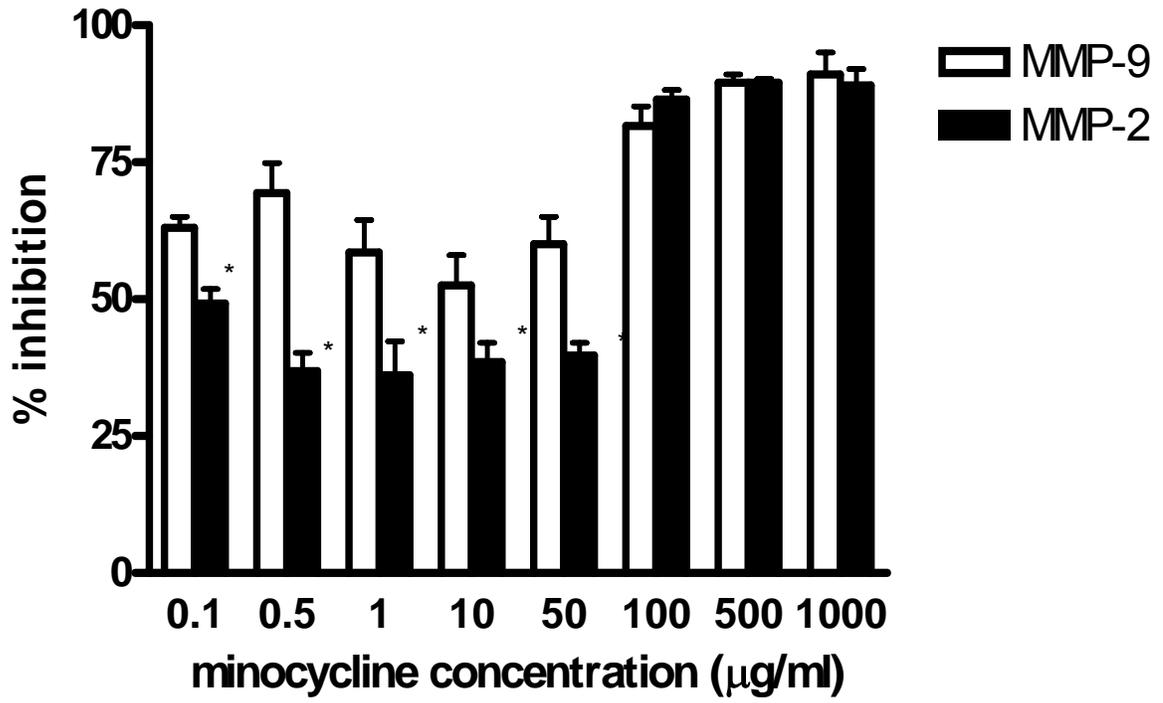


Figure 2.4



## CHAPTER 3

### MINOCYCLINE AND TISSUE PLASMINOGEN ACTIVATOR: PRECLINICAL EVALUATION PRIOR TO TRANSLATION TO STROKE PATIENTS<sup>1</sup>

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<sup>1</sup> Livia S. Machado, Irina Sazonova, Anna Kozak, Daniel C. Wiley, Azza B. El-Remessy, Adviye Ergul, David C. Hess, Jennifer L. Waller, Susan C. Fagan.  
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## **ABSTRACT**

**Background and purpose:** New treatment strategies for acute ischemic stroke must be evaluated in the context of effective reperfusion. Minocycline is a neuroprotective agent that inhibits proteolytic enzymes and therefore could potentially both inactivate clot lysis and decrease the damaging effects of tissue plasminogen activator. This study determined the effect of minocycline on tPA clot lysis and tPA induced hemorrhage formation after ischemia.

**Methods:** Fibrinolytic and amidolytic activities of tPA were investigated *in vitro* over a range of clinically relevant minocycline concentrations. A suture occlusion model of three hour temporary cerebral ischemia in rats treated with tPA and two different minocycline regimens was used. Blood brain barrier basal lamina components, MMPs, hemorrhage formation, infarct size, edema and behavior outcome were assessed.

**Results:** Minocycline did not affect tPA fibrinolysis. However, adjuvant treatment with minocycline 45 mg/ kg intraperitoneally decreased tPA-induced MMP-9 activity to below control levels ( $p = 0.0136$ ). Minocycline 3 mg/kg intravenous treatment decreased total protein expression ( $p = 0.001$  for 92 kDa and  $p=0.0084$  for 87 kDa). It also decreased incidence of hemorrhage ( $p = 0.019$ ), mortality, and improved neurological outcome ( $p=0.0001$  for Bederson score and  $p=0.0391$  for paw grasp test). MMP inhibition was associated with decreased degradation in collagen IV and laminin- $\alpha 1$  ( $p=0.0001$ ).

**Conclusions:** Combination treatment with minocycline is beneficial in tPA-treated animals and does not compromise clot lysis. These results also suggest minocycline neurovascular protection after stroke may involve the direct protection of the blood brain barrier during thrombolysis with tPA.

## **BACKGROUND**

The scientific community and regulatory bodies demand that all new acute treatments of ischemic stroke be evaluated for their potential to interact with the only approved therapy, tissue plasminogen activator (tPA). Intravenous treatment with tPA within three hours of onset has been shown to be beneficial in achieving better outcomes <sup>1</sup> and recent information suggests that carefully selected patients may benefit when treated even up to 4.5 hours after the onset of symptoms <sup>2</sup>. However, there is still a great need to develop treatments that complement and enhance the safety and efficacy of tPA.

Recent data have shown that tPA has deleterious effects that are independent of its fibrinolytic activity. tPA leads to increased chances of hemorrhagic transformation by amplifying the matrix metalloproteinase (MMP) cascade triggered by ischemic damage in the brain <sup>3</sup>. MMPs 2 (72 kDa) and 9 (92 kDa) have been shown to be elevated early after experimental stroke in rats <sup>4, 5, 6</sup> and the formation of edema and hemorrhagic transformation associated with thrombolysis with tPA has been linked to MMPs <sup>7, 8, 9</sup>. MMP inhibition might represent a target for decreasing the risks of thrombolysis <sup>10</sup>.

Minocycline treatment has been shown to decrease the disruption and leakage of the blood brain barrier <sup>11</sup> and attenuate the enzymatic activity of the proteolytic enzyme MMP-9 following stimulation with vascular endothelial growth factor <sup>12</sup>. Furthermore, minocycline has been shown to decrease microvascular permeability associated with MMP-2 and MMP-9 activity <sup>13</sup>. Our previous studies showed that post – injury

minocycline treatment decreases the activation of MMPs after temporary cerebral ischemia<sup>14</sup>.

Although minocycline is a promising neuroprotective strategy, a thorough investigation of its interaction with tPA after stroke has not been pursued. Minocycline could reduce tPA's fibrinolytic activity through its enzyme inhibitory effect. However, it could also prevent reperfusion– induced MMP activation and vascular damage. This study aimed to determine whether the interaction of minocycline with tPA was significant *in vitro* and in an experimental model of stroke.

## **MATERIALS AND METHODS**

### **tPA *in vitro* clot lysis and amidolytic activity**

tPA activity was measured by *in vitro* fibrinolytic and amidolytic assays. For the fibrinolytic assay, whole blood was obtained from 4 healthy individuals. This study was approved by the Human Assurance Committee of the Medical College of Georgia. Whole blood (50  $\mu$ l) from healthy individuals was mixed with trace amounts of <sup>125</sup>I-labeled human fibrinogen (100,000 cpm) and clotted. The formed clots were washed, suspended in plasma (1 ml) and placed in a water bath at 37°C. A wide range of clinically relevant concentrations of minocycline (0-30  $\mu$ g/ml) were added to the supernatant, and clot lysis was immediately initiated by t-PA 2nM (Activase; Genentech). The degree of fibrinolysis was measured at various times of incubation by counting the percent soluble <sup>125</sup>I-fibrin degradation products<sup>15, 16</sup>. The kinetic parameters of amidolysis were measured in the presence or absence of minocycline

with H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide dihydrochloride (S-2288; Chromogenic) as substrate. 100 nM tPA was added to the microtiter plate containing assay buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 8.4), minocycline (30 µg/ml) and S-2288 (150- 1500 µM) at 37°. The generation of amidolytic activity was measured at 405 nm for 7 minutes in a microplate reader (Synergy HT, Bio-Tech). The data was plotted as velocity of p-nitroanilide release over substrate concentration and analyzed by hyperbolic curve fitting with GraphPad Prism software <sup>17</sup>.

### **Animal procedures and experimental stroke**

The Institutional Animal Care and Use Committee (IACUC) of the Veterans Affairs Medical Center approved our study protocol. Male Wistar rats in a range of 270-300 g of body weight purchased from Charles River Laboratory (Wilmington, MA) were used. Cerebral ischemia was induced by intraluminal suture occlusion of the middle cerebral artery occlusion (MCA) for 3 hours <sup>14</sup>. Immediately before removal of the suture, the jugular vein was exposed and the tip of a rat jugular vein catheter (Braintree Scientific R-JVC) plugged with a catheter port plug (Braintree Scientific 23-PP) was inserted into the lumen. After removing the occlusion suture from the internal carotid artery, the jugular catheter was connected to a polyethylene tube 50 and the bolus injection containing 10 % of the tPA dose was pushed, followed by a 20 minute infusion (Harvard Apparatus Infusion Pump). Animals were randomized between 4 groups: control (saline treated), tPA (10 mg/kg) alone, tPA plus minocycline (Sigma Aldrich CO.) intravenously (IV) and tPA plus minocycline intraperitoneally (IP) groups. Minocycline 45 mg/kg was

injected IP 5 minutes after the onset of reperfusion and the second one 12 hours later. Sacrifice occurred 24 hours after stroke.

### **Gelatin Zymography and MMP immunoblotting**

After extraction, the brain was washed with PBS and placed in a coronal matrix to be sliced and homogenized as previously described by Heo and collaborators<sup>5, 14</sup>. Gelatin Zymography and MMP 2 and 9 immunoblotting were performed as reported in our previous study [14]. The bands were quantified with the use of the GelPro image analysis software.

### **Collagen type IV and laminin- $\alpha$ 1 slot blot analysis**

The basal lamina components laminin- $\alpha$ 1 and collagen type IV were used to determine the health of the blood brain barrier in the brains of the experimental animals. Both collagen IV and laminin proteins were studied with slot blotting and semi quantification by densitometric analysis. The same brain tissue homogenate used for MMP analysis was used. Nitrocellulose membranes were used. Samples were loaded into the Bio – Dot apparatus (Bio-Rad Laboratories, CA) and a slow vacuum was applied. Collagen type IV antibody (rabbit polyclonal anti – collagen IV, Santa Cruz Biotechnology) and laminin- $\alpha$ 1 (goat polyclonal anti – laminin- $\alpha$ 1, Santa Cruz Biotechnology) were used. Secondary antibodies anti- rabbit IgG and anti – goat IgG horseradish peroxidase conjugated antibodies were used, respectively. The membranes were developed and exposed to autoradiography films (Hyblot CL, Denville Scientific Inc.). The semi

quantification of the bands was performed with the use of the GelPro image analysis software.

### **Infarct size and edema determination**

The infarct volume were measured using 2,3,5 – triphenyltetrazolium chloride (TTC) stained brain slices as previously described <sup>18</sup>. The image of the slices were captured and analyzed with the Zeiss KS300 software. The total infarct volume was reported as percent volume to the total ischemic hemisphere. The edema was quantified by the percent difference of volume between the stroke and contralateral hemispheres.

### **Hemorrhage formation and enzyme linked immunosorbent assay for hemoglobin**

The presence of visible hematoma or hemorrhagic transformation was recorded and the quantification of hemorrhage was done by assessing the hemoglobin content in the tissue after complete perfusion of the brain. This was accomplished by an ELISA method for hemoglobin, as has been previously reported <sup>19</sup>.

### **Neurological examination**

The animals are examined immediately prior reperfusion and immediately prior sacrificing for motor function. The Bederson method <sup>20</sup> and paw grasp test were used. The paw grasp test measures in a scale of 0 to 3, the use and grasping strength of the ipsilateral forelimb. The occurrence of death – or near death, unable to perform tests – was also recorded.

## Data analysis

To examine differences in various outcome measures between treatment groups, chi-square tests (if the variable was categorical) and one-way ANOVA (if the variable was continuous) were performed. Because not all post hoc pair wise comparisons were warranted between treatment groups, a Bonferroni adjustment to the overall alpha level for the number of post hoc comparisons were used. All statistical analyses were performed using SAS 9.1.3 and overall statistical significance was assessed using an alpha level of 0.05.

## RESULTS

### tPA activity

Kinetic studies were performed to determine whether minocycline may directly affect tPA proteolytic activity. The kinetic parameters of cleavage of the tripeptide substrate S-2288 at pH 7.4 and 37°C by tPA are shown in Table 1. Minocycline (30 µg/ml) did not change either the apparent Michaelis-Menten constant ( $K_m$ ) or the catalytic constant ( $K_{cat}$ ). Thus, minocycline does not affect amidolytic efficiency of tPA. To test fibrinolytic activity of tPA, *in vitro* clot lysis assay was used. In the presence of different minocycline concentrations (1-30 µg/ml), the rate of clot lysis by tPA (2 nM) remained to be 17%, 38% and 55% after 35, 75 and 120 minutes, respectively, regardless of minocycline concentration (Fig.1). At no concentrations tested did minocycline decrease fibrinolysis by tPA.

## **Matrix metalloproteinases 2 and 9**

Only two bands were detected by gelatin zymography: 85 kDa and 67 kDa, corresponding to active MMP-9 and active MMP-2 respectively. tPA treatment during reperfusion increased the activity of both MMP-2 and MMP-9 in the brain. In the minocycline IP – but not in the IV group – animals had significantly decreased tPA-induced exacerbation of MMP activity compared to untreated stroke animals ( $p=0.0136$ ). MMP-9 activity was decreased below control levels. IV treatment however, impacted the protein content of MMP-2 (detected at 72 kDa) and MMP-9 (92 and 87 kDa bands) in the brain (Fig. 2B). Minocycline delivered through intravenous injection significantly decreased all the bands detected ( $p= 0.0034$  for MMP-2,  $p=0.0084$  for MMP-9 87 kDa and  $p=0.001$  for MMP-9 92 kDa). Treatment with intravenous minocycline appeared, however to decrease plasma MMP-9 activity but not MMP-2 when compared to animals treated with tPA alone.

## **Minocycline plus tPA and vascular outcome**

We measured vascular integrity using four different parameters: 1) incidence of visible hemorrhagic transformation, 2) the content of hemoglobin in the brain parenchyma after complete perfusion, 3) the formation of brain edema and 4) basal lamina protein degradation. Although the content of tissue hemoglobin in tPA treated animals was only slightly larger than in untreated animals (not shown), the occurrence of bleeding observed macroscopically in these animal brains was two fold higher than control and minocycline IV treated animals ( $p = 0.0190$ ) (Fig. 3A). Inspection of animals that died prematurely also revealed that tPA-treated animals had developed large brain

hematomas as illustrated by Fig. 3B. Furthermore, edema in the ischemic hemisphere was slightly elevated in the tPA group and decreased in combination with IV minocycline (Fig. 3C). Stroked brains had significantly reduced collagen type IV compared to sham brains ( $p=0.0011$ ). tPA treatment further reduced the amount of collagen and combination treatment with 3 mg/ kg IV minocycline preserved this protein to above stroked control animals (Fig. 4A). Likewise, IV minocycline robustly prevented laminin- $\alpha$ 1 degradation in the brain ( $p=0.0001$ ) (Fig. 4B).

### **Infarct size and neurological evaluation**

tPA increased infarct volume when compared to stroked control animals (infused with just saline) ( $p=0.0035$ ). Intravenous minocycline animals had decreased infarct size compared to tPA alone animals, but it remained above control levels and it was not statistically significant (Fig. 5A). Only animals that had a maximum deficit before reperfusion, demonstrating successful MCAO prior to reperfusion, were included in these results. Minocycline treatment acutely (IV) at low dose resulted in improvement of the performance of animals ( $p=0.0001$  for the Bederson score and  $p=0.0391$  for paw grasp) (Figs. 6 A and B). Although intraperitoneal treatment with minocycline seemed to decrease the impairment, the effect was not statistically significant, suggesting early delivery is critical to achieve optimal improved functional outcome.

