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Original Article

Negative pressure wound therapy accelerates rats diabetic wound by promoting agenesis

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Abstract: Negative Pressure Wound Therapy (NPWT) has become widely adopted to several wound treatment over the last 15 years, including diabetic foot ulcer (DFU). Much of the existing evidence supports that NPWT increase in blood flow, reduce in edema, decrease bacterial proliferation and accelerate granulation-tissue formation. However, the accurate mechanism is not clear till now. The aim of the present study was to further elucidate the effects of NPWT on angiogenesis of diabetic wound model. As result, our data showed: 1) NPWT promoted the wound healing and blood perfusion on both diabetic and normal wound compared with control, 2) The NPWT increased wound vessel density, and the wound treated with NPWT showed well developed and more functional vessels at day 7 post operation compared with control 3) NPWT up regulated the expression of VEGF at day 3 and Ang1 at day 7 on RNA and protein level. 4) Ang2 was up regulated in diabetic rats but NPWT attenuated this affection. Our data indicated that NPWT increased vessel density and promoted the maturation of neovascular over the potential mechanism of up regulated VEGF and Ang1 and down regulated of Ang2.

Keyword: Negative pressure wound therapy, diabetic wound, angiogenesis, wound healing

Introduction

The main concern with diabetic wounds is delayed healing caused by the peripheral arterial diseases and lack of blood circulation, especially in the extremities. Angiogenesis and functional vessels play critical role in the formation of granulation tissue and wound healing [1, 2]. NPWT is an option for management of diabetic foot ulcers and got good results [3, 4] as it promote the more rapid and robust granulation tissue response [5], and reduced risk for a second amputation of diabetic foot [6]. Other essential components of this therapy are the continuous evacuation of wound fluid which removed inhibitory angiogenic factors such as matrix metalloproteinase (MMPs), further alerts new vessel formation [7]. Despite the widespread use of NPWT, little is known about the molecular mechanisms, especially about of the mechanisms that regulate neovascularization in the treated wounds.

Among the variety of factors and pathways involved in the control of angiogenesis, vascular endothelial growth factor (VEGF) family members and the angiopoietins are considered to play the pivotal role. VEGF is essential for early blood vessel formation and angiogenesis. Mice deficient in even one allele for VEGF die in embryogenesis due to decreased in endothelial cell number and severe defects in blood vessel formation [8]. Angiopoietin-1 is essential for a later stage of blood vessel formation. Mice deficient for Ang1 die by embryonic day 12.5 due to defects in vessel remodeling and maturation [9].

With the present study, we aimed at identifying the functional and anatomical characteristics of newly formed vessels as well as to investigate angiogenic factors expression profiles under the effect of different treatment patterns of NPWT.

Materials and methods

Ethics statements

All animal experiments were carried out in strict accordance with the guidelines of the Administration of Animal Experiments for Medical

Research Purposes issued by the Ministry of Health of China. The protocol was approved by the Animal Experiment Administration Committee of Fourth Military Medical University. All surgical procedures were performed under sodium pentobarbital anesthesia and in a clean surgical room with sterilized instruments. All efforts were made to minimize the suffering of the mice during the experiments.

Animal

80 male SD rats (8 weeks old) were conducted in the study. The animals were obtained from the Animal Centre of Experimental Animals, the Fourth Military Medical University, China. Rats were housed with water ad libitum. After 24 h, 40 rats were randomized into 2 groups, the normal-NPWT group (n=20), and normal-control group (n=20). The other 40 rats were used to induce diabetic rat model by intra-peritoneal injection of streptozotocin (STA, 65 mg/kg in 50 mmol/l citrate buffer, pH 4.0; Sigma, USA). After 48 h, blood glucose levels were assessed, and monitored each week till 4 weeks; rats were included in the study if glucose concentrations were greater than 280 mg/dl in heparinized tail vein blood (measured by glucometer, Johnson & Johnson medical Devices Co., Ltd. China) [10]. Two weeks later, the diabetic rats were randomized into two groups, the diabetic-NPWT group (n=20) and diabetic-control group (n=20).

