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Effects of minocycline on apoptosis and angiogenesis-related protein expression in a rat model of intracerebral hemorrhage☆

Ruizhi Wang[1], Dongning Hao[2], Wei Shi[1], Jingnan Pu[1], Zizhang Wang[3]

1*Department of Neurosurgery, Second Affiliated Hospital, Medical School of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China*
2*Department of Neurosurgery, Yulin First People's Hospital, Yulin 718000, Shaanxi Province, China*
3*Department of Head and Neck Surgery, Tumor Hospital of Shaanxi Province, Xi'an 710061, Shaanxi Province, China*

Abstract

In the present study, a rat model of non-traumatic intracerebral hemorrhage was established by type IV collagenase injection into the right globus pallidus. Bax and Bcl-2 expression in tissues surrounding hematomas was significantly increased within 14 days after injury, and it then gradually decreased. Vascular endothelial growth factor, Flk-1 and Flt-1 mRNA expression gradually increased over time. After intraperitoneal injection with minocycline, Bax expression was decreased 1 day after intracerebral hemorrhage. Flk-1 and Flt-1 mRNA expression was decreased after minocycline injection, but Bcl-2 expression was increased, and vascular endothelial growth factor mRNA expression was decreased between 4–14 days. These results indicated that protective effects of minocycline on nerve tissues were associated with increased Bcl-2 expression and decreased Bax expression in the early stage after intracerebral hemorrhage. In the late stage, minocycline downregulated vascular endothelial growth factor and its receptor expression to inhibit brain tissue self-repair.

Key Words: intracerebral hemorrhage; minocycline; vascular endothelial growth factor; apoptosis; inflammation; Bax; Bcl-2; Flk-1; Flt-1; neural regeneration

Abbreviations: ICH, intracerebral hemorrhage; VEGF, vascular endothelial growth factor; RT-PCR, reverse transcription-PCR

Ruizhi Wang☆, M.D., Attending physician, Department of Neurosurgery, Second Affiliated Hospital, Medical School of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China

Corresponding author: Wei Shi, Professor, Doctoral supervisor, Department of Neurosurgery, Second Affiliated Hospital, Medical School of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China
wrz510@163.com

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INTRODUCTION

The pathophysiology of intracerebral hemorrhage (ICH) can be considered as primary injury and secondary injury is caused by inflammatory reactions[1]. The inflammatory response involves inflammatory cell migration and activation, enzyme up-regulation, brain tissue breakdown, and repair[2]. Increasing evidence indicates that various cellular and molecular components of inflammation are involved in ICH-induced brain injury[3-5]. Strategies need to be designed to reduce the inflammatory cascade associated with ICH to decrease the morbidity and mortality. Vascular endothelial growth factor (VEGF) is a potent mitogen for endothelial cells, and its receptors are required for VEGF signal transduction[6]. Flt-1 is important for the control of cell proliferation and Flk-1 plays a pivotal role in vascular development and regulation of vascular permeability[7]. Physiologically, both VEGF and its receptors can hardly be detected in the adult brain, but their overexpression is induced by hypoxia and ischemia[8]. Minocycline is a second-generation, semi-synthetic tetracycline derivative, which is able to easily penetrate the blood-brain barrier. It is currently used against bacterial infections[9]. Minocycline can reduce inflammation by inhibiting macrophages/microglia and neutrophils, and by downregulating some cytokines that are involved in blood-brain barrier damage after ICH in animal models[10-14]. Some experimental ICH studies have reported that minocycline treatment reduces matrix metalloproteinase-12 expression at 7 days and improves functional recovery[15]. In the present study, minocycline was delivered 6 hours after ICH to simulate the clinical situation. We examined the effect of minocycline on apoptosis and angiogenesis-related proteins (VEGF, Flt-1 and Flk-1) using immunohistochemistry and reverse transcription-PCR (RT-PCR) following ICH.

