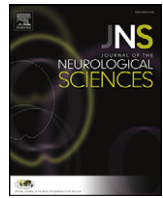




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## Effects of atorvastatin on the inflammation regulation and elimination of subdural hematoma in rats<sup>☆</sup>



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## ABSTRACT

**Background and purpose:** It is well known that inflammation influence chronic subdural hematoma (CSDH) formation to a large extent. Atorvastatin has pleiotropic effects on restraining inflammation and promoting angiogenesis besides its cholesterol-lowering function. Hence, atorvastatin may induce anti-inflammation effects and facilitate therapeutic effects for subdural hematoma (SDH).

**Methods:** Adult male Wistar rats were subjected to SDH and successful establishment of SDH was confirmed by magnetic resonance imaging (MRI). The treatment was initiated 6 hours after SDH induction. For the treatment, rats suffering SDH were randomly divided into saline group (the control group, rats were treated by saline,  $n = 29$ ) and atorvastatin group (rats were treated by atorvastatin, 3 mg/kg/day,  $n = 30$ ). The volume of lesion before treatment as well as on day 2 and day 7 after initial treatment was measured by MRI, respectively. The behaviors before SDH induction and on the days 1, 3, 5 and 7 after the initial treatment were dynamically evaluated. Gene expression, cytokine secretion and the number of neutrophilic granulocyte and vascular density were measured in both neomembrane and SDH lesion on the day 2 and day 7 after the initial treatment.

**Results:** It was found that the SDH rats treated by atorvastatin had a better behavior recovery compared to the ones treated by saline ( $p < 0.05$ ). By virtue of MRI scanning, it was revealed that SDH volumes were eliminated at a high speed by administration of atorvastatin than that of saline. With the help of the microscopic examination in the neomembrane, it was detected that the density of CD31 + neovasculars in the atorvastatin group was significantly higher than that in the saline group and the number of neutrophilic granulocyte in the atorvastatin group is less than that in the saline group. In comparison with saline treatment, the atorvastatin treatment did not change IL-10 expression and secretion, but it significantly decreased TNF- $\alpha$  and IL-6 level as well as VEGF gene expression. **Conclusions:** Atorvastatin treatment may eliminate SDH and improve the neural function of the rats through its anti-inflammatory effects. Hence, it indicated that statin induced inflammatory modulation might play a significant role in rats' SDH elimination and the functional recovery.

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### 1. Introduction

Generally, subdural hematoma (SDH) could be classified into acute, sub-acute and chronic subtypes based on the time between injury and appearance of hematomas. Acute SDH (ASDH) refers to the hematomas which form within 3 days after injury, while chronic SDH (CSDH) is considered to be the hematomas generated 3 weeks after injury. Mostly, acute traumatic SDH, whose mortality reaches up to 50%, are treated by an emergency operation [1–3]. However, CSDH is mainly found in the aged and resulted from a cephalic trauma or indirect head injury. Up to now, detailed mechanisms of CSDH have not been clearly clarified and therapeutics for CSDH have been controversial [4]. Some studies

suggest that CSDH is caused by an increasing localized inflammatory reaction [5–7]. Recent reports have revealed that inflammatory factors in hematoma fluid of CSDH patients are highly elevated [8]. It was shown that administration of dexamethasone which acts as a potent anti-inflammatory drug [9] successfully cures CSDH, the hypothesis that localized chronic inflammatory reaction participates in formation of CSDH is reasonable. However, it is worth mentioning that dexamethasone has many side effects including insomnia, increased weight, agitation, acne, thrush, nausea, vomiting and so on, which influence its clinical use [10].

As cholesterol-lowering drugs, statins and HMG-CoA reductase inhibitors are widely used to prevent and treat coronary artery disease [11]. It should be noted that statins are pleiotropic medicines which induce angiogenesis and have anti-oxidation and inflammatory modulation effects. A series of *in vitro* and *in vivo* experiments have confirmed that statins have ability to suppress inflammation, which is independent of their potent function on blood lipid reduction [12–14]. To verify that inflammation involved in the formation and elimination of CSDH, we studied effects of atorvastatin on regulating inflammatory cytokines inside SDH and changes in the volumes of SDH based on the rat model of SDH established previously [15].

## 2. Materials and methods

### 2.1. Animal model

Eighty adult male Wistar rats (350–400 g, obtained from Military Medical Academy of China) were housed in animal facility of Tianjin Medical University General Hospital. All animal procedures were performed in accordance with the institutional animal care guidelines approved by Chinese Small Animal Protection Association.

#### 2.1.1. SDH models

The rat models of SDH were established according to the procedures which had been published before with a little modification [15]. The detailed procedures were shown as follows. First of all, to expose its coronal and sagittal suture, the rat was positioned in a stereotaxic frame (Stoelting, Wood Dale, IL) after satisfactorily intraperitoneal anesthetization with 10% of chloral hydrate solution. Then, a small sphenoid burr hole (0.9 mm in diameter) was performed by using a sphenoid drill on the left coronal suture whose lateral distance to the sagittal suture was 3 mm. Next, endocranium of the rat was scuffed with a small hooked needle (with a diameter of 0.3 mm) under the microscope. Then, 300  $\mu$ l autologous venous blood collected from angular vein of the rat was injected into its subdural space at a rate of 50 ml/min by using a 20-gauge Venflon catheter with a tapered tip (BD Venflon, Helsingborg, Sweden).

#### 2.1.2. Magnetic resonance imaging (MRI) and criteria for selection

Two hours after SDH induction, the magnetic resonance imaging (MRI) (GE3.0 T) was applied to demonstrate whether SDH was successfully established or not. Meanwhile, volume of the lesion was measured. The rat was fixed on the coil (3 T, Shanghai Chengguang Technology Co. Ltd CG-MUC19-H300-AG) and positioned into the MRI (GE3.0 T). MRI session started with T2WI along the coronal view of the rat's head without contrast injection, with 1-mm slice thickness. Inclusion and exclusion of SDH rats were based on results of MRI scanning. Specifically, with respect to the exclusion of SDH model, rats that failed in induction of the SDH, SDH rats which had cortex contusion or laceration as well as the ones suffered severe edema around the hematoma were excluded. In typical SDH models, hypointensity hematoma and cortex ischemia areas could be observed between skull and cortex in the prophase after the induction of SDH models. However, cortex contusion, laceration and severe edema around the hematoma could not be observed. In this study, we successfully established 59 out of 80 SDH rat models, with a successful rate of 73.75%.

