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ORIGINAL PAPER

Aspirin Inhibits Degenerative Changes of Aneurysmal Wall in a Rat Model

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Abstract Aneurysmal subarachnoid hemorrhage still has a high mortality and morbidity despite notable advances in surgical approaches to cerebral aneurysm (CA). We examined the role of aspirin in vascular inflammation and degeneration. CA was induced in male Sprague–Dawley rats by ligating left common carotid artery and bilateral posterior renal arteries with or without aspirin treatment. The right anterior cerebral artery/olfactory artery (ACA/OA) bifurcations were stripped and assessed morphologically after Verhoeff's Van Gieson staining. Blood sample was obtained to examine circulating $CD34^+$ $CD133^+$ endothelial progenitor cells (EPCs), platelet aggregation and platelet counts. Macrophages infiltration in aneurysmal wall was evaluated by immunohistochemistry. Expression of matrix metalloproteinase-2 and 9 (MMP-2 and 9), nuclear factor kappa B (NF-κB), macrophage chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) was examined by RT-PCR. 2 months after CA induction, surgically treated rats manifested aneurysmal degeneration in ACA/OA bifurcations. Aspirin-treated rats exhibited a significant decrease in degradation of internal elastic lamina (IEL), medial layer thinning, CA size and macrophages infiltration with reduced expression of MMP-2 and 9 compared with rats in the CA group. RT-PCR demonstrated that the upregulation of NF-κB, MCP-1 and VCAM-1 after CA induction was reversed by aspirin treatment. Aspirin treatment following CA induction increased circulating EPCs to near control levels and reduced platelet aggregation without changing platelet counts. The evidence suggested that aspirin significantly reduced degeneration of aneurysm walls by inhibiting macrophages-mediated chronic inflammation and mobilizing EPCs.

Keywords Aspirin · Cerebral aneurysm · Endothelial progenitor cells · Inflammation · Platelet

Abbreviation

CA	Cerebral aneurysm
EPCs	Endothelial progenitor cells
PRP	Platelet-rich plasma
PPP	Platelet-poor plasma
MNCs	Mononuclear cells
ACA/OA	Anterior cerebral artery/olfactory artery
IEL	Internal elastic lamia
SMCs	Smooth muscle cells
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
NF-κB	Nuclear factor kappa B
MCP-1	Macrophage chemoattractant protein-1
VCAM-1	Vascular cell adhesion protein-1
ANOVA	One-way analysis of variance
ECs	Endothelial cells
PGE2	Prostaglandin E2
EP2	Prostaglandin E receptor 2
ICAM	Intercellular adhesion molecule

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PECAM	Platelet endothelial cell adhesion molecule
TNF-α	Tumor necrosis factor-α
ROS	Reactive oxygen species

Introduction

Cerebral aneurysm (CA) rupture is the leading cause of subarachnoid hemorrhage which comprises 5–10 % of all strokes [1]. CA is characterized by chronic inflammation and abnormal vascular remodeling [2–4]. Following endothelial dysfunction at bifurcations caused by sustained hemodynamic shear stress, inflammation is activated and aggravates the impairment of vessel wall [5]. Chyatte et al. [6] reported the frequent presence of macrophages, T lymphocytes and B lymphocytes in aneurysm tissue. Similar findings were reported by Kataoka et al. [7] that inflammatory cells infiltration was found in 50 % of unruptured CA (10/20) and 100 % of ruptured CA (40/40). Accordingly, modulation of the inflammatory process may be a promising treatment option to prevent aneurysmal degeneration. Currently, microsurgical clipping and endovascular coiling are the main therapeutic options for unruptured CA. However, due to the unnegligible complications caused by these invasive surgical procedures, it is meaningful to seek a noninvasive medical treatment, as a powerful treatment supplement, to limit aneurysm growth and rupture.

Aspirin, a well-established antiplatelet and nonsteroidal anti-inflammatory drug, is widely used for secondary prevention of cardiovascular and cerebrovascular disease [8]. Hasan et al. [9] found that aspirin tends to reduce the risk of CA progression to rupture based on the prospective cohort aspect of the International Study of Unruptured Intracranial Aneurysms. In addition, Hasan et al. [10] reported that aspirin reduced inflammatory response in the walls of human CA. However, the precise protective mechanism has not been clarified in their studies and there is no direct experimental evidence supporting the epidemiologic findings. Endothelial progenitor cells (EPCs) play a critical role in neovasculogenesis and maintenance of vascular integrity [11–13]. Our previous study showed that the circulating level of EPCs was reduced in CA patients and rats, which correlates with the pathogenesis of CA [11, 14]. Aspirin has been reported to increase the proangiogenic potential of EPCs [15]. Thus, we hypothesized that aspirin could confer protection against degeneration of aneurysmal wall by a combined effect of modulating inflammation and mobilizing EPCs.

