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3-Aminobenzamide protects against cerebral artery injury and inflammation in rats with intracranial aneurysms

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This study aimed to investigate the treatment effects and molecular mechanism of 3-aminobenzamide (3-AB) on intracranial aneurysms (IA). The IA model was established in male Sprague-Dawley (SD) rats and sham group was set up without ligation. The rats were intraperitoneally injected with normal saline in sham and model control groups and 10 mg/kg, 20 mg/kg and 40 mg/kg 3-AB for low, middle and high 3-AB groups for 3 months, respectively. The rates in and blood pressures of caudal artery were measured and anterior cerebral artery and olfactory artery were stained with hematoxylin and eosin (HE) for morphology observation. Besides, the effects of 3-AB on inflammatory cells, macrophages, neutrophils and T cells, were evaluated using immunohistochemistry. Gene expressions of TNF-α, MMP-9, MMP-2, iNOS, TLR4, PARP-1 and p65 were measured using qRT-PCR and the protein levels of TLR4, PARP-1 and p-p65 were evaluated using western blotting. Blood pressures of rats in 3-AB treatment groups were decreased in a dose-dependent manner. The damage of cerebral artery wall was alleviated and the inflammatory cells (macrophages, neutrophils and T cells) were reduced to some extent in 3-AB high-dose groups. The gene expression of TNF-α, MMP-9, MMP-2, iNOS, TLR4, PARP-1 and p65, as well as the protein expression of TLR4, PARP-1 and p-p65 in 3-AB treatment groups were decreased in a dose-dependent manner ($P < 0.01$).3-AB exhibited therapeutic effects on IA through inhibiting the secretions of inflammatory cytokines and MMPs.

1. Introduction

Intracranial aneurysm (IA) is a cerebrovascular disorder caused by local intracranial vascular abnormalities and it is one of the most common humanity cerebrovascular diseases (Nuki et al.2009). IA is characterised by high morbidity and mortality owing to clinical symptoms like neurological dysfunction, hemiplegia, aphasia, blindness and mental disorder (Van Gijn et al.2007). Genetic factors, environmental factors, high blood pressure and hemodynamic changes have been identified to be associated with the formation of aneurysms (Jia et al.2014). The vessel endothelial damage and vessel wall inflammation caused by hemodynamics changes can lead to the occurrence of IA.

Surgical clipping of artery aneurysms and interventional embolization are widely used for IA treatment. Surgical treatments including neck of the aneurysm clipping, ligation, aneurysm wall reinforcement surgery, isolated surgery, aneurysm surgery seam (aneurysmorrhaphy) and intravascular embolization have been used for IA (Seibert et al. 2011). However, there are some risks of aneurysm surgery, such as hyperemia, edema, brain trauma and even surgical aneurysm. Though several agents have been developed as adjuvant therapies and widely used for IA treatment, such as the anti-angiogenic therapy with bevacizumab (Sadeghi et al.2008), epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) of Polycystin 1 (Belz et al.2001), and treatment of primary central nervous system (CNS) vasculitis with rituximab (Salvarani et al.2014), patients with IA are still with poor prognosis. Therefore, it is of great importance to find some new type drugs for effective treatment of IA.

Poly (ADP-ribose) polymerase-1 (PARP-1) is an enzyme that catalyzes post-translational modifications with ADP-ribose polymers. This modification plays pivotal roles in the regulation of various fundamental cellular processes, including DNA repair, chromatin remodeling and transcription (Kirkland 2010). PARP-1 is simultaneously activated in systemic inflammatory response syndrome and myocardial inflammation (Pacher et al. 2007; Hekimoglu et al. 2014). Inflammatory response induced by hemodynamic changes has been certified to precede IA formation. Therefore, it might be very crucial to inhibit the excessive activation of PARP-1 in IA. Since 3-aminobenzamide (3-AB) could inhibit the activity of PARP-1, it has been used in the treatment of multi-organ tissue ischemic injury, experimental subarachnoid hemorrhage vascular disease and inflammation (Czapski et al.2013; Satoh et al.2001). However, the role of 3-AB in IA has not been thoroughly reported. Studies have shown that the deletion of PARP-1 gene could block the toll-like receptor 4 (TLR4)-induced translocation in NF-κB and DNA binding activity thereby affecting the synthetic of downstream factors (such as NO) and the expression of intercellular cell adhesion molecule 1 (ICAM-1) in TLR4/NF-κB signaling pathway (Ahmad et al. 2014; Zerfaoui et al. 2010). There are many kinds of inflammatory cytokines, such as tumor necrosis factor α (TNF-α), involved in the growth and rupture process (Jayaraman et al. 2008). Matrix metalloproteinases 2 (MMP-2) and MMP-9 are crucial for homeostasis of extracellular matrix metabolism and they are closely related to intracranial aneurysm (Li et al.2013). Nitric oxide synthase (iNOS) which is the rate-limiting enzyme in the synthesis of NO also plays an important role in the occurrence of IA (Liaw et al.2014). Therefore, in our study, IA rat model was established to investigate the effects of 3-AB on IA lesions. In addition, the expression levels of TNF-α, MMP-9, MMP-2, iNOS TLR4, p65/p-p65 and PARP-1 were also detected to study the potential signal mechanisms of 3-AB on IA lesions.

