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Original Article

Expression of connective tissue growth factor and periostin of wound tissue in patients with diabetes who had vacuum sealing drainage

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Abstract: This study aimed to examine clinical effect of vacuum sealing drainage in treating the wound tissue of patients with diabetes; and investigate the expression of periostin and connective tissue growth factor in wound tissue. We selected 40 patients with diabetes and chronic ulcers, and assigned randomly into two groups: the vacuum sealing drainage group and routine dressing group (controls). Tissues were cut at day 1 before treatment and day 7 after treatment. Immunohistochemistry was used to observe periostin and connective tissue growth factor expression. Cure time and cure rate between the two groups was observed. Connective tissue growth factor and periostin were rarely expressed in wound tissue before treatment, but were increased after treatment. The mean count of periostin-positive cells was 25.2±3.50 in the vacuum sealing drainage group and 12.9±1.8 in the control group ($P < 0.05$). The mean count of connective tissue growth factor-positive cells was 19.7±2.54 in the vacuum sealing drainage group and 6.8±1.58 in the control group ($P < 0.05$). The mean duration of hospitalization of the vacuum sealing drainage group was 33.4±7.91 days, and 17 (85%) patients were cured. The mean duration of hospitalization of the routine treatment group was 50.45±6.77 days, and 13 (65%) patients were cured (both $P < 0.05$). This study shows that vacuum sealing drainage can shorten the healing time of diabetic chronic wounds and was addition to the mechanism of the vacuum sealing drainage treatment of diabetic wound.

Keywords: Diabetic ulcer, connective tissue growth factor, periostin, vacuum sealing drainage

Introduction

Chronic diabetic ulcers are a major complication of diabetes [1]. General characteristics of chronic diabetic ulcers include a longer sustained course, higher medical costs, and a unique pathology, such as hyperglycemia (glycosylated metabolites), microcirculation disorders, and peripheral neuropathy [2-4]. In healing of diabetic wounds, there is difficulty in achieving proliferation and the remodeling phase, which results in losing the ability to rebuild and restore integrity of skin barrier function [5, 6]. In recent years, the role of the extracellular matrix in mouse skin healing has been well studied, but only one prospective study examined impaired wound healing and the role of the extracellular matrix [7].

A previous study showed that periostin (PN) and connective tissue growth factor (CCN2/CTGF) expression in chronic wounds is absent [8]. PN is a novel secreted protein. PN is a collagen-rich connective tissue protein that is commonly expressed in the periosteum, and is essential for synthesis of the extracellular matrix [9]. CCN2 provides support for a range of physiological activities of endothelial cells, to ensure that they can function properly and to accelerate formation of blood vessels [10]. Furthermore, CCN2 promotes vascular growth factor expression and enhances their activity, such as basic

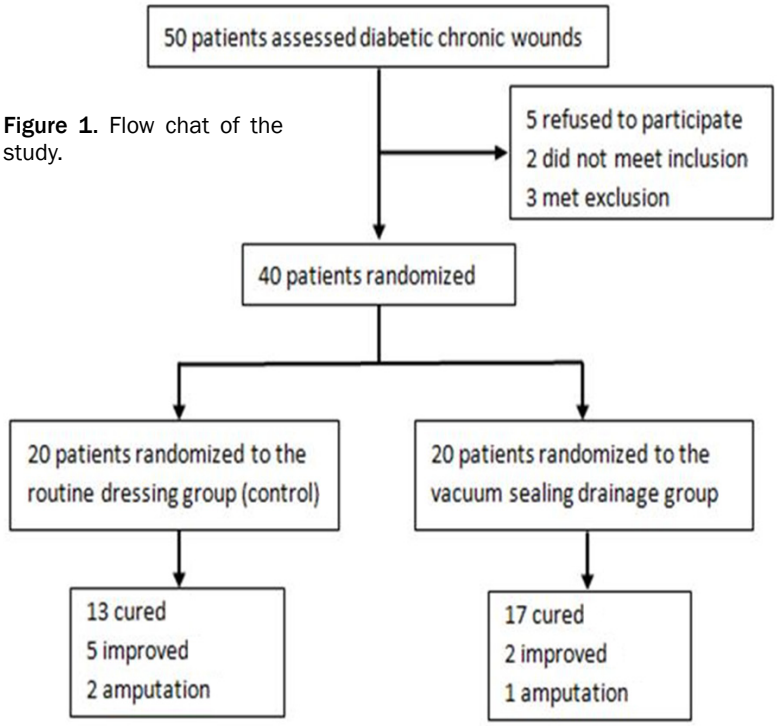

Figure 1. Flow chat of the study.