Materials and Methods

Preparation of CA Model and Experimental Groups

Animals were used in compliance with the US National Institutes of Health's Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Tianjin Medical University General Hospital. 7-week-old male Sprague–Dawley rats were obtained from Military Medical Academy of China. CA induction was performed as previously described [14, 16]. Briefly, animals were anesthetized with 10 % chloral hydrate (0.3 ml/100 g, i.p.) and underwent surgical procedures to expose and ligate left common carotid artery and posterior branches of bilateral renal arteries. Then rats were housed in institutional standard facilities with free access to a high-salt diet containing 8 % sodium chloride for 2 months. The

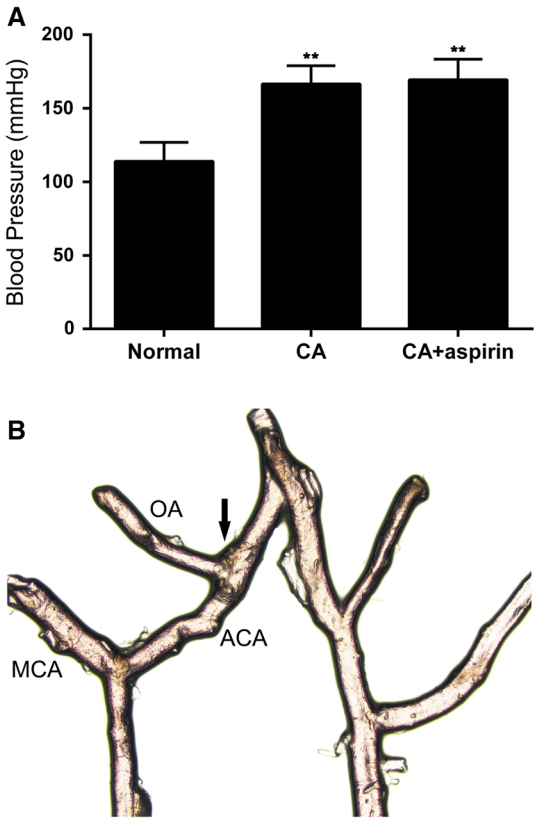

Fig. 1 Blood pressure monitoring and a representative view of cerebral arteries. **a** Blood pressure was elevated 2 months after CA induction, and showed no significant difference between CA group and CA + aspirin group. Results are presented as the mean ± SD (n = 10/group). $^{**}P < 0.01$ versus normal group. **b** A schematic view of cerebral arteries branches. *Black arrow* indicates aneurysm formation at the right ACA/OA bifurcation. Magnification ×4. ACA, anterior cerebral artery; OA, olfactory artery; MCA, middle cerebral artery

blood pressure of unanesthetized rats (n = 10/group; Fig. 1a) was measured using a non-invasive tail cuff method (Kent Scientific Corporation, Torrington, CT, USA).

To clarify the role of aspirin in degenerative changes of aneurysmal walls, rats (n = 20/group) were randomly divided into blank control group (not undergoing surgical procedures), CA group (untreated control) and CA + aspirin group (dissolved aspirin in their drinking water, concentration: 0.067 mg/ml, purchased from Bayer Health Care, Beijing, China). Aspirin was applied from 24 h post-surgery and continued every day for 2 months before sacrifice. A water consumption of 15 ml/100 g/24 h was assumed providing an estimated daily intake of 10 mg/kg aspirin per rat [17]. This dose was chosen because it was previously reported to play an anti-inflammatory role in the brain [18].

Platelet Aggregation Measurements

Whole blood (1.8 ml) (n = 6/group) was collected in 3.2 % sodium citrate (volume ratio of citrate to blood is 1:9) from deeply anesthetized rats through the abdominal aorta using a 22-G needle. Platelet-rich plasma (PRP) was prepared by centrifugation at 70*g* for 15 min. The PRP was then transferred to fresh plastic tubes. Platelet-poor plasma (PPP) was obtained by centrifuging the remaining blood at 2000*g* for 10 min. Platelet counts of PRP were normalized to 2.5×10^8/mL with homogeneous PPP. Aggregation was initiated by adding 250 μg fibrillar type I collagen (Helena Laboratories, Beaumont, TX, USA) to 25 ml PRP to get a final concentration of 10 μg/ml. Platelet aggregation was continuously monitored for 5 min at 37 °C on an optical aggregometer (AggRAM, Helena Laboratories) with constant stirring at 1200 rpm. The platelet counts were measured on a Sysmex XT-1800i Hematology Analyzer (Sysmex Corporation, Kobe, Japan).

