Int J Clin Exp Med 2016;9(6):11476-11484
www.ijcem.com /ISSN:1940-5901/IJCEM0021111

Original Article

Treatment of seawater-immersed blast-injury wounds of pig skin soft tissues using vacuum assisted closure technology

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Received December 3, 2015; Accepted March 2, 2016; Epub June 15, 2016; Published June 30, 2016

Abstract: The aim of this study was to dynamically observe and compare the effects of vacuum-assisted closure (VAC) technology on the healing of seawater-immersed blast-injury wounds (SIBIW) of pig skin soft tissues, and to summarize the mechanisms of VAC technology in promoting such SIBIW healing, with the goal of providing new experimental evidence for the rescue of such wounds. The bilateral scapular regions and hips of 5 small pigs underwent SIBIW with detonators. After the debridement, the pigs were randomly divided into control and experiment groups (VAC-120 mmHg group, VAC-180 mmHg group, VAC-240 mmHg group), with 5 wounds in each group. The control group's dressings were changed daily, and the experiment group's dressings were changed every other day. All wounds were treated for 9 days, and were observed until they had all healed (i.e., on the 58th day). We then compared and observed the macroscopic and microscopic changes in the wounds of each group. Compared with the control group, the experimental groups showed more active proliferation of peri-wound cells, and faster crawling of the epithelium. In the early stage, the expression of type I collagen was low, while that of type III collagen was higher, the bacterial index decreased rapidly, the bacterial transition was faster, and the wound healed faster. In treating SIBIW, VAC technology had obvious advantages over conventional therapy.

Keywords: Vacuum assisted closure, seawater immersion, blast injury, tumor necrosis factor-α, type I collagen, type III collagen

Introduction

The rising tide of domestic and international terrorist bombing attacks has resulted in a large number of blast injuries previously seen only in military conflicts. The incidence of blast injuries is increasing in the civilian setting; thus, clinicians need to understand the spectrum of injury and management strategies [1]. Wounds caused by blast injuries often have larger skin soft tissue defects, irregular wound edge, and are severely contaminated or associated with infections. The surgical closure cannot be performed immediately, and early re-dressing is very important. Vacuum-assisted closure (VAC) is a relatively new technology that has applications for a variety of difficult to manage acute and chronic wounds. It involves the application of open cell foam to a suitable wound, the addition of a seal of adhesive drape, and then the controlled application of subatmospheric pressure to the wound [2]. As a special method of wound treatment, compared with conventional therapy, VAC technology exhibits obvious advantages in treating various complex wounds, and has also been successfully applied to blast-injury wounds, with significant effect [3, 4]. To date, there are no reports regarding the application of this technology in seawater-immersed blast-injury wounds (SIBIW). In the present study, we simulated sea war-explosion-induced skin soft tissue defects, which were also associated with seawater immersion, thus, establishing the pig skin soft tissue SIBIW model. The objective was to investigate the effects of VAC technology on the healing of such wounds, and to observe whether there were differences when using different vacuum pressures. Here, we used mini pigs as the animal model and established the first model of SIBIW, with the goal of comparing

the macro (general body) and micro (pathology, type I collagen, type III collagen, tumor necrosis factor-α [TNF-α], and bacteria) changes of the wounds under different negative pressures, thus providing effective experimental evidence for the treatment of such wounds. This study not only scientifically supplements the changes in pathological patterns of SIBIW, but also adds partial mechanisms of VSD in treating such wounds; therefore, this study had its own specific characteristic and innovations.

Methods

Animals

We selected 5 small experimental white pigs, 4-6 months old, without gender limit. The pigs weighed 23-25 kg, and the average weight was 24 kg. Pigs were domesticated one week before the experiment to allow their adaption, then deprived of food and water 12 h before the experiment; atropine was administrated to inhibit the glandular secretion-induced suffocation, 10-15 min before combined anesthesia with ketamine (0.05 mg/Kg) and xylazine (0.2 mL/Kg) was intramuscularly injected.