fibroblast growth factor (b-FGF) and vascular endothelial growth factor (VEGF). CCN2 promotes the integrity and stability of extracellular matrix-related molecules (collagen, matrix metalloproteinases (MMPs), tissue inhibitor of matrix metalloproteinases (TIMPs)) by increasing expression or enhancing activity by a direct or indirect role in the regulation of angiogenesis [11]. Reports on these two factors are mostly on fibrosis or tumor-related diseases, and there are only a few studies on their role in wounds [11-13]. Three days after injury, CCN2 expression in granulation tissue is decreased, tumor necrosis factor-alpha (TNF-α) levels are decreased, and transforming growth factor (TGF)-β1 expression is increased. PN and CCN2 are upregulated by TGF-β1, and in a low-pressure environment, PN protein promotes myofibroblast differentiation and proliferation of granulation tissue [8].

In the present study, we investigated the effect of vacuum-assisted closure on CCN2 and PN protein expression in diabetic wound tissue.

Material and methods

Subjects

A randomized, controlled trial (**Figure 1**) was carried out in the burn wound center of our hospital. All eligible patients (age > 18 years) with a diabetic chronic wounds corresponding to Wagner 2 to 4 that has continuously existed for a minimum of 1 month who meet all inclusion criteria and no exclusion criteria (**Table 1**), may be included in the study. We included 40 patients. The patients were divided into the routine dressing group (controls) and the vacuum sealing drainage group (VSD). The demographic information and clinic feature of both groups were collected (**Table 2**). All patients provided written informed consent.

Diabetes was diagnosed according to the 2010ADA diabetes guidelines standard. Criteria for the diagnosis of diabetes were as follows. (1) Hemoglobin A1c (HbA1c) was ≥ 6.5%. (2) Fasting plasma glucose levels were ≥ 7.0 mmol/l. (3) Two-hour plasma glucose levels were ≥ 11.1 mmol/l during an oral glucose tolerance test. (4) In patients with classic symptoms of hyperglycemia or hyperglycemic crisis, plasma glucose levels were ≥ 11.1 mmol/l [14].

Diabetic wound grading was determined by the Wagner classification. Wounds were divided into six grades: grade 0, a high risk of ulcers; grade 1, no infection, foot ulcer surface; grade 2, a full-thickness ulcer, which was often associated with soft tissue infection; grade 3, a deep ulcer, where the depth tended to affect bone tissue, and may have been associated with deep abscesses and osteomyelitis; grade 4, local gangrene; and grade 5, foot gangrene. According to the diagnostic criteria of Armstrong for diabetic foot ulcers, ulcers were divided into three grades: I, the ulcer encroached the skin only; II, the ulcer invaded deep tissue and the muscle layer, but it did not spread to the bone and joints; and III, the ulcer invaded the bone and joints [15].

Materials and reagents

Disposable negative pressure drainage and protecting material were provided by Wuhan

Table 1. Inclusion and exclusion criteria of the study

Inclusion and exclusion criteria
Inclusion criteria
(1) Clinical diagnosis of type 2 diabetes mellitus;
(2) The wound was consistent with the diagnosis of a chronic wound;
(3) 2 ≤ Wagner grade ≤ 4.
(4) Continuous existence of the diabetic foot lesion for a minimum of 1 month.
Exclusion criteria
(1) Refuse to written informed consent
(2) Age < 18 years
(3) Pregnancy
(4) Present of expected non-compliance with the requirements of the study estimated by investigator at time point of inclusion
(5) Necrotic tissue with present that cannot be debrided
(6) Malignancy of the wound
(7) Severe heart disease, heart failure, unstable angina pectoris, myocardial infarction or severe systemic infection;
(8) Severe renal insufficiency, with a serum creatinine level > 106 umol/L;
(9) Liver dysfunction, with alanine aminotransferase levels > 125 U/L or glutamic-oxalacetic transaminase level > 87.5 U/L;
(10) Application of immunosuppressive agents and growth factors;
(11) Poor compliance, death, or unable to complete the course of treatment (during treatment);
(12) Contraindications for surgery or patients did not agree to having surgery.

