

Original Article

Multifaceted effects of astragaloside IV on promotion of random pattern skin flap survival in rats

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Abstract: Random pattern skin flap transplantation is frequently applied in plastic and reconstructive surgery, but the distal part of skin flaps often suffers necrosis due to ischemia. Astragaloside IV (AS-IV), a natural saponin purified from *Astragalus membranaceus*, may have beneficial functions for flap survival. In this study, rats were divided into a control group and an AS-IV treatment group, and underwent surgery using a modified "McFarlane flap" model. After intragastric administration of vehicle control or AS-IV for their respective groups, flap survival area and water content were measured 7 days after surgery. Flap tissue was separated to test protein expressions related to angiogenesis, inflammation, oxidative stress and autophagy via western blot, immunohistochemistry, and immunofluorescence. Results showed that AS-IV improved flap survival area and reduced tissue edema. AS-IV also increased mean vessel densities and upregulated levels of VEGF protein, both of which indicate increased angiogenesis. Furthermore, AS-IV depressed leukocyte infiltration, decreased expressions of inflammatory proteins TNF- α , IL1 β and IL6, increased SOD activity, decreased MDA content, and stimulated autophagy. Overall, our results suggest that AS-IV promotes skin flap survival via inducing angiogenesis, depressing inflammation and dampening oxidative stress; it also activates autophagy, which may be an underlying mechanism for oxidative stress depression.

Keywords: Astragaloside IV, random skin flap survival, angiogenesis, inflammation, oxidative stress, autophagy

Introduction

Random pattern skin flap transplantation is frequently applied in plastic and reconstructive surgery to repair superficial skin defects caused by traumatic skin injury or body surface tumor removal [1, 2]. Skin flap viability and survival rely on adequate blood supply, which is delivered via the vasoganglion at the pedicle bed of the flap [3]. However, blood supply gradually decreases along the length of the skin flap towards the distal end [4], therefore, the distal part of random pattern skin flaps often suffers necrosis due to ischemia [5, 6]. Despite improved flap surgical techniques, the length-to-width ratio of flaps still cannot exceed 2:1 [7], significantly limiting the clinical application of random pattern flap transplantation. Previous studies have shown that inadequate angiogenesis, inflammatory reactions, and oxidative

stress are crucial factors that contribute to flap necrosis [8-10]. These findings prompted the investigation of promising pro-angiogenesis, anti-inflammatory, and oxidative stress reducing agents to improve flap survival [5, 11, 12].

Currently, Traditional Chinese Medicine (TCM) is widely used to treat ischemic diseases in the Chinese population [13]. Astragaloside IV (AS-IV, 3-O- β -D-xylopyranosyl-6-O- β -D-glucopyranosyl cycloastragenol [14]), a natural saponin purified from *Astragalus membranaceus* and a popular TCM [15], is commonly utilized for the prevention and treatment of cardiovascular and cerebrovascular ischemia [16]. AS-IV's underlying mechanism was found to involve a series of biological activities, such as promoting angiogenesis via activation of VEGF *in vitro* [17]. It also depresses the level of intracellular oxidative stress via the activities of antioxidant

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enzymes such as GSH-Px and SOD [18]. Furthermore, AS-IV has the capacity to depress inflammation by regulating the NF- κ B signaling pathway [19]. More recently, studies have indicated that AS-IV enhances cell autophagy [15], which is thought to protect the viability of cells and tissues under ischemic conditions [20-22]. Since random pattern skin flaps are prone to ischemia, we hypothesized that AS-IV can improve flap survival via activation of the aforementioned pathways.

In this study, the effects of AS-IV on random flap survival were explored in rats. The underlying mechanisms involved in the pro-angiogenesis properties of AS-IV, along with the ability of AS-IV to depress inflammation, dampen oxidative stress, and activate autophagy, were analyzed using histological and protein analyses.

Materials and methods

Ethics statement

Surgical procedures, treatments, and post-operative care were carried out with the permission of the Wenzhou Medical University Ethical Committee and in strict accordance with the International Health and Medical Research Guidelines for Animal Welfare.

Reagents

Astragaloside IV (C₄₁H₆₈O₁₄, HPLC > 98%) was acquired from Tauto Biotechnology (Shanghai, China). H&E staining kit, DAB developer, and pentobarbital sodium were purchased from Solarbio Science & Technology (Beijing, China). SOD, GSH, and MDA testing kits were acquired from Jiancheng Technology (Nanjing, China). Primary antibodies against VEGF and CD34 were purchased from Bioss Biotechnology (Beijing, China). Primary antibodies against LC3, Beclin1, IL1 β and IL6 were acquired from Cell Signaling Technology (Danver, USA). DMSO and primary antibody against p62 were purchased from Sigma-Aldrich Chemical Company (Milwaukee, USA). Primary antibodies against CTSD, TNF- α , SOD and β -action, and HRP-conjugated IgG second antibody were purchased from Santa Cruz Biotechnology (California, USA). FITC-conjugated IgG second antibody was obtained from Boyun Biotechnology (Nanjing, China). VEGF mRNA in situ hybridization kit was obtained from Biological Technology (Wuhan, China). DAPI staining solution was pur-

chased from Beyotime Biotechnology (Jiangsu, China). BCA kit was acquired from Thermo Fisher Scientific (Rockford, USA). ECL-plus reagent kit was purchased from perkinelmer life sciences (Waltham, USA).

Animals and experimental protocol

72 male Sprague-Dawley rats (250 g-275 g) were purchased from Wenzhou Medical University (license no. SCXK [ZJ] 2005-0019). All rats in this experiment were housed with a 12 h light/dark cycle and provided with regular food and water for 1 week before any experimental procedure. Rats were randomly divided into the Control group (n=36) and the AS-IV (Astragaloside IV) group (n=36). AS-IV was dissolved in DMSO (250 mg/ml), and further diluted with normal saline to a concentration of 5 mg/ml. Before surgery, rats in the AS-IV group received Astragaloside IV (50 mg/kg/day, ig) each day for 7 consecutive days. Rats in the control group received an equal volume of DMSO-saline solution (vehicle control) for 7 consecutive days. After the administration of AS-IV and vehicle control, surgery was performed as follows.