Model preparation

The detonator (model 660929F48841-56) was used to prepare 4 blast injury wounds, with relatively equal degree, on the bilateral scapulae and buttocks of each pig; then the injuries were soaked for 1 h in seawater obtained from the sea area near Bohai Bay. After the debridement, the 4 wounds were randomly divided into the control group and the experimental groups (VAC-120 mmHg, VAC-180 mmHg, and VAC-240 mmHg groups), with five wounds in each group. The control group wounds were dressed daily, and the experimental group wounds were redressed with VAC every other day. In all groups, before redressing, routine disinfection (disinfection using 0.5% iodophor, followed by washing with sterile saline) was performed. Treatment was performed for a total of 9 days, and the dynamic contrast observation was carried out until all the wounds healed (i.e. the 58th day). At this time, general changes, collagen synthesis, bacterial capacity, and other indicators of the wounds of each group were detected dynamically, and the effects of VAC technology towards the healing of seawater-immersed blast wounds were summarized.

Hematoxylin-eosin and immunohistochemical staining

According to reported methods [5-7], 0.5 cm × 0.5 cm × 0.5 cm tissue masses were cut from different locations of each wound edge and of each group, before treatment and 1, 3, 5, 7, 9, 16, 30, and 58 days after treatment. Masses were fixed in 10% formalin solution, then, samples were removed and progressively dehydrated until transparent in the xylene, embedded in paraffin, and sliced. Finally, samples underwent H&E staining or immunohistochemical staining for type I and III collagen.

Wound bacterial index

According to the reported method [8], 0.5 cm × 0.5 cm × 0.3 cm tissue masses were cut from different locations of each wound surface from each group before treatment, and 1, 3, 5, 7, and 9 days after treatment. Masses were then weighed by one electronic balance; 99-volumes of saline were added for homogenization. The homogenate was then diluted 10 times (10 ×, 100 ×, 1000 ×) and 100 μL diluent was inoculated onto a 90 mm blood agar plate, and incubated at 37°C for 24 h. Then, the number of bacterial colonies on the blood agar plate were counted, and the colony characteristics were evaluated. The number of bacteria per gram of tissue = number of colonies × 10^3 × dilution time, which were then converted to a logarithmic index.

Enzyme-linked immunosorbent assay detection

According to the reported methods [7, 9, 10], the TNF-α level of swine was detected by the double antibody sandwich method. Before and 1, 3, 5, 7, and 9 days after treatment, a granulation tissue section of 0.5 cm × 0.5 cm × 0.3 cm was taken from different locations of the wounds. From these sections, a sample of 100 mg was weighed by electronic balance, rinsed twice in 4°C pre-cooled PBS solution (pH = 7.4), and was then immediately placed into the liquid nitrogen for rapid freezing. When these sections were thawed, the tissue was first placed in an ice bath for 30 min, then kept at 2-8°C, before the appropriate amount of 4°C pre-cooled PBS solution (pH = 7.4) was added for full homogenization by one hand homogenizer. The homogenized sample was centrifuged for