2. Investigations and results

2.1. Changes of blood pressure in different groups

No difference in the blood pressure was found among these five groups before surgery ($P > 0.05$, Table 1). Compared with the shame group, the blood pressures of rats in the model control group

Table 1: Blood pressures of rats before surgery and at one, two and three months after surgery (mmHg, $\bar{x} \pm s$)

Group	Before surgery	One month	Two months	Three months
Sham	108 ± 6	113 ± 10	117 ± 8	119 ± 9
Model control	106 ± 8	187 ± 12*#	197 ± 10*#	210 ± 11*#
3-AB low dose	108 ± 7	178 ± 10*#	185 ± 8*#&	196 ± 9*#&
3-AB middle dose	107 ± 10	166 ± 8*#&$	170 ± 10*#&$	181 ± 9*#&$
3-AB high dose	107 ± 9	153 ± 11*#&$@@	162 ± 8*#&$@	170 ± 7*#&$@@

*: $P < 0.01$, compared with the blood pressures before surgery; #: $P < 0.01$, compared with the sham group; &: $P < 0.01$, compared with the model control group; $: $P < 0.01$, compared with the low dose group; @: $P < 0.05$, @@: $P < 0.01$, compared with the middle dose group.

and 3-AB treatment groups were significantly increased one, two and three months after surgery ($P < 0.01$, Table 2). Two and three months postoperatively, the blood pressures of rats in the low dose group were obviously lower than those of rats in the model control group ($P < 0.01$). The blood pressures of rats in the middle dose group were significantly reduced as compared with those in the low dose group one, two and three months after surgery ($P < 0.01$). The blood pressures of rats in the high dose group were decreased to 153±11 mmHg, 162±8 mmHg, and 170±7 mmHg, with significant differences when compared with those in the middle dose group ($P < 0.01$). Though the blood pressures of rats in the high dose group were still higher than those of rats in the sham group, the blood pressures of rats in 3-AB treatment groups were decreased in a dose-dependent manner compared with those in the model control group.

2.2. The effect of 3-AB on morphology of IA

The rat model of IA in ACA/OA junction was successfully induced. No aneurysm occurred in the sham group. The incidence of IA was 66.7 % (6/9) in model control group and 54.8 % (17/31) in 3-AB treatment groups (6/10, 6/10 and 5/11 for low, middle and high dose group, respectively). According to the HE staining, ACA-OA cerebral artery wall of rats in sham group had no obvious defects, convex or phenomenon of vessel wall injury (Fig. 1A). In the IA model control group, there was different degree of stratification in cerebral artery wall, besides, the smooth muscle cells decreased and obvious inward depressing exited in vascular wall (Fig. 1B). However, the damage of cerebral artery wall was keeping alleviated and the inward depressing became weaker and weaker to some extent with the increasing of 3-AB concentration (Fig. 1C-E).