### 2.1.3. Experimental groups

SDH rats were randomly divided into 2 groups. In detail, 30 rats were included in the atorvastatin group (3 mg/kg/day, Pfizer, USA) and 29 in the saline group (the control group). The therapy was carried out through intragastric tubes 6 hours after the induction of SDH and was daily continued in a 7-day period. Neurological functional outcome was detected dynamically on the 1st, 3rd, 5th and 7th days after the initial treatment. The change in the volumes of hematoma was assayed on the 2nd and 7th days after the treatment by using MRI and calculated via the Volume Viewer 4.3 (GE Healthcare, USA).

## 2.2. Histological assessment and tissue extraction

Rats were sacrificed on the 2nd ( $n = 4/\text{group}$ ) and 7th ( $n = 4/\text{group}$ ) days after the treatment. The rats were perfused by 0.9% NaCl and then euthanized.

### 2.2.1. Brain tissue dissection

The cranium (except for the basilar region) was removed to expose the whole brain. In order to delineate the surface of the hematoma, this procedure was imaged by a high-resolution camera. Along an artificial line that was 3 mm posterior to the burr hole and perpendicular to the sagittal suture, the whole brain including basal bone was divided into two parts which were approximately equal. Initial procedures of tissue extraction for each certain assessment were the same.

### 2.2.2. Hematoxylin and eosin (H&E) staining

Firstly, the dura was fixed in place by inserting 3 stylets of 25G needles into the cortex before the brain was immersed in 4% paraformaldehyde for 48 hours at room temperature since it was critical to keep the intact dura in place over a hematoma for subsequent histological examination. Then, the brain tissue was selected and cut into a 10-mm block. Next, the block was dehydrated with a graded ethanol series, cleared in dimethylbenzene and then embedded in paraffin. Meanwhile, the 10-mm coronal block was further cut into 0.5 mm sections where H&E staining was performed according to standard procedures.

## 2.3. Immunohistochemical staining and quantification

The brain tissue blocks were embedded in OCT medium (Sakura Finetek USA, Inc.) and then cut into 10  $\mu$ m sections displayed in slides. In consideration of immunohistochemical staining, the slides were first treated by 0.3%  $\text{H}_2\text{O}_2$  (20 min at room temperature) to quench endogenous peroxidase activity. Next, they were incubated with 5% bovine serum albumin to block nonspecific staining (30 min at room temperature) and then incubated overnight at 4 °C with a mouse monoclonal antibody against CD31 (an endothelial cell marker, 1/200 dilution, Abcam ab64543). At the same time, the treated slides were refrigerated at 4 °C overnight, which was followed by 30-min incubation with HRP-conjugated streptavidin (Zymed Laboratories, South San Francisco, CA) at 37 °C. The antibody binding was visualized by 3,3'-diaminobenzidine tetrahydrochloride, and the slides were counterstained with the help of Mayer's hematoxylin. Similarly, negative controls were processed without the primary antibody.

In addition, brain paraffin blocks were also obtained and then cut into 6  $\mu$ m sections displayed in slides. After deparaffinization and rehydration, nonspecific endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide in methanol for 30 min. Antigen was recovered by boiling the sections for 10 min in 10 mM citrate buffer (pH 6.0). Nonspecific binding was blocked with 1% non-immune serum in PBS for 30 min. The sections were then incubated with rabbit polyclonal antibody against myeloperoxidase (1/100 dilution, Abcam ab9535) overnight at 4 °C. They were then washed with PBS, incubated with a biotinylated anti-rabbit IgG (1:100, Santa Cruz Biotechnology) for 2 hours at 37 °C, then washed and incubated with an avidin peroxidase conjugate solution (1:100, Santa Cruz

Biotechnology) for 1 hour. Finally, the sections were developed with diaminobenzidine for 5 min. Negative controls were similarly processed, but without the primary antibody.

In accordance with the previous method [15], CD31+ cells and neutrophilic granulocytes were counted. Briefly, five areas (four corners and one central area) on each slide were chosen to count high density of CD31+ (an endothelial cell marker) staining and neutrophilic granulocytes under 100× magnification. In detail, endothelial cells or their clusters that were stained positive for CD31 as well as neutrophilic granulocytes were counted under 400× magnification by two independent investigators who were blinded to the treatment of the animals in each chosen area.

#### 2.4. Sample collection for real-time PCR and ELISA assay

Brain tissue with intact dura and hematoma was preserved in a freezer at  $-80^{\circ}\text{C}$  for total RNA and protein extraction. The front part of brain tissue with intact dura and hematoma preserved in the freezer where the temperature was  $-80^{\circ}\text{C}$  was processed for PCR or ELISA assay.

#### 2.5. RT-PCR

The total RNA was isolated by using a Tissue Total RNA Extraction Reagent (GenePharma, Hi-Tech Park, Shanghai, China) in accordance with the manufacture's direction. A real-time polymerase-chase reaction (RT-PCR) was performed under the condition that RT-PCR Kit (GenePharma, Hi-Tech Park, Shanghai, China) was utilized. ACTB was used as an internal control. For primers, they included TNF- $\alpha$ : 5'-CACCACGCTCTTCTGTACTG-3' and 5'-GCTACGGGCTTGTCACTCG-3'; IL-6: 5'-TGGAGTTCCGTTTCTACTCG-3' and 5'-AATGACTCTGGCTTTGTCTTCT-3'; IL-10: 5'-AGGCAGTGGAGCAGGTGAA-3' and 5'-ACGTAGGCTTCTATGCAGTTGA-3'; VEGF: 5'-GAGCAGGAGCCGAAGCC-3' and 5'-GAGCCAGAAGTTGGACGA-3'; ACTB: 5'-GAAGTACCCATTGAACACGG-3' and 5'-TGGGTATCTTTTACCGGTTG-3'. The condition under which PCR was implemented was that the temperature was  $95^{\circ}\text{C}$  and duration was 3 min followed by 40 cycles at  $95^{\circ}\text{C}$  for 15 seconds and  $62^{\circ}\text{C}$  for 40 seconds. We performed three independent measurements on six samples for each group.