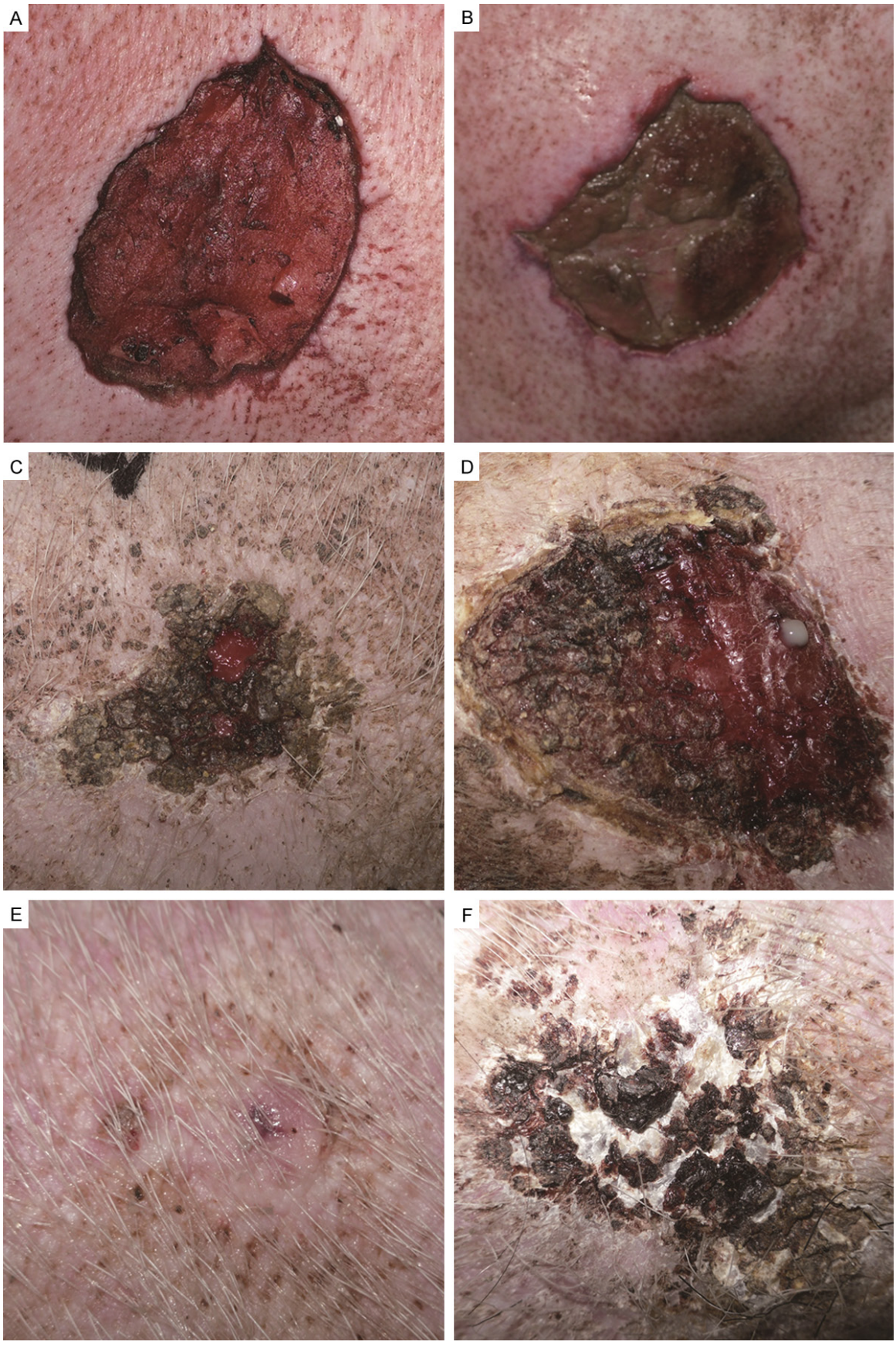
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Figure 1. Treatment of wounds. (A, B) At 1st day of treatment of wounds (A was VAC-120 mmHg group; B was the control group). (C, D) At 30th day of wounds (C was VAC-120 mmHg group; D was the control group). (E, F) At 58th day of wounds (E was the VAC-120 mmHg group; F was the control group).

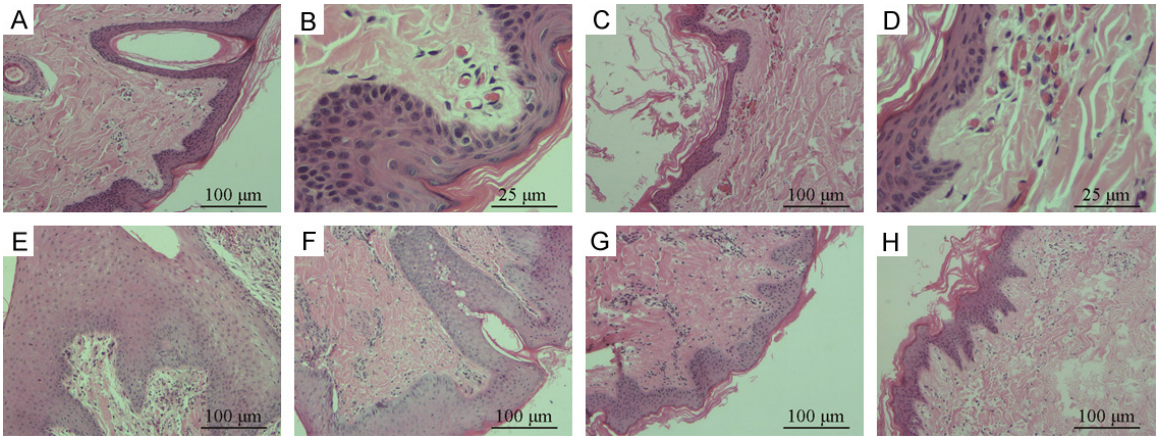

Figure 2. Pathological observation of peri-wound tissues. (A, B) Normal skin tissue pathology; (C, D) Skin tissue pathology before treatment; (E, F) At 5th day of peri-wound skin tissue pathology (E was VAC-120 mmHg group; F was the control group); (G, H) Skin tissue pathology at 58th day of VAC-120 mmHg group (G) and control group (H) wound.

