Topically Applied Metal Chelator Reduces Thermal Injury Progression in a Rat Model of Brass

Comb Burn

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Abbreviations: reactive oxygen species (ROS), lipid aldehydes (LA), 4-hydroxynonenal (HNE), malonaldehyde (MDA) and acrolein (ACR), Aldehyde dehydrogenase (ALDH), ethylenediaminetetraacetic acid (EDTA), methyl sulfonyl methane (MSM), livionex formulation(LF)

Abstract

Oxidative stress may be involved in the cellular damage and tissue destruction as burn wounds continues to progress after abatement of the initial insult. ***Since iron and calcium ions play key roles in oxidative stress, this study tested whether topical application of Livionex formulation (LF) lotion, that contains disodium EDTA as a metal chelator and methyl sulfonyl methane (MSM) as a permeability enhancer, would prevent or reduce burn injury.***

Methods: We used an established brass comb burn model with some modifications. Topical application of LF lotion was started 5 minutes post-burn, and repeated every 8 hours for 3 consecutive days. Rats were euthanized and skin harvested for histochemistry and immunohistochemistry. Formation of protein adducts of 4-hydroxynonenal (HNE), malonadialdehyde (MDA) and acrolein (ACR) and expression of aldehyde dehydrogenase (ALDH) isozymes, ALDH1 and ALDH2 were assessed.

Results: LF lotion-treated burn sites and interspaces showed mild morphological improvement compared to untreated burn sites. Furthermore, the lotion significantly decreased the immunostaining of lipid aldehyde-protein adducts including protein -HNE, -MDA and -ACR adducts, and restored the expression of aldehyde dehydrogenase isozymes in the unburned interspaces.

Conclusion: This data, for the first time, demonstrates that a topically applied EDTA-containing lotion protects burn injury progression with a concomitant decrease in the accumulation of reactive lipid aldehydes and protection of aldehyde dehydrogenase isozymes. Present studies are suggestive of therapeutic intervention of burn injury by this novel lotion.

Keywords: thermal injury, burn progression, iron chelation, brass comb burn, oxidative stress, reactive aldehydes, wound healing

Introduction

A typical burn wound was initially described to have three concentric zones [1]. The central zone is the region of coagulation that undergoes irreversible necrosis as a result of direct injury from heat energy. The outer zone is the region of hyperemia which invariably recovers. The intermediate zone correlates with stasis that does not initially undergo necrosis, but experiences complete cessation of blood flow within the first 24 hours. The intermediate zone can consequently become necrotic and eventually indistinguishable from the zone of coagulation [1, 2]. The natural history of the zone of stasis brought about the concept that tissue destruction in an untreated burn wound continues to progress even after abatement of the initial burn insult [1, 2]. Clinically, the injury progression is seen as the expansion of the necrotized wound to adjacent unburned areas. Microscopically, the burn progression is observed to increase in its burn depth. An increase in burn depth can be demonstrated by the progression of a deep partial-thickness burn into a full-thickness burn [1, 2]. During the past several decades, extensive efforts have been made to explore the various mechanisms that are responsible for burn progression [3, 4].

Oxidative stress is one of the most important mechanisms proposed to be responsible for burn injury progression [3-6]. It is well documented that major burns are accompanied with a significant local and systemic release of inflammatory cytokines such as IL-6 and TNF- α , as well as with the release of chemokines such as IL-8 [7]. These bioactive inflammatory molecules, in turn, generate a subsequent cascade of reactive oxygen species (ROS) [8-10]. Aside from their protective effects, the generated ROS may also induce vital cellular injuries via lipid peroxidation [11-13]. The polyunsaturated fatty acids (PUFAs) in cell membranes are especially susceptible to ROS activity, yielding of reactive lipid aldehydes

(LA) such as 4-hydroxynonenal (HNE), malondialdehyde (MDA) and acrolein (ACR) [14, 15]. Although the mechanisms by which the metals initiate LA production is not entirely understood [16], trace metals such as iron and copper are recognized as effective catalysts in the initiation of LA production [17,]. In addition, our previous studies have demonstrated that the topical application of metal chelators such as EDTA (ethylenediaminetetraacetic acid) ameliorated oxidative and inflammatory markers in rat models of glaucoma [18] and diabetic cataract [19].

