KERATIN BIOMATERIAL TREATMENTS FOR BURN INJURY AND
MECHANISMS OF TISSUE SURVIVAL

BY

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DEDICATION

Dedicated to

Daddy & Amma
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ABBREVIATIONS

µm: Micrometer

ACUC: Animal Care and Use Committee

APC: Activated protein C

cm: Centimeter

DE: Dermal-epidermal

DI: Deionized water

DoD: Department of Defense

EGF: Epidermal growth factor

FGF2: Fibroblast growth factor 2

GM-CSF: Granulocyte monocyte colony stimulating factor

H&E: Hematoxylin and Eosin

h: Hour

H2O: Water

HBSS: Hanks balanced salt solution

hr: hour

IPA: Isopropyl alcohol
KAP: Keratin associated proteins

KDa: Kilo dalton

kg: Kilograms

KOS: Keratose

mcg/h: Micrograms per hour

mg: Milligrams

min: Minutes

MKH: Modified keratose hydrogel

mL: Milliliters

MRad: Mega radiation absorbed dose

ºC: degrees celsius

PBS: Phosphate buffer saline

PCR: Polymerase chain reaction

PEG: Polyethylene glycol

RCF: Relative centrifugal force

rpm: Revolutions per minute

r-TPA: Recombinant tissue type plasminogen
s: Seconds

SD: Standard Deviation

sec: Second

SIRC: Statens Serum Institut Rabbit Cornea

SSD: Silver sulfadiazine

TBSA: Total body surface area

TCP: Tissue culture plastic

w/v: weight/volume

WFIRM: Wake Forest Institute for Regenerative Medicine

WFU IACUC: Wake Forest University Institutional Animal Care and Use Committee
ABSTRACT
Deepika Rani Poranki

KERATIN BIOMATERIAL TREATMENTS FOR BURN INJURY AND MECHANISMS OF TISSUE SURVIVAL

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Thermal burns typically display an injury pattern dictated by the transfer of the thermal energy into the skin and underlying tissues. There are often three zones of injury represented by a necrotic zone of disrupted cells and tissue, an intermediate zone of injured and dying cells, and a distant zone of stressed cells that will recover with proper treatment. There are currently no approved clinical therapies that target the zone of stasis and can reduce the need for excision and grafting by salvaging potentially viable cells and tissue and thereby contributing to spontaneous healing. In this project, we endeavored to investigate the potential of keratin biomaterials to mediate such healing. Pilot studies testing treatment of chemical (mouse model) and thermal (swine model) burns showed that a native mixture (termed “crude keratose” throughout this thesis) of keratose was able to promote wound healing by stabilizing the size of the burn, suggesting that cell survival was being facilitated in the zone of stasis. These results led us to hypothesize that the interaction of the keratose biomaterial with cells and tissue promoted survival and reduced the total body surface area burned. As crude keratose is a heterogeneous mixture of proteins representing alpha and gamma protein fractions, we determined the differential activity on cell survival associated with these fractions by using an in vitro thermal stress model and showed that gamma keratose contributed to cell survival while alpha keratose did not. In additional studies, the specific mechanism of cell survival associated with the gamma keratose fraction was investigated. These results showed that gamma keratose was able to downregulate the genes involved in cell death pathways. Later, an in vivo porcine deep partial thickness burn model was developed to study the wound healing properties of a modified keratose hydrogel (MKH) that contained a reduced amount of gamma keratose, reflecting the same concentrations used in in vitro thermal stress model. The MKH showed a faster re-epithelialization rate and wound closure compared to other treatments. Finally, to investigate the potential clinical relevance to actual burn treatment, a delayed treatment thermal burn study was performed. These results showed that MKH was able to promote faster wound healing compared to other treatments but not with the same efficacy compared to more immediate post-burn treatment. This work suggested the potential benefit of using keratin biomaterial in burn therapy and provided informative data for developing second generation keratin biomaterial treatments.
CHAPTER 1

INTRODUCTION
Introduction:

Skin is the largest organ of the body and acts primarily as a homeostatic tissue maintaining body temperature and fluid balance. There are two main layers of the skin: epidermis and dermis. Cells in the basal layer of the epidermis give rise to proliferating keratinocytes that differentiate as they move up the strata [1, 2]. As they reach the surface of the skin, they undergo programmed cell death [3] and these flattened, enucleated cells form the outer stratum corneum, a heavily keratinized layer of highly cross-linked proteins that provide a structural barrier to keep pathogens out and fluid in. This entire process is relatively fast and the epidermis turns over every 2 weeks. The dermis consists of blood vessels, nerves, hair follicles, smooth muscle, lymphatic tissue, and elastin fibers, along with loose connective tissue collagen. Fibroblasts, adipocytes and macrophages are the main cell types found in dermis. The renewing property of the epidermis helps in burn healing or any other skin injury.

Burns:

Burns are defined as injuries caused by heat, friction, electricity, radiation or chemicals. They are classified into first degree (1°), second degree (2°), third degree (3°) and fourth degree (4°) depending on the depth of the burn. First degree burns mainly involve the epidermal layer of the skin. Second degree burns are further divided to superficial or deep partial thickness burns. Superficial partial thickness burns do not extend entirely through the dermis and leave behind epithelial-lined dermal appendages including sweat glands, hair follicles and sebaceous glands. Deep partial thickness burns extend to the lower layers of dermis and usually heal in 3-9 weeks. Third degree burns are full thickness burns that involve all layers of the dermis and subcutaneous
adipose tissue. Fourth degree burns involve all the layers of dermis, subcutaneous tissue and the underlying fascia or muscle.

According to Douglas Mac G Jackson, intensity of the burn is characterized by 3 concentric zones – the Zones of hyperemia, stasis and coagulation [4] (Figure 1). The outer zone of hyperemia is characterized by the presence of blood circulation and active metabolism. The intermediate zone of stasis is where the blood circulation is not reduced but metabolism is diminished. If proper treatment is provided, the viable cells in this zone will survive. The central zone of coagulation is the zone where injury occurred and is characterized by complete destruction of the blood vessels. The cells in this zone are completely necrosed [4].

**Figure 1: Three Zones in Burn Wounds:** The central zone of coagulation is where injury occurred and cells are completely necrosed, intermediate zone of stasis is where the blood circulation is not reduced but metabolism is diminished and outer zone of hyperemia is characterized by the presence of blood circulation and active metabolism. (www.indiasurgeons.com)
The majority of burn wounds are healed either by re-epithelialization, scar formation, contraction, skin grafting, or combinations thereof. Re-epithelialization occurs mainly in first and second degree burns where remnants of epithelium are present. Epithelial cells migrate from the wound edges and regions around hair follicles to the wound bed and help in resurfacing the residual dermis [4-7].

In scar formation, collagen deposition occurs in the wounded region. Scarring can be divided into 3 phases – 1) Inflammatory phase where high number of inflammatory cells are present in the wounded region, 2) Proliferative/collagen phase where there is rapid increase in the collagen content, and 3) Maturation phase where the tensile strength of the skin increases but there is no increase in the collagen content [8]. Contraction of the wound is mainly due to myofibroblasts that express α-actin. The last form of healing is skin grafting which can be divided into 3 phases – 1) Phase of imbibition where skin absorbs nutrients from the wound bed, 2) Phase of neovascularization where blood vessels invade the graft, and 3) Phase of maturation where collagen bridges are formed between the wound bed and the graft [9].

Some burns are superficial at presentation and do not require extensive treatment and re-epithelialize on their own, while some are full thickness burns and are best treated with excision and grafting. However, many burns are indeterminate with mixed burn depth which may require grafting. A typical protocol for burn care is to allow the wound to re-epithelialize if it can occur by itself, preventing further tissue injury and avoiding infection of the wounded area.

As a standard of care, the patient is topically treated with antimicrobial agents like silver sulfadiazine cream or silver impregnated dressings [10]. However, silver sulfadiazine has been shown to inhibit re-epithelialization [11, 12]. In burns that are superficial and re-epithelializing, other antimicrobial agents like topical antibiotic creams are preferred [13].
Skin grafts are used in treating partial thickness and full thickness burns. **Autografts** are the best skin grafts and are harvested from appropriate areas of the patient’s unburned skin. When large areas of the patient are burned, harvesting sufficient autograft can be a major challenge, particularly when unburned areas are inaccessible or undesirable (e.g. soles of the feet or palms). In these cases there are temporary skin substitutes or permanent skin substitutes available in the market.

**Temporary skin substitutes** provide transient physiologic wound coverage, help to control pain and can absorb modest amounts of wound exudate. These include human allograft, human amnion, xenografts and synthetic membranes. These are usually applied on a cleaned, debrided wound. Other than human allograft, temporary skin substitutes do not vascularize but provide effective temporary wound coverage and control pain. **Human allograft** is the most often used temporary biological dressing. Allograft can either be viable with live cells (keratinocytes, fibroblasts, endothelial cells and langerhans cells) or non-viable without any live cells and usually is glycerolized or gamma irradiated, freeze dried or ethylene oxide treated [14-16]. **Human amnion** is derived from the amniotic membrane. It acts as a temporary dressing for clean wounds like partial thickness burns, donor sites or full thickness wounds [17-19]. One of the main drawbacks of amnion is the difficulty of screening for viral diseases. **Xenograft** is porcine derived and is used as temporary coverage for clean superficial second degree burns and donor sites [20]. **Synthetic membranes** are semi-permeable membrane dressings that provide a barrier for bacteria, vapor and also control pain until the underlying wound heals [21, 22]. Biobrane is a two layer nylon mesh that helps in fibrovascular in-growth and has an outer silastic layer that act as vapor and bacterial barriers. One type of hydrocolloid dressings is a three layered structure that has a porous inner layer, absorbent methyl cellulose middle layer, and semi
permeable outer layer. This provides a moist environment for the wound to heal and absorbs wound exudate [23, 24].

Permanent skin substitutes are also available but are not ideal. Epicel, Integra and Alloderm are marketed as permanent skin substitutes because some or all of their components become integrated into the healing wound. **Epicel** is cultured epidermal autograft. Epithelial cells are isolated from a small skin biopsy and are cultured in vitro until there are enough cells to form a dressing of useful size [25, 26]. These epithelial cell sheets are attached to petroleum coated gauze which acts as a carrier and then applied on burn wounds [27]. **Integra** is the first dermal substitute approved by the US Food and Drug Administration. Integra is also known as artificial skin [28]. It contains an inner bovine collagen fibrous layer and outer silicone layer with vapor transmission characteristics similar to normal epithelium. Once full thickness wounds are covered with Integra and become vascularized, the outer silicone layer is replaced with thin epithelial autograft 2-3 weeks later [29]. **Alloderm** is an acellular human dermal allograft. It is devoid of epidermis and must be covered by a split thickness autograft at the time of initial operation. It helps in reducing post operative scarring [30, 31].

**Keratin Biomaterial:**

Keratins are intermediate filament proteins that provide mechanical stability and integrity for epithelial cells and tissues [32]. Mammalian keratins are classified into two groups – hard keratins and soft keratins based on their structure, function and regulation. Hard or trichocytic keratins, have high sulfur content and are found in a number of external appendages such as wool and hair fibers. These have ordered arrays of intermediate filaments embedded in a matrix of cysteine rich proteins (Figure 1). Soft or cyto keratins, are characteristic of epithelial cells with low sulfur content [33-36]. Hair keratins and cyto keratins consist of obligate heterodimers:
type I and type II proteins with non-helical N-terminal and C-terminal domains and a central coiled rod domain. Type I and II chains differ in their molecular weight [36].

The hair follicle is a remarkably proliferative and regenerative organelle. To produce new hair, the follicle undergoes a series of cycles – anagen (growth), catagen (regression) and telogen (rest) [37]. During anagen follicles produce the entire hair shaft. During catagen and telogen, stem cells are activated; the lower cycling portion of the hair follicle regresses in growth and is in a resting phase. Stem cells activated in this phase trigger the new hair growth cycle again. More than 30 growth factors and cytokines are involved in the hair cycling process, and have also been shown to play a role in the regeneration of other tissues [38].

Figure 2: Schematic diagram of Wool fiber: Human hair has a similar superstructure to wool fiber with 80% alpha- to 20% gamma-keratin. Alpha-keratins are the high molecular weight (40-60kDa), low sulfur containing proteins that are mainly composed of the right-handed, alpha-helical proteins. Gamma-keratins are the low molecular weight (10-25kDa), high sulfur

Hair fiber consists of 3 components – cuticle, cortex and medulla (Figure 2). Cuticle is the thin outer surface of the fiber and consists of β-keratins that help to protect the hair fiber from external physical and chemical damage. Cortex is composed of spindle shaped cells with keratin filaments contained within them. Type I (acidic) and Type II (basic to neutral) intermediate filament (IF) keratins form coiled coil obligatory heterodimers through the interaction of their α-helical rod domains [39]. Two dimers assemble in a staggered anti-parallel fashion to make a tetramer. Two tetramers form an octamer and four octamers join to form a cylindrical unit length filament (ULF). Individual ULF join to form short filaments which fuse end to end followed by a compaction phase that leads to the formation of 10nm diameter IF [40]. The hair keratins have high sulfur content and are further reinforced by highly cross linked matrix proteins called keratin associated protein (KAPs) [32, 41]. Medulla is present occasionally in the center of the hair fiber. The many different structures and keratin sub-types lead to the complexity of the hard keratin protein family.

**Keratins Classification:** Keratins constitute two classes of the intermediate filament (IF) family proteins – type I acidic keratins and type II basic keratins [42, 43]. Type I keratins are smaller in size with acidic isoelectric point (pl) and type II keratins are larger in size with neutral to slightly basic pl. Genome analyses demonstrated that there are a total of 54 functional keratin genes in humans – 28 type I keratins on chr 17q21.2 and 26 type II keratins on chr 12q13.13 [44-46]. In 2006, Schweizer et al., published a new consensus nomenclature for mammalian keratins
by dividing the keratins into 3 categories: 1) epithelial keratins/genes 2) hair keratins/genes, and 3) keratin pseudogenes.

The cortical proteins in the hair fiber can be divided into two groups – a cystine poor fraction called α-keratin and cystine rich fraction called γ-keratins. The α-keratins are the main structural component of the hair with average molecular weight 40-60KDa [47]. In the terminology of past wool literature, the cystine rich fraction is termed γ-keratins with a molecular weight range of 10-25KDa [47]. However, more recent publications state that the cysteine rich fraction is more appropriately termed keratin associated proteins (KAP) and that these matrix proteins in human hair are coded by 85 genes [41]. To add to the nomenclature confusion, γ-keratin is often used to describe an acid-soluble fraction isolated after extraction of oxidized keratin proteins, or so-called “keratorese”. KAP can account for as much as 20-30% of hair fiber proteins. The α-keratins assemble together to form keratin intermediate filaments (KIF’s) that impart toughness to the fiber. The matrix proteins functions as disulfide cross linkers that hold the KIF’s together. In humans, there are total 17 hair keratin genes – 11 type I and 6 type II [46] and more than 85 KAP genes [41].

Using chemical methods, soluble keratin proteins are extracted from the hair fiber by breaking the disulfide crosslinks. If an oxidant is used cystine is converted to cysteic acid derivatives and the product is termed “keratorese”, and if a reductant is used cystine is converted to cysteine and the product is termed “kerateines”. Keratoreses are hygroscopic, non-disulfide cross linkable, water soluble, and susceptible to hydrolytic degradation at extreme of pH due to polarization of the backbone caused by the electron withdrawing properties of cysteic acid. These characteristics cause relatively quick degradation (days to weeks) of keratoreses in vivo.
Kerateines are less polar, more stable at extremes of pH and can be re-crosslinked through oxidative coupling of cysteine groups. These can persist in vivo for weeks to months.

**Hydrogel Treatments for Burns:**

A burn dressing should serve three principle functions: comfort, metabolic and protective [48]. They act as a protective barrier, absorb excess wound exudate, and maintain a moist environment, which in turn helps in pain reduction. A moist wound environment is known to accelerate wound healing [49-51], and there is abundant literature on various hydrogel treatments. Collagen and chitosan hydrogels, and a combination of these two with other hydrogels like alginate or dextran are the commonly used treatments. Collagen sheets were introduced commercially in the 1960’s for the covering of large excised wound areas[52]. Research and variations soon followed, with one study showing that modified collagen membrane was permeable to topical antibiotics with no significant antigenicity and was superior to homograft and heterograft in autograft uptake [53]. In vitro experiments have shown that human keratinocytes were able to grow extensively on human dermal collagen and differentiate within a few days into columnar conformation [54]. Porous collagen sponges were able to provide three dimensional matrix for tissue infiltration in vivo and also cell growth in vitro [55]. Collagen sheets were shown to promote proliferation and attachment of neonatal rat keratinocytes in vitro and promote faster re-epithelialization when these sheets were used to cover full thickness burns on the dorsum of rats [56]. More recently, collagen hydrogel has been used synergistically with other hydrogel systems in the treatment of burns. Collagen and chitosan porous scaffolds cross linked with glutaraldehyde have shown an increase in proliferation in vitro, and when tested in animals have shown that the scaffold was able to support and accelerate
fibroblast cell infiltration from surrounding tissue, suggesting its use as a potential dermal equivalent [57]. Chitosan –collagen hydrogel incorporated with lysostaphin was shown to control methicillin-resistant *Staphylococcus aureus* infection in third degree burn patients and promoted healing [58].

Chitosan is a de-acetylated derivative of chitin that has been shown to facilitate burn wound healing in vivo [59-67] and cell adhesion and viability in vitro in primary rat fibroblast cells [59]. Full thickness burn wounds that were treated with high molecular weight, high de-acetylated chitosan showed faster re-epithelialization and wound closure in rats compared to 2% Fucidin treatment [68]. Chitosan hydrogel was also used as a carrier for biopharmaceuticals, antimicrobials and growth factors. A chitosan gel formulation with epidermal growth factor (EGF) was shown to significantly increase cell proliferation and had a faster re-epithelialization rate in second degree burns in rats [60, 67]. It was also used as a fibroblast growth factor 2 (FGF2) carrier that induced angiogenesis and collateral blood circulation in impaired diabetic mice [69]. In Wistar-Albino rats, Chitosan gels with controlled slow release granulocyte-monocyte colony stimulating factor (GM-CSF) assisted burn wound healing with better collagen fibril organization [70]. Chitosan acetate bandages were shown to control the growth of *Pseudomonas aeruginosa* and *Pseudomonas mirabilis* bacteria, and control the development of systemic sepsis in third degree burns in mice [64]. A hydrogel sheet composed of alginate, chitosan and fucoidan was shown to promote wound healing in healing impaired rats [71]. Dextran hydrogel scaffolds were shown to promote early inflammatory cell infiltration in full thickness burns, which led to the degradation of the scaffold and in turn promoted the infiltration of endothelial cells into the wounded region. This enhanced neo-vascularization and restoration of hair fibers and epidermal morphology similar to normal mouse skin [72].
Alginates are polysaccharides derived from seaweed and there are a wide variety available commercially that have been shown to promote wound healing [73-78]. Alginate dressings are typically made from calcium alginate, which has cytotoxicity and immune reactions associated with it. [79]. Development of alginate dressing with less calcium ions reduced the cytotoxicity and foreign body reaction in both in vitro and in vivo experiments [79, 80]. Hydrocolloid dressings are used in partial thickness and small burns where the wound exudate is comparatively less [81, 82]. Compared to hydrocolloid dressings, alginate dressings remain gelled longer as the presence of calcium ion crosslinks decreases the biodegradability of alginate gels [83]. The antimicrobial properties of the above mentioned alginate dressings have been increased by the addition of silver ions [84-90].

**Cell Death Pathways:**

In burns, one of the main reasons for the increase in total body surface area (TBSA) burned is the increase in cell death after the thermal insult. Apoptosis and Necrosis are the two main modes of cell death that occur when the tissues are exposed to heat. There are two apoptotic pathways – extrinsic or death receptor pathway, and intrinsic or mitochondrial pathway (Figure 3). In both these pathways, cysteine aspartyl-specific proteases (caspases) are activated, which further leads to the morphological and biochemical changes associated with apoptosis. The death receptors usually belong to the tumor necrosis factor super family, comprising a subfamily with a characteristic intracellular death domain [91]. When the ligands of the tissue necrosis factor (TNF) family bind to their respective death receptors such as CD-95 and TRAIL-R1, they are activated and attract intracellular Fas-associated death domain (FADD), which further recruits caspases [92]. Caspase 8 and 10 function as initiator caspases that are recruited to the death inducing signaling complex (DISC) [93]. The DISC cleaves the pro-caspase 8 and 10
and yield active initiator caspases 8 and 10 [94]. Active caspase 8 cleaves BH3 interacting domain (BID), a BCL2 family protein which translocates to mitochondria and initiates the intrinsic pathway [95, 96]. Activation of mitochondria leads to the release of Cyt C into the cytosol, which binds to apoptotic protease activating factor 1 (APAF1) to form the apoptosome. Apoptosome, along with TP, cleave procaspase 9 and activate it. Any of the BCL2 family proteins: BAD, BID, BIM can activate the intrinsic pathway of apoptosis. Once the initiator caspases are activated, they cleave and activate executioner caspases 3, 6 and 7, which cleave the cellular substrates downstream [92]. Once the phosphatudylerine are exposed on the cell membrane, the remains of the cell are engulfed by phagocytosis [97].
Figure 3: Apoptotic Pathways: The 2 major pathways of apoptosis: the extrinsic (Fas and other TNFR superfamily members and ligands) and the intrinsic (mitochondria-associated) pathways. Both pathways lead to activation of caspase-3, giving rise to apoptotic cell death. (Used with permission from Seminars from Arthritis and Rheumatism, Schultz DZ, Apoptosis: Programmed cell death at a molecular level. Semin Arthritis Rheum. 2003 Jun;32(6):345-69)

There are some other cell death pathways that are not well defined and do not require caspase activation [98-100]. Apoptotic pathways can be regulated at the initial receptor level, by inhibiting caspase activation, or by influencing the permeability of the mitochondrial membrane. Receptor level regulation is performed by FLIPs (FADD-like interleukin-1 β-converting enzyme-like protease (FLICE/caspase-8)-inhibitory proteins) [101]. The members of BCL2 family proteins regulate apoptosis at the mitochondrial level and may be either pro-apoptotic or anti-apoptotic [95, 96]. Inhibitor of Apoptotic Proteins (IAPs) bind and inhibit activation of caspases [102]. IAPs are inhibited by SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pI), which is released from mitochondria along with cyt c [103, 104].