### **DISCUSSION**

This study demonstrated that the MMP inhibition effect of minocycline did not impair the ability of tPA to cleave plasminogen and exert its fibrinolytic effect. This was confirmed

in vivo by Murata and collaborators <sup>21</sup>. In their study, minocycline treatment did not appear to have significant effect on the cerebral perfusion restored by tPA after embolic stroke in rats. However, the clot lysis effect of the treatment interaction was not studied directly.

We demonstrated in this study that post-reperfusion treatment with both low dose IV minocycline and high dose IP minocycline inhibited MMPs that are upregulated by treatment with tPA. These two drug dosing regimens were based on previous studies demonstrating therapeutic efficacy of minocycline in experimental stroke <sup>18, 22</sup>. Intraperitoneal administration has been shown to achieve a *delayed* but constant plasma concentration <sup>23</sup>. However, because systemic delivery of minocycline is delayed, we also tested whether acute delivery of minocycline (by IV administration) would achieve improved or comparable results. Both treatments decreased the tPA induced MMP-9 to below control levels, but only the IV treated group achieved a significant reduction in protein expression. These findings reinforce our previous results demonstrating that MMP-9 is more sensitive to minocycline inhibition <sup>14</sup> and that earlier delivery of minocycline may achieve better MMP inhibition.

The major endpoint in our study was the formation of hemorrhage in the brain. Image analysis of macroscopic hemorrhages in the animal brains showed a two fold increase in bleeding and an increase in brain swelling in tPA treated animals when compared to untreated animals. For both parameters, treatment with intravenous minocycline proved

beneficial. There was a corresponding increase in mortality among tPA treated animals and a decreased mortality with minocycline.

Our results also showed that minocycline decreased infarct size, even after corrected for edema, and improved overall outcome as shown by the behavior tests. It could be argued that the decreased mortality and improved behavior outcome in the combination therapy group derived from minocycline's ability to decrease lesion volume rather than its direct protection of the vasculature<sup>18, 24</sup>. However, because hemorrhage formation has been shown to be directly related to increased mortality and minocycline prevented basal lamina degradation, it is likely that minocycline MMP inhibition and vascular protection contribute at least partially to the decreased mortality and overall protection after acute ischemic stroke.

Minocycline seems a logical addition to reperfusion therapy with tPA in acute ischemic stroke. Although known as an inhibitor of proteases, minocycline, in a wide range of clinically relevant concentrations, does not negatively impact the ability of tPA to lyse clots *in vitro*. In addition, minocycline's pleiotropic effects in the brain include structural protection of the vasculature to prevent leakiness and hemorrhagic transformation. Since tPA has the potential to exert multiple negative actions in the brain vasculature – directly or via MMPs or fibrin degradation products<sup>25</sup>, combination therapy is likely to at least be additive in benefit.

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## TABLE AND FIGURE LEGENDS

### Table 3.1

Effect of minocycline on amidolytic parameters of tPA. The hydrolysis of S-2288 was monitored for 7 minutes at 37°C and analyzed by Michaelis-Menten curve fitting. Minocycline did not affect tPA amidolytic efficiency. Values represent the mean  $\pm$  standard error of the mean (SEM) (n=6).

### Table 3.2

Mortality rate observed in all cohorts of animals. Mortality was three times higher in tPA animals compared to both stroke saline control animals and IV (early delivery) minocycline-treated (3 mg/ kg) animals, although it was not statistically significant. When minocycline was delivered late (through IP injection), the mortality was similar to that of the tPA treated group.

### Figure 3.1

Impact of minocycline on fibrinolytic activity of tPA. The lysis of blood clots was initiated by 2nM tPA at 37°C. The amount of fibrinolysis was determined by measuring the release of soluble <sup>125</sup>I-fibrin degradation products at various time intervals (35, 75 and 120 minutes). The fibrinolytic activity of tPA was not affected by any concentration tested of minocycline (1 through 30 ug/ ml). The means  $\pm$  SEM (n=4) are shown.

### Figure 3.2

(A) Brain MMP-2 and MMP-9 *activities* after stroke and tPA treatment 24 hours after stroke as measured by gelatin zymography. MMP-2 was elevated by tPA compared to stroked saline controls. MMP-9 activity was also elevated by treatment with tPA 10 mg/kg. Minocycline IP group significantly decreased MMP-9 activity to below control levels ( $p = 0.0136$ ). (B) Brain MMP-2 (72 kDa) and MMP-9 (87 kDa and 92kDa) protein contents 24 hours after stroke as determined by immunoblotting. IV Minocycline group had significantly lower MMP-2 protein, MMP-9 92 kDa and MMP-9 87 kDa proteins compared to tPA animals ( $p=0.0034$ ;  $p=0.001$  and  $p=0.0084$ , respectively). Vertical bars indicate SEM.

### Figure 3.3

(A). Hemorrhagic transformation was increased ( $p = 0.0190$ ) in animals treated with tPA 10 mg/ kg. Adjuvant treatment with minocycline 3 mg/ kg intravenously prevented this increase. (B) Bleeding in an animal in the tPA treated group (10 mg/ kg). Hematomas were seen in the animals that died. (C) Minocycline treatment (3 mg/ kg intravenously) decreased the tPA-induced increase in brain swelling. Vertical bars indicate SEM.

### Figure 3.4

(A) Effect of treatment on collagen type IV protein content in the brain. Stroke significantly decreased baseline value ( $p=0.0011$ ). Treatment with tPA 10 mg/ kg further decreased collagen IV. Treatment with 3 mg/ kg minocycline intravenously diminished collagen degradation. (B) Effect of treatment on laminin- $\alpha$ 1 protein content in the brain.

Stroke significantly decreased laminin and treatment with 3 mg/ kg minocycline strongly prevented laminin degradation ( $p = 0.0001$ ). Vertical bars indicate SEM.

### **Figure 3.5**

(A) Infarct size after correction for edema. tPA significantly increased infarct size ( $p=0.0035$ ). Adjuvant treatment with 3 mg/ kg of minocycline intravenously decreased infarct size. Vertical bars indicate SEM. (B) Illustration with a representative brain of each of the treatment groups.

### **Figure 3.6**

Effect treatment in behavioral outcome. ( A) Improvement in the Bederson scale of animals treated with IV minocycline compared to tPA only treated animals ( $p=0.0001$ ). (B) tPA + Mino IV treatment group performed better in the paw grasp task ( $p=0.0391$ ). Vertical bars in all graphs represent SEM.

## TABLES

Table 3.1

Minocycline	$K_m$ , $\mu\text{M}$	$k_{cat}$ , $\text{s}^{-1}$	$K_m/k_{cat}$ , $\text{mM}^{-1}\text{s}^{-1}$
none	$1332 \pm 203$	$6.2 \pm 0.5$	4.7
30 $\mu\text{g/ml}$	$1330 \pm 227$	$6.0 \pm 0.6$	4.5

Table 3.2

Group	Stroke Saline Control n = 25	tPA n = 28	tPA + Mino IP n = 11	tPA + Mino IV n = 27
Dead (mortality rate)	2 (8%)	7 (25%)	2 (18%)	2 (7%)

# FIGURES

Figure 3.1

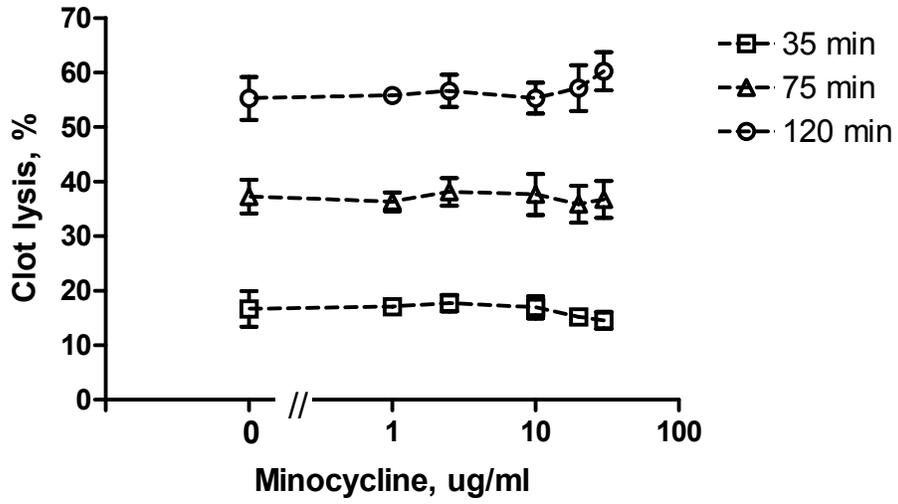


Figure 3.2 A

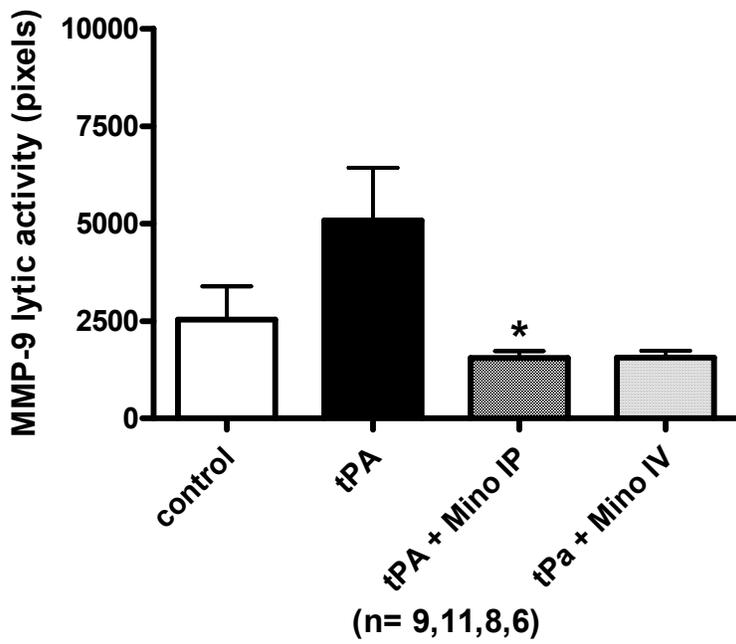
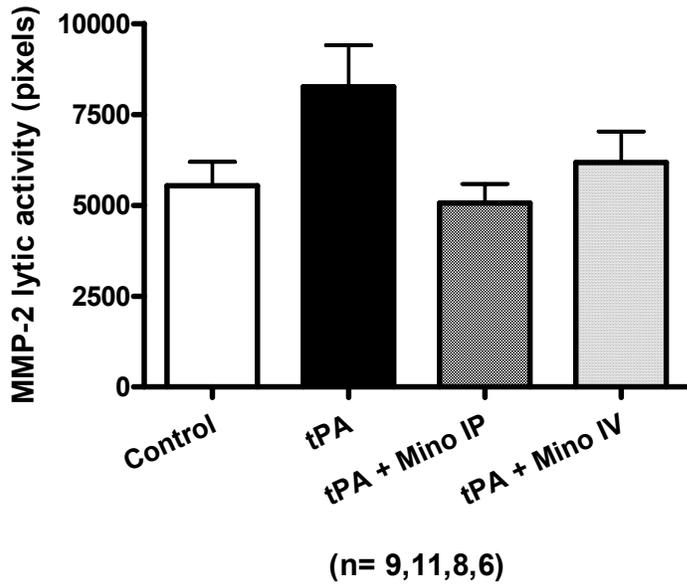
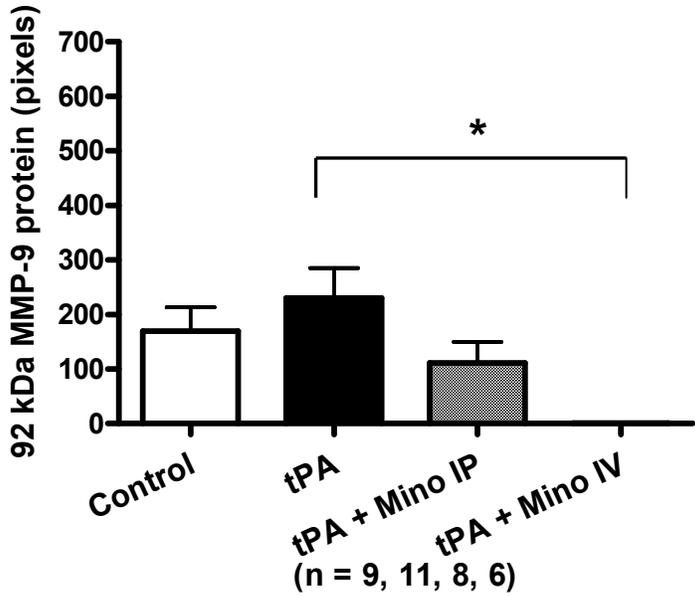
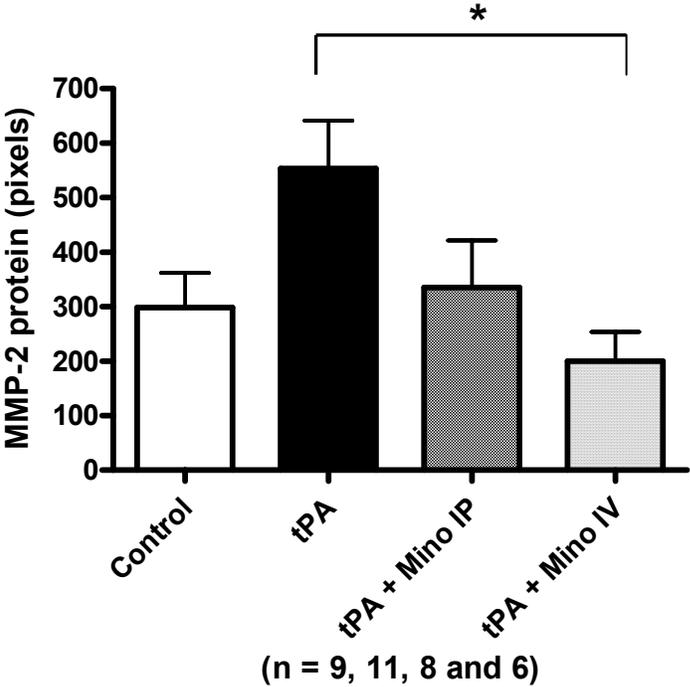


