

Bensal HP Treatment for Burn and Excision Wounds: An In-Vivo Assessment of Wound Healing Efficacy and Immunological Impact

Jamie Rosen BA,[a*] Angelo Landriscina BA,[a*] Anjana Ray PhD,[b] Lydia Tesfa PhD,[b] Joshua D. Nosanchuk MD,[b] and Adam J. Friedman MD[c,d]

[a]Department of Medicine (Division of Dermatology), Montefiore Medical Center, Bronx, NY
[b]Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY
[c]Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY
[d]Department of Dermatology, George Washington School of Medicine and Health Sciences, Washington, DC
*These authors contributed equally to the production of this work.

ABSTRACT

Natural ingredients are of increasing interest within the field of dermatology. Bensal HP, an ointment containing 3% oak bark extract, 3% salicylic acid, and 6% benzoic acid, is believed to be efficacious against a variety of inflammatory and infectious dermatidites. Here we evaluate Bensal HP's ability to influence wound healing, which has yet to be studied in this setting. Bensal HP applied to burn wounds on the dorsal surface of BALB/c mice significantly attenuated wound expansion in the first few days post-injury as compared to controls. Histological analysis mirrored these findings with accelerated maturation of the wound bed and increased collagen deposition by the end of the study period. Cytokine analysis revealed decreased IL-6 and TNFα secretion in the Bensal HP-treated burns as compared to controls. Similarly, excisional wounds treated with Bensal HP demonstrated comparable wound healing as compared to controls with positive histologic features and increased collagen deposition. Furthermore, IL-6 production was attenuated in the Bensal-HP treated wounds at day 3, with no differences appreciated in IL-6 at day 7 or in TNFα at either time point. While Bensal-HP represents a therapeutic strategy to enhance the histologic and immunologic milieu in burn and excisional wounds, further study is needed to fully elucidate the full potential of this treatment.

J Drugs Dermatol. 2015;14(11):1322-1326.

INTRODUCTION

Natural ingredients are the basis for many widely used pharmaceutical agents. Some of the most commonly used medications, including aspirin (initially extracted from the white willow tree), came about as the result of the study of plant extracts. Natural ingredients continue to be of great interest in dermatology, with new applications being developed for conditions ranging from wound infections to melanoma.[1,2]

Bensal HP, an ointment containing 3% oak bark extract (QRB7), 3% salicylic acid and 6% benzoic acid was developed in the 1950s by Dr. Harry Stanley as a botanical treatment. It has been used as a topical therapy for the treatment of inflammation and irritation associated with a variety of dermatidites and bacterial and fungal infections, and has been studied in the setting of diabetic foot ulcers.[3] However, there is a lack of data regarding Bensal HP's influence on general wound healing, and the mechanism by which it exerts this influence. The following study seeks to characterize the effect of Bensal HP on both burn and excisional wounds, and describes preliminary immunological data that may help to explain its mechanism.

MATERIALS AND METHODS

In-Vivo Wound Model

All animal experiments were approved by the Institutional Animal Care and Use Committee at Albert Einstein College of Medicine. The dorsal surface of 90 BALB/c mice were shaved and depilatory cream was applied and washed. Two 5mm full-thickness burns were induced on each of 45 mice by applying a calibrated 160C metal bar to the dorsal surface for 10 seconds. These were split into three groups: untreated control, silver sulfadiazine(SSD)-treated, and Bensal HP-treated. Four 5mm excision wounds were induced on the dorsal surface of the remaining mice, and these were split into three treatment groups: untreated control, petrolatum-treated, and Bensal HP-treated. Treatments (0.05cc per wound) were applied daily. Wounds were photographed daily and measured using ImageJ Software (NIH, Bethesda, MD).

Histology

Mice were sacrificed for histology on day 7 and 11 for excisional wounds, and days 7 and 17 for burn wounds. Specimens were stained with hematoxylin and eosin (H&E) and Masson's

trichrome in order to visualize wound morphology and collagen deposition, respectively.