Assessment of Circulating EPCs Level

Circulating EPCs were examined using flow cytometry according to our previous study [14]. Briefly, 1.5 ml whole blood (n = 6/group) was collected through the retro-orbital venous plexus and mononuclear cells (MNCs) were isolated using a Ficoll gradient centrifugation method. The isolated MNCs were washed twice with phosphate buffer solution and subsequently incubated with 1 ml blocking buffer for 30 min at 4 °C. To identify circulating EPCs, MNCs were incubated with PE-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, Shanghai, China) against CD34 (stem cell lineage-marker) and FITC-conjugated antibody (Abcam, Cambridge, MA, USA) against CD133, which was recognized as a marker of immature EPCs with high differentiating capability [19, 20]. EPCs were defined as double positive cells and quantified using flow cytometry (BD FACS ArisTM, Beckman-Dickinson, San Jose, CA, USA).

Vessel Harvest and Verhoeff's Van Gieson Staining

2 months after CA induction, rats were euthanized by deep anesthesia and cervical dislocation, and then perfused transcardially with 50 ml phosphate buffered saline and 4 % paraformaldehyde. The right anterior cerebral artery/olfactory artery (ACA/OA) bifurcations (Fig. 1b) were stripped, washed, dehydrated in a graded ethanol series, paraffin-embedded and cut into 5 μm sections through artery vertical axis. Verhoeff's Van Gieson staining (n = 8/group) was performed to assess degenerative changes of vascular walls. Evaluation system included the degradation of internal elastic lamia (IEL), aneurysm size and thickness of medial smooth muscle cells (SMCs) layer, which were examined by an independent blinded investigator. The degradation of IEL was graded as: 0 = continuous IEL, 1 = fragmented IEL and 2 = completely disappeared IEL [21]. The aneurysm size was quantified as an average of the maximal longitudinal diameter and the maximal transverse diameter while thickness of medial layer was determined by calculating the ratio of the minimal thickness in an aneurysmal wall to the media thickness in its surrounding normal arterial wall.

Immunohistochemistry

Infiltration of macrophages in aneurysmal walls was assessed by immunohistochemistry (n = 6/group). All sections were blocked with 5 % goat serum (30 min, 37 °C) before incubation with primary antibodies. Then sections were triple-labeled with goat polyclonal anti-CD68 antibody (macrophages; Santa Cruz Biotechnology), rabbit polyclonal anti-smooth muscle α-actin antibody (SMCs; Santa Cruz Biotechnology) and DAPI (nuclei; Sigma-Aldrich, St. Louis, MO, USA) over night at 4 °C, followed by corresponding TRITC or FITC-conjugated secondary anti-goat and anti-rabbit IgG (Sigma-Aldrich) 1 h at room temperature. The number of CD68-positive cells in aneurysmal walls was counted by a blinded observer in a 100-μm-square field via a fluorescence microscopy (IX81, Olympus, Tokyo, Japan).

RNA Isolation and RT-PCR

Total RNA from the whole Willis ring was isolated using the Trizol reagent (Life Technologies, Grand Island, NY, USA). RT-PCR was performed using All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA) and All-in-One™qPCR Mix (GeneCopoeia) (n = 6/group).

The primer sets used were: forward 5′-CTGATAACCT GGATGCAGTCGT-3′, reverse 5′-CCAGCCAGTCC GATTTGA-3′ for matrix metalloproteinase-2 (MMP-2); forward 5′-TTCAAGGACGGTCGGTATT-3′, reverse 5′-CTCGAGCCTAGACCCAACTTA-3′ for matrix metalloproteinase-9 (MMP-9); forward 5′-ACGATCTGTTTCCCC TCATC-3′, reverse 5′-TGCTTCTCTCCCCAGGAATA-3′ for nuclear factor kappa B (NF-κB); forward 5′-CCTCCA CCACTATGCAGGTCTC-3′, reverse 5′-GCACGTG GATGCTACAGGC-3′ for macrophage chemoattractant protein-1 (MCP-1); forward 5′-GCGAAGGAAACTGGA GAAGACA-3′, reverse 5′-ACACATTAGGGACCGTGC AGTT-3′ for vascular cell adhesion protein-1 (VCAM-1); forward 5′-AAGAAGGTGGTGAAGCAGGC-3′, reverse 5′-TCCACCACCCTGTTGCTGTA-3′ for GAPDH. The reaction profile was 95 °C for 10 min followed by 40 cycles at 95 °C for 20 s, 53 °C for 20 s and 72 °C for 20 s. The mRNA content was determined as a relative value normalized to GAPDH mRNA level. All experiments were repeated three times.

