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Negative pressure wound therapy promoted wound healing by suppressing inflammation via down-regulating MAPK-JNK signaling pathway in diabetic foot patients

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ABSTRACT

Aims: Negative pressure wound therapy displayed significant clinical benefits in the healing of diabetic foot wounds. In the present study, we investigated the mechanism of regulation of MAPK-JNK (Mitogen-activated protein kinase- c-Jun N-terminal kinase) signaling pathway by negative pressure wound therapy on these wounds.

Methods: Twenty-six type 2 diabetes patients with foot ulceration were randomly assigned to the two groups, thirteen treated with negative pressure wound therapy and the others treated with traditional debridement therapy. Skin samples were harvested and histologically and immunohistochemical analyzed in both groups. Immunofluorescence stain, Enzyme-linked immunosorbent assay and Western blotting were performed for inducible nitric oxide synthase, inter leukin-6, tumor necrosis factor-α, P-c-Jun N-terminal kinase and c-Jun N-terminal kinase. Real time-polymerase chain reaction was performed to evaluate expression of c-Jun N-terminal kinase, extracellular signal regulated kinase1/2 and p38.

Results: Negative pressure wound therapy could effectively alleviate inflammatory reaction and reduce inter leukin-6 and inducible nitric oxide synthase production after 7 days treatment. The level of tumor necrosis factor-α, inter leukin-6 and P-c-Jun N-terminal kinase were significantly decreased. However, there was no statistical difference in messenger ribonucleic acid expression of p38, extracellular signal regulated kinase1 and 2.

Conclusions: Negative pressure wound therapy possibly suppress the wound inflammation by inhibiting inter leukin-6, tumor necrosis factor-α and inducible nitric oxide synthase in diabetic foot patients. This effect is maybe mediated at least in part by suppression of Mitogen-activated protein kinase- c-Jun N-terminal kinase signaling pathway.

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1. Introduction

Inflammation is a host protective response to tissue injury and infection. Upon recognition of a pathogen, resident macrophages initiate an early inflammation response that recruits immune system including neutrophils and monocytes to produce inflammatory cytokines, such as interleukin-β (IL-β) and tumor necrosis factor-α (TNF-α) [1,2]. This overwhelming "cytokine storm" can result in deregulation of tissue matrix metalloproteinases (MMPs), and limit the formation and maturation of granulation tissue [3]. Meanwhile, the inflammatory response termed ischemia / reperfusion injury can lead to tissue damage during wound healing in type 2 diabetes patients, the main pathological mechanisms of this injury involve the pathological leucocyte-endothelium interaction and production of the reactive oxygen species (ROS) which can enhance the tissue damage [4]. Thus, the normal wound healing process can be delayed by the prolonged inflammation [5].

Negative pressure wound therapy (NPWT) uses negative pressure to evacuate infected fluid from wound through a tube and dressing which is connected to a vacuum pump to keep a sealed environment [6,7]. A great many randomized controlled studies had reported that NPWT not only was a safe and effective treatment, but also leads to a lower amputation rate, higher proportion of healed wound, and faster healing rate than standard care in type 2 diabetes patients [8–10]. The underlying reason was that NPWT could attenuate the inflammatory reaction in the wound [11,12]. However, the precise physiological mechanism that NPWT suppresses inflammation is still not clear. Meanwhile, MAPK family of serine kinases was an important component of the signaling pathways that regulated varieties of intracellular processes [13]. MAPK activation also played a pivotal role in the synthesis of pro-inflammatory cytokines, especially, JNK signaling pathway in regulating responses to inflammation [14]. At present, there is no related report about the relationship between anti-inflammatory effect of NPWT and JNK MAPK signaling pathway. Therefore, we assessed the possible molecular mechanism of the NPWT's regulation on JNK MAPK pathway and responding inflammatory effect.

2. Research design and methods

2.1. Study population

In this study, twenty-six type 2 diabetes patients with foot ulcerations from inpatient were enrolled in the clinical trial from July 2017 to April 2018. Inclusion criteria for the study were: the patient age $\geq$ 18 years diagnosed with type 2 diabetes mellitus, and ulcer categorized Wagner grade 2–4 and mild moderate ischemia (ABI > 0.6). We excluded patients with wounds resulting from venous insufficiency, untreated cellulitis or untreated osteomyelitis. And also excluded patients who received treatment of growth factors, immunosuppressive drugs, chemotherapy and percutaneous transluminal angiography (PTA) therapy within 30 days. Patients were also excluded if they refusal to participate or missing samples who quit therapy for their poor medicare. The patients were randomly assigned to NPWT group (After debridement) and traditional debridement (control group). The experimental group was composed of 13 patients who treated with NPWT after debridement, while thirteen patients in the control group received gauze dressings change twice a day after debridement. After the procedure, all patients diagnosed bacterial infection for the high level of CRP and inflamed hot pain symptom. Meanwhile, they were received hypoglycemic treatment (insulin regimen using rapid-acting insulin three times a day and glargine once a day). The antibiotic therapy was conducted according to the result from wound tissue of bacterial culture and susceptibility test. And second and third-generation cephalosporin were selected on the solid basis of differential leukocyte count and classification, and gauze dressings were changed twice a day in the control group. All patients received systematic treatment according to standardized treatment guidelines. This study was approved by the Ethics Committee of Qingpu Branch of Zhongshan Hospital Affiliated to Fudan University, and informed consent was obtained from all patients.

