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# Medical management of cutaneous sulfur mustard injuries

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ABSTRACT

*Background:* Sulfur mustard (2,2′-dichlorodiethyl sulfide; HD) is a potent vesicating chemical warfare agent that poses a continuing threat to both military and civilian populations. Significant cutaneous HD injuries can take several months to heal, necessitate lengthy hospitalizations, and result in long-term complications. There are currently no standardized or optimized methods of casualty management. New strategies are needed to provide for optimal and rapid wound healing.
*Objective:* The primary aim of this research was to develop improved clinical strategies (treatment guidelines) for optimal treatment of superficial dermal (second degree) cutaneous HD injuries, with the goal of returning damaged skin to optimal appearance and normal function in the shortest period of time.
*Methods:* Superficial dermal HD injuries were created on the ventral abdominal surface of weanling pigs. At 48 h post-exposure, lesions were laser debrided and a treatment adjunct applied. Cultured epithelial allografts and 11 commercial off-the-shelf (COTS) products were examined for their efficacy in improving wound healing of these injuries. Clinical evaluations and a variety of non-invasive bioengineering methods were used at 7 and 14 days post-surgery to follow the progress of wound healing and evaluate various cosmetic and functional properties of the wounds. Measurements included reflectance colorimetry to measure erythema; evaporimetry to examine transepidermal water loss as a method of evaluating barrier function; torsional ballistometry to evaluate the mechanical properties of skin firmness and elasticity; and two-dimensional high frequency ultrasonography (HFU) to monitor skin thickness (e.g., edema, scar tissue). Histopathology and immunohistochemistry were performed 14 days following surgery to examine structural integrity and quality of healing. Logical Decisions® for Windows was used to rank the 12 treatment adjuncts that were studied.
***Results:* The most efficacious treatment adjuncts included (1) Vacuum Assisted Closure™, V.A.C.®, involving application of topical negative pressure, (2) Amino-Plex® Spray (bi$O_2$ Cosmeceuticals International, Inc., Beverly Hills, CA), a nutritive cosmeceutical product that is designed to increase oxygen in cells, stimulate ATP synthesis, improve glucose transportation, stimulate collagen formation, and promote angiogenesis, and (3) ReCell® Autologous Cell Harvesting Device (Clinical Cell Culture Americas LLC, Coral Springs, Florida), an innovative medical device that was developed to allow rapid harvesting of autologous cells from a thin split-thickness biopsy followed by spray application of a population of skin cells onto wounds within 30 min of collecting the biopsy, without the need of culturing the keratinocytes in a clinical laboratory.**
***Conclusions:* Complete re-epithelialization of debrided HD injuries in 7 days is possible. In general, shallow laser debridement through the basement membrane zone (100 μm) appears to provide better results than deeper debridement (400 μm) with respect to early re-epithelialization, cosmetic appearance, functional restoration, and structural integrity. Of the 12 treatment adjuncts examined, the most promising included Vacuum Assisted Closure™, Amino-Plex® Spray, and ReCell® Autologous Cell Harvesting Device.**

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## 1. Introduction

Sulfur mustard (2,2′-dichlorodiethyl sulfide; HD) is a potent vesicating chemical warfare agent that poses a continuing threat to both military and civilian populations. This agent is inexpensive, easily obtainable or synthesized, frequently stockpiled, easily disseminated, can negatively impact combat effectiveness by forcing military forces to don protective gear, and has the potential to be used by terrorists (Khateri et al., 2003; Saladi et al., 2006). HD primarily affects the lungs, eyes and skin. The chemical properties, proposed mechanisms of action, toxicokinetics, pathogenesis of injury, acute toxic effects, and delayed toxic effects have been extensively reviewed (Balali-Mood et al., 2005; Graham et al., 2005; Kehe and Szinicz, 2005; McManus and Huebner, 2005; Mellor et al., 1991; Momeni et al., 1992; Naraghi et al., 2005; Somani and Babu, 1989; Willems, 1989).

Long-term complications in HD casualties from the Iran–Iraq War (1980–1988) have recently been reported. An extensive review of medical records of Iranian casualties indicated that of the 100,000 HD casualties one third are suffering effects today (Shohrati et al., 2007a). A review of late complications of 34,000 casualties indicated that the incidences of lung, eye, and skin problems were 42.5%, 39.3%, and 24.5%, respectively (Khateri et al., 2003). Common cutaneous problems being reported include hyperpigmentation, hypopigmentation, atrophy, multiple cherry angiomas, sensory loss, burning, pruritis, xerosis, hypohidrosis, local hair loss, erythematous papular rash, eczema, scaling, desquamation, sensitivity to mechanical injury with recurrent blistering and ulceration, and dermal scarring (Balali-Mood et al., 2005; Balali-Mood and Hefazi, 2006; Emadi et al., 2008; Hafazi et al., 2006; Khateri et al., 2003; Momeni et al., 1992; Panahi et al., 2007; Shohrati et al., 2007a,b). Chronic cutaneous symptoms are generally more severe during colder seasons (Hafazi et al., 2006). Microscopic examinations have noted mild papillomatosis and acanthosis in the epidermis with pigmentation in the basal layer, atrophy of adnexal structures, non-specific dermal fibrosis and sclerosis, marked epidermal atrophy, orthokeratotic hyperkeratosis, perivascular mononuclear infiltrate throughout the papillary dermis, melanosomes within the epidermis, and melanophages in the upper dermis (Balali-Mood et al., 2005; Emadi et al., 2008; Hafazi et al., 2006). These patients have a higher incidence of psoriasis and autoimmune diseases such as vitiligo and discoid lupus erthematosis than the general population (Balali-Mood and Hefazi, 2006; Hafazi et al., 2006). Lower incidences of acne vulgaris, folliculitis, and tinea versicolor have been reported, probably due to chronic dry skin (Hafazi et al., 2006).

Significant cutaneous HD injuries can take several months to heal (Balali-Mood and Hefazi, 2006; Graham et al., 2005, 2006; Kehe and Szinicz, 2005; Momeni et al., 1992; Newmark et al., 2007; Willems, 1989), necessitate lengthy hospitalizations, and result in considerable cosmetic (e.g., scarring, dyspigmentation, dry skin) and/or functional (e.g., contractures over joints that limit motion, fragile skin easily damaged by trauma, hypersensitivity, chronic ulceration) deficits. There are currently no standardized or optimized methods of casualty management (Graham et al., 2005). Current treatment strategy consists of symptomatic management and is designed to relieve symptoms, prevent infections, and promote healing. The current strategy primarily involves deroofing large blisters, disinfecting and applying antibiotic creams or ointments, conducting frequent dressing changes, administering systemic analgesics and antihistamines, applying topical or systemic antipruritics, and close monitoring of fluids and electrolytes (Balali-Mood and Hefazi, 2006; McManus and Huebner, 2005; Mellor et al., 1991; Newmark et al., 2007; Saladi et al., 2006; Willems, 1989, and recommendations by the U.S. Centers for Disease Control and Prevention available at http://www.cdc.gov). An in-depth review of the currently recommended treatment regimens as they appear in a variety of military textbooks and handbooks has been provided (Graham et al., 2005).

In spite of the symptomatic management strategies currently employed, lengthy healing periods and long-term complications still occur. New strategies are needed to provide for optimal and rapid wound healing, and to ameliorate long-term complications. Such strategies have recently been formulated by an international working group (Graham et al., 2005). The research described in this paper was guided by these strategies. In short, adequate wound debridement of partial-thickness injuries is needed, with subsequent treatment of the lesions using contemporary medical approaches similar to those applied for the treatment of chronic cutaneous ulcers or partial-thickness thermal burns.

Previous animal studies have shown that surgically aggressive approaches are needed to prevent or minimize significant cosmetic and functional deficits that result from deep HD injury (Graham et al., 2002a,b). [Superficial (first degree) injuries involve only the epidermis, superficial dermal (second degree) injuries involve the epidermis and upper third of the dermis, deep dermal injuries involve the epidermis and most of the dermis, and full-thickness injuries involve the destruction of all skin elements and sometimes involve underlying muscle, tendon, or bone (Arturson, 1996).] For the best outcome, deep dermal/full-thickness cutaneous HD injuries require full-thickness debridement followed by skin grafting. While past HD wound healing research has concentrated on deep dermal/full-thickness injuries, superficial and superficial dermal HD injuries may have greater clinical relevance on the battlefield or in terrorist attacks on civilian populations. Superficial dermal HD injuries will likely not require such surgically aggressive approaches (e.g., serial tangential excisions followed by autologous split-thickness skin grafting). The research described in this paper focuses on the treatment of superficial dermal HD injuries.

In general, superficial dermal burns heal within 21 days without treatment. In weanling pigs, untreated superficial dermal HD injuries often have a thin dry eschar present 14 days after exposure that is just beginning to separate from the underlying, regenerating epithelium. While the lesions have clinically re-epithelialized by 21 days, there are significant histological abnormalities present (Graham et al., 2006). It has not been determined how long it would take tissue architecture to return to normal. The primary aim of this research was to develop improved clinical strategies (treatment guidelines) for optimal treatment of superficial dermal cutaneous HD injuries, with the goal of returning damaged skin to optimal appearance and normal function in the shortest period of time. Superficial dermal HD injuries were created on the ventral abdominal surface of weanling pigs. At 48 h post-exposure, select lesions were laser debrided and a treatment adjunct applied. Cultured epithelial allografts and a variety of commercial off-the-shelf (COTS) products were examined for their efficacy in improving wound healing of these injuries.