Anesthesia of 2% (w/v) pentobarbital sodium (40 mg/kg, ip) was administered for all rats. Then, a caudal-based 3 cm \times 9 cm skin/panniculus carnosus flap was separated from the underlying fascia on the back of rats using the "McFarlene Flap" model. Both sacral arteries supporting the blood supply of this flap model were sectioned completely. Then, the separated flaps were sutured back into its original position with 4-0 silk and a wedged-on cutting needle. The flap area was divided into three equal zones: proximal area (Area I), intermediate area (Area II), and caudal area (Area III).

Following these procedures, rats in both groups were housed individually in standard experimental cages, and continued their pre-surgery treatment regimen (50 mg/kg/day AS-IV for the AS-IV group and equal volume of vehicle control for the control group) consecutively for 7 additional days. 7 after surgery, all rats were euthanized with pentobarbital sodium overdose for histological and protein analyses.

Flap survival assessment

After surgery, macroscopic changes of the random skin flaps were observed each day. Changes in appearance, color, texture, and hair

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condition were noted. 7 days after the operation, the surviving areas of flaps in each group (n=6) were measured via superimposition of photographs on graph paper. All results were presented as percentages of surviving area calculated as: surviving area \times 100%/total area (3 cm \times 9 cm). Tissue edema is a key factor causing the necrosis of ischemic flaps, therefore, the extent of edema is an important indicator of necrosis tendency. Extent of tissues edema can be measured by water content. Thus, 7 days after the operation, skin flap tissues were weighed and dehydrated in an autoclave at 50°C. The tissues were then weighted daily until the weight remained constant for at least 2 days. The percentage of water content in flap was determined by the following formula:

Tissue% water content = [wet weight - dry weight]/wet weight \times 100%

H&E staining

Samples (1.0 cm \times 0.2 cm) from the middle part of Area II in each group (n=6) were collected after rats were euthanized. The samples were fixed in 4% (v/v) paraformaldehyde for 24 h and then embedded in paraffin wax for transverse sectioning. Then, the samples were sectioned into 10 μ m thick slices and mounted on poly-L-lysine-coated slides for H&E staining. Leukocyte infiltration was examined under light microscopy (\times 200 magnifications), and the number of microvessels per unit area (per mm², an indicator of microvascular density) was calculated. Six random fields of three random sections from each tissue sample were used for counting.

Immunohistochemistry

Sections from the middle part of Area II in each group (n=6) were deparaffinized in xylene, and then rehydrated using graded ethanol after rats were euthanized. After washing, 3% (v/v) H₂O₂ was added on the sections to block endogenous peroxidase. Then, the sections were treated with a 10.2 mM sodium citrate buffer at 95°C for 20 min, blocked with 5% (w/v) bovine serum albumin and 1% (v/v) Tween-20 in PBS for 10 min, and incubated with primary antibodies: CD34 (1:100), VEGF (1:100), and SOD (1:200) at 4°C overnight. Sections were then further incubated with the HRP-conjugated IgG

second antibody (1:1000) and stained with a DAB detection kit. Next, all sections were counterstained with hematoxylin. Finally, the flap sections were imaged at \times 200 magnification with a DP2-TWAN image-acquisition system (Olympus Corp, Tokyo, Japan). IA values were calculated using Image-J software (NIH, USA) to indicate protein expression of VEGF and SOD. The number of CD34-positive blood vessels per unit area (mm²) was calculated. Six random fields of three random sections from each tissue sample were used for counting.

Western blotting

After rats were euthanized, samples (0.5 cm \times 0.5 cm) from the middle of Area II flaps (n=6) in each group were separated and stored at -80°C for western blot analysis. After homogenization, the concentration of protein in homogenate was determined using the BCA assay. An equivalent amount of 55 μ g of proteins was separated on a 12% (w/v) gel and transferred on PVDF membranes (Roche Applied Science, Indianapolis, IN, USA). After blocking with 10% (w/v) non-fat milk for 2 h, the membranes were incubated with the following primary antibodies at 4°C overnight: VEGF (1:1000), TNF- α (1:500), IL1 β (1:1000), IL6 (1:1000), Beclin1 (1:1000), p62 (1:500), LC3 (1:500), and β -actin (1:1000). Then, membranes were incubated with the HRP-conjugated IgG second antibody (1:5000) for 2 h at room temperature. Bands on the membranes were visualized using the ECL-plus reagent kit. Finally, band intensity was quantified via the Image Lab 3.0 software (Bio-Rad, Hercules, CA, USA).

SOD activity and MDA content test

SOD and MDA testing kits were used to detect the oxidative stress status of the skin flaps. After rats were euthanized, tissue samples (0.5 cm \times 0.5 cm) were separated from the middle area of Area II flaps in each group (n=6), weighed, homogenized, and diluted to 10% (v/v) in an ice bath. Then, the homogenate was centrifuged at 3500 r.p.m for 15 min. Next, the supernatant solution of homogenate was collected, and SOD activity is tested via a xanthine oxidase method. 0.1 ml homogenate was mixed with 1.4 ml xanthine oxidase and then maintained in a water bath at 37°C for 40 min. Then, 2.0 ml of developer was added for shade selection at 550 nm following a 10 min incuba-