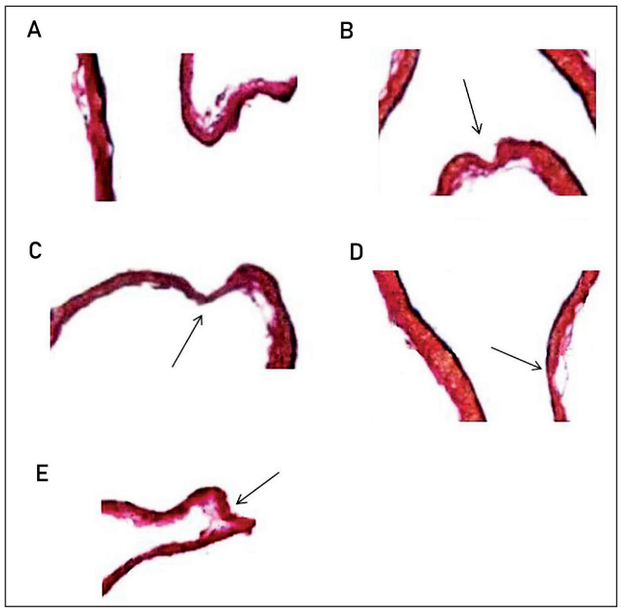

Fig. 1: Effects of 3-AB on morphology of intracranial aneurysms. Hematoxylin and eosin (HE) staining of anterior cerebral artery and olfactory artery (ACA-OA) bifurcations was conducted to observe the histological changes (200 ×). A: sham group (n=12); B: model control group (n=6); C: 3-AB low dose group (10 mg/kg) (n=6); D: 3-AB middle dose group (20 mg/kg) (n=6); E: 3-AB high dose group (40 mg/kg) (n=5).

2.3. 3-AB obviously reduced the proportion of inflammatory cells

The effects of 3-AB on inflammatory cells, macrophages, neutrophils and T cells, were evaluated by detecting CD68, CD11b and CD3 expression using immunohistochemistry, respectively (Fig. 2). Compared with the sham group, macrophages were predominantly infiltrated in aneurysmal walls and the number of infiltrating macrophages (CD68) was increased in IA model. Meanwhile, small amounts of neutrophils (CD11b) and T cells (CD3) were also accumulated in aneurysmal walls in IA model. However, 3-AB treatment obviously reduced the proportion of these three kinds of inflammatory cells in IA model as a dose-depended manner.

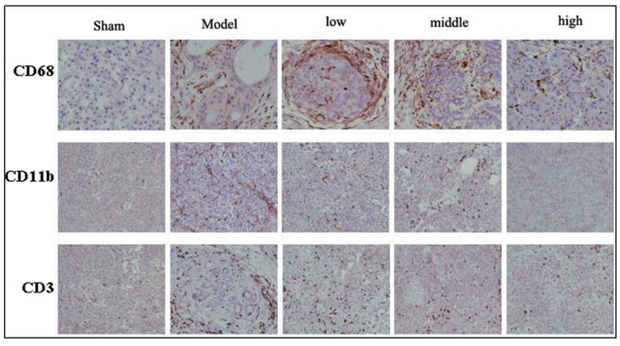

Fig. 2: 3-AB obviously reduced the proportion of inflammatory cells. The effects of 3-AB on inflammatory cells, macrophages, neutrophils and T cells, were evaluated by detecting CD68, CD11b and CD3 expression using immunohistochemistry, respectively (100 ×). Sham group (n=12); Model control group (n=6); 3-AB low dose group (10 mg/kg) (n=6); 3-AB middle dose group (20 mg/kg) (n=6); 3-AB high dose group (40 mg/kg) (n=5).

2.4. 3-AB significantly inhibited the mRNA levels of inflammatory cytokines

The changes of TNF-α, iNOS, MMP-9 and MMP-2 mRNA levels in different groups are shown in Fig. 3. According to the results, the mRNA levels of TNF-α, iNOS, MMP-9 and MMP-2 were significantly higher in IA model control group than those in sham group ($P < 0.01$). The low, middle and high doses of 3-AB all remarkably suppressed the expression levels of TNF-α, iNOS, MMP-9 and MMP-2 in IA model ($P < 0.01$) and a dose-dependent manner was also certified ($P < 0.01$).

2.5. 3-AB inhibited inflammatory cytokines expression via TLR4 and NF-κB pathways

The mRNA of TLR4, PARP-1 and p65 as well as protein expression of TLR4, PARP-1 and phosphorylated p65 in each group were measured by qRT-PCR and western blot, respectively (Figs. 4 and 5). The expressions of these factors were significantly enhanced in the model control group both in gene and protein levels ($P < 0.01$). As expected, 3-AB significantly reduced the mRNA and protein levels of TLR4, PARP-1 and p65/p-p65 in a dose-dependent manner ($P < 0.05$).

