

Daphnetin suppresses experimental abdominal aortic aneurysms in mice via inhibition of aortic mural inflammation

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Abstract. Rupture of abdominal aortic aneurysm (AAA) is a devastating event that can be prevented by inhibiting the growth of small aneurysms. Therapeutic strategies targeting certain events that promote the development of AAA must be developed, in order to alter the course of AAA. Chronic inflammation of the aortic mural is a major characteristic of AAA and is related to AAA formation, development and rupture. Daphnetin (DAP) is a coumarin derivative with anti-inflammatory properties that is extracted from *Daphne odora* var. However, the effect of DAP on AAA development remains unclear. The present study investigated the effect of DAP on the formation and development of experimental AAAs and its potential underlying mechanisms. A mice AAA model was established by intra-aortic infusion of porcine pancreatic elastase (PPE), and mice were intraperitoneally injected with DAP immediately after PPE infusion. The maximum diameter of the abdominal aorta was measured by ultrasound system, and aortic mural changes were investigated by Elastica van Gieson (EVG) staining and immunohistochemical staining. The results demonstrated that DAP significantly suppressed PPE-induced AAA formation and attenuated the depletion of aortic medial elastin and smooth muscle cells in the media of the aorta. Furthermore, the density of mural macrophages, T cells and B cells were significantly attenuated in DAP-treated AAA mice. In addition, treatment with DAP resulted in a significant reduction in mural neovessels. These findings indicated that

DAP may limit the formation and progression of experimental aneurysms by inhibiting mural inflammation and angiogenesis. These data confirmed the translational potential of DAP in clinical AAA inhibition strategies.

Introduction

Abdominal aortic aneurysm (AAA) is a lethal, age- and sex-related chronic inflammatory degenerative disease, with a prevalence ranging from 5-10% amongst men >65 years old, and increasing with age (1-3). Recently, endovascular aneurysm repair (EVAR) is a new technology that is used to treat patients with AAA when the anatomy is suitable (4,5). Greenhalgh *et al* (4) reported that the 30-day mortality of AAA in the EVAR group was 1.7 vs. 4.7% in the open repair group within British hospitals. Although treatment methods for AAA have improved significantly in the last few decades, the mortality rate of patients with ruptured AAA remains very high worldwide (5,6). At present, surgical repair is an effective treatment for advanced AAAs (diameter >55 mm). Public screening programmes frequently detect AAA at an early stage (7). However, surgical repair does not provide a significant benefit for AAA at an early stage (8), and the current practice is to watch and wait until the diameter exceeds 55 mm, resulting in a risk of rupture or subsequent complications. AAA has a natural process of progressive growth, which indicates that early stage AAA can grow and subsequently require surgical treatment (9). To date, no pharmacological inhibition strategies have been effective in suppressing AAA development or eventual rupture (7). Discovering novel drugs that could inhibit the development of AAA is therefore crucial.

AAA is characterized by increased mural inflammation, neoangiogenesis, degeneration of smooth muscle cells (SMCs), degradation of extracellular matrix, activation of matrix metalloproteinases (MMPs) and accumulation of intraluminal thrombi (10). Therapeutic strategies for stopping or decelerating AAA progression must target the underlying events that promote its development (11). Mural inflammation has been implicated in AAA formation, development and rupture. Lindberg *et al* (12) reported that numerous inflammatory biomarkers are significantly elevated and positively correlated with aortic diameter in abdominal aortic aneurysms. Furthermore, anti-inflammation-based therapy has been demonstrated to attenuate aneurysmal development in