Full-thickness skin defect model [5]

The dorsum of rat was clipped and depilated 24 h before the experiment, anesthetized with 3% isoflurane 5 minutes before the surgery. After disinfecting the dorsum with alcohol patches, a piece of 2 cm×2 cm skin were removed to create a full-thickness wound. Rats in NPWT groups (normal, diabetic) were treated with medical foam and occlusive dressing. Connected foam to the RNPT-type negative pressure therapy instrument (Enoch Shanxi medical Technology Co., Ltd.) with a drainage tube, and applied continuous 120 mmHg negative pressure for 4 hours per day; Wound of rats in control groups (normal, diabetic) were covered with medical foam without suction. The animals were euthanized at the indicated times (post-surgery day 1, 3, 7, 14), and 1-1.5 mm of the tissue from wound edges were harvested and bisected, with half placed in 10% formalin and the other half snap-frozen in liquid nitrogen and stored at -80°C.

Wound perfusion analysis

The wound blood flow was detected by PeriScan PIM3 type laser Doppler imager (Perimed AB, Sweden) at the indicated time (post-surgery day 0, 1, 3, 7, 14). The distance from the probe to the wound is 14 cm, and the size of the scanning window is 100 mm×100 mm. Result of detection was record as the perfusion units (PU).

Histology analysis of vessels [11]

The formalin-fixed tissue were embedded in paraffin, cut into 5 μm sections, placed on to glass slides, and stained for routine haematoxylin and eosin (H&E) staining. Vasculature in the wounds was assessed by staining for CD34 (Santa Cruz, USA). The stained blood vessels on each slide were counted by investigators in the most vascular area of the tissue section (the hotspot) using a method described by Weidner et al [12, 13]. Briefly, this method involves scanning tissue sections under low magnification to identify the hotspot. Within the hotspot, the number of vessels in a high-power field of 200× over 10 non-overlapping areas was used as the micro vessel density (MVD) of that tissue. The vessels were counted by two investigators in a blinded fashion.

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from harvested wound tissue using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instruction. The RT reaction was incubated at 37°C for 15 min, 85°C for 5 seconds, inactivated at 99°C for 5 min and cooled at 58°C for 5 min. A total of 9 μl of complementary DNA (cDNA) from the RT reaction was added to 11 μl real-time quantitative polymerase chain reaction (qPCR) mixture containing 10 ml SYBR Green PCR Master (TaKaRa, Japan). PCRs were carried out in a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each sample was triplicated. The measurement was repeated four times to ensure reliability of results. The thermal profile for SYBR Green real-time RT-PCR was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 92°C for 12 s and 60°C for 60 s. A single amplification product was confirmed by running a melting curve for all PCRs. Primers were synthesized by Invitrogen Technologies (Shanghai, China). The primers

