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Topical Nanoemulsion Therapy Reduces Bacterial Wound Infection and Inflammation Following Burn Injury

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Abstract

Background—Nanoemulsions are broadly antimicrobial oil-in-water emulsions containing nanometer-sized droplets stabilized with surfactants. We hypothesize that topical application of a nanoemulsion compound (NB-201) can attenuate burn wound infection. In addition to reducing infection, nanoemulsion therapy may modulate dermal inflammatory signaling and thereby lessen inflammation following thermal injury.

Methods—Male Sprague-Dawley rats underwent a 20% total body surface area (TBSA) scald burn to create a partial thickness burn injury. Animals were resuscitated with Ringer's lactate and the wound covered with an occlusive dressing. Eight hours after injury, the burn wound was inoculated with 1×10^6 CFU of *Pseudomonas aeruginosa*. NB-201, NB-201 placebo, 5% mafenide acetate solution or 0.9% saline (control) was applied onto the wound at 16 and 24 hrs following burn injury. Skin was harvested 32 hrs post-burn for quantitative wound culture and determination of inflammatory mediators in tissue homogenates.

Results—NB-201 reduced mean bacterial growth in the burn wound by a thousand fold, with only 11% animals having *P. aeruginosa* counts greater than 10^5 CFU/g tissue versus 91% in the control group ($p < 0.0001$). Treatment with NB-201 attenuated neutrophil sequestration in the treatment group as measured by myeloperoxidase assay and by histology. It also, significantly reduced levels of pro-inflammatory cytokines (IL-1 β and IL-6) and the degree of hair follicle cell apoptosis in skin when compared to saline-treated controls.

Conclusions—Topical NB-201 substantially reduced bacterial growth in a partial thickness burn model. This reduction in the level of wound infection was associated with an attenuation of

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the local dermal inflammatory response and diminished neutrophil sequestration. NB-201 represents a novel potent antimicrobial and antiinflammatory treatment for use in burn wounds.

Keywords

Apoptosis; Cytokine; Myeloperoxidase; Partial thickness burn; Tissue edema

INTRODUCTION

Contemporary burn wound management involves early debridement and reconstruction of clearly non-viable skin coupled with provision of supportive care and topical antimicrobial dressing changes to partial thickness burn wounds. The goal of modern burn wound care is to provide an optimal environment for epidermal renewal. During the period of epidermal renewal it is important to avoid further injury to the skin, abrogate burn wound progression, and minimize secondary complications such as wound infection.¹

Popular topical antimicrobial agents include silver sulfadiazine (Silvadene), mafenide acetate (Sulfamylon), and colloidal silver impregnated dressings (Acticoat, Silverlon). Each of these agents has potential limitations such as variable ability to penetrate eschar, uneven efficacy against both Gram-negative and Gram-positive bacteria, and potential toxicity to host immune cells.² There exists a need to develop a new generation of broad spectrum topical antimicrobial agents that can penetrate deeper into the burn wound. These agents could potentially be combined with antiinflammatory drugs to minimize early burn wound inflammation and tissue edema. In addition to local effects, severe dermal burns are known to induce the systemic inflammatory response syndrome (SIRS), which results in a high-risk of end-organ dysfunction.³

Antimicrobial nanoemulsions are mixtures of oil-in-water droplets where the droplets range from 200–600 nm in size. These emulsions are stabilized by surfactants and alcohol. The active ingredients are approved for over-the-counter human applications and are on the FDA ‘Generally Recognized as Safe’ (GRAS) list. A high-energy state is formed in the particle during manufacture using a high-speed mixer. In-vitro testing of these agents has confirmed that they have broad antimicrobial activity against Gram-negative and Gram-positive bacteria, enveloped viruses, fungi, spores, and protozoa. The liquid nanoemulsion particles are thermodynamically driven to fuse with lipid-containing organisms. Fusion with cell membranes is enhanced by the electrostatic attraction between the cationic charge of the emulsion and the anionic charge of the pathogen. When critical concentrations of nanoparticles fuse with the cell membrane, they release energy trapped within the emulsion which destabilizes the pathogen lipid membrane and results in cell lysis and death.^{4–8}

NB-201 is an antimicrobial nanoemulsion formulation consisting of emulsification of vegetable oil and water with surfactants and alcohol. We hypothesized that treatment of burn wounds with this innovative nanoemulsion compound would attenuate the development of wound infection based on quantitative wound culture, with a positive result defined as growth of bacteria at greater than 1×10^5 colony forming units per gram of tissue.^{9–11} Treatment with topical nanoemulsion will also result in less dermal inflammation and reduce hair follicle apoptosis in an animal model of partial thickness scald injury.