Statistical Analysis

The IEL score was analyzed by 2 × 3 contingency table with Fisher's exact test. Other data were presented as mean ± SD. Student's *t* test was used to determine the differences between two groups while one-way analysis of variance (ANOVA) followed by Bonferroni correction was used for the comparison of three groups. $P < 0.05$ was considered statistically significant.

Results

Effect of Aspirin on Platelet Aggregation and Platelet Counts

No difference was found in platelet aggregation between normal group and CA group (81.8 % ± 3.1 % versus 80.1 % ± 2.7 %, n = 6; $P = 0.68$; Fig. 2a). Compared with the CA group, aspirin treatment significantly reduced platelet aggregation (80.1 % ± 2.7 % versus 40.5 % ± 6.4 %, n = 6; $P < 0.01$). The platelet counts showed no significant differences among groups (normal group: 706.34 ± 116.57 × 10^9/L; CA group: 713.48 ± 126.94 × 10^9/L; CA + aspirin group: 720.63 ± 113.87 × 10^9/L; Fig. 2b).

Aspirin Enhanced EPCs Mobilization

To examine the effects of aspirin on EPCs mobilization, we detected the level of circulating $CD34^+CD133^+$ EPCs 2 months after CA induction using flow cytometry. EPCs level was significantly decreased in CA rats as compared with that in normal rats (34.3 ± 8.7/2 × 10^5 MNCs vs 65.5 ± 8.2/2 × 10^5 MNCs, n = 6; $P < 0.01$; Fig. 3d). Aspirin treatment significantly increased circulating EPCs level compared with CA group (61.5 ± 6.4/2 × 10^5 MNCs vs 34.3 ± 8.7/2 × 10^5 MNCs, n = 6; $P < 0.01$).

Aspirin Reduced Aneurysmal Degeneration

Aspirin treatment following CA induction reduced the IEL score (n = 8; Pearson $\chi^2 = 8.500$, DF = 2, $P = 0.019$; Fig. 4d). The media thinning was significantly decreased in the CA + aspirin group compared with that in the CA group (0.62 ± 0.12 vs 0.46 ± 0.06, n = 8; $P < 0.01$; Fig. 4e). The aneurysm size was also smaller in the CA + aspirin group than that in the CA group (43.9 ± 6.7 μm vs 58.2 ± 8.8 μm, n = 8; $P < 0.01$; Fig. 4f).

Aspirin Reduced Inflammatory Response in Aneurysmal Walls

Immunohistochemistry revealed that macrophages infiltration into the aneurysmal walls was reduced in the CA + aspirin group compared with the CA group (2.5 ± 0.7cells/100 μm^2 vs 4.5 ± 1.4 cells/100 μm^2, n = 6; $P < 0.01$; Fig. 5d). In the CA group, the mRNA expression of MMP-2, MMP-9, NF-κB, MCP-1 and VCAM-1 were upregulated in the aneurymal walls compared with that in the normal group (MMP-2, $P < 0.01$, Fig. 5e; MMP-9, $P < 0.01$, Fig. 5f; NF-κB, $P < 0.01$ Fig. 5g; MCP-1, $P < 0.01$, Fig. 5h; VCAM-1, $P < 0.01$; Fig. 5i). In contrast, the mRNA expression level of these molecules was significantly lower in the CA + aspirin group compared with CA group (MMP-2, $P < 0.01$; MMP-9, $P < 0.01$; NF-κB, $P < 0.01$; MCP-1, $P < 0.01$; VCAM-1, $P < 0.01$).

Discussion

Dysfunction and destruction of endothelial cells (ECs) are the early pathological feature in both experimental and human CA [16, 22, 23]. In response to sustained hemodynamic stress, ECs at arterial branch points undergo functional and morphological changes [24], which drives local vascular inflammation and remodeling. EPCs, a specific subpopulation of hematopoietic stem cells, play an important role in neovasculogenesis and vascular repair [25]. Evidence indicates that number and function of circulating EPCs correlate inversely with cardiovascular risk factors [26]. Our previous studies also demonstrated that the number and potential of EPCs were closely related to CA formation [11, 14]. Although the efficacy of aspirin in cardiovascular disease has been reported, few studies have investigated the effect of aspirin on the number and function

