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Effects and mechanism of urinary kallidinogenase in the survival of random skin flaps in rats

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ABSTRACT

Objective: This study explored the effects of urinary kallidinogenase (UK) on ischemia and necrosis of random skin flaps in rats, and the mechanisms thereof.
Methodology: Ischemia and necrosis of random skin flaps were induced by constructing a modified McFarlane flap model on the dorsa of rats. UK (0.016 PNA/kg) or normal saline (10 ml/kg, control) was administered through the tail vein immediately after flap ischemia model construction and then daily for 7 days. After sacrifice, the flap tissue was harvested and stained with hematoxylin and eosin (H&E), and histopathological changes were observed. Lead oxide/gelatin angiography and laser Doppler imaging were performed to demonstrate angiogenesis and changes in blood flow. Immunohistochemical analysis of the H&E-stained slices was performed to detect the expression of vascular endothelial growth factor (VEGF), interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α). The TNF-α and IL-6 levels were also detected by enzyme-linked immunosorbent assays. The activity of superoxide dismutase (SOD) and the malondialdehyde (MDA) content were measured to represent the oxidative damage level.
Results: UK significantly alleviated ischemia and necrosis of random skin flaps, as evidenced by improved general results and histopathological manifestations, and markedly increased the mean survival area. UK prompted angiogenesis, increased blood flow and VEGF expression. The levels of TNF-α, IL-6, and IL-1β were declined. Furthermore, UK increased SOD activity and decreased MDA content, suggesting that it has the capacity to alleviate oxidative damage.
Conclusion: These findings suggest that UK sufficiently attenuated flap ischemia and increased the survival of random skin flaps in rats.

1. Introduction

Random skin flap necrosis is a critical issue in the field of plastic and reconstructive surgery; it results from the lack of an arteriovenous system and depends primarily on the perfusion of subcutaneous capillary networks. Flap necrosis remains a critical concern despite advances in surgical techniques, as the mechanism underlying flap ischemia and necrosis is not fully understood [1–3].

Ischemia and necrosis of random skin flaps occur predominantly in the distal regions as consequences of insufficient arterial blood perfusion and/or venous disorders [4,5]. Inflammation and oxidative stress also contribute to the failure of skin flaps [6]. Based on these findings, we sought to identify a medicine that could inhibit inflammation and oxidative stress and improve vascularity and blood flow, thereby accelerating wound healing and improving flap viability. Tissue kallikreins constitute a series of acid glycoproteins with similar gene and protein structures, which have been confirmed to be engaged in a variety of pathophysiological processes, including suppression of the inflammatory response, oxidative stress, and apoptosis [7]. Urinary kallidinogenase (UK), a tissue kallikrein, has been reported to inhibit the production of inflammatory factors, such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), and IL-6 [8]. Moreover, accumulated clinical evidence confirms that UK is a promising therapeutic drug for acute ischemic stroke, as it promotes post-ischemic angiogenesis and increases cerebral blood perfusion [9]. We hypothesized that UK would enhance the viability of ischemic skin flaps.

In this study, we explored the efficacy of UK in ischemic and necrotic random dorsal skin flaps (McFarlane flaps) in rats.

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2. Material and methods

2.1. Animals and procedures

Specific pathogen-free male Sprague–Dawley rats (weight, 200–250 g) were selected from the Laboratory Animal Center of Wenzhou Medical University (license no. SCXK[ZJ]2005-0019). All animal experiments were approved by the Laboratory Animal Ethics Committee of Wenzhou Medical University (wydw2017-0509). All rats were housed in standard cages kept at constant temperature (20–24 °C) and humidity (50%–60%), and they were given free access to feed and water. In this study, according to the previous research [7,10] and preliminary experiments, a total of 36 rats was divided randomly into a control group and a UK group, with 18 rats in each group. After 5 days of adaptive feeding, the modified McFarlane flap model was established on the dorsum of each rat, as described previously [11]. Briefly, after the induction of anesthesia with 10% chloral hydrate, each rat was placed in the prone position and the entire back was shaved of hair. A rectangular random skin flap (width, 3 cm; length, 9 cm) was designed and constructed on the dorsum of each rat. The intercrestal line was used as the base of the flap. The skin and superficial fascia were dissected completely from the deep fascia, and the bilateral iliac arteries were ablated with preservation of the subdermal capillary network. The flap was restored to the original site using 4-0 medical sutures. Following the operation, 0.016 PNA U/kg UK was injected through the tail vein in the treatment group, and the control rats received the same volume of normal saline. UK and normal saline were administered daily for 7 days. To reduce errors, the same person performed all experimental operations. Each experiment was repeated at least three times.