RESULTS

Quantitative analysis of experimental animals

A total of 110 rats were randomly assigned to sham-surgery (n = 10), ICH (n = 50) and minocycline (n = 50) groups. The model of

ICH was established in ICH and minocycline groups by injecting type IV collagenase. Six hours after ICH, animals in the minocycline group were intraperitoneally injected with minocycline. Ten rats from each of the ICH and minocycline groups were sacrificed at 1, 2, 4, 7, and 14 days after ICH for immunohistochemistry and RT-PCR analysis. Brain samples of rats from the sham-surgery group were harvested at 4 days after ICH. All 110 rats were included in the final analysis.

Morphological changes in rat brain tissue after ICH

One day after ICH, a hematoma core, which was spherical in shape, formed in the corpus striatum but did not extend into the adjacent white matter. This was accompanied by swelling of brain tissue surrounding the hematoma, compression of the ventricle and midline shift (Figure 1). Hematomas were not observed in the sham-surgery group (Figure 1B).

Effect of minocycline on Bax and Bcl-2 expression in brain tissue surrounding the hematoma of rats after ICH

Bcl-2 and Bax positive cells were stained yellow-brown around the hematomas. A few Bcl-2- and Bax-positive cells were observed in the sham-surgery group. Both Bcl-2- and Bax-positive cells peaked at 1 day after ICH and gradually decreased thereafter. Minocycline increased Bcl-2 expression at all time points and decreased Bax expression at 1, 2, and 4 days after ICH ($P < 0.05$; Figures 2–4).

Effect of minocycline on VEGF, Flk-1 and Flt-1 expression

VEGF, Flk-1 and Flt-1 were mainly expressed in brain tissue surrounding the hematomas as shown by immunohistochemistry. Their expression was mainly detected at the late stage following ICH (7–14 days; Figure 5).

RT-PCR showed that the expression of VEGF, Flk-1 and Flt-1 mRNA began to increase 1 day after ICH and peaked at 14 days. Minocycline treatment significantly decreased VEGF mRNA at all time points after ICH ($P < 0.05$). Flk-1 mRNA expression (7 and 14 days) and Flt-1 mRNA expression (4, 7 and 14 days) were downregulated following minocycline treatment ($P < 0.05$; Figure 6).

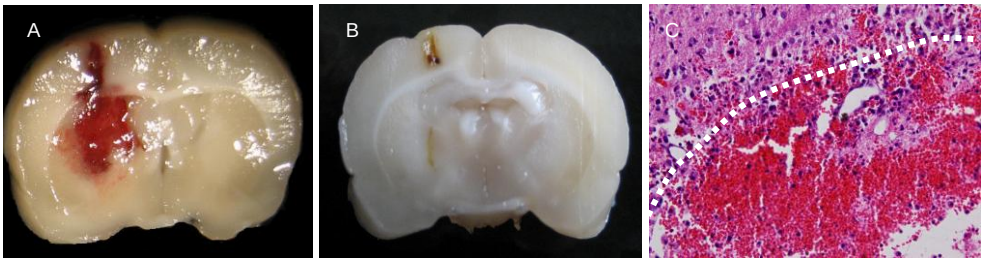

Figure 1 Morphology of brain tissues in rats with intracerebral hemorrhage.

(A) Representative brains are shown, which were dissected 1 day after injury, and they were sliced coronally through the middle of the hematoma.

(B) Hematomas occupied a large area and were located primarily in the corpus striatum. Hematomas were not observed in the sham-surgery group except for the needle track.

(C) There was a demarcation between the hematoma and brain tissue, which had accumulated with infiltrated cells. The area below the dotted line is a hematoma (hematoxylin-eosin staining, × 400).