## 2. Materials and methods

### 2.1. Animal model

Sixty-six female Yorkshire crossbred pigs (weanlings), *Sus scrofa*, 8–15 kg (mean 11.8), were used (Country View Farms, Shanksville, PA). Research was conducted in compliance with Animal Welfare Regulations (7 USC, 9 CFR, Ch 1 Subchapter A parts 1–4) and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Animals were quarantined for 1 week prior to use on the study to screen for signs of disease. Pigs were lightly anesthetized with intramuscular injections of xylazine

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HCl (Xyla-Ject®, Phoenix Pharmaceutical, Inc., St. Joseph, MO; 2.2 mg/kg) and a combination of tiletamine HCl and zolazepam HCl (Telazol®, Fort Dodge Animal Health, Fort Dodge, IA; 6.0 mg/kg) for blood collection. Blood (8 ml) was collected from the anterior vena cava for routine clinical pathology before agent exposure and just prior to euthanasia.

Each efficacy study consisted of six pigs. Surgical procedures (laser debridement and application of treatment adjunct) were conducted at 48-h post-exposure. A 14-day healing period followed surgery, during which clinical observations, body temperature measurements, and non-invasive bioengineering methods were conducted on a weekly basis. At the end of the healing period, animals were humanely euthanized for histopathological and immunohistochemical evaluation of skin sites.

### 2.2. Sulfur mustard exposure

Eighteen to 24 h before agent exposure, a pre-exposure body temperature was measured and the ventral abdominal skin clipped with an electric clipper, and then depilated as previously described (Graham et al., 2002b). Immediately after hair removal, 4 exposure sites were demarcated on the ventral abdominal surface, two sites per side parallel to and approximately 2.5 cm lateral to the teat line and located between the axillary and inguinal areas. The inguinal fold and sharply curved areas of the rib cage were avoided. A plastic template was used for even spacing and consistent anatomical positioning of the sites among animals. Small dots were placed at each corner using a permanent marker to delineate each site. Each site within the grid measured approximately 5 cm by 5 cm. Four exposure sites were demarcated on the ventral abdominal surface. Three of the four sites on each animal were exposed to undiluted liquid sulfur mustard for 8 min to produce superficial dermal injuries. The fourth site was sham exposed (no HD). The location of the sham site was rotated among animals to preclude sensitivity biases based upon anatomical location. The exposure procedures used to generate 3-cm diameter superficial dermal injuries in the center of each HD-exposed site were the same as those previously described (Graham et al., 2002b); however, in place of the tile floats a calibration weight (300 g) was placed on top of the rubber stopper and manually held in place for the duration of the exposure to ensure even downward pressure and complete contact of the wetted filter paper with the skin. Prior to exposure, animals were lightly anesthetized with Xyla-Ject® and Telazol® as described above and placed in the agent hood in dorsal recumbency, supported by a stainless steel pig sling. A therapeutic heating pad (Gaymar Industries, Inc., Orchard Park, NY), with the circulating water temperature set at 41 °C, was placed under the animal during the exposure period to minimize hypothermia.

### 2.3. Debridement and treatment procedures

At 48 h post-exposure, animals were lightly anesthetized as described above, intubated, and placed on 1.0–2.5% isoflurane in oxygen at a flow rate of 0.8–1.5 L/min using an Excel 210SE anesthetic machine with an Isotec 5 isoflurane vaporizer (Datex-Ohmeda, Madison, WI). At the end of the debridement and treatment applications, the concentration of isoflurane was reduced gradually until the animal was on 100% oxygen and then room air. Rectal temperature, pulse rate, respirations, and pulse oximetry were monitored throughout all procedures.

Following induction into a deep plane of anesthesia, treated wounds were first debrided slightly beyond the visible borders of the lesions to a depth of 100, 300 or 400 μm with an erbium:yttrium–aluminum–garnet (Er:YAG) laser (Sciton Profile Laser Surgical System with Scanner, Sciton, Inc., Palo Alto, CA), then a treatment adjunct immediately applied. The laser system, configured as a high-powered dual mode long pulse Er:YAG laser, offers independent control of both depth of coagulation (to control blood loss) and depth of ablation (for tissue removal) to specified, uniform depths with minimal residual thermal damage. Operating parameters for the laser were as follows. Percent overlap of spots was 50%. Scanning pattern was set to a square measuring 3 cm × 3 cm. For those sites debrided in multiple passes, eschar was wiped off using dry gauze between each pass. For those lesions debrided to a depth of 100 μm, one pass of the laser was used with the following ablation/coagulation settings (in μm): 100/0. For those sites debrided to a depth of 300 μm, four passes of the laser were used with the following settings (in sequential order): 80/0, 80/0, 80/50, and 60/0 (30% overlap, last pass only). For those sites debrided to a depth of 400 μm, five passes of the laser were used with the following settings: 100/0, 80/0, 80/0, 80/50, and 60/50. Ablation settings of 100, 80, and 60 μm corresponded to fluences of 25, 20, and 15 J/cm$^2$, respectively.

On each animal, one HD-exposed site was left untreated (positive control), and the other two HD-exposed sites were debrided with the Er:YAG laser and a treatment adjunct applied. The fourth experimental (sham-exposed, untreated) site served as a negative control. As the location of the sham site was rotated among animals, treatment sites were also rotated; however, the two treated sites on any given animal were at the same cranial–caudal level to facilitate circumferential application of compression bandages. The positive and negative control sites (also at the same cranial–caudal level) were dressed with a single layer of sterile gauze secured in place with surgical tape and staples. After all sites were dressed, a protective cotton stockinette was then put in place over the animal's torso, secured with surgical staples and elastic tape. All dressings were removed on post-surgical day 7 (PS07) just prior to clinical observations. Sites remained undressed for the remainder of the experiment.

### 2.4. Treatment adjuncts

The following treatment adjuncts were tested.

1. *Flexzan Foam Adhesive Dressing* (*Bertek Pharmaceuticals Inc., Morgantown, WV*) is an ultra-thin, semi-occlusive polyurethane foam adhesive dressing that is frequently used following laser facial resurfacing. Excess wound fluid is absorbed into open cells of the foam dressing and evaporates through a closed cell outer surface. A light compression dressing was placed over the Flexzan using elastic tape that was wrapped around the circumference of the animal's torso and held in place by surgical staples. Dressings (including fresh Flexzan) were changed at four days post-surgery. This dressing was tested in parallel with frozen cultured epithelial porcine allografts (#2 below) on the same animal, where two HD-exposed sites were laser debrided to a depth of 400 μm with one debrided site dressed in Flexzan and the other in allograft.
2. *Frozen cultured epithelial porcine allograft* (*Living Skin Bank, State University of New York, Stony Brook, NY*) was prepared on a petrolatum backing from pig keratinocytes collected from a naïve set of animals as previously described (Graham et al., 2006; Randolph and Simon, 1993; Rheinwald and Green, 1975), and thawed at room temperature prior to use. A moderate compression dressing was placed over the allograft by placing Kerlix™ Super Sponges (Tyco Healthcare/Kendall, Mansfield, MA) and Reston™ Self-Adhering Foam Pads (3M Health Care, St. Paul, MN) over each treated site, followed by elastic tape that was wrapped around the circumference of the animal's torso held in place with surgical staples. Dressings (including fresh allograft) were changed at four days post-surgery. This graft material was tested in parallel with Flexzan Foam Adhesive Dressing (#1 above) on the same animal, where two HD-exposed sites were laser debrided to a depth of 400 μm with one debrided site dressed in Flexzan and the other in allograft.
3. *DuoDERM Signal* (*ConvaTec, Princeton, NJ*) is a hydrocolloid dressing designed to provide a moist wound healing environment, manage exudate, and have a longer wear time than typical hydrocolloid dressings. The dressing was applied to sites laser debrided to a depth of either 300 or 400 μm. Edges of the dressings were secured with surgical tape that was further secured with surgical staples.
   For this study, two different debridement depths were chosen (300 and 400 μm) to ascertain if depth of debridement affected outcome. As no differences were noted in outcome using these two depths, a decision was made to widen the depth range to 100 and 400 μm in subsequent experiments using the treatment adjuncts described below.
4. *Amino-Plex® Spray* (*bi$O_2$ Cosmeceuticals International, Inc., Beverly Hills, CA*) is a nutritive cosmeceutical product that is designed to increase oxygen in cells, stimulate ATP synthesis, improve glucose transportation, stimulate collagen formation, and promote angiogenesis. It is a mixture of over 100 low-molecular weight ingredients, including amino acids, trace minerals, nucleotides, nucleosides, oligopeptides, electrolytes, glycosaminoglycans, and glycolipids. The ingredients are dissolved in de-ionized water, along with minute quantities of preservatives dissolved in a small percentage of propylene glycol. The product is provided in a pump spray bottle without propellants. According to the company, this product has been clinically shown to reduce irritation and improve results in laser resurfacing, chemical peels, microdermabrasion, hair transplantation, and hair removal. Amino-Plex® was lightly sprayed on the surface of wounds that were laser debrided to a depth of either 100 or 400 μm. Treated sites were then covered with Tegaderm™ Ag Mesh (3M Health Care, St. Paul, MN). An Argyle Salem Sump Tube (Tyco Healthcare Group LP, Mansfield, MA) was sutured in place over the Tegaderm™ Ag Mesh to allow daily applications of the nutritive mixture directly onto the wound without the need to change dressings. The tubing was routed to the area over the dorsal spine and held in place with Mefix® self-adhesive fabric tape (Mölnlycke Health Care Inc., Norcross, GA), further secured with surgical staples. Next, a foam dressing (3M Health Care, St. Paul, MN) was cut to size and placed over the tubing (which was sitting on the Tegaderm Ag Mesh), and the entire dressing covered by Tegaderm™ Transparent Dressing (3M Health Care, St. Paul, MN). Edges of the Tegaderm Transparent Dressing were further secured with surgical tape and staples. Ten milliliters of Amino-Plex® were injected into each tube (5 ml on subsequent days), followed slowly by 5 ml air to saturate the foam dressing with the material. Five pieces of sterile 4″ × 4″ gauze were then put over each transparent dressing, which was held down with strips of surgical tape. Light compression was then applied by elastic tape that was circumferentially wrapped around the animal and held in place by surgical staples. Every 24 h after surgery for 7 days (including the day that the dressings were removed), animals were lightly sedated with 0.5 ml Telazol and additional Amino-Plex® treatments applied as described above. Following injections, the port ends of the tubing were capped with Parafilm M® (Alcan Packaging, Neenah, WI).
5. *ReCell® Autologous Cell Harvesting Device* (*Clinical Cell Culture Americas LLC, Coral Springs, FL*) is an innovative medical device that was developed to allow rapid harvesting of autologous cells from a thin split-thickness biopsy followed by

