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Effects of batroxobin treatment on the survival of random skin flaps in rats☆

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ABSTRACT

Objective: Batroxobin is a medicinal preparation extracted from the venom of the Fer-de-Lance snake, and is used to lower blood viscosity, promote blood fibrinogen decomposition, and inhibit thrombosis. This research is to investigate whether batroxobin can improve the survival of random skin flaps in a rat model.

Materials and methods: Dorsal McFarlane flaps were harvested from 36 rats divided into two groups. Experimental group: Batroxobin was administered *via* the tail vein once daily. Control group: The same amount of normal saline was injected instead. On day 2, superoxide dismutase (SOD) and malondialdehyde (MDA) levels were measured. On day 7, tissue slices were stained with haematoxylin and eosin. Expression of vascular endothelial growth factor (VEGF) was immunohistochemically evaluated. Microcirculatory flow was measured by laser Doppler flowmetry. Flap angiography, using the lead oxide-gelatin injection technique, was performed with the aid of a soft X-ray machine.

Results: The batroxobin group exhibited a greater mean flap survival area, a better microcirculatory flow, and higher-level expression of SOD and VEGF compared with the control group. However, the MDA level was significantly reduced.

Conclusion: Batroxobin effectively improved the survival of random skin flaps.

1. Introduction

As random flaps afford maximal design flexibility, they are widely used during orthopedic repair of tissue defects and deformities. However, flap necrosis is a common complication of orthopedic surgery. To improve flap survival, the aspect ratio must be considered (optimally 1.5–2:1) [1]. If the aspect ratio exceeds that range, the flap may become necrotic after transfer due to an insufficient blood supply, ischaemia-reperfusion injury [2], inflammation, apoptosis [3], or some other cause.

Batroxobin is a snake venom produced by *Bothrops atrox* and *Bothrops moojeni*, venomous species of pit viper (Fer-de-Lance) found east of the Andes in South America. Batroxobin isolated from *B. atrox* venom has procoagulant properties and can be used for hemostasis; while batroxobin isolated from *B. moojeni* venom has defibrinogen action and can be used for thrombolysis [4]. The application is different, but they have been referred to as batroxobin.

Batroxobin reduces blood viscosity, breaks down blood fibrinogen, inhibits thrombosis, and dissolves thromboses that form [5,6]. Batroxobin is used to treat acute ischemic cerebrovascular disease and sudden deafness [7,8].

The clinical applications of batroxobin are listed below:

1) As a neuroprotective drug, batroxobin reduces nerve cell apoptosis and inflammatory reactions during the acute phase of necrosis [9], consistent with its use to increase the flap aspect ratio.
2) Batroxobin reduces hyperviscosity, thus lowering vascular resistance and improving blood circulation [10], increasing vascular blood flow in general, and enhancing the blood supply. This allows the blood to attain the distal end of the flap, again allowing the

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aspect ratio to be increased.

3) Batroxobin may counter ischemia-reperfusion injury [11]. Zhang L et al. found that the superoxide dismutase (SOD) level fell with prolongation of ischemic time [12]. Batroxobin reduces SOD activity, thus probably countering ischemia-reperfusion injury.
4) Batroxobin may promote angiogenesis [13] and inhibit apoptosis.

During treatment of acute cerebral infarctions with batroxobin [14], the vascular endothelial growth factor (VEGF) level is significantly higher than that during conventional treatment. Batroxobin further increases the serum VEGF level in patients with acute cerebral infarctions. Thus, we speculate that batroxobin promotes angiogenesis and inhibits apoptosis.

When batroxobin is applied to rat blood vessels, the drug promotes angiogenesis and reduces vascular ischemia-reperfusion injury and inflammation, conducive to flap survival. Therefore, we explored the effects of batroxobin on random flap survival and the mechanisms involved.

2. Materials & methods

2.1. Animal preparation

In this study, we used the batroxobin produced by *B. moojeni* snake venom, whose molecular weight (MW) is approximately 36,000 Unit. 1 BU corresponds approximately to 0.18 NIH (thrombin) units. Batroxobin (purity HPLC-UV: ≥95%, No: H20031074) was purchased from Beijing Tobishi Pharmaceutical Co., Ltd. (Beijing, China).

36 adult male Sprague-Dawley rats weighing 200–250 g were used in this research. Rats were randomly distributed into batroxobin-treated (experimental group, $n = 18$) and saline-treated groups (control group, n = 18). All animals were housed individually in separate cages maintained at a constant temperature with standard day/night cycles and access to food and water *ad libitum*. To reduce operator error, all surgical procedures were conducted by the same researcher.