"These results suggest a role for Bensal HP in burn treatment, especially in the first few days post-injury, when unchecked inflammation results in wound expansion."

Cytokine Analysis

Mice were sacrificed on days 3 and 7 for cytokine analysis in all groups. Tissue was flash-frozen using liquid nitrogen, homogenized in ice-cold phosphate buffered saline (PBS) with Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). Samples were centrifuged at 10,000 G for 20 minutes to remove debris. Aliquots of the supernatants were assayed for total protein content by the BCA method. Cytokine analysis was carried out using BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA) according to the instruction manual. Samples were examined using BD FACS Canto II flow cytometer at 485 and 633nm and results were analyzed with Flowjo software.

RESULTS

Clinical Wound Closure

Topical Bensal HP applied to burns (Figure 1a) inhibited wound expansion during the first six days post-injury with significant differences over the untreated group on days 1-3, and over SSD on days 1-4,6, and 17. The majority of wounds in both the untreated and Bensal HP-treated mice reached closure by day 17. In excisional wounds (Figure 1b), Bensal HP showed a significant improvement in wound healing over the untreated group by the final day of the study period, with petrolatum significantly outperforming Bensal HP on days 5 and 7 post-injury. Wounds in all three study groups reached closure by day 11 post-injury.

Histology

H&E staining of burn wounds on day 7 (Figure 2a) revealed a serous neutrophil rich crust overlying a homogenized inflammatory dermis/granulation in both control and bensal treated wounds. Interestingly, the SSD treated wound revealed minimal inflammation, rather a fully homogenized wound area

FIGURE 1. Wound size analysis of BALB/c mice skin lesions. Bensal HP attenuates wound expansion in burn wounds (A) and enhances wound healing in excisional wounds. (B) *P<0.05 in comparing Bensal HP to untreated control. Triangle P< 0.01 in comparing Bensal HP to SSD. Error bars denote SEM.

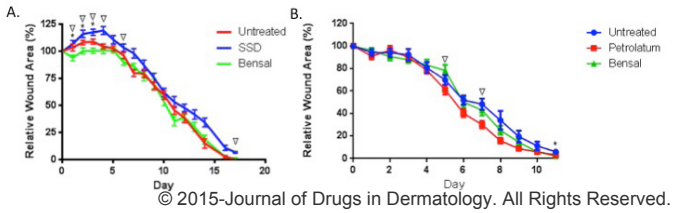
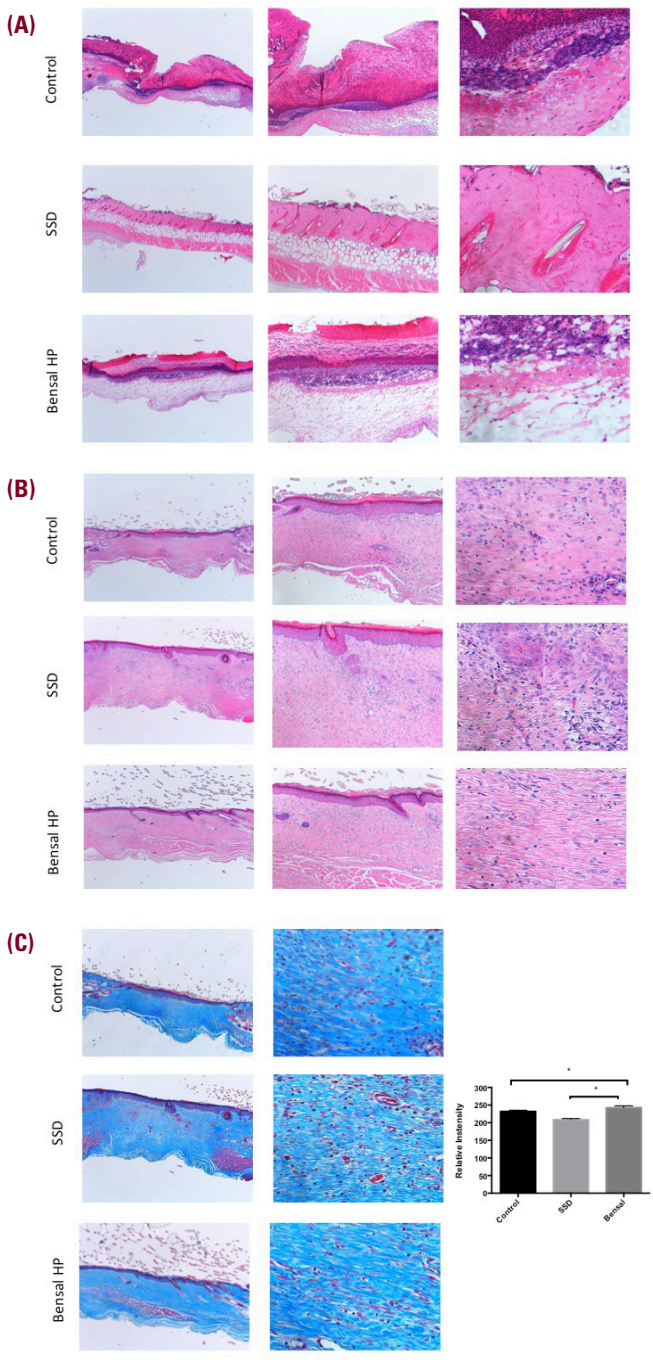