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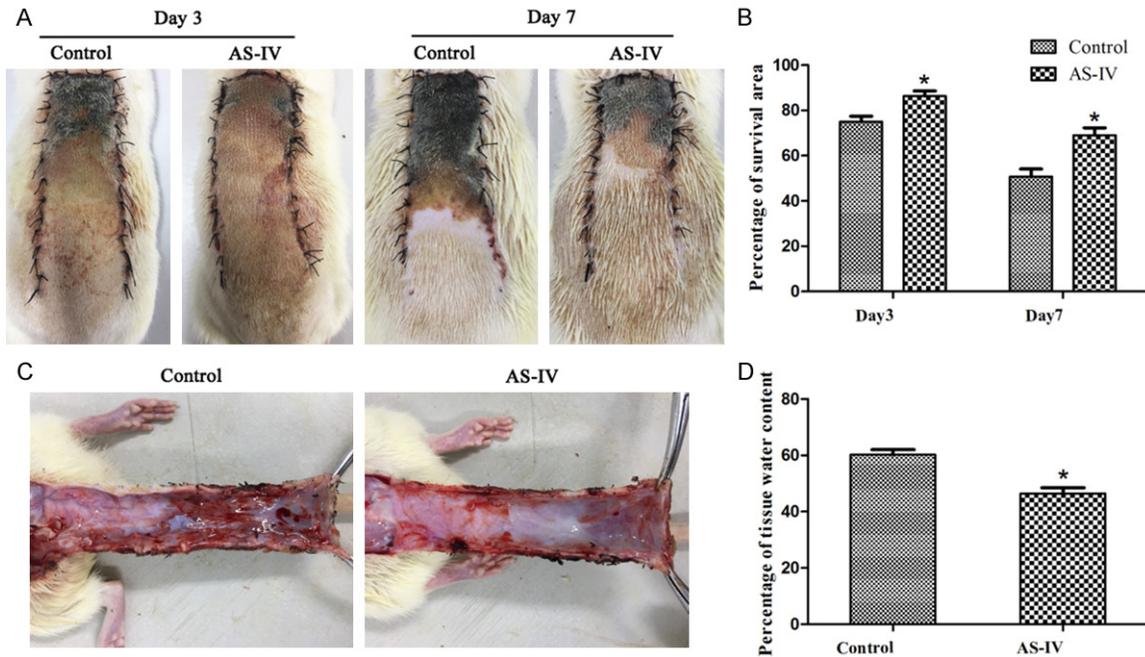


Figure 1. Astragaloside IV improves flap survival area and ameliorates tissue edema. The appearance in the exterior and the degree of edema of the random skin flap were assessed after a modified “McFarlane flap” model was established. A. Digital photographs of flap survival in the Control and AS-IV groups were taken on day 3 and day 7. B. Flap survival percentages for each group were then quantified and analyzed. C. Digital photographs of the inner side of the skin flap were taken from each group to show the degree of tissue edema. D. Quantification and analysis of the tissue water content percentages in each group are shown. Significance: * $p < 0.05$ versus the Control group. Data represent Mean values \pm SEM, $n=6$ per group.

tion at room temperature. MDA content was tested via a modified TBA test: 0.1 ml homogenate was mixed with 0.1 ml dehydrated alcohol, 0.1 ml TBA, and 4.0 ml developer, and maintained in a water bath at 95°C for 40 min. Finally, this mix solution was cooled in running water and centrifuged at 4000 r.p.m for 10 min. The supernatant was collected and its absorbance was measured at 532 nm.

Statistical analysis

Statistical analyses were carried out by using SPSS19 software (Chicago, USA). All data are presented as mean \pm SEM. Comparisons of means between two groups in each analysis were performed using Student’s t-test. In all analyses, p values < 0.05 were considered statistically significant.

Results

Astragaloside IV improved flap survival area and ameliorated tissue edema.

One day after the random pattern skin flap surgery, the appearance of flaps became pale

and swollen to some extent in each group. Area III of the flaps in both groups were purple in color and edematous, indicating ischemia at the distal parts. On day 3, the previously ischemic parts underwent necrosis, evidenced by the appearance of dark and brown nidus. As shown in **Figure 1A**, necrosis of the flap was limited in appearance for Area III in both the Control group and the AS-IV group on day 3. However, there was a significant difference in survival area between the Control group and the AS-IV group. Mean survival area percentages in the AS-IV group ($86.40 \pm 2.20\%$) was significantly higher than that of the Control group ($75.02 \pm 2.47\%$, $p < 0.05$; **Figure 1B**). On day 7, Area I of all flaps survived, whereas the necrosis in Area III became darker and started to spread to Area II, with scabbing and hardening in both groups (**Figure 1A**). The difference of survival area between each group was more obvious than before, with the AS-IV group having significantly higher mean survival area percentage than the control group ($69.00 \pm 3.30\%$ and $50.80 \pm 3.29\%$, respectively; $p < 0.05$; **Figure 1B**). To observe the extent of edema, flap tissues from both groups were