#### 2.6. ELISA assay for inflammation-related cytokines, the blood counting, clotting functions and lipid assay

Three milliliters of blood was collected from angular vein of each SDH rat on the 2nd ( $n = 8/\text{group}$ ) and 7th ( $n = 8/\text{group}$ ) days after the initial treatment. Then, 1 ml of the blood was stored in a coagulant tube and its serum was transferred into freezing tubes and kept at  $-80^{\circ}\text{C}$  after centrifugation at 3000 rpm for 10 min. At the same time, the left blood was stored in EDTAK2 anticoagulant tubes (0.5 ml) and sodium citrate anticoagulant tubes (1.5 ml), respectively. Next, they were sent to a clinical laboratory for blood counting (0.5 ml) and clotting functions assay (1.5 ml) by an automatic blood analyzer (XT-1800i Sysmex Corporation Japan) and a Thrombosis/Hemostasis Analyzer (CA-1500 Sysmex Corporation Japan), respectively. The pieces of the neomembrane, which were taken out from the brain and stored at  $-80^{\circ}\text{C}$ , were micro-ground. After being dissolved with 0.01 mol/L PBS in a geometric proportion and mixed thoroughly, they were centrifuged at 5000 rpm for 5 min to separate supernatant fluid. Then, the blood serum and the supernatant fluid of the SDH brain neomembrane were used to measure the cytokines related to angiogenesis (TNF- $\alpha$ , IL-6 and IL-10) according to the manufacture's directions and with the help of commercial ELISA kits (R&D Systems, USA). For ELISA, we performed three independent measurements on eight samples in each group. On the other hand, eight samples would be applied to the blood counting and clotting functions assay for each group.

By using the CardioChek P●A analyzer and total cholesterol and triglycerides check strips (Polymer 285 Technology System, Inc., Indianapolis, IN) according to the manufacturer's instructions, level of blood lipids was measured 7 days after the treatment in both groups. Fifteen microliters of blood was collected from vein of rats' tails. The data are presented by taking mg/dl as a unit.

#### 2.7. Neurological behavioral tests

Neurological Severity Scores (NSS) functional test was performed before induction of SDH models and repeated on the 1st, 3rd, 5th and 7th days after the initial treatment. Specific procedures were based on previous studies [16] and shown in Table 1. All testers were well trained and blinded to assignments of the experimental treatment.

#### 2.8. Statistical analysis

All data were analyzed by using SPSS (V16.0; SPSS, Inc., Chicago, IL) and expressed as means  $\pm$  SEM. To analyze the volume of the hematoma, MVD in the neomembrane, expression of factors related to neovascularization and inflammation in peripheral blood and neomembrane, as well as outcomes of behavioral tests, one-way analysis of variance (ANOVA) with a post hoc Bonferroni test was used. A paired Student's T test was adopted to analyze indexes at different time points in the same group. In addition, Pearson's correlation coefficient was utilized to examine the relationship between neomembrane neovascularization and the change in the volume of hematoma. A p value that was less than 0.05 was considered to be statistically significant.

### 3. Results

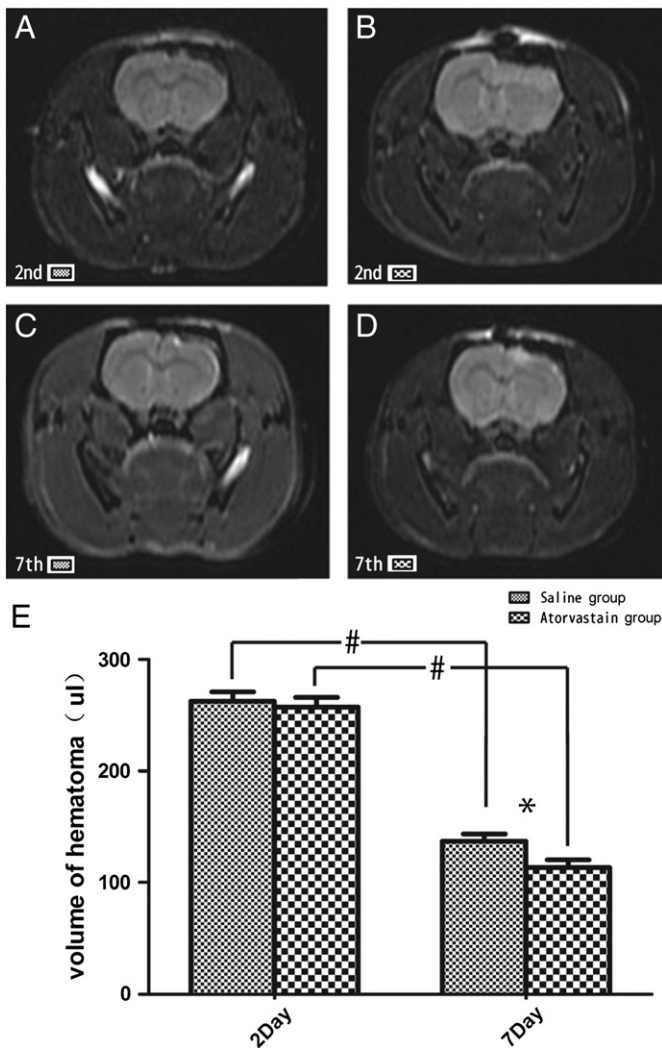
In typical SDH models, the hypointensity hematoma and cortex ischemia areas could be observed between the skull and cortex in the prophase after the induction of SDH models. As hematoma was absorbed gradually and neomembrane was formed, hyperintense areas circulating the hematoma appeared and the volume of the hematoma decreased as time went on (Fig. 1A–D).