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AAA models (11,13-15). Xiong *et al* (13) demonstrated that blocking TNF- α expression can attenuate aneurysm formation in a murine model. Li *et al* (11) reported that cold-inducible RNA-binding protein (CIRP) is a novel proinflammatory cytokine, and that anti-CIRP-based therapy could attenuate aneurysm formation by inhibiting mural inflammation in an experimental murine model. Numerous studies have therefore focused on exploring anti-inflammatory agents that could alleviate AAA development. Previous studies have demonstrated that some natural products can be used to treat coronary heart disease, atherosclerosis, diabetes mellitus due to their powerful anti-inflammatory activities (16-18). Furthermore, previous studies reported that natural products have antioxidant and anti-inflammatory effects. Fan *et al* (19) found that curcuma, which is the source of the spice turmeric widely used in Asian countries, can attenuate rat thoracic aortic aneurysm formation. In addition, Hao *et al* (16) reported that curcumin attenuates AAA by inhibiting the inflammatory response in a murine model. Importantly, Kaluza *et al* (20) demonstrated that an anti-inflammatory diet is associated with a reduced risk of AAA, which is an association that was even more significant for AAA rupture. Natural products are therefore considered as ideal sources of potential agents capable of managing AAA because of their low toxicity and the absence of clear side effects.

Daphnetin (7,8-dihydroxycoumarin; DAP) is a natural anti-inflammatory, anti-oxidative and anti tumour product (21-25) that is extracted from *Daphne odora* var. Liu *et al* (26) reported that DAP has some protective effects on severe acute pancreatitis in a rat model by inhibiting the expression of inflammatory cytokines. Subsequently, DAP was found to prevent and treat numerous diseases, including cerebral ischaemia/reperfusion injury, rheumatoid arthritis, colitis and endotoxin-induced lung injury, due to its powerful anti-inflammatory activities (24,27-32). At present, DAP is an anti-inflammatory agent used in some inflammation-related diseases (33-35). Li *et al* (33) reported that DAP inhibited inflammation in the systemic lupus erythematosus murine model via inhibition of NF- κ B activity. Wang *et al* (34) demonstrated that DAP alleviated experimental autoimmune encephalomyelitis via regulating dendritic cell activity. Chronic inflammation of the aortic mural is a major characteristic of AAA and is related to AAA formation, development and rupture (1). However, the anti-inflammatory effect of DAP on AAA remains to be determined.

The present study hypothesized that DAP could prevent aneurysm development by inhibiting the inflammatory response. To investigate this hypothesis, this study established an AAA mice model induced by intra-aortic infusion of porcine pancreatic elastase (PPE) and the effect of DAP on AAA mice were evaluated.

Materials and methods

Experimental animals. The present study used 14 male C57BL/6 mice weighing 24-27 g (Beijing Vital River Laboratory Animal Technology Co., Ltd.) aged 8-10 weeks. All experimental protocols were approved by the Medical Ethics Committee of Shandong Shanxian Central Hospital. All animals were cared for in accordance with the recommendations of the Guide

for the Care and Use of Laboratory Animals of the National Institutes of Health (36).

Animal model establishment. The AAA model was induced by intra-aortic infusion of type I porcine pancreatic elastase (PPE) as described previously (3,37-39). In the present study, mice were anesthetized using 4% isoflurane prior to surgery followed by 2.5% isoflurane to maintain the anaesthesia. Once mice were anesthetized, a vertical midline abdominal incision was performed. After exposing the posterior abdominal wall, the abdominal aorta from the left renal vein to the bifurcation was isolated, and the lumbar arteries were ligated under sterile conditions. The proximal and distal abdominal aortas were temporarily blocked with 6-0 silk sutures. Subsequently, a polyethylene catheter (PE-10, Instech Laboratories, Inc.) was inserted into the controlled aorta above the bifurcation, and the aorta was infused with type 1 PPE (1.5 U/ml in saline; Sigma-Aldrich; Merck KGaA) for 5 min under constant pressure (100 mm Hg).

