ULTRAVIOLET-A LIGHT AND NEGATIVE PRESSURE WOUND THERAPY TO ACCELERATE WOUND HEALING AND REDUCE BACTERIAL PROLIFERATION

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Background: Ultraviolet-A therapy is a simple, inexpensive, and effective modality for wound healing with tremendous potential to improve healing and reduce clinical infections in several clinical settings. To date, application of UV-A relies on bulky and hard to dose lamps that provide inconsistent therapy, thus making it difficult to apply therapy that is appropriate for the patient.

Methods: This study was designed to test the effectiveness of a novel wound therapy device that combines UV-A with traditional negative pressure wound therapy.
promote wound healing. Further, we tested the ability of fiber optic UV-A delivery to inhibit bacterial proliferation. Finally, we assayed the level of DNA damage that results from UV-A as compared to established UV-C therapies. Wound healing studies were performed in a porcine model using an articulated therapy arm that allows for continued therapy administration over an extended time course. Negative pressure wound therapy was administered alone or with ultraviolet-A fiber optic therapy for 2 weeks. Dressings were changed twice a week at which time wound area was assessed.

Results: Data demonstrate that UV-A with NPWT treatment of wounds results in greater healing than NPWT alone. Using the same therapy device, we demonstrate that exposure of *Staphylococcus aureus* and *Pseudomonas aeruginosa* to fiber optic UV-A results in decreased colony area and number of both bacterial strains. Finally, we show that UV-A induces minimal DNA damage in human fibroblasts and no more DNA damage in wound tissue as compare to intact skin.

Conclusions: These data demonstrate that UV-A can decrease bacterial proliferation and promote wound healing when coupled with NPWT.

There is an international epidemic of Type II diabetes. The estimated prevalence of diabetes in the United States is 12-14%, equating to approximately 37-44 million Americans (1). Lower extremity wounds are one of the most common complications in diabetic patients leading to amputation and hospitalization (2, 3). The direct cost of foot
ulcers and amputations has been estimated to be approximately $9.1-13.2 billion dollars a in addition to the direct cost of diabetes (4). Further, the annual mortality rate for diabetics who develop a foot ulcer is about 11%, and after a lower extremity amputation annual mortality is doubled to about 22% a year (5).

In diabetes, infected foot ulcers are one of the most common contributing causes of hospitalization and amputation. In a large cohort study (n=1,666), over half (56%) of the patients were treated for an infection of their ulceration over the course of 24 months (6). Infections (>10^5 organisms per gram of tissue) are associated with delayed wound healing in chronic wounds (7-9). But, routine use of antibiotics in uncomplicated ulcers does not improve healing rates or decrease bacterial colonization (10, 11). Wound therapies that could be used in conjunction with current modalities, such as negative pressure wound therapy (NPWT), can accelerate wound healing and reduce bacterial bioburden could have wide reaching implications to treat chronic, non-healing wounds.

NPWT has become a mainstay of complex lower extremity wound management where its use has been shown to increase chances of healing by nearly 6 fold and decrease amputation risk by more than 4 times (12, 13). While NPWT has been shown to improve outcomes of patients with soft tissue deficits, by improving wound perfusion and accelerating granular tissue ingrowth, further advances in wound management with
NPWT should include methods to address bacterial colonization and infection such as ultraviolet light therapy (12-14).

Ultraviolet light-A therapy (UV-A) is a simple, inexpensive, and effective modality for wound healing with tremendous potential to improve healing and reduce clinical infections in a number of clinical settings. There are several prospective clinical studies that report improved wound healing in surgical wounds, venous stasis ulcers and burns with UV-A phototherapy (15-20). Disinfection by UV-A is accomplished via the byproducts of a photo-reactive chemical process (21), where UV-A phototherapy initiates a chemical cascade ending in the creation of bactericidal hydroxides and reactive oxygen species (21-23). To this point, UV-A phototherapy devices have been bulky lamps that have an inconsistent output. Their output levels depend on distance from the wound, angle of incidence, and age of the bulb. Because of this, patients can only receive treatment in a clinical setting where the frequency and accuracy of dosing is difficult and places an additional transportation burden on the patient. The only UV device currently approved for use in wound care is the Thera-Wand Model C-100 Ultraviolet C (UV-C) Treatment Lamp (Biomation, Canada) (24-26), which uses UV-C versus UV-A.
The aims of this study were threefold. The first aim was to test the capacity of UV-A to inhibit growth of two bacterial pathogens (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) commonly found in wound environments. Secondly, this study examines a novel wound healing therapy, NPWT with UV-A fiber optic therapy (NPWT+UVA) against NPWT alone. Finally, we test the level of DNA damage that results from continuous treatment with UV-A therapy as compared to a UV-C device currently approved for treatment in the wound care setting.

