Combined negative pressure wound therapy with open bone graft for infected wounds with bone defects: An experimental study

Ramesh Kumar Jha, Chengyan Xia, Zonghuan Li, Weiyang Wang, Kai Deng

ABSTRACT

Background: Bone and soft-tissue defects in infected wound have been an intractable problem to many surgical consultations. Infected wounds with bone defects are physical and financial burden to society. Nowadays, infected wounds with compound defect of bone and soft tissues are common in orthopedics department. Currently, no simple and efficient treatment has been found to solve this problem. This study investigates the effects of combining negative pressure wound therapy (NPWT) with open bone graft on this focus.

Materials and Methods: Twenty four rabbits with bone and soft tissue defects accompanied infected wounds were randomized into experimental (combined NPWT with open bone graft) and contrast group (only open bone graft). Treatment efficacy was assessed by the wound condition; wound healing time, bacterial bioburden, and bony callus were evaluated by X-ray. Furthermore, samples of granulation tissue from wounds on the 3rd, 7th, and 14th days of healing were evaluated for blood vessels and expression of vascular endothelial growth factor.

Results: Wounds in the experimental group tended to have shorter healing time, healthier wound conditions, lower bacterial bioburden, better bony callus, and more blood supply than those in the controlled group.

Conclusions: In conclusion, NPWT combined open bone graft can act as a feasible and valuable method to treat combined infected bone and soft-tissue defects.

Key words: Bone and soft-tissue defect, bone graft, infected wound, negative pressure wound therapy

MeSH terms: Grafting, bone, surgical wound infection, wound healing

INTRODUCTION

Infected wounds with bone defects are physical and financial burden to society. Nowadays, infected wounds with compound defect of bone and soft tissues are common in orthopedics practice. Currently, no simple and efficient treatment has been found to solve this problem. Fleischmann et al.1 introduced an open bone graft surgery, it has vastly shortened the course compared with traditional treatments. However, ensuing dressing changes could not control infection efficiently, especially for those free bone fragments without blood supply, which results in longer time for wound healing. Then, one-stage cancellous bone grafting method2 was applied to treat infected bone defects. This therapy union merges the bone graft and tissue flap, so the risks of surgery become a Gordian knot.

This study originated from the successful experience of negative pressure wound therapy (NPWT). Basic science studies of the benefits of NPWT mainly focus on the removal of edema, increasing blood supply, decreasing bacterial count, and fast formation of good granulation tissues.3 We combined NPWT with traditional bone graft to gain a new simple and efficient therapy. We analyzed animal models...
with bone and soft-tissue defects in infected wounds can be treated more effectively with NPWT than treated with traditional dressing changes. An infected wound model with radius and surface tissue defect in rabbit was made to analyze this new therapy. The local expression of vascular endothelial growth factor (VEGF), regarded as one of the most important molecules involved in the process of wound healing,\(^4\) was detected to confirm the increasing blood and the efficiency of NPWT.

**Materials and Methods**

**Animal model**
Twenty four rabbits (New Zealand) weighing 3.0–3.5 kg were procured from an approved animal care facility (The Center of Animal Experiments of Wuhan university). They were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (30 mg/kg). The forearm hair of all the rabbits was shaved off with 8% NAS solution before each surgical procedure. An incision, 2.5 cm in length including skin and subcutaneous tissue, was made in the middle of the radial side of each forearm. Radius was partially amputated with a length of 1 cm in the middle [Figure 1]. Then, sufficient autologous ilium was taken and grafted into the defects. The wounds were inoculated with *Staphylococcus aureus* (1 × 10\(^6\)/ml) to develop infected wounds. After 3 days, all wounds were debrided and bacterial counting test was performed to determine whether the model was success or not. Two forearms were randomized to be treated by either experiment group or control group. Wounds of the rabbits in the experiment group were treated with vacuum-sealing drainage (VSD) foam (VSD Inc., Wuhan, Hubei province, China). The value of negative pressure was –75 mmHg. Wounds in the contrast group were covered by conventional gauze. The day when this surgery was performed was defined as day 0. All dressings were renewed on days 3, 7, and 14, and granulation tissue with a volume of 2 mm × 5 mm × 10 mm was harvested under aseptic conditions and then divided in triplicate. The triplicate was then analyzed for bacterial counting immediately, stored in −80°C for Western blot analysis, and immersed in 4% paraformaldehyde for immunohistochemical (IM) analysis.

