

Original Research Article

Minocycline inhibits nerve cell apoptosis caused by intracerebral hemorrhage in young mice via TRAIL signaling pathway

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Abstract

Purpose: To investigate the influence of minocycline on nerve cell apoptosis caused by intracerebral hemorrhage (ICH) in young mouse model, and the mechanism of action involved.

Methods: C57BL/6 mice were divided into control group, ICH group and minocycline treatment group (MC group, 25 mg/kg). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was conducted to determine nerve cell apoptosis in the brain tissues. The expression levels of genes and proteins related to apoptosis and TRAIL signaling pathway were measured by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting.

Results: The levels of Glu, Cr, Na⁺, IL-6, IL-1 β and TNF- β were significantly increased in ICH group, and the content of K⁺ was significantly raised in MC group ($p < 0.05$). TUNEL staining showed that there were more apoptotic cells, dominated by glial cells in ICH group, and fewer apoptotic cells in MC group. Gene assay results indicate that ICH group exhibited markedly raised mRNA levels of caspase-3, TNF- β and TRAIL1, as well as lowered levels of B-cell lymphoma-2 (Bcl-2) ($p < 0.05$). The results of protein assay showed that the protein levels of caspase-3 and TRAIL1 rose while that of Bcl-2 declined significantly in ICH group. However, the expression trends of the genes and proteins in MC group were the opposite of those in the ICH group.

Conclusion: Minocycline inhibits nerve cell apoptosis caused by ICH in the young mouse model by repressing the expression of the TRAIL signaling pathway. The findings may provide new insight into the treatment of ICH.

Keywords: Minocycline, TRAIL signaling pathway, Intracerebral hemorrhage, Nerve cells, Apoptosis, Inflammatory responses

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INTRODUCTION

Intracerebral hemorrhage (ICH) is the most common cerebrovascular disease and the most refractory subtype of hemorrhagic stroke [1], and

stroke is one of most common central nervous system diseases. Although ICH only accounts for a third of all the stroke cases, the prognosis of patients is usually poor.

Currently, there is no efficacious drug therapy to improve the survival rate or living quality of the survivors [2]. Hemorrhagic encephalopathy in neonates sometimes can result in cerebral palsy, mental retardation and epilepsy [3].

The occurrence of ICH is associated with cerebral amyloid angiopathy seriously affecting the quality of life [4]. Sudden and spontaneous ICH has close correlations with high mortality and morbidity rates. ICH-induced neuroinflammation can activate the residual glial cells and infiltrating plasma-derived immune cells, destroy the blood-brain barrier, form edema and exacerbate the neurobehavioral function through the inflammatory mediators produced in the blood lysate [5]. It has been demonstrated in a study that claudin-5, ZO-1 and other key proteins maintain the integrity of blood-brain barrier and limit cell permeability [6].

So far, the molecular mechanism of ICH has been extensively reported [7]. However, the in-depth molecular mechanism involved in ICH has not been completely elaborated. Hence, it is essential for the treatment of ICH to deeply understand its molecular regulatory network. Therefore, seeking the novel targets of ICH has become the problem to be urgently solved.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) family induces cell apoptosis by binding to death receptors (DRs), and represses cell apoptosis by binding to decoy receptors (DcRs) [8]. It was stated in a study that ischemia around the focus and hypoxia response after ICH attack, stimulates pathological changes and activate the TRAIL response pathway, further resulting in secondary inflammatory injury [9]. Minocycline, a semisynthetic tetracycline, possesses independent anti-inflammatory functions, and exerts neuroprotective effects on brain injury models [10]. Therefore, it is often applied in the treatment of ICH. Because the exact action mechanism of the TRAIL signaling pathway in neuronal apoptosis in ICH mice is not very clear, the influence of minocycline on nerve cell apoptosis in ICH mice on TRAIL signaling pathway was investigated using multiple molecular approaches. This research aims to explore the effects of minocycline on the nerve cell apoptosis caused by ICH in a young mouse model, via TRAIL signaling pathway.