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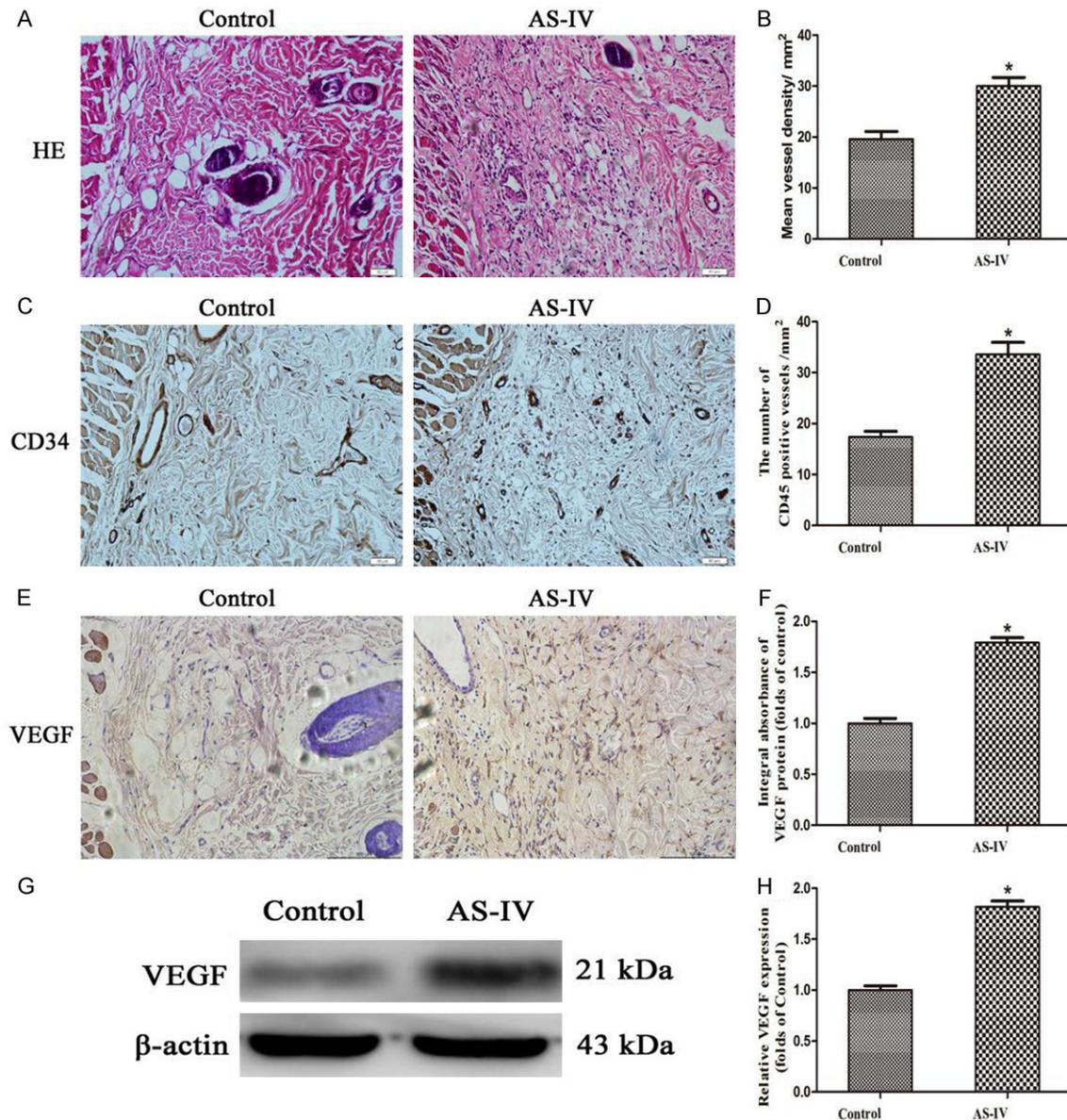


Figure 2. Astragaloside IV promoted angiogenesis in the ischemic areas of the flap. On day 7, angiogenesis in the ischemic area (Area II) of the flap was assessed via H&E staining, Immunohistochemistry for CD34, and Western blotting for VEGF expressions. A. H&E staining to observe the neovascularization in Area II of the Control group and the AS-IV group (original magnification, $\times 200$; scan bar, 50 μm). B. The mean vessel densities (MVDs) in each group were quantified and analyzed. C. Immunohistochemistry for CD34 to visualize vessels in Area II of the Control and AS-IV groups (original magnification, $\times 200$; scan bar, 50 μm). D. CD34-positive vessel densities in each group are quantified and analyzed. E. Immunohistochemistry for VEGF protein expression in the Area II of the Control group and the AS-IV group (original magnification, $\times 200$; scan bar, 100 μm). F. Optical density values of VEGF expressions are quantified and analyzed for each group. G. Western blotting for VEGF protein expressions in Area II tissue in the Control and AS-IV groups. Gels were run under the same experimental conditions, and cropped blots are used here. The original images are available in [Figure S1A](#). H. Optical density values of VEGF expressions in each group are quantified and analyzed for each group. Significance: * $p < 0.05$ versus Control group. Data presented as Mean \pm SEM, $n=6$ per group.

detached to display the inner side. As shown in **Figure 1C**, the distal part of flaps was swollen and bruised, with subcutaneous venous blood

stasis in the Control group. These signs were less apparent in the AS-IV group compared to the control group. To quantify edema, testing

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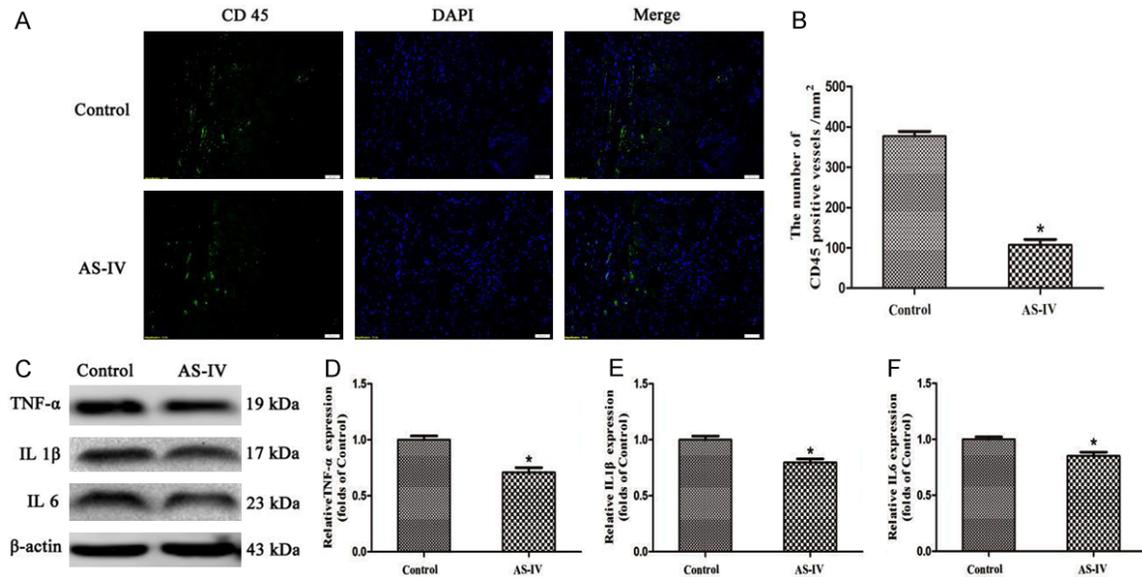