MATERIALS AND METHODS

Reagents

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Animals

Male specific pathogen-free Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing approximately 250–300 g were used in all experiments. All experiments were performed in accordance with National Institutes of Health guidelines for care and use of animals. Approval for the experimental protocol was obtained from the University of Michigan Animal Care and Use Committee.

Burn model

The procedure was performed according to a previously established method to produce partial thickness burn injury.^{12,13} Briefly, animals were anesthetized with a 40 mg/kg intraperitoneal (ip) injection of sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL). Dorsal hair was closely clipped and removed using Nair depilatory cream (Church & Dwight Inc., Princeton, NJ). Each rat was placed in an insulated, custom-made mold, which exposes the dorsal region over 20% of the total body surface area. Partial thickness scald burn injury was achieved by placing the exposed skin of the rat in a 60°C water bath for 25 seconds. Sham burn animals received the same treatment except they were immersed in room temperature water (21–24°C). The burn wound was scrub-debrided with dry sterile gauze and rinsed with 0.9% sterile NaCl. Each animal was resuscitated with 4 mL Ringer's lactate / % total body surface area burn / kg body weight. One half of this fluid volume was given intraperitoneally and half subcutaneously immediately following the burn injury. After drying, an occlusive dressing of sterile Telfa (Kendall Co., Tyco Healthcare Group LP, Mansfield, MA) and Tegaderm HP (3M Health Care, St Paul, MN) was applied to prevent wound contamination. During the experiment each rat was singly housed and received 0.01 mg/kg buprenorphine subcutaneously at the time of burn and at 16 hours for postburn pain control.

Local wound treatment

Stock nanoemulsion compound NB-201 was obtained from NanoBio Corporation (Ann Arbor, MI). This nanoemulsion was manufactured by emulsification of super-refined soybean oil and water with surfactants and alcohol. The resultant droplets had a mean particle diameter of 350 nm. The experimental solution was made by diluting 1 mL of the 60% stock formulation with 4.88 mL sterile saline and adding 120 µL of 1 M ethylenediaminetetraacetic acid (EDTA) giving a final concentration of 10% NB-201 and 20 mM EDTA. A placebo nanoemulsion compound was manufactured in the same manner as the NB-201, but one of the active ingredients was deleted from the formulation (benzalkonium chloride). 5% Sulfamylon (UDL Laboratories, Inc., Rockford, IL) solution was formulated by mixing 50 g of mafenide acetate powder in 1 L of 0.9% sterile saline. The control reagent used was 0.9% sterile saline. Experimental groups consisted of sham, burn, burn + NB-201, burn + bacteria + saline, burn + bacteria + placebo, burn + bacteria + NB-201, and burn + bacteria + Sulfamylon. Sixteen hours following burn injury animals were anesthetized with inhaled isoflurane. The occlusive dressing and Telfa was removed. Nanoemulsion (NB-201), placebo, Sulfamylon or sterile saline was applied in a uniform fashion to the burn wound surface using a spray bottle. Animals in the sham or burn group received no topical treatment, but did undergo dressing change under anesthesia. The burn wound was then redressed with Telfa and a Tegaderm occlusive dressing. This treatment and dressing change was repeated at 24 hours following burn injury.

Bacterial culture and inoculation

Pseudomonas aeruginosa isolated from a human burn patient was provided by the Department of Pathology at the University of Michigan. This bacterial isolate is sensitive to the topical agent Silvadene and Sulfamylon. A bacterial inoculum was prepared by thawing

