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Granulocyte colony-stimulating factor protected against brain injury in a rat cerebral hemorrhage model by modulating inflammation

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Abstract: Granulocyte colony-stimulating factor (G-CSF) has been reported to exert a protective effect against secondary brain damage, but the underlying mechanisms remain unknown. We explored the ability of G-CSF to protect the brain from injury in a rat autologous blood-induced model of intracerebral hemorrhage (ICH), with a special focus on the anti-inflammation effect. An ICH was induced in 8-week-old male rats by an infusion of autologous blood, and the rats were then randomly assigned to five treatment groups: sham, ICH, and ICH+ low-dose (25 µg/kg), middle-dose (50 µg/kg), and high-dose (75 µg/kg) G-CSF. We then evaluated the levels of brain inflammation-related genes and proteins. The levels of tumor-necrosis factor-alpha (*TNF-α*) and interleukin-6 (*IL-6*) mRNA increased between days 1 and 14 post-ICH, with the highest expression on day 3. These changes were rectified by G-CSF in a dose-dependent manner. At day 3 post-injury, an elevation of the nuclear factor-kappa B (NF-κB) p65 protein level and a reduction of the inhibitor of NF-κB alpha (IκBα) protein level were observed; G-CSF treatment exerted a beneficial effect on both protein expressions. The expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins were increased; these changes were rectified by the highest dose of G-CSF. The brain-protecting effects of G-CSF are likely to be attributable, at least in part, to attenuation of the *TNF-α*, *IL-6*, iNOS, and COX-2 expressions induced by NF-κB activation in the brain tissues of this autologous blood-induced ICH rat model.

Key words: autologous blood, granulocyte colony stimulating factor, inflammation, intracerebral hemorrhage

Introduction

The precise mechanisms that underlie the various brain injuries that occur after an intracerebral hemorrhage (ICH) are not known, and effective treatments for an ICH remain to be identified. The widely accepted main macromechanisms of brain injury after an ICH are as follows: (i) Direct damage, i.e., the cerebral blood vessel rupture and bleeding, (ii) a space-occupying effect, and (iii) secondary brain damage. The events involved in secondary brain injury after an ICH includes

the following: (i) Thrombin induces brain edema and neurocyte death [1]. (ii) Red blood cell destruction increases inflammation as well as the production of iron and hemoglobin, leading to the aggravation of the brain injury [2]. (iii) A release of cytotoxic substances aggravates ischemic brain injury [3]. (iv) The production of oxygen free radicals causes brain inflammation and cell apoptosis [4]. The current treatments for an individual who has suffered an ICH include the control of blood pressure, glucose and hypothermia, and the prevention of rebleeding and complications. However, there

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is still no clear consensus on the indications, methods, or timing of surgery for an ICH.

The activation of the nuclear factor kappa B (NF- κ B) p65 signaling pathway plays a vital role in the process of secondary brain injury due to an ICH. The high levels of thrombin and inflammatory factors related to ICH activate the phosphorylation of inhibitor of NF- κ B kinase-beta (IKK- β) and the subsequent phosphorylation of inhibitor of NF- κ B alpha (I κ B α), which facilitates increased NF- κ B binding to the target DNA, leading to an aggravation of secondary brain injury by the production of downstream inflammatory cytokines [5]. The specific inhibitor of the NF- κ B signaling pathway has thus gained considerable interest as a therapeutic target for brain injury after an ICH in humans and animals.

Granulocyte colony-stimulating factor (G-CSF), a small polypeptide growth factor that passes freely across the blood-cerebrospinal fluid barrier, induces the proliferation and differentiation of hematopoietic stem cells by stimulating their expression of adhesive molecules [6]. At the same time, G-CSF inhibits hematopoietic stem cell apoptosis, and thus G-CSF has been widely used in the management of various diseases such as neutropenia [7]. The clinical application of G-CSF in oncology has become more common over the past 20 years. For example, G-CSF is used to treat cancer patients who have granulocytopenia after radiotherapy or chemotherapy toward the goal of reducing the incidence of infection. It was reported that G-CSF suppressed inflammation and apoptosis and promoted angiogenesis [8]. These G-CSF functions are consistent with protective effects against secondary brain injury after an ICH, and the functional characteristics of G-CSF provide a basis for its potential use in the treatment of ICH-related brain injury.

Here we conducted the present study to investigate the effects of G-CSF on brain injury in a rat autologous blood-induced ICH model, with a focus on the expressions of NF- κ B and downstream inflammation-related genes and/or proteins, i.e., inducible nitric oxide synthase (iNOS), cyclo-oxygenase (COX)-2, tumor necrosis factor-alpha (TNF- α), interleukin (IL)-6, and I κ B α .

Materials and Methods

Reagents and antibodies

The following commercially available antibodies were used: Recombinant human G-CSF was purchased from Sihuan Pharmaceutical Holdings Group (Beijing, China). Anti-COX-2, anti-NF- κ B p65, and anti-I κ B α antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-iNOS and anti-IL-1 β were purchased from Abcam (Cambridge, UK). Anti-cleaved-

caspase-1 (anti-C-casp-1) was purchased from (Affinity Bioscience LDT, Changzhou, China). The BCA Protein Assay Kit was purchased from Beijing Solarbio Science & Technology Co. (Beijing, China). RIPA lysis buffer was purchased from Beijing Beyotime Biotechnology (Beijing, China). Goat anti-rabbit IgG-HRP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TRIzolTM was purchased from Thermo Fisher (San Jose, CA, USA). Agarose was purchased from Invitrogen (Carlsbad, CA, USA). The one-step reverse transcript and polymerase reaction chain (RT-PCR) kit was from Takara (Dalian, China).