Table 2. Clinical characteristics of the study subjects

Variables	Control	VSD	*P* value
N	20	20	/
M/F	14/6	14/6	/
Age (years)	65.35±10	67.85±12.21	0.4830
BMI (kg/m^2)	21.07±2.04	21.46±1.19	0.0978
FBG (mmol/L)	5.5±1.3	5.7±1.4	0.324
PBG (mmol/L)	8.5±1.1	8.7±1.3	0.525
HbA1c (%)	6.39±0.75	6.68±1.31	0.065
C-Peptide (pmol/L)	476.16±50.21	465.39±87.07	0.153
Insulin (pmol/L)	79.2±0.70	74.79±6.66	0.0605
TC (mmol/L)	5.16±0.81	5.44±0.96	0.0802
TG (mmol/L)	1.59±0.82	1.33±0.55	0.4101
LDL-C (mmol/L)	2.28±0.87	2.30±0.72	0.0620
HDL-C (mmol/L)	1.07±1.03	1.18±0.20	0.2001
APO-A (g/L)	1.30±0.16	1.29±0.18	0.0882
APO-B (g/L)	0.85±0.10	0.84±0.19	0.3203
Hs-CRP (mg/L)	2.35±0.13	2.75±4.06	0.0643
Hcy (μmol/L)	5.53±0.56	5.49±0.57	0.2102

Data were expressed as means ± SD. Differences between multiple groups were tested by variance (ANOVA) for continuous variables. N, number; M/F, male/female; BMI, body mass index; FBG, fasting blood glucose; PBG, post challenge blood glucose; HbA1c, hemoglobin A1c; TC, total cholesterol; TG, total triglyceride; LDL-C, low-density, lipoprotein cholesterol; HDL-C, high-density lipopretion cholesterol; APO-A, Apolipoprotein A; APO-B, Apolipoprotein B; Hs-CRP, High sensitivity C reactive protein; Hcy, Hyperhomocysteinemia. *P < 0.05, compared with control.

VSD Medical Science & Technology Co. Ltd (World Trade Organization plaza room 3188, Jiefang Road, No. 686, Jianghan District, Wuhan, Hubei Province, China). Specifications were divided into two types of 15 × 5 × 1 cm and 15 × 10 × 1 cm (length L1 × width W1 × thickness H1). Reagents that were used included anti-PN antibody (Abcam ab14041) and anti-CCN2 (CTGF) antibody (Abcam ab6992).

Management of patients and wounds

Patients were managed with basic information registration, treatment of underlying disease, controlling of blood glucose and antibiotics to cure (**Table 3**).

The following procedure was performed in the operating room under sterile conditions. In the negative pressure treatment group, the wound was cleaned and disinfected by repeatedly washing with sterilized physiological saline, hydrogen peroxide, and iodine solution (dosage > 2000 ml). We covered the wound with negative pressure material according to the shape and size after debridement. The plastic tube has a side hole into the medical sponge, and the other end of the suction device and is connected with the connection. Finally, the wound and the edge of the wound were covered with sterile film (**Figure 2**). Negative pressure was maintained at -120-400 mmHg. Dressing should be

Table 3. Management of patients

Management of patients
1. Basic information registration
2. Treatment of underlying disease
3. Fasting blood glucose levels ≤ 8 mmol/l
4. Postprandial blood glucose levels ≤ 12 mmol/l
5. Antibiotics were provided according to general culture sensitivity wound select sensitive antibiotics