Necrosis used to be considered a disorganized mode of cell death, especially when the cells are exposed to severe physiochemical conditions. But recently it was shown that necrosis occurs during normal physiological development [105, 106]. There are published in vitro studies showing that even though apoptosis is completely inhibited, cell death still occurs and is usually independent of caspases, following a specific signaling pathway where the cell morphology looks more like necrotic cell death [107-111]. FADD (Fas-associated death domain) plays a crucial role in propagating apoptotic or necrotic signals as it has both a death domain that can trigger necrosis and a death effector domain that can propagate apoptosis [112, 113]. When
caspase activation is impeded, FADD is not recruited when the TNF ligand binds to the death receptor TRAIL-R1 [114-116]. Instead, a complex 1 is formed at the plasma membrane consisting of TNF-R1, TRAF2 and RIP1 that activates NF-κB and MAPKs [117, 118]. After receptor endocytosis, a second complex with TRADD that recruits FADD and procaspase 8 is formed [119, 120]. If complex 1 is not able to induce sufficient anti-apoptotic proteins, then caspase 8 is activated leading to caspase induced apoptosis [121]. If the anti-apoptotic proteins block the caspases, then necrosis occurs through the caspase independent pathway [109]. Holler et al. have shown that RIP is required for necrotic cell death induced by TNF and TRAIL, and that it has its own kinase activity that activates the cell death pathway when phosphorylated [122]. In Rip null (Rip−/−) mouse fibroblast cells, it is shown that RIP is required for TNF-induced activation of the MAPKs extracellular-signal-related kinase (ERK), p38 and c-Jun amino-terminal kinase (JNK) [123].

Autophagy refers to a catabolic process involving degradation of the cell’s own components and is usually activated by oxidative, nutritional or toxic stresses. Macroautophagy is an evolutionarily conserved, genetically controlled multi-step process in which intracellular organelles are sequestered within characteristic double or multi-membrane autophagic vacuoles termed autophagosome, and finally delivered to lysosomes for bulk degradation [124] (Figure 4). Embryonic fibroblasts from double knock out Bax−/− Bak−/− mice are resistant to apoptotic inducers. When treated with etoposide, the cells failed to undergo apoptosis and instead manifested a massive autophagy followed by cell death [125]. But knocking down the autophagy related gene (Atg) products beclin-1 and Atg5 reduced etoposide induced cell death [125]. Using other apoptotic inhibitors did not trigger the autophagy cell death pathway, indicating that Bax and Bak absence played an important role in autophagy activation [125]. In other cell types like
L929 mouse fibrosarcome, Human Jurkat T lymphoma and U937 monocytoid cells, inhibition of cystein proteases (caspases) was shown to induce autophagy [126, 127]. In stress conditions like nutrition depletion or loss of growth factors, autophagy gets activated by the inhibition of apoptosis and promotes cell survival [128, 129]. If autophagy is inhibited in cell stress conditions then the cells usually die through apoptosis. This cell death can be postponed by inhibiting BAX or BAK or caspases [130]. In vitro and in vivo experiments have shown induced apoptotic cell death by the inactivation of Atg genes – Atg 5 or 7, beclin-1[131-134].

**Figure 4: Autophagy and its inhibitors:** Autophagy starts with the stepwise engulfment of cytoplasmic material (cytosol and/or organelles) by the phagophore (also called isolation membrane), which sequesters material in double-membraned vesicles named autophagosomes (also called autophagic vacuoles). There are 4 regulatory process: (1) De-repression of the
mTOR Ser/Thr kinase (2) Activation of mammalian Vps34 in the initial steps of vesicle nucleation (3) Two ubiquitin-like conjugation systems that are part of the vesicle elongation process (4) Autophagosomes maturation by the fusion with lysosomes to create autolysosomes. (Used with permission from Nature Publishing Group, Maiuri MC, Zalckvar E, Kimchi A, Kroemer G Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007 Sep;8(9):741-52)

Bcl2 and Bcl-XL are regulators of beclin-1. Lamp2, lysosome-associated membrane glycoprotein-2. Autophagy may develop as a primary response to stress stimuli, which then triggers either apoptosis or necrosis [135, 136]. There is crosstalk between all the cell death pathways, apoptosis, necrosis and autophagy, depending on the external and internal conditions to which the cells are exposed.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-95</td>
<td>Fas ligand</td>
<td>Cytokine that binds to TNFRSF6/FAS, a receptor that transduces the apoptotic signal into cells.</td>
</tr>
<tr>
<td>TRAIL-r1</td>
<td>tumor necrosis factor receptor superfamily</td>
<td>Receptor for the cytotoxic ligand TNFSF10/TRAIL.</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
<td>Apoptotic adaptor molecule that recruits caspase-8 or caspase-10 to the activated Fas (CD95) or TNFR-1 receptors.</td>
</tr>
<tr>
<td>BID</td>
<td>BH3-interacting domain death agonist</td>
<td>This gene encodes a death agonist that heterodimerizes with either agonist BAX or antagonist BCL2.</td>
</tr>
<tr>
<td>Cyt C</td>
<td>Cytochrome C</td>
<td>Electron carrier protein. Plays a role in apoptosis. Suppression of the anti-apoptotic members or activation of the pro-apoptotic members of the Bcl-2 family leads to altered mitochondrial membrane permeability resulting in release of cytochrome c into the cytosol. Binding of cytochrome c to Apaf-1 triggers the activation of caspase-9, which then accelerates apoptosis by activating other caspases</td>
</tr>
<tr>
<td>APAF1</td>
<td>Apoptotic protease activating factor 1</td>
<td>This gene encodes a cytoplasmic protein that initiates apoptosis. This protein contains several copies of the WD-40 (beta transducin)domain, a caspase recruitment domain (CARD), and an ATPase domain (NB-ARC).</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL2-associated agonist of cell death</td>
<td>Promotes cell death. Successfully competes for the binding to Bcl-X(L), Bcl-2 and Bcl-W, thereby affecting the level of heterodimerization of these proteins with BAX. Can reverse the death repressor activity of Bcl-X(L), but not that of Bcl-2 (By similarity). Appears to act as a link between growth factor receptor signaling and the apoptotic pathways</td>
</tr>
<tr>
<td>BIM</td>
<td>BCL2-like 11</td>
<td>Induces apoptosis.</td>
</tr>
<tr>
<td>Caspases 3,6,7,8,10</td>
<td>cysteiny1 aspartate proteases</td>
<td>Caspases are involved in the signal transduction pathways of apoptosis, necrosis and inflammation. Initiator caspases include Caspases 1,-4,-5,-8,-9,-10,-11,-12 and effector caspases include Caspase -3,-6,-7</td>
</tr>
<tr>
<td>FLIP</td>
<td>FADD-like interleukin-1 - converting enzyme-like protease (FLICE/caspase-8)- inhibitory proteins</td>
<td>Apoptosis regulator protein which functions as a crucial link between cell survival and cell death pathways in mammalian cells.</td>
</tr>
<tr>
<td>------</td>
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</tr>
<tr>
<td>SMAC, DIABLO</td>
<td>second mitochondria-derived activator of caspase/direct IAP binding protein with low pI</td>
<td>Promotes apoptosis by activating caspases in the cytochrome c/Apaf-1/caspase-9 pathway.</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNF receptor-associated factor 2</td>
<td>Regulates activation of NF-kappa-B and JNK and plays a central role in the regulation of cell survival and apoptosis.</td>
</tr>
<tr>
<td>RIP1</td>
<td>receptor (TNFRSF)-interacting serine-threonine kinase</td>
<td>Serine-threonine kinase which transduces inflammatory and cell-death signals (necroptosis) following death receptors ligation.</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
<td>NF-kappaB (nuclear factor-kappa B) is a rapidly acting primary transcription factor involved in cellular responses to stimuli such as cytokines and stress and plays a key role in regulating the immune response to infection.</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFRSF1A-associated via death domain</td>
<td>Adapter molecule for TNFRSF1A/TNFR1 that specifically associates with the cytoplasmic domain of activated TNFRSF1A/TNFR1 mediating its interaction with FADD. Overexpression of TRADD leads to two major TNF-induced responses, apoptosis and activation of NF-kappa-B</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
<td>Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR.</td>
</tr>
<tr>
<td>ERK</td>
<td>MAPKs extracellular-signal-related kinase</td>
<td>Involved in both the initiation and regulation of meiosis, mitosis, and postmitotic functions in differentiated cells by phosphorylating of transcription factors</td>
</tr>
<tr>
<td>P38, JNK, MAPK9</td>
<td>p38, c-Jun amino terminal kinase, Mitogen activated protein kinase</td>
<td>Three major MAPKs include ERKs (Extracellular signal-Regulated Kinases), JNKs (c-Jun NH(2)-terminal protein Kinases), and p38 Kinases. These are activated by environmental stresses and inflammatory cytokines</td>
</tr>
<tr>
<td>Beclin-1</td>
<td>autophagy-related</td>
<td>Plays a central role in autophagy.</td>
</tr>
<tr>
<td><strong>Gene</strong></td>
<td><strong>Description</strong></td>
<td><strong>Function</strong></td>
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<tr>
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</tr>
<tr>
<td>ATG5</td>
<td>autophagy related 5 homolog</td>
<td>Required for autophagy. Its expression is a relatively late event in the apoptotic process, occurring downstream of caspase activity.</td>
</tr>
<tr>
<td>ATG7</td>
<td>autophagy related 7 homolog</td>
<td>Functions as an E1 enzyme essential for multi substrates such as ATG8-like proteins and ATG12.</td>
</tr>
<tr>
<td>BAX, BAK1</td>
<td>Bcl-2-associated X protein</td>
<td>Accelerates programmed cell death by binding to, and antagonizing the apoptosis repressor BCL2.</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>B-cell lymphoma-extra large</td>
<td>Positive regulator of apoptosis</td>
</tr>
</tbody>
</table>
Rationale for the hypothesis that the interaction of keratin biomaterial with burned tissue promotes cell survival and will reduce total body surface area (TBSA) burned:

Previous work in our group has focused on testing the wound healing capacities of keratin biomaterials in animals. Two wound healing burn studies were performed – a mouse chemical burn study and a swine thermal burn study. Both of the animal studies have shown that keratin biomaterials promote tissue salvage after burn injury and speed healing. Generally, hydrogel treatments are able to provide a moist environment, help control exudation of wound fluid, which in turn reduces pain. As previously discussed, there are many hydrogel treatments available for burn patients, but none of those treatments were able to show that they facilitate cell survival and tissue salvage by any of the aforementioned pathways. From our previous wound healing studies, it was shown that a hydrogel made from a “crude” preparation of keratose (i.e. not otherwise refined or purified) was able to reduce TBSA burned and accelerate wound healing. As crude keratose is a heterogeneous mixture of alpha, gamma and KAPs, we hypothesized that separating the different fractions and testing their cell salvage abilities would be helpful in the development of better keratin formulations for the treatment of burns. To test this hypothesis, three specific aims were undertaken and form the basis of this thesis project:

**Specific Aim 1:** To develop an in vitro thermal injury model that mimics the Jackson model [4] of burn injury and determine the effect of keratin subtype on cell survival and recovery from thermal injury.

**Specific Aim 2:** To understand the molecular mechanism by which keratose biomaterial promotes cell survival.

**Specific Aim 3:** To test the wound repair capabilities of meta-keratose in a partial thickness pig burn injury model.
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CHAPTER 2

A KERATIN BIOMATERIAL PROMOTES SKIN REGENERATION AFTER BURNS IN VIVO AND RESCUES CELLS AFTER THERMAL STRESS IN VITRO

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The following manuscript has not yet been submitted for publication. Deepika Poranki prepared the manuscript. Dr. Mark Van Dyke acted in an advisory and editorial capacity.
Abstract:

Thermal burns typically display an injury pattern dictated by the transfer of the thermal energy into the skin and underlying tissues. There are often three zones of injury represented by a necrotic zone of disrupted cells and tissue, an intermediate zone of injured and dying cells, and a distant zone of stressed cells that will recover with proper treatment. Burn treatment is directed toward removal of clearly necrotic skin and therapeutic support for remaining tissue that may spontaneously heal without complication. Excised areas of the wound then require grafting. Decisions regarding when and how much damaged tissue to excise are largely determined empirically, and there is substantial debate about the relative merits of early or late excision and grafting. Early grafting carries the risk of removing too little tissue, as cells in the intermediate zone continue to die up to 10 days after injury. Late excision can expose the patient to many inflammatory mediators produced by the dead and dying tissue. Strategies to facilitate the survival of cells and tissue in the intermediate zone of burn injury may be capable of mediating spontaneous healing, or reducing the overall extent of excision and grafting, but to date no such therapies are available for clinical use.

The wound healing capabilities of a keratin biomaterial hydrogel were studied in two pilot studies, one using a chemical burn model in mice and the other a thermal burn model in swine. In both studies, an oxidized form of keratin biomaterial called “keratose” was shown to prevent enlargement of the initial wound area and promote faster wound closure. This keratose hydrogel was provided as a heterogeneous mixture of proteins representing an “alpha” fraction and a “gamma” fraction. These different fractions of keratin have disparate properties including molecular weights, amino acid content, and solubility characteristics. They can be easily
separated by techniques such as dialysis or isoelectric precipitation. To determine if there was a
differential wound healing activity associated with these two keratose fractions, an in vitro heat
stress model mimicking the different zones of burn injury was developed. Treating thermally
stressed dermal fibroblast with growth media containing alpha, gamma or the native mixture
(termed “crude keratose” throughout this manuscript) showed that gamma keratose was able to
maintain cell viability and promote cell proliferation. By the third day of treatment, the cell
viability for gamma keratose-treated cells was 125% ± 15.6, whereas with other treatments the
cell viability was 65% ± 20.7 (crude), 46% ± 32.5 (alpha) and 37% ± 20 (fibroblast media).

Electrophoretic separation of the gamma keratose fraction revealed sub-fractions of
different molecular weights ranging from 110kDa to 8kDa. Mass spectrometry analysis
identified keratins 81, 83, 85, 86, 31, 33A, 33B, 34 in the excised bands. Epithelial keratins 1 and
10 were identified in the excised bands with molecular weights ~60 kDa and ~8 kDa,
respectively. Keratins 81, 83, 85, 86, 31, 33A, 33B and 34 were also identified with lower than
expected molecular weights, suggesting that these were degraded alpha keratose peptides.
Keratin-associated protein 19-5, galectin-7, calmodulin-like protein 3, thioredoxin, and cystatin
A were also identified in the ~8 kDa excised band.

These results suggest that the gamma fraction of keratose is comprised essentially of
degraded alpha keratose proteins, and that these peptides may facilitate the rescue of cells from
thermal injury. Treatment of burns with gamma keratose may therefore represent a potential
therapy for wounds with an intermediate zone of damaged tissue that has the potential to
contribute to spontaneous healing.
Keywords: keratin, biomaterial, burn, wound, heat shock, hydrogel, keratose, alpha keratin, gamma keratin, proteomics, mass spectrometry
1. Introduction:

Burn wound repair is a complex process with a series of overlapping phases: Inflammation; matrix deposition; and tissue remodeling. The duration of these individual phases varies depending on the intensity and depth of the burn. Most burn dressing treatments aim to facilitate these stages of wound healing by providing a moist environment, control excessive exudate buildup, and protect against infection that would perturb normal healing. It has been suggested that a burn dressing should serve three principle functions: Provide comfort by protecting the wound surface from external air currents; absorb wound secretions and reduce water loss from exposed burns; and protect from microorganisms [1]. In recent years, hydrogel wound dressings have drawn increased attention as they serve all of these purposes. They act as a protective barrier, absorb excess wound exudate, and maintain a moist environment, which in turn helps in pain reduction. A moist wound environment is known to accelerate wound healing [2-4].

There is abundant literature on various hydrogel treatments, among them chitosan, alginate, collagen and hydro-colloid dressings that have been shown to promote burn wound healing. Chitosan is a de-acetylated derivative of chitin that has been shown to promote burn wound healing in vivo [5-13]. Chitosan did not appear to have a detrimental effect on primary rat fibroblast viability in vitro [5]. In another study, full thickness burn wounds treated with high molecular weight, highly de-acetylated chitosan showed faster re-epithelialization and wound closure in rats compared to 2% Fucidin treatment. [14]. Chitosan hydrogel has also been used as a carrier for biopharmaceuticals, antimicrobials and growth factors. Chitosan gel formulation with epidermal growth factor (EGF) was shown to significantly increase cell proliferation and to
promote faster re-epithelialization in second degree burns in rats [6, 13]. It was also used as a fibroblast growth factor 2 (FGF2) carrier that induced angiogenesis and collateral blood circulation in impaired diabetic mice [15]. Another study using pluronic/chitosan hydrogels with recombinant human EGF enhanced epidermal cell differentiation in second degree burns in mice with impaired wound healing [16]. Also, chitosan gels with controlled slow release granulocyte-monocyte colony stimulating factor (GM-CSF) assisted in better collagen fibril organization in Wistar-Albino rats [17]. Chitosan acetate bandages were shown to control the growth of *Pseudomonas aeruginosa* and *Pseudomonas mirabilis* and to prevent the development of systemic sepsis in mice with third degree burns [10]. Despite these positive findings, chitosan has no apparent utility beyond providing a moist wound healing environment and a physical barrier, and typically is combined with another therapeutic to enhance its efficacy.

Wound dressings containing alginate, a polysaccharide derived from sea weed, have also been investigated. In 1987, Barnett and coworkers showed that a calcium alginate dressing was an effective hemostat and facilitated wound repair [18]. A wide variety of alginate dressings are available commercially that promote wound healing by providing a moist environment [19-24]. However, like chitosan, alginates have no intrinsic capacity for facilitating wound healing beyond their physical attributes. Moreover, a disadvantage of alginate dressings containing calcium alginate is that some cytotoxicity and immune reactions have been noted [25]. Development of alginate dressings containing less calcium has reduced the cytotoxicity and foreign body reaction in experiments in vitro and in vivo [25, 26], but there are no market-leading burn dressings based on alginate biomaterials.
Dressings containing collagen have also been used in the treatment of burns. They provide a moist wound environment and act as a barrier to microorganisms. Collagen dressings have been shown to promote faster re-epithelialization, early granulation tissue formation [27-29], and when used as a composite wound dressing along with other biomaterials or antibiotics, were shown to promote faster wound healing with a reduced rate of infection [30-32]. Lyophilized bovine collagen has been shown to help in the management of pain and faster epithelialization of skin graft donor sites [33]. However, these findings do not differ substantially than those for other hydrogel biomaterials and have no specific efficacy for burn wound healing. An additional limitation to the use of collagen dressings for burn wounds is that the environment of a burn is highly proteolytic, and protein-based dressings such as collagen can breakdown rapidly [34].

Hydrocolloid dressings are primarily used in partial thickness burns and small burns with less wound exudate [35, 36]. In their intact state hydrocolloid dressings are impermeable to water vapor and provide an effective barrier to transepidermal moisture loss, whereas in presence of wound exudate the dressings absorb the liquid and form gel, making them permeable to vapor [36]. Hydrocolloid dressings along with silver sulfadiazine have been shown to promote faster wound closure and also help in pain management [37]. The principal benefit of using these dressings for burns is pain management.

Recognizing the limited functionality of the above mentioned dressings, additional enhancements have been made by commercial vendors of these products. Prominent among them is the addition of antimicrobial properties by the addition of silver ions [37-43]. However, the underlying limitation of all of these products is that they have no particular efficacy for burn
wounds and the complex physiology of skin that has been damaged by the application of external energy (thermal and electrical) or agents (chemical). The opportunity therefore exists to develop new treatments that can specifically address the pathophysiology of burns.

Keratin biomaterial hydrogels may represent a burn-specific wound dressing technology. In addition to possessing the physical attributes for a wound dressing (facilitates a moist environment, protects the wound, absorbs excess wound fluid, etc.), keratins may facilitate the survival of thermally and chemically damaged cells. Keratins represent a class of biomaterials that have been used in a variety of biomedical applications, but the understanding of how keratin proteins function in these applications is only at its nascent stages.

The major proteins contained in human hair fibers are classified as alpha keratins [44], beta keratins and keratin-associated proteins (KAP) [45]. They can be extracted using oxidative or reductive chemical techniques, and separated using various methods into purified fractions with different molecular weights, amino acid content, and solubility characteristics [46]. Another fraction derived from the oxidative extraction of these proteins from hair fibers is often referred to in the literature as “gamma” keratin [47].

The general biocompatibility and utility of keratin biomaterials in potential medical and biotechnology applications has been demonstrated in numerous studies showing proof of principle for cell culture, wound healing, bone and nerve regeneration, drug delivery, hemostasis, corneal tissue engineering, cardiac repair, and wound healing [48-61]. Recently, keratin dressings were shown to enhance wound healing by the activation of keratinocytes in a porcine partial thickness burn model [54]. However, no data was provided on the type of keratin used in these dressings, or the underlying mechanism of wound repair. Given this body of evidence, we
hypothesized that keratose hydrogels would facilitate both chemical and thermal burn repair, and that there may be differential efficacy among the different keratose fractions present in a mixed protein hydrogel. To test this postulate, we utilized a mouse chemical burn model and a swine thermal burn model to conduct pilot studies using a hydrogel prepared from an un-purified sample of crude keratose extract. We then investigated the differential effect of alpha and gamma keratose fractions on cell survival in an in vitro model of thermal insult.

2. Materials and Methods:

2.1. Keratin Biomaterial Extraction: Keratin biomaterial was extracted from hair using an oxidative protocol described by de Guzman et al. [62]. This heterogeneous mixture of cortical proteins is referred to as crude keratose. For crude keratin hydrogel preparations, the extract solution was dialyzed against endotoxin free water using a 5kDa spiral wound cartridge (Millipore, Billerica, MA) for 5 volume washes. Finally, the solution was concentrated to minimal volume, adjusted to pH 7.4 and lyophilized.

2.2. Separation of Alpha and Gamma Keratose fractions: Crude keratose was further separated into alpha and gamma keratose fractions by isoelectric precipitation. Concentrated hydrochloric acid was added to the crude keratose solution with stirring until the pH dropped to 4.2, facilitating the precipitation of the insoluble alpha keratose. The soluble gamma keratose was separated by fixed angle centrifugation at 1,500 rpm (252 RCF) for 15min at 4°C and filtered. After neutralizing the gamma solution to pH 8.5, it was dialyzed against endotoxin free water using a 5kDa spiral wound cartridge (Millipore) for 5 volume washes. Finally, the solution was concentrated to minimal volume, adjusted to pH 7.4 and lyophilized. The precipitated alpha keratose was
reconstituted to a concentrated solution using a minimum of 0.1M sodium hydroxide and then dialyzed against endotoxin free water using a 30kDa spiral wound cartridge (Millipore) for 5 volume washes. Finally the solution was concentrated to minimal volume, adjusted to pH 7.4 and lyophilized.

2.3. Hydrogel Preparation: Crude keratin hydrogel was prepared by reconstituting the lyophilized powder at 15 percent by weight with normal saline. It was not sterilized prior to use. Chitosan (Sigma-Aldrich) was similarly prepared by reconstituting the dry powder at 9 weight percent with saline. Chitosan also was not sterilized prior to use. Gels were loaded into syringes so that they could be accurately applied to the wounds.