The current study was designed to test our hypothesis that the topical application of a lotion containing an iron chelator might prevent or reduce burn injury progression in a modified brass comb burn model in rats. The lotion, formulated by Livionex Inc (Los Gatos, CA), is referred to as the “Livionex Formulation”, or “LF lotion”. It consists of two components: EDTA disodium as the active, chelating agent, and methyl sulfonyl methane (MSM) as a permeation enhancer. EDTA has been shown to penetrate the cornea, conjunctiva, and iris/ciliary body from a topically applied dose (Grass et al, 1985). Additionally, previous studies from this laboratory have shown that MSM dramatically increased C¹⁴-EDTA penetration into rat eye. When topically applied in conjunction with MSM, C¹⁴-EDTA penetrated various rat ocular tissues, including the aqueous zone, cornea, lens, vitreous and retina plus choroid. This indicated the feasibility and efficacy of delivering the active chelating agent to specific, intended tissue sites. [20]. ***The mechanism of how MSM increases permeation is still unclear, however we hypothesize that it may interact with the cell membrane and modulate the tight junctions between cells, allowing molecules to pass between adjacent cells [20].*** The modified brass comb model provides “burn sites” that represent the classic central zone of “coagulation” and unburned “interspaces” that depict the intermediate zone of “stasis”. The model produced a burn size of about 2% of the total body surface area (TBSA), which limits the impact that

massive systematic factors such as hypoperfusion, hypoxemia and infection can have on local injury progression. We evaluated and compared pathological characteristics of the “burn sites” and the “interspaces” to determine the effect that the lotion had on injury progression. We then carried out immunohistochemistry (IHC) labeling of known reactive aldehydes (HNE, MDA, ACR) in order to determine the effect of topical application of the metal chelator on lipid aldehyde production. Aldehyde dehydrogenases (ALDH1A1 and ALDH2) efficiently detoxify these aldehydes [21-24] and therefore play an important role, to combat toxicity ,especially under oxidative stress [20, 21, 25, 26]. Expression of ALDH1 and ALDH2 was evaluated in order to demonstrate the effect of LF lotion on the production of reactive aldehydes through maintaining cellular defense abilities against LA production after burn injury.

MATERIALS AND METHODS

LF lotion

The lotion was provided by Livionex Inc (Los Gatos, CA) and consists of two generally regarded as safe (GRAS) components: EDTA disodium as the chelating agent, and methyl sulfonyl methane (MSM) as a permeability enhancer.

Animals

Male Sprague–Dawley rats weighing ~350 gm were obtained from Charles River Laboratories International, Inc. The rats were individually housed with a 12-hour light–dark cycle (lights on at 07.00 h, off at 19.00 h) in a temperature- and humidity-controlled environment. Each rat was given a standard diet ad libitum for at least a week before experiment. The rats weighed ~420 gm at the initiation of the experiment. All animal manipulations were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston. Housing and care of the rats met the National Research Council guidelines.

Brass comb

The brass comb model (Fig. 1A) used in the present study has three 10-mm teeth separated by two 10-mm (instead of 5-mm) notches and is a modified version of the Regas and Ehrlich model [27]. This brass comb produced a rectangular space consisting of three 10x19 mm burned sub-rectangles separated by two unburned 10x19 mm sub-rectangles (Fig. 1B). While the burn sites represent the zone of coagulation, the unburned interspaces represent the zone of stasis or ischemia.

Experimental protocol

The rats were anesthetized with an intraperitoneal injection of 90 mg/kg of ketamine (10%) and 10 mg/kg of xylazine (2%). The dorsum of each rat was completely shaved with an electrical clipper. A rectangle of 19x50 mm was bilaterally marked between the caudal end of scapula and rostral end of ilium. The rectangle was about 10 mm distal and parallel

to the dorsal middle line. Three of the anesthetized animals were randomly selected to serve as controls (CTR) and received no further insult. The other rats received thermal injury. The brass comb was preheated in boiling water (100°C) for 3 minutes and applied with minimal pressure for 30 seconds on the pre-marked rectangle on one side. This led to 3 burn sites separated by 2 unburned interspaces (Fig 1B). The brass comb was reheated and similarly applied to the other side of the back, leaving bilateral comb burn wounds on the back of each rat. After the burn procedure, animals were observed, given oxygen, and placed into cages after full recovery from the anesthesia. The rats were subsequently randomly divided into two groups: burn alone with no further treatment (Burn), and burn with topical LF lotion application (Burn+LF). ***A bundle of three cotton swabs was employed to carry the lotion (~1ml) and carefully lay it onto each burn wound including burn sites and unburned interspaces. The burn wounds were not covered by bandage or gauze.*** Lotion application was started 5 minutes post-burn, and repeated every 8 hours for 3 days. All animals were treated with 0.01 mg/kg buprenorphine (IM) for the first 24 hours after the burn to relieve pain and discomfort. Animals were euthanized by decapitation 72 hours after the burn injury followed by harvesting of skin samples. ***In our initial studies on the placebo groups (no burn+carrier lotion and burn+carrier lotion) showed no effect of the carrier lotion on the skin morphology (data not shown)***