2.2. Procedures

Patients in NPWT-group were received negative pressure wound therapy system (VSD Medical Science and Technology Co. Ltd, Wuhan, China) after debridement. This therapy system uses medical-grade polyurethane foam, with adhesive drapes for sealing and connector tubing that were connected to a wall-mounted suction device (negative pressure of −125 mmHg) 13 to create a closed microenvironment. Samples (10 mm in diameter and 5 mm in depth) were taken at the center of wound beds during debridement and dressing change (post-surgery after one week) in both groups. One sample for each patient was divided into the two parts, one half sample was stored in 10% neutral buffered formalin at 4 °C for histological analysis and immunofluorescence analysis for iNOS, while the other half was stored in refrigerator at −80 °C for IL-6, TNF-α, JNK, ERK1/2 and p38 expression.

2.3. Histopathological analysis

The tissue samples were fixed in 10% buffered formalin and embedded in paraffin, and standard 3 μm sections were cut and stained with hematoxylin & eosin (Boster Biological Technology, Wuhan, China) [15]. Slides were examined under a normal light microscope (Nikon YS100). The severity of the inflammatory response was determined by two independent observers who had been evaluated for the presence or absence of inflammatory cells, Image J software was used to quantify the neutrophil cells, these features were recorded for intensity: mild (absence of inflammatory cells); moderate: (a small amount of inflammatory cells); serious: (a large amount of inflammatory cells).

2.4. Immunohistochemical analysis for IL-6

The tissue samples were fixed in 10% buffered formalin and embedded in paraffin, and standard 3 μm sections were cut and stained with immunohistochemical staining for IL-6 as described by MetaMorph software (MDS analytical technologies molecular devices, USA) [16]. The primary and secondary antibodies were obtained from Sigma (St. Louis, MO). The stained sections were randomly observed with a microscope (Olympus, Japan). And results of immunohistochemical staining were recorded as an average positive area (yellowish-brown color area) by a medical image analysis system (Medical 5.0 Digital Medical Image Analysis System).

2.5. Immunofluorescence analysis for iNOS

The samples were blocked with normal serum at 37 °C for 30mins. Subsequently, these samples were incubated in anti-iNOS antibody (Novus Biologicals, Colorado) for one hour at room temperature and then overnight. Secondary antibodies (goat-anti rabbit antibody. Life Technologies, California) were applied in the dark for 1 h. Finally, fluorescence was visualized by a fluorescence microscope (Carl Zeiss, Jena, Germany).

2.6. Enzyme-linked immunosorbent assay (ELISA) assay for IL-6 and TNF-α

The level of IL-6 and TNF-α in diabetic foot wound were determined with ELISA kits (R&D, Minneapolis, MN, USA) according to the manufacturer's directions.

2.7. Western blotting for JNK MAPK expression

The level of JNK MAPK in wound tissue was assayed by Western blotting [17]. Equal amounts of JNK from each sample were resolved by 8% SDS-PAGE and transformed to nitrocellulose (NC) membranes (Amersham, Little Chalfont, UK). Blocked with 5% nonfat milk for 1 h at room temperature, and probed with the primary antibody (mouse antibody, 1:1000 dilution, Cell Signaling Technology, Inc.) at 4 °C overnight. After washing, the membranes were subsequently incubated with secondary antibody (goat anti-rabbit antibody, 1:1000 dilution, Beyotime Institute of Biotechnology) at room temperature for 1 h. The membrane-bound antibody was visualized by Phototope-HRP western detection kit (Cell Signaling Technologies Inc., Danvers, MA). Densitometry was performed using Semi-quantitative analysis software (FluorChem E, ProteinSimple, CA, USA).

2.8. Real-time PCR for JNK, ERK1/2 and p38 expression

Total RNA was extracted from wound skin tissue using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and quantified using ultraviolet absorption method according to the manufacture's instruction. First-stand complementary DNA (cDNA) was added to real-time quantitative polymerase chain reaction (qPCR, Reverse Transcription System, promega Cat, A3500) mixture containing a SYBR Green PCR Master (TaKaRa, Otsu, Japan). PCRs were carried out in a StepOnePlus Sequence Detection System (Biosystems, Foster City, CA, USA) to examine the mRNA expression of JNK, ERK1/2 and p38 [18]. The relative amounts of JNK, ERK1/2 and p38 were normalized to the amount of GAPDH and calculated by $2^{-\triangle\triangle}$Ct methods. The primers sequences were described in Table 1.

Table 1 – Primers used in the Real-time PCR assay.