During the 14 days following the operation, mice were intraperitoneally injected with DAP or an equal volume of vehicle every day. On the 14th day after the operation, mice were euthanized under CO₂ exposure (flowrate of CO₂, 2 l/min; air displacement rate, 20%/min). Death was confirmed by the absence of breathing, pulse, corneal reflex, response to firm toe pinch, heart beat and respiratory sounds, the graying of the mucous membranes and rigor mortis. Exception of rigor mortis, none of these signs can independently confirm death. Blood was collected after mice euthanasia cardiac puncture. Blood was collected (0.6 ml) and stored in 1.5 ml tube for 2 h at 4°C. Then, 0.2 ml serum was collected after centrifugation at 3,000 x g at 4°C for 10 min. Aortic infused segments (about 10 mm long) were also collected, embedded in optimal cutting compound media (cat. no. 4583; Sakura Finetek USA, Inc.) and then stored in -80°C.

Experimental groups and DAP treatment. Mice were randomly assigned into two groups as follows: The AAA+ vehicle group (n=7, surgery was not performed on one mouse as a abdominal aorta aberrance which was identified via ultrasound, which measured the maximum diameter of the abdominal aorta before surgery) and the AAA+DAP group (n=7). Briefly, DAP (purity >98%) was obtained from Sigma-Aldrich; Merck KGaA. DAP was dissolved at the concentration of 1 mg/ml in a solvent composed of 5% DMSO and 95% saline. Mice were intraperitoneally injected with DAP (20 mg/kg/day) or an equal volume of vehicle immediately after PPE infusion and lasted for 14 days. The dose of DAP was determined according to a previous study demonstrating that improved survival in a rodent cerebral ischaemia/reperfusion model (24). Aortic segments were subsequently collected on day 14 following surgery operation.

Aortic size measurements via ultrasound. The maximum diameter of the abdominal aorta was measured in anesthetized mice by using a 30 MHz ultrasound system (VisualSonics, Inc.) before surgery and on days 3, 7 and 14 following surgery. Two experienced operators who were blinded to study group assignments independently completed the full-scale measurement and quantitative analysis of the ultrasonic data. AAA

was defined as a 50% or greater increase in infrarenal aortic diameter compared with the baseline assessment (Vevo 770 ultrasound system).

Elasticvan Gieson staining. Aortas were collected on the 14th day after the operation and embedded in a straight strip shape in optimal cutting compound media (cat. no. 4583; Sakura Finetek USA Inc.). To observe changes in the aortic tissues at the morphological level, 8- μ m sections of aortic tissue were sectioned and fixed with cold acetone for 8 min at 4°C. To evaluate the integrity of elastin, aortic tissue sections were stained with Elasticvan Gieson (EVG; cat. no. G1042, Wuhan Servicebio Technology Co. Ltd.) according to the manufacturers' protocol. As previously described, the analysis of medial elastin destruction was graded from mild (I) to severe (IV) (3).

Immunohistochemical staining. Immunohistochemical staining was performed as described previously (3). Briefly, 8- μ m sections of aortic tissue were prepared as afore mentioned for EVG staining. The sections were incubated with antibodies against α -smooth muscle actin (α -SMA; 1:400; cat. no. ab32575; Abcam), CD68 (1:400; cat. no. ab125212, Abcam), CD8 (1:100; cat. no. ab22378; Abcam), B220 (1:100; cat. no. ab64110; Abcam) and CD31 (1:200; cat. no. ab28364; Abcam) overnight at 4°C. Then, sections were incubated with a biotinylated secondary antibody kit (cat. nos. PV-9001 and PV-9004; Beijing zhongshan Jinqiao Biotechnology Co., Ltd.), and immune complexes were visualized using a 3-amino-9-ethylcarbazole (AEC) peroxidase substrate kit (cat. no. A2010; Beijing Solarbio Science & Technology Co., Ltd) according to the standard procedure. Subsequently, the sections were counterstained with haematoxylin. After dehydration overnight at 25°C incubator, clearing and mounting using aqueous non-fluorescing mounting media (cat. no. HS-106; National Diagnostics), sections were visualized under x10 magnification by microscopy (CX31; Olympus Corporation). The degeneration of SMCs was graded from mild (I) to severe (IV) as described previously (3). The infiltration of macrophages was graded as follows: i) I, infiltration limited to the adventitia or $<1/4$ of the aortic circumference; ii) II, infiltration involving the medial layer and $\leq 1/2$ of the aortic circumference; iii) III, infiltration involving the medial layer and $>1/2$ and $\leq 3/4$ of the aortic circumference; and iv) IV, infiltration involving all aortic layers and expanded $>3/4$ of the aortic circumference. Mural T cell infiltration, B cell infiltration and angiogenesis were quantified as CD8⁺, B220⁺ and CD31⁺ blood vessels, respectively, per aortic cross section analysis.