Figure 3. Astragaloside IV depressed inflammation in the ischemic area of flap. On day 7, the inflammation in Area II was assessed via Immunofluorescence for CD45 and Western blotting for TNF- α , IL1 β , and IL6 expressions. A. Immunofluorescence showing leukocyte infiltration in Area II of the Control and AS-IV groups; leukocytes were labeled with CD45 (green), nuclei was labeled with DAPI (blue) (original magnification, $\times 200$; scan bar, 50 μm). B. CD45-positive cell densities were quantified and analyzed in ischemic flaps for each group. C. Western blot results for TNF- α , the IL1 β , and IL6 protein expression in Area II tissue of the Control group and the AS-IV group. Gels were run under the same experimental conditions, and cropped blots are used here. The original images are available in Figure S1B, S1C. D-F. Optical density values of TNF- α , IL1 β , and IL6 expressions are quantified and analyzed in each group. Significance: * $p < 0.05$ versus Control group. Data presented as Mean \pm SEM, $n=6$ per group.

for percentages of tissue water content was carried out. Percent tissue water content was significantly lower in the AS-IV group ($46.40 \pm 2.11\%$) than in the Control group ($60.20 \pm 1.85\%$; $p < 0.05$; **Figure 1D**), indicating that tissue edema was ameliorated with the treatment of AS-IV.

Astragaloside IV promoted angiogenesis in the ischemic area of flap

H&E staining was performed to observe the histology of Area II of all flaps. As shown in **Figure 2A**, the AS-IV group flap presented more neovascularization than the control group flap. From H&E staining results, mean vessel densities (MVDs) were calculated to quantify angiogenesis. The MVD of Area II in the AS-IV group was $30.02 \pm 1.70/\text{mm}^2$, which was significantly larger than that in the Control group ($19.60 \pm 1.50/\text{mm}^2$; $p < 0.05$; **Figure 2B**). For further quantification of MVDs in each group, immunohistochemistry for CD34 was used to label endothelial cells in vessels. As shown in **Figure 2C**, AS-IV treatment increased the number of CD34-positive vessels, with densities of 33.20

$\pm 5.17/\text{mm}^2$ in the AS-IV group compared to $16.48 \pm 2.87/\text{mm}^2$ in the Control group ($p < 0.05$; **Figure 2D**). VEGF is a highly specific mitogen for vessel endothelial cells, and is able to promote angiogenesis. Thus, VEGF expression was tested in order to quantify the capacity of neovascularization in both groups. Immunohistochemistry for VEGF was performed to show protein expression. As shown in **Figure 2E**, VEGF was expressed in vessels and stromal cells, and the expression was higher in the AS-IV group compared with the Control group as measured by optical density values ($p < 0.05$; **Figure 2F**). Moreover, western blotting showed that the AS-IV group expressed more VEGF than the control group ($p < 0.05$; **Figure 2G-H**).

Astragaloside IV depressed inflammation in the ischemic area of flap.

Immunofluorescence staining was carried out for CD45 to observe leukocyte infiltration in Area II of random skin flaps. Leukocytes were labeled with CD45 (green), and nuclei were labeled with DAPI (blue) (**Figure 3A**). Moreover,

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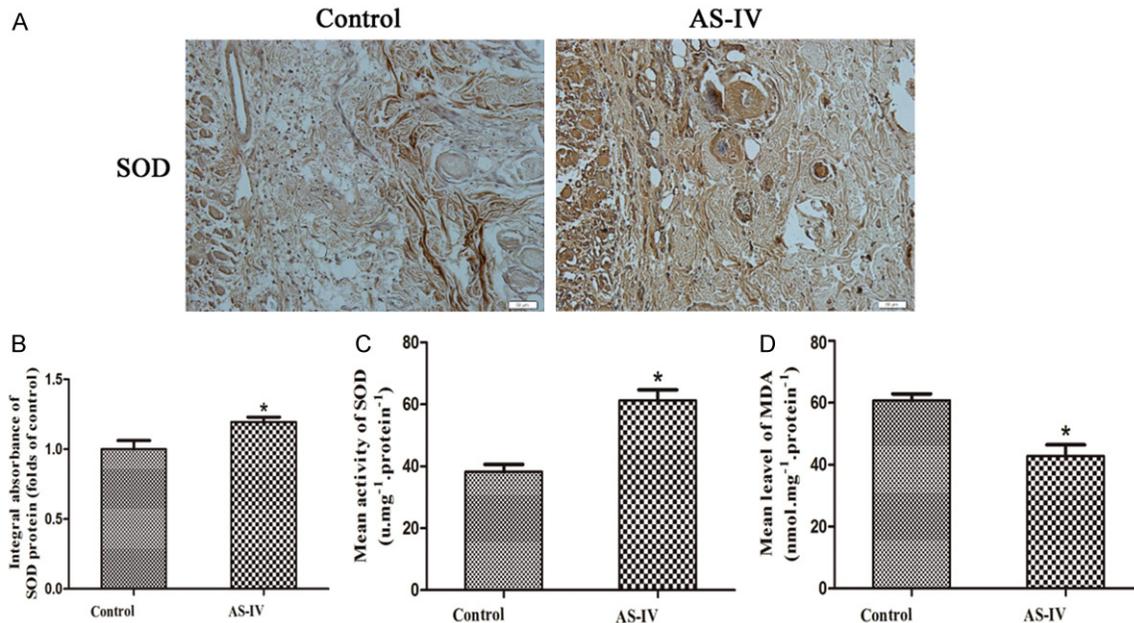