Six rats from each group were selected for surgical examination. On postoperative day 2, flap tissue samples were punctured to measure oxidative stress indicators (SOD and malondialdehyde [MDA]). On postoperative day 7, another six rats were used to visually evaluate flap survival, H&E staining, and immunohistochemistry. The remaining six rats in each group were subjected to gelatin-lead oxide angiography after using laser Doppler angiogenesis, which were used as inter-group controls.

For surgical procedures, rats were anesthetized with 1% pentobarbital sodium (40 mg/kg). Using the two iliac crest lines as a guide, a rectangular flap (9 × 3 cm) was drawn on the back of each rat [15], and the skin and subcutaneous tissue were cut to reach the deep fascia, keeping the subdermal capillary network intact. After blunt dissociation, the two iliac arteries were cut off at the base of the flap. After complete hemostasis, the flap was sutured using a 4–0 mousse medical suture. Following the procedure, rats were injected intraperitoneally with 40,000 units of gentamicin, and an erythromycin ointment was rubbed on the wound to prevent infection.

For the experimental group, batroxobin was administered *via* the tail vein once daily (5 BU/kg/day) for 7 days, with the control group receiving an equal volume of normal saline (0.5 mL/kg/day) in place of batroxobin. After surgery, all animals were fitted with a cardboard neck cover to prevent rats from biting the wound (Fig. 1).

2.2. Superoxide dismutase and malondialdehyde expression

On postoperative day 2, a 0.5 cm × 0.5 cm whole layer punch was collected from in the middle of the flap (zone 2). After removing the muscle layer, samples were assessed for quality. After dilution, samples were diluted 1:10 in an ice bath. Superoxide dismutase (SOD) and Malondialdehyde (MDA) content were then quantified using SOD and MDA test kits, according to the manufacturer's instructions.

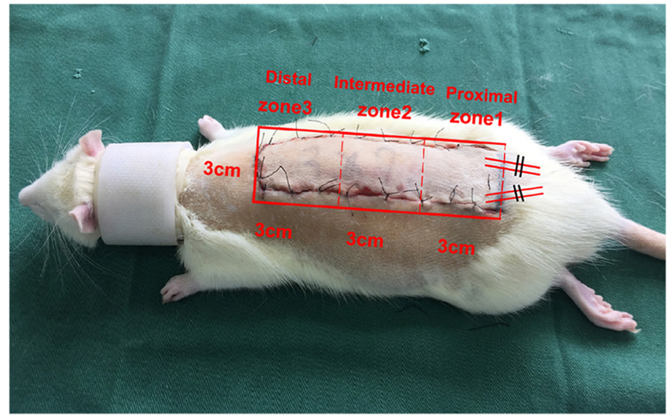

Fig. 1. Experimental animal model.

2.3. Flap survival

To assess the overall health of the flaps, each flap was monitored for color, tissue elasticity, and texture. On postoperative day 7, rats were anesthetized, after which transparent paper was applied to each flap to accurately trace healthy and necrotic areas. Trace images were then captured with a Sony digital camera using relevant scale marks, and the resulting images were imported into a computer. The length of flap survival was measured with Image-Pro Plus software. Assessment of flap survival was determined based on the average maximum and minimum flap lengths. Similar comparisons were performed for area, comparing survival area and total area and with results presented as a ratio (flap survival area ratio = flap survival surface area / flap total surface area ×100%). Criteria for assessing necrotic tissue included black flap color, tissue retraction, poor elasticity, hard texture, and a lack of bleeding when cut.

2.4. Histopathological examination

Histopathological examinations were performed as described by Mandriota et al. [16]. Briefly, the rat flap was divided into three equal parts: proximal (zone 1), intermediate (zone 2) and distal (zone 3). Seven days after operation, samples were collected from the midpoint of the flap (zone 2), fixed in 10% formaldehyde, and embedded in paraffin. Blocks were then cut into 4 μm-thick slices and analyzed by haematoxylin-eosin staining (H&E staining). Sections were assessed based on the extent of granulation, structural changes of the capillaries, tissue edema, necrosis, and the presence of inflammatory infiltrates under light microscopy (100×). Microvessel density (MVD) was measured for flaps using the following counting method: one slice was selected for each group. For each slice, five random fields (200×) were selected from the central and peripheral areas, and the total number of blood vessels was counted. Each high magnification field of view was 0.305 mm^2. The number of microvessels per unit area (/mm^2) was calculated as an indicator of MVD.

