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ORIGINAL RESEARCH

Roles of Oxidative Stress and Raftlin in Wound Healing Under Negative-Pressure Wound Therapy

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Background: Negative-pressure wound therapy (NPWT) is an effective way to promote wound healing. However, its mechanisms have not been investigated thoroughly. Growing evidence suggests that oxidative stress and Raftlin levels play important roles in wound healing. However, whether NPWT promotes wound healing through this mechanism remains unclear.

Purpose: Our study focuses on the different levels of oxidative stress and antioxidant response between wounds treated by NPWT and routine dressing change. The objective of this study was to measure the differences in Raftlin levels between the two groups, which is a new biomarker related to wound healing.

Methods: We divided 48 male Sprague-Dawley rats with identical full-thickness skin defects into two groups. At specific times (0, 3, 5, 7, 9, 11, and 13 days after surgery), wound tissue samples were obtained for immunohistochemistry and biochemical analysis. The expression of Raftlin and levels of oxidative stress, including malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) levels were measured by biochemical analysis. Wound-healing times were also compared.

Results: In the NPWT group, MDA levels were significantly decreased on days 3, 5, and 7. Furthermore, the expressions of SOD and CAT were significantly reduced on days 3 and 5. Our data also revealed that Raftlin was significantly upregulated across the whole period of wound healing. Moreover, wound healing in the NPWT group was significantly more rapid (16 days on average) than in the control group (24 days on average). On day 13 post surgery, the wound-healing percentage in the NPWT group was 91%, while that in the control group was 48%.

Conclusion: NPWT may promote wound healing by upregulating Raftlin and inhibiting oxidative stress levels.

Keywords: SOD, CAT, MDA, Raftlin, negative-pressure wound therapy

Introduction

Complex wounds are clinically common and difficult to treat. They therefore impose heavy economic pressure on individuals and society worldwide. Epidemiological data indicate that the incidence of wounds with various causes is gradually increasing, and the medical costs associated with them are also rising year by year.[1] According to one report, in 2013, 20.8% of global years lived with disability were caused by musculoskeletal disorders.[2] In the past five years, the cost of diabetic wound repair alone has increased by more than ten times.[3] Negative-pressure wound therapy (NPWT) is a method in which negative pressure

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is applied to a wound to promote healing. Some studies have revealed that NPWT has a positive effect on wound healing,[4,5] and the use of NPWT in various clinically complex wounds has been reported.[6,7] Some scholars believe that NPWT can remove the surplus wound exudate to reduce tissue edema,[5] promote angiogenesis and the growth of granulation tissue[8] and ultimately promote wound healing.[9] However, in clinical work, we have observed various complications of NPWT including local hemorrhage, local indentation, the formation of blisters, allergies to the adhesive drape, excoriation of the skin, restricted mobility, adherence of the tissues to the foam and skin necrosis.[10] As previously reported, the short-term use of NPWT is accompanied by the risk of bleeding, infection,[10] pain, heart rupture,[11] and even death. Long-term use can reduce the quality of life, increase anxiety,[12] and even lead to malnutrition.[13,14] Although many mechanisms of NPWT promoting wound healing have been proposed, they are still incomplete and controversial;[15,16] only by clarifying its specific functional mechanisms can we put it into effective use in clinical work and avoid adverse reactions. Therefore, to minimize the incidence of complications from NPWT and apply it more appropriately, it is important to further clarify the specific mechanisms of the wound-healing process under negative pressure.

Oxidative stress is an internal imbalance of the body's pro- and antioxidants. In the process of oxidative stress, the production of reactive oxygen species (ROS) increases, and these are very important in wound healing. Low concentrations of ROS participate in the regulation of many signal transduction pathways in cells, and they also provide energy for phagocytes to phagocytose bacteria.[17,18] Although an appropriate amount of active oxygen is essential for wound healing, in most cases, the harmful biological effects of active oxygen are more profound. High concentrations of ROS can directly react with cell lipids, proteins and DNA, causing cell damage and death.[19] For example, lipid peroxidation, the production of malondialdehyde (MDA), is a manifestation of the damage of ROS to the plasma membrane of cell membranes and organelles.[20]

To cope with excessive oxidation reactions, the body will produce enzyme antioxidants, of which superoxide dismutase (SOD) and catalase (CAT) are the most important two. For complex wounds that are difficult to heal, supplementation with antioxidants can help to protect cells from oxidative damage and can improve wound healing.[21]

