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Effects of adiponectin on random pattern skin flap survival in rats

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ABSTRACT

Background: Random flaps are commonly used to repair wounds and improve the clinical appearance. However, flap necrosis is frequently encountered in the clinical setting. Adiponectin is a biologically active endogenous polypeptide secreted by adipocytes that can reduce oxidative stress, inflammation, and apoptosis. This study was performed to explore the effects of adiponectin on the survival of random flaps in rats.

Materials and methods: Thirty-six healthy rats were divided into two groups, *i.e.*, an adiponectin group and a control group. A modified McFarlane flap was created on the backs of the rats. The area of flap survival was gauged after sacrifice of the rats on day 7 after surgery, and the tissue samples were subjected to histological analysis. Angiogenesis was assessed by oxide-gelatin angiography, laser Doppler imaging, and immunohistochemistry. Pathological changes in the flaps were examined by hematoxylin and eosin staining. The level of oxidative stress was evaluated using malondialdehyde (MDA) and superoxide dismutase (SOD) kits.

Results: The adiponectin group had a larger tissue survival area and less edema compared with the control group. VEGF expression and SOD activity were markedly increased, but the MDA level was significantly decreased, in the adiponectin group. Histological analysis showed that adiponectin promoted angiogenesis and inhibited inflammation.

Conclusions: Adiponectin is useful for improving random skin flap survival.

1. Introduction

Flaps are used to repair soft tissues and skin defects in plastic surgery. A flap is an ideal material for organ and body repair, such as nasal reshaping, correction of lip deformities, and breast reconstruction [1–3]. Flap transplantation, particularly local rotating flap transfer, is also indispensable for scar repair after excision and scalp defect repair during head and face surgery [4,5]. Previous studies have demonstrated that flap necrosis can be caused by a variety of factors, such as insufficient blood supply, ischemia–reperfusion injury, oxidative stress, and the inflammatory response [6–8]. Therefore, new strategies to improve flap survival are needed to alleviate these effects.

Adiponectin is an endogenous biologically active polypeptide secreted by adipocytes that has a wide range of biological effects. Previous studies have confirmed that adiponectin plays a key role in preventing ischemia–reperfusion injury [9–12]. In addition, one study also showed that adiponectin reduces oxidative stress, inflammation, and apoptosis [13]. It also improves the production of vascular endothelial growth factor (VEGF) [14,15]. Despite these biological properties, it is still unknown whether adiponectin promotes the survival of random flaps. Therefore, we investigated the influence of adiponectin on the survival of random flaps in rats, along with the underlying mechanism.

2. Methods

2.1. Animals and reagents

Thirty-six healthy male Sprague–Dawley (SD) rats (260–310 g) were provided by the Animal Experimental Center of Wenzhou Medical University (SYXK [Zhe] 2015–0009). All protocols in this research were in compliance with relevant rules and regulations. SD rats were housed in a suitable environment and were divided randomly into two equal groups: an adiponectin group ($n = 18$) and a control group (normal saline, $n = 18$) groups.

Adiponectin (purity ≥95%, Lot: AG11028278) was obtained from Shanghai Wildlife Technology Co., Ltd. (Shanghai, China). Test kits for superoxide dismutase (SOD) and malondialdehyde (MDA) were acquired from the Nanjing Jiancheng Biology Institution (Nanjing, China). Anti-VEGF antibody (Lot: 16c5074) was purchased from