**Materials and Methods**

**Articulated Arm for Continued Therapy**

Previous studies have always been short term, up to a few days, in a restrained animal, or in a manner that is obstructive to the animal causing undue stress. We developed a novel method to study wound-healing therapies in a porcine model for extended periods of time that involves an articulated freedom-of-motion therapy arm. The arm attaches to the side of the animal cage, has movement in all 3 cardinal planes, and allows the therapy mechanism to swivel with the pig (Figure 1A). This design allows for a safe and stress-free environment during therapy. The animal connects to the arm using a pin-locking mechanism with the D-ring on the dorsal side of a standard dog harness (Figure 1B). This allows for a quick-release in the event that the animal needs to
be unattached. This novel method for therapy delivery allows our group to accurately and reliably deliver therapies to a large animal without undue stress or intrusion.

**Aim 1: UV-A Administration and Wound Therapy Patches**

Although medical applications of UV light have existed for many years, our therapeutic approach has several novel advantages. The older systems used lamps above the treatment area, have uncontrolled output, and often lead to cross contamination.

Previous UV-A phototherapy studies used a large device, so therapy was limited to treatments for short periods of time in a clinic or hospital setting. UV-A was delivered using the UVITEK system (ThermoTek, Inc, Flower Mound, TX). This system uses a novel method to administer UV-A phototherapy using fiber optics (Figure 2A). These fibers are designed to only allow UV-A to escape in a single direction, thus facilitating more accurate dosing. These fibers transmit UV-A overtime with minimal (~6% loss per day) degradation (Figure 2B). For therapy administration, these fibers are incorporated into a patch that can be laid on top of the wound prior to dressing administration (Figure 2C). This system allows for ease of application and administration with other therapies, such as NPWT.
**Animals and Surgical Procedures**

A porcine model was used for this study. Five female pigs with a weight between 90-120 lbs. were purchased from K-Bar Livestock (Sabinal, TX). Animals were kept in a singly housed temperature-controlled environment. During a minimum of 5 days acclimation and experimental procedures, pigs were fed ad libitum chow (Harlan, Houston, TX). The animal was acclimated to the articulated arm that supported the therapeutic devices for 7 days prior to surgery to ensure minimal distress.

All animals were fasted 12 hours prior to surgery. Animals were anesthetized using (3mg/kg) Telazol (Ft Dodge Veterinaria, Ft Dodge, Iowa) and Xylazine (10mg/kg) by intramuscular injection and the animal was kept on a constant flow of an isoflurane/oxygen mixture via facemask followed by intubation. A pre-surgical injection of Atropine (0.04mg/kg) was also administered I.M. IV access was established for hydration with lactated ringer. A water blanket and Bair Hugger (Arizant Healthcare, Eden Prairie, NJ) was used to maintain body temperature. The dorsum of the animal, from below the shoulder blades to the hip bones, was cleaned, shaved and treated with Veet (Reckitt Benckiser, Parsippany, NJ) for hair removal. The animal was disinfected with chlorhexidine and alcohol, repeated three times and draped with sterile surgical drapes. Aseptic technique was used to place 3, 3cm diameter full thickness excisional wounds to the level of the muscle fascia on the dorso-lateral surface of the animal. After surgery, animals were monitored twice daily for the duration of the study. Wounds were
photographed prior to dressings being applied. To each of these wounds, 1 of 3 therapies were applied: -125mm Hg NPWT (NPWT), -125mm Hg NPWT + UV-A fiber optic therapy (NPWT+UVA), or saline-moistened gauze (CONT) (n=5 for each treatment group). Care of all animals and procedures were approved by the UT Southwestern Medical Center Institutional Animal Care and Use Committee.

Dressing Change, Wound Assessment and Sacrifice

Dressings were changed on days 3, 7, and 10. All animals were fasted for 12 hours prior to dressing change. Animals were anesthetized using Telazol (3mg/kg) and Xylazine (10mg/kg) by intramuscular injection and kept on a constant flow of isoflurane/oxygen via facemask. The skin surrounding the wound sites was cleaned with saline soaked gauze and treated with Veet for hair removal. Wounds were assessed for inflammation in or around the wound bed, excessive discharge, even granulation tissue formation, induration, and general appearance (color, bleeding before or during debridement, etc) in a blinded manner. After assessment, wounds were lightly debrided, and dressings were replaced. Photos were taken of each wound at each dressing change. Photos were scaled and analyzed by NIH ImageJ (Bethesda, MD) to obtain wound area. Wound area reduction was calculated as percent change from surgical incision area. Therapies were kept the same for each wound for the duration of the study. Animals were sacrificed 21 days post-operatively. Animals were anesthetized with an intramuscular
Injection of Telazol (6mg/kg) and sacrificed with Euthasol (Verbac AH, inc, Ft Worth, TX) (320mg/kg) injection intravenously.