2.2. General observations and flap survival calculation

From 1 to 7 days after the operation, a researcher observed and recorded the general manifestations of the skin flaps. The following characteristics were examined to define flap necrosis: flap color, tissue elasticity, and tissue texture. Black skin flaps, and those with poor elasticity and a hard texture, were considered to be necrotic. We accurately measured the surviving and total areas of the skin flaps with transparent paper. The survival area relative to the total area was recognized as the amount of the flap that survived.

2.3. Histopathological analysis of flap tissue

Fresh flap tissues in each group were fixed in 4% paraformaldehyde, dehydrated, and paraffin embedded. The tissue samples were cut into 5-μm sections and placed on microscopic slides. After deparaffinization and dehydration, each slice was stained with hematoxylin and eosin. A blinded examiner observed the stained slides under a light microscope (×100 and ×200 magnifications).

2.4. Lead oxide/gelatin injection technique

Lead oxide/gelatin (150 ml/kg) was injected into the right common carotid artery of each rat through a no. 22 silicon indwelling catheter. The perfused rat cadavers were kept at −20 °C overnight to allow the gelatin oxide mixture to solidify. Each skin flap was stripped and photographed under radiography (40 kV, 50 mA, 100 ms) on the second day, and vascular changes attributable to the flap were observed.

2.5. Flap blood flow assessment

To investigate the effect of UK on revascularization, cutaneous blood perfusion was assessed using a laser Doppler flowmeter (Moor Instruments Ltd., London, UK), according to the manufacturer's instructions. Blood perfusion images were obtained and quantified using the manufacturer's software (ver. 6.1; Moor Instruments Ltd.).

2.6. Immunostaining of VEGF, IL-1β, IL-6 and TNF-α

Formaldehyde-fixed paraffin-embedded skin flap samples of 5 μm thickness were processed for the immunohistochemical analysis. The slices were deparaffinized using xylene and dehydrated through a graded ethanol series. Each section was incubated for 10 min with 3% H_2O_2 at room temperature to block endogenous peroxidase activity, and then rinsed three times in phosphate-buffered saline (PBS; 3 min each time). Then, the slices were treated with goat serum blocking solution for 10 min at room temperature to block non-specific sites, followed by incubation with primary antibodies specific for vascular endothelial growth factor (VEGF), interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) at 4 °C overnight. On the second day, the slides were rewarmed to room temperature for 30 min, incubated with specific secondary antibodies for 2 h at room temperature, and rinsed three times in PBS. The tissue sections were counterstained with hematoxylin, and images were captured under a light microscope at 400× magnification.

2.7. Pro-inflammatory cytokine analysis

After 7 days, serum was obtained, and expression levels of pro-inflammatory cytokines, including TNF-α and IL-6, were detected using specific enzyme-linked immunosorbent assay (ELISA) kits.

2.8. Superoxide dismutase and malondialdehyde levels

After 7 days, 0.3 × 0.3-cm samples of flap tissue were removed from the boundary between regions II and III in all groups. The harvested samples were homogenized and centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was collected, and superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were measured using specific detection kits according to the manufacturer's instructions.

2.9. Statistical analysis

All data are expressed as means ± standard deviations and were analyzed with SPSS software 20.0 (IBM SPSS, Armonk, NY, USA). Data from the treatment and control groups were compared by one-way analysis of variance. Data charts were constructed using GraphPad Prism v. 6.0 (GraphPad Software Inc., La Jolla, CA, USA). *P*-values < 0.05 were considered to be significant.

3. Results

3.1. Effects of UK on viability and general flap features

The morphology of the skin flaps was observed on postoperative day 7. The flap tissue in the necrotic area, particularly in the distal zone, tended to have a black crust, hard texture, and poor elasticity. After in situ removal, flaps in the treatment group presented less active bleeding, and less hematocele, effusion, and inflammatory secretion under the sarcolemma. The necrotic area was smaller in the treatment group than in the control group (Fig. 1A). A significant increase in flap viability (74.12% ± 4.13%) was observed in the flaps treated with UK compared with the control group (52.017% ± 4.13%; $P < 0.01$; Fig. 1B).

