Application of Hyperosmotic Nanoemulsions in Wound Healing: Partial Thickness Injury Model in Swine

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Objective: In this work, we introduce a novel hyperosmotic nanoemulsion (HNE) topical agent for use in wound healing. These topical emulsion complexes combine a lipophilic thymol nanoemulsion with a hyperosmotic saccharide matrix. This combination has been previously shown to possess synergistic antimicrobial activity against a host of common and drug-resistant pathogens *in vitro*.

Approach: In this study, we present additional data to assess the safety and efficacy of these emulsions in a partial thickness injury model in swine. Ten wounds sized 2×3.5 cm were created in 18 pigs using an electrodermatome set at a depth of 0.76 mm. The wounds were subsequently contaminated with a cocktail of *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Candida albicans* at 5×10⁷ total colony forming unit per wound. Treatments were subdivided in the control group and emulsion concentrations at 0.0%, 0.01%, 0.03%, and 0.063% thymol content. Longitudinal metrics for wound healing included rate of reepithelialization, wound bed color measurements, amount of wound exudate, wound swab culture data, and histological examination at 4, 7, and 14 days. The cosmetics of the healed wound were obtained at day 14 with three-dimensional photogrammetry.

Results: Experimental results showed that HNE reduced the wound level bacteria count by ~0.5–1 log versus controls after 24 h. The amount of pathogen reduction was weakly correlated to the concentration of the emulsion. In addition, all HNE groups maintained a moist wound environment and showed increased fibrin formation and improved hemostatic response.

Innovation: No significant difference in the rate of reepithelialization or wound closure was found between treatment concentrations and control groups. HNE treatment did not demonstrate any adverse host tissue response.

Conclusion: These results suggest HNE may be a candidate for reducing wound bacterial counts without compromising reepithelialization.

Keywords: hyperosmotic, nanoemulsion, contaminated wounds, swine

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Submitted for publication March 15, 2016.
Accepted in revised form April 25, 2016.

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INTRODUCTION

WOUND HEALING IS A finely orchestrated process that consists of an inflammatory, proliferative, and remodeling phase. While there are many factors that mediate the rate

and quality of wound healing, microbial contamination and subsequent infection have been implicated as a critical component in delayed healing. Wound site infection induces a localized inflammatory state

that encourages the overproduction of cytokines, which inhibit keratinocyte proliferation, perturb the formation of new matrix, impede wound closure, and initiate possible secondary injury and dermal scarring.^{1–3} These suboptimal conditions protract healing times and increase healthcare costs. Current topical therapeutics to treat infected wounds include antibiotics and silver ion-based regimes. However, there is a growing concern regarding the emergence of multidrug-resistant pathogens and the diminished efficacy of traditional antibiotics. Alternatively, silver, an antimicrobial agent frequently indicated for burns and acute injuries, has well-documented biocidal properties. There is significant evidence that shows various silver preparations to be effective antimicrobial treatments for wound healing.⁴ However, recent literature suggests that silver may have cytotoxic effects to both fibroblasts and keratinocytes and impair healing.^{5–7} Therefore, the impetus for developing new classes of antimicrobial agents with minimal adverse host responses has led us to explore a new therapeutic platform called hyperosmotic nanoemulsions (HNEs).

The HNE concept was developed as an extension of nonionic hyperosmotic therapies similar to topical honeys and sugars. Recently, hyperosmotic treatments have gained attention in treating chronic and malodorous wounds.^{8–10} While many of these investigations were case studies or non-controlled experiments, hyperosmotic saccharides appear to reduce bioburden through a cellular dehydration effect. That is, the low water activity (a_w) of sugars discourages microbial growth.^{11,12} The strong hyperosmotic gradients may further improve tissue debridement and microcirculation through osmotic pull of wound fluids.^{12,13} Our goal with HNE was to enhance the bacteriostatic properties of the native sugars. Subsequently, HNEs utilize two main components: (1) a high energy lipophilic nanoemulsion composed of thymol and (2) a hyperosmotic saccharide matrix. The HNE's bactericidal action is based on a biomechanical mode of action in which the thymol emulsion disrupts bacterial membranes while the hyperosmotic component dehydrates the "leaky" cells.¹⁴ HNEs were shown to deactivate common and drug-resistant bacteria within a matter of seconds *in vitro*. We currently extend the line of investigation to an *in vivo* swine model of wound healing. To emulate the clinical setting, a polyflora consisting of common skin bacteria and fungi was used to infect the wounds.¹⁵ Our main objectives were to characterize both the dose-dependent antimicrobial properties of HNE and the subsequent