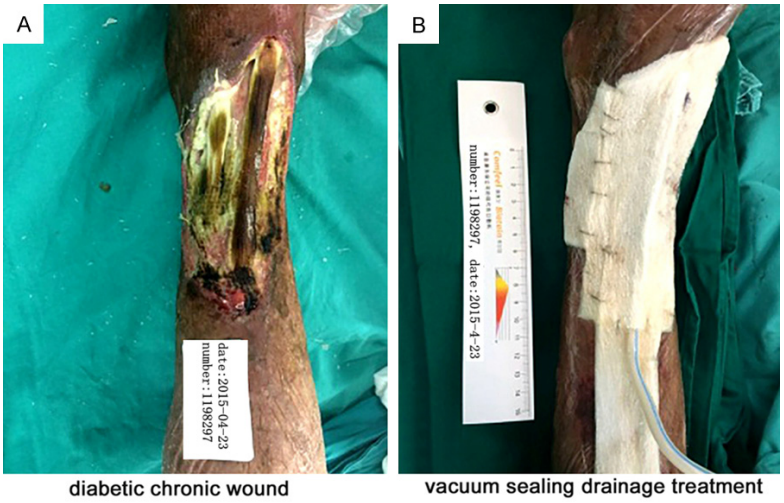

Figure 2. Picture of the VSD treatment. A: Diabetic chronic wound before treatment; B: Diabetic choronic wound with vacuum sealing drainage treatment, the wound and the edge of the wound were covered with sterile film. Negative pressure was maintained at -120-400 mmHg. Negative pressure drainage and protecting material were provided by Wuhan VSD Medical Science & Technology Co. VSD: vacuum sealing drainage.

changed every 7 days. In the conventional dressing group, negative pressure sealing materials were not used. We applied a 0.5% dilute iodoform gauze and Vaseline gauze dressing. The dressing was changed every other day.

Specimen collection

In the negative pressure treatment group, we cut a margin of full-thickness skin tissue, approximately 6 × 5 × 2 mm, in the wound the day before VSD treatment and at 7 days after VSD treatment. Tissue was fixed with 10% formaldehyde, embedded in paraffin, sliced, and used for detection of immunohistochemistry. In the conventional dressing change group, we cut a sample before debridement and at 7 days after changing the dressing. The remaining steps were the same as those in the negative pressure treatment group.

Observations and immunohistochemistry

We observed infiltration of the wound surface, granulation tissue growth, and epithelium of the wound surface every 7 days, a total of 1 months.

With regard to immunohistochemistry, we observed PN and CCN2 protein expression in fibroblasts. Fibroblasts were stained brown-yellow if they were positive. We used an optical microscope with a magnification of 400 × to observe the relative content of PN and CCN2. We randomly selected five fields and counted the number of positive cells. Cells were categorized as follows: negative, no positive cells; weakly positive (+), 1-10 positive cells; moderately positive (+ +), 11-30 positive cells; and strongly positive (+ + +), more than positive cells. The average cell count in five fields in each group was used for analysis.

Statistical analysis

All measurement data are expressed as mean ± sd. Data were analyzed with the independent-samples t test and paired-samples t test by using SPSS 22.0 statistical software. $P < 0.05$ was considered statistically significant.

Results

Observation of wounds

In the conventional dressing treatment group (control), before debridement, exudation of the wound surface was increased, granulation tissue was aging and dark (**Figure 3A**). One week after ordinary gauze dressing, the granulation tissue on the wound was not fresh, and infection, obvious edema, and exudation were usually observed (**Figure 3B**). These wounds could not be skin grafted 1 month after treatment (**Figure 3C**).