Animals

Eight-week-old male Sprague-Dawley (SD) rats were purchased from Yanbian University Animal Center (Yanji, China). They were fed a standard diet and housed three per cage under standard conditions ($22 \pm 2^\circ\text{C}$, $50 \pm 5\%$ humidity) with a 12-h light-dark cycle at the Animal Research Center of Yanbian University. All animal protocols were approved by the Institutional Animal Care and Use Ethics Committee of Yanbian University and were performed according to the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

ICH Model and G-CSF treatment groups

ICH model establishment

A slow injection of autologous blood to the caudate nucleus was used to generate the ICH model in SD rats. For this procedure, the rat was first anesthetized with an intraperitoneal injection of 10% chloral hydrate (0.3 ml/100 g). The rat's general state was then observed to confirm that the anesthesia was successful; i.e., the rat's breathing became regular and slow, its movements stopped, it did not close its eyes when its cornea was touched, and there was no response to pain stimulation.

After skin preparation, the rat was fixed in the supine position on a brain stereotaxic instrument (RWD Life Science Co., Shenzhen, China). A longitudinal incision was made in the middle of the head to expose the bregma and posterior fontanelle. The skull was drilled; the drilling point was located 1 mm before the bregma and 3 mm on the right side of the bregma [9]. A 50- μ l microinjection needle was then inserted 5.5–6 mm to the caudate nucleus, and 20 μ l of autologous blood obtained from the rat's tail artery was slowly injected at first; the needle was maintained in its position for 5 min. Another 30 μ l of autologous blood was then injected, and the needle was maintained in position again for 5 min before withdrawal. Finally, the cut tissue was sutured and closed, and an iodine solution was used to disinfect

the surgical wound. The rats in the ICH model groups and the sham group underwent the same surgery, but the same volume of saline was injected into the caudate nucleus of the sham surgery group to mimic the surgical injury.

Experiments 1 and 2

The following two experiments were performed. First, for the assessment of the impact of the ICH on brain inflammation, rats that underwent the above-described ICH surgery were randomly assigned to one of day 0, 1/24 (1 h), 1/4 (6 h), 1/2 (12 h), 1, 3, 5, 7, and 14 groups (n=8 for each group) after surgery. Second, to explore the beneficial effect of G-CSF on brain injury, rats that had undergone the ICH protocol were randomly assigned to one of four groups (n=6, each group): Sham, ICH+saline (50 μ l), ICH+G-CSF low-dose (25 μ g/kg), ICH+G-CSF medium-dose (50 μ g/kg), or ICH+G-CSF high-dose (75 μ g/kg). At various time points after the induction of an ICH, following the rat's sacrifice with an overdose of 10% chloral hydrate, the brain tissues were flushed clear of blood with normal saline at physiological pressure. For the gene assay, the brain tissues were put in TRIzol and stored at 20°C. For the Western blot analysis, the brain tissues were snap-frozen in liquid nitrogen and stored at 70°C.

Gene assay

Total RNA was isolated from the brain tissues with 1 ml of TRIzol, and we then measured the concentration of mRNA according to the manufacturers' instructions. The sequences of the investigated primers are as follows: β -actin: sense primer: 5'-CAC CCG CCA CCA GTT CGC CA-3'; antisense primer: 5'-CAG GTC CCG GCC AGC CAG GT-3'; *IL-6*: sense primer 5'-CGG AGA GGA GAC TTC ACA GAG GA-3' antisense primer 5'-GGA GAG CAT TGG AAG TTG GGG GA -3'; *TNF- α* : sense primer: 5'-TGC CTC AGC CTC TTC TCA TT-3' antisense primer: 5'-GTG GGT GAG GAG CAC GTA GT -3'. A one-step RT-PCR kit (Takara) was applied to the cDNA synthesis and amplification of the target genes (*IL-6*, *TNF- α* , and β -actin) using a Bio-Rad PCR system (Bio-Rad Laboratories, Hercules, CA, USA) [10]. The RT-PCR program was as follows: one cycle at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 58°C for 20 s, and 72°C for 30 s. Following electrophoresis, the agarose gels were set to take the related bands by the UVP Imaging System (Skit Instrument Equipment Co., Shanghai, China), and the band intensities were analyzed with a FluorChem HD2 imaging system (ProteinSimple, San Jose, CA, USA). β -actin was used as an internal reference.

Western blot analysis

Western blotting assay was performed as described [11]. After the extraction of total protein from the tissues with a RIPA lysis buffer, the amounts of protein (40 μ g/line) were transferred to polyvinylidene difluoride membranes and immunoreacted with the following targeted primary antibodies: NF- κ B p65, I κ B α , C-casp-1, COX-2 (1:1,000), IL-1 β , iNOS (1:500), and β -actin (1:2,000). The determination of targeted proteins was performed using the Amersham ECL Prime Western Blotting Detection kit (Amersham Biosciences, Piscataway, NJ, USA). Quantifications of targeted protein amounts from the western blots were normalized by loading internal β -actin as the control.

Statistical analysis

Data are presented as the mean \pm SEM. Student's *t*-test (for comparisons between two groups) or a one-way analysis of variance (ANOVA) (for comparisons of three or more groups) followed by Tukey's post hoc tests were used for the statistical analyses. SPSS software ver. 22.0 (SPSS, Chicago, IL, USA) was used. Probability (*P*)-values <0.05 were considered significant.

Results

Effects of ICH on the expressions of *IL-6* and *TNF- α* genes and NF- κ B and I κ B α protein in the brain tissue

We first evaluated the impact of ICH on brain inflammation. Representative RT-PCR images and quantitative data (Figs. 1 and 2) showed that the *TNF- α* and *IL-6* mRNA levels had significantly increased between day 1 and 14 after ICH, with the highest expression occurring at day 3; these changes were rectified by the low dose of G-CSF. As anticipated, ICH significantly elevated the NF- κ B p65 protein level on days 1 and 7 after ICH injury, with the highest expression occurring on day 3 (Figs. 3A and B). In contrast, with the exception of day 5, ICH significantly decreased the level of I κ B protein between days 1 and 7 after ICH injury, with the lowest expression occurring on day 3 (Figs. 3A and C). We also observed that the iNOS and COX-2 protein levels had also significantly increased between 12 h and day 7 (for iNOS) and between 6 h and 24 h (for COX-2) after ICH, with the highest expression occurring at 24 h (Fig. 4).