host–tissue response. Based on prior *in vitro* data, we hypothesize that the hyperosmotic matrix will reduce wound microflora and provide a moist microenvironment that encourages wound healing. Wound healing was assessed visually, with wound bed color measurements, culture swabs, and histological examination. Together, the preclinical data provide proof of concept for topical use of HNEs in acute wound management.

CLINICAL PROBLEM ADDRESSED

The proposed research provides proof of concept for HNEs as a topical prophylaxis or in treatment of contaminated wounds. These emulsions are non-antibiotic based and further provide a moist environment for wound healing. The emulsions further reduce bioburden without impairment to wound closure or reepithelialization.

MATERIALS AND METHODS

Animal surgery

18 Yorkshire swine, weighing 42 to 50 kg, were used for the study. Two pigs were used for an initial pilot study of 7 days, followed by a study of 16 experimental pigs involving explants at day 4 ($n=4$), day 7 ($n=6$), or day 14 ($n=6$). Experiments were conducted following approval and in compliance with the Purdue University Institutional Animal Care and Use committee. Animals were received and given an intramuscular injection of Ceftiofur 1 ml/20 kg, an antibiotic for treatment of swine respiratory disease that maintains therapeutic levels for 7 days. The pigs were then kept a minimum of 7 days before beginning the study. Before the study, animals were fasted for 12 h, but given access to water. Each animal was anesthetized with a mixture of TELAZOL (Tiletamine HCL and Zolazepam HCL), xylazine and ketamine given intramuscularly. The animals were then intubated and maintained under general anesthesia with isoflurane and oxygen. The animal was positioned in ventral recumbency and the back and sides were clipped with a #40 blade. The surgical sites were scrubbed with chlorhexidine gluconate and saline, and a sterile drape was applied. A template with five 2.5×2.5 cm squares spaced 5 cm apart was used to draw squares on the skin with a sterile marker (Fig. 1). A Padgett® electric dermatome (Integra LifeSciences) with a 2.5 cm guard and the depth set at 0.76 mm was used to harvest a partial thickness skin graft at each square site. Sterile mineral oil was used as a lubricant, and the skin was stretched taut. Considerable manual pressure was applied to the dermatome to create wounds of

Figure 1. Schematic of experimental design: (A) Ten partial thickness dermal injuries $\sim 20 \times 35$ mm in size were created on the dorsal side of pigs. (B) The wounds were made with an electric dermatome set at a constant depth of 0.76 mm. Treatments included (1) control, (2) 0.00%, (3) 0.01%, (4) 0.03%, and (5) 0.063% W/W of the thymol active component. Per each side, treatments were randomly assigned. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

consistent depth. Generally, each wound measured ~ 20 mm wide by 35 mm long, and dimensions of the thickness of the harvested skin were validated with a micrometer. Following injury, the wounds were blotted with a saline soaked sponge and then inoculated with a mixture of common wound micro-organisms. The solution contained *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 8739, and *Candida albicans* (Carolina Biologic) combined at a 1:1:1:1 ratio in a 50 μ L inoculation volume at a final concentration of $\sim 1 \times 10^9$ colony forming unit (CFU)/mL using the McFarland standard. Therefore, a total of 5×10^7 CFU were inoculated into each wound. After contamination, the wounds were allowed to incubate for 5–20 min before bandage application.

