

Vacuum-assisted Closure Therapy Attenuates the Inflammatory Response in a Porcine Acute Wound Healing Model

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Porcine full-thickness wounds were treated with V.A.C.® Therapy (KCI, San Antonio, Tex) or moist wound dressing without negative pressure. V.A.C. Therapy related systemic effects included post-wound reduction in the number of peripheral blood monocytes and neutrophils during the inflammatory phase of wound healing, as well as reduced serum levels of the pro-inflammatory cytokines IFN-g and IL-6. Local effects included reduced concentrations of IL-8, TGF-b1, and TNF-a in wound fluid following V.A.C. Therapy. Collectively, these responses suggest that V.A.C. Therapy may attenuate the pro-inflammatory response following cutaneous wounding.

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Initial injury to the skin triggers coagulation and an acute inflammatory response followed by cell recruitment, proliferation, and matrix synthesis. Disruption in any of these stages of the healing response can either delay or significantly impair wound healing. For example, failure to resolve inflammation can lead to chronic nonhealing wounds, whereas uncontrolled matrix accumulation, often involving aberrant pathways, can lead to excess scarring and fibrotic sequelae. Pro-inflammatory cytokines, growth factors, and proteases have been implicated as key regulators of wound healing.^{1,2} By investigating the complex roles of biomolecules in wound healing, the pathways that result in their inhibition or enhancement can be better understood and wound healing better controlled or modulated.

Vacuum-assisted Closure® (V.A.C.® Therapy, KCI, San Antonio, Tex), also referred to as V.A.C.® Brand Negative Pressure Wound Therapy (NPWT), is a relatively new and increasingly established treatment for promoting wound healing.³⁻⁵ Considerable interest has centered on elucidating the mechanism of action for this treatment modality. Early clinical evidence that NPWT affects the amount of cytokines and proteases in wounds includes altered expression of genes for inflammatory cytokines,⁶ and reduced matrix metalloproteases.⁷⁻⁹ More recently, it has been shown that levels of tumor necrosis factor-alpha (TNF-a), a pro-inflammatory cytokine, decreased in wound fluid from adults with chronic pressure ulcers treated with NPWT over a 7-day period.⁸

Application of NPWT to full-thickness excisional wounds in young, otherwise healthy swine has been shown to accelerate the rate of granulation tissue formation, allowing for earlier wound closure.^{10,11} Preliminary studies in swine have indicated that the levels of selected cytokines are altered by NPWT,^{12,13} suggesting that this treatment may have systemic effects on cytokine levels in the blood. Immediate use of NPWT on pigs with large scald burns (> 15% body surface area) prevented the typical post-burn decrease in mesenteric artery blood flow, which correlated with changes in serum concentrations of IL-6 and IL-8 over the first 4 hours after wounding.¹² More recently, Kilpadi et al¹³ reported that increases in serum levels of IL-10 occurred within the first 2 hours following creation of an excisional wound, whereas IL-6 levels were maintained throughout the 4 hours of treatment with NPWT.

In the present study using a porcine wound healing model, the potential systemic effects of NPWT were measured between 12 and 180 hours after wounding. The primary focus of this work was to examine the effect of NPWT on the pro-inflammatory phase of wound healing. Levels of selected pro-inflammatory cytokines, namely interferon-gamma (IFN-g), IL-1b, IL-4, IL-6, IL-8, transforming growth factor-beta1 (TGF-b1), and tumor

necrosis factor-alpha (TNF-a), in serum and in wound fluid were assessed in this study in an effort to characterize how NPWT may attenuate the acute pro-inflammatory response.

Table 1

Group	Treatment	Number of Swine	Wound Site 1	Wound Site 2
1	Control	4	Quilten (Q2) P Tenderloin (T2)	Quilten (Q2) P Tenderloin (T2)
2	Experimental	4	Quilten (Q2) P Tenderloin (T2)	Quilten (Q2) P Tenderloin (T2)

Quilten (Q2) P and Tenderloin (T2) were provided by KCI, Inc. (San Antonio, TX). Quilten (Q2) P and Tenderloin (T2) were provided by KCI, Inc. (San Antonio, TX). Quilten (Q2) P and Tenderloin (T2) were provided by KCI, Inc. (San Antonio, TX).

