Influence of Simvastatin on Microthrombosis in the Brain after Subarachnoid Hemorrhage in Rats: A Preliminary Study

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Abstract. Although previous studies indicate that simvastatin can attenuate cerebral vasospasm after subarachnoid hemorrhage (SAH), its effect on the secondary pathophysiological changes after SAH has not been investigated. Accumulating evidence demonstrates that SAH-induced microthrombosis plays important roles in the pathogenesis of delayed cerebral ischemia. To date, however, no study focused on the treatment of microthrombosis in SAH models. The purpose of this study was to determine the impact of simvastatin on microthrombi formation after SAH in rats. Adult male SD rats were divided into four groups: (1) control group (n = 6); (2) SAH group (n = 6); (3) SAH+vehicle group (n = 6) and (4) SAH+simvastatin group (n = 6). SAH was induced by injecting 0.3 ml of fresh arterial, non-heparinized blood into the prechiasmatic cistern in 20 sec with a syringe pump. In the SAH+simvastatin group, simvastatin was administered ip at a dose of 20 mg/kg/d after SAH. Brain samples were excised after perfusion fixation at 7 days after SAH. The cross-sectional areas of the middle cerebral artery and anterior cerebral artery were measured. Microclots were evaluated by H&E staining. Microthrombi formation was measured by fibrin(ogen) immunostaining. The results showed that administration of simvastatin prevented vasospasm on day 7 following SAH (p <0.01). The number of microthrombi was significantly increased in both cerebral cortex and cerebellar cortex at 7 days after SAH (p <0.01). Simvastatin treatment down-regulated the formation of microclots in this SAH model and the number of microthombi was decreased significantly in the SAH+simvastatin group compared to the SAH or SAH+vehicle groups (p <0.01). In conclusion, simvastatin administration attenuates cerebral vasospasm and alleviates microthrombosis in the late phase of SAH in this prechiasmatic blood injection model.

Keywords: simvastatin, microthrombosis, subarachnoid hemorrhage, vasospasm

Introduction

Delayed cerebral ischemia (DCI) occurring as a result of chronic cerebral vasospasm following subarachnoid haemorrhage (SAH) is the most important factor influencing morbidity and mortality [1]. Efforts to elucidate the mechanisms of DCI have focused on vasospasm of the large conducting cerebral arteries, but it is clear that this type of vasospasm cannot entirely account for the occurrence of delayed ischemic neurological deficits. Clinical symptoms of DCI (eg, decreased cerebral blood flow and oxygen consumption) can occur without angiographic evidence of vasospasm [2]. Conversely, severe angiographic vasospasm is often found in the patients without obvious clinical signs of cerebral ischemia [3]. An explanation for these observations could be that the cerebral microcirculation and its regulatory mechanisms are directly affected by SAH.

Cerebral microthrombi were detected for the first time in 1983 upon autopsy in a patient with DCI after SAH, and were later confirmed in other studies [4-6]. In an autopsy study investigating six
SAH patients, including four patients who supposedly died from DCI, it was observed that patients who die from DCI have significantly more microthrombi in clinically ischemic regions and in areas showing cerebral infarction on computed tomographic scans, when compared with patients who die from rebleeding, acute hydrocephalus, or other SAH-related complications [5]. In a recent report by Romano et al. [7], bilateral microembolic signals were detected in 38% of SAH patients at a mean of 6.7 days after hemorrhage, which shows a close association with DCI symptoms [7]. These data suggest that microthrombosis may play a complementary role in the pathogenesis of DCI.

Simvastatin is a potent inhibitor of cholesterol synthesis and has proven beneficial in the prevention of coronary heart disease. Recent experimental and clinical evidence has demonstrated beneficial effects that extend beyond simvastatin’s cholesterol-lowering activity. Simvastatin improves endothelial function and blood flow by reducing vascular inflammation, inhibiting vascular smooth muscle cell proliferation and platelet aggregation, and promoting vasodilation by up-regulating endothelial nitric oxide synthase (eNOS) [8]. Simvastatin has been demonstrated to ameliorate SAH-induced delayed vasospasm in mice [8], rats [9], rabbits [10], and SAH patients [11]. Nevertheless, it is still unknown whether simvastatin prevents or attenuates the formation of microthrombi after SAH. The aim of the current study was to determine the effect of simvastatin on microthrombosis in the late phase (day 7) of SAH, using a prechiasmatic blood injection model in rats.