Each set of wounds (five on the left and five on the right side of each pig) was randomized to receive one of the following five treatments: (1) no treatment control, (2) HNE with 0.0% thymol, (3) HNE with 0.01% thymol, (4) HNE with thymol at 0.03%, or (5) HNE with thymol at 0.063%. Xtrasorb[®] (Derma Sciences) foam pads were cut slightly larger than the wound and applied to each site. Uro-bond[®] brush on silicone adhesive (Urocare Products, Inc.) was painted around the periphery of the wound, and a Tegaderm[™] transparent dressing (3M) was then placed over each wound. Hypafix[®] (BSN medical) was then placed to cover all five wounds on each side. When wounds on both sides of the animal were created, treated, and bandaged, a snug-fitting shirt was applied and the animal was allowed to recover from anesthesia. Buprenorphine

0.005 mg/kg, tramadol 1–4 mg/kg per os, or butorphanol 0.1 mg/kg intramuscularly was given for postoperative pain control. When the animal was ambulatory, it was fed and monitoring continued until evening when additional postoperative analgesic was given. The following morning, each animal was given the injectable anesthetic agent and maintained with an oxygen mask with or without the isoflurane inhalant. Bandages were changed and treatments were applied daily or until the wounds were fully scabbed (~ 10 days). Following bandage change each day, animals were monitored until recovered from anesthesia and given feed and water. Additional analgesics were given if the animal appeared to be in pain by showing signs of vocalization, shivering, reluctance to rise, or refusal to eat. After 3 days of giving buprenorphine 0.005 mg/kg twice daily, it was determined that additional analgesics were not needed and they were discontinued.

Exudate estimation

Bandages from each wound during the first 3 days were used to calculate the exudate from each wound. Xtrasorb (Derma Sciences) foam bandages were removed from the wounds and weighed using an analytical balance. The size of the bandages was also measured with a ruler. The amount of wound exudate was calculated by subtracting the dry weight (estimated from size and density) from the wet weight of the used bandage.

Microbiological culture

Microbiological culture was performed at 1, 7, and 14 days postinjury/infection. Briefly, the foam bandage was removed, and each wound was gently lavaged with sterile saline to remove debris, exudate and blotted with a sterile sponge. A sterile cotton tipped applicator was pressed and swabbed across the surface from margin to margin in a 10-point zigzag manner.^{16,17} The swab tips were then homogenized and serially diluted and plated onto tryptic soy broth agar. At 20–48 h postplating, the culture dishes were digitally scanned and analyzed for colony counting. In the day 14 samples, a 6 mm biopsy punch was used to obtain a skin sample of the healed wound. The skin sample was then homogenized and serially plated on tryptic soy media for colony enumeration.

Wound imaging

Wounds were photographed using a Nikon D200 DSLR and 55 mm f2.8 Micro Nikkor lens. The lens was fixed at 0.4 m subject to plane focal distance. The camera was calibrated at 5,900 K color temperature and constant flash output (manual setting, full power) at f22. Pictures of the wound, along with a calibrated ruler, were taken daily. The

wound bed color was obtained through the images in Photoshop CS6. Briefly, the color was taken by an averaging of a square ($\sim 2 \times 2$ cm) section of pixels from the center of the wound. The colors were reported in the CIE $L^*a^*b^*$ color space. In this color system, L^* pertains to luminance or brightness, while a^* refers to values in the green–red color spectrum and b^* refers to blue–yellow spectrum, respectively.

Histology

Subjects were sacrificed at day 4 ($n=6$), day 7 ($n=6$), or day 14 ($n=4$) for histologic analysis. Following general anesthesia and removal of the bandages, photographs and final wound culture biopsies were taken. Each animal was given an intravenous injection of euthanasia solution containing barbiturate and monitored until all vital function ceased. The entire section of lateral body wall was removed down to the level of the deep fascia. Next, an ~ 8 mm wide transverse strip of the wound bed and an additional 5 mm of uninjured skin were taken from the midsection of the wound. Tissues were fixed in 4% paraformaldehyde for 48 h and embedded in paraffin blocks cut to 5 μ m thick. Histological sections were taken from opposing end faces of the 8 mm strip. Sections were then stained with hematoxylin and eosin (H&E) or gram stained for histologic examination. Histologic slides were imaged through a Nikon Diaphot 200 microscope and CCD (Diagnostic Instruments, Inc.) imager. Sections were blindly graded by a board-certified veterinary pathologist using a modified semiquantitative histomorphologic grading scale, as shown in Table 1. Histologic grading scale adapted from Lomash *et al.*¹⁸ The maximum histologic score is 16.