spray application of a population of skin cells onto wounds within 30 min of collecting the biopsy, without the need of culturing the keratinocytes in a clinical laboratory. This single-use device is designed for injuries up to 2% total body surface area (TBSA). This product is designed for use in superficial dermal, deep dermal, and full-thickness burns, donor sites, scar treatment, chronic ulcers, pigment loss, and cosmetic skin rejuvenation following laser resurfacing, dermabrasion, or chemical peels. Following processing of the skin biopsy, the resulting cell suspension provides autologous keratinocytes, epidermal stem cells, melanocytes, fibroblasts, and Langerhans cells. Briefly, a split-thickness biopsy (250 μm thick) measuring approximately 5 cm × 5 cm was aseptically removed from the right paravertebral area of each animal using a nitrogen-driven dermatome (Zimmer, Inc., Warsaw, IN) following subcutaneous injection of approximately 35 ml sterile saline to stiffen up the area. This donor site was dressed in xeroform petrolatum dressing (Sherwood Medical, St. Louis, MO) and sterile gauze, secured by surgical staples and left in place for 7 days. The biopsy was trimmed into two pieces each measuring approximately 1.5 cm × 1.5 cm. Lyophilized trypsin (0.75%) was reconstituted with 10 ml of sterile water, dispensed into a warming chamber in the ReCell® kit, and pre-heated to 37 °C. Each piece of trimmed tissue was placed into the heated trypsin solution for 25 min (using separate kits) to allow the epidermis to be separated from the dermis prior to further processing. After 25 min, the tissue was removed from the trypsin solution and briefly rinsed in a sterile sodium lactate solution to stop the trypsinization process. Using sterile fine tipped forceps, the epidermis was separated from the dermis in the kit's sterile petri dish. A few drops of fresh sodium lactate solution were then dripped onto the dermal–epidermal junction of both layers. The cells from the junctional surfaces were then scraped with a sterile scalpel to develop a plume of cells. The petri dish was then rinsed with 1.5 ml of fresh sodium lactate solution. The plume of cells, suspended in the sodium lactate solution, was then drawn up into a sterile syringe fitted with a blunt-tipped 18-gauge needle and aspirated several times to create a cell suspension. The final cell suspension was then dispensed through a cell strainer into a conical well in the kit. The entire filtered cell suspension from each piece of tissue was drawn up into a new sterile syringe fitted with a blunt-tipped 18-gauge needle and then dripped onto an HD-exposed site that had been laser debrided to a depth of either 100 or 400 μm. Immediately following application of the cell suspension, each treated wound was covered with SURFASOFT Fixative for Skin Grafts (MEDIPROF, Holland), secured with surgical staples, over which was placed sterile gauze secured with surgical tape and staples. Light compression was then applied by elastic tape that was circumferentially wrapped around the animal and held in place by surgical staples.

6. *Application of topical negative pressure* (*also known as Vacuum Assisted Closure*™, *V.A.C.®*) involves placing an open cell foam into the wound bed (cut to conform to the shape of the wound), sealing it with an adhesive drape, and applying subatmospheric pressure (125 mmHg below ambient) that is transmitted via an evacuation tube by a computerized vacuum pump. Several V.A.C.® therapy systems are available from Kinetic Concepts, Inc. (KCI), San Antonio, TX. The unit used in these experiments was the V.A.C. ATS® System. Laser debridement was conducted to a depth of 100 or 400 μm. Prior to application of the V.A.C.® dressings according to manufacturer's instructions, areas surrounding the treated sites were close shaved with a razor, washed with 20% soap solution, and rinsed with saline. Mastisol® liquid adhesive (Ferndale Laboratories, Inc., Ferndale, MI) was blotted around the treated areas (after debridement) where the V.A.C.® drape was to be adhered to the surrounding skin. Debrided sites were covered first with TELFA™ clear non-adhesive wound dressing (Tyco Healthcare/Kendall, Mansfield, MA) then a GranuFoam silver dressing (KCI, San Antonio, TX) that had been bisected width-wise. The purpose of the TELFA™ dressing was to prevent ingrowth of cells into the V.A.C.'s GranuFoam dressing. After both treated lesions had been dressed, a bridge was constructed of V.A.C.® drape material and GranuFoam silver dressing to connect the two sites. Both treatment areas and the bridge were covered with a single V.A.C.® drape, which had been trimmed to proper width before application. The edges of the drape were further secured with Mefix® self-adhesive fabric tape (Mölnlycke Health Care Inc., Norcross, GA). A small hole was cut in the drape in the center of the bridge, and a V.A.C.® trac pad put in place over the hole with tubing routed to the back in a manner similar to that described for the Amino-Plex® experiment. 3M™ Vetrap™ bandaging tape (3M Health Care, St. Paul, MN) was then coiled over the draped area using light compression, further secured with strips of elastic tape. Following dressing of the positive and negative control sites with sterile gauze and placement of a protective cotton stockinette over the animal's torso, each animal was then placed in a specially designed V.A.C.® harness to prevent damage to or entanglement in the evacuation tubes. The tubes were then connected, and the individual pumps started at 125 mmHg (pump and wound pressure). The pumps were run 24 h per day. The dressings were changed at 48 and 96 h post-surgery per manufacturer recommendation, and were removed on PS07 just prior to clinical observations.
7. *Biobrane®* (*Bertek Pharmaceuticals Inc., Morgantown, WV*) is a bi-layered composite consisting of a synthetic epidermal analog and a biologic (collagen-based) dermal analog designed for partial-thickness wounds. It is commonly used in treating partial-thickness thermal burns. Following laser debridement at either 100 or 400 μm, treated sites were covered with Biobrane with minimal overlap onto perilesional skin, and stapled in place. Five single 4″ × 4″ sterile gauze pads were placed over the Biobrane®, held in place with surgical tape and staples. Light compression was then applied by elastic tape that was wrapped circumferentially around the animal's torso and held in place by surgical staples.
8. *AQUACEL® Ag* (*ConvaTec, Princeton, NJ*) is a silver impregnated hydrofiber dressing designed for use on partial-thickness burns, donor sites, and chronic skin ulcers. It may be left *in situ* for up to 14 days, has a large fluid absorption capacity, and kills a broad spectrum of wound pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus* (VRE). Dressings were applied to sites that had been laser debrided to a depth of 100 or 400 μm. The dressings were covered with sterile gauze. Light compression was then applied by elastic tape that was wrapped circumferentially around the animal's torso and held in place by surgical staples.
9. *ACTICOAT 7 Day Antimicrobial Dressing* (*Smith & Nephew, Inc., Largo, FL*) consists of two layers of an absorbent, rayon/polyester inner core between three layers of silver-coated polyethylene mesh, designed for use on partial-thickness burns, donor sites, and chronic skin ulcers. The inner core maintains a moist wound healing environment. The dressing may be left *in situ* for up to 7 days, and kills a broad spectrum of wound pathogens. Following laser debridement to a depth of 100 or 400 μm, ACTICOAT 7 dressings were placed over the prepared wound beds and wetted with sterile water to activate the dressing. The ACTICOAT 7 dressings were then covered with Allevyn Adhesive hydrocellular polyurethane dressings (Smith & Nephew, Inc., Largo, FL). Light compression was then applied by elastic tape that was wrapped circumferentially around the animal's torso and held in place by surgical staples.
10. *Silon-TSR® Temporary Skin Replacement* (*Bio Med Sciences, Inc., Allentown, PA*) is a complex weave of biopolymers that produce a thin protective membrane, and is designed for use on partial-thickness burns, donor sites, and laser resurfaced skin. The dressing is perforated to allow exudate to escape. Following laser debridement to a depth of 100 or 400 μm, Silon-TSR® dressings were placed on the prepared wound beds and secured with surgical tape and staples. The dressings were then covered with Kerlix™ super sponges (Tyco Healthcare/Kendall, Mansfield, MA). Light compression was then applied by elastic tape that was wrapped circumferentially around the animal's torso and held in place by surgical staples.
11. *APLIGRAF®* (*Organogenesis, Inc., Canton, MA*) is a living bi-layered skin substitute designed for the treatment of venous leg ulcers and diabetic foot ulcers. According to the manufacturer, "like human skin Apligraf consists of living cells and structural proteins. The lower dermal layer combines bovine type 1 collagen and human fibroblasts (dermal cells), which produce additional matrix proteins. The upper epidermal layer is formed by promoting human keratinocytes (epidermal cells) first to multiply and then to differentiate to replicate the architecture of the human epidermis. Unlike human skin, Apligraf does not contain melanocytes, Langerhans' cells, macrophages, and lymphocytes, or other structures such as blood vessels, hair follicles or sweat glands." APLIGRAF® was applied to sites that were laser debrided to a depth of 100 or 400 μm. The product was stapled in place using surgical staples and then covered with a single piece of xeroform petrolatum dressing (Sherwood Medical, St. Louis, MO) that was folded into quarters. Five single 4″ × 4″ sterile gauze pads were placed over the xeroform, held in place with Durapore tape and surgical staples. Light compression was then applied by elastic tape that was wrapped circumferentially around the animal and held in place by surgical staples.
12. *Promogran* Matrix Wound Dressing* (*Johnson & Johnson Wound Management, Somerville, NJ*) is a freeze dried composite of 45% oxidized regenerated cellulose (ORC) and 55% collagen for use on partial-thickness burns, donor sites, and chronic skin ulcers. It is designed to bind matrix metalloproteases and protect growth factors. Treated sites were laser debrided to a depth of 100 or 400 μm. Promogran* dressings were then cut to shape, wetted with sterile water, placed on the prepared wound beds, and covered with Allevyn Adhesive hydrocellular polyurethane dressings (Smith & Nephew, Inc., Largo, FL). Light compression was then applied by elastic tape that was wrapped circumferentially around the animal's torso and held in place by surgical staples.

