

# Isoliquiritin Pre-Treatment Promotes Multi-Territory Perforator Flap Survival in Rats: An Experimental Study

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## Research article

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# Abstract

**Background** The present study was design to investigate the effect of isoliquiritin (ISL) pretreatment on multi-territory perforator flap survival and blood vessels of Choke II zone in rats.

**Methods** A total of 80 adult Sprague-Dawley (SD) rats were randomly divided into ISL group and normal saline group, and subsequently and subjected to multi-territory perforator flap operations on the left flank. Afterwards, rats in ISL group were intraperitoneally injected with ISL, and rats from normal saline group were intraperitoneally injected with equal amount of normal saline. After seven days, the surviving flap area was calculated, the density of microvessels and (vascular endothelial growth factor,VEGF)were measured in Choke II zone. In addition, blood vessels of the flap were subjected to lead oxide-gelatin radiography.

**Results** The flap survival area was significantly enhanced in rats from the ISL group compared with that from the saline group ( $P < 0.01$ ). HE staining indicated significantly higher microvascular density in Choke II zone in the ISL group ( $P < 0.01$ ). Immunohistochemistry and Western blot assays showed that the expression of VEGF in the ISL group was significantly higher than that in the control group ( $P < 0.01$ ). Moreover, the vascular structure in Choke II zone of the flap was clearer, with more new blood vessels and more complete vascular structure in the potential zone from the ISL group, in comparison with those from the normal saline group.

**Conclusion** ISL is beneficial to the multi-territory perforator flap survival in rats.

## 1.0 Background

Multi-territory perforator flaps are commonly used for soft tissue defect repair in the extremities [1], however, distal necrosis of the flap remains challenging. The angiosomes are the basic anatomical unit of the perforator flaps [2], which can be divided into three regions, including the anatomical region from the source artery, the dynamic region adjacent to the anatomical region and the potential region adjacent to the dynamic region. The flap is relatively stable in the anatomical and dynamic regions, while necrosis generally occurs in the potential region. A choke vessel is defined as the vascular connection between adjacent angiosomes with gradually decreased caliber, which is of great significance to the multi-territory perforator flap survival. The occluded vascular anastomosis area between the anatomical region and the dynamic region is called as the Choke I zone, and the occluded vascular anastomotic area between the dynamic area and the potential area is called as the Choke II zone. The vascular dilation and insufficient angiogenesis of the Choke II zone is one of the important potential reasons for flap necrosis [3]. Thus, improving blood supply in the Choke II zone plays a critical role in flap survival [4–6].

In recent years, to improve the survival rate of multi-territory perforator flaps, delayed surgery [7, 8] drug intervention [4–6] and etc., have been proposed to dilate vessels and/or promote angiogenesis in the Choke II zone, to improve the blood supply in the Choke II zone, thereby increasing the flap survival area. However, delayed surgery has the disadvantage of increasing the number of operations and increasing

pain [8]. In addition, drug intervention has become a research hotspot due to high safety, small trauma and little pain [4–6].

Liquorice is a widely-used herb in southern Europe and certain parts of Asia [9]. Isoliquiritin (ISL) is a water-soluble isoflavone component in liquorice root. ISL has been reported to exert an additional pro-angiogenic effect on damaged angiogenesis in zebrafish embryos, which might be potentially used to treat disorders caused by insufficient angiogenesis [9]. Liu YY, et al. [10] have investigated the effect and mechanism of ISL on wound healing activity in zebrafish, revealing that ISL can promote inflammatory response and angiogenesis, which plays a key role in promoting wound healing in zebrafish. Therefore, ISL is a promising candidate to promote wound healing. However, it remains largely undefined whether ISL could promote angiogenesis in Choke II zone of the flaps to subsequently improve the survival of distal multi-territory perforator flaps in rats. Compared with vertebrates, mammalian skin wound healing is a complicated, multi-step process that involves various stages, including formation of blood clot, inflammation, re-epithelialization, formation of granulation tissue, neovascularization and remodeling, generally with scar left [11–12]. To this end, the present study was design to assess the effects of ISL pretreatment on the survival of flap transplantation and angiogenesis in Choke II zone of the flap in rats by establishing the multi-territory perforator flap model.

## 2.0 Methods

### 2.1 Chemicals

ISL (CAS no. 5041-81-6, purity  $\geq$  98%) was purchased from Pufei De Bio Co.(Chengdu, China), its chemical structure is shown in Fig. 1. Dimethyl sulfoxide(DMSO), tricaine (ethyl 3-aminobenzoate methane- sulfonate) and hematoxylin and eosin (H&E) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trizols Reagent was purchased from Invitrogen (Carlsbad, CA, USA).

### 2.2 Animals and group assignments

Adult Sprague-Dawley (SD) female rats (weight 210–240 g) were purchased from the Experimental Animal Center of Suzhou University. Rats were housed in the single cage, fed with standard rat chow at an appropriate temperature (23–25 °C).

In total, 80 rats were randomly divided into ISL group (n = 40) and normal saline group (n = 40). All surgical procedures and protocols in this study were approved by the Animal Ethics Committee of Suzhou University. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals described by the National Research Council Drug Administration.