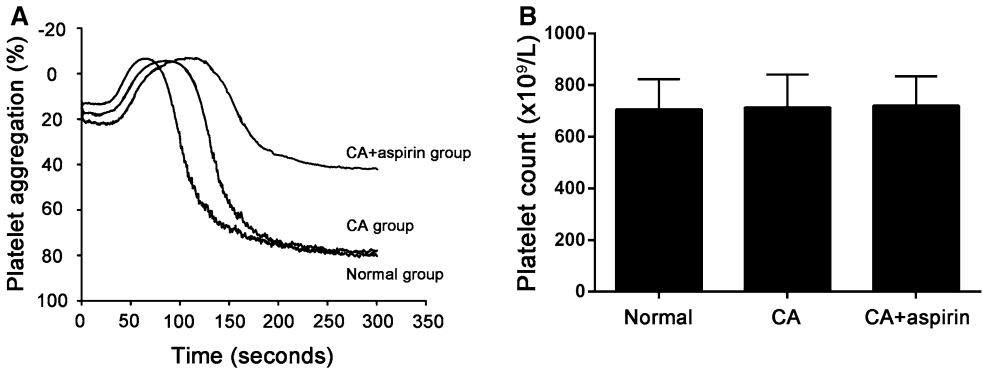

Fig. 2 Effect of aspirin on platelet aggregation and platelet counts. **a** Representative images of platelet aggregation analysis. Aspirin treatment following CA inhibited the formation platelet aggregation (n = 6/group). **b** No difference in platelet counts was found among groups. Results are presented as the mean ± SD (n = 6/group)

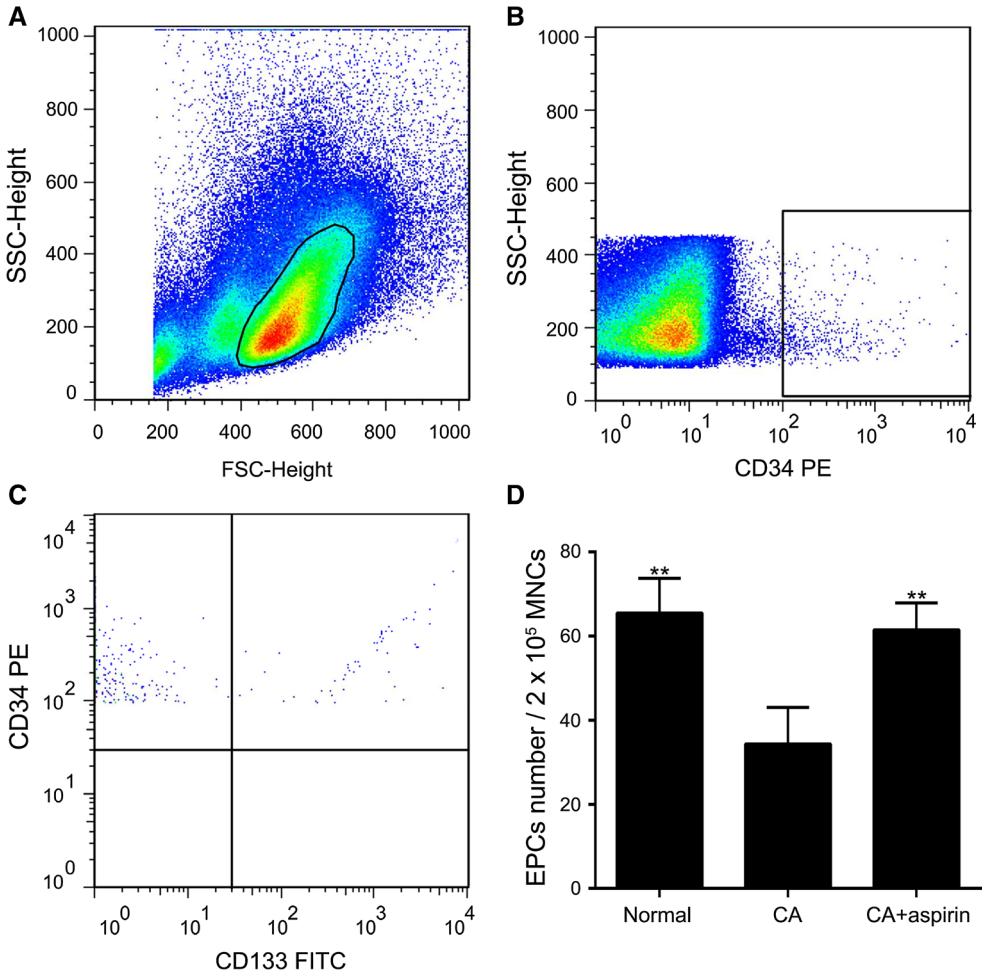

Fig. 3 Effect of aspirin on circulating levels of EPCs. **a**–**c** Representative photographs showing the flow cytometry analysis of circulating $CD34^+$ and $CD133^+$ double-positive EPCs. **d** The level of circulating $CD34^+CD133^+$ EPCs was decreased 2 months after CA induction and improved by aspirin treatment. Results are presented as the mean ± SD (n = 6/group). $^{**}P < 0.01$ versus CA group

