Mitigating Burn Severity and Infection Rates Using a Novel Dressing that Targets Multiple-Burn Related Pathologies

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DECLARATION OF INTEREST

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NOTICE AND SIGNATURE PAGE

EVALUATION OF PROLONGED FIELD CARE RESUSCITATION GUIDED BY BLOOD PRESSURE VERSUS CEREBRAL PERFUSION IN A SWINE MODEL OF HEMORRHAGE AND TRAUMATIC BRAIN INJURY

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13. SUPPLEMENTARY NOTES

14. ABSTRACT-

Thermal injuries pose a risk for service members in prolonged field care (PFC) situations or to civilians in levels of lower care. Without access to prompt surgical intervention and treatment, potentially-salvageable tissues are compromised, resulting in increases in both wound size and depth. Immediate debridement of necrotic tissue enhances survivability and mitigates the risks of burn shock, multiple organ failure, and infection. However, due to the difficulty of surgical removal of the burn eschar in PFC situations and lower levels of care, it is of utmost importance to develop alternative methods for burn stabilization. Studies have indicated that cerium (III) nitrate may be used to prolong the time before surgical intervention is required. The objective of this study was to incorporate cerium (III) nitrate into an electrospun dressing that could provide burst release. Select dosages of cerium(III) nitrate were dissolved with either pure solvent or polyethylene oxide (PEO), for coaxial or traditional electrospinning set-ups, respectively. The solutions were coaxially electrospun onto a rotating mandrel, resulting in a combined nonwoven mesh, and then compared to traditionally spun solutions. Additionally, the dressings were evaluated *ex vivo* and *in vivo*. The dressings were first evaluated *ex vivo* using a lactate dehydrogenase (LDH) assay to confirm that no cytotoxic effects were present. Then, one female Yorkshire swine (Midwest Research Swine) received ten 5 cm x 5 cm contact burns with a brass burn device that was heated to 100°C. The deep-partial thickness wounds were randomly assigned to one of five treatment groups: 1) a single layer of the PEO/CeN dressing, 2) four layers of the PEO/CeN dressing, 3) four layers of a control electrospun PEO dressing, 4) Flammacerium® cream (silver sulfadiazine 1%, cerium nitrate 2.2%), or 5) the PFC standard of care (gauze). Wounds were observed over an 18-day period, with surgical debridement occurring on Day 4. Transepidermal water loss, depth to debridement, and histologic measurements of necrosis were utilized to assess the burns.

15. SUBJECT TERMS-

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1.0 EXECUTIVE SUMMARY

Thermal injuries pose a risk for service members in prolonged field care (PFC) situations or to civilians in levels of lower care. Thermal burns account for 5-10% of casualties sustained in present-day military conflicts and are expected to be one of the most common wounds to occur in future conflicts [1, 2]. Without access to prompt surgical intervention and treatment, potentially-salvageable tissues are compromised, resulting in increases in both wound size and depth. Immediate debridement of necrotic tissue enhances survivability and mitigates the risks of burn shock, multiple organ failure, and infection. However, due to the difficulty of surgical removal of the burn eschar in PFC situations and lower levels of care, it is of utmost importance to develop alternative methods for burn stabilization. Studies have indicated that cerium(III) nitrate may be used to prolong the time before surgical intervention is required. The objective of this study was to incorporate cerium(III) nitrate into an electrospun dressing that could provide burst release. Additionally, the proof-of-concept dressing was characterized for cytotoxicity and its ability to impact burn eschars, and its potential to improve deep-partial thickness burns outcomes when combined with delayed standard surgical interventions.

2.0 INTRODUCTION

Thermal burns that require medical intervention affect nearly 500,000 people and account for approximately 3,400 deaths annually in the US [3]. Patients who have received major burn damage, total burn surface area (TBSA) > 20%, typically undergo care at a specialized burn center, with a survival rate of above 97% [4]. However, in cases where immediate transport to a burn center is not possible, or, for burns resulting from military conflict, survival rates drop as low as 80% [5, 6]. In these situations, the removal of necrotic tissue and access to IV fluid resuscitation may be delayed for more than 72 hours, which increases the chances of sepsis, burn shock, multiple organ failure, and mortality [7]. To combat the aforementioned issues and minimize the burn morbidities associated with prolonged field care scenarios, novel wound dressings are needed to help extend the time needed for patients to receive specialized care.

The severity of a thermal burn is classified by its depth, and it is generally accepted that thermal burns consist of three zones that vary in both the extent and depth of tissue damage [3, 7, 8]. The zone of necrosis (coagulation) is the area that sustains the greatest damage from the thermal trauma, and suffers irreversible destruction of its vascular system. It is characterized by dead cells and necrotic tissue that is collectively known as the burn eschar. The zone of hyperemia, conversely, will eventually recover without additional treatment. The middle area, the zone of stasis, contains highly stressed tissue that, without intervention, will die and allow for wound progression. As such, it is of vital importance to focus burn care on cultivating the tissue in each of these zones, especially in prolonged field care scenarios [1].

The gold standard for burn treatment is immediate debridement, or surgical removal, of the dead tissue [3]. Unfortunately in extended field situations, surgical debridement methods are not readily available [5]. For the burn eschar in particular, the dying cells leach toxic metabolites that can have detrimental effects on the surrounding tissue, and studies have shown that delayed grafting leads to reduced survivability [7-10].

Topical treatment of the burn area with cerium (III) nitrate (Ce (III)), either by itself or in combination with silver sulfadiazine, has been shown to delay the need for debridement [9-13]. Burn eschars treated with Ce (III) become firm and leather-like, slowing the leaching of toxic metabolites, but do not separate from the wound area. However, once excised, the tissue beneath the eschar is generally healthy, and has a high rate (>90%) of graft acceptance. Additionally, multiple studies have shown Ce (III) may maintain late stage burn immune responses and reduce levels of immunomodulatory cytokines by binding and denaturing the lipid protein complex[9, 11, 14-17]. For delivery, Ce (III) has been used in both commercially available creams and incorporated into gelatin- and chitosan-based film dressings [10-12, 18]. Creams and hydrogels, unfortunately, are not suitable for prolonged field care use, due to the amount of space they require in a medic's pack. An alternative delivery method would be the incorporation of Ce (III) in a nonwoven fiber dressing resembling traditional gauze [19-21].

Nanomaterials are increasingly becoming more popular for use as wound dressings due to their high surface area-to-volume ratios, variable degradation rates, and ability for controlled drug delivery [22-31]. Additionally, the nonwoven fibers fabricated through electrospinning exhibit fiber diameters on the nanoscale (< 1μm) range, which is consistent with collagen fibril diameters in the extracellular matrix (< 500nm). Furthermore, electrospinning fabrication techniques are compatible with a wide range of polymers, both natural and synthetic, allowing researchers to pick the polymer with the most optimal properties for the application. Due to these characteristics, electrospinning is at the forefront for fabricating functionalized nanomaterial scaffolds for burn wound dressing applications.

Electrospinning also allows for the use of various protective techniques in order to not only retain the moiety bioactivity, but also reduce environmental effects on that moiety. Nanoparticles are most commonly used in conjunction with electrospinning in order to control drug release profiles [25, 26, 30-33]. Alternately, coaxial electrospinning can be used to encase a sensitive moiety "core" within a protective polymer "shell" [22, 24, 33-38]. This method traditionally consists of electrospinning one polymer inside of another resulting in a core-shell arrangement, with the drug contained within the core.

In order to combat the aforementioned issues associated with the treatment of burn pathologies in areas without a burn center, the objective of this study was to incorporate Ce (III) into an electrospun dressing that could provide a burst release of treatment. The polymer vehicle for the dressing was polyethylene oxide, which is known for having a very rapid degradation rate. In addition, a modified coaxial-electrospinning method was utilized to encapsulate cerium within the fibers while simultaneously stopping hygroscopic effects. The resulting nanofibers were characterized and evaluated for their biocompatibility, Ce (III) loading capacity, and release kinetics using *in vitro* methods.

3.0 MATERIALS AND METHODS

MATERIALS

Acetone (97%), polyethylene oxide (PEO, MW = 300,000), cerium (III) nitrate hexahydrate (Ce (III)), phosphate buffered saline, sodium triphosphate, Tris-buffered saline (TBS), and dichloromethane (DCM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human dermal fibroblasts (PCS-201-012) and cell culture reagents were purchase from American Type Culture Collection (ATCC; Manassas, VA, USA). CYQUANT cell proliferation and LDH cytotoxicity assay kits were purchased from Thermo Fisher Scientific (Walthem, MA, USA).