### 2.3 Operative technique

All rats were anesthetized with 2% pentobarbital sodium (40 mg/kg, intraperitoneal injection). After anesthesia, rats were fixed in the prone position on the sterile operating table, hair was removed using a

pet electric shaver, and rats were deiodinated with alcohol after iodophor disinfection. The dorsal flap was designed according to Miyamoto [11]. To be specific, a trivascular perforator flap based on a perforating artery of deep iliac circumflex artery was design on the left dorsal of the rat, including two choke zones. In addition, the flap borders were as follows: medial border, the midline of the dorsum; the cranial border, subscapular angle; caudal border, the spina iliaca posterior superior, and the dorsal flap measuring 10 × 2.5 cm was designed. Firstly, the skin was cut along the medial border of the flap, and the subcutaneous tissue was separated just from the meat layer. The flap was gradually lifted to separate trivascular pedicles. The arteriathoracodorsalis perforator and the intercostal artery perforator were ligated. Finally, the arteriae circumflexa ilium profunda perforator was preserved and the flaps were completely separated, followed by identification of Choke I and II zone (Fig. 2) and corresponding marking on the epidermis using a marker pen (Fig. 2). After complete hemostasis, the flap sutured back into its original position with 4 – 0 silk. Wounds were post-operatively coated with chlortetracycline ointment to prevent infection. The flap was resected by the same surgeon to ensure the uniform thickness of each flap and to reduce the factors that might affect flap survival due to the different thickness of the flap.

## **2.4 Dosage and administration of drug**

Rats in the experimental group were intraperitoneally injected with ISL solution at a dose of 100,200 µg/kg two days before operation, two hours before operation and two days after operation according to the pre-experiment results. Rats in the control group were intraperitoneally injected with the same volume of saline solution accordingly.

## **2.5 Gross observation of flap and calculation of flap survival rate**

All flaps were grossly observed and recorded 1, 3, 5, and 7 days after operation, including the flap color, tissue elasticity, texture, skin and hair growth, infection, necrosis, and etc. After post-operative observation for seven days,ten rats were anesthetized and high-quality photographs of flap were obtained using a digital camera and imported into Image-Pro Plus v6.0 software to calculate the percentage of flap survival area. The flap survival rate in each group was calculated according to the following formula: flap survival rate = flap survival area / total flap area x100%. The standard of flap necrosis was as follows: the flap color was black, the tissue was hard, shrunk, dry necrosis, inelastic, and no blood outflow when cutting.

## **2.6 Perforator flap angiography**

On the 7th day after operation, 10 rats were randomly selected from the control and ISL groups (n = 10 each, 20 in total), and subjected to gelatin-lead oxide angiography of the flap vessels. (1) Preparation of the perfusion solution: The preparation ratio of perfusion solution was: industrial gelatin 1 g, warm water 20 ml 40 °C, lead oxide 20 g. First, the gelatin was dissolved in warm water for 3–4 h. After the gelatin was completely dissolved, lead oxide was added and stirred. Because the gelatin-lead oxide mixture was

prone to solidify under low temperature, the entire procedure must be carried out in water bath at 40 °C. The amount of perfusion depended on the size of rats, generally ranging from 40–50 ml / kg, not exceeding 50 ml / kg. (2) Perfusion process: rats were fixed in a supine position after anesthesia, and unilateral carotid artery was bluntly separated. Afterwards, No.24 indwelling catheter was inserted into the carotid artery, the catheter was ligated with silk thread for fixation. The blood from was completely drained from the rat, rinsed with heparin saline. When the effluent was clear, the prepared gelatin-lead oxide mixture was slowly and uniformly injected into the carotid artery until the sclera and the distal limbs of the rat showed dot-like or patchy orange-red. Afterwards, rats were placed in a supine position in the refrigerator at 4 °C for about 24 h for gelatin solidification. (3) X-ray radiography: 24 hours after perfusion, the entire back skin of the rat was peeled off and photographed under X-ray camera (40kv, 50 mA and 100 ms exposure time). (4) Image analysis: The PACS system of our hospital was used to visualize and assess the blood vessels in Choke II zone of the flaps in rats from both groups.

## **2.7 Histological examination of microvessel density (MVD) in the Choke II zone of the flap**

Seven days after operation, six rats from each group were anesthetized according to the previously described procedure. The flap tissue (2.0 cm × 0.5 cm in size) of Choke II zone was obtained, fixed in 40 g/L paraformaldehyde, routinely dehydrated, sliced into 5 µm-thick sections and subjected to HE staining. Afterwards, the staining results of the tissue sections were observed under the microscope. As a result, the cytoplasm was stained as pink by eosin, and the nuclei were stained as blue by hematoxylin. Firstly, the dense area of microvessels was found under 4 × 10 low magnification. Afterwards, five fields of view were randomly selected under 10 × 10 magnification microscope, and the number of blood vessels was counted manually for average to calculate the number of microvessels per unit area (number/mm<sup>2</sup>), which was used as an indicator for MVD.

## **2.8 Immunohistochemistry (IHC) for VEGF expression in Choke II zone of the flap**

The sections prepared in Sect. 2.7 (one for each rat) were routinely dewaxed, dehydrated, microwaved for antigen retrieval, incubated with 3% (volume fraction) peroxide at 37 ° C in water bath for 10 min to inactivate endogenous peroxidase, blocked with 5% (volume fraction) goat serum at 37 ° C for 30 min. The sections were subsequently incubated with anti-rat VEGF primary antibody (dilution 1:200) at 4 ° C overnight, reacted with HRP-conjugated goat anti-rat IgG secondary antibody at 37 ° C for 1 h, and incubated with HRP-conjugated streptomycin avidin at 37 ° C for 45 min. Afterwards, the sections were visualized by DAB, counterstained with hematoxylin, dehydrated with ethanol, transparented with xylene, sealed with neutral resin. The distribution of VEGF (brown yellow particles) in Choke II zone of the flap was observed under light microscope at 400 magnification and photographed under five randomly selected fields of view. Images were imported into Image Pro Plus 6.0 software to detect VEGF expression and the results were shown as integral absorbance values.