Figure 4. Astragaloside IV dampened oxidative stress in the ischemic area of flap. The oxidative stress in Area II of flaps was assessed via immunohistochemistry for SOD, SOD level, and MDA content test 7 days after surgery. **A.** Immunohistochemistry for SOD protein expression in Area II of flaps in the Control and AS-IV groups (original magnification, $\times 200$; scan bar, 100 μm). **B.** Optical density values of SOD expressions are quantified and analyzed in each group. **C.** The level of SOD activity in Area II of the Control group and the AS-IV group measured via the xanthine oxidase method is quantified and analyzed. **D.** The values of MDA content in Area II in each group was quantified and analyzed via the modified TBA test. Significance: * $p < 0.05$ versus Control group. Data presented as Mean \pm SEM, $n=6$ per group.

the AS-IV group exhibited less CD45-positive cells than the Control group. The number of leukocyte per mm^2 was $377.00 \pm 12.00/\text{mm}^2$ in the AS-IV group, which is significantly larger than that in the Control group ($107.60 \pm 13.26/\text{mm}^2$; $p < 0.05$; **Figure 3B**). Furthermore, expressions of inflammation related proteins (TNF- α , IL1 β and IL6) were evaluated via western blotting. As shown in **Figure 3C-D**, levels of TNF- α , IL1 β and IL6 were significantly lower in the AS-IV group than in the Control group ($p < 0.05$ for all).

Astragaloside IV dampened oxidative stress in the ischemic area of flap.

Immunohistochemistry for the SOD protein was performed to indicate oxidative stress status in Area II of random skin flaps. As shown in **Figure 4A**, higher SOD expression was observed in the AS-IV group compared with the Control group, with a higher optical density in the AS-IV group than in the Control group ($p < 0.05$; **Figure 4B**). Furthermore, SOD activity was tested via the xanthine oxidase method and MDA content was

detected via the modified TBA test. Results showed that the AS-IV group had a much higher mean level of SOD ($61.20 \pm 3.48 \text{ U.mg}^{-1}.\text{protein}^{-1}$) than the control group ($38.20 \pm 2.41 \text{ U.mg}^{-1}.\text{protein}^{-1}$; $p < 0.05$; **Figure 4C**). The mean level of MDA in the AS-IV group was $42.75 \pm 3.68 \text{ nmol.mg}^{-1}.\text{protein}^{-1}$, which was significantly less than $60.75 \pm 2.17 \text{ nmol.mg}^{-1}.\text{protein}^{-1}$ in the control group ($p < 0.05$; **Figure 4D**).

Astragaloside IV activated autophagy in the ischemic area of flap.

Immunofluorescence was performed for autophagosomes in cells from Area II of flaps in both groups. As shown in **Figure 5A**, autophagosomes were labeled via LC3II punctate dots (green), and nuclei were labeled with DAPI (blue) in both groups. In the AS-IV group, the number of LC3II-positive dots in the cytoplasm of flap cells was greater than that in the Control group. Expressions of autophagosome formation related proteins, Beclin1 and LC3II/II, were evaluated via western blotting (**Figure 5B**). Optical density values showed that the

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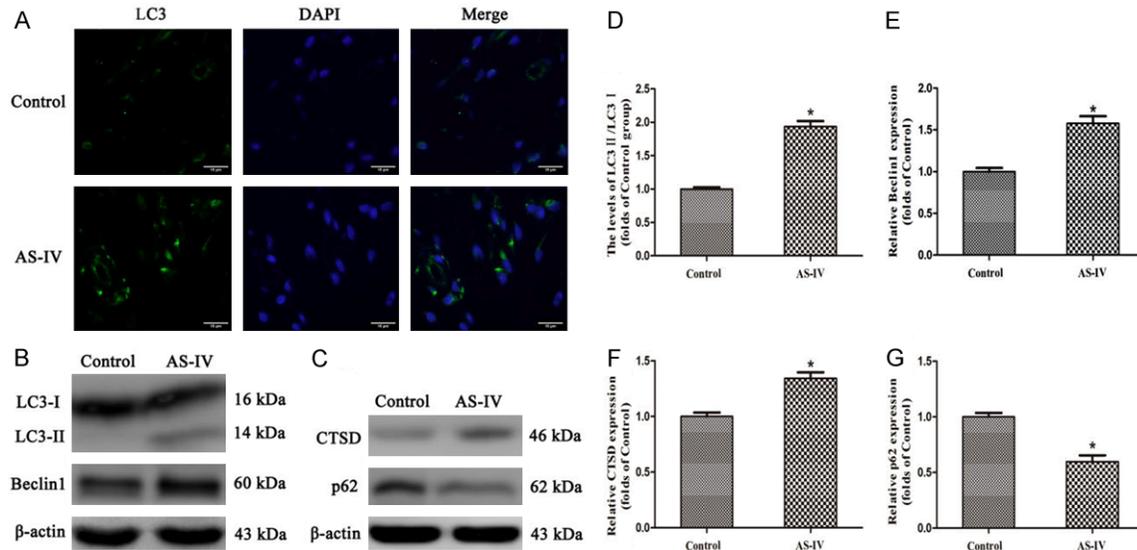


Figure 5. Astragaloside IV activated autophagy in the ischemic areas of flaps. The autophagy activity in Area II of flap was assessed via Immunofluorescence for LC3 and western blotting for LC3II/I, Beclin1, CTSD, and P62 protein expressions 7 days after surgery. A. Immunofluorescence for the autophagosomes in the cells in Area II of the Control and the AS-IV groups; autophagosomes were labeled via LC3II punctate dots (green), nuclei were labeled with DAPI (blue) (scan bar, 15 μ m). B, C. Western blotting was performed for LC3II/I, Beclin1, CTSD and P62 protein expressions in Area II tissue from the Control group and the AS-IV group. Gels were run under the same experimental conditions, and cropped blots are used here. The original images are available in [Figure S1D](#). D-G. Optical density values of LC3II/I, Beclin1, CTSD, and p62 expressions were quantified and analyzed in each group. Significance: * $p < 0.05$ versus Control group. Data presented as Mean \pm SEM, $n=6$ per group.