FIGURE 2. Histologic analysis of untreated controls, SSD-treated and Bensal HP-treated burn wounds. (A and B) H&E staining of control vs petrolatum-treated vs Bensal HP-treated wounds at 4x, 10x and 40x (left to right) at day 7 and 17, respectively. (C) Blue staining indicates collagen in Masson's Trichrome stain at 4x, 10x and 40x (left to right) at day 17. Intensity measures from 10 high powered fields per sample. Error bars denote SEM. P< 0.05.



overlying the panniculus. On day 17 (figure 2b) following the initial injury, Bensal treated burns demonstrated a more organized and less inflammatory dermis in the wound bed as compared to both control and SSD treated burns. Quantitative analysis of Masson's trichrome staining on day 17 (Figure 2c) showed a statistically significant increase in collagen deposition in the Bensal HP-treated group compared to both untreated control and SSD-treated wounds (242.9 vs 232.2 vs 208.6, respectively), which correlates with the visible and parallel arranged collagen bundles.

In excisional wounds, H&E staining on day 7 (Figure 3a) demonstrated apparent scale crust, and a thin reepithelialized epidermis overlying a moderately infiltrated granulation tissue with engorged vessels in the control group. This contrasted with findings in the petrolatum-treated group which exhibited a well formed epidermis with a compact, orthokeratotic stratum corneum, and a mild to moderate inflammatory infiltrate in the dermis with signs of parallel deposition of collagen bundles. Bensal HP-treated wounds exhibited a well formed epidermis, compacted stratum corneum, with a mixture of maturing dermis and inflammatory granulation tissue. On day 11 (Figure 3b), control tissue revealed a mildly contracted wound with overlying crust and persistent inflammatory infiltrate in the dermis. Petrolatum-treated wounds exhibited a contracted mildly inflammatory wound bed with a mature overlying epidermis and parallel arrangement of collagen bundles. Bensal HP treated tissue was notable for a completely contracted scar, visualized by the patchy homogenized collagen seen in high power fields and incomplete muscle healing beneath the panniculus. Masson's trichrome stain revealed a significant difference in intensity of staining between Bensal HP-treated and control wounds on days 7 and 11 (Figures 3c and 3d, respectively), though no difference was seen between Bensal HP and petrolatum treatment on either day.