Preparation of Electrospun Cerium (III) Nitrate Containing Polyethylene Oxide Dressings

Solutions containing PEO and Ce(III) were made using three different methods, corresponding to the electrospinning technique used to fabricate the nonwoven mesh dressing. The electrospinning set ups utilized are shown in Fig 1, above. For use in traditional electrospinning, PEO and Ce(III) were dissolved at a mass ratio of 1:1 in a 2:1 (v/v) ratio of acetone to DCM to obtain a total polymer content of 5 % w/v. The resulting combined solution was loaded onto a syringe pump. The solution was electrospun through an 18 gauge spinneret, with a flight distance of 15 cm and relative humidity of 10%. Supplied voltage ranged from 10-18 kV and the associated flow rate ranged from 1.05-5 mL/hr. Fibers were collected on a grounded mandrel rotating at 25 rpm.

For dressings fabricated using the coaxial set-up, PEO solutions were made by following the steps stated above. A separate solution of Ce (III) was made by dissolving 5% w/v Ce(III) in acetone. Following this, each solution was loaded onto a separate syringe pump connected to a different inlet of an 18/16 gauge coaxial needle, and flow rates were initially set to 1.5 mL/hr and 0.15 mL/hr for PEO and Ce (III) solutions, respectively, and slowly increased to 5 mL/hr and 0.5 mL/hr for PEO and Ce(III) solutions, respectively. For these samples, variability was mitigated by electrospinning the same volume of polymers for each batch. The solutions were spun at 28kV with a flight distance of 12 cm onto a mandrel rotating at 25 rpm.

Dressing Characterization

Polymer electrospinning solutions were evaluated for conductivity using a Malvern Zetasizer Nano (Malvern Panalytical, United Kingdom), following manufacturer protocols. Electrospun dressings were analyzed for porosity and fiber diameter using field emission scanning electron microscopy (FSEM) (Zeiss Sigma VP-40) following sputter-coating with gold palladium. Three dressings were analyzed per dressing composition, with 30 fibers evaluated for each, using ImageJ. Additionally, dressings were analyzed using Fourier Transform Infrared Spectroscopy (FTIR) at a range of 4000-500 cm^{-1} to confirm the presence of Ce (III).

In vitro Dressing Degradation and Loading Potential

Dressing degradation was determined in deionized water at room temperature. Ce(III) has been shown to interact negatively with phosphate-based buffers and cell culture medium [19, 39]. Scaffolds (n=3) were immersed in 10 mL of deionized (DI) water and incubated them for up to 3 hours.

Cerium (III) Release Rate

The amount of Ce (III) released over time by the electrospun dressings was evaluated using a PermeGear in-Line diffusion system (Twin-Flow, Dual In-Line; Hellertown, PA, USA). In brief, a total of 9 Ce (III) containing electrospun wound dressings were cut to a diameter of 25 mm and applied to a Durapore® hydrophilic polyvinylidene fluoride (PVDF) 0.45 µm filter. The wound dressings and filter were placed over a support in the PermeGear® in-line flow-through diffusion cell, pre-wet with Tris Buffered Saline (TBS) and clamped into position. The inlet port of the diffusion cell was attached to a multichannel peristaltic pump and TBS was continuously pumped through each cell at a flow rate of 10-20 µL/min at 37ºC. Samples were collected at 1, 3, and 16 hours through the in-line diffusion cell outlet port using an automated fraction collector. Ce(III) concentrations of samples were measured using a rapid analytical fluorometric detection method that was developed to determine trace amounts of Ce(III) in aqueous samples[40, 41].

In Vitro Cell Cytotoxcity

Cytotoxicity of the dressings was evaluated using CYQUANT assay kits following manufacturer recommended methods. Human dermal fibroblasts were cultured to 80% confluency, trypsinized, resuspended at a concentration of 10,000 cells/mL of media, and seeded into 6-well plates. After a 24 hour seeding period, media was refreshed and the study groups (no treatment control, PEO scaffold control, pure Ce(III) control, coaxially spun scaffold, and dual-spun scaffold) were placed directly into the wells (n=3). At 24 hours, dressings were removed, cells were briefly washed with buffer, and cultured for an additional 48 hours. This resulted in a 72 hour culture period with a 24 hour treatment.

Ex vivo Viability Assay

Full-thickness pig skin was resected within one hour of euthanasia and preserved in ice-cold sterile saline until processing. Samples either remained unburned or received a 15 second contact burn from a 100°C thermo-coupled brass block [42, 43]. Burned tissue was allowed to cool for 15 minutes. Tissue biopsies (6 mm) were taken and placed in a 96-well plate (Corning) containing Hanks Balanced Salt Solution (Gibco) [44]. The epidermal surface was treated with one of six treatments and incubated at 37°C with 5% CO_2. Optimization was required to prevent post application CeN precipitation, which is known to occur in the presence of phosphate buffers and cell culture media [11, 45, 46].

Tissue viability was assessed using the Pierce LDH Cytotoxicity Assay® (ThermoFisher). LDH absorbance was read on a Synergy HTX multimode plate reader (Agilent Technologies, Santa Clara, CA). LDH values were normalized and compared to their respective untreated controls using a two-way analysis of variance (ANOVA) with QQ and homoscedastity post-hoc analyses. Technical triplicates were taken from each tissue sample.

Animals

One female Yorkshire swine (50kg) (Midwest Research Swine) was used in this pilot study. The animal was housed, with *ad libitum* access to water, and was acclimated to the facilities for at least seven days before any procedures. Research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility's Institutional Animal Care and Use Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by the AAALAC.

Anesthesia and Analgesia

The Animal was fasted the night before anesthetic events. On the day of thermal insult, the animal was pre-medicated with glycopyrrolate (0.01mg/kg, IM), induced with tiletamine-zolazepam (4-6mg/kg, IM) and anesthetized with 3-5% isoflurane in oxygen via a facemask. Anesthesia was maintained with 1-3% isoflurane in oxygen. Analgesia was administered prior to wounding and/or dressing changes, with sustained release buprenorphine (0.1-0.24mg/kg) subcutaneously in the lateral neck.

Thermal Injury and Treatment

The burn wound procedure has been described previously [47, 48]. Briefly, hair was removed and the skin was rinsed. Then ten contact burns were made 4cm from the spine and 3cm from each other. Five 5cm x 5cm burns were made on each side of the spine of the anesthetized animal with a brass burn device (100°C for 15 seconds) [42-44, 49, 50]. A 1.7 kg ring was added to the device to deliver constant and consistent pressure during insult (~0.4 kg/cm^2).

Wounds were allowed to cool for one hour and randomly assigned to one of five treatment groups: 1) 1-LayerPEO/CeN dressing, 2) 4-LayersPEO/CeN dressing, 3) four layers of a control electrospun PEO dressing, 4) Flammacerium® (silver sulfadiazine 1%, cerium nitrate 2.2%), or 5) gauze. All wounds were then covered with an occlusive dressing (Tegaderm™, 3M) and sterile nonadherent gauze (Telfa, Kendall, Mansfield, MA). Vetwrap (3M) was wrapped around the trunk of the body to cover the entire wounded area. Finally, a fabric vest (DeRoyal, Powell, Tennessee) was applied for additional protection.

Two days later, wounds were cleaned, patted dry, and measured for transepidermal evaporative water loss (TEWL), treatments re-applied, and the animal was re-dressed.

Debridement and Wound Care

On Day 4 post-burn, all burns were tangentially excised to punctate bleeding using a dermatome (Zimmer Biomet, Warsaw IN). Punctate bleeding is indicative of a viable tissue and is the clinical standard to indicate complete removal of dead tissue. Total debridement depth was defined as the thickness of tissue removed until punctate bleeding was observed.

Debrided wounds were covered with Silveron® (Argentum Medical, Geneva IL), wet gauze, and an occlusive dressing. This was followed by gauze, Vetwrap, and a fabric vest. Silverlon® dressings were changed twice per week. At each dressing change, wounds were rinsed with diluted 4% chlorhexidine gluconate, sterile water, and patted dry with sterile gauze. Wounds were imaged during each dressing change.

Wounds were biopsied using an 8 mm biopsy punch 14 days after debridement. A strip biopsy (4.0 x 0.5cm) spanning the wound bed was also taken on the final day of the experiment.