3.2. Effects of UK on histological changes

The histological features of the skin flaps were assessed by light microscopy. Significant infiltration of inflammatory cells was detected, and these pathological changes were markedly reversed by UK administration (Fig. 2A). The treatment group showed a change in the microvessel density per area, characteristic of angiogenesis, relative to the control group (12.94 ± 2.94/mm^2). A notable increase in the

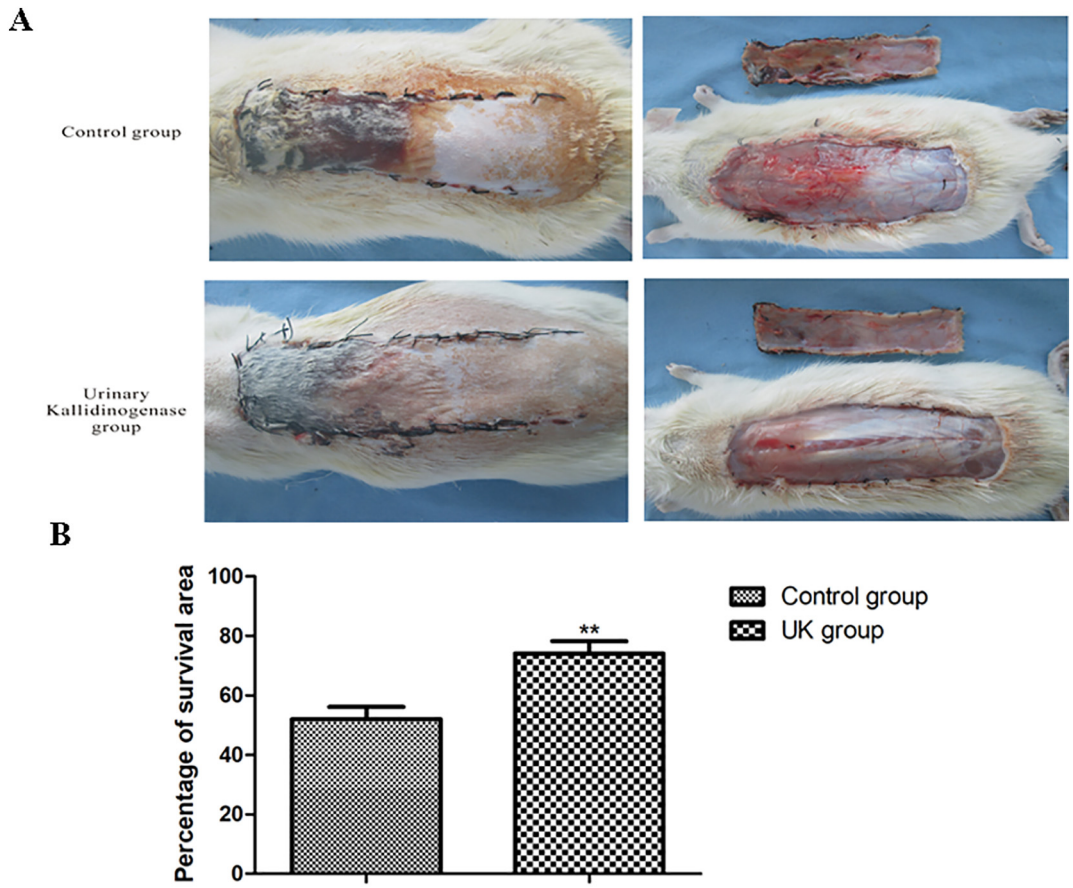

Fig. 1. (A) Surviving areas of skin flaps from the urinary kallidinogenase (UK) group and the control group were determined macroscopically on postoperative day 7. The morphology of the inner sides of skin flaps from the two groups. (B) Quantification of the percentage of surviving skin flap. $^{**}P < 0.01$ vs. control.

number of microvessels was observed in the UK-treated group ($29.46 \pm 6.32/mm^2$; $P < 0.01$; Fig. 2B).