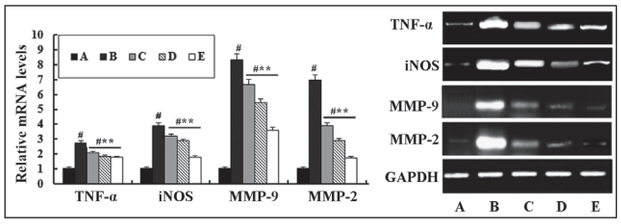

Fig. 3: Effects of 3-AB on mRNA expressions of TNF-α, iNOS, MMP-9 and MMP-2. A: sham group (n=12); B: model control group (n=6); C: 3-AB low dose group (10 mg/kg) (n=6); D: 3-AB middle dose group (20 mg/kg) (n=6); E: 3-AB high dose group (40 mg/kg) (n=5). The effects of 3-AB on mRNA expressions of TNF-α, iNOS, MMP-9 and MMP-2 were detected using quantitative real-time polymerase chain reaction (qRT-PCR) (left) and agarose gel electrophoresis (right). Columns, mean (n=3); bars, SD; #P<0.01 vs sham group, *P<0.05 vs model control group, **P<0.01 vs model control group.

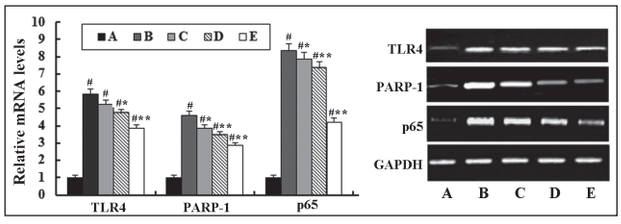

Fig. 4: The effects of 3-AB on mRNA expressions of TLR4, PARP-1and p65. A: sham group (n=12); B: model control group (n=6); C: 3-AB low dose group (10 mg/kg) (n=6); D: 3-AB middle dose group (20 mg/kg) (n=6); E: 3-AB high dose group (40 mg/kg) (n=5). The effects of 3-AB on mRNA expressions of TLR4, PARP-1 and p65 were detected using quantitative real-time polymerase chain reaction (qRT-PCR) (left) and agarose gel electrophoresis (right). Columns, mean (n=3); bars, SD; #P<0.01 vs sham group, *P<0.05 vs model control group, **P<0.01 vs model control group.

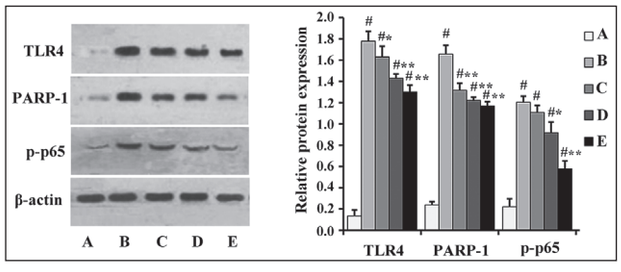

Fig. 5: The effects of 3-AB on protein expressions of TLR4, PARP-1 and p-p65. A: sham group (n=12); B: model control group (n=6); C: 3-AB low dose group (10 mg/kg) (n=6); D: 3-AB middle dose group (20 mg/kg) (n=6); E: 3-AB high dose group (40 mg/kg) (n=5). Columns, mean (n=3); bars, SD; #P<0.01 vs sham group, *P<0.05 vs model control group, **P<0.01 vs model control group.

3. Discussion

Hemodynamic changes, inflammation and vascular wall damage are the major pathophysiological factors for the occurrence of IA. Thus, seeking the treatment to inhibit these adverse factors is of great importance. In our study, we aimed to investigate the treatment effects and molecular mechanism of 3-AB on IA. There are two main types of animal models of IA: one is established using the rapid injection of trypsin to cause the damage of vessel wall; the other one is established using one side carotid artery ligation, bilateral renal artery branch ligation and high-salt diet to build an IA model which has been widely used in basic research since it is much similar to the pathological features of human IA (Morimoto et al. 2002; Fukuda et al. 2000). In this study, we successfully established a renal hypertension induced aneurysm model by ligation of the left carotid artery and bilateral renal artery posterior branch. Many studies have confirmed that an increase in blood flow can lead to elevated hemodynamic stress and impinging flow may play important roles in the pathogenesis of IA (Singh et al. 2010; Tobe et al. 2014). The blood pressures of rats in the model control group were significantly enhanced one, two and three months after the surgery, as compared with those in the sham group in our study. Though the blood pressures of rats in the high dose group were still higher than the normal level, the blood pressures of rats in 3-AB treatment groups were decreased in a dose-dependent manner. Moreover, the pathological changes also revealed that the damage of cerebral artery wall in 3-AB high-dose group was alleviated to some extent as inward depressing in the arterial wall was obviously weakened. Therefore, it could be concluded that the 3-AB might have some effective treatments on IA.