## 2.9 Western blot for VEGF expression in Choke II zone of the flap

Seven days after operation, total tissue protein was extracted from the Choke II zone of the flap from ten rats in each group. After adjusting protein concentration, equal amount protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to membranes and blocked with 50 g / L skim milk solution at room temperature for 2 h. The membranes were incubated with mouse anti-rat VEGF primary antibody (dilution 1:300), rabbit anti-mouse GAPDH primary antibody (dilution 1:500) overnight at 4 °C, followed by incubation with HRP-conjugated goat anti-mouse IgG secondary antibody (dilution 1:4000) or HRP-conjugated goat anti-rabbit IgG secondary antibody (dilution 1:5000) at room temperature for 2 h. Afterwards, the membranes were visualized using chemiluminescence, and grayscale scanning analysis was performed using gel image analysis system. The band intensity of VEGF was quantified and presented relative to GAPDH (internal control). This assay was performed in triplicate.

## 3.0 Treatment Of Rats After Experiment

All rats were killed by cervical dislocation after the experiment.

## 3.1 Statistical analysis

Statistical analysis was performed using SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL, USA). All normally distributed data were expressed as mean standard deviation (SD). Independent student-t test was used for comparison between two groups when the data were normally distributed. Otherwise, Wilcoxon rank sum test was used. A P value  $\leq 0.05$  was considered as statistically significant.

## 4.0 Results

### 4.1 Gross observation and flap survival rate

Seven days after operation, rats in both groups were alive, without flap infection. There was no abnormal behavior of rats throughout the experiment, and the body weight of rats were not significantly changed before and after the experiment. Seven days after operation, the necrotic area of the flap of rats in the ISL group was approximately located far away from the thoracodorsal artery perforator, the scar was dark yellow, with soft texture and easy to peel off. The necrotic area of the flap of rats in the control group was approximately located far away from the Choke II zone, and the scar was brownish black, with hard texture and difficult to peel off (Fig. 3). Seven days after operation, the flap survival rate was significantly higher in the ISL group ( $89 \pm 2\%$ ) than that in the saline solution group ( $81 \pm 3\%$ ) ( $P < 0.01$ ).

## 4.2 Vascular condition in Choke II zone of flap and potential region

Seven days after operation, the vascular structure of Choke II zone of flap of rats in the ISL group was relatively clear, with more neovascularization and relatively complete vascular structure in the potential region. However, in the saline solution group, the vascular structure of Choke II zone of flap of rats was ambiguous, with less neovascularization and disordered or disappeared vascular structure in the potential region (shown in Fig. 4).

## 4.3 MVD of Choke II zone of flap

Seven days after operation, there was more neovascularization in the Choke II zone of flap in rats of the ISL group, with MVD of  $30.2 \pm 2.2/\text{mm}^2$ . In rats of saline solution group, there was fewer neovascularization in the Choke II zone of flap, with MVD of  $21.5 \pm 3.2/\text{mm}^2$ . The difference was statistically significant between groups ( $P < 0.01$ ) (shown in Fig. 5).

## 4.4 VEGF expression in Choke II zone of flap

Seven days after operation, IF showed that the expression of VEGF in the Choke II zone of flap in rats from ISL group was  $7050 \pm 143$ , which was significantly higher than that of the saline solution group ( $3512 \pm 146$ ) ( $P < 0.01$ ) (shown in Fig. 6).

Seven days after operation, Western blot showed that the expression of VEGF in the Choke II zone of rats from the ISL group was  $0.52 \pm 0.02$ , which was significantly higher than that of the saline solution group ( $0.24 \pm 0.03$ ) ( $P < 0.01$ ) (shown in Fig. 7).

## 5.0 Discussion

In this study, we validated for the first time that ISL pretreatment can promote multi-territory perforator flap survival in rats and promote angiogenesis in the Choke II zone of the flap. Vascular endothelial growth factor (VEGF) is one of the most important regulatory factors in angiogenesis, which can specifically stimulate the proliferation and regeneration of vascular endothelial cells. VEGF is generally considered as an improving factor of the survival ability of ischemic flaps [12]. In addition, VEGF has been validated to induce angiogenesis and improve the survival rate of muscle flaps [13]. Fichter, et al. [14] have demonstrated that single dose injection of VEGF into the flap wound area can significantly decrease the necrotic area. Kane, et al. [15] have demonstrated that NO from iNOS can promote ischemic flap survival through angiogenesis, possibly by increasing the expression of VEGF in mast cells in the angiogenesis zone. In this study, Western blot and IHC staining were used to detect the expression of VEGF in Choke II zone of the flaps in rats, both of which showed that the expression of VEGF in the Choke II zone of the rat flap in the ISL group was significantly higher than that in the normal saline group.

Therefore, we consider that ISL can induce angiogenesis in the Choke II zone by promoting VEGF expression, thereby promoting flap survival.

Skin tissue, blood vessels and other accessory structures are generally formed seven days after flap transplantation [16–18], therefore, this time point was selected for observation. Zhao G, et al. [16] has confirmed that irisin can protect the perforator flap from ischemia-reperfusion injury by promoting the proliferation of vascular endothelial cells. In their study, the flap survival area after ischemia-reperfusion treatment in rats was significantly increased, compared with the saline group. Tao XY, et al. [17] have investigated the effects of iNOS on the multi-territory perforator flap survival and the blood vessels in the Choke II zone in rats, revealing that flap survival area was significantly enhanced in the iNOS intervention group than the control group. Cao B, et al. [18] have observed the effect of lidocaine on the flap survival area in rats, who found that the flap survival area in the lidocaine group was significantly larger than that in the control group. Herein, in the present study, we aimed to evaluate whether intraperitoneal injection of ISL could promote flap survival in rats. The calculating method of flap survival rate was consistent with the above studies. As a result, the flap survival area of the ISL group was significantly larger than that of the control group.

