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Original Article

Atorvastatin suppresses CD147 and MMP-3 expression and improves histological and neurological outcomes in an animal model of intracerebral hemorrhage

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Abstract: Intracerebral hemorrhage (ICH) poses serious health concerns in the Chinese population. Few treatment options are available for managing ICH. However, atorvastatin has been shown to improve functional outcomes after ICH. The present study investigated the possible mechanism by which atorvastatin protects against tissue damage in an animal model of ICH. Rats were injected with collagenase in the globus pallidus to induce ICH. Six hours after ICH induction, rats in treatment group A orally received 2 mg/kg and rats in treatment group B orally received 10 mg/kg atorvastatin, followed by once daily administration of atorvastatin for seven days. The ICH model group did not receive any medication and the sham surgery group was injected with saline. Expression of cluster of differentiation 147 (CD147) and matrix metalloproteinase-3 (MMP-3) and histological and neurological outcomes were assessed at 24, 48, 72 h, and seven days post ICH induction. Compared to the sham surgery group, the ICH group showed a significant increase in CD147 and MMP-3 levels, as well as neuronal loss and impaired neurological functions ($p < 0.01$). Treatment with atorvastatin at both dosages significantly suppressed the increase in CD147 and MMP-3 levels, and the treated animals exhibited significantly improved histological and neurological outcome in a dose-dependent manner. From our data, we concluded that atorvastatin exerts neuroprotective effects through suppressing CD147, which leads to MMP-3 down-regulation, thereby reducing inflammatory responses secondary to ICH and improving histological and neurological outcomes.

Keywords: Intracerebral hemorrhage (ICH), atorvastatin, cluster of differentiation 147 (CD147), rat

Introduction

Intracerebral hemorrhage (ICH) is the second most common type of stroke, with an overall incidence of 24.6 per 100,000 person-years [1]. ICH is associated with a high fatality rate, and the median case fatality at 1 month is 40.4% [2]. Moreover, the prevalence of ICH in the Chinese population is high, accounting for 27.5% of all stroke cases [3] and has the lowest survival rate among all types of strokes [4]. Because ICH is associated with high mortality and disability rates, effective treatments for the condition are essential for improving the quality of life of ICH patients and relieving the burden of the disease. However, despite vigorous research efforts, very few therapeutic options are available for ICH.

The inflammatory response following ICH is particularly harmful to the brain because the disruption in the blood brain barrier is amplified, and infiltrating leukocytes can damage the surrounding tissue. Matrix metalloproteinases (MMPs), enzymes that play a key role in the degradation of basal membrane and extracellular matrix, are inflammatory mediators in the pathogenesis of ICH. MMPs are upregulated after ICH [5, 6]. Cluster of differentiation 147 (CD147), also known as EMMPRIN or basigin, is a glycoprotein and a member of the immunoglobulin superfamily. CD147 has multiple binding partners, including cyclophilins, S100A9, platelet glycoprotein VI, GLUT1, CD44, and CD98, and thereby participates in a variety of physiological processes [7]. In the central nervous system, CD147 is associated with various disease pathologies, including stroke, Alzheimer's disease, and gliomas [8]. Recently, CD147 has been identified as a novel inflammatory modulator [9] that contributes to the migration of inflammatory leukocytes [10].

Atorvastatin, a member of the statin family, is traditionally used to treat high cholesterol. Subsequent studies found that atorvastatin is potentially beneficial for treating neurological diseases such as Alzheimer's disease [11]. Furthermore, treatment with atorvastatin improves neurological outcomes after ICH. The mechanisms by which atorvastatin mediate neuroprotective effects, however, are not fully known. Of note, a study reported that atorvastatin, in combination with methylprednisolone, was effective in treating relapsing multiple sclerosis, and this therapeutic effect was associated with modulation of interleukins [12]. As multiple sclerosis is an autoimmune disease, atorvastatin might exert its neuroprotective effects through modulating inflammatory and immune responses, which usually occur secondary to ICH. In this study, we used an animal model of ICH to test the hypothesis that atorvastatin protects the brain tissue by suppressing the inflammatory response secondary to ICH through inhibiting CD147 and MMPs.