2.4. Mouse Chemical Burn Model: Forty eight CD1 mice were used for this study under a protocol approved by the Wake Forest University Institutional Animal Care and Use Committee (WFU IACUC). A few minutes prior to the surgery and under general anesthesia (2-3% halothane by nose cone inhalation), hair at the dorsal mid-line and below the shoulders of the mice was removed with a commercial depilatory cream. The area of exposed skin was washed with soap and water, then disinfected with iodine and cleaned with saline soaked gauze. Chemical burns were created with 90% phenol in a 1 cm x 2 cm area of skin exposed for 60 sec. Phenol was removed with copious amounts of phosphate buffered saline. Twenty minutes after creation of the phenol burns, the treatments were applied, covered with a commercial adhesive bandage, and the animals placed in a protective jacket to keep the dressings in place. Treatments included crude keratose hydrogel, chitosan hydrogel, and saline only. Dressings were changed every 2 days for 20 days. Digital pictures were taken with a ruler in the field and wound size measurements made using digital image analysis software (ImageJ, NIH, Bethesda,
Wound size values for each treatment group were averaged (e.g. n=12 on day 0, n=11 on day 2, n=10 on day 4, etc.) and reported as mean ± standard error of the mean (SEM).

2.5. Swine Thermal Burn Model: Four Yorkshire swine were used for this study under a protocol approved by the WFU IACUC. Anesthesia was introduced intramuscularly with ketamine (20mg/kg), xylazine (2.2mg/kg), acepromazine (1mg/kg) and then maintained by nose cone with 2-3% halothane throughout the surgery and during dressing changes. Several minutes prior to producing the burn, the animals were shaved and washed with soap and water. Thermal burns were created using 3.5 cm diameter heated brass blocks with a contact time of 12 sec [63]. Sixteen such wounds were created, eight on each side of the dorsal mid-line between the shoulders and hip. Treatments were applied within 30 minutes after burn creation and included crude keratose hydrogel, chitosan hydrogel or saline soaked gauze. Treatment areas were covered with dry gauze followed by an occlusive dressing (adhesive Ioban™), a cotton stocking, a plastic saddle over the back, and a nylon jacket to keep all dressings in place. Dressing changes were performed on anesthetized swine every 3 days. Digital pictures were taken with a ruler in view and wound size measurements made using digital image analysis software (ImageJ). Wound size values for each treatment group were averaged (e.g. n=16 on days 0, 3, 6, and 9; n=12 on days 12 and 15; n=8 on day 18; n=4 on days 21, 24, and 27) and reported as mean ± SEM.

2.6. In Vitro Thermal Injury Model:

2.6.1. Primary Mouse Skin Fibroblast Isolation: Primary mouse fibroblasts were isolated from adult CD1 mouse ear pads. The ear pads were incubated in Dispase II
overnight and then the dermis was peeled off using forceps and minced before incubating in 0.35% type I collagenase reconstituted in DMEM high glucose for 30 min at 37°C on a shaker. The suspension was filtered using a 100 micron mesh filter and cells pelleted by centrifugation. The cell pellet was washed once with Hank’s balanced salt solution (HBSS) to remove the residual collagenase. Finally, the fibroblasts were seeded in a 150 mm tissue culture plastic (TCP) dish at 2.5 x 10^6 cells/mL in fibroblast media [DMEM high glucose plus 10% Fetal Bovine Serum (Hyclone® Laboratories, Inc., Logan, Utah) and 1% Antibiotic/Antimycotic solution (Hyclone® Laboratories, Inc.)].

2.6.2. **In Vitro Thermal Stress Method:** Primary mouse fibroblasts from passages 3 to 6 were used for these experiments. Fibroblasts were seeded in the range of 1.83 x 10^5 to 2.36 x 10^5 cells/mL in a 100 mm TCP dish. The cells were grown in fibroblast media until 95% confluent. Media changes were performed every 3 days. For a thermal injury experiment, a media change was performed one day prior to heating the cells. On the day of the experiment, fibroblasts were moved from a 37°C, 5% CO₂ incubator to a 44°C, 5% CO₂ incubator for 60 minutes. After the thermal stress, culture dishes were moved back to a 37°C, 5% CO₂ incubator to allow for reattachment of the cells and for the media temperature to equilibrate. Six hours after heat exposure, various treatments were applied in fresh media. Treatments included 0.01 mg/mL crude keratose, 0.001 mg/ml gamma keratose, 0.001 mg/ml alpha keratose and fibroblast media. All keratose samples were sterilized by gamma irradiation at 1 MRad before adding to the media. This experiment was performed
with three replicates. All the data were normalized to 0 hours (before heat shock) and reported as mean ± standard error of the mean (SEM).

2.6.3. **Trypan Blue Assay:** Equal amounts of cell suspension and 0.4% trypan blue (Invitrogen, Gibco, Grand Island, NY) were mixed together and stained cells were counted using a hemocytometer. Live cells with intact cell membrane excluded the trypan blue dye whereas dead cells retained the dye and were blue.

2.7. **Detection and Identification of Gamma Keratose proteins:** Proteins associated with the gamma keratose fraction were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained protein bands were recovered and analyzed by mass spectrometry as previously described [62].

2.8. **Statistical Analysis:** Two-way analysis of variance (ANOVA) and Bonferroni post-hoc analyses were performed using Graph pad Prism at 95% confidence intervals for both the in vivo and in vitro studies.

3. **Results:**

3.1. **Mouse Chemical Burn Study:** Compared to the other treatments, keratose hydrogel treated burns showed no increase in wound size over the first four days post-injury and faster wound closure. There was a statistically significant reduction in wound area with keratose treatment relative to either chitosan or saline at days 4 through 16 (**Figure 1**). Digital pictures of representative burn wounds at day 0, 8 and 16 showed faster wound healing progression and closure (**Figure 2**). A well-defined eschar (sloughed skin) was observed early in the keratose treated wound at day 8 compared to day 16 for the chitosan and saline soaked gauze treatments (occlusive dressing).
3.2. Swine Thermal Burn Pilot Study: Compared to the other treatments, keratose hydrogel treatment again showed no increase in burn wound size and resulted in faster wound closure (Figure 3). There was a statistically significant reduction in wound area at days 3, 6, and 12 in the keratose treated group compared to both chitosan and saline. Digital pictures of representative burn wounds at day 3, 9 and 24 shows wound healing progression and faster wound closure (Figure 4).

3.3. In Vitro Thermal Stress Model: Control cultures were examined for their ability to mimic the phases of heat induced cell damage. Immediately after heat stress, 40-50% cell death was noted by cell counting using trypan blue staining. Further cell death was evident in the following 24 hours if proper treatment was not provided. After 7 days, cultures grew back to a confluent state (Figure 5). Using this in vitro heat injury model, different keratose treatments were compared to each other and to control treatment (i.e. fibroblast growth media containing serum). Fibroblasts that received a single treatment of gamma keratose at 0.001mg/mL dissolved in the fibroblast media and were able to maintain their viability, whereas all other treatments resulted in additional cell death between the time of treatment (6 hours) and 24 hours. Even by day 3, the other treatments were only able to maintain their post-treatment cell numbers [65% ± 20.7 (crude), 46% ± 32.5 (alpha) and 37% ± 20 (fibroblast media)], while the gamma keratose treated fibroblasts had 125% ± 15.6 cell viability (Figure 6). The gamma keratose treatment resulted in statistically significant cell growth compared to all other treatments at day 3, and compared to the alpha keratose treatment at day 5.

3.4. Identification and separation of gamma keratose proteins: Electrophoretic separation of gamma keratose revealed proteins of different molecular weights ranging from
110 kDa to 8 kDa. Protein bands at 110 kDa, 40-60 kDa, ~30 kDa, ~15-20 kDa, and ~8-10 kDa that were excised for mass spectrometry analysis were identified as containing keratins 81, 83, 85, 86, 31, 33A, 33B, 34 in all the excised regions. Epithelial keratins 1 and 10 were identified in the excised regions with molecular weight 60kDa and 8kDa. The excised region at ~110 kDa band was identified as alpha keratin heterodimers, which were previously reported by de Guzman et al [62]. Keratin-associated protein 19-5, galectin-7, calmodulin-like protein 3, thioredoxin, and cystatin A were identified in the band at ~8 kDa (Figure 7). Mass spectrometry analysis of the excised bands with protein identities, molecular weights and protein scores is provided as supplementary data (Tables 1-6).

4. Discussion:

Hydrogels are gaining more interest as wound dressings, especially for the treatment of burns, ulcers, and other necrotic wounds [64]. They have inherent properties such as the ability to absorb wound exudate and provide a moist wound environment that enhances healing [3, 64]. Keratin proteins provide a readily available, inexpensive biomaterial hydrogel that has been shown to be beneficial for regenerating tissues [51, 54, 56-58, 65-67]. Recently, a keratin biomaterial extracted from wool was shown to promote faster re-epithelialization in deep partial thickness burns [54]. Moreover, keratose, the oxidized form of keratin protein extracted from hair and wool fibers, was previously purported to be beneficial for wound healing [68]. However, neither of these accounts attempts to describe a plausible mechanism for improved wound healing, nor do they describe the keratin in sufficient detail to provide much insight into its structure or composition. The ability of a hydrogel made from a structural protein such as
keratin to provide beneficial wound healing is not surprising, given the general nature of hydrogels and the proven efficacy of providing a protected wound environment where moisture can be controlled. However, in this study, we sought to demonstrate the particular efficacy of a keratose biomaterial hydrogel for the treatment of chemical and thermal burns, then investigate a potential mechanism of cell rescue that was tied to a specific fraction of keratin biomaterial that has until now, been uncharacterized in the literature. In both pilot animal studies, a crude form of keratose was used that contained both gamma and alpha fractions. While this mixture of proteins was efficacious in both models, we were not able to determine if either of these fractions individually or synergistically produced differential effects on the damaged tissues. To better understand the relative contributions of these different forms of keratin biomaterial, a heat stress model that mimics thermal injury in vivo was developed.

The injury to tissue that results from thermal insult was first described by Jackson [69]. According to Jackson’s burn model, burn injury is characterized by three zones: 1) the inner zone of coagulation, which is characterized by necrotic tissue, 2) an intermediate zone of stasis, which is characterized by damaged cells that may live or die depending on treatment and other factors, and 3) an outer zone of hyperemia where cells are stressed but will likely survive if the wound does not get infected and standard treatment is given [69]. Similar to Jackson’s model, the heat stress culture system was optimized to produce a population of cells that died from necrosis during the heat treatment phase, a second population of cells that died from apoptosis in the hours following thermal injury, and a third population that recovered and could re-establish the culture. Using this model, we were able to show that gamma keratose was better able to maintain
cell viability compared to crude keratose, alpha keratose, and fibroblast growth media. By day 5, gamma keratose treated fibroblast growth was so robust that the culture was over-confluent.

Heat stress models have been used previously to isolate cells into a more simplistic system and study molecular mechanisms of damage and survival [70]. In this study we not only showed that a biomaterial made from oxidized keratin protein can improve fibroblast survival, but that this phenomenon is associated only with the gamma keratose fraction. As this is the first study to show that this keratose fraction has particular efficacy for cell salvage after heat stress, we performed mass spectrometry analysis to understand the composition of proteins present in the gamma keratose fraction. Mass spectrometry analysis of the excised bands showed that they contain keratins 81, 83, 85, 86, 31, 33A, 33B, and 34. Because molecular weights between 26 and 46 kDa do not correspond to any known, intact human hair alpha keratin or KAP, and because there are no alpha keratin proteins with molecular weights below 46kDa, we conclude that all proteins identified by mass spectroscopy in the 30kDa, 15-20kDa, and 8kDa electrophoretic bands must represent fragments of degraded alpha keratose proteins. In much of the early published literature on keratins, the gamma fraction has been described as being derived from the cortical matrix [71], and this concept has become dogma in the current keratin biomaterials field. However, given the oxidation and extraction conditions employed in our protein isolation method, it is not surprising that the generation of peptide fragments occurs, especially when considering recent evidence that shows a number of weak bonds in alpha keratin proteins [72].

The concept that fragments of larger proteins have a biological function different than that of the parent molecule is not new. Extra cellular matrix (ECM) derived from various tissues
contain growth factors that promote tissue remodeling and angiogenesis [73]. Badylak et al. showed that bone marrow derived stem cells actively participated in the remodeling of ECM scaffolds in vivo. They also suggested that portion of these bone marrow derived stem cells might have participated in angiogenic response [74]. In another study, it was shown that a low molecular weight peptide fraction of ECM (5-16kDa) has chemoattractant properties for mature endothelial cells both in vitro and in vivo assays. This low molecular weight peptide fraction was derived from small intestine sub mucosa ECM. The ECM containing structural proteins (collagens, proteoglycans, glycoproteins, glycosylaminoglycans) and growth factors was reduced to particulate form and further separated into seven fractions based on molecular weight. These biologically active peptides were not purified or characterized completely but have been shown to attract endothelial cells that might have promoted angiogenesis when ECM scaffolds were implanted in vivo [75].

It has been shown that proteolytic degradation exposes cryptic sites on resulting peptide fragments, imparting biological function that was not possessed by the intact protein [76]. Understanding more about the composition and structural nature of gamma keratose and the active peptide region(s) that may be responsible for promoting survival in thermally stressed cells may assist in the development of better keratin-based treatments for burn injuries.

5. **Conclusions:**

Pilot burn studies in mice and pigs have identified the potential for a keratose biomaterial to spare burned tissue and facilitate skin regeneration. A closer examination of this observation has revealed that gamma keratose, identified through proteomic techniques as peptide fragments cleaved from the structural alpha keratins during extraction, were primarily responsible for this
phenomenon. The gamma keratose fraction was better able to rescue cells after thermal insult in an in vitro model system and therefore may represent a potential therapy for burn injury.

Conflict of interest statement

Mark Van Dyke, Ph.D. holds stock and is an officer in the company, KeraNetics LLC, which has provided partial funding for this research. Wake Forest Health Sciences has a potential financial interest in KeraNetics, LLC through licensing agreements.
References:


Figures:

**Figure 1. Mouse Chemical Burn Wound Area**

Digital Image analysis shows that crude keratose hydrogel treatment of chemical burns results in a significant difference in wound area compared to saline and chitosan hydrogel control treatments at days 4 through 16 (indicated by asterisks).
Figure 2. Digital Images from Mice Chemical burn study

Digital pictures of representative burn wounds at day 0, 8 and 16 show enhanced wound healing and closure with keratose treatment compared to chitosan or saline (occlusive dressing).
Figure 3. Swine Thermal Burn Wound Area

Digital Image analysis shows that crude keratose hydrogel treatment of thermal burns results in a significant difference in wound area compared to saline and chitosan hydrogel control at days 3, 6 and 12.
Figure 4. Digital Images from Swine thermal burn study

Digital pictures representing burn wounds at day 3, 9 and 24 shows wound healing progression and faster wound closure.
Figure 5. In Vitro Thermal Stress Model

An in-vitro heat shock model was developed that produced three populations of cells: necrotic, apoptotic, and survivable. This graph represents the growth curve of the survivable cells treated with fibroblast growth media after heat treatment at 44°C for 60 minutes.
Figure 6. Percent Cell Viability Curve

Gamma keratose treated cells did not show a characteristic drop in viability at 24hrs and were able to grow to 125% ± 15.6 confluency by day 3, whereas with other treatments the fibroblasts grew to only 65% ± 20.7 (crude), 46% ± 32.5 (alpha) and 37% ± 20 (Fibroblast Media) of pre-treatment levels. P<0.001 - ***, P<0.01 - **, P<0.05 - *
Mass spectrometry analysis of gamma keratose identified keratin 81, 83, 85, 86, 31, 33A, 33B, 34 in all the excised regions. Epithelial keratins – 1 and 10 were identified in the excised regions with molecular weight 60 kDa and 8 kDa. Keratin-associated protein 19-5, galectin-7, calmodulin-like protein 3, thioredoxin, and cystatin A were also identified in the ~8 kDa excised band.
Supplementary Mass Spectrometry Analysis Data:

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CHAPTER 3

GAMMA KERATOSE MAINTAINS CELL VIABILITY IN VITRO AFTER THERMAL STRESS BY REGULATING THE EXPRESSION OF CELL DEATH PATHWAY SPECIFIC GENES

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The following manuscript has not yet been submitted for publication. Deepika Poranki prepared the manuscript. Dr. Mark Van Dyke acted in an advisory and editorial capacity.
Abstract:

When skin is thermally burned, transfer of heat energy into the skin results in the destruction of cells. Some of these skin cells are damaged but may be capable of self-repair and survival, thereby contributing to spontaneous healing of the wound. This phenomenon can be replicated in cell cultures through models that utilize mild heating, where cells are thermally stressed and their response to injury studied in the presence and absence of therapeutic treatments. Keratin protein-based biomaterials have been suggested as potential treatments for burn injury. Isolation of cortical proteins from hair fibers results in an acid soluble fraction of keratin proteins referred to as “gamma” keratose. In the present study, treatment with this fraction dissolved in media was able to maintain cell viability after thermal stress in an in vitro model using primary mouse dermal fibroblasts. PCR array analysis identified that at 12hr compared to the pre-treatment control (6hr), gamma keratose treated cells has a significant (p<0.01) up regulation of Ulk1 (autophagy) and Casp3 (apoptosis and autophagy). Whereas in the growth media treated cells although there is relatively high expression (fold change) of the genes, significant difference was not observed. At 18hr, necrotic (Parp2) and autophagic (Atg16l2) genes were highly expressed in growth media treated cells with statistically significant (p<0.01) difference compared to the pre-treatment control (6hr), whereas none of the cell death related genes were highly expressed in the gamma keratose treated cells. Moreover, gamma keratose treatment significantly (p<0.01) down regulated autophagic (Esr1) and necrotic (Parp2) genes compared to the pre-treatment control (6hr). By 24 hours, there was similar expression in both the treatment groups. These results suggest that gamma keratose treatment may assist in the survival and salvage of thermally stressed cells, maintaining their viability by regulating cell death pathway related genes. Gamma
keratose may be a promising material for burn treatment that aids in spontaneous wound healing from viable tissue surrounding the burn.

**Keywords:** gamma keratose, thermal stress, PCR array, apoptosis, necrosis, autophagy
1. Introduction

When skin is thermally burned, transfer of the heat into the tissue results in cell destruction. Some damaged cells may be capable of self-repair and survival, if properly treated, thereby contributing to spontaneous healing of the wound. Complete tissue necrosis is often observed in the region where the injury occurs, beginning with the surface of the skin and radiating downward into the tissue. The tissue surrounding this necrotic zone can be termed a “thermally stressed zone”, which has received the energy as an initiating factor but is susceptible to several further insults that result in cell death [1]. This phenomenon can be replicated in cell culture by models that utilize mild heating (i.e. 42-48°C), resulting in a heterogeneous population of cells, some of which die as a direct result of the thermal injury and others that die secondary to the thermal insult due to the inflammatory cytokines released into the media. There are also some cells that survive both insults and are capable of self repair. Heating cells has been shown to induce various cell death pathways [2-8]. Depending on the intensity and duration of the exposure, apoptosis, necrosis or autophagy pathways are triggered. Apoptosis is a genetically programmed cell death mechanism that is associated with the activation of cysteine-dependent aspartate-specific proteases called caspases and is involved in the development of homeostasis [9]. Necrosis is morphologically characterized by cellular swelling, swelling of organelles, plasma membrane rupture and loss of intracellular contents [10]. Until recently, it was considered as non-programmed and unregulated cell death, but recent evidence indicates that programmed necrosis occurs through the activation of death receptors in a caspase independent manner termed “necroptosis”[11]. Autophagy is a catabolic process involving degradation of the cell’s own components and is usually activated by oxidative, nutritional or toxic stresses [12]. In
contrast to other cell death pathways, autophagy reflects a cell survival mechanism by maintaining normal cellular function during nutrient deficient conditions or by removing damaged organelles and aggregated proteins [8, 13].

Numerous studies have been published in which in vitro models have been used to investigate thermally-induced cell death. In one study the log-phase cultures of mouse mastocytoma cells exposed to heat from 42ºC - 44ºC showed an increase in apoptosis; at 45ºC both necrosis and apoptosis were observed, and at 46º and 47ºC only necrosis was observed [2]. In another study, prolonged energy deprivation followed by thermal stress (43ºC for 10 min) of murine P3 O1 myeloma and Ehrlich ascites carcinoma cells induced high expression levels of Hsp70 that protected the cells from necrosis [4]. A third study used osteoblasts exposed to 48ºC for 10 min and showed a 15-20% increase in necrotic cells within minutes after heating. After a 12 hour recovery at 37ºC, there was a 10% increase in apoptotic cells compared to control cells maintained at 37ºC, indicating that thermal stress induced necrosis in the early phase and apoptosis in the late phase [5]. These studies have shown that mild heating of cells in culture can induce both necrosis and apoptosis at different stages, thus serving as a basis for further investigation of these pathways at the cell and molecular level.

More extensive studies have been published in which pathway analysis at the gene level has been conducted. For example, exposure of NIH3T3 cells to 45ºC for 15 min induced the activation of Akt mediated by PI3Kinase. Phosphotidylinositol 3 kinase (PI3 kinase) activates Akt (a serine threonine kinase) downstream in the signaling pathway of growth factors and cytokines [14-16]. Akt is also activated by PI3Kinase in cell stress conditions such as hydrogen peroxide (H2O2) and heat shock [17]. Phosphorylated Akt gradually decreased to basal levels in
9 hours after insult and a high rate of cell proliferation was observed within 24 hours. Whereas when the cells were exposed to prolonged heating at 45ºC for 60min, apoptosis was increased by about 70% compared to untreated cells at 37ºC. This treatment failed to activate Akt, indicating that cells might be irreversibly damaged when exposed to high temperatures for prolonged periods[6]. In another study, heat treatment of Statens Seruminstitut Rabbit Cornea (SIRC) cells at 42ºC for 30 min triggered a mitochondrial apoptotic pathway indicated by a change in Bax/Bcl-2 ratios, resulting in caspase-9 activity [7]. Thermal stress to cells was also shown to trigger death receptor apoptotic pathways by a change in Fas ligand expression, which in turn resulted in the activation of caspase 8. Addition of an antioxidant decreased heat mediated apoptosis, suggesting that ROS plays a critical role in the mitochondrial and death receptor pathways of these cells [7].

The aforementioned studies shed further light on the time/temperature dependence on cell death pathways, and begin to elucidate some of the molecular pathways involved in necrosis and apoptosis. However, autophagy has been less widely studied. There are published in vitro studies showing that in spite of apoptosis being completely inhibited, cell death still occurs and is usually independent of caspases, following a specific signaling pathway where the cell morphology resembles necrotic cell death [18-22]. If anti-apoptotic proteins block the caspases, then necrosis occurs through the caspase independent pathway [20]. Embryonic fibroblasts from double knock out Bax/Bak−/− mice are resistant to apoptotic inducers. Bax and Bak regulate the intrinsic pathway of apoptosis by controlling the activation of caspases [23]. Bax/Bak−/− double knockouts are used in research to study the different mechanisms of cell death (autophagy and necrosis) that occur when apoptosis is completely inhibited. When treated with etoposide, the
cells failed to undergo apoptosis and instead manifested a massive autophagy response followed by cell death, but knocking down the autophagy related gene (Atg) products beclin-1 and Atg5, reduced etoposide induced cell death [24]. Using other apoptotic inhibitors did not trigger the autophagic cell death pathway, indicating that Bax and Bak absence played an important role in autophagy activation [24]. In other cell types like L929 mouse fibrosarcoma, human Jurkat T lymphoma, and U937 monocyteid cells, inhibition of cystein proteases (caspases) was shown to induce autophagy [25, 26]. In stress conditions like nutrition depletion or loss of growth factors, autophagy gets activated by the inhibition of apoptosis and promotes cell survival [27, 28]. If autophagy is inhibited in cell stress conditions, the cells usually die through apoptosis. This cell death can be postponed by inhibiting Bax or Bak or caspases [29]. In vitro and in vivo experiments have shown induced apoptotic cell death by the inactivation of Atg genes Atg 5 or 7, and beclin-1[30-33]. Autophagy may develop as a primary response to stress stimuli, which then triggers either apoptosis or necrosis [34, 35].