an aliquot (0.5 mL, stored in 50% skim milk at -80°C) in 40 mL of Trypticase soy broth (Becton Dickinson, Franklin Lakes, NJ) and grown overnight at 37°C with constant shaking at 275 rpm. A sample of the resulting stationary-phase culture was transferred to 35 mL of fresh Trypticase soy broth and incubated for 2.5 hours to reach the log-phase. This subculture was transferred to a 50 mL conical polystyrene tube and centrifuged for 10 minutes at 4°C and 880 g. The bacterial pellet was washed with 0.9% sterile saline, and resuspended in 10 mL of ice-cold saline. The optical density of the suspension was measured at 620 nm and bacterial concentration (colony forming units [CFU] /mL) calculated using the formula $\text{OD}_{620} \times 2.5 \times 10^8$. The bacterial suspension was diluted with 0.9% sterile saline to a final concentration of 1×10^6 CFU per 100 μL . Eight hours following burn injury animals were anesthetized with inhaled isoflurane. The rats then underwent topical application of 1×10^6 CFUs of log-phase *Pseudomonas aeruginosa* in 100 μL of sterile saline pipetted onto a piece of Telfa in a uniform fashion followed by coverage with a Tegaderm occlusive dressing.

Tissue harvest

Thirty-two hours after thermal injury the animals were sacrificed and skin tissue samples were harvested using sterile technique. Skin samples were used immediately or frozen in liquid nitrogen.

Quantitation of bacterial wound infection

A 100 mg piece of excised skin tissue was mechanically homogenized in 1 mL of 0.9 NaCl. This homogenate was then further diluted with 9 mL of sterile saline. Serial dilutions were performed and skin homogenates plated in triplicate on blood agar plates (Becton Dickinson, Franklin Lakes, NJ). Culture plates were incubated for 24 hours at 37°C and CFUs counted.

Dermal cytokine analysis (ELISA)

A 100 mg sample of dorsal skin was homogenized in 1 mL of ice-cold lysis buffer consisting of 50 mL of PBS and protease inhibitor (Complete X, Roche, Indianapolis, IN) and 50 μL of Triton X (Roche). Homogenates were centrifuged at 3000g for 5 minutes and the supernatants collected and stored frozen at -80°C until use. Rat IL-1 β , IL-6, TNF- α , CINC-1, CINC-3, IL-10 and TGF- β were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using antibodies and reagents from R&D Systems, Inc. (Minneapolis, MN). Results were adjusted for previous dilution and expressed as pg/mL.

Detection of neutrophil sequestration (Myeloperoxidase assay)

100 mg of skin tissue was mechanically homogenized in 1 mL ice cold potassium phosphate buffer consisting of 115 mM monobasic potassium phosphate (Sigma Aldrich, Milwaukee, WI). Homogenates were centrifuged at 3000g for 10 min at 4°C , the supernatants were removed and the pellets were re-suspended in 1 mL C-TAB buffer consisting of dibasic potassium phosphate, cetyltrimethylammonium bromide, and acetic acid (Sigma Aldrich, Milwaukee, WI). The suspensions were sonicated (Branson Sonifier 250, Danbury, CT) on ice for 40 seconds. Homogenates were centrifuged at 3000g for 10 min at 4°C and the supernatant collected. Supernatants were incubated in 60°C water bath for 2 hours (Shaker Bath, 2568; Forma Scientific, Marietta, OH). Samples were stored at -80°C until needed or assayed immediately.

20 μL standards (Calbiochem, Gibbstown, NJ) or samples were added to a 96- well immunosorbent micro-plates (NUNC, Rochester, NY), followed by the addition of 155 μL of 20mM TMB/DMF consisting of 3,3',5,5'-tetramethylbenzidine/N,N-dimethylformamide in 115 mM potassium phosphate buffer (Fischer Scientific, Pittsburgh, PA) to each well.

The samples were mixed well, after which 20 μL of 3 mM H_2O_2 was rapidly added to each well. The reaction was stopped immediately by adding 50 μL /well of 0.061 mg/mL Catalase (Roche, Indianapolis, IN). The plates were read using a microplate reader at 620 nm. Myeloperoxidase (MPO) concentrations were calculated using a linear standard curve and adjusted for previous dilution. The final concentrations were expressed as $\mu\text{g/mL}$.

Determination of dermal capillary leak and tissue edema (Evans blue)

Burn wounds are associated with significant levels of capillary leak. This can lead to depletion of the intravascular volume and a need for large amounts of intravenous crystalloid fluid administration. To assess whether our therapy reduced capillary leak in conjunction with reducing inflammation we utilized the Evans blue assay, which is a measure of vascular permeability.¹³ Animals were anesthetized 90 minutes before tissue harvest. 50 mg/kg body weight of 10% Evans blue (Merck KgaA, Darmstadt, Germany) was injected ip into the burned animal at time 30.5 hours following thermal injury. At the tissue harvest time point animals were exsanguinated by incision of the inferior vena cava. Systemic Evans blue was flushed out with a total of four times the blood volume (7.46 mL/100 g body weight) of 0.9 NaCl with 100 units/mL heparin. Dorsal skin samples were harvested and a 100 mg sample was placed in 4 mL 99.5% formamide in polyethylene tubes. Tubes were placed on a shaker at room temperature for 48 hours for Evans blue extraction. Supernatants were collected and the absorbance read on a microplate reader at 620 nm. Concentrations were calculated from an Evans blue in formamide standard curve. Results are expressed as micrograms of Evans blue per mg of skin tissue.