Raftlin is the main lipid raft protein found in B cells, and it is responsible for the signal regulation of B-cell antigen receptors.[22] Raftlin is also involved in the nuclear capture complex, the induction of TLR3 activation and the autoimmune response.[23,24] Lipid raft proteins play a major role in the pathophysiology of vasculitis.[25–27] In addition, Raftlin can be recruited into vascular endothelial growth factor receptor 2 to control pro-angiogenic signals.[28]

It has been reported that Raftlin is related to the vascular inflammatory response and induction of the autoimmune response, and these are important parts of the complex biological process of wound healing.[29,30] Similarly, ROS participate in many phases of wound including hemostasis, inflammation, proliferation, and remodeling.[17,31] Growing evidence suggests that oxidative stress and Raftlin levels are associated with wound healing.[32] However, no study has focused on comparing the levels of oxidative stress and Raftlin in wounds treated by NPWT with that treated by routine dressing change. Thus, the purpose of this research was to explore the roles of oxidative stress and Raftlin in wound healing under negative pressure.

Materials and Methods

Ethics Statement

The experimental program was approved by the Committee on the Ethics of Animal Experiments of Zhongnan Hospital (approval number WP20210011). All operations were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee of Wuhan University. Every effort was made to minimize animal suffering.

Animals and Clean Wound Model

We obtained eight-week-old male Sprague-Dawley rats, each weighing about 250–300 g, from the animal experiment center of Wuhan University. These were housed in a clean environment, in which fresh water and food were not limited. The rats were given at least 3 days to adapt to the environment. On the day of surgery, all rats were anesthetized with 1% sodium pentobarbital (40 mg/kg; intraperitoneal). After the backs of the rats were shaved and disinfected, we removed a 1.5×1.5 cm full-thickness skin and subcutaneous panniculus carnosus section from each.

Study Groups

A total of 48 male Sprague-Dawley rats were randomly divided into two groups: the NPWT group and the control group (n=24 in each group). The rats of the control group were only covered with ordinary gauze. For the NPWT-group rats, we covered the wounds with a medical foam (VSD Medical Technology Inc., Wuhan, China) and used a vacuum-assisted closure device (VSD Medical Technology Inc., Wuhan, China) to produce 120-mmHg suction. For both groups, the dressings were changed every two days.

Wound Closure Measurement and Wound Tissue Collection

At 0, 3, 5, 7, 9, 11, and 13 days after the operation, three rats from each group were killed for wound photography and sample collection. In each case, the wound area was measured with a sterile 15-cm steel ruler, and the wound size was calculated using the ImageJ software package (National Institutes of Health, Bethesda, USA) as follows. A 1-cm line segment was drawn on graph paper according to the scale of the steel ruler, and the wound margin was traced on the photograph. The ImageJ software then calculated the size of each wound according to the 1-cm line segment. The final value of wound closure (%) = (wound size on day zero − wound size on day x)/(wound size on day zero) × 100%.

For each sample, the whole wound was harvested, including a 3-mm margin of surrounding skin. Each sample was divided into two parts: one was fixed in 4% paraformaldehyde for immunohistochemistry; the other was placed in an Eppendorf tube and stored in liquid nitrogen for later biochemical analysis. The remaining rats were raised until their wounds had completely closed, and the wound-healing times were recorded.

Immunohistochemical Analysis

Cluster of differentiation 31 (CD31) is regarded as an endothelial cell marker to measure angiogenesis. Similarly, CD68 has been used to mark and measure macrophages, which take part in inflammation. The percentages of CD31-positive and CD68-positive area were measured using ImageJ in six randomly selected areas from three animals at 200× magnification.

Biochemical Analysis

In the biochemistry laboratory, we evaluated the MDA level (as a measure of free oxygen radicals), SOD and CAT levels (as a measure of antioxidant enzymes), and Raftlin level of each tissue sample. We divided the tissue samples into small pieces and washed off the residual blood with phosphate-buffered saline. After weighing, we added 90 μL of ice-cold Western and IP cell lysate (P0013, Beyotime, Wuhan, China) for each 10 mg tissue to homogenize the samples. The supernatants obtained after centrifugation at 14.000g for 5 min at 4°C were used to measure the levels of MDA, SOD, CAT, and Raftlin.

The MDA level was measured using the Lipid Peroxidation MDA Assay Kit (Beyotime, Wuhan, China) according to the manufacturer's instructions. According to the method described by Ohkawa et al,[33] lipid peroxidation can be detected when MDA reacts with thiobarbituric acid to form a complex. When the mixture was fully reacted, it was centrifuged at 1000g for 10 min, and the reaction products were spectrophotometrically measured at 525 nm. The MDA level is expressed as nmol/mg protein.