Materials and methods

Animals

Male Sprague-Dawley rats (Department of Experimental Animal, Central South University) were randomly assigned to control, sham surgery, ICH model, ICH treatment A, and ICH treatment B groups. All animals were cared for in accordance with the guidelines set by Central South University, and the animal protocol was approved by the IACUC of Central South University.

ICH induction and atorvastatin administration

ICH was induced with the method developed by Rosenberg *et al.* [13]. Briefly, male rats (250-300 g) were weighed and anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3-0.4 ml/100 g, Xiangya Hospital). When anesthetized, the animals were mounted on a stereotaxic frame (RWD Life Science Inc), and then the skulls were exposed and a small hole was drilled 1.4 mm posterior and 3.2 mm to the right of the bregma. A needle attached to a microinjector (Shanghai Gaoge Industrial and Trading Co., LTD) was inserted 6.0 mm vertically through the hole and 2.7 mL collagenase (0.2 U/mL dissolved in saline, Sigma) was injected into the globus pallidus. Sham operation group animals were injected with an equal volume of saline (0.9% NaCl), whereas the control group of animals did not undergo any procedure. Six hours after ICH surgery, animals were examined for neurological deficits on a 1-5 grade described by Longa [14]: grade 1, no neurological deficit; grade 2, a mild neurological deficit (difficulty in stretching forepaws on the paralyzed side); grade 3, moderate neurological deficits (circling when walking); grade 4, severe neurological deficits (falling to the paralyzed side when walking); and grade 5, could not walk spontaneously and exhibited decreased level of consciousness. Animals that scored 2 to 4 on the grade scale were considered to meet the requirements of the ICH model and included in this study. Atorvastatin was administered intragastrically six hours after the surgery in the treatment groups (2 mg/kg for treatment group A and 10 mg/kg for treatment group B) followed by daily administration of atorvastatin for seven days or until rats were sacrificed at appropriate study end points. Expression of CD147 and MMP-3, brain tissue damage, and neurobehavioral function were assessed at 24, 48 and 72 hr, and seven days after the surgery in all groups.

Histology and immunocytochemistry

At the end of the study, the animals were anesthetized and perfused with 4% paraformaldehyde (pH = 7.4, Wuhan Boster Biological Technology, LTD) that was dissolved in 0.1 mol/L phosphate buffered saline (PBS); the brains were then collected and embedded in paraffin. DAB staining was performed according to the manufacture's instructions (Beijing ZSGB-BIO Origene Company). Briefly, the brain tissues were sectioned into 4 μm slices, blocked in goat serum (1:20, BIO OriGene Inc.) for one hour, and incubated with primary antibody against MMP-3 (1:400, Abcam) or CD147 (1:500, Abcam) at 4°C overnight. The slices were then washed with 0.1 mol/L PBS for 10 min, three times. Reagents B and C of the Streptavidin-Peroxidase (SP) kit were used to stain CD147 and reagents 1 and 2 of the PV 2-step plus Poly-HRP kit were used to stain MMP3. Slices were incubated for 20 min and then developed with DAB. Images were acquired at 400 × magnification with a microscope camera (Leica). Mean optical density (MOD) of CD147 and MMP-3 were measured with Image