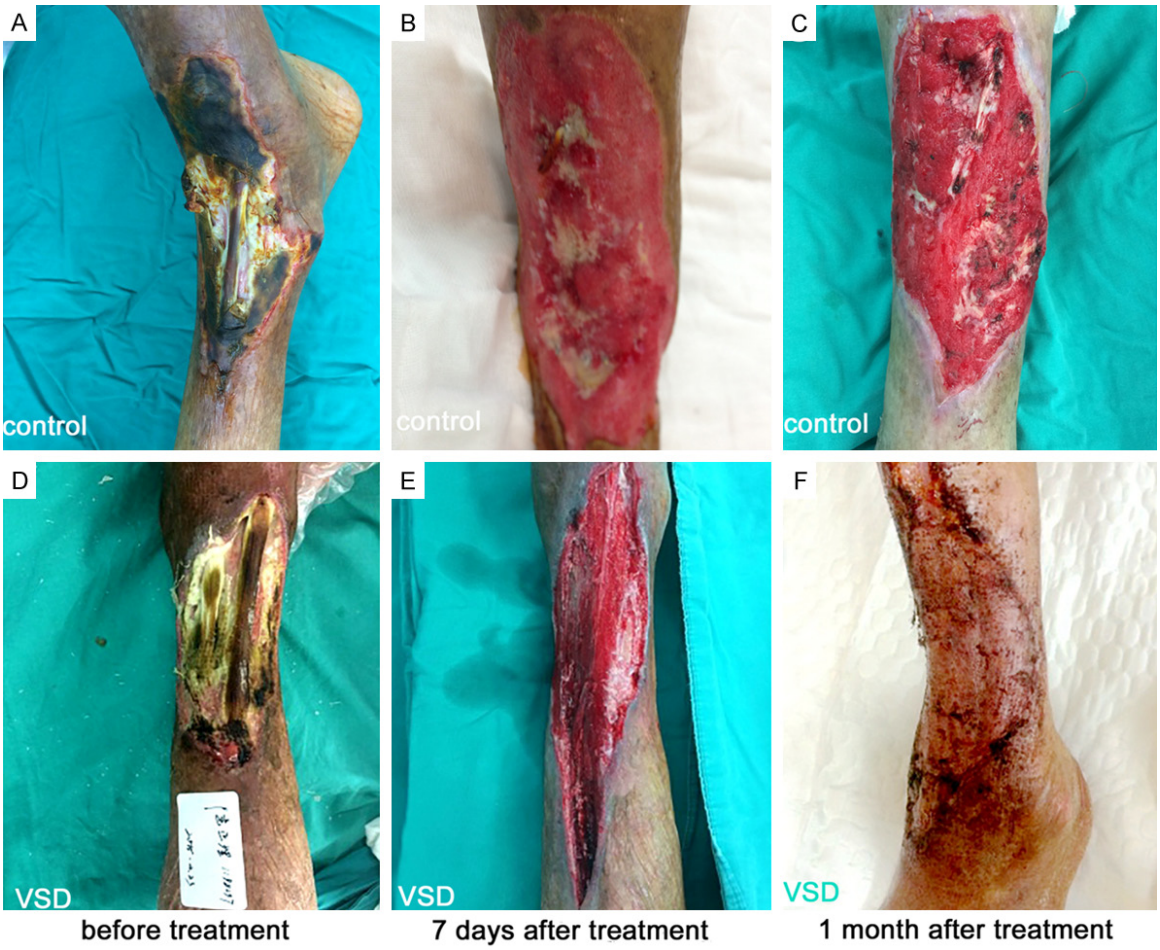

Figure 3. Observations of necrotic tissue in both group before and after treatment. A-C: Necrotic tissue treatment with the conventional dressing (control group); D-F: Necrotic tissue treatment with the vacuum sealing drainage (VSD group); A, D: Necrotic tissue before treatment; B, E: Necrotic tissue 7 days after treatment; C, F: Necrotic tissue 1 month after treatment. A: Exudation of the wound surface was increased, granulation tissue was aging and dark. B: The granulation tissue was not fresh, and infection, obvious edema, and exudation were usually observed. C: The wound could not be skin grafted. D: Necrosis involved the skin and fascial tissue, granulation tissue was dull and thin, and there was more exudation. E: The wound exudate was reduced showed a large amount of fresh red granulation, bleeding of granulation tissue, and visible wound again epithelial and white transparent epithelial cells. F: The wound was healed by a skin graft.

We observed necrotic tissue in the vacuum sealing drainage group (VSD group). Necrosis involved the skin and fascial tissue, granulation tissue was dull and thin, and there was more exudation (**Figure 3D**). One week after VSD treatment, the wound exudate was reduced compared with before treatment. The wound showed a large amount of fresh red granulation, bleeding of granulation tissue, and visible wound again epithelial and white transparent epithelial cells (**Figure 3E**). The wound was healed by a skin graft 1 month after treatment (**Figure 3F**).

CCN2 and PN expression was detected by immunohistochemistry

PN expression: Before treatment, PN protein expression was rare in the wound in the two groups (**Figure 4A**, **4B**, observed by light microscopy at 10 × 10). In the control group after treatment, PN expression in the cytoplasm of fibroblasts in granulation tissue was increased, and was weakly positive or positive compared with before treatment (shown by the arrows in **Figure 4C**, 10 × 40). In the VSD group after treatment, PN expression was strongly positive