2.5. Immunohistochemical investigation

The remaining paraffin slices were subjected to Elivison two-step staining. First, tissue sections were observed under light microscope to identify areas of high VEGF expression. Next, each section was photographed under high magnification (400×) five times using a DP2-TWAIN image acquisition system (Olympus) with parameters such as white balance, aperture, and shutter time kept consistent between fields. Images were then analyzed using Image-Pro Plus software. Select the index “Accumulated Absorbance IA” to be analyzed. Read the IA value for measurement as an indicator of vascular endothelial growth factor (VEGF) expression levels.

2.6. Neovascularization

Flap blood flow perfusion was measured on postoperative day 7 using blood perfusion unit (PU) values as the basic index of laser Doppler flowmeter (LDF) score. PU is indicative of the flow of erythrocytes produced by the Doppler shift value but is also a measure of the depth of the local tissue microcirculation in terms of the relative units of blood flow. Changes in PU values are indicative of changes in microcirculation blood flow in flap tissue, and are therefore indirectly reflective of the neovascularization of the flap. After this step, gelatin-lead oxide angiography was performed on each rat.

2.7. Gelatin-lead oxide angiography

On postoperative day 7, 0.9% saline was injected into the carotid artery of the rats after anesthesia. At the same time, blood was collected from the ipsilateral jugular vein, and the gelatin-lead oxide perfusate fluid (100 mL/kg) was slowly injected into the carotid artery. Injection was stopped when the rat's sclera and limbs began to exhibit the same color as the perfusate. After perfusion, the rats were frozen for 12–24 h to make the gelatin agglutinate. Finally, the dorsal skin flap and the surrounding skin were dissected and examined by X-ray.

2.8. Statistical analysis

All experimental data are expressed as means ± standard deviation (SDs). The data were analyzed using SPSS statistical software (SPSS Inc.). Differences between the experimental group and control groups were analyzed by two-way ANOVA, with p values < 0.05, considered statistically significant.

3. Results

3.1. SOD and MDA expression

In the batroxobin group, the average SOD content was 62.09 ± 4.03 units/mg protein, significantly higher than that of the normal saline group (23.37 ± 2.59 units/mg protein) ($p < 0.01$). The average MDA level in the batroxobin group was 22.75 ± 3.94 nmol/mg protein, significantly lower than that of the normal saline group (59.21 ± 5.90 nmol/mg protein) ($p < 0.01$) (Fig. 2C,D).

3.2. Flap survival

On postoperative day 1, the two groups exhibited significant swelling near the margins of the three-zone incisions, with dark brown patches, an inability to feel stimuli, and no sense of movement. No tissue swelling was obvious in zones 1 and 2. A few days later, the color of the zone 3 patches gradually deepened to purple/black, accompanied by dry crusting, whereas zone 2 also exhibited necrosis that was not obvious in zone 1. The extent of flap necrosis of the control group was significantly greater than that of the experimental group. The flap color was darker and the necrotic flap area larger (Fig. 3).

The survival areas of the flaps were calculated as percentages for both groups: 76.26 ± 3.43% for the experimental group, 48.27 ± 6.69% for the control group. The between-group difference in survival area was statistically significant ($p < 0.01$) (Fig. 2A).

3.3. Histopathological examination

Seven days after flap placement, subcutaneous fibroblast proliferation was evident in the experimental group. The granulation tissue was thin. Low-grade tissue edema and scattered subcutaneous hemorrhage were evident, as was diffuse neutrophil infiltration or scattered focal infiltration. Flap sections exhibited more neovascularization than controls. In contrast, control flaps were thicker and exhibited less fibroblast proliferation and neovascularization, more edema, and more inflammatory cell infiltration as observed microscopically (Fig. 4).

The neovascularization data were as follows: the microvessel densities (MVDs) of zone 2 in the experimental group (30.83 ± 2.86/mm^2) were higher than in the control group (14.44 ± 3.56/mm^2). The difference was statistically significant ($p < 0.01$) (Fig. 2B).

3.4. Immunohistochemical evaluation

We calculated cumulative VEGF absorbance values (IAs). The VEGF expression level in the experimental group (3975.00 ± 474.42 IA) was higher than that in the control group (1854.50 ± 251.45 IA). The difference between the two groups was statistically significant ($p < 0.01$) (Fig. 5).

3.5. Neovascularization

The LDF images were shown in Fig. 6. The blood perfusions in the batroxobin group in zones 1 (422.83 ± 28.87 PU), 2 (123.74 ± 36.62 PU), and 3 (66.66 ± 15.10 PU) were much higher than those in the control group (327.84 ± 23.80 PU, 51.77 ± 17.05 PU, and 14.48 ± 5.26 PU). The differences were statistically significant (all $p < 0.01$; Fig. 6).