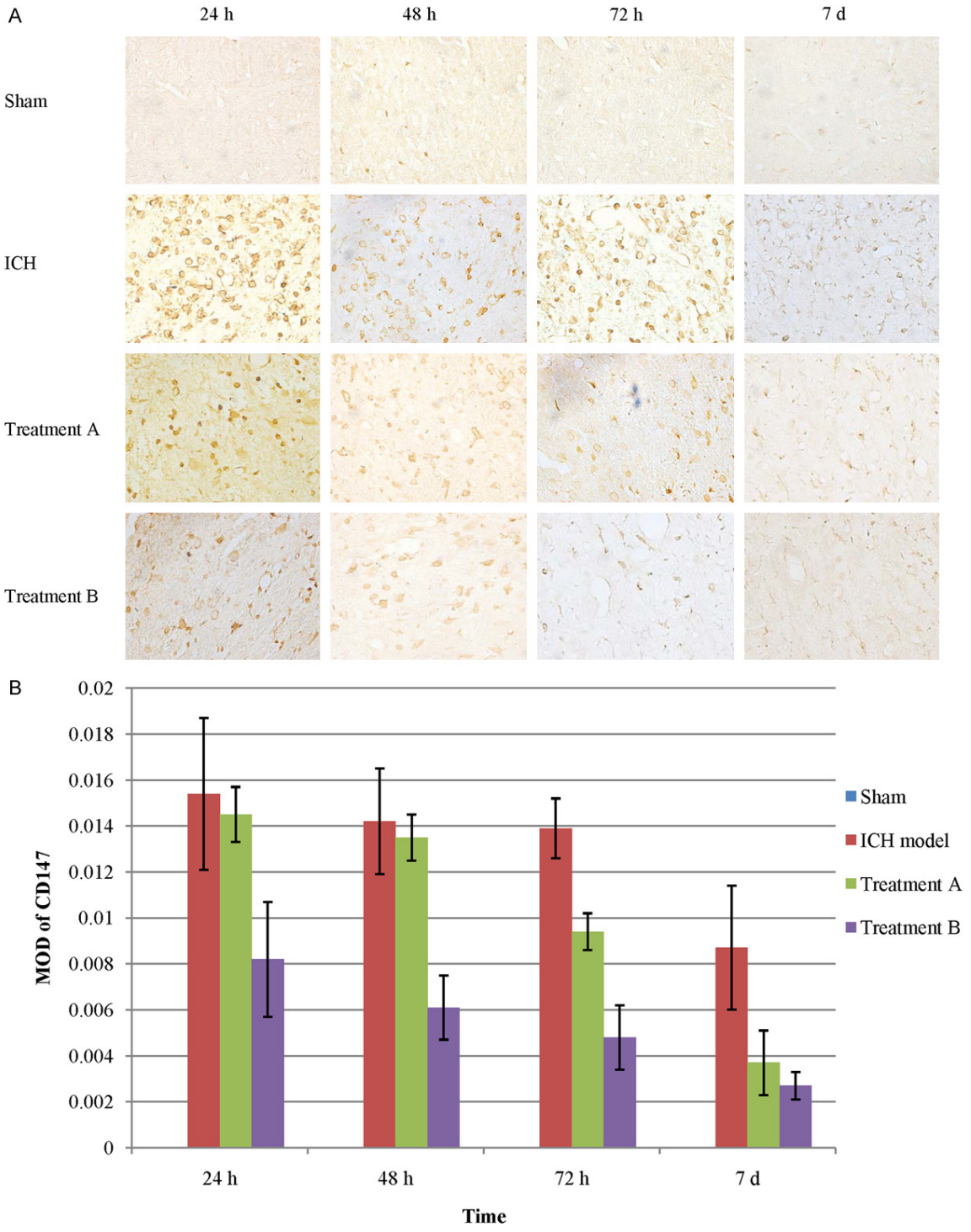

Figure 1. Treatment with atorvastatin was associated with reduced CD147 expression, CD147 immunohistochemistry (× 400). A. Representative slides stained for CD147 from each group at the site of ICH induction at different time points. B. CD147 expression level represented by MOD. CD147 level in the sham group (n = 5 at each time point) was undetectable, and significantly increased in the ICH model group (n = 5 at each time point). Treatment with 2 mg/kg atorvastatin (treatment A, n = 5 at each time point) and treatment with 10 mg/kg atorvastatin (treatment B, n = 5 at each time point) significantly reduced the expression of CD147 ($p < 0.01$, treatment groups *vs* ICH group, one-way ANOVA). Further, treatment group B showed significantly lower CD147 expression compared with treatment group A.

Pro Plus 6.0. Hemotoxylin and eosin (HE) stain was used to assess brain tissue damage. From each animal, three slices were selected for analyses, and each slice had been selected for three complete and non-overlapping views.

Modified neurological severity score

Modified neurological severity score (mNSS) was used to evaluate neurological functions of the rats. The mNSS evaluates the neurological functions on a 0-18 scale (0 being normal and 18 the most impaired) with a composite of motor, sensory, beam balance, and reflex and unusual behavior tests. Briefly, motor tests included raising the animals by the tails and observing the presence of flexion of forelimb (one point) and hind limbs (one point), head movements of greater than ten degrees (one point), and placing the animals on the ground to observe their walking behavior (zero point for normal walk, one for inability to walk straight, two for circling while walking, and three for falling when walking). Sensory tests included visual and tactile tests (one point for ablepsia and anaphia) and a proprioceptive test (one point for loss of deep sensations). The beam balance test assesses the ability of the animals to balance on a beam on a 0 to 6 scale (0, balanced with steady posture; 1, balanced by holding the beam; 2, a limb fell while holding the beam; 3, two limbs fell or spun, but stayed on the beam for longer than 60 s; 4, attempted to balance but fell after 40 s; 5, attempted to balance but fell within 20-40 s; 6, attempted to balance but fell in less than 20 s). In the reflex and usual behavior test, the animals were given one point for deficiency in ear reflex, one point for deficiency in corneal reflex, one point for deficiency in startle reflex, and one point for epileptic seizures, myoclonus and dystonia.