**Bacterial counting**
The samples were immediately weighed, cut and then homogenized and diluted. Five microliter diluents were placed on an agar plate. The dilutions were placed on normal agar and incubated at 37° with 5% CO\(_2\) for 48 h. The number of bacteria in each wound was calculated by the colony-forming units (CFUs) on each plate.

**X-ray imaging**
Both upper extremities’ lateral film was performed in each rabbit on the 0, 7\(^{th}\), 14\(^{th}\), 21\(^{st}\), and 28\(^{th}\) days. The fracture condition and the healing rate of fracture on the 28\(^{th}\) day were recorded.

**Immunohistochemical analysis**
All samples fixed in 4% paraformaldehyde were embedded in paraffin and sectioned 4 μm routinely. Staining was performed by SABC method. Primary rabbit antiporcine monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:1000) with primary polyclonal was applied to the sections and incubated for 1 h at room temperature, rinsed again with PBS in triplicate, and then the sections were incubated with fluorescein isothiocyanate or rhodamine-conjugated secondary antibody (Santa Biotechnology Inc.,) for 30 min. Antibodies were visualized by treating with avidin–biotinylated enzyme complex, and then with peroxidase substrate solution for 2 min. The positively stained micro-blood vessels were counted in the most vascularized area on each section. In brief, this method involves scanning tissue sections under high magnification to identify the hotspot. Within the hotspot, the number of vessels in a high-power field of ×200 over six nonoverlapping areas was counted.

**Western blot analysis**
All samples were homogenized adequately in buffer with an added protease inhibitor cocktail (Roche Inc., Switzerland), 10 mM NaCl, 1% NP40, 0.02% sodium azide, and 50 mM Tris. Homogenates were then centrifuged at 12,000 rpm for 10 min at 4°C. Supernatant was stored in −20°C before use. The volume of loading sample was 50 μg, and the proteins separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis were then transferred to polyvinylidene difluoride membrane. Membranes were blocked with 5% milk at 37°C for 1–2 h, and then incubated by the shaker for 2 h. The membranes were incubated with a primary goat-anti-rabbit antibody at 4° overnight for either VEGF (Santa Biotechnology Inc., 1:200) or β-actin (Santa Biotechnology Inc., 1:10000) as a loading control. Blots were then washed for 5–10 min with

![Figure 1: The model of bone and soft-tissue defect in rabbit](image)
Tris-buffered saline containing 0.05% Tween-20 (TBST) for several times. After washes, the blots were incubated for 1 h with a horseradish-peroxidase (HRP)-coupled secondary antibody, and then washed again with TBST. Blots were incubated with a luminescent HRP substrate for 1 min till positive signals were detected. A high resolution flatbed scanner (Hewlett Packard Company, Palo Alto, CA, USA) and Image J densitometry software were used for performing densitometric quantification of the immunoblot bands. The optical densities of at least three replicates of each sample were quantified. Samples were stored at −80°C before use.

**Statistical analysis**

All data were collected as the mean ± standard deviation. The values were compared between NPWT group and gauze-covering group using Student’s *t*-test. The significant of *P* values was fixed at 0.05 for the statistical analysis.

**RESULTS**

**Wound condition**

On days 3, 7, 14, and 21 after the surgical procedure, wounds in the experiment group were cleaned and dried with less exudate; also the quality of granulation tissue was better in the NPWT group [Figure 2]. The wound healing rate increased significantly on every recorded time and wound healing time (21.6 ± 3.7 vs. 29.5 ± 3.9) reduced in experimental group as shown in Figure 3 and Table 1.