Previous studies (Chapter 2) have shown that gamma keratose was able to maintain cell viability post thermal stress compared to the other treatments in an in vitro model using primary mouse fibroblasts. It was evident in these studies that a large number of cells died from the initial heat treatment, additional cells died within hours after heating, and surviving cells re-established the culture. Thus, we concluded that all three pathways of cell death may be present in our cell culture model, and that keratin treatment may affect one or more of these pathways. In the present study, we hypothesize that treating primary dermal fibroblast cells with gamma keratose six hours post thermal stress will induce differential expression of genes related to the cell death pathways. The RT² Profiler™ PCR array was used to investigate cell death pathway related gene
expression in thermally stressed cells treated with gamma keratose dissolved in the media compared to cells treated with fibroblast growth media only.

2. Methods

2.1. Fibroblast cell Isolation: Primary mouse fibroblasts were isolated from adult CD1 mouse ear pads. The ear pads were incubated in Dispase II overnight and then the dermis was peeled off using forceps and the remaining tissue minced before incubating in 0.35% type I collagenase reconstituted in DMEM high glucose for 30 min at 37°C on a shaker. The suspension was filtered using a 100 micron meshed filter and the cells were pelleted by centrifugation. The cell pellet was washed once with Hank’s balanced salt solution (HBSS) to remove the residual collagenase. Finally, the fibroblast cells were seeded in a 150 mm tissue culture plastic (TCP) dish at 2.5 x 10⁶ cells/mL in fibroblast growth media [DMEM high glucose plus 10% Fetal Bovine Serum (Hyclone® Laboratories, Inc. Logan, Utah)] and 1% Antibiotic/Antimycotic solution (Hyclone® Laboratories, Inc. Logan, Utah)).

2.2. Gamma Keratose Extraction: Keratin biomaterial was extracted from human hair using the oxidative protocol specified by de Guzman et al. [36]. The extracted keratin biomaterial was termed crude keratose. The gamma keratose fraction was further separated from crude keratose by isoelectric precipitation. Concentrated hydrochloric acid was added dropwise to the crude keratose solution with stirring until a pH 4.2 is reached. At this pH, the alpha keratose precipitates leaving the acid-soluble gamma fraction in solution. The insoluble alpha keratose was separated by fixed angle centrifugation at 1500 rpm (252 RCF) for 15 min at 4°C. After neutralizing the gamma-
containing supernatant to pH 8.4, it was dialyzed against endotoxin free water using a 5kDa spiral wound cartridge (Millipore, Billerica, MA) for 5 volume washes. Finally, the solution was concentrated to minimal volume, pH adjusted to 7.4, lyophilized, and sterilized by gamma irradiation at 1 MRad prior to use.

2.3. **In vitro thermal stress model:** For this experiment, primary mouse fibroblasts between passages 3 to 6 were seeded at approximately $8 \times 10^5$ cells/mL in 100mm TCP dishes. The cells were allowed to grow in fibroblast growth media until they were 95% confluent. Media changes were performed every 3 days. For a thermal stress experimental run, a media change was performed a day prior to heating the cells. On the day of the experiment, cultures were moved from a 37˚C, 5% CO$_2$ incubator to a 44˚C, 5% CO$_2$ incubator and heated for 150 minutes. After the heat treatment, culture dishes were moved back to a 37˚C, 5% CO$_2$ incubator to allow re-attachment of cells and the media temperature to equilibrate. Six hours post heat treatment, cultures were randomized and treatment was applied in fresh media. Treatments included 0.1mg/mL gamma keratose supplemented to fibroblast growth media and fibroblast growth media alone (control containing 10% FBS). The former will be termed gamma keratose treatment and the latter growth media treatment in the rest of the chapter.

2.4. **Trypan Blue Assay:** Cell counting with a hemocytometer was performed using 0.4% trypan blue reagent (Invitrogen, Gibco, Grand Island, NY), which stains cells with a disrupted plasma membrane. Cells were counted at 6hr, 12hr, 18hr and 24 hr post heat treatment. Out of two separate in vitro experiments performed, three samples showing the highest percent cell viability after gamma keratose treatment and three samples showing the lowest percent cell viability with growth media treatment were selected for
further analysis using PCR arrays. Cell viability data from these samples was presented as mean ± SEM (standard error of the mean).

2.5. **RNA extraction & quantification:** RNA extraction was performed using 5 prime perfect pure RNA cultured cell kit (5 Prime Inc., Gaithersburg, MD 20878). After trypsinization and cell counting, the cell pellet obtained after centrifugation was lysed with a solution provided in the kit. The suspension was loaded onto a column with a filter and centrifuged, followed by two washes with wash solution and RNA elution from the column filter was performed using an elution buffer. Extracted RNA was quantified using a Thermoscientific Nanodrop 2000 spectrophotometer. Out of the two separate thermal stress experiments that were performed, RNA was extracted from three individual (10cm) tissue culture plates for each treatment that showed the highest percent cell viability after gamma keratose treatment and the least percent cell viability after growth media treatment.

2.6. **PCR arrays:** cDNA synthesis was performed with the RT² First Strand Kit (SABiosciences) using an AB Applied Biosystems Veit 96 well thermocycler (850 Lincoln Center Drive, Foster City, CA) following the protocol specified by the manufacturer. After the cDNA synthesis was performed, RT² Profiler™ PCR microarray mouse cell death pathway finder (SABiosciences) was used to determine the expression of genes that are involved in cell death. RT² Sybr green ROX qPCR master mix (SABiosciences) was used to perform the gene array experiment using an AB Applied Biosystems 7300 Real time PCR system (850 Lincoln center drive, Foster City, CA) following the protocol specified by the manufacturer. RT² Profiler™ PCR array mouse cell death pathway finder analyzes the real-time expression of 86 genes
(Supplementary Table 1) related to cell death pathways, which includes apoptosis, necrosis and autophagy.

3. **Data Analysis:** Data was analyzed using the Auto Ct settings provided by the 7300 Real Time PCR system software. Further data analysis was done using RT² profiler PCR array web based software provided by the manufacturer that follows ΔΔCₜ method of calculation [37]. This provided a comparison of post-treatment (12hr, 18hr, and 24hr) to the pre-treatment control (6hr). To compare the relative up or down regulation between the gamma keratose and fibroblast growth media treatment groups, the ratio of gene expression (fold change) for gamma keratose treatment to growth media treatment was calculated. Using this calculation, if the ratio is less than 1, it indicates that the gene expression is higher in growth media treated cells, if it is equal to 1, the expression is the same in both the treatments, and if it is greater than 1, the expression is greater in the gamma keratose treated cells.

4. **Results:**

4.1. **In vitro thermal stress model:** Six hours after heating, the average percent cell viability was 53.7%±4.35. At 12hr, there is a significant difference (p<0.05) in the percent cell viability between gamma keratose treated cells and growth media only treated cells. The average percent cell viability for gamma keratose treated cells was 37.8%±0.85, whereas growth media treated cells was 24.41%±2.20. At 18 hr, gamma keratose treated cells were able to maintain cell viability at 39.83%±5.54, whereas in growth media treated cells, additional cell death was noted with an average percent cell viability of only 20.18%±4.58. This difference was statistically significant (p<0.01). At 24 hr, the average percent cell viability for gamma keratose treated cells was 36.9%±3.74 compared to
growth media treated cells at 24.83%±0.69. This difference was not statistically significant (p>0.05) (Figure 1).

4.2. RNA quantification: The total amount of RNA extracted from all the 24 samples is listed in Table 2. The A260:280 and A260:230 ratios were approximately 2.1 and 2.0 indicating the purity of extracted RNA (Table 1).

4.3. PCR array data analysis: Gene expression of gamma keratose and growth media treated cells at 12hr, 18hr and 24hr was compared to that of post thermal stress control (i.e. cells at 6 hours post-heating, but immediately prior to treatment with fresh growth media or gamma keratose supplemented media).

At 12hr compared to the pre-treatment control (6hr), the growth media treated cells have no genes that were statistically significant even though their gene expression (fold change) was high. In the gamma keratose treated cells, 29 genes were significantly (p<0.05) up-regulated compared to the pre-treatment control. Among these 29 genes, 19 genes were down-regulated, six genes were up-regulated in gamma keratose treated cells and four genes had a similar expression when compared to growth media treated cells. The 19 genes that were down-regulated were involved in necrosis (9430015G10Rik, Bmf, Txnl4b, Parp2, Pvr, Jph3), autophagy (Atg7, Atg5, Atg12, Map1lc3a), both apoptosis and autophagy (Akt1, Bcl2, Bax) and apoptosis (anti-apoptotic [Traf2, Xiap] and pro-apoptotic [Dffa, Fasl, Apaf1, Birc2]). The six up-regulated genes were involved in autophagy (Htt, Ulk1, Atg16l1, Irgm1), both apoptosis and autophagy (Casp3) and apoptosis (anti-apoptotic [Mcl1]). The genes that had similar expression between both
growth media and gamma keratose treated cells were involved in autophagy (App, Mapk8, Rps6kb1) and apoptosis (pro-apoptotic [Cflar]) (Table 2).

At 18hr, compared to the pre-treatment control (6hr), 34 genes in the growth media treated cells were statistically significant (p<0.05). Of them seven genes were down-regulated and 27 genes were up-regulated in the growth media treated cells. In the gamma keratose treated cells, 18 genes had a gene expression that was statistically significant (p<0.05) compared to pre-treatment control (6hr). Of these, six genes were down-regulated and 12 genes were up-regulated in the gamma keratose treated cells. When the 34 genes that were significantly expressed in growth media treated cells were compared to the gamma keratose treated cells, six genes involved in necrosis (Galbt5, Foxi1, Olfr1404), autophagy (Ins2, Esr1) and apoptosis (anti-apoptotic [Tnfrsf11b]) were down-regulated and six genes involved in necrosis (Parp2, Rab25, Bmf, S100a7a), autophagy (Atg16l1) and apoptosis (pro-apoptotic [Fasl]) were up-regulated in growth media treated cells. Twenty two genes involved in necrosis (Commd4, Txnl4b, 9430015g10Rik), autophagy (Htt, Atg7, Pik3c3, Atg5, Ulk1, Mapk8, Map1lc3a), apoptosis (anti-apoptotic [Xiap, Birc3] and pro-apoptotic [Dffα, Birc2, Casp1, Apaf1, Gadd45a]) and both apoptosis and autophagy (Casp3, Bcl2l11, Bcl2, Bax, Akt1) had a similar expression in both the groups. Comparison of the 18 genes that were expressed in gamma keratose treated cells to growth media have shown that three genes involved in necrosis (Rab25, Parp2) and autophagy (Atg16l1) were down-regulated and three genes involved in necrosis (Galnt5), autophagy (Esr1) and anti-apoptosis (Sqstm1) were up-regulated in gamma keratose treated cells. The 12 remaining genes involved in necrosis
(Txnl4b), autophagy (Htt, Atg7, Atg5, Ulk1, Mapk8, Map11ca), apoptosis (anti-apoptotic [Xiap, Birc3] and pro-apoptotic [Birc2]) and both apoptosis and autophagy (Casp3, Bax) had a similar expression between both the groups (Table 3 & 4).

At 24hr compared to the pre-treatment control (6hr), 21 genes were expressed in growth media treated cells with statistical significance (p<0.05). Of them, 10 genes were down-regulated and 11 genes were up-regulated in the growth media treated cells. Similar gene expression was noticed in the gamma keratose treated cells. Twenty one genes in gamma keratose treated cells had significant difference (p<0.05) compared to pre-treatment control. Of them, 10 genes were down-regulated and 11 genes were up-regulated in the gamma keratose treated cells. When the 21 genes that were significantly expressed in growth media treated cells were compared to gamma keratose treated cells, seven genes involved in necrosis (Galnt5, Bmf, Txnl4b), autophagy (Esr1), apoptosis (pro-apoptotic [Gadd45a] and anti-apoptotic [Tnfrsf11b]), apoptosis and necrosis (Sycp2) were up-regulated. The remaining 14 genes involved in necrosis (Olfr1404, Commd4, S100a7a, Parp2), autophagy (Ifng, Ctsb, Atg16l1, Gaa), apoptosis (anti-apoptotic [Xiap] and pro-apoptotic [Birc2, Nol3, Casp1]) and both apoptosis and autophagy (Bcl2l1, Bax) were expressed similarly between the two groups. Comparison of the 21 genes that were significantly expressed in gamma keratose treated cells to the growth media treated cells have shown that six genes involved in necrosis (Galnt5), autophagy (Esr1, Ins2), apoptosis (anti-apoptotic [Tnfrsf11b] and pro-apoptotic [Gadd45a]) and both apoptosis and necrosis (Sycp2) were down-regulated and one gene involved in apoptosis (pro-apoptotic [Abl1]) were up-regulated in gamma keratose treated cells. The remaining 14
genes involved in necrosis (Rab25, Olfr1404, Commd4, S100a7a, Parp2), autophagy (Ifng, Ctsb, Atg16l1, Gaa), apoptosis (anti-apoptotic [Xiap] and pro-apoptotic [Birc2, Nol3, Casp1]) and both apoptosis and autophagy (Bcl2l1) were similarly expressed between the groups (Table 5 & 6).

5. Discussion

Thermal burns typically display an injury pattern dictated by the transfer of the thermal energy into the skin and underlying tissues. According to Jackson’s burn model, the zone of stasis contains viable cells initially but due to the inflammatory cytokines released by the surrounding necrotic tissue, more cell death occurs [38]. Necrosis is the major mechanism of cell death that occurs as thermally damaged tissue converts to dead tissue and the wound progresses from second degree to third degree. This process has sometimes been described as burn wound progression [39]. This concept was shown by Clarke et al. using a validated porcine hot comb model in which high mobility group box 1 (HMGB-1) protein was used as a marker for cellular necrosis and activated cleaved caspase-3 as a marker for apoptosis. The authors showed that cellular necrosis in the zone of stasis occurred between one and four hours and apoptosis was responsible for cell death only after 24 hr. They also suggested that an early intervention within the first four hours is necessary to limit the irreversible cellular changes that account for burn injury progression. However, there have been relatively few therapies that specifically target cessation of burn injury progression by treating the thermally stressed tissue immediately following injury. In one rare example, c-jun amino terminal kinase, a member of the mitogen-activated protein kinase family involved in both apoptosis and necrotic pathways was investigated as a potential target [40, 41]. In a mouse thermal injury burn model, a peptide
inhibitor of c-jun was shown to promote re-epithelialization faster and also reduced cell death surrounding the wounded region by 24 hr post injury [42]. Clearly, treatments that can arrest the death of potentially viable cells are feasible and represent a novel therapy that may provide tissue sparing treatment, but surprisingly little research has been conducted in this field.

Previous studies (Chapter 2) showed that gamma keratose was able to reduce the TBSA in in vivo studies and maintain cell viability in an in vitro thermal stress model. Using the same thermal stress model, we attempted to understand whether gamma keratose induces differential expression of cell death pathway genes as a possible mechanism of its apparent tissue-sparing activity. There are four main findings from the present experiments:

1. As expected, heat treatment of mouse dermal fibroblasts up regulates cell death pathway related genes.

2. Gene expression was more consistent (i.e. smaller p values) in cells treated with gamma keratose compared to cells treated with fibroblast growth media at the early time point post thermal stress (12 hr).

3. A single gamma keratose treatment at 0.1mg/mL appeared to influence gene expression at 12 and 18 hr post-thermal stress (6 and 12 hr post-treatment, respectively), but this effect was diminished by 24 hr.

4. In general, treating these thermally stressed cells with gamma keratose substantially diminishes the gene up regulation compared to treatment with fresh fibroblast growth media.

To further explain the first point, real time PCR array data analysis showed that there was an overall up regulation of the genes involved in cell death pathways (necrosis, autophagy,
apoptosis) six hours post thermal stress, and the cell viability data from the in vitro thermal stress model correlates with this point. After the 6hr post thermal stress interval, there was a drop in the cell viability from 100% to 53.7%±4.35 (Figure 1).

To further explain the second point, at 12hr post thermal stress, gamma keratose treated cells showed statistically significant (p<0.05) alterations in the expression of 29 genes compared to the pre-treatment control (6hr). Whereas in the growth media treated cells although there was high expression (fold change) of the genes compared to the pre-treatment control, no statistical significance was observed (p>0.05) due to the variability in the sample replicates for this treatment group (Table 2).

As mentioned in point three, treating the thermally stressed cells once with 0.1mg/mL gamma keratose was able to maintain cell viability by regulating the cell death related genes at 12hr and 18hr. At 12hr, 29 genes had a significant (p<0.05) gene expression in gamma keratose treated cells compared to the pre-treatment control (6hr). Of these, 19 genes were highly expressed by the growth media treated cells. By 18hr, only 18 genes were significantly (p<0.05) expressed in gamma keratose treated cells, whereas 34 genes were significantly (p<0.05) expressed in growth media treated cells compared to the pre-treatment control (6hr). The regulation of cell death related genes by gamma keratose strongly correlates with the in vitro thermal stress model cell viability results. In the in vitro model at 12hr post thermal stress, gamma keratose treated cells were able to maintain cell viability at 37.8%±0.85 compared to growth media treated cells at 24.41%±2.20 and the difference was statistically significant (p<0.05). At 18 hr, gamma keratose treated cells were able to maintain cell viability at 39.83%±5.54, whereas in growth media treated cells, additional cell death was observed with an
average percent cell viability of only 20.18%±4.58. This difference was statistically significant (p<0.01). By 24hr, there was no significant difference in the cell viability and the gene expression was similar between the two treatment groups (Figure 1 and Table 7 respectively).

To further explain the fourth point, at 12hr, 10 genes in gamma keratose treated cells showed a statistically significant difference (p<0.01) compared to the pre-treatment control. Among these 10 genes, two genes involved in autophagy (Ulk1) and both apoptosis and autophagy (Casp 3) were up-regulated, 8 genes involved in necrosis (Parp2, Pvr), autophagy (Atg5, Map1lc3a, Atg7), apoptosis (pro-apoptotic[Fas1]) and both apoptosis and autophagy (Bax, Akt1) were down-regulated in the gamma keratose treated group compared to the growth media treated cells (Table 2).

Tozte et al. screened embryonic kidney 293 cells by kinase specific si-RNA library using autophagy assay and identified Ulk1 as a novel candidate for autophagy [43]. In mammalian cells, Ulk1/2 forms a complex with Atg13, Atg101 and FIP200 that was positively regulated by AMPK (AMP-activated protein kinase) and negatively regulated by mTOR (mammalian target of rapamycin) [44, 45]. AMPK is an evolutionarily conserved energy sensing kinase that is activated by metabolic stress [45]. mTOR regulates growth, proliferation and protein synthesis of cells in response to growth inducing stimuli [46]. Caspase 3 (Casp 3) is involved in the cleavage of Atg4D (regulates autophagosome formation) and the truncated Atg4D is highly toxic and couples both apoptosis and autophagy [47]. Fasl is a member of tumor necrosis family (TNF) of type II transmembrane proteins. It is prototypic death factor that induces apoptosis when it binds to its receptor Fas [48]. Atg5 and Atg7 are involved in the formation of autophagosome [49]. Starved mouse embryonic fibroblasts lacking the autophagy gene Atg7 failed to undergo cell
cycle arrest. Whereas prolonged metabolic stress resulted in augmented DNA damage and increased p-53 mediated apoptotic cell death [50].

The gene expression data suggests that gamma keratose treatment was able to control the up regulation of most of the cell death genes at 12hr compared to the growth media. The genes that were up regulated in the gamma keratose treatment are involved in the autophagy pathway, suggesting that the cell death observed in the gamma keratose treatment from 6 to 12 hrs post thermal stress was due to autophagy. In vitro studies have shown that in stressed conditions like starvation and energy deprivation, cell death occurs through autophagy when other cell death pathways (like apoptosis) were inhibited [24-26]. At 18hr compared to the pre-treatment control, gamma keratose treated cells had a significant (p<0.01) expression of 4 genes. Of the four genes, two genes involved in autophagy (Esr1) and necrosis (Parp2) were down-regulated compared to growth media treated cells (Table 3). The remaining two genes had a similar expression between both the groups. Even though down regulation of Esr1 was observed in our thermal stress model, no studies were published that suggest that Esr1 expression regulates autophagy when the cells are exposed to stress conditions. Parp2 was shown to be involved in programmed necrosis (also called necroptosis). Hitomi et al. treated L929 cells with TNF-α (tumor necrosis receptor family involved in apoptosis by the activation of caspase 3) and zVAD.fmk (caspase inhibitor) and performed a genome wide siRNA screen to identify the regulators of necroptosis. They identified Parp-2 as a key regulator of necroptosis and showed that knockdown of Parp-2 inhibits necroptosis [51]. This suggests that gamma keratose treated cells were able to maintain their cell viability (39.83%±5.54) at 18hr when compared to 12hr (20.18%±4.58) by controlling the down regulation of genes involved in autophagy and necrosis. By 24 hrs, the gene expression between
The two treatment groups was similar, suggesting that the effect of the gamma keratose treatment had diminished (Table 5 & 6). Although the mechanism of interaction of the gamma keratose with the cell was not investigated in this study, the metabolic demand of thermally stressed cells is known to be pathologically high [52-55], so it would not be surprising if the keratose were consumed rapidly and its effects short lived.

6. Conclusion:

This study has shown that a single treatment of gamma keratose at 0.1mg/mL after thermal stress can mediate survival of mouse dermal fibroblasts and faster recovery of the culture. At 12hr compared to pre-treatment control (6hr), there was approximately 15% decrease in cell viability and an increase in expression of autophagy related genes in the gamma keratose treated cells. However, by 18hr gamma keratose was able to maintain cell viability similar to that observed at 12 hrs and also down regulated the genes related to autophagy. This study suggests that along with apoptosis and necrosis, autophagy plays an important role in the viability of thermally stressed cells. Importantly, gamma keratose treatment was able to maintain cell viability by down regulating the expression of the genes involved in apoptosis, necrosis and autophagy. Gamma keratose, as a component in a keratin biomaterial hydrogel, may therefore have been beneficial as a burn treatment as shown in earlier studies due to this mechanism. Further detailed studies are necessary to determine if this is indeed the case, as the processes of cell death are complex and any potential role of gamma keratose would need to be examined in more detail, particularly with regard to how the cell interacts with the gamma keratose molecules.
Conflict of interest statement

Mark Van Dyke, Ph.D. holds stock and is an officer in the company, KeraNetics LLC, which has provided partial funding for this research. Wake Forest Health Sciences has a potential financial interest in KeraNetics, LLC through licensing agreements.
References:


Figures & Tables:

Figure 1: In vitro Heat shock Model

Gamma keratose treated cells were able to maintain cell viability at 12hr, 18hr post-thermal stress compared to the fibroblast growth media treated cells with statistical significance. P<0.01**, P<0.05*
Table 1: RNA Quantification

The 260/280 and 260/230 ratios were close to 2.1 and 2.0 indicating the purity of the extracted RNA.