20 min (3000 rpm/min), before the level of TNF-α in the supernatant was detected using a swine TNF-α ELISA immunoassay kit.

Statistical analysis

Data analyses were performed using SPSS 11.5 statistical software. Results were expressed as mean ± standard deviation (SD). The analysis of variance of multiple dependent variables was performed, with $P<0.05$ considered as statistical difference, and $P<0.01$ considered significant statistical difference.

Results

General observations

Wound healing processes in each group were observed and contrasted by dynamically comparing naked-eye observations and digital images. Before treatment, the wound base of each group was pale, the area around the wound was flushed, while the skin inside the flush was pale and swelling, and the boundary of necrotic range was unclear, tough, and inelastic. After treatment, the experiment group (**Figure 1A**, **1C**, **1E**) exhibited cleaner wounds, with less exudation at the surface. The wounds were red, the proliferation of granule tissues active, the wound margin clearly contracted, and the epithelium crawled faster. The VAC-120 mmHg treatment group exhibited the fastest wound healing, followed by the VAC-180 mmHg group. In contrast, the control group (**Figure 1B**, **1D**, **1F**) exhibited dirtier wounds, with more exudation on the surface. Their wound color was dull, and the proliferation of granule tissues, wound margin contraction, and epithelial crawling were not obvious. Indeed, they exhibited the trend of expansion and deepening of wound in the early stage, few wounds appeared purulent (**Figure 1F**), the wound healing was poor, and the healing time was the longest.

Pathological observation of peri-wound tissues

The normal skin (**Figure 2A**, **2B**) included the epidermis, dermis, and subcutaneous tissue; the dermis was divided into the papillary and reticular layers. The papillary layer was thinner, located in the superficial layer of the dermis, with relatively thinner fibers, and exhibited light pink H&E staining. The reticular layer was thicker, located below the papillary layer, the fibers were somewhat thicker, and the H&E staining was red and corrugated. Before treatment (**Figure 2C**, **2D**), the dermal collagen fibers had edema, the gaps were widened and disorganized, the capillaries were expanded and congested, the red blood cells spilled out, and inflammatory cell infiltration could be seen around the vessels. In the early stage of wound