FIGURE 3. Histologic analysis of untreated controls, SSD-treated and Bensal HP-treated excision wounds. (A and B) H&E staining of control vs SSD-treated vs Bensal HP-treated wounds at 4x, 10x and 40x (left to right) at day 7 and 11, respectively. Blue staining indicates collagen in Masson's Trichrome stain at 4x, 10x and 40x (left to right) at day 7 (C) and day 11 (D). Intensity measures from 10 high powered fields per sample. Error bars denote SEM. $P < 0.05$.

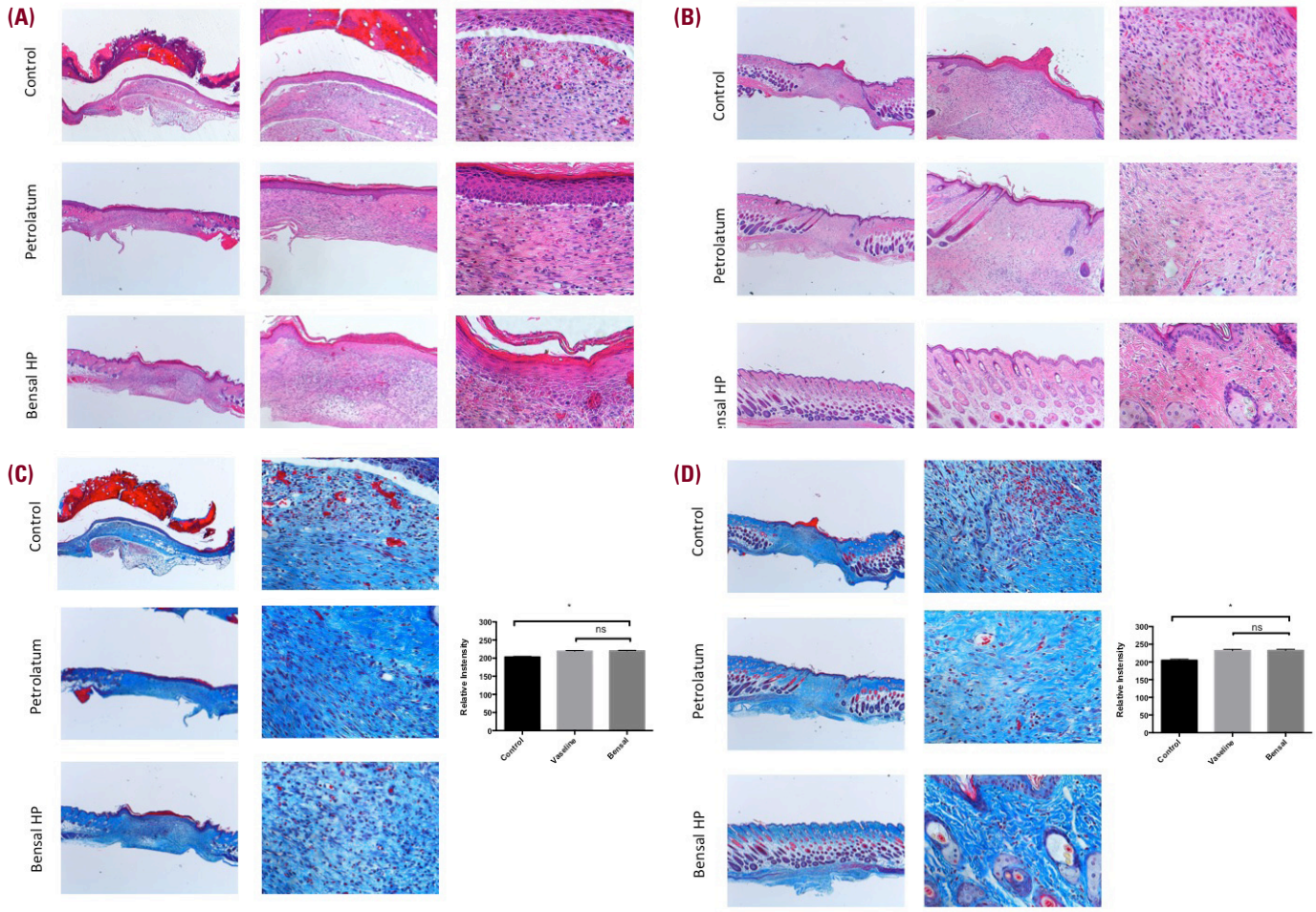
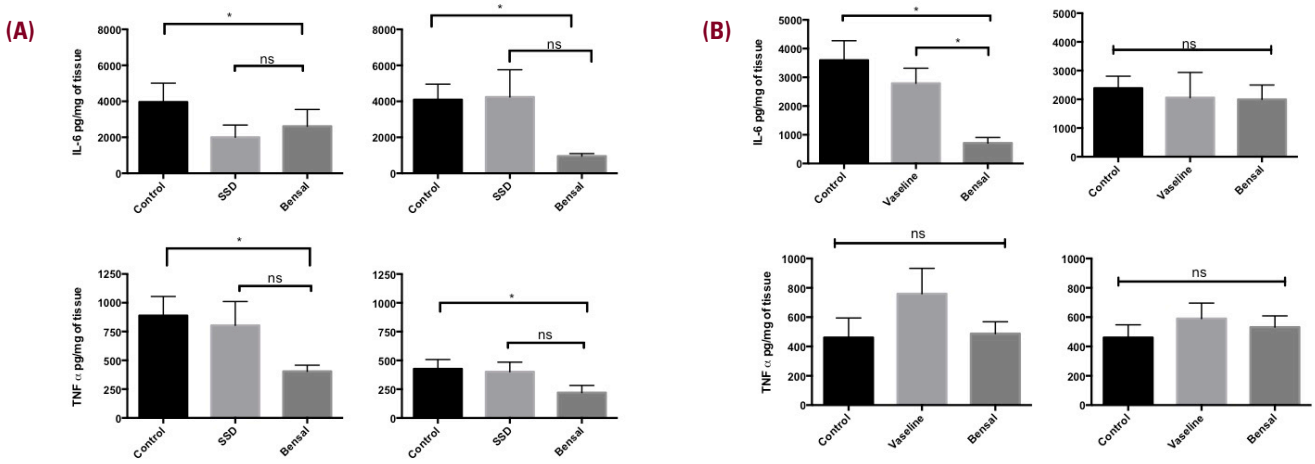


