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Laboratory Investigation

Prior Use of 3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A Reductase Inhibitor, Simvastatin Fails to Improve Outcome after Experimental Intracerebral Hemorrhage

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Objective : Contrary to some clinical belief, there were quite a few studies regarding animal models of intracerebral hemorrhage (ICH) *in vivo* suggesting that prior use of statins may improve outcome after ICH. This study reports the effect of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase inhibitor, simvastatin given before experimental ICH.

Methods : Fifty-one rats were subjected to collagenase-induced ICH, subdivided in 3 groups according to simvastatin treatment modality, and behavioral tests were done. Hematoma volume, brain water content and hemispheric atrophy were analyzed. Immunohistochemical staining for microglia (OX-42) and endothelial nitric oxide synthase (eNOS) was performed and caspase-3 activity was also measured.

Results : Pre-simvastatin therapy decreased inflammatory reaction and perihematomal cell death, but resulted in no significant reduction of brain edema and no eNOS expression in the perihematomal region. Finally, prior use of simvastatin showed less significant improvement of neurological outcome after experimental ICH when compared to post-simvastatin therapy.

Conclusion : The present study suggests that statins therapy after ICH improves neurological outcome, but prior use of statins before ICH might provide only histological improvement, providing no significant impact on neurological outcome against ICH.

Key Words : Inflammation · Intracerebral hemorrhage · Neuroprotection · Outcome · Rat · Statins.

INTRODUCTION

Intracerebral hemorrhage (ICH) is a lethal form of stroke with the mortality rate of 23 to 58%[23]. No effective surgery and neuroprotective drugs have been identified in this entity thus far. The initial mechanism by which tissue injury occurs after ICH includes mechanical destruction or displacement caused by the hematoma itself. Then, subsequent inflammation and impairment of blood flow around the blood clot contribute to delayed cell death[31]. The suppression of inflammation has been reported to reduce brain edema and tissue injury as well as improve functional outcome after experimental ICH[13,14,25].

Statins are structural analogs of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, a restrictive enzyme in the cholesterol synthesis. It has been proposed that pleiotropic properties mediate neuroprotective effects through endothelial function, immunomodulation, and impeding excitotoxic cell injury via anti-inflammatory actions and angiogenesis or neurogenesis[1,4,7].

Statins have been identified as modulators of lesion size and outcome in animal models of ischemic stroke[7,15], head injury[17], subarachnoid hemorrhage[19] and ICH[13,14,25]. Prior use of statins also improves neurological outcome in animal models of ischemic stroke[2,3]. Clinical studies regarding the effects of statins in patients with ICH have been limited and inconclusive, with some studies reporting protective effects[9,16,20,28] while others failing to find such effects[6,8]. No animal studies ever exist as yet, which demonstrate the possible usefulness of prior administration of statins in the acute phase of ICH. We hypothesized that prior administration of simvastatin would decrease inflammation and improve neurological outcome after experimental ICH in rats.

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MATERIALS AND METHODS

Induction of ICH

All protocols were approved by Institutional Animal Care and Use Committee of the author's University. Experimental

ICH was induced in male Sprague-Dawley rats (180 to 200 grams; Orient Bio, Korea). Study groups consisted of pre-simvastatin treated rats (n=21), vehicle-treated rats (n=21) and post-simvastatin treated rats (n=9 : only for behavioral testing). The ICH was created by stereotaxic infusion of bacterial collagenase type IV (Sigma, St. Louis, MO, USA) into the striatum[12,13,27]. After an intraperitoneal injection of tiletamine/zolazepam 27.78 mg/kg (Zoletil®; Virbac Laboratories, Carros, France) and 2% xylazine hydrochloride 0.647 mg/kg (2% Rompun®; Bayer Korea, Seoul, Korea), rats were placed prone in a stereotaxic frame (Stoleting Co, Wood Dale, IL, USA). After a midline incision, a 1-mm bone hole on the skull was made and a 30-gauge Hamilton syringe needle was inserted into the left striatum (location : 3.0 mm left lateral to the midline, 0.2 mm posterior to the bregma, 6 mm in depth below the skull). The administration of 1 uL of saline containing 0.23 U of collagenase was sustained over 5 minutes. Then, the needle was slowly withdrawn 4 minutes after infusion. The bone hole was sealed with bone wax, and the scalp wound was sutured. Rectal temperature was maintained at 37±0.5℃ using a thermistor-controlled heating blanket during ICH induction. The rats were placed in cages with free access to food and water. The rats were kept in air-ventilated cages at 24±0.5℃ for the duration of the experiment. The body weights of all rats were checked regularly for consecutive 6 weeks.