EXPERIMENTAL

Animal grouping and modeling

After anesthesia with 10 % chloral hydrate (0.3 mL/100 g) (Sigma, Louis, MO, USA) through

intraperitoneal injection, the C57BL/6 mice aged 2 days were fixed on an operation table in the supine position, 10 μ L of the autologous blood was drawn from the femoral artery of the mice, and the mice were placed on the brain stereotaxic instrument in the prone position immediately after hemostasis. Next, autologous blood from the femoral artery was injected slowly (1 μ L/min) into the mice to establish the ICH model in accordance with previous methods [10]. The mice in treatment group (MC group, n = 20) were intraperitoneally injected with 25 mg/kg minocycline every day, while those in normal control group (Control group, n = 20) were administered the same dose of normal saline. Mouse blood was collected at the end of the experimental period and then centrifuged to collect serum which was stored at -80 °C for the detection of serum biochemical indices. The mice were anesthetized by injecting pentobarbital sodium, and two portions of appropriate brain tissues were taken carefully, one of which was used for morphology examination, and the other for preservation at -80 °C and determination of gene and protein expressions.

Ethical approval

The current study was approved by the Laboratory Animal Ethics Committee of North China University of Science and Technology, and all the animal procedures were performed according to the relevant regulations in the guide for the laboratory animals by NIH. All procedures were conducted in accordance with the 'Animal Research: Reporting in Vivo Experiments guidelines 2.0' [11].

Assessment of neurological scores of mice

The neurological function of the mice in each group was scored by special personnel using blind and 6-point methods before the mice were sacrificed. The detailed scoring rules are shown in Table 1. The mice with the highest and the lowest scores, as well as those which had other clinical manifestations during the experiment were discarded and substituted by others randomly.

Evaluation of injury indices after ICH

In order to predict the incidence of ICH in clinical practice and provide important references for early diagnosis, such indexes as glucose (Glu), K⁺, creatine (Cr) and Na⁺ were examined. A biochemistry analyzer was used for the determination of the changes in the above indexes.

Table 1: Detailed scoring rules

Grade	Manifestation
0	Walked voluntarily
1	Rotated to the contralateral side of lesion under the condition of free movement
2	Rotated to the contralateral side of lesion
3	Have weakened resistance to the pressure applied on the contralateral side of lesion
4	Have general flexion to the contralateral side
5	Have severe neurological deficit with apparent symptoms

Enzyme-linked immunosorbent assay (ELISA)

Serum inflammatory factors were determined via ELISA (Hanbio Biotechnology Co., Ltd., Wuhan, China). The serum samples previously collected and frozen at -80 °C were taken out, slowly thawed at 4 °C and centrifuged again at a low speed to harvest the supernatant. Changes in the indexes were tested according to the manufacturer's instructions. Finally, the absorbance was measured via a microplate reader.

TUNEL apoptosis assay

The apoptosis assay kit (Roche, Basel, Switzerland) was performed to determine the cell apoptosis in paraffin-embedded sections, as follow: The paraffin-embedded sections were deparaffinized, washed in phosphate buffer saline, then added with proteinase K working solution and soaked into blocking buffer, followed by fixation, rinsing and permeation. TUNEL assay kit was employed to detect the apoptotic DNA fragments subjected to fluorescein isothiocyanate (FITC)-end labeling. A fluorescence microscope was used for the observation of the TUNEL-positive cells.

Determination of expression of relevant genes by RT-PCR

The total RNA was extracted from a total of 100 mg sterile brain tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). The Quantitative real time polymerase chain reaction (qRT-PCR) system (20 µL) was designed and the reaction conditions are as follows: reaction at 50°C for 2 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 50°C for 30 s, a total of 45 cycles.

ABI 7500 fluorescence PCR amplification instrument was used for quantitative analysis. With GAPDH as the endogenous control, the $2^{-\Delta\Delta CT}$ method was used to calculate the mRNA expression levels of each target gene. The primer sequences are shown in Table 2.

Table 2: Primer sequences used for RT-PCR

Target gene	Primer sequence
GAPDH	F: 5'-TGACTTCAACAGCGACACCCA-3' R: 5'-CACCCCTGTTGCTGTAGCCAAA-3'
TNF-β	F: 5'-CGCTACGACCGCCAGATTG-3' R: 5'-ACACCGTTCACCAGCAAGTC-3'
Caspase-3	F: 5'-CTACCGCACCCGGTTACTAT-3' R: 5'-TTCCGGTTAACACGAGTGAG-3'
B-cell lymphoma-2 (Bcl-2)	F: 5'-GGTGCTCTTGAGATCTCTGG-3' R: 5'-CCATCGATCTTCAGAAGTCTC-3'
TRAIL1	F: 5'-TTCCATTGTGGGTAGGTGG-3' R: 5'-CTTACAGCTCCGCTCAG-3'