Figure 3.2 B



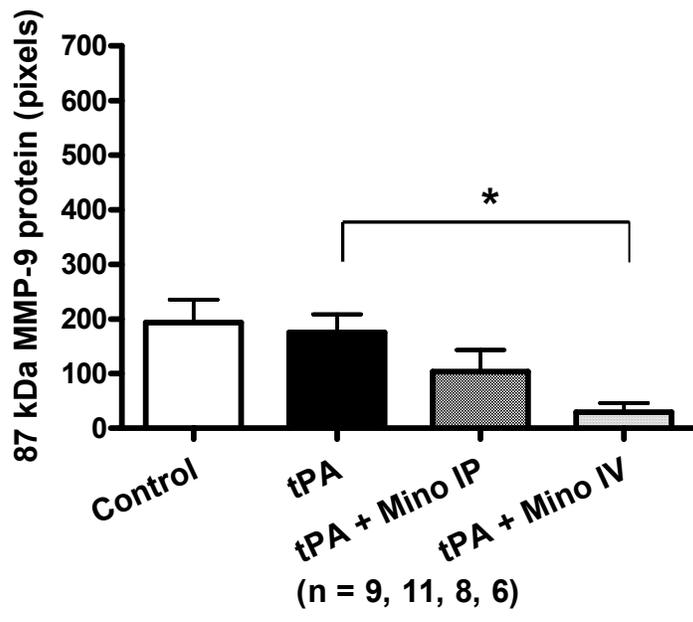


Figure 3.3 A

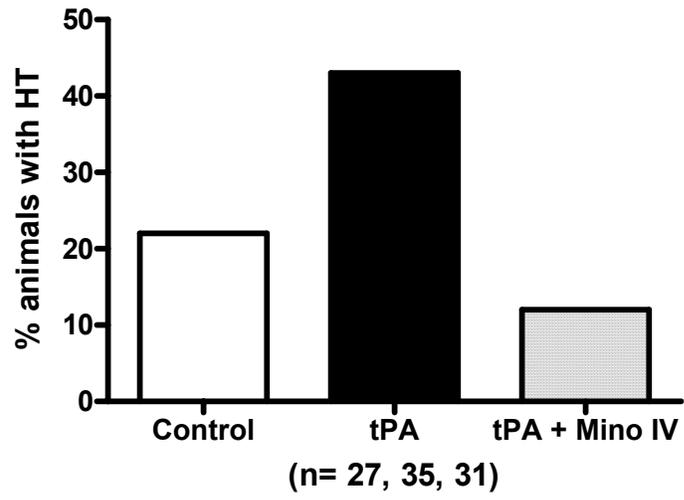


Figure 3.3 B



Figure 3.3 C

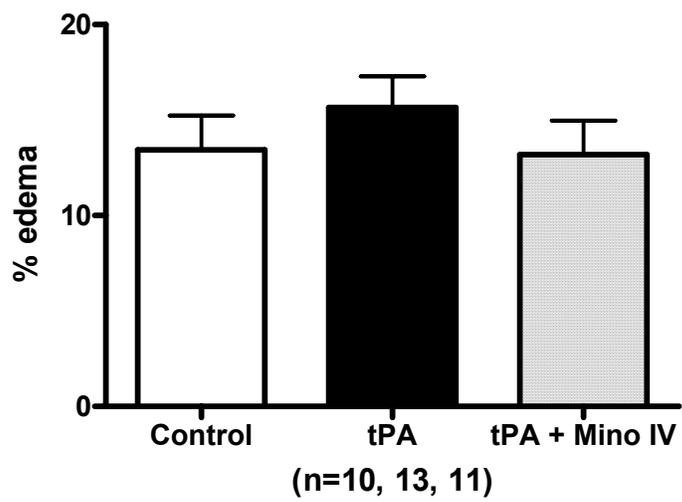


Figure 3.4 A

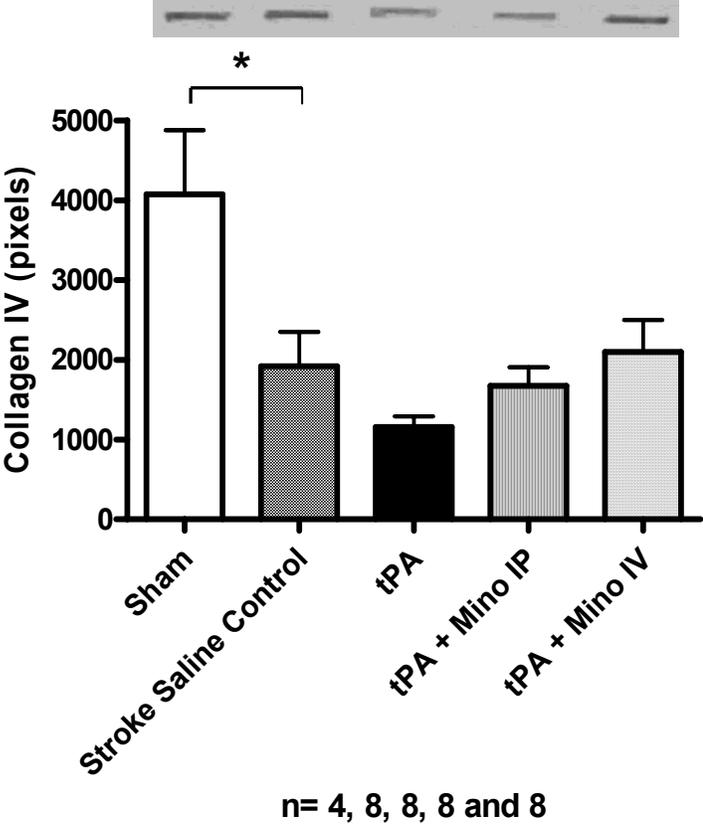
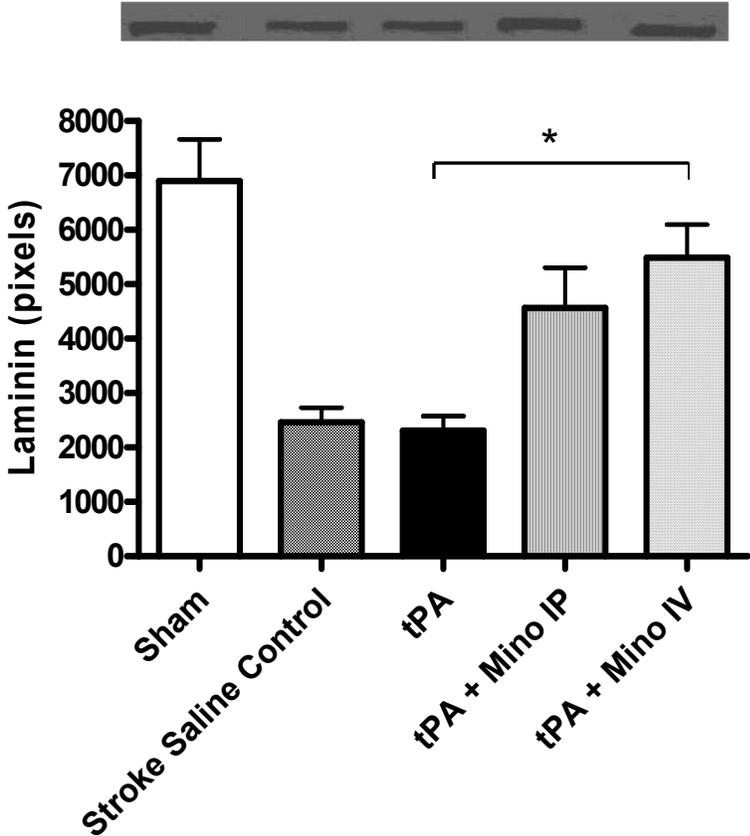


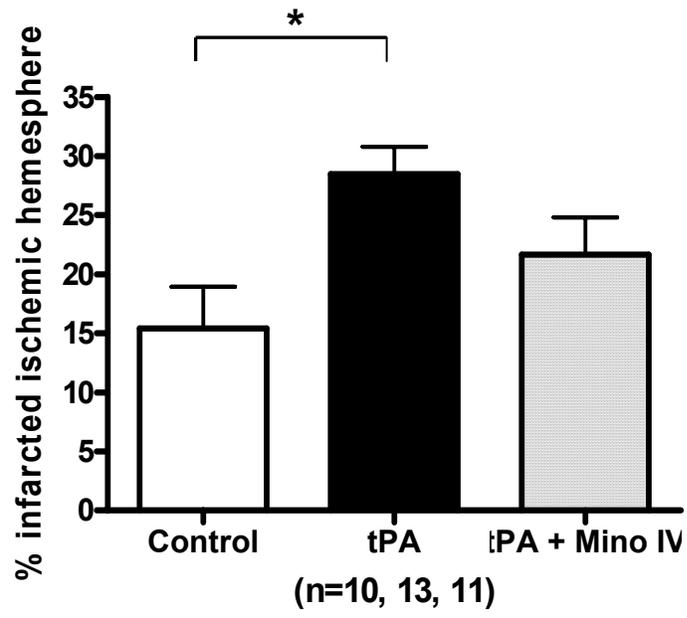
Figure 3.4 B



(n= 4, 8, 8, 8 and 8)

Figure 3.5

A



B

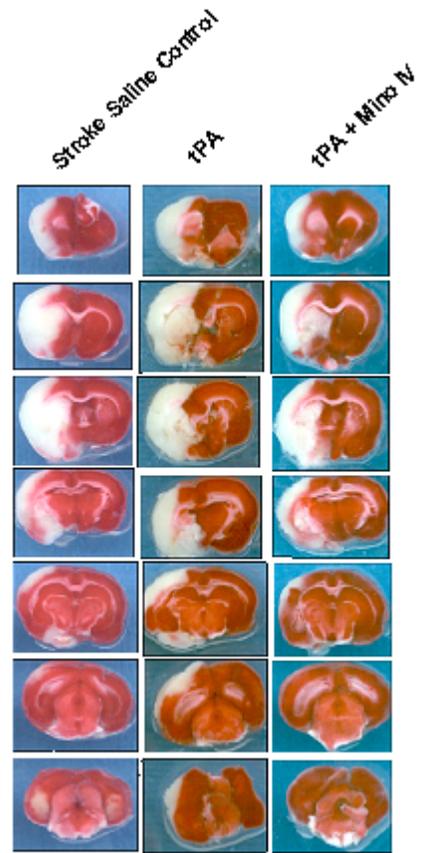


Figure 3.6 A

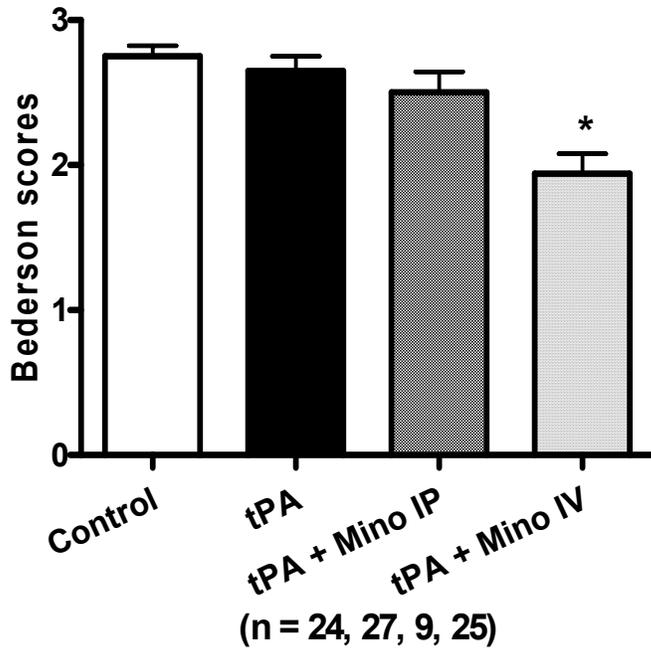
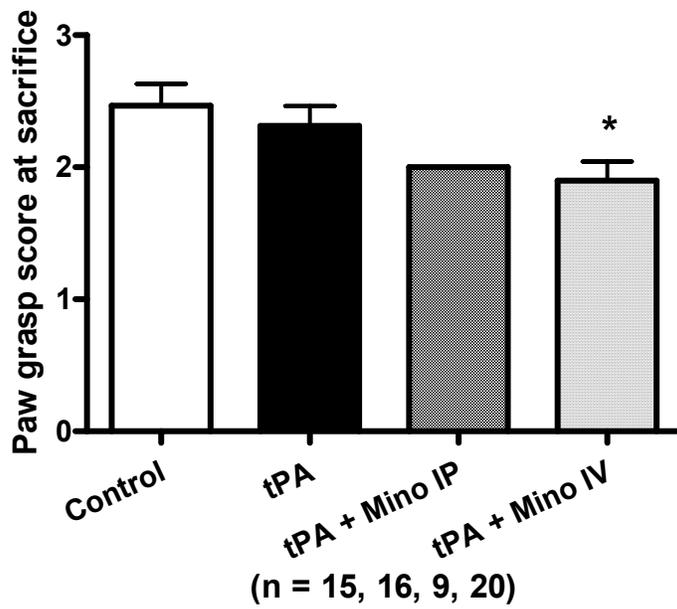


Figure 3.6 B



## CHAPTER 4

### UNPUBLISHED DATA

#### A. Minocycline vascular protection study

Prior to beginning the studies, determining the appropriate length of survival after stroke and treatment was important in order to have a maximal MMP activation (and vascular degradation) induced by ischemia in the acute period. We tested whether three hours of reperfusion was ideal to study MMP activation and found that at three hours after ischemic injury, MMP-2 activity was unchanged from baseline and MMP-9 activity was not maximal in our three hour occlusion model (Fig. 4.1) but at 24 hours it had a ten fold increase.

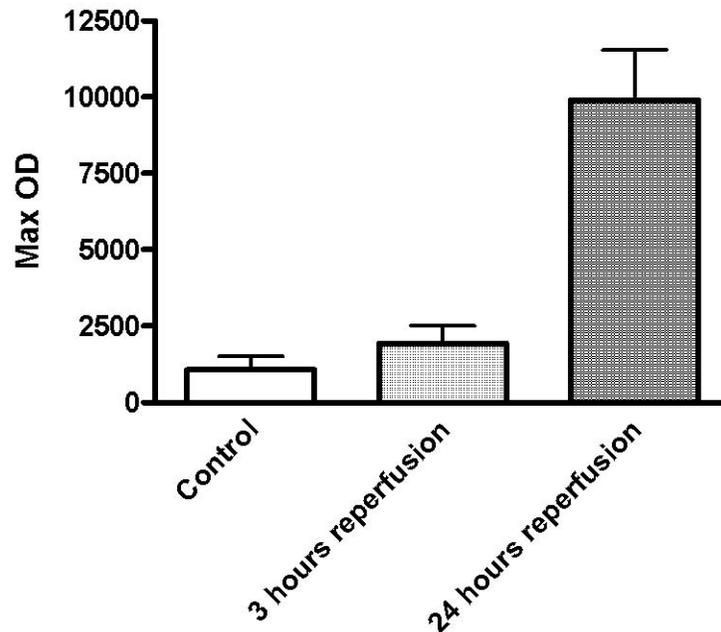


Figure 4.1: MMP- 9 activity in stroke hemisphere in Wistar rat brains after 3 hours occlusion and 3 or 24 hours reperfusion expressed as maximum optical density. Sample sizes are 4 each. Vertical error bars indicate standard error of the mean.

After determining that reperfusion length was ideal at 24 hours, we also studied the impact of minocycline treatment (initially chosen to be one IP injection of 45 mg/kg of body weight every twelve hours) on plasma MMP activity after stroke. Those experiments revealed that this treatment regimen did not affect circulating MMP-2 activity but decreased MMP-9 (Figs. 4.2.A and 4.2.B).

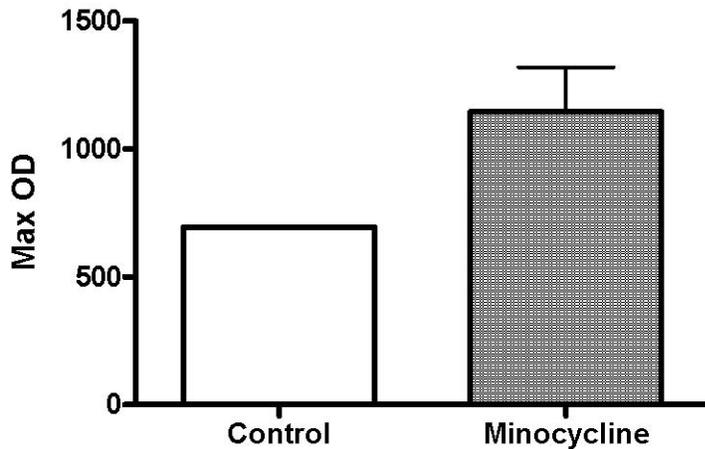


Figure 4.2.A: MMP-2 activity in the plasma of Wistar rats after 3 hour occlusion and 24 hour reperfusion and treatment with minocycline 45 mg/kg (expressed as maximum optical density). Sample sizes are 1 and 5, respectively. Vertical error bar indicates standard error of the mean.

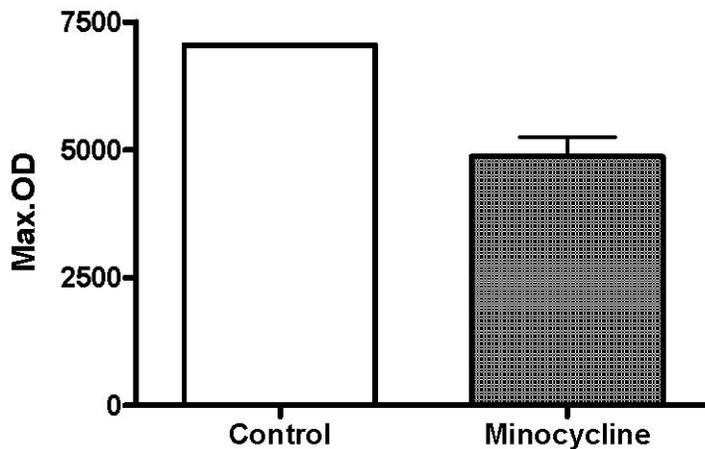


Figure 4.2.B: MMP-9 activity in the plasma of Wistar rats after 3 hour occlusion and 24 hour reperfusion and treatment with minocycline 45 mg/kg (expressed as maximum optical density). Sample sizes are 1 and 5, respectively. Vertical error bar indicates standard error of the mean.