**Table 1**  
Neurologic severity scores (NSS).

Motor test	points
<i>Raising the rat by the tail</i>	3
1 Flexion of forelimb	
1 Flexion of hindlimb	
1 Head moved more than $10^{\circ}$ to the vertical axis within 30 seconds	
<i>Walking on the floor (normal = 0; maximum = 3)</i>	3
0 Normal walk	
1 Inability to walk straight	
2 Circling toward the paretic side	
3 Falling down to the paretic side	
<i>Beam balance tests (normal = 0; maximum = 6)</i>	6
0 Balances with steady posture	
1 Grasps side of beam	
2 Hugs the beam and one limb falls down from the beam	
3 Hugs the beam and two limbs fall down from the beam, or spins on beam (>30 seconds)	
4 Attempts to balance on the beam but falls off (>20 seconds)	
5 Attempts to balance on the beam but falls off (>10 seconds)	
6 Falls off: No attempt to balance or hang on to the beam (<10 seconds)	
<i>Reflexes absence</i>	2
1 Pinna reflex (a head shake when touching the auditory meatus)	
1 Corneal reflex (an eye blink when lightly touching the cornea with cotton)	
<i>Maximum points</i>	14

We performed three independent measurements on eight samples per group.





**Fig. 1.** Images of MRI. The MRI images obtained on the 2nd and the 7th days after the saline treatment (A and C) and the atorvastatin treatment (B and D). "E" represents the changes in the volume of the hematomas between the saline group and the atorvastatin group. # indicates the change in the volume between the 2nd and 7th days after the initial treatment was markedly different ( $p < 0.001$ , Paired-Samples T Test). \* stands for a statistically significant difference between the two groups ( $p = 0.037$ , one-way ANOVA).

### 3.1. Atorvastatin induced an apparent shrinkage of the SDH

Fig. 1A–D showed MRI measurement 2 and 7 days after SDH in animals treated by saline (A and C) and atorvastatin (B and D). We found that a time-dependent decreasing tendency appeared in the volumes of hematomas in both groups. There was no significant difference between the two groups 2 days after the treatment ( $257.125 \pm 24.52 \mu\text{l}$  vs  $262.25 \pm 24.53 \mu\text{l}$ ,  $p > 0.05$ , one-way ANOVA). The volumes of the SDH animals treated by atorvastatin were significantly decreased 7 days after treatment ( $113.375 \pm 19.50 \mu\text{l}$ ) in comparison with SDH animals accepting treatment of saline ( $137 \pm 18.85 \mu\text{l}$ ,  $p < 0.05$ , Fig. 1E).

This indicated that the shrinkage of SDH in the rats was time dependent but a more significant shrinkage was induced by atorvastatin treatment.

### 3.2. Atorvastatin treatment improved neurological outcomes after SDH

On the one hand, all rats with SDH had severe unilateral sensorimotor deficits. On the other hand, a decreased tendency in the mNSS was

revealed after the treatment in both groups. However, atorvastatin induced a significant decrease in the mNSS on the 5th and 7th days after the treatment compared with the animals treated by saline, which indicated that the neurological function was apparently improved by atorvastatin treatment (Fig. 2).

### 3.3. Atorvastatin increased the microvessel density (MVD) of the neomembrane

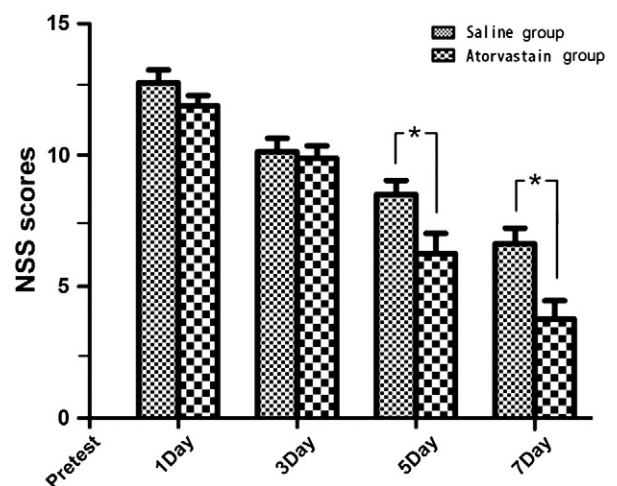
An increased MVD stained by CD31 was induced in the neomembrane of SDH in both groups. However, the increasing degree of the MVD was not significant between the two groups on the 2nd day after the treatment ( $10.63 \pm 1.13$  vs  $9.75 \pm 0.55$ ,  $p > 0.05$ , one-way ANOVA). On the 7th day after the treatment, an apparent increasing in the MVD in the neomembrane was observed in the atorvastatin group compared with the saline group ( $67.13 \pm 2.77$  vs  $51.13 \pm 1.66$ ,  $p < 0.05$ ,  $n = 8$ , one-way ANOVA) (Fig. 3F). On the 7th day after the treatment, it was confirmed that there was a significant association between the MVD in the neomembrane and the changes in volume of the hematoma in both groups. The coefficients were 0.731 in the saline group and the atorvastatin group ( $p < 0.05$ , Pearson's correlation coefficient). These suggested that the intensity of CD31 expression was positively related to absorption of the SDH. The atorvastatin induced more MVD marked by CD31 along with a significant shrinkage of the SDH.