3.3. Effects of UK on angiogenesis

Angiography performed 7 days after surgery showed significantly more new blood vessels in the treatment group than in the control group, particularly in region III (Fig. 3).

3.4. Effect of UK on flap vascularity

The physiological evaluation of flap vascularity was carried out by laser Doppler flowmetry (Fig. 4A). The microvascular perfusion rate was significantly higher in flaps from UK-treated rats (213.96 ± 49.75 PU), whereas increased skin blood perfusion was attenuated in flaps from rats treated with normal saline (96.02 ± 56.14 PU; $P < 0.01$; Fig. 4B).

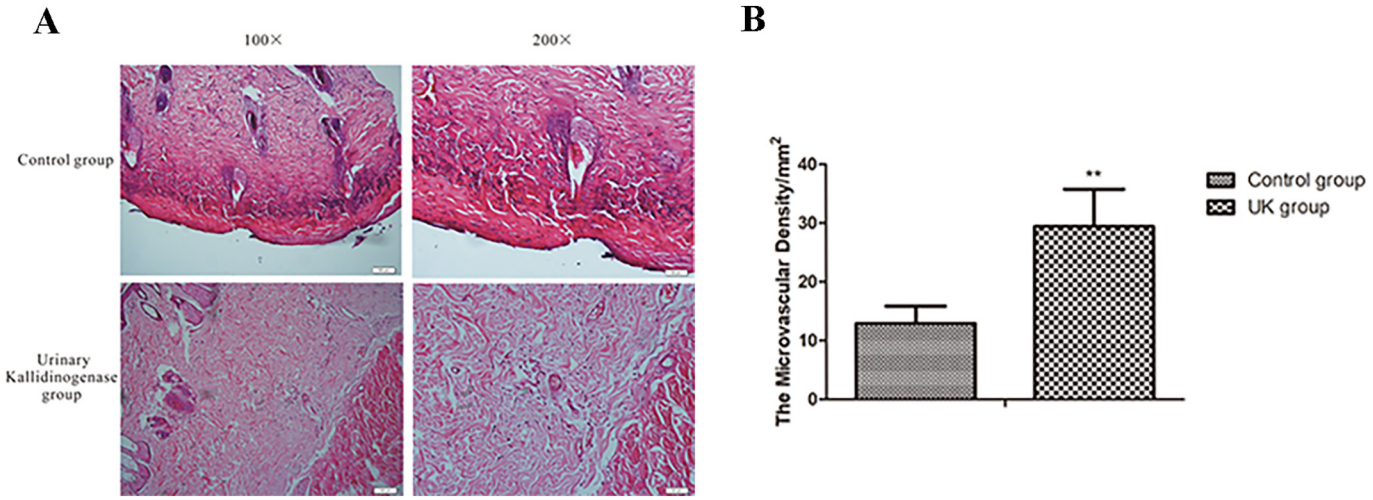

Fig. 2. (A) Hematoxylin and eosin (H&E) staining was used to observe the morphological features of flap tissues under a light microscope (100× and 200×). (B) Microvessel density (MVD) in the flap area on the mouse dorsum was assessed. $^{**}P < 0.01$ vs. control.