Histology

Skin samples were fixed in 10% buffered formalin and embedded in paraffin. Eight μm thick sections were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to assess morphologic changes.

Detection of hair follicle cell apoptosis (TUNEL assay)

Animals were anesthetized and underwent creation of a 20% partial thickness scald burn wound or sham injury. Treatment groups consisted of sham, burn + saline, burn + placebo, and burn + NB-201. Treatment and dressing changes were performed at 0 and 8 hours post-burn. No bacterial infection was created in this experiment. Full-thickness skin samples were taken from three locations across the entire burn wound at 12, and 24 hours post thermal injury for determination of hair follicle cell apoptosis. There were four animals per treatment group per time sample.

As described previously, apoptosis was detected in situ with fluorescein based labeling of DNA strand breaks using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (ApopTag, CHEMICON International, Inc, Temecula, CA).¹³ The three fresh skin samples for each animal were placed in disposable vinyl cryomolds filled with optimal cutting temperature compound (Sakura Finetek, U.S.A., Inc., Torrance, CA), and frozen at -80°C until ready for use. Frozen embedded skin specimens were cut into 4- μm -thick serial sections in a cryostat and collected on Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PN). Sections were fixed and stained according to the manufacturer's instructions.

The TUNEL assay slides were blinded to groups, and under the microscope appropriate hair follicle cells in a randomly chosen high-power field were identified. Appropriate hair follicles for analysis were those sectioned in the mid-sagittal or midcoronal plane. A total of 3–6 hair follicles were selected from among the three skin samples present on a slide. Fluorescent-labeled TUNEL slides were captured digitally at identical time post-labeling to

control fading of fluorescence using an Olympus BX-51 fluorescence microscope at fixed image capture settings and 40× magnification. Each hair follicle was selected and first digitally captured by visualizing counterstained nuclei present using the DAPI excitation/emission channel. Then for each hair follicle analyzed, the excitation/filter channel was changed to visualize the fluorescein-labeled TUNEL-positive cells, and images again digitally captured. Within the captured images a region of interest (ROI) was digitally defined, set to include only hair follicle cells and exclude bright fluorescing hair shafts and surrounding cells (NIH Image J software, NIH, Bethesda, Md). Fluorescence of TUNEL-positive cells was quantified, normalized to ROI size, and expressed as pixels/area fraction, controlling for differences in ROI size.

Statistical Methods

All statistical analysis and graphs were performed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). Results are presented as mean values $\pm$ the SEM unless otherwise noted. Continuous variables were analyzed using an unpaired two-tailed Student's *t*-test and/or One-way ANOVA followed by Tukeys posttest comparisons. The Kruskal-Wallis test with Dunn's multiple comparisons was used to evaluate differences in medians for data with a non-parametric distribution. Discrete variables were compared using Fisher's exact test. Statistical significance was defined as a *p*-value < 0.05 .

RESULTS

Topical application of NB-201 nanoemulsion reduces *P. aeruginosa* growth in burn wounds