FIGURE 4. Cytokine analysis of burn and excisional wounds. (A) Burn wound quantitative cytokine analysis IL-6 (top row) and TNF-α (bottom row) at day 3 and 7 (left and right, respectively). (B)Excisional wound quantitative cytokine analysis of IL-6 (top row) and TNF-α (bottom row) at day 3 and 7 (left and right, respectively). *P*< 0.01. Error bars denote SEM.



Cytokine Analysis

Cytokine analysis (Figure 4a) of burn wounds treated with Bensal HP showed a significant reduction of both IL-6 and TNFα at days 3 and 7 as compared to untreated controls (2617.0 vs 3966.9 and 954.1 vs 4099.3 pg/mg for IL-6, and 405.1 vs 889.5 and 220.1 vs 426.3 pg/mg for TNFα at days 3 and 7, respectively), with no significant difference when compared to SSD-treated wounds (2617.0 vs 2004.2 and 954.1 vs 4240.2 pg/mg for IL-6, and 405.1 vs 803.2 and 220.1 vs 400.8 pg/mg for TNFα at days 3 and 7, respectively). For excisional wounds (Figure 4b), Bensal HP treatment resulted in a statistically significant decrease in IL-6 expression at day 3 when compared to untreated controls and petrolatum (714.0 vs 3597.0 and 2792.0 pg/mg, respectively). There was no statistical difference appreciated in the expression of IL-6 at day 7 or TNFα at days 3 and 7.