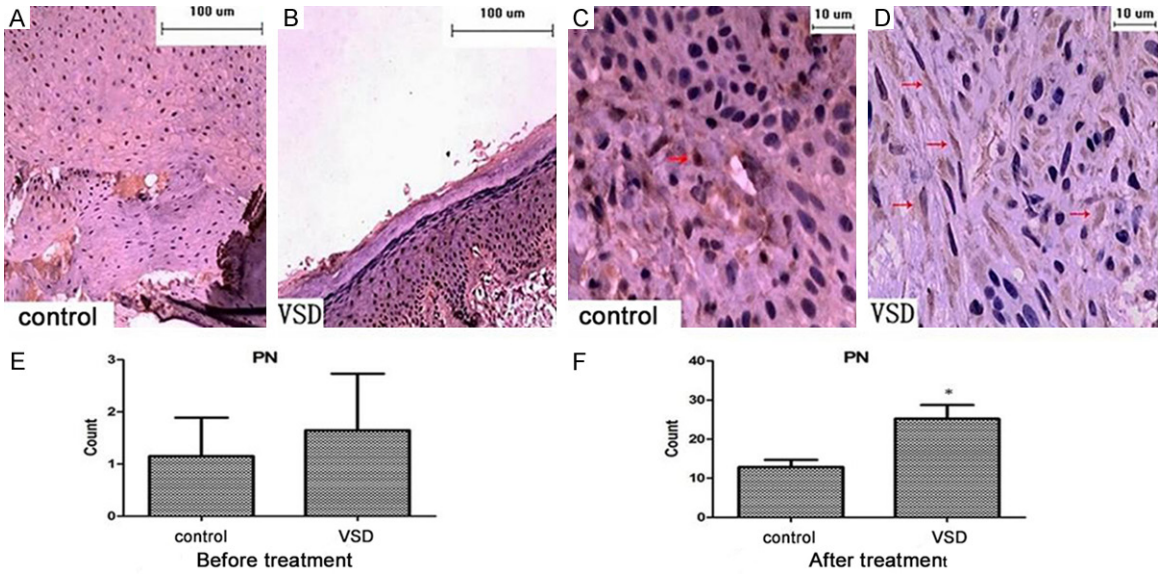

Figure 4. PN protein expression in the VSD and control groups. A, B: PN protein expression before treatment, observed by light microscopy at 10 × 10, Bar = 100 um; C, D: PN protein expression after treatment, observed by light microscopy at 10 × 40, Bar = 10 um; A, C: PN protein expression in the conventional treatment (control group); B, D: PN protein expression in the VSD group; E: The mean count of PN protein positive cells in two groups before treatment, There was no significant difference in control and VSD group (1.15±0.74 vs 1.65±1.08, P=0.098); F: The mean count of PN protein positive cells in two groups after treatment, The mean count of positive cells in the VSD group was significantly higher than that in the control group (25.2±3.50 vs 12.9±1.80, P=0.001); Data are shown as the average cell count of PN positive cells relative to the control group. Fibroblasts were stained brown-yellow if they were positive.We randomly selected five fields and counted the number of positive cells. The average cell count in five fields in each group was used for analysis. All data are shown as mean ± sd. Differences between two groups were tested by t test. *P < 0.05, versus control. VSD: vacuum sealing drainage (therapy group). PN: periostin.

in epidermal cells and dermal fibroblasts. Additionally, the number of macrophages, monocytes, and fibroblasts in granulation tissue was significantly increased compared with that before treatment, and expression was strongly positive (shown by the arrows in **Figure 4D**, 10 × 40).

There was no significant difference in the mean count of positive cells before treatment between the two groups (**Figure 4E**, P > 0.05). The mean count of positive cells in the VSD group was significantly higher than that in the conventional treatment group (control group) after treatment (25.2±3.50 vs 12.9±1.80, P < 0.01, **Figure 4F**).

CCN2 expression: CCN2 expression was negative and in the two groups before treatment (**Figure 5A**, **5B**, 10 × 10). A small amount of CCN2 expression was observed in the cytoplasm of epidermal cells in the conventional treatment group after treatment (shown by the arrows in **Figure 5C**, 10 × 40). CCN2 was strongly expressed in the cytoplasm of dermal fibroblasts in the VSD group after treatment (shown by the arrows in **Figure 5D**, 10 × 40).

There was no significant difference in the mean count of positive cells before treatment between the two groups (**Figure 5E**, P > 0.05). The mean count of positive cells in the VSD group was significantly higher than that in the conventional treatment group after treatment (19.7±2.54 vs 6.8±1.58, P < 0.01, **Figure 5F**).