**Aim 2: In Vitro UV-A Treatment of Staphylococcus aureus and Pseudomonas aeruginosa**

Suspended cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (ATCC, Manassas, VA) were streaked onto trypticase soy agar (TSA) plates. Plates were inverted and cultured at 37°C for 24 hours. Half of the plates were incubated over fiber optic patches delivering UV-A and half were incubated over patches with no UV-A delivery. UV-A light was administered at a daily dose equivalent to what was used in the animal study below.

**Aim 3: In vitro UV-A and UV-C Treatment of human fibroblasts**

The UV-A fiber optic system was calibrated and set to a level that would deliver 750µW/cm² to the cells plated in a plastic tissue culture dish. Levels were assessed using a UV-A measuring device (General Tools Corp. Model Number: UV513AB). Early passage (~10x) fibroblasts isolated from human foreskin were grown to 70-80% confluence and exposed to UV-A fiber optics for 48 hours (constant exposure), with samples being taken at 0, 24, and 48 hours. UV-C was delivered using the Thera-Wand Model C-100 Ultraviolet C Treatment Lamp (Biomation, Canada). The wand was used
per manufacturer’s instructions at an output of 4mW/cm². Cells were exposed for 30s with the wand ~1in above the dish of cells. Cells were then placed back in the incubator and samples were taken at 0 (prior to exposure), 2h, 6h, 24 and 48hr time points. The 48-hour data is not presented due to the level of cell death by this time point and the inability to obtain a sample.

**Comet Assay**

The comet assay to detect DNA damage was performed using the method of Sasaki et al. with some modifications (27). Briefly, fibroblasts were exposed to UV-A or UV-C as noted above. At the end of the treatment, cells were removed from the plates with trypsin. Trypsin was inactivated with serum-containing phosphate buffered saline (PBS). Fifty microliters of the cell suspension (500,000 cells/ml) were diluted in 500μl low-melting-point agarose (0.5% w/v in PBS). The resulting suspensions were embedded in previously prepared normal-melting-point agarose (1% w/v in PBS) on frosted slides followed by the addition of 75μl of normal-melting-point agarose (1%). The slides were then immersed in lysis buffer (2.5 M NaCl, 100mM Na₂EDTA, 10mM Tris-HCl, pH 10) for 0.5 hr at 4°C in the dark. After lysis slides were placed in alkaline electrophoresis buffer (0.3 M NaOH, 1mM Na₂EDTA) for 20 min at 4°C to denature DNA and express alkali-labile sites. Electrophoresis was carried out at 4°C for 20 min at 21 V and approximately 300mA. The slides were then washed twice in neutralizing buffer (0.4 M Tris-HCl, pH 7.4)
for 5 min. The DNA was stained by adding 45 μl of ethidium bromide (20 μg/ml) to each slide.

**Western Blot Analyses**

Cells were harvested by trypsinization, washed once in phosphate-buffered saline, and resuspended in lysis buffer (10 mM sodium phosphate buffer [pH 7.2], 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, and 1% NP-40, supplemented with 10 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 mM β-glycerophosphate, 10 mM sodium orthovanadate, and 10 μg/ml leupeptin and aprotinin). Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories). Samples containing equal amounts of protein were mixed with an equal volume of 2× Laemmli sample buffer (125 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate (SDS), 20% glycerol) containing 5% β-mercaptoethanol, boiled, and proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose and probed with antibodies against ATM pS1981 (Epitomics Inc.) and gamma H2AX (Millipore Inc.). Ku80 (Cell Signaling Technology Inc.) was assessed for equal protein loading.

**Statistics**

The data are presented as mean ± SEM. After confirming normal distribution of data. Comparisons between conditions were made by the unpaired two-tailed Student’s t-
test; repeated-measures ANOVA were used to compare changes over time between conditions. A p value less than 0.05 is considered to be statistically significant. Significance to control is denoted by a (*) and significance to NPWT is denoted by a (#).