The blood vessels in the Choke II zone of the flaps were macroscopically observed by lead oxide radiography, and microvascular density was determined by histological detection. As a result, the microvascular density of the flaps was significantly enhanced in rats from the ISL group than that of the normal saline group; and lead oxide radiography showed that the vascular structure of the flaps was clearer, and the vascular structure in the potential area was more complete in rats from the ISL group compared to those from the normal saline group. Xie LZ, et al. [19] improved the survival of rat flaps by local injection of bone marrow mesenchymal stem cells (BMSCs)-derived exosomes. In their study, HE staining of Choke II zone showed higher microvascular density and more angiogenesis in the exosome group. By using the macroscopic observation via lead oxide angiography for vascular structure in the Choke II zone in previous literature [20, 5], we demonstrate that ISL can promote angiogenesis in the Choke II zone of rat flaps.

In this study, we have validated that ISL promotes the multi-territory perforator flap survival by promoting angiogenesis in the Choke II zone in rats. There was no rat death or flap infection in this study, no abnormal behavior in rats from the ISL group. The body weight of rats was basically the same at the beginning and the end of the experiment, indicating the safety and tolerability of ISL. However, there are still certain limitations in this study. We failed to investigate the specific mechanism of ISL in promoting angiogenesis in Choke II zone after flap transplantation. Previous studies have shown that the up-regulation of VEGF/VEGFR2 and Ang/Tie signaling pathways by ISL may be associated with the angiogenic activity observed on zebrafish embryos [10]. Therefore, in the future, we will further study the specific mechanism of ISL in promoting flap survival. Although accumulative evidence has supported the beneficial effects of ISL on the cardiovascular system, the potential negative effects still need to be explored. Our present findings only suggest that ISL has a certain role in promoting the perforator flap survival in rodents, which should be cautiously extrapolated to humans. In addition, the dose, route of

administration, and timing of ISL for flap transplantation in clinical practice should also be further investigated. Nevertheless, our study provides a potential beneficial drug to promote flap survival.

## 6.0 Conclusion

In this study, we show for the first time that ISL can promote the survival of multi-territory perforator flaps in rats and promote angiogenesis in the Choke II zone of the flaps, which has enriched the medicinal value of liquorice again. Hopefully, our findings could provide a potential drug candidate to promote flap survival.

## Abbreviations

ISL  
isoliquiritin; SD:Sprague-Dawley; DMSO:Dimethyl sulfoxide; H&E:hematoxylin and eosin;  
MVD:microvessel density;  
IHC  
Immunohistochemistry; VEGF:Vascular endothelial growth factor

## Declarations

### Ethics approval and consent to participate

The study was approved by the ethics committee of Wuxi No.9 People's Hospital Affiliated to Soochow University.

### Consent to publish

Not applicable.

**Availability of data and materials**Not applicable.

**Competing interests**The authors declare that they have no competing interests.

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**Authors' contributions**YW1 designed the study, prepared the manuscript. XZ and YW2 contributed to the data acquisition. YM contributed to the quality control of data and algorithms. JL, YR contributed to the data analysis and interpretation and reviewed the manuscript. All authors read and approved the final manuscript.