Furthermore, the rate of wound healing was quantified by determining the percentage of reepithelialization across the insult as seen in his-

tological cross sections (midwound) at the sacrificial time points. Briefly, images of H&E sections were taken at 20 $\times$ magnification and stitched together (Adobe Photoshop CS6). The % of reepithelialization was determined by tracing the length of the new epithelium (both from the margins and hair follicles) divided by the total wound length $\times 100$, as determined by locating the wound margins. Results were averaged for all sections ($n=8-12$) per treatment group. Tracing was conducted through ImageJ software (NIH).

Wound topography

The surface topography of the day 14 wounds was assessed with close range photogrammetry. A model cast of the wound was created with liquid plastic resin. Multiple high-resolution images of the plastic model were captured using an Olympus OMD-EM5 camera with a 12–50 mm macro lens at various low camera angles. Photographs were then processed with reconstructive software (PhotoModeler) in the automated mode using nontarget feature points to create a three-dimensional (3D) rendition of the wound.

Statistics

All data are reported in the form of $AVG \pm SEM$. Statistical analysis was conducted using a Student's *t*-test or one-way analysis of variance. The Tukey test was used for *post hoc* evaluation. A *p*-value <0.05 was considered significant (denoted by “*”).

RESULTS

Surgery

Eighteen animals successfully underwent the procedure for wound creation and survived to the time of necropsy without complications, injury, or illness. Two of the eighteen animals were used as pilot pigs for range finding of the initial pathogen

Table 1. Scoring criteria for tissue sections

Criterion	Score	Description	Criterion	Score	Description
Reepithelialization	0	Absent	Collagenization	0	Abnormal lower reticular dermis
	1	<50% intact epidermis		1	Abnormal upper reticular dermis
	2	>50% intact epidermis		2	Abnormal papillary dermis
	3	Intact epidermis		3	Normal dermis
Epidermal hyperplasia	0	Absent	Fibroblast proliferation	0	Absent
	1	Present		1	Haphazard arrangement
Dermal Separation	0	Absent		2	Parallel to epithelial surface
	1	Present		3	Normal fibroblasts
Inflammatory Cells	0	Absent	Vascular Proliferation	0	Absent
	1	Present		1	Present
Adnexal Structures	0	Absent	Hemorrhage	0	Present
	1	<100% present		1	Absent
	2	Present and normal			

The maximum histologic score is 16.

inoculation and optimization of the bandage treatments. Data from these pigs were not included in the assessment of treatment effect. All wounds created were within 10% of the target depth (0.76 mm, data not shown) based on micrometer measurements of the excised tissue.

Visual appearance of wound

Representative photographs of the wounds at various time periods are shown in Fig. 2. All wounds appeared as expected the day after surgery and infection. At 24 h, the control wounds were characterized by bloody exudate. The amount of wound exudate decreased over time, but the control foam bandages adhered to the wounds, especially near the perimeter. Care was used to remove the foam bandage, and saline was used to facilitate the process, but it was evident that bandage removal was painful and minor bleeding occurred. By day 5, there was a noticeable decrease in wound area in all wounds; however, afterward, the control wounds began to form a thin fibrin film, which masked the underlying wound margins. In contrast, all HNE-treated wounds had achieved hemostasis within 24 h. Approximately 2–3 days

postsurgery, coagulated exudate overlying the wound was observed on the HNE-treated sites. After day 4, the HNE wound surfaces shifted toward a brown color and evolved into a pliable scab consisting of serum, fibrin, and blood. It was then difficult to observe wound size since wound healing was occurring underneath this fibrin layer. By day 7–8, all wounds had formed a superficial scab. By day 14, the scab was sloughing off the animal and loosely attached to newly regrown hair. These scabs were gently brushed away from the surface and the underlying surface imaged (Fig. 2).

Exudate estimation

The amount of exudate produced by each wound was estimated by weighing the saturated foam pads during bandage changes. The exudate level was a surrogate indicator of the moisture level maintained between bandage changes. Results showed that control bandages had a net weight (exudate weight) of 5.94 ± 0.4 , 4.02 ± 0.2 , and 3.78 ± 0.2 g for the first 3 days (~hemostatic phase) following insult, respectively. In comparison, every concentration of the HNE treatment produced significantly (** $p < 0.001$) higher levels of moisture

Figure 2. Representative photographs of wound healing over the course of 14 days. All HNE-treated wounds demonstrated a quicker hemostatic response and less bleeding as well as a moist appearance. HNE wounds also showed a faster tendency to form a soft scab on the surface of the wound versus controls. By day 14, the wounds were completely reepithelialized. Scale bar: 1 cm. HNE, hyperosmotic nanoemulsion. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

Figure 3. Absorbed fluid weight of spent bandages as a function of treatment. All HNE concentrations showed a significant increase in the amount of absorbed liquids compared to respective time controls. $**p < 0.001$.

content versus control. However, there was no direct correlation between the amount of exudate produced and the thymol concentration (Fig. 3).