The SOD activity was measured using the Total Superoxide Dismutase Assay Kit (Beyotime, Wuhan, China) according to the manufacturer's instructions. In brief, this was measured based on the superoxide radicals generated by reaction od hypoxanthine and xanthine oxidase with WST-8 to form formazan dye. Once the mixture had been incubated at 37°C for 30 min, the absorbance of each sample at 450 nm was measured. The SOD level is expressed as U/mg protein.

The CAT activity was measured by using the Catalase Assay Kit (Beyotime, Wuhan, China) according to the manufacturer's instructions. Catalase can catalyze the decomposition of hydrogen peroxide into oxygen and water when the level of hydrogen peroxide is great enough. The CAT activity can thus be determined by measuring the amount of water and oxygen produced by CAT in a specific time and space. After the mixture was incubated at 25°C for 15 min, the absorbance of each sample 540 nm was measured. The CAT activity is expressed as U/mg protein.

Raftlin levels in the tissue samples were measured using a Rat Raftlin ELISA kit (ELK Biotechnology, Wuhan, China) following the manufacturer's instructions. We measured the absorbance of the samples at 450 nm, and the Raftlin level is expressed as U/mg protein.

Statistical Analysis

All results are shown as average values and standard deviations (SDs). Data were analyzed using two-sample t-tests in GraphPad Prism 9 (GraphPad Inc., San Diego, CA, USA). A value of $p < 0.05$ was considered to indicate a statistically significant difference.

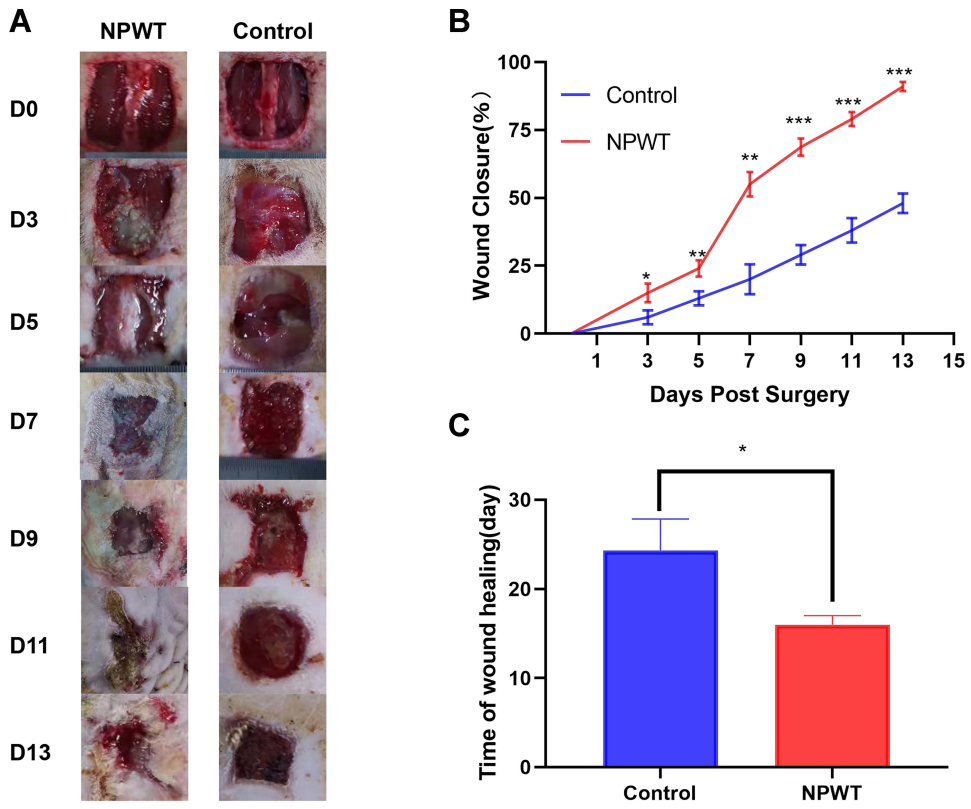

Figure 1 (**A**) Wound images of the NPWT and control groups on days 0, 3, 5, 7, 9, 11, and 13 post surgery. (**B**) Percentage of wound closure. (**C**) Wound-healing times. *p < 0.05, **p < 0.01, ***p < 0.001.
Abbreviations: NPWT, negative pressure wound therapy group; Control, control group; D X, day X post-surgery.