### 3.4. Atorvastatin decreased the neutrophilic granulocytes in neomembrane

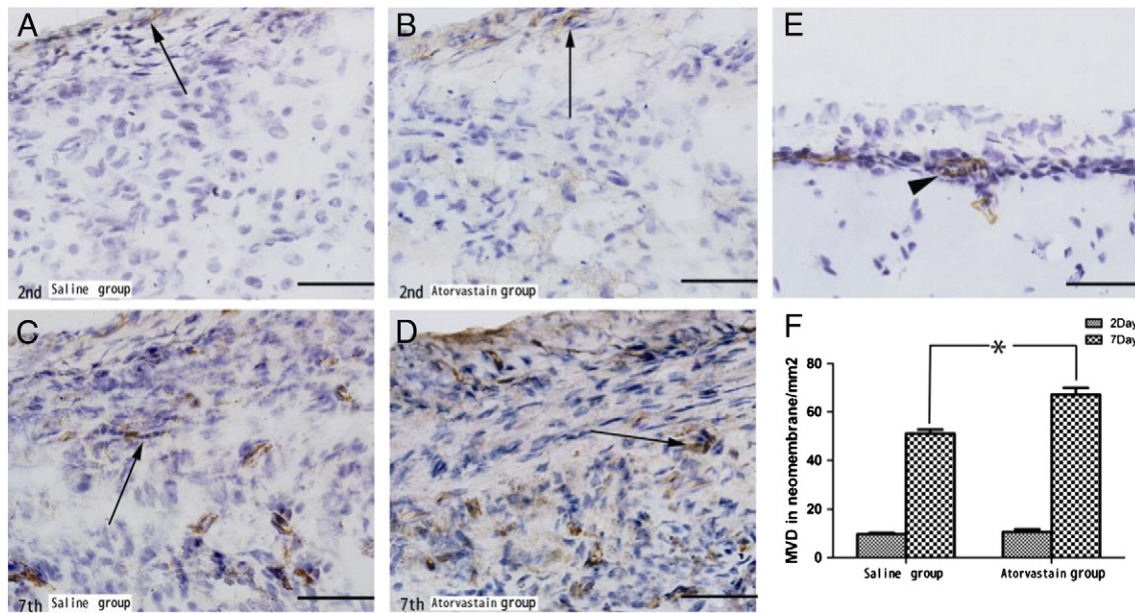
As progression of inflammation, increased neutrophilic granulocytes were induced in the neomembrane of SDH in both groups. However, on the 7th day after the treatment, an apparent decreasing in the number of neutrophilic granulocytes in the neomembrane was observed in the atorvastatin group compared with the saline group ( $56.5 \pm 1.76$  vs  $45.1 \pm 2.58$ ,  $p < 0.05$ ,  $n = 8$ , one-way ANOVA) (Fig. 4F). The atorvastatin decreases the number of neutrophilic granulocytes along with a significant shrinkage of the SDH.

### 3.5. Atorvastatin induced significant anti-inflammatory effects and decreased $\text{TNF-}\alpha$ and $\text{IL-6}$ cytokines in brain tissue after SDH instead of in the circulating blood

To generally observe the changes in hematomas and neomembrane, Fig. 5 showed there was a smaller amount of hematoma left in the



**Fig. 2.** Changes in Neurological Severity Scores (NSS). We performed three independent measurements on eight samples for each group. \* represents a statistically significant distinction between the two groups ( $p = 0.014$  and  $p = 0.003$  on the 5th and the 7th days after the treatment respectively, one-way ANOVA).

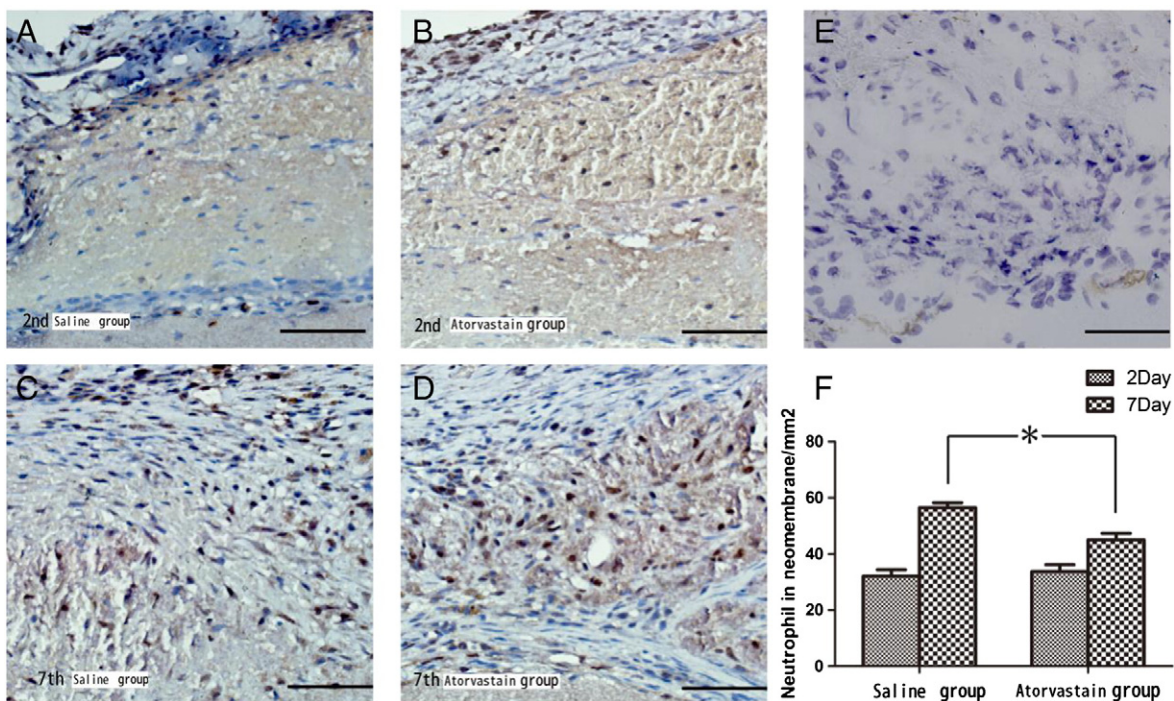


**Fig. 3.** MVD in the membrane of hematoma. The IH staining images of CD31 in the saline group (A and C) and the atorvastatin group (B and D) taken on the 2nd and the 7th days after the treatment. Arrows indicate the CD31 + cells. \* represents the statistically significant distinction between the two groups ( $p = 0.004$ , one-way ANOVA) and E refers to CD31 staining of the opposite side of trauma.

atorvastatin group or the saline group on the 7th day, but the neomembrane became thicker compared with that on the 2nd day.