of EPCs. Xu et al. studied the effect of intravenous immunoglobulin and aspirin (5 ~ 50 mg/kg/day) on EPCs function in children with Kawasaki disease and found that the adhesion, proliferation and migration activities were increased after 7 days of treatment [27]. Hu et al. found that low-dose (0.1 ~ 100 μmol/L) aspirin improved migration and adhesion capacity, and prevented senescence of EPCs, without affecting proliferation, apoptosis and eNOS expression of EPCs from healthy male volunteers [15]. On the other hand, Lou et al. reported chronic aspirin (81 mg/day or 325 mg/day) administration in patients with coronary artery disease resulted in reduced levels of circulating EPCs [28], while Bulut et al. found that circulating EPCs remained unchanged after chronic aspirin (100 mg/day) treatment [29]. Chen et al. demonstrated that aspirin (1, 2, 5 and 10 mmol/L) not only decreased the number of EPCs from healthy adults, but also inhibited the proliferative, adhesive, migratory, in vitro vasculogenesis,

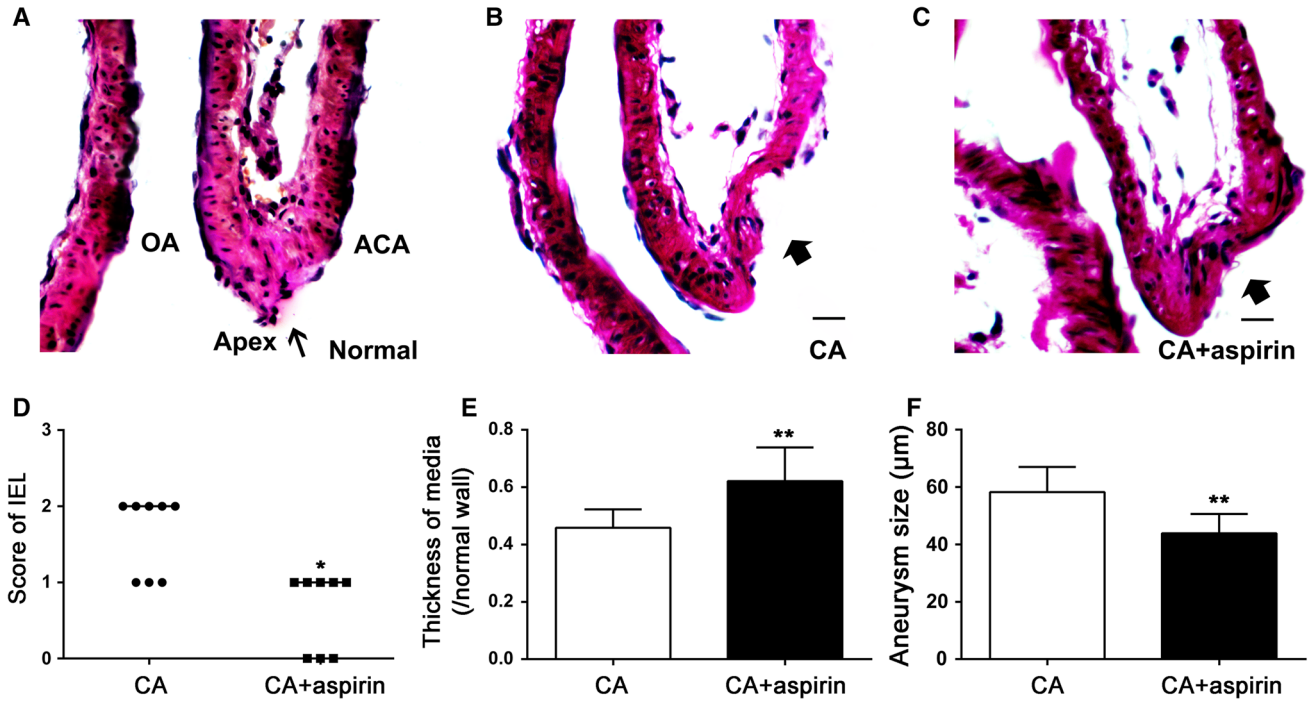

Fig. 4 Effect of aspirin on vascular degeneration after CA induction. **a–c** Representative Verhoeff's Van Gieson staining of vascular wall at the right ACA/OA bifurcations. The elastic fibers and nuclei shows *black* while collagen displays *red*. The *thin arrow* shows the direction of blood flow and the *thick arrow* indicates the outward aneurysmal lumen. Magnification ×40. Scale bar = 30 μm. *ACA* anterior cerebral artery, *OA* olfactory artery. **d** IEL score. Data are presented as scatter plots with a median line (n = 8/group). $*P < 0.05$ versus CA group. **e** Thickness of media. **f** Aneurysm size. Results **e–f** are presented as the mean ± SD (n = 8/group). $**P < 0.01$ versus CA group (Color figure online)

and iNOS expression capacity of EPCs [30]. Colleselli et al. reported that 1 mmol/L lysin acetylsalicylate significantly inhibited EPCs proliferation while lower concentrations did not affect EPCs proliferation in vitro [31]. Though the above findings are contradictory, our present study showed that long-term therapy with relatively high-dose (10 mg/kg/day) aspirin improved the impaired circulating levels of EPCs after CA induction. This positive effect of aspirin on EPCs biology is promising, but warrants further study.