Name		Sequence (5′ to 3′)
JNK	F	AATGTTGCCTCTCCGTATCC
	R	GCTTCAATGCCCTTTACCTC
p-38	F	CTACAGAGAACTGCGGTTAC
	R	AGATGGGTCACCAGATACAC
ERK1	F	CTGGTCTGTGGGCTGCATTC
	R	CAGCAGGTCAAGGGCTTTGG
ERK2	F	CTATTCTTGCCCTGAAATAC
	R	GAAGAGGACTCCAAATTAAC
GAPDH	F	CACCCACTCCTCCACCTTTG
	R	CCACCACCCTGTTGCTGTAG

2.9. Statistical analysis

The continuous demographic dates are expressed as the mean ± standard error of the mean. Statistically significant differences between groups were determined using *t*-test using SPSS 14.0 software. Categorical demographic variables compared with Chi-square test. The expression of IL-6, TNF-α, JNK, p38 and ERK1/2 were compared with paired or unpaired student *t* test. p-value < 0.05 was considered significant.

3. Results

3.1. Patient characteristics

In this study, thirteen patients in NPWT group were received Vacuum Assisted Closure therapy, while the others received traditional debridement (Surgical removal of dead tissue and pus from the wound by scalpel, surgical scissors and so on) in the control group. The demographics and laboratory results of diabetic foot patients in the two groups were shown in Table 2. And there were no statistically significant differences in the demographics and laboratory results between the two treatment groups.

3.2. Inflammation analysis of diabetic foot wounds

As shown in Fig. 1, after 7 days treatment, the wounds treated with NPWT were well healing with densely cell deposited matrix with the mild and moderate inflammation; while the inflammation in control group was still serious (Fig. 1A and B). Quantitative analysis documented a significantly smaller number of neutrophil in NPWT-group, when compared to the control-group (126.3 ± 26.60 *vs* 191.2 ± 20.10, P = 0.001) (Fig. 1C). Qualitative inspection of immunohistochemical result showed that the level of IL-6 was decreased (711.1

Table 2 – Demographics and laboratory parameters of diabetic foot patients.

	Control group	NPWT group	X^2 or t value	P value
Patients characteristics	(n = 13)	(n = 13)		
Age (years)	55.6 ± 8.6	56.0 ± 12.8	0.082	0.935
Sex (male/female)	8/5	9/4	0.170	0.680
Fast blood glucose (mM)	11.4 ± 3.5	11.3 ± 3.3	0.046	0.964
Current smoker (n/%)	6/46.2	5/38.5	0.158	0.691
Current use alcohol (n/%)	5/38.5	4/30.8	0.170	0.680
Diabetes duration (years)	14.7 ± 7.1	11.4 ± 7.0	1.053	0.306
Hypertension (n/%)	7/53.8	8/61.5	0.158	0.691
Congestive heart failure (n/%)	3/23.1	3/23.1	0.000	1.000
Albumin (g/L)	29.7 ± 5.0	31.5 ± 6.3	0.704	0.491
Creatinine (mmol/L)	60.9 ± 18.0	64.0 ± 18.5	0.379	0.709
ALT (U/L)	31.1 ± 9.3	35.4 ± 8.3	1.087	0.292
AST (U/L)	27.9 ± 9.6	28.4 ± 10.2	0.113	0.911
CRP (mg/L)	67.3 ± 39.4	63.1 ± 31.7	2.263	0.796
HbA1c (mmol/mol)	113.1 ± 42.9	103.7 ± 35.9	0.532	0.601
Lesion features				
Ulcer duration before treatment (days)	43.0 ± 25.9	36.7 ± 29.4	0.508	0.618
Ischemic/neuroischemic/neuropathic	3/6/4	4/6/3	0.286	0.867
Toe/dorsal/plantar/others	4/3/4/2	3/2/5/3	0.654	0.884
Baseline wound area (cm^2)	17.3 ± 7.8	16.9 ± 9.7	0.101	0.920
Infected/uninfected	10/3	11/2	0.248	0.619
Wagner grade < 3 (n/%)	5/38.5	5/38.5	0.000	1.000
Wagner grade ≥ 3 (n/%)	8/61.5	8/61.5	0.000	1.000
Ankle-brachial index	0.60 ± 0.13	0.61 ± 0.15	1.194	0.848
Loss of protective sensation (n/%)	11/84.6	12/92.3	0.000	1.000

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CRP: c-reactive protein.

± 86.60 *vs* 538.3 ± 94.27, P = 0.000) in the wound tissue of NPWT-treated patients after 7 days treatment (Fig. 2A and B).

3.3. *Immunofluorescence assay for expression of iNOS in wound granulates*

The immunofluorescence analysis revealed that the expression of iNOS was decreased, and they were mainly expressed in the cytoplasm after the treatment of NPWT. DAPI (4, 6-diamino-2-phenylindole) counterstaining helped to identify non-damaged nuclei. These findings indicated that NPWT inhibits iNOS activation after 7 days treatment (Fig. 3).

