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Original Research Article

Simvastatin suppresses cerebral aneurysm in rats through suppression of release of pro-inflammatory cytokines

Congcong Chen[1], Hongdan Wei[2], Jiawen Song[3], Xiaona Zhang[4]*

[1]Department of Pharmacy, The 2nd Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000, [2]Department of Pharmacy, Huzhou Third People's Hospital, Huzhou, Zhejiang 313000, [3]Department of Pharmacy, Lishui People's Hospital, Lishui, Zhejiang 323000, [4]Department of Stomatology, The 906th Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army, Wenzhou, Zhejiang 325000, China

For correspondence: **Email: xianazhang84@gmail.com; **Tel:** 0086-0577-56989991*

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Abstract

Purpose: *To investigate the protective effect of simvastatin (SA) against onset of cerebral aneurysm-mediated inflammatory cytokine release and subsequent inflammation and degradation of the extracellular matrix of smooth muscle cells.*
Methods: *Cerebral aneurysm was induced in male Wistar rats using elastase injection, and aneurysm growth was monitored for one month following SA treatment. Inhibition of aneurysm growth was determined along with the expression levels of chemokines and pro-inflammatory cytokines, including TNF-α, IL-8, IL-1β, IL-17, IL-6, macrophage chemoattractant protein-1 (MCP-1), and matrix metalloproteinases 2 and 9, using enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR).*
Results: *Aneurysm size decreased in rats treated with SA, relative to values obtained for control rats ($p < 0.05$). The corresponding expressions of inflammatory cytokines and chemokines were also reduced following pre-treatment with SA ($p < 0.05$). The results indicate a reduction in the aneurysm area in rats pre-treated with SA, when compared to the untreated animals ($p < 0.05$).*
Conclusion: *SA treatment inhibits the progression of cerebral aneurysms via its protective effect against inflammation, indicating its potential for use in the prevention and treatment of cerebral aneurysms.*

Keywords: *Simvastatin, Intracranial aneurysm, TNF-α, Cerebral*

INTRODUCTION

Intracranial aneurysm is a cerebrovascular disease that arises from vascular abnormalities in the arteries, leading to subarachnoid haemorrhage which results in high mortality and morbidity [1]. An understanding of the mechanisms involved in the pathogenesis of the disease and vascular rupture would help in devising a strategy for therapeutic intervention. Hemodynamic stress has been closely linked to vascular remodeling in aneurysm as a response aimed at reducing shear stress in the vascular wall [2]. The early stages of aneurysm formation are associated with degradation of elastic lamina. These stages are artificially mimicked in the animal models by injecting elastase with a high-salt diet into the cerebrospinal fluid of the right basal cistern, thereby inducing hypertension [3]. This procedure has achieved aneurysms similar to human aneurysms, with degeneration of the elastic lamina and infiltration of inflammatory cells [3].

Infiltration of inflammatory cells such as macrophages, T-cells, and monocytes in aneurysm is associated with degradation of collagen in the aneurysm walls, as well as increased expressions of cytokines, collagenase and elastase which are the hallmarks of patients with cerebral aneurysm [4]. Tumor necrosis factor (TNF-α) is primarily associated with aneurysm formation, and due to its inflammatory properties, it ensures the build-up of vascular inflammation in the blood vessels, leading to plaque formation [5]. Tumor necrosis factor alpha (TNF-α) is secreted by T-cells and other immune cells which stimulate chronic inflammation by synergistic action in increasing the degeneration of the internal elastic lamina, the endothelial cells, and the medial smooth muscle. These processes render the aneurysm walls more permeable to inflammatory cytokines and the successive invasion of macrophages [6]. These events alter the blood-brain barrier, thereby increasing the accumulation of fluids and blood flow which favor subarachnoid haemorrhage.

Simvastatin (SA) is a well-known statin which is widely used in the attenuation of inflammation through regulation of the expressions of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [7]. Studies have shown that the administration of SA reduced inflammation in animal models, as was evident in decreased level of inflammation and reduced infiltration of polymorphonuclear leukocytes [8].

In the present study, cerebral aneurysm was induced in rats via stereotaxic injection of elastase into the cerebrospinal fluid so as to degrade the elastic lamina through TNF-α-mediated activation of MMP-9. Then, the effect of SA on aneurysm was investigated in terms of its efficacy in controlling inflammation and immune modulation with respect to the expression levels of proinflammatory cytokines, TNF-α, and MMP-9.