As shown in chapter 2 by our published data, we determined that a treatment protocol of minocycline 45 mg/kg intraperitoneally immediately after reperfusion followed by another one twelve hours later can prevent ischemia-induced MMP activation in our temporary model of focal cerebral ischemia in Wistar rats. We followed those studies with experiments to test the impact of this MMP inhibition observed in the overall outcome after stroke and speculated that MMP inhibition would lead to neurovascular protection. However, the results of these experiments showed that this treatment regimen was neither vascular nor neuroprotective (Figs. 4.3 and 4.4) as measured by the formation of edema, the development of hemorrhage (by tissue hemoglobin), infarct size and behavior outcome. The quantification of hemorrhage was done by assessing the hemoglobin content in the tissue after complete perfusion of the brain. This was accomplished by an ELISA method for hemoglobin. In this method, after the image of the TTC stained slices has been captured, the 2 mm slices B through E (+ 2 to -6 from the bregma) were divided into the stroke and contra-lateral hemispheres. The same hemisphere slices were combined and prepared for homogenization. The tissue were homogenized mechanically with 10 % glycerol TTBS for 10 minutes. The samples were then sonicated for 1 minute and the supernatant were collected after low speed centrifugation. The protein content was determined with the Bradford method. The samples were blocked with bovine serum albumin and incubated with a rabbit anti- rat hemoglobin antibody (AbD Serotec, Inc.). Next, a donkey anti-rabbit horseradish conjugated antibody was used. Spectrophotometric detection was used at 450 nm.

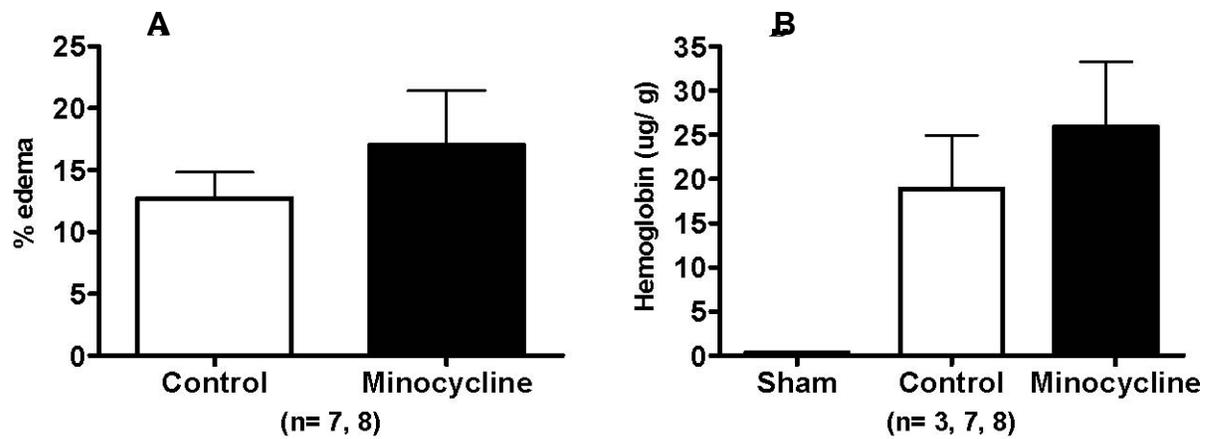


Figure 4.3: (A) Brain edema formation after 3 hours occlusion and 24 hours reperfusion and treatment with minocycline 45 mg/kg. Vertical error bars indicate standard error of the mean. (B) Hemorrhage transformation measured by brain tissue hemoglobin after 3 hours occlusion and 24 hours reperfusion and treatment with minocycline 45 mg/kg. Vertical error bars indicate standard error of the mean.

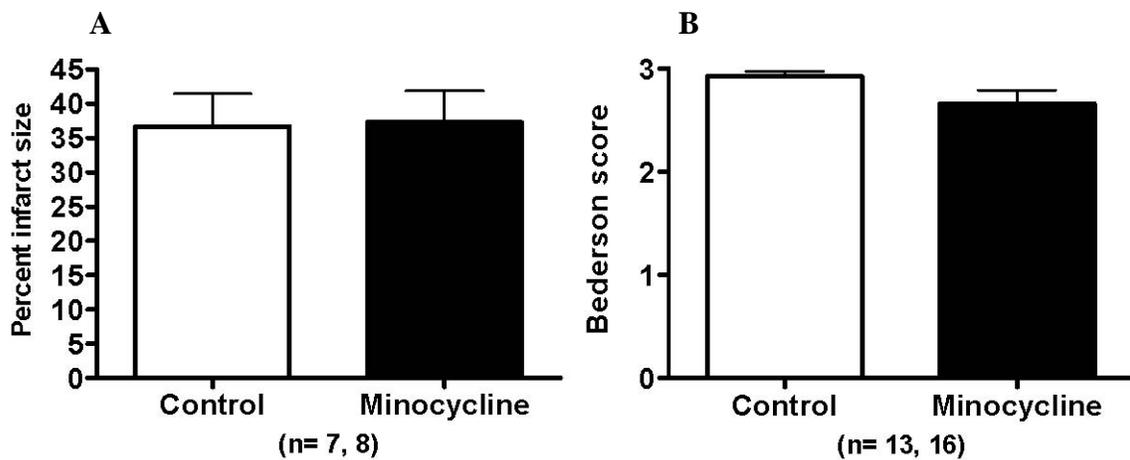


Figure 4.4: (A) Brain infarct size after 3 hours occlusion and 24 hours reperfusion and treatment with minocycline 45 mg/kg. (B) Neuro-behavior outcome measured by the Bederson scale after 3 hours occlusion and 24 hours reperfusion and treatment with minocycline 45 mg/kg. Vertical error bars indicate standard error of the mean.

In these animals treated with minocycline, we collected central blood immediately prior to sacrifice to measure the minocycline serum concentration. We found that the mean minocycline concentration was 10.78  $\mu\text{g/ml}$  (Fig. 4.5).

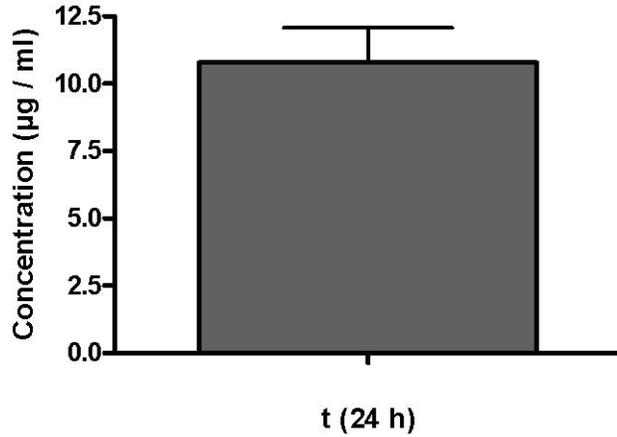


Figure 4.5: Minocycline serum concentration at the time of sacrifice after the minocycline 45 mg/kg IP treatment regimen. Sample size is 5 animals. Error bar indicates standard error of the mean.

During the *in vitro* experiments shown in chapter 2, we tested the lowest concentration of minocycline able to inhibit the two gelatinases. As previously discussed, MMP-9 seems to be more susceptible to minocycline inhibition. Graph 4.6 depicts that difference in MMP inhibition to the same concentrations of minocycline. At lower concentrations of minocycline, MMP-9 inhibition was approximately 70%, while MMP-2 activity was inhibited by 40%. At higher concentrations, minocycline loses its specificity to MMP-9 and both enzymes appear to be inhibited at a similar rate.

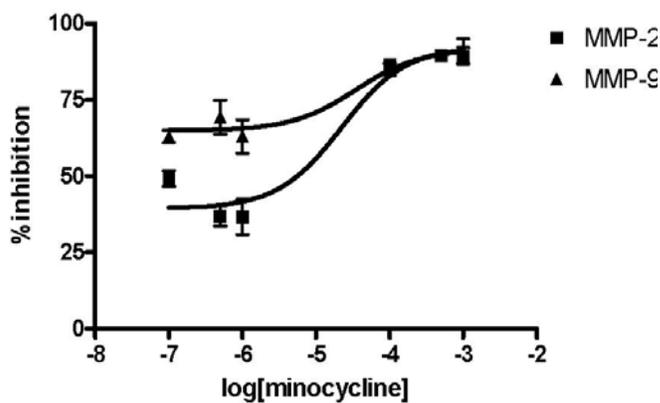
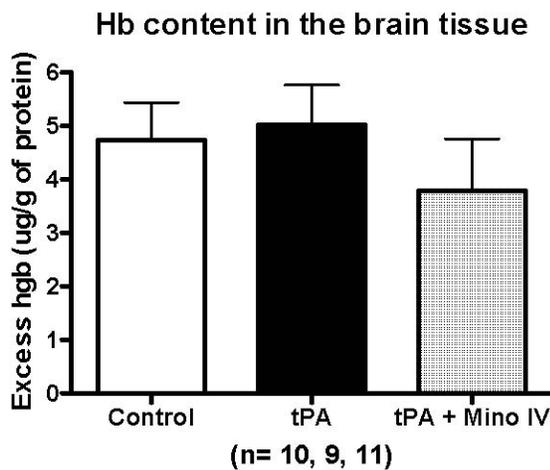


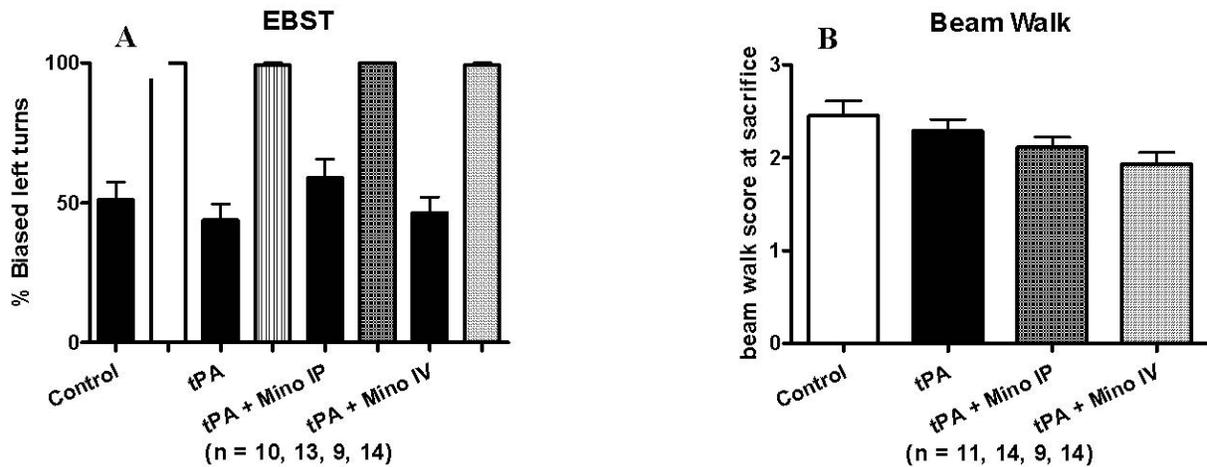
Figure 4.6: Dose-response graph showing the direct inhibitory effect of minocycline in concentrations ranging from 0.1 to 1000 µg/ml on recombinant gelatinase activity after normalization to respective standard. Error bars indicate standard error of the mean.

Continuing with the next arm of the study when we tested the interaction of minocycline treatment with tPA, we showed that minocycline prevented some of the tPA-induced injury in the brain. Figure 4.7 shows the hemoglobin concentration in the brain parenchyma. This measurement of vascular breakdown was not statistically different between any of the groups, although an increased incidence of bleeding was observed in the tPA group (Fig. 3.3.A).



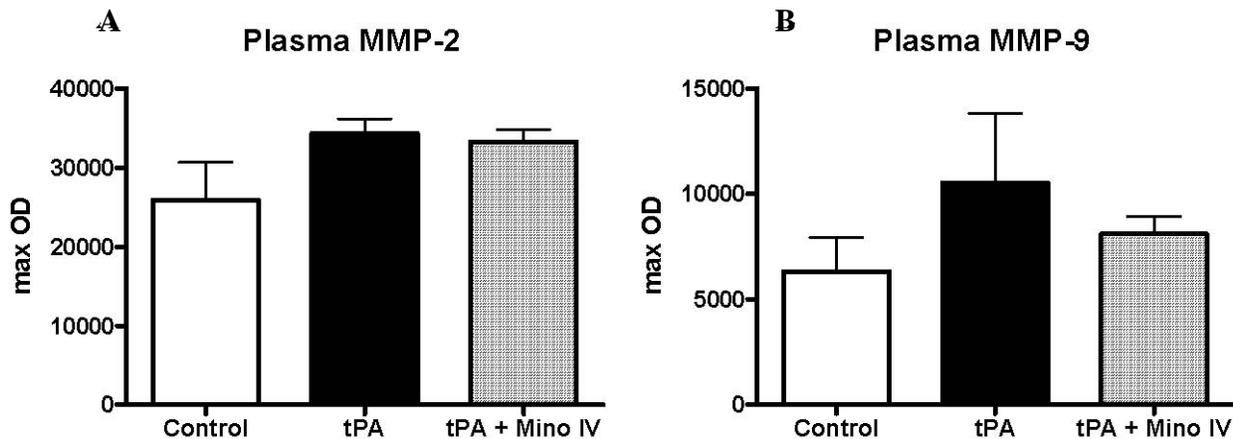
**Figure 4.7: Quantification of vascular breakdown by brain tissue hemoglobin (as measured by Hb ELISA) after stroke and treatment. Error bars indicate standard error of the mean.**

To study the extent of minocycline neurovascular protection, we tested the animals' motor-behavior with four different tests: the Bederson scale, the paw grasp test, the beam walk test and the elevated body swing test. The Bederson scale and paw grasp were sensitive enough to detect the differences between treatment groups. However, although there seems to be a similar trend in the beam walk test, both the Elevated body swing and beam walk tests were not able to detect differences between treatment groups (Fig. 4.8 A and B).



**Figure 4.8: (A) Elevated body swing test shows pre and post stroke performance as measured by unilateral preference in body swing when elevated by the tail. Baseline values are approximately 50%, whereas after stroke the swings become biased. (B) Beam walk test measures the ability to stay on and traverse a beam between two points. Points range from 0 (no difficulty in performing the task) to 3 (unable to move or stay on the beam). Error bars indicate standard error of the mean.**

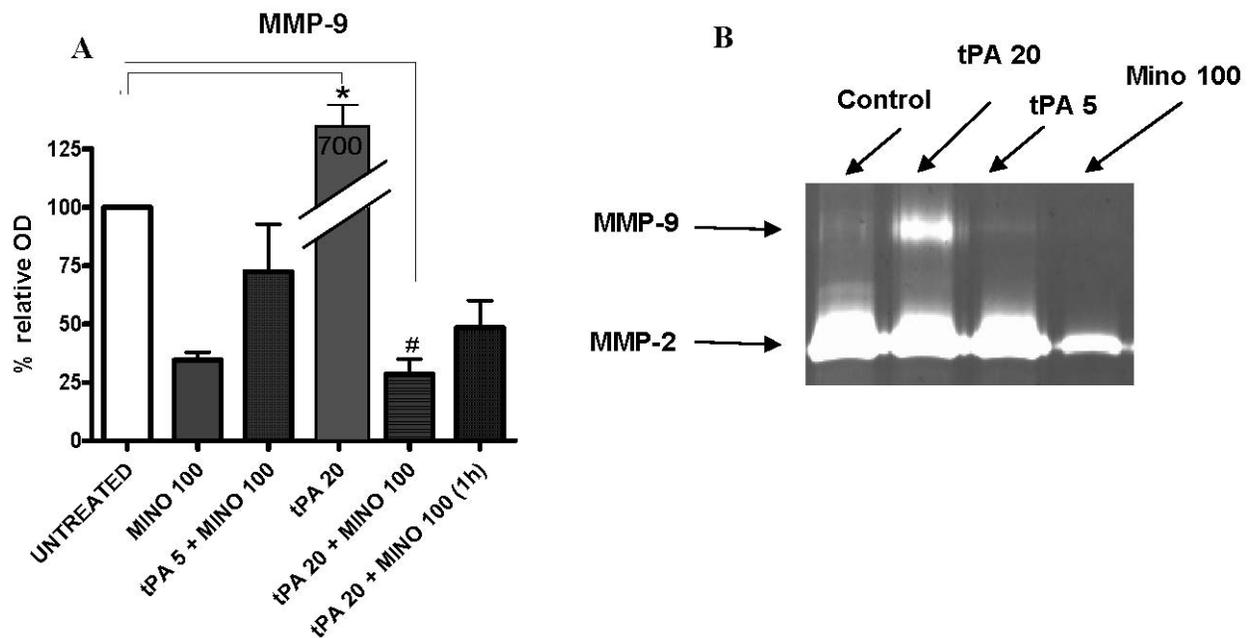
In a few animals of the tPA/ minocycline study arm, we collected central blood immediately prior sacrifice to analyze the circulating MMPs in response to tPA and minocycline treatment. Although there seems to be a trend in increased MMP with tPA treatment, the sample sizes were too small to detect any difference in activity between the different treatment groups (Fig. 4.9 A and B).