3.4. *ELISA assay for levels of TNF-α and IL-6 in wound granulates*

ELISA kits were used to measure the level of TNF-α and IL-6 in the lysate of newly granulation tissue. As shown in Fig. 4a and b, after 7 days treatment, the expressions of TNF-α and IL-6 in NPWT-group were significantly decreased (239.20 ± 24.91 ng/L *vs* 215.00 ± 22.41 ng/L, *P* = 0.035 and 4.10 ± 0.62 ng/L *vs* 3.33 ± 0.63 ng/L, *P* = 0.013). Compared with control group, the concentration of TNF-α and IL-6 in NPWT-group had no statistical significance (215.00 ± 22.41 ng/L *vs* 223.90 ± 25.38 ng/L, *P* = 0.417 and 3.33 ± 0.63 ng/L *vs* 3.78 ± 0.53 ng/L, *P* = 0.101).

3.5. *JNK expression in wound skin tissue by Western blot*

The result of Western blotting showed that there is no statistically significant difference in P-JNK expression between the two groups before treatment. After 7 days treatment, level of P-JNK was significantly down-regulated (0.886 ± 0.449 *vs* 0.504 ± 0.236, *P* = 0.029), however, without statistical difference compared with control group (0.735 ± 0.339 *vs* 0.504 ± 0.236, *P* = 0.094) (Fig. 5). Also, the ratio of P-JNK/JNK in NPWT-group was significantly down-regulated after 7 days treatment (0.878 ± 0.146 *vs* 0.601 ± 0.258, *P* = 0.008) (Fig. 5e).

3.6. *The mRNA level of JNK, ERK1/2 and p38 by Real-time PCR*

As shown in Fig. 5f, after 7 days treatment, the mRNA level of JNK was significantly down-regulated (0.293 ± 0.1121 *vs* 0.114 ± 0.0593, *P* = 0.000), and decreased compared with control group (0.114 ± 0.0593 *vs* 0.196 ± 0.0991, P = 0.037); However, there was no statistical difference in expression of p38 (0.007 ± 0.0027 *vs* 0.008 ± 0.0035, *P* = 0.282) (Fig. 4f), ERK1 (0.020 ± 0.0178 *vs* 0.025 ± 0.0121, *P* = 0.495) (Fig. 3c) and ERK2 (0.006 ± 0.0048 *vs* 0.005 ± 0.0040, *P* = 0.528) (Fig. 4d) after 7 days treatment. Also, there was no significant difference in ratio of ERK1/ERK2 in NPWT group after treatment (13.793 ± 24.6312 *vs* 12.765 ± 15.4687, *P* = 0.912). (Fig. 4e).

4. Discussion

In the early phase of wound healing, the elevated impermanent inflammatory response could accumulate neutrophils which could accelerate the clearance of bacterial and tissue necrosis [11]. In diabetic patients, the normal continuum of wound healing is disrupted, the wound enters a chronic and non-healing state featured by sustaining inflammation [5]. However, the long-term excessive of inflammatory cells could