Wound cultures

Representative cultures acquired from wound swabs 24 h postinfection are shown in (Fig. 4). There was a significant reduction in bacteria CFUs for HNE-treated wounds in comparison to the control. Total CFUs at 24 h post-treatment in the control wounds were $5.66 \pm 0.85 \times 10^3$. Conversely, 0.063% HNE treatment significantly ($p < 0.05$) decreased the population to $4.83 \pm 0.18 \times 10^2$ CFU, representing a 91.5% reduction in comparison to the control and a >99.999% reduction in comparison to the starting inoculum. Other HNE concentrations also reduced the superficial bacteria versus controls.

Wound swabs at days 4 and 7 and homogenized tissue biopsies at day 14 were also plated and enumerated. Histologic examination of the wounds revealed that the bacteria resided in the scab layer, not in the wound bed (data not shown).

Wound bed color

Wound color has been used as a semiquantitative marker for wound health and viability,¹⁹ and similar methods were used in this study to assess wound color transitions. Wound bed color was obtained from the color temperature calibrated digital photographs (Fig. 5). Scab formation after the third day began to interfere with wound bed measurements, and therefore, color values were only reported for the initial three days. Over this time period, the control injuries demonstrated a

Figure 4. Wound swab culture data for each treatment. Swabs were taken at 24 h postinfection/treatment. **(A)** Data show that all treatment groups were statistically different from control ($*p < 0.05$), whereas the 0.063% and 0.01% groups were different from each other ($^{\dagger}p < 0.05$). Increasing the concentration of the emulsion component marginally decreased the number of recovered pathogens. **(B)** Representative images of the cultured plates demonstrating the number of recovered CFU (1:40 dilution). CFU, colony forming unit.

Figure 5. Wound bed color measurements were used as a marker for wound viability. Color was represented by the CIE L*a*b* color space (left color map), in which luminosity (L*) and color direction (a*, b*) are shown. The scale for luminosity ranges from 0 (black) to 100 (white), while the a* and b* hues range from -128 to +127. The top table reports the average wound L*a*b* color values and the corresponding color swatch as a function of treatment and time. The lower table refers to changes (Δ) in the L*a*b* values with respect to day 0. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

continuous reduction in luminosity ($L^* = -\Delta 8$) and slight transition toward red ($a^* = \Delta 7.0$). HNE-treated wounds expressed higher luminosity ($L^* = -\Delta 3.0$ on average) and a chromaticity shift away from red ($a^* = +\Delta 1.0$). That is, all HNE wounds were of a paler red color when compared to control. All wounds looked to be relatively healthy within the first three days, and no stark shifts toward nonviable tissue color were observed.

Histologic assessment and rate of reepithelialization

Stained histology samples acquired at day 4, 7, and 14 were graded by a board-certified veterinary pathologist masked to the treatment. The individual grading criteria included multiple key metrics such as reepithelialization, presence of inflammatory cells, degree of collagen fibril formation, and angiogenesis. The cumulative scores are shown in Fig. 6B. The histological grading showed no significant differences in cumulative scores between the untreated controls and HNE-treated groups at 4, 7, 14 days. All samples showed increasingly better scores as a function of time, with no signs of reinfection. The untreated control samples received cumulative scores of 8.7, 12.3, and 13.5 for the 4-, 7-, and 14-day time points, respectively. In comparison, HNE at the lowest concentration scored an 8.1, 11.8, and 14.5. HNE at 0.01% scored an 8.0, 11.9, and 14. Samples treated with HNE at 0.03% scored 6.9, 11.5, and 12.1 for the same time points. Similarly, the highest concentration of HNE at 0.063% scored 7.3, 11.5, and 12.1.