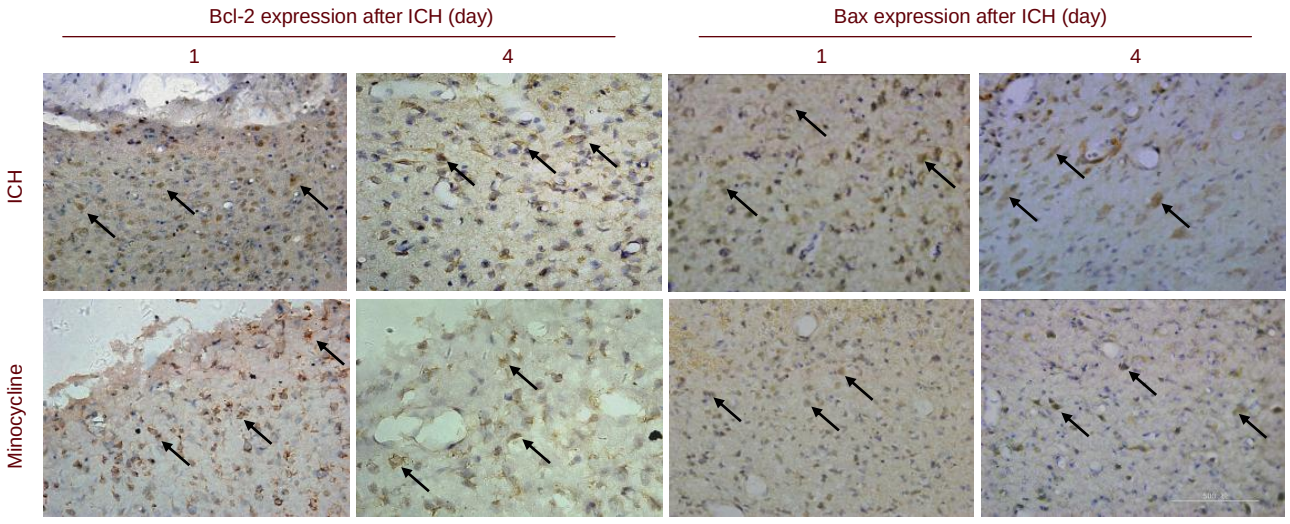

Figure 2 Bcl-2 and Bax expression around the hematoma after intracerebral hemorrhage (ICH; immunohistochemical staining, × 400). Positive cells are stained as yellow-brown.

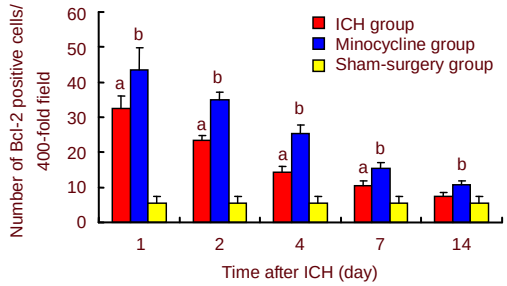

Figure 3 Quantification of Bcl-2-positive cells after intracerebral hemorrhage (ICH).

The number of Bcl-2 positive cells reached a peak at 1 day after ICH and then gradually decreased. Minocycline treatment increased Bcl-2 expression at each time point.

[a]*P* < 0.05, *vs*. sham-surgery group; [b]*P* < 0.05, *vs*. ICH group.

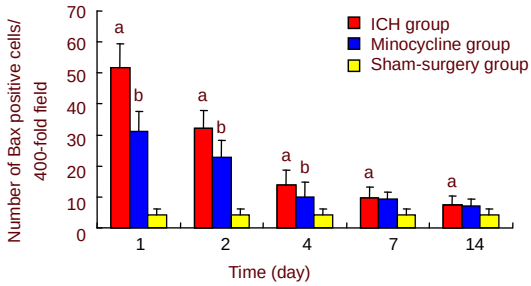

Figure 4 Quantification of Bax-positive cells after intracerebral hemorrhage (ICH).

The number of Bax-positive cells reached a peak at 1 day after ICH and then gradually decreased. Minocycline treatment increased Bax expression at 1, 2 and 4 days.

[a]*P* < 0.05, *vs*. sham-surgery group; [b]*P* < 0.05, *vs*. ICH group.