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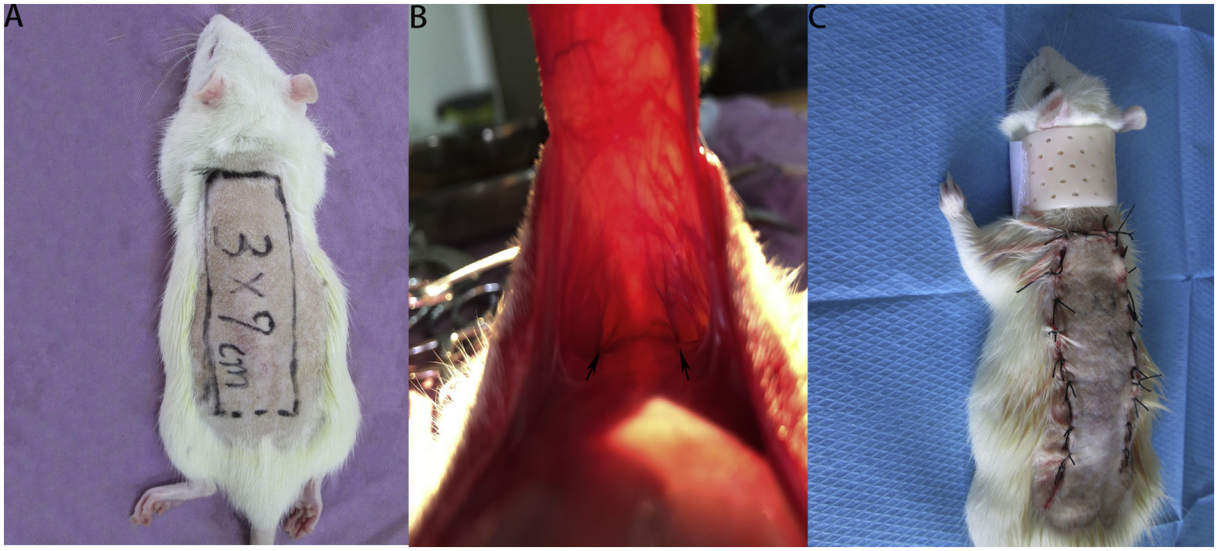

Fig. 1. (A) A random 3 × 9 cm flap was established in the middle of the back of each rat. (B) Both sacral arteries (indicated by the arrow) were severed. (C) All rats were fitted with a neck strap to avoid self-mutilation.

Affinity Biosciences (Cincinnati, OH, USA).

2.2. Animal flap model

SD rats were anesthetized by injection of 10% chloral hydrate (3 mL/kg) into the abdominal cavity. The back of the rat was depilated and the animal was fixed in the supine position. Iodophor was used to sterilize the skin, and a surgical drape was used to cover the animal. The modified McFarlane flap model was used for the procedure. We designed a random 3 × 9 cm flap in the middle of each rat's back [16]. The skin was cut along the design line and the flap was completely separated from the deep fascia; both sacral arteries were severed and rapidly sutured *in situ*. The flap region was divided into three equal regions: the proximal region (region I), intermediate region (region II), and distal region (region III) (Fig. 1).

The adiponectin group received an intraperitoneal injection of adiponectin (dissolved in 40 μg/kg/d deionized water), daily for 7 days, starting on the day after surgery, while the control group received an equal volume of sterile saline according to the same schedule. All rats were reared in individual cages with collars to avoid injury. On day 7, the animals were euthanized by intraperitoneal overdose of sodium pentobarbital.

2.3. Observation and assessment of flaps

Flap survival was examined visually and the flap was assessed in terms of color and texture. Photographs of the flaps were taken daily. After 7 days, clear cellophane was used to precisely gauge the survival area of each flap and total area of the flap. The cellophane was weighed on an electronic scale and counted. The area of flap survival was calculated according to the following formula: (weight of surviving flap/total weight of entire flap area) × 100% [17]. Tissue was considered necrotic if the flap was black in color and there was tissue retraction, poor elasticity, hard texture, and lack of bleeding when cut.

2.4. Hematoxylin and eosin staining

Animals were euthanized with an excess of sodium pentobarbital. Three tissue samples (1 cm × 1 cm) were harvested from the same location of each flap area (regions I–III), fixed with 4% paraformaldehyde for 24 h, and embedded in paraffin. The paraffin blocks were cut into 4—μm-thick sections and mounted on poly-L-lysine-coated slides, followed by staining with hematoxylin and eosin (H&E). Light microscopy (BH51; Olympus, Tokyo, Japan) was used to examine neutrophil infiltration. Five fields of view were randomly selected in the central and peripheral regions of each slice (100× magnification), and the number of neutrophils in each field of view was counted three times and averaged to calculate the total number. The number of neutrophils per unit area (/mm^2) was taken to indicate the degree of neutrophil infiltration. In addition, we calculated the number of microvessels per unit area (/mm^2).