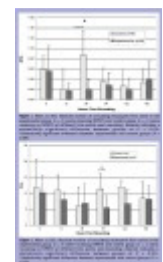
Materials and Methods

This study was performed with approval of the animal care and use committee. Ten domestic Yorkshire swine, 3–6 months old, weighing approximately 40–60 kg each, were quarantined for 4–7 days before placement on study. Food and water was available ad libitum, and the room temperature and humidity were monitored daily. The swine were premedicated with glycopyrrolate (0.01–0.02 mg/kg) administered intramuscularly (IM), then anesthetized with Telazol® (3.0–5.1 mg/kg) and xylazine (1.6–2.3 mg/kg) IM. Following intubation, a surgical plane of anesthesia was achieved with isoflurane. Each anesthetized animal was placed in a sling for the procedure. The hair was removed from the entire back, and 2 paraspinal surgical sites were outlined by tattoo using a template 5 cm in diameter. An intravenous (IV) catheter was placed, and lactated Ringer’s solution was administered intravenously at an appropriate rate to support intra-operative blood pressure. During the procedure, blood pressure and pulse oximetry were monitored. Heart activity was monitored using electrocardiogram.

The area was disinfected by sequential scrubbing for at least 4 minutes with Betadine® scrub. The Betadine was allowed to air dry and was then removed with 70% isopropyl alcohol. The area was draped using aseptic surgical techniques. Two full-thickness excisional wounds (5 cm diameter each) were created between the crest of the shoulders and the coccygeal tuberosity by vertically excising the skin immediately interior to the tattoo markings. An incision was made down to the first layer of adipose tissue above the dorsal muscle, and the dermal tissue was removed. Direct pressure was used to obtain hemostasis, after which photographs of the wound were taken.

The study was designed in order to assess both systemic and local effects of NPWT. Animals were assigned to either an experimental or control group as shown in Table 1. One of the wounds in the experimental group was treated with NPWT, consisting of GranuFoam® Dressing and T.R.A.C.® Pad Connector with tubing (KCI, San Antonio, Tex), to which the V.A.C. Brand ATS® System (KCI, San Antonio, Tex) was connected and set to deliver 125 mmHg of continuous negative pressure to the wound (designated as the “EVAC” site). The contralateral wound in the experimental group received DuoDERM® CGF (ConvaTec, Skillman, NJ), a moist wound dressing (MWD), and was designated as the “EC” site. For the control group, both wounds received the MWD and were designated as the “CC” site. None of the wounds treated with MWD received negative pressure treatment. A flexible, protective cover was applied over the wound dressings, and secured in place with V.A.C.® Drape (KCI, San Antonio, Tex) and surgical tape. Animals that received NPWT were maintained on treatment by securing the pump to a customized aluminum turntable placed above the cage, allowing the animals to move freely within their cages.

Figures 1-2



Animals were allowed to recover from anesthesia and buprenorphine was administered IM at 0.01–0.02 mg/kg immediately prior to recovery from anesthesia. Additional buprenorphine was administered IM at 0.01–0.03 mg/kg over the next 24–48 hours.

Sample collection and testing. Dressings were changed at 12, 36, 84, and 132 hours after wound creation. These time points corresponded to specimen collection times predetermined to target the early and later stages of the inflammatory phase of wound

healing. Prior to dressing changes, animals fasted a minimum of 12 hours. Animals were premedicated and intubated as previously described, and maintained on oxygen and isoflurane. Each animal was placed in a sling and the overlying bandage and any other overlying dressings were removed. Wound fluid was collected from control sites with a 10-mL syringe and 18 gauge needle inserted through the dressing. For wound sites that received NPWT, wound fluid was collected directly from the canister. Protease inhibitor was added to all fluid, which was snap frozen in liquid nitrogen and stored at -70°C to -80°C . All wound sites were visually monitored during the course of the study for erythema, edema, percent (%) granulation, and re-epithelization, and photographed using a Nikon Coolpix 4100 digital camera. A ruler and an identification label were positioned adjacent to the wound to use as references. Digital images were viewed within SigmaScan Pro® Version 5.0 (SPSS Inc, Chicago, Ill) and wound area was determined by tracing the wound circumference using the computer mouse. Each image was calibrated by measuring a distance of 1 cm on the ruler in each image. The skin surface was shaved as necessary, and the wounds were re-dressed and treatment re-initiated.

