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;42:00-00.)

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

Intracerebral hemorrhage (ICH) is a subtype of stroke with high morbidity and mortality. Evidence suggests that iron is involved in ICH-induced brain injury. 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. 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, aged rats and pigs. 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. 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 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. For example, it inhibits macrophage/microglia activation after ICH in rats. Evidence indicates that there is an inflammatory component to ICH-induced brain injury. However, a recent study has shown that minocycline also attenuates iron neurotoxicity in cortical neuronal cultures by chelating iron. 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 catheterized 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 intracaudate 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.14 Rats were euthanized 1, 3, and 7 days later for serum total iron determination, immunohistochemistry, and Western blot assay (n=9 for each group). 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), FeCl3 (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 separated, and total iron levels measured by a Quantichrom Iron Assay Kit (Bioassay Systems).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described.3 Primary antibodies were polyclonal rabbit antihuman ferritin IgG (DACO; 1:500 dilution) and monoclonal mouse antirat neuronal trans ferrin receptor (1:2000 dilution; Invitrogen), polyclonal sheep antirat ferritin-H-chain (1:2000 dilution; Abnova), polyclonal rabbit antirat ferritin-L-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 antirat 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).

**Western Blot Analysis**

Western blot analysis was performed as described earlier.3 The primary antibodies were polyclonal goat antiferritin-α-chain (1:1000 dilution; Abnova), polyclonal rabbit antiferritin-H-chain (1:2000 dilution; Cell Signaling), polyclonal rabbit anti human transferrin (1:2000 dilution; Dako), monoclonal mouse antihuman transferrin receptor (1:2000 dilution; Invitrogen), polyclonal sheep antirat ceruloplasmin (1:2000 dilution; Abcam), or polyclonal goat antirat 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 antishsheep 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.3

**Brain Water and Ion Contents**

Animals were reanesthetized, the brain was removed, and a coronal tissue slice (4 mm thick) around the injection needle tract was cut. Five tissue samples from each brain were obtained: the ipsilateral and contralateral cortex, the ipsilateral and contralateral basal ganglia, and the cerebellum. Brain samples were then dried at 100°C for 24 hours to obtain the dry weight and water content calculated as: (wet weight−dry weight)/wet weight. The dehydrated samples were digested in 1 mL of 1 mol/L nitric acid for 1 week. Sodium and potassium contents of this solution were measured by flame photometry. Sodium and potassium ion contents were expressed in milliequivalents per kilogram of dehydrated brain tissue (mEq/kg dry weight).

**Fluoro-Jade C Staining**

Brain sections were kept in 0.06% potassium permanganate for 15 minutes and rinsed in distilled water, sections were stained by gently shaking for 30 minutes in working solution of Fluoro-Jade C composed of 10 mL 0.01% Fluoro-Jade C in distilled water and 90 mL 0.1% acetic acid, then rinsed in distilled water 3 times. After being dried with a blower, slides quickly dipped into xylol and covered after being mounted by DPX (Electron Microscopy Sciences, Inc).

**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-end-labeling-positive cells were counted on 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 perihematomal 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 minocycline-treated group at both Day 3 and Day 7 (Figure 2C–D).

Transferrin, transferrin receptor, and ceruloplasmin are involved in iron metabolism and our previous studies have shown an increase of those iron-handling proteins in the brain after ICH. Minocycline reduced transferrin,
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

Figure 3. A, Transferrin (Tf), (B) transferrin receptor (TfR), and (C) ceruloplasmin (CP) protein levels in the ipsilateral basal ganglia at Days 1, 3, and 7 after ICH treated with or without minocycline (MC). Values are mean±SD; n=4, #\( P<0.01 \), *\( P<0.05 \), compared with the ICH+vehicle group. ICH indicates intracerebral hemorrhage.

Figure 4. Cells positive for neuronal nuclei (NeuN; A) at Days 1, 3, and 7 and Fluoro-Jade C (B) at Day 1 in the ipsilateral basal ganglia after ICH in rats treated with vehicle or minocycline (MC). Values are mean±SD; n=5, #\( P<0.01 \), compared with the ICH+vehicle group. Scale bar=20 μm. ICH indicates intracerebral hemorrhage.

**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%±0.4% 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-

Figure 5. Brain water (A) and sodium (B) contents 24 hours after the injection of saline, FeCl2, FeCl2+MC, or FeCl2+MIF into the right basal ganglia. Values are mean±SD; n=6, #P<0.01 vs saline or Fe+MC group. Protein levels of albumin (C–D) in the ipsilateral basal ganglia 24 hours after the injection. Values are means±SD; n=4, #P<0.01 vs other groups, ##P<0.01 vs saline. MC indicates minocycline; MIF, microglia inhibitory factor; Fe, iron.

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 perihematomatous 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.

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Disclosures

None.

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ミノサイクリンによる実験的脳内出血後の鉄過剰症および脳損傷低減

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.

背景および目的：脳における鉄過剰は、脳内出血（ICH）後の脳損傷に悪影響を及ぼす。最近の研究では、ミノサイクリンが鉄キレート剤として機能し、鉄による神経細胞死を低減することがin vitroで明らかとなった。本研究では、ミノサイクリンがICH後の鉄過剰および鉄による脳損傷をin vivoで低減するか否かを検討した。

方法：本研究は次の4部にて分けて実施された：(1) ICHの大きさに異なるラットを3日後に安楽死させ、血清鉄濃度および脳浮腫を定量した。(2) ミノサイクリンまたは溶媒対照をICHのラットに投与し、血清鉄濃度、脳内鉄濃度、および脳内鉄結合蛋白を測定した。(3) 生理食塩水、鉄、鉄+ミノサイクリン、または鉄+マクロファージ/ミクログリア抑制因子をラットの尾状核内に注入し、脳浮腫および神経細胞死を定量した。(4) 鉄をラットの尾状核内に注入した後、ミノサイクリンを投与し脳浮腫を定量した。

結果：ICH後、血清鉄および脳内非ヘム鉄濃度は上昇し、これらの変化はミノサイクリンの投与により低下された。また、ミノサイクリンは、ICHが誘発する脳内鉄結合蛋白の増加および神経細胞死を低下させた。鉄の尾状核内注入により、脳浮腫、血管脳間膜浸出、および神経細胞死が出現し、これらのすべてがミノサイクリンの併用投与により著しく減弱した。

結論：本研究では、ミノサイクリンはICH後の鉄過剰および鉄による脳損傷を低減することが明らかとなった。ミノサイクリンはミクログリア活性化の阻害薬としてもよく知られている。鉄の蓄積およびミクログリア活性化はICH後の脳損傷の一因であることから、ミノサイクリンはICH患者の治療に非常に有用かもしれない。

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注：Fluoro-Jade C は蛍光色素で、組織染色においてすべての変性神経細胞を染色するとされる。