### 2.5. Pharmacologic treatment

Thirty minutes prior to surgery, animals were administered a prophylactic antibiotic (cefazolin sodium, Ranbaxy Pharmaceuticals Inc., Jacksonville, FL; 20 mg/kg i.m.). For analgesia, buprenorphine HCl (Buprenex® Injectable, Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA; 0.01 mg/kg i.m.) was administered immediately after HD exposure, the morning following agent exposure, and at the conclusion of the surgical procedures. Beginning on the morning following surgery, animals were provided 160 mg acetaminophen once daily for 7 days *per os*.

For any required dressing changes or just prior to conducting clinical observations on PS07 and PS14, animals were sedated with Xyla-Ject® (2.2 mg/kg) and Telazol® (6.0 mg/kg). Repeated injections were provided if needed. For those ani-

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mals in the Amino-Plex® study, animals were lightly sedated with 0.5 ml Telazol® i.m. for daily administration of the Amino-Plex®.

Euthanasia on PS14 (following clinical observations) was accomplished under Xyla-Ject®/Telazol® sedation (as described above) by an injection of an overdose of sodium pentobarbital-based euthanasia solution (Fatal-Plus® Solution, Vortech Pharmaceuticals, Ltd., Dearborn, MI; 78 mg/kg i.v.) administered into the anterior vena cava.

### 2.6. Post-surgical procedures

#### 2.6.1. Clinical evaluations

Lesions were gently cleansed with sterile saline and gauze to remove dried and loosely adhered exudate prior to clinical evaluation of each treated site for re-epithelialization. Re-epithelialization of debrided HD wounds was subjectively scored on PS07 and PS14 using the following scale: 0 = none, 1 = less than 25% of the original HD-exposed area had re-epithelialized, 2 = at least 25% but less than 50% of the original area had re-epithelialized, 3 = at least 50% but less than 75% of the original area had re-epithelialized, 4 = at least 75% but less than 100% of the original area had re-epithelialized, and 5 = 100% of the original area had re-epithelialized. An area was considered to be re-epithelialized only if it was visible (i.e., not covered by adherent eschar or scab) and petechial hemorrhaging was absent.

#### 2.6.2. Non-invasive bioengineering methods

A variety of non-invasive bioengineering methods were used to follow the progress of wound healing and evaluate various cosmetic and functional properties of the wounds. Measurements included reflectance colorimetry (RC) to measure erythema; evaporimetry to examine transepidermal water loss (TEWL) as a method of evaluating barrier function; torsional ballistometry (TB) to evaluate the mechanical properties of skin firmness and elasticity; and two-dimensional high frequency (20 MHz) ultrasonography (HFU) to monitor skin thickness (e.g., edema, scar tissue). Measurements were made before agent exposure and on PS07 and PS14 for all methods except HFU and TB, which were only conducted before exposure and on the last day of each study (PS14). HFU and TB were not conducted on PS07 to avoid disruption of the fragile neoepidermis. HFU was not conducted in the DuoDERM study due to equipment failure. Bioengineering methods were performed as previously described (Graham et al., 2002b).

#### 2.6.3. Histopathology

On the last day of each study, animals were euthanized with an overdose of Fatal-Plus Solution®. Full-thickness excisions (including panniculus carnosa) of each entire lesion plus surrounding skin were removed, stapled onto labeled, laminated index cards, and placed into 10% neutral buffered formalin. Sections were later trimmed, paraffin embedded, cut on a microtome into 5-μm thick sections, and stained with hematoxylin and eosin (H&E) for routine histopathology. Serial sections were also stained with Masson's trichrome to highlight dermal collagen, and Movat's pentachrome to highlight elastic fibers. A veterinary pathologist scored the sections in a blinded fashion based on a published histomorphologic scale for rating burn scars (Singer et al., 2000) modified for evaluation of the tissues (Table 1). Maximum total score for best possible outcome (e.g., normalcy) was 14.

#### 2.6.4. Immunohistochemistry

Eight-mm biopsies were collected from each excised site and processed for immunohistochemical staining. They were trimmed of excess subcutaneous fat, placed in cotton gauze, flash frozen in chlorodifluoromethane refrigerant (Freon 22) for approximately 30 s, placed in foil packages, and stored at −70 °C. Immunohistochemistry was conducted on frozen tissue sections to characterize the progression of cutaneous wound healing by localizing proteins associated with granulation tissue formation, neovascularization, basement membrane zone remodeling, and re-epithelialization. Sections of frozen tissues were cut at 10 μm in a cryostat and stained using the avidin–biotin–peroxidase complex (ABC) method for labeling of primary antibodies (VECTASTAIN® Elite ABC kits, Vector Laboratories, Burlingame, CA). The chromagen used was 3,3-diaminobenzidine tetrahydrochloride (DAB). The following cross-reactive human antibodies were used (all with a 30-min incubation period): CD49f (for staining alpha 6 integrin in the basement membrane zone, 1:50 dilution, Chemicon International, Inc., Temecula, CA), collagen IV (for staining anchoring plaques and the lamina densa in the basement membrane zone, 1:50 dilution, ICN Biomedicals, Inc., Irvine, CA), collagen VII (for staining anchoring fibrils in the basement membrane zone, 1:100 dilution, Sigma Chemical Company, St. Louis, MO), laminin 5 (for staining anchoring filaments within the basement membrane zone, 1:40 dilution, Chemicon International, Inc., Temecula, CA), filaggrin (for staining epithelium in the stratum granulosum, 1:50 dilution, Lab Vision Corp., Fremont, CA), laminin (for staining the lamina lucida of the basement membrane zone and basal lamina of endothelium, 1:100 dilution, Sigma Chemical Company, St. Louis, MO), vimentin (to stain intermediate filament proteins of fibroblasts, endothelial cells, and macrophages, 1:50 dilution, BioGenex Laboratories, Inc., San Ramon, CA), and von Willebrand factor (to stain endothelial cells, 1:100 dilution, Chemicon International, Inc., Temecula, CA). For the von Willebrand factor and vimentin stains, the area of staining was quantified by image analysis using Image-Pro® Plus 5.0 for Windows 2000/XP Professional (Media Cybernetics, Silver Spring, MD). For all other stains, the percent area of normal localization found across the entire section was subjectively graded using the following scale: 0 = none, 1 = 5%, 2 = 10–40%, 3 = 50–80%, 4 = 90–95%, and 5 = 100%.

**Table 1**
Histomorphologic scale

| Parameter | Score |
|---|---|
| Re-epithelialization | 1 = complete<br>0 = not complete |
| Epidermal hyperplasia | 1 = absent<br>0 = present |
| Epidermal/dermal separation | 1 = absent<br>0 = present |
| Inflammatory cells | 1 = absent<br>0 = present |
| Hair follicles | 1 = present<br>0 = absent |
| Glands | 1 = present<br>0 = absent |
| Elastic fibers | 1 = normal<br>0 = reduced size and number |
| Smooth muscles | 1 = present<br>0 = absent |
| Collagen orientation | 3 = normal dermis<br>2 = abnormal papillary dermis<br>1 = abnormal upper reticular dermis<br>0 = abnormal lower reticular dermis |
| Fibroplasia | 1 = absent<br>0 = present |
| Vascular proliferation | 1 = absent<br>0 = present |
| Hemorrhage | 1 = absent<br>0 = present |

Tissue sections were trimmed, paraffin embedded, cut on a microtome into 5-um thick sections, and stained with hematoxylin and eosin (H&E) for routine histopathology. Serial sections were also stained with Masson's trichrome to highlight dermal collagen, and Movat's pentachrome to highlight elastic fibers. A veterinary pathologist scored multiple parameters in each section in a blinded fashion. For each tissue section, scores for each individual parameter were added together. Maximum total score for best possible outcome (e.g., normalcy) was 14.