Results

Better Healing Situation in NPWT Group

Photographs of wound areas form each group on days 0, 3, 5, 7, 9, 11, and 13 after surgery are shown in Figure 1A, and B intuitively shows the percentage of wound closure at the different time points. These results suggest that the NPWT group had significantly better healing rates (p < 0.05) throughout all the wound healing stages compared with the control groups. The p values in Figure 1B are ∞, 0.023246, 0.008885, 0.001096, 0.000142, 0.000178, and 0.000049, respectively. Figure 1C shows wound-healing times of the two different groups. The results show that the wound healing time of the NPWT group was significantly shorter (p = 0.0168) than the control.

Increased Angiogenesis and Reduced Inflammation in NPWT Group

The percentage of CD31-positive area was used to assess the capillary density, and the results of this are revealed in Figures 2 and 4A. As shown in Figure 4A, the vessel density significantly increased (p < 0.05) in the NPWT group compared with the control group on days 3, 5, 7, 9, 11, and 13. The p values of Figure 4A are 0.543371, 0.022041, 0.014781, 0.012670, 0.002649, 0.001258, and 0.001109, respectively. The percentage of CD68-positive area was adopted to assess inflammation, and the results of this are revealed in Figures 3 and 4B. As shown in Figure 4B, compared with the control group at each time point (3, 5, 7, 9, 11, 13 days post-surgery), the percentage of CD68-positive area were significantly lower (p < 0.05) in the NPWT group. The p values of Figure 4B are 0.203754, 0.012237, 0.021234, 0.023588, 0.011484, 0.031687, and 0.004025, respectively.

Reduced MDA Level in NPWT Group

The level of MDA in both groups on days 0, 3, 5, 7, 9, 11, and 13 are shown in Figure 5. The differences in the level of MDA were not statistically significant on days 0, 9, 11 or 13. The level of MDA in the NPWT group was significantly lower (p < 0.05) than the control group on days 3, 5, and 7 of wound healing. The p values of Figure 5 are 0.345303, 0.012039, 0.036822, 0.047096, 0.856179, 0.699760, and 0.597544, respectively.