2.5. Immunohistochemistry

Tissue samples were collected from flap region II and subjected to immunohistochemical staining using the streptavidin–peroxidase method [18]. We added one drop of 3% hydrogen peroxide solution to each section and incubated the sections for 10 min at room temperature to block endogenous peroxidase activity, followed by rinsing with phosphate-buffered saline (PBS). The slides were then incubated with normal goat serum for 20 min at room temperature to block nonspecific binding, followed by the addition of 50 μL of anti-VEGF antibody (working concentration, 1:100). All slides were incubated overnight at 4 °C. The slides were rewarmed to 37 °C for 45 min and washed with PBS. The slides were then immunostained with 50 μL of goat anti-rat antibody (working concentration, 1:50) for 1 h at room temperature and rinsed with PBS. For color development, 3,3-N-diaminobenzidine tetrahydrochloride was used. The sections were counterstained with hematoxylin and mounted. We examined regions showing strong VEGF expression under low magnification, and the number of VEGF-positive cells was quantified in five random fields per specimen under higher magnification (400×). The parameters of interest (white balance, aperture, shutter speed, time) were consistent. Images were saved using Image-pro Plus software (ver. 6.0; Media Cybernetics, Rockville, MD, USA), and integral absorbance (IA) was used as an indicator of protein expression level [19].

2.6. Flap angiography

Systemic angiography was performed on postoperative day 7 using the modified lead oxide-gelatin injection technique [20]. First, we injected 1.5 mL of 1% heparin saline into the right carotid artery, followed by injection of the contrast agent consisting of lead oxide, gelatin, and water at 150 mL/kg. Rats were maintained in the supine

position overnight, and the flaps were radiographed with an X-ray machine.

2.7. Laser Doppler imaging

The blood supply to the whole flap was measured on postoperative day 7 using a laser Doppler imaging camera (PIMII; Lisca, Stockholm, Sweden). Mean blood flow is expressed in perfusion units [21].

2.8. SOD activity and MDA content

Using a random number table, five samples (55 mm) obtained from region II in each group were processed according to a battery of procedures, including weighing on postoperative day 7, homogenization, and dilution. The oxidative stress level in the flaps was measured using MDA and SOD test kits in accordance with the manufacturer's instructions.

The activity of total SOD (T-SOD) was determined using the xanthine–xanthine oxidase system to produce superoxide ions, which reacted with 2-(4- iodophenyl)-3-(4-nitrophenol-5-phenlyltetrazoliom chloride) to form a red formazan dye; the absorbance at 550 nm was measured. The values are expressed as units per mg of protein, and protein concentration was determined by BCA protein assay, where one unit of SOD was defined as the amount of SOD inhibiting the rate of reaction by 50% at 25 °C. Lipid peroxidation was evaluated by measuring MDA concentrations according to the thiobarbituric acid (TBA), method as recommended by the manufacturer. The method was based on spectrophotometric measurement of the color produced during the reaction of TBA with MDA. MDA concentrations were calculated based on the absorbance of TBA-reactive substances (TBARS) at 532 nm [22,23].

2.9. Statistical analysis

Data are expressed as means ± standard deviation, and statistical analysis was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The results were analyzed by Student's *t*-test. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. General observations

From postoperative day 1 to day 7, each flap was examined by the same observer and conditions (fold color, tissue elasticity, texture, and necrotic area) were recorded. On the first day after surgery, the flaps in both groups showed some swelling, and region III was dark purple, but no obvious necrosis had occurred. On day 3 after surgery, region III of the flap was blackened and hardened, and some areas were necrotic. On day 7, the boundaries between the areas of survival and necrosis in both groups tended to be stable. The necrotic area of the flap was dark with a hard texture, and no bleeding occurred when the tissue was cut (Fig. 2).