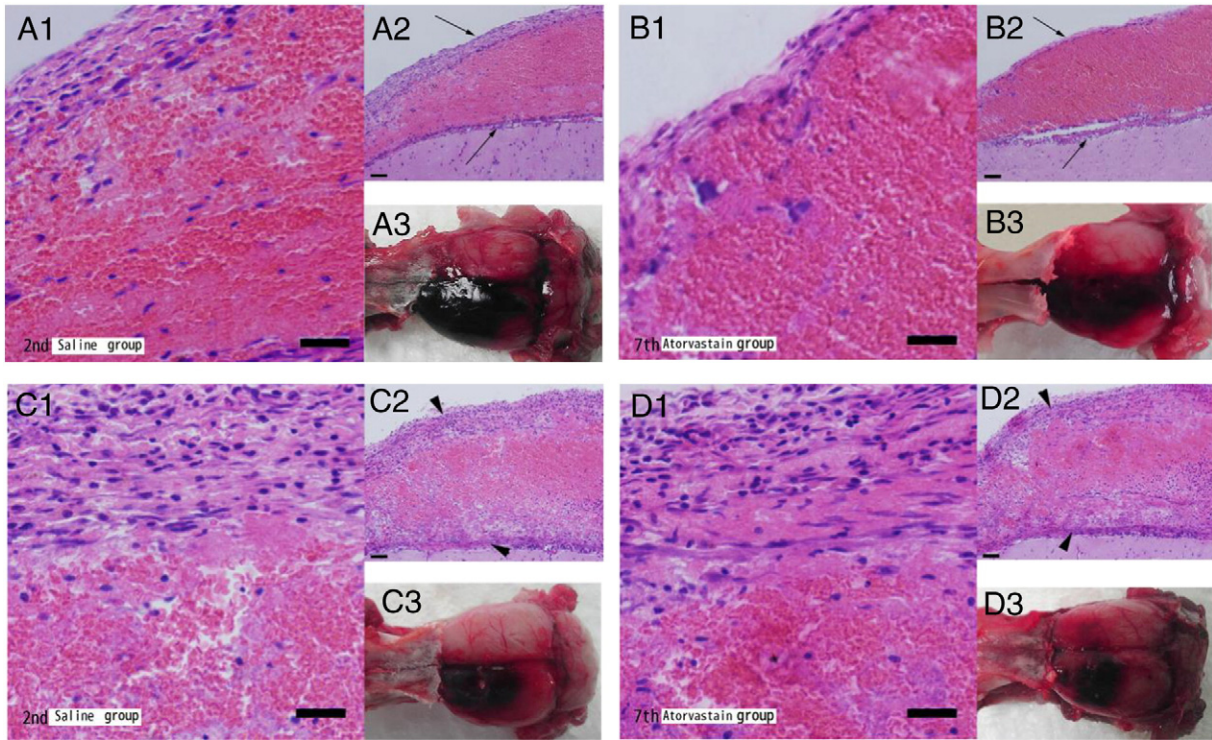
To test the underlying mechanisms which were related to the situation that atorvastatin induced decrease in the volume of hematoma after SDH, the inflammatory factors secretion (TNF- $\alpha$  and IL-6 and IL-10) were measured by ELISA assay. As a result, no notable change was detected in the level of the inflammation-related cytokines (TNF- $\alpha$ , IL-6 and IL-10) in the peripheral blood between the 2nd day and 7th day

after SDH in both groups. Furthermore, there was no diversity in these cytokines in the peripheral blood between the two groups either on the 2nd day or the 7th day after the treatment (Table 2). However, it was found the TNF- $\alpha$  and IL-6 level in the damaged brain tissue of neomembrane was significantly decreased on the 7th day, while the level of IL-10 of the brain neomembrane did not show significant difference between the two groups (Fig. 6). The results indicated that SDH contained several inflammation-related cytokines, and the atorvastatin



**Fig. 4.** Neutrophilic granulocytes in the membrane of hematoma. The IH staining images of neutrophilic granulocytes in the saline group (A and C) and the atorvastatin group (B and D) taken on the 2nd and the 7th days after the treatment. \* represents the statistically significant distinction between the two groups ( $p = 0.02$ , one-way ANOVA) and E refers to neutrophilic granulocytes staining of the opposite side of trauma.





**Fig. 5.** Morphological changes in subdural hematomas. The HE staining sections from the saline group (A1 and C1) and the atorvastatin group (B1 and D1) (400×). The bar represents 25 μm. The HE staining sections of the saline group (A2 and C2) and the atorvastatin group (B2 and D2) (200×). Arrows indicate the neomembrane obtained on the 2nd day after the treatment and arrows represent the neomembrane obtained at the 7th day post-treatment. The bar represents 50 μm. The images of the hematomas of the SDH rats in the saline group (A3 and C3) and the atorvastatin group (B3 and D3).

treatment induced a significant decrease in TNF-α and IL-6 but did not regulate the IL-10 expression in the brain neomembrane compared to the saline treatment.

**3.6. Atorvastatin induced the mRNA expression of the inflammation and angiogenesis-related cytokines in SDH**

To test whether atorvastatin regulated inflammatory factor gene expression, the total RNA was extracted from the neomembrane of the SDH on the 2nd day and the 7th day after the treatment. Then, mRNA abundances of the inflammation-related cytokines (TNF-α, IL-6 and IL-10) and the angiogenesis-related cytokines (VEGF) in these neomembrane were semi-quantitatively measured by using RT-PCR. In consequence, it was showed that the changes in the mRNA amount of all inflammatory cytokines were consistent with corresponding proteins measured by virtue of ELISA. Besides, it was indicated that the mRNA amount of TNF-α and IL-6 showed a descending tendency with

time in both groups and such a tendency was more obvious in the atorvastatin group. In addition, compared with the saline group, the atorvastatin group also had significantly decrease in TNF-α and IL-6 gene expression 7 days after the treatment ( $p < 0.05$ ,  $n = 6$ , one-way ANOVA). However, there was no significant difference in IL-10 gene expression between the two groups. Besides, the atorvastatin group also had significantly decrease in VEGF gene expression 7 days after the treatment ( $p < 0.05$ ,  $n = 6$ , one-way ANOVA), while the saline group had an adverse change (Fig. 6.).

**3.7. Atorvastatin did not change the blood counting, functions of blood clotting or blood lipids**

To observe effects of atorvastatin and inflammatory reaction of the peripheral blood, both the blood counting and functions of blood clotting were measured. On the basis of Table 3, it was revealed that the blood counting and functions of blood clotting did not significantly change in the SDH rats when the atorvastatin treatment was used compared with the saline treatment. In addition, the detected lowest level of the total cholesterol check strips was 100 mg/dl. We found that the total cholesterol level of both groups was less than 100 mg/dl. Triglyceride was measured 7 days after the treatment (atorvastatin treatment:  $72.1 \pm 4.3$  mg/dl; the saline group:  $76.8 \pm 4.1$  mg/dl). There was no significant difference in the total level of cholesterol and triglyceride between the two groups ( $p > 0.05$ , one-way ANOVA).