Simvastatin administration

Simvastatin (Zocor®; MSD Korea, Seoul, Korea) crosses the blood brain barrier, and its neuroprotective potency was greater than other statins[14]. Pre-simvastatin treated rats were fed 2 mg/kg simvastatin, using 20 G feeding needles, dissolved in phosphate-buffered saline, daily for 7 days prior to induction of ICH and post-simvastatin treated rats were fed 2 mg/kg simvastatin for 7 days after induction of ICH[14]. Control rats were fed by vehicle (phosphate-buffered saline) alone daily for 7 days prior to induction of ICH.

Behavioral testing

Behavioral testing was performed weekly up to 42 days for pre-simvastatin treated rats, post-simvastatin treated and vehicle-treated rats (n=9 per each group) altogether using the modified limb placing test[22] and the corner turn test[10,11], which were monitored by one blinded investigator for group allocation. Total score of 7 points denoted maximal neurological deficit and 0 point indicated normal performance. The baseline behavioral tests were performed in all experimental rats prior to induction of ICH.

Measurement of hematoma volume and hemispheric atrophy

At 3 days after ICH, brains from pre-simvastatin treated rats and vehicle-treated rats (n=4 per each group) were extracted and cut coronally through the needle entry site (identifiable on the brain surface) on rat brain matrix (Harvard Bioscience, Holliston, MA, USA). Then, serial slices (1-mm thickness) were obtained. Digital photography of the serial slices was taken and hematoma volume was measured using image analyzer program (analySIS® Pro 3.2; Soft Imaging System GmbH, Münster, Germany). The total hematoma volume (mm^3) was calculated by summing the clot area in each section and multiplying it by the distance between sections[27]. At 42 days after ICH, the rats (n=4 per each group) used for behavioral test were sacrificed to measure hemispheric atrophy. Three sections through the needle entry site, and sites 1.0 mm anterior and 1.0 mm posterior to plane were obtained. The total hemispheric area of each section was traced and measured using image analyzer. Hemispheric atrophy was expressed as a percentage of contralateral hemispheric area[12].

Analysis of brain water content

At 3 days after ICH, analysis of brain water content and immunohistochemistry were performed because inflammatory reaction has been known to be maximal at 48 to 72 hours after ICH[31]. Pre-simvastatin treated and vehicle-treated ICH rats (n=4 per each group) were sacrificed to measure brain water content. The brain was divided into 2 hemispheres along the midline, and the cerebellum and the brain stem were removed. The brain sample was immediately weighed on an electronic analytical balance to obtain the wet weight. The sample was then dried in a gravity oven at 100℃ for 24 hours to obtain the dry weight. Brain water content was expressed as a percentage of wet weight : the formula for calculation was (wet weight-dry weight)/(wet weight)×100[27].

Immunohistochemistry

Pre-simvastatin treated and vehicle-treated rats (n=4 per each group) were used for immunohistochemistry. Each animal was first anesthetized and the chest was opened and perfused through the aorta with 300 mL cold saline and 300 mL of 4% paraformaldehyde dissolved in 0.1 mol/L phosphate-buffered saline. An incision was made in the right atrium to allow for outflow of perfused solution. The brain was harvested and fixed in 4% paraformaldehyde for 7 hours. Thereafter, the brain was cryoprotected with 30% sucrose for 48 hours and were cut by a cryostat (Leica CM 1800; Leica Inst., Nussloch, Germany) into 30-um sections. Tissue sections were mounted on glass slides for immunohistochemical study. Immunohistochemical staining was processed as described by Jeong et al.[12]. Rabbit anti-human myeloperoxidase (1 : 100; DAKO Corporation, Carpinteria, CA, USA) and mouse anti-rat OX-42 primary antibodies (1 : 500; Chemicon, Temecula, CA, USA) were used as a marker for neutrophil and microglia, respectively and thus double-label staining was made on the same tissue section. Endothelial nitric oxide synthase (eNOS) immunohistochemistry was performed with anti-eNOS antibody (1 : 500, rabbit polyclonal; BD Transduction Laboratories, Lexington, KY, USA). Cleaved anti-caspase-3 antibody (1 : 100, mouse, Cell Signaling Technology,