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Not applicable

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## Figures

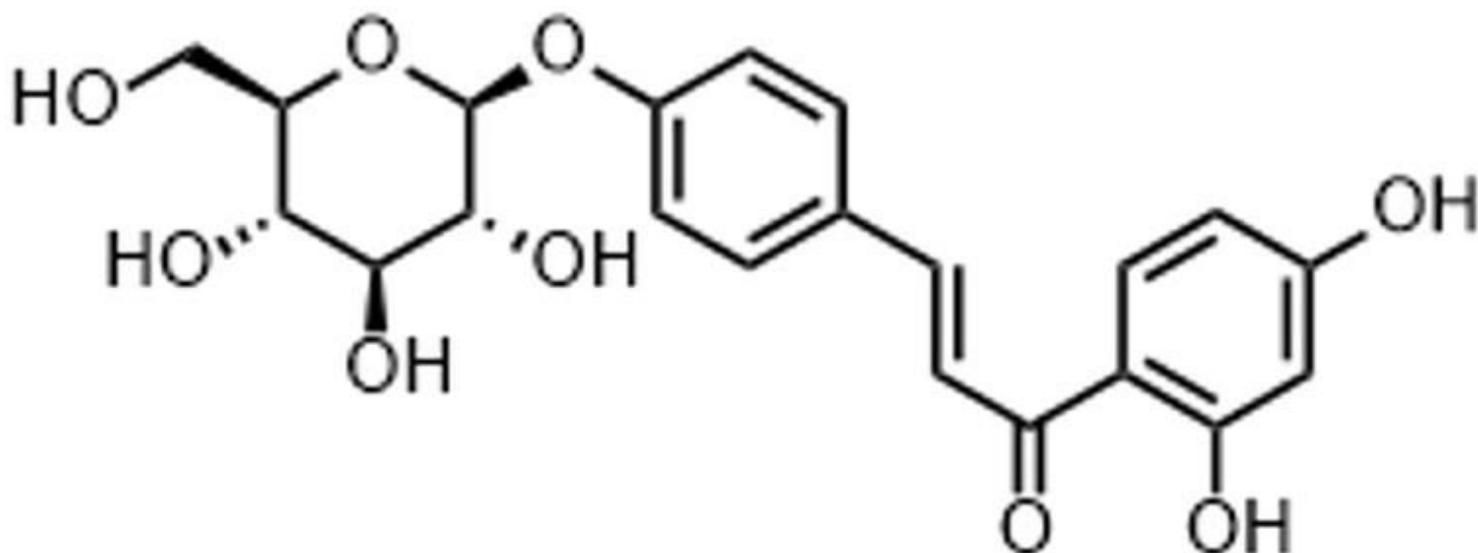
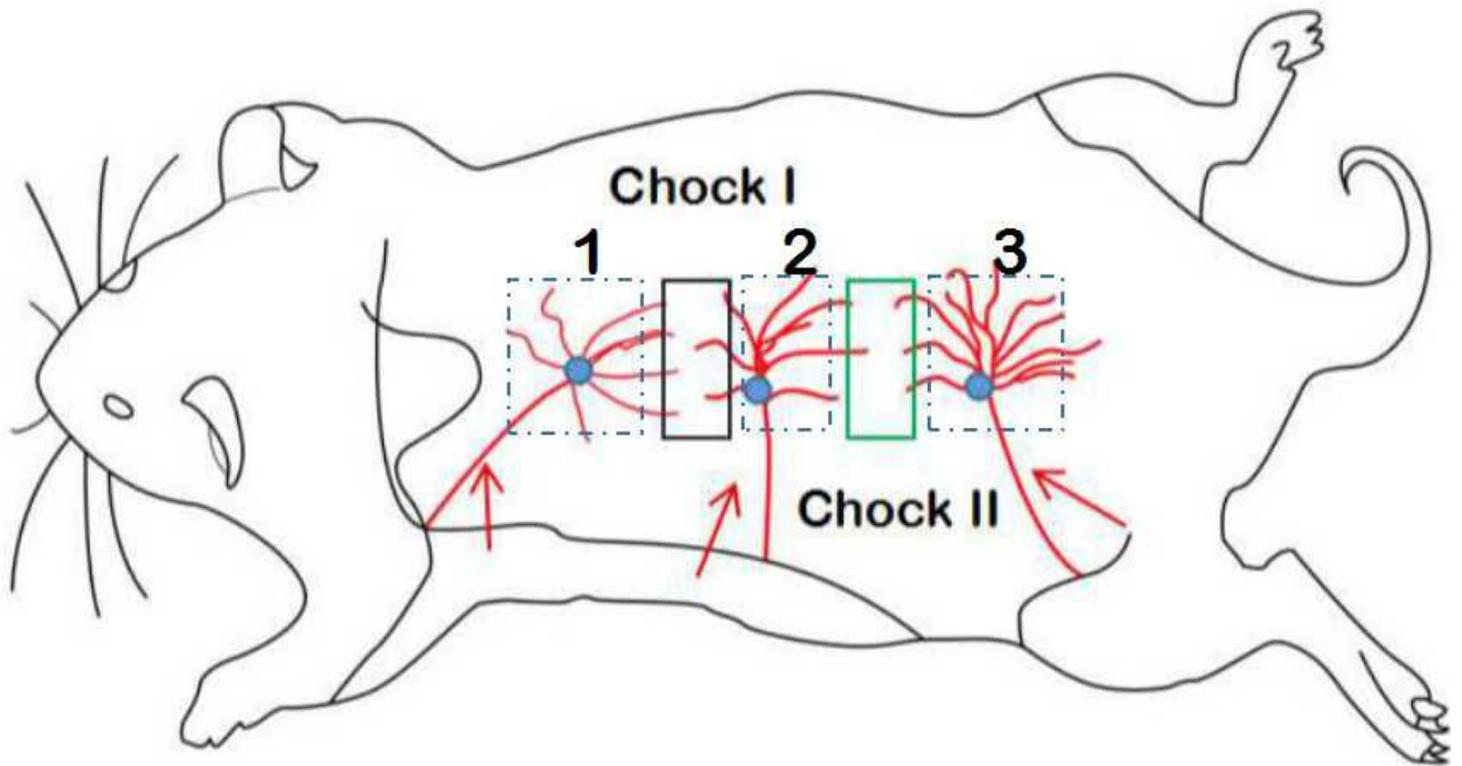


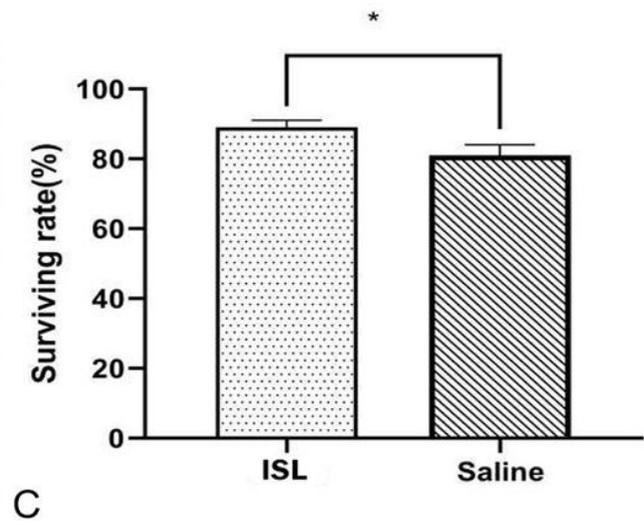
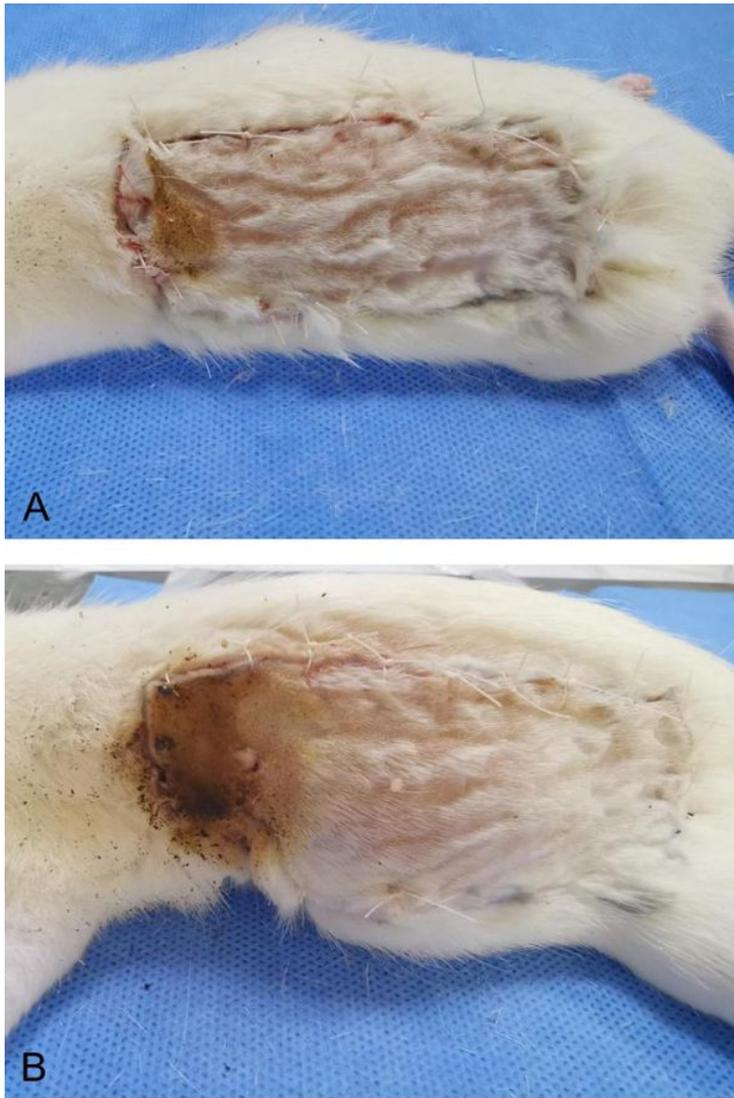
Figure 1