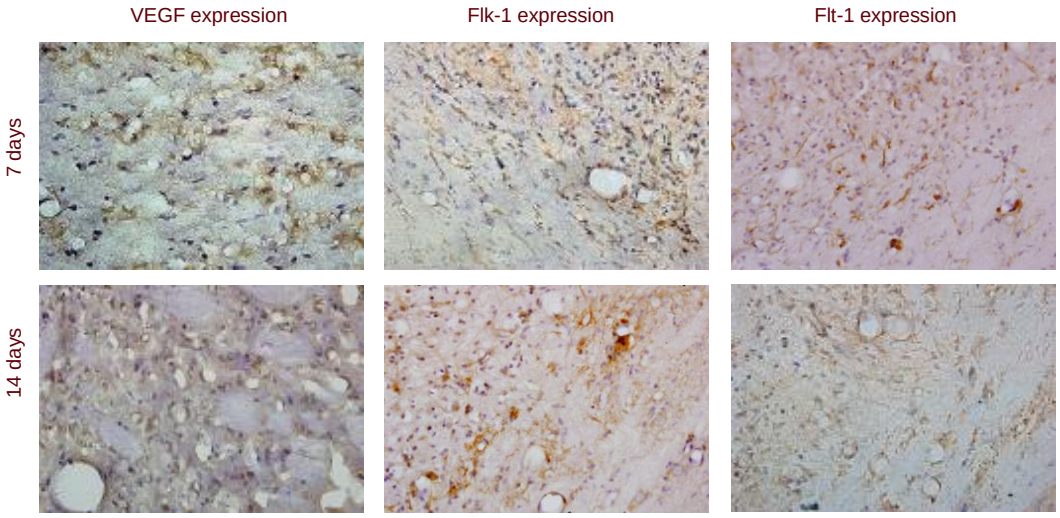

Figure 5 Vascular endothelial growth factor (VEGF), Flk-1 and Flt-1 expression in brain tissue after intracerebral hemorrhage (× 400). Representative micrographs from the corpus striatum show VEGF, Flk-1 and Flt-1 expression 7 days after intracerebral hemorrhage. Positive cells are stained yellow-brown. Vessel cavities can be clearly observed in the field of view.

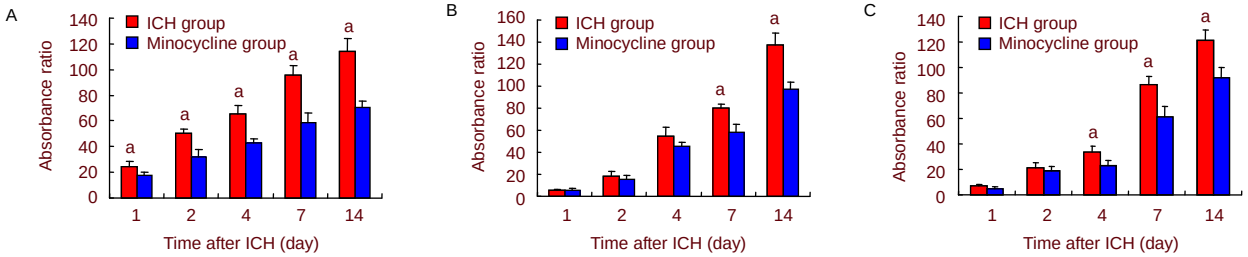

Figure 6 Effects of delayed minocycline treatment on vascular endothelial growth factor (VEGF), Flk-1 and Flt-1 mRNA expression in rats.

VEGF (A), Flk-1 (B) and Flt-1 (C) mRNAs were expressed at low levels after ICH and peaked at 14 days. Minocycline decreased VEGF, Flk-1 and Flt-1 expression after ICH. [a]*P* < 0.05, *vs*. minocycline group. ICH: Intracerebral hemorrhage. Target gene expression were expressed as absorbance ratio of target gene/beta-actin gene to sham-surgery group target gene/beta-actin gene.

DISCUSSION

In the present study, minocycline treatment protected brain tissue by increasing Bcl-2 and decreasing Bax expression after ICH in rats. Recent evidence has shown that the ratio of Bcl-2 to Bax determines whether cells are sensitive or resistant to death to a large extent[16]. Minocycline can cause Bcl-2 overexpression under oxidative stress[17] with ICH. Oxygen free radical release is one of the mechanisms involved in secondary brain injury[3]. Wang *et al* [18] reported that downregulation of Bcl-2 abolishes the protective effects of minocycline. In contrast, injection of Bcl-2 protein into injured brain tissue significantly increases neuronal survival[19].