To investigate the regular mechanisms of 3-AB, the macrophages, neutrophils and T cells were evaluated by detecting CD68, CD11b and CD3 expression using immunohistochemistry, respectively. 3-AB obviously reduced the infiltrating macrophages and inflammatory cells proportion induced by IA as a dose-depended manner, which suggested the anti-inflammatory infiltration effects of 3-AB on IA. We further measured the expression levels of some inflammatory-related genes and metalloproteinases. The results indicated that the gene levels of TNF-α, MMP-9, MMP-2, iNOS, TLR4, PARP-1 and p65, as well as protein expression of TLR4, PARP-1 and p-p65 in 3-AB treatment groups were significantly decreased compared with those in the model control group. These results are in consistence with previous studies, which also revealed that 3-AB could reduce the expression of inflammatory mediators and improve the histological inflammatory damage in several diseases, such as arthritis, sepsis and wound healing (Deng et al. 2013; El-Hamoly et al.2014). Furthermore, 3-AB, a PARP-1 inhibitor, is considered as a mechanistic target for drug development in various forms of inflammation, ischemia and cancer therapy (Sethi et al.2014). Meanwhile, it has been reported that iNOS was involved in the generation process of aneurysms (Zhang et al.2003). TNF-α not only shows cytotoxicity of selective killing tumor cells but also plays an important role in the intracellular signal transduction, infection and inflammatory response. It has been also reported that TNF-α was involved in the inflammatory response of IA and finally activated the NF-κB pathway (Aoki et al.2014). In our study, iNOS and TNF-α had a high expression value in model control group and then were dose-dependently inhibited by 3-AB treatment, which suggested that 3-AB might have treatment effects on IA by inhibiting the expressions of TNF-α and iNOS.

MMPs are hydrolytic enzymes which destruct the extracellular matrix of zinc and calcium-dependent protein and MMPs are divided into collagenase, stromelysin and gelatinase. 3-AB has been reported to stimulate angiogenesis by regulating the expression of MMP2 to potentiate anticancer therapy (Caldini et al.2011). Wu et al. (2014) demonstrated that 30 mg/kg of 3-AB could protected the blood-brain barrier against hyperpermeability by downregulating MMP-9 expression. In addition, increased expression of MMP-2 and MMP-9 may cause injury to internal elastic membranes which can maintain the toughness of cerebral artery walls, and this injury is one of the important reasons for the formation of IA (Yan et al.2013). In our study, the expression of MMP-2 and MMP-9 was increased in model control group and then decreased by 3-AB treatment as a dose-dependent manner. These results suggested that 3-AB might relieve the damage of IA by downregulating the expressions of MMP-2 and MMP-9.

Furthermore, we also detected the TLR4 expression level since it is an important member of Toll-like receptor family and it could recognize pathogen-associated molecular patterns. Besides, TLR4 plays important roles in the activation of innate immunity. When the body tissues were damaged by external stimulation, the expression of TLR4 was elevated, which further activated P38-MAPK, JNK, NF-κB and several other signal pathways and ultimately induced the production of a variety of inflammatory cytokines (Bowie and Haga 2005). NF-κB is an important gene transcription regulatory factor in the downstream of TLR4-mediated signaling pathways and participates in the process of chronic inflammation and tissue damage. Meanwhile, treatment with 3-AB could attenuate lung-kidney crosstalk partly by inhibiting proinflammatory cytokines depended on NF-κB (Si et al.2013). 3-AB significantly reduced p65 nuclear accumulation and subsequently downregulated the expression of ICAM-1 and iNOS in response to TLR4 stimulation in LPS-treated smooth muscle cells (Zerfaoui et al.2010). TLR4

Table 2: Primer sequences of TNF-a, iNOS, MMP-9, MMP-2, TLR4, PARP-1 and NF-κB.