DISCUSSION

The above results represent the first pre-clinial evaluation of Bensal HP on wound healing. In the setting of burn wounds Bensal HP treatment resulted in limited wound expansion within the first days post-injury when compared to controls and SSD, though reached closure at the same day as controls. These results were mirrored by histological findings that showed accelerated maturation of the wound bed by day 17 as compared to other treatment groups, with a statistically significant increase in collagen deposition in the wound bed in Bensal treated groups. Lastly, both IL-6 and TNFα secretion were decreased in Bensal HP-treated burns when compared to the other groups, though this difference was only statistically significant when compared to controls. In excisional wounds, Bensal HP treatment resulted in comparable wound healing times as compared to controls and petrolatum. Positive histologic features were seen in both Bensal HP and petrolatum-treated wounds, with both groups exhibiting increased collagen deposition when compared to controls. Additionally, IL-6 production was significantly attenuated in the Bensal HP-treated wounds at day 3, with no significant difference seen at day 7. No difference in TNFα expression was appreciated throughout the study interval.

These results are of considerable interest, given the fact that Bensal HP has not been extensively studied as a wound healing adjuvant, even in spite of decades of use. One study by Jacobs et al. found that Bensal HP application to diabetic ulcers resulted in a statistically significant improvement in wound closure when compared to SSD in a human clinical trial.[3] Investigators posited that the observed effect could be due to a host of factors including the antimicrobial efficacy of benzoic acid, and the keratolytic effect of salicylic acid.[3] While these results are promising, they are limited and offer no insight into mechanism of action.

Wound healing can be understood in several phases. The first phase post-injury is hemostasis. With the formation of a fibrin clot and platelet aggregation and degranulation, a number of pro-inflammatory cytokines and growth factors are allowed to concentrate in the area of the wound bed. These mediators help to attract various cell types, including neutrophils whose predominance defines the inflammatory stage of wound healing. Neutrophils help to disinfect the wound and clear damaged tissues through both phagocytosis and the release of proteases and pro-inflammatory cytokines. It is important to note that these proteases and inflammatory mediators can diffuse into surrounding tissue, allowing wounds (especially burns) to expand within the first few days following injury. Next is the proliferative phase which is characterized by a predominance of macrophages. Macrophages carry out remodeling of the extracellular matrix in concert with fibroblasts. Fibroblasts first lay down a provisional extracellular matrix composed of

glycans.[4] This provisional matrix is later replaced by type III collagen.[4] This process is vital for several reasons including wound contraction and strength of new tissue.

"In excisional wounds, Bensal HP treatment resulted in comparable wound healing times as compared to controls and petrolatum."

As outlined above, the signals carried by cytokines and other molecular mediators are extremely important to proper wound healing. The activity and interactions between these mediators are complex, with many levels of regulation. Our results demonstrate some significant differences in two cytokines with Bensal HP treatment: IL-6 and TNFα. IL-6 is a pro-inflammatory cytokine released by a variety of cell types including macrophages and fibroblasts.[5] Its effects have been shown to have a great impact on wound healing, with in vivo studies demonstrating that exogenous IL-6 induces persistent leukocyte infiltration and delayed wound healing.[6] In contrast, IL-6 knockout mice demonstrated delayed wound healing compared to controls with decreased gene expression of a multitude of pro inflammatory cytokines and cellular adhesion molecules, diminished leukocyte infiltration and decreased collagen deposition.[5] These studies show that while IL-6 is essential to proper wound healing, dysregulation by either over or underexpression results in delayed wound closure. Our results showed that IL-6 expression was attenuated in Bensal HP-treated wounds, though was not completely extinguished. The results of this change were more pronounced in the burn wounds, with less inflammatory infiltrate, resulting in dampened wound expansion and increased collagen deposition by the end of the study period.