Chemical structure of isoliquiritin (ISL).



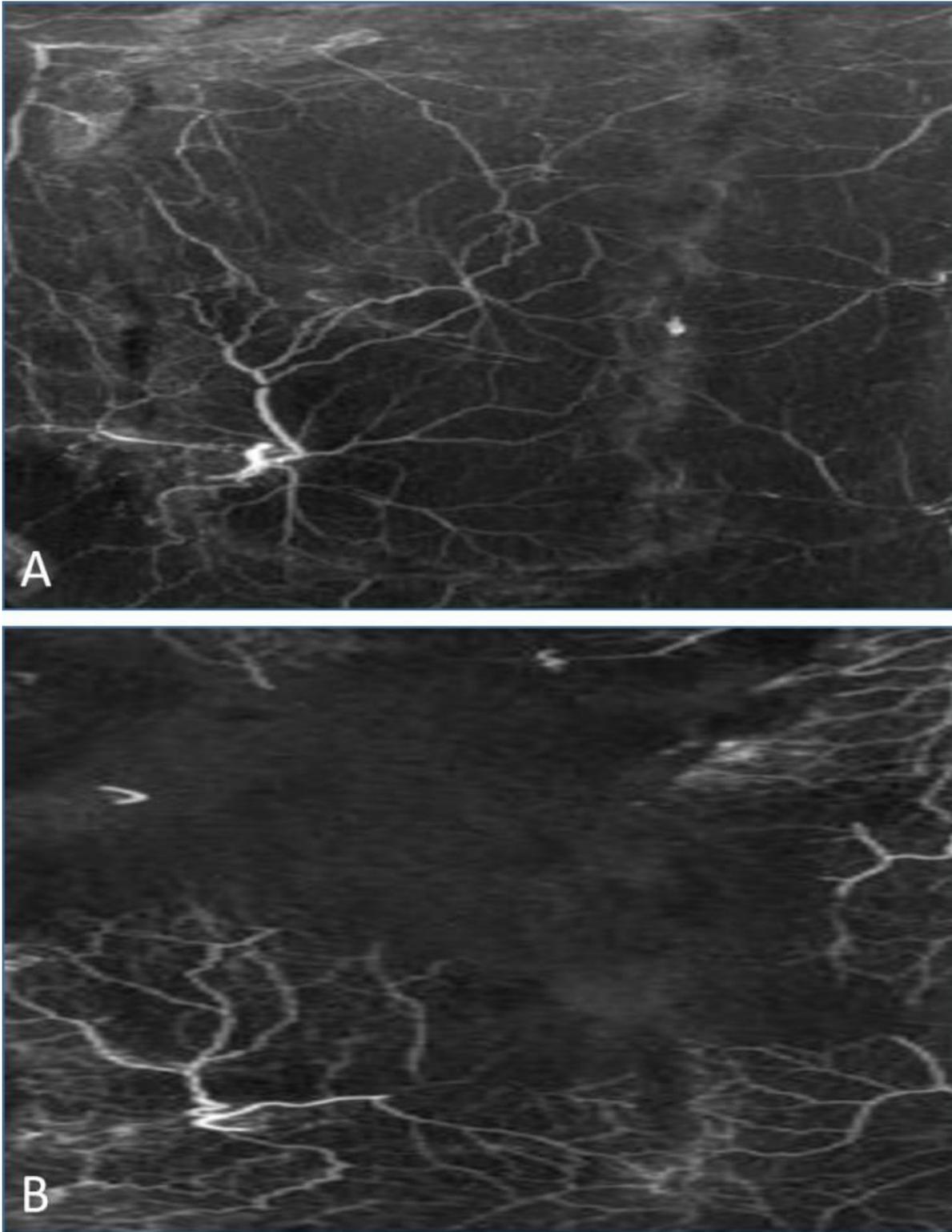
**Figure 2**

Schematic diagram of dorsal perforator flap in rats. The flap side was exposed to show the deep circumflex artery perforator (↑), intercostal artery perforator (↑), thoracodorsal artery perforator (↑), three vascular pedicles, Choke I zone (between anatomical region and dynamic region), Choke II zone (between dynamic region and potential region). The midlines of the three vascular pedicles and two choke zones were marked in the flap surface using a marker pen, and the flap sutured back into its original position.