Beverly, MA, USA) was used as a marker of apoptosis. Primary antibodies were incubated overnight at room temperature with the slide-mounted method. Cy3-conjugated anti-mouse IgG antibodies (1 : 100; Jackson ImmunoResearch, West Grove, PA, USA) and Alexa488-conjugated anti-rabbit IgG (1 : 200; Invitrogen, Carlsbad, CA, USA) were used for secondary antibodies. Secondary antibodies were incubated for 3 hours at room temperature. Negative control slides from each animal were prepared for detecting autofluoroscence in the identical manner except primary and secondary antibodies were omitted.

Cell quantification

The immuno-positive cells were identified and counted in the perihematomal region (1-mm width) by one investigator who was blinded for the group allocation. Total counts in the measured sections were converted into cell densities for comparison between the ICH groups.

Statistical analysis

All data in this study are presented as the mean and standard deviation (SD). Data were analyzed by Mann-Whitney U test or Kruskal-Wallis test. Two-tailed value of $p<0.05$ was considered significant. All statistical analyses were conducted with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Physiological parameters

All rats tolerated the surgical procedure well and there was no surgical mortality. The physiological parameters, including body weights and body temperatures, were not significantly different in any experimental groups before, during or after ICH.

Behavioral test

The post-simvastatin treated group showed neurological improvement on the modified limb placing test and the corner turn tests after 2 weeks and showed better final outcome when compared with other two groups (Fig. 1A; $p=0.003$ and Fig. 1B; $p<0.001$, respectively, Kruskal-Wallis test). However, there was no significant neurological improvement in the pre-simvastatin treatment group after 42 days when compared with the vehicle treated group ($p=0.426$ in the modified limb placing test and $p=0.546$ in the corner turn test, Mann-Whitney U test). The initial body weights and those over the course of 6 weeks were similar.

Brain water content and hemispheric atrophy

The hematoma volumes were 26.1 mm^3 (SD 2.16) in the pre-simvastatin treated rats and 27.2 mm^3 (SD 2.03) in the vehicle-treated rats and were not different between two groups ($p=0.564$, Mann-Whitney U test) (Fig. 2A). Brain water content of the lesioned side was decreased in the pre-simvastatin treated group, but it did not reach statistical difference ($p=0.309$, Mann-Whitney U test) (Fig. 2B). Brain water content of the non-lesioned side was not different between two groups ($p=0.655$). The hemispheric atrophy was significantly decreased in the post-simvastatin treated group, but not in the pre-simvastatin treatment group ($p=0.021$, Kruskal-Wallis test) (Fig. 2C).

Immunohistochemistry

Increased infiltration of cells positive for OX-42 in the perihematomal region was detected 3 days after ICH. The numbers of OX-42 were decreased by pre-simvastatin treatment (Fig. 3A) compared with those of post-simvastatin treatment (Fig. 3B). However, the eNOS expression in the perihematomal region was not detected both in the pre- and post-simvastatin treated group. Quantitative analysis revealed 29% reduction of OX-42 positive cells in the pre-simvastatin treated rats compared with the vehicle-treated rats ($p=0.021$, Mann-Whitney U test) (Fig. 3C).

Caspase-3 activity

After 3 days, caspase-3 positive cells were present around the hematoma area in the blood clot in the pre-simvastatin treated rats (Fig. 4A) and in the vehicle-treated rats (Fig. 4B). "Merging"

