Minocycline-Induced Attenuation of Iron Overload and Brain Injury After Experimental Intracerebral Hemorrhage

Fan Zhao, MD; Ya Hua, MD; Yangdong He, MD; Richard F. Keep, PhD; Guohua Xi, MD

Background and Purpose—Brain iron overload plays a detrimental role in brain injury after intracerebral hemorrhage (ICH). A recent study found that minocycline acts as an iron chelator and reduces iron-induced neuronal death in vitro. The present study investigated if minocycline reduces iron overload after ICH and iron-induced brain injury in vivo.

Methods—This study was divided into 4 parts: (1) rats with different sizes of ICH were euthanized 3 days later for serum total iron and brain edema determination; (2) rats had an ICH treated with minocycline or vehicle. Serum iron, brain iron, and brain iron handling proteins were measured; (3) rats had an intracaudate injection of saline, iron, iron+minocycline, or iron+macrophage/microglia inhibitory factor and were used for brain edema and neuronal death measurements; and (4) rats had an intracaudate injection of iron and were treated with minocycline. The brains were used for edema measurement.

Results—After ICH, serum total iron and brain nonheme iron increased and these changes were reduced by minocycline treatment. Minocycline also reduced ICH-induced upregulation of brain iron handling proteins and neuronal death. Intracaudate injection of iron caused brain edema, blood–brain barrier leakage, and brain cell death, all of which were significantly reduced by coinjection with minocycline.

Conclusions—The current study found that minocycline reduces iron overload after ICH and iron-induced brain injury. It is also well known minocycline is an inhibitor of microglial activation. Minocycline may be very useful for patients with ICH because both iron accumulation and microglia activation contribute to brain damage after ICH. (Stroke. 2011;42:3587-3593.)

Key Words: brain edema ■ cerebral hemorrhage ■ iron ■ minocycline

Intracerebral hemorrhage (ICH) is a subtype of stroke with high morbidity and mortality.1 Evidence suggests that iron is involved in ICH-induced brain injury.2 After ICH, iron concentrations in the surrounding brain can reach very high levels. Thus, our previous studies showed an increase in brain nonheme iron after ICH in rats, and this remains high for at least 1 month.3 Brain iron overload after ICH causes brain edema in the acute phase and brain atrophy later. We have now demonstrated that an iron chelator, deferoxamine, reduces ICH-induced brain edema, neuronal death, brain atrophy, and neurological deficits in young rats.4–6 aged rats7 and pigs.8 Clinical data also suggest a role of iron in ICH-induced brain injury. Recent studies found that high levels of serum ferritin, an iron storage protein, are independently associated with poor outcome and severe brain edema in patients with ICH.9,10

Minocycline is a semisynthetic second-generation derivative of tetracycline. It is a highly lipophilic compound and penetrates the brain–blood barrier (BBB) easily. It has a clear neurovascular protective effect in animal models of ICH and cerebral ischemia.11–14 and it is in current clinical trial for patients with ischemic stroke. Minocycline has been reported to provide neuroprotection by reducing the inflammatory response to injury, including inhibiting microglia, matrix metalloproteinase, and poly(ADP-ribose) polymerase-1 activation.15,16 For example, it inhibits macrophage/microglia activation after ICH in rats.17 Evidence indicates that there is an inflammatory component to ICH-induced brain injury.18 However, a recent study has shown that minocycline also attenuates iron neurotoxicity in cortical neuronal cultures by chelating iron.19

Therefore, the present study investigated whether minocycline can attenuate iron overload and brain injury after ICH and whether minocycline reduces iron-induced brain injury in vivo.

Materials and Methods

Animal Preparation and Intracerebral Injection

Animal use protocols were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 160 male Sprague-Dawley rats (weighed 275–300g, Charles River Laboratories, Portage, MI) were used in this study. Septic precautions were used in all surgical procedures and body temperature was maintained at 37.5°C. Rats were anesthetized with pentobarbital (45 mg/kg, intraperitoneally) and the right femoral artery was cannulated for...
continuous blood pressure monitoring and blood sampling. Blood from the catheter was used to determine pH, PaO₂, PaCO₂, hematocrit, and glucose. It was also the source for the intracerebral blood injection. The animals were positioned in a stereotactic frame (Kopf Instruments). Rats received an injection into the right basal ganglia and the coordinates were 0.2 mm anterior to bregma, 5.5 mm ventral, and 4.0 mm lateral to midline.