Harvesting samples

Burned wounds were sampled immediately after the decapitation of each animal. Two skin tissue blocks were harvested from each rectangle of the wound by placing cut lines parallel to one side and perpendicular to the other side of the rectangle. In total, four skin tissue blocks were collected from each animal resulting in 12 blocks per group. Each skin tissue block was 9mm x 30mm (10mm burn site + 10mm interspace+10mm burn site) (Fig 1B). All skin tissue blocks were fixed in 10% neutral buffered formalin. All four skin blocks from each animal were embedded in one cassette with paraffin and checked to

ensure that the side of each tissue block was evenly laid on the bottom. Embedded tissues were cut into 5- μ m sections with each containing four segments of skin tissues. The sections were then kept at -20°C until further processing for histochemistry and immunohistochemistry.

Histochemistry and immunohistochemistry (IHC)

For histochemistry, frozen formalin-fixed paraffin-embedded skin sections were de-paraffinized, rehydrated and stained with Hematoxylin-Eosin (Hematoxylin Stain Harris Formulation, cat. #SL90-16 and Eosin Y, Cat. #SL98-16, StatLab, McKinney, TX) and Masson's trichrome (Cat. #HT15-1KT, Sigma-Aldrich Corporation, St. Louis, MO).

For immunohistochemistry, frozen formalin-fixed paraffin-embedded skin sections were de-paraffinized, rehydrated and treated with citrate buffer. Sections were then washed with 0.01M PBS, quenched for 10 minutes in 3% hydrogen peroxide/methanol, washed with PBS, and blocked for 1 hr with blocking solution (3% normal goat serum/2% BSA/0.1% cold fish skin gelatin/0.1% Triton X-100/0.05% Tween 20/0.05% Sodium azide in 0.01M PBS). The sections were washed again, blocked and then incubated overnight at 4°C with the primary antibodies listed below. After being washed with PBS, the sections were incubated for 90 minutes with secondary biotinylated goat anti-rabbit or goat-anti-mouse antibodies, washed, and processed in the dark for 1 hour with ABC reagents (standard Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA). After washing with PBS, the sections were developed with a mixture of DAB substrate (brown) (Vector Laboratories, Burlingame, CA) followed by counterstaining with 0.5% methyl green. Determination of the optimal concentration of primary antibody and negative control was carried out at the beginning of each IHC experiment. In the negative control, the primary antibody step was omitted. The primary antibodies tested in the present study include: rabbit anti-4 hydroxynonenal (protein-HNE) (#HNE11-S, Alpha Diagnostic International, San Antonio, TX), rabbit anti-malondialdehyde (protein-MDA) (#ab6463, abcam, Cambridge, MA), mouse monoclonal

anti-acrolein (protein-ACR) (#ab48501), rabbit anti-ALDH1A1 (#ab52492, abcam, Cambridge, MA), and rabbit anti-ALDH2 (#ab108306, abcam, Cambridge, MA).

All histochemical (H&E and Masson's trichrome staining) and immunohistochemical stained skin sections were visualized by using an Olympus BX53 digital microscope. Images were acquired and morphometric measurements were carried out by using the Olympus CellSense program.

Measurements and microscopic scoring

Burn injury progression to interspaces was monitored by microscopic measurement of the length (mm) of survived interspace epidermis on H&E-stained sections. Survived epidermis was determined by the existence of continuous viable epidermal cells observed under a higher power objective (20x). The length was measured under a lower power objective (2x) by the aid of the Olympus CellSense Program. The survived interspaces of all four skin segments from each animal were measured and averaged into one value for each animal.