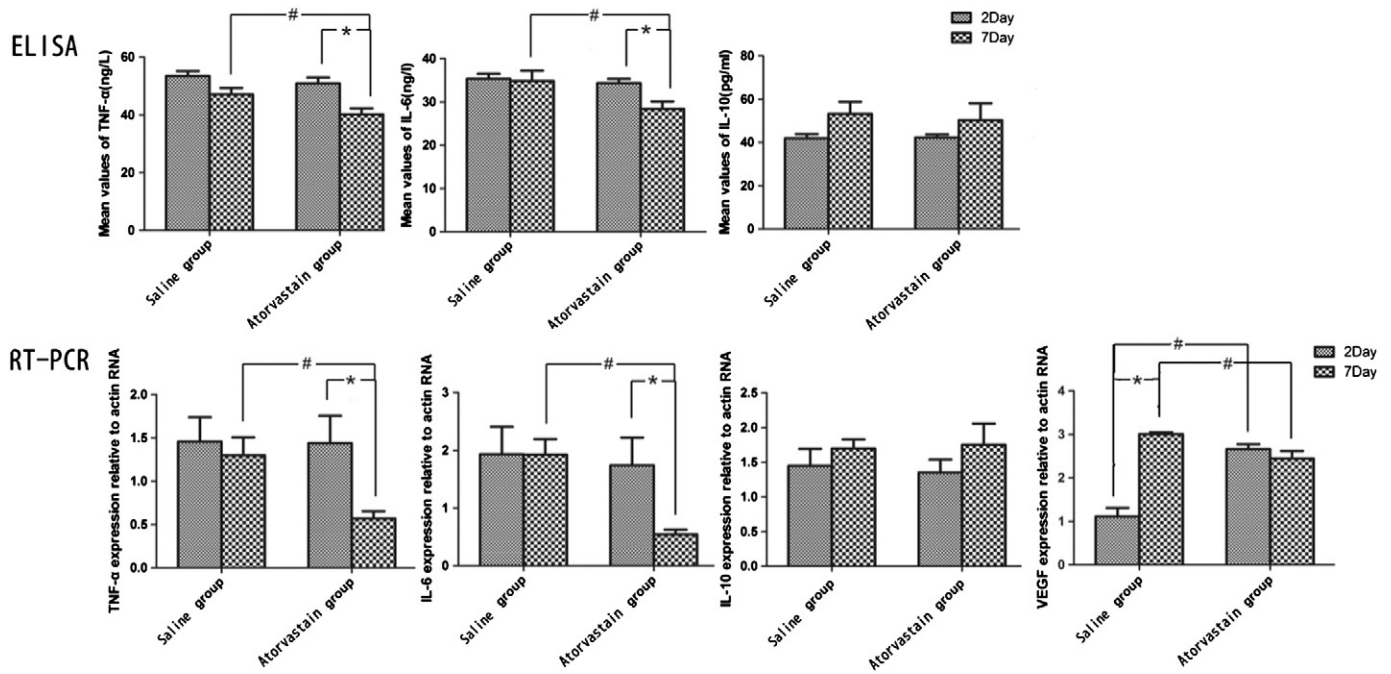
**Table 2**  
ELISA changes of factors in peripheral blood.

	Control group		Experimental group	
	2 day	7 day	2 day	7 day
TNF-α (ng/L)	36.8 ± 5.57	37.43 ± 4.82	34.97 ± 4.82	36.52 ± 2.49
IL-6 (ng/L)	21.98 ± 1.86	22.31 ± 2.32	23.18 ± 2.27	21.01 ± 1.71
IL-10 (pg/ml)	29.28 ± 4.81	29.61 ± 2.9	31.17 ± 4.15	30.56 ± 4.19

The levels of the cytokines in the peripheral blood of the SDH rats receiving atorvastatin or saline remained stable between the 2nd day and 7th day after treatment initiation ( $p > 0.05$ , one-way ANOVA). Furthermore, the different treatment did not induce a diversity change in the levels of the cytokines in the peripheral blood at the same time points ( $p > 0.05$  Paired-Samples T Test). We performed three independent measurements on eight samples per group. Eight samples were included in each group.

**4. Discussion**

The mechanisms of formation of CSDH have been explored for several decades, but the detailed mechanism still needs to be fully elucidated. Some researchers have advocated that CSDH is resulted from repeated bleeding of nascent capsule [17,18]. Furthermore, it has shown that there is an imbalanced regulation of plasminogen activator in hematoma cavity, which disturbs the fibrinolysis and thrombomodulin



**Fig. 6.** Expression of the inflammation cytokines and their mRNA detected by RT-PCR and ELISA. We performed three independent measurements on eight samples (ELISA) and six samples (PCR) for each group. # represents a statistically significant distinction in the same group at different days ( $p < 0.05$  Paired-Samples T Test). In addition, \* represents a statistically significant distinction between the two groups ( $p < 0.05$ , one-way ANOVA).

coagulation system [19–22]. Our previous study has revealed that the shrinkage speed of rats' SDH is associated with enhanced angiogenesis to a large extent [15]. Furthermore, in accordance with many clinical pathology studies, it can be found that rich and disordered vessels are developed in the neomembrane of hematomas and form a number of sinusoidal vessels with high permeability and fragility [8,23–26]. It seems that angiogenesis plays a key role in both the formation and elimination of CSDH. However, a high inflammatory reaction level has been detected in the liquid of the CSDH with apparent increase in TNF- $\alpha$  and IL-6 [8] and fluctuation of IL-10 [27]. The inflammatory reaction could change osmotic pressure of hematoma liquid, which has impacts on angiogenesis by modifying maturation of vessels [28]. Statins have been shown to have pleiotropic effects including anti-inflammation, neuroprotection and angiogenesis regulation [29–31]. Since we have performed researches on angiogenesis in SDH rats in another study (data not shown), this research focused on inspecting both the role of inflammatory cytokines in the formation and elimination of SDH and the impact of the statin on this effect.