3.2. Survival area

The region of flap survival was decreased in both groups on day 7 compared with day 1. The survival area was 73.76% ± 2.09% in the adiponectin group and 46.96% ± 4.38% in the control group. The mean area of flap survival was significantly larger in the adiponectin group than the control group (**$p < 0.01$) (Fig. 3).

3.3. Hematoxylin and eosin staining

As shown in Fig. 4, region I of the flaps was histologically similar between the control and experimental groups on day 7 after surgery, with numerous infiltrating inflammatory cells and abundant tissue edema. Many inflammatory cells were seen in region II in the control group, whereas relatively few inflammatory cells were observed in the adiponectin group, indicating a relatively weak inflammatory response in the adiponectin group. The microvessel density values in region II of the experimental and control groups were 30.72 ± 3.28/mm^2 and 14.22 ± 1.57/mm^2, respectively (Fig. 4, **$p < 0.01$). The adiponectin group showed a degree of edema in flap region I, and the levels of vasodilation and inflammatory cell infiltration were lower than in the control group.

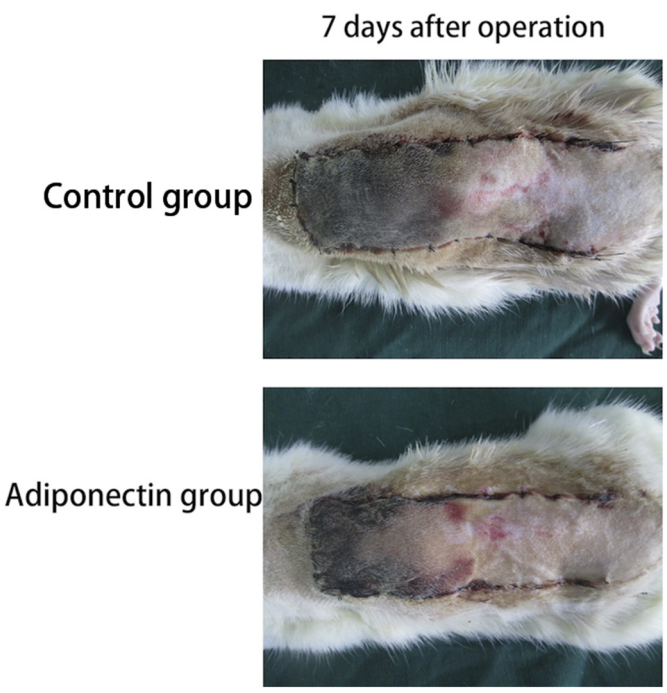

Fig. 2. Postoperative morphological observations of the rats in both groups.

3.4. VEGF immunohistochemistry

According to the IA value, the level of VEGF expression was significantly higher in the adiponectin group (4253.33 ± 128.32) than the control group (1878.33 ± 166.75) (Fig. 5, **$p < 0.01$).

3.5. Flap angiography

On day 7 after surgery, the microvasculature of flaps could be clearly seen on X-ray images. In the adiponectin group, the microvascular appearance of the flaps was larger than in the control group (Fig. 6).

3.6. Laser Doppler imaging

We used a laser Doppler system to assess the blood supply to the random skin flaps. The levels of blood perfusion in zones I (449.49 ± 26.82 perfusion units [PU]), II (219.27 ± 16.08 PU), and III (71.66 ± 10.26 PU) in the adiponectin group were much higher than those in the control group (344.51 ± 32.39 PU, 58.45 ± 15.58 PU, and 22.82 ± 4.58 PU, respectively, all **$p < 0.01$) (Fig. 7).

3.7. SOD activity and MDA content

The adiponectin group showed significantly higher SOD activity (58.33 ± 2.85 U/mg protein) than the control group (25.11 ± 2.58 U/mg protein) (**$p < 0.01$). The mean MDA level in the adiponectin group was 28.56 ± 2.05 nmol/mg protein, which was