Blood was collected either in tubes containing EDTA (for counting circulating blood cells) or in tubes containing no additives for processing of serum for cytokine analysis. Analysis of blood samples was performed at Laboratory Corporation of America (San Diego, Calif). Serum and wound fluid were snap frozen in liquid nitrogen and shipped to Bio-Quant, Inc. (San Diego, Calif) for analysis in triplicate of the following pro-inflammatory cytokines: IFN-g, IL-1b, IL-4, IL-6, IL-8, TGF-b, and TNF-a. These samples were tested using customized enzyme-linked immunoassays developed specifically for porcine analytes by Bio-Quant, Inc.

Necropsy. The study was terminated at 180 hours post-wounding. Blood and wound fluid collection and wound observations were performed. Animals were then administered IV Euthasol (390 mg/mL pentobarbital and 50 mg/mL phenytoin) at a dose of at least 1 mL per 10 lb.

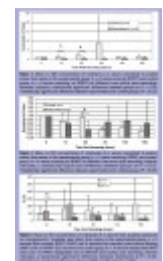
Statistical methods. Granulation, erythema, and edema results were analyzed using the Wilcoxon Rank Sum Test, and repeated measures ANOVA was used for wound area data. Univariate and 1-factor ANOVA were performed on the absolute numbers of neutrophils and monocytes and on cytokine data. The mean value for serum cytokines at each time point post-wounding was first normalized to the mean pretest value. Univariate analysis was performed on the \log_{10} transformed cytokine data by parameter, treatment group, and observation time. The probability value reflects a difference from zero; consistent with $\log_{10}[1] = 0$. P values < 0.05 were considered significant, while values of $0.05 \leq P < 0.10$ were considered highly suggestive.

Results

The systemic effects of NPWT were examined by comparing the results of the blood counts and serum cytokine levels between the experimental and control groups with the assumption that any differences between these 2 groups would be attributable to the effect of NPWT, since both groups had similarly treated control sites. Local effects of NPWT were examined by comparing the results of cytokine levels in wound fluid from EVAC versus EC sites, and EVAC versus CC sites. In addition, any significant differences in cytokine levels between EC and CC wound sites might suggest there is a systemic effect of NPWT on the contralateral wound in the experimental group.

Wound assessment. Wound area for the EVAC sites was significantly less than EC sites at 84, 132, and 180 hours and CC sites at 36, 84, 132, and 180 hours post-wounding

Figures 3-5



($P < 0.05$; data not shown). No differences were seen in wound area between the control wound sites. All wound sites reached approximately 50%–100% granulation on the day of necropsy, and epithelization, as visually assessed during dressing changes, was $< 15\%$.

Leukocyte numbers. Statistically significant ($P < 0.05$) differences between the experimental and control groups were seen in the mean number (\pm SD) of circulating monocytes at 36 hours post wounding (0.2 ± 0.2 versus 0.9 ± 0.5 per nL, respectively; Figure 1) and neutrophils at 84 hours post wounding (5.4 ± 3.6 versus 13.6 ± 6.1 per nL; Figure 2), with the values for the experimental group being consistently lower than those for the control group. At all other time points tested, the values for both groups were similar.

Cytokine analyses. Since this study involved the comparison of 2 different treatment modalities, namely V.A.C.® Therapy (NPWT) versus DuoDERM®, the data for sera were analyzed by treatment group—experimental versus control—while wound fluid data were analyzed by wound site (EVAC versus EC, EC versus CC, and CC1 versus CC2). It was not deemed feasible to normalize values for EVAC wounds to control wounds because the individual treatments were unique and did not share any similarities either in material properties or NPWT. No sham controls were used in this study.

Serum. Significantly lower serum levels of IFN-g were seen at 12 and 36 hours ($P < 0.05$; Figure 3) for the experimental group compared to the control group. Serum IL-6 levels were also significantly decreased in the experimental group at 36 hours ($P < 0.05$ [1-factor ANOVA]; Figure 4) relative to the control group. At this time, the mean level of IL-6 in the experimental group had decreased more than 50% below baseline (pretest, $P = 0.055$ [univariate analysis]). However, beginning at 84 hours post wounding the mean IL-6 level for the control group had fallen below 50% of baseline ($P = 0.015$), while the mean level of IL-6 in the experimental group had returned to approximately the pretest level ($P > 0.10$). No significant differences were seen between the control and experimental groups for IL-1b, IL-4, IL-8, TGF-b, or TNF-a at any of the time points tested (data not shown).