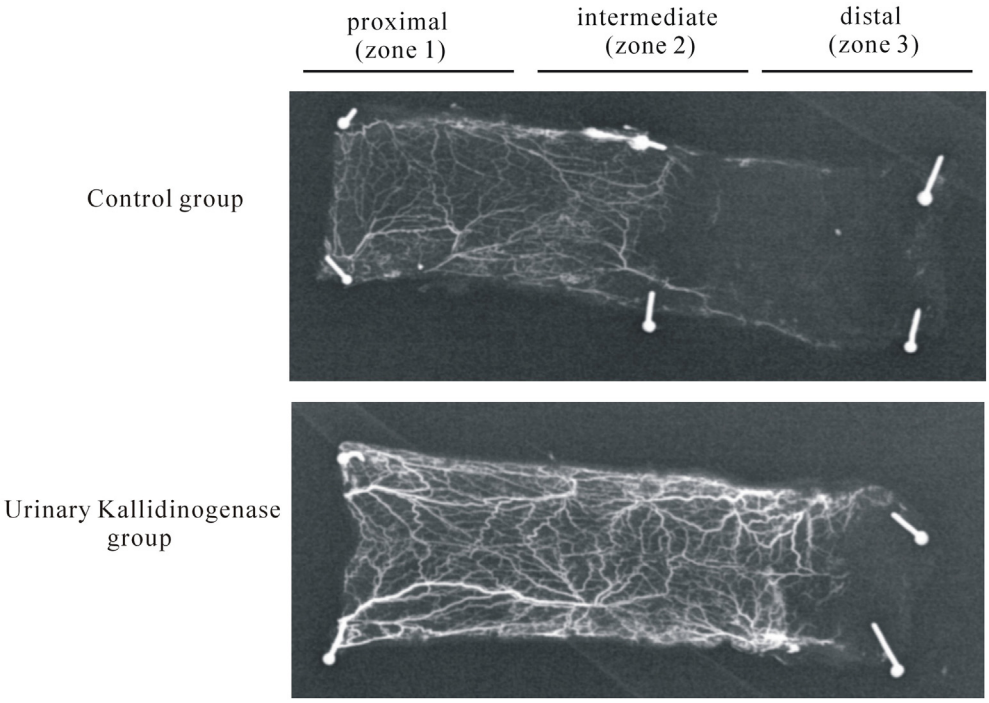

Fig. 3. Lead oxide/gelatin angiography imaging analysis.

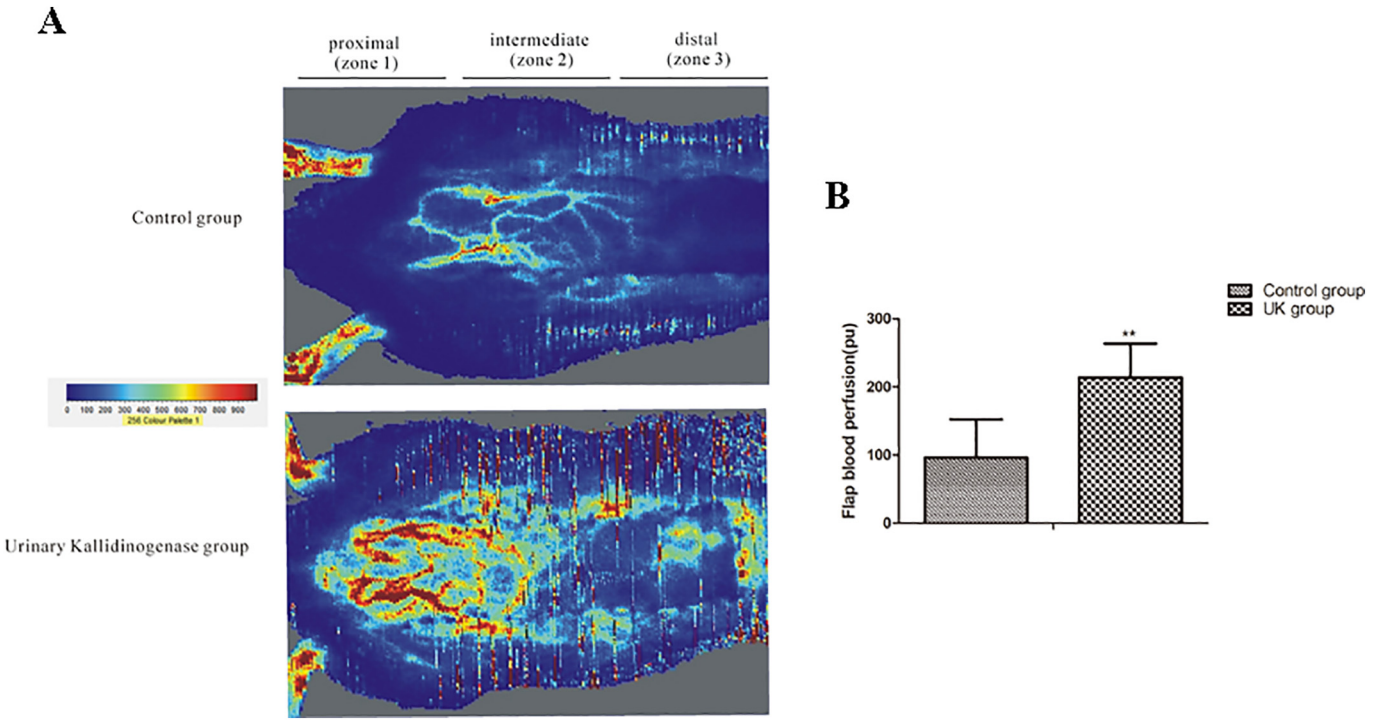

Fig. 4. (A) Flap blood perfusion was checked by laser Doppler perfusion imaging. (B) Blood flow was quantified. $^{**}P < 0.01$ vs. control.