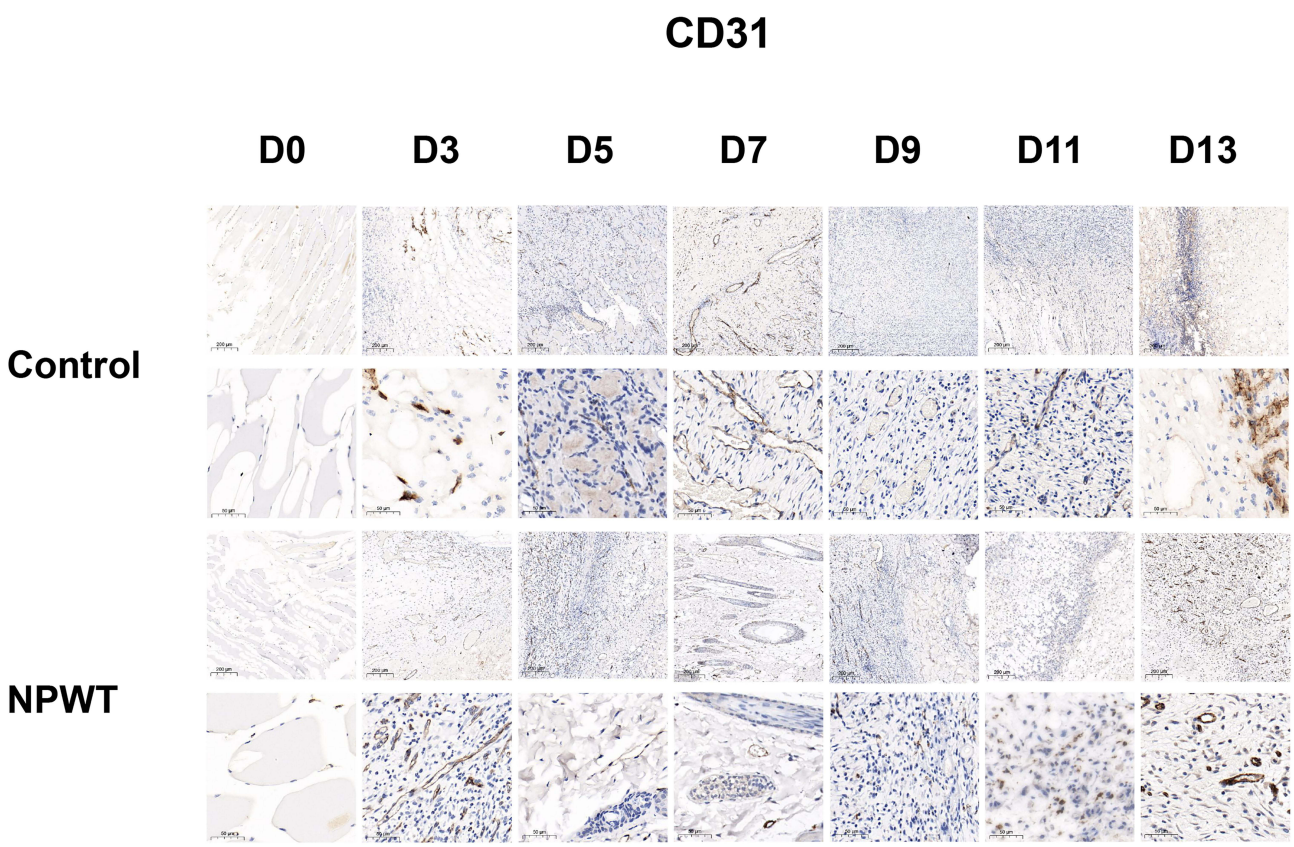

Figure 2 Anti-CD31 immunohistochemistry assays. Scale bar = 200 μm or 50 μm.
Abbreviations: NPWT, negative-pressure wound therapy group; Control, control group; D X, day X post-surgery.

Reduced SOD and CAT Activities in NPWT Group

We tested the SOD activity in both groups on days 0, 3, 5, 7,9, 11, and 13, and the results are shown in Figure 6A. These results reveal that the differences in SOD activity were not statistically significant on days 7, 9, 11, and 13. However, on days 3 and 5 post surgery, the SOD activity in the NPWT group was significantly lower ($p < 0.05$) than in the control group. The p values of Figure 6A are 0.628969, 0.047421, 0.019592, 0.863500, 0.608240, 0.219014, and 0.275152, respectively.

The results of the CAT activity in both groups on days 0, 3, 5, 7, 9, 11, and 13 are shown in Figure 6B. On days 3 and 5 post surgery, the CAT activity in the NPWT group was significantly lower ($p < 0.05$) than in the control group. The p values of Figure 6B are 0.143598, 0.027856, 0.011362, 0.469068, 0.870513, 0.122343, and 0.291332, respectively.

Increased Raftlin Level in NPWT Group

The Raftlin levels in both groups are shown in Figure 7. The Raftlin levels in the NPWT group were significantly higher ($p < 0.05$) than in the control group at all time points. The p values of Figure 7 are 0.643175, 0.023104, 0.001204, 0.008148, 0.015871, 0.013755, and 0.010094, respectively.

Discussion

In brief, our experiments revealed significant increases in Raftlin level, which regulates the inflammatory and autoimmune response in wounds treated with NPWT. This may be one of the mechanisms by which NPWT promotes wound healing. Furthermore, significant decreases in the activities of MDA, SOD, and CAT in the NPWT group suggest that there are fewer free oxygen radicals and antioxidant enzymes, and less cellular damage is caused by oxidative stress. Therefore, further research examining oxidative stress and Raftlin levels may be a way to study the mechanism of NPWT.

The use of NPWT has been reported for the treatment of various wounds,[7,34,35] and it has active effects for various acute and chronic wounds.[9,36] Although there has been a large amount of research in this area in the past two decades, the specific mechanism of NPWT is still unclear.[37,38]