G-CSF mitigated the levels of the inflammation-related genes and proteins in the brain tissue in response to ICH

We next explored whether the administration of G-CSF could exert a protective effect against brain injury

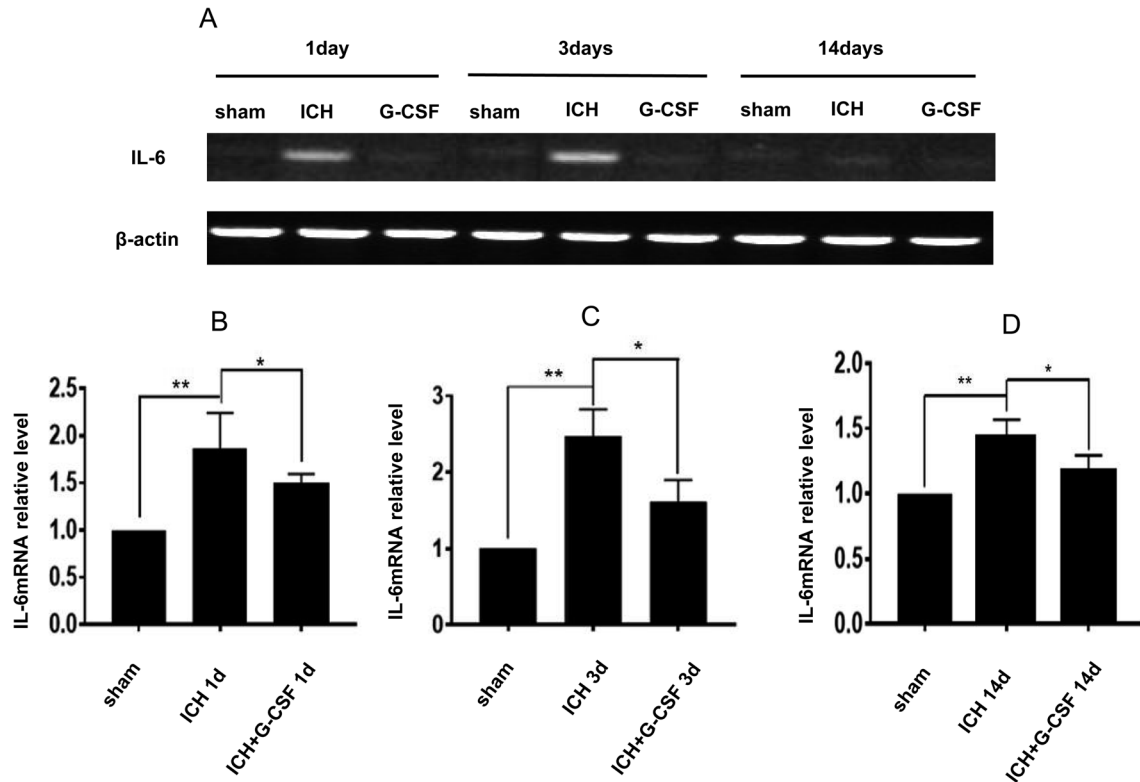


Fig. 1. The impact of ICH on the expression of *IL-6* gene in the rat brain tissues. A: Representative PCR images showing the changes in the levels of *IL6* mRNA in a time-dependent manner. B–D: Quantitative data show the levels of *IL-6* in the injured brain tissues of three experimental groups (Sham, ICH, and ICH-G-CSF 25 μ g). Values are mean \pm SEM (n=5). * P <0.05 *** P <0.001 vs. corresponding controls by one-way NOVA followed by Tukey's post hoc tests.

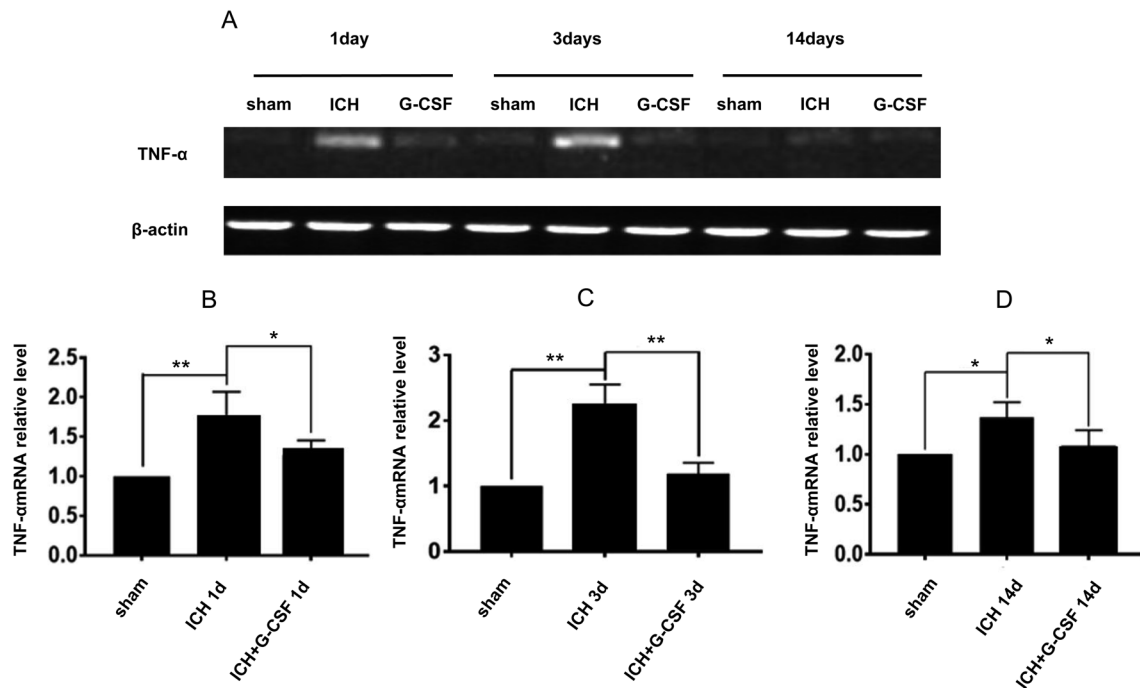


Fig. 2. The impact of ICH on the expression of *TNF- α* gene in the brain tissues. A: Representative PCR images showing the changes in the levels of *TNF- α* mRNA in a time-dependent manner. B–D: Quantitative data of the levels of *TNF- α* in the injured brain tissues of three experimental groups (Sham, ICH, and ICH-G-CSF 25 μ g). Values are mean \pm SEM (n=5). * P <0.05, ** P <0.01 *** P <0.001 vs. corresponding controls by one-way ANOVA followed by Tukey's post hoc tests.