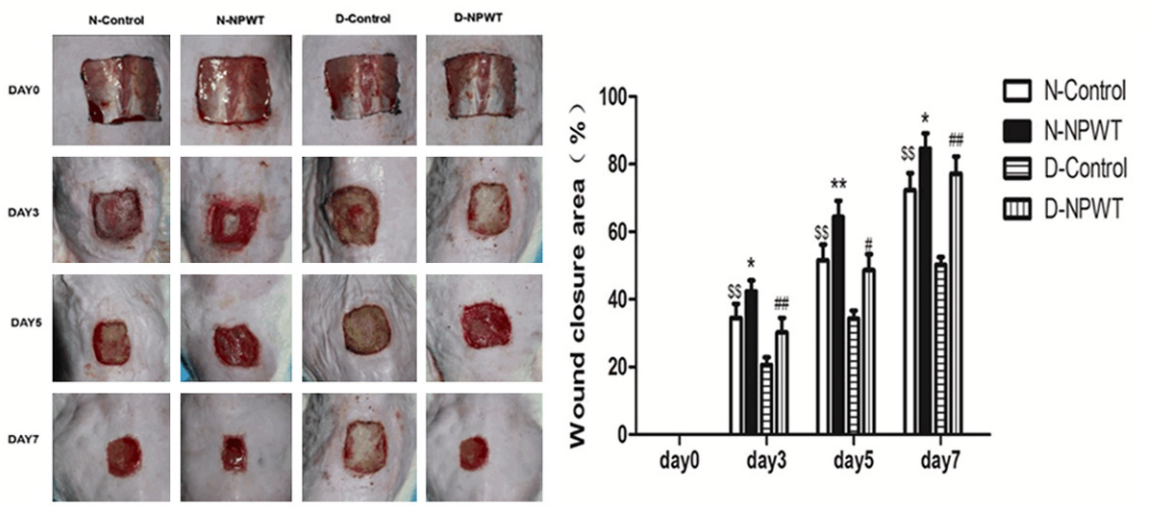

Figure 1. Wound closure in 4 groups: NPWT-treated wound showed significantly increased closing area at day 3, day 5 and day 7 post-surgery, and attenuate delayed would healing over Diabetic wound. *NPWT treated normal wound compared with control normal wound. #NPWT treated diabetic wound compared with control diabetic wound. $Normal wound control compared with diabetic control. "**"P<0.01, "*"P<0.05, "##"P<0.01, "#"P<0.05, "$$"P<0.01.

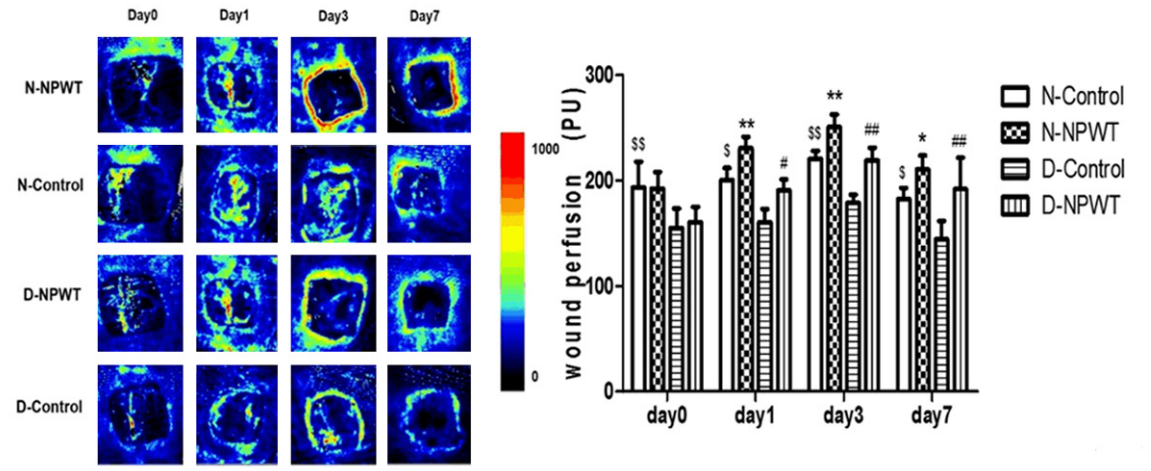

Figure 2. Wound perfusion in 4 groups measured by laser Doppler imager. NPWT-treated wound showed significantly increased wound perfusion at day 1, day 3 and day 7 post-surgery on both control and diabetic wound. *NPWT treated normal wound compared with control normal wound. #NPWT treated diabetic wound compared with control diabetic wound. $Normal wound control compared with normal diabetic control. "**"P<0.01, "*"P<0.05, "##"P<0.01, "#"P<0.05, "$$"P<0.01, "$"P<0.05.