Wound fluid. Lower levels of IL-8 were seen in EVAC wound fluid compared to EC wounds at all time points tested. However, these results were statistically significant only at the 12-hour time point (6.1 ± 8.2 g/mL for EVAC versus 133.6 ± 133.4 g/mL for EC, $P < 0.05$; Figure 5). The concentration of TGF-b was significantly lower in EVAC wounds relative to the EC wounds at 132 (507 ± 599 pg/mL versus 1120 ± 869 pg/mL, respectively) and 180 hours (1208 ± 1305 pg/mL versus 3236 ± 3472 pg/mL, respectively) post-wounding ($P < 0.05$; Figure 6). In addition, a highly suggestive decrease in TNF-a was also seen in EVAC wound sites (30 ± 16 pg/mL) compared to EC sites (250 ± 64 pg/mL) at 180 hours post-wounding ($P = 0.055$; Figure 7). No differences were seen between the control and experimental groups for IFN-g, IL-1b, IL-4, or IL-6 at any of the time points tested (data not shown).

Discussion

The results of this study support the hypothesis that NPWT (V.A.C.® Therapy) attenuates the early inflammatory response in a porcine acute wound healing model. Wound healing is a complex process characterized by a series of overlapping phases, namely inflammation, proliferation, and remodeling. The porcine full-thickness acute wound healing model is considered the model of choice by most investigators,¹⁴⁻¹⁶ since healing can be monitored by a variety of endpoints. However, the granulation tissue response in young, healthy swine is robust, and the rapid healing makes it rather difficult to determine differences between treatment groups.¹⁷ Therefore, a strict, standardized protocol must be followed in order to derive the most meaningful interpretation when using this model. This study employed a rigorous timetable for dressing changes, clinical assessment of wound healing, and collection of blood and wound fluid specimens.

During the early inflammatory phase of wound healing, cytokines and chemokines play

an important role in the recruitment of leukocytes (ie, neutrophils and monocytes), and stimulation of fibroblasts and epithelial cells. For example, TNF promotes neutrophil release from the marrow and stimulates expression of endothelial leukocyte adhesion molecules, enhancing neutrophil recruitment to the area of injury.^{18,19} IL-8 is a chemokine that attracts neutrophils,²⁰ while other chemokines act on monocytes and macrophages.^{21,22} In addition, MCP-1 (monocyte chemoattractant protein) is a chemokine with potent macrophage recruitment and activating functions.^{23,24} In this study, there was no apparent correlation between serum levels of either TNF- α or IL-8 and decreased numbers of circulating neutrophils and monocytes.

While inadequate inflammation can impair wound healing, increased severity, duration, and intensity of inflammation have been associated with scarring.²⁵⁻²⁸ Neutrophils are first to arrive at the site of injury, followed by macrophages that have differentiated from blood-borne monocytes.^{29,30} The main function of neutrophils is to decontaminate the open wound by destroying invading microbes.³¹ After neutrophils complete their task, tissue repair and restoration may commence. Therefore, it is critical for neutrophils to undergo apoptosis and be removed via phagocytosis by macrophages.³² Persistence of neutrophils may lead to over-production of bioactive substances (eg, proteases that cleave extracellular matrix proteins). In a genetically diabetic murine model, it has been shown that depletion of neutrophils significantly accelerates wound re-epithelization, suggesting that neutrophils can delay normal wound closure.²⁰ Macrophages are generally believed to play a positive role in augmenting wound healing because of their ability to release cytokines and growth factors that in turn influence a variety of other cells. Among the major bioactive molecules produced by macrophages are TNF- α , IL-1, IL-6, IL-8, IFN- γ , IL-10, TGF- β 1, platelet-derived growth factor, fibroblast growth factor, insulin-like growth factor, and epidermal growth factor.³⁰ As can be readily discerned, unless carefully controlled, macrophage production of pro-inflammatory cytokines and growth factors can be detrimental to wound repair.