Similar results were seen when analyzing expression of TNFα, a pro inflammatory cytokine primarily produced by macrophages, whose overexpression has been observed in a variety of inflammatory disease states.[7] TNFα has multiple roles in the wound healing process and is important not only for the recruitment of inflammatory cells and for the release of inflammatory cytokines (such as IL-6), but also for angiogenesis and the formation of extracellular matrix.[4] As with IL-6, overabundance of TNFα has been linked to delayed wound healing, with persistence of inflammatory infiltrates and decreased collagen deposition.[7,8] Furthermore, investigators have shown that knockout of the p55 TNFα receptor results in accelerated wound healing, reduced leukocyte infiltration and enhanced collagen deposition in vivo.[9] Results of our study showed decreased TNFα at both time points investigated for burn wounds with Bensal HP treatement. Again, this correlates well to our findings of decreased inflammatory infiltrates and increased collagen deposition when compared to controls.

These results suggest a role for Bensal HP in burn treatment, especially in the first few days post-injury, when unchecked inflammation results in wound expansion. Though clinical wound closure was achieved with comparable efficacy to controls, increased collagen deposition in both burns and excision wounds may represent stronger tensile strength, and higher quality of tissue. In general, wounds only achieve 80% of the initial strength of the tissue, making a stronger wound bed desirable.[10] Furthermore, given previously described data, we hypothesize that the modulation of cytokine expression seen with Bensal HP treatment may be beneficial to wound healing, especially in burns which expand due to over exuberant inflammation.

CONCLUSION

Our results have several implications for the role of Bensal HP treatment in the setting of excisional and burn wounds. While Bensal HP treatment resulted in positive histologic and altered immunologic features, the full potential of these effects has yet to be characterized. Additionally, more accurate immunologic studies may help to fully elucidate the mechanism by which Bensal HP exerts its immunologic influence and wound healing efficacy. Further human trials may also be useful in ascertaining the utility of this treatment in human subjects.

DISCLOSURES

The authors acknowledge funding from Valeant Pharmaceuticals, and no other financial disclosures.

REFERENCES

1. Krausz AE, Adler BL, Cabral V, et al. Curcumin-encapsulated nanoparticles as innovative antimicrobial and wound healing agent. *Nanomedicine* 2015. 11(1): 195-206.
2. Rosen J, Landriscina A, Adler BL, et al. Characterization and assessment of nanoencapsulated sanguinarine chloride as a potential treatment for melanoma. *J Drugs Dermatol.* 2015;14(5):453-458.
3. Jacobs AM, Tomczak R. Evaluation of Bensal HP for the treatment of diabetic foot ulcers. *Adv Skin Wound Care.* 2008;21(10):461-465.
4. Broughton G, Janis JE, Attinger CE. The basic science of wound healing. *Plast Reconstr Surg.* 2006;117(7 Suppl):12S-34S.
5. Lin Z-Q, Kondo T, Ishida Y, Takayasu T, Mukaida N. Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. *J Leukoc Biol Suppl.* 2003;73(6):713-721.
6. Saba AA, Kaidi AA, Godziachvili V, et al. Effects of interleukin-6 and its neutralizing antibodies on peritoneal adhesion formation and wound healing. *The American surgeon.* 1996;62(7):569-572.
7. Buck M, Houglum K, Chojkier M. Tumor necrosis factor-alpha inhibits collagen alpha1 (I) gene expression and wound healing in a murine model of cachexia. *Am J Patho.* 1996;149(1):195.
8. Siwik DA, Chang DL-F, Colucci WS. Interleukin-1β and tumor necrosis factor-α decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. *Circ Res.* 2000;86(12):1259-1265.
9. Mori R, Kondo T, Ohshima T, Ishida Y, Mukaida N. Accelerated wound healing in tumor necrosis factor receptor p55-deficient mice with reduced leukocyte infiltration. *FASEB J.* 2002;16(9):963-974.
10. Levenson SM, Geever EF, Crowley LV, Oates JF, Berard CW, Rosen H. Healing of Rat Skin Wounds. *Ann Surg.* 1965;161(2):293-308.

AUTHOR CORRESPONDENCE

Adam J. Friedman MD
E-mail:..... Ajfriedman@mfa.gwu.edu