According to our study, it was confirmed that there were plenty of inflammatory cytokines (TNF- $\alpha$ , IL-10, IL-6) in the SDH *in vivo* with a consistent change in both protein and mRNA levels, while there was not a notable change in inflammation in the circulating peripheral blood. At the same time, it was revealed that a significant shrinkage of the SDH induced by atorvastatin was accompanied by the level

modulation of these inflammatory cytokines and the decreasing number of neutrophilic granulocytes. Both the pro-inflammatory cytokine (TNF- $\alpha$ ) and the dual role cytokine (IL-6) were down-regulated by the atorvastatin treatment, while the anti-inflammatory cytokine (IL-10) maintained constant.

TNF- $\alpha$ , a cytokine produced by activated macrophages in response to pathogens and other injurious stimuli, was necessary and sufficient for mediation of local and systemic inflammation [32]. IL-6 was a potent pleiotropic Th2 cytokine that regulated immune defense response and played a dual role in pro-inflammatory and anti-inflammatory responses. Release of IL-6 was triggered by tissue damage or infection. IL-6 played a key role in the transition from an acute phase to a chronic one of the inflammatory process [33,34]. Though the function of IL-6 was a cytokine with a dual function, a significant increase in IL-6 has been confirmed in published clinical literatures [8,26] and significant atorvastatin-inducing inhibition of IL-6 was correlated with the increasing speed of hematoma shrinkage in SDH models. It indicated that TNF- $\alpha$  and IL-6 might play a pro-inflammatory role in the formation of CSDH, while the inhibition of TNF- $\alpha$  and IL-6 contributed to eliminating the hematoma. IL-10 not only acted as an anti-inflammatory cytokine released by monocytes and macrophages but also suppressed production of inflammatory cytokines by monocytes [35,36]. Increasing level of IL-10 was related to less recurrence of CSDH [8]. Although the level of IL-10 was not increased significantly by application of atorvastatin in our

**Table 3**

Routine analysis of blood and functions of blood clotting.

	Control group		Experimental group	
	2 day	7 day	2 day	7 day
Number of white cells ( $\times 10^9/L$ )	12.28 $\pm$ 0.53	12.83 $\pm$ 0.93	11.79 $\pm$ 1.15	10.67 $\pm$ 0.66
Number of red cells ( $\times 10^{12}/L$ )	7.04 $\pm$ 0.42	6.95 $\pm$ 0.28	7.32 $\pm$ 1.06	6.78 $\pm$ 0.31
Number of platelets ( $\times 10^9/L$ )	721.88 $\pm$ 39.18	765.87 $\pm$ 59.51	717.13 $\pm$ 42.31	651 $\pm$ 46.01
Prothrombin time (seconds)	10.63 $\pm$ 0.54	9.79 $\pm$ 0.44	9.49 $\pm$ 0.66	9.48 $\pm$ 0.38
Fibrinogen (g/L)	2.64 $\pm$ 0.31	2.48 $\pm$ 0.27	2.23 $\pm$ 0.27	1.88 $\pm$ 0.19

The blood counting and coagulation function of the SDH rats receiving atorvastatin or saline remained stable between the 2nd day and 7th day after treatment initiation ( $p > 0.05$ , one-way ANOVA). Furthermore, the different treatment did not induce a diversity change in these items in the blood at the same time points ( $p > 0.05$  Paired-Samples T Test). Eight samples were included in each group.



study, an increasing tendency was observed. It was anticipated that a significant change in IL-10 might be observed if the treatment was extended. By combining our data with known information, it was suggested that there was a correlation between the anti-inflammatory modulation induced by atorvastatin and the shrinkage of the SDH volume.

Actually, it has been known that modulation of inflammation is related to both the angiogenesis and the permeability of vessels. TNF- $\alpha$ , one of the most potent pro-inflammatory factors, regulated vascular endothelial cell permeability through formation of stress fiber and interruption of cellular junctions, which could be confirmed further by using transendothelial electrical resistance (TER) [37–40]. IL-6 promoted permeability of vessels by expanding endothelium interspace [41]. Since high permeability of vessels was detected in the neomembrane of CSDH [18,42–46], the correlation between the level change in both IL-6 and TNF- $\alpha$  and the formation and elimination of SDH strongly indicated that these cytokines played a critical role in repair of vascular permeability. However, to confirm this hypothesis, it will be essential to carry out a research in future.

Furthermore, it has been acknowledged that atorvastatin possesses dual functions, i.e., regulating inflammatory reaction and promoting angiogenesis. Matsumura has concluded that low doses of atorvastatin could enhance expression of VEGF in rats' ischemic hindlimbs [47]. In this study, the MVD marked by CD31 was enhanced by atorvastatin, which was also correlated with the change in TNF- $\alpha$  and IL-6. Consequently, the role the angiogenesis along with the inflammation played in the formation and elimination of SDH was apparent. However, it has been known that excessive expression of VEGF could not only lead to more blood vessels with high permeability [48] but also result in immature and unstable vessels, which would not benefit the absorption of hematomas [49]. In this study, it was observed that the gene of VEGF markedly increased and then showed a descendant tendency in the atorvastatin group. On the contrary, the gene of VEGF in saline group showed an ascent tendency. Then, we could make a hypothesis that the maturity of blood vessels would also participate in the absorption of hematoma. Meanwhile, a future research should be performed to confirm this hypothesis.

Our study was only designed to observe the change in inflammatory cytokines in the formation and absorption of SDH. The SDH model could not ideally mimic the chronic course of the CSDH. The reason for this is that the outcome had only been observed for 1 week. Thus, to confirm the role that the inflammation plays in CSDH, more detailed data and a modified SDH model should be provided in the future. Since there is a clear clue to imply the role of both angiogenesis and inflammation in CSDH, it is necessary to find out which one is the initial role and whether both of them work simultaneously in the same process. However, a therapeutic strategy based on atorvastatin for CSDH is potentially suggested.

## 5. Conclusion

In summary, based on SDH models *in vivo*, we confirmed that atorvastatin (3 mg/kg/day) could not only modulate the inflammatory cytokines but also promote the shrinkage of SDH as well as improve neural functions of the SDH rats. The therapeutic results of atorvastatin treatment might be partially ascribed to normalization of the inflammatory reaction and enhancement in potential angiogenesis.

## Conflict of interest

None.

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