3.6. Gelatin-lead oxide angiography

Angiography clearly revealed that the number and quality of regenerated blood vessels in the experimental group were significantly higher than in the control group. Revascularization of the flap, recipient site, and margin was good, and batroxobin clearly increased the blood supply to the tissue (Fig. 6).

4. Discussion

The color and texture of random flaps were similar to those of the affected tissue [17,18], suggesting the possibility of greater flexibility in flap design. Random flaps are commonly used in plastic surgery and hand surgery to repair tissue defects and other deformities [18]. Unlike the well-defined vasculature associated with axial flaps, random skin flaps rely solely on the existing blood vessels of the surrounding tissues [19], freeing them from the limitations of conventional flaps, including design site, axial direction, axial vascular distribution, and traversing direction [17].

There are many kinds of flaps used to study the effects of drugs on skin flap survival, the most common of which are prefabricated [20] and perforator flaps [21]. Prefabricated flaps represent a subset of axial flaps that are often used to repair larger wounds. In this model, tissues receiving little to no blood supply are reconnected to the surrounding vasculature, enabling successful transplantation of and wound closure [22]. Similarly, the use of perforator flaps has gained significant traction in recent years. The concept of the design is based on the clinician's intention for the skin flap to provide a strong blood supply to the musculocutaneous flap, resulting in less swelling and secondary damage.

We chose random flaps as the preferred model for this investigation. As prefabricated and axial flaps contain muscle or well-known blood vessels, these factors may confound our interpretation of the clinical outcomes. In contrast, random skin flaps contain only skin and subcutaneous tissue with no additional vasculature, meaning that all blood and nutrients would have to be provided by the pedicle capillary network. This approach limits the potential confounding effects of the different vascular networks, making it a superior choice for experimental research models.