The degree of reepithelialization was compared between groups, and there were no significant differences (Fig. 6A). The untreated control group had reepithelialized by $62.14\% \pm 7.62\%$ after 4 days, $93.26\% \pm 3.61\%$ after 7 days, and completely after

14 days. The wounds treated with HNE 0.0% had reepithelialized $65.19\% \pm 11.32\%$ after 4 days, $94.29\% \pm 3.78\%$ by day 7, and 100% by day 14. HNE treatment at 0.01% produced similar results with reepithelialization of $55.04\% \pm 13.61\%$, $95.72\% \pm 2.21\%$, and 100% at the same time points. HNE at 0.03% had reepithelialized $57.77\% \pm 6.50\%$ after 4 days, $98.58\% \pm 1.42\%$ by day 7, and 100% by 14 days, and wounds treated with HNE 0.063% had reepithelialized $53.76\% \pm 6.09\%$ after 4 days, $91.99\% \pm 5.06\%$ by day 7, and 100% by 14 days (Fig. 7).

Corresponding gram staining at days 4 and 7 showed no discernible pathogens in the wound space (Fig. 8). Day 14 slides also did not exhibit any positive gram staining within the wound (data not shown). A few pockets of microorganisms were localized in the dessicated scab, but these microbial zones were apical to the keratinized layer.

Qualitative analysis of wound topography

Images of the wound bed at day 14 were used to reconstruct the 3D geometry of the lesion for topographical measurements. Representative topographic models are shown in Fig. 9. Analysis revealed that all HNE-treated wounds produced a well-filled lesion. Slight contraction lines were observed on the wound perimeter. The control cohort showed a comparable degree of remodeling. Hypergranulation in one untreated control wound was seen. In this sample, the volume of tissue fill exceeded the height of the original skin, causing a raised defect (Fig. 9). In contrast, there was no evidence of hypergranulation in any of the HNE samples.

DISCUSSION

The prevalence of antibiotic resistance and the ambiguity in the cytotoxic effects of silver have

Figure 6. (A) Quantitation of the degree of reepithelialization; (B) histological scores for all treatment groups and reepithelialization are shown. Scores were made from representative tissue sections at days 4, 7, 14. No significant differences were found between groups for their respective timeframes. Reepithelialization percentage was measured from the histological cross sections of H&E-stained slides. Results showed no statistical significance between the control and treatment groups. H&E, hematoxylin and eosin.

warranted new strategies to address the issue of wound infection. While new drugs offer a potential solution, the many challenges in drug discovery severely limit the availability of new treatments in a timely manner. One particular concept that has gained attention is the use of nonionic hyperosmotic agents such as sugars and honey.^{8–10} These hyperosmotic treatments have been used extensively to treat acute and chronic wounds with some level of experimental and clinical success.^{10,20,21} Previously, we demonstrated that the combination of a membrane disrupting emulsion (*i.e.*, thymol) with hyperosmotic stress synergistically deactivates common and drug-resistant pathogens *in vitro*.¹⁴ The mode of action was biomechanical through membrane damage and enhanced cellular dehydration. Extending on these data, we presently describe a swine model of partial thickness injury to assess the effects of topical HNE administration. A swine model was chosen due to the close anatomic and physiologic similarities between pig and human skin.²²

Experimental data confer several distinct characteristics of HNE topical treatment. First, HNE-

treated wounds were associated with an increase in wound exudate and a moist wound environment. All of the HNE material and exudate were fully absorbed into the primary foam dressings. The control wounds were slightly dry and adhered to the foam dressing during bandage changes. In contrast, the HNE sites were hydrated and showed no signs of bandage adhesion. This is consistent to previous reports that hyperosmotic sugars help maintain a moist wound environment.²³ Wound bed color, which has been used as an indicator of chronic wound viability,¹⁹ was used here to detect possible differences in the acute wound healing phase. Color measurements showed some slight differences, whereby the controls exhibited a more red tone, which may be indicative of either improved vascularization or suboptimal hemostasis. For example, the control sites were often still bleeding at day 3, while most HNE achieved hemostasis within 24 h postsurgery. Neither the control nor HNE wounds appeared grossly inflamed, and the color was within the bounds of healthy wounds. This trend was consistent until day 4, at which the HNE groups began to darken