**Figure 4.9: MMP activity (A is MMP-2 and B is MMP-9) in the blood of animals treated with tPA 0.9 mg/kg or tPA plus minocycline combination (3 mg/kg IV) at the time of sacrifice. Sample sizes are 4, 2 and 6 respectively. Error bars indicate standard error of the mean.**

To study the effect of ischemia-like injury and treatment with minocycline and tPA *in vitro* in endothelial cells, a model of oxygen-glucose deprivation method was also used. Bovine retinal endothelial cells (BREC) and primary isolated human brain endothelial cells (HBMEC) were used. These cells were exposed to low oxygen (below 0.2 percent), low glucose (1 g/L, minimum essential Eagle's medium from ATCC) conditions during the course of the experiments. The cells were kept in normal oxygen, normal glucose medium (M199 cell culture medium, Invitrogen) containing 10% volume fetal bovine serum, 5% donor calf serum (Cellgro) and 150 mg/l endothelial cell growth factor (Sigma) during the growth period and replaced with serum-free normal glucose medium 24 hours before the beginning of experiments (when cells achieved above 80 % confluence). The cells were transferred from a normal cell culture incubator to an anoxic chamber with controlled oxygen. In both normoxic and hypoxic conditions, the carbon dioxide is kept at 5%. The hypoxic/ hypoglycemic (otherwise named ischemic) cells were replaced with the serum free EMEM medium and placed in the anoxic chamber for a time period varying from 3 to 12 hours. They were then returned to normoxic/ normoglycemic conditions for 24 hours before collection of the media and cell lysates. These studies found that tPA (20 µg/ml) has an activation effect on MMP-9 but not on MMP-2 in BRECs after the ischemia-like injury (Fig. 4.10). Treatment with minocycline 100 µg/ml reversed this MMP activation.

**Figure 4.10: (A) MMP-9 activity in the supernatant of cultured BRECs after oxygen glucose deprivation injury and treatment with tPA 5 or 20 µg/ml and minocycline 100 µg/ml alone or in combination with tPA. Minocycline treatment occurred at the same time of tPA treatment or one hour later. Sample sizes are of three per group. Error bars indicate standard error of the mean. (B) Illustration of a zymogram of these treated cells.**



In these cells, we also measured the cell death as the release of lactate dehydrogenase to the supernatant, collected 24 hours after the onset of OGD. This experiment demonstrates that minocycline is protective against cell death induced by OGD only when treatment occurs one hour prior returning the cells to normoxic normoglycemic conditions. tPA appears to increase the LDH release.

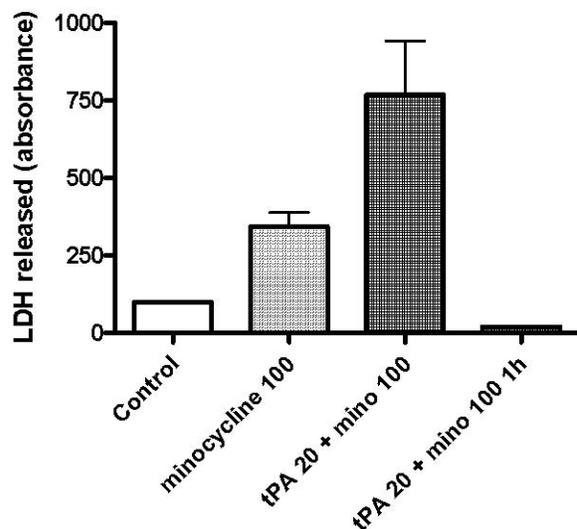


Figure 4.11: Measure of cell death as the absolute release of lactate dehydrogenase to the supernatant of BREC cells exposed to OGD and treatment. Sample sizes are of three per group. Error bars indicate standard error of the mean.

When we did the same experiments with isolated human brain endothelial cells, the cells did not respond in the same manner to OGD damage. There was only one band/ gelatinase detected at the supernatant of these cultures. This band was detected at 74 kDa and remained at similar levels in all conditions. MMP activity remained unchanged after OGD with or without re-exposing them to normoxic/ normoglycemic conditions.

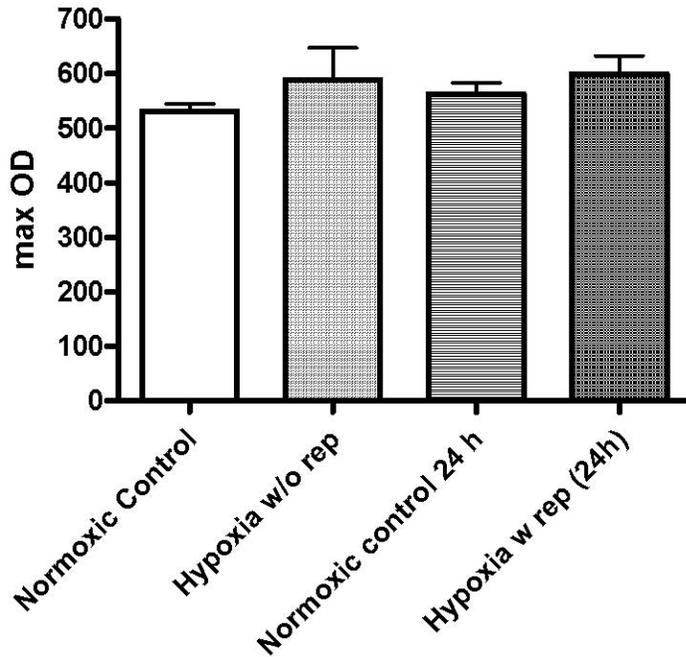


Figure 4.12: MMP activity (74 kDa) in the supernatant of HBMECs after exposure to OGD with or without re-exposure to normoxic/ normoglycemic conditions. Sample sizes were three per group. Error bars indicate standard error of the mean.

## CHAPTER 5

### DISCUSSION

The aims of this project were to evaluate the potential of minocycline as a vascular protective drug in acute ischemic stroke and investigate its MMP inhibition properties in stroked Wistar rats. We also proposed to study minocycline effects in clinically relevant concentrations. As we had anticipated, the 24 hour reperfusion window was ideal for detecting acute MMP activity elevation and studying the effect of MMP inhibition in our stroke model. This reperfusion time window together with a 3 hour occlusion provides a good model to test the vascular events after acute stroke. Previous studies by our group have shown that approximately 50% of animals will undergo hemorrhage transformation after a 3 hour occlusion and 24 hour reperfusion acute ischemic stroke model<sup>1</sup>.

This project demonstrated for the first time that delayed treatment with minocycline inhibits MMPs that are elevated in the brain after temporary experimental cerebral ischemia. As demonstrated by the studies reported in chapter 2, the MMP inhibitory effect was present *in vitro* in clinically relevant concentrations of minocycline. Unlike previous reports of therapeutic efficacy of minocycline in achieving neuroprotection<sup>2-4</sup>, we also tested a weight based dose of minocycline that is currently used in humans (chapter 3). This low dose was also efficient in providing MMP inhibition after ischemia and tPA treatment.

Another major finding of this study was that minocycline protects the vasculature after acute ischemic stroke. Although treatment with minocycline 45 mg/kg intraperitoneally was not successful in our studies, as displayed in chapter 4, the treatment protocol of minocycline 3 mg/kg intravenously decreased the incidence of hemorrhage transformation and had a trend in decreasing brain edema that was further increased by tPA treatment (Figs. 3.3A and 3.3C, respectively). This difference in results is likely due to the nature of the delayed delivery of minocycline to the brain that is inherent to intraperitoneal injections. Contrary to the intraperitoneal delivery, the intravenous minocycline had an immediate delivery to the site of injury that was likely to have an impact on the course of the acute stroke cascade of events. As we have previously reviewed and illustrated, several deleterious events occur in the first minutes to a few hours after the onset of ischemia<sup>5</sup>. The more delayed the treatment, the less likely it is to have a substantial impact on the course of events and ultimately on the extent of injury. Furthermore, in the delayed phases after injury, MMPs may play a beneficial role rather than deleterious as thought to be in the acute phase<sup>6</sup>. It is thought that MMPs and other proteases may be needed during the stroke recovery phase for neurovascular remodeling. In that sense, early delivery of minocycline is not only critical for decreasing damage but also for avoiding potential impairment of the recovery process. Lastly, although the decrease in edema formation was not statically significant as shown in figure 3.3C, it is possible that same treatment protocol (of minocycline 3 mg/kg IV) would have a significant positive impact in non tPA-treated animals as others have shown that minocycline can decrease the edema that follows experimental stroke<sup>7</sup>.

The third original finding of this project is that minocycline treatment decreases the degradation of blood brain barrier components collagen IV and laminin  $\alpha$ -1 following experimental ischemic stroke (Figs. 3.4A and 3.4B). Although others have previously addressed minocycline's positive impact on the stability of the blood brain barrier, this is the first evidence that minocycline treatment reduces the degradation of basal lamina components collagen type IV and laminin  $\alpha$  1 following ischemic stroke injury and treatment with tPA. There is supportive evidence however, that minocycline decreases the blood brain barrier breakdown after experimental stroke in rats demonstrated by magnetic resonance imaging<sup>8</sup>. In this study the authors explored the additive effect of minocycline treatment with hypothermia after stroke and showed that the volume of BBB breakdown correlated with presence or absence of MMP-9 in the brain after minocycline treatment. Furthermore, Yenari et al demonstrated that minocycline treatment reduces Evans blue extravasation into the brain after stroke in mice<sup>2</sup>. Murata et al. have also shown that adding minocycline treatment to tPA treatment after embolic stroke in rats reduces brain hemorrhage that is observed in tPA-only treated animals<sup>9</sup>.

Although the observations of this study show that minocycline is a strong candidate for acute stroke therapy, the MMP inhibition role in its early neurovascular protective effects could only be dissected from minocycline pleiotropic effects in the brain by determining its direct impact on the structural and functional components of the vasculature that maintain the blood brain barrier and prevent leakiness and hemorrhage formation. The stability of the BBB is essential to prevent further exposure of the brain to inflammatory stress.

It may be argued that the decrease in vascular breakdown is secondary to a decrease in infarcted area (and hence a milder injury) attributed to minocycline's neuroprotection properties rather than a direct protective effect on the degradation of the blood brain barrier. However, there is evidence that there can be a dissociation between area of the brain ischemic lesion and hemorrhage formation<sup>10</sup>. Our own previous studies showed that treatment with tPA accelerated the formation of hemorrhage without impacting the infarct size after ischemic stroke of different occlusion lengths<sup>1</sup>. Furthermore, collagen type IV is a known direct substrate for matrix metalloproteinases 2 and 9.

In order to study the direct effect of minocycline MMP inhibition on the vasculature after stroke, we studied known direct substrates of MMPs that are involved in maintaining the blood brain barrier<sup>11</sup>. The most classically recognized substrate for MMPs 2 and 9 is collagen type IV (and denatured collagen), which is an important component of the basal lamina along with laminin- $\alpha$ 1. Together, they provide a strong and flexible structural matrix for the endothelial cell layer. When one of these proteins is degraded, the endothelial cell – matrix homeostasis is disrupted. It is speculated that this is one of the multiphasic roles of MMPs during acute stroke<sup>12</sup>. In fact MMP-9 reduction has been associated with decreased collagen IV degradation in the brain<sup>13, 14</sup>. In our studies, minocycline treatment was able to decrease the basal lamina degradation. This reinforces a role of MMPs to vascular breakdown and the relevance of MMP inhibition for the positive effects of minocycline after stroke.

The relationship between MMP activity and basal lamina collagen degradation after stroke has been previously demonstrated in the primate brain<sup>15</sup>. Fukuda et al. also showed that MMP inhibition with synthetic non specific inhibitors reverses the degradation of collagen<sup>16</sup>. Guo et al. showed that pre-ischemic exercise downregulated and decreased the activity of MMPs 2 and 9 and that lead to decreased collagen IV immunoreactivity. They also showed that blocking MMP-9 preserved collagen IV in microvessels<sup>13</sup>. Lastly, basal lamina collagen IV degradation has been shown to be associated with increased MMP-9 activity after stroke and preceded hemorrhage formation<sup>14</sup>. There is also evidence that laminin  $\alpha$ -1 can be rescued from proteolysis when a highly specific inhibitor of MMP-9 is used after experimental stroke. In this study the authors showed that gelatinolytic activity was spatially associated with laminin  $\alpha$ -1 degradation in the brain of mice after transient MCAO<sup>17</sup>. Another important component of the blood brain barrier is tight junction proteins. During ischemia/ reperfusion, the tight junction proteins within and between endothelial cells are disrupted<sup>18</sup>. Interestingly, MMP inhibition with synthetic inhibitors has also been associated with disruption of tight junction proteins in the cerebral vessels after stroke in rats. Tight junction proteins claudin-5 and occludin were shown to be increased when animals were treated with BB-1101 – a specific MMP inhibitor with no known pleiotropic effects – when compared to stroked control animals<sup>19</sup>. These data point to a direct and causal relationship between MMP activity and blood brain barrier failure. Altogether, this evidence suggests that the direct minocycline MMP inhibition property may be at least partially responsible for preserving the vascular integrity and preventing further tissue injury.

As seen on the infarct size data (Fig. 3.5A and B), tPA seemed to worsen the infarct when compared to controls. This finding is not surprising and reinforces the finding of others that tPA is neurotoxic and amplifies the ischemic excitatory damage when the brain is exposed by a weakened BBB and tPA is no longer confined in the vascular space<sup>20, 21</sup>. Although the primary action of tPA is fibrin lysis within the blood vessel, it exerts multiple effects in the brain – directly or via MMP or thrombotic degradation products<sup>22</sup> – specially in the presence of weakened vessels<sup>23</sup>. As tPA is the only FDA approved drug for acute ischemic stroke and there has been evidence showing minocycline is a promising neuroprotective agent with MMP inhibition properties<sup>24-26</sup>, the combination treatment seems logical. To determine if minocycline would be safe in tPA treated patients in not affecting tPA thrombolytic efficacy and compromising clot lysis and artery recanalization, we studied the interaction of the two drugs in a controlled *in vitro* clot lysis assay. This study demonstrated that the MMP inhibition effect of minocycline did not impair the ability of tPA to cleave plasminogen and exert its fibrinolytic effect. This has also been suggested by the data of Murata and collaborators<sup>9</sup>. In their study, minocycline treatment did not appear to have significant effect on the cerebral perfusion restored by tPA after embolic stroke in rats. However, the clot lysis effect of the treatment interaction was not studied directly.

Furthermore, although minocycline possesses multiple beneficial effects<sup>27</sup>, the ability to directly inhibit MMPs suggests that minocycline may not only be “neurovascularprotective” in general but also increase the safety and improve outcome of thrombolytic therapy with tPA. This would possibly allow for a longer safety time

window for tPA treatment. The study of Murata et al. also demonstrated that addition of minocycline treatment to animals treated with tPA 6 hours after the onset of stroke reduced infarct sizes to levels similar to those of animals treated with tPA after 1 hour. Similarly, hemorrhagic transformation – which was increased in animals only treated with tPA after 6 hours – was significantly reduced. Our results provide further evidence that minocycline is safe to use in combination with tPA after stroke and demonstrates that it does not impair tPA clot lysis. These results also suggest that this combination therapy may benefit a larger stroke patient population than tPA alone.