The inflammatory response triggered by abnormal hemodynamic stresses and endothelial injury is essential in the pathogenesis of CA [3]. Shear stress causes endothelial dysfunction and activates prostaglandin E 2 (PGE2)-prostaglandin E receptor 2 (EP2) signaling in ECs, which induces CA formation through an amplifying loop via NF-κB [24]. NF-κB activation in vascular walls evokes upregulation of MCP-1 and VCAM-1 expression, which are prerequisites for monocyte/macrophage recruitment [32–34]. The infiltrated inflammatory cells, especially macrophages [35, 36], are responsible for the degradation of aneurysmal wall through the release of MMPs [36, 37]. Recent studies reveal that frequent aspirin use tend to reduce the risk of CA rupture [9] by inhibiting inflammatory response in aneurysmal wall [10]. The present study provided direct evidence that chronic aspirin administration dramatically inhibited the expression of NF-κB, MCP-1 and VCAM-1 in aneurysmal wall, which consequently led to reduced macrophages infiltration and subsequent MMP-2 and 9-driven wall destruction. Growing evidence indicates that aspirin can limit monocyte/macrophage infiltration into damaged tissue by improving endothelial function and interfering with the interaction between ECs and monocyte/macrophages. In an experimental model of denuded artery, local sustained delivery of aspirin (5 and 25 μg/mm^2) reduced adhesion of platelet and monocyte, and promoted re-endothelialization of the injured artery [38, 39]. In a rat model of neuroinflammation, chronic therapeutic doses of aspirin (10 and 100 mg/kg/day) decreased the expression of pro-inflammatory PGE2 in bacterial lipopolysaccharide-stimulated brain [18]. In a hyperlipidemic rat model, aspirin (5 mg/kg/day) downregulated the expression of intercellular adhesion molecule (ICAM), VCAM, platelet endothelial cell adhesion molecule (PECAM), E-selectin and MCP-1 in thoracic aorta tissue [40]. An ex vivo study by Weber et al. showed that aspirin (2 ~ 10 mmol/L) inhibited NF-κB activation, expression of VCAM-1 and E-selectin, and subsequent monocyte adhesion to ECs stimulated by tumor necrosis factor-α (TNF-α) [41]. Yang et al. found that aspirin (10 ~ 500 μg/mL) reduced the upregulated expression of MCP-1 and IL-8 in TNF-α-stimulated ECs, and monocyte adhesion and transmigration [42]. Dragomir et al.