LC3II/LC3I ratio and Beclin1 expressions were significantly greater in AS-IV group than in the Control group ($p < 0.05$ for both; **Figure 5D-E**). Expressions of autophagic flux related proteins, CTSD and p62, were also tested via western blotting (**Figure 5C**). The optical density values show that CTSD expression was much higher in the AS-IV group than in the Control group ($p < 0.05$; **Figure 5F**), whereas p62 expression was much lower in the AS-IV group than in the Control group ($p < 0.05$; **Figure 5G**).

Discussion

Astragaloside IV is the main constituent of *Astragalus membranaceus*, and has been widely used for treatment of vascular ischemic diseases in China for thousands of years [17]. Ischemia and necrosis in the distal part of random pattern skin flap induced by the lack of blood supply is a common problem in plastic and reconstructive surgery. However, few studies have paid attention to Astragaloside IV's potential therapeutic functions in the context of random pattern skin flap transplantation. The results of the present study showed that Astragaloside IV significantly enhances random

skin flap survival. Furthermore, we showed that its pharmacological mechanism of action involved promoting angiogenesis, depressing inflammation, dampening oxidative stress, and activating autophagy.

Previous studies have shown that Astragaloside IV exerts pro-angiogenic effects on human umbilical vein endothelial cells (HUVECs) *in vitro* [14, 17, 23], and even in animal models of myocardial infarction [24]. Improving angiogenesis and increasing blood supply to ischemic areas was considered to promote the survival of random skin flap [25]. In our study, MVD (an indicator of angiogenesis) results from H&E staining and immunohistochemistry results for CD34 showed more neovascularization in skin flaps from the AS-IV treatment group. Thus, these findings demonstrate that Astragaloside IV promotes survival of random skin flap via increased angiogenesis. Moreover, we also showed that Astragaloside IV enhanced the expressions of vascular endothelial growth factor (VEGF). VEGF is secreted by keratinocytes, fibroblasts in the cutis, and dermal vascular structures [5], and its increased endogenous or exogenous expression can promote vascular-

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ization of random skin flaps [12, 26]. Therefore, we hypothesized that Astragaloside IV promoted angiogenesis in random skin flap via the enhancement of VEGF expressions. In the present study, levels of VEGF in vessels and stromal cells in the dermis was up-regulated after AS-IV treatment. Western blotting also revealed higher levels of VEGF in the AS-IV group than the control group. In short, Astragaloside IV promotes random skin flap survival via the enhancement of VEGF-induced angiogenesis.

Literature has reported that inflammation is an important factor predisposing flaps to ischemia and necrosis [27]. The greater the extent of inflammation, the more pronounced the necrosis, ultimately compromising flap success [5]. Recently, Astragaloside IV was found to have an anti-inflammatory activity in the treatments of various diseases such as myocardial infarction, necrotizing enterocolitis, and ischemic brain injury. In the present study, leukocyte infiltration and expression of inflammatory factors (TNF- α , IL1 β and IL6) in flap tissue were evaluated to further elucidate the impact of AS-IV on the inflammatory response. Immunofluorescence results showed that leukocyte infiltration was significantly attenuated in ischemic flap tissue after the treatment of Astragaloside IV. Moreover, western blotting results indicated that TNF- α , IL1 β and IL6 expressions were reduced in the ischemic flap tissue after treatment with Astragaloside IV. Thus, we conclude that Astragaloside IV had a strong anti-inflammatory effect in ischemic areas of random skin flaps.

Part of the tissue damage in random skin flap is thought to be due to post-ischemic reperfusion, which occurs when oxygen returns to the tissue following initial ischemia, resulting in the generation of reactive oxygen species [28]. In early stages of oxidative stress, these reactive oxygen species react with the lipids of cell membranes and proteins, triggering peroxidation and destruction of cells and tissues [29]. As a marker of lipid peroxidation, MDA production can reflect the extent of tissue injury [30]. Furthermore, SOD is one of the body's defenses against reactive oxygen species [31], and its level is an indicator of antioxidant status to some extent. Thus, SOD activity and MDA content are important biomarkers that reflect oxidative stress status. In studies of acute kidney injury, Astragaloside IV has been shown to

inhibit oxidative stress [32]. Consistent with other studies, our results demonstrated that Astragaloside IV reduced oxidative stress in ischemic parts of skin flaps, as reflected by an increased expression of SOD and lower MDA levels. In short, we found that Astragaloside IV dampened oxidative stress in ischemic areas of random skin flaps.

In recent years, it has also been reported that Astragaloside IV exerts cell protective effects via enhancing autophagy to eliminate dysfunctional mitochondria [15]. Autophagy, a major pathway for bulk cytosolic degradation and efficient turnover under stress, has an important role in maintaining cellular homeostasis by degrading and recycling damaged organelles and unwanted proteins through the autophagosomal-lysosomal pathway [22]. Growing evidence has shown that autophagy acts as a pro-survival mechanism in various vascular diseases [33], particularly in ischemia-reperfusion injury [20]. For example, the stimulation of autophagy provides protective functions for neurons in cerebral ischemia-reperfusion injury via inhibition of apoptosis [34]. Enhancing autophagy also acts as a protective mechanism in cardiac myocytes after ischemia-reperfusion injury [35]. In random skin flaps, ischemia-reperfusion injury greatly impacts flap survival [36]. However, the role of autophagy in random skin flap is unclear, and to our knowledge, our present study is the first report of Astragaloside IV-mediated activation of autophagy in the context of random skin flap. In our study, immunofluorescence results revealed that more LC3II punctate dots were present in the cytoplasm of cells after treatment Astragaloside IV. Western blotting also showed that LC3II/LC3I and Beclin1 were up-regulated, indicating that the number of autophagosomes increased in the ischemic area of flaps after treatment with AS-IV. However, the generation of autophagosomes does not necessarily indicate an increase of autophagic flux. To further show an increase in overall autophagic activity, we further investigated the protein p62 (a substrate of the autophagic process [37]) and CTSD (a common maker of lysosomal activity [38]), which better reflect the intensity of autophagic flux [39]. From western blotting results, CTSD expression in the AS-IV group was much higher than in the Control group, whereas p62 expression in the AS-IV group was much lower compared with the Control group,

together indicating that autophagy flux was enhanced in the AS-IV group. Overall, our results show that Astragaloside IV activated autophagy in ischemic areas of random skin flaps.