Statistical analysis. Data are presented as the means \pm standard deviation. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). Student's *t*-test was used to evaluate the significance of differences between two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

DAP treatment attenuates experimental AAA formation and development. The maximum aortic diameter was measured with by ultrasound before the operation and on days 3, 7 and 14 after operation (Fig. 1A). In the control group, the infused

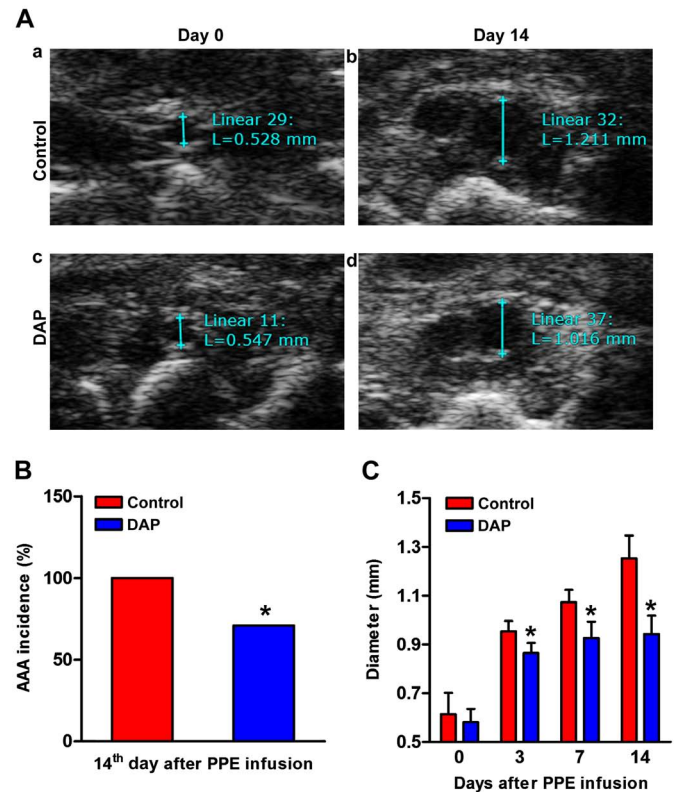


Figure 1. Effect of DAP treatment on experimental AAA formation and development. (A) Representative ultrasonographic images of aortas in the control and DAP treatment groups prior to and 14 days after intra-aortic infusion of PPE. (B) AAA incidence in each group on day 14 after PPE administration. (C) Maximum diameters of each group prior to and at days 3, 7 and 14 after PPE infusion. $n=6-7$ mice/group. * $P < 0.05$ vs. control group. AAA, abdominal aortic aneurysm; DAP, Daphnetin; PPE, porcine pancreatic.

abdominal aorta sustained enlargement in a time-dependent manner. In the DAP treatment group, aortic enlargement was significantly decreased compared with the control group. The maximum aortic diameter decreased significantly compared with that of the control group at each checkpoint after the operation (Fig. 1C). In the DAP treatment group, aneurysm incidence ($>50\%$ baseline diameter increase) was significantly decreased compared with the control on the 14th day after operation (71 vs. 100%, DAP group vs. vehicle group; $P < 0.05$; Fig. 1B).