Transepidermal Evaporative Water Loss (TEWL)

On Day 2 post-burn, TEWL data was obtained from the top, middle, and bottom of each wound using a digital, multiple probe adapter (MPAS-6) system (Courage Khazaka Electronic, Cologne, Germany). This probe system uses the MPA software to operate the Tewameter® TM 300 and Mexameter® MX 18 probes (Courage Khazaka Electronic, Cologne, Germany).

Histologic Wound Assessment

Tissue samples were fixed in 10% neutral buffered formalin for at least 48 hours and processed for paraffin embedding. Tissue sections (~4µm thick) were cut, cleared in xylene, and rehydrated with an ethanol gradient (100%, 95%, 70% ethanol and deionized water). Sections were stained with hematoxylin and eosin (H&E) or Caspase-3 (Cas-3) to observe remodeled collagen and re-epithelialization or the depth of necrotic tissue, respectively. A blinded, clinical pathologist scored the slides for cell phenotypes and indicators of wound healing.

Statistical Analysis

Scaffold Fabrication Statistical Analysis

Statistical analyses were conducted using GraphPad Prism (San Diego, CA, USA). Quantitative data is represented as the mean ± standard deviation, and was calculated using analysis of variance (ANOVA) with a posthoc Tukey test. $p < 0.05$ was considered statistically significant.

In Vivo Statistical Analysis

Data is presented using means and standard deviation, unless otherwise noted. All statistical comparisons use a 2-way analysis of variance (ANOVA). Cytotoxicity measurements employed a linear regression analysis with data normalcy validated by QQ and homoscedasticity plots.

4.0 RESULTS

Properties of PEO/Ce(III) Solutions. Control PEO, Ce(III) nitrate, and PEO/Ce(III) nitrate solutions were evaluated for conductivity (Table 1). PEO in acetone, DCM solvent is widely considered one of the most stable solutions for electrospinning [25, 51, 52], and this is supported by the observed low conductivity shown in Table 1. Conversely, Ce(III) had a relatively high conductivity. This was observed during the electrospinning process by its difficulty incorporating into polymer fibers, resulting in a wide range of fiber diameters as seen in Table 2. Also of note, the addition of Ce(III) with PEO in acetone, DCM solutions causes the PEO to drop out of solution.

Morphology and Properties of PEO/Ce(III) Nanofibers

SEM micrographs were taken of electrospun control PEO, dual-spun PEO/Ce(III) nanofibers, and coaxial-spun nanofibers (Figure 2), and evaluated for fiber diameter and membrane porosity (Table 2). Surprisingly, coaxially-spun fibers had the smallest mean fiber diameter of all groups, 1.09 ± 0.25, not normally seen in the literature [22, 24, 34, 36-38]. Additionally, this group had the lowest mean dressing porosity, 36.1 %. Most importantly, SEM imaging confirmed the presence of the PEO/Ce(III) core-shell architecture. The magnified insert in Fig. 2 shows a PEO polymer shell that has been stretched open to show its interior.

Dressing Chemical Composition

The chemical composition of the electrospun samples was evaluated using FTIR. The arrows point to various peaks specific to Ce(III). The peak seen just above 700 indicates the presence Ce(III), while the peaks at 3386 and 1641 are OH groups from bound moisture [19]. Bound moisture is present on FTIR spectra due to the hygroscopic characteristics of Ce(III). This moisture is not present in the coaxially-spun samples, further supporting the success of encapsulation.

Cerium(III) Nitrate Loading Potential

The loading potential, amount of Ce(III) contained within each electrospun dressing, was evaluated on each dressing type. After drying, dressings were weighed then dissolved in TBS; results were then compared on a per weight basis. Figure 4 shows the resulting loading capacity across various electrospinning batches. Ce(III) loading capacities average 80 µg/mg for coaxially spun dressings and just shy of 60 µg/mg for dual-spun dressings. The lower amount of Ce(III) detected in the dual-spun samples may be due to the interactions between Ce(III) and PEO. First, Ce(III) has a very high conductivity , which causes it to carry the electrical charge throughout the electrospinning process, drying the solution [53, 54]. This is coupled with its limited solubility in PEO. The less polymer in solution would then result in less polymer to carry Ce(III) across the air gap and less Ce(III) in the final fiber mat. Coaxial setups solve this issue by keeping the Ce(III) in the interior of the fibers, and thereby mitigating these undesirable interactions.

Ce(III) Release Rate

Cumulative release of Ce(III) was demonstrated in samples collected from coaxially spun, cerium(III) nitrate containing scaffolds using the PermeGear® ILC-07 automated system (Table 3). The results of the in vitro diffusion study demonstrated a burst release of Ce(III) at 1 hour. The amount of Ce(III) collected decreased at 3 hours and only trace amounts of Ce(III) was observed at 16 hours. Additionally, there is dose-dependent release control, seen by the increase in overall release amounts for dressings with a higher initial loading (Figure 5).

Viability of Human Dermal Fibroblasts

Viability of primary human fibroblasts was performed on each dressing type and compared against PEO control dressing and Whatman paper soaked in 5% (w/v) Ce(III) in acetone (Figure 6). When comparing the controls, there are no significant differences in cell viability. Over 72 hours, there are no significant differences between the controls and the coaxially-spun samples; however, the dual-spun group shows a statistically significant decrease in proliferation (possibly due to the exposed Ce(III), as discussed previously).

Dressing Fabrication and *Ex Vivo* Evaluation

Preliminary analyses demonstrated that dressings released ≥90% of the CeN payload within the first hour of solubilization, which was similar to previously characterized dressings. All dressings were sterilized via UV irradiation, which did not significantly decrease CeN content per gram of dressing (data not shown) [55].

To test for cytotoxicity, treatments were placed atop freshly excised porcine tissue. The media was assessed for LDH levels 24 or 72 hours post treatment. These treatments included: 1) no treatment, 2) 1 layer PEO/CeN, 3) 4 layers PEO/CeN, 4) four layers of the electrospun PEO dressing, 5) Flammacerium®, and 6) an aqueous solution of 2% CeN. None of the treatments produced significant increases or decreases in LDH at either time point, indicating the dressings did not convey cytotoxic or cytoprotective effects.

Effect of CeN on Burn Wound Eschars

Deep partial thickness (DPT) burn wounds were made along the dorsum of a female Yorkshire pig. Two days later, dressings were removed and the burns were assessed for TEWL (Figure 8). Unburned (control) skin had a low TEWL score, whereas the gauze-treated burn had a much higher TEWL score. This is indicative of the difference in moisture retention between undamaged skin and burned skin. Burns treated with Flammacerium® showed a TEWL score similar to unburned skin ($36.4 gm^{-2}h^{-1}$ and $22.3 \pm 18.2 gm^{-2}h^{-1}$, respectively), as did those treated with the PEO dressing ($57.4 \pm 38.6 gm^{-2}h^{-1}$). Burns treated with either the 1-Layer PEO/CeN or the 4-Layer PEO/CeN dressing also had significantly lower TEWL readings ($77.1 \pm 5.32 gm^{-2}h^{-1}$ and $73.9 \pm 14.7 gm^{-2}h^{-1}$, respectfully) than the burns treated with gauze ($101.2 gm^{-2}h^{-1}$), albeit still greater than the control group. After TEWL readings, a biopsy sample was taken from each wound, and new dressings were applied. Two days later, the dressings were removed, the wounds were imaged, and then the wounds were surgically debrided until punctate bleeding was evident.

Depth of Necrotic Burn Tissue

The average depth to achieve punctate bleeding was recorded for each burn (Figure 9A). The SOC treated burn required the greatest amount of debridement (2159μm) and was the only treatment that necessitated more than 2000μm of tissue to be removed. As the amount of PEO/CeN dressing applied increased from 1-Layer to 4-Layers, the depth to debridement decreased from 1981±76μm to 1778±0μm, respectively. There was no significant difference among the burns treated with the PEO/CeN dressings, the PEO dressing (1727±279 μm), and the Flammacerium® cream (1778μm). Of note, the debridement surgeon (blinded to treatment groups) noted that two of the treated burns felt “desiccated and leathery.” These burns were later identified as being treated with either 1-layer PEO/CeN or 4-layers PEO/CeN.

As an independent measure of necrotic tissue depth, biopsies collected prior to debridement were assessed for Cas-3 expression. Cas-3 is a crucial mediator of apoptosis, and is frequently utilized to delineate the depth of severe tissue damage following thermal insult [49, 56, 57]. Burns treated with the PEO dressing marginally reduced the depth of tissue necrosis compared to the SOC gauze treatment (2348±653μm and 2692±382μm respectively) (Figure 9B). As the PEO/CeN dressing treatment increased from 1-Layer to 4-Layers, the depth of necrotic tissue decreased (2185±301μm and 1247±791μm, respectfully). The Flammacerium® cream, which contained the largest composition of cerium nitrate (2.2% w/v), conferred the shallowest depth of necrotic tissue (1028±59μm), suggesting that cerium-based products can have a considerable therapeutic effect on burn progression.