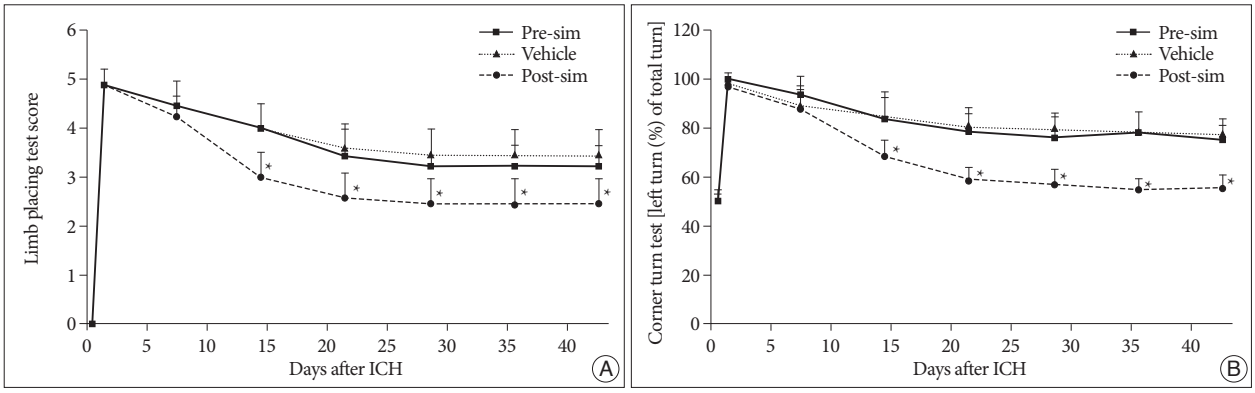

Fig. 1. Behavioral tests. The modified limb placing test (A) and the corner turn test (B) reveal that the post-simvastatin treated group shows better neurological outcome on behavioral tests when compared with other two groups, but there is no significant neurological improvement in the pre-simvastatin treatment group; bars represent mean and standard deviation (SD); n=9 per each group; *$p<0.05$; Kruskal-Wallis test. ICH : intracerebral hemorrhage.

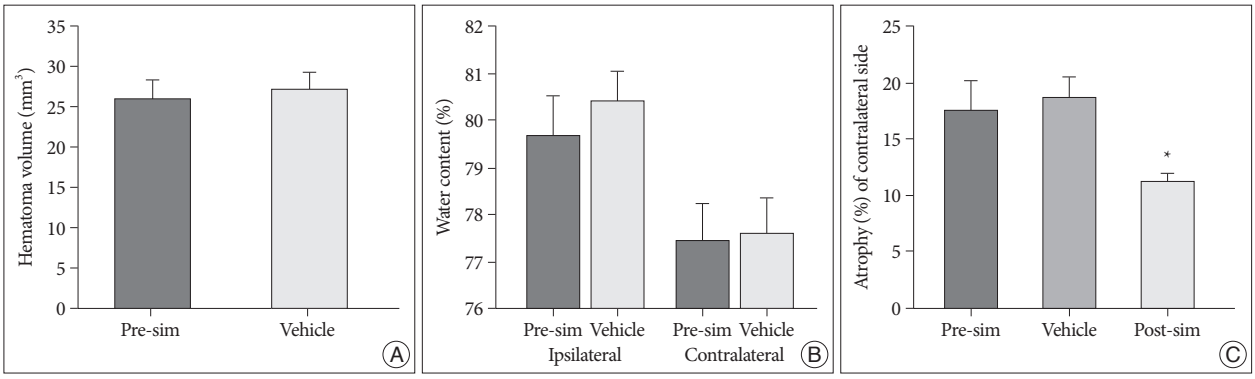

Fig. 2. Hematoma volume, brain water content and hemispheric atrophy. Measurement of hematoma volume (A) shows no difference between two groups. Analysis of brain water content (B) reveals that prophylactic adminstration of simvastatin decreased brain edema after ICH, albeit not statistically significant (p=0.309). Measurement of hemispheric atrophy (C) shows that there was a significant decrease of atrophy in the post-simvastatin treated group compared with others; n=4 per each group; bars represent mean and SD; *p<0.05; Mann-Whitney U test or Kruskal-Wallis test. SD : standard deviation.