DAP treatment attenuates medial elastin and SMC destruction. To further investigate DAP-mediated protection against experimental AAA, changes in the aortic tissues at the morphological level were observed. Medial elastin and SMC destruction are the main histological characteristics of clinical and experimental AAAs (10,40). In the DAP treatment group, SMCs stained with α -SMA were degenerated and had almost fully disappeared in the aortic mural (Fig. 2A). The degeneration of SMCs was significantly decreased in the presence of DAP (20 mg/kg/day). Furthermore, in the control group, elastin stained with EVG was degraded and had almost fully disappeared in the aortic mural (Fig. 2B). However, in the DAP treatment group, the EVG-stained elastic lamellae exhibited partial aortic elastin preservation, which was determined by semi-quantitative analysis ($P < 0.05$).

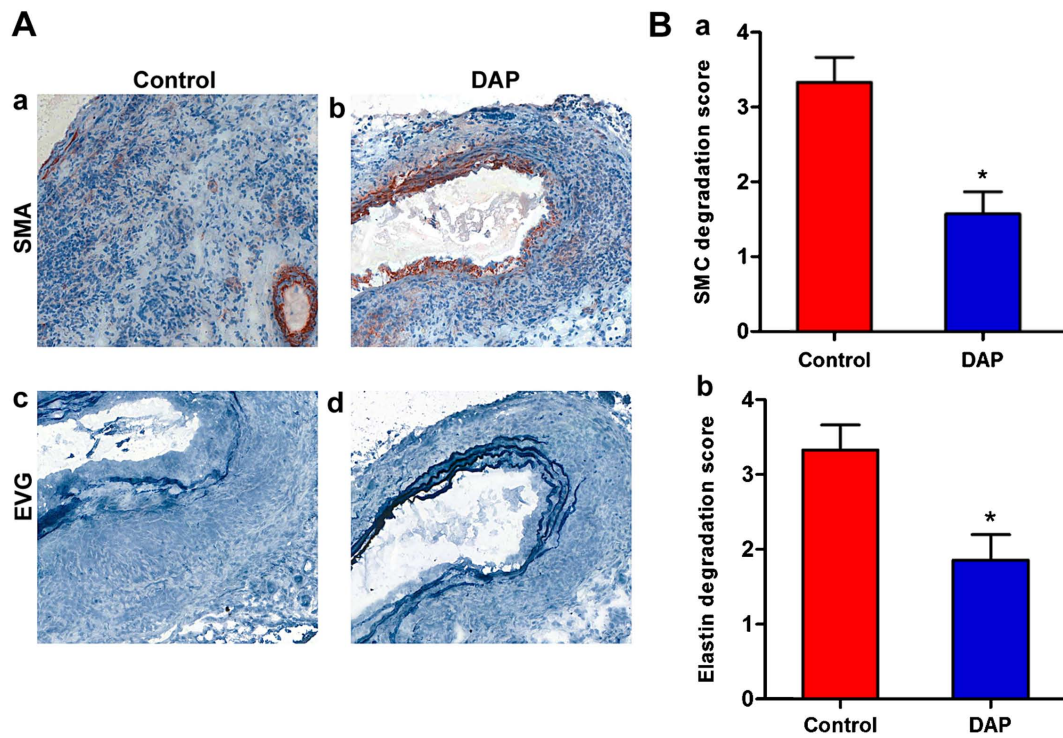


Figure 2. Effect of DAP treatment on medial SMCs and elastin destruction in the experimental AAA model. Mural elastin and smooth muscle cell degradation were graded from I (mild) to IV (severe). (A) Representative images of α -SMA (a, b) and EVG (c, d) staining of SMCs and elastin, respectively, in the control and DAP treatment groups. (B) Mean scores of mural SMC degradation (a) and elastin degradation (b) in each group. $n=6-7$ mice/group. * $P<0.05$ vs. control group. AAA, abdominal aortic aneurysm; DAP, Daphnetin; SMC, smooth muscle cell; EVG, Elastica van Gieson; α -SMA, α -smooth muscle actin.