Biopsies were collected 0, 2, and 4 days post-burn and stained for H&E. There were no discernable differences in burn depth or acute inflammation among the treatment groups (Supplemental Figure 1).

Post-Debridement Wound Healing

After surgical debridement, all burns were covered with a silver-based dressing. During the twice-per-week dressing changes. Burns were also biopsied once per week to assess histological indicators of wound healing, such as granulation tissue, fibroplasia, and epidermal hyperplasia (Figure 4). Three days after debridement, granulation tissue was present in all groups, and epidermal regeneration/hyperplasia was present in all groups except for the PFC SOC. Except for the Flammacerium®-treated burn, there was moderate fribroplasia three days after debridement (Day 7), which increased to marked fibroplasia at 7 and 10 days after debridement. Typically, fibroplasia is more severe in more extensive dermal injury due to the amount of denatured collagen that needs to heal. Conversely, epidermal hyperplasia, which progresses from epidermal regeneration, was noted at 7 and 10 days after debridement for the Flammacerium®-treated samples and may suggest faster wound healing compared to the other groups. Decreases in neutrophils and lymphocytes were also observed in all treatments, compared to the PFC SOC, beginning as early as 10 days after debridement (Figure 10).

Finally, H&E stained sections of tissue at Day 18 demonstrate the formation of a near complete or progressing layer of epidermis in all treatments (Supplemental Figure 2). All three post-debridement burns treated with the 4-Layer PEO/CeN dressings displayed prominent bands of remodeled collagen that span virtually the entire wound space. However, few conclusions can be drawn from this data until a larger sample size is achieved.

5.0 MAJOR EVENTS/MILESTONES/SUCCESS

In preparation for the execution of this project;

- Project Kick Off Meeting – June 2018
- Develop Protocol – June 2018
- Obtian IRB approval (NRD) – 07/2018
- Execution of protocol – 06/2019
- Data Analysis and Interpretation –01/2020
- Development of final report/manuscript – Journal of Trauma and Acute Care Surgery published Dec 2020: 02/2021

6.0 RISK ASSESSMENT

6.1 Risk Analysis:

Tested additional polymers to determine compatibility with cerium nitrate electrospinning.
- Developed two scaffold formulations that increased the cerium nitrate loading capacity of previous formulations by three-fold.

- Established lab safety protocols for working with antioxidant scaffold components.
- Antioxidant candidates down-selected to include two antioxidant compounds .

- Tested cerium-based scaffolds for antimicrobial activity against Pseudomonas a eruginosa, Methicillin-resistant Staphylococcus aureus, and Acinetobacter baumannii.
- Conducted in vitro biocompatility studies with electrospun cerium nitrate scaffolds.

-In vitro studies to approximate in vivo release kinetics of multiple layers of cerium nitrate scaffold completed July 2019.
-Scaffold has been electrospun to commence in vivo studies July 2019.
- In vivo studies to test cerium nitrate scaffold effect on burn eschar: Jul 2019.

-Presented an oral presentation at the 2019 MHSRS held in August in Orlando, Florida.
-In vivo studies to test cerium nitrate scaffold effect on a porcine burn eschar are currently ongoing.

FY20Q1 - No comments

FY20Q2 - N/A

FY20 Q3 - A manuscript titled "Cerium(III) Nitrate Containing Electrospun Dressing for Mitigating Infection and Burn Severity" has been drafted and routed to collaborators for comments/edits. Once the PAO review process has completed the manuscript will be submitted for publication.

FY20Q4-
The manuscript has been drafted and routed to collaborators for comments/edits.

FY21Q1-
The manuscript edits/comments were incorporated into the manuscript and routed through the command PAO. The manuscript was also routed through the ISR PAO. Currently awaiting PAO approval to submit the manuscript for publication.

FY21Q2-The manuscript has been submitted for publication to ACS Biomaterials Science and Engineering, and awaiting publication decision. A manuscript titled "An Electrospun Poly-Ethylene Oxide/Cerium (III) Nitrate Dressing for Delayed Debridement and Improved Wound Healing of Warfighter Contact Burns" is currently routing through the NAMRU-SA PAO for publication submission.

FY21Q3- Awaiting the decision of the Journals for one manuscript, and re-submitting the first manuscript to a different journal.

6.2 Technical Challenges

Development of a medical supply material to treat thermal injuries at point-or-injury. The material is anticipated to reduce severity of burn injuries, enhance casualty recovery time, and lower overall treatment costs.

The aim of our study is to develop and test a multifunctional, electrospun burn wound dressing that contains antioxidants, cerium nitrate, and/or chitosan.

FY20Q1 -

No comments

FY20Q2 -
During this quarter the labs at NAMRU-SA were shut down due to the COVID-19 pandemic affecting the nation. This shut down will delay the histopathology studies performed by collaborators at the ISR. Mitigation strategies will are being discussed with collaborators. A more definite plan will be implemented once we are notified the labs at NAMRU-SA will be re-opened.

FY20Q3 -
The labs at NAMRU-SA remain shutdown due to the COVID-19 pandemic. During this time, a manuscript titled "Cerium(III) Nitrate Containing Electrospun Dressing for Mitigating Infection and Burn Severity" has been drafted and routed to the ISR collaborators for comments/edits. The shutdown has delayed the completion of the histopathology experiments. Once the analysis is completed a manuscript will be drafted.

FY20Q4- The labs at NAMRU-SA were re-opened on a limited basis. Staff was allowed to return to the lab in shifts to minimize exposure to the virus. The manuscript titled "Cerium(III) Nitrate Containing Electrospun Dressing for Mitigating Infection and Burn Severity" was returned by the ISR collaborators with edits/suggestions.

FY21Q1-The labs at NAMRU-SA remained re-opened on a limited basis, and staff continued to be allowed in the lab in shifts. The manuscript edits/comments were incorporated into the manuscript and routed through the command PAO. The manuscript was also routed through the ISR PAO. Currently awaiting PAO approval to submit the manuscript for publication.

FY21Q2- The labs at NAMRU-SA remained re-opened on a limited basis and staff were allowed to return to the lab on a more consistent basis on 22 March 2021. The manuscript was submitted to ACS Biomaterials Science and Engineering, and we are currently awaiting the editors decision. a second manuscript has been completed by the ISR collaborators titled "An Electrospun Poly-Ethylene Oxide/Cerium (III) Nitrate Dressing for delayed Debridement and Improved Wound Healing of Warfighter Contact Burns" has been routed through the ISR PAO, and currently routing through NAMRU-SA PAO.

FY21Q3- The labs at NAMRU-SA have been opened on a consistent basis and personnel have been working in the labs. The first manuscript submitted did not fit in the journal that it was submitted, and we are in the process of routing the manuscript to submit to another journal. The manuscript submitted by our collaborators is still awaiting the decision of the reviewers.

7.0 TRANSITION PLAN

7.1 Military Relevance

Thermal burns account for 20% of casualties sustained in present-day conflicts and are expected to be one of the most common wounds to occur in future conflicts [2, 3]. Despite the significant progress that has been made in burn wound treatment, effective treatment of thermal wounds remains a challenging problem in the combat arena. Access to resuscitation fluids and immediate debridement of dead burn tissue can greatly reduce the chances of mortality and late-stage complications such as burn shock and multiple organ failure. However, future conflicts are anticipated to occur in austere environments where evacuations could be delayed up to 72 hours. Warfighters and combat-support personnel located in these PFC situations will have limited access to medical facilities and standard burn wound treatments.

Without access to prompt surgical interventions and standard burn treatments, burn casualties are at risk of wound progression, a process whereby burn wounds become deeper and more extensive [5, 6]. Burned tissue is also highly susceptible to infection, which can cause detrimental complications to wound healing and significantly increase the chances of mortality; even in modern burn units, complications associated with infection account for more than 50% of late-onset mortality in patients with >20% total burn surface area (TBSA) [58-60]. Together, these situational complications highlight the significant unmet need for a method to quickly and efficiently stabilize and protect acute burn injuries for service members in PFC scenarios.