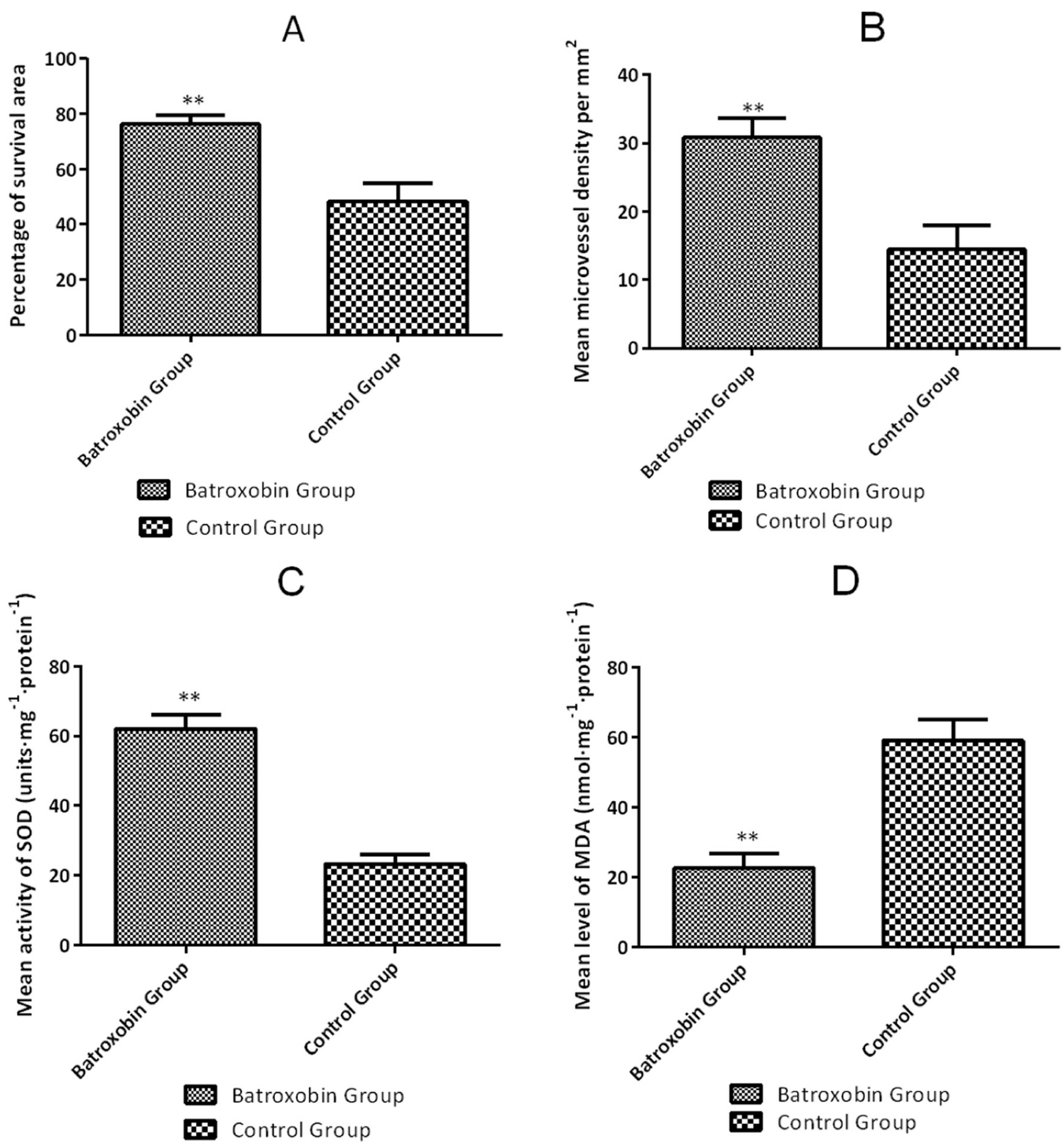

Fig. 2. Percentage of survival area (A). Mean microvessel densities of the two groups (B). Mean superoxide dismutase activities of the two groups (C). Mean malondialdehyde levels of the two groups (D). All values are expressed as means ± standard deviations (SDs). $n = 6$ per group.

Batroxobin is derived from the venom of the Fer-de-Lance, and has been shown to reduce blood viscosity, as well as break down blood fibrinogen, inhibit thrombosis, and dissolve new thromboses [5,6]. In a previous study by Yitao et al., batroxobin showed efficacy for treating acute cerebral stroke in a clinical setting [23]. Similarly, Wang et al. found that batroxobin plus aspirin was able to reduce restenosis after angioplasty for arterial occlusive disease in diabetic patients with lower-limb ischemia [24]. Other clinical applications include treatment of sudden deafness at full frequency [25], reduction of blood loss in spinal fusion surgery [26], treatment of deep venous thrombosis following arthroscopic posterior cruciate ligament reconstruction [27], and mobilization of circulating endothelial progenitor cells in patients with deep vein thrombosis [28].

Taken together, these mechanisms are all conducive to the survival of skin flaps. We therefore sought to examine the effects of batroxobin on the survival of random ischemic flaps. The goal of this experiment is to provide more feasible choices for clinical postoperative skin flap application.

We found that batroxobin promoted VEGF production. The MVD was higher in the experimental group than the control group. Flap sections exhibited more neovascularization after drug treatment. Thus, batroxobin may improve flap survival by promoting VEGF expression, accelerating angiogenesis, and inhibiting ischemia-reperfusion injury. Earlier studies showed that the flap VEGF content greatly influenced the formation of flap vessels [29–31]. During early angiogenesis, VEGF promotes vasodilation and extracellular matrix formation. Also, at this time, VEGF regulates vascular endothelial cell proliferation, differentiation, and migration, contributing to the formation of new capillaries. VEGF also promotes the formation and maturation of the neovascular lumen, also increasing the number of capillaries. VEGF can improve the survival rate of full-thickness flaps after transplantation by reducing flap necrosis. VEGF promotes angiogenesis, enhances the anti-infective capacity of the flap, reduces inflammatory infiltration of flap tissue, and promotes wound-healing.

The experimental results show that the SOD level of the batroxobin group was higher than that of the saline group, and the MDA level of

Fig. 3. Digital photographs showing the overall appearance of the flaps in the experimental and control groups.