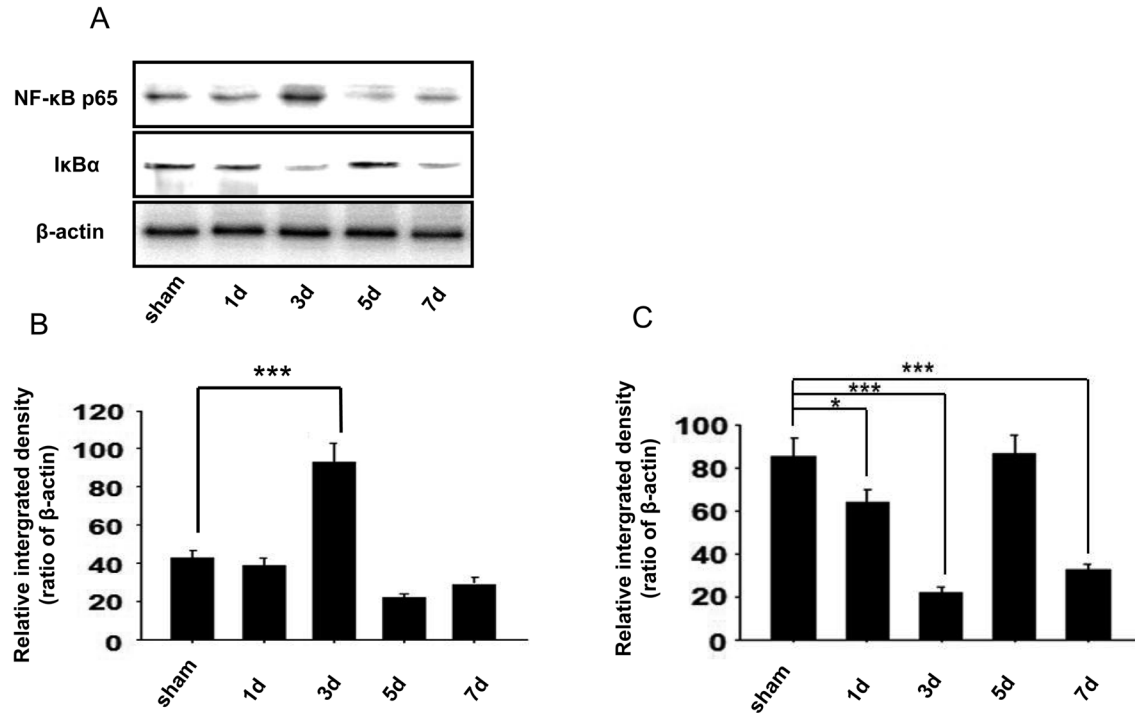


Fig. 3. The impact of ICH on the levels of NF-κB p65 and IκBα proteins in the brain tissues. A–C: Representative western blotting images and quantitative data revealing the changes in the levels of NF-κB p65 and IκBα proteins in the brain tissues in response to ICH at the indicated timepoints. Values are mean ± SEM (n=4). * P <0.05 *** P <0.001 vs. corresponding controls by one-way ANOVA followed by Tukey's post hoc tests.

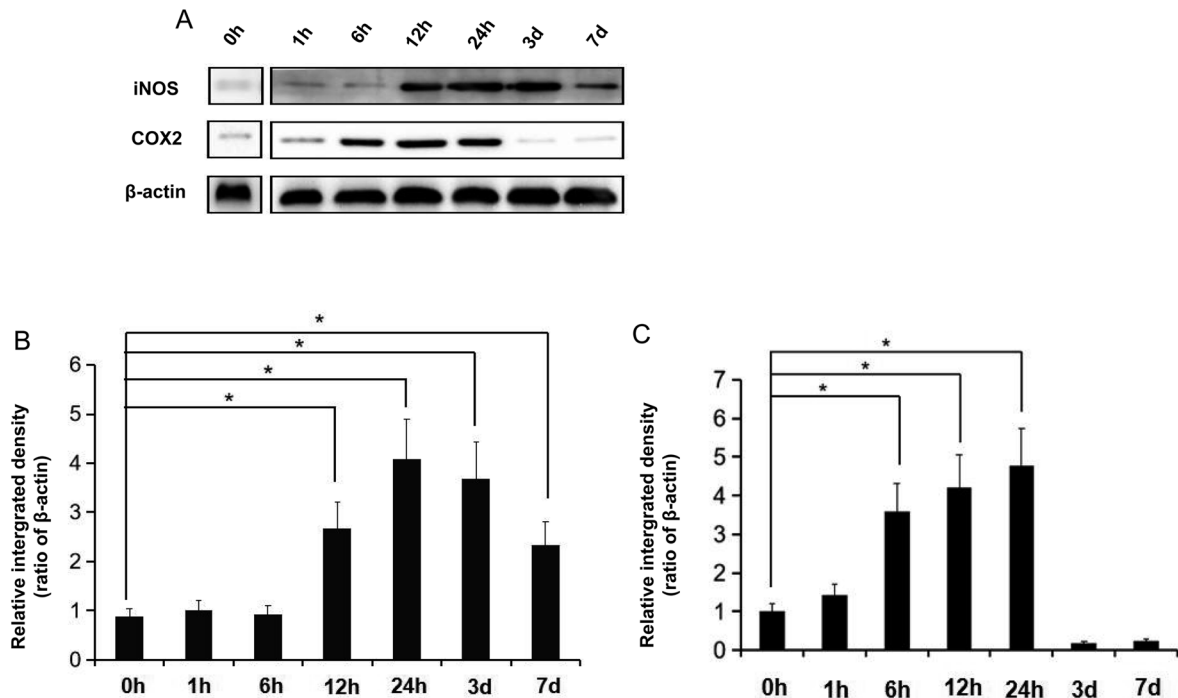


Fig. 4. The impact of ICH on the expressions of iNOS and COX-2 proteins in the brain tissues. A–C: Representative western blotting images and quantitative data showing the changes in the levels of iNOS and COX-2 proteins in the brain tissues in response to ICH at the indicated timepoints. Values are mean ± SEM (n=3). * P <0.05, ** P <0.01, *** P <0.001 vs. corresponding controls by one-way ANOVA followed by Tukey's post hoc tests.