Gene	Sequences of primers	Ta	Length
TNF-a	5'- TAC TGA ACT TCG GGG TGA TTG GTC C-3' (forward)	60°C	692 bp
	5'-CAG CCT TGT CCC TTG AAG AGA ACC-3' (reverse)		
iNOS	5'-CAG CCC TCA GAG TAC AAC GAT -3' (forward)	51°C	364 bp
	5'-CAG CAG GCA CAC GCA ATG AT -3' (reverse)		
MMP-9	5'-GTT CCC GGA GTG AGT TGA-3' (forward)	48°C	362 bp
	5'-TTT ACA TGG CAC TGC AAA GC-3' (reverse)		
MMP-2	5'-CCA CTG CCT TCG ATA CAC-3' (forward)	57°C	150 bp
	5'-GAG CCA CTC TCT GGA ATC TTC AAA-3' (reverse)		
TLR4	5'-TGG ATA CGT TTC CTT ATA AG-3' (forward)	56°C	623 bp
	5'-GAA ATG GAG GCA CCC CTT C-3' (reverse)		
PARP-1	5'-TTG AAA AAG CCC TAA AGG CTC A-3' (forward)	58°C	156 bp
	5'-CTA CTC GGT CCA AGA TCG CC-3' (reverse)		
NF-κB	5'-AGG TCG GTG TGA ACG GAT TTG-3' (forward)	56°C	364 bp
	5'-TGT AGA CCA TGT AGT TGA GGT CA-3' (reverse)		

was also been reported to be involved in the occurrence of IA and activated NF-κB expression (Aoki et al.2010). In this present study, the expression of p65 and TLR4 showed a dose-dependent decrease after 3-AB treatment in IA model. Thus, we hypothesized that 3-AB inhibited the secretions of inflammatory cytokines and matrix metalloproteinases by regulating TLR4-mediated NF-κB signaling pathway.

In conclusion, our study demonstrated that 3-AB exhibited beneficial effects on IA probably by inhibiting the production of inflammatory cytokines and MMPs. These findings suggest that 3-AB could be developed as a novel approach to prevent the occurrence of IA.

4. Experimental

4.1. Animals

Male Sprague-Dawley (SD) rats (7 weeks and weighing 200±20 g) provided by the Animal Center of Chinese Academy of Sciences were raised in the specific pathogen free (SPF) barrier system at constant temperature (25±1 °C) with the relative humidity of 40 – 60 % under a 12:12 light/dark cycle. The rats were fed with sterilized water and forage freely. This study was conducted strictly in accordance with the recommendations of the national guidelines for the use of animals. All the experiments were also approved by the local ethics committee of East Hospital, Tongji Medical College (permit number 2011-008). All protocols were rapidly performed under pentobarbital sodium anesthesia and all efforts were made to minimize the suffering of animals.

4.2. Intracranial aneurysm animal model and treatments

The IA animal model was built according to the modified method described by Hashimoto et al. (Aoki et al.2014). Briefly, the rats were firstly anesthetized with intraperitoneal injection of 50 mg/kg pentobarbital sodium. Then, the right common carotid artery and the posterior branches of both renal arteries were ligated to establish the IA model. Twelve SD rats were selected as controls and underwent exactly the same procedure (anesthetized and surgery) as the IA animals except for the ligation. One week after the surgery, all the rats were fed with 0.5 % saline instead of drinking water.

From the day after surgery, rats received daily intraperitoneal injections of 3-AB (Sigma, USA) at doses of 10 mg/kg (low dose group, n = 12), 20 mg/kg (middle dose group, n = 12) and 40 mg/kg (high dose group, n = 12) for 3 months, respectively. The rats in the sham group (n = 12) and model control group (n = 12) were intraperitoneally injected with an equal volume of normal saline. Blood pressure of caudal artery for rats in each group was measured using rat-tail pressure measuring instrument (Kent, USA) preoperatively and at 1, 2 and 3 months postoperatively.