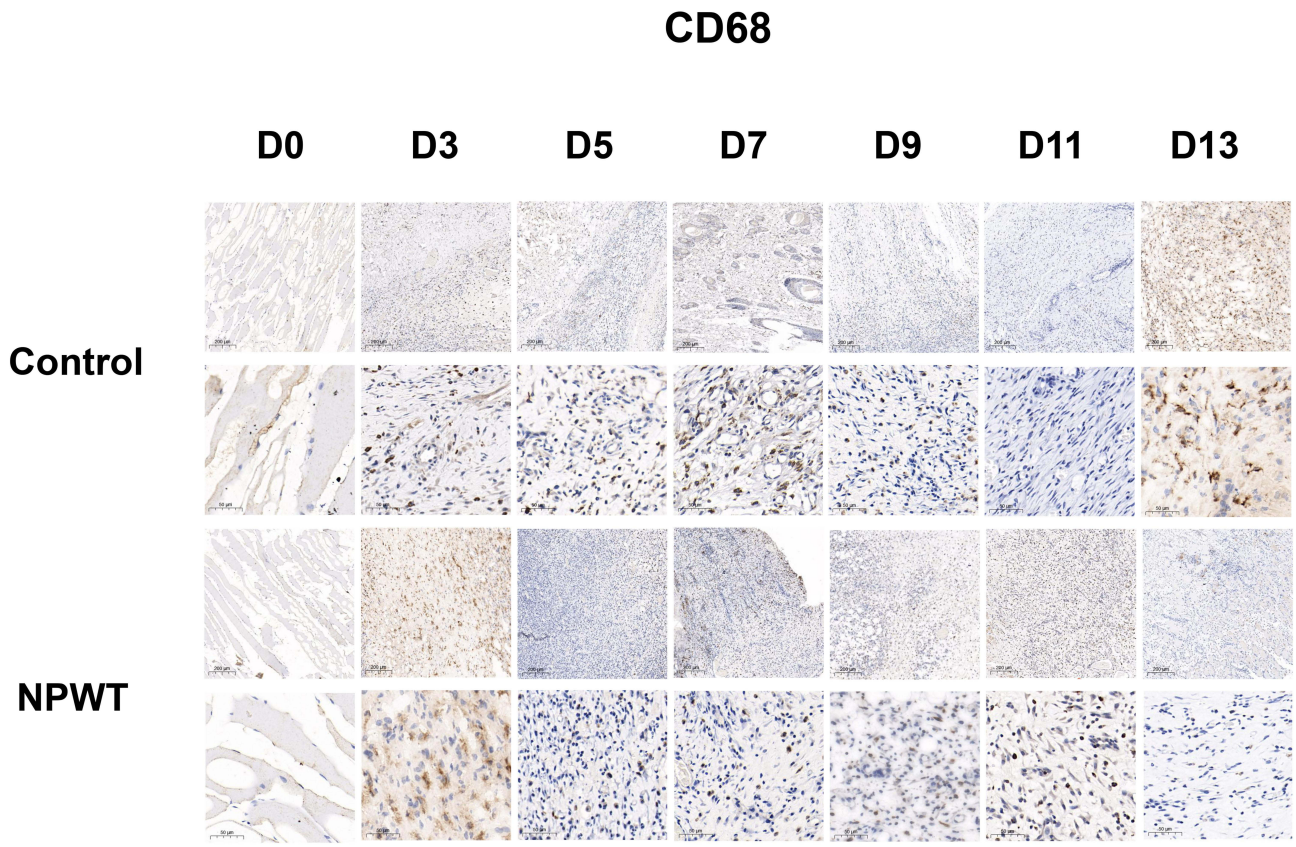

Figure 3 Anti-CD68 immunohistochemistry assays. Scale bar = 200 μm or 50 μm.
Abbreviations: NPWT, negative-pressure wound therapy group; Control, control group; D X, day X post-surgery.

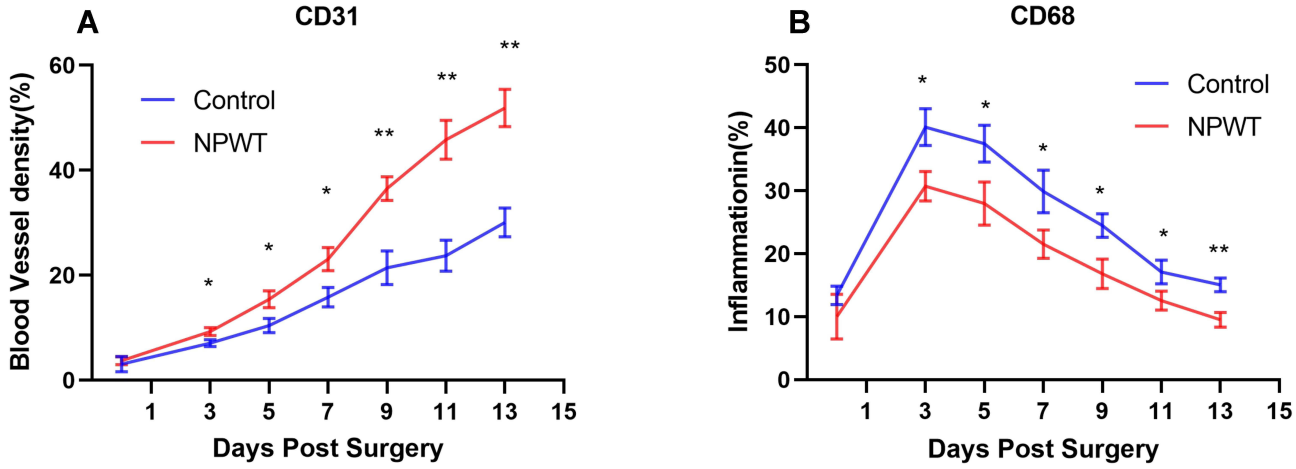

Figure 4 (**A**) Quantification of blood-vessel density by measuring the percentage of CD31-positive area on days 0, 3, 5, 7, 9, 11, and 13 post surgery. (**B**) Quantification of inflammation by measuring the percentage of CD68-positive area on days 0, 3, 5, 7, 9, 11, and 13 post surgery. *$p < 0.05$, **$p < 0.01$.
Abbreviations: NPWT, negative-pressure wound therapy group; Control, control group.