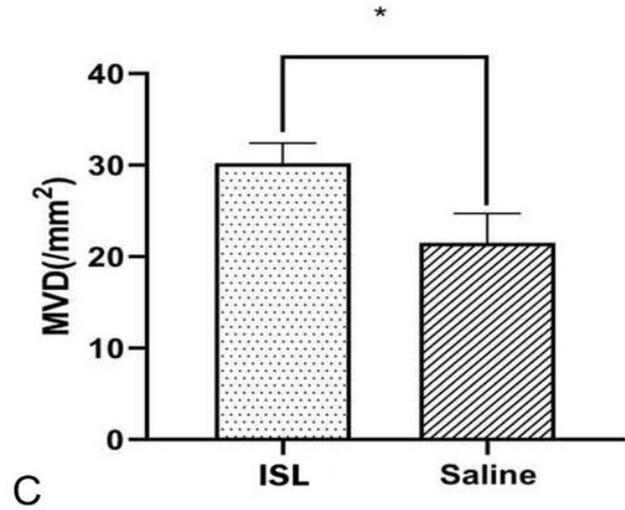
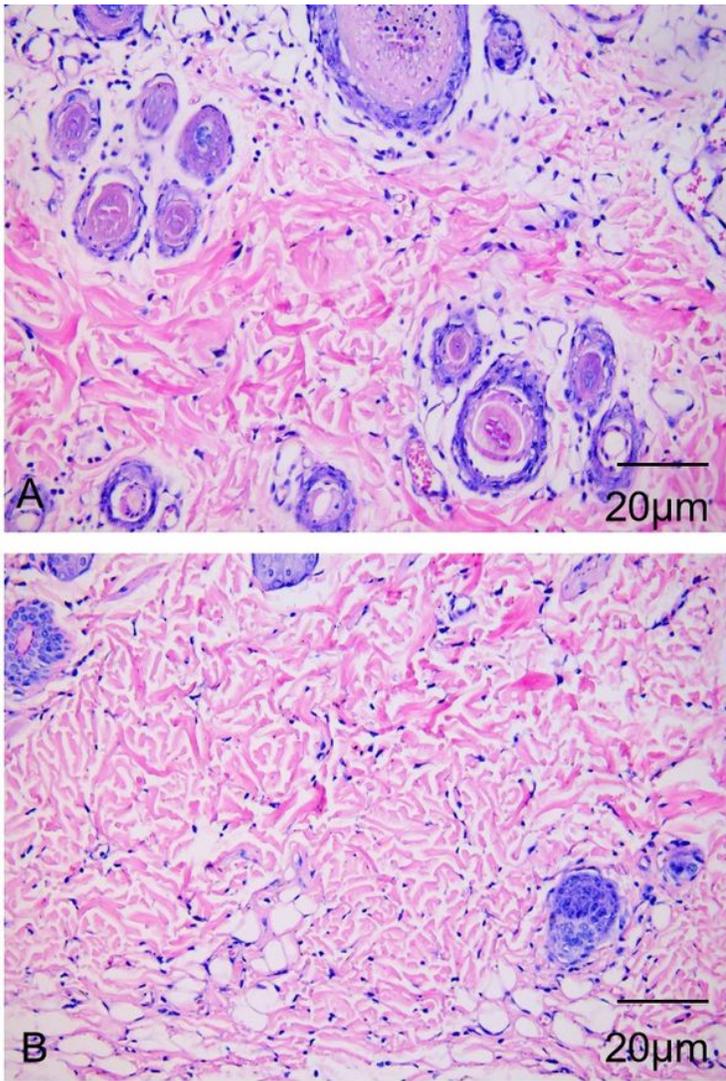
**Figure 3**

Gross observation of flaps in two groups of rats seven days after operation. A. The necrotic area of the flap of rats in the ISL group was approximately located far away from the thoracodorsal artery perforator, and the scar was dark yellow. B. The necrotic area of the flap of rats in the saline solution group was approximately located far away from the Choke II zone, and the scar was brownish black. C. The flap survival rate of rats was significantly higher in the ISL group than that in the saline group; \*Statistical significance ( $P < 0.01$ )