The present study showed that expression of VEGF and its receptors was very weak as detected by immunohistochemistry and RT-PCR in the sham-surgery group. After ICH, there was overexpression of VEGF mRNA and its receptors around the lesioned region. It has been shown that the expression of VEGF and its receptors is correlated with vascular generation after ICH. A previous study indicated that VEGF is induced by hypoxia-inducible factor 1-alpha, which is released by macrophages, and is upregulated in inflammatory reactions[20]. However, in the present study, minocycline treatment inhibited VEGF mRNA as well as Flt-1 and Flk-1 mRNA, which was evident in the first week after ICH. Furthermore, it has been shown that hypoxia-inducible factor 1-alpha induced by ischemia at 48 hours leads to cellular survival[21]. Therefore, even though minocycline protects the brain from secondary injury in the first week following ICH, it might ultimately inhibit angiogenesis and brain repair after this time.

In conclusion, minocycline plays a protective role in brain damage following ICH by increasing Bcl-2 expression and decreasing Bax expression. Minocycline treatment also inhibits expression of VEGF and its receptors at a later period following ICH, which might depress the self-repairing function of the brain.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The study was performed at the Animal Experimental Center and Institute of Neurobiology, Laboratory of Molecular Biology and Laboratory of Human Anatomy, Medical School of Xi'an Jiaotong University, China, from October 2008 to March 2010.

Materials

Animals

A total of 110 healthy, clean level, male Sprague-Dawley rats weighing 200 to 250 g, were provided by the Animal Experimental Center, Medical School of Xi'an Jiaotong University (license No. SCXK (Shaan) 2007-001), housed under identical conditions and allowed free access to food and water. The protocol was performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[22].

Drugs

Minocycline ($C_{23}H_{27}N_3O_7$ HCl) was purchased from Sigma-Aldrich, St. Louis, MO, USA, product No. M9511.

Methods

ICH model establishment

The animals were anesthetized with 2% chloral hydrate (350 mg/kg) and the ICH model was prepared as previously described[23]. The head was placed in a stereotaxic frame (Narishige, SN-2 N, Tokyo, Japan). After a midline scalp incision was made, a cranial hole was drilled and 0.2 U collagenase (type IV, Sigma-Aldrich) and 2 U heparin in 1 μL 0.9% sterile saline was injected into the right globus pallidus (1 mm posterior to and 3 mm lateral to bregma, with a 6.0 mm depth below the surface of the skull[24]) with a 5-μL Hamilton syringe (Hamilton Company, Reno, NV, USA) for 5 minutes. The needle was maintained for 10 minutes. The bone hole was sealed with bone wax, and the scalp wound was sutured. For the sham-surgery group, the same amount of sterile heparin saline was injected into the brain in the same manner. During the procedure, the animal body temperature was maintained at 36.5–37.5°C with a feedback controlled heating pad. A successful model was identified by contralateral limb dysfunction in the ICH rats[23] (supplementary Figure 1 online).

Minocycline intraperitoneal injection

The minocycline group was intraperitoneally injected with 45 mg/kg minocycline for the first injection at 6 hours after ICH, followed by 22.5 mg/kg every 12 hours for 14 consecutive days[25].

Preparation of samples and morphology of brain tissue

ICH, minocycline and sham-surgery group animals were sacrificed by an overdose of 2% chloral hydrate at 1, 2, 4, 7 or 14 days after surgery and then perfused through the heart with 0.9% saline followed by 100 mL ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and postfixed for 2 hours and then transferred to 20%, 30% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4 °C until sinking. Coronal sections (20 μm-thick) of the brain were prepared with a microsyringe needle tract as the center at −20°C through a cryostat (CM1800 Leica, Wetzlar, Germany). Hematomas in the ICH and minocycline groups were observed at 1 day after ICH and changes in hematomas after hematoxylin-eosin staining were observed by a microscope (Olympus, Tokyo, Japan).