Our results also showed that minocycline decreased infarct size, even after corrected for edema, and improved overall outcome as shown by the behavior tests. These findings reinforce the results of others that minocycline is neuroprotective<sup>4, 28, 29</sup>. It could be argued that the decreased mortality and improved behavior outcome in the combination therapy group derived from minocycline's ability to decrease lesion volume rather than its direct protection of the vasculature. However, because hemorrhage formation has been shown to be directly related to increased mortality (as also shown in our observation of increased prevalence of hematomas in post-mortem analysis), and minocycline prevented basal lamina degradation, it is likely that minocycline vascular protection contribute to the decreased mortality and overall protection after acute ischemic stroke.

Together, the vascular and neuroprotective properties of minocycline – along with its safety profile – provide this drug strong potential for therapeutic use and may explain

the results of studies that show that minocycline treatment improves long-term functional outcome in rats<sup>30</sup>.

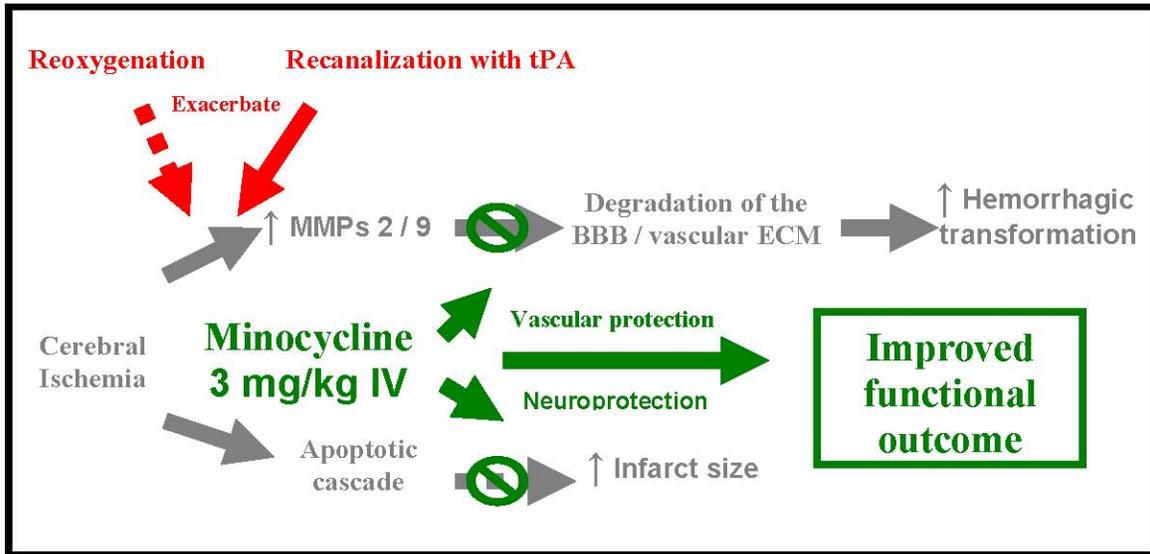


Figure 5.1: Conceptual scheme that outlines the summary of the project results

In light of this, the results of this dissertation helped assess if minocycline could potentially be useful for acute stroke patients, especially those who are in higher risk of developing hemorrhagic transformation. Results presented here show that minocycline treatment, particularly with the early intravenous 3 mg/kg dose regimen, is neurovascular protective after stroke in rats in our mechanical model. This neurovascular protection and matrix metalloproteinase inhibition occurs in the same weight-based dose approved for minocycline in humans. Importantly, minocycline remained safe and protective when it was used in combination with tissue plasminogen activator. These results point to a potential for minocycline treatment in acute ischemic stroke patients. However, whether these pre-clinical findings will be translated into human studies remains to be seen.

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## APPENDICES

Included: 2 items

Published manuscript:

Susan C. Fagan, David C. Hess, Livia S. Machado, Elizabeth J. Hohnadel, David M.

Pollock, and Adviyeh Ergul

Tactics for vascular protection after acute ischemic stroke

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Published manuscript:

Hazem Elewa, Hend Hilali, Livia S. Machado and Susan C. Fagan

Minocycline for short term neuroprotection.

Pharmacotherapy 2006 Apr;26(4):515-21

## **APPENDIX A**

### **TACTICS FOR VASCULAR PROTECTION AFTER ACUTE ISCHEMIC STROKE**

# REVIEWS OF THERAPEUTICS

## Tactics for Vascular Protection After Acute Ischemic Stroke

Susan C. Fagan, Pharm.D., David C. Hess, M.D., Livia S. Machado, B.S., Elizabeth J. Hohnadel, M.S.,  
David M. Pollock, Ph.D., and Advije Ergul, M.D., Ph.D.

**Background.** The vascular events that happen during ischemic stroke worsen outcomes in patients by causing edema, hemorrhagic transformation, and general neurologic tissue compromise. In the past 2 decades, clinical trials in patients after ischemic stroke focused on neuroprotection, but these strategies have failed in providing actual benefit. Vascular protection represents a new field to be explored in acute ischemic stroke in order to develop new approaches to therapeutic intervention.

**Purpose.** We identified tactics likely to provide vascular protection in patients with ischemic stroke. These tactics are based on knowledge of the molecular processes involved.

**Summary of Review.** The pathologic processes due to vascular injury after an occlusion of a cerebral artery can be separated into acute (those occurring within hrs), subacute (hrs to days), and chronic (days to mo). Targets for intervention can be identified for all three stages. In the acute phase, superoxide is the predominant mediator, followed by inflammatory mediators and proteases in the subacute phase. In the chronic phase, proapoptotic gene products have been implicated. Many already-marketed therapeutic agents (statins, angiotensin modulators, erythropoietin, minocycline, and thiazolidinediones), with proven safety in patients, have been shown to have activity against some of the key targets of vascular protection.

**Conclusion.** Currently available pharmacologic agents are poised for clinical trials of vascular protection after acute ischemic stroke.

**Key Words:** acute ischemic stroke, vascular protection, molecular processes.  
(*Pharmacotherapy* 2005;25(3):387–395)

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From the Program in Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia (Drs. Fagan and Ergul, Ms. Machado, and Ms. Hohnadel), the Department of Neurology (Drs. Fagan and Hess) and the Vascular Biology Center (Drs. Pollock and Ergul), Medical College of Georgia, and Specialty Care Service Line, Veterans Administration Medical Center (Drs. Fagan and Hess and Ms. Machado), Augusta, Georgia.

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Address reprint requests to Susan C. Fagan, Pharm.D., University of Georgia Clinical Pharmacy, CJ-1020 Medical College of Georgia, 1120 15th Street, Augusta, GA 30912-2450; e-mail: sfagan@mail.mcg.edu.

### OUTLINE

Pathophysiologic Processes  
Tactics for Vascular Protection  
    Reduction in Hemorrhagic Transformation  
    Reduction in Cerebral Edema  
Clinical Trials of Agents for Vascular Protection  
    Statins  
    Angiotensin-Converting Enzyme Inhibitors and  
    Angiotensin II Receptor Blockers  
    Erythropoietin  
    Minocycline  
    Thiazolidinediones  
Conclusion

**Table 1. Vascular Molecular Mediators and Protectors, Their Abbreviations, and Functions**

Mediator/Protector Name	Abbreviation	Function
Superoxide	O <sub>2</sub> <sup>-</sup>	Free anion radical that is involved in mechanisms of aging and peroxidation of lipids.
Endothelin-1	ET-1	Potent vasoconstrictor that, balancing with nitric oxide, controls tonus of vascular smooth muscle. It is implicated also with the release of hormones, thrombin, cytokines, and reactive oxygen species.
Nitric oxide	NO	Vasodilator secreted by endothelial cells.
Angiopoietin-1 and -2	Ang-1 Ang-2	Angiogenic factors thought to be involved in endothelial development and vascular remodeling.
Vascular endothelial growth factor	VEGF	Important survival factor for the endothelium, angiogenesis, and endothelial cell proliferation.
Matrix metalloproteinases 9 and 2	MMP-9 MMP-2	Zinc-dependent enzymes that degrade extracellular matrix proteins and play an important role in tissue remodeling.
Interleukin-1	IL-1	Cytokine involved in fever, prostaglandin synthesis, and lymphocyte activation.
Tumor necrosis factor- $\alpha$	TNF- $\alpha$	Proinflammatory cytokine that is pyrogenic and is believed to mediate neuronal death.
Basic fibroblast growth factor	bFGF	Angiogenic molecule that plays a role in inflammation, wound healing, and tumor cell proliferation.
Caspases	—	Family of apoptotic proteases that trigger a cascade of events that cleave proteins critical for cell survival.
B cell-associated X protein	Bax	Its ratio with B cell lymphoma leukemia 2 determines cell survival or death after an apoptotic stimulus. It acts by converting procaspase 9 into the active caspase 9.
Transformation-related protein 53	Trp53	Important controller of cell cycle, this protein interferes with cell response to stress, cell growth, stability, and apoptosis.
B cell lymphoma leukemia 2	Bcl2	Homologous partner of B cell-associated X protein that holds a prosurvival role in the cell.
Inhibitor of apoptosis protein	Iap	Antiapoptotic family of proteins that act by inhibiting the caspase family activation.

In acute ischemic stroke, both vascular and neuronal tissues are damaged. The vascular damage caused by ischemia contributes to the ultimate degree of brain injury. The vasculature is affected by a series of processes that lead to the degradation of the endothelial barrier, and this in turn results in the loss of vascular integrity. Edema and hemorrhage into the brain tissue are both thought to be related to reduced vascular integrity during ischemia and after reperfusion. Vascular endothelium is the first component of the blood vessel damaged after ischemia. Since the endothelium is responsible for providing the adjacent tissue with metabolic signaling<sup>1, 2</sup> by releasing molecular mediators of cell survival (Table 1), stability, growth, and migration, ischemia interferes with these processes, leading to further brain injury.<sup>3</sup> There has been a recent call to develop means to protect the vasculature to improve stroke outcome.<sup>4</sup>

The purpose of this review was to identify tactics to focus on for the development of

vascular protection after acute ischemic stroke. Ovid searches were performed using the key words “vascular protection,” “ischemic stroke,” and “vascular.” We identified English-only literature from 1966–2004.

### Pathophysiologic Processes

Damage to the vasculature after an ischemic stroke can be separated into the acute phase (occurring within hrs), subacute phase (hrs to days), and chronic phase (days to mo). The targets for vascular protection after acute ischemic stroke recently were reviewed in detail.<sup>5</sup> In the acute phase, neutrophils adhere to the endothelium and within hours the blood-brain barrier begins to leak. Mediators of vascular damage in the acute phase include superoxide and endothelin-1. Endogenously produced protectors of the vasculature in the acute phase include nitric oxide, angiopoietin-1, and, potentially, vascular endothelial growth factor (VEGF).

In the subacute phase (hrs), frank edema occurs, and if the injury is of sufficient magnitude, hemorrhagic transformation can occur. Mediators of this damage include matrix metalloproteinase (MMP) 9, interleukin-1, tumor necrosis factor- $\alpha$ , and potentially MMP-2. Protectors in the subacute phase include VEGF, angiopoietin-2, and basic fibroblast growth factor (bFGF).

During the chronic phase of vascular injury, the mechanisms that are mostly involved are endothelial cell apoptosis, antiapoptotic processes, and angiogenesis. In response to various factors released in the acute and subacute phases, proapoptotic gene products such as caspases, B cell-associated X (Bax) protein, and transformation-related protein 53 (Trp53) are stimulated. As a compensatory mechanism, antiapoptotic proteins B cell lymphoma leukemia 2 (Bcl2) and inhibitor of apoptosis protein (Iap) are also upregulated, suggesting that a balance of pro- and antiapoptotic proteins may be critical for the survival of both endothelial and neuronal tissue. In addition, VEGF may promote angiogenesis and improve perfusion of the damaged area. Eventually, the injured blood vessel may either promote recovery by angiogenesis or undergo apoptosis or atherosclerosis, depending on its size and function.

Figure 1 summarizes the major mediators, protective factors, and events that are involved in the ischemic damage of the cerebral vasculature.

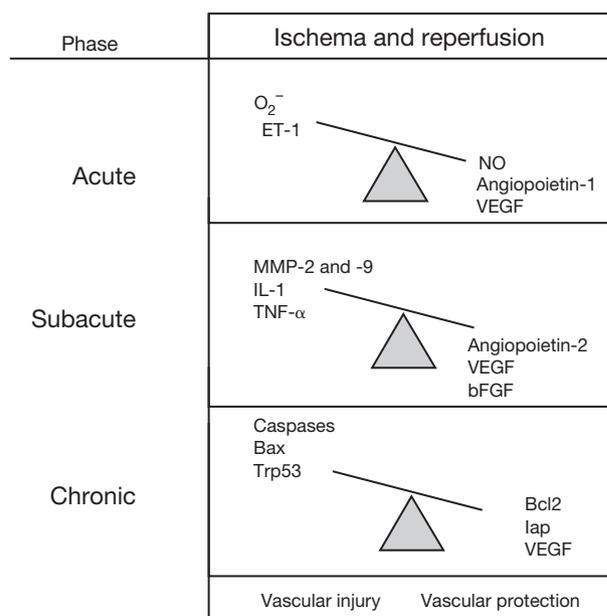
### Tactics for Vascular Protection

The only effective and widely accepted strategy for improving clinical outcomes after ischemic stroke is reperfusion therapy with thrombolysis.<sup>6-8</sup> Whether protection of the vasculature during acute cerebral ischemia will result in improved outcomes in human stroke patients is unclear. However, when an occluded artery is reopened, not only is the ischemic neuronal tissue spared, but also the vessel itself receives benefits associated with reperfusion. Neuroprotective strategies, although successful in preclinical investigation, have repeatedly failed to improve outcome in human stroke patients.<sup>9-11</sup> One possible reason for the failure of neuroprotective therapies in humans is that the damaged vasculature is unable to supply the necessary nutrients to the jeopardized tissue, nor is it able to deliver the requisite concentration of neuroprotectant to the tissue at risk. Vascular protection may not only preserve the delivery of protective substances to the injured tissue, but also prevent the two major

consequences of vascular damage in the brain—hemorrhagic transformation and ischemic cerebral edema.

However, vascular protection may not be the optimal therapeutic target in the acute phases of ischemic stroke. Some investigators have argued that vessel breakdown is necessary for the initiation of angiogenesis and the recovery process after ischemic damage.<sup>12, 13</sup> Infiltration of circulating stem cells, which may release trophic factors necessary for neurogenesis, may depend on metalloproteinase activity to facilitate passage through the microvasculature.

### Reduction in Hemorrhagic Transformation



**Figure 1.** Targets for vascular protection after acute ischemic stroke. After a period of ischemia followed by reperfusion, a cascade of events occurs that results in further damage to the cerebral vasculature by upsetting the balance between mediators of injury and protectors against injury. Illustrated is the increase in mediators of injury at each phase after the onset of ischemia. In the acute phase, superoxide ( $O_2^-$ ) and endothelin-1 (ET-1) are the predominant mediators of injury. Also in this phase, neutrophils adhere to the endothelium and initial blood-brain barrier breakdown occurs. In the subacute phase, frank hemorrhage and/or significant edema can occur if the injury is severe. In the chronic phase, both apoptosis and angiogenesis can occur, determining the ultimate fate of the tissue. NO = nitric oxide; VEGF = vascular endothelial growth factor; MMP = matrix metalloproteinase; IL-1 = interleukin-1; TNF- $\alpha$  = tumor necrosis factor- $\alpha$ ; bFGF = basic fibroblast growth factor; Bax = B cell-associated X protein; Trp53 = transformation-related protein 53; Bcl2 = B cell lymphoma leukemia 2; Iap = inhibitor of apoptosis protein.

Table 2. Potential Agents for Vascular Protection in Acute Ischemic Stroke

Agent	Proposed Mechanism of Action	Phase of Injury Targeted
Statins <sup>28, 29, 36-43</sup>	Increased endothelial NOS	Acute
	Antioxidant	Acute
	Decreased MMPs	Subacute
	Antiinflammatory	Subacute
	Decreased smooth muscle cell proliferation	Chronic
ACE inhibitors and ARBs <sup>28, 44-49</sup>	Antiapoptotic	Chronic
	Increased nitric oxide	Acute
	Decreased superoxide	Acute
	Decreased endothelin 1	Acute
Erythropoietin <sup>50-58</sup>	Decreased smooth muscle cell proliferation	Chronic
	Decreased proteases	Subacute
	Decreased apoptosis	Chronic
Minocycline <sup>59-71</sup>	Decreased glutamate toxicity	Acute
	Antiinflammatory	Subacute
	Decreased VEGF and bFGF	Subacute
	Decreased MMPs	Subacute
	Antiapoptotic	Chronic
Thiazolidinediones <sup>72-78</sup>	Antiapoptotic	Chronic
	Increased nitric oxide	Acute
	Decreased superoxide	Acute
	Decreased endothelin 1	Acute
	Antiinflammatory	Subacute
	Decreased MMPs	Subacute

NOS = nitric oxide synthase; MMP = matrix metalloproteinase; ACE = angiotensin-converting enzyme; ARB = angiotensin II receptor blocker; VEGF = vascular endothelial growth factor; bFGF = basic fibroblast growth factor.