### 2.7. Data analysis

Initial comparisons of treatment groups were made on the two principal histopathological (re-epithelialization and collagen orientation) and immunohistochemical (alpha 6 and collagen IV) variables measured. This was performed using an analysis of variance and a Kruskal–Wallis test on the scores. If significant treatment effects were observed, then a Tukey's or Mann–Whitney's test was used to compare pairs of treatment groups. For binomial response data, a Chi-square analysis was used to compare the treatment groups and if significant, further Chi-square or Fisher's exact tests were used for pairs of treatment group comparisons. Statistical significance was defined as $p < 0.05$ for all tests. These initial analyses provided little information regarding consistent treatment group differences (data not shown). Since there were many more histopathological, immunohistochemical, bioengineering, and clinical variables measured, a decision analysis was considered the best method to incorporate all the information collected to determine the best overall treatment groups. Therefore, further statistical analyses comparing treatment groups for individual variables were not performed.

Logical Decisions® for Windows V6.0 (Fairfax, VA) was used to rank the 12 treatment adjuncts that were studied. Four main areas of examination and scoring were defined, routine histopathology, bioengineering, clinical judgment, and immunohistochemistry. In each of these areas, there were at least two and up to 12 distinct parameters recorded. Parameters were recorded at PS07 and/or PS14. The mean or median score for each parameter was used for each treatment. When scores were missing, either the positive control value or the mean of all scores was used. Vimentin and von Willebrand factor measurements were normalized based on the total score across all treatments to bring the large continuous measurements more in line with the categorical scores given to the other parameters under immunohisotchemistry. To maintain consistency across parameters, all parameters were scored similarly; a high score was considered the best response. If a parameter's

original scoring did not follow this, the parameter's score was transformed using an inverse of the score (e.g. original score 0.5, inverse is 1 divided by 0.5 which equals 2). This was used for bioengineering categories alpha (TB), delta $a^*$ and delta $E^*_{ab}$ (RC), TEWL and skin thickness (HFU). It was also used for the normalized vimentin and von Willebrand factor measurements.

Each parameter was assigned a weight by the Logical Decisions® software based on the number of parameters in their respective area and the parameter's relative importance. The sum of the parameter weights equaled one for each area. The four main areas were also weighted based on their order of importance using the software. Table 2 displays the relative importance of each area and each distinct parameter within each area along with their respective weights. Structural integrity was considered the most important indicator of wound healing; thus, routine histopathology and immunohistochemistry were assigned a relative importance of one. Functional data (bioengineering results) were considered second in importance, and subjective clinical assessments the least important.

Regarding routine histopathology, parameters related to the main structural components of the skin (epidermis, elastic fibers, collagen fibers, and blood vessels) were considered of highest importance. Of least importance were parameters related to adnexal structures (hair follicles, glands, smooth muscles) that could potentially be absent in any given section due to the exact location of the histological section that was cut from the paraffin block. Of intermediate importance was the presence of inflammatory or red blood cells outside of the vasculature.

Regarding immunohistochemistry, antigens related to the basement membrane zone (BMZ) were considered of highest importance; without an intact BMZ, recurrent blistering of the skin is more likely. Regarding bioengineering and clinical judgment, data collected on PS14 were considered of higher value than those collected on PS07 immediately after bandage removal.

**Table 2**
Areas/parameters measured, relative importance, and weights

| Main areas/parameters | Relative importance | Weights |
|---|---|---|
| **Routine histopathology (PS14)** | 1 | 0.361 |
| Re-epithelialization | 1 | 0.107 |
| Epidermal hyperplasia | 1 | 0.107 |
| Epidermal/dermal separation | 1 | 0.107 |
| Inflammatory cells | 2 | 0.071 |
| Hair follicles | 3 | 0.036 |
| Glands | 3 | 0.036 |
| Elastic fibers | 1 | 0.107 |
| Smooth muscles | 3 | 0.036 |
| Collagen orientation | 1 | 0.107 |
| Fibroplasia | 1 | 0.107 |
| Vascular proliferation | 1 | 0.107 |
| Hemorrhage | 2 | 0.071 |
| **Bioengineering (PS07 and PS14)** | 2 | 0.194 |
| TEWL – PS07 | 2 | 0.067 |
| TEWL – PS14 | 1 | 0.133 |
| RC – delta $E^*_{ab}$ PS07 (skin color) | 2 | 0.067 |
| RC – delta $E^*_{ab}$ PS14 (skin color) | 1 | 0.133 |
| RC – delta $a^*$ PS07 (erythema) | 2 | 0.067 |
| RC – delta $a^*$ PS14 (erythema) | 1 | 0.133 |
| TB – indentation PS14 (hardness) | 1 | 0.133 |
| TB – alpha value PS14 (elasticity) | 1 | 0.133 |
| HFU – skin thickness PS14 | 1 | 0.133 |
| **Clinical judgment (PS07 and PS14)** | 3 | 0.083 |
| HD area re-epithelialization PS07 | 2 | 0.33 |
| HD area re-epithelialization PS14 | 1 | 0.67 |
| **Immunohistochemistry (PS14)** | 1 | 0.361 |
| Filaggrin | 8 | 0.028 |
| Laminin | 5 | 0.111 |
| Alpha 6 | 1 | 0.222 |
| Collagen VII | 4 | 0.139 |
| Collagen IV | 2 | 0.194 |
| Laminin 5 | 3 | 0.167 |
| von Willebrand factor | 6 | 0.083 |
| Vimentin | 7 | 0.056 |

Logical Decisions® for Windows V6.0 (Fairfax, VA) was used to assign relative weights to various measured parameters. Four main areas of examination and scoring were defined, routine histopathology, bioengineering, clinical judgment, and immunohistochemistry. In each of these areas, there were at least two and up to 12 distinct parameters recorded. Parameters were recorded at 7 days and/or 14 days post-surgery (PS). Each parameter was assigned a weight based on the number of parameters in their respective area and the parameter's relative importance. The sum of the parameter weights equaled one for each area. RC, reflectance colorimetry; TEWL, transepidermal water loss; TB, torsional ballistometry; HFU, high frequency ultrasound.

## 3. Results

All 66 animals remained healthy in appearance and behavior throughout the course of the study. Mean pre-exposure body temperature was 101.2 °F. Post-exposure, temperatures remained within two standard deviations of the mean with the exception of slight, transient elevations noted on two separate occasions in different animals. No signs of infection within the experimental sites were seen. Occasional loose stools or diarrhea, likely stress related, were noted. Skin scratches and mild inflammation around some surgical staples were occasionally noted. These signs did not correlate to elevated temperatures.

Clinical pathology examinations indicated that several clinical chemistry parameters were elevated or depressed (data not shown). Depressions were not clinically significant. Clinically significant increases were defined as a three-to-five-fold increase over published reference ranges. Alkaline phosphatase (ALP), an analyte whose increase may be associated with hepatobiliary disease, was elevated in 39 pigs. An increased level of this analyte is common in weanling pigs, and increased levels were not consistently related to treatment. Alanine transaminase (ALT), often used as an indicator of liver function, was slightly increased in 60 animals and tended to be higher after exposure. However, none of the measured levels were clinically significant. Aspartate aminotransferase (AST), another indicator of liver function, was elevated in only six animals, and in all cases there was also an increase between pre- and post-exposure levels, but no change was clinically significant. Lactate dehydrogenase, another important indicator of liver function in this species, was elevated in 56 animals, but levels were never clinically significant, and changes that occurred between pre- and post-exposure values did not trend in one direction. When taken together, liver enzyme values (ALT, AST, LDH) reflect no notable effects on liver function during the study. Amylase, a pancreatic enzyme and potential indicator of pancreatic or renal compromise, was clinically elevated but within the range typically reported at this institute for this species and age animal, and may be a reflection of the feeding regimen. In 32 cases, levels increased 5–20% over baseline after treatment. However, because samples were taken 2 weeks apart, the increases seen could also have been age-related, since this enzyme increases with age in this species. Creatine kinase (CK) is an indicator of muscle damage. While levels were increased in 51 animals, in all except 7 cases levels were not clinically significant; increases in 5 of those 7 cases were seen after exposure. All of these were within the range of increase potentially caused by intramuscular injection-associated trauma, since multiple needle sticks occurred to maintain light anesthesia between depilation and euthanasia. All other clinical chemistry values out of reference range were few and not clinically significant.