Animals treated with nanoemulsion had a decreased mean (6.5×10^4 vs. 7.9×10^7 , $p=0.07$) and median (0 vs. 4.4×10^6 , $p<0.05$) number of CFUs of bacteria per gram of skin tissue when compared to the saline treated controls (Figure 1). A similar reduction in skin bacterial counts was found for NB-201 treated animals vs. those treated with the placebo (mean: 6.5×10^4 vs. 5.5×10^6 , $p=0.02$). When performing quantitative wound culture on clinical tissue samples a positive result is generally considered to be growth of organisms at greater than 1×10^5 CFUs per g of tissue.^{9–11} Using these criteria, 29 of 32 animals in the control group exhibited evidence of a positive quantitative wound culture and only 3 of 23 animals in the nanoemulsion group demonstrated proof of this level of wound infection (91% vs. 13%, $p<0.0001$). Positive quantitative wound culture results for the placebo group were 9 of 12 animals (75%) and for the Sulfamylon group were 2 of 10 animals (20%). The Sulfamylon treated animals also demonstrated a significant reduction in both the median wound bacterial level and positive quantitative wound culture rate as compared to the saline controls (3×10^4 vs. 4.4×10^6 , $p<0.05$ and 20% vs 91%, $p<0.0001$). Treatment with nanoemulsion or Sulfamylon produced a similar reduction in the level of *Pseudomonas* cultured from the burn wound when compared to the saline treated animals. However, there was no statistically significant difference between the placebo and Sulfamylon groups whereas there was a difference for the NB-201 group compared to the placebo.

NB-201 treatment following burn injury attenuates dermal proinflammatory cytokine levels

Scald injury resulting in a partial thickness burn produced differences in dermal levels of IL-1 β and cytokine-induced neutrophil chemoattractant-3 (CINC-3) within skin homogenates obtained 32 hours post-injury compared to sham injured animals (Figure 2A & E). Treatment with NB-201 at 16 and 24 hours post-burn reduced the dermal level of these two inflammatory mediators back down to the baseline (sham) in the absence of bacterial infection. In experiments where a bacterial wound infection was not created, a difference in neutrophil sequestration as measured by myeloperoxidase assay was not observed despite the rise in the rat CXC chemokine CINC-3 within burned skin. A difference was found

between all three groups (sham, burn, and burn + NB-201) for CINC-1 ($p=0.04$, ANOVA), however the values for intergroup comparison did not reach statistical significance.

NB-201 treatment following burn wound infection with *P. aeruginosa* attenuates dermal cytokine levels and results in reduced neutrophil sequestration

Skin homogenates from the nanoemulsion treated group had levels of IL-1 β and IL-6 that were considerably diminished when compared to the levels measured in the saline treated animals (Figure 3A & B). There was no statistically significant difference seen in the level of TNF- α between the two experimental groups of animals (Figure 2C & 3C). Treatment of the infected burn wound with Sulfamylon did not result in any significant alteration of dermal levels of the measured proinflammatory cytokines (IL-1 β , IL-6, TNF- α , CINC-1 or CINC-3) when compared to controls. Treatment with either NB-201 or Sulfamylon reduced the level of myeloperoxidase found in the infected burn wound at 32 hours post-injury. This suggests that treatment with an antimicrobial reduces the level of neutrophil sequestration into the partial thickness burn wound.

Burn injury caused a rise in the level of the anti-inflammatory cytokine TGF- β , but not IL-10 when compared to the sham injured animals (Figure 4). NB-201 treatment reduced the amount of TGF- β present in the infected burn wound as compared to the level found in the burn wound alone. This finding suggests that NB-201 not only alters acute burn wound dermal inflammation, but that it could potentially reduce the eventual immunosuppression created by thermal injury.

On histological examination of skin from the saline control animals, there is loss of most of the epidermis and a diffuse cellular infiltrate in the subepidermal region, extending into the lower dermal connective tissue in which collagen fibrils are separated by the infiltrating leukocytes and edema fluid (Figure 5A). At a higher power (not shown), the cellular infiltrate between the collagen bundles consists almost entirely of neutrophils. Edema fluid causes separation of the collagen fibrils. In Figure 5B, the skin was subjected to thermal injury followed by application of *P. aeruginosa* after which the nanoemulsion was topically applied to the burned area. The keratin layers of the epidermis are separating and some of the keratin has been lost. There is a barely detectable intradermal presence of neutrophils together with neutrophils that are adhering to the wall of a venule, which has been longitudinally sectioned (in the center of the microphotograph). The changes in this microphotograph are substantially less extensive than those seen in Figure 5A.

Quantification of capillary leak and tissue edema

Quantitative measurement of the amount of Evans blue dye leaching out of the blood stream and into the skin tissue revealed that the nanoemulsion treated animals had less evidence of post-burn capillary leak and tissue edema than the saline treated controls (Figure 6).