Statistics

All data were expressed as mean ± standard deviations (SD). Statistical analyses (one-way ANOVA) were performed with SPSS 20.0, and a *p* value of less than 0.05 was considered significant.

Results

The effect of atorvastatin on CD147 expression

We first examined the effect of atorvastatin on the expression of CD147 following ICH induction. CD147 was stained a yellow or light brown color in the slices, and the expression level of CD147 was estimated by measurements of MOD at 24, 48, 72 h, and seven days after ICH induction. CD147 expression was negligible at all time points assessed in the sham surgery group (injected with saline, n = 5 for each time point), and CD147 levels were undetectable in the control group (no procedure performed, n = 5 for each time point). In contrast, CD147 expression was typically found in glias and inflammatory cells in the perihematoma area in the ICH model group (n = 5 for each time point). CD147 expression level was the highest at 24 h after ICH induction, but decreased progressively with time. However, the CD147 expression levels remained significantly higher in the ICH model group compared with the sham surgery group ($p < 0.01$ at all time points). At seven days after ICH induction, we were still able to detect high levels of CD147 in neutrophils and macrophages in the perihemotoma area.

Treatment group A (2 mg/kg of atovasatatin daily, n = 5 for each time point) exhibited a similar pattern in CD147 expression as the ICH model group, such that the level of CD147 was highest at 24 h after ICH induction and decreased progressively over time, but remained significantly higher compared with the sham surgery group at all time points ($p < 0.05$). At 72 h and seven days after ICH induction, the CD147 expression levels were significantly lower compared with the ICH model group ($p < 0.05$). Treatment group B (n =5 for each time point) exhibited decreased levels of CD147 compared with the ICH group, that is, CD147 levels were significantly lower compared with the ICH group at all time points ($p < 0.01$), although still significantly higher than those in the sham surgery group ($p < 0.01$). Furthermore, CD147 expression was significantly lower in treatment group B than that in the treatment group A at all time points ($p < 0.01$). Thus, treatment with atorvastatin dose-dependently decreased CD147 expression after ICH induction (**Figure 1**).

The effect of atorvastatin on MMP-3 expression

Since CD147's effects are associated with MMPs activation, we next measured MMP-3 expression by MOD. MMP-3 expression was negligible in the control and sham surgery