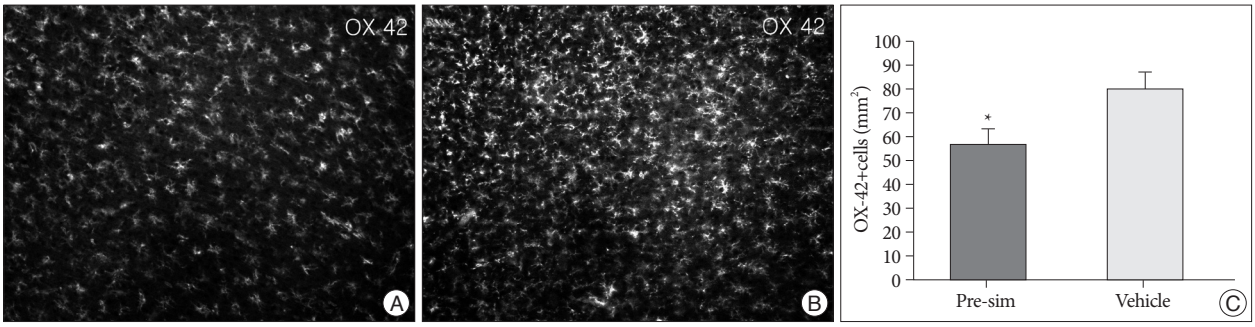

Fig. 3. Immunohistochemistry for OX-42 around the hematoma. OX-42 positive microglia were clustered around the hematoma in the pre- (A) and post-simvastatin treated rats (B). The numbers of OX-42 are decreased by pre-simvastatin treatment. Quantitative analysis (C) shows a significant reduction in OX-42 positive cells in pre-simvastatin treated group than in the vehicle-treated group; n=4 per each group; magnification ×100; bars represent mean and SD; *p<0.05; Mann-Whitney U test. SD : standard deviation.

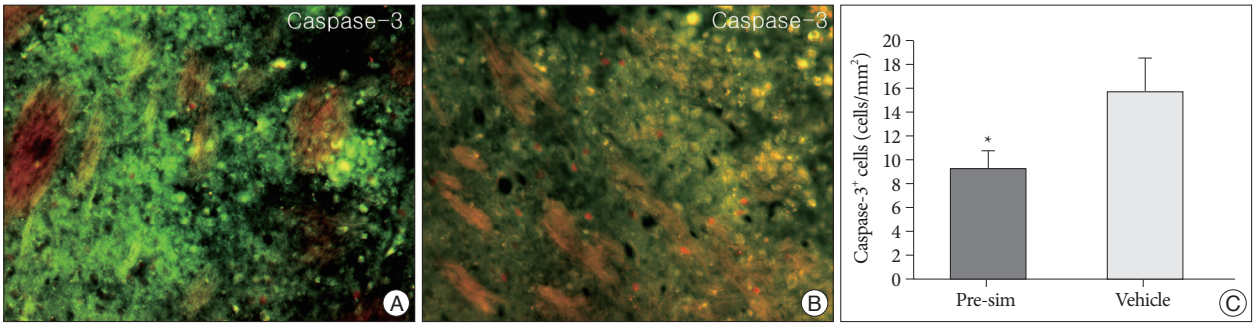

Fig. 4. Perihematomal cell death. Caspase-3 assay (merged images) shows that less abundant caspase-3 positive cells (orange-tinged color) were observed around the blood clot in the pre-simvastatin treated rats (A) than in the vehicle-treated rats (B). Quantitative analysis (C) shows less caspase-3 positive cells in the pre-simvastatin treated rats than in the vehicle-treated rats; n=4 per each group; bars represent mean and SD; *p<0.05; Mann-Whitney U test. SD : standard deviation.

method was used to avoid interference associated with autofluorescence around the hematoma. The caspase-3 positive cells in the pre-simvastatin treated rats were significantly decreased by 41% compared with the vehicle-treated rats (p=0.020, Mann-Whitney U test) (Fig. 4C).

DISCUSSION

The major finding of this study is that simvastatin therapy after ICH ameliorates neurological deficits, but prior use of simvastatin exerts only histological improvement against ICH without providing functional benefit. This is consistent with some previous clinical studies[6,8] that prior use of statins before ICH is not associated with good outcome. In this study, simvastatin therapy after ICH decreased hemispheric atrophy and promoted neurological recovery. On the other hand, prior use of simvastatin decreased inflammation and perihematomal cell death during the acute phase of ICH, but it did not finally result in fa-

vorable outcome. This result provides initial evidence that prior use of statins provides only partial neuroprotective action against ICH by modulating inflammation, contrary to statins therapy after ICH.

