Minocycline is Effective in Intracerebral Hemorrhage by Inhibition of Apoptosis and Autophagy

Zehan Wu¹ MD; Xiang Zou¹ MD; Wei Zhu¹ PhD, MD; Ying Mao¹ PhD, MD; Liang Chen¹ PhD, MD; Fan Zhao^{1*} PhD, MD

1: Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai, China

The first two authors contribute equal to this work.

***Corresponding author**

Fan Zhao: Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai 200040, China

Tel: 0086-21-52887030

Fax: 0086-21-62489191

Email: drfanzhao@163.com

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ABSTRACT

Background: Intracerebral hemorrhage is the least treatable type of stroke and affects millions of people worldwide. Treatment for ICH varies from medicine to surgery, but the rate of mortality and mobility still remains high. Minocycline is a tetracycline antibiotic increasingly recognized for its neuroprotective potential. In earlier studies, we demonstrated that many secondary injuries caused by ICH could be significantly reduced by injection of minocycline in rat models. The following research investigates the role of minocycline in reducing brain injury.

Methods: Twenty-four rats were administered 100ul autologous arterial blood injections into the right basal ganglia, treated with minocycline or vehicle and euthanized on the 1st, 3rd, and 7th day. Immunohistochemistry, Tunel, and western blot analysis were performed to analyze the effects of minocycline on apoptosis and autophagy.

Results: After the injection of minocycline, Tunel-positive cells were remarkably reduced on day 1, 3 and 7; Beclin-1, LC3BII/I, caspase-3/8 were all suppressed after treatment. The relationship between Cathepsin D and minocycline remained unknown.

Conclusions: Our studies suggest the potential medicinal value of minocycline, through both anti-autophagy and anti-apoptosis pathways.

KEY WORDS

intracerebral hemorrhage, minocycline, apoptosis, autophagy, rats

INTRODUCTION

Intracerebral hemorrhage (ICH) is a common stroke subtype with high morbidity and mortality[1]. ICH, induced by primary injury, is a rapid process that leads to spontaneous bleeding into the brain tissue. Current research shows that after the initial injury, physical and chemical damage from the expanding hematoma, neurotransmitter release, and calcium influx will create two major outcomes: cerebral edema and cellular damage or death[2, 3]. Of which, neuronal death is a critical endpoint of the secondary injury phase of the ICH. Thrombin, infiltrating inflammatory cells, and hemoglobin and its degradation products contribute to the neuronal death pathways[4].

Minocycline is a tetracycline antibiotic, increasingly recognized for its neuroprotective potential. As a highly lipophilic compound, it has the ability to penetrate the brain-blood barrier (BBB) and emerges frequently in neurovascular studies. Recent studies have shown that single dose of minocycline, 60 minutes before the cerebral ischemia, in the middle cerebral artery occlusion rat model can inhibit neuronal apoptosis and autophagy in rats[5]. Our previous studies demonstrate that brain edema, blood-brain barrier leakage, and neuronal death caused by experimental intracerebral hemorrhage, can be significantly reduced by injection of minocycline.

The present follow up study, experimental ICH rat models are used to investigate whether a daily dose of minocycline would reduce the neuronal apoptosis and autophagy in vivo.

METHODS

All experiments were approved by the Animal Care and Use Committee of Huashan Hospital, Fudan University. Twenty-four male Sprague-Dawley rats, weighing 300 to 350 g, were used in the experiment. Three rats were contained in each cage, under controlled condition of temperature ($22\pm 2^{\circ}\text{C}$) and humidity ($50\pm 5\%$). The cages were kept on a 12 hour light cycle (commencing at 7.00 am).

Surgical and Intervention Protocols

All of the rats underwent surgical procedures following an intraperitoneal injection of 1.5% pentobarbital sodium at a dose of 0.2 mL per 100g. The anesthetic was supplemented as needed during the procedure. All surgical procedures were

performed using standard sterile techniques. First, the right femoral artery was catheterized for arterial blood sampling. The rats were then positioned in a stereotactic frame, a 1-2mm burr hole was drilled 0.2 mm anterior to bregma and 4.0 mm lateral to midline. The rats received a 100 μ l autologous arterial blood injection into the right basal ganglia at a depth of 5.5 mm in 10 minutes, using a microinjection syringe. The needle was held in place for an additional 60 seconds to prevent reflux. Afterwards, 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 for 3 and 7 days) or vehicle. Rats were euthanized on the 1st, 3rd and 7th day according to observation intervals.