Figure 7. H&E-stained sections of the injury area at 4, 7, and 14 days. Histological examination showed that the controls healed with less fibrin accumulation than the HNE groups. At day 4, controls exhibited bleeding, with red blood cells (RBC) present at the lesion. In contrast, all HNE wounds demonstrated a thick layer of fibrin covering the dermis. Black arrows denote migration edge of keratinocytes. By day 7, nearly all wounds were completely reepithelialized. Infiltrating fibroblasts and a loose arrangement of collagen can be observed in the granulation tissue (G). By day 14, all wounds were healed and showed a thin keratinized layer (K) with the presence of rete pegs (RP). Granulation tissue and a newly deposited collagen matrix (Col) could be observed. Scale bar: 200 μ m. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

with the formation of a soft scab. Photographs of the wounds were taken daily to monitor wound size. However, changes in wound size were not practical to measure as the soft scab limited visualization of the wound periphery. As a result, histological measurements of the epithelium were used to track keratinocyte migration.

Wound swabs taken at 24 h postsurgery showed that all treatments had much lower CFU counts than the original inoculation dose of 5×10^7 CFU. While all wounds were contaminated with microflora, they were not above thresholds typically as-

sociated with an infection [*i.e.*, $<1 \times 10^6$ CFU/g of tissue or 1×10^6 CFU per swab²⁴ (see Dai *et al.*²⁵ for review of injury models)]. This early rapid reduction may be attributed to the action of the wound exudate, which carries nonadherent microorganisms into the absorbent foam pad. The HNE concentrations demonstrated lower CFU counts, with approximately a 0.5–1.0 log reduction over control. The degree of reduction was most noticeable at the highest thymol concentration. We again suspect that the hygroscopic nature of HNE aids in flushing debris and bioburden into the foam dressing, as

Figure 8. Gram-stained sections of the injury area at 4 and 7 days post-injury. Higher magnified images of the wound region did not show bacterial infiltration into the epithelium or granulation tissue. *Black arrows* denote leading edge of keratinocyte migration. Scale bar: 200 μ m. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

noted by the saturated bandages during dressing change. Follow-up wound swabs were taken beyond the first 24 h to assess changes in wound flora. Cultures at days 4 and 7 and skin biopsies at 14 days showed that the microorganism counts increased (data not shown) as healing progressed. However, histological examination revealed that the bacteria were superficial and the population increase was localized to the nonviable crust of the wound scab. No signs of deeper penetration or re-infection were present.

Histologically, it was evident that the hemostatic response varied between the control and HNE cohorts. All HNE-treated wounds possessed a thick fibrin covering while the reepithelialization front was observed bisecting through the fibrin layer. In contrast, the keratinocytes in the control group followed a migration path above the dermal

layer. Only a small amount of fibrin was present at day 4 and was accompanied by areas of nude dermis. Note that part of the fibrin may also have been removed due to its adherence to the bandage, but the foam was moistened with saline to minimize wound damage during dressing changes. This delayed hemostatic response may predispose the control group to additional contamination, although no such cases were found. All wounds reepithelialized at a comparable rate, $\sim 60\%$ by day 4 and 90% by day 7. All wounds were completely healed by day 14. Granulation tissue was observed at day 7 and progressed throughout day 14. Histomorphologic grading that took into account multiple variables did not show any statistical significance between the control and HNE groups. Indeed, with the exception of fibrin thickness, it was histologically difficult to differentiate between treatment groups.

Moreover, there have been associations that hyperosmotic treatments may reduce inflammation and dermal scarring.^{26,27} It has been hypothesized that the sugars may enhance the production of hyaluronic acid or direct collagen formation into a more reticular structure.²⁸ At day 14, collagen reorganization was apparent from the parallel oriented fibers, but no conclusions regarding scar formation could be made at such an early time frame. Three-dimensional photogrammetry was used to qualitatively assess wound topography.²⁹ Results showed all wounds were well filled, with visible contraction lines at the wound margins. Nonetheless, one control wound did exhibit hypergranulation tissue formation and hyperreepithelialization. Additional research is needed to clarify whether the hypergranulation tissue was a statistical anomaly or a low-frequency outcome.