sequence were described as follows: GAPDH, left primer 5'-GGCACAGTCAAGGCTGAGAATG-3, right primer 5-CCAG GAGTGCAAATGGATGAAG-3; Fit-1, left primer 5-CCAGGAGTGCA AATGGATGAAG-3, right primer 5-CCAGGAGTGCAAATGGATGAA G-3; VEGF, left primer 5-GCACGTTGGCTCACTTCCAG-3, right primer 5-TG GTCGGAACCAGAATCTTTATCTC-3; Ang-1, left primer 5-ACCGTGAGGATGGAAGCCTAGA-3, right primer 5-AATG AACT CGTTCCCAAGCCAATA-3; Ang-2, left primer 5-CTTCAAGTCAGGA CTCACCACCA-3, right primer 5-RCCACCGTCACAG-3; Tie-2, left primer 5-TTGGATTGTCACGAGGTCAAGAA-3, right primer 5-ATGT CATGCCGCAGTATGGAG-3.

Western blot

The frozen wound tissues were grinded in liquid nitrogen, then lysed with a RIPA lysis buffer (50 mM Tris-Cl [pH 7.6], 150 mM NaCl, 1% NP-40,

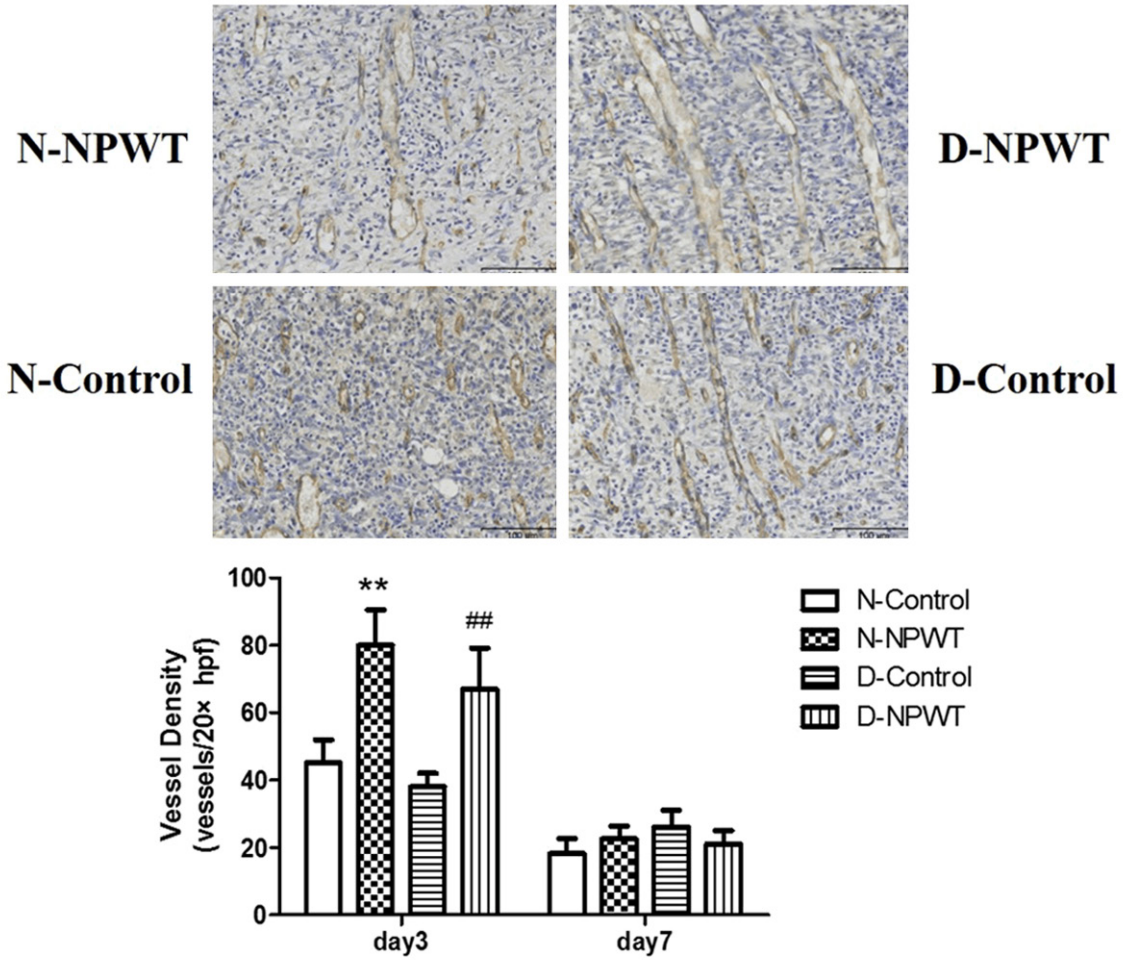

Figure 3. Microvessel density in wounds showed a statistically significant higher in NPWT group wounds as compared to control group wounds on day 3. This difference was lost on day 7. However, NPWT-treated wounds had well-developed and more functional looking vessels by CD34 staining (top, magnification 200×). *NPWT treated normal wound compared with control normal wound. #NPWT treated diabetic wound compared with control diabetic wound. "**"P<0.01, "##"P<0.01.

0.1% SDS, 0.5% deoxycholic acid, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride) and were centrifuged at 12,000 g at 4°C for 30 min to obtain proteins in the supernatant. Equal amounts of proteins from each sample were resolved by 6%-15% SDS-PAGE, transferred to NC membranes, blocked with 5% milk for 1 h at room temperature, and probed with the following primary antibodies at 4°C overnight: VEGF, Fit-1, Ang-1, Ang-2, β-actin (Santa Cruz Biotechnology), the blots were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) at 1:1,000-1:5,000. Immunoreactive bands were visualized using enhanced chemiluminescence, and densitometry was performed using Quantity One software (Bio-Rad Laboratories, Hercules, California, USA).

Statistical analysis

Data were presented as mean ± standard error. Statistical significance was assessed by one-way analysis of variance (ANOVA) and differences between groups were considered statistically significant at $P<0.05$ or $P<0.01$.

Result

NPWT accelerated wound healing of both normal and diabetic wounds

The wound area treated with or without NPWT were measured at day 0, day 3, day 5 and day 7 to calculate the wound healing speed. As result, NPWT accelerated the wound healing of both normal and diabetic wound significantly com-