Results

_Ultraviolet-A Fiber Optic Therapy combined with Negative Pressure Wound Therapy Dramatically Increases Wound Healing Over Negative Pressure Wound Therapy Alone_

Using a porcine model, we tested the effects of UV-A fiber optic therapy on wound healing in aseptic full-thickness wounds. After 14 days of treatment with NPWT or NPWT+UVA, neither treatment showed any adverse effects, excessive induration, or inflammation (Figure 4A, representative images). With all treatments, the wounds had significant closure over the time course compared to day 0. Wound area was calculated as a percent of day 0 (Figure 4B). The control wound demonstrated some expansion at day 7, which was not seen in the two groups treated with NPWT. By day 14, however, the wounds treated with NPWT+UV-A demonstrate a significant reduction in wound area compared to NPWT or Control treated wounds. These data demonstrate the greater effectiveness of UV-A therapy to heal wounds as compared to NPWT.

_Ultraviolet-A Fiber Optic Decreases the Growth of Staphylococcus aureus and Pseudomonas aeruginosa in vitro_
After 24 hours of UV-A (or control) therapy, plates were removed and photographed (Figure 3A). Analyses of colony size demonstrate that UV-A fiber optic treatment results in significantly smaller colonies of Staph and Pseudomonas than Control treated plates (Figure 3B). Further, colony number of both strains was reduced with UV-A fiber optic therapy (Figure 3C). These data demonstrate that UV-A fiber optic therapy reduces proliferation of two skin-borne pathogens, of Staphylococcus aureus and Pseudomonas aeruginosa, commonly found in wound sites.

_Ultraviolet-A Fiber Optic Therapy induces minimal DNA damage as compared to established UV based therapies._

Comet assays were performed on human fibroblasts to assess DNA damage occurrence over 48 hours of exposure to UV-A and UV-C therapy. The levels of DNA damage are correlated to the length and density of the ‘comet tails’ (denoted with orange arrows, Figure 5A) and inversely correlated to size and density of the comet heads (yellow arrows, Figure 5A). The comet tails are visibly longer and denser in the UV-C treated samples than in the UV-A treated samples. The 48-hour sample from UV-C treated fibroblasts could not be analyzed due to cell death. The 24-hour UV-C treated cells display less dense ‘comet heads’, which means severe DNA damage occurred in the nuclei. Further, the UV-A treated samples show only a small number of cells with comet tails comparable to the Sham control. The density of comet heads is similar between
the UV-A treated samples and the Sham control, even at the 48hr time point. These data suggest that significantly more DNA damage occurs with UV-C treatment than with UV-A or Sham. To more sensitively assess the degree of DNA damage response, expression of phosphorylated ATM (ATM pS1981) and histone H2A variant H2AX (γH2AX) (Figure 5B). Data demonstrate that UV-C induces significant phosphorylation (activation) of ATM 2 hours after exposure and this activation continues through 24 hours. There is a slight detectable upregulation of ATM phosphorylation after 24 hours in the UV-A treated cells but it is far below what is observed in the UV-C treated condition. These data correlate with the degree of phosphorylated H2AX (γH2AX) observed in the UV-C versus the UV-A treated cells. Together these data further support the conclusion that UV-C treatment with the Thera-Wand Model C-100 Ultraviolet C Treatment Lamp for 30s induces far more DNA damage than continuous use of UV-A.

Discussion

The data presented here demonstrate a novel and effective method to administer UV-A phototherapy using fiber optic technology. In addition, we performed safety and efficacy studies on a novel wound therapy system that combines the benefits of NPWT with UV-A phototherapy. Unlike other therapy devices that use lamps, which are bulky and provide an inconsistent dosage, the NPWT-UV device tested here allows for UV-A
therapy to be delivered alongside NPWT 24 hours a day. Further, with this device, the levels of UV-A can be changed to fit the patient’s needs.

There are a few other studies that demonstrate UV effectiveness in promoting wound healing. Wills and colleagues reported the efficacy of UV light therapy (mixed spectrum of 200-400 nm) from a randomized, placebo controlled clinical trial (n=16) in patients with pressure ulcers. Patients treated with active therapy healed their ulcers faster than sham therapy (6.3 vs. 8.4 weeks, p<0.02) (28). Likewise, Nussbaum and colleagues reported the results of a randomized, placebo controlled trial (n=43) of patients with pressure ulcers. The percent wound area reduction in Stage 2 buttock pressure ulcers was significantly smaller in patients treated with active UV-C therapy compared with sham therapy at weeks 3, 5, and 7. For all pressure ulcers the percent wound area reduction was 37% for patients that received active UV therapy and 6% for patients that received sham (p=0.09) (29). The data presented here show that fiber optic delivery of UV-A results in decreased wound area in a porcine model of aseptic wound healing and this improvement continues over 2 weeks of therapy.