**Bacterial colonies’ counts**

Tissues harvested from the center of wounds were cultured and analyzed after every covering was changed. *S. aureus* with density more than $1 \times 10^5$ CFU/ml was detected in all wounds on day 0, and the differences of the two groups had no statistical significance ($P = 0.44$). This suggested that the infected wound model was successfully established. However, on days 3, 7, and 14, bacterial count in gauze group (control group) was significantly more than NPWT, $P = 0.004$, 0.001, and 0.001, respectively [Figure 4 and Table 2].

**Immunohistochemical analysis**

On days 3, 7, and 14, microvessels of wounds were counted by IM staining of CD34 molecule [Figure 5].

### Table 1: The comparison of the wound healing rate

| Group     | Wound healing rate (%) | Days needed for wound healing |
|-----------|-------------------------|------------------------------|
|           | Day 3 | Day 7 | Day 14 | Day 21 |                              |
| NPWT group| 17.2±8.5 | 30.5±6.8 | 69.4±14.6 | 89.5±16.2 | 21.6±9.7                     |
| Gauze group| 13.1±5.1 | 27.3±7.9 | 58.3±11.9 | 76.1±22.1 | 29.5±8.9                     |
| *P*       | 0.001 | 0.005 | 0.002 | 0.007 | 0.008                        |

NPWT = Negative pressure wound therapy

**Figure 2:** (a and b) Wound surface on the 7th day for vacuum-assisted closure group and gauze group (control group), respectively

**Figure 3:** A bar diagram showing (a) The comparison of the wound healing rate in both groups. On the 3rd, 7th, 14th, and 21st day, (b) the time needed for wound healing in vacuum-assisted closure group was less than the gauze group
The results showed that the number of microvessels in NPWT group was higher than that in gauze group during the whole process. Moreover, the number of microvessels increased over time. And, the difference was statistically significant ($P = 0.012, 0.007, \text{and } 0.011$ on days 3, 7, and 14, respectively).

**Western blot for vascular endothelial growth factor expression**

Higher expression of VEGF was observed in wound treated by NPWT compared to gauze group on every detected point. The values were all significantly different ($P < 0.003$ all) [Table 3]. On the 7th day of experimental group, VEGF expressed the highest level but gauze group had the lowest at the same time [Figure 6].

![Figure 4: A bar diagram showing bacterial counting of wounds at different time points](image)

**Table 2: Bacterial counting of wounds at different time points**

| Group               | Day 0 bacterial count (CFU/ml) | Day 3 bacterial count (CFU/ml) | Day 7 bacterial count (CFU/ml) | Day 14 bacterial count (CFU/ml) |
|---------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| NPWT group          | $12.5\times10^5\pm3.5$        | $3.7\times10^5\pm2.1$        | $0.8\times10^5\pm0.3$        | $0.04\times10^5\pm0.02$       |
| Gauze group (control)| $12.9\times10^5\pm2.7$        | $9.8\times10^5\pm1.7$        | $2.3\times10^5\pm1.2$        | $0.58\times10^5\pm0.12$       |
| $P$                 | 4.48                          | 0.004                         | 0.001                         | 0.001                         |

NPWT=Negative pressure wound therapy, CFU=Colonies-forming unit

**X-ray examination**

The fracture healing processes for all rabbits were monitored by X-ray examination on the 0, 7th, 14th, 21st, and 28th days [Figure 7]. The results showed that bone callus growth in experimental group was much better than contrast group. On the 28th day, the healing rate of NPWT was 50% (6/12), which was much higher than 8.3% (1/12) in gauze group.