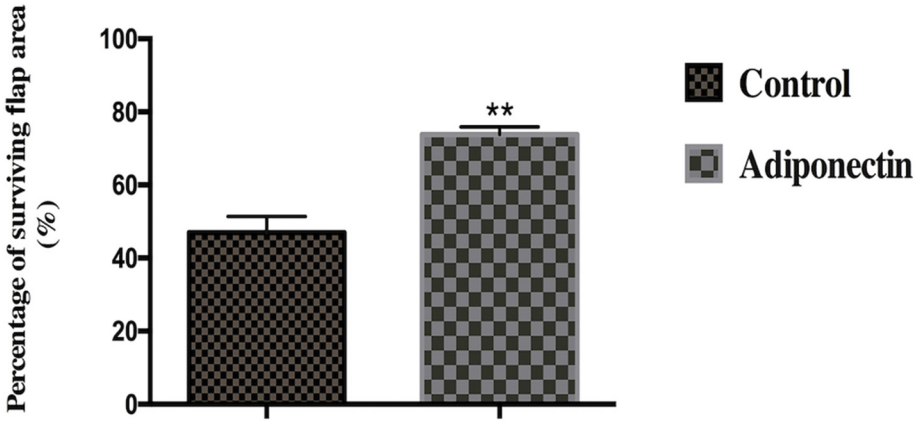

Fig. 3. Percentage of the flap survival area on day 7 after surgery in the adiponectin (73.76% ± 2.09%) and control groups (46.96% ± 4.38%). The ratio was significantly higher in the adiponectin group than in the control group (**$p < 0.01$).

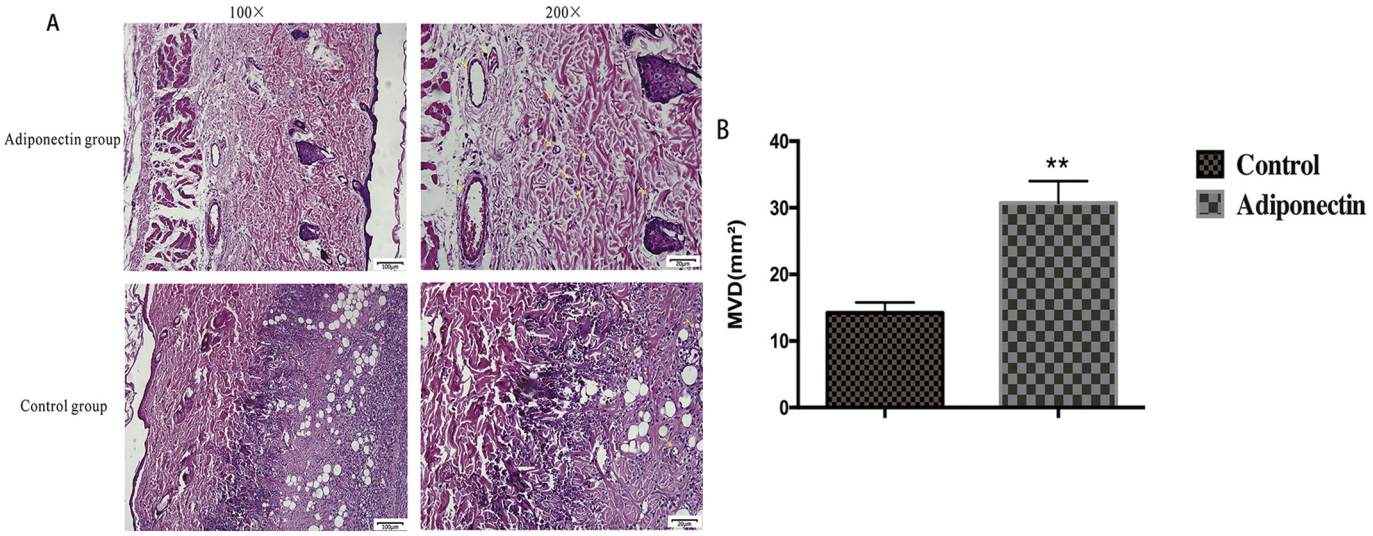

Fig. 4. (A) Histological changes in flap region II of the two groups revealed by hematoxylin and eosin staining. (B) Microvessel density in the middle of region II was significantly lower in the control group (14.22 ± 1.57/mm^2) than the adiponectin group (30.72 ± 3.28/mm^2) (**$p < 0.01$). Angiogenesis is indicated by yellow arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