EXPERIMENTAL

Animals

Male Wistar male of mean weight 120 ± 10 g were used in the present study. All animals were kept in cages in a temperature-controlled room with a 12-h light/2-h dark cycle, with temperature and humidity maintained at 25 ± 2 °C and 55 ± 5 %, respectively. The rats received free access to regular standard rat chow and RO water. This research was approved by the Animal Ethical Committee of the 2nd Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (approval no. 202015), and it was conducted according to the guidelines of Principles of Laboratory Animal Care [9].

Aneurysm induction in rats

The induction of aneurysm in rats and subsequent experiments were approved by the institutional animal care and use committee in line with the institutional guidelines.

Animal grouping and treatments

The animal groups were assigned to vehicle control (group 1), rats induced with aneurysm (group 2, SA-treated rats (group 3), and TNF-α inhibitor (adalimumab)-treated rats (group 4). Male Wistar rats aged 7 weeks were used in this study. Ketamine (75 mg/kg) was used for induction of IA, with 16 animals in control group, and the same number of animals was used for IA induction and SA treatment (100 mg/kg/day, orally). The SA treatment was given one week prior to IA induction, and the treatment was continued throughout the experimental period. Similarly, adalimumab (50 mg/kg) was used in the treatment of group 4 rats, while SA treatment was given to group 3. Briefly, the induction of IA in the animal model was carried out by ligation of the left internal carotid artery and injection of elastase with stereotaxic delivery into the cerebrospinal fluid at the right basal cistern. Animals in all the groups were fed a high salt diet (8 %) for 6 weeks to induce hypertension. The systolic arterial blood pressure in rats was measured using the tail-cuff method before the treatment and after the surgery, every 4 weeks till 16 weeks. The rats were euthanized after 120 days, after which the aneurysm grades were assigned blindly from the circle of Willis (COW) and major branches using arterial perfusion of bromophenol dye.

Determination of expression levels of cytokines

The levels of expression of inflammatory cytokines (TNF-α, IL-2, IL-17, IL-8, IL-6, and IL-1β) in the tissues around the circle of Willis from all groups of animals were measured using their respective commercial ELISA kits (Fine Biotech Co. Ltd, Hubei, China), as per the manufacturer's instructions. Infiltration of macrophages was calculated from the IA areas of 5-um sections of brain tissue which were stained with CD69 primary antibody and secondary antibodies (Santa Cruz Biotech, CA, USA). The tissue sections were counterstained with hematoxylin. The number of macrophages in the aneurysm area was calculated by counting the CD68+ cells in 100-μm square field area [10].

Fluorometric determination of MMP-9 activity

The measurement of MMP-9 activity was performed fluorometrically [11]. Rat monoclonal anti-MMP-9 (Thermo Fisher Scientific, Catalogue No: MS-817-P) was added to wells that were already coated with Protein G to orient the antibody to bind to the MMP-9 present in the biological samples. The setup was then incubated with plasma from each rat group, for tight binding. An intact FRET peptide containing QXLTM520 was added to wells containing the anti-MMP antibody and plasma samples. The wells corresponding to the plasma samples of rats the from different groups showed differences in the capacity to cleave the FRET peptide because of differential expressions of MMP-9 in them. The resulting fluorescence from the cleaved 5-FAM fragment was read at 485/528nm, and the results were interpreted indirectly in terms of the expressions of MMP-9 in the various rat groups.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the tissues of the circle of Willis from all the experimental animals after the completion of experiments, and stored at -80 ^{O}C. The total RNA was isolated by homogenization in TRIzol® reagent. The tissue homogenates were treated with chloroform, and mixed and centrifuged at 12000 g for 15 min at 4 °C for phase separation of RNA. The upper aqueous phase that contained the RNA was treated with isopropanol and incubated for 10 min at 25 °C, followed by centrifugation at 12000 g for 10 min at 4 °C. The RNA pellet thus obtained was washed with 70 % ethanol and its concentration was determined using a spectrophotometer. The extracted RNA was reverse-transcribed to cDNA using an iScript cDNA synthesis kit, and RT-PCR was performed using SYBR master mix. The sequences of the primers used for specific amplification of the genes are shown Table 1. Bio-rad PCR system was used for the experiment, and the fold increase in the gene expression was calculated using the comparative Ct method ($2^{-\Delta\Delta CT}$), with GAPDH gene as endogenous control.