3.5. Effect of UK on VEGF, IL-1β, IL-6, and TNF-α expression

The expression levels of VEGF, IL-6, IL-1β, and TNF-α were measured by immunohistochemistry. Rats treated with UK exhibited significantly increased VEGF expression (4099 ± 361 IA) compared with rats administered normal saline (1974 ± 252 IA; $P < 0.01$; Fig. 5). In contrast, a markable decrease in IL-6 level was observed in the UK treatment group (1403 ± 308 IA) in comparison with the control group (6053 ± 370 IA; $P < 0.01$; Fig. 5). Similarly, IL-1β level in the UK treatment group (1418 ± 358 IA) was dramatically declined compared with the control group (2801 ± 265 IA; $P < 0.01$; Fig. 5), and TNF-α level in the UK treatment group (1403 ± 270 IA) was also significantly declined compared with the control group (2352 ± 357 IA; $P < 0.01$; Fig. 5).

3.6. Effects of UK on pro-inflammatory cytokine levels

The ELISA results showed a significant decline in the IL-6 level in

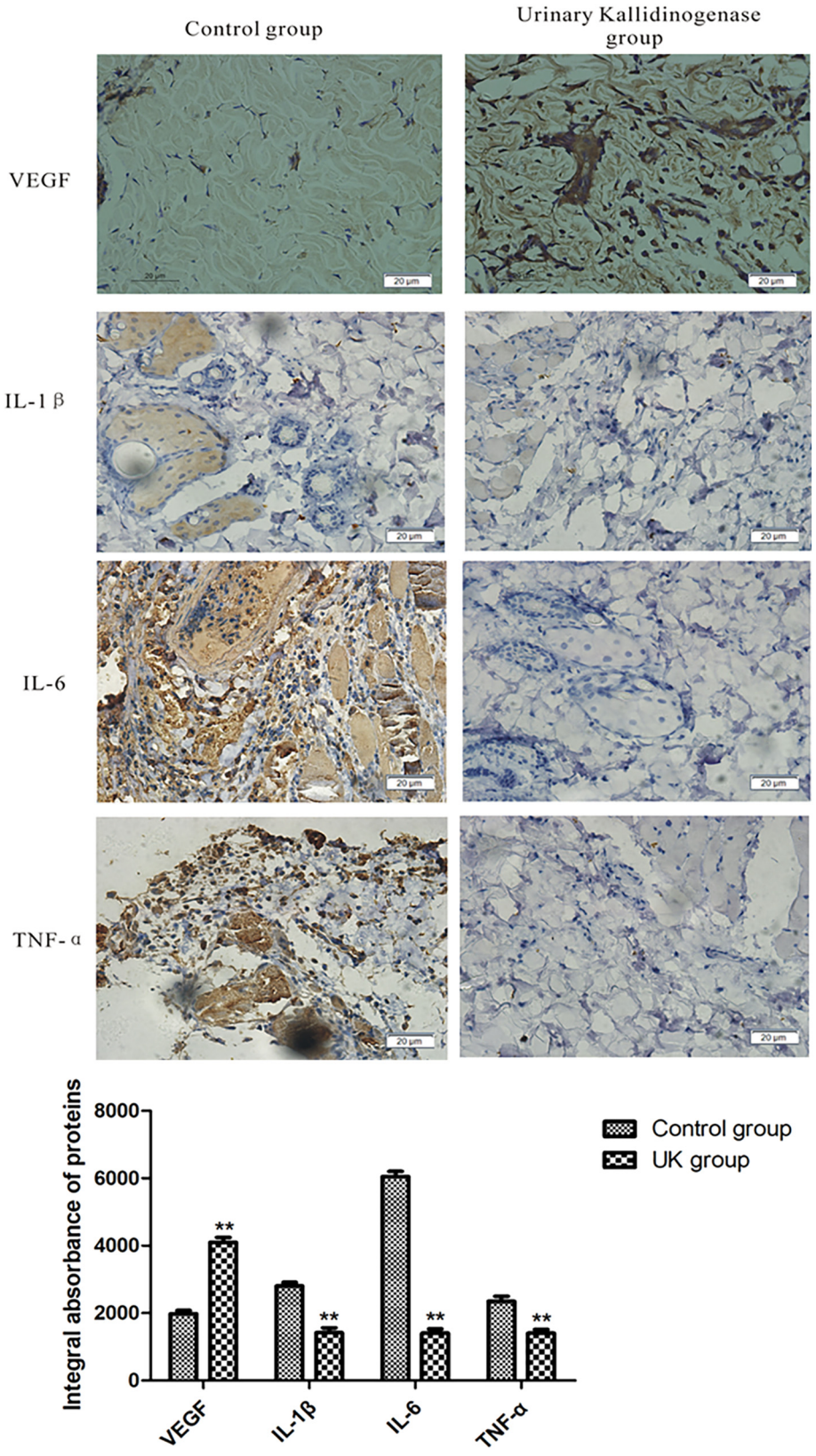