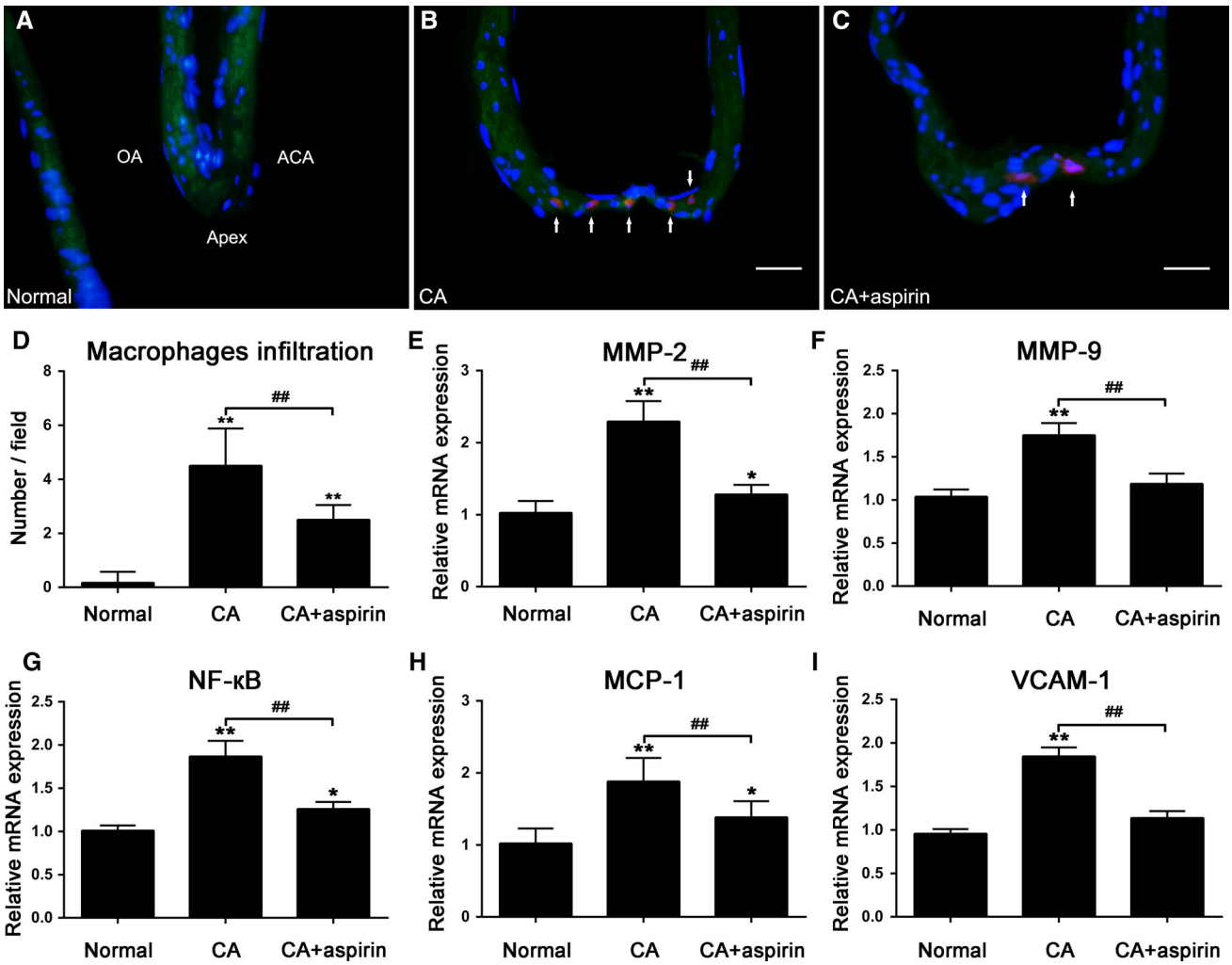

Fig. 5 Effect of aspirin on inflammatory response in aneurysmal walls. **a–c** Fluorescent staining of macrophages infiltrated into aneurysmal walls. The *white arrows* denote infiltrated macrophages. Magnification ×40. Scale bar = 30 μm. *Red*: CD68; *green*: smooth muscle α-actin; *blue*: DAPI. *ACA* anterior cerebral artery, *OA* olfactory artery. **d** The number of infiltrated macrophages in the CA + aspirin group was significantly lower than that in the CA group. Results are presented as the mean ± SD (n = 6/group). $^{**}P < 0.01$ versus normal group; $^{\#\#}P < 0.01$ versus CA group. **e–i** The gene expression of MMP-2 (**e**), MMP-9 (**f**), NF-κB (**g**), MCP-1 (**h**) and VCAM-1(**i**) in aneurysmal walls. Results are presented as the mean ± SD (n = 6/group). $^{*}P < 0.05$, $^{**}P < 0.01$ versus normal group; $^{\#\#}P < 0.01$ versus CA group (Color figure online)

demonstrated that aspirin (1 mmol/L) suppressed the upregulation of MCP-1 expression through the inhibition of reactive oxygen species (ROS) and NF-κB activation in high glucose-stimulated ECs [43].

The intraluminal thrombi can facilitate leukocytes recruitment and interfere with vascular stem/progenitor cells colonization [44, 45], causing abnormal vascular remodeling and distention of aneurysmal wall. In this study, we failed to find intraluminal thrombi 2 months after CA induction. A rational explanation is that the occurrence of spontaneous CA thrombosis is a rare event in non-giant saccular CA despite approximately 50 % incidence in giant CA [46, 47]. The second explanation is that intraluminal thrombosis may be washed off by perfusion solution.

There are several limitations to our study. First, we failed to examine the dose-dependent effect of aspirin on degeneration of aneurysmal walls. Second, we only investigated the effect of aspirin on local inflammatory response in aneurysmal walls without clarifying the effect on systemic inflammatory response.

In summary, we have demonstrated that aspirin, by augmenting EPCs mobilization and inhibiting macrophage-mediated chronic vascular inflammation, appeared to play a vasoprotective role in a rat model of CA. Further study in humans is necessary to determine safety, appropriate dosage and timing of aspirin administration.

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Conflict of interest The authors have no conflicts of interest to declare.

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