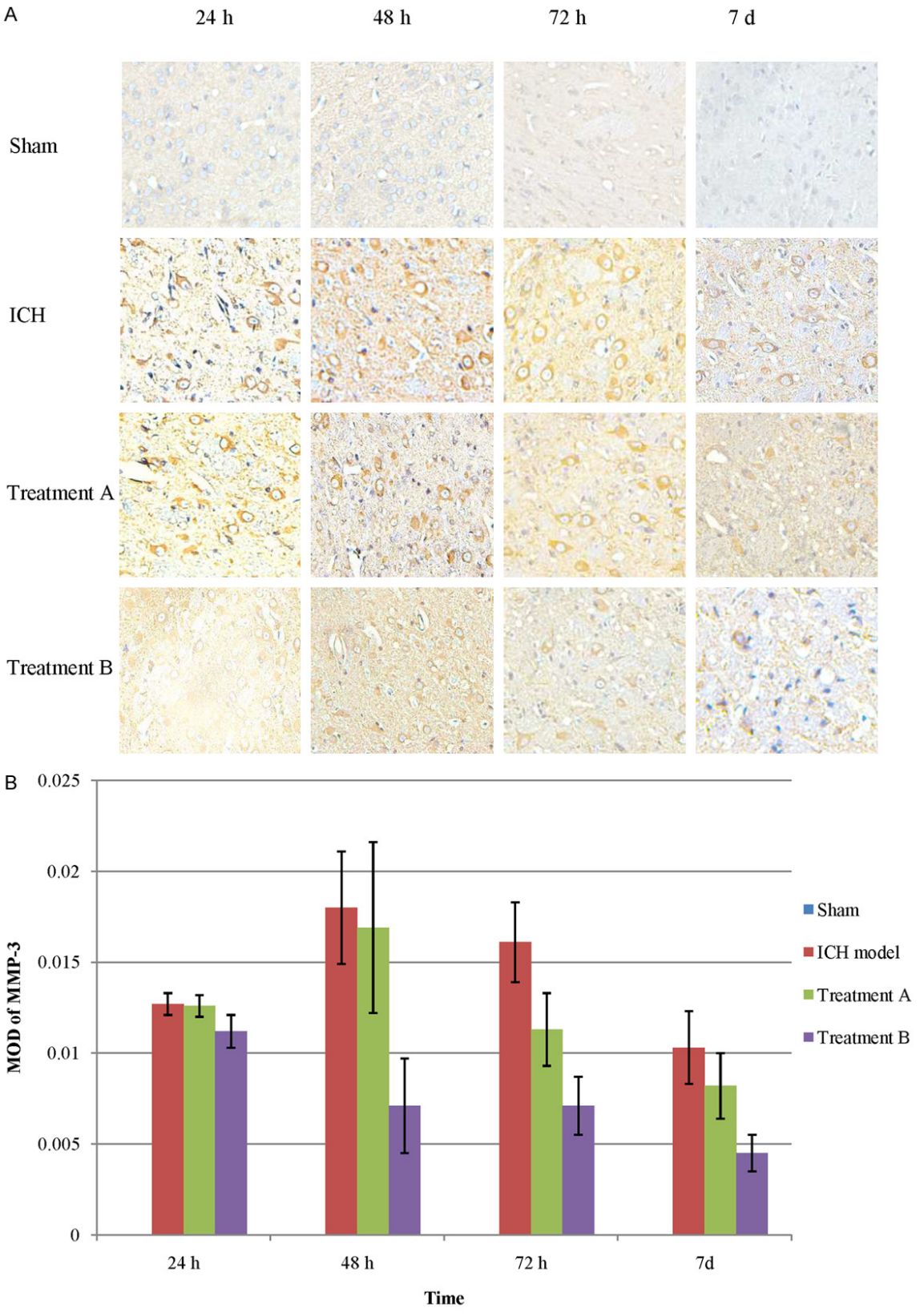

Figure 2. Treatment with atorvastatin was associated with reduced MMP3 expression, MMP-3 immunohistochemistry (× 400). A. Representative slides stained for MMP3 from each group at the site of ICH induction at different time points. B. MMP3 expression level represented by MOD. CD147 level in the sham group (n = 5 at each time point)

was undetectable, and significantly increased in the ICH model group (n = 5 at each time point). Treatment with 2 mg/kg atorvastatin (treatment A, n = 5 at each time point) and treatment with 10 mg/kg atorvastatin (treatment B, n = 5 at each time point) significantly reduced the expression of MMP3 ($p < 0.01$, treatment groups *vs* ICH group, one-way ANOVA). Further, treatment group B showed significantly lower MMP3 expression compared with treatment group A.