ICH causes tissue damage by multiple mechanisms. Direct mechanical destruction by the hematoma itself occurs immediately. This is followed by subsequent development of edema, ischemic damage due to raised intracranial pressure or impairment of cerebral circulation[31]. Delayed injury can result through a variety of mechanisms including perihematomal ischemia, inflammation, apoptosis and excitotoxicity[5]. Inflammation is recognized as the most important key factor in delayed brain injury and outcome in ICH[13,14,25,30]. When ICH occurs, toxic blood components including red blood cells, neutrophils, macrophages and plasma proteins can induce an inflammatory response in and around the hematoma[29,30]. Infiltrating inflammatory cells or activated microglia in particular, enhance the production of proinflammatory cytokines, cyclooxygenase-2, and inducible nitric oxide synthase (iNOS), presumably inducing the signal pathway to mediate the apoptosis[18,21]. Statins may inhibit recruitment and migration of leukocytes into the brain[24] and can suppress the proinflammatory mediators such as tumor necrosis factor-α, interferon-γ, and iNOS[11]. Therefore, statins therapy has neuroprotective effect by exerting anti-inflammatory action[4,19].

Perihematomal cell death may mediate some of brain injury after ICH. Apoptosis after ICH begins at 24 hours, peaks at 72 hours, and continues for 4 weeks[18]. Ischemia, inflammatory responses and free radicals in the perihematomal region have been suggested to induce apoptosis of neuron and glial cells[18,31]. Thus, modulation of inflammation has been reported to reduce brain edema and delayed brain injury and improve functional outcome in experimental ICH. Statins have been showed to decrease perihematomal apoptosis by modulating inflammation after ICH, giving functional recovery[13,14,25].

Statins also exert neuroprotective action on ICH via augmentation of cerebral blood flow[1,7]. Statin therapy has been reported to enhance cerebral blood flow by up-regulating of eNOS in experimental ischemic stroke[1,7,15], experimental subarachnoid hemorrhage[19] and experimental ICH[13]. In this study, functional recovery by prior simvastatin was not present and this may be attributed to the absence of eNOS expression. Neuroprotective effect and augmentation of cerebral blood flow by statins were never observed in eNOS-deficient mice[7]. These indicate that up-regulation of eNOS by statins is the key mechanism of neuroprotective action.

In clinical point of view, the therapeutic window of neuroprotective action of statins is very important, but there were only few studies applied in ICH. Two animal studies of ischemia have shown that prophylactic but not delayed administration of simvastatin significantly protected from brain damage[2,3]. In an ischemic rat model, neuroprotective effect of simvastatin was observed, administered 6 hours from ischemic attack and lasted for 48 hours, but neuroprotection was no longer afforded, administered 10 hours after the ictus[26]. These data indicate that statins might represent powerful tools in preventing delayed brain injury during acute inflammatory period. This neuroprotective effect of prophylactic administration of statins has been known to be long-lasting in ischemia[2,3], but delayed neuroprotective effect might not be observed in ICH. Some of the injury after ICH are attributable to toxic blood components or factors, and may be different from those of ischemic stroke. Compared with ischemic process, a long-standing hematoma itself can cause inflammatory reaction and may continue for a longer time. Perihematomal cell death and axonal loss can continue up to 3 months[31]. Thus, prior adminstration of statins may not offer substantial recovery after ICH contrary to the ischemic counterpart.

The reasons for the absence of functional benefit of prior use of statins are not known; however, some possible mechanisms can be postulated. First, prior administration of statins can suppress early inflammation after ICH, but, in this study, it did not seem to reduce brain edema or atrophy significantly. Statins therapy before ICH does not seem to be long-lasting contrary to ischemic counterpart. Next, the absence of blood flow augmentation by up-regulation of eNOS can also contribute to the lack of functional benefit.

The current study has several limitations. First, immunohistochemical study was not performed in post-simvastatin treated rats. Thus, the degree of neuroprotective potency in the histological aspects after ICH was not investigated between presimvastatin and post-simvastatin treated groups. Second, this study did not check the dose-response effect of statins therapy. The dosage of simvastatin was selected based on previous animal studies which investigated the neuroprotective effect of statins after ICH[14,25]. The low dose of atorvastatin (2 mg/kg) was effective, providing functional benefit in ICH of rats, on the other hand, higher dose (8 mg/kg) of atorvastatin did not improve neurological recovery[25].

CONCLUSION

The present study suggests that statins therapy after ICH improves neurological outcome, but prior use of statins before ICH might provide only histological improvement, providing no significant impact on neurological outcome against ICH. The mechanism of this action may be related to the absence of blood flow augmentation by up-regulation of eNOS or the lack of delayed neuroprotective effect by statins against the long-standing hematoma. To clarify mechanism and neuroprotective effect of statins, further investigations are needed.

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