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Table 2: Gene expression of gamma keratose treated cells at 12hr

These are list of genes that were having significant p-value <0.05 for the gamma keratose treated cells at 12hrs compared to the post heat shock pre-treated cells. Red color represents the up regulated genes and blue color represents down regulated genes.

<table>
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<th>Gene Symbol</th>
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<th>Gamma keratose treated cells fold change</th>
<th>Gamma keratose treated cells fold change/Growth media treated cells fold change</th>
<th>Pathway Involved</th>
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Table 3: Gene expression of gamma keratose treated cells at 18hr

These are list of genes that were having significant p-value <0.05 for the gamma keratose treated cells at 18hrs compared to the post heat shock pre-treated cells. Red color represents the up regulated genes and blue color represents down regulated genes.

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<th>Gamma keratose treated cells fold change</th>
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**Table 4: Gene expression of growth media treated cells at 18hr**

These are list of genes that were having significant p-value <0.05 for the growth media treated cells at 18hrs compared to the post heat shock pre-treated cells. Red color represents the up regulated genes and blue color represents down regulated genes.

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<td>P.A.</td>
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<td>4.74</td>
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</tr>
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<td>7.75</td>
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<td>0.61</td>
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</tr>
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<td>9.00</td>
<td>6.27</td>
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<td>2.33</td>
<td>0.71</td>
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</tr>
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<td>1.60</td>
<td>0.80</td>
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<td>0.47</td>
<td>1.10</td>
<td>Pro-Apoptotic</td>
</tr>
</tbody>
</table>
Table 5: Gene expression of gamma keratose treated cells at 24hr

These are list of genes that were having significant p-value <0.05 for the gamma keratose treated cells at 24hrs compared to the post heat shock pre-treated cells. Red color represents the up regulated genes and blue color represents down regulated genes.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gamma keratose treated cells p-value &lt;0.05</th>
<th>Growth media treated cells fold change</th>
<th>Gamma keratose treated cells fold change</th>
<th>Gamma keratose treated cells fold change/Growth media treated cells fold change</th>
<th>Pathway Involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnfrsf11b</td>
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<td>0.34</td>
<td>0.24</td>
<td>0.73</td>
<td>Anti Apoptotic</td>
</tr>
<tr>
<td>Xiap</td>
<td>0.02</td>
<td>3.71</td>
<td>3.59</td>
<td>0.97</td>
<td>Anti Apoptotic</td>
</tr>
<tr>
<td>Sycp2</td>
<td>0.03</td>
<td>0.23</td>
<td>0.13</td>
<td>0.58</td>
<td>Apoptosis and Necrosis</td>
</tr>
<tr>
<td>Bcl211</td>
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<td>2.93</td>
<td>3.09</td>
<td>1.06</td>
<td>Apotosis and Autophagy</td>
</tr>
<tr>
<td>Esr1</td>
<td>0.00</td>
<td>0.10</td>
<td>0.04</td>
<td>0.41</td>
<td>Autophagy</td>
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<td>Ins2</td>
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<td>0.21</td>
<td>0.13</td>
<td>0.64</td>
<td>Autophagy</td>
</tr>
<tr>
<td>Ifng</td>
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<td>0.14</td>
<td>0.13</td>
<td>0.95</td>
<td>Autophagy</td>
</tr>
<tr>
<td>Ctsb</td>
<td>0.00</td>
<td>0.46</td>
<td>0.45</td>
<td>0.96</td>
<td>Autophagy</td>
</tr>
<tr>
<td>Atg16l1</td>
<td>0.04</td>
<td>3.74</td>
<td>3.84</td>
<td>1.03</td>
<td>Autophagy</td>
</tr>
<tr>
<td>Gaa</td>
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<td>0.47</td>
<td>0.57</td>
<td>1.20</td>
<td>Autophagy</td>
</tr>
<tr>
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<td>0.13</td>
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</tr>
<tr>
<td>Rab25</td>
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<td>5.51</td>
<td>0.79</td>
<td>Necrosis</td>
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<tr>
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<td>0.13</td>
<td>0.95</td>
<td>Necrosis</td>
</tr>
<tr>
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<td>2.21</td>
<td>1.01</td>
<td>Necrosis</td>
</tr>
<tr>
<td>S100a7a</td>
<td>0.01</td>
<td>7.87</td>
<td>8.18</td>
<td>1.04</td>
<td>Necrosis</td>
</tr>
<tr>
<td>Parp2</td>
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<td>2.04</td>
<td>2.50</td>
<td>1.23</td>
<td>Necrosis</td>
</tr>
<tr>
<td>Gadd45a</td>
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<td>0.46</td>
<td>0.33</td>
<td>0.71</td>
<td>Pro Apoptotic</td>
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<tr>
<td>Birc2</td>
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<td>0.76</td>
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<td>Nol3</td>
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<td>3.76</td>
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### Table 6: Gene expression of growth media treated cells at 24 hr

These are list of genes that were having significant p-value <0.05 for the growth media treated cells at 24hrs compared to the post heat shock pre-treated cells. Red color represents the up regulated genes and blue color represents down regulated genes.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Growth media treated cells p-value &lt;0.05</th>
<th>Growth media treated cells fold change</th>
<th>Gamma keratose treated cells fold change</th>
<th>Gamma keratose treated cells fold change/Growth media treated cells fold change</th>
<th>Pathway Involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnfrsf11b</td>
<td>0.00</td>
<td>0.34</td>
<td>0.24</td>
<td>0.73</td>
<td>Anti Apoptotic</td>
</tr>
<tr>
<td>Xiap</td>
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<td>3.71</td>
<td>3.59</td>
<td>0.97</td>
<td>Anti Apoptotic</td>
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<tr>
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<td>Apoptosis and Necrosis</td>
</tr>
<tr>
<td>Esr1</td>
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<td>0.10</td>
<td>0.04</td>
<td>0.41</td>
<td>Autophagy</td>
</tr>
<tr>
<td>Ifng</td>
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<td>0.13</td>
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<td>Autophagy</td>
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<tr>
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<td>0.45</td>
<td>0.96</td>
<td>Autophagy</td>
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<td>3.84</td>
<td>1.03</td>
<td>Autophagy</td>
</tr>
<tr>
<td>Gaa</td>
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<td>0.47</td>
<td>0.57</td>
<td>1.20</td>
<td>Autophagy</td>
</tr>
<tr>
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<td>0.13</td>
<td>0.51</td>
<td>Necrosis</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Commd4</td>
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<td>Necrosis</td>
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<tr>
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<td>1.04</td>
<td>Necrosis</td>
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<tr>
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<td>2.55</td>
<td>1.05</td>
<td>Pro Apoptotic</td>
</tr>
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</table>
Table 7: Total number of genes significantly expressed in gamma keratose treated cells and growth media treated cells with p<0.05

The third column in the table indicates the total number of genes showing significant differential expression in each of the treatments at that particular time point. The columns 4, 5 and 6 are breakdown numbers of column 3. Columns 4 and 6 show how many of the total differentially expressed genes were expressed higher in gamma keratose treatment and in growth media treatment respectively. Column 5 represents the number of genes that have similar expression in both the treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment hours</th>
<th>Total number of genes showing significant differential expression</th>
<th>Number of genes expressed higher in gamma keratose treatment</th>
<th>Number of genes showing similar expression in both the treatments</th>
<th>Number of genes expressed higher in growth media treatment</th>
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</thead>
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<tr>
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<td>18hrs</td>
<td>18</td>
<td>3</td>
<td>12</td>
<td>3</td>
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<tr>
<td></td>
<td>24hrs</td>
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<td>6</td>
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### Supplementary Table 1: Genes included in the PCR array

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<th>Gene Name</th>
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<td>Necrosis</td>
</tr>
<tr>
<td>Abl1</td>
<td>C- abl oncogene 1, non-receptor tyrosine kinase</td>
<td>Pro-Apoptotic</td>
</tr>
<tr>
<td>Akt1</td>
<td>Thymoma viral proto-oncogene 1</td>
<td>Apoptosis and Autophagy</td>
</tr>
<tr>
<td>Apaf1</td>
<td>Apoptotic peptidase activating factor 1</td>
<td>Pro-Apoptotic</td>
</tr>
<tr>
<td>App</td>
<td>Amyloid beta (A4) precursor protein</td>
<td>Autophagy</td>
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</tr>
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<td>Autophagy-related 16-like 1 (yeast)</td>
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</tr>
<tr>
<td>Atg3</td>
<td>Autophagy-related 3 (yeast)</td>
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<td>Atg5</td>
<td>Autophagy-related 5 (yeast)</td>
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<tr>
<td>Atg7</td>
<td>Autophagy-related 7 (yeast)</td>
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</tr>
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<td>Apoptosis and Autophagy</td>
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<tr>
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<tr>
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DEVELOPMENT OF A PORCINE DEEP PARTIAL THICKNESS BURN MODEL

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Abstract:

Swine are the preferred animal models to study the effects of burns on dermal wound healing. Various studies have been published in which little emphasis was placed on minimizing burn variability and inconsistency. We developed a novel method to create deep partial thickness burns that are highly consistent.

A custom-made burn device was fabricated to control the pressure applied on the swine skin during burn creation. Cylindrical brass blocks, measuring 3 cm in diameter, are used to create the burns. A stainless steel post extends from the block for insertion into the device holder. In this study, burns were created in four female Yorkshire swine. Heating of the brass blocks was conducted using a boiling azeotropic solution of 80% polyethylene glycol (PEG) and 20% water and boiling water alone. Contact times ranging from 12 to 20s were used. At 24h and 7d post-injury, two swine were euthanized and tissues collected for histological assessment using Gomori trichrome staining. Histological analysis showed that burns created using boiling water were superficial compared to those created using the boiling PEG:H₂O solution. With a burn contact time of 20s, 48.5% ± 5.7 tissue damage was demonstrated at 24h when the PEG:H₂O solution was used, whereas only 11.9% ± 1.3 was observed with boiling water.

Keywords: contact burn, polyethylene glycol, azeotropic mixture, deep partial thickness burn, swine, skin
1. Introduction

The structural similarities of swine and human skin render swine as preferred subjects for in vivo burn therapy research [1, 2]. Additionally, the large bodies of swine allow for ample surface area for multiple wound creation and clinical evaluation [3]. Of the many contact injury models published [4-6], few studies have considered the incidence of burn wound variability and inconsistency as a function of burn technique. The use of models with such inherent sources of error may give an inaccurate picture of the treatment being employed in studies where differences between treatments is being quantified and statistically compared.

Kempf et al. [7] reported the occurrence of variable deep partial thickness scald burn wounds upon creation, despite full adherence to a consistent burn procedure. Consequently, reddened, superficially-burned regions within the wounds healed faster than the surrounding eschar-covered regions by 14 days post-injury (Figure 1). Papp et al. [8] reported similar difficulties in burn wound creation, displaying obvious signs of inconsistency in a representative photo (Figure 2). In the aforementioned studies, a single operator created all burn wounds with only the weight of gravity on the heat source serving as applied pressure. Singer et al. [9] have shown that the amount of pressure applied on the animal has a direct effect on the intensity and depth of burn. As such, maintaining a constant pressure every time is important in creating consistent burns. The observed incidence of burn wound variability suggests that better control over the pressure applied by the heat source on the skin combined with a more effective heat transfer technique may produce wound uniformity. A large portion of the published literature describes the use of boiling water to heat the brass blocks employed in wound creation [8, 10-12]. Boiling water
produces air bubbles that adhere to the brass blocks and can create non-uniform heating (i.e. “cold spots”). Uniform thermal energy transfer is then compromised when the brass block is in contact with the skin of the animal. The cold spots can produce superficially-injured skin regions that, over time, develop into regenerated ‘islands’ of new skin within the wound at earlier time points than the rest of the burn. As migration of healthy cells from the periphery of normal skin to the wounded area is part of the wound healing process, the wounds that have these islands of less damaged, faster regenerating skin heal faster compared to wounds without islands [13-15].

Our lab has developed a model in which a custom-made, spring-loaded burn device was fabricated to standardize the pressure applied on the swine skin during burn creation, independent of the operator. Cylindrical brass blocks served as our heat source for wound creation. Preliminary evaluations of the brass block temperature upon immersion in boiling water indicated that the blocks did not thermally equilibrate in the water bath and reach an optimal temperature. Use of a boiling azeotropic mixture of deionized water and polyethylene glycol raised the equilibration temperature of the brass blocks and also appeared to eliminate the formation of cold spots. This allowed for the creation of highly consistent burn wounds and facilitated the determination of an optimal contact time to achieve the desired burn depth.

2. Materials and methods

0.1. Animal model: This swine study was approved by the Wake Forest University Animal Care and Use Committee. Six female, Yorkshire swine (Baux-Mountain, Winston-Salem, NC) weighing 20-25 kg at the time of surgery were used in this study. The animals were housed in individual pens upon their arrival and allowed to become
acclimated for at least 7 days. The animals were washed and shaved one day prior to surgery, at which time they were pretreated with a transdermal fentanyl patch (50mcg/h) for pain management. While on study, the animals were fasted each night prior to the administration of anesthesia.

0.2. General anesthesia and surgical monitoring: Anesthesia was induced intramuscularly with ketamine (10mg/kg) and dexmedetomidine (0.05mg/kg), and was later continued with isoflurane during surgery. The previously applied fentanyl patch was removed and a new patch (75mcg/h) applied during surgery, which was replaced every 3 days. Blood pressure, heart rate and body temperature were monitored during surgery for any complications. Ten burn wounds were to be created with 3 cm diameter cylindrical brass blocks, 5 on each side of the mid-dorsal line between the shoulder and the hip.

0.3. Wound creation

2.3.1 Pressure-controlled burn device: Cylindrical brass blocks (360 grade), measuring 3 cm in diameter and 5 cm in height, were used to create the burn wounds (Figure 3). The blocks were heated in a boiling solution until used. A stainless steel post protruded from the top of each block to allow for easy insertion into the burn device holder, made of insulative Delrin®. An adjustable spring was housed within the otherwise hollow body of the holder to enable a consistent force response when the block was pressed onto the animal’s body (Figure 4). During burn creation, the operator positioned the device holder over the brass block in solution, dislodging a pin connector to allow for insertion of the steel post into the holder. Release of the pin connector ensured secure locking of the block within the holder. The holder was then used to remove the block from solution, residual liquid was quickly removed with a towel, and the block placed onto the animal’s
skin in a randomly assigned location. Enough force was applied so that the bottom of the device holder was flush with the brass block, allowing for a consistent block pressure controlled by the spring. Prior to use on animal subjects, the burn device was tested for operational consistency by two separate researchers using pressure-sensitive film (Sensor Products Inc., Madison, NJ). This was further verified under experimental conditions through visual observation within the current study. A total of 3 identical brass blocks were rotated for use during the burn surgery to assure temperature equilibration and minimize the amount of time needed to maintain the animal under general anesthesia.

2.3.2 **Metal block surface temperature validation:** Initial studies conducted by our lab have shown that the brass blocks reached a temperature range of only 88-92°C when equilibrated in boiling water. A potential solution was to raise the temperature of the water through the addition of PEG. An 80:20 ratio of PEG:H₂O produced a negative azeotropic mixture that raised the boiling temperature to 115-125°C. Temperature of the bottom surface of the brass blocks after equilibration in the boiling liquids was tested using temperature sensing strips (Omega Engineering, Stamford, CT). An unanticipated effect of the use of this azeotropic liquid was its wetting characteristics and the potential to reduce the prevalence and adherence of vapor bubbles on the under side of the brass blocks. As the azeotropic solution contained mostly PEG, we evaluated the difference in surface tension compared to water alone. A contact angle meter (KSV Cam100 Instruments Ltd, Helsinki, Finland) was used to determine the average contact angle measurements of water and the azeotropic PEG:H₂O mixture on polished brass metal coupons. The coupons were of the same composition and surface finish as the brass blocks used with the burn device. Contact angles were measured in 5 places along the
diameter of the metal coupons and the difference between average values was evaluated using a Student’s T-test with p<0.05 considered significant.

2.3.3 **Burn wound creation:** Two Yorkshire swine were used for each boiling solution - water alone and the 80:20 PEG:H₂O mixture. Ten burn wounds were created on each animal using the custom-made device. Brass block contact times from 12-20s, in 2s increments, were used in wound creation. Two replicate burns were created for each contact time and were randomly assigned locations on the animal. Sixty minutes after burning, the wounds were covered with saline soaked non-adhesive gauze (Telfa, Tyco Healthcare, Mansfield, MA) followed by an occlusive adhesive dressing (Ioban™ 2, 3M, St.Paul, MN), a protective plastic shield and a nylon jacket to keep the dressings in place. Atipamezole (0.05mg/kg) was administered intramuscularly to reverse the effect of the dexmedetomidine post-surgery. Two animals were euthanized after 24h and the wounds were harvested for further histological analysis. The other two animals were euthanized after 7days. Dressing changes were performed every 3 days for the 7 day animals.

2.4 **Data acquisition**

2.4.1 **Digital wound capture:** Digital pictures were taken at 0, 1, 3 and 7 days post-surgery (Nikon-D90, Nikon Inc., Melville, NY) to document the presence or absence of islands of normal tissue. A ruler and color wheel were located within each photograph for later processing of the image.

2.4.2 **Histology:** The entire wound was excised from the animal at the designated time point and half of the tissue was fixed in 10% neutral buffered formalin for 2 days. After fixation, a tissue cross section closest to the center of the wound was cut and processed in 70% isopropyl alcohol (IPA), 80% IPA, 95% IPA, 100% IPA and xylene (Leica
ASP300S Tissue processor, Buffalo Grove, IL) overnight. The tissue was embedded in paraffin and cut in 5 μm sections using a microtome (Leica CM 1850, Leica Microsystems Inc., Buffalo Grove, IL) and stained with Gomori trichrome. Representative sections of each wound were evaluated by an experimental pathologist blinded to the protocol. At pre-determined distance intervals from the edge of the wound, tissue morphology was scored according to the following scale:

**Epidermis**

0 Normal
1 Flattening of the dermal-epidermal (DE) junction
2 Flattening and disruption of the DE junction
3 Absence of epidermis

**Dermis**

0 Normal
1 Loss of collagen architecture (papillary dermis)
2 Loss of collagen architecture and dye intensity color change
3 Loss of collagen architecture in reticular dermis (mid dermis)
4 Loss of collagen (full thickness)

Digital images of stained tissue sections were captured (Axio Imager M1 Microscope, Carl Zeiss USA, Thornwood, NY) and the burn areas (i.e. demonstrating infiltration of inflammatory cells, edema, and damaged collagen) and total area of the skin from the epidermis down to the dermis-fat junction were measured (ImageJ, National Institutes of Health). Total percent tissue loss was determined by calculating the area of burn (as indicated by a change from green to red color and denaturation of the collagen bundles
[16]) divided by the total area of the skin. Average percent tissue loss was calculated from two replicate wounds and reported as mean ± standard deviation.

3 Results

3.1 Brass block temperature validation and surface chemistry: The brass blocks heated in boiling azeotropic mixture of 80:20 PEG:H₂O reached a temperature range of 99-103°C (Figure 5B), whereas the blocks heated with water only reach 88-92°C (Figure 5A). The contact angle analysis showed a relatively lower average contact angle for the PEG:H₂O mixture compared to water alone (Figure 6). During heating, it was observed that vapor bubbles did not adhere to the surface of the brass blocks.

3.2 Visual differences due to heating method: When water alone was used, burn wounds can be uneven as shown in other studies. Islands of superficially-burned skin are evident, causing an unwanted increase in burn edges that would be expected to affect the rate of healing. These islands presumably arise from the presence of vapor bubbles that adhere to the bottom of the brass blocks, thereby insulating the surface and creating cold spots. The PEG:H₂O azeotropic mixture has low surface tension compared to water alone, creating a continuous liquid interface along the bottom of the block surface. In addition, the PEG:H₂O mixture does not boil as vigorously as water alone, resulting in noticeably fewer vapor bubbles. The combination of fewer bubbles and better wetting mitigated the formation of cold spots and created uniform heating when the PEG:H₂O azeotrope was used. This difference can be seen visually with digital pictures of wounds taken on days 0, 3 and 7 post-surgery (Figure 7). Wounds created by two separate operators and using the PEG:H₂O azeotropic mixture showed greater consistency (Figure 8), as verified by an initial experiment using pressure-sensitive film (data not shown).
3.3 Histological analysis

3.3.1 Wound variability evaluation: Wound scoring data for day 1 is shown graphically in Figure 9. Each bar represents the mean value for contact time with the burning device, and the error bars provide a measure of the variance seen between the two replicate burns (i.e. large relative error bars indicate larger variance). As can be seen in the graphs, the damage created by the water heated blocks (Figure 9A) was relatively less severe than those created by the PEG:H₂O heated blocks (Figure 9B) in the epidermis. In addition, the PEG:H₂O burns were more consistent as suggested by the small error bars. Evaluation of the dermis showed similar results (Figures 9C and 9D) in that the PEG:H₂O burns were relatively more severe and more consistent across replicates and contact times. By day 7, the epidermis in both groups was necrotic and had been completely removed by debridement during dressing changes. In the dermis, the extent of damage in the PEG:H₂O burns was extensive as these wounds had progressed to nearly full thickness in most cases and were not scored, while the water burns showed only modest damage and continued variability (scoring data not shown).

3.3.2 Percent dermal tissue damage: Gomori trichrome staining was used to measure the burn depth. When water alone was used for wound creation, the burns were more superficial, whereas the PEG:H₂O heating system created a more severe partial thickness burn (Figure 10). Importantly, there was no correlation of burn depth with the contact time as has been reported in earlier studies [8]. When the PEG:H₂O mixture was used, the burns were more consistent and deep and there was a measurable correlation between the contact time and intensity of the burn. For a 20s contact time, 48.5% ± 5.7 of tissue loss
was observed with the PEG:H$_2$O boiling solution but only 11.9% ± 1.3 tissue loss was observed with boiling water alone (Figure 11).

4. Discussion

The creation of highly-reproducible burn wounds is essential in the assessment of healing progression in therapeutic models. Researchers have recognized the need to create consistent burn wounds, but have cited the change in underlying tissue structure of the animal as a hindrance. While we acknowledge the inherent variability encountered in working with animal subjects, the present model offers control over variability through two simple means, a burn device that creates operator-independent pressure, and a heating system that ensures consistent and uniform heating of the brass blocks. Several other groups have limited their attempts at controlling variability to designating a single operator to create burn wounds during surgery. While this method has led to slightly better control, this study demonstrates that a higher level of control can be achieved. Through the customized design of our spring-loaded device, any operator can create the burn wounds without concern for uneven pressure when applied appropriately. Pressure applied from the operator is only transferred along the periphery of the device holder, where the device holder hits the animal’s skin along its insulated bottom surface. This portion of the holder does not directly encounter the hot bath and does not achieve a temperature as high as the actual brass block. In addition, the device and mode of operation is potentially less hazardous to users than some other methods such as scalding [7].