The decreased numbers of circulating neutrophils and monocytes in the experimental group where 1 wound received NPWT compared to the control group of animals whose wounds received only MWD suggests an attenuation of the pro-inflammatory response involving these 2 cell types by NPWT. In fact, it appeared that the control group showed a rebound effect at 36 hours post wounding for monocytes and later for neutrophils, whereas no such effect was observed for the experimental group. Since 1 study showed that NPWT reduced the number of neutrophils in partial-thickness and burn wounds,³³ it would be interesting to determine the relative number of neutrophils and monocytes in the wound at various time points during healing.

The significantly lower numbers of circulating neutrophils and monocytes seen in the NPWT group compared to the control group could be explained by an increased mobilization of these cells into the injured tissue due to the presence of the chemoattractants IL-8 or MCP-1.³⁴ Studies are planned to determine the levels of TNF- α and IL-8 in wound tissue that could account for the mobilization of neutrophils. Support for a causal relationship was observed when monocytes isolated from the NPWT group were capable of releasing IL-8 upon stimulation with lipopolysaccharide (LPS), a bacterial endotoxin (K. Norbury, unpublished data, 2006). Therefore, this study suggests that circulating monocytes transmigrate from circulating blood into wounds where they become macrophages activated to release IL-8, which in turn attracts neutrophils into the wound. The domestic swine used in this study were not certified to be pathogen-free, thus it is not unreasonable to presume that bacterial LPS might have been present in the wounds. In addition, the reduction in circulating monocytes in the NPWT group may be due to the higher motility of monocytes in the experimental group compared to the control group, or continued promotion of monocyte chemotaxis into the site of injury. Alternatively, NPWT may mitigate the production or effect of granulocyte colony stimulating factor (CSF) and/or granulocyte-macrophage CSF, which mobilize these inflammatory cells from the bone marrow,^{35,36} perhaps through the effects of

IL-10.³⁷ Additional studies will be necessary to confirm these hypotheses, including whether NPWT may cause a reduction in PMN oxidative burst.

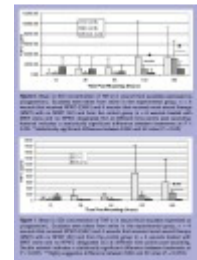
The analysis of serum cytokines likewise revealed a decrease in the pro-inflammatory cytokines IFN- γ and IL-6 in the sera of NPWT-treated animals compared to controls. Interferon- γ has been shown to play a role in the regulation of the immune response³⁸ by activating macrophages during inflammation³⁹ and inhibiting TH2 CD4+ cells.⁴⁰ It has been shown in mice that TH1-mediated inflammation is characterized by tissue damage, while TH2 directs wound healing.⁴¹ More work is needed to determine whether NPWT may shift the balance away from the TH1 phenotype in blood in favor of the TH2 phenotype. The data for serum IL-6 support the previous findings that NPWT reduced the level of IL-6 in the systemic circulation post burn in swine¹³ and in chronic wounds found in humans.^{42,43} Whereas interleukins, such as IL-1 and IL-10 are seemingly pleiotropic in their effects, IL-6 may be considered the prototypic pleiotropic cytokine.^{44,45} IL-6 plays a crucial role in wound healing, as demonstrated in knockout mice by delayed reduction in wound area with attenuated leukocyte infiltration, re-epithelization, angiogenesis, and collagen accumulation.⁴⁶ IL-6 exerts its biological activities through interaction with specific receptors expressed on the surface of target cells.⁴⁷ Such activation renders cells capable of responding to IL-6-receptor complexes, resulting in diverse activities ranging from control of immune response to involvement in pathological states.⁴⁸ Therefore, the possible influence of NPWT on IL-6 could be important during the course of wound healing in controlling against the negative effects of this inflammatory mediator. It would be important for future studies to analyze IL-6 levels in wound tissue.