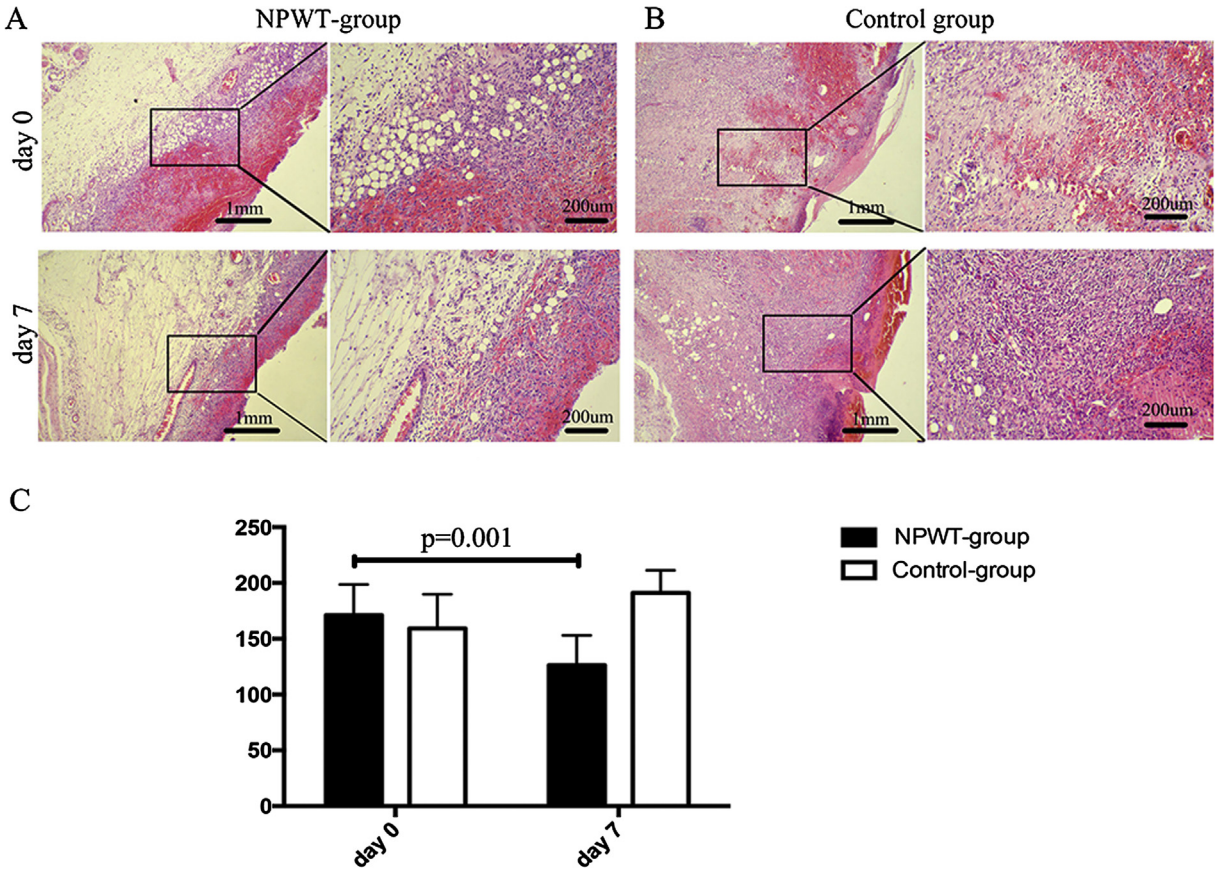

Fig. 1 – Examples of H&E stained the diabetic foot wound in the two group. (A): the wounds treated with NPWT were well-healing with densely cell deposited matrix with the mild and moderate inflammation after 7 days (left: ×40; right: ×100); (B): While the inflammation in Control-group was still serious (left: ×40; right: ×100). (C): Quantitative analyses of neutrophil cells were evaluated through image J in the biopsy sections. NPWT: negative pressure wound therapy.

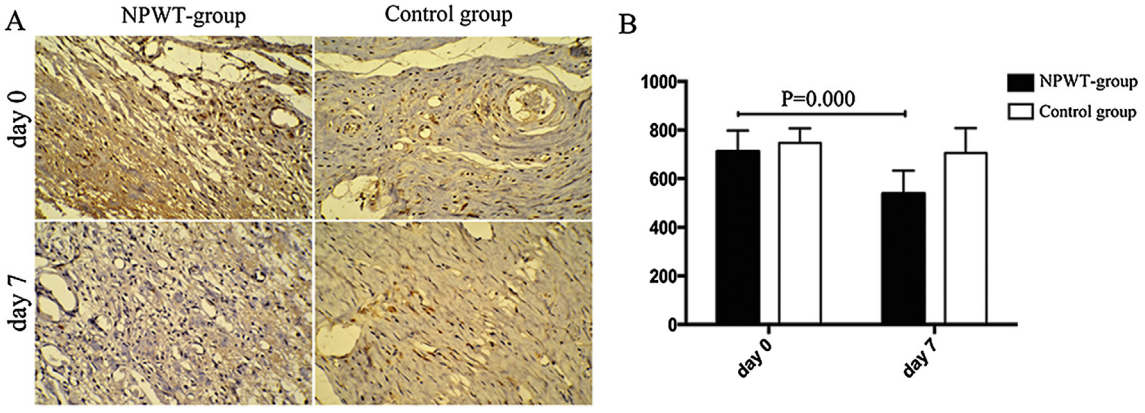

Fig. 2 – Examples of immunohistochemical stained the diabetic foot wound in the two group. (A): Wound sections in the both groups were immunostained for IL-6 on day 0 and 7, the yellowish-brown color denotes positive area (×200); (B): Quantitative analyses of IL-6 expression was evaluated through measuring positive areas in the biopsy sections. NPWT: negative pressure wound therapy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cause sustaining generation of pro-inflammatory cytokines which deregulated tissue matrix metalloproteinases (MMPs), and obviously restrict the formation and maturation of granulation tissue [3]. Also it would affect formation of granulation tissue if the inflammation has been extremely suppressed. Thus, optimization of this process is therefore