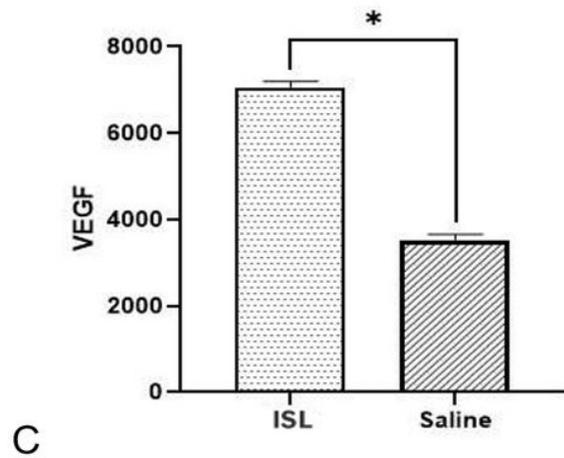
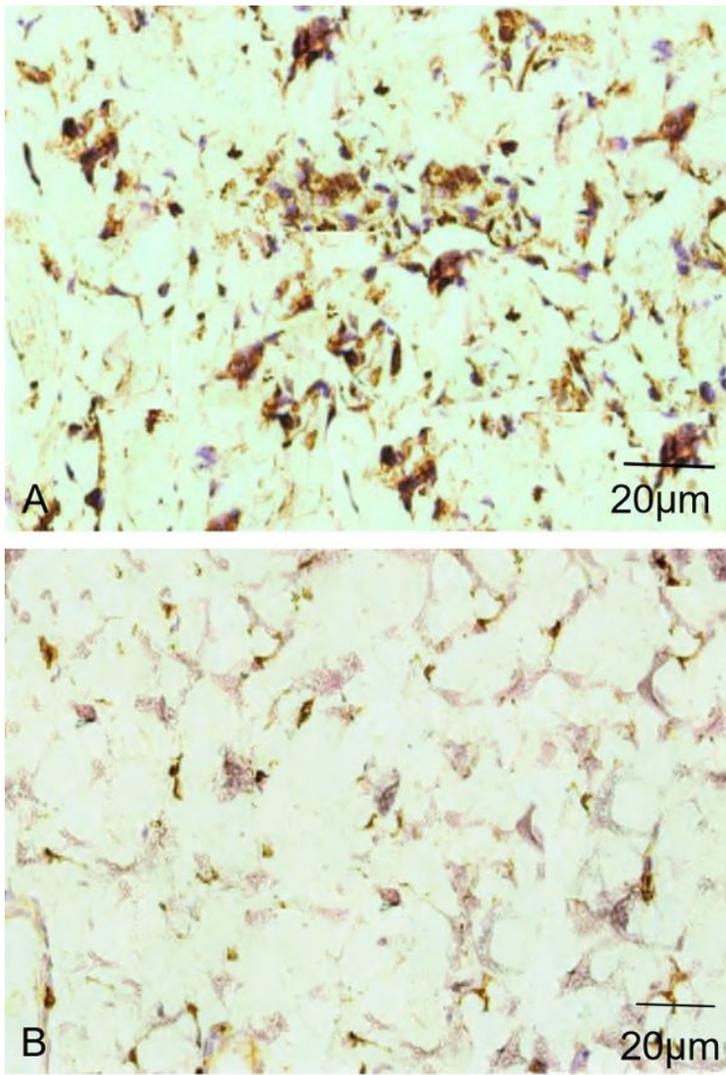
**Figure 4**

Radiography of flaps in rats from both groups seven days after operation. A. In the ISL group, the vascular structure of Choke II zone of flap of rats was relatively clear, with more neovascularization and relatively complete vascular structure in the potential region B. In the saline solution group, the vascular structure of Choke II zone of flap of rats was ambiguous, with less neovascularization and disordered or disappeared vascular structure in the potential region.



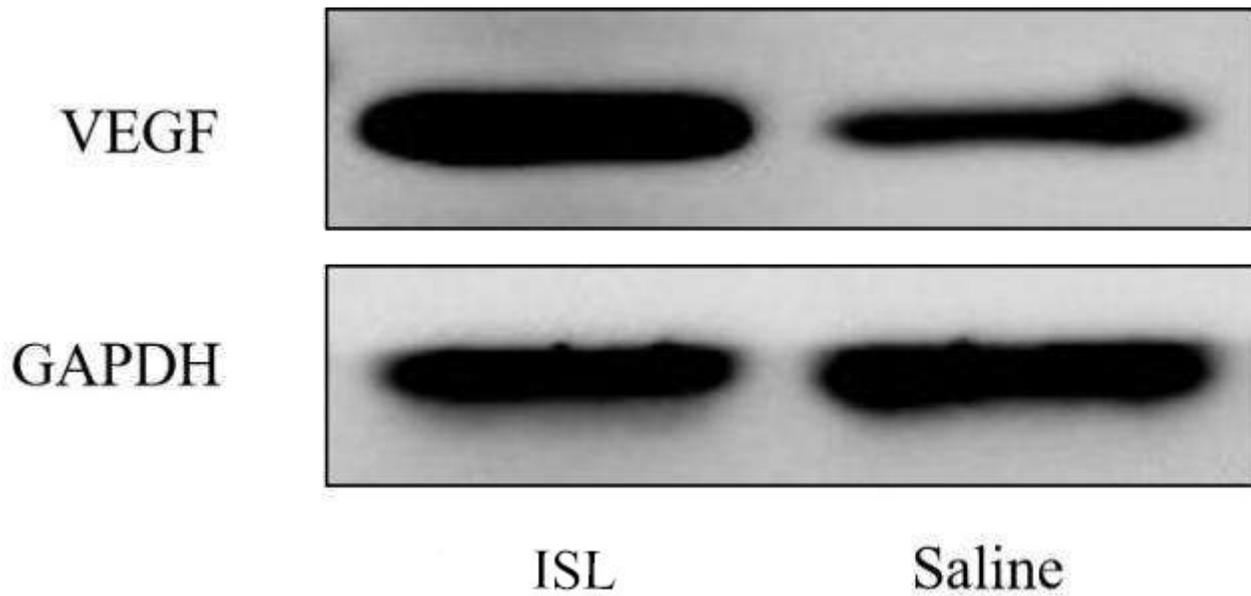
**Figure 5**

Histological observation of the neovascularization of Choke II zone of flap in rats of both groups seven days after operation. HE ×100. A. There was more neovascularization in the Choke II zone of flap in rats from the ISL group; B. There was fewer neovascularization in the Choke II zone of flap in rats from the saline solution group; C. The MVD in the irisin group was significantly higher than that in the saline solution group seven days after operation. \* Statistical significance (P < 0.01)



**Figure 6**

Seven days after operation, IF showed the expression of VEGF in the Choke II zone of rats from two groups (brown), diaminobenzidine-hematoxylin x100. A: The expression of VEGF was obvious in ISL group; B: the expression of VEGF in saline solution group was weaker than that in ISL group.



**Figure 7**

Western blot of the expression of VEGF in Choke II zone in rats from both groups.

## Supplementary Files

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