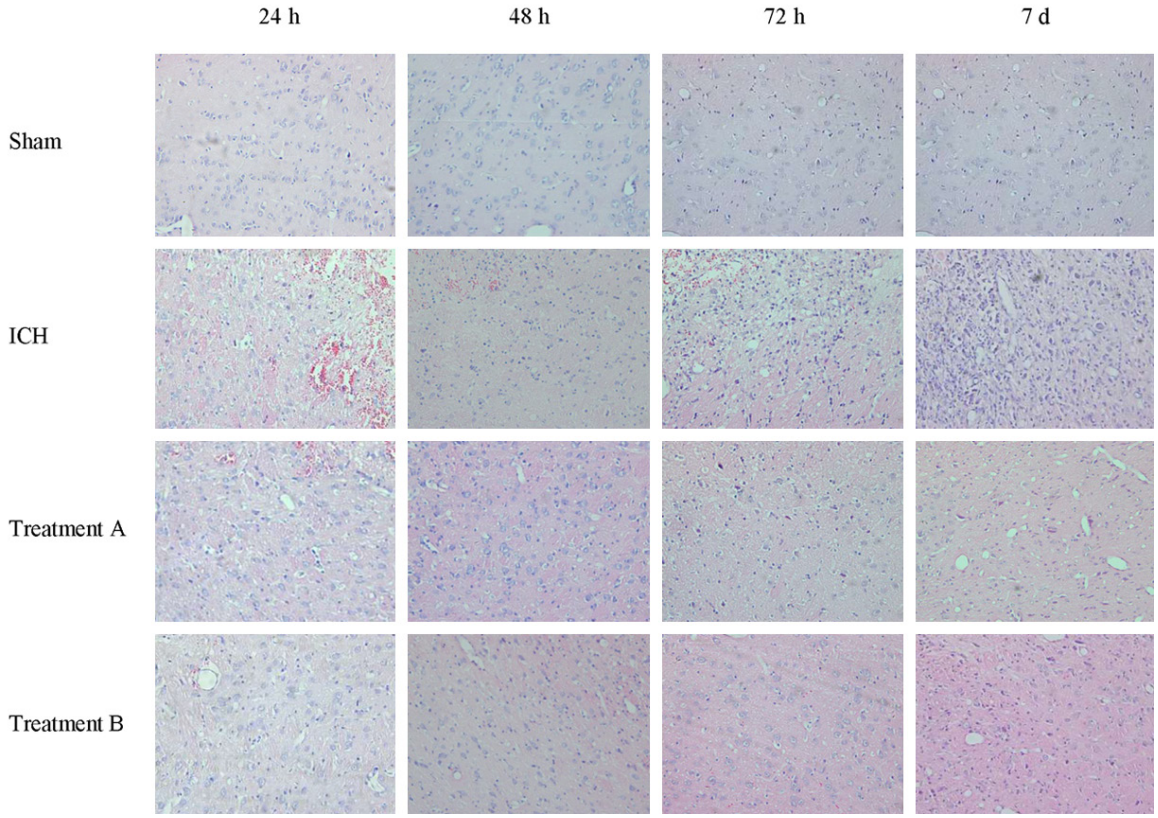

Figure 3. Atorvastatin-treated groups exhibited less tissue damage, HE staining (× 200). ICH induction was associated with tissue damage. Treatment with atorvastatin at both dosages ameliorated tissue damage.

groups. In the ICH model group, MMP-3 was detected in damaged neurons, glias, and inflammatory cells in the pereihematoma area; some MMP-3 was also detected in the extracellular matrix. A substantial amount of MMP-3 was detected at 24 h. MMP-3 expression peaked at 48 h after ICH induction and then progressively decreased. However, MMP-3 expression in the ICH model group remained significantly higher than that in the sham surgery group at all time points ($p < 0.01$).

Treatment group A exhibited a similar MMP-3 expression pattern as the ICH model group. MMP-3 expression was significantly increased compared with the sham surgery group at all time points. MMP-3 was detected at a relatively high level at 24 h and peaked at 48 h after ICH induction. The expression level decreased progressively, although it remained significantly higher than that in the sham surgery group. Furthermore, MMP-3 expression levels at 72 h and seven days were significantly decreased compared with the ICH model group ($p < 0.05$). In contrast, MMP-3 levels in treatment group B were the highest 24 h after ICH induction and progressively decreased over time, although the levels remained significantly higher than that in the sham surgery group ($p < 0.05$). Furthermore, MMP-3 levels were significantly lower than both the ICH model group ($p < 0.01$) and treatment group A ($p < 0.01$) at all time points (**Figure 2**).

The effect of atorvastatin on histological outcomes after ICH

In order to evaluate morphological changes induced by ICH, we observed histological changes in the injected brain area by using HE