To minimize thermal injury morbidities and mortalities in PFC scenarios, solutions must be focused on patient-level interventions and outcomes rather than the broader trauma system. The components of the proposed dressing can simultaneously stabilize the burn wound, prevent infection, and reduce the risk of late-stage burn complications by targeting the burn eschar. Additionally, the combination of cerium and chitosan (CS) is anticipated to provide superior antimicrobial protection against bacterial species that commonly infect military burn wounds, including *S. aureus* and *P. aeruginosa*. The electrospun dressing is a lightweight, dry dressing that can be easily transported into austere environments and rapidly applied to large surface areas. Given these characteristics, the proposed burn wound dressing is expected to provide superior support for individuals suffering from combat or combat-support thermal injuries in PFC scenarios. The dressing is also expected to simplify burn wound treatment techniques for the first responders and be extremely beneficial in mass trauma situations.

7.2 Transition Strategy

Transition Plan: Additional collaborators and/or industry partners will be sought to conduct further pre-clinical tests and evaluate scale-up methodologies. ROI: The burn wound dressing will be created using electrospinning technology and will result in a durable, lightweight burn dressing that can be applied at the time of thermal injury**.**

8.0 CONCLUSION/DISCUSSION

Scaffold Fabrication Conclusion

In this work, Ce(III) was encapsulated within electrospun polyethylene oxide nanofibers, and characterized for its potential use as a treatment containing burn dressing. Results shown herein indicated that coaxial electrospinning was capable of containing Ce(III) within the interior of the nanofibers, while also shielding the Ce(III) from hygroscopic effects. Additionally, this finding was supported in *in vitro* loading capacity studies, wherein coaxially spun dressings contained statistically more Ce(III) than the traditionally dual spun dressings. Although the dual-spun Ce(III)-containing dressings experienced decreased cell proliferationy in *in vitro* tests, coaxially-spun dressings performed statistically equivalent to the control groups. Finally, coaxially spun dressings were capable of delivering a burst release of Ce(III). In conclusion, the work presented demonstrates the applicability of coaxial electrospinning for the encapsulation and subsequent fabrication of difficult to handle moieties without the use of a secondary polymer vehicle. Further, this study lays the groundwork for the development of Ce(III) dressings for thermal injury treatment and its use as a point-of-injury treatment for thermal injuries and a potential means of mitigating the deleterious effect of delayed surgical debridement.

In vivo Discussion/Conclusion

Burns treated with CeN creams such as Flammacerium® can convert burn eschars into a leathery layer that prevents the invasion of external factors while retaining valuable wound moisture [42, 45, 61]. Similarly, the proof-of-concept studies in this paper strongly suggest that an electrospun PEO/CeN dressing can achieve similar effects. The electrospun dressing did not negatively impact cell viability *ex vivo* and, when compared to the PFC SOC *in vivo*, the dressing 1) reduced the TEWL of DPT burn wounds and 2) decreased the overall amount of necrotic tissue that developed. There were also no significant differences in acute inflammatory cell infiltrate. Interestingly, the PEO-only dressings facilitated wound moisture retention to a greater extent than CeN-loaded dressings (Figure 2). This may be due to the absorptive properties of PEO, which exists in a higher overall fraction in unloaded dressings compared to CeN loaded dressings, as well as the high surface area-to-volume ratio found in electrospun dressings [62, 63]. However, in contrast to the burn treatments with higher CeN concentrations the PEO-only dressing did not reduce the amount of necrotic tissue formed by the burn (Figure 3). . Together, these data strongly suggest that the lightweight, electrospun dressing effectively delivered CeN to the necrotic burn tissue and reduced the overall tissue loss associated with thermal injury.

While these results strongly advocate for further evaluation of this CeN dressing for combat casualty care in PFC scenarios, there are two significant drawbacks to note. First, the limited number of test subjects precludes us from drawing any statistically powered conclusions from the *in vivo* study. The limited number of wounds also put a constraint on which wounds received the various treatments. Because porcine skin can differ in thickness and healing capacity along the cranio-caudal axis, it is possible that the perceived differences in necrotic tissue depth and/or healing capacity could be dependent on where the burns were located. To address this possibility, a blinded pathologist scored the burn depths and found no differences amongst the individual wounds.

The second drawback of these studies is that the CeN content the electrospun dressings was considerably lower than that of Flammacerium®. Each layer of electrospun PEO/CeN dressing contained 11.0 ± 2.9mg of Ce(III). This means that burns treated with the 1-Layer CeN dressing received 11.0mg Ce(III) and the burns treated with the 4-Layer dressing received approximately 44.0mg Ce(III). This is in sharp contrast to the burn treated with Flammacerium®, which received approximately 110mg Ce(III). An equivalent Ce(III) delivery would have required treatment with 10 layers of the electrospun fiber. Nonetheless, the ~44 mg Ce(III) delivered by the CeN dressings was sufficient to cause a decrease in necrotic tissue depth. Given the limited sample size of this pilot study, it is not possible to statistically determine if the electrospun dressing can achieve similar results as the Flammacerium® cream. However, there is a strong inverse correlation between the amount of CeN used to treat the burn and the final depths of the burn injury. Increased concentrations of CeN is inversely correlated with the depth of Cas-3 staining, which suggests that the electrospun dressing and Flammacerium® have the capacity to reduce the amount of necrotic tissue that develops after a thermal injury. These results also correlate with the recorded depths of surgical debridement. Furthermore, these studies demonstrate that DPT burns are not adversely impacted by PEO/CeN dressings during the critical period for immediate interventions in PFC scenarios. Compared to the current SOC all treatments decreased the depth of debridement to achieve punctate bleeding, by approximately 250µm. This is a substantial difference in debridement depth considering that uninjured dermal tissue thickness ranges between 2-3mm, and that there is a strong correlation between debridement depth and the rate of wound healing.

9.0 DELIVERABLES

9.1 Publications:

1. Thompson MA, Kowalczewski C, Roy J, Nathan Wienandt MAJ, Williams C III, Chambers-Wilson R, Martinez LA, Christy R, Jockheck-Clark AR. An Electrospun Poly-Ethylene Oxide/Cerium (III) Nitrate Dressing for Delayed Debridement and Improved Wound Healing of Warfi ghter Contact Burns. J Biomed Res Environ Sci. 2021 June 24; 2(6): 509-515. doi: 10.37871/jbres1267, Article ID:JBRES1267
2. Williams, C., III; Chambers-Wilson, R.; Roy, J.; Kowalczewski, C.; Jockheck-Clark, A.R.; Christy, R.; Martinez, L.A. Cerium(III) Nitrate Containing Electrospun Wound Dressing for Mitigating Burn Severity. *Polymers* 2021, *13*, 3174. https://doi.org/10.3390/polym13183174

9.2 Presentations:

1. Williams, C., Roy, J., Kowalczewski, C., Jockheck-Clark, A. R., Christy, R., Martinez, L. Cerium (III) Nitrate Containing Electrospun Dressing For Mitigating Infection and Burn Severity. Oral Presentation Military Health System Research Symposium (MHSRS) Aug 2019.

2. Williams, C., Roy, J., Chambers-Wilson, R., Kowalczewski, C., Jockheck-Clark, A., Christy, R., and Martinez, L. Cerium (III) Nitrate Containing Electrospun Dressing For Mitigating Infection And Burn Severity. Oral Presentation. Southern Region Burn Conference (SBRC) 2019

10.0 COST

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1. FIGURES AND TABLES:

Table 1: Conductivity of PEO/Cerium(III) nitrate solutions

sample	conductivity ± SD (ms/cm)
5% w/v PEO in 1:1 acetone,DCM	0.00399 ± 0.000915
5% w/v Ce(III) nitrate in acetone	2.12 ± 0.114
5% w/v PEO in 1:1 acetone,DCM + 5% w/v Ce(III) nitrate in acetone	0.132 ± 0.00651

Table 2: Fiber diameter and membrane porosity of electro-spun scaffolds

sample	average fiber diameter ± SD (µm)	average porosity
PEO, control	1.80 ± 0.10	56.6
Dual-spun PEO/Ce(III)	1.16 ± 0.21	49
Coaxially-spun PEO/Ce(III)	1.09 ± 0.25	36.1

Table 3: Cumulative Cerium(III) Nitrate Release

PEO/Ce(III) Scaffold Cumulative Release ± SD (µg/mg)		
time (hours)	50 $\mathrm{mg}_{\mathrm{scaffold}}/\mathrm{mL}_{\mathrm{diluent}}$	200 $\mathrm{mg}_{\mathrm{scaffold}}/\mathrm{mL}_{\mathrm{diluent}}$
1	29.0 ± 0.73	47.5 ± 1.77
3	44.3 ± 0.51	98.5 ± 1.91
16	44.4 ± 0.38	98.6± 1.90