after ICH. As depicted in Figs. 5 and 6, on operative day 3 after ICH, representative RT-PCR images and quantitative data showed that G-CSF mitigated the *TNF- α* and *IL-6* mRNA expressions in brain tissues in response to ICH in a dose-dependent manner. Likewise, G-CSF also mitigated harmful changes in the levels of NF- κ B p65 and I κ B protein in a dose-dependent manner on day 3 after ICH (Fig. 7). Consistently, with the exception of the low dose of G-CSF, G-CSF had a beneficial effect on the expressions of both of these proteins at 24 h after ICH (Fig. 8). On operative day 7 after ICH, we also observed that the administration of G-CSF at the middle and high doses resulted in a significant reduction in the level of iNOS protein (Figs. 9A and B). Finally, we found that ICH resulted in an increase in the level of IL-1 β protein at day 1 after injection as well as its converting enzyme C-casp-1; and these changes were rectified by the high dose of G-CSF (Figs. 9C and D).

Discussion

The results of this study demonstrate that the injured brain tissues of rats had increased levels of NF- κ B protein and its downstream inflammatory genes or/and proteins (i.e., *TNF- α* , *IL-6*, iNOS, and COX-2) after ICH at specific timepoints, and these changes were rectified by G-CSF administration in a dose-dependent manner. The G-CSF-mediated brain protective effect might therefore be due to the modulation of an NF- κ B-dependent *TNF- α* /*IL-6* and iNOS/COX-2 axis in brain tissues in a rat-autologous blood-induced ICH mode.

As a sequence-specific transcription factor, NF- κ B is widely present in the central nervous system and has the function of transmitting information inside and outside the cell nuclei [12]. It plays an important role in synaptic signal transduction, nerve shaping, nerve development and degeneration, immunity, stress response, inflammation, and apoptosis [13]. I κ B generally binds with NF- κ B/I κ B- β complex to mask its function, whereas once its level is out of control, NF- κ B induces inflammatory gene expressions in various pathophysiological conditions [14]. Our present findings revealed that the ICH injury increased the expression of NF- κ B protein and decreased that of I κ B α protein, suggesting that the imbalance of NF- κ B and I κ B α may contribute to brain inflammation and damage after an ICH.

G-CSF is known as a growth factor that can pass through the blood-cerebrospinal fluid barrier, and it exerts an anti-inflammatory role by binding to hematopoietic progenitor cells [15]. G-CSF has been widely used in the treatment of granulocytopenia caused by tumor radiotherapy and chemotherapy. Kang and colleagues

reported that G-CSF improved cardiac fibrosis and cardiac function via the stimulation of angiogenesis [16]. G-CSF has also been applied to treat neurological diseases such as stroke, amyotrophic lateral sclerosis, and Parkinson's disease [17–19]. In our present investigation, G-CSF treatment suppressed NF- κ B p65 protein expression and increased I κ B α protein expression in a dose-dependent manner. G-CSF thus appears to mitigate brain inflammation via the rectification of the imbalance of NF- κ B and I κ B α after an ICH.

The inflammatory responses of damaged brain tissues after an ICH include mainly the activation of microglia, the infiltration of inflammatory cells, and the release of mediators that promote the inflammatory responses [20]. IL-1 β is major pro-inflammatory cytokines that are activated after an ICH and further activate the NF- κ B signaling pathway, resulting in the degradation of I κ B α . The released NF- κ B p65 translocates to the cell nucleus, promotes the transcription of target genes, and produces IL-1 β [21]. We have shown that ICH elevated the levels of IL-1 β and C-casp-1 proteins in the injured brain tissues; and these changes were rectified by the G-CSF therapy. Taken together, these findings suggest that the brain protective actions of G-CSF are mediated, at least in part, via NF- κ B/I κ B α -dependent mechanism that is modulated by IL-1 β /caspase-1 axis in our experimental conditions.

We also observed that the overall experimental results regarding *IL-6* tended to be similar to those of the expression of *TNF- α* mRNA, which is probably correlated with a cascade reaction of *TNF- α* which can promote the production of proinflammatory cytokines such as *IL-6*. It was reported that after an ICH, immune cells and neuronal cells are activated to express *TNF- α* , and then *TNF- α* further promotes the secretion of *IL-6* [22–24] (which is consistent with the results of our experiment), and then these changes were reversed by the application of G-CSF. Considering the past and present findings together, we propose that after an ICH, G-CSF acts a key protective mediator of secondary damage to the brain via the reduction of *TNF- α* /*IL-6*.

iNOS, which is one of the downstream molecules of the NF- κ B signaling pathway, is distributed mainly in microglia, astrocytes, and vascular endothelial cells [25–27]. iNOS can be activated by endotoxins, lipopolysaccharides, etc. and generates a large amount of nitric oxide (NO), which has toxic effects on cells [28, 29]. There are two subtypes of cyclooxygenases, i.e., COX-1 and COX-2, which are the key rate-limiting enzymes of the prostaglandins converted from arachidonic acid and other lipid mediators. COX-1 can be expressed in various tissues [30, 31], and COX-2 is induced mainly