Since the introduction of reperfusion therapy for the treatment of acute ischemic stroke, clinicians and scientists have been very interested in preventing the major adverse event associated with the therapy, hemorrhagic transformation. Hemorrhagic transformation is the development of bleeding into an ischemic lesion in the brain and can be associated with a poor outcome in 5–10% of stroke patients who receive thrombolytic therapy to achieve reperfusion.<sup>6-8</sup> Hemorrhagic transformation is thought to result from ischemic damage to the microvasculature,<sup>14</sup> leading to destruction of the basal lamina, and this is amplified by the presence of the thrombolytic tissue plasminogen activator.<sup>15-19</sup> Early in development, it was reported that acute hypertension increased the likelihood of hemorrhagic transformation in experimental animals,<sup>20</sup> whereas a reduction in blood pressure reduced this risk.<sup>21</sup> Although the relationship of hemorrhage to elevated blood pressure has been demonstrated in human stroke patients,<sup>22, 23</sup> to our knowledge, intervention to lower blood pressure and reduce hemorrhagic transformation has not been studied in a randomized clinical trial.

Other agents that have been shown to be

vascular protective and reduce hemorrhage after thrombolysis in experimental stroke include a metalloproteinase inhibitor,<sup>24</sup> a potent platelet inhibitor,<sup>25</sup> and a free radical-trapping agent.<sup>26</sup> These strategies, with distinct pharmacologic targets, point to the many ways in which damage occurs to the vasculature after cerebral ischemia.

#### Reduction in Cerebral Edema

Malignant cerebral edema is associated with large cerebral infarcts and is the most common cause of death in the first few days after an ischemic stroke.<sup>27</sup> Passage of water and proteins through the damaged cerebrovasculature, often within hours of the onset of symptoms, leads to neurologic deterioration and even fatal herniation. No clinical trial evidence exists to guide the treatment of ischemic cerebral edema. Although osmotic agents, hyperventilation, and hemicraniectomy are attempted in stroke patients, it is without definitive evidence of improved outcomes.

#### Clinical Trials of Agents for Vascular Protection

Many pharmacologic strategies have vascular protective properties. Since many have demonstrated safety in humans for other indications,

they are attractive candidates for clinical trials in ischemic stroke. The agents and their molecular targets are summarized in Table 2.<sup>28, 29, 36–78</sup>

### Statins

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have generated the most excitement surrounding their potential to provide vascular protection. The statins were initially developed for their ability to reduce serum cholesterol safely and effectively in order to reduce the risk of cardiovascular morbidity and mortality. Recent evidence strongly suggests that the health benefits of the statins go well beyond those associated with lowering of lipid levels.<sup>28, 29</sup> In fact, early in clinical investigation, statins were associated with reductions in cardiovascular events when compared with patients who had similar reductions in cholesterol levels achieved by other means.<sup>29</sup> Clearly, statins have many vascular effects, including stimulation of endothelial nitric oxide synthase (NOS), action as an antioxidant, reduction in blood viscosity and platelet aggregation, augmentation of endogenous thrombolysis, inhibition of smooth muscle cell proliferation and migration, and inhibition of the MMPs responsible for plaque instability. In addition, the statins have antiinflammatory effects that may be beneficial in both acute ischemia and the long-term progression of atherosclerotic disease.

In humans, there is mounting evidence, as with the angiotensin-converting enzyme (ACE) inhibitors, that the statins reverse endothelial dysfunction in patients with nephrotic syndrome,<sup>30</sup> coronary artery disease,<sup>31</sup> and heart transplantation.<sup>32</sup> Although it is difficult to definitively separate the effects on lipids from the benefits to the endothelium<sup>33</sup> when performing studies in humans, most believe that the vascular protection effects contribute to the improved cardiovascular outcomes in patients taking statins.<sup>34, 35</sup>

Benefits of statins unrelated to lowering of lipid levels appear to include the upregulation of endothelial NOS and subsequent increased production of nitric oxide.<sup>36</sup> Statin treatment prevents mevalonate formation but also leads to inhibition of the important signaling molecules Rac, Rho, and Cdc42 through the disruption of the isoprenoids farnesyl pyrophosphate and geranylgeranyl pyrophosphate formation. Accumulation of Rac, Rho, and Cdc42 in the cytoplasm results in upregulation of endothelial NOS and tissue plasminogen activator as well as

a decrease in the amounts of plasminogen activator inhibitor-1, endothelin-1, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cell proliferation, and cell migration. Separate activation of the phosphatidylinositol kinase–protein kinase Akt pathway leads to the increased NOS activity and upregulation of antiapoptotic proteins Bcl2 and nuclear factor- $\kappa$  B<sup>37</sup> while inhibiting caspase 9.<sup>38</sup> Recent studies reveal that activation of this pathway by statins encourages angiogenesis, synaptogenesis, and neurogenesis.<sup>39</sup> Of interest, one study found that treatment with statins for 14 days before ischemic stroke reduced infarct size in mice, but the effect was lost in endothelial NOS knock-out mice (compared with wild type), suggesting that statins exert their protective effects through nitric oxide regulation.<sup>40, 41</sup> In both human and animal studies, withdrawal of statin treatment resulted in a rebound effect characterized by insufficient nitric oxide and a loss of vascular protective effects.<sup>40, 41</sup> Some researchers postulate that the neuroprotective properties of statins are vascular in nature and are due to the upregulation of the fibrinolytic system and endothelial NOS activation.<sup>42</sup> It is important to note that all statins are not equally potent<sup>42</sup> and their actions may vary in different vascular beds.<sup>43</sup>

### The Angiotensin-Converting Enzyme Inhibitors and Angiotensin II Receptor Blockers

Evidence supporting the vascular protective properties of ACE inhibitors was recently reviewed.<sup>44</sup> It is likely that the benefits of ACE inhibition are realized through both the reduction in production of angiotensin II and the enhancement of bradykinin, a known stimulant of nitric oxide and prostacyclin activity.<sup>44</sup> The angiotensin II receptor blockers (ARBs) also have been touted to bestow many of the same benefits on the vasculature as those of the ACE inhibitors.<sup>28</sup> Through blocking the damaging effects of angiotensin II on endothelial relaxation as well as smooth muscle cell proliferation and migration, the ARBs may compare favorably with the more studied ACE inhibitors.

Angiotensin II has been shown to stimulate superoxide production in human internal mammary arteries and saphenous veins.<sup>45, 46</sup> This process is known to be mediated by NADPH oxidase through an angiotensin II type 1 (AT<sub>1</sub>) receptor–dependent mechanism. Recently, local angiotensin II was found to cause time- and concentration-dependent endothelial dysfunction

in cerebral arteries that was entirely prevented by administration of a superoxide scavenger and an NADPH oxidase inhibitor.<sup>47</sup> When the AT<sub>1</sub>-receptor blocker irbesartan was compared with amlodipine and hydrochlorothiazide-hydralazine *in vivo*, irbesartan was found to be more effective in decreasing superoxide and increasing nitric oxide bioavailability in carotid arteries, despite similar changes in blood pressure.<sup>48</sup> Studies have also shown that angiotensin II stimulates endothelin-1 production, which in turn could reduce endothelial-dependent vasorelaxation and increase superoxide.<sup>49</sup> These findings are important because they point to the potential for angiotensin II blockade to be vascular protective in the acute phase.

### Erythropoietin

Erythropoietin is an endogenous trophic factor and a known mediator of blood cell maturation and survival. In the central nervous system, endogenous erythropoietin is a mediator of the physiologic response to hypoxia and confers neuroprotection in many different models of brain injury.<sup>50–54</sup> The ability of systemically administered erythropoietin to effectively cross the blood-brain barrier has been demonstrated in several models,<sup>51</sup> and the presence of erythropoietin receptors on brain endothelial cells<sup>52</sup> may make this possible. In a small study of intravenous erythropoietin, administered to 20 patients with ischemic stroke, compared with placebo, erythropoietin was shown to enter the cerebrospinal fluid successfully and was associated with improved functional outcome at 30 days.<sup>55</sup>

It is likely that erythropoietin is a vascular protectant in addition to being neuroprotective. In an animal model of subarachnoid hemorrhage, subcutaneously administered erythropoietin was associated with preservation of cerebral autoregulation.<sup>56</sup> In cultured brain endothelial cells, erythropoietin has been shown to prevent DNA fragmentation and membrane phosphatidylserine exposure leading to apoptosis, and this effect was dependent on the activation of protein kinase B (Akt1).<sup>57</sup> In addition, erythropoietin inhibited the activation of the caspases and mitochondrial cytochrome c release.<sup>58</sup> Therefore, administration of erythropoietin to patients with ischemic stroke may improve outcomes by both neuroprotection and vascular protection.

### Minocycline

Minocycline is an antibiotic and a very promising neuroprotective agent.<sup>59</sup> Initially studied for its antiinflammatory effects in central nervous system disorders,<sup>60</sup> it soon became evident that minocycline has multiple beneficial effects in brain injury. Minocycline inhibits microglial activation,<sup>61–63</sup> prevents glutamate toxicity,<sup>60, 61</sup> prevents caspase 1-activated apoptosis,<sup>64</sup> and decreases the activity of inducible NOS and p38 mitogen-activated protein kinase.<sup>65, 66</sup>

In the cerebral vasculature, minocycline may be expected to have protective effects by virtue of its antiangiogenic properties. Minocycline has been shown to inhibit VEGF and bFGF,<sup>67</sup> and to decrease the activity of cysteine proteases.<sup>68</sup> Finally, minocycline is a potent inhibitor of MMP activity,<sup>69, 70</sup> and this inhibition correlated to improved outcome in an animal model of intracerebral hemorrhage.<sup>71</sup>

### Thiazolidinediones

The thiazolidinediones are peroxisome proliferator-activated receptor  $\gamma$  agonists used extensively in the management of insulin resistance in type 2 diabetes mellitus. In addition, pioglitazone and rosiglitazone appear to have many additional vascular protective effects.<sup>72</sup> The potential for vascular protection in the acute stroke period is related to their ability to reduce oxidative and nitrative stress. In a rabbit model of hypercholesterolemia, rosiglitazone was shown to inhibit the production of superoxide and to reduce the degradation of nitric oxide.<sup>73</sup> In addition, other investigators have found that the thiazolidinediones downregulate AT<sub>1</sub> receptors and upregulate AT<sub>2</sub> receptors, decreasing inflammation.<sup>74</sup> Finally, the thiazolidinediones decrease macrophage activation, MMP-9 activity,<sup>75, 76</sup> and endothelin-1 production.<sup>77</sup>

As evidence of how these agents are ready for clinical trials in stroke, pioglitazone has been shown to be safe and to improve markers of metabolic syndrome and inflammation in patients with recent (but > 2 mo) stroke or transient ischemic attack.<sup>78</sup>

### Conclusion

Protection of the cerebral vasculature after an ischemic stroke appears to be a logical approach to prevent edema and hemorrhage and to

enhance recovery. Enhancement of nitric oxide and angiopoietin production and the inhibition of VEGF, angiotensin II, endothelin-1, the MMPs, and inflammatory mediators are all potential pharmacologic targets for vascular protection. Although experimental evidence of the beneficial effects of vascular protection exists for both acute and chronic phases, only vascular protection in the chronic phase in human stroke patients has been proved as a therapeutic intervention. To initiate clinical trials of vascular protection in acute ischemic stroke, some questions remain to be answered. For example, what are the proper end points for a clinical trial of vascular protection (computed tomography, magnetic resonance imaging, functional outcome)? What type of patient should be included (only those with reperfusion or those with large infarcts with risk of edema and hemorrhagic transformation)? How long should the patient be treated (3 vs 7 days or longer)? Many of these questions can be answered with well-designed translational research projects and pilot studies.

Statins, ACE inhibitors, and ARBs have been shown to reduce cardiovascular morbidity, including recurrent stroke in patients with ischemic stroke; are already prescribed to many patients with acute ischemic stroke; and are ready for clinical trials. The thiazolidinediones pioglitazone and rosiglitazone are also frequently used in diabetic stroke patients and can be safely administered in dosages shown to have favorable effects on systemic markers of inflammation. Minocycline and erythropoietin are not frequently administered to patients with ischemic stroke and may require further dose-finding and safety studies before vascular protection trials are performed in patients with acute stroke. Use of these and other available agents for vascular protection in the acute phase needs to be explored in humans as a strategy to improve neurologic outcomes after an acute ischemic stroke.

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## **APPENDIX B**

### **MINOCYCLINE FOR SHORT TERM NEUROPROTECTION**

# REVIEWS OF THERAPEUTICS

## Minocycline for Short-Term Neuroprotection

Hazem F. Elewa, B.S., Hend Hilali, B.S., David C. Hess, M.D., Livia S. Machado, B.S., and Susan C. Fagan, Pharm.D.

Minocycline is a widely used tetracycline antibiotic. For decades, it has been used to treat various gram-positive and gram-negative infections. Minocycline was recently shown to have neuroprotective properties in animal models of acute neurologic injury. As a neuroprotective agent, the drug appears more effective than other treatment options. In addition to its high penetration of the blood-brain barrier, minocycline is a safe compound commonly used to treat chronic infections. Its several mechanisms of action in neuroprotection—antiinflammatory and antiapoptotic effects, and protease inhibition—make it a desirable candidate as therapy for acute neurologic injury, such as ischemic stroke. Minocycline is ready for clinical trials of acute neurologic injury.

**Key Words:** minocycline, stroke, neuroprotection.  
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### OUTLINE

Background  
Neuroprotective Properties  
Mechanisms of Action as a Neuroprotective Agent  
    Antiinflammatory Effects  
    Antiapoptotic Effects  
    Inhibition of Matrix Metalloproteinases  
Summary  
Pharmacokinetic Issues  
Adverse Effects  
Conclusion

Numerous studies have been conducted to develop an effective neuroprotective agent for acute brain injury, particularly ischemic stroke. To date, no pharmacologic agent has been effective in a context of short-term intervention. Agents such as calcium channel blockers and glutamate antagonists do not provide a benefit in

acute brain injury for several reasons, including poor penetration of the blood-brain barrier, dose-limiting toxicity of the agent,<sup>1–4</sup> and a time window of effectiveness that is too short for clinical use.<sup>3–6</sup> After carefully reviewing studies of failed agents, we clearly found a need to investigate new approaches to acute brain injury.

### Background

Minocycline is a widely used semisynthetic tetracycline antibiotic<sup>7</sup> with known antiinflammatory, antiapoptotic, and glutamate-antagonist properties in several models of brain injury. The drug has been used for decades to treat infections caused by a variety of gram-negative and gram-positive organisms. Minocycline is indicated for the treatment of several diseases including acne vulgaris,<sup>8</sup> central nervous system and urinary tract infections, gonorrhea, meningitis, shigellosis, conjunctivitis, psittacosis, Q fever, relapsing fever, and syphilis. Minocycline is a generic drug, available in oral or intravenous formulations in humans, and subgingival sustained-release microspheres are used in adults with periodontitis.<sup>9</sup>

Like other tetracycline compounds, minocycline interferes with bacterial protein synthesis by binding to the 30S ribosomal subunit, inhibiting messenger RNA–transfer RNA interaction and

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From the Program in Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Athens, Georgia (all authors); the Department of Neurology, Medical College of Georgia, Augusta, Georgia (Drs. Hess and Fagan); and the Specialty Care Service Line, Veterans Administration Medical Center, Augusta, Georgia (all authors).

Address reprint requests to Susan C. Fagan, Pharm.D., University of Georgia Clinical Pharmacy, CJ-1020 Medical College of Georgia, 1120 15th Street, Augusta, GA 30912-2450; e-mail: sfagan@mail.mcg.edu.