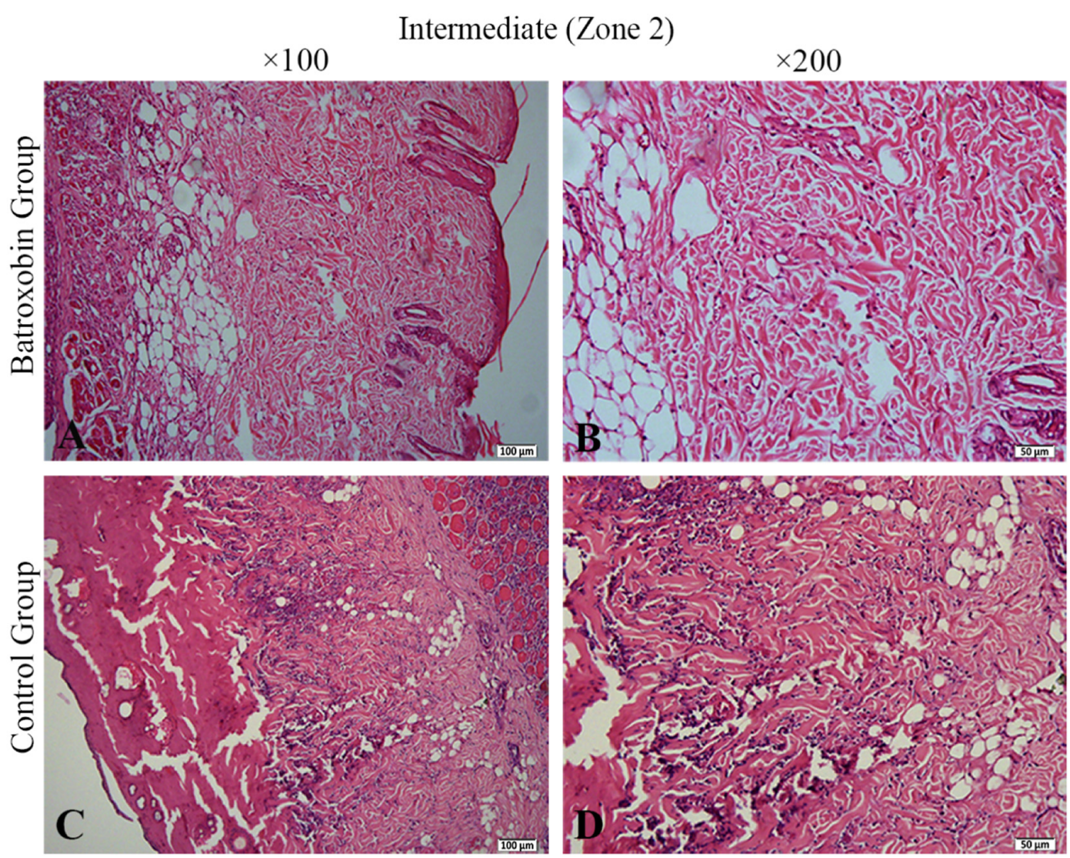

Fig. 4. Histopathological examination of zone 2 of the flaps in the batroxobin group and control group. Haematoxylin and eosin staining. Magnifications: 100× and 200×.

the batroxobin group was lower than that of the saline group. The superoxide anion (O^{2-}) is an intermediate produced during physiological reactions. As one of the most important factors underlying biological oxygen toxicity, the O^{2-} anion is strongly oxidative. SOD transforms O^{2-} into H_2O_2 [32], protecting cells from damage [33]. MDA, a product of lipid peroxidation, is closely associated with free radical metabolism. SOD and MDA levels are often associated [34]. The MDA level indirectly reflects the severity of free radical attack. The SOD level indirectly reflects the ability of tissue to scavenge free oxygen radicals.