Immunohistochemistry

Brain tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μ m thicknesses, and stained with anti-Beclin-1 (Rabbit monoclonal, Abcam, HK Ltd), Cathepsin D, Caspase-3/8, and Microtubule-associated protein B-light chain 3 (LC3B) antibodies (Rabbit polyclonal, Abcam, HK Ltd.). The secondary antibody used was goat anti-rabbit immunoglobulin. Five random areas were selected in low power field (100x) in 5 sections, and the numbers of Caspase-3, Caspase-8, cathepsinD, Beclin-1 positive cells were counted in 200x fields. Assessment of the immunohistochemistry was performed by two independent observers who were blinded to each group.

Cells Death Measurement

We analyzed cell death by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (Tunel) technique. Experimental procedures were performed on brain sections according to the introduction of the Roche kit. Cell counting was performed on brain coronal sections. Tunel-positive cells were counted blind on these 5 random areas at a 200x field around the hematoma or iron injection site from each rat brain section. The assessment of the immunohistochemistry was performed by two independent observers who were blinded to each group.

Western Blot Analysis

Bilateral basal ganglia tissue of ICH site and symmetrical normal sites were prepared for further analysis. The total, nuclear and cytoplasmic proteins (100 μ g) of the

bilateral basal ganglia tissue were size-fractionated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P membranes (Millipore). The blotted membranes were incubated with primary antibodies against Beclin-1, CathepsinD, Caspase-3, LC3B and GAPDH (Abcam, HK Ltd), followed by incubation with an HRP-conjugated secondary antibody (Jackson). The immunoreactivity was detected using an enhanced chemiluminescence reaction system (Amersham Pharmacia Biotech).

Statistical Analysis

All of the data is presented as the means $\pm$ SD. Data were analyzed using independent-samples t-tests and one-way analysis of variance. P-value <0.05 was considered as statistically significant. All statistical analyses were performed using SPSS 16.0 for Windows.

RESULTS

Minocycline Reduces Brain Neuronal Death after ICH

To verify the neuro-protective effect of minocycline after ICH, we used Tunel to demonstrate neuronal death in the basal ganglia after ICH. We found that Tunel-positive cells were remarkably reduced on day 1, 3 and 7 by the intervention of minocycline (168.33 ± 25.10 vs. 60.27 ± 12.86 , 112.20 ± 18.36 vs. 39.13 ± 11.83 , 70.47 ± 13.36 vs. 10.13 ± 4.50 , $p < 0.001$, Figure 1).

Minocycline Suppressed the Expression of Autophagy Related Factors Induced by ICH

Immunohistochemical staining demonstrated the number of Beclin-1 highly positive cells was declined in the minocycline treatment group around the injection area on the Day 1 and 3 (11.60 ± 1.40 vs. 19.60 ± 2.03 and 22.20 ± 2.34 vs. 38.07 ± 3.20 , $p < 0.001$, Figure 2B). Western blot analysis was performed for semi-quantitative assessment of the autophagy related factors in the bilateral basal ganglia (Figure 3). As expected, Beclin-1 protein levels was elevated in the vehicle treated ICH site compared with the normal site after one day (0.654 ± 0.153 vs 0.351 ± 0.090 , $p = 0.034$, Figure 3A). By minocycline intervention, Beclin-1 level was significantly suppressed in the ICH site, compared to the vehicle treated ICH site (0.226 ± 0.084 vs 0.654 ± 0.153 , $p = 0.021$, Figure 3A). On the 3rd and 7th day, there is no difference in Beclin-1 levels among

groups. For LC3B, we calculated the LC3B-II/I ratio to show the autophagy activity, and found that LC3B-II/I ratio in the ICH site was remarkably increasing on the day one (0.092 ± 0.013 vs 0.007 ± 0.003 , $p<0.001$, **Figure 3B**), and after minocycline treatment the ratio was significantly decreased compared to the vehicle group (0.092 ± 0.013 vs 0.007 ± 0.006 , $p<0.001$, **Figure 3B**). On the 3rd and 7th day, the LC3B II bands were not found in each group.