**Experimental Groups**

This study was divided into 4 parts. In the first part, rats (n=6 for each group) had an intracaudate injection of 10 µL or 100 µL autologous whole blood. Rats were euthanized at Day 3 for serum total iron and brain water content determination. Normal or sham operation rats (n=4) were used as controls. In the second part, rats had an intracerebral injection of 100 µL autologous whole blood, and the rats were treated with minocycline (45 mg/kg, intraperitoneally, at 2 and 12 hours after ICH followed by 22.5 mg/kg twice a day up to 7 days) or vehicle. This dose of minocycline can reduce ICH-induced brain edema. In addition, rats (n=6 for each group) were euthanized 3 days later for brain nonheme iron determination. In the third part, rats (n=15 each group) had intracaudate injection of 50 µL of saline, FeCl₂ (0.5 mmol/L), FeCl₂ (0.5 mmol/L)+minocycline (0.5 mmol/L; Sigma) or FeCl₂ (0.5 mmol/L)+macrophage/microglia inhibitory factor (MIF; 0.5 mmol/L; American Peptide Co, Inc). Rats were euthanized at 24 hours and the brains were used for brain edema, BBB disruption, and brain cell death measurements. In the fourth part, rats had intracaudate injection of 50 µL of FeCl₂ (0.5mmol/L), and the rats were treated with minocycline (45 mg/kg, intraperitoneally, immediately and 12 hours after iron injection) or vehicle (n=5 each group). Rats were euthanized at 24 hours for brain edema measurement.

**Serum Total Iron Determination**

Venous blood samples were drawn for total serum iron measurement before euthanasia. The blood samples were centrifuged after clotting, the serum separated, and total iron levels measured by a QuantChrom Iron Assay Kit (Bioassay Systems).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described. Primary antibodies were polyclonal rabbit antihuman ferritin IgG (DAKO; 1:500 dilution) and monoclonal mouse antirat neuronal nuclei IgG (Millipore; 1:500). Normal rabbit IgG or mouse IgG was used as negative controls.

**Western Blot Analysis**

Western blot analysis was performed as described earlier. The primary antibodies were polyclonal goat antiferritin-l-chain (1:1000 dilution; Abnova), polyclonal rabbit antiferritin-H-chain (1:2000 dilution; Cell Signaling), polyclonal rabbit antihuman transferrin (1:2000 dilution; Dako), monoclonal mouse antihuman transferrin receptor (1:2000 dilution; Invitrogen), polyclonal sheep antiserum ceruloplasmin (1:2000 dilution; Abcam), or polyclonal goat antimouse albumin antibody (1:20000 dilution; BETHYL Laboratories Inc). The secondary antibodies were goat antirabbit IgG, goat antimouse IgG, rabbit antigoat IgG (1:4000 dilution; Bio-Rad), and rabbit antishheep IgG (1:4000 dilution; Millipore).

**Nonheme Brain Tissue Iron Determination**

Rats were euthanized 3 days after ICH and the brains were perfused with phosphate-buffered saline. A coronal slice (4 mm thick) around the injection needle tract was cut, divided into ipsilateral and contralateral sides, and weighed. Nonheme brain tissue iron was determined according to the method described previously.

**DNA Damage Measurements**

The DNA polymerase I-mediated biotin-dATP nick-translation assay and the terminal deoxynucleotidyl transferase-mediated dUTP nick...
end-labeling technique were performed on brain sections to detect DNA single- and double-strand breaks according to the method we used in our previous studies.20

Cell Counting

Cell counting was performed on brain coronal sections. Three high-power images (×40 magnification) were taken around the hematoma or iron injection site using a digital camera. Fluoro-Jade C, neuronal nuclei, polymerase I-mediated biotin-dATP nick-translation, and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling-positive cells were counted on these 3 areas from each rat brain section.

Statistical Analysis

All the data in this study are presented as mean±SD. Data were analyzed by Student t test and 1-way analysis of variance. A level of P<0.05 was considered statistically significant.

Results

Physiological Variables

All physiological variables were measured immediately before the injection. Mean arterial blood pressure, blood pH, PaO₂, PaCO₂, and blood glucose level were within normal ranges (mean arterial blood pressure, 80–120 mm Hg; pO₂, 80–120 mm Hg; pCO₂, 35–45 mm Hg; hematocrit, 38%–43%; blood glucose, 80–120 mg/dL).

Minocycline Reduces Increased Total Iron Levels in Serum After ICH

In the normal rats, serum iron concentration was 143±32 μg/dL. To test the effects of hematoma size on serum iron levels, rats had an intracaudate injection of 10 or 100 μL autologous blood. Three days after ICH, serum total iron increased. The bigger clot resulted in higher serum iron levels (238±17 versus 182±44 μg/dL in the 10-μL blood group, P<0.05; Figure 1A). They also caused more severe perihematomatous brain edema (79.7±0.6 versus 78.4±0.3% in the 10-μL blood group, P<0.01) at Day 3. Control water content was approximately 78%.