Analysis of human wound fluid from healing wounds and chronic ulcers has shown that nonhealing wounds have high concentrations of certain cytokines (eg, pro-inflammatory).^{1,49} Therefore, it is generally accepted that lowering the levels of pro-inflammatory cytokines in wounds can lead to a reversal from a nonhealing to healing paradigm. Differences between wounds treated with NPWT and control wounds receiving a moist wound dressing without negative pressure treatment (MWD, DuoDERM alone) were determined in this study by measuring cytokine levels in wound fluid. It is important to note that there were no significant differences in MWD-treated wounds between the experimental and control groups (EC versus CC). Thus, any changes in cytokine concentrations in this study were strictly due to the effect of treatment with NPWT (V.A.C.® GranuFoam® Dressing plus V.A.C.® Brand NPWT) and not treatment with MWD. The results of this study also suggest that there is no systemic effect of NPWT on the contralateral wound in the experimental group.

The decreased levels of IL-8, TGF- β 1, and TNF- α in wound fluid from NPWT treated wounds provide additional support for the hypothesis that NPWT may attenuate the pro-inflammatory phase of wound healing. These results correlate well with the results reported by Stechmiller et al⁸ for decreases in TNF- α , but not IL-1 β in human patients that received NPWT even though they studied a different type of wound, namely pressure ulcers. TGF- β 1 has both pro-inflammatory and anti-inflammatory properties.^{50,51} Clinical studies have shown TGF- β 1 levels peak in the first 24 hours post-wounding,⁴³ while in pigs, TGF- β 1 concentrations peaked between 7–9 days.^{52,53} In addition, Kilpadi et al⁵⁴ showed higher levels of TGF- β 1 in fluids from human wounds treated with NPWT at Day 3 when compared to Day 1. Results from the present study corroborate these findings^{43,52-54} at 12- and 36 hours post-wounding, although statistically significant differences between NPWT treated wounds versus controls ($P > 0.05$) were not seen. Furthermore, the results at later time points (132- and 180 hours post-wounding) support earlier reports by several groups who have also shown a significant reduction in inflammatory cytokine profiles following the administration of NPWT.^{6,55} Obviously, interpretation of wound fluid data can be challenging because decreases in cytokine concentrations in the NPWT-treated wounds may have been the result of a dilution effect caused by greater amounts of fluid exudate being pulled through the wound tissue by the application of negative pressure, and collection methods may vary. Such issues are being addressed in subsequent studies.

The lack of appreciable differences between the NPWT-treated and control groups for the other biomolecules examined may be due in part to the time points chosen, animal-animal variation, the relatively small number of animals used in this study, and/or the fact that they do not play a major role in this model system. In mice, it has been shown that IL-1 plays a critical role in oral, but not dermal, wound healing.⁵⁶ Alternatively, other factors may play a role, such as wound etiology, wound size, degree of inflammatory stimulus is insufficiently strong to cause systemic effects, and the age, genetic background, and general health of the animals. There was no evidence to suggest that a systemic inflammatory response syndrome characterized by fever, leukocytosis, infection, or organ dysfunction had developed as a result of wounding in this study. Increases from baseline seen in pro-inflammatory cytokine serum levels, including TNF- α , IL-6, and IL-1,⁵⁷ analyzed during the course of the study were typically ≤ 2 fold. A rise in serum C-reactive protein levels was less than 5-fold above baseline (pretest) values, but there were no statistically significant differences between the experimental and control groups.⁵⁸ Studies of cytokine levels in wound tissue are now underway to examine local biological response, and further work will be necessary to elucidate the complexity of cytokine interactions during acute versus chronic wound healing, as well as following therapeutic intervention with NPWT.

Figures 6-7



Conclusion

This study using an acute, porcine wound healing model provides evidence of a systemic effect attributed to NPWT (V.A.C. Therapy) beyond the initial 1–4 hours post-wounding that others have investigated, and further demonstrates that application of NPWT can result in local effects. Specifically, the systemic effects related to NPWT included post-wound reduction in the number of peripheral blood monocytes and neutrophils during the inflammatory phase of wound healing, as well as changes in certain pro-inflammatory cytokines present in serum compared to the control group. Local effects included reduced levels of certain pro-inflammatory cytokines in wound fluid following NPWT. Collectively, these responses corresponded to increased wound closure, suggesting that NPWT may attenuate the pro-inflammatory response following cutaneous wounding. Additional studies are necessary to measure the function of circulating neutrophils, monocytes, as well as other immune cells, such as T and B lymphocytes, in order to further define the extent of the effects of NPWT on the inflammatory response associated with wound healing.

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