Eight pigs displayed a mild anemia (hematocrit, HCT, of less than 30%) that did not change when measured after exposure. One pig's hematocrit decreased after exposure to a mildly anemic level. Fourteen pigs had a mild-moderate anemia (HCT less than 25%) that in all cases either remained the same or improved when measured after exposure. Four animals had a moderate anemia (HCT less than 20%), which in all cases had increased to above 22% in post-exposure samples. All anemias noted were microcytic and normochromic in nature and not considered clinically significant since no animals appeared pale, weak or ill in any way at any time. Total protein was slightly low for all animals. These combined results may reflect a residual mild iron deficiency, common in weanling pigs, which caused no clinical effects. The leukogram revealed that 61 animals had a mild monocytosis, and in half of these cases a

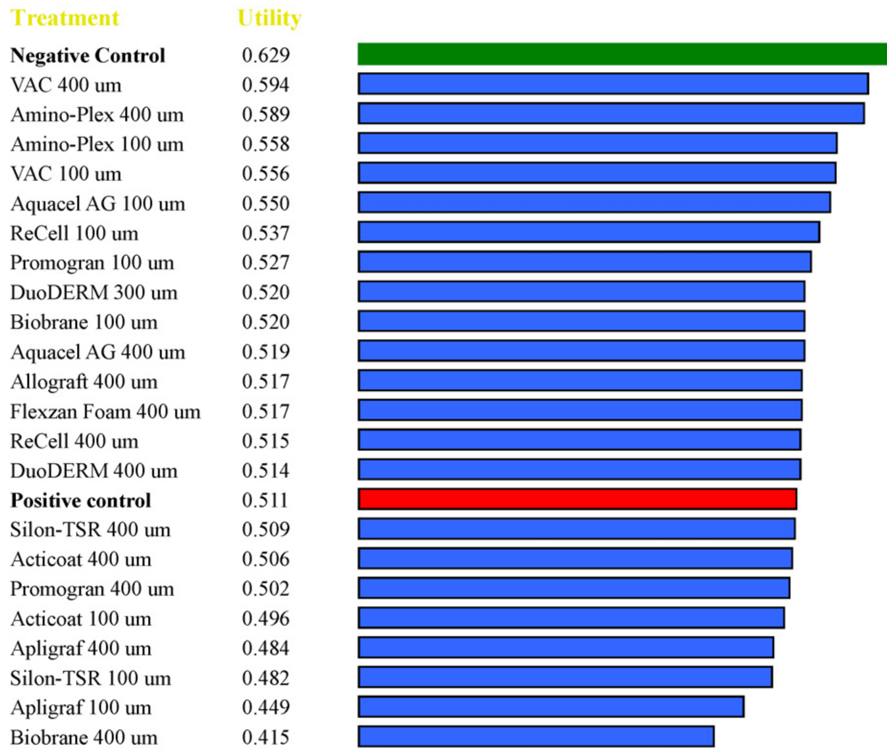


**Fig. 1.** Treatment rankings. Logical Decisions® for Windows V6.0 (Fairfax, VA) was used to rank the treatments applied to sulfur mustard injuries at 48 h post-exposure, utilizing the relative importance and ranks of each measured parameter listed in Table 1. The mean or median score for each parameter was used for each treatment.

lymphopenia also occurred; these changes probably reflect a stress response to handling and/or exposures, since no trend associated with exposure was seen. No neutrophilia was seen, corresponding to the lack of clinical signs of significant inflammation in these animals.

There is the possibility that the acute nature of this study prevented full manifestation of any clinical chemistry or hematological changes directly related to the treatments.

No TB or HFU data were collected from 15 of the 264 sites evaluated on PS14 due to the presence of hardened, adherent eschar or scab. These sites were primarily untreated positive controls.

Results of the decision analysis comparing all treatments are displayed in Fig. 1, listed from the most efficacious to least efficacious method. The top six treatments (in decreasing order of efficacy) were V.A.C.® (400 μm debridement), Amino-Plex® (400 μm debridement), Amino-Plex® (100 μm debridement), V.A.C.® (100 μm debridement), Aquacel® AG (100 μm debridement, and ReCell® (100 μm debridement). As use of V.A.C.® devices would not be practical in mass casualty scenarios and a silver impregnated dressing was included in the Amino-Plex® treatment, the remaining figures and subsequent discussion are focused on Amino-Plex® and ReCell®.

Sites treated with many of the adjuncts had not fully re-epithelialized by PS07. Most sites treated with Amino-Plex® and ReCell® had re-epithelialized by that time point. Some pinpoint petechial hemorrhaging was occasionally noted with these treatment adjuncts where the primary dressings in contact with the wound bed remained adherent to the neoepidermis and hair that was regrowing; removal of the dressings denuded minute patches of skin. Gross photographs of sites that were shallowly debrided (to 100 μm) and treated with Amino-Plex® or ReCell® vs. positive controls can be seen in Fig. 2a and b, respectively. Lesions treated with Amino-Plex® were typically erythematous but fully re-epithelialized by 7 days post-surgery. Appearance was near normal by 14 days post-surgery. Lesions treated with ReCell® were similarly erythematous but had fully re-epithelialized by 7 days post-surgery, and appearance was also near normal by 14 days post-surgery. In general, for HD-exposed sites treated with any adjunct, those that were deeply debrided (300–400 μm) were not as near normal in cosmetic appearance as were those shallowly debrided (100 μm, not shown); skin tone tended to be darker over the entire area treated with the laser.

Routine H&E stains of HD-exposed tissues collected on PS14 from the lesions shown in the gross photographs are seen in Fig. 3a (untreated positive control), b (treated with Amino-Plex®), and c (treated with ReCell®). Epidermal hyperplasia and abnormal collagen orientation limited to the papillary dermis were noted in all three sections. Re-epithelialization was complete in the treated sites. Incomplete re-epithelialization, abnormal collagen orientation extending into the lower reticular dermis, fibroplasia, vascular congestion, and hemorrhage can be seen in the untreated positive control.

Immunohistochemical localization of collagen type VII and CD49f (alpha 6) is shown in Figs. 4 and 5. Untreated sites showed an overall decrease in immunospecificity for collagen type VII within the sublamina densa of the basement membrane zone (Fig. 4a). In addition, portions of the basement membrane zone were also found to be diffusely localized or absent of collagen VII staining altogether. Fig. 4b (Amino-Plex® treatment) shows robust immunolocalization of collagen type VII (arrows) to anchoring fibrils within the sublamina densa of the basement membrane zone, which is characteristic of normal cytoarchitecture. Untreated sites also showed diffuse or interrupted immunolocalization of alpha 6 within the basement membrane zone (Fig. 5a). Localization of alpha 6 to cell membranes of hypertrophic basal and supra basal epithelial cells was also observed. Fig. 5b (ReCell® treatment) shows normal immunolocalization patterns of alpha 6 to the cell membranes of basal and supra

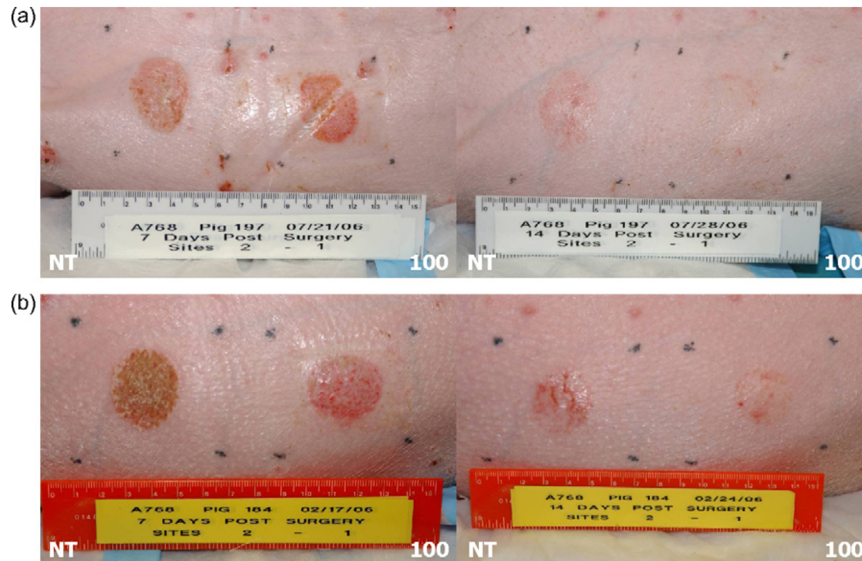


**Fig. 2.** Clinical photographs. (a) HD exposed, 100 μm debridement, treated with Amino-Plex® Spray vs. not treated. Treated lesion was erythematous and had an indentation from the tubing used to deliver the Amino-Plex®, but was fully re-epithelialized by 7 days post-surgery (left panel). Appearance was near normal by 14 days post-surgery (right panel). NT: not treated, 100: debrided to 100 μm and treated. (b) HD exposed, 100 μm debridement, treated with ReCell® vs. not treated. The treated lesion was erythematous but had fully re-epithelialized by 7 days post-surgery (left panel), and appearance was near normal by 14 days post-surgery (right panel). NT: not treated, 100: debrided to 100 μm and treated.

basal cells within the epithelium and to the basement membrane zone.

## 4. Discussion

The importance of wound debridement in the healing process is well established. This principal should apply to cutaneous sulfur mustard injuries as it does to thermal burns, chronic leg ulcers, diabetic foot ulcers, and decubitis ulcers. Powered dermabrasion (Rice, 1995; Rice et al., 2000) and laser debridement (Evison et al., 2006; Graham et al., 1997, 2002a,b) have been particularly successful in improving the rate of healing of cutaneous HD injuries in pigs. A review of previous research involving various methods of debridement of vesicant injuries is available (Graham et al., 2005). The type of laser (Er:YAG) used in this study for debridement is particularly suited for cutaneous resurfacing (Graham et al., 2005).