Minocycline Suppressed the Expression of Apoptosis Relative Proteins Induced by ICH

To analyze the pathway minocycline participated in during ICH induced apoptosis, we examined the caspase-3/8 and cathepsin D changes. Immunohistochemical staining showed the number of caspase-3 highly positive cells were all decreased around the injection area on the Day 1, 3 and 7 by minocycline treatment (24.53 ± 5.49 vs. 41.33 ± 6.37 , $p<0.001$; 16.00 ± 4.21 vs. 71.80 ± 8.97 , $p<0.001$; 44.87 ± 8.06 vs. 50.60 ± 6.49 , $p=0.41$, **Figure 4B**). The effect of minocycline was also remarkable on caspase-8 (5.67 ± 0.49 vs. 14.93 ± 1.16 ; 12.40 ± 2.03 vs. 39.47 ± 5.99 ; 5.00 ± 0.76 vs. 12.40 ± 1.12 , $p<0.001$, **Figure 5B**) and cathepsin D (29.33 ± 4.29 vs. 42.40 ± 4.10 ; 30.13 ± 3.20 vs. 60.07 ± 5.86 ; 23.53 ± 3.11 vs. 31.67 ± 4.29 , $p<0.001$, **Figure 6B**) on day 1, 3, and 7. On the 3rd day, western blot demonstrated that caspase-3 level increased in the ICH site compared to the normal site (0.606 ± 0.093 vs 0.324 ± 0.062 , $p=0.021$, **Figure 3C**). For cathepsin D, levels among each groups showed no difference (**Figure 3D**).

DISCUSSION

Autophagy is a critical intracellular pathway, involving degradation and recycling of aged proteins and entire organelles[6, 7]. Beclin-1 activation is the first step in autophagy. As a component of class III phosphatidylinositol kinase (PI3K), beclin-1 starts autophagy through interaction with the other components of the PI3K pathway. LC3s are structural proteins of autophagosomal membranes. LC3s identified in humans include LC3A (variant-1, variant-2), LC3B, and LC3C[8, 9]. LC3 proteins are recognized as major markers of autophagy in various studies. The best-studied endogenous autophagic marker is LC3B[10-12]. In the current and our previous study, Tunel-staining has demonstrated the anti-neuronal death effect of minocycline. To analyze the role minocycline participated in during ICH induced cell death, we

examined the Beclin-1 and LC3B-II/I changes. Immunohistochemical staining and Western blot analysis together showed the suppressing of Beclin-1 by minocycline on day 1 and 3. For LC3B, LC3B-II/I ratio in the ICH site was decreased by minocycline during the observation interval. These results suggest the anti-autophagy role of minocycline in this ICH model.