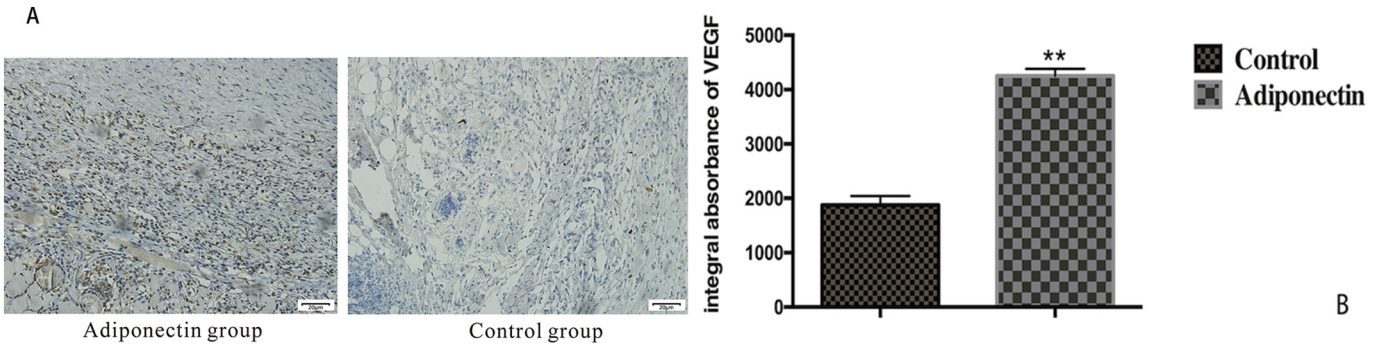

Fig. 5. (A) Vascular endothelial growth factor (VEGF) expression in region II of the flaps in the two groups was determined by microscopic observations under 400× magnification. (B) The integral photometric value was significantly higher in the adiponectin group (4253.33 ± 128.32) than the control group (1878.33 ± 166.75) (**$p < 0.01$).

significantly less than in the control group (59.52 ± 5.63 nmol/mg protein, **$p < 0.01$) (Fig. 8).

4. Discussion

Flap transfer is commonly used for wound repair in orthopaedics. The application of flaps can preserve limbs and provide a satisfactory appearance and function of certain organs, but flap necrosis remains a challenge, especially for distal flaps. Flap failure can be caused by tissue hypoperfusion, ischemia–reperfusion injury, and inflammatory responses. The results of the present study showed that adiponectin had a positive influence on the survival of random skin flaps by alleviating the

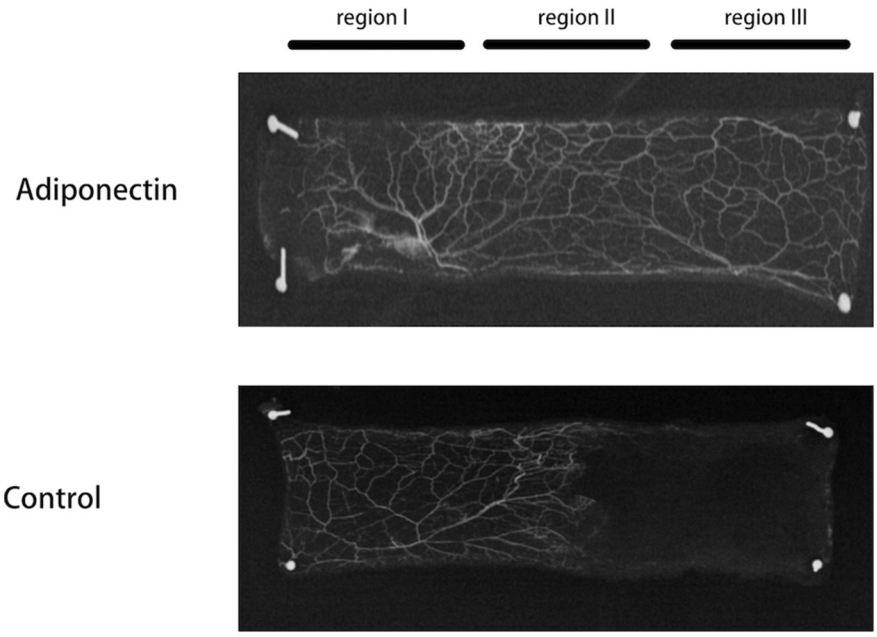

Fig. 6. Flap angiography in the control and adiponectin groups.