The use of the PEG:H$_2$O azeotrope improved the heat transfer efficiency within our system. Moreover, the azeotropic mixture appears to maintain a contiguous coating along the contact surface of the brass blocks, even in the presence of boiling fluid. Water vapor bubbles adhere more strongly to the brass surface and are more difficult to dislodge through wetting
because of the hydrophobicity of the brass block. The lower surface tension of the PEG:H$_2$O-brass interface minimizes the insulating effects of adhered vapor bubbles and thus the formation of cold spots. If such uneven heating is present, a more superficial burn is created in this area, accounting for the ‘islands’ that many researchers have encountered. The use of a non-toxic, biocompatible polymer such as PEG for thermal burn models not only increases overall maximum temperature, but provides an added benefit of ensuring more even distribution of heat to allow for more consistent burning and the elimination of lateral and depth temperature gradients. PEG is used in a variety of applications such as coating the surfaces of nanoparticles to increase the amount of surface active reactive groups [17], and in many cosmetic products [18]. It has also been used to neutralize the residual phenol left in a chemical burn [19-21]. Therefore it is unlikely that residual PEG on the brass block surface would induce a chemical burn or toxicity tissue reaction.

Histological analysis with Gomori trichrome staining showed that using PEG:H$_2$O as a heating solution created more consistent and deep burns compared to boiling water alone. For a 20s burn, more tissue loss was observed with PEG:H$_2$O compared to water after 24 hours. One of the possible reasons might be that the brass blocks are not being heated to 100°C with water alone, as shown by other investigators [8]. Also, given the dimensions of the brass blocks used in this study, they did not provide an adequate heat sink for 20s when the temperature reached using water alone was only 88-92°C. This is demonstrated by the fact that we expected to see more superficial burns with a contact time of 12s compared to 20s. However, when boiling water was used the burns were more variable overall and the 20s burns were more superficial compared to the 12s burns.
There are many variables that need to be considered when creating a burn wound, and controlling those variables will help in creating a consistent burn. Several prior studies have discussed the use of animals of similar size/age, controlling contact time, and using the same operator. Randomization and the use of a large number of replicate wounds is also advocated. The need for randomization is evident in the data for the 18s contact time in the water only group where the percent burn was unusually high. Despite randomly locating all wounds, these two burns ended up over the abdomen, which likely contributed to a high value for this test condition.

Heating brass blocks to a consistently high temperature and avoiding the formation of cold spots are also important considerations in developing a burn model with consistent, efficient thermal energy transfer. This study demonstrates that these aspects can be addressed through the application of simple surface chemistry and thermodynamic principles.

**Conflict of interest statement**

Mark Van Dyke, Ph.D. holds stock and is an officer in the company, KeraNetics LLC, which has provided partial funding for this research. Wake Forest Health Sciences has a potential financial interest in KeraNetics, LLC through licensing agreements.

**Acknowledgements**

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The authors would like to thank Christina Ross and Mary Ellenburg (WFIRM) for their assistance with the burn surgeries.
References:


Figure 1. Differences in healing of deep partial thickness scald burns due to inconsistent burning. Consistent, deep partial thickness burn wound (A) compared to a wound containing red, superficially-burned areas (inside dashed lines; B) will heal more uniformly (C) compared to the superficially-burned areas which were healed by day 14 (inside dashed lines; D). Used with permission [7].
Figure 2. Burns with non-uniform severity created with brass block heated in boiling water along the right flank of the animal. A burn wound created with a contact time of 12s is indicated by the arrow. Used with permission [8].
Figure 3. 360 grade, 3 cm diameter brass block used for burn wound creation. Notched stainless steel post extends from block for easy insertion into the device holder.
Figure 4. Burn device operation. The brass block is held within the device holder by engaging a pin connector at the notched end. Upon depression of the fully-extended assembly, the spring contracts exerting an even force onto the block with no extraneous forces from the operator.
Figure 5. Heated brass blocks with temperature sensors. Equilibrium temperatures of the block contact surfaces upon heating in boiling water alone (A; up to 88-92°C; arrow) and the PEG:H$_2$O azeotropic mixture (B; 99-103°C; arrow).
Figure 6. Contact angle analysis using water and PEG:H2O azeotropic mixture to determine surface wetting. The addition of PEG caused a significant drop in surface tension and consequently, static contact angle (n=5, p<0.05, Student’s T-test).
Figure 7. Healing progression of wounds with and without islands. The presence of superficially burned skin in the lower left of the water heated block would be expected to heal more non-uniformly. The PEG:H₂O azeotropic mixture avoided the island formation and created a more uniform burn.
Figure 8. Visually consistent burn wounds created by using boiling PEG:H₂O azeotropic mixture. Each row of wounds was created by a different operator.
**Figure 9.** Histological scoring data from day 1 wounds. Wounds created using water heating caused less severe damage to the epidermis and were more variable (A) compared to wounds created using PEG:H$_2$O (B). This trend continued in the dermis (C and D, respectively).
Figure 10. Tissue sections stained with Gomori trichrome stain showing a variation in the burn depth for a 20s contact burn after 24h when PEG:H$_2$O azeotropic mixture and boiling water alone were used – Boiling water alone has created superficial burn compared to PEG:H$_2$O
Figure 11. Percent dermal tissue damage. For a 20s contact time, 48.5% ± 5.7 tissue loss was observed using PEG:H₂O boiling solution for heating whereas only 11.9% ± 1.3 tissue loss was observed with boiling water after 24h.
CHAPTER 5

ASSESSMENT OF DEEP PARTIAL THICKNESS BURN TREATMENT WITH KERATIN BIOMATERIAL HYDROGELS IN A SWINE MODEL

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The following manuscript has not yet been submitted for publication. Deepika Poranki prepared the manuscript. Dr. Mark Van Dyke acted in an advisory and editorial capacity.
Abstract:

Partial thickness burns can advance to full thickness burns due to inadequate tissue perfusion and increased production of inflammatory cytokines, even with proper hospitalization and treatment. This advancement of wound severity in the first several days after injury has been referred to as burn wound progression and is the result of a cascade of events that results in further tissue loss in the periphery of the original burn. There are currently no commercially available treatments that can control burn wound progression. In our previous work (Chapters 2 and 3), we demonstrated that a keratin biomaterial hydrogel placed over a burn wound appeared to reduce progression and that this may be due to a particular fraction of keratin, gamma keratose, that showed the ability to regulate cell death pathway genes.

In the present thermal burn study, we tested the hypothesis that a modified formulation of keratin, hereafter referred to as modified keratose hydrogel or MKH, which contained a reduced amount of the gamma keratose fraction would heal wounds faster compared to the original formulation of crude keratose. Crude keratose was used in the original pilot studies and was included here as a control, as well as a collagen hydrogel (Coloplast®) and silver sulfadiazine cream (SSD; clinical standard of care).

Fourteen female Yorkshire swine weighing 20-25 kg were used in the study. Standardized burn wounds were created and treated within 60 minutes. Two animals were euthanized on days 1, 3, 6, 9, 12, 15 and 30; their tissues collected. Digital images of the wounds immediately prior to sacrifice were captured and analyzed using ImageJ software. The tissue sections were stained and analyzed for burn depth, granulation of tissue thickness and re-epithelialization.
Wound assessment from digital images showed a significant difference between the MKH and SSD on days 9 (p<0.05), 12 (p<0.001) and 15 (p<0.05). Significant differences were also found between crude keratose and SSD on days 12 (p<0.001) and 15 (p<0.05), and between Coloplast and SSD on days 9 and 12 (p<0.01). Rates of re-epithelialization at early time points showed a 220% increase for the MKH compared to SSD. A Linear regression model predicted that MKH and crude keratose had a time to wound closure of 19 days while Coloplast and SSD treatments took 24 days. However, these data were not statistically significant due to high variability in the SSD treated wounds. Measurements of burn depth and granulation tissue thickness showed that by day 9, vertical burn progression had occurred in all wounds. Moreover, crude keratose treatment appeared to result in a relatively smaller increase in burn depth (5.56%) compared to other treatments (SSD - 9.81%, MKH - 20.01%, Coloplast - 22.5%). SSD treatment appeared to show a higher difference (1.115cm) in the thickness of granulation tissue from days 9 to 15 compared to other treatments, while crude keratose appeared to show the least difference (0.746cm). Again, no statistically significant differences between treatment groups were noted.

These results did demonstrate a smaller wound area with treatment using the MKH at mid-time points during healing, and appeared to show a faster healing rate for this biomaterial compared to the standard of care, SSD. However, the inconsistency of healing in the SSD treated wounds contributed to a loss of statistical significance in several outcome measures. Additional studies using keratin biomaterials that more specifically monitor burn wound progression, preferably in a non-spontaneously healing burn injury model, are warranted.

**Keywords:** keratin biomaterial, deep partial thickness burns, hydrogel, crude keratose, alpha keratose, gamma keratose, collagen, silver sulfadiazine, re-epithelialization, granulation tissue.
1. **Introduction:**

Burns are one of the most catastrophic injuries to treat. Often, partial thickness burns convert to full thickness burns due to inadequate tissue perfusion and increased production of inflammatory cytokines leading to protein denaturation and necrosis [1]. The phenomenon of burn injury progression occurs when events downstream of the original damage cause further tissue loss. As a result, wounds increase in surface area and deepen, progressing from second degree to third degree burns and increasing overall total body surface area (TBSA) burned, thereby increasing risk to the patient, healing time, and treatment expense. In 1953, Jackson described a model of burn injury in terms of three zones of tissue damage: 1) the inner zone of coagulation, which is characterized by necrotic tissue, 2) an intermediate zone of stasis, which is characterized by damaged cells that may live or die depending on treatment and other factors, and 3) an outer zone of hyperemia where cells are stressed but will likely survive if the wound does not get infected and standard treatment is given [2].

Superficial and partial thickness burns heal from the remaining epithelial structures in the dermis [3], so the zone of stasis represents an opportunity for treatment as these cells are capable of survival if an appropriate therapy can be developed that increases their survival, yet limited clinical research has been directed in this area. In vivo studies however, have shown that treatments like cerium nitrate, curcumin, activated protein C (APC), recombinant tissue type plasminogen (r-TPA), TAK-044 (nonselective endothelin receptor antagonist), simvastatin, and beraprost sodium (prostaglandin I2 analogue) were able to control burn injury progression in rats by preventing tissue necrosis in the zone of stasis [4-11]. In general these studies have made use of drug compounds that require intravenous or enteric administration, thus complicating their
clinical development. Topical application of an efficacious therapeutic agent represents a simplified, local treatment modality.

Previous studies (Chapter 2) in our lab have shown that crude keratose, which is a heterogeneous mixture composed of alpha keratose, gamma keratose and keratin associated proteins (KAPs) fractions increased tissue sparing and enhanced wound healing in both mice chemical and swine thermal burn studies. Another study in an in vitro thermal injury model using primary mouse dermal fibroblasts showed that the gamma keratose fraction was able to maintain increased cell viability compared to the other treatments post thermal stress (Chapter 2). Finally, gamma keratose was shown to salvage the cells by down regulating many genes involved in cell death pathways (Chapter 3). Mass spectrometry analysis identified the major constituents of the gamma fraction as keratins 81, 83, 85, 86, 31, 33A, 33B, 34 with lower than expected molecular weights, suggesting that these were degraded alpha keratin proteins (Chapter 2). As such, they represent peptides with biological function different than that of their parent molecules. In the present study, we hypothesized that the peptides found in the gamma keratose fraction would provide a therapeutic benefit when used in a topical keratin biomaterial hydrogel formulation. This postulate was tested by comparing a “crude” keratose hydrogel with a re-formulated hydrogel, modified keratose hydrogel or MKH, that contained a reduced amount of gamma keratose, in a swine burn injury model. The amount of gamma keratose was reduced to reflect the concentration at which gamma keratose was effective in the heat stress cell culture model. A collagen hydrogel, Coloplast®, and the clinical standard of care, silver sulfadiazine cream (SSD) were used as controls.
2. Methods:

2.1. Keratin Protein Extraction: Keratin protein was extracted from human hair using the oxidative protocol specified by de Guzman et al. [12]. The extracted keratin is referred to as crude keratose.

2.2. Separation of Alpha and Gamma Keratose Fractions: Crude keratose was further separated into alpha and gamma keratose fractions by isoelectric precipitation. Concentrated hydrochloric acid was added dropwise to the crude keratose solution with stirring until a pH 4.2 was reached. At this pH, the alpha keratose precipitates leaving the acid-soluble gamma fraction in solution. The insoluble alpha keratose was separated by fixed angle centrifugation at 1500 rpm (252 RCF) for 15min at 4ºC. After neutralizing the gamma-containing supernatant to pH 8.4, it was dialyzed against endotoxin free water using a 5kDa spiral wound cartridge (Millipore, Billerica, MA) for 5 volume washes. Finally, the solution was concentrated to minimal volume, pH adjusted to 7.4, lyophilized, and sterilized by gamma irradiation at 1 MRad prior to use.

The precipitated alpha keratose was re-dissolved in 0.1M sodium hydroxide (NaOH) solution and dialyzed against endotoxin free water using a 5kDa spiral wound cartridge (Millipore) for 5 volume washes. Finally, the solution was concentrated to minimal volume, pH adjusted to 7.4, lyophilized, and sterilized by gamma irradiation at 1 MRad prior to use.

2.3. Hydrogel Preparation: The MKH was prepared using a proprietary mixture of alpha and gamma keratose powder and reconstituted with phosphate buffered saline (PBS) to form a hydrogel. Crude keratose powder was similarly reconstituted with PBS to form a 15% w/v hydrogel. The gels were made under sterile conditions, centrifuged to remove
air bubbles and incubated in warm room (37°C) overnight on a shaker. Gels were loaded into syringes for easy application onto the burn wounds. Collagen hydrogel (Woun’Dress® Collagen Hydrogel, Coloplast) and SSD (Watson Pharmaceuticals) were obtained from commercial sources and used as received.

2.4. Swine burn study: The swine study was approved by the Wake Forest University Animal Care and Use Committee (ACUC). Fourteen female, Yorkshire swine (Baux-Mountain, Winston-Salem, NC) weighing 20-25 kg at the time of surgery were used in this study. The animals were housed in individual pens upon their arrival and allowed to become acclimated for at least 7 days.

2.5. General Anesthesia and Monitoring: The animals were washed and shaved one day prior to the surgery, at which time they were pretreated with a transdermal fentanyl patch (50mcg/h) for pain management. While on study, the animals were fasted each night prior to the administration of anesthesia. Anesthesia was induced intramuscularly with ketamine (10mg/kg) and dexmedetomidine (0.05mg/kg). Isoflurane was used to maintain the animal in an anesthetic state during the surgery and during dressing changes. The previously applied fentanyl patch was removed and a new patch (75mcg/h) was applied during surgery, which was replaced every 3 days. Blood pressure, heart rate and body temperature were monitored during surgery for any complications.

2.6. Burn Wound Creation: Wounds were created using a pressure controlled burn device (described in Chapter 4). This spring loaded device was made of insulative Delrin®. Cylindrical brass blocks (360 grade), measuring 3 cm in diameter and 5 cm in height, were used to create the burn wounds. The blocks were heated in a boiling solution of polyethylene glycol (PEG; M_n 400; Sigma-Aldrich) and deionized (DI) water. A
stainless steel post protruded from the top of each block to allow for easy insertion into the burn device holder. A total of three identical brass blocks were rotated for use during the burn surgery to assure temperature equilibration and minimize the amount of time needed to maintain the animal under general anesthesia. As described previously in Chapter 4, twelve burns were created on each swine, six on either side of the mid dorsal line between shoulder and the hip. For each time point, twenty four wounds (on two swine) were randomly assigned to four treatment groups and treatments were administered 60 minutes after creating burns. Treatments included Coloplast (Collagen Hydrogel), Crude Keratose, MKH, and SSD. Each treatment had six replicates. Following the treatments, the wounds were covered with saline soaked non-adhesive gauze (Telfa, Tyco Healthcare, Mansfield, MA), followed by an occlusive adhesive dressing (IobanTM 2, 3M, St.Paul, MN), a fabric stocking, a protective plastic shield, and a nylon jacket to keep the dressings in place and prevent the animal from rubbing directly on the wounds. Atipamezole (0.05mg/kg) was administered intramuscularly to reverse the effect of the dexmedetomidine after surgery. Two animals were euthanized at 1, 3, 6, 9, 12, 15 and 30 days after the surgery and tissues harvested for histological analysis after euthanasia on those respective days. Dressing changes were performed every three days.

2.7. Digital wound capture: Digital pictures were captured on 0, 1, 3, 6, 9, 12, 15, 21 and 30 days post-surgery during the dressing changes, immediately after cleaning and debriding the wounds (Nikon-D90, Nikon Inc., Melville, NY). All the images were taken with the camera placed exactly at the same distance and at the same angle (90º, perpendicular) to the wound. A ruler and grey scale were located within each photograph for later
processing of the image. Wound area was measured using ImageJ software (National Institutes of Health, Bethesda, MD). These data were represented as mean ± standard deviation.

2.8. Histology: The entire wound was excised from the animal at the designated time point and half of the tissue was fixed in 10% neutral buffered formalin for 2 days. After fixation, a tissue cross section closest to the center of the wound was cut and processed in 70% isopropyl alcohol (IPA), 80% IPA, 95% IPA, 100% IPA and xylene (Leica ASP300S Tissue processor, Buffalo Grove, IL) overnight. The tissue was embedded in paraffin, cut in 5 µm sections using a microtome (Leica CM 1850, Leica Microsystems Inc., Buffalo Grove, IL), and stained with hematoxylin and eosin (H&E). Representative sections of each wound were evaluated histomorphometrically by a blinded reviewer.

Burn depth measurement: Digital images of stained tissue sections (1.5cm in length) were captured (Axio Imager M1 Microscope, Carl Zeiss USA, Thornwood, NY). The thickness of the normal skin section from the epidermis down to the dermis-fat junction and the residual dermis in the burned region were measured. An average of three measurements was taken, one at the burned-normal dermis junction, one in the center of the burned region, and another on the opposite end of the burned region. Burn depth was determined by calculating the difference of the thickness of the residual dermis in the burned region from the thickness of the normal skin section.

Re-epithelialization measurement: The burned region without epithelial cell coverage (defined as at least one layer of cells staining dark pink) was measured from the stained tissue sections. The percent re-epithelialization was determined by subtracting the burned region from the measured radius of the wound (as determined from the digital image for
the same wound at that time point). Re-epithelialization data was analyzed in two phases: Early time points that included day 3 and 6, and later time points that included day 9, 12 and 15. This was done because the re-epithelialization curves demonstrated two phase healing behavior, suggesting that wound healing rates were different between these two time ranges and that distinct phases of healing (tissue salvage and cell survival followed by matrix deposition and tissue remodeling) were present.

**Granulation tissue thickness measurement:** The thickness of the new granulation tissue (characterized by connective tissue and blood vessels stained pink with H&E) was determined by taking an average of three measurements obtained throughout the length of the stained tissue sections as previously described.

3. **Data Collection and Statistical Analysis:** For visual wound assessment digital images, wound size values for each treatment group were averaged (e.g. n=36 on day 3, n=30 on day 6, n=24 on day 9, etc.) and reported as mean ± standard deviation (SD). For burn depth, re-epithelialization and granulation tissue assessment, values for each treatment group from six replicates were averaged and represented as mean ± standard deviation (SD). Two-way ANOVA and Bonferroni analyses were performed using Graph pad Prism at 95% confidence intervals for all the data analyses.

4. **Results:**

4.1. **Visual wound assessment:** The eschar (dead skin) typically sloughs off completely within 9-12 days, so re-epithelialization was observed most distinctly in the digital images of the wounds from 9 to 30 days. To this end, image analysis data showed a significant difference between MKH and SSD on days 9 (p<0.05), 12 (p<0.001) and
15\((p<0.05)\), and crude keratose and SSD on days 12 \((p<0.001)\) and 15 \((p<0.05)\). Also, a significant difference \((p<0.01)\) was noted between Coloplast and SSD on days 9 and 12. 

(Figure 1)

4.2. Histology:

**Percent burn depth:** On day 3, the average burn depth was minimal in Coloplast treatment group (26.6%) compared to the other treatment groups (MKH - 30.9%, Crude KOS – 34.51%, SSD – 34.98%). By day 9, vertical progression was evident in all the treatments. Crude keratose treatment (5.56%) had a minimal increase in burn depth compared to the other treatments (Coloplast - 22.5%, MKH - 20.01%, SSD - 9.81%) (Figure 2 & Table 1). There was no statistically significant difference in burn depth between the treatments.

**Re-epithelialization:** Burns treated with crude keratose and MKH demonstrated an upward trend in re-epithelialization at early time points (days 3 and 6) and later time points (days 9, 12 and 15) (Figure 3&4). The rate of re-epithelialization was relatively higher in the MKH group \((4.1+/-.96)\) compared to other treatments at early time points (Coloplast - 3.4 +/- 1.1, crude keratose - 3.4 +/- 1.2, SSD - 1.3 +/- 1.7; Table 2). This observation corresponded with previous findings that SSD can impede re-epithelialization [13, 14]. In addition, healing was generally inconsistent across the replicates in the SSD treatment group as indicated by the higher standard deviations.

At later time points (days 9, 12 and 15), the rate of re-epithelialization was relatively higher in the MKH treatment group \((7.6 +/- 1.2)\) compared to other treatments (Coloplast - 6.9 +/- 1.1, crude keratose - 7.3 +/- 1.4, SSD- 5.9 +/- 1.2; Table 3), although not as markedly as at the early time points. Healing was generally more consistent across
replicates as indicated by the relatively smaller standard deviations compared to early stages of healing. When the data was extrapolated to determine a predicted value for the number of days taken to complete wound closure, crude and MKH treatments required 19 days, whereas both Coloplast and SSD treatments required 24 days (Table 4).

Thickness of granulation tissue: Compared to the other treatments, SSD had a larger difference (1.115 cm) in the thickness of granulation tissue from days 9 to 15 and crude keratose had the least difference (0.746 cm). Differences in all the treatment groups were not statistically significant (Figure 5).

5. Discussion:

Due to tissue necrosis and burn wound progression, deep partial thickness burns can convert to full thickness burns, thereby increasing risk to the patient, length of hospital stay, and the costs associated with treatment. At the early stages after burn injury, the processes of healing and wound progression are in opposition to each other, and most often progression prevails due to a lack of specific treatments that can address tissue loss at the level of cellular pathways. Currently there are no approved treatments that can control burn injury progression by regulating death signals within the cell. The standard of care is generally focused on avoiding infection while allowing the burn to take its natural course, followed by excision of dead tissue after some short time period to facilitate healing from the healthy periphery of the burn. However, the standard of care, SSD, did not perform as well as the other treatments in this study. One of the more striking findings was the variability in healing observed in this treatment group. Despite its beneficial antimicrobial properties [15, 16], the use of SSD as a standard of care has been questioned due to its reported interference with re-epithelialization [13, 14, 17, 18]. Tian et al.
suggested that the use of silver nano-particles as a more effective alternative to SSD [17].
Crystalline silver has also been shown to have beneficial wound healing properties [19], but to
our knowledge no comparative testing of these different forms of silver in the same burn model
have been published.