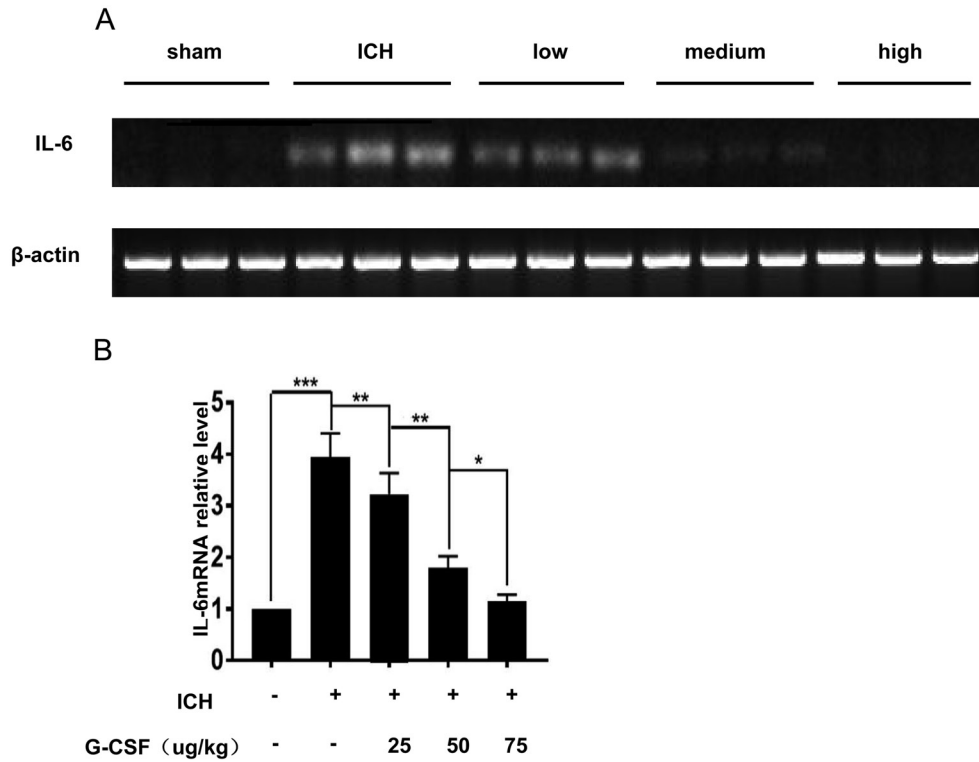


Fig. 5. The effect of G-CSF on *IL-6* gene expression in the brain tissues of the five experimental groups (Sham, ICH, ICH-G-CSF 25 μ g, ICH-G-CSF 50 μ g, and ICH-G-CSF 75 μ g) at day 3 after ICH. A, B: Representative PCR images and combined quantitative data showing the changes in the levels of *IL6* in a dose-dependent manner. Values are mean \pm SEM (n=3). * P <0.05, ** P <0.01, *** P <0.001 vs. corresponding controls by one-way ANOVA followed by Tukey's post hoc tests.

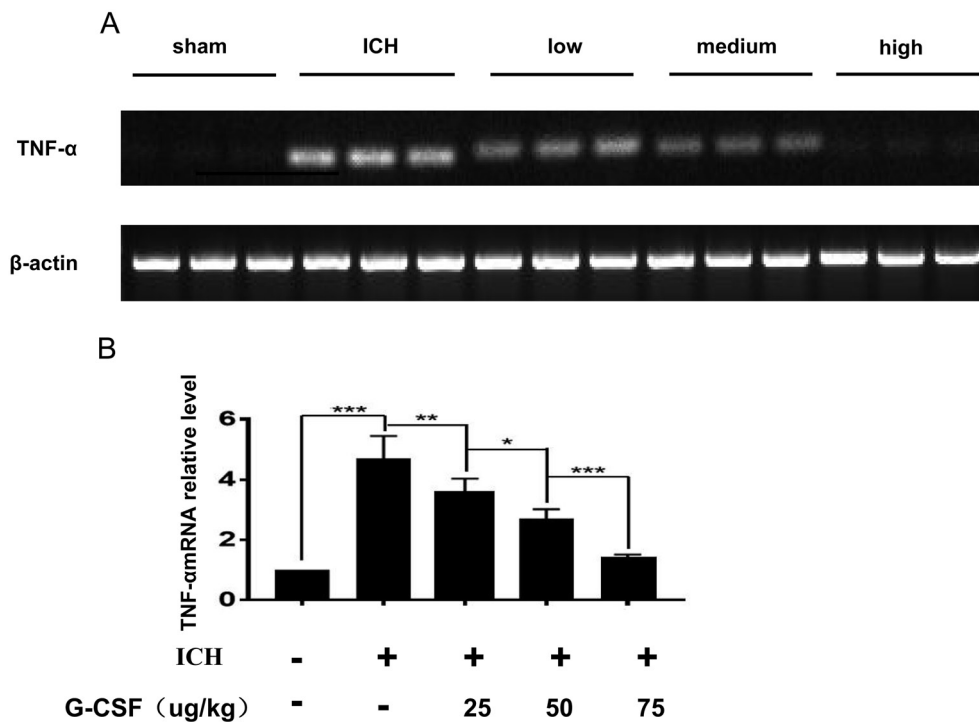


Fig. 6. The effect of G-CSF on *TNF- α* gene expression in the brain tissues of the five experimental groups (Sham, ICH, ICH-G-CSF 25 μ g, ICH-G-CSF 50 μ g, and ICH-G-CSF 75 μ g) at day 3 after ICH. A, B: Representative PCR images and combined quantitative data showing the changes in the levels of *TNF- α* in a dose-dependent manner. Values are mean \pm SEM (n=4). * P <0.05, ** P <0.01, *** P <0.001 vs. corresponding controls by one-way ANOVA followed by Tukey's post hoc tests.