Vacuum Assisted Closure™ is becoming widely used for the closure of chronic wounds such as stage III and IV pressure ulcers; venous, arterial, and neuropathic ulcers; and subacute and acute wounds such as dehisced incisions, split-thickness meshed skin grafts, and muscle flaps (Banwell and Teot, 2003; Joseph et al., 2000; Mendez-Eastman, 1998). V.A.C.® is also gaining popularity in the management of complex orthopedic wounds. While results were promising, use on the battlefield or in a mass casualty scenario is not practical; application and maintenance of the dressings is very labor intensive. While GranuFoam silver dressings were used as part of the V.A.C.® dressing procedure, the silver ions released into the wound bed by the GranuFoam were likely not the primary inducers of improved wound healing, as the V.A.C.® procedure performed better than AQUACEL® Ag and ACTICOAT 7. Additional testing with a non-silver ion delivering foam dressing would be needed to determine whether the subatmospheric pressure applied by the V.A.C.® procedure was solely responsible for the improved healing noted.

Amino-Plex® is a nutritive cosmeceutical product that is designed to increase oxygen in cells, stimulate ATP synthesis, improve glucose transportation, stimulate collagen formation, and promote angiogenesis. Boyce et al. (1995) noted that application of topical nutrients supports keratinocyte viability during graft vascularization of cultured skin substitutes and inhibits wound contraction. In this study, Amino-Plex® showed great promise in improving wound healing of superficial dermal HD injuries at both depths of laser debridement studies (100 and 400 μm). While Tegaderm™ Ag Mesh was utilized in conjunction with this nutritive product, the silver ions released into the wound bed by this dressing were likely not the primary inducer of improved wound healing, as other silver-ion delivering dressings tested (AQUACEL® Ag and ACTICOAT 7) did not perform as well. Additional testing without the incorporation of the Tegaderm™ would be needed to determine whether Amino-Plex® was solely responsible for the improved wound healing noted.

In this study, debridement to a depth of 100 μm followed immediately by application of a suspension of skin cells processed by the ReCell® kit showed great promise in improving wound healing of superficial dermal HD injuries. Cells applied to lesions debrided to a depth of 400 μm did not produce results by PS14 as good as those produced using the shallow debridement, but performed better than many of the other treatment adjuncts tested. Application of keratinocytes in suspension has been shown to improve epidermal wound healing in pig (Currie et al., 2003; Navarro et al., 2000; Svensjo et al., 2001) and mouse (Horch et al., 1998; Voigt et al., 1999) models. Keratinocyte suspension technology shows promise in that it does not require the length of time necessary to produce cultured epidermal sheets. Use of this technology has proven efficacious in the treatment of thermal burns in humans (Gravante et al., 2007; Wood, 2003; Wood et al., 2006) and appears to be as efficacious as conventional melanocyte–keratinocyte transplantation for the treatment of vitiligo (Mulekar et al., 2008). Collection of autologous biopsy material for use in ReCell® kits is less invasive than harvesting of classic skin grafts yet provides similar aesthetic and functional outcomes (Gravante et al., 2007). Use of spray keratinocyte technology has also been shown to reduce total length-of-stay per %TBSA over that seen for patients treated with confluent sheets of cultured epithelial autografts (Wood et al., 2006). More recently, its concurrent use in conjunction with Integra® Dermal Regeneration Template (Integra Lifesciences Corp.,

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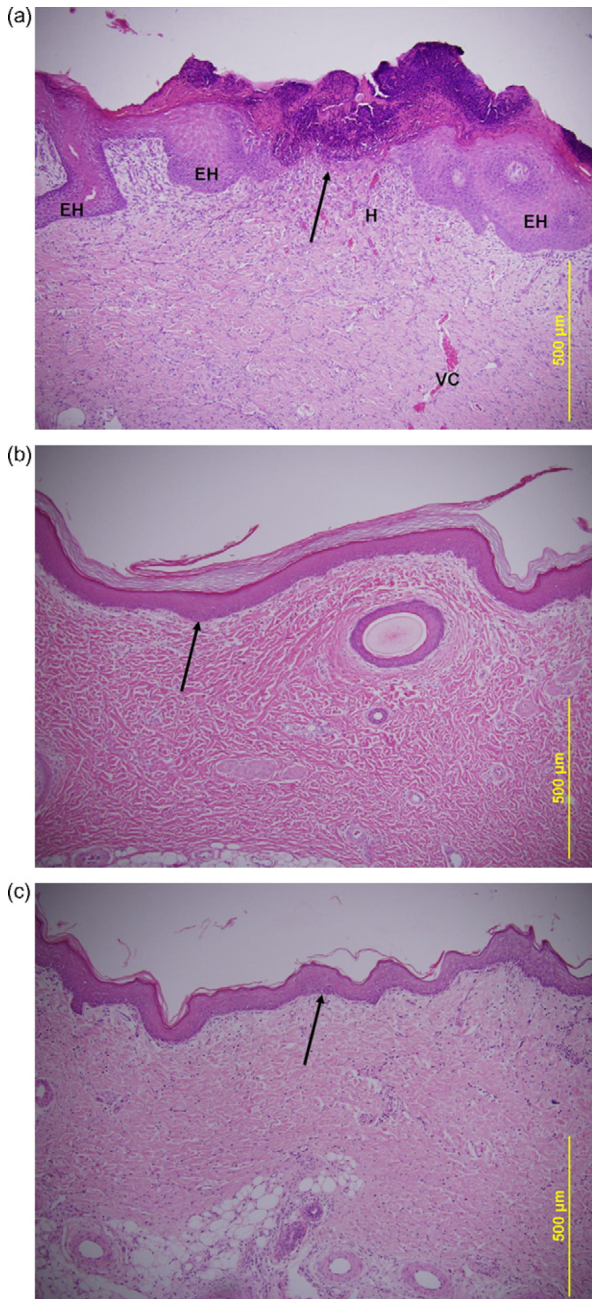


**Fig. 3.** (a) Histopathology, 14 days post-surgery, HD-exposed, untreated positive control (H&E stain). Epidermal hyperplasia (EH), vascular congestion (VC), hemorrhage (H), and fibroplasia are evident. Abnormal collagen orientation from the papillary dermis to the lower reticular dermis was noted. Re-epithelialization was not complete (arrow). (b) Histopathology, 14 days post-surgery, HD-exposed, 100 μm debridement, treated with Amino-Plex® Spray (H&E stain). Epidermal hyperplasia was noted in portions of this tissue section, along with abnormal collagen orientation limited to the papillary dermis. Re-epithelialization was complete (arrow). (c) Histopathology, 14 days post-surgery, HD-exposed, HD-exposed, 100 μm debridement, treated with ReCell® (H&E stain). Epidermal hyperplasia was noted in portions of this tissue section, along with abnormal collagen orientation limited to the papillary dermis. Re-epithelialization was complete (arrow).

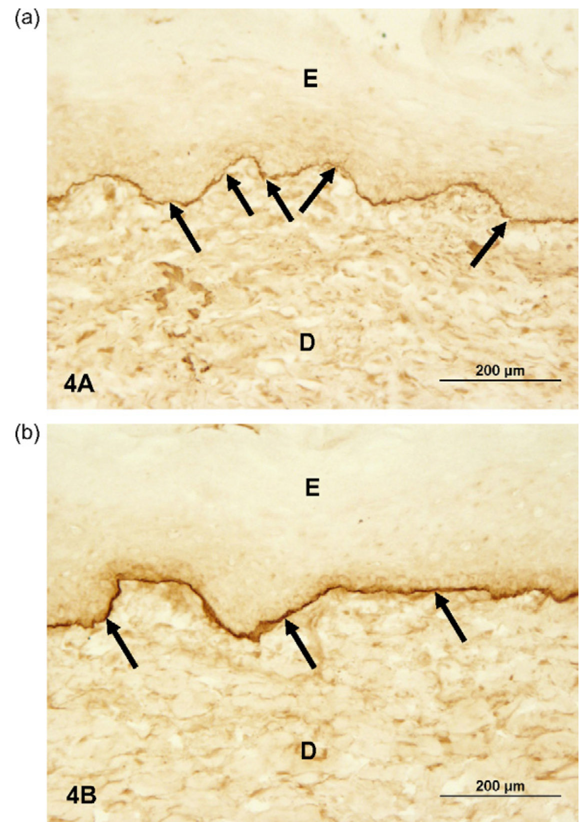


**Fig. 4.** Immunohistochemistry of collagen type VII for Amino-Plex® Spray, 14 days post-surgery. (a) Light micrograph of pig skin exposed to HD and left untreated showing an overall decrease in immunospecificity for collagen type VII within the sublamina densa of the basement membrane zone. In addition, portions the basement membrane zone were also found to be diffusely localized or absent of collagen VII staining altogether (arrows). Epidermis (E). Dermis (D). (b) Light micrograph of pig skin exposed to HD, laser debrided to a depth of 100 μm, and treated with Amino-Plex® Spray showing robust immunolocalization of collagen type VII (arrows) to anchoring fibrils within the sublamina densa of the basement membrane zone, which is characteristic of normal cytoarchitecture. Epidermis (E). Dermis (D).

Plainsboro, NJ) for the treatment of experimental full-thickness excisional wounds in pigs demonstrated enhanced epithelialization at early time points (1–2 weeks) compared to controls, facilitating one-step process skin reconstruction (Wood et al., 2007).