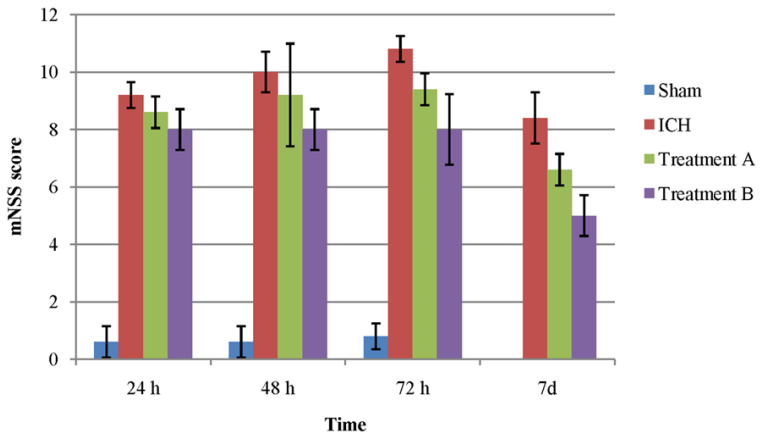

Figure 4. Atorvastatin-treated groups showed significantly improved neurological outcomes. Neurological functions were assessed by mNSS scores. The sham injection group exhibited near normal neurological functions, as indicated by very low mNSS scores. The ICH model group, in contrast, exhibited severely impaired neurological functions at all time points assessed. Treatment with 2 mg/kg atorvastatin attenuated the neurological impair, and 10 mg/kg atorvastatin exhibited even greater beneficial effects on the neurological outcomes as shown by significantly decreased mNSS scores.

staining. In the ICH model group, hematoma and accumulation of red blood cells were observed at 24, 48, and 72 h after ICH induction. Furthermore, the brain tissue in the perihematoma area showed signs of disintegration that were characterized by low cell density. In addition, edema and inflammatory cell infiltration were also observed. However, seven days after ICH induction, the hematoma was resolved and few red blood cells were found in the perihematoma area; edema was not observed, while new glias growth was observed. In addition, neutrophils decreased significantly, whereas hemosiderin-containing macrophages increased in the area, indicating ongoing phagocytosis of the red blood cells. Atorvastatin at both dosages ameliorated the morphological changes (reduced hematoma and edema, decreased accumulation of red blood cells and inflammatory cell infiltration, and diminished severe nervous tissue damage) associated with ICH (**Figure 3**).

The effect of atorvastatin on neurological outcomes after ICH

Neurological functions in all groups were assessed with mNSS scale, which rated function on a 0-18 scale, with 0 for normal and 18 for the most impaired. The sham surgery group scored closely to the normal level at all time points, showing that the surgery itself did not impair neurological function. The ICH group exhibited significantly impaired neurological function (substantially reduced and limited activities, extremely slow response to stimuli), and the mNSS scores were the highest at 48 and 72 h after ICH. The mNSS score decreased somewhat but remained significantly higher than that in the sham surgery group at seven days after ICH induction ($p < 0.05$). Treatment group A also exhibited significantly impaired neurological functions and scored significantly higher on the mNSS scale than that in the sham surgery group. However, at 72 h and seven days after ICH induction, the mNSS score was significantly reduced compared with the ICH group ($p < 0.05$). Treatment group B also exhibited significantly impaired functions; however, the mNSS score in group B was significantly decreased compared with both the ICH group ($p < 0.01$) and treatment group A ($p < 0.05$). Furthermore, treatment group B showed recovery, as evidenced by the decrease in the mNSS score over time (**Figure 4**).

Discussion

In this study, we used an animal model to examine the effects of atorvastatin on ICH. We found that treatment with atorvastatin exerted neuroprotective effects in a dose-dependent manner. Histological and neurological outcomes significantly improved in the atorvastatin-treated groups. Since tissue damage and neuronal loss often result from inflammatory responses secondary to ICH, and CD147 and MMPs are critical signaling proteins in this process, we therefore hypothesized that the neurological improvements correlated with suppression of ICH-induced increase in these CD147 and MMP-3. Indeed, we found that atorvastatin dose-dependently reduced expression of CD147 and MMP-3 in our ICH animal model. Treatment with 2 mg/kg atorvastatin significantly decreased CD147 and MMP-3 levels at