Ischemia-reperfusion injuries usually occur 24–48 h after ischemia

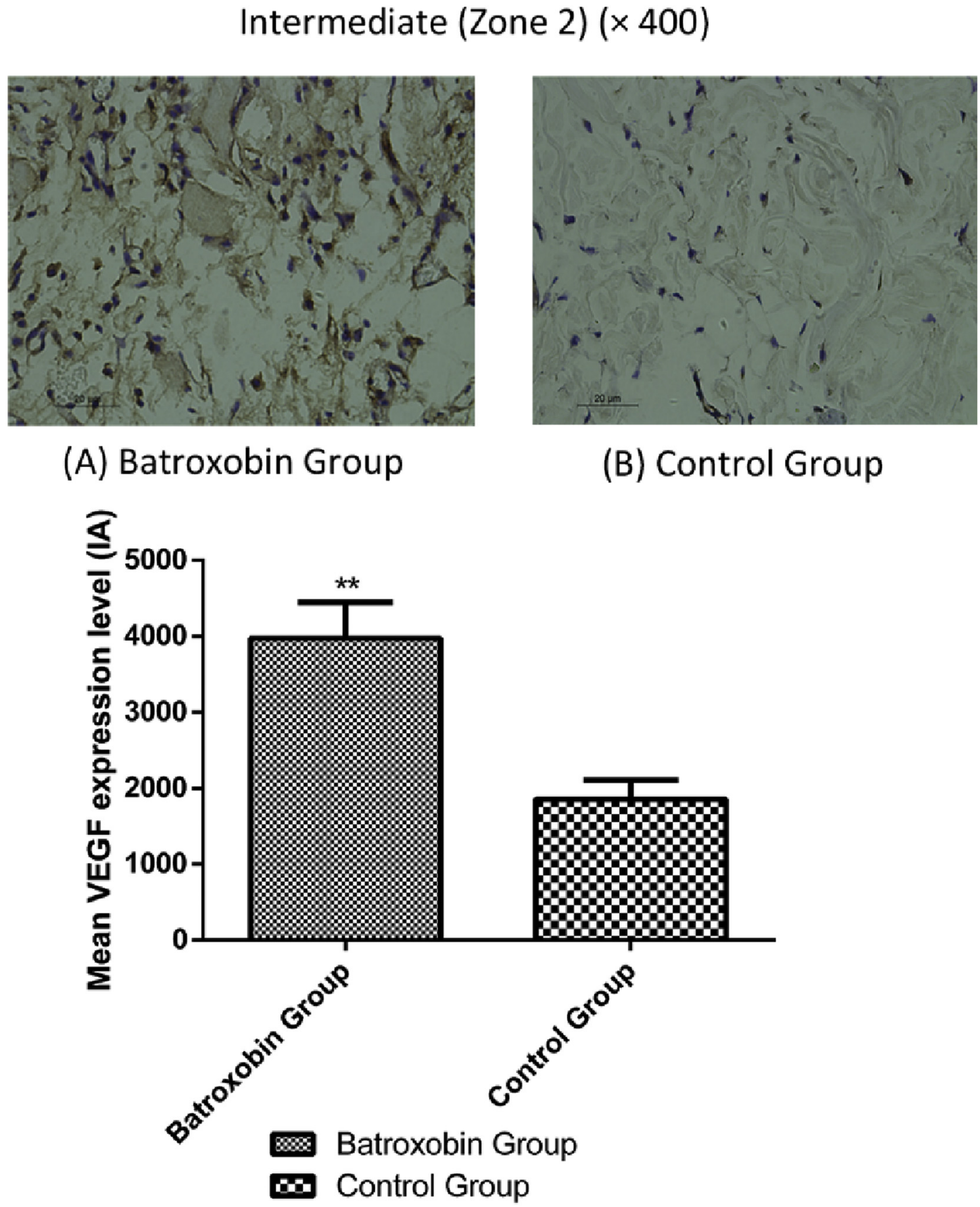

Fig. 5. Expression levels of vascular endothelial growth factor (VEGF) in zone 2 of the flaps of the batroxobin and control groups. Immunohistochemical staining. Magnification: 400×. Integral absorbances (IAs) of the VEGF expression levels were expressed as means ± standard deviations (SDs). $n = 6$ per group.

recanalization [35], with the resulting blood flow capable of aggravating tissue damage. To better assess the role of oxidative stress in this model, we examined the oxidation markers SOD and MDA on postoperative day 2 [36]. In one such study, Wu et al. reported the effects of batroxobin on neuronal apoptosis in rats with focal cerebral ischemia and reperfusion. They found that a large number of apoptotic cells began to appear at 12 h after reperfusion and peaked between 24 and 48 h [37]. Therefore, use of SOD and MDA on postoperative day 2 provides the best opportunity to assess the severity of ischemia-reperfusion injury.

Ischemia-reperfusion injury is an early event in flap procedures, and an important process underlying necrosis of ischemic random skin flaps. Tissues produce a large amount of reactive oxygen species (ROS), including oxygen ions, free radicals, and peroxides during ischemia and reperfusion. The resulting free radicals primarily activate mitochondria, as well as other important downstream targets such as endogenous endonucleases and protein kinases, resulting in programmed cell death and distal necrosis of the flap. *In vivo*, the production and clearance of ROS are in a constant state of dynamic equilibrium. During tissue ischemia, a large amount of ROS are produced. When the blood flow is finally reconnected, the accumulated free radicals attack the cells in the tissue, resulting in an ischemic reperfusion injury. At the same time, the ability of ischemic tissue to scavenge oxygen free radicals is hindered, which intensifies the damage caused by free radicals to ischemia-reperfusion tissue.

Gelatin-lead oxide angiography and LDF angiography showed that batroxobin effectively stimulated neovascularization. The number and quality of regenerated blood vessels in the experimental group were significantly higher than those in the control group. Batroxobin increased the blood supply to flap tissue. To a certain extent, a higher