Materials and Methods

Animal model. Thirty male Sprague Dawley rats (350 to 400 g) were purchased from the Animal Center of the Chinese Academy of Sciences, Shanghai, China. The rats were housed in temperature- and humidity-controlled animal quarters with a 12-hr light/dark cycle. All procedures were approved by the Institutional Animal Care Committee and were in accordance with the guidelines of the National Institutes of Health on the care and use of experimental animals.

Following ip anesthesia with a mixture of ketamine (100 mg/kg) and xylazine (0.5 mg/kg), the rat’s head was fixed in a stereotactic frame. The experimental SAH model involved stereotactic insertion of a needle with a rounded tip and a side hole into the prechiasmatic cistern as previously described [12,13]. The needle was tilted 45° in the sagittal plane, and placed 7.5 mm anterior to the bregma in the midline, with the hole facing the right side. It was lowered until the tip reached the base of the skull, 2-3 mm anterior to the chiasma (about 10 to 12 mm from the brain surface), and was retracted 0.5 mm. Loss of CSF and bleeding from the midline vessels were prevented by plugging the burr hole with bone wax before inserting the needle. Then 0.3 ml of non-heparinized fresh autologous arterial blood was slowly injected into the prechiasmatic cistern for 20 sec with a syringe pump under aseptic technique. Control animals were injected with 0.3 ml of saline. The animals were allowed to recover 45 min after SAH. After the operation, rats were returned to their cages and the room temperature was kept at 23 ± 1°C. Twenty ml of 0.9% NaCl solution was injected sc immediately after the operation to prevent dehydration. Heart rate and rectal temperature were monitored, and the rectal temperature was kept at 37 ± 0.5°C by physical cooling (ice bag) when required throughout the experiment. Rats were killed by fixation-perfusion while under anesthesia at 7 days after SAH.

Experimental groups. We established 4 experimental groups in a randomized fashion: (a) the control group (n = 6); (b) the SAH group (n = 6); (c) the SAH+vehicle group (n = 6); and (d) the SAH+simvastatin group (n = 6). In the SAH+simvastatin group, simvastatin was administered ip at a dose of 20 mg/kg/day after SAH. Rats of the SAH+vehicle group received equal volumes of 10% ethanol in normal saline solution with the same schedule. The dose was chosen according to previous studies in which the authors observed beneficial effects (e.g., preventing cerebral vasospasm and improving neurological function) in the rat SAH model [8,9].

Perfusion-fixation and tissue preparation. The rats scheduled for death were anesthetized with an ip injection of ketamine (100 mg/kg) and xylazine (0.5 mg/kg). Perfusion-fixation was then performed. The thorax was opened with a cannula placed in the left ventricle, the descending thoracic aorta was clamped, and the right atrium was opened. Perfusion was begun with 250 ml of physiological phosphate buffer solution (PBS, pH 7.4) at 37°C, followed by 250 ml of 10% buffered formaldehyde under a perfusion pressure of 120 cm H₂O. After perfusion-fixation, the whole brain was removed and immersed in the same fixative solution. The whole brain was dissected 48 hr after fixation, as described in Fig. 1.

H&E staining and cross-sectional area measurement. The degree of cerebral vasospasm was evaluated by measurements of the lumenal cross-sectional areas of the middle cerebral artery (MCA) and the anterior cerebral artery (ACA). The formalin-fixed and paraffin-embedded sections (4 μm thick) were deparaffinized, hydrated, washed, and stained with H&E. Then micrographs of the arteries were put into the computer. Cross-sectional areas of the blood vessels were determined by an investigator (who was blinded to the experimental groups) using ImageJ 1.41 software (National Institutes of Health, Bethesda, MD, USA). The areas were calculated by measuring the perimeter of the actual vessel lumen and then calculating the area of an equivalent circle (area = πr², where r = radius) based on the calculated equivalent.
r value from the perimeter measurement \( r = \frac{\text{perimeter}}{2\pi} \), thus correcting for vessel deformation and off-transverse sections. For each vessel, three sequential sections (midpoint of proximal, middle, and distal segments) were taken, measured, and averaged. The number of microclots in each section was counted in 10 microscopic fields (at \( \times200 \) magnification) and averaged to determine the number of microclots per field.