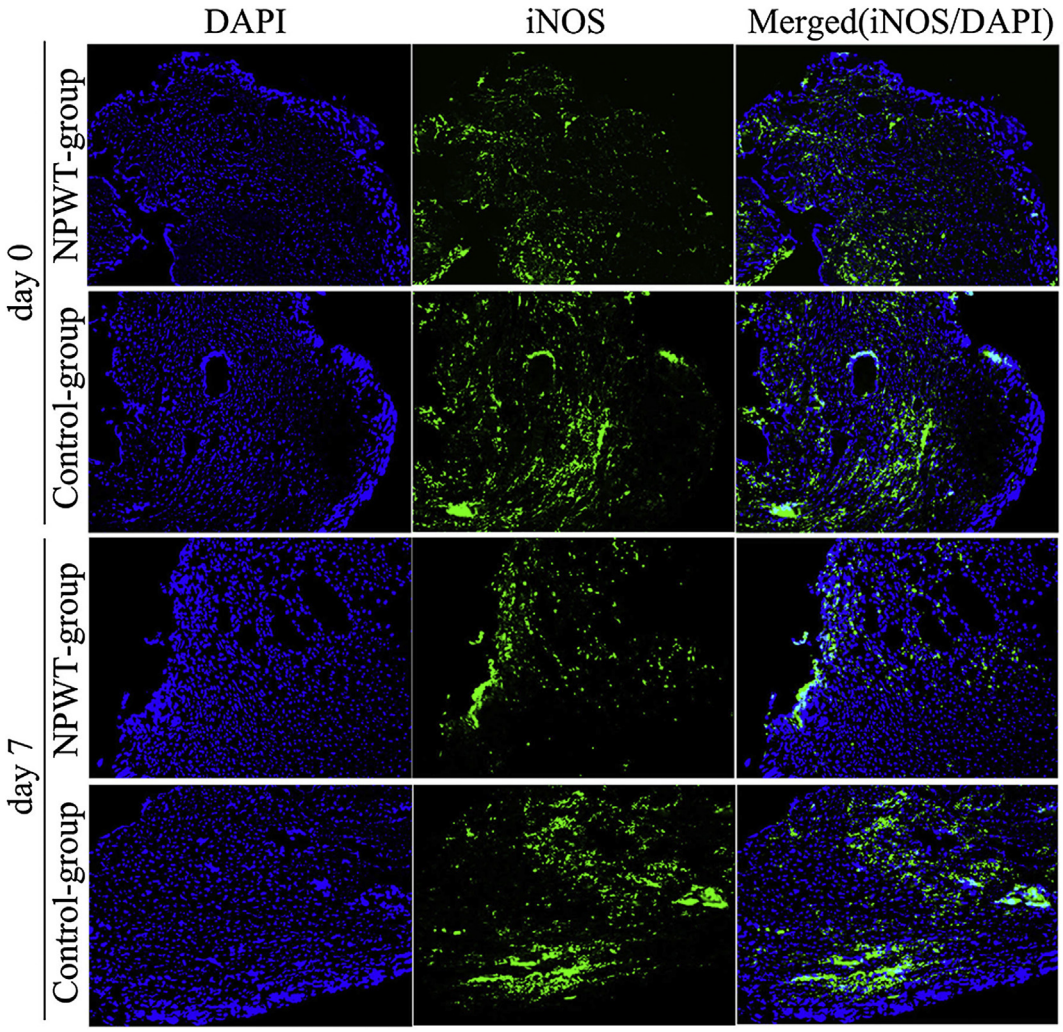

Fig. 3 – Immunofluorescence results of iNOS after NPWT treatment (magnification ×100). The nuclei of the corresponding cells were visualized in blue by DAPI nuclear stain. iNOS positive labeled cells appear green. The right panels show the merged image of the blue and green channels. NPWT: negative pressure wound therapy; iNOS: inducible nitric oxide synthase; DAPI: 4, 6-diamino-2-phenylindole. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

important, the suitably decrease of pro-inflammatory cytokines is likely useful for reducing the inflammatory states of diabetic foot wound.

The focus of the current study was the possible molecular mechanism of anti-inflammatory effect of NPWT. And we at first time provided evidence that NPWT's anti-inflammatory effect associated with the down-regulation of JNK signaling, which inhibited pro-inflammatory enzymes and cytokines during diabetic foot healing. Moreover, the clinical data showed that diabetic foot patients were more susceptible to the hyper-inflammation, which was associated to the elevated levels of TNF-α and IL-6 [19]. In the present study, TNF-α and IL-6 were regarded as representative pro-inflammatory cytokines, and high levels of pro-inflammatory cytokines amplified and prolonged inflammatory reaction [20].

Similarly, in the present study, ELISA analysis revealed that NPWT displayed a same tendency with a decrease in IL-6 and TNF-α levels after 7 days treatment. The decrease of IL-6 was as accurate as could be obtained by the immunohistochemistry analysis. The similar result was observed in Liu et al's experiment. They created a full-thickness wound on the back of rabbit, and found that NPWT could significantly decrease the level of IL-6 and TNF-α 4 days after surgery [11]. Chan et al considered that the probably reasons of NPWT contributed to successful wound healing were that the reduction of TNF-α in wound tissue could improve the angiogenesis [21].

NO (nitric oxide) which is a reactive produced from the guanidine nitrogen of l-arginine by NOS is crucial for immune responses to pathogenic disease. The overexpression of NO could result in the chronic inflammatory response process [22]. On the other hand, the down-regulating NO level may be an effective therapy to the diabetic foot wound healing [23]. In our study, we found that iNOS level in wound bed was diminished after NPWT treatment (Fig. 3). Different from Hitomi et al study, they created wounds on the mouse ear, and then applied NPWT to the wounds. They observed that