On the other hand, apoptosis is a cell death program that is essential for getting rid of damaged or aged cells. Intrinsic and extrinsic pathways are mainly responsible for the activation of the apoptosis. In the extrinsic one, cell death signals often initiated by the trauma, tumor or others. The upstream signals bind to their receptors and Fas-associated protein with death domain(FADD). After that, the procaspase (procaspase-8, -10) can be initiated as caspase-8, -10 by the death-inducing signaling complex. And that will activate the executioner caspase. As for intrinsic pathway, it's regulated by the B-cell lymphoma-2(BCL-2) family protein. When intrinsic signals occur, the pro-apoptotic BCL-2 family protein like BAX and BAK can be inhibited and the anti-apoptotic function of BCL-2 is deactivated. Which makes the mitochondrial membrane release the mitochondrial activator of caspase and cytochrome c. Together cytochrome c, procaspase-8, -9 and other signal protein make the "apoptosome" to activate procaspase-8, -9 into the active caspase-8, -9. Here, the caspase-8, -9, 10 as above mentioned are the initiator caspase, after being activated, the downstream executioner caspase-3, -6, -7 can initiate enzymes for DNA, RNA and ribose. Both extrinsic and intrinsic pathway will pass the cell death signal to the executioner caspase which is caspase-3, -6, -7. After converting procaspase to caspase, cell death will be programmed. Of all the caspase families, casp-3 and -8 is considered to be most significant. Caspase-3 has the characteristic structure as -6 and -7: a short pro-domain connected with a caspase catalytic region, which allows these three to become the executioners of the apoptotic process[13]. Moreover, as the main effector caspase, caspase-3 is activated by caspase-8 and -9. Casp-8 serves as the main mediator carrying apoptosis signals from the cell surface receptor to casp-3. Caspase-3 staining rate around the injection area had decreased on day 1, 3 and 7 by minocycline treatment. The same effect of minocycline was also found on caspase-8. The results imply inhabitation of caspase-3 and -8 in the therapeutic group of the ICH model. Inhabitation of caspase-8 will cause incomplete complex of the apoptosome"[14], and the cell death signal of the extrinsic pathway will not pass

down. With inhabitation of caspase-3, the executioner caspase, cell death will not be performed. This study focus on the caspase-8 and -3, as description above, that is the beginner of extrinsic pathway and the common downstream pathway. There will be follow-up study of BCL-2 family, like BAK and BAX for the intrinsic pathway[15]. Study shows activation of caspase-8 by cathepsin D is crucial step in the inflammatory response mediated neutrophils apoptosis. In a vitro cell-free assay, after pre-cleaved by cathepsin D, recombinant human caspase-8 protein can initiate caspase-3 activation[16]. As known, cathepsin D is ubiquitously expressed in both immune and nonimmune cells, promising relationship between cathepsin D and caspase is worthy of our study[17]. Our data about cathepsin D shows no difference in the western-blot, but obvious difference in the immunohistochemical staining. Sebastien Conus[18] find cathepsin D directly cleave caspase-8 at pH 3.0 as well as in neutrophil cytosolic extracts, but at pH 5.0, cathepsin D successfully process caspase-8. They think pH5.0 may be the neutral pH to increase the activity of cathepsin D, just like the pH in the lysosomes. Because of the known pH sensitivity, we predict that, it is because of the pH of western blot inhabiting the activity of the cathepsin D and we can see no difference between the control and therapeutic group. Due to its lipophilic nature that allows it to pass through the BBB, minocycline has gradually shown to be the most effective neuroprotective tetracycline, appearing in research of experimental models of cerebral ischemia, brain injury, and several neurodegenerative diseases. Our findings give evidence that minocycline achieves this goal by suppressing both pathways, which are the major causes of cellular death. We believe that there may be a dose-effect relationship and it is worth of our effort for further research.

CONCLUSIONS

Despite the limitations of this study, our findings collectively imply the potential therapeutic value of minocycline in this ICH rat model, through both anti-autophagy and anti-apoptosis pathway. This deserves further investigation to determine its clinical effect on ICH patients.

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DISCLOSURE

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

FIGURE LEGENDS

Figure 1. The number of TUNEL C -positive cells in the ipsilateral basal ganglia on day 1, 3 and 7 after ICH. n=6, **P<0.001, compared with the vehicle group. Scale bar: 50um.

Figure 2. The number of Beclin-1 positive cells in the ipsilateral basal ganglia on day 1, 3 and 7 after ICH. n=6, **P<0.001, compared with the vehicle group. Scale bar: 25um.

Figure 3. Western blot analysis of Beclin-1, LC3B-II/I, caspase-3 and cathpsinD in Basal Ganglia.

(A) Representative bands relative amounts of Beclin-1. *p<0.05 compared to vehicle treated ICH site on day 1, n=6. (B) Representative bands relative amounts of LC3B-II/I. *p<0.05, compared to vehicle treated ICH site on day 1, n=6. (C) Representative bands relative amounts of caspase-3. *p<0.05 compared to vehicle treated ICH site on day 3, n=6. (D) Representative bands relative amounts of cathpsinD.