Hospitalization time and cure rate

Vacuum sealing treatment group, the average duration of hospitalization was 33.4±7.91 d, 17 cases were cured, 2 cases improved, 1 case of amputation, the cure rate was 85%. The average hospitalization time of the control group was 50.45±6.77 d, 13 cases were cured, 5 cases improved, 2 case of amputation, the cure rate was 65%. The mean hospitalization time in the VSD group was significantly shorter than that in the routine dressing change group (33.4±7.91 vs 50.45±6.77 days,

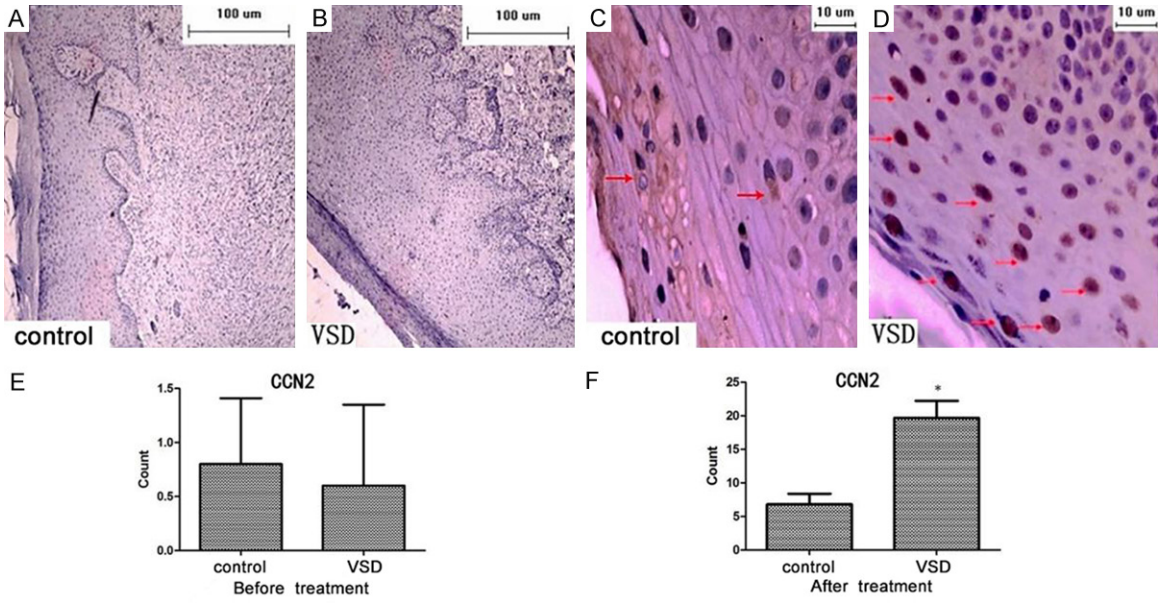

Figure 5. CCN2 expression in the VSD and control groups. A, B: CCN2 expression before treatment, observed by light microscopy at 10 × 10, Bar = 100 um; C, D: CCN2 expression after treatment, observed by light microscopy at 10 × 40, Bar = 10 um; A, C: CCN2 expression in the conventional treatment group (control group); B, D: CCN2 expression in the VSD group; E: The mean count of CCN2 (CTGF) positive cells in two groups before treatment, There was no significant difference in control and VSD group (0.80±0.61 vs 0.60±0.75, P=0.364); F: The mean count of CCN2 (CTGF) positive cells in two groups after treatment, The mean count of positive cells in the VSD group was significantly higher than that in the control group (19.7±2.54 vs 6.8±1.58, P=0.001). Fibroblasts were stained brown-yellow if they were positive. We randomly selected five fields and counted the number of positive cells. The average cell count in five fields in each group was used for analysis. All data are shown as mean ± sd. Differences between two groups were tested by t test. *P < 0.05, versus control. VSD: vacuum sealing drainage; CCN2 (CTGF): connective tissue growth factor.

Table 4. Hospitalization time and cure rate in both groups

Group	Hospitalization time (d)	Treatment effect			Cure rate (%)
		Cure	Improve	Amputation	
Control	50.45±6.77	13	5	2	65%
VSD	33.40±7.91**	17	2	1	85%*
t	-7.32				-7.79
P	0.003				0.02

Data were expressed as means ± sd. Differences between two groups were tested by t test. *P < 0.05, compared with control; **P < 0.01, compared with control. Cure rate = number of (cure + improve)/total number *100%. VSD: vacuum sealing drainage.