Assessment of digital images of wound areas did show that MKH had a significantly
greater effect on minimizing wound size at days 9, 12, and 15 compared to SSD, but not so
compared to crude keratose and Coloplast. Even though significant difference between MKH
and SSD was observed in the visual wound assessment, histological analyses have not shown
any significant difference at the time points 9, 12 and 15. This might be due to the parameters
considered when measuring a wound visually from digital images. Visually a wound is
considered to be re-epithelialized if it is covered with a thin pink or white opalescent non glossy
covering, but there is a chance that such a coating is present even in the absence of neo-
epidermis [20]. Additionally there is a higher chance for the fragile neo-epidermis to be
accidentally removed during the processing and sectioning of the tissue samples. In the present
study, percent re-epithelialization as determined using stained tissue sections showed that crude
keratose and MKH treated wounds were able to re-epithelialize faster compared to SSD,
especially at early time points where the rate for MKH was 220% higher than SSD. However,
these data were not statistically significant, largely due to the high standard deviation in the SSD
treatment group (the percent relative standard deviation was 131% for SSD versus 23% for
MKH; the R squared for the least squares linear regression of these data was 0.03 for SSD versus
0.54 for MKH). Later stages of re-epithelialization appeared to be similar in all treatment groups.

As expected, the injury model did demonstrate burn injury progression in all treatment
groups, but it was the crude keratose that showed the smallest increase in burn depth compared
to the other treatments. This finding may indirectly support our hypothesis as the crude keratose formulation contained the highest level of gamma keratose, which may be the reason that wound progression was relatively lower. However, this outcome measure also failed to demonstrate statistical significance.

Granulation tissue depth, a measure of later stages of healing, was highest in the SSD treatment group and minimal in the crude keratose treatment. However, this may have been due to the fact that this treatment group experienced the most wound progression, and consequently had the most extensive tissue loss. Higher amounts of granulation tissue may have been the normal response to tissue loss and not necessarily indicative of better healing. Regardless, the differences in this outcome measure were again found to not be statistically significant when comparing the different treatments.

In order to better understand the apparent lack of statistical power in our experimental design, power calculations were performed based on the re-epithelialization data. This showed that two more replicates would be required to show a significant (p<0.05) difference in re-epithelialization rate between MKH and SSD, and more than 100 replicates would be required to show a significant difference between MKH and crude keratose or Coloplast. These numbers point toward improvement in arguably the most important outcome in burn treatment, re-epithelialization, but they do not necessarily support the conclusion that the amount of gamma keratose was optimal, and/or that the route of administration was ideal in this study. As was shown in Chapter 2, the gamma keratose fraction contains large peptide molecules, and delivery of these compounds through the skin and especially through a thick layer of eschar, would be expected to be difficult and inefficient. Interestingly, Nunez et al. have shown that soluble keratose has vasodilation properties when administered intravenously or topically [21].
Considering that the zone of stasis is characterized by reduced blood circulation due to the clogging of blood vessels after burn injury [2], perhaps part of the mechanism of tissue sparing may be due to keratin’s ability to dilate blood vessels in the tissue surrounding the burn. Moreover, a different route of keratin administration such as intravenous injection may show even greater efficacy, as has been the case with other compounds tested in burn models. However, as discussed previously, local delivery is often preferred so a higher dose of gamma keratose, such as was demonstrated using crude keratose, may represent a viable solution. Regardless, a better understanding of the mechanism of action, optimal dose, and efficient administration of the most efficacious compounds will require more research.

6. Conclusion:

In this study, we tried to understand if a particular formulation of keratin hydrogel containing a reduced concentration of gamma keratose, MKH, could have a beneficial effect on burn wound healing. We compared MKH to the crude keratose that was previously shown to promote burn wound healing (Chapter 2) and that contained a fraction of protein, gamma keratose, which rescued thermally stressed cells (Chapter 2 and 3). The amount of gamma keratose was reduced in MKH to reflect the concentration that demonstrated efficacy in a cell culture model of thermal stress. A statistically significant difference in wound size was observed, but this did not translate to the histological measurements. The rate of early re-epithelialization appeared faster in the MKH treatment group, particularly at early time points where it was expected to have its greatest effect, but statistical significance was lost due to variance in the SSD treatment group. Regardless, the outcome measures in this study were not able to differentiate MKH (i.e. low gamma keratose concentration) from crude keratose (high keratose
concentration). More research to investigate the mechanism of keratins in burn wound healing, and more specifically in burn injury progression, are needed.

**Conflict of interest statement**

Mark Van Dyke, Ph.D. holds stock and is an officer in the company, KeraNetics LLC, which has provided partial funding for this research. Wake Forest Health Sciences has a potential financial interest in KeraNetics, LLC through licensing agreements.
References:


Figures and Tables:

Figure 1: Visual wound assessment

MKH has a significant difference compared to silver sulfadiazine (SSD) at days 9 (p<0.05), 12 (p<0.001) and 15 (p<0.05). Crude keratose has a significant difference compared to SSD on days 12 (p<0.001) and 15 (p<0.05) and coloplast has a significant difference to SSD days 9 (p<0.01) and 12 (p<0.01).
Figure 2: Burn depth

Vertical progression in burn depth was observed for all the treatments by day 6. Error bars indicate the variance in the data. Significant difference was not observed between the treatments.
Figure 3: Early time points rates of re-epithelialization

Burns treated with keratose (KOS) treatments demonstrated a general upward trend in re-epithelialization at early stages of treatment, whereas Coloplast and SSD generally trended downward. There was no statistically significant difference between treatment groups (n=6, mean +/- standard deviation, p>0.05)
Figure 4. Rates of re-epithelialization during later stages of wound healing

Compared to the other treatments, keratose (MKH and CrudeKOS) treatments promote faster re-epithelialization. There was no statistically significant difference between treatment groups (n=6, mean +/- standard deviation, p>0.05)
Figure 5: Thickness of granulation tissue

Silver sulfadiazine has a higher difference in the thickness of granulation tissue from day 9 to 15 compared to other treatments. No significant difference was observed between treatments.

Table 1: Percent increase in burn depth from day 3 to 6

Crude KOS showed the minimal increase in burn depth (5.57%) and coloplast showed the maximum increase in burn depth (22.59%). No significant difference was observed between the treatments (p>0.05).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Percent increase in burn depth from day 3 to 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKH</td>
<td>20.016%</td>
</tr>
<tr>
<td>Crude KOS</td>
<td>5.570%</td>
</tr>
<tr>
<td>Coloplast</td>
<td>22.590%</td>
</tr>
<tr>
<td>SSD</td>
<td>9.812%</td>
</tr>
</tbody>
</table>
Table 2. Rates of re-epithelialization at day 3 and day 6.

Compared to silver sulfadiazine, MKH has the highest difference in the rate of re-epithelialization in the early stages of wound healing (days 3 and 6).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate (% Re-Epithelialization/Day)</th>
<th>R Squared</th>
<th>% Difference vs. SSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coloplast</td>
<td>3.4 +/- 1.1</td>
<td>0.40</td>
<td>+160%</td>
</tr>
<tr>
<td>Crude KOS</td>
<td>3.4 +/- 1.2</td>
<td>0.35</td>
<td>+160%</td>
</tr>
<tr>
<td>MKH</td>
<td>4.1 +/- 0.96</td>
<td>0.54</td>
<td>+220%</td>
</tr>
<tr>
<td>SSD</td>
<td>1.3 +/- 1.7</td>
<td>0.03</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 3. Rates of re-epithelialization between days 9 and 15.

Compared to silver sulfadiazine, MKH has the highest difference in the rate of re-epithelialization in the later stages of wound healing (days 9 to 15).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate (% Re-Epithelialization/Day)</th>
<th>R Squared</th>
<th>% Difference vs. SSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coloplast</td>
<td>6.9 +/- 1.1</td>
<td>0.72</td>
<td>17%</td>
</tr>
<tr>
<td>Crude KOS</td>
<td>7.3 +/- 1.4</td>
<td>0.64</td>
<td>24%</td>
</tr>
<tr>
<td>MKH</td>
<td>7.6 +/- 1.2</td>
<td>0.72</td>
<td>29%</td>
</tr>
<tr>
<td>SSD</td>
<td>5.9 +/- 1.2</td>
<td>0.62</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 4. Days to wound closure.

Keratose (Crude KOS and MKH) treatments have a faster wound closure compared to coloplast and silver sulfadiazine.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Days to Wound Closure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coloplast</td>
<td>24.2</td>
</tr>
<tr>
<td>Crude KOS</td>
<td>19.5</td>
</tr>
<tr>
<td>MKH</td>
<td>19.2</td>
</tr>
<tr>
<td>SSD</td>
<td>23.4</td>
</tr>
</tbody>
</table>
CHAPTER – 6

DELAYED TREATMENT OF PORCINE DEEP PARTIAL THICKNESS BURNS
SUBJECT TO THERAPEUTIC KERATIN BIOMATERIALS

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This manuscript was not yet submitted for publication. Deepika Poranki assisted in the design and execution of the animal study and data analysis. Dr. Mark Van Dyke acted in an advisory and editorial capacity.
Abstract

Burn wound healing outcomes rely heavily upon the timely administration of first aid treatments and can affect the quality of later healing. The previously-reported wound healing capabilities of keratin biomaterials makes them a viable treatment option as they may be capable of influencing cell survival in the peri-burn region. In this study, the healing efficacy of deep partial thickness burns subject to keratin biomaterial treatments 10 hrs after injury was evaluated. While burn wound digital image examination suggested that keratin biomaterials enhance wound closure rates at 12 days post-injury, histological evaluation suggested that the standard of care silver sulfadiazine (SSD) encouraged faster reepithelialization rates for the first 15 days of the 30-day study. By Day 30, the extent of reepithelialization due to keratin biomaterial treatments was significantly greater in comparison to SSD (p≤0.001). Measurement and evaluations of additional endpoints during the second half of the study are needed to determine the true downstream healing efficacy of keratin biomaterials for the delayed treatment of deep partial thickness burn wounds.

Keywords: deep partial thickness burns, keratin biomaterials, delayed treatment, swine model, reepithelialization
1. Introduction

Burn wound healing outcomes rely heavily upon the timely administration of first aid treatments. Arguably the most precarious burn wounds to care for, deep partial thickness burns, are those in which damage extends through the epidermis and well into the reticular dermis. Unmediated treatment of these wounds is likely to result in burn wound progression, in which tissue damage moves radially from a centralized region and evolves into a full thickness burn condition. Within this centralized “zone of coagulation,” damage to blood vessels and dermal appendages result in an ischemic state that starts within 48 hrs of injury [1-3] and lasts up to 3-7 days post-injury [4, 5]. Fibroblasts, keratinocytes and affiliated dermal cells within the adjacent “zone of stasis” will succumb to either cell death effectively extending the zone of coagulation and increasing the size and severity of the wound or revitalization and proliferation based upon decided treatment modalities. Proper assessment of burn severity dictates the optimal course of treatment for challenging conditions encountered downstream such as contracture, hypertrophic scarring and microbial infection that often accompany full thickness burns.

Timely access to first aid burn treatments is often limited by physical proximity to appropriate medical personnel or facilities specializing in burn care. Burn victims are responsible for 45,000 hospitalizations in the US, 55% of who were transferred to specialized “burn units [6].” At the time of injury, the victim may be located hundreds of miles away from assistance, as often seen in rural communities nationwide or with members of the armed forces serving overseas needing specialized care. With a total of 128 self-reported burn centers (51 American Burn Association-verified) dispersed throughout the US, Canada and Australia [7], easy access by the multitudes of severely burned victims may not be easily attainable. While
Klein [7] reported that approximately 80% of the US lives within 1-2 hrs of ground or air transport of a verified burn center, many cover large geographic areas in which initial treatment is transferred to a closer local hospital for initial assessment. As such, actual transfer to a burn center may result in delays, ultimately leading to longer hospital stays, increased complications and possible mortality. Sheridan [8] reported that a 5-day delay in transporting pediatric burn victims with severe burns at >20% total body surface area (TBSA) burned to a designated burn center resulted in significantly greater incidences of bacterial infection, wound sepsis and longer wound closure times. MacKenzie [9] found that the in-hospital and one year mortality rates for patients seen at a trauma center vs. non-trauma center was significantly lower. A study conducted at the University of Washington Burn Center reported an average transport time post-injury of 7.2 hrs; initial assessments described wounds with a 17% TBSA [10]. In this study, the estimated burn size by non-trauma care personnel was overestimated, as TBSA of 15% is the threshold for fluid resuscitation needed.

According to the United States Army Institute of Surgical Research (USAISR) Burn Center, hand and facial burns were the most prevalent seen in military personnel, with approximately 73-76% of combat victims experiencing this type of injury within a 2-year timeframe [11-13]. As perhaps the most difficult regions of the body to treat, inadequate healing may lead to impaired functionality and in some cases removal from previous military duties [14]. From 2003 to 2007, transport time for injured soldiers from Iraq and Afghanistan to the USAISR was up to 4 days from time of injury to admission to the burn center [12]. While 5.6% of the victims died from their injuries at the USAISR, the ventilation support provided to over one-third of the victims in-flight likely attributed to the relatively low casualty rate. These studies suggest that preventative
measures taken to stabilize burns prior to more extensive treatments are crucial to limiting negative outcomes.

Spontaneous wound healing largely depends on the following conditions: a) systemic hydration via fluid resuscitation, b) local hydration of the wound bed to encourage reepithelialization and c) wound infection control. Microbial control may have the most critical local effects on progressive wound healing. Stander [15] suggests that microbial control within the wound bed be maintained if delayed transport to a burn center is over 12 hrs. D’Avignon [16] suggested the application of silver sulfadiazine (SSD) within 8 hrs of injury in combat-related burns. Topical application of SSD cream has long been considered the ‘gold standard’ for microbial control [17, 18]. In deep partial thickness burns, sweat glands and hair follicles may still be present in deeper layers of the dermis. If left untreated, gram-positive bacteria will migrate from these appendages to colonize the wound within 48 hrs post-injury [19, 20]. By 7-days post-injury, gram-positive and gram-negative microbes migrate from other regions in the body to inhabit the burn wound eschar, creating a ripe environment for infection [21-23]. Additionally, researchers suggest that the application of SSD along with gentle debridement is most effective in containing wound progression if patients cannot be transported for at least 72 hrs [17, 24]. While infection control is important, SSD alone is inadequate to keep the wound on the path of healing. In fact, some studies have reported that SSD has a deleterious effect on healing through exhibiting cytotoxic effects that delays wound closure. A study conducted by Fraser [25] suggested that keratinocytes grown in culture exhibited a significant reduction in cell number when exposed to SSD for 72 hrs.

An optimal first-line treatment for burns would be to encourage cell proliferation within the wound bed without causing further infection to the wound. Burns have been characterized as
having an exaggerated inflammatory phase [19]. Growth factor (i.e. transforming growth factor beta or TGF-β) infiltration into the wound bed accounts for the initial influx of fibroblasts during early wound healing, and is often responsible for scarring and contracture [26].

The first line of defense in burn care is the use of topical treatments. From a physiological standpoint, the primary goals of such treatments are to prevent further tissue loss and infection, and to promote spontaneous healing. In order for this to occur, revascularization of the wound must happen within a few days post-injury. One such benefit of topicals is their convenience and ease of use. This is especially important for victims to be able to treat themselves at home or on the battlefield. In lieu of SSD, biologic dressings that exhibit some bioactivity have been proven to enhance wound healing [27-31]. Common themes among these treatments are non-immunogenicity, biocompatibility, non-toxicity and biodegradability.

A potential wound healing treatment that embodies the aforementioned characteristics, as well as the added benefit of prompting no spontaneous proteolytic activity within the body, are keratin biomaterials. Keratin proteins derived from human hair have shown promise in a variety of regenerative medicine applications, and is comprehensively outlined in Rouse et al. [32]. Within cell culture models, keratin hydrogels encouraged cell adhesion and proliferation on par with collagen hydrogels [33]. Keratin dressings have also been shown to accelerate wound healing and closure through the stimulation of keratinocyte migration and proliferation along the wound margin within a porcine partial thickness model [34]. Previous studies conducted by our lab demonstrate enhanced wound healing rates in a swine contact burn model treated with a crude extract of keratin hydrogel (Chapter 2). Additionally, keratin hydrogel-treated chemical burns within a rodent model showed containment of the burn wound (Chapter 2). The present study was designed to test the healing efficacy of delayed treatment of deep partial thickness
burns with a crude keratose hydrogel and a modified keratose hydrogel (MKH) within a tightly controlled swine contact burn model. This delayed treatment scheme was conducted to compliment a standard treatment paradigm evaluated in our lab (Chapter 5) and more closely represents cases encountered by first responders in an attempt to provide initial care for burn wounds and prevent the need for more invasive surgical treatments.

2. Materials & Methods

2.1. Animal study logistics: This study was approved by the Wake Forest Animal Care and Use Committee. Fourteen female, Yorkshire swine (Baux Mountain, Winston-Salem, NC) weighing 20-25 kg at the time of surgery were used. The animals were housed in individual pens and allowed to acclimate to their surroundings for at least 7 days prior to surgery. Deep partial thickness burns were created on pairs of swine treated and monitored for 1, 3, 6, 9, 12, 15 or 30 days relative to the date of injury. The order in which these designated endpoints occurred were randomly selected. One day prior to surgery, the swine were cleaned and shaved along the dorsum. At this time, a fentanyl patch (50 mcg/h) was adhered to the shoulder or hip of each animal as an analgesic in preparation for the upcoming surgery. The animals were fasted the night before the administration of anesthetization for each procedure conducted throughout the study.

2.2. Burn surgery protocol: Details of the burn surgery protocol used were reported previously [35]. Briefly, the animals were anesthetized intramuscularly with a combination of ketamine (10 mg/kg) and dexmedetomidine (0.05 mg/kg), intubated and sustained on isofluorane during the surgical procedure. Vitals, such as blood pressure and body temperature, were closely monitored during surgery to ensure animal safety. Three cm diameter wounds were created using an innovative burn system in which a
custom-made, pressure-controlled burn device housed a detachable 360 grade brass block. As the designated heat source, the brass block was immersed in a boiling negative azeotropic mixture of 80:20 polyethylene glycol (M_n = 400; Sigma-Aldrich, St. Louis, MO):deionized water to ensure proper heat transfer from the source to the animal. For each wound, the brass block made contact with the animal’s skin for 20s. Six wounds were created on each side of the dorsal mid-line, between the shoulder and hip. A total of 24 wounds were created, 12 per animal (Figure 1). The wounds were allowed to set for 1 hr prior to being covered with saline-soaked gauze and bandaged as described previously [35]. For the Day 30 endpoint, black dots were tattooed in the 4 corners surrounding each wound as a means to monitor contracture over time. Post-surgical care consisted of the intramuscular administration of atipamezole (0.05 mg/kg) to reverse the sedative effects of the dexmedetomidine and careful monitoring during recovery.

2.3. Bandage changes: A typical bandage change occurred approximately every 3 days. Under general anesthesia, all bandaging was removed and the wounds were lightly debrided with saline-soaked gauze. Digital images of each wound were captured (Nikon D60, Nikon, Melville, NY); a gray scale (Kodak, Rochester, NY) was included in the field of view for image optimization along with a ruler to ensure proper scaling during analysis. After treatment application, the animals were re-bandaged and monitored during recovery. Saline was administered intravenously every other bandage change to ensure proper systemic hydration. A new fentanyl patch (75 mcg/h) was placed on each animal for pain management.
2.4. Delayed treatment: Test and control treatments were randomly applied to wounds over the swine pair for each endpoint. Test treatments consisted of Crude and modified keratose formulations of keratin-based hydrogels, which will be referred to as Crude KOS and MKH throughout the paper. Keratin proteins were extracted from a commercial source of human hair via oxidative methods as described in de Guzman [36]. Crude keratose was further separated into alpha and gamma keratose fractions by isoelectric precipitation. Concentrated hydrochloric acid was added dropwise to the crude keratose solution with stirring until a pH 4.2 was reached. At this pH, the alpha keratose precipitates leaving the acid-soluble gamma fraction in solution. The insoluble alpha keratose was separated by fixed angle centrifugation at 1500 rpm for 15min at 4°C. After neutralizing the gamma-containing supernatant to pH 8.4, it was dialyzed against endotoxin free water using a 5kDa spiral wound cartridge (Millipore, Billerica, MA) for 5 volume washes. Finally, the solution was concentrated to minimal volume, pH adjusted to 7.4, lyophilized, and sterilized by gamma irradiation at 1 MRad prior to use. The precipitated alpha keratose was re-dissolved in 0.1M sodium hydroxide (NaOH) solution and dialyzed against endotoxin free water using a 5kDa spiral wound cartridge (Millipore) for 5 volume washes. Finally, the solution was concentrated to minimal volume, pH adjusted to 7.4, lyophilized, and sterilized by gamma irradiation at 1 MRad prior to use. Crude KOS and MKH formulations were sterilized via gamma irradiation (2 MRad). Under sterile conditions, powdered formulations were reconstituted in saline and agitated overnight at 37°C to form 15 weight% hydrogels. Control treatments consisted of a collagen-based hydrogel wound dressing indicated for the treatment of superficial and partial thickness burns (Woun’Dres™ Collagen Hydrogel, Coloplast,
Minneapolis, MN) and 1 wt% silver sulfadiazine (SSD) cream (Watson Pharmaceuticals, Parsippany, NJ), long considered the standard of care for burn injuries [18]. All topical treatments were transferred to 10 cc syringes for controlled application, with approximately 2 cc applied to each wound. Treatment was delayed for approximately 10 hrs post-surgery and administered under general anesthesia. The animals were then bandaged and allowed to recover as previously stated. All hydrogel treatments were applied for 15 days, and SSD for 9 days, and was replaced by saline-soaked gauze for protection during additional healing.

3. Data analysis

3.1. Digital Image Capture: Digital images of the burn wounds at progressive stages of healing were captured on surgery day and at every subsequent bandage change and endpoint. The margin of each wound was traced using Image J software (NIH, Bethesda, MD); the area of the open wound was calculated. For each series of wounds corresponding to an endpoint, the average area value was normalized to the surgery day area value. The data set was analyzed via 2-way ANOVA and Bonferroni post hoc analyses using GraphPad Prism (GraphPad Software, La Jolla, CA). Additionally, the series of Day 30 wounds were evaluated for contracture where the circumference of the “boxed” area around each of the wounds was measured by connecting the dots using Image J. The data set was analyzed using 2-way ANOVA and Bonferroni post hoc analyses using GraphPad Prism.