Therefore, the preservation of medial elastin and SMC cellularity of aortic wall are the mural structural factors for explaining reduced aortic diameter enlargement in DAP-treated mice. These findings suggested that DAP may protect against AAA by attenuating aortic elastin and SMC destruction.

DAP treatment attenuates mural leukocyte infiltration. Mural leukocyte accumulation is another pathologic hallmark of AAA disease (10,40). DAP is an anti-inflammatory agent used in inflammation-related diseases. To evaluate the influence of DAP treatment on mural inflammation, leukocyte infiltration was detected. CD68, CD8 and B220 staining was performed on aortic samples. As presented in Fig. 3, significant accumulation of macrophages and T and B lymphocytes was present in the media and adventitia in the control group. However, in the DAP treatment group, the increased numbers of mural macrophages, T cells and B cells were significantly decreased, which indicated that mural inflammation was attenuated. These results indicated that DAP treatment may limit experimental AAA progression in part by diminishing aortic accumulation of proinflammatory leukocytes.

DAP treatment attenuates mural angiogenesis. Neoangiogenesis is an important histopathological feature of AAA (41-43). The influence of DAP treatment on mural angiogenesis was therefore evaluated, and CD31 staining was performed on aortic samples. As presented in Fig. 4, the quantity of mural CD31⁺ neovessels decreased significantly in the DAP treatment group compared with the control group. These results suggested that DAP treatment may suppress AAA by impairing mural angiogenesis during aneurysm formation and progression.

Discussion

DAP is a natural product extracted from *Daphne odora* var with anti-inflammatory, antioxidant and antitumour effects (21-25). Liu *et al* (24) reported that DAP protects against cerebral ischaemia/reperfusion injury in mice via inhibition of inflammatory responses. Yao *et al* (44) demonstrated that DAP has therapeutic effects on an autoimmune arthritis model by modulating inflammation. Yu *et al* (31) found that DAP possesses some anti-inflammatory activities in endotoxin-induced lung injury. Furthermore, Liu *et al* (26) reported that DAP has protective effects on severe acute pancreatitis in a rat model by inhibiting the expression of inflammatory cytokines. Ji *et al* (32) demonstrated that DAP ameliorates experimental colitis by modulating microbiota composition and the Treg/Th17 balance. Currently, an increasing number of studies have demonstrated that DAP serves a crucial role in the prevention and treatment of inflammation-related diseases due to its powerful anti-inflammatory activities (33-35).

The pathogenesis of AAA is characterized by inflammation with leukocyte infiltration, degradation of extracellular matrix and depletion of vascular smooth muscle cells (10,40). Local chronic inflammation of the aortic wall has been implicated in the formation, development and rupture of AAA (1). Leukocytes are the principal effector cells of aneurysmal disease, contributing to aortic degradation via the production of extracellular MMPs, reactive oxygen species and proinflammatory cytokines. Leukocyte accumulation in the aortic wall is an early feature of PPE-induced AAAs and is present throughout the process of AAA. The present study demonstrated that high numbers of macrophages and T and