However, whether Astragaloside IV promotes random skin flap survival via autophagy activation is still unclear. From the evidences of other studies, when ROS are produced by damaged mitochondria under conditions of oxygen stress, spontaneous autophagy is activated to sequester and degrade damaged mitochondria and its ROS, helping cells escape death [40]. Based on our results of autophagy enhancement along with oxidative stress depression in the AS-IV group, we hypothesize that Astragaloside IV may reduce oxidative stress by activating autophagy in random skin flaps. However, autophagy was also found to be a mechanism of programmed cell death, termed autophagic cell death [41]. Therefore, stimulated autophagy after the treatment of Astragaloside IV in random skin flap may be a side effect which accelerates cell death and necrosis in ischemic tissue. Therefore, further investigation is needed to elucidate the role of autophagy following treatment with Astragaloside IV for random skin flap ischemia and necrosis.

In conclusion, Astragaloside IV induced angiogenesis, depressed inflammation, and dampened oxidative stress, contributing to a significant increase in random skin flap survival. Autophagy was activated in the ischemic area of random skin flaps after Astragaloside IV treatment, which may be an underlying mechanism for oxidative stress depression.

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Disclosure of conflict of interest

None.

Abbreviations

GSH-Px, Glutathione peroxidase; NF- κ B, Nuclear factor- κ B; HPLC, High performance liquid chromatography; H&E, Hematoxylin-eosin; DAB, Diaminobezidin; SOD, Superoxide dismutase; GSH, Glutathione; MDA, Malondialdehyde; VEGF, Vascular endothelial growth factor; LC3, Microtubule-associated 1 protein light chain 3; IL6, Interleukin 6; IL1 β , Interleukin 1 β ; TNF- α , Tumor necrosis factor- α ; DMSO, Dimethyl sulfoxide; CTSD, Cathepsin D; HRP, Horse radish peroxidase; FITC, Fluorescein isothiocyanate; IgG, Immunoglobulin G; BCA, Bicinchoninic acid; ECL, Electrochemiluminescence; ip, Intraperitoneal injection; ig, Intragastric administration; AS-IV, Astragaloside IV; mM, mmol/L; PBS, Phosphate buffer saline; IA, Integral absorbance; DAPI, 4,6-Diamidino-2-Phenylindole; PVDF, Polyvinylidene fluoride; DTNB, Dithiobis-nitrobenzoic acid; TBA, Thiobarbituric acid; r.p.m, Revolutions per minute; SEM, Standard error of the mean.

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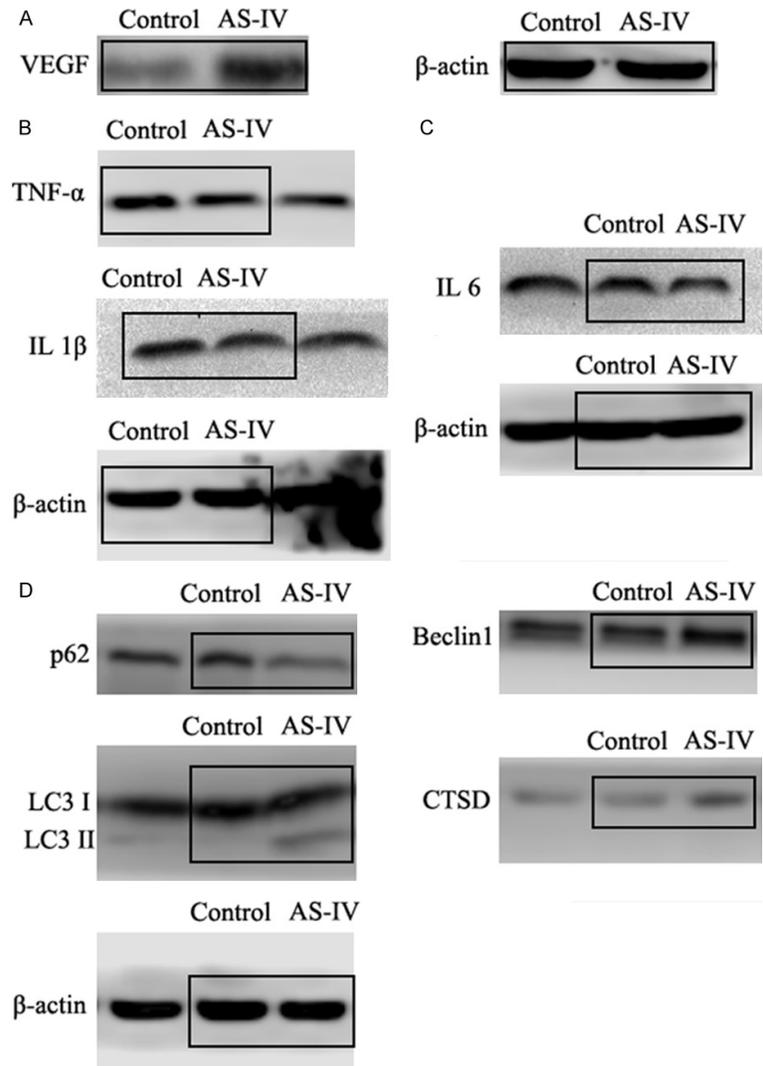


Figure S1. Original western blot images for each western blot in the study. A. The original gel image for **Figure 2G**. B, C. The original gelimages for **Figure 3C**. D. The original gelimage for **Figure 5B, 5C**.