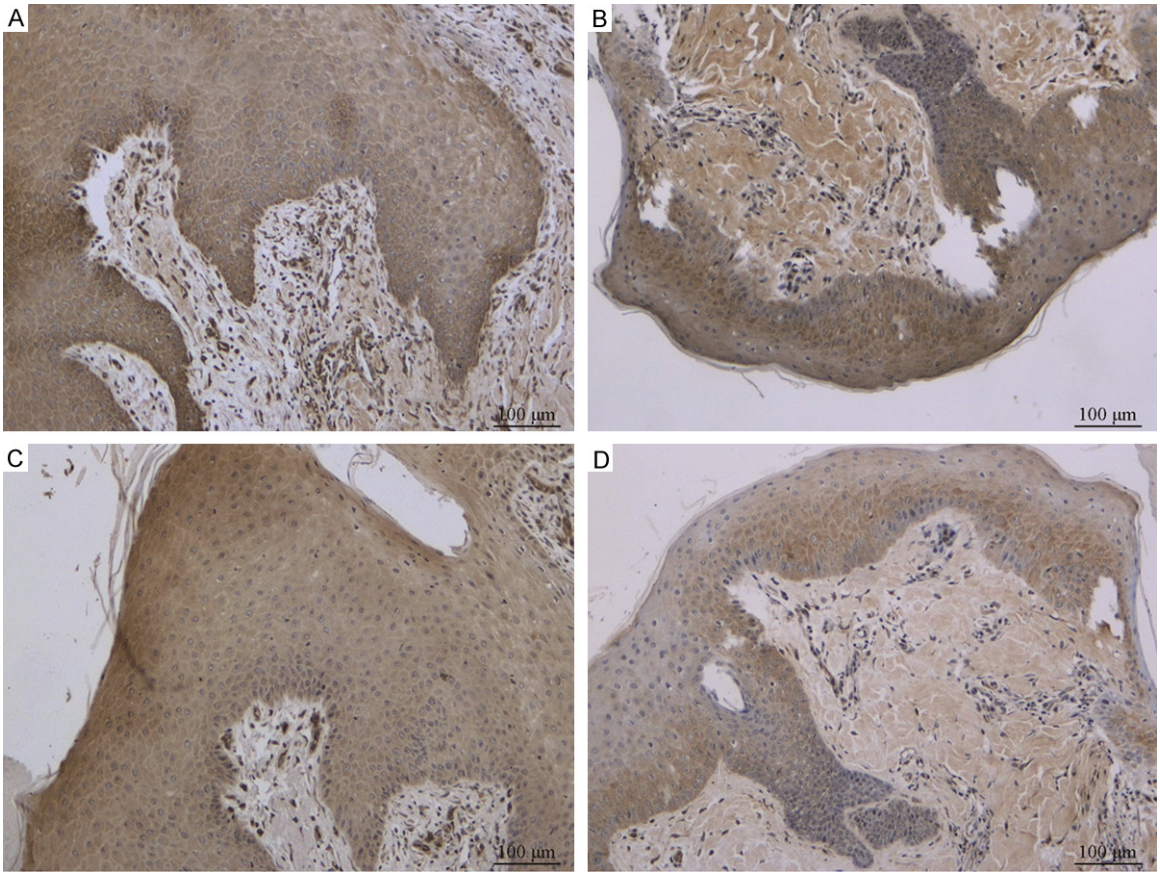

Figure 3. Immunohistochemical expressions of type I and III collagen in the peri-wound tissues. (A, B) Collagen type I positive expression in the VAC-120 mmHg group (A) and in the control group (B); (C, D) Collagen type III positive in the VAC-120 mmHg group (C) and the control group (D).

healing, the peri-wound cell proliferation in the experiment group (**Figure 2E**) was much more active than that in the control group. It mainly exhibited increased and thickened epidermal prickle cell layer, the edema of dermal fibers became lighter, and the fibers were more regular. In contrast, in the control group (**Figure 2F**), peri-wound cell proliferation was poor, the changes in the prickle cell layer were not significant, and the edema of dermal fibers was obvious and relatively irregularly arranged, even appearing to have focal or lamellar necrosis. In the late stage of wound repair, the differentiation of epidermal layers of the experiment group (**Figure 2G**) was mature, the distribution of epidermal processes was normal, and the dermal fibers were arranged neatly. However, the control group (**Figure 2H**) exhibited long, thin, and densely distributed epidermal processes, which were even bifurcated in the distal end, the gaps among the dermal fibers were wider, and their arrangement was relatively disordered.

Immunohistochemical expressions of type I and III collagen

In normal skin tissues, the expression of type I and III collagen was rare, but increased after the trauma. After treatment, the expression of type III collagen in the experiment group was higher than that in the control group, while the expression of type I collagen was lower. After the cessation of therapy, the expression of type I and III collagen in the wound tissues of each group gradually declined. However, the decrease of collagen expression in the experiment group was more rapid, with the fastest decline observed in the VAC-120 mmHg group, followed by the VAC-180 mmHg group. Collagen expression of the experiment groups was normal by the 58th day, but the expression remained high until day 58 in the control group. In the early period of rehabilitation, the level of collagen type III positive expression in the VAC-120 mmHg group (**Figure 3C**) was higher than that in the control group (**Figure 3D**), but the

Table 1. Dynamic changes of TNF-α levels in the wounds of each group at different time points ($\bar{x}$±SD, ng/L, n = 5)

Time point (day)	VSD-120 mmHg	VSD-180 mmHg	VSD-240 mmHg	Control
0	192.76±5.46	201.19±3.70	200.92±3.77	201.76±3.21
1	235.86±6.48	221.49±3.19	218.25±3.77	208.92±3.13
3	257.47±12.6	241.69±3.10	230.05±3.26	215.51±2.89
5	344.15±2.14	323.63±7.25	305.39±8.90	250.58±2.72
7	263.36±5.91	273.40±2.30	280.45±7.08	290.24±4.54
9	245.05±9.66	250.43±9.21	269.94±8.54	286.82±3.45

The statistical analysis revealed that after the 5 day of treatment, there existed the statistical significance between the experiment group and the control group (P<0.01), as well as between the VAC-120 mmHg group and the VAC-240 mmHg group (P = 0.032), while no statistical significance between the adjacent VAC groups (i.e.: the comparison between the VAC-120 mmHg group and the VAC-180 mmHg group, P = 0.131; the comparison between the VAC-180 mmHg group and the VAC-240 mmHg group, P = 0.461).