**Immunohistochemistry.** Immunohistochemical studies were conducted on formalin-fixed, paraffin-embedded sections. Chicken-anti-rat monoclonal antibody of fibrin(ogen) (diluted 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. For immunohistochemistry, sections were incubated in phosphate-buffered saline (PBS) with 5% normal goat serum for 30 min at room temperature. Sections were washed three times with PBS and incubated with primary antibody for 30 min at room temperature. After washing with PBS, sections were incubated with biotinylated second antibodies for 30 min at room temperature. Sections incubated in the absence of primary antibody were used as negative controls. Microscopy of the stained tissue sections was performed by a pathologist blinded to the experimental groups. The extent of microthrombosis was evaluated by the average number of

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Fig. 1. Schematic representation of tissue cutting and embedding for the whole brain. Upper, 'A' section was for detecting the histological changes of middle cerebral artery (MCA) and anterior cerebral artery (ACA). 'B' section was for evaluating the microthrombi or microclots in the cerebral cortex. 'C' section was for counting the number of microthrombi or microclots in the cerebellar cortex. Bottom, specifically for 'A' part, 3 cuttings were made for detecting MCA and ACA, respectively. The arrows indicate the cutting direction.
fibrin(ogen)-positive vessels in each section counted in 10 microscopic fields (at ×200 magnification).

Statistics. All data were presented as mean ± SD. SPSS 12.0 was used for statistical analysis of the data. The measurements were subjected to one-way ANOVA. Differences between experimental groups were determined by Fisher’s LSD post-test. Statistical significance was inferred at p <0.05.

Results

General observations. There were no significant differences in body weight, temperature, or arterial blood gas data among the experimental groups (data not shown). After induction of SAH, all animals stopped breathing for about 30 sec. Six rats died after the blood injection; the mortality in SAH animals was 6/30 (20%).

Fig. 2. Changes in the cross-sectional area of middle cerebral artery (MAC) and anterior cerebral artery (ACA) in the control group (n = 6), SAH group (n = 6), SAH+vehicle group (n = 6), and SAH+simvastatin group (n = 6). Upper panel: representative images of cross-sectional areas of the MAC and ACA of control rats, rats subjected to SAH alone, SAH plus injection with vehicle, or simvastatin. Severe vasospasm could be detected in the SAH group and SAH+vehicle group, which was attenuated in the SAH+simvastatin group (bar = 200 μm). Bottom panel: histogram of the average cross-sectional area of MCA and ACA from the 4 groups. There is a significant difference in the artery cross-sectional area between the SAH and control groups. The basilar artery cross-sectional area was significantly increased in the SAH+simvastatin group vs SAH or SAH+vehicle groups (** p <0.01 vs control group; ns, p >0.05 vs SAH group; ## p <0.01 vs SAH+vehicle group.)
**Influence of simvastatin on cerebral vasospasm after SAH.** As shown in Fig. 2, there was a significant difference in the cross-sectional areas of MCA and ACA among all the groups on day 7 following SAH (p < 0.01). A significant difference was detected between the SAH and control groups (p < 0.01). There was also a significant difference in arterial cross-sectional area of the SAH+simvastatin vs the SAH+vehicle groups (p < 0.01). No significant difference was seen between the SAH group and the SAH+vehicle group.

**Effect of simvastatin on microclot formation after SAH.** The H&E staining images are shown in Figs. 3 and 4. Few microclots were observed in the control group rat brain (Figs. 3A, 4A). In the SAH group, the number of microclots was greatly increased in the cerebral and cerebellar cortex (Figs. 3B, 4B) compared to controls (p <0.01). Compared to the SAH+vehicle group (Figs. 3C, 4C), the number of microclots in the SAH+simvastatin group (Figs. 3D, 4D) was decreased (p <0.01).

**Fig. 3.** Representative histological images of microclots in cerebral cortex in control group (n = 6), SAH group (n = 6), SAH+vehicle group (n = 6), and SAH+simvastatin group (n = 6). Upper panel: (A) control rats showing few microclots; (B) SAH rats showing more microclots, indicated by arrows; (C) in the SAH+vehicle group, more microclots were seen, as demonstrated by arrows; (D) the SAH+simvastatin rats show fewer microclots than SAH rats (bar = 100 μm). Bottom panel: In the SAH and SAH+vehicle groups, the numbers of microclots are greater than the control group (p <0.01). The number of microclots in the SAH+simvastatin group was significantly less than that of rats in SAH or SAH+vehicle groups (p <0.01); (** p <0.01 vs controls; ns, p >0.05 vs SAH group; ## p <0.01 vs SAH+vehicle group.)
Simvastatin inhibited microthrombosis after SAH.