Figure 4. The number of Caspase-3 positive cells in the ipsilateral basal ganglia on day 1, 3 and 7 after ICH. n=6, **P<0.001, *P<0.05, compared with the vehicle group. Scale bar: 25um.

Figure 5. The number of Caspase-8 positive cells in the ipsilateral basal ganglia on day 1, 3 and 7 after ICH. n=6, **P<0.001, compared with the vehicle group. Scale bar: 25um.

Figure 6. The number of CathpsinD positive cells in the ipsilateral basal ganglia on day 1, 3 and 7 after ICH. n=6, **P<0.001, compared with the vehicle group. Scale bar: 25um.

REFERENCE

1. Aguilar, M.I. and B.M. Demaerschalk, Intracerebral hemorrhage. Semin

- Neurol, 2007. 27(4): p. 376-84.
2. Nakamura, T., et al., Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage. *J Neurosurg*, 2004. 100(4): p. 672-8.
3. Hua, Y., et al., Long-term effects of experimental intracerebral hemorrhage: the role of iron. *J Neurosurg*, 2006. 104(2): p. 305-12.
4. Zhao, X., et al., Toxic role of prostaglandin E2 receptor EP1 after intracerebral hemorrhage in mice. *Brain Behav Immun*, 2015. 46: p. 293-310.
5. Nagel, S., et al., Minocycline and hypothermia for reperfusion injury after focal cerebral ischemia in the rat: effects on BBB breakdown and MMP expression in the acute and subacute phase. *Brain Res*, 2008. 1188: p. 198-206.
6. Klionsky, D.J. and S.D. Emr, Autophagy as a regulated pathway of cellular degradation. *Science*, 2000. 290(5497): p. 1717-21.
7. DE DUVE, C., et al., Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem J*, 1955. 60(4): p. 604-17.
8. He, H., et al., Post-translational modifications of three members of the human MAP1LC3 family and detection of a novel type of modification for MAP1LC3B. *J Biol Chem*, 2003. 278(31): p. 29278-87.
9. Bai, H., et al., A transcriptional variant of the LC3A gene is involved in autophagy and frequently inactivated in human cancers. *Oncogene*, 2012. 31(40): p. 4397-408.
10. Barth, S., D. Glick, and K.F. Macleod, Autophagy: assays and artifacts. *J Pathol*, 2010. 221(2): p. 117-24.
11. Ferguson, C.J., G.M. Lenk, and M.H. Meisler, Defective autophagy in neurons and astrocytes from mice deficient in PI(3,5)P2. *Hum Mol Genet*, 2009. 18(24): p. 4868-78.
12. Aoki, H., et al., Monitoring autophagy in glioblastoma with antibody against isoform B of human microtubule-associated protein 1 light chain 3. *Autophagy*, 2008. 4(4): p. 467-75.
13. Sakamaki, K. and Y. Satou, Caspases: evolutionary aspects of their functions in vertebrates. *J Fish Biol*, 2009. 74(4): p. 727-53.
14. Qureshi, A.I., et al., Apoptosis as a form of cell death in intracerebral hemorrhage. *Neurosurgery*, 2003. 52(5): p. 1041-7; discussion 1047-8.
15. Zaman, S., R. Wang, and V. Gandhi, Targeting the apoptosis pathway in hematologic malignancies. *Leuk Lymphoma*, 2014. 55(9): p. 1980-92.
16. Pranjol, M.Z., et al., The Potential Role of the Proteases Cathepsin D and Cathepsin L in the Progression and Metastasis of Epithelial Ovarian Cancer. *Biomolecules*, 2015. 5(4): p. 3260-79.
17. Roberg, K., K. Kågedal, and K. Öllinger, Microinjection of Cathepsin D Induces Caspase-Dependent Apoptosis in Fibroblasts. *The American Journal of Pathology*, 2002. 161(1): p. 89-96.
18. Conus, S., et al., Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the resolution of inflammation. *J Exp Med*, 2008. 205(3): p. 685-98.

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