Fig. 5. Immunohistochemical expression of vascular endothelial growth factor (VEGF), interleukin (IL)-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) proteins in skin flap samples, detected by light microscopy at 400× magnification. The quantitative levels of VEGF, IL-1β, IL-6, and TNF-α are presented. $^{**}P < 0.01$ vs. control.

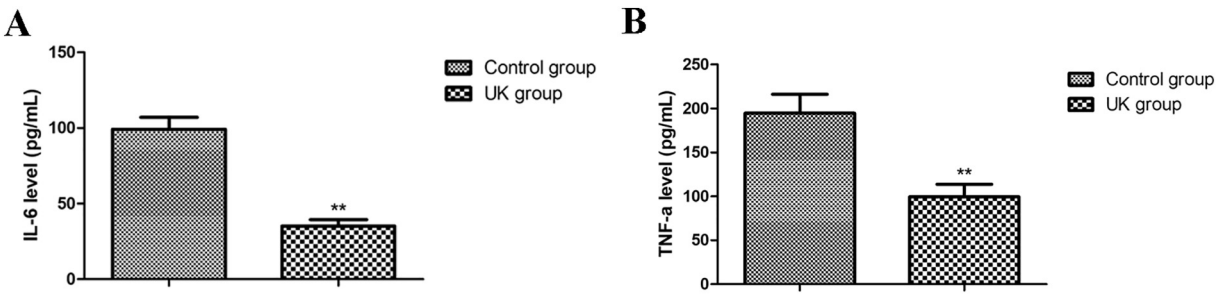

Fig. 6. Levels of tumor necrosis factor-alpha (TNF-α) (A) and interleukin (IL)-6 (B) in serum were measured by enzyme-linked immunosorbent assay. $^{**}P < 0.01$ vs. control.

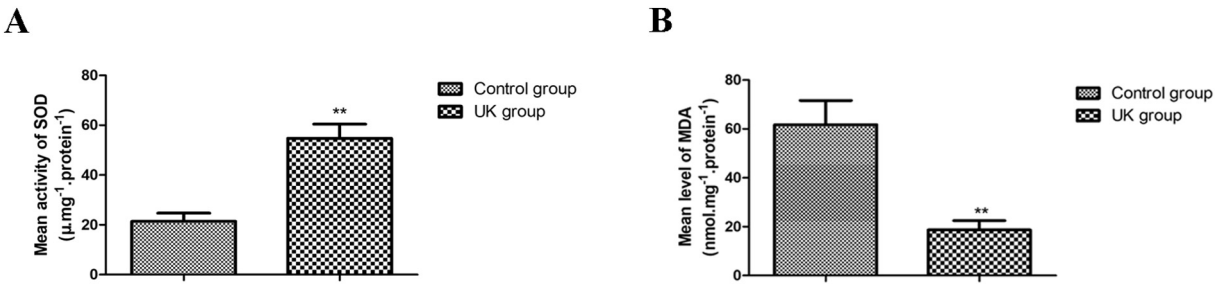

Fig. 7. Superoxide dismutase (SOD) (A) activity and malondialdehyde (MDA) (B) content in the control and treatment groups. $^{**}P < 0.01$ vs. control.

the UK treatment (35.16 ± 4.34 pg/ml) group compared with the control group (99.17 ± 7.86 pg/ml; $P < 0.01$; Fig. 6A). Similarly, a significant decline in the TNF-α level after UK treatment (99.47 ± 14.42 pg/ml) relative to the control treatment was observed (194.63 ± 21.59 pg/ml; $P < 0.01$; Fig. 6B).