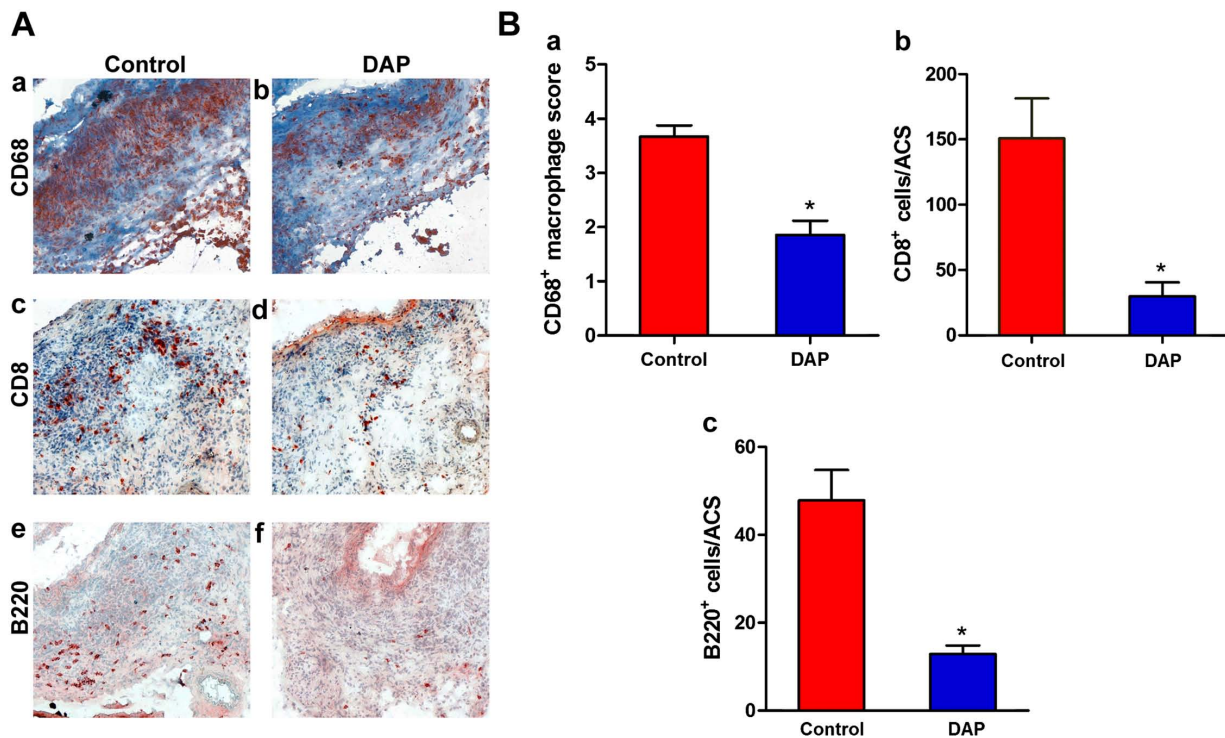


Figure 3. Effect of DAP treatment on mural leukocyte infiltration in the experimental AAA model. Mural macrophage accumulation was graded from I (mild) to IV (severe). (A) Representative immunostaining images of CD68+ macrophages (a, b), CD8+ T cells (c, d) and B220+ B cells (e, f) in the control and DAP treatment groups. (B) Mean scores of mural macrophages (a), T (b) and B cells (c) in each group were quantified as CD68+ cells, CD8+ cells and B220+ cells per ACS. n=6-7 mice/group. *P<0.05 vs. control group. ACS, aortic cross section; DAP, Daphnetin.

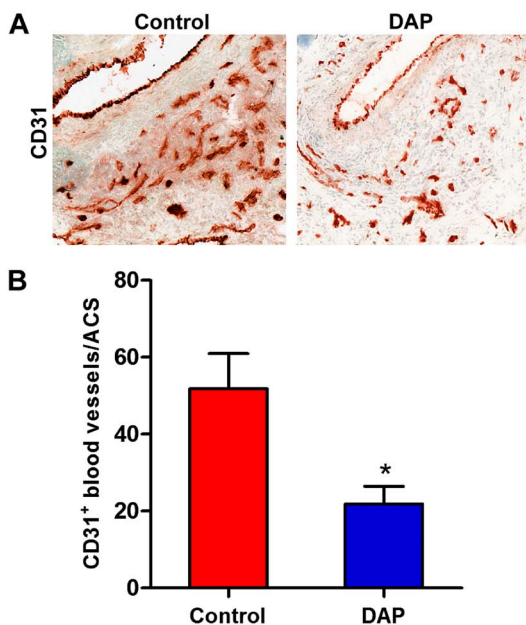


Figure 4. Effect of DAP treatment on aortic wall angiogenesis. (A) Representative immunostaining images of CD31+ blood vessels in the control and DAP treatment groups. (B) Mean scores of mural angiogenesis in each group were quantified as CD31+ blood vessels per ACS. n=6-7 mice/group. *P<0.05 vs. control group. ACS, aortic cross section; DAP, Daphnetin.