Treatment with NB-201 reduces burn induced hair-follicle apoptosis

Dermal apoptosis occurs in the hair follicle cells following thermal injury. Using a fluorescence labeled TUNEL assay the burn wounds treated with saline showed evidence of intense FITC-TUNEL positive cells which appear green (Figure 7). The DAPI nuclear stain allows identification of coronal or sagittally sectioned hair follicles with the cells staining blue. FITC-TUNEL positive cells appear green and are representative of apoptotic cells. In the merged images, the apoptotic hair follicle cells are evident in slides from the burn + saline animals and these changes are diminished in the burn + NB-201 treated animals. Counting the pixels of TUNEL positive cells within a hair follicle region of interest allowed quantification of the reduction in hair follicle cell apoptosis by treatment with topical NB-201 (Figure 8). The saline treated control animals had an increased amount of TUNEL

positive cells when compared to the sham burn animals. Both the NB-201 and placebo treatment resulted in a decrease in hair follicle cell apoptosis following partial thickness burn injury in tissue harvested 12 hours following thermal injury. This difference was not evident in the dermal skin sampled at 24 hours post-burn. To summarize, treatment with NB-201 reduced apoptotic cell death in hair follicles in the early post-burn period.

DISCUSSION

NB-201, the nanoemulsion formulation utilized in these experiments is capable of reducing and in some cases eradicating a *P. aeruginosa* wound infection within a partial thickness burn wound. We found that this reduction in microbial infection was coupled with generation of lower levels of local dermal pro-inflammatory cytokines and evidence of reduced neutrophil sequestration into the burn wound. This decrease in burn wound bacterial growth and inflammation also produced less capillary leak in the early post-thermal injury time-period. Having the ability to clinically reduce capillary leak and tissue edema in the immediate post-burn time-period could result in less need for large volume crystalloid fluid resuscitation and a reduction in the associated sequela of physiologic volume overload, pulmonary dysfunction, and abdominal compartment syndrome.

Skin that is damaged by thermal injury loses its ability to protect the host against infection from both the loss of physical barrier function and the secondary immunosuppression caused by the thermal injury. Increased production of TGF- β and IL-10 during the postburn period can result in immunosuppression.^{14,15} It has been established that treatment of burn injured animals with anti-TGF- β can improve local and systemic clearance of *P. aeruginosa*.¹⁶ Inhibition of TGF- β also results in increased survival following bacterial challenge. In our experiments, we found a significant elevation of TGF- β , but not IL-10 in the skin following partial thickness burn injury. Topical application of NB-201 to the burn wound inoculated with bacteria did result in a reduction of the level of TGF- β when compared to the untreated burn wound.

Onset of a bacterial infection within a burn wound can delay or even reverse the tissue healing process.¹⁷ Topical antimicrobial therapy is used to reduce the microbial load in the burn wound and reduce this risk of infection. Current topical agents include silver nitrate (AgNO₃), silver sulfadiazine, mafenide acetate, and nanocrystalline impregnated silver dressings. Thermal injury initiates dermal inflammatory and pro-apoptotic cell signaling.¹³ None of the above listed agents acts principally to reduce burn wound inflammation. In this study topical application of NB-201 resulted in reduced hair follicle cell apoptosis within the dermis of burned skin. This suggests that NB-201 may be helpful in reducing conversion of the partial thickness burn wound within the “zone of stasis” to regions of full thickness burn. A limitation of our experimental model is that we created a partial rather than full thickness burn wound. This was specifically done so that we could investigate the hair follicle response to the topical treatment agents.

In patients without evidence of inhalational injury, the burn wound itself is the primary source triggering the systemic inflammatory response via generation of pro-inflammatory cytokines and sequestration of neutrophils into the burn wound.^{18–20} Topical application of SB 202190, an inhibitor of activated p38 MAPK, can control the source of inflammation at the level of the dermis, resulting in lower levels of pro-inflammatory mediators, reduced neutrophil sequestration and microvascular damage, and less epithelial apoptosis in burn wound hair follicle cells.¹³ Dermal source control of inflammation also reduces bacterial growth and attenuates the systemic inflammatory response resulting in less acute lung injury and cardiac dysfunction following partial thickness burn injury in a rodent model.^{21–24} Our search for a potential vehicle in which to deliver this compound led us to consider

nanoemulsion technology. The ultimate future therapeutic goal is to couple an antimicrobial vehicle (NB-201) with a synergistic antiinflammatory agent (SB 202190 or similar inhibitor of activated p38 MAPK) to reduce local dermal inflammation and the risk of infection within early burn wounds.