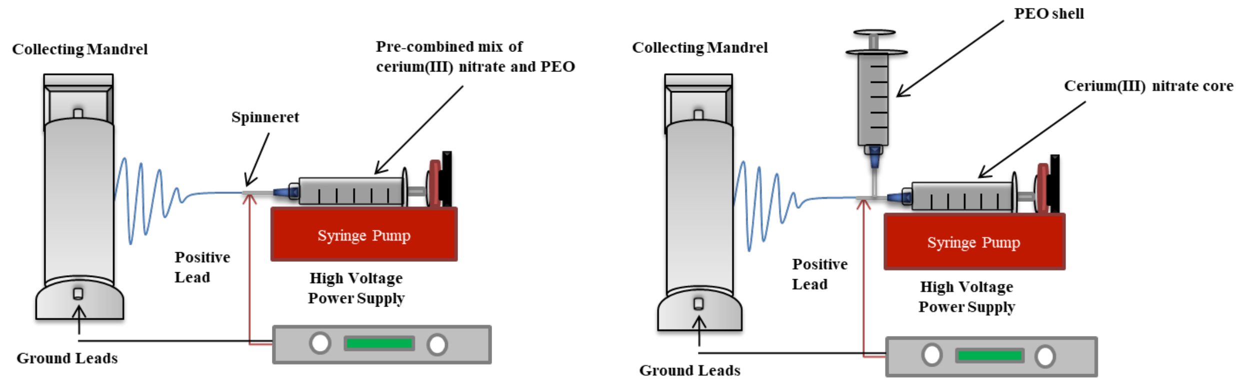


Figure 1: 5 Page Trauma Resuscitation Flow Sheet and T6 Application on an iPAD

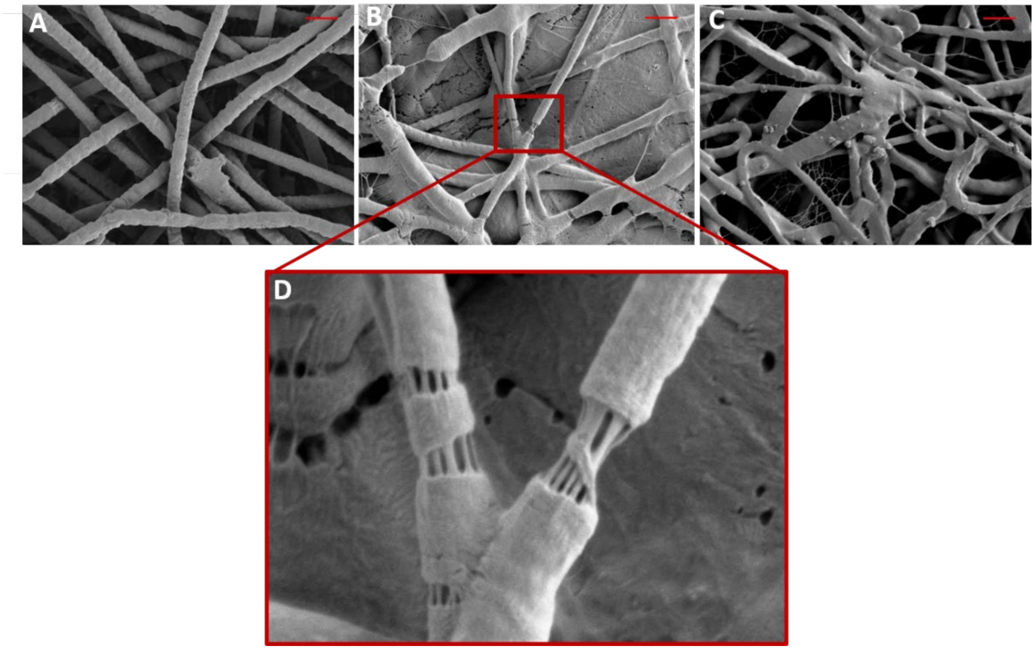


Figure 1: Representative SEM images of (A) electrospun PEO, (B) dual-spun PEO/Cerium(III) nitrate, (C) coaxially electrospun PEO/Cerium(III) nitrate scaffolds, (D) core-shell formation in coaxially electrospun PEO/Cerium(III) nitrate. Scale bar = 4 µm; Red area = 64 μm^2

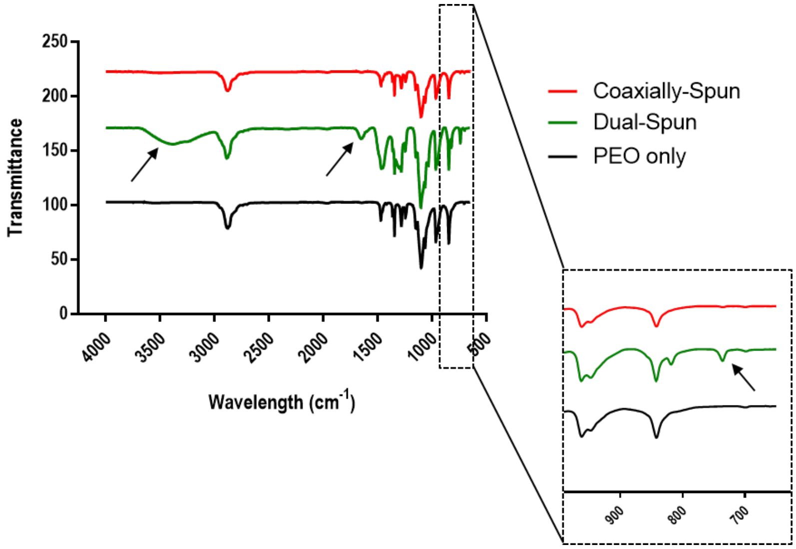


Figure 2: FTIR spectra of electrospun PEO (black), dual-spun PEO/Cerium(III) nitrate (green), and coaxially-spun PEO/Cerium(III) nitrate scaffolds. Arrows indicate known peaks (3387 and 1641 are O-H groups of bound moisture, and ~700 is Ce(III)) [14].

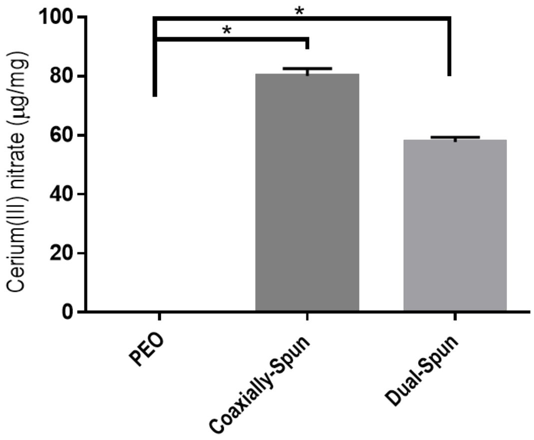


Figure 3: Loading potential of electrospun dressings. (n=9)

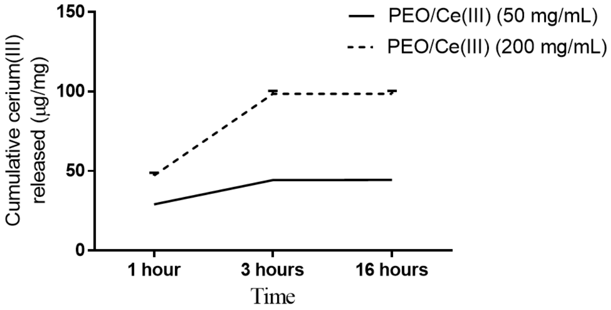


Figure 4: Cumulative Ce(III) released per scaffold (µg/mg) over 16 hours for 50 $mg_{scaffold}/mL_{diluent}$ and 200 $mg_{scaffold}/mL_{diluent}$ PEO/Ce(III). (n=9)

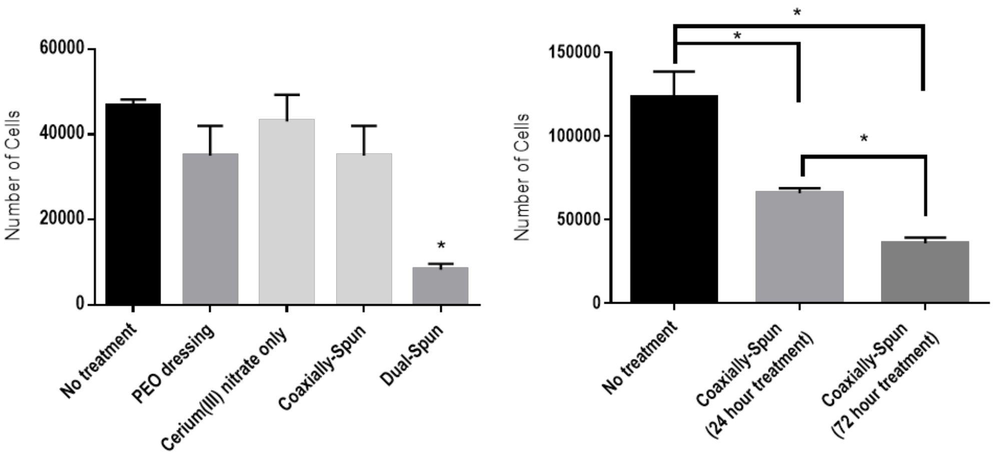


Figure 6: Cell viability of human dermal fibroblasts over 24 hours (left, n=3). Comparison of cell viability of human dermal fibroblasts between 24 hour and 72 hour scaffold incubations (right, n=3).