4.3. Morphology observation of IA

Three months after surgery, rats were deeply anesthetized and perfused with phosphate buffer solution (PBS, 0.1 mol/L, pH 7.4) followed by 4 % paraformaldehyde solution. The brain was removed to obtain anterior cerebral artery and olfactory artery (ACA-OA) bifurcations for histologic examination. The stripped ACA-OA bifurcations were fixed in 4 % formaldehyde, dehydrated with concentration gradient of ethanol, and embedded in paraffin. Then the specimens were cut into sections as the thickness about 4 μm and stained with hematoxylin and eosin (HE). Finally, the pathological changes were observed under the light microscopy (Nikon, Japan). Aneurysm refers to an outward bulging or an inward depressing of the arterial wall detected by light microscopy. Slight aneurysmal change refers to a focus of infection with discontinuous internal elastic lamina but without apparent outward bulging or inward depressing in the arterial wall. Advanced aneurysm refers to an obvious outward bulging or inward depressing in the arterial wall with a fragmentation or disappearance of the internal elastic lamina. Three independent researchers who were blind to the experiment grouping design assessed the histopathological changes. Inter-examiner agreement was assessed using weighted coefficient Kappa and a high Kappa score higher than 0.9 was obtained, which indicated a high inter-observer agreement.

4.4. Immunohistochemistry

An immunohistochemistry assay was conducted to detect the levels of macrophages (CD68), neutrophils (CD11b) and T cells (CD3) using streptomyces avidin - peroxidase link (SP) method. Briefly, paraffin sections were equilibrated to room temperature, dewaxed using xylene and rehydrated with different concentrations of ethanol. After antigen retrieval and washed with PBS, 50 μL normal non-immune serum was added at room temperature for 10 min. The sections were incubated with rabbit anti-human primary antibodies of CD68,CD11b and CD3 (1:100; AbD Serotec, Kidlington, UK) at 4 °C overnight, followed by secondary antibodies at room temperature for 10 min, 50 μl SP solution at room temperature for 10 min. After washed with PBS, they were stained with 100 μL DAB solution and counterstained with hematoxylin. Finally, the sections were observed using amicroscope (Olympus, Japan) and five random images were taken for each section.

4.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

The cerebral artery tissue (ACA/OA bifurcation; 100 mg) from the rat was collected for RNA extraction using Trizol reagent (Invitrogen, USA) and cDNA was synthesized by cDNA synthesis kit (Promega, USA) according to the manufacturer's instructions. The qRT-PCR reaction was carried out in a reaction volume of 25 μl containing 12.5 μl SYBR Green permix Ex TaqTM, 2 μl of cDNA reaction as a template, 1 μl forward primer, 1 μl reverse primer, and 8.5 μl of sterile deionized water. The PCR conditions were as follows: 95 °C for 1 min followed by 40 cycles of 95 °C for 30 s, annealing for 30 s and 72 °C for 1 min. The sequences of PCR primers, annealing temperature (Ta) and product length of target genes (TNF-a, iNOS, MMP-9, MMP-2, TLR4, PARP-1 and p65) are shown in Table 2. The relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. Besides, the PCR products of TNF-a, iNOS, MMP-9, MMP-2, TLR4, PARP-1 and p65 were also analyzed using 1.5 % agarose gel electrophoresis.

4.6. Western blotting assay

A total of 100 mg cerebral artery tissue samples from rats were treated with RIPA lysis buffer (Beyotime, China) on ice for 45 min, and the samples were then centrifuged at 12000 × g for 10 min at 4 °C. The supernatant was quantified and equal amount of protein (20 μg) in each experimental group was separated by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated protein bands were transferred onto polyvinylidene fluoride (PVDF) membrane and then blocked with 3 % bovine serum albumin (BSA) for 1 h at room temperature. After blocking, the membranes were incubated with primary antibodies of TLR4 (1:1000; Santa Cruz, USA), PARP-1 (1:100; Sigma, USA) and p65 NF-κB (1:1000; Cell Signaling Technology, USA) overnight at 4 °C, respectively. The membranes were then washed

three times by Tris-buffered saline plus Tween 20 (TBST) solution and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:1000; Santa Cruz, USA) for 1 h at room temperature. Finally, the bands were visualized with chemiluminescence. β-Actin was used as an internal control.

4.7. Statistical analysis

All the data were shown as mean±standard deviation (SD) and the statistical analysis of data was performed with SPSS 19.0 software (SPSS Inc, Chicago, IL). Bonferroni test of one-way analysis of variance (ANOVA) was used for the comparison of different samples. A value with *P* less than 0.05 was considered as statistically significant difference.

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Conflicts of interest: None declared.

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