Western blotting assay

Lysis buffer was used for protein extraction from the fresh bone tissues. Then the products were centrifuged at 14,000× g for 5 min. Bicinchoninic acid method (Pierce, Rockford, IL, USA) was used for protein quantification. After the protein was denatured, 50 µg was taken for SDS-PAGE electrophoresis (140 V), and then transferred to a polyvinylidene fluoride (PVDF) membrane at 300 mA for 1 h. The membrane was washed with TBST buffer for 5 min × 3 times. The membrane was then blocked in 5% defatted milk for 2 h at room temperature. After washing with TBST for 3 times, Caspase-3, Bcl-2 and TRAIL1 antibodies (1:1000) were added respectively for incubation overnight at 4 °C, followed by adding the secondary antibody (1:200) for incubation for 1 h at room temperature.

The membrane images were captured using the chemiluminescent reagent in the dark via the gel imaging system. ImageLab (Media Cybernetics, Silver Springs, MD, USA) software was used for analysis. The expression level of the target protein was expressed as the ratio of the gray value of the target protein band to the gray value of the internal reference protein (GAPDH) band.

Statistical analysis

The raw experimental data recorded were processed using SPSS statistical analysis software (version 26.0). Data were presented as

mean \pm standard deviation (mean \pm SD), and $p < 0.05$ suggested statistically significant differences. GraphPad Prism 7.0 (La Jolla, CA, USA) was used to prepare the histograms plotted.

RESULTS

Neurological scores of mice in each group

The mice in the ICH group were unable to walk and generally flexed to the contralateral side, while there were no abnormalities in the control group. The MC group showed remarkably alleviated symptoms ($p < 0.05$). The specific scores are shown in Figure 1.

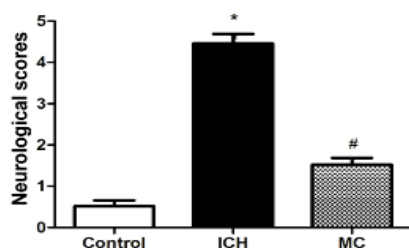


Figure 1: Neurological scores. Some mice in the ICH group were unable to walk and generally flexed to the contralateral side, while there were no abnormalities in the control group, and remarkably alleviated symptoms in MC group. * $p < 0.05$ vs. Control group, # $p < 0.05$ vs. ICH group

Serum test results

As shown in Table 3, the levels of Glu, Cr and Na^+ were markedly increased, while K^+ level was decreased significantly in ICH group. The opposite of these changes in those levels were observed in the MC group ($p < 0.05$), signifying the occurrence and development of the disease.

Serum levels of TNF- β , IL-1 β and IL-6

According to Table 4, the ICH group manifested higher levels of IL-1 β , IL-6 and TNF- β ($p < 0.05$),

Table 3: Changes in levels of Glu, Cr, K^+ and Na^+

Group	K^+ (mmol/L)	Glu (U/L)	Na^+ (mmol/L)	Cr ($\mu\text{mol/L}$)
Control	20.48 \pm 2.31	4.10 \pm 2.04	101.10 \pm 6.14	20.33 \pm 4.12
ICH	4.56 \pm 1.27*	15.47 \pm 1.74*	180.89 \pm 2.09*	75.45 \pm 2.10*
MC	15.24 \pm 3.12#	6.24 \pm 1.85#	114.87 \pm 3.18#	28.28 \pm 3.65#

* $p < 0.05$ vs. control group, # $p < 0.05$ vs. ICH group

Table 4: Contents of serum TNF- β , IL-1 β and IL-6

Group	TNF- β (fmol/mL)	IL-6 (mg/L)	IL-1 β (mg/L)
Control	15.25 \pm 3.19	21.38 \pm 6.17	29.80 \pm 5.14
ICH	39.28 \pm 2.10*	89.64 \pm 3.47*	91.68 \pm 2.14*
MC	19.34 \pm 3.01#	33.21 \pm 3.14#	40.85 \pm 3.89#

* $p < 0.05$ vs. control group, # $p < 0.05$ vs. ICH group

while MC group showed lower levels of these factors ($p < 0.05$).