A time course showed that total serum iron levels after 100 μL ICH were low at Day 1, increased significantly at Day 3, and stayed at high levels at Day 7 (Figure 1B). Sham operation did not increase serum iron levels significantly at Days 1 and 7 (eg, Day 7: 169.4±5.9 μg/dL). Minocycline reduced serum total iron levels at both Day 3 (158±36 versus 245±22 μg/dL in the vehicle-treated group, P<0.01) and Day 7 (206±45 versus 341±53 μg/dL in the vehicle-treated group, P<0.01).

Minocycline Reduces Brain Iron Overload and Neuronal Death After ICH

Lysis of erythrocytes resulted in a buildup in nonheme iron in brain tissue. Minocycline reduced brain nonheme iron accumulation 3 days after ICH (ipsilateral/contralateral: 115.0%±18.2% versus 160.2%±28.7% in the vehicle-treated group, P<0.05; Figure 2A).

Ferritin, an iron storage protein, was upregulated after ICH. Ferritin-positive cells were less in minocycline-treated animals (Figure 2B; eg, Day 7: 643±80 versus 1238±75 cells/mm² in the vehicle-treated group, P<0.01). Western blot analysis showed that both ferritin-L-chain and ferritin-H-chain protein levels were lower in the vehicle group. ICH indicates intracerebral hemorrhage; MC, minocycline.
transferrin receptor, and ceruloplasmin levels significantly (Figure 3).

Neuronal nuclei staining and Fluoro-Jade C staining were used to assess live and dead neurons, respectively. The number of neuronal nuclei-positive neurons in the ipsilateral basal ganglia was significantly higher in the minocycline-treated group (eg, Day 3: 743+/−11006 × 33 versus 295+/−11006 × 16 cells/mm² in the vehicle-treated group, P < 0.01; Figure 4A). Fluoro-Jade C-positive cells were less in the minocycline-treated group at Day 1 (254+/−11006 × 29 versus 419+/−11006 × 75 cells/mm² in vehicle group, P < 0.01; Figure 4B).

Our previous study showed that minocycline also reduces perihematomal brain edema.14 Coinjection of Minocycline, But Not MIF, Attenuates Iron-Induced Brain Edema, BBB Disruption, and Brain Cell Death

Intracerebral injection of iron caused brain edema. Coinjection of iron with minocycline reduced iron-induced brain edema in the ipsilateral basal ganglia at Day 1 (78.3+/−1.1% versus 81.9+/−1.1% in the iron group, P < 0.01; Figure 5A). This was associated with a decrease of brain sodium content (212+/−44 versus 391+/−129 mEq/kg dry wt in the iron group, P < 0.01; Figure 5B) and less loss of potassium content (451+/−49 versus 353+/−66 mEq/kg dry wt in the iron group) in the ipsilateral basal ganglia. The coinjection of iron with MIF, however, did not reduce iron-induced brain edema (Figure 5A).

Brain albumin, a marker of BBB disruption, was measured by Western blot analysis. Albumin in the ipsilateral basal ganglia was markedly increased 1 day after iron injection. Minocycline, but not MIF coinjection, reduced iron-induced BBB leakage (P < 0.01; Figure 5C–D).

Intracerebral injection of iron also caused neuronal death and DNA damage. Fluoro-Jade C, polymerase I-mediated
biotin-dATP nick-translation, and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling-positive cells in the ipsilateral basal ganglia were markedly decreased in the iron and minocycline coinjection group at Day 1 (eg, Fluoro-

Jade C: 189±34 versus 508±98 cells/mm² in the iron alone group, P<0.01; Figure 6). MIF also reduced iron-induced single chain DNA damage (576±216 versus 867±146 cells/mm² in FeCl₂ group, P<0.05; Figure 6). However, MIF did

Figure 6. The number of Fluoro-Jade C (A), polymerase I-mediated biotin-dATP nick-translation (PANT; B), and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL; C) positive cells in the ipsilateral basal ganglia at 24 hours after the injection of saline, FeCl₂, FeCl₂+MC, and FeCl₂+MIF. Values are means±SD; n=5, #P<0.01, compared with the saline or Fe+MC group; *P<0.05, vs Fe group. Scale bar=20 μm. MC indicates minocycline; MIF, microglia inhibitory factor; Fe, iron.
not reduce the number of Fluoro-Jade C and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling-positive cells (Figure 6).