It has been found that ROS are closely related to wound healing, and they directly participate in many stages of the process.[39,40] Low levels of ROS provide energy for wound healing, while high levels of ROS cause cell damage.[20] In summary, striking a balance between the positive and negative effects of ROS and reducing the damage caused by oxidative stress are essential for wound healing.

Growing evidence suggests Raftlin is involved in the B-cell and T-cell immune responses.[22–25] In addition, lipid

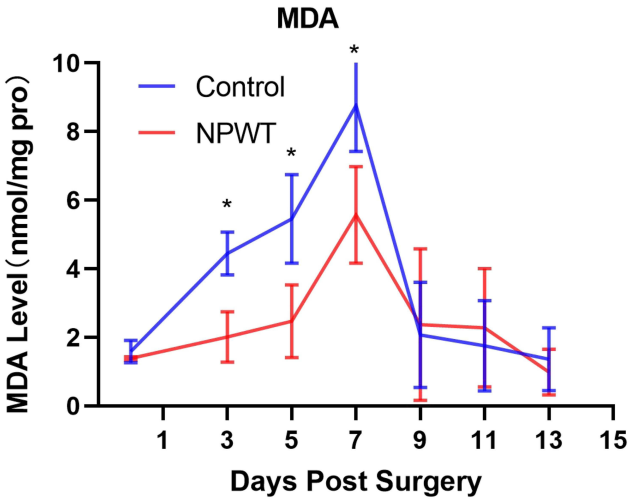

Figure 5 The level of MDA (as a measure of free oxygen radicals) of the control and NPWT-treated groups was tested on days 0, 3, 5, 7, 9, 11, and 13 post surgery. *$p < 0.05$.
Abbreviations: MDA, malondialdehyde; NPWT, negative-pressure wound therapy group; Control, control group.

raft proteins play a major role in the pathophysiology of vasculitis.[25–27] Recently, it has also been reported that Raftlin can participate in regulating angiogenesis.[28] Based on previous research, we speculated that NPWT may regulate immunity and inflammation, participating in angiogenesis by affecting the levels of Raftlin and oxidative stress.[25,32]

The levels of oxidative stress in wound tissues under negative pressure were examined in this study. The results showed that on days 3, 5, and 7 after surgery, the MDA levels of the NPWT group were significantly lower than those of the control group, which indicates less cellular damage caused by oxidative stress. This result is in accordance with the view that negative pressure appears to be an effective treatment for wound healing.[41] In addition, on days 3 and 5 after surgery, the levels of the antioxidant enzymes SOD and CAT in the NPWT group were significantly lower than those in the control group. This may be due to the low level of ROS in the wound treated by NPWT, and the body does not need to produce a large amount of antioxidant enzymes to cope with ROS. In summary, NPWT was associated with lower level of oxidative stress in wound tissues.

This study also established the levels of Raftlin in wound tissues under negative pressure, and this is not something that has been previously reported. The results indicate that the level of Raftlin in the NPWT group was significantly higher than that in the control group. This protein plays a role in the immune-regulation phase and can also promote angiogenesis, which means it might participate in both the inflammation and repair phases of the wound. This study also revealed that the protein is significantly increased during all stages of wound healing (days 3, 5, 7, 9, 11 and 13 post surgery) in the NPWT group. This leads us to infer a preliminary conclusion that NPWT may promote wound healing by regulating Raftlin, further regulating immunity and promoting angiogenesis. More experiments are needed to confirm this.