Immunohistochemical photomicrographs of brain sections of cerebral and cerebellar cortex are shown in Figs. 5 and 6. In cerebral cortex and cerebellar cortex, control rats had few microthrombi after saline injection. In both SAH (Figs. 5B, 6B) and SAH+vehicle rats (Figs. 5C, 6C), the number of microthrombi was greater than in the controls (Figs. 5A, 6A) (p<0.01). The rats in SAH+simvastatin group had fewer microthrombi (Figs. 5D, 6D) than the SAH or SAH+vehicle rats (p<0.01).

Discussion

The most important finding of this study is that SAH induced significant up-regulation of microthrombosis in rat brain, which was inhibited by ip administration of simvastatin. Treatment with simvastatin also ameliorated cerebral vasospasm in MCA and ACA in this SAH model, which is in agreement with previous clinical and experimental studies [8-10]. Based on these results, it appears that simvastatin, a 3-hydroxy-3-methylglutaryl
coenzyme A reductase inhibitor, not only prevents the vasospasm in large cerebral arteries, but is beneficial for the microcirculatory pathological changes after experimental SAH.

In the current study, we used a model created by Prunell et al. [12,13], but we made some modifications. First, we injected 0.3 ml of non-heparinized fresh autologous arterial blood, instead of 0.2 ml. In a preliminary experiment, we found that the arterial contraction was mild on day 7 after 0.2 ml blood injection, which was not appropriate for evaluating the effect of simvastatin on cerebral vasospasm. Second, the clinical symptoms of the rats with 0.3 ml blood injection were more severe than those with 0.2 ml blood injection. Most SAH rats in this present study stopped breathing for approximately 30 sec after the blood injection, which is closer to the clinical situation and was not observed in the studies by Prunell et al. [12,13]. We suggest that this phenomenon was due to the

Fig. 5. Representative photomicrographs of fibrinogen immuno-histochemistry in the cerebral cortex in the control group (n = 6), SAH group (n = 6), SAH+vehicle group (n = 6), and SAH+simvastatin group (n = 6). (A) In controls, few microthrombi were observed; (B) more microthrombi are seen in the SAH group as demonstrated by the arrows; (C) the number of microthrombi is still large in the SAH+vehicle group; (D) after simvastatin treatment, microthrombosis was down-regulated in the SAH+ simvastatin rats (bar = 50 μm). (** p <0.01 vs control group; ns, p >0.05 vs SAH group; ##p <0.01 vs SAH+vehicle group).
Inceased blood volume injected. Finally, we injected the blood with a syringe pump in 20 sec, instead of manual injection. In the original model, the authors increased intracranial pressure (ICP) to the level of MABP during injection, which meant that cerebral perfusion pressure (CPP) was zero. In their study, the mortality was 100% for 0.3 ml blood injection. But in our experiment, the mortality was only 20%, which may be caused by our modifications of this SAH model.

There have been previous studies of microthrombosis after SAH [4,7,14]. As summarized by Vergouwen et al. [14], activation of the coagulation cascade, impaired fibrinolytic activity, and inflammatory processes, all lead to the formation of microthrombi, which ultimately result in DCI. The cerebral microcirculatory changes inside the parenchyma after SAH are controversial [15-17]. However, for both small and large vessels, endothelial function plays an important role in the

![Fig. 6. Representative photomicrographs of fibrin(ogen) immunostaining in the cerebellar cortex in the control group (n = 6), SAH group (n = 6), SAH+vehicle group (n = 6), and SAH+simvastatin group (n = 6). (A) Few microthrombi show in the cerebellum of control rats; (B) microthrombosis was induced in the SAH group and the microthrombi are indicated by the arrows; (C) in the SAH+vehicle, the number of microthrombi are similar to the SAH group; (D) post-SAHT administration of simvastatin repressed the microthrombosis in the cerebellar cortex (bar = 100 μm; ** p < 0.01 vs controls; ns, p >0.05 vs SAH group; ### p <0.01 vs SAH+vehicle group).](image-url)
whole pathological process. A number of previous reports have shown that simvastatin can improve endothelial function in patients with different diseases [18-20]. We believe that iv administration of simvastatin may dilate the arterioles and venules, improving endothelial function in the brain when microcirculation is spasmodic after SAH.

