IMPROVED HEALING IN TISSUE CULTURE MODEL OF VESICANT INJURY SHOWN BY HIGH FEATURE ANTIMICROBIAL DRESSING

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Abstract

Quick-Med Technologies (QMT) has developed a dressing for the treatment of vesicant injuries following debridement under an army Small Business Innovation Research (SBIR) program grant. This dressing is designed to enable optimal wound healing by providing a moist wound healing environment with antimicrobial protection and protease inhibiting properties, through sustained delivery of an antibiotic (doxycycline) and a growth factor (EGF). Sustained antimicrobial activity and protease inhibition have been previously documented through *in vitro* experiments. Research detailed here describes tissue culture models as used to assess healing of chemical injuries.

Epiderm FT (Mattek Corporation, MA) full thickness dermal tissue cultures were chemically injured by vapor phase exposure to 'half-mustard' (CEES) gas. Injured tissue constructs treated with the experimental dressing improved healing in a statistically significant manner as evaluated by cell proliferation assay and confirmed by histopathological evaluation. In the untreated groups pathology showed inhibited cell growth and increased detachment. Treatment with the dressings appeared to prevent the characteristic dermal – epidermal separation induced by vesicant injury. These results have prompted progress of the research project to animal experiments.

Background

Sulfur Mustard (SM) is a vesicant (blistering) agent that was widely used in WWI, and was more recently used in Iraq by Saddam Hussein. The mechanisms by which sulfur mustard creates injury on skin is thought to involve proteases (and likely other inflammatory agents), which illustrates significant similarity in the biochemistry of chemical and thermal burns.

Exposure of skin to sulfur mustard (also often called HD or SM) is thought to disrupt the balance between basement membrane protein synthesis by keratinocytes and their degradation by proteinases. This disturbance causes a loss of adherence between epidermis and dermis due to more net protein degradation than synthesis. This effect is observed macroscopically as vesication. Mortality resulting from direct SM exposure is relatively low, but blistering predisposes victims to secondary bacterial infection, which represents the greatest hazard to SM victims.

This research is the result of a US Army SBIR solicitation for a treatment that provides a moist wound healing environment combined with fluid handling, antimicrobial protection and protease inhibition while delivering nutritive factors and growth factor to stimulate healing. The research described here involves a tissue culture skin model that was chemically insulted using 'half mustard' (CEES), on which the dressing design was evaluated for impact on wound healing.

Antimicrobial dressings can help lower levels of bacteria in wounds, and can serve as the delivery vehicle for controlled release of the auxiliary compounds. The NIMBUS-SAP dressing treated with doxycycline has redundant dual-mode microbial control capacity, as well as providing protease inhibition. The MMPI doxycycline is a potent antibiotic, while the base dressing material provides intrinsic antimicrobial efficacy. The dressing matrix is designed to provide continuous barrier function, and prevent reinoculation of the wound from the dressing itself. Experiments have shown that the NIMBUS-SAP loaded with doxycycline is effective against repeated inoculation with *Pseudomonas aeruginosa*, identified by CDC as one of the most common burn wound pathogens, (PA – inoculation with 500 µl of 106 cfu/ml onto a 1" square dressing) for at least 7 consecutive days (**Figure 2**). This protection can be critical for SM exposed patients with severely compromised skin surfaces.

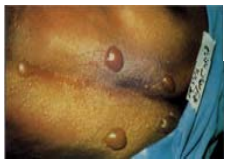


Figure 1 (above). Vesicant injury after sulfur mustard exposure. Blisters on the back 16 h after SM exposure. (from Willems, Annals Med Militar Belg, 1989).

	Doxycycline-SAP	NIMBUS-SAP
Day 1	7.48*	5.65
Day 2	7.64*	7.64*
Day 3	7.90*	6.23
Day 4	8.82*	8.82*
Day 5	9.03*	6.93
Day 6	8.99*	8.99*
Day 7	9.01*	4.31

* Denotes full kill. Numbers variation due to individual controls used.