Compared with the control group, we found that the expression of CD31 in the NPWT group was significantly increased; this indicates that the NPWT group had higher angiogenesis. This may be related to the higher level of Raftlin in the NPWT group, and this result is in line with

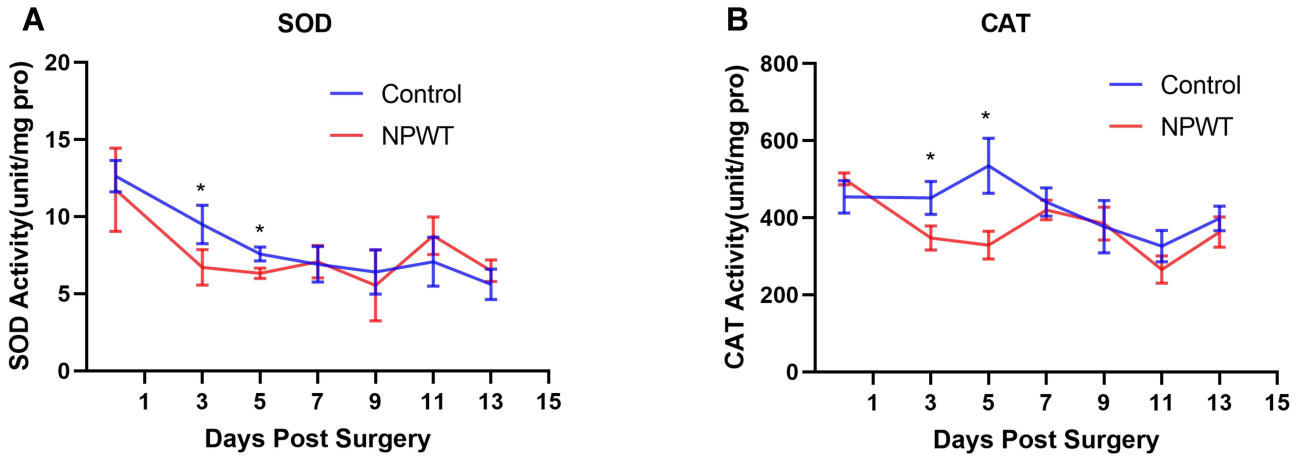

Figure 6 (**A**) SOD activity of the control and NPWT-treated groups was tested on days 0, 3, 5, 7, 9, 11, and 13 post surgery. (**B**) CAT activity of the control and NPWT-treated groups was tested on days 0, 3, 5, 7, 9, 11, and 13 post surgery. * $p < 0.05$.
Abbreviations: SOD, superoxide dismutase; CAT, catalase; NPWT, negative-pressure wound therapy group; Control, control group.

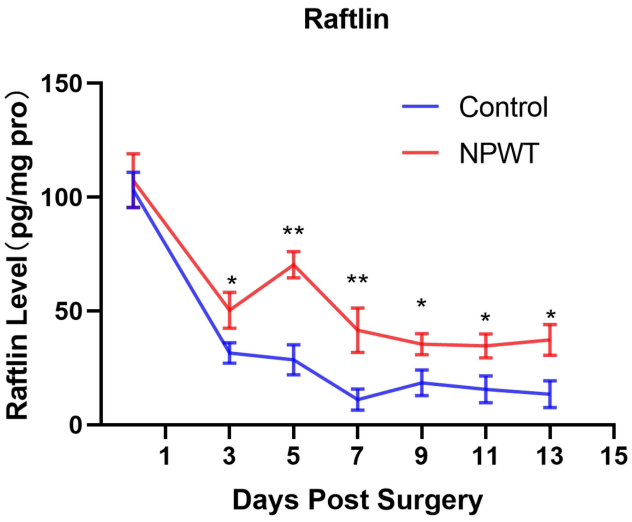

Figure 7 Raftlin levels of the control and NPWT-treated groups were tested on days 0, 3, 5, 7, 9.11, and 13 post surgery. * $p < 0.05$, ** $p < 0.01$.
Abbreviations: Raftlin, lipid raft protein; NPWT, negative-pressure wound therapy group; Control, control group.

our previous speculation. In addition, the expression level of CD68 in the NPWT group was significantly reduced, which indicates a lower inflammatory response. This may be due to the lower oxidative stress level in the NPWT group.[39,42]

Overall, our study indicated that NPWT may promote wound repair by affecting oxidative stress and Raftlin levels. However, considering the large differences between animals and humans, the approach used in this study cannot completely simulate human wound healing, especially the healing of complex clinical wounds. In addition, the relatively small sample size may restrict the validity of the experimental results. Therefore, expanding the sample size or conducting clinical research is necessary to clarify the concrete mechanisms of oxidative stress and Raftlin under negative pressure.

Conclusion

Our study showed that NPWT is associated with lower level of oxidative stress and higher levels of Raftlin. This provides a new direction for studying the mechanisms of NPWT in wound healing.

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Disclosure

The authors report no conflicts of interest in this work.

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