Statistical analysis

One-way analysis of variance (ANOVA) was used for multiple group comparison, followed by post hoc (Bonferroni) test, for determination of statistically significant differences. Student *t*-test was used for comparison between two groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

The IA rat groups had significantly higher mean aneurysm size (68 ± 8 μm) that the control group (mean size, 33.2 ± 3 μm), and significantly higher aneurysm size when compared to the aneurysm sizes observed in the animal groups that were earlier treated with SA (mean size, 25 ± 3.4 μm).

Table 1: Sequences of primers used in PCR

Gene	Primer	Sequence	Annealing
TNF-α	F	CGTGTTCATCCGTTCTCTAC	56
	R	GAAAGCCCATTGGAATCCTT	
IL-6	F	ATGAACAGCGATGATGCACT	58
	R	ATAGCACACTAGGTTTGCCG	
INF-γ	F	GAACTGGCAAAAGGACGGTA	57
	R	ACTTGGCGATGCTCATGAAT	
MMP-2	F	TTTGCTCGGGCCTTAAAAGT	58
	R	TACTCGGACCACTTGTCCTT	
MMP-9	F	CAGCTGACTACGACACAGAC	58
	R	ATTGGCTTCCTCCGTGATTC	
Transgelin	F	AATGGCGTGATTCTGAGCAA	58
	R	GAATTGAGCCACCTGTTCCA	
GAPDH	F	GTGCCAGCCTCGTCTCATA	59
	R	CGTTGATGGCAACAATGTCC	

Indeed, the mean aneurysm size of rats pretreated with SA was comparable to that of control rats. These results are presented in Figure 1 A. The aneurysm enlargement observed in the animals was due to the increased systemic blood pressure which was absent in the SA-treated group which showed values similar to the blood pressure of the control animals (Figure 1 B). Infiltration of immune cells was significantly increased at the end of 4 weeks of aneurysm induction, but it decreased with the treatment of SA, when compared with the IA rats (Figure 1 C).

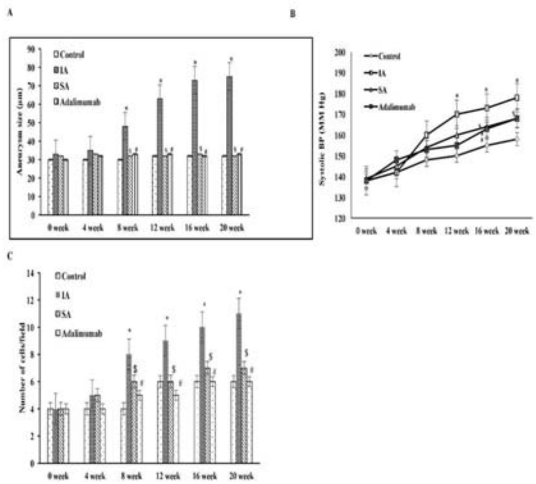

Figure 1: A. The progression of cerebral aneurysm (CA). **B**: Systolic blood pressure. **C**: Infiltration of macrophages, as represented with the number of cells shown in control, IA induced, simvastatin (SA)-treated in the group of rats over a 24-week period (mean ± standard error of mean; n=6). *P < 0.05, vs vehicle-treated control rats; $$p$ < 0.05, SA-treated groups vs rats with cerebral aneurysm; #p < 0.05, adalimumab-treated rats vs CA rats

Furthermore, the activity of MMP-9 was assayed in control, IA, SA-treated, and adalimumab-treated groups using the FRET peptide-based immunocapture assay. There was higher fluorescence of FAM from the IA-group, but the fluorescence was quenched in the SA-treated and the adalimumab-treated groups as in the control animal group, indicating aneurysm in the IA group. The SA pre-treated rats demonstrated reduced MMP-9 activity and expression, indicating that MMP-9 expression was inhibited in this group of rats (Figure 2 A). Results from ELISA (Figure 2B) corroborated the changes in MMP with TIMP expressions (Figure 2 C) which increased in SA-treated group in tandem with decreases in expressions of MMPs in the same group, when compared to the CA group of animals.