During the 1990's the Michigan Nanotechnology Institute developed a composite material that resulted in a new class of antimicrobial agents with broad activity against Gram-positive and Gram-negative bacteria, spores, fungi, and viruses.⁴⁻⁷ These nanoemulsions are oil-in-water mixtures containing high energy nanometer-sized droplets stabilized by surfactants. The spectrum of antimicrobial activity can be altered depending upon which detergents and solvents are added to stabilize the emulsion.⁶ Addition of EDTA permeabilizes the outer membrane of Gram-negative bacteria, and enhances the intrinsic activity of the nanoemulsion against *P. aeruginosa*. The dilute emulsions are milky white in consistency and appearance. Thickeners can be added to increase viscosity and reduce running. This was not done in the current experiment because we wished to avoid mechanical manipulation of the skin surface following inoculation with bacteria.

Pathogen killing is a function of the nanoemulsion particles being thermodynamically driven to fuse with lipid-containing cell walls of bacteria or other organisms. This fusion is enhanced by electrostatic attraction between the cationic charged emulsion particles and the anionic charge of the pathogen cell wall. When a critical amount of nanoparticles fuse with the pathogen they release part of the energy trapped within the emulsion. Both the active ingredient and released energy act to destabilize the pathogen lipid membrane resulting in cell lysis and death.⁵⁻⁷ The nanoemulsion material is selectively toxic to microbes at concentrations that are non-irritating to human skin or mucous membranes. An FDA phase 2b clinical trial for topical treatment of *Herpes labialis* using a similar formulation has been completed.^{25,26} No safety issues were identified and the treatment was both efficacious and well tolerated.²⁷ Because of their small particle size and surface-active properties nanoemulsions are believed to traverse skin pores and hair follicles, while being excluded from entering the tight junctions of the epithelium. Fluorescent labeling of a similar nanoemulsion confirmed that it is distributed preferentially in the hair follicles and sebaceous glands following application to human cadaver skin.²⁸ Thus, the nanoemulsion compound can be highly bioavailable in the dermal tissues without disrupting the normal epithelial matrix. This property, coupled with its antimicrobial activity, and low toxicity makes NB-201 a highly desirable topical agent to be used alone or as a vehicle to deliver additional agents to enhance antiinflammatory treatment of burn wounds.

CONCLUSION

Topical nanoemulsion therapy with NB-201 significantly reduced bacterial growth in a partial thickness burn model. Killing of the inoculated pathogen, *Pseudomonas aeruginosa*, resulted in attenuation of the local dermal inflammatory response and reduced neutrophil infiltration into the burn wound. NB-201 is a novel potent antimicrobial treatment for potential use in clinical burn wounds.

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Figure 1.

Topical application of NB-201 reduces *P. aeruginosa* growth in burn wounds. Male Sprague-Dawley rats received partial thickness burn wounds. At 8 hours post-injury, animals were inoculated with 10^6 CFU *P. aeruginosa*. At 16 and 24 hours post-burn the animals were treated with topical saline (Control), placebo (NB-201 without benzalkonium chloride), NB-201 (Nanoemulsion), or Sulfamylon (mafenide acetate). At 32 hours, animals were sacrificed, skin samples obtained and homogenized, plated, and CFUs counted. The scatter plot represents cultured CFUs for each individual animal. The median value for each group is plotted as a horizontal line. There was minimal pathogen growth in 12 out of 23 of the NB-201 treated animals. A majority of the control (29/32) and placebo (9/12) animals with burn injury had evidence of wound infection based on a positive quantitative wound culture with significantly more bacteria present in the wound than those animals treated with NB-201. $p < 0.0001$, Kruskal-Wallis test, $p < 0.05$ for saline vs. NB-201, placebo vs. NB-201, and saline vs. Sulfamylon, Dunn's multiple comparison test.

Figure 2.

NB-201 treatment following burn injury attenuates dermal proinflammatory cytokine expression. Groups were sham, burn, and burn with NB-201 treatment (n=8–10 per group).

A) IL-1 β ($p=0.02$, 1-way ANOVA), **B)** IL-6 ($p=0.8$), **C)** TNF- α ($p=0.5$), **D)** CINC-1 ($p=0.04$), **E)** CINC-3 ($p=0.005$), and **F)** Myeloperoxidase ($p=0.07$). Burn injured animals treated with nanoemulsion had decreased levels of IL-1 β (1773 ± 516 vs. 5625 ± 1743 pg/mL) and CINC-3 (225 ± 66 vs. 1589 ± 527 pg/mL) when compared to untreated partial thickness burned animals. * $p < 0.05$, t-test or Tukey's multiple comparison test.