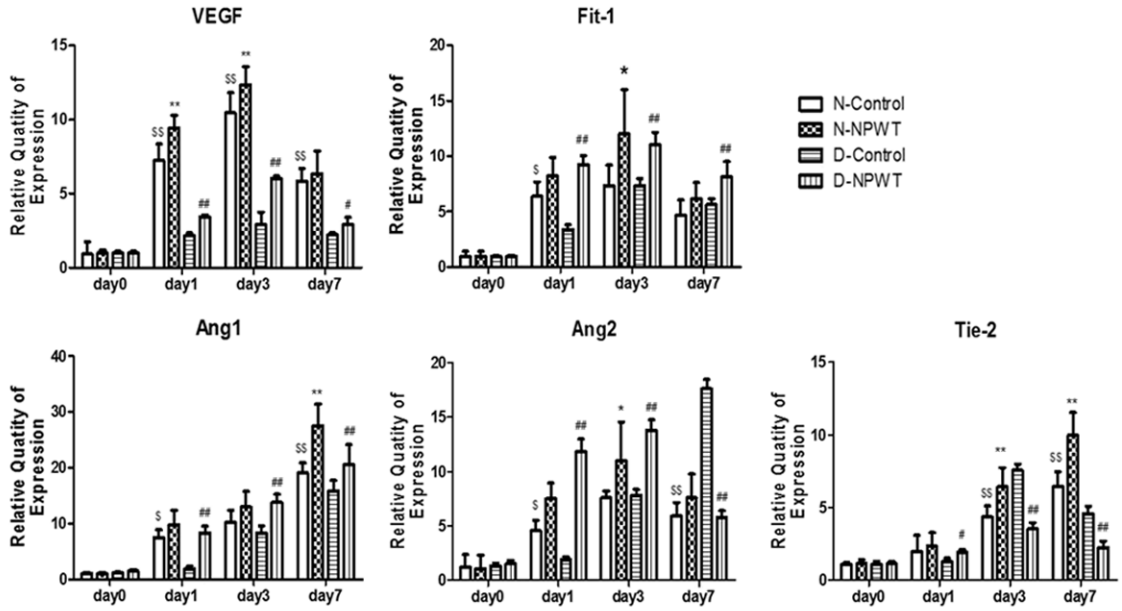

Figure 4. Relative expression mRNA of regulating factors and receptors by RT-PCR in 4 groups. *NPWT treated normal wound compared with control normal wound. #NPWT treated diabetic wound compared with control diabetic wound. $Normal wound control compared with diabetic control. "**"P<0.01, "*"P<0.05, "##"P<0.01, "#"P<0.05, "$$"P<0.01, "$"P<0.05.

pared with control group. The wound treated with NPWT became more hyperemic with a red granulation tissue, while the control diabetic wound was covered with much necrotic tissue (**Figure 1**).

NPWT promoted wound blood perfusion

As previously noted, diabetes impaired the blood perfusion and angiogenesis in the wound healing cascade. We measured wound perfusion by laser Doppler imager, as result, NPWT-treated wound showed significantly increased wound perfusion at all the time points (post-surgery day 1, 3, 7) compared to the control group, especially at day 3 and day 7. The wound perfusion of diabetic rats was significantly decreased at the indicated time (post-surgery day 0, 1, 3, 7) compared to the normal rats, certified that blood supply is one of critical point for delayed wound healing of diabetic wound, and NPWT attenuated this process (**Figure 2**).

NPWT improved the angiogenesis of the diabetic wounds

As result of CD34-staining, both normal and diabetic wound treated NPWT showed a significantly higher microvessel density at day 3, which was lost by day 7. However, the NPWT-treated wound had well-developed, larger-caliber vessels compared to the control. Diabetic wound showed impaired vascularization by histological analysis compared with normal rats wound (**Figure 3**).

RT-PCR assay

Assayed the regulating factors of angiogenesis in the wound healing cascade. VEGF and Fit-1, the receptor of VEGF, were significantly up-regulated in the both normal and diabetic wound treated with NPWT at day 3. Ang1 mRNA expression was increased on the day 3 and day 7 compared to the control group, Angiopoietin-2, the natural antagonist of Ang-1, mRNA expression was significantly decreased than the control group (**Figure 4**).

Western blot assay

To further check the level of regulating factors on protein, we harvested wound tissue and did western blot assay. As result, the expression of VEGF, Fit-1, Ang1, Angiopoietin-2 were consistent with PCR (**Figure 5**).