P < 0.05). The cure rate in the VSD group was significantly higher than that in the control group (85% vs 65%, P < 0.05) (**Table 4**).

Discussion

Treatment of chronic ulcers in diabetes is difficult because the wound is easily infected, and growth of granulation tissue is slow. Our study showed that PN and CCN2 expression levels in the wounds of the vacuum sealing drainage group (VSD group) improved compared with those in the conventional dressing change group. Additionally, granulation tissue in the wounds of the VSD group increased more rapidly, there was less edema and infection, and the time of performing a skin graft to repair the wound was shorter compared with the conventional dressing change group.

A lack of growth factors and changes in the extracellular matrix, into fibroblast function reduced, leukocyte dysfunction, inappropriate systemic application of antibiotics, disorder of the microcirculation, and other factors might result in delayed healing of wounds [16]. The principle of wound treatment is to reduce local tissue pressure, restore blood supply to the skin, control infection, appropriate treatment of wounds, treat complications, and guide the correct treatment of diseases [17]. Newly developed trauma-recovery technology, such as hyperbaric oxygen, biological tis-

sue engineering, recombinant platelet-derived growth factor BB, extracellular matrix production, and the granulocyte colony-stimulating factor method should be integrated using multidisciplinary treatment for wounds. This approach could eliminate the risk factors of ischemia and osteomyelitis in treating limbs and decrease the rate of amputation [18]. Several studies have shown that recombinant human platelet-derived growth factor, recombinant basic fibroblast growth factor, and recombinant human granulocyte-macrophage growth factor have definite therapeutic effects on chronic wound healing and have been approved in many countries [19-21].

Elliott showed that expression was upregulated in mouse skin 3 days after injury [15]. They regulated the various stages of proliferation, including infiltration of bone marrow mesenchymal cells and differentiation of muscle fibroblasts in wounds. Other studies showed that CCN2 and PN expression was not present at the edge of human chronic refractory wounds, and was missing in the wound bed [15]. The present study showed that protein CCN2 and PN expression was rare, and increased after treatment compared with before treatment. In the VSD group, expression levels were significantly increased, and the healing time was significantly shorter after treatment compared with before treatment. Therefore, CCN2 and PN expression levels might be positively correlated with wound healing.

The VSD technique is safe and effective in treatment of wounds, and is easy to operate [22]. VSD promotes wound healing, but the exact mechanism of VSD promoting wound healing is unclear. Possible mechanisms of VSD are accelerated wound blood flow, reduction of local edema, and a reduction in accumulation of wound exudate. The wound should be continually washed to inhibit proliferation of bacteria on the surface of the wound and to promote growth of granulation tissue. The wound environment should also be kept moist to modulate regulation of collagenase and gelatinase activity, and promote a variety of growth factors, such as CCN2 (CTGF) and VEGF secretion [23, 24].

In the present study, we found that application of VSD significantly improved PN and CCN2 expression in wound tissue. This resulted in acceleration of growth of granulation tissue, a reduction in wound healing time of diabetic chronic ulcers, and improvement in the cure rate. We also found that CCN2 and PN protein expression in chronic ulcer wounds was inhibited by the other hand, which is an important factor in formation of chronic wounds. VSD promotes healing of chronic ulcers in diabetes, shortens the treatment time. Therefore, VSD is one of the preferred treatments of diabetic ulcers in the clinical setting. All stages of wound healing involve many growth factors, regulated by related genes.

Obtaining a good understanding of the treatment mechanism of VSD by investigating the effect of VSD on regulation of gene expression in chronic wound healing is important. This understanding will likely offer a new target for clinical treatment of refractory chronic wounds in the future.

The pathogenesis of diabetic ulcers remains unclear, and its healing process involves many molecular mechanisms. Our study shows that VSD may increase PN and CCN2 protein expression in diabetic wounds to promote wound healing. However, the association between expression levels of these factors and blood glucose levels requires further research.

This study was carried out in a single-center small numbers survey including some deficiency, so the multicentre large sample study was desired to establish.

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Disclosure of conflict of interest

None.

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