Some previous studies in patients with aneurysmal SAH showed that levels of serological coagulation markers correlate with the development of DCI and cerebral infarction [15,21,22]. Up-regulated platelet activation and aggregation are suggested by increased levels of β-thromboglobulin, thromboxane B₂, soluble platelet selectin (P-selectin), and platelet-activating factor [21-23]. Pastuszczak et al. [24] analyzed the effect of prior simvastatin treatment on thrombin generation and platelet activation in patients with myocardial infarction. They found that prior simvastatin use was associated with lower thrombin generation and platelet activation following vascular injury in the early phase of myocardial infarction. Nomura et al. [25] studied the effects of simvastatin on circulating levels of platelet activation markers. They found that administration of simvastatin inhibits platelet activation and reduces cardiovascular complications in hypertensive and hyperlipidemic patients. Since platelet activation is an important cause for formation of microthrombi after SAH, we tentatively envision that simvastatin suppresses microthrombosis probably by inhibiting platelet activation.

The natural antagonist of thrombin generation is fibrinolysis, which results in the degradation of fibrin by plasminogen. The activity of plasminogen is regulated by a positive regulator, tissue-type plasminogen activator (t-PA), balanced by a negative modulator, plasminogen activator inhibitor-1 (PAI-1). The concentration of PAI-1 antigen in the cerebrospinal fluid (CSP) is significantly higher in patients with DCI after SAH than that in patients without DCI, which suggests that overactive inhibition of fibrinolysis is associated with DCI [26]. Guven et al. [19] studied the effects of simvastatin on fibrinolysis by measuring concentrations of thrombin activatable fibrinolysis inhibitor (TAFI) in hypercholesterolemic patients. The plasma TAFI levels were significantly decreased after simvastatin treatment [median 17.0 (range 0.4-93.7) μg/ml vs median 6.9 (range 0.8-63.0) μg/ml, p <0.001], which means that simvastatin increased fibrinolysis in these patients [19]. The data from the present study suggest that simvastatin decreases the number of microthrombi after experimental SAH, one mechanism of which might be that simvastatin up-regulates fibrinolysis secondary to SAH.

Besides the coagulation and fibrinolytic cascade, inflammatory processes also play an important role in the pathogenesis of microthrombosis after SAH. Clinical and experimental studies have shown increased levels of cytokines and adhesion molecules in the cerebrospinal fluid and serum after SAH [27]. During an inflammatory reaction, stimuli such as thrombin lead to the mobilization of P-selectin from the endothelium, which can be stimulated by pro-inflammatory cytokines such as tumor necrosis factor-α. Adhesion molecules bind to leukocytes, which leads to slower leukocyte rolling and subsequent leukocyte adhesion. Accordingly, leukocytes can penetrate the vessel wall. Moreover, there is well-known evidence that inflammatory responses induce coagulation and down-regulate anti-coagulatory pathways [28]. Recent data from studies concerning simvastatin and inflammation suggest that simvastatin treatment has anti-inflammatory effects in in vivo models [29,30]. In the present study, our results showed that simvastatin reduced the formation of microthrombi in the cortex of both cerebrum and cerebellum, which may be mediated partially by the anti-inflammatory potential of simvastatin.

The current research was focused on the cerebral and cerebellar cortex. As reported by a previous study, microthrombi can be easily found in the choroid plexus in a murine cancer model [31]. However, we did not see microthrombi or thrombi in the choroid plexus of any of the experimental groups in the present study. The time-point we chose was day 7 after SAH, which is a peak time of vasospasm in this rat model. Thus, this time-point is appropriate for analyzing both vasospasm and microthrombosis. In humans, the duration of delayed vasospasm is from day 3 to day 14 following SAH, which means multiple time-
points may be more persuasive for this research. In any event, our preliminary study has demonstrated the effect of simvastatin on cerebral vasospasm and microthrombosis after experimental SAH.

Nowadays, whether statins reduce morbidity and mortality following aneurysmal SAH has become a controversial topic in many studies including animal experiments and clinical trials [8-11,32]. A large, multicenter, randomized, controlled trial is currently ongoing in the UK, USA, and Canada (http://www.stashtrial.com). For simvastatin's side effects, exploratory safety analyses demonstrated that simvastatin did not increase the incidence of myocardial infarction, congestive heart failure, fever, bacteremia, sepsis, and hypokalemia [33]. The authors did not observe more bleeding issues in the simvastatin-treated patients, even though our data show that simvastatin can inhibit microthrombi formation. The possible explanation may be that simvastatin did not affect the blood coagulation system so much as to cause bleeding. In the present study, we did not analyze platelet activation and fibrinolysis. The mechanism concerning simvastatin and SAH-induced cerebral microthrombosis calls for further research.

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References