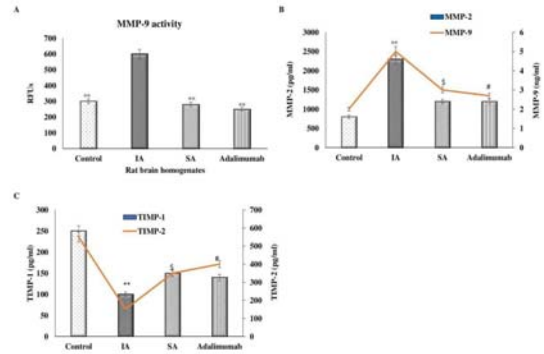

Figure 2: A: Plasma activities of MMP-9 in rat groups after 48 h of induction of aneurysm, as measured using FRET peptide-based immunocapture assay. Total MMP-9 level was recorded for control, aneurysm, SA and Adalimumab-treated aneurysm rats. The addition of SA led to significant reduction of MMP-9 levely. **P < 0.001, vs aneurysm rats (n = 5). **B**: ELISA results for the expressions of MMP-2 and MMP-9. **C**: ELISA results for the expression of TIMP-1 and TIMP-2. #P < 0.05, adalimumab vs CA rats; **p < 0.01, vs vehicle-treated control rats; $$p$ < 0.001, SA-treated groups vs rats with cerebral aneurysm

The levels of expression of inflammatory cytokines measured after treatment with elastase are depicted in Figure 3. The protective influence of SA against aneurysm was determined by measuring the mRNA levels of the inflammatory cytokines and matrix proteins, using real-time quantitative PCR, with GAPDH gene as the control.

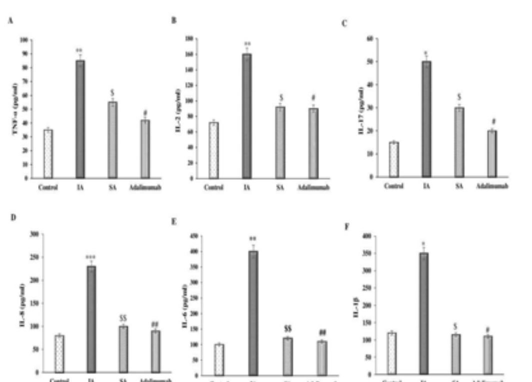

Figure 3: Expression levels of inflammatory cytokines in control, CA-induced and SA-administered rats (**A-F**). Values are expressed as mean ± SEM (n = 6). *P < 0.05, **p < 0.01, ***p < 0.001, vs vehicle-treated control; $$p$ < 0.05, $$$p$ < 0.01, SA-administered rats vs CA rats; #p < 0.05, ##p < 0.01, adalimumab group vs with CA rats

As presented in Figure 4, the mRNA levels of TNF-α, IL-6, and MMP-3, 9 were increased in the IA rats, when compared with the control group. However, the levels of these mRNAs were reduced in rats pre-treated with SA. This was evident in the normalization of the mRNA levels, suggesting that SA protected the rats from aneurysm. The results presented in Figure 4 show significant increases in mRNA expressions of TNF-α, IL-6, IFN-γ in the cerebral aneurysm rats, when compared to the control. The expressions of these mRNA were reduced in the SA-treated rats, indicating that SA protected the rats from developing aneurysm by restoring the mRNAs to near-normal levels after treatment (Figure 4). This provides a mechanism for the treatment of aneurysm. The effect was significant with respect to the expression of transgelin in SA-treated rats (Figure 4 F).

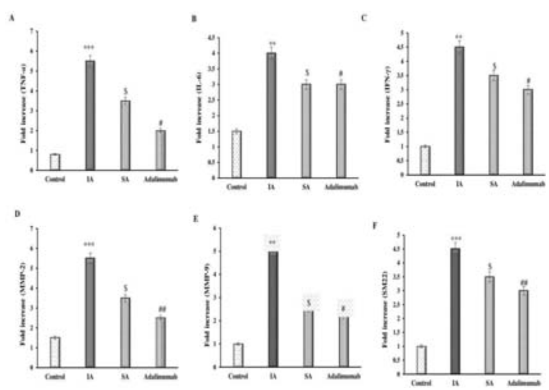

Figure 4: Expression levels of pro-inflammatory cytokines in rats in the control, CA, SA, and adalimumab groups (A - F). The fold-increase represents the change in gene expression relative to that of the housekeeping gene (GAPDH). Results are expressed as mean ± standard error of the mean (SEM) (n = 6). $^{**}P < 0.01$, $^{***}p < 0.001$, vs vehicle-treated control; $\$p < 0.05$, SA-treated groups vs CA rats; $\#p < 0.05$, $\#\#p < 0.01$, adalimumab group vs CA rats

DISCUSSION

The induction of IA in rats using elastase is an established model which was successfully established in this study with injection of the enzyme into the cerebrospinal fluid. All rat groups had IA except the control group. This was evident from the size of the aneurysm developed due to the action of elastase which resulted in the loss of elasticity of the blood vessels in the tissues of the aneurysm walls, leading to significant swelling in the aneurysm area which was absent in the control group [12].