The cumulative data suggest that application of HNE did neither impede nor improve the rate of wound closure in this partial thickness injury model. Interestingly, we did not find any statistical significance between increasing thymol concentration and wound healing. Therefore, thymol is well tolerated, and we conclude that the primary wound healing differences between the control and HNE groups were from the hyperosmotic matrix. While all animals demonstrated an efficient response in coping with the initial inoculum, higher virulence pathogens could prove more challenging. In such cases, the inclusion of thymol may have a more therapeutic impact.

The current study has several limitations and potential sources of error. For example, an electrodermatome was used to create wounds with an average depth of 0.76 mm. These depth measurements were confirmed by micrometer measurements of the excised skin to vary by no more than

Figure 9. Topographical analysis of the healed wounds at day 14. Superficial molds of the wounds were obtained through polymeric casting and subsequently imaged through three-dimensional photogrammetry. Representative inverted reconstructions are shown. Qualitative cosmetic analysis revealed wounds that were mostly well filled, with some perimeter contraction lines. No significant differences in the cosmetics were observed, although one control wound (*) demonstrated slight hypergranulation and ridged areas. *Black arrows* denote wound perimeter. Scale bar: 1 cm. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

10% (data not shown). However, slight localized differences in wound depth may contribute to increased variability in healing. In addition, we have not made comparisons with standard treatments such as silver sulfadiazine or topical antibiotics, and it is unclear if the current daily dosing frequency is optimized for the HNE. Hyperosmotic sugar and honey dressing changes vary from every other day up to two to three times per day, with daily changes being most common.^{21,30,31} Future studies will investigate how standard treatments compare with HNE both in antimicrobial activity and in wound healing. Follow-up studies may further investigate the role of HNEs in ameliorating wound biofilm in chronic wounds (colonization vs. contamination), wound flora identification, as well as the wound molecular biology in response to hyperosmotic gradients.

In summary, HNEs offer an innovative agent to wound care. This study, while not demonstrating accelerated healing, showed an early reduction in pathogen numbers following wound contamination. HNE assisted in maintaining a moist wound environment as measured by wound exudate and kept the bandage from adhering to the wound. Subsequently, the bandages were easier and less painful to remove and resulted in satisfactory

healing and cosmetic outcomes in the porcine partial thickness wound model. HNE promoted fibrin formation and improved hemostasis, but it is unclear if this excessive fibrin is beneficial. Furthermore, the threshold value of 0.063% thymol and below did not have deleterious effects on wound healing. The proposed mechanism of antimicrobial action is a combination of microbial inhibition and osmotic fluid draw that cleanses the wound of surface contaminants and bioburden. Much of that action was attributed to the osmotic component while the emulsion aspect served as a preservative. Therefore, we conclude that the HNEs as presented are well tolerated as topical agents for acute wound management.

INNOVATION

Currently, there is a growing concern regarding the emergence of antibiotic-resistant pathogens due to the ubiquitous use of antibiotics. Furthermore, alternative topical biocides often pose cytotoxic side effects and may delay the wound healing process. In this study, we propose a novel hyperosmotic emulsion that can be used to reduce wound bioburden without delays in wound healing. The emulsions discourage microbial growth while simultaneously providing a moist clean wound bed

conductive to healing. The HNEs do not utilize antibiotics and may therefore be utilized as a prophylaxis or topical agent in the management of acute wounds.

ACKNOWLEDGMENTS AND FUNDING SOURCES

Technical help—Catherine Schmidt, and technicians from BME. Mohammad Zaroura for colony counting and microbiology. This study was funded by the National Science Foundation SBIR Phase 1 grant 1248852 made to Medtrix, LLC, 2013.

AUTHOR DISCLOSURE AND GHOSTWRITING

J.L. and S.C. are officers at Medtrix Biotech, LLC, and may potentially benefit from the outcomes of this research. The content of this article was expressly written by the authors listed. No ghostwriters were used to write this article.

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KEY FINDINGS

- HNEs maintain a moist and well-debrided wound bed while promoting early stage fibrin formation.
- HNEs reduced wound bioburden compared to control when wounds were contaminated with multiflora

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Abbreviations and Acronyms

a_w	=	water activity
CFU	=	colony forming unit
H&E	=	hematoxylin and eosin
HNE	=	hyperosmotic nanoemulsion