Figure 2 (above). Repeated inoculation with *P. aeruginosa (PA)* per AATCC method 100-1999. Results reported as log reduction

Experimental

Experimental dressing. Previous research (reported at previous WHS meetings including 2006 and 2007) described the NIMBUS-SAP dressing system (Quick-Med Technology, Inc), characterized by laboratory assays. The dressing system was shown to have broad antimicrobial activity as tested per AATCC method 100-1999. Additionally, protease inhibition at high levels was demonstrated using Azocoll and TNO-212 assays.

Experimental model and evaluation methods. Epiderm FT (full thickness) skin model features dermal and epidermal structures composed of normal human-derived dermal fibroblasts and epidermal keratinocytes, and has a well defined basement membrane. Tissue cultures were acquired from MatTek (Ashland, MA), and had been specially cultured in media free of epidermal growth factor (EGFF media). EGFF media was also utilized throughout the conditioning process. In all cases standard treatment for the tissue constructs was followed: media changes at arrival and after 24h, and then at 48-72h intervals depending on the experiment. Samples were evaluated per MTS (Cell Titer 96 Aqueous one solution) cell proliferation assay (from Promega). Selected samples were also evaluated histologically with standard fixation and H&E staining.

Chemical Insult with CEES

Injury Model. In order to assess wound healing, a reproducible injury model was devised. **Figure 3** shows the downregulation of cell proliferation generated by the two final candidate exposure amounts – either 20 µl for 10 min or 40 µl for 20 min of CEES (2-chloroethyl ethyl sulfide), applied onto a Whatmann 24mm filter disk that was placed atop the cell well insert holding the tissue construct. The larger dose was selected because it achieved an injury that more accurately reproduced the basement membrane detachment characteristic of SM injury, although cell proliferation measured by MTS assay gave the same average result for both exposures. **Figure 4** shows histology for unexposed vs. exposed tissue constructs.

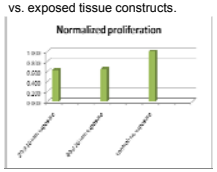


Figure 3 (above). Normalized results from cell proliferation assay (MTS) comparing chemical injury protocols to controls.

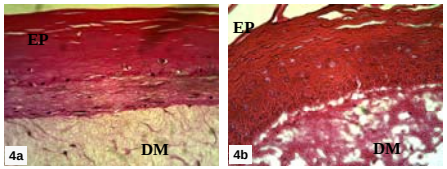


Figure 4 (above). Histology of tissue constructs comparing exposed and unexposed controls. The basal cellular layer is firmly attached to the insert for the most of the [unexposed] control sample (**4a**). After 20 minutes of CEES vapor exposure (**4b**) the tissue substrate is completely detached. The number of the cells is severely depleted, and cellular morphology of the dermal matrix is also changed. **Legend: EP-epidermis; DM-dermis**

Testing details

Treatment groups assessed were as follows:

- Controls, untreated = unexposed, untreated control tissues
- Controls, exposed and untreated = exposed and sham treated by placing a dressing of equal size (6mm diameter of J&J Sof-Wick nonwoven gauze) on the construct
- Treated with NIMBUS-SAP with Doxy (doxycycline and vitamins)
- Treated with NIMBUS–SAP with doxy + EGF (and vitamins)

In all cases where treatment was provided, a 6mm punch (12mg) of NIMBUS-SAP dressing material was treated with 200µl of treatment solution, in which the concentration of active agents was: 1ng/ml of EGF, 50mg/ml doxycycline hyclate and 100mg/ml each of vitamin C and vitamin E analog Trolox.

Cellular Proliferation, per MTS assay demonstrated that CEES exposure has caused ~ 40% depression in the metabolic activity of the exposed untreated controls (**Figure 5**). A statistically significant upregulation of 10-15% was shown between exposed control and both exposed treated groups (DOXY+EGF and DOXY). No significant difference was noted between the two treated groups (DOXY+EGF) vs. (DOXY).