Long-term complications of cutaneous HD injury include hypopigmentation and hyperpigmentation. Hypopigmentation is noted in areas where severe HD damage induced local destruction of melanocytes. Otherwise, post-inflammatory hyperpigmentation predominates (Kehe and Szinicz, 2005; Khateri et al., 2003). Hyperpigmentation can be treated with laser resurfacing or pharmacologically treated under UVA/UVB protection with hydroquinone, kojic acid, azelaic acid, ascorbic acid, tretinoin, or topical glucocorticoids. Treatment for hypopigmentation is a much more challenging task. Utilization of ReCell®, which provides living and functional melanocytes in addition to other skin cells, may restore pigment to hypopigmented or depigmented skin previously exposed to HD. If the primary aim of using this technology on a patient is to address hyperpigmentation by applying autologous melanocytes (along with other skin cells), as in treating vitiligo (Mulekar et al., 2008), the biopsy material should be taken from an area of the patient's body with similar pigmentary qualities as is found in unaffected skin immediately surrounding the treatment site. Patients should be protected from UVA/UVB and undergo periodic examination by a dermatologist.

In this study we noted that complete re-epithelialization of debrided superficial dermal HD injuries in 7 days is possible. Debrided HD wounds were moderately exudative, more so in the lesions debrided to 300 or 400 μm than the lesions shallowly debrided (100 μm). The dressings applied in this study were adequately able to manage the exudate. In general, shallow Er:YAG laser debridement through the basement membrane zone (100 μm)

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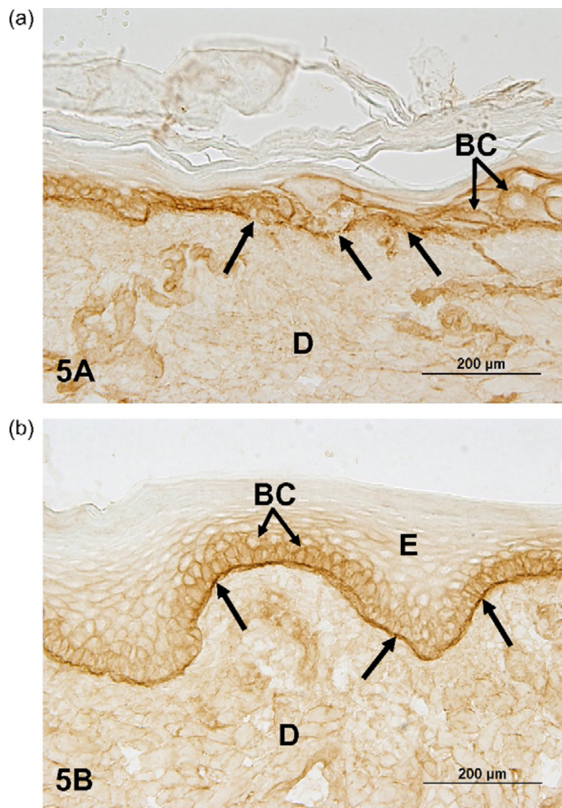


**Fig. 5.** Immunohistochemistry of CD49f (alpha 6) for ReCell® Autologous Cell Harvesting Device, 14 days post-surgery. (a) Light micrograph of pig skin exposed to HD and left untreated showing diffuse or interrupted immunolocalization of alpha 6 within the basement membrane zone (arrows). Localization of alpha 6 to cell membranes of hypertrophic basal and supra basal epithelial cells (BC) was also observed. Dermis (D). (b) Light micrograph of pig skin exposed to HD, laser debrided to a depth of 100 μm, and treated with ReCell® showing normal immunolocalization patterns of alpha 6 to the cell membranes of basal and supra basal cells (BC) within the epithelium (E) and to the basement membrane zone (arrows). Dermis (D).

appears to provide better results than deeper debridement (300–400 μm) with respect to early re-epithelialization, cosmetic appearance, functional restoration, and structural integrity as determined by clinical evaluations, non-invasive bioengineering methods, histopathology and immunohistochemistry.

Should both ReCell® and Amino-Plex® be unavailable for use when needed, using either xeroform petrolatum dressing or scarlet red dressings will be beneficial (Graham et al., 2006). Given a choice of using either ReCell® or Amino-Plex®, Amino-Plex® will likely prove to be the most practical modality as it is relatively inexpensive, is simple to apply, and can be self-administered without the need for intense nursing care. Although it is much more labor intensive than Amino-Plex® to apply and requires the presence of a physician, ReCell® would be better indicated for use on the hands and face where dyspigmentation poses a greater cosmetic concern.

In addition to vesication and death of epidermal keratinocytes, HD exposure results in sublethal damage to keratinocytes along the periphery of the gross lesion. Damage to the basement membrane zone and underlying collagen in the papillary dermis has also been noted. Deroofing frank blisters followed by timely removal of this adjacent and subjacent damage will likely improve the rate of re-epithelialization (Graham et al., 2005). Sublethal damage is clearly noted at the periphery of cutaneous HD lesions and has been reported previously (Braue et al., 1997; Graham et al., 1999; Reid et al., 2000). Sublethally injured cells at the periphery of an HD lesion and in hair follicles and other adnexal structures may be partly responsible for the slow rate of re-epithelialization seen in these injuries. Rice et al. (2000) suggested that the level of damage to cellular DNA at the margins of HD lesions may be sufficient to delay or prevent effective replication of those keratinocytes. In the current study, removal of these sublethally damaged keratinocytes at the margins of the wounds by debriding beyond the visible borders of the lesions likely helped to speed up the re-epithelialization process. In addition, HD induces damage to the BMZ at the level of the lamina lucida (Petrali et al., 1993). The floor of the blister retains portions of the damaged BMZ and needs to be removed to provide an adequate scaffold over which keratinocytes feeding the re-epithelialization process can migrate. Simply deroofing a blister, as has been often done in the past, is likely inadequate and may partially explain why these injuries have taken so long to heal. At minimum, debridement needs to proceed down into the papillary dermis after removal of the blister roof as was done in the current study. Beyond the BMZ, dermal collagen itself is affected by HD exposure and can itself impede the wound healing process (Brown and Rice, 1997; Lindsay and Rice, 1995; Rice et al., 2000). Brown and Rice (1997) reported coagulation and hypereosinophilia of the papillary dermis in Yucatan minipig skin 12–24 h following saturated HD vapor exposure, with the deeper reticular dermis unaffected. Rice et al. (2000) and Lindsay and Rice (1995) suggested that following exposure to HD, papillary dermal collagen is altered and may no longer function normally as a healthy scaffold over which epidermal cells can migrate.

Within the first week following debridement, the neoepidermis appears to be very fragile and easily removed. Care must be taken during bandage changes, and a non-adherent dressing that could be left in place for a long period of time (e.g., 7 days) would be beneficial, both to the patient and medical logistical burden (e.g., nursing care). In the experiment involving Flexzan foam dressings and cultured epithelial allografts, where the dressings were changed after four days, we noted that the neoepidermis was very fragile during the first week following debridement; the fewer bandage changes the better. Newmark et al. (2007) reported the treatment history of a U.S. serviceman who received partial-thickness injuries on his left arm and hand (6.5% TBSA) after demilitarizing a 75-mm munition. The injury progressed as expected from a cutaneous HD exposure and large blisters appeared in a classic “string of pearls” appearance. The blisters were filled with clear fluid that tested positive for the HD breakdown product thiodiglycol, along with HD-protein adducts. The blisters were not unroofed but were allowed to resorb as much as possible. Painful epidermal sloughing occurred after discharge from a burn unit 10 days post-exposure. Following discharge, the wounds underwent thrice-weekly dressing changes, including gentle debridement and application of silver sulfadiazine cream followed by application of wet-to-dry dressings. These procedures were repeated for 6 weeks before re-epithelialization was complete. It is very likely that the combination of (1) lack of early removal of the sublethally damaged keratinocytes along the periphery of the blisters and the blister floors (e.g., damaged BMZ), (2) frequent bandage changes, and (3) frequent gentle debridement with use of wet-to-dry bandages, which likely caused repeated removal of the neoepidermis, significantly contributed to the delay in healing in this patient.

General recommendations for medical management of cutaneous HD injuries include: (1) provide a high quality of care, which may be just as important as the modality that is chosen, (2) debride beyond the visible borders of the lesion and into the papillary dermis, (3) avoid the use of wet-to-dry dressings, (4) choose a modality that maintains a moist, clean wound healing environment, (5) change secondary dressings as frequently as needed, but do not disturb primary dressings that are in direct contact with the wound bed for a week unless there are indications of a possible

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infection, (6) protect the wounds from mechanical injury, and (7) after dressing removal protect the sites from ultraviolet radiation (UV-A and UV-B).

## 5. Conclusions

A number of treatment adjuncts were evaluated following Er:YAG laser debridement for their efficacy in improving wound healing of superficial dermal (second degree) sulfur mustard injuries in a weanling pig model. Complete re-epithelialization of debrided HD injuries in 7 days is possible. In general, shallow laser debridement through the basement membrane zone (100 μm) appears to provide better results than deeper debridement (300–400 μm) with respect to early re-epithelialization, cosmetic appearance, functional restoration, and structural integrity as determined by clinical evaluations, non-invasive bioengineering methods, histopathology and immunohistochemistry. Of the 12 treatment adjuncts examined, the most promising included Vacuum Assisted Closure™, Amino-Plex® Spray, and ReCell® Autologous Cell Harvesting Device.

## Conflict of interest statement

None.

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