Immunohistochemistry for expression of Bcl-2, Bax, VEGF, Flt-1 and Flk-1

The sections were first washed by phosphate buffered saline, and then incubated with 3% H_2O_2 (50 μL), drained and blocked against endogenous biotin activity with 5% bovine serum albumin and drained. Primary rabbit

anti-rat Bcl-2 (1:100; Biosynthesis Biotechnology, Beijing, China), Bax (1:100; Biosynthesis Biotechnology), VEGF (1:100; Biosynthesis Biotechnology), Flt-1 (1:100; Biosynthesis Biotechnology) and Flk-1 (1:100; Biosynthesis Biotechnology) antibodies were used. After incubation with primary antibodies at 4°C overnight, sections were incubated with biotinylated goat anti rabbit IgG (1:200; Boster, Wuhan, China) at room temperature for 90 minutes. The sections were colored with diaminobenzidine, dehydrated, cleared and mounted with neutral gum. Sections were washed in Tris-buffered saline after each incubation step. Bcl-2- and Bax-positive cells in five fields of view around the hematoma cavity were quantified by 400 × microscopy (Olympus) and the distribution of VEGF, Flt-1 and Flk-1 expression was also observed.

Quantitative real-time RT-PCR for VEGF, Flt-1 and Flk-1 mRNA expression

Rats (n = 5, per time point) were sacrificed by an overdose of chloral hydrate. Brain tissue was rapidly harvested, and then perfused through the heart with 100 mL phosphate buffered saline. Dissected brains were cut coronally, 2 mm anterior and 2 mm posterior to the needle track and used for RNA extraction. Total RNA was extracted using TRIzol Reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Primers of VEGF, Flt-1, Flk-1 and β-actin for rat were obtained through the use of software Primer Premier 5.0 (PRIMER Biosoft International, Palo Alto, CA, USA), and are listed as follows. The primers were prepared by Runde Biotechnology Ltd. (Xi'an, China).

Gene	Sense	Antisense	Product size (bp)
VEGF	5'-TGC AGA TCA TGC GGA TCA AAC-3'	5'-TTT CTC CGC TCT GAA CAA GGC-3'	360
Flt-1	5'-TAC CCG CAA CGG AGA A-3',	5'-GGC TTG GAA GGG ACG A-3'	555
Flk-1	5'-AAC GCT TGC CTT ATG AT-3'	5'-AAG TCG CTG TCT TGT CG-3'	537
β-actin	5'-TCA CCC ACA CTG TGC CCA TCT ATG A-3'	5'-CAT CGG AAC CGC TCA TTG CCG ATA G-3'	432

Reverse transcription was performed using the RevertAid ^{TM} First Strand cDNA Synthesis kit (K1621) by following the manufacturer's instructions (Fermentas Life Sciences, Glen Burnie, MD, USA). Amplification was performed using the two-step RT-PCR kit AMP260802 (Lifesontech Medical Tech Inc., Xi'an, China). Melting curves of all samples were always performed as controls of specificity. All gene expression data were calculated as $2^{-\Delta\Delta CT}$, which indicates an n-fold change in gene expression relative to the sham-surgery sample and standard curves were obtained. The absorbance ratio of the target gene/beta-actin mRNA in the ICH or minocycline group to target gene/beta-actin mRNA in the sham-surgery group was calculated.

Statistical analysis

Data are expressed as mean ± SD. Normal distribution and homogeneity-of-variance were analyzed before random analysis of variance (SPSS, Chicago, IL, USA) to compare means. A P value < 0.05 was considered statistically significant.

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Author contributions: Ruizhi Wang conducted the experiments, wrote and revised the manuscript. Dongning Hao conducted the PCR part of animal experiments and wrote the manuscript. Wei Shi was in charge of funds and collected and analyzed the data. Zizhang Wang and Jingnan Pu conducted the experiments.

Conflicts of interest: None declared.

Ethical approval: The study gained full ethical approval from the Animal Ethics Committee of Xi'an Jiaotong University, China.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org, and entering Vol. 7, No. 8, 2012 after selecting the "NRR Current Issue" button on the page.

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