72 h and seven days after ICH, whereas treatment with 10 mg/kg atorvastatin significantly reduced CD147 and MMP-3 levels as early as 24 h and all subsequent time points after ICH. CD147 activates MMPs, and therefore it is possible that the decreased MMP-3 level observed in this study was caused by a reduction in CD147. We found that CD147 levels peaked at 24 h after ICH while MMP-3 levels peaked 48 h after ICH, which suggests that CD147 plays a role in MMP-3 activation. In the atorvastatin-treated animals, however, CD147 and MMP-3 levels did not correlate in a linear fashion, suggesting that MMP-3 levels might not depend solely on CD147 activity. Nevertheless, a higher dosage of atorvastatin was associated with a greater reduction in both CD147 and MMP-3 levels. Furthermore, histological studies showed that treatment with atorvastatin reduced inflammatory cell infiltration, decreased tissue damage, and attenuated neuronal loss, while neurological functions assessed with mNSS tests showed that atorvastatin improved neurological outcomes. In addition, these favorable effects were more pronounced in animals treated with a higher dose of atorvastatin. Therefore, we propose that atorvastatin inhibits CD147 expression, which in turn suppresses MMP-3, resulting in an attenuated inflammatory response that is associated with better histological and neurological outcomes.

Our findings that atorvastatin improves neuronal survival and neurological outcomes after ICH are consistent with previous studies that found atorvastatin to be neuroprotective. Although most studies agree that atorvastatin likely protects the brain tissue after injury through its anti-inflammatory and antioxidant effects [15-19], the molecular mechanisms by which atorvastatin exerts these effects have not been elucidated. Our results pointed to the possibility that atorvastatin inhibits CD147, thus downregulating MMPs, the downstream effecters of CD147. Very few studies have reported the effects of statins on CD147 expression, and the results from these studies are inconsistent. While one study reports that simvastatin has no effect on CD147 levels [20], others report that atorvastatin and fluvastatin inhibit CD147 expression [21-23]. In our study, we found that atorvastatin treatment decreases CD147 expression in the brain after ICH. Thus, it is possible that different types of statins have varying physiological effects, and atorvastatin, in particular, inhibits CD147 expression.

Atorvastatin treatment has been reported to be associated with decreased levels of MMP-9 [24]. Here we reported that MMP-3 levels in atorvastatin-treated rats were lower than the levels in the untreated rats. As MMP-3 can activate MMP-9, these results implied that it is possible for atorvastatin to inhibit MMP-9 signaling via CD147. Of note, while a correlation between CD147 and MMP-3 levels was reported in tooth germs [25, 26], human uterine fibroblasts [27], peripheral blood monocytes [28], tumor cells [29, 30], and other tissues [31], few studies have reported the association of these two proteins in the brain. This study is one of the first reports that provides evidence for a correlation between CD147 expression and MMP-3 levels in the brain following ICH.

In addition to the CD147 signaling pathway, atorvastatin could affect other signaling pathways that ameliorate tissue damage secondary to ICH. Many studies report that atorvastatin improves neurological outcome after brain injury via Akt-dependent pathways [32-35], as well as modulating molecules that are responsible for immune response, such as tumor necrosis factor-α (TNF-α) and interleukins [36, 37]. Others have reported that atorvastatin upregulates brain derived neurotrophic factor (BDNF) [38], preserves the integrity of the blood brain barrier [39], blocks the mitochondrial permeability transition pore [40], and employs the GluN2B-dependent signaling pathway [41] to exert its neuroprotective effects. Thus, it is likely that the neuroprotective effects of atorvastatin are a combination of multiple signaling pathways.

The main limitation of this study is that the molecular signaling analysis was solely based on animal studies, and interactions between molecules were not assessed directly. Even though we were able to describe a correlation between CD147 and MMP-3 levels in an *in vivo* ICH model, the exact molecular mechanisms were not fully investigated. Further studies are required to confirm a causal relationship between CD147 expression and increased MMP-3 levels. In addition, the animals were assessed 6 hr after surgery, which is considered a short recovery period for the anesthetic

used in the study. Therefore, our neurological assessments could have been complicated by the remaining effects of anesthetics, and the true severity of ICH might have been obscured by including animals that did not meet ICH criteria. In addition, although hematoma size correlates with colleganase dosage [42], colleganase itself can cause tissue damage and bleeding, which might not mimic ICH in clinical settings.

Moreover, as accumulating studies described atorvastatin as neuroprotective in animal models, further clinical studies are necessary to explore atorvastatin's therapeutic effects.

Conclusion

Atorvastatin showed neuroprotective effects, and these effects were possibly mediated by reducing CD147 and MMP-3 levels, which contribute to ameliorate inflammatory response in the brain and protect against brain tissue damage secondary to ICH. The clinical implications of the finding will be further explored.

Disclosure of conflict of interest

None.

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