Table 2. Dynamic changes of bacteria index in the wounds of each group at different time points ($\bar{x}$±SD, n = 5)

Time point (day)	VSD-120 mmHg	VSD-180 mmHg	VSD-240 mmHg	Control
0	7.49±0.21	7.44±0.19	7.44±0.27	7.41±0.24
1	7.01±0.07	7.40±0.09	7.45±0.12	8.19±1.04
3	6.13±0.12	6.64±0.05	6.81±0.11	8.93±0.17
5	4.15±1.09	4.72±0.13	5.29±0.17	7.57±0.29
7	3.02±0.19	3.32±0.15	4.03±0.31	7.13±1.06
9	2.23±0.09	3.12±0.24	3.79±0.12	6.41±0.11

The statistical analysis revealed that in the first 3 days of treatment, the difference of bacterial index between the experiment group and the control groups was not significantly different; while after 3 days treatment, the difference was statistically significant (P<0.01), as well as that between the VAC-120 mmHg group and the VAC-240 mmHg group (P = 0.047), while not significant between the adjacent VAC groups (P>0.05).

level of collagen type I positive expression in the VAC-120 mmHg group (**Figure 3A**) was less than that in the control group (**Figure 3B**).

Changes of tumor necrosis factor-α levels

As shown in **Table 1**, during treatment, the granule tissues of each group showed changes in TNF-α levels that exhibited a single-peak shape. Compared with the control group, the experiment group exhibited more rapid increases and decreases of TNF-α levels. The peak expression of TNF-α in the experiment group appeared not only earlier but also higher than that in the control group. In the experiment group, the peak TNF-α levels were observed on the 5th day of treatment; the peak TNF-α expression of the VAC-120 mmHg group was the highest, followed by the VAC-180 mmHg group, while the control group reached the peak on the 7th day of treatment. The statistical analysis revealed that after the 5th day of treatment, while no statistical significance between the adjacent VAC groups (P>0.05).

Dynamic changes in the bacterial index of each group

As shown in **Table 2**, the bacterial index of the experiment group showed a trend of rapid decline after treatment. Indeed, the bacterial index decreased most rapidly in the VAC-120 mmHg group, to less than 5 on the 5th day of treatment, followed by the VAC-180 mmHg group. Conversely, the bacterial index of the control group exhibited a rising trend in the first 3 days of treatment, which increased from 7.4 to 8.9, and slowly decreased 3 days later; however, the index remained high (up to 6.4) on the 9th day of treatment. Statistical analyses revealed that in the first 3 days of treatment, there were no significant differences in the bacterial index between the experiment groups and the control group; however, after 3 days of treatment, differences in the bacterial index between the groups were statistically significant (P<0.01), but not those between the different VAC groups (P>0.05).

Changes of bacteria in the wounds of each group

Before treatment, bacterial cultures showed the main bacteria in each wound to be *Proteus vulgaris*. One-to-two days later, the bacteria in the wound exhibited greater diversity, mainly sea-based bacteria. With respect to bacterial colony formation, the order of abundance was *Proteus vulgaris*, *Escherichia coli*, *Pseudomonas fluorescens*, *Klebsiella oxytoca*, and *Vibrio parahaemolyticus*, etc. With time, there

was a gradual increase in the abundance of pig skin parasites (e.g., *Staphylococcus aureus*, *Staphylococcus lentus*). Three days later, the count of sea-based bacteria in the experiment group was significantly reduced, while that of pig skin parasites had significantly increased. Five days later, the wounds in the experiment group contained only pig skin parasites. The trend in the control group was generally consistent with that in the experiment group, but the changes took place at a slower speed. Indeed, the control group exhibited mainly pig skin parasites on the 7th day of treatment, and this trend was consistent with the bacterial Gram staining results of the wound.