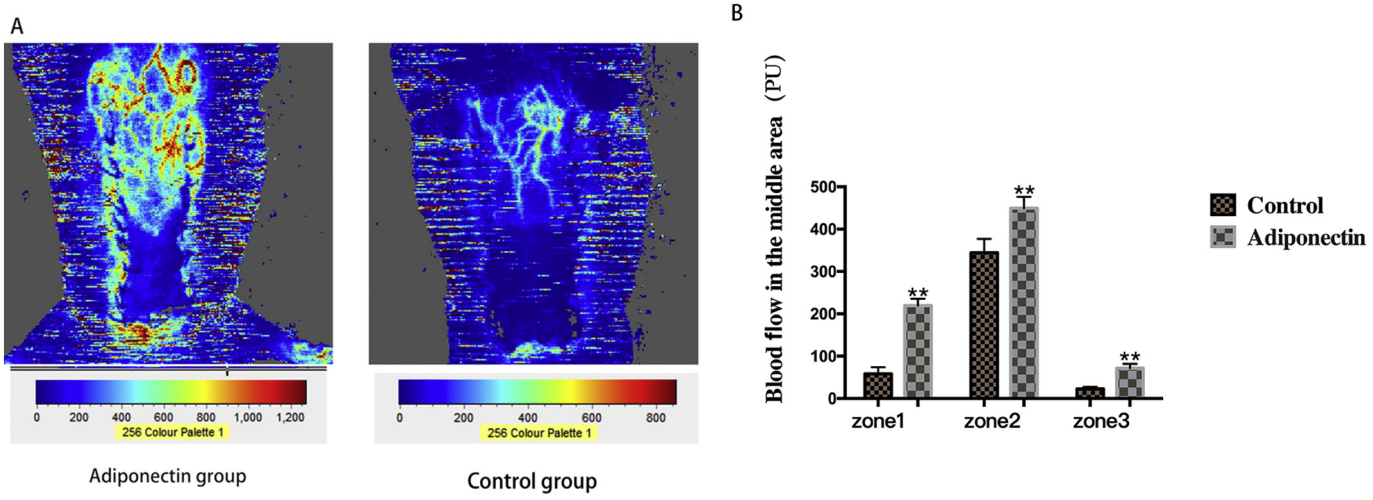

Fig. 7. (A) Blood perfusion in the flaps of the two groups was determined by laser Doppler imaging. (B) On day 7 after surgery, the mean levels of blood perfusion in the adiponectin group, in zones I (449.49 ± 26.82 PU), II (219.27 ± 16.08PU), and III (71.66 ± 10.26 PU), were much greater than those in the control group (344.51 ± 32.39 PU, 58.45 ± 15.58 PU, and 22.82 ± 4.58 PU, respectively) (**$p < 0.01$).