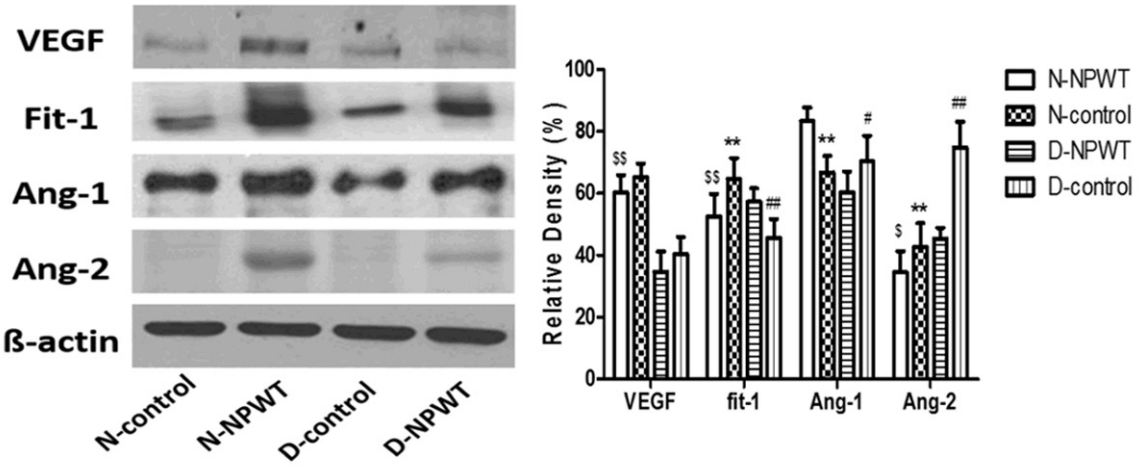

Figure 5. Relative expression of regulating factors and receptors by western blot in 4 groups at day 3 post-surgery. *NPWT treated normal wound compared with control normal wound. #NPWT treated diabetic wound compared with control diabetic wound. $Normal wound control compared with diabetic control. "**"P<0.01, "*"P<0.05, "##"P<0.01, "#"P<0.05, "$$"P<0.01, "$"P<0.05.

Discussion

The use of negative pressure was proposed by Argenta et al and Morykwas et al [14, 15] firstly in 1997, and then was widely used. This consisted of developing a mechanical system to help the healing process. Negative pressure is created by a machine that is connected by a plastic tube to a sponge placed over the wound bed. The pressure is adjusted to between -50 and -125 mmHg continuously or intermittently [16]. The wound bed should be completely covered by the sponge, thus creating an environment under vacuum when the machine is switched on. Although research suggests that NPWT shown to positively affect angiogenesis, a detailed analysis of the mechanisms controlling neovascularization and molecular changes in the expression of angiogenic factors is not clear till now.

We established the full-thickness skin defect wound of normal rats, measured the wound perfusion at the different time point during the whole wound healing process. The NPWT treated wound showed higher wound perfusion and historically, had higher vessel density at day 3 and well developed vessels at day 7. These result confirmed that NPWT has the positive effect on the angiogenesis functionally and anatomically. Angiogenesis, including endothelial proliferation, migration and capillary tube-like formation, is regulated by many angiogenic factors. Assessment of angiogenic factors expression showed that the expression of VEGF, Fit-1and Ang2 were all up-regulated in the NPWT treated wound at day 3. The expression of Ang1 was upregulated at day 7. According to the previous report, high expression of VEGF and Ang2 can promote the neovascular formation, and high expression of Ang1 will be helpful for the neovascular maturation and stability [17].

At the flowing study, we induced the diabetic rats and created the full thickness skin defect wound on the back as the chronic wound model. STZ-induced diabetic rat is a commonly used rat model of diabetes [18]. STZ destroys islet β-cells, to reduce the synthesis and secretion of insulin, and glucose metabolism is disordered so as to cause the occurrence of diabetes [19]. In our study, the diabetic wound showed the decreased wound perfusion during the whole wound healing process compared to the normal wound. This result indicated diabetes impaired the angiogenesis of the wound.

At the further study, we investigated the effect of NPWT on the angiogenesis of diabetic wounds. The result showed that the NPWT treated wound have the higher vessel density at day 3 and more functional looking vessels at day 7. Wound perfusion was measured by laser Doppler imager at different time points, the result showed that NPWT increased wound perfusion of diabetic rats at all-time points. These

finding, confirmed that NPWT is beneficial for angiogenesis of diabetic wound. Real-time PCR demonstrated that up regulated mRNA levels of Ang1 and down regulated expression of Ang2 in NPWT treated wound at day 7. Ang1 stabilizes the endothelium and promotes per-endothelial cell recruitment to the newly formed vessels. Ang-2 is a natural antagonist of Ang-1, inhibiting Ang-1 induced Tie-2 phosphorylation and thereby promoting vascular remodeling [20-22]. VEGF is the most important growth factor in angiogenesis. It promotes endothelial cells' proliferation and migration. In our study, we found that NPWT increased the mRNA expression of VEGF in diabetic rats wound.

Conclusion

NPWT not only increased vessel density, but also promoted the maturation of neovascular. The potential mechanism maybe related to up regulation of VEGF and Ang1, and down regulation of Ang2.

Acknowledgements

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Disclosure of conflict of interest

None.

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