Discussion

Wound healing involved the interaction of multiple biological systems at the molecular, cellular, and organizational levels, and formed a series of complex dynamic pathophysiological processes, related with the local and systemic factors of the wound [11]. Generally, the skin soft tissues had no defect or the defects were minimal, thus, the wound could be directly sutured or performed after the subcutaneous sneak peel. The healing process tended to progress through three stages: 1) inflammatory exudation, 2) collagen formation, and 3) mature. The open wound had larger defects of skin soft tissues in the wound's cavity and surface, which could not be directly sutured, and the scar healing process was much more complex. It included not only the aforementioned pathological processes, but also the following aspects: formation of granule tissues, concentric contraction of wound edge, epithelial crawling, etc. [12-15]. We chose 4-to-6-month-old minipigs as the subjects, because the structure of their skin soft tissues was similar to that in humans [16, 17]. We ignored the individual differences, and mainly observed the effects of local disturbance factors towards wound healing. The extracellular matrix occupies a considerable proportion of the skin organization, and could affect the morphology, differentiation, migration, proliferation, and other functions of cells [18]. Collagens are the main components of the extracellular matrix, and are indispensable in wound healing. In normal skin, type I collagen accounted for about 80-90% of collagens, and type III collagen accounted for about 10-20%. During wound healing, the main process was observed as the deposition of fibroblasts, which then formed the type I and III collagens. Type I collagen mainly reflects the late stage of wound healing, while type III collagen mainly reflects the early stage of wound healing [19-21]. The amount of collagen synthesis could also reflect the extent of wound healing, because increased collagen synthesis could accelerate wound healing [22]. Generally, 5 to 6 days after injury, the fibroblasts synthesize collagen, one of a handful of proteins that contains the hydroxyl proline. Wang reported that VAC could improve the hydroxyl proline contents of blast-injury wounds and promote wound healing [23]. Shi et al. [24] reported that the VAC technology could reduce the activity of collagenase, thus, preventing collagen degradation, and promoting wound healing. In a study of Wistar rats' full-thickness skin wounds on the back, Ponrasu et al. [12] found that type I and type III collagen levels in the granule tissues were increased after injury. In the early stage of wound healing, the synthesis of type III collagen was relatively higher than that of type I collagen, and its synthesis was similar to that of type III collagen in the embryonic stage, which was closely related with conditions, such as edge contraction and wound epithelialization. Wounds with greater synthesis of type III collagen in the early stage had shorter healing time. Both domestic and international studies have successfully established a variety of animal post-trauma scar models, especially the pig skin hypertrophic scar model [13]. Combined with the results of this study, these findings suggest that after injury, the expression of type I and III collagens in the peri-wound tissues of each group were increased. In the early stage of wound healing, the expression of type III collagen in the peri-wound tissues of the VAC group was higher than the control group, among which the VAC-120 mmHg group exhibited the highest expression, followed by the VAC-180 mmHg group. However, the expression of type I collagen was lower in the experiment group than in the control group. In the late stage of wound healing, the expression of type I and III collagens in the peri-wound tissues of the VAC group approached normal levels, while the control group still exhibited higher expression. Combined with the peri-wound pathological findings of each group, the general observation of epithelialized wounds and the histopathological examination of peri-wound tissues were

normal, and the hair distribution was also normal. These findings might indicate that the healing process of SIBIW of pig skin soft tissues was similar to the non-scar healing process of embryonic skin; however, the mechanisms of the two processes are different. Satish and Kathju [25] found that the early inflammatory healing reaction of embryonic skin was low or absent, the peri-wound skin did not shrink or the shrinkage was not obvious. In the present study, we found that compared with the control group, the VAC group exhibited lighter wound inflammatory reactions, with shorter durations, obvious shrinkage of peri-wound tissues, and the healing time was shorter. Thus, our findings might indicate that the wound healing mechanism of pig skin exhibits certain differences compared to the non-scar healing process of embryonic skin and adult skin healing, and such differences might be related to pig skin not being prone to form scars after wound healing.

In summary: 1) During the treatment of SIBIW, the VAC technology had obvious advantages compared to the conventional medication therapy. The application of VAC technology in processing wounds should comprehensively consider the factors that resulted in the wounds, the local and systemic wound factors, and effects of the external environment, before selection of the ideal negative vacuum. In this research, the -120 mmHg pressure was the most ideal. 2) The experimental data were statistically processed: the comparison between the adjacent pressure groups (wound healing time, bacteria index, TNF-α level) had no statistical significance, which was considered to be related with an insufficient pressure span (60 mmHg) between the adjacent groups. Conversely, the absence of statistical significance between groups might be related to the relatively low number of experimental samples. 3) In this study, no additional pressures were set for the control group. The main reason was that the animal was the minipig, with limited surface skin; thus, the animal model exhibited a larger trauma area, resulting in their intolerance to further wounds.

Disclosure of conflict of interest

None.

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