**DISCUSSION**

Management of bone defect that occurs after severe trauma of open fractures continues to challenge orthopedic surgeons. Our study reported the method of NPWT combined with open bone graft treated for defects of both bone and soft tissue in infected wound in an animal model. We confirm that it is a feasible treatment reflected by a significant reduction of healing time, eliminating bacteria, increasing microvascular vessels, and better bone healing. Rhinelander and Papineau first reported that open cancellous bone grafting could be applied to patients with bone defect. However, this surgery cannot be performed when an infected wound with accompanying soft-tissue defect occurs. The traditional treatment was debridement and conventional gauze dressing changing until the clearance of infection and healing of wound, and then open bone graft as a second-stage repair. This method generally needs more than 6 months of hospital stay. Although composite tissue flap transplantation could achieve this goal in one stage, this surgery is too difficult to be popularized and also flap harvest from donor site...
additionally damage the patient. Hence, this study found a new method for this troublesome illness.

NPWT had shown a significant effect in facilitating wound healing, especially for wounds that are difficult to treat.6 This device consists of VSD foam that is placed on the wound and covered with an occlusive dressing.7 The mechanisms of NPWT contain restraining bacterial growth, eliminating infection, completing wound debridement, reducing the time needed to produce a healthy granulation bed and increasing wound contraction, removing edema, increasing blood flow, promoting granulation tissue growth, influencing expression of endogenous epidermal cell growth factor, enhancing the expression of calcitonin gene, and substance P secretion on peripheral nerve endings.8-10 In our study, wounds of NPWT group had a better granulation tissue condition and less healing time. Wound healing rate was significantly increased compared to gauze group at every recorded time.

Diminishing bacteria of infected wound is necessary for wound healing process. Whether NPWT is useful in reducing bacteria in infected wound is still debatable. In Gabriel et al’s.11 clinical studies, NPWT could decrease the bacterial number in infected wound, but on the contrary, Assadian et al.12 reported that the bacterial number has no difference between NPWT and without negative pressure in an in vitro wound model. In our study, bacteria in NPWT group were removed actively and infection was controlled effectively in the experiment group. Because of mechanical suction, mononuclear cells and neutrophils are migrating earlier into wound, causing local inflammatory cells against inflammation rapidly.

In our study, IM results showed that blood flow was increased after NPWT and the number of microvessels increased over time during NPWT. To explore whether it was exactly the mechanism in this study, Western blot of VEGF was performed. In all time points we detected, the expression of VEGF was higher in experiment group than that in gauze group, this was in line with our earlier reports of a swine experimental model.13 NPWT can result in cellular strain and microdeformations,14 which can induce cellular proliferation and angiogenesis in wound. VEGF, which is a mitogenic factor acting on endothelial cells, plays a crucial role in vasculogenesis and angiogenesis,15 and it is regarded as one of the most important molecules involved in the process of angiogenesis.4 VEGF receptor remains only in vascular endothelial cells, so VEGF is the key factor for the formation of blood vessels, increasing the number of capillaries and promoting vascularization in wound. Labler has reported that VEGF in wound was significantly higher in patients treated by NPWT than that treated by gauze.17 In our view, increasing blood flows also contribute to eliminate infection.

Researches of NPWT on fracture or bone healing were seldom reported, for that NPWT has no influence on bone healing. Recently, Babjak18 has a report that NPWT combined with recombinant human bone morphogenetic protein 7 can accelerate bone healing. However, in our
study, bone healed better on NPWT group and needed less time compared to gauze group. We conjecture that it was related to the following cause. Autogenous cancellous bone with a diameter <0.5 cm can survive easily due to its large specific surface area, which help gain more nourishment connections with the surrounding tissues. Then, under negative pressure, local blood flow accelerated and blood supply increased relatively, the ability of anti-infection was enhanced, and mechanical suction enhanced the assimilation of sequestrum, all these facilitated the healing process of bone.

A limitation of our study was that we failed to record the exact time needed for bone healing of all animal models which is largely because of taking X-ray images everyday on all animal was nearly impractical for both the rabbits-self and our device configuration. Furthermore, the conclusion of our study was only based on a rabbit model, whether this method could achieve the same result in human remains unknown, so further study needs to be conducted to clarify this problem.

Financial support and sponsorship
This work was financed by Province Science Funds of Hubel, China (Project code: 2012FFB04311).

Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. Fleischmann W, Strecker W, Bombelli M, Kinzl L. Vacuum sealing as treatment of soft tissue damage in open fractures. Unfallchirurg 1993;96:488-92.
2. Lei H, Yi L. One-stage open cancellous bone grafting of infected fracture and nonunion. J Orthop Sci 1998;3:318-23.
3. Arslan E, Ozturk OG, Aksoy A, Polat G. Vacuum-assisted closure therapy leads to an increase in plasma fibronectin level. Int Wound J 2011;8:224-8.
4. Kadowaki I, Ichinohasama R, Harigae H, Ishizawa K, Okitsu Y, Kameoka J, et al. Accelerated lymphangiogenesis in malignant lymphoma: Possible role of VEGF-A and VEGF-C. Br J Haematol 2005;130:869-77.
5. Papineau IJ, Alfageme A. Chronic osteomyelitis of long bone resection and bone grafting with delayed skin closure. J Bone Joint Surg (Br), 1976;58:138.
6. de Laat EH, van den Boogaard MH, Spauwen PH, van Kuppevelt DH, van Goor H, Schoonhoven L. Faster wound healing with topical negative pressure therapy in difficult-to-heal wounds: A prospective randomized controlled trial. Ann Plast Surg 2011;67:626-31.
7. Armstrong DG, Lavery LA, Abu-Rumman P, Espensen EH, Vazquez JR, Nixon BP, et al. Outcomes of subatmospheric pressure dressing therapy on wounds of the diabetic foot. Ostomy Wound Manage 2002;48:64-8.
8. Webb LK, Pape HC. Current thought regarding the mechanism of action of negative pressure wound therapy with reticulated open cell foam. J Orthop Trauma 2008;22 10 Suppl:S135-7.
9. Chen SZ, Li J, Li XY, Xu LS. Effects of vacuum-assisted closure on wound microcirculation: An experimental study. Asian J Surg 2005;28:211-7.
10. Torbrand C, Wackenfors A, Lindstedt S, Ekman R, Ingemansson R, Malmsjö M. Sympathetic and sensory nerve activation during negative pressure therapy of sternotomy wounds. Interact Cardiovasc Thorac Surg 2008;7:1067-70.
11. Gabriel A, Shores J, Bernstein B, de Leon J, Kampealli R, Wolvos T, et al. A clinical review of infected wound treatment with vacuum assisted closure (V.A.C.) therapy: Experience and case series. Int Wound J 2009;6 Suppl 2:1-25.
12. Assadian O, Assadian A, Stadler M, Diab-Elschahawi M, Kramer A. Bacterial growth kinetic without the influence of the immune system using vacuum-assisted closure dressing with and without negative pressure in an in vitro wound model. Int Wound J 2010;7:283-97.
13. Zhou M, Yu A, Wu G, Xia H, Xu X, Qi B. Role of different negative pressure values in the process of infected wounds treated by vacuum-assisted closure: An experimental study. Int Wound J 2013;10:508-15.
14. Scherer SS, Pietramaggiore G, Mathews JC, Prsa MJ, Huang S, Orgill DP. The mechanism of action of the vacuum-assisted closure device. Plast Reconstr Surg 2008;122:786-97.
15. Lizarov GA. The tension-stress effect on the genesis and growth of tissues: Part II. The influence of the rate and frequency of distraction. Clin Orthop Relat Res 1989;239:263-85.
16. Ferrara N. Molecular and biological properties of vascular endothelial growth factor. J Mol Med (Berl) 1999;77:527-43.
17. Labler L, Rancan M, Mica L, Härter L, Mihic-Probst D, Keel M. Vacuum-assisted closure therapy increases local interleukin-8 and vascular endothelial growth factor levels in traumatic wounds. J Trauma 2009;66:749-57.
18. Babiak I. Open tibial fractures grade IIIC treated successfully with external fixation, negative-pressure wound therapy and recombinant human bone morphogenetic protein 7. Int Wound J 2014;11:476-82.
19. Burchardt H. The biology of bone graft repair. Clin Orthop Relat Res 1983;174:28-42.