3.7. Effects of UK on SOD activity and MDA content

Rats administered UK exhibited a significant increase in SOD activity (54.79 ± 5.66 U/mg prot) compared with the control group (21.40 ± 3.00 U/mg prot; $P < 0.01$; Fig. 7A). In contrast, a sharp increase in MDA content was observed in the control group (61.71 ± 9.08 nmol/mg prot), which was notably reversed by the administration of UK (18.73 ± 3.47 nmol/mg prot; $P < 0.01$; Fig. 7B).

4. Discussion

Random-pattern flaps are used in plastic and reconstructive surgery to cover skin defects. However, flap survival, particularly in the distal area, tends to be unstable and unreliable, so flaps have been restricted to a strict length-to-width ratio to ensure viability [12]. In previous studies, a variety of drugs and techniques were investigated, such as resveratrol [13], exhibits proangiogenic, antiapoptotic, and anti-oxidative abilities in protecting ischemic skin flaps; calcitriol [14] promotes skin flap survival by ameliorating inflammatory responses, reducing oxidative stress, and promoting autophagy; in addition to drug therapy, electroacupuncture at the Zusanli point [15], can also alleviate ischemia or necrosis of random skin flaps. However, none of these methods has achieved clinical application. In this study, according to general observations and histopathological examination, the flap survival rate was significantly enhanced in response to UK, indicating that UK promoted skin flap viability in rats.

Ischemia and necrosis can directly damage vascular endothelial cells, affecting their angiogenic ability [16]. Moreover, inadequate vascularity-induced hypoxic stress in distal flaps has been accepted as a critical risk factor for skin flap ischemia and necrosis [17]. Therefore, the generation of new vasculature from existing blood vessels in ischemic skin flaps is an important step during wound healing. UK has been reported to have post-ischemic angiogenic functions, with increased vessel density due to upregulated VEGF expression in MCAO (cerebral artery occlusion) mice [10]. In this study, MVD was increased significantly in the skin flaps of UK-treated rats compared with the control group. Moreover, lead oxide/gelatin angiography showed a similar pattern of change after treatment, as new blood vessels developed, particularly in the distal flaps. Blood perfusion of the skin flaps was markedly enhanced by UK-induced new vasculature formation, according to laser Doppler imaging.

VEGF is a widely known growth factor that regulates vascular development and maturation [18]. Accumulating evidence demonstrates that VEGF stimulates angiogenesis, thereby improving wound healing and ameliorating flap failure [19,20]. With the aim of identifying the mechanism through which UK improved angiogenesis, we examined changes in VEGF levels by immunohistochemistry. Our results revealed that the VEGF level was significantly upregulated after UK treatment, which might play a role in the angiogenic effect of UK in ischemic skin flaps.

Locally high accumulation of reactive oxygen species can result from blood vessel injury and tissue cell apoptosis [21]. Cumulative studies have demonstrated that oxidative stress and inflammation are interrelated biological events in the pathogenesis of flap ischemia and necrosis, and antioxidant therapy is accepted as an effective treatment for ischemic disorders [22]. SOD is an antioxidant enzyme that ameliorates oxidative injury [23]. MDA is a vital marker of tissue damage, and oxidant and inflammatory stimuli upregulate MDA levels [24]. Based on the potent anti-inflammatory and anti-oxidative abilities of UK, we determined the levels of pro-inflammatory cytokines, SOD activity, and MDA content in the tissue flaps. Consistent with our expectations, the production of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, decreased significantly. SOD activity was significantly promoted, whereas MDA content decreased significantly, suggesting that UK prompted the survival of random skin flaps by alleviating inflammatory and oxidative damage.

5. Conclusion

Our results demonstrate that UK protected against ischemia and necrosis in random skin flaps by attenuating inflammatory and

oxidative damage, and prompted angiogenesis by upregulating VEGF expression.

Declaration of Competing Interest

None.

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