The increase in swelling is an indication of the extent of aneurysm developed over the weeks after elastase injection. Elastase reduced the elasticity of the extracellular matrix around the tissues, but this was not observed in the SA-treated animals and those that were treated with adalimumab. This effect was primarily due to the increased expression of TNF-α which increased the inflammatory lesions in the injected rats. The increased size of the cerebral aneurysm altered the intracranial hemodynamics, thereby increasing the infiltration of inflammatory cells and cytokine release [12,13]. The high systolic blood pressure observed in the aneurysm rats was a result of the altered hemodynamics [12]. However, the systolic blood pressure was reduced by SA pre-treatment as well as treatment with adalimumab.

The infiltration of macrophages was followed by matrix metalloproteinase-catalyzed degradation of ECM components and vascular remodelling of the smooth muscle cells [15]. These are the major contributors in the development of aneurysm. Indeed, MMP-2 and MMP-9 are highly expressed in the aneurysmal tissues of experimental animals, relative to controls [16]. The excessive proteolytic activity of the MMPs degrades the ECM components and initiated vascular remodelling. A study has reported MMP-9 secretion and infiltration of macrophages after vascular inflammation with elastase [3]. The increased secretion of MMP-9 by macrophages was observed only in the rats in which cerebral aneurysm was induced: it and was not observed in the SA-treated group due to increased expressions of tissue inhibitors of metalloproteinase-1 (TIMP-1) [17]. Further changes in the expression of MMP-2 or the expression of other TIMPs were not observed.

The development of cerebral aneurysm has been attributed to release of several cytokines, with TNF-α primarily involved in activation of MMP-9, reduction of the expression of TIMP-1, and the initiation of apoptosis [18]. These findings were not observed in rats treated either with adalimumab or with SA which counteracted the proinflammatory TNF-α-induced apoptosis in the CA area, thereby exerting protective effect [19]. Measurement of pro-inflammatory cytokines after elastase treatment indicated up-regulated expressions of IL-1β, IL-6, and TNFα arising from activated macrophages [20]. In contrast, there were no such increases in pro-inflammatory cytokine expressions in SA-pretreated and adalimumab-treated groups. The anti-inflammatory properties of SA protected the rats against cerebral stroke and its complications [21]. The reduction in IL6 levels is an indication of absence of cerebral injury due to aneurysm. Reduction in IL-1β expression protected the vasculature from degradation, ECM biosynthesis was not inhibited by IL-1β, and progression of the aneurysm was prevented [22].

Apart from its effect on the secretion of cytokines by immune cells, the effect of SA on IL-17 production was determined. The brain lesions observed in aneurysmal animals treated with elastase only (group 2) was associated with increased expressions of IL-17 [23] and IL8. However, SA treatment suppressed the expressions of IL-17 and IL-8, and hence prevented accumulation of monocytes and neutrophils, respectively, at the site of inflammation. Thus, cytokine production and inflammation in the cerebral blood vessels of rats pre-treated with SA were prevented [23].

The generation of TNF-α in stroke is an inflammatory response. Thus, the signal route activated by TNF-α is important in the present study. The brain lesions produced after elastase treatment are positively correlated with TNF-α levels and corresponding increases in TNF-α receptors [24]. This is consistent with the observed increase in TNFR in the aneurysmal rat group. This effect was not observed in the SA-treated group or in the group treated with the TNF-α inhibitor, adalimumab. Thus, the TNF-α receptor axis is important in the development of aneurysm in the cerebrum, and in the subsequent inflammatory events.

CONCLUSION

These results indicate that TNF-α, through binding to its receptor, is important in the development and maturation of aneurysms in elastase-induced aneurysmal rats. Moreover, the findings suggest that SA can potentially be used to control aneurysm and prevent its rupture in the brain.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Congcong Chen, Hongdan Wei, and Jiawen Song conducted the experiments, and Xiaona Zhang designed the experiments and wrote the manuscript. All authors read and agree on the final manuscript.

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