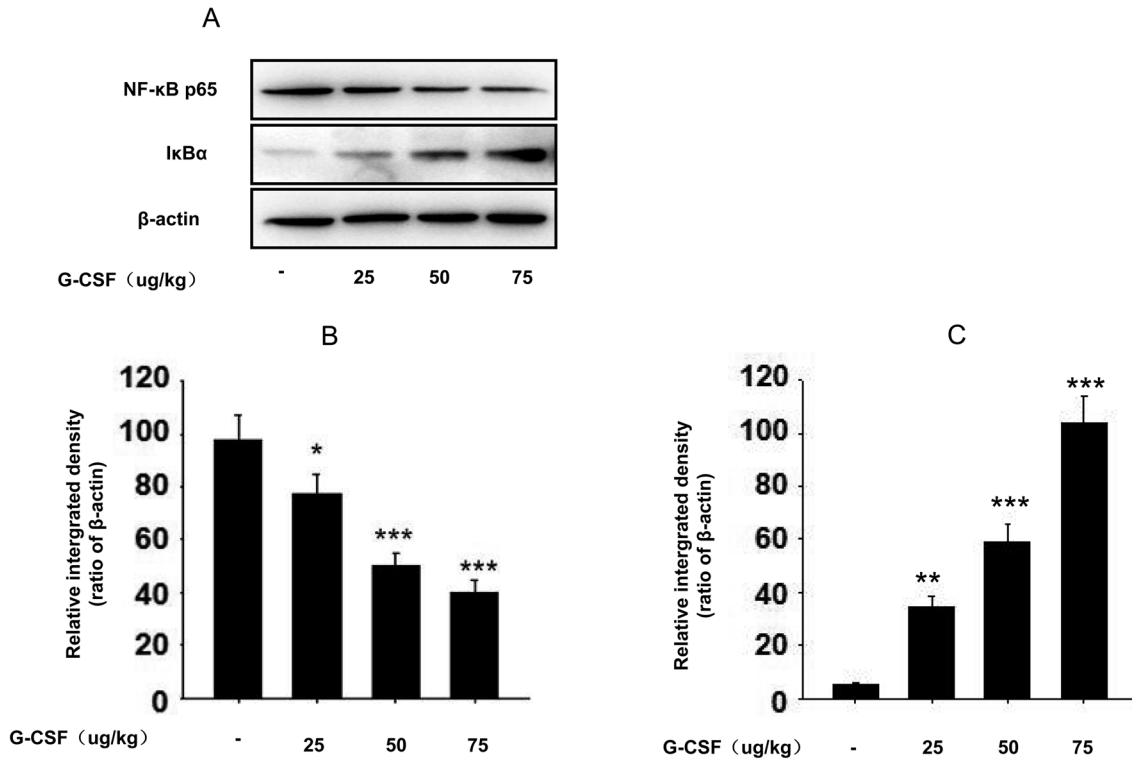


Fig. 7. The effect of G-CSF on the levels of NF-κB p65 and IκBα proteins in the brain tissues of four experimental groups (ICH, ICH-G-CSF low dose, ICH-G-CSF middle dose, and ICH-G-CSF high dose) at day 3 after ICH. A–C: Representative western blotting images and combined quantitative data showing the changes in the levels of NF-κB p65 IκBα proteins in a dose-dependent manner. Values are mean ± SEM (n=3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. corresponding controls by one-way ANOVA followed by Tukey’s post hoc tests.

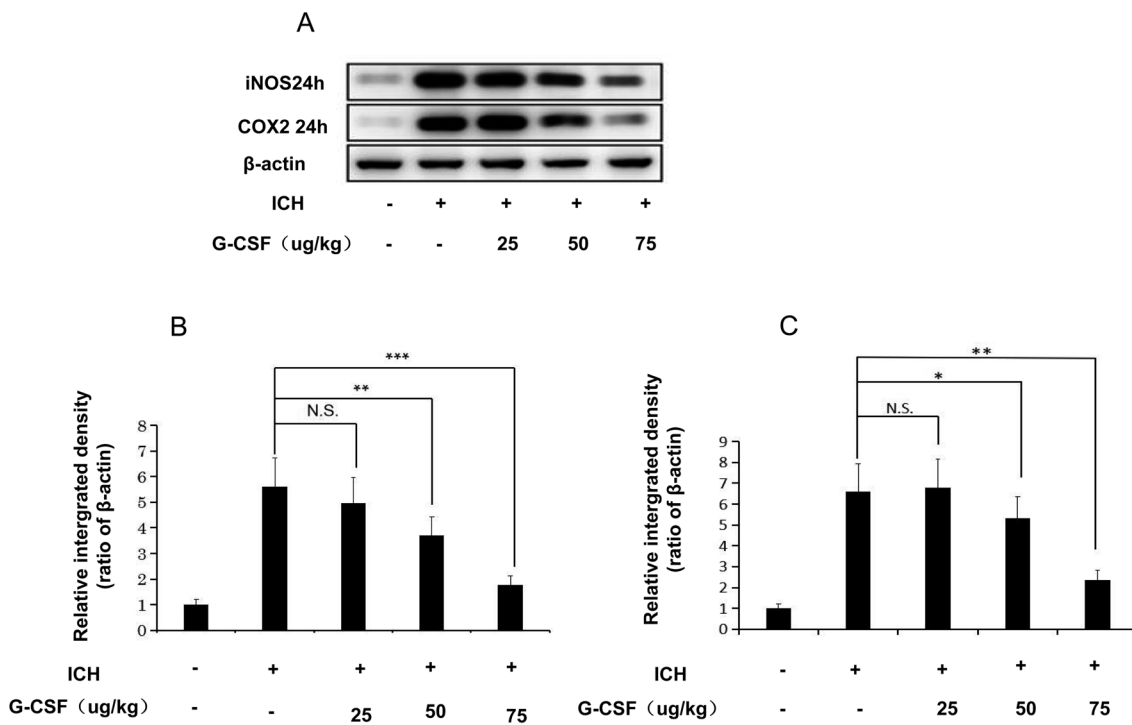


Fig. 8. The effect of G-CSF on the levels of iNOS and COX-2 proteins in the brain tissues of five experimental groups (Sham, ICH, ICH-G-CSF 25 μg, ICH-G-CSF 50 μg, and ICH-G-CSF 75 μg) at 24 h after ICH. A–C: Representative western blotting images and combined quantitative data showing the changes in the levels of TNF-α in a dose-dependent manner. Values are mean ± SEM (n=3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. corresponding controls by one-way ANOVA followed by Tukey’s post hoc tests. NS: no significant difference.