There are several animal and human studies that report the effectiveness of UV phototherapy to improve or prevent infections [23, 31-39]. In this study we show UV-A delivered through fiber optics can decrease the proliferative capacity of Staphylococcus aureus and Pseudomonas aeruginosa, pathogens commonly found in wounds.
Interestingly, the colony formation was not drastically affected by exposure to UV-A.

Several things could explain this. It is possible that higher doses of UV-A could have a more dramatic impact on Staphylococcus colony formation. To determine this, further dosing studies will need to be performed. In addition, it is possible that the antibacterial effects of UV-A would be more potent in an in vivo wound environment. The presence of blood components and other organic molecules have demonstrated to be critical in the capacity of UV-A to reduce bioburden [18].

We also show that fiber optic delivery of UV-A is effective in reducing proliferation of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Studies have demonstrated that the presence of bioburden in wounds results in delayed healing. In a study by Xu et al, 32 patents with diabetic foot wounds were evaluated for 28 days. The rate of healing had a strong inverse relationship with log colony forming units (CFU). For every log order of CFU there was a 44% delay in wound healing (30). Infections complicate the treatment of wounds and impede the healing process by damaging tissue, reducing wound tensile strength and inducing an undesirable inflammatory response (31, 32). It is generally believed that wound infection advances in stages from contaminated, to colonized, to critically colonized, and to infected (33). When wound surface bacteria begin to replicate and increase their metabolic activity, the resulting byproducts, such as endotoxins and metalloproteinases (MMPs), negatively impact all
phases of wound healing (34). It has been established that UV therapy can reduce bioburden.

NPWT has changed how large, complex wounds are treated clinically and data here demonstrate that addition of UV-A fiber optic therapy to 'traditional' NPWT increases its effectiveness. Early animal studies suggest that NPWT accelerates wound healing and reduces bacteria and biofilm in the wound bed (35, 36). However, in clinical studies, there has not been any difference in the incidence of adverse events or infections. In fact, in a randomized clinical trial of diabetic foot wounds treated with NPWT or standard moist wound therapy, patients treated with NPWT had more infections (16.7% vs. 9.8%); however the results were not statistically significant (12). The addition of UV-A fiber optic therapy may improve on NPWT outcomes by improving the ability of the therapy to reduce wound bioburden. This combination of NPWT and UV-A fiber optic therapy would be beneficial in the post-operative period in patients undergoing staged debridement or amputations in order to treat residual infection or bacterial colonization. This may also have an application outside of the immediate perioperative period for patients requiring extended NPWT courses for large deficits in an effort to reduce colonization and post-operative infections.
Conclusions

Together these data demonstrate that UV-A promotes wound healing and inhibits growth of Staphylococcus and Pseudomonas. These results are likely to be even more impressive in a chronic wound environment, as often seen in the clinical setting. In fact, NPWT studies with pig models demonstrated at best a 20% reduction in wound healing. The clinical significance of NPWT on healing rates is far more significant. The next step for this type of combination therapy is a translational step to treat complex wounds in humans.

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Figure 1. Freedom-of-Motion Therapy Arm. A. Schematic of therapy arm. B. Arm in use for the study.
Figure 2. **Fiber Optic Ultraviolet-A Delivery Patches.** A. Fiber optic design allows for UV-A to escape in a unidirectional manner. B. Delivery of UV-A degradation over time. C. UV-A fiber optic patches are integrated into a patch that also delivers NPWT.
Figure 3: Fiber Optic Ultraviolet-A Delivery Inhibits Staphlococcus aureus and Pseudomonas aeruginosa. A. Photographs of bacterial plates incubated for 24hrs. B. Average colony area (n=5). C. Average colony number per plate (n=5). Data are presented as mean ± SEM, and *P<0.05
Figure 4. Improved Wound Healing with Ultraviolet-A+NPWT. A. Representative wound images at day 0, 7, and 14 of NPWT, NPWT+UV and control therapies. B. Change in wound area over time (n=5). Data are presented as mean ± SEM, and *P<0.05.
Figure 5. *Minimally detectable DNA damage from Ultraviolet-A Therapy.* A. Comet assay on human fibroblasts treated with UV-A, UV-C, or control therapy for the indicated times. B. Western blot analyses of human fibroblast cell extract treated with UV-A, UV-C or control therapies for the indicated times.