Figure 5 (below). Cell proliferation assay data from exposed and treated tissue constructs

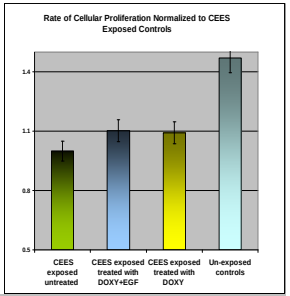


Figure 6 (below). Exposed, placebo treated tissue constructs. The placebo treated samples exhibited various degrees of epidermal-dermal separation, with or without complete loss of all connections between basal cells and the basement membrane zone and microblister formation after CEES exposures. Diffuse **(6a)** or complete **(6b, c)** epidermal-dermal separation was present in all CEES exposed placebo treated specimens. Clear signs of morphological epidermal keratinocyte damage, such as ballooning degeneration, acantholysis, and nuclear pyknosis in basal and suprabasal layers were present. Epidermal-dermal separation is marked by arrows.
Legend: EP-epidermis; DM-dermis

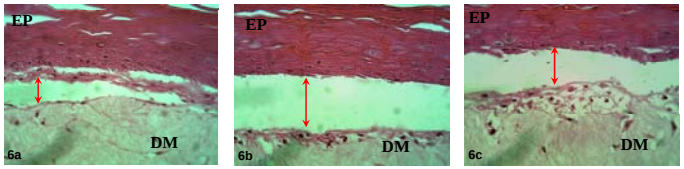


Figure 7 (below). Exposed, NIMBUS-SAP with doxy and vitamins. Features seen with DOXY+EGF and only DOXY treatment were similar at the upper layers of the epidermis. The most notable variation in cellular response to the treatments (compared to untreated exposed control) was at the epidermal/dermal junction as shown in **Figures 7** and **8** below. Histopathological features included focal depletion of the epidermal layer, presence of pyknotic nuclei in the basal and suprabasal layers, acantholytic cells in basement membrane zone and varying degree of cytoplasmic vacuolations and spongiosis extending to deeper dermal layers. Minor focal microblistering was observed (**7b, c** marked by *). Involvement of the upper dermal zone has been a histopathological finding in all treated samples, suggesting that the effect of CEES in EpiDerm FT tissue constructs may have extended beyond the basement membrane zone. Dermal damage may be attributable to the absence of vasculature in the *in vitro* skin tissue model, and abnormally high levels of accumulated by-products of cellular degradation.
Legend: EP-epidermis; DM-dermis

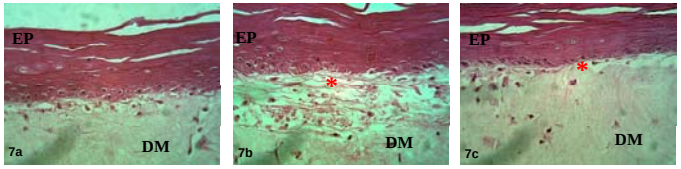
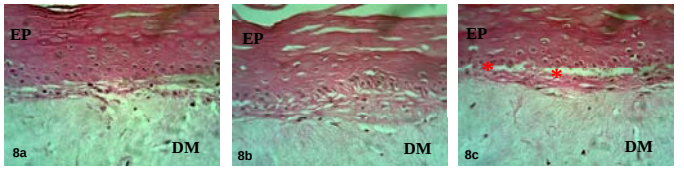


Figure 8 (below). Exposed tissue constructs treated with full system: doxycycline, EGF and vitamins. All samples in which EGF+DOXY treatment was applied had appearance similar to examples shown on **Image 8** below. Cells with pyknotic nuclei located mainly in the basal and suprabasal layers of the epidermis were evident in all specimens (**8a, b, c**). Several samples featured mild spongiosis at epidermal/dermal junction, and acantholysis of some basal cells with widening of intercellular spaces (**8a,b**). There did not appear to be any epidermal-dermal separation with complete loss of all connections between basal cells and the basement membrane zone; but focal microblister formations were present (**8c** marked by *). **Legend: EP-epidermis; DM-dermis**



Conclusions

The results presented showed that the NIMBUS-SAP enhanced dressing system significantly improved healing during the 3 day exposure period tested *in vitro* using Epiderm EFT-400 tissue model. Pronounced vesication due to the CEES injury, which manifested as epidermal-dermal separation, was mitigated by application of NIMBUS-SAP treatments. These results have warranted progress to an animal exposure model to substantiate these results *in vivo*. Animal tests involve a well characterized weanling pig model, developed by and tested at Battelle Memorial Institute.

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