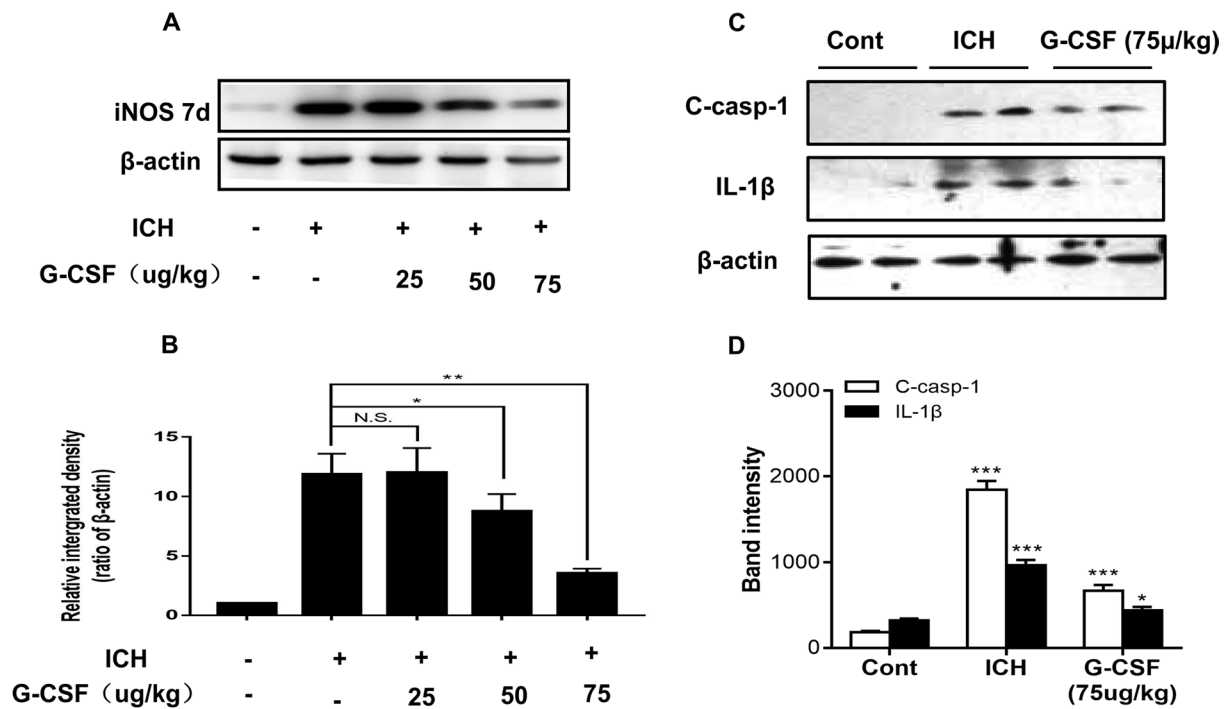


Fig. 9. The effect of G-CSF on the levels of iNOS protein in the brain tissues of the five experimental groups (Sham, ICH, ICH-G-CSF 25 μ g, ICH-G-CSF 50 μ g, and ICH-G-CSF 75 μ g) at day 7 after ICH. A, B: Representative western blotting images and combined quantitative data showing the changes in the levels of iNOS in a dose-dependent manner. C, D: Representative western blotting images and combined quantitative data showing the changes in the levels of IL-1 β and C-casp-1 proteins in three experimental groups at day 1 after ICH. Values are mean \pm SEM (n=3). * P <0.01 *** P <0.001 vs. corresponding controls by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. NS: no significant difference.

by inflammatory stimuli; activated NF- κ B translocates to the cell nucleus and expresses COX-2 [32, 33]. In the central nervous system, COX-2 is expressed mainly by microglia (which is one of the signs of microglia activation), which can cause damage to neurons [34]. iNOS and COX-2 are important inflammatory enzymes that respond to neuroinflammation, and they are also downstream molecules of the NF- κ B signaling pathway [35]. Our present observations revealed that iNOS and COX-2 increased in different degrees at different time points after the ICH and reached a peak at 24 h after injury; these changes were rectified by the medium and high doses of G-CSF. The reduction in the levels of iNOS and COX-2 by G-CSF treatment could therefore present a potential mechanism in the protection against secondary injury to the brain after an ICH.

One major potential limitation of the present study is that although current studies contains the detail biological data of the pharmacological interventions targeted toward NF- κ B/I κ B α and TNF- α /IL-1 β -caspase-1 axes ameliorates ICH-induced brain injury, we could not explore an direct evidence of how G-CSF modulates both signals to contribute to brain protective actions in our experimental conditions. Furthermore, it was not included the data of brain cell experiments. Further studies

will be needed to investigate these issues.

Taken together, the previous and present findings indicate that G-CSF might prevent secondary brain damage via the reductions of TNF- α , IL-6, iNOS, and COX-2-related inflammation that is mediated by the negative modulation of the NF- κ B signaling pathway in rats that have been subjected to ICH injury, suggesting a novel therapeutic strategy for the control of ICH-related brain damage by G-CSF therapy. Further neuronal morphological and functional studies are needed to clarify the pathophysiological significance of G-CSF in the reduction of the inflammatory response to an ICH.

Authors' Contributions

YL: Main contributor to the collection and assembly of data, manuscript drafting, biological and morphological analyses, data statistical analysis and interpretation. TX and BZ: Collection and assembly of samples and data. XP and XS: Editing of the manuscript. XWC: Financial support; design and editing of the manuscript. SZ: Main contributor to financial support; design and editing of the manuscript. All authors approved the final version of submission.

Ethics Approval and Consent to Participate

The animal study protocol (YM2013-17) was approved by the Ethics Committee on Animal Research at Yanbian University Medical College. All experiments were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals of the Chinese Pharmacological Society.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this manuscript.

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