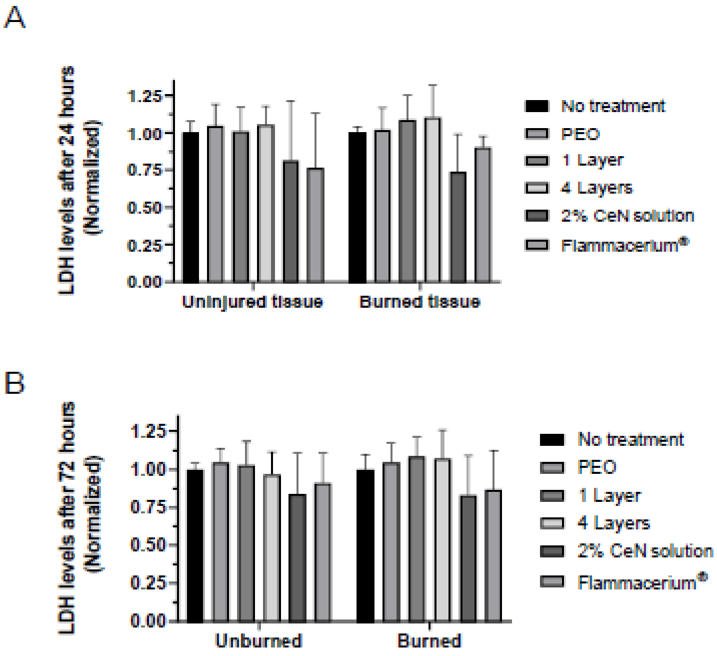


Figure 7. *Ex vivo* treatment with cerium nitrate-containing treatments does not significantly affect cell viability. Treatments were applied to the epidermal surface of isolated pig tissue and incubated at 32℃ with 5% CO2 for 24 (A) or 72 (B) hours. Data are representative of four independent experiments with technical triplicates; mean with standard deviation. Polyethylene oxide (PEO); cerium(III) nitrate (CeN); lactose dehydrogenase (LDH).

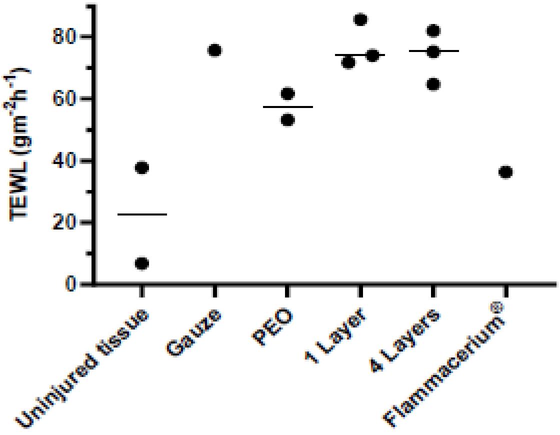


Figure 8. Electrospun cerium nitrate dressings reduce transepidermal water loss (TEWL) by Day 2. Burns were assessed for TEWL after one dressing treatment. Measurements greater than those of the uninjured tissue suggest that the wound surface is damaged and/or more susceptible to dehydration. Data points show the average value of three measurements taken from each wound; mean values are indicated by solid lines. Polyethylene oxide (PEO).