Expressions of inflammatory and apoptotic genes and pathway molecules

No obvious positive cells were observed in the control group. The number of TUNEL-positive cells in the ICH group was evidently larger than that in the control group, which were mainly distributed around the hemorrhagic focus and dominated by glial cells. There were reduced apoptotic cells in MC group ($p < 0.05$), illustrating that ICH promotes the abnormal apoptosis of nerve cells. The detection of gene expression levels (Figure 2) showed that the messenger RNA (mRNA) levels of Caspase-3, TNF- β and pathway-related gene TRAIL1 were elevated in the ICH group, but they were clearly lowered in the MC group. The opposite was detected in the expression trend of Bcl-2 ($p < 0.05$), suggesting that the pathway-related genes and inflammatory factors may be activated in the process of an ICH attack, thus indicating the further development of ICH.

Expressions of apoptosis- and pathway-related proteins

The results of the assessment of proteins related to neuronal apoptosis and the pathway (Figure 3) indicated that the ICH group exhibited increased levels of apoptotic protein caspase-3 and pathway-related protein TRAIL1, while the MC group displayed decreased levels of those proteins. Moreover, Bcl-2 had the opposite expression trend to those proteins ($p < 0.05$), implying that minocycline is capable of inhibiting the apoptosis of the pathway and nerve cells, and accelerating the recovery of injury after ICH.

DISCUSSION

Intracerebral hemorrhage is a latent fatal disease without efficacious clinical therapies. It is a rapid

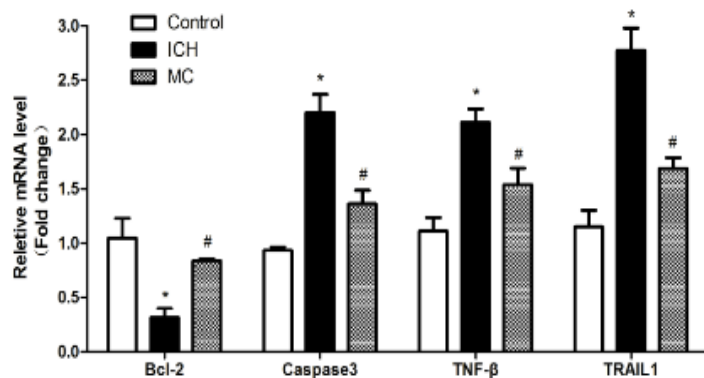


Figure 2: Expression levels of key genes. The mRNA levels of Caspase-3, TNF- β and pathway-related gene TRAIL1 are elevated in the ICH group, but they are clearly lowered in the MC group. The opposite expression trend of Bcl-2 is detected ($p < 0.05$); * $p < 0.05$ vs. control group, # $p < 0.05$ vs. ICH group

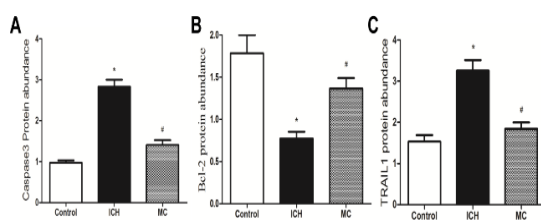


Figure 3: Protein expression levels of (A) Caspase-3, (B) Bcl-2 and (C) TRAIL1. The ICH group exhibited increased levels of apoptotic protein Caspase-3 and pathway-related protein TRAIL1, while the MC group displayed decreased levels of those proteins. Moreover, Bcl-2 had the opposite expression trends to those proteins ($p < 0.05$); * $p < 0.05$ vs. control group, # $p < 0.05$ vs. ICH group

process triggered by primary injuries, which can lead to spontaneous hemorrhage in the brain tissues, with relatively high morbidity and mortality rates [12]. Intracerebral hemorrhage can result in a series of life-threatening pathophysiological responses such as accumulation of edema, release of inflammatory cytokines and neurobehavioral deficits, whose morbidity rate is rising year by year. Large quantities of neuropathological, neuroimaging and molecular investigations have manifested that the neuroinflammation-induced brain injury is a common pathological characteristic of ischemic and hemorrhagic strokes [13]. The neuronal death is a crucial end point of secondary injury following ICH, and thrombin, infiltrating inflammatory cells, hemoglobin and their degradation products are involved in the pathway of neuronal death.

Studies have demonstrated that the infiltration of inflammatory cells, edema and cell apoptosis in the diseased brain tissues are important predisposing factors of secondary brain injury following ICH. The theories of inflammatory responses and cell apoptosis have attracted

widespread interest from clinical research. After ICH, the degree of brain edema is one of the critical factors for brain injury deterioration. Current evidence has indicated the presence of infiltrating neutrophils in the rat model of ICH, thereby increasing the inflammatory factors [14].