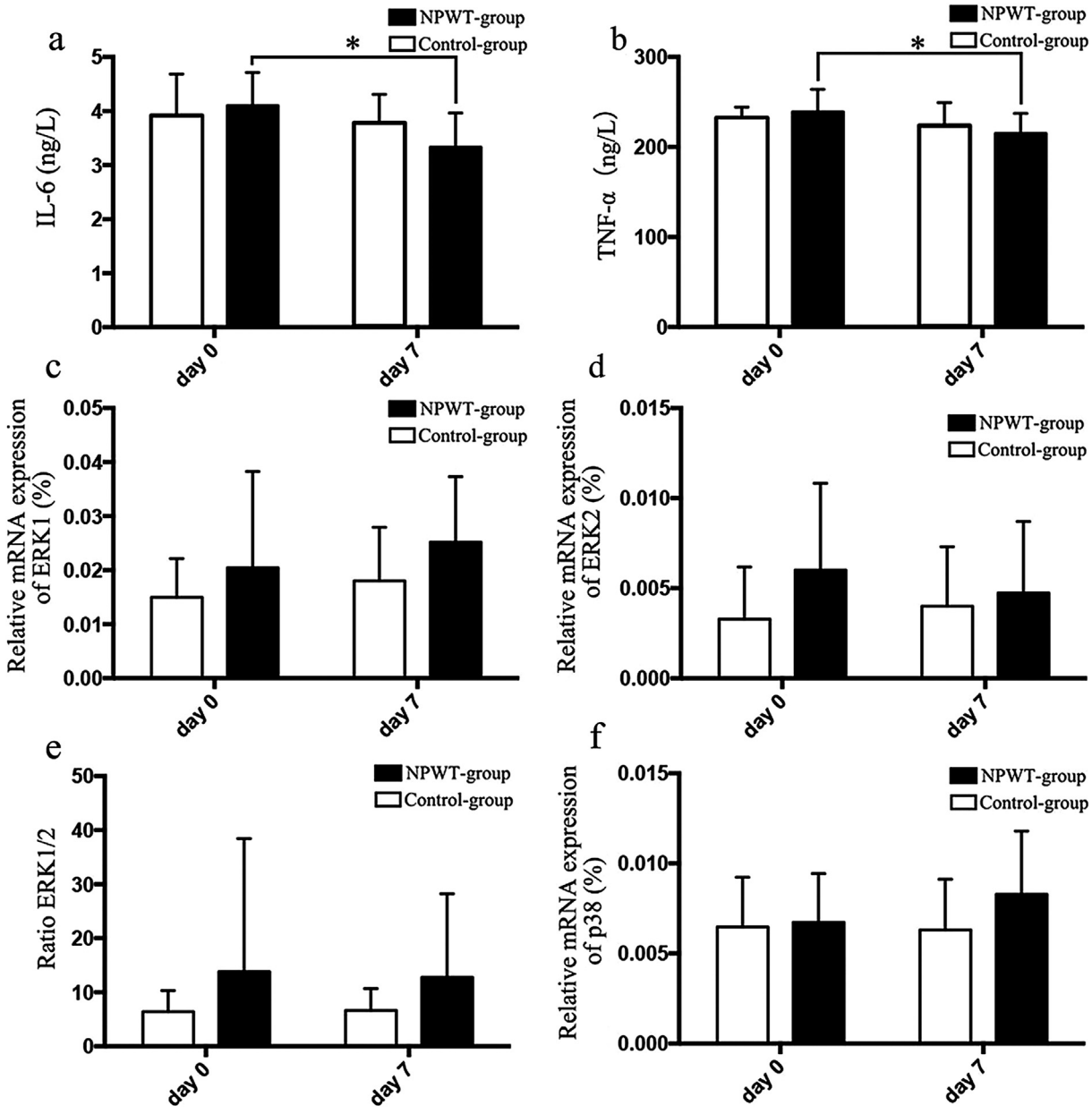

Fig. 4 – The expressions of TNF-α and IL-6 by eLISA analysis and ERK1, ERK2, ERK1/2 and p38 by rt-PCR in two groups. *P < 0.05. NPWT: negative pressure wound therapy.

NPWT could increase the level of NO which involved in the wound bed microcirculatory change [24].

The MAPK family of serine kinases is an important component of the signaling pathways that regulates varieties of intracellular processes [13]. MAPK activation also plays a pivotal role in the synthesis of pro-inflammatory cytokines. Especially, JNK signaling pathway plays a key role in regulating responses to inflammation [14]. These important MAPK pathways (P38, ERK1/2, JNK) were investigated in the current study. And we found that NPWT blocked the phosphorylation of JNK but not p38 or ERK1/2 kinase. JNK signaling pathway was activated in response to several transcription factors including NF-kB, which induce the transcription of a various of inflammatory genes like iNOS and cytokines [25]. These data provided the first evidence that NPWT performs an anti-inflammatory role through reducing the JNK pathway. The potential mechanism is NPWT works on the kinase upstream, and then JNK regulate downstream. In the previous study, the reason of down-regulation of JNK probably directly interacts with the activity of peroxisome proliferator activated receptors (PPARs). The activation of PPARs suppresses c-Jun- and p65-induced transcription of the IL-6 promoter and further represses the inflammatory gene response [26]. And IL-6 stimulation strongly activates JNK which in turn mediates the phosphorylation of c-Jun [13,27]. Jing et al also suggested that the transcription factor zinc-finger protein Miz1 suppresses TNF-α-induced JNK activation, which may account for the temporal control of TNF-α-JNK signaling [28]. The similar result was reported in Lee's study. They studied the mechanism of regulation of airway inflammation by