3.2. Histological analysis: At each specified endpoint, a pair of swine was euthanized and each of the wounds was harvested in full as reported previously [35]. Briefly, the wounds were cut into quadrants and fixed in 10% neutral buffered formalin for 48 hrs.
A 2-cm thick slice from the innermost portion of the quadrant of each wound was sampled and paraffin processed as previously reported. The slice represented the region of the wound with the greatest degree of damage. The tissue samples were embedded in paraffin and sectioned into 5 µm thick slices. One tissue section was evaluated per wound, representing a sample size of 6 for each treatment. Sections stained with hexatoxylin and eosin (H&E) were used to qualitatively access burn depth and the relative density of granulation tissue. The extent of reepithelialization was calculated by subtracting the length of the non-epithelialized burn region of the tissue sample from the measured radius of the corresponding surgery day digital image of the wound to reveal the length of the reepithelialized region. The length of the reepithelialized region was normalized to the surgery day wound radius to determine the % reepithelialization. All measurements were taken with Image J. Each data set was analyzed via linear regression up to Day 15. At Day 30, the data was analyzed via 1-way ANOVA and Bonferroni post hoc analysis. All statistical evaluations were done using GraphPad Prism.

4. Results

4.1. Digital Image Capture: An approximate 10% increase in measured wound size for up to 6 days post-injury was observed for all treatments, followed by a steady decrease to <25% of the original wound size by Day 30 (Figure 2). The fastest rates of size reduction were seen between Days 9 and 12. The healing patterns among treatments were not significantly different (p>0.05) over the course of 30 days, except for significant differences in the extent of healing in wounds treated with the keratin hydrogels vs. SSD (p<0.001) and Coloplast vs. SSD (p<0.05) at Day 12. In measuring the progressive
occurrence of wound contracture, the mean distance surrounding each wound expanded in all treatment groups within the first 6 days of injury (Figure 3). Thereafter, these “borders” steadily contracted to near baseline with some variation at the later time points. There was no significant difference in the degree of wound contracture among treatment groups at any specified time point (p>0.05). On average, MKH and Coloplast exhibited the least amount of contracture over 30 days of healing, while Crude KOS and SSD exhibited slightly higher amounts.

4.2. Histological analysis:

**Burn Depth:** Representative images of the degree of tissue damage incurred as a result of a deep partial thickness burn wounds at Day 1 post-injury are depicted in Figure 4. In all cases, the epidermis had been completely destroyed, leaving a gradient of dermal damage relative to the proximity of the heat source throughout the depth of the tissue. The upper to mid dermal regions revealed fully coagulated tissue, as defined by the damaged appendages (e.g., hair follicles, sweat glands) and the absence of distinct collagen bundles and blood vessels. In the lower dermal regions, next to the adipose layer of skin, was dermis that incurred a slightly lesser degree of damage than the upper layers. While distinct collagen bundles were present, they were loosely packed and show evidence of potentially early coagulation. The depth of burn damage remained relatively constant over all treatment groups.

**Inflammatory Cell Infiltration:** At Day 6 post-injury, inflammatory cells infiltrated the wound margin as evidenced by the purple bands of nuclei depicted in Figure 5. For SSD-treated wounds, evidence of this band is barely visible and appears to be hidden within
the adipose layer. The deposition of collagen and new blood vessel formation within interstitial spaces along and below the lower dermis encouraged cells from fatty layer to disperse and spread throughout the entire dermal region. Coagulated tissue initially found toward the top of the dermal layer eventually sloughed off, leaving behind extensive granulation tissue by Day 12 (Figure 6). Lower densities of granulation tissue were observed in the Crude KOS and SSD treated wounds relative to the other treatments.

**Re-epithelialization:** Minimal amounts of regenerated epithelium were present up to Day 6 post-injury. Within the first 6 days of treatment, rates of re-epithelialization among treatment groups showed a general downward trend hovering at 20% or lower (Figure 7). The MKH treatment showed the greatest stability after Day 3 at approximately 10% wound closure. Re-epithelialization has progressed to the greatest degree in wounds treated with SSD as indicated by a sharp initial slope to Day 3 and at least a 40% greater re-epithelialization rate as compared to that of the keratin treatments and 28% compared to Coloplast (Table 1). Although healing is generally inconsistent across replicates as indicated by the large error bars, the tightest control is seen at Day 3 with the SSD treatment. Overall, no statistically significant difference in healing rate was observed between treatment groups (p>0.05).

Re-epithelialization rates during the later stages of healing indicated an upward trend among all treatment groups, with the most linear progression demonstrated by MKH (Figure 8). SSD maintains the fastest re-epithelialization rate from Days 9 to 15, which is 40% greater than that of the MKH and Coloplast treatments and 15% greater than Crude
KOS (Table 2). Healing was more consistent across replicates in comparison to the earlier stages of wound healing as evidenced by the smaller error bars, although the tightest control in this case was demonstrated by the Crude KOS treatment. Qualitatively, well-organized, multilayered epithelium was present with all treatments at Day 12 (Figure 9). Distinct rete ridges were seen in the Coloplast and SSD treatments. In the keratin treatments, the papillary dermis appears to be loosely packed in comparison to other treatments and may be indicative of mechanical weakness. No statistically significant difference in healing rate observed between treatment groups was reported (p>0.05). The degree of re-epithelialization shifts dramatically at Day 30 post-injury as the hydrogel treatments exhibit significantly greater wound closure than SSD at p≤0.001 (Figure 10).

5. Discussion

Delays in the administration of adequate first aid treatments for burn wounds may have a deleterious effect on downstream wound healing processes. In our study, keratin biomaterial treatments were applied to deep partial thickness burns at 10 hrs post-injury in order to evaluate a more realistic scenario during which potential complications that may arise from delayed burn care. The 10-hr threshold was chosen based on discussions with burn care clinicians, logistical constraints for conducting the study within our animal care facility and the desire to inflict minimal stresses onto the animals during the study.

Quantitative analyses of the burn wound digital images showed significant decreases in wound size over time in all treatment groups (Figure 2). Wound closure along the burn surface is a function of the migration and proliferation of keratinocytes originating from healthy dermal tissue along the wound margin and appendages. The keratin biomaterial treatments showed the
greatest reduction in wound size at Day 12 post-injury in comparison to SSD. From a histological standpoint, there was no significant difference in the degree of re-epithelialization over time between treatment groups (Figure 7). Interestingly, the data exposes a slightly greater re-epithelialization rate in SSD between Days 9 and 12 in comparison to all other treatment groups (Table 2). While in direct conflict with recent reports on the stagnated closure of burn wounds when treated with SSD as mentioned previously, its potential usefulness in healing cannot be ignored [37, 38]. In the case of the present study, the latter concept was evident at Days 9-15. To address the apparent discrepancy between the statistically significant wound size evaluation and non-significant histological evaluations, attention must be brought to the integrity of the reepithelialized layer [39]. While the hydrogel materials may have encouraged a greater degree of re-epithelialization at Day 12 as demonstrated by the wound digital images, perhaps portions of the re-epithelialized layers as defined by migrating keratinocytes were lost during histological processing [40]. Mechanical stresses incurred during gross tissue preparation or paraffin block sectioning may be contributing factors. The multilayered appearance of the emerging epithelium and presence of distinct rete ridges and density of the papillary dermis may be considered in determining epithelial integrity. The representative H&E images of the Coloplast- and SSD-treated burns at Day 12 (Figure 9) showed a more robust papillary dermis to support epithelial growth as compared to the other treatment groups. Interestingly, wound closure as exhibited by the hydrogel treatments was significantly greater than that of SSD by Day 30 (Figure 10). This suggests that perhaps the keratin and Coloplast treatments showed the greatest activity at time points between Days 15 and 30, and that accurate re-epithelialization rates cannot be determined without more definitive data from this timeframe.
Beneath the open wound, granulation tissue at various stages of remodeling was present at Day 12 (Figure 6). An abundance of cells from the fatty layer was dispersed within the granulation tissue with the Coloplast and MKH treatments. Evidence of densely-packed collagen deposits with relatively smaller regions of cells from fatty layer dispersed was seen with the Crude KOS and SSD treatments. The latter condition suggests that these treatments may be further along in the wound healing process, creating a dermal foundation of greater integrity to support closure of the open wound.

Up to Day 6 post-injury, the influence of all treatments appears to be minimal. After a slight increase in the degree of re-epithelialization at Day 3, all treatment groups trended downward upon reaching Day 6. This was likely due to the formation of wound eschar requiring cleaning at each bandage change prior to treatment application. Although careful and deliberate debriding techniques were used, it is possible that growing epithelium at the interface between the eschar and healthy wound margin was removed as well. Overall, the lack of significant differences between treatment groups raises the question of how well, or if, penetration of the topical treatments occur through the eschar to more centralized regions of the burns [41]. Additionally, H&E images indicate relatively consistent initial burn depths across treatments at Day 1 and a similarly-emerging presence of an inflammatory cell band to begin the processes of removing damaged tissue and debris from the wounds and collagen deposition.

In contrast to a similar burn study conducted at our lab in which treatments were applied 1 hr post-injury, the treatment of deep partial thickness burns after 10 hrs with keratin biomaterials for enhanced wound healing prior to Day 15 cannot be validated based on the outcomes of this study. In the former case, the mean rates of re-epithelialization during the earlier (up to Day 6) and later (Days 9-15) endpoints were 220% and 29% greater than those for
SSD, respectively (Chapter 5). The current study demonstrated outcomes in favor of SSD up to Day 15, with a striking end result of significant wound closure using Crude KOS, MKH and Coloplast by Day 30. While our surgical protocol was fully qualified to produce consistent deep partial thickness burns and the wounds were randomly treated as indicated in Figure 1, the thickness and microstructure of the underlying tissue along the dorsum of the swine may have also introduced variation that was exposed in our results. Additional endpoints between Days 15 and 30 post-injury needed to provide greater insight into the re-epithelialization activity during the second half of the study.
References


Figures and Tables:

Figure 1. Schematic of randomized placement of treatments. The dorsal of a pair of swine is depicted, with burn wounds color-coded to represent the type of treatment applied. Replicates are numbered from 1 to 6 to represent a sample size of 6 for each treatment group.
**Figure 2. Extent of burn wound size reduction over time.** At Day 12, wounds treated with Crude KOS and MKH exhibited significantly greater healing than those treated with SSD (p<0.001). Additionally, wounds treated with Coloplast exhibited significantly greater healing than those treated with SSD (p<0.05) at this time point. Otherwise, there was no statistically significant difference between treatment groups (n=6, mean +/- standard deviation, p>0.05)
Figure 3. **Degree of wound contracture as a function of time.** There was no statistically significant difference between treatment groups (n=6, mean +/- standard deviation, p>0.05). On average, MKH and Coloplast exhibited the least amount of contracture over 30 days of healing, while Crude KOS and SSD exhibited slightly higher amounts.
Figure 4. **H&E images of deep partial thickness burn depth at Day 1.** Representative images of burned dermal tissue subject to A) Coloplast, B) Crude KOS, C) MKH  D) SSD treatments at Day 1. Fully coagulated tissue is labeled in the upper to mid dermal region, while gradients of thermal damage is depicted by arrows within the lower dermal regions. The extent of dermal damage remains consistent at this end point.

Figure 5. **H&E images of deep partial thickness burns at Day 6.** Representative images of burned dermal tissue subject to A) Coloplast, B) Crude KOS, C) MKH  D) SSD treatments at Day 6. Emerging band of inflammatory cells is depicted by arrows within the mid to lower dermal regions. Cell infiltration in the SSD-treated wounds is barely present in the adipose region.
Figure 6. **H&E images of deep partial thickness burns at Day 12.** Representative images of granulation tissue subject to A) Coloplast, B) Crude KOS, C) MKH  D) SSD treatments at Day 12. Fatty tissue that has been dispersed through the dermal region by the deposition of collagen via fibroblastic activity is indicated, with scant regions present in the Crude KOS- and SSD-treated wounds.
Figure 7. Initial rates of re-epithelialization in deep partial thickness burns. All wounds treated at 10 hrs post-burn demonstrated a general downward trend in reepithelialization at early stages of treatment, with the greatest stability observed after Day 3 using MKH. Healing was generally inconsistent across replicates as indicated by the large error bars, with the greatest consistency observed at Day 3 using the SSD treatment. There was no statistically significant difference between treatment groups (n=6, mean +/- standard deviation, p>0.05).
Figure 8. Rates of re-epithelialization in second degree burns during late stages of healing. All treatments demonstrated an upward trend in healing. Healing was generally more consistent across replicates as indicated by the relatively smaller error bars compared to early stages of healing (see Figure 7). There was no statistically significant difference between treatment groups (n=6, mean +/- standard deviation, p>0.05).
Figure 9. H&E images of deep partial thickness epithelium at Day 12. Representative images of emerging epithelium subject to A) Coloplast, B) Crude KOS, C) MKH D) SSD treatments at Day 12. All images show relatively well-organized, multilayered epithelium. Well defined rete ridges are seen in the Coloplast and SSD treatments. The papillary dermis (as indicated by arrows) for all treatments except Coloplast and SSD appear to be loosely packed due to potential mechanical stress or the density of adipose tissue.

Figure 10. Degree of reepithelialization at Day 30 post-burn. Wounds treated with Coloplast, Crude KOS and MKH KOS exhibited significantly greater healing than those treated with SSD by Day 30 (n=6, mean +/- standard deviation, p≤0.001).
Table 1. Initial rates of reepithelialization between Days 0 and 6. SSD exhibited a rate of 1.9 ± 1.4 % re-epithelialization/day within the first 6 days of injury, at rates of 28% greater than Coloplast, 42% greater than Crude KOS and 48% MKH.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate (% Reepithelialization/Day)</th>
<th>R²</th>
<th>% Difference vs. SSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coloplast</td>
<td>2.6 +/- 1.7</td>
<td>0.12</td>
<td>-28%</td>
</tr>
<tr>
<td>Crude KOS</td>
<td>2.1 +/- 1.2</td>
<td>0.17</td>
<td>-42%</td>
</tr>
<tr>
<td>MKH</td>
<td>1.9 +/- 1.4</td>
<td>0.10</td>
<td>-48%</td>
</tr>
<tr>
<td>SSD</td>
<td>3.6 +/- 1.0</td>
<td>0.44</td>
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</table>

Table 2. Rates of reepithelialization between Days 9 and 15. SSD exhibited a rate of 5.7 ± 0.78 % re-epithelialization/day between Days 9 and 15, at rates of 43% greater than Coloplast, 15% greater than Crude KOS and 41% MKH.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate (% Reepithelialization/Day)</th>
<th>R²</th>
<th>% Difference vs. SSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coloplast</td>
<td>3.2 +/- 1.1</td>
<td>0.37</td>
<td>-43%</td>
</tr>
<tr>
<td>Crude KOS</td>
<td>4.8 +/- 1.0</td>
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<td>-15%</td>
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<td>MKH</td>
<td>3.4 +/- 1.1</td>
<td>0.36</td>
<td>-41%</td>
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<td>SSD</td>
<td>5.7 +/- 0.78</td>
<td>0.77</td>
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CHAPTER 7

SUMMARY, CONCLUSIONS AND PROSPECTIVE RESEARCH
SUMMARY

The overall goal of this dissertation work was to test the wound healing properties of human hair derived keratin biomaterials. Keratins represent a class of biomaterials that have been used in a variety of biomedical applications [1-9], but the understanding of how keratin proteins function in these applications is only at its nascent stages. Keratin biomaterial hydrogels possess all the physical attributes of a burn wound dressing like providing a moist environment, acting as a protective barrier and absorbing excess wound fluid. As part of this work, pilot in vivo studies in mice and swine showed that an oxidized form of keratin biomaterial termed “keratose hydrogel” was able to promote faster wound healing by preventing an initial increase in wound area that often accompanies a burn (known a burn wound progression). This keratose hydrogel was provided as a heterogeneous mixture of proteins representing “alpha” and “gamma” fractions of keratin. These different fractions have disparate properties including molecular weights, amino acid content, and solubility characteristics and can be separated by techniques such as dialysis or isoelectric precipitation. Burn progression is mainly due to tissue necrosis and therefore, we hypothesized that the interaction of keratin with burned tissue would promote cell survival and reduce the total body surface area burned. The preceding chapters provide insight into the differential wound healing activity associated with keratin fractions (alpha and gamma) and the molecular mechanism by which the oxidized form of keratin biomaterial promotes cell survival after thermal injury.

The first research aim, as presented in Chapter II, identified the keratose fraction that displayed differential activity in salvaging thermally stressed cells. An in vitro thermal stress model similar to Jackson’s burn model was developed in which a large number of cells died from
the initial heat treatment, additional cells died within hours after heating, and surviving cells re-established the culture. Using this in vitro thermal stress model, the efficacy of different keratose preparations (alpha, gamma and crude keratose) in cell survival was tested. Our results showed that gamma keratose was better able to maintain cell viability compared to crude keratose, alpha keratose, and fibroblast growth media. As this is the first study to show that this keratose fraction has particular efficacy for cell salvage after thermal stress, mass spectrometry analysis was performed to understand the composition of proteins present in the gamma keratose fraction. Mass spectrometry analysis results suggested that gamma keratose was a degraded protein product of alpha keratose.

Chapter III provided an in depth analysis of the potential cell survival mechanism(s) when gamma keratose treatment was given. Using the same thermal stress model (developed in Chapter II), we attempted to understand whether gamma keratose induces differential expression of cell death pathway genes as a possible mechanism of its apparent cell salvage activity. RT² Profiler™ PCR array (specific to cell death pathways: apoptosis, necrosis and autophagy) was used to investigate gene expression in thermally stressed cells treated with gamma keratose dissolved in the media compared to cells treated with fibroblast growth media only. Our results showed that gene expression was more significant (i.e. smaller p values) in cells treated with gamma keratose compared to cells treated with fibroblast growth media at the early time point post thermal stress. Additionally, we observed that treating the thermally stressed cells once with gamma keratose was able to maintain cell viability by down regulating the genes involved in apoptosis, necrosis and autophagy.
The creation of highly-reproducible burn wounds is essential in the assessment of healing progression in therapeutic models. Chapter IV explains in detail the development of a porcine deep partial thickness burn model. This model offered control over variability through two simple means, a burn device that creates operator-independent pressure, and a heating system that ensures consistent and uniform heating of the brass blocks. Heating the brass blocks with an azeotropic solution of polyethylene glycol (PEG) and water created deeper burns compared to boiling water only. Using the same in vivo burn model, a modified keratose hydrogel (MKH) that contained a reduced amount of gamma keratose was tested for its wound healing properties along with crude keratose, collagen hydrogel and silver sulfadiazine. Results showed that MKH promoted faster re-epithelialization and wound closure compared to the other treatments. To demonstrate potential clinical relevance and real-world practicality, a delayed treatment study was performed with the same treatment groups wherein treatment was delayed 10 hours, thereby representing transportation and admission to a burn unit. Results showed that MKH was still able to promote faster re-epithelialization compared to the other treatments, but not with the same efficacy as more immediate treatment.

LIMITATIONS AND PROSPECTIVE RESEARCH

Further investigation of the composition of the gamma keratose fraction by gel electrophoresis and mass spectrometry identified degraded alpha keratose proteins with different molecular weights. As gamma keratose showed differential activity in cell salvage after thermal stress, further work needs to be done to elucidate whether the differential activity is associated with a single excised band or multiple alpha keratose peptides. Additionally, the interaction of gamma keratose with cells needs to be further investigated to better understand the mechanism of
cell survival (for example, how do(does) the alpha keratose peptide(s) “communicate” with the cell?). Ultimately, this knowledge will help to develop second generation keratin biomaterials for wound healing.

Gamma keratose showed an overall down regulation of the genes involved in cell death pathways (apoptosis, necrosis and autophagy). Autophagy, unlike apoptosis and necrosis involves cell survival mechanism by maintaining normal cellular function during nutrient deficient conditions or by removing damaged organelles and aggregated proteins [10, 11]. Therefore, additional molecular studies need to be performed to understand whether autophagy helps in survival of thermally stressed cells when treated with gamma keratose. Also, in vitro studies have shown that in stressed conditions like starvation and energy deprivation, cell death occurs through autophagy when other cell death pathways (like apoptosis) are inhibited [12-14]. Further work needs to be done to understand if the down regulation of apoptotic and necrotic genes is triggering the activation of the autophagic pathway. In addition, the effect of gamma keratose on other skin cell types (e.g. endothelial cells, keratinocytes) should be investigated to gain insight into the total wound healing mechanism(s) of gamma keratose.

In the in vivo studies we performed, there were some limitations that need to be addressed in future work. Although an in vivo burn model using a burn device and an azeotropic mixture solution successfully created highly consistent deep partial thickness burns, 3 cm burns were not critical size wounds that convert to full thickness if proper treatment was not provided. To study whether keratose treatment is able to prevent the conversion of the zone of stasis region to necrotic tissue and further limit burn progression, using the contact burn comb model would have provided more definite information. Moreover, a non-spontaneously healing model could
have been used in which full wound closure would only happen if autografting is performed. In addition, the experimental design for the in vivo studies used 24 hours as the earliest time point for euthanasia, but according to Clark et al. cellular necrosis in the zone of stasis occurs between one and four hours and apoptosis is responsible for cell death only after 24 hours. Therefore, including early time points within hours after creation of the burn would provide more insight into the tissue salvage mechanism of gamma keratose. Another interesting facet of this research that should be investigated relates to the fact that the zone of stasis is characterized by reduced blood circulation due to the clogging of blood vessels after burn injury [15]. Nunez et al. have shown that alpha keratose and KAPs (referred to as crude keratose in this work) had vasodilation properties when administered intravenously or topically [1]. Future investigations utilizing keratin as an IV administered fluid may therefore be worth pursuing. In addition, performing immunohistochemical staining specific to blood vessel may provide more insight into the role of blood vessel plugging (early time points), or even new blood vessel formation (later time points) when keratin treatment is given. Like many other compounds, there is likely a limit to the beneficial dose of gamma keratose. In vitro studies in the lab have shown that very high concentrations of gamma keratose (i.e. >10mg/mL) can be detrimental for thermally stressed cells. Determining an optimum ratio of alpha and gamma keratose fractions in pilot studies and then using that ratio to study burn wound healing may have provided more statistical difference between treatment groups. Finally, determining the optimum time in which keratose treatment can promote tissue salvage would be important information for clinical application as most changes at the cell and molecular level are irreversible.
CONCLUSIONS

In summary, this dissertation work outlines the importance of keratose biomaterial in the treatment of burns. In vitro studies demonstrated that the gamma keratose fraction was able to promote survival of thermally stressed cells by down regulating the genes involved in cell death pathways. In vivo studies showed that keratose biomaterial was able to promote faster re-epithelialization and wound closure in deep partial thickness burns. Although further work is needed to understand the specific mechanism(s) of wound healing in both in vitro and in vivo studies, particularly those related to burn wound progression, the results so far have indicated the potential benefit of using keratin biomaterial in burn therapy.
References


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ABSTRACTS – PODIUM PRESENTATIONS


ABSTRACTS – POSTER PRESENTATIONS


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