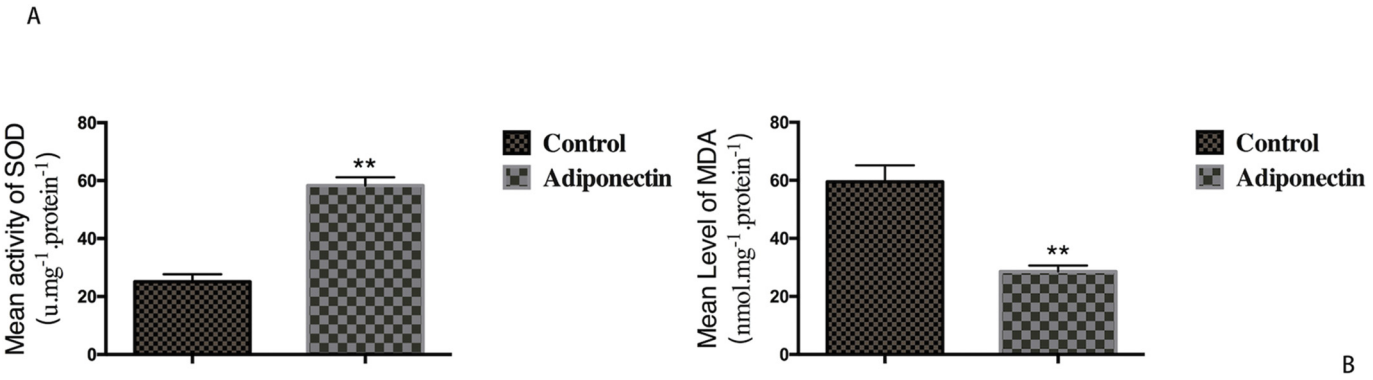

Fig. 8. (A) Superoxide dismutase (SOD) activity was significantly higher in the adiponectin group (58.33 ± 2.85 U/mg protein) than the control group (25.11 ± 2.58 U/mg protein) (**$p < 0.01$). (B) The average malondialdehyde (MDA) content was significantly lower in the adiponectin group (28.56 ± 2.05 nmol/mg protein) than the control group (59.52 ± 5.63 nmol/mg protein) (**$p < 0.01$).

inflammatory response, promoting neovascularization, and ameliorating ischemia–reperfusion injury.

One study [24] showed that adiponectin at 10 μg/kg reduced lung ischemia–reperfusion injury *via* antiinflammatory, antiapoptotic, and antioxidative stress activities in a type 2 diabetic rat model of lung ischemia–reperfusion injury. Another study [25] used a therapeutic adiponectin dose of 20 μg/kg/day to treat type 2 diabetic rats with a no-reflow coronary injury, and showed that adiponectin protected endothelial cells and improved myocardial microcirculation, providing a new target and theoretical basis for decreasing myocardial ischemia–reperfusion injury. In this study, a therapeutic adiponectin dose of 40 μg/kg/day increased flap survival in rats by promoting angiogenesis, reducing inflammation, and ameliorating ischemia–reperfusion injury.

VEGF can stimulate endothelial cell proliferation and promote angiogenesis, thereby promoting flap survival [26,27]. Previous studies suggested that adiponectin promotes the expression of VEGF-A in human chondrosarcoma cells [15] and VEGF-C in human macrophages [14]. In addition, adiponectin stimulates endothelial cell migration by activating AMPK signaling and differentiation into capillary-like structures *in vitro* [28].

In the present study, the mean vascular density in region II and the number of vessels revealed by laser Doppler imaging and flap angiography were increased in the adiponectin group compared with the control group. In addition, VEGF expression based on the IA value was significantly higher in the adiponectin group, suggesting that adiponectin promotes angiogenesis by improving blood supply and VEGF expression, as well as increasing the number of blood vessels in damaged tissues. However, further studies are required to determine the underlying mechanism.

The level of oxygen free radicals increases rapidly during ischemia–reperfusion, which is a common cause of lipid peroxidation and tissue damage. SOD is an important metalloproteinase that eliminates harmful substances produced by organisms during metabolism [29]. MDA is one of the final products of peroxidation of polyunsaturated fatty acids in cells, and its level is often used as a marker of oxidative stress [30]. We found that the SOD activity increased, and the MDA content decreased significantly, in the adiponectin group in comparison to the control group, indicating that the level of oxidative stress was markedly lower in the adiponectin group. These results suggest that adiponectin can reduce oxidative stress and the inflammatory response, thereby efficiently preventing ischemia–reperfusion injury.

This study had some limitations. Further studies are needed to determine the optimal dose and route of administration of adiponectin, as well as the specific mechanism underlying its protective effect. Finally, this study only achieved satisfactory results in animal experiments; further research is required to determine whether adiponectin can be used the clinical setting.

5. Conclusion

This study showed that adiponectin improves the survival of random skin flaps.

Acknowledgments

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Declaration of competing interest

No.

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