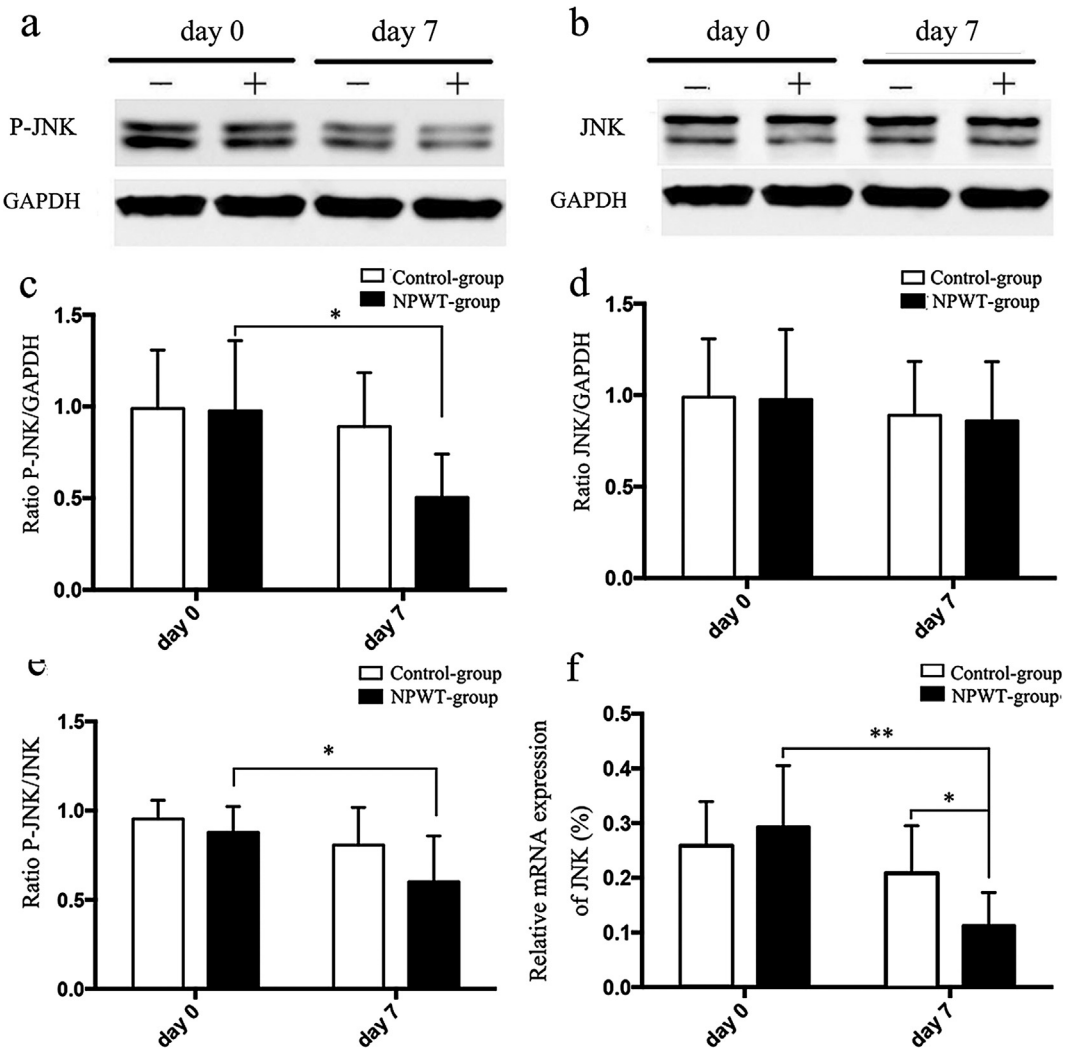

Fig. 5 – The expressions of P-JNK, JNK and ratio of P-JNK/JNK by eLISA analysis and mRNA of JNK by rt-PCR between the two groups. $^{*}P < 0.05$; $^{**}P < 0.01$. NPWT: negative pressure wound therapy.

interrupting the MAPK and NF-kB pathways in IL-1-treated lung epithelial cells (A549) and revealed that a JNK inhibitor clearly reduced IL-6 level, and the JNK pathway was crucial for IL-6 production in cells, whereas an ERK inhibitor did not affect production of IL-6 [29].

5. Conclusions

The present study confirmed that the inflammatory response is mitigated significantly by NPWT treatment, the NPWT's anti-inflammatory effect results from the inhibition pro-inflammatory enzymes and cytokines including iNOS, IL-6 and TNF-α, and the pro-inflammatory active is possibly caused by JNK pathway inhibition, thus the NPWT's anti-inflammatory effect contributes a lot to the success of diabetic foot treatment.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2019.02.024.

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