Minocycline is a tetracycline antibiotic to which increased importance has been attached because of its potential neuroprotective effect. Because it is a type of highly lipophilic compound, minocycline is capable of penetrating the blood-brain barrier, and it is often used in neurovascular studies. According to the latest studies, single-dose administration of minocycline represses neuronal apoptosis and autophagy in the middle cerebral artery occlusion in rat models, 60 min before cerebral ischemia [15]. In this research, the neurological score was evaluated by establishing ICH in the mice model. The results indicated that the mice in the ICH group were unable to walk, and generally flexed to the contralateral side, while those in control group had no abnormalities, and those in the MC group exhibited remarkably alleviated symptoms. Subsequently, indices such as Glu, Cr, K^+ and Na^+ were determined, and it was found that the levels of Glu, Cr and Na^+ were increased, but the K^+ level was decreased in the ICH group. However, the MC group displayed opposing levels, signifying the occurrence and development of the disease, and suggesting the successful establishment of the model. In this study, the contents of IL-1 β , IL-6 and TNF- β were raised in the ICH group, but declined in the MC group; and the inflammatory factors were activated, thereby indicating the further progression of ICH. These results are consistent with the aforementioned findings.

Cell apoptosis, as a type of programmed cell death, is able to eliminate the junk generated by the cells in maintaining their life activities timely,

so it is a metabolic pathway [16]. The endogenous and exogenous pathways are responsible for the activation of cell apoptosis, and among the exogenous death signals, the cell death signals are generally induced by trauma, tumor or other factors. The intrinsic pathway is regulated by the Bcl-2 family proteins, and the generated intrinsic signals inhibit the pro-apoptotic Bcl-2 family proteins such as Bax and Bak, thereby inactivating the anti-apoptotic function of Bcl-2. Both extrinsic and intrinsic pathways transmit the cell death signals to caspase-3, and caspase-3 and caspase-8 are regarded as important pro-apoptotic factors [17].

It has been revealed in a study that the caspase-3 level and nerve cell apoptosis are increased in the ICH model. Apoptosis responds to the invasion of cell bodies, and the apoptotic response is initiated rapidly in the case of lethal threats, so as to maintain cell stability [18]. Apoptosis and autophagy have become the hotspots of studies in the biological field so far [17]. However, the mechanism of action of apoptosis in physiological metabolism in organisms has not been completely explored. The TUNEL apoptosis assay results in this experiment showed that there were no apparent positive cells in the control group. The ICH group had more TUNEL-positive cells than the control group, with the cells mainly distributed around the hemorrhagic focus and dominated by glial cells. Besides, the number of apoptotic cells declined in the MC group, further buttressing the fact that ICH enhances the abnormal apoptosis of nerve cells. In addition, the gene and protein levels of apoptotic Caspase-3 were elevated in the ICH group, while they were lowered in MC group, and Bcl-2 showed the reverse trend in the results.

TRAIL is a pro-apoptotic ligand in the TNF superfamily, and a key player in the regulation of neuronal apoptosis. The gene and protein assay results in this research indicated that the TRAIL1 level rose in the ICH group, and declined in the MC group, suggesting that minocycline can inhibit the TRAIL activation and nerve cell apoptosis, thereby accelerating injury recovery after ICH, which is in line with the findings by Wu *et al* [19] and Wang *et al* [20]. The specific mechanism of minocycline in influencing the nerve cell apoptosis by repressing the TRAIL signaling pathway was confirmed through multiple molecular means in this research, providing novel potential targets for the treatment of injury after ICH. Despite the results obtained, there are probably still some deficiencies in this research. Hence, an in-depth and detailed subsequent study of the regulatory mechanism in

the nerve cell apoptosis in ICH mice will need to be conducted using cell culture experiments *in vitro*, as well as various molecular biological methods.

CONCLUSION

The findings of this study show that minocycline controls the development of nerve cell apoptosis in ICH mice by inhibiting the TRAIL signaling pathway, and that TRAIL plays a vital role in the regulatory mechanism of nerve cell apoptosis in ICH. Thus, the results provide a theoretical basis for the prevention and treatment of ICH and related diseases. In subsequent studies, more cell lines will be introduced, and multiple techniques, including gene knockout, will be adopted to further investigate other possible mechanisms of action.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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