B lymphocytes assembled in the media and adventitia in experimental AAAs. Mural macrophages and lymphocytes secrete various types of inflammatory cytokines and induce mural injury and subsequent aneurysm formation (10,12,40). Previous studies have demonstrated that increased levels

of inflammatory cytokines, including interleukin (IL)-1 β , IL-6 and tumour necrosis factor (TNF)- α , are positively correlated with AAA formation and expansion (12,16,45,46). Lindberg *et al* (12) investigated the relationship between AAA and the inflammatory biomarkers IL-6, TNF- α , endothelin-1 and soluble urokinase-type plasminogen activator receptor and reported that inflammatory cytokines play important roles in AAA progression. Xiong *et al* (13) reported that blocking TNF- α attenuates aneurysm formation in a murine model. De *et al* (14) found that systemic blockade of monocyte chemoattractant protein 1 inhibits aortic aneurysm formation in Ang II-induced AAA in apolipoprotein E-deficient mice. Mural inflammation serves therefore a crucial role in AAA formation and progression.

In the present study, following DAP treatment, the increased numbers of mural macrophages, T and B cells were significantly attenuated in the aortic wall. Previous studies also reported that DAP inhibits the infiltration of inflammatory cells to alleviate inflammatory injury in mice (25,31,44). The results of DAP inhibiting the infiltration of inflammatory cells from the current study were consistent with previous studies. These results indicated that DAP may prevent the formation and progression of AAA via anti-inflammatory effects. Furthermore, treatment with DAP resulted in a significant reduction in mural neovessels. Neoangiogenesis is an important histopathological feature of AAA (41-43). Previous studies demonstrated that aortic mural neovascularization serves a key role in AAA progression and rupture (47-49). Kaneko *et al* (50) found that inhibition of vascular endothelial growth factor A (VEGF-A) by soluble VEGF-A receptor-1 attenuates experimental AAA

development. Vijaynagar *et al* (48) reviewed studies on the role of angiogenesis in AAA and demonstrated that angiogenesis plays important roles in AAA progression. Recently, anti-angiogenesis therapy has been used as a potential intervention to treat AAA (48,51,52). DAP treatment may therefore suppress AAA via impaired mural leukocyte infiltration and angiogenesis during aneurysm formation and progression.

The present study aimed to examine the influence of DAP on the formation and progression of experimental AAAs; however, this study had some limitations. A previous study reported that DAP inhibits the TLR4/NF- κ B signalling pathway (24,53). Liu *et al* (24) found that DAP protects against cerebral ischaemia/reperfusion injury in mice via inhibition of the TLR4/NF- κ B signalling pathway. Song *et al* (53) demonstrated that DAP downregulates the activation of concanavalin A-induced NF- κ B signal transduction pathways in mouse T lymphocytes. A previous study has also found that TLR4/NF- κ B signalling pathway is activated in AAA and mediates AAA formation and progression (54). DAP may therefore suppress experimental AAA by inhibiting the TLR4/NF- κ B-mediated inflammatory signalling pathway, and further investigation is thus required. In addition, the dose-range experiments and the clinical dose for humans was not determined in the current study. Therefore, substantial work will be required before clinical trials.

In conclusion, the findings from the present study suggested that DAP attenuated AAA formation and progression. This inhibitory effect may be mediated by inhibition of mural leukocyte infiltration and angiogenesis. These results suggested that DAP may be considered as a clinical candidate in early AAA disease suppression.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CH and LW conceived and designed the experiments. SX, LM, HG, SG and LW performed the experiments. SX and CH analysed the data. CH wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental protocols were approved by the Medical Ethics Committee of Shandong Shanxian Central Hospital (Shanxian, China; approval no. IACUC A1027). All animals

were cared for in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (28).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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