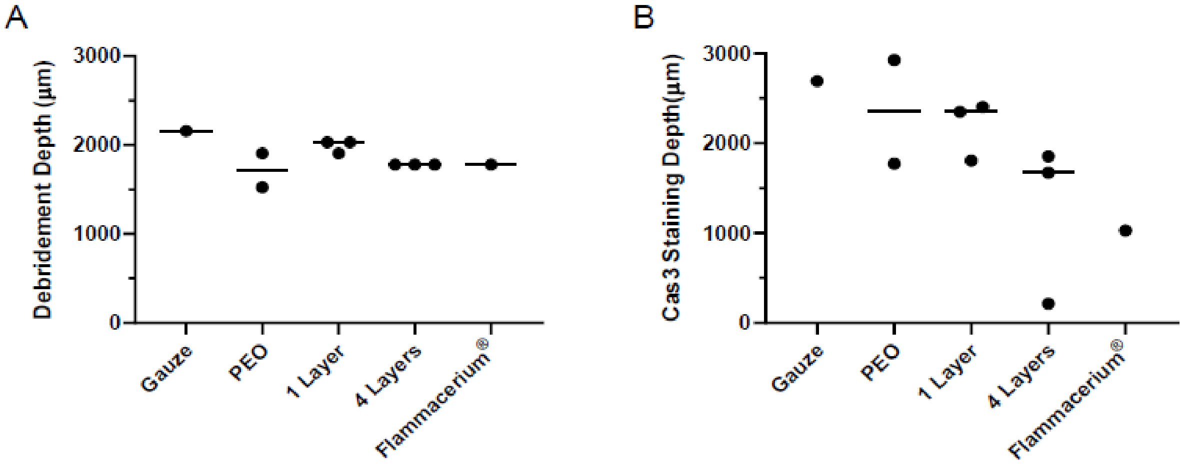
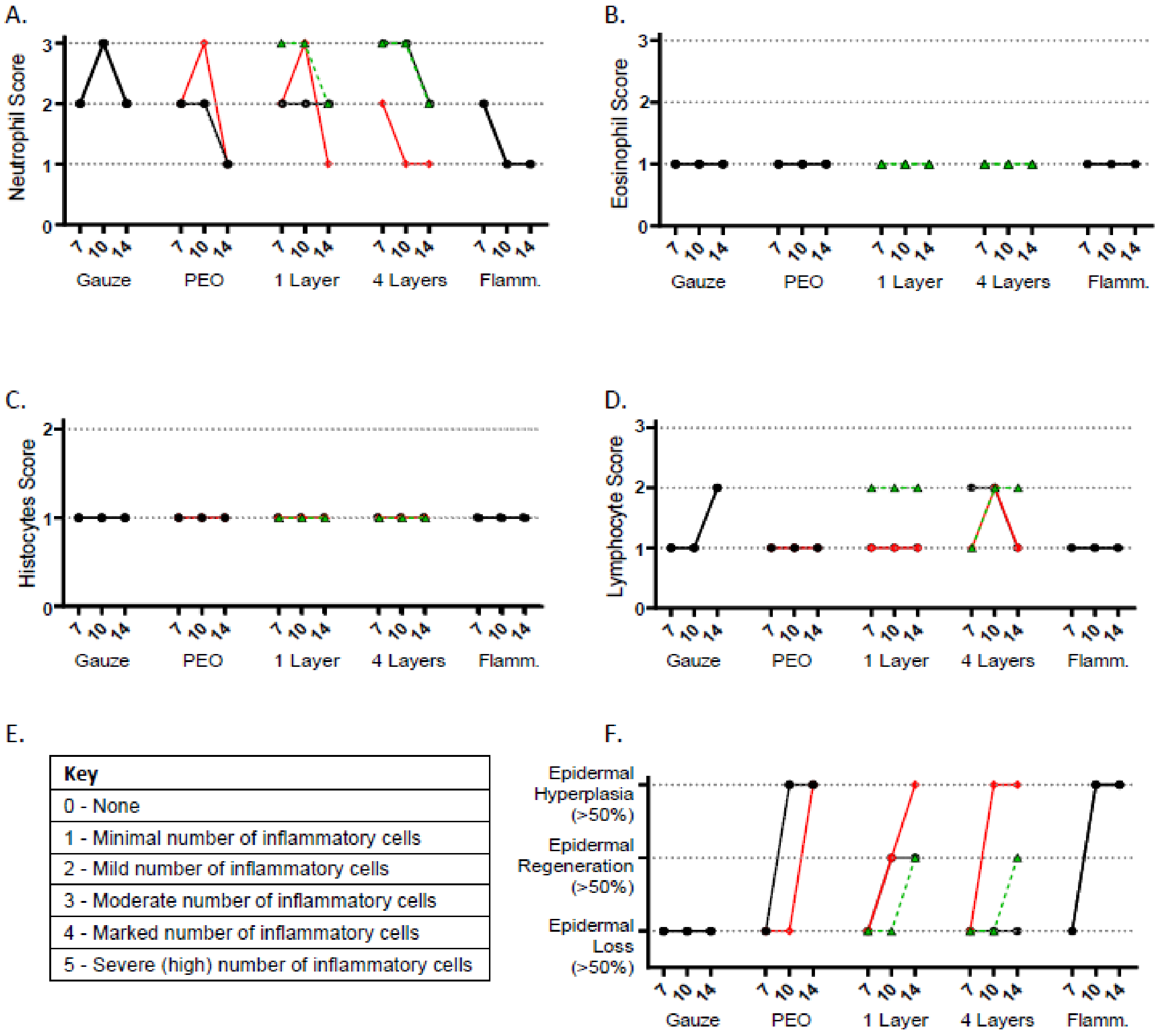
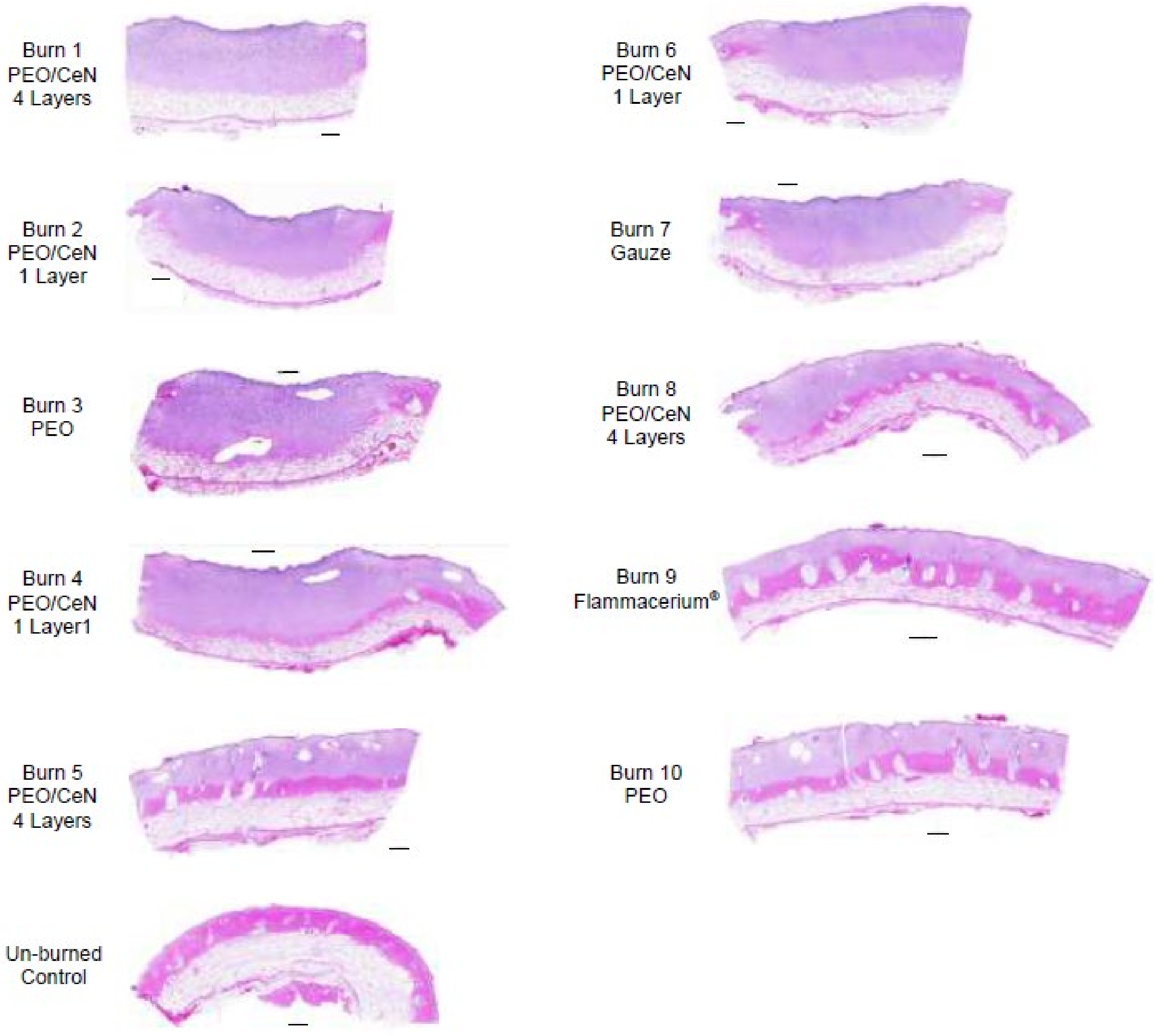


Figure 9. Comparison of surgical debridement depths and depths of necrotic tissue. (A) Four days after injury, a blinded operator surgically debrided all burns until punctate bleeding was observed. The total depth of debridement was recorded for each wound. (B) Immediately before surgical debridement, each burn was biopsied with an 8 mm biopsy punch. Samples were stained for Caspase 3 (Cas3). The distance from the wound surface to the bottom of Cas3 staining were recorded for each sample. Polyethylene oxide (PEO).



Key
0 - None
1 - Minimal number of inflammatory cells
2 - Mild number of inflammatory cells
3 - Moderate number of inflammatory cells
4 - Marked number of inflammatory cells
5 - Severe (high) number of inflammatory cells

Figure 10. Electrospun cerium nitrate dressings reduce pro-inflammatory cell infiltrates and may heal faster than to the prolonged field care standard of care. Biopsy samples were collected 3, 6, and 10 days after surgical debridement. A blinded pathologist scored all samples for neutrophil infiltrate (A), eosinophil infiltrate (B), histocyte infiltrate (C), lymphocyte infiltrate (D), and extent of epidermal regeneration (F). The histological scorecard is included (E). Individual wounds are indicated by the different colored lines. No difference in histology scores were observed prior to surgical debridement on Day 4. Polyethylene oxide (PEO); Flammacerium® (Flamm.).



Supplemental Figure 1. Hematoxylin and eosin staining of deep-partial thickness burn wounds, 14 days after debridement. Burns were made on the dorsum of an anesthetized Yorkshire pig. Wounds were treated one hour after injury, and again two days later. Burns were surgically debrided to punctate bleeding on Day 4 post-burn. Two weeks later biopsy strips that spanned the entire wound length were harvested. Images are ordered with Burns 1 and 6 (cranial) at the top and Burns 5 and 10 (caudal) at the bottom. Polyethylene oxide

12.0 LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

µm	Micrometer
AAALAC	American Association for Accreditation of Laboratory Animal Care
ANOVA	Analysis of Variance
Cas-3	Caspase 3
Ce(III)	Cerium (III)
CeN	Cerium Nitrate
cm	Centimeter
DI	Deionized
DOD	Department of Defense
DPT	Deep Partial Thickness
FSEM	Field Emission Scanning Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
H&E	Hematoxylin and Eosin
hr	Hour
IM	Intramuscular
ISR	Institute of Surgical Research
IV	Intravenous
Kg	Kilogram
kv	Kilovolts
LDH	Lactate dehydrogenase
mg	Microgram
MHSRS	Military Health Science Research Symposium
mL	Milliliter
mm	Millimeter
MW	Molecular Weight
NAMRU-SA	Naval Medical Research Unit San Antonio
nm	Nanometer
OH	Hydroxyde
PAO	Public Affairs Officer
PEO	Polyethylene Oxide
PFC	Prolonged Field Care
PVDF	Polyvinylidene
ROI	Return on Investment
rpm	Revolutions per Minute
SEM	Scanning Electron Microscopy
SOC	
TBS	Tris-Buffered Saline
TBSA	Total Burn Surface Area
TEWL	Transepidermal Evaporative Water Loss
US	United States
UV	Ultraviolet
v/v	Volume/Volume
w/v	Weight/Volume