However, systemic minocycline treatment starting at the time of iron injection did not reduce iron-induced brain edema (82.8% ± 0.3% versus 82.7% ± 0.7% in the vehicle-treated group, *P* > 0.05).

**Discussion**

The major findings of current study are: (1) serum total iron levels were increased after ICH and this was reduced by systemic use of minocycline; (2) minocycline reduced brain iron overload after ICH; (3) minocycline treatment reduces ICH-induced neuronal death; and (4) minocycline attenuates iron-induced brain edema formation and BBB disruption, an effect not found with a microglia inhibitor, MIF.

It is well known that brain iron overload occurs after experimental ICH and causes perihematomatal brain edema, neuronal death, brain atrophy, and neurological deficits. Clinically blood levels of ferritin, an iron storage protein, are increased in patients with ICH and associated with brain edema development and functional outcome. In this study, we found that serum total iron is increased after ICH and minocycline can reduce this increase. The causes of higher serum iron levels after ICH are unknown and could be related to: (1) iron released from the hematoma; (2) complement system activation, as occurs after ICH, which might cause systemic hemolysis; and (3) iron redistribution from tissues after ICH. Future studies should determine whether serum iron levels are correlated with ICH-induced brain injury and whether serum iron is a new biomarker of ICH-injury brain injury.

Minocycline acts as an iron chelator and reduces ICH-induced brain iron overload. Both brain nonheme iron and brain iron handling protein levels are decreased after minocycline treatment. Evidence shows that minocycline is an iron chelator. For example, absorption of minocycline is significantly decreased by administration with iron supplements and skin hyperpigmentation, an adverse effect of long-term minocycline therapy, may be related to insoluble minocycline–iron chelation products.

Recent evidence has also shown that minocycline can attenuate iron neurotoxicity in cortical neuronal cultures. Treatment of cultured cortical neurons with 10 μM ferrous sulfate for 24 hours caused significant neuronal death and increases in malondialdehyde. Minocycline prevents this injury with near-complete protection at the concentration of 30 μmol/L. To test whether minocycline can reduce iron-induced brain injury in vivo, rats received an intracerebral injection of iron with or without minocycline. In the proof of concept study, 50 μL of iron (0.5 mmol/L) was injected because the concentration of iron in rat red blood cells is approximately 10 mmol/L. Minocycline is an inhibitor of microglial activation; therefore, MIF was used as a control. We found that minocycline, but not MIF, attenuates iron-induced brain edema and BBB disruption. We have previously found that MIF, with the dose and route of administration used here, is capable of inhibiting ICH-induced microglial activation.

Iron is not the only cause of brain injury after ICH. There is considerable evidence for there being an inflammatory component including that linked to microglia activation. Minocycline is a potent inhibitor of microglia activation and has been reported to provide neuroprotection by inhibiting microglia. It is a highly lipophilic compound that penetrates the BBB easily. Minocycline has been found neuroprotective in both hemorrhagic and ischemic animal models. In the current study, although MIF did not reduce iron-induced brain edema, both minocycline and MIF reduced single-strand DNA damage caused by iron suggesting a role of microglia in iron-induced neuronal death.

In summary, minocycline reduces iron overload after ICH and iron-induced brain injury. These effects, along with microglia and other actions, suggest that minocycline may be a new treatment for patients with ICH.

**Sources of Funding**

This study was supported by grants NS-039866, NS-052510, and NS-057539 from the National Institutes of Health (NIH) and 0840016N from the American Heart Association (AHA). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH and AHA.

**Disclosures**

None.

**References**

Minocycline-Induced Attenuation of Iron Overload and Brain Injury After Experimental Intracerebral Hemorrhage

Fan Zhao, Ya Hua, Yangdong He, Richard F. Keep and Guohua Xi

*Stroke*. 2011;42:3587-3593; originally published online October 13, 2011; doi: 10.1161/STROKEAHA.111.623926

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/42/12/3587

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2012/08/21/STROKEAHA.111.623926.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/
Abstract

Minocycline-Induced Attenuation of Iron Overload and Brain Injury After Experimental Intracerebral Hemorrhage

Fan Zhao, MD; Ya Hua, MD; Yangdong He, MD; Richard F. Keep, PhD; Guohua Xi, MD

Department of Neurosurgery, University of Michigan, Ann Arbor, MI.

Minocycline-Induced Attenuation of Iron Overload and Brain Injury After Experimental Intracerebral Hemorrhage

Stroke 2011; 42: 3587-3593