**Table 1. Efficacy of Minocycline in Animal Models of Acute Neurologic Injury**

Animal Model and Type of Injury	Minocycline Dosage	Therapeutic Window	Outcome
Rat focal ischemia, TMCAo (90 min) <sup>14</sup>	45 mg/kg i.p. b.i.d. on day 1, then 22 mg/kg i.p. b.i.d. for 2 days	Pretreatment: 2–4 hrs after insult:	76% infarct reduction (72 hrs) 63% reduction
Rat focal ischemia, embolic clot <sup>15</sup>	45 mg/kg i.p. b.i.d. on day 1, then 22.5 mg/kg i.p. b.i.d. on day 2	1 hr after insult	42% infarct reduction (48 hrs)
Mouse focal ischemia, PMCAo <sup>16</sup>	90 mg/kg i.p.	Pretreat 60 min before or 30 min after insult	Reduced infarction and brain swelling
Rat focal ischemia, TMCAo (90 min) <sup>17</sup>	3 and 10 mg/kg i.v.	4 hrs (3 mg/kg), 5 hrs (10 mg/kg)	40–50% infarct reduction (24 hrs)
Gerbil global ischemia <sup>18</sup>	45 mg/kg i.p. x 1, then 90 mg/kg i.p. b.i.d. on day 1, then 45 mg/kg after 36 hrs	Pretreatment: 30 min after insult:	Increased survival of CA1 neurons from 10% to 77% Increased survival to 71%
Rat neonatal hypoxia-ischemia <sup>19, a</sup>	45 or 22.5 mg/kg i.p.	Immediately before or after insult	Robust protection before and at 30 min, but not at 3 hrs
Rat spinal cord injury <sup>20</sup>	50 mg/kg i.p. b.i.d. for 2 days	30 min after insult	Improved function
Rat spinal cord injury <sup>21</sup>	90 mg/kg i.p. x 1, then 45 mg/kg i.p. for 5 days	1 hr after insult	Enhanced long-term hind-limb locomotion, coordinated motor function, and hind-limb reflex recovery
Mouse spinal cord injury <sup>22, b</sup>	50 mg/kg	1 hr after insult	Improved hind-limb function and strength, axonal sparing, superior to methylprednisolone
Mouse traumatic brain injury <sup>23</sup>	90 mg/kg i.p. x 1, then 45 mg/kg i.p. b.i.d. until sacrifice	Pretreatment or 30 min after insult	Improved function (Rotorod test), decreased lesion size
Rat intracerebral hemorrhage <sup>24, c</sup>	45 mg/kg i.p. b.i.d. on day 1, then 22.5 mg/kg i.p. x 1	1 hr after insult	Improved function

TMCAo = temporary occlusion of the middle cerebral artery; i.p. = intraperitoneally; PMCAo = permanent occlusion of the middle cerebral artery.

<sup>a</sup>Carotid occlusion plus hypoxia.

<sup>b</sup>Extradural compression with aneurysm clip.

<sup>c</sup>Collagenase.

protein translation.<sup>10</sup> In addition to the anti-bacterial properties, evidence supports anti-inflammatory actions of tetracyclines.<sup>11, 12</sup> Because of its anticollagenase, immunosuppressive, and immunomodulating effects, minocycline hydrochloride has been used to manage rheumatoid arthritis.<sup>13</sup>

### Neuroprotective Properties

Over the past 5 years, numerous reports have demonstrated the efficacy of minocycline in a variety of animal models of acute neurologic injury (Table 1).<sup>14–24</sup> The drug had a broad neuroprotective effect unrivaled by those of other agents. Minocycline was effective in animal models of global cerebral ischemia,<sup>18, 19</sup> focal cerebral ischemia,<sup>14, 17, 25</sup> traumatic brain injury,<sup>23</sup> spinal cord injury,<sup>20–22</sup> and intracerebral hemorrhage.<sup>24</sup> All of these injuries share common pathophysiologic mechanisms and the need for early (probably within 3–6 hrs of onset)

interventions and treatment. Minocycline not only reduced tissue injury but also improved functional recovery.

Minocycline is likely to be more successful than other studied neuroprotective compounds in that it avoids the common pitfalls stated above. Minocycline has superior penetration of the blood-brain barrier,<sup>26</sup> and it was protective for longer than 3 hours in the studied animals.<sup>14, 17, 24</sup> In addition, because it is a safe compound, minocycline is particularly well suited for a clinical trial. The drug appears to be an ideal candidate as a therapy that will overcome the issues identified in neuroprotective trials of failed agents.

### Mechanisms of Action as a Neuroprotective Agent

#### Antiinflammatory Effects

The antiinflammatory actions of tetracyclines

have been demonstrated in both acute and chronic brain injury. Minocycline has anti-inflammatory effects on neutrophils, monocytes, microglial cells, and neurons. It inhibits neutrophil-mediated tissue injury by inhibiting neutrophilic migration and degranulation and by suppressing the formation of oxygen radicals.<sup>27</sup> In a model of focal cerebral ischemia, minocycline inhibited enzymes that contribute to inflammation, such as the inducible form of nitric oxide synthase and interleukin-1 $\beta$  converting enzyme,<sup>18</sup> it suppressed apoptosis, and it reduced microglial activation.<sup>14, 18</sup> Minocycline inhibited nitric oxide release (likely by suppressing the expression of nitric oxide synthase) from monocytic cells induced by lipopolysaccharide or interferon- $\gamma$  expression.<sup>28, 29</sup>

In an acute toxin model of Parkinson's disease, minocycline protected neurons (induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in an oxygen radical-based mechanism of injury), where inflammation prominently contributed to neuronal injury.<sup>30</sup> In this model, minocycline prevented microglial activation and expression of interleukin-1 and the inducible form of nitric oxide synthase.

Minocycline was also studied in a rat model of immune-inflammatory encephalitis in which microglia, monocytes, and T-cell activation mediated neuronal injury by means of several inflammatory mediators.<sup>31</sup> The drug delayed and reduced the progression of disease (including demyelination) as well as the infiltration of inflammatory cells.

At nanomolar concentrations, minocycline inhibited glutamate excitotoxic effects in mixed neuron-glial cell cultures in correlation with the inhibition of p38 phosphorylation and interleukin-1 release.<sup>32</sup> The drug protected rat neurons (cerebellar granules) from excitotoxic injury induced by reactive oxygen species and nitric oxide. Minocycline neuroprotection *in vitro* was associated with the inhibition of inflammatory signaling kinases, such as p38.<sup>33</sup> In a model of immune-inflammatory encephalitis, minocycline reduced the release of tumor necrosis factor from activated oligodendrocytes while enhancing the release of interleukin-10, an antiinflammatory cytokine.<sup>31</sup>

The antiinflammatory effects of minocycline have also been demonstrated in humans. At doses commonly used for indications other than neuroprotection, minocycline provided antiinflammatory benefits in rheumatoid arthritis that was not treated with other disease-modifying

agents.<sup>34, 35</sup> In a small pilot clinical trial of multiple sclerosis, minocycline 200 mg/day reduced the number of gadolinium-enhancing lesions on magnetic resonance imaging,<sup>36</sup> demonstrating its ability to decrease the inflammatory damage associated with the disease.

In summary, compelling evidence from the last few years suggests that minocycline modulates inflammation and that this drug might be a novel therapeutic approach to diseases characterized by the stimulation of inflammatory cascades, such as acute ischemic brain injury.

### Antiapoptotic Effects

Apoptosis, or program cell death, is thought to play a role in both acute and chronic brain injury. Minocycline prevented apoptosis and the release of cytochrome c from mitochondria in both *in vitro* and *in vivo* models. Minocycline delayed the progression of amyotrophic lateral sclerosis-like syndrome in superoxide dismutase-1 mutant mice and inhibited mitochondrial release of cytochrome c *in vitro* and *in vivo*.<sup>37</sup> The drug inhibited mitochondrial cell death, both caspase dependent (cytochrome c and Smac/Diablo release) and caspase independent (apoptosis inducing factor release), in a Huntington striatal-cell model.<sup>38</sup> Minocycline also protected the renal proximal tubule cells from apoptosis on exposure to azide, hypoxia, staurosporine, and cisplatin.<sup>39</sup>

Furthermore, minocycline induced the upregulation of the antiapoptotic protein bcl-2 at the messenger-RNA level. In fact, the antiapoptotic effects of minocycline were lost when cells were pretreated with bcl-2 antisense; this finding suggested that the antiapoptotic action of minocycline depended on bcl-2. Moreover, bcl-2 was upregulated in neurons *in vitro* when they were incubated with equivalent doses of clinically therapeutic concentrations of minocycline.<sup>40</sup> In cardiomyocytes exposed to anoxia and reoxygenation, minocycline inhibited the release of cytochrome c and Smac/Diablo from mitochondria and inhibited both caspase activation and apoptosis.<sup>41</sup>

### Inhibition of Matrix Metalloproteinases

Tetracyclines are known to inhibit matrix metalloproteinases.<sup>42</sup> Low-dose doxycycline, the first matrix-metalloproteinase inhibitor the United States Food and Drug Administration approved, is used in periodontal disease.<sup>43</sup>

In a rat model of adjuvant arthritis, doxycycline and tetracycline (two close analogs of minocycline) reduced joint swelling and inflammation and improved radiologic evidence of damage when they were given with a standard nonsteroidal antiinflammatory agent. In this model, the arthritic syndrome was associated with the suppression of matrix metalloproteinase-2 (gelatinase) activation in the inflamed joints.<sup>44</sup>

Minocycline also reduced levels of matrix metalloproteinase-9 in a model of immune-inflammatory encephalitis.<sup>31</sup> In a collagenase-induced model of intracerebral hemorrhage, minocycline reduced MMP-12 and improved functional outcomes.<sup>24</sup> In addition, minocycline reduced renal microvascular leakage in a rat model of ischemic renal injury. This action was probably due to diminishing the activity of matrix metalloproteinases.<sup>45</sup>

Matrix metalloproteinases are increasingly associated with diseases that involve degeneration of extracellular proteins and matrix in the brain.<sup>46</sup> For this reason, the inhibition of these proteases with minocycline seems to be an attractive experimental therapy.

## Summary

Minocycline has several mechanisms of action, including antiinflammatory, matrix-metalloproteinase inhibitory, and antiapoptotic effects, that make it an attractive neuroprotective agent. It has demonstrated activity in many acute and chronic animal models of neurologic disease, and it is one of few agents that has been shown to be efficacious in animal models of spinal cord injury, traumatic brain injury, intracerebral hemorrhage, or global or focal cerebral ischemia.

These observations point to a key element that distinguishes minocycline from other neuroprotective agents, namely, the diversity of cellular mechanisms affected. Minocycline may likely act by means of vascular mechanisms as well. These mechanisms have been correlated with the intensity of the inflammatory response to injury and with the severity of damage to the brain parenchyma.<sup>47</sup> Finally, in humans, minocycline appears to exert antiinflammatory properties with the same dosage regimens as those clinically used for antibacterial treatment. Therefore, minocycline is a likely candidate as a drug for neuroprotection in acute brain injury in its present human formulation and dosage.

## Pharmacokinetic Issues

In acute brain injury, the ability to rapidly deliver a potential neuroprotective agent to the systemic circulation is a necessity. In this setting, intravenous administration is most often required.

Because minocycline has been used for decades, its clinical pharmacokinetics are well described in humans. After a 200-mg intravenous dose, the mean peak concentration is 4.0 mg/L,<sup>48</sup> and steady-state concentrations after a dose of 100 mg given orally twice/day for 3 days are 1.4–1.8 mg/L.<sup>26</sup> Minocycline is the most lipophilic of the commonly used tetracycline antibiotics, and its concentration in the cerebrospinal fluid is 11–56% of plasma concentrations.<sup>26</sup> Therefore, concentrations in cerebrospinal fluid after long-term dosing are expected to be approximately 0.5 mg/L. In addition, urinary excretion is lower with minocycline than with other tetracyclines; therefore, minocycline is safer than the other tetracyclines for patients with renal insufficiency.

Since 1999, most reported studies of the neuroprotective effects of minocycline in rodent models of brain injury used large intraperitoneal doses of 10–90 mg/kg.<sup>14, 16, 17, 19, 23–25, 39, 49, 50</sup> Even in stroke models, in which timely cerebral neuroprotection is important, intraperitoneal administration was used.<sup>14, 19, 24, 39</sup>

Because the pharmacokinetics of large intraperitoneal doses of minocycline in rodents were unknown but necessary to extrapolate experimental results to humans, we studied this issue. We found that the intraperitoneal route resulted in widely variable serum concentrations of minocycline and that it delayed absorption in the systemic circulation, with peak concentrations achieved at a mean of 2.5 hours after injection. Compared with intravenous administration, intraperitoneal administration resulted in a bioavailability of 10–80%, which was probably due to the frank deposition of the drug in the peritoneal cavity.<sup>51</sup> The intraperitoneal route of administration probably accounts for the wide range of high doses reported in the literature. Intravenous dosing was needed to determine the true therapeutic window and the dose-response relationship in focal cerebral ischemia.<sup>51</sup> We determined that peak serum concentrations above 3.5 mg/L and trough concentration above 2 mg/L were neuroprotective in temporary focal cerebral ischemia in rats.<sup>17</sup> Low doses are being studied.

When intravenous administration was used to overcome the absorption problems of both oral and intraperitoneal administration, the volume of distribution of minocycline was similar in rats and in humans when adjusted by weight.<sup>51</sup> In other words, the intravenous administration of 3 mg/kg in humans and in rats is expected to achieve peak concentrations of the same magnitude (3–5 mg/L). The main difference in the pharmacokinetic parameters between the species is the half-life, which is approximately 17 hours in humans<sup>26</sup> and only 3 hours in rats.<sup>51,52</sup>

In summary, intravenous doses of minocycline commonly used in humans should achieve serum and cerebrospinal fluid concentrations that were neuroprotective in animal models.

### Adverse Effects

Most available information on the tolerability of minocycline was obtained after long-term oral administration. In studies of ambulatory patients taking minocycline long term, increasing the dosage above 100 mg twice/day was problematic because of the common adverse effect of dizziness (affecting 26–78% of patients).<sup>8, 53</sup> More recently, most adverse effects of oral minocycline therapy in patients with amyotrophic lateral sclerosis were gastrointestinal.<sup>54</sup> The mean tolerated dose was 387 mg/day, and all patients could tolerate at least 300 mg/day. No patient had dizziness, but elevated concentrations of blood urea nitrogen and liver enzymes were reported over the 6-month treatment period.

Doses of up to 400 mg given intravenously have safely been used to treat serious infections in humans. In a case series of 119 patients who received intravenous minocycline 200–400 mg for 2–24 days to treat an infectious disease, 21 (18%) had adverse effects, 50% of which were gastrointestinal.<sup>55</sup> Only one patient discontinued therapy prematurely. This patient developed azotemia, but a chronic urinary tract infection complicated its attribution.

In a search for adverse effects associated with intravenous minocycline that have been reported to the World Health Organization Collaborating Center for International Drug Monitoring (Uppsala, Sweden) since 1975, we found 122 case reports of adverse drug reactions. No assessment of causality was given, and the reports do not represent the opinion of the World Health Organization. The most common event was abnormal hepatic function (19 reports). Thrombocytopenia was reported 11 times, and

injection-site reaction was reported once. The data of the World Health Organization were limited because no denominator could be ascertained and because the dosage and duration of intravenous minocycline treatment were unknown.

The dose of minocycline that is neuroprotective and tolerable in humans is still unknown. In addition, the feasibility of rapidly administering intravenous doses of minocycline to patients and the preliminary evidence of the activity of the compound should be determined before minocycline is further developed as a treatment for acute neuroprotection. The question of optimal duration should be addressed by assessing a biomarker of inflammation and by measuring serum levels of minocycline in the patient. In addition, further translational studies in animals will contribute to our understanding of the optimal duration of minocycline treatment for neuroprotection.

### Conclusion

Minocycline is already in clinical trials for the chronic brain injury of amyotrophic lateral sclerosis and multiple sclerosis and has a strong potential for treating brain diseases that require acute intervention, such as stroke. Minocycline has long been established as a safe drug for clinical use, it has several mechanisms of action, and it had a delayed therapeutic window in experimental models. Minocycline is ready for clinical trials as a short-term neuroprotectant.

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