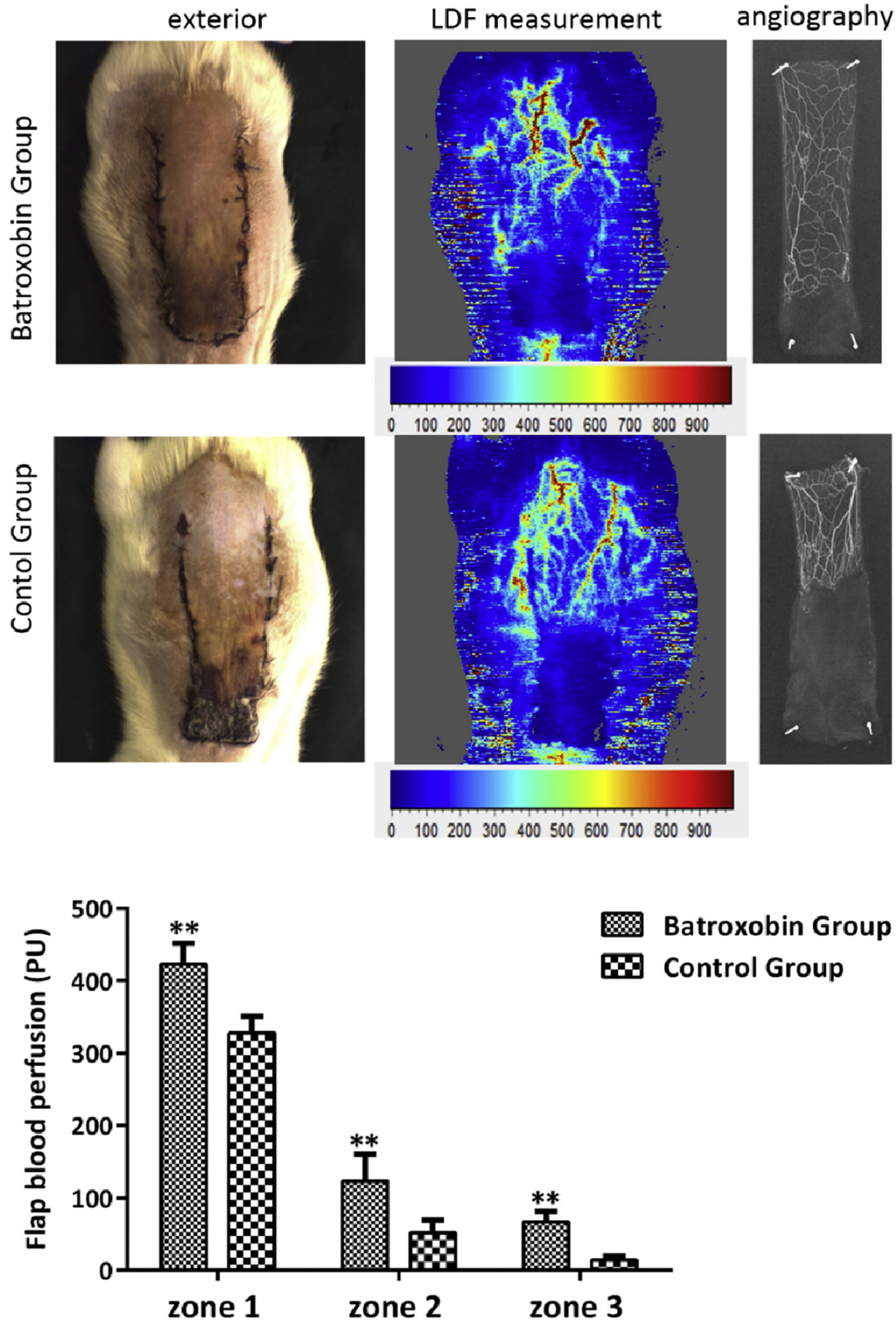

Fig. 6. Laser Doppler flowmetry angiography and x-ray angiography of the flaps of the batroxobin group and the control group. The mean blood perfusion levels in zones 1, 2, 3 of the two groups. All values are expressed as means ± standard deviation (SDs). $n = 6$ per group.

blood supply eases an inflammatory response, thereby improving the flap survival rate [38].

Other studies have shown that anticoagulant drugs, such as heparin, can also promote flap survival [39,40]. However, the effects of batroxobin differ from those of heparin, where batroxobin is able to decompose fibrinogen and dissolve thrombus, while heparin exerts a more limited anticoagulant effect. Evidence of these differences can be seen in a study by Tomaru et al., which examined the antithrombotic effects of three different types of antithrombotic agents (argatroban, heparin, and batroxobin) in canine coronary and iliac arteries [41]. The results showed that batroxobin significantly reduced fibrinogen, levels, while argatroban increased the antithrombin content; however, both showed significant antithrombotic effects. In contrast, the heparin group exhibited no significant reduction in thrombosis. Based on these data, we speculate that batroxobin may promote angiogenesis by dissolving blood clots and reducing blood flow resistance.

Further studies will be necessary to determine the optimal dose and injection method of batroxobin, as well as to yield a more detailed understanding of the drug's mechanisms of action. Finally, this study is an experimental investigation performed exclusively in rats; whether batroxobin can be applied to the recovery and reconstruction of human skin flaps transplantation requires further trials.

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