Figure 3.

NB-201 treatment following burn wound infection with *P. aeruginosa* attenuates dermal proinflammatory cytokine expression. All animals received burn injury and groups were saline, placebo, NB-201, and Sulfamylon (n=10–30 per group). **A)** IL-1 β ($p=0.001$, 1-way ANOVA), **B)** IL-6 ($p=0.07$), **C)** TNF- α ($p=0.3$), **D)** CINC-1 ($p=0.8$), **E)** CINC-3 ($p=0.4$), and **F)** Myeloperoxidase ($p=0.0001$). Burn wound infected animals treated with nanoemulsion had decreased levels of IL-1 β (1007 ± 157 vs. 3054 ± 499 pg/mL) and IL-6 (244 ± 51 vs. 485 ± 73 pg/mL). The NB-201 and Sulfamylon treatment groups demonstrated reduced dermal neutrophil sequestration when compared to saline and placebo as evidenced

on myeloperoxidase assay (NB-201: 0.09 ± 0.02 , Sulfamylon: 0.08 ± 0.02 , Saline: 0.40 ± 0.06 , Placebo: 0.45 ± 0.11 $\mu\text{g/mL}$). $*p < 0.05$, Tukey's multiple comparison test.

Figure 4.

Burn injury upregulates dermal TGF- β expression and treatment with NB-201 in the setting of burn wound infection decreases the level of TGF- β in the wound. Dermal levels of the anti-inflammatory cytokines IL-10 and TGF- β were measured in the burn wound 32 hours after thermal injury in animals treated with nanoemulsion, and in animals exposed to bacteria and treated with NB-201 or Sulfamylon. **A)** IL-10 ($p=0.4$, 1-way ANOVA), and **B)** TGF- β ($p=0.0001$). Partial thickness burn increased the presence of dermal TGF- β as compared to sham (624 ± 55 vs. 232 ± 17 pg/mL). Treatment of an infected burn wound with NB-201 significantly reduced the dermal level of TGF- β compared to the untreated burn group (404 ± 43 vs. 624 ± 55 pg/mL). Sulfamylon treatment did not suppress the level of IL-10 or TGF- β in the infected burn wound. * $p<0.05$, Tukey's multiple comparison test.

Figure 5. Cross sectional histology of burn skin following infection with *P. aeruginosa* and treatment with saline or NB-201. Both views of skin are 32 hours after burn injury. **A)** Representative section from saline (control) treated animal (Hematoxylin and eosin×40). **B)** Representative cross-section from NB-201 (nanoemulsion) treated animal (Hematoxylin and eosin × 40).

Figure 6.

Evans blue assay to quantify capillary leak and tissue edema. Evans blue is a dye that binds to serum albumin and can be quantitated to determine vascular permeability. Topical NB-201 treatment resulted in less capillary leak following thermal injury and bacterial inoculation of the wound when compared to saline treated control animals (1.26 ± 0.05 vs. 1.93 ± 0.24 $\mu\text{g Evans blue/mg tissue}$, $n=8$ per group). $*p=0.02$, t-test.

Figure 7.

Photomicrographs of partial thickness burned skin with fluorescence labeled TUNEL staining to detect hair follicle cell apoptosis. Skin samples were harvested at 12 hours post-burn. Treatment was performed at 0 and 8 hours following thermal injury. All images are at 40× magnification.

Figure 8.

TUNEL assay for hair follicle cell apoptosis following a partial thickness burn injury. Skin samples from sham, burn + saline (control), burn + placebo (NB-201 without benzalkonium chloride), and burn + NB-201 (Nanoemulsion) were sectioned for fluorescein-labeled TUNEL assay. Treatment was performed at time 0 and 8 hours post injury. No bacterial inoculation of the burned skin was done in this experiment. There was a significant reduction in the degree of hair follicle apoptosis among burn injured and treated animals for tissue samples harvested at 12 hours post injury ($p=0.006$, 1-way ANOVA). Differences were found for sham vs. burn + saline, burn + saline vs. burn + placebo, and burn + saline vs. burn + NB-201 ($*p<0.05$, Tukey's multiple comparison test).