Burn injury progression in burn sites was defined by scoring the microscopic depth of vessel blockade and, necrosis of epithelial and endothelial cells [29]. The depth of the vessel blockade was defined as the vertical (from epidermal basement) length of visibly dilated venules and/or arterioles filled with denatured clots. The depth of the necrosis of epithelial and endothelial cells was defined as the vertical location of nuclear pyknosis. The scores of the depth of vessel blockade and cell necrosis were determined as follows: 0 for no lesion at all; 1 for lesions limited at or above epidermis; 2 for lesions extended down to dermis but above the bottom of hair follicles; 3 for lesions extended down to the hypodermis but above skeletal muscle; 4 for lesions extended down to skeletal muscle; 5 for lesions extended down below the skeletal muscle. The scoring was performed in the middle of both burn sites for each segment, resulting in 8 scores for each animal. Each animal's scores were then averaged to one value for that animal.

The burn depth progression in the burn sites was also evaluated by scoring the microscopic depth of collagen discoloration via Masson trichrome staining. Scoring was defined as follows: 0, indicated no discoloration; 1, discoloration limited within the first half of dermis; 2, discoloration extended down below hair follicles; 3, discoloration extended down through 25% of the hypodermis; 4, discoloration extended down through 50% of the hypodermis; and 5, the discoloration extended down throughout the entire hypodermis.

The measurements and microscopic scoring were carried by two operators one of them was blinded to the group designation. Each of the microscopic scoring was recorded when the two operators agreed on that scoring.

Statistical analysis

For the “interspaces”, each segment of skin sample had one measurement and yielded four measurements per animal, resulting in 12 data values per group (n=3). For “burn sites”, each segment of skin sample had two measurements, yielding eight measurements per animal and a total of 24 data values per group (n=3). Each animal's specific measurement values or scores were averaged into one value for each animal. Quantitative group data was presented as the mean $\pm$ SD. One-way ANOVA in conjunction with Tukey's post hoc test was used to stratify and determine differences among groups by using Prism from GraphPad (San Diego, CA). An unpaired t-test (two-tailed) was used to determine differences between two groups at a time. Differences were considered significant at $p < 0.05$.

RESULTS

Burn size and wound appearance

The burn sizes (% of total body surface area (TBSA)) were 2.05 ± 0.03 and 2.02 ± 0.02 in the Burn and Burn+LF, respectively. There was no significant difference in the TBSA between these groups ($p > 0.05$). TBSAs were calculated according to Meeh's formula ($TBSA = kW^{2/3}$) with a k constant of 9.83 [30].

At 72 hours post burn injury the burn wound (with no lotion treatment) had its zone of necrosis expand from the burn sites to the unburned interspaces (Fig. 2B). This led to an increase in the area of the burn sites, and subsequently a marked decrease in the size of the two interspace rectangles when compared to the same wound 5 min after the injury (Fig. 2A). After 72 hours, the burn treated with LF lotion (Fig. 2D), displayed sizes of both burn sites and unburned interspaces similar to those of the same wound at 5 minutes after the injury (Fig. 2C). This suggests that the LF lotion blocked the expansion of burn wound necrosis while preserving the unburned interspaces.

Microscopic characteristics of burn sites

On H&E stained sections, the untreated burn sites harvested 72 hours after thermal injury showed the following typical microscopic characteristics: 1) the entire epidermis was necrotic, lost or remained (Figs. 3A-B, 4B); 2) the cell necrosis featured nuclear pyknosis of all cell types and extended from the superficial epidermis down to the skeletal muscles (Figs. 3A, 4B); 3) the lumen of the vessels in the capillary loops and subpapillary plexus was completely obliterated and filled with denatured blood clots (Fig 3A-C, 4B); 4) scattered inflammatory cell infiltration appeared in the interstitial spaces around the vessels above or below the skeletal muscles (Figs. 3A, 3C, 4B); 5) collagen discoloration (denaturation) extended from the dermis down to the hypodermis (Fig. 4B); 6) all skeletal muscles under the necrotic epidermis were destructed and discolored (Figs. 3A, 3C, 4B); 7) all the pathological changes listed above including cell necrosis, vessel blockade, inflammatory cell

infiltration, collagen denaturation and muscle destruction and discoloration appeared to progress to the interspaces leading to much deeper damaged area of the burn sites compared to the interspaces between them; and 8) skin layers were thinner than that of the middle of the interspaces due to tissue contraction of the burn sites or swelling of the interspaces or both (4B).

The LF lotion-treated burn sites showed an irrefutable improvement specifically in the hypodermal, muscular and sub-muscular levels. There was less vessel blockade (Fig. 3D vs. 3A, Fig. 3F vs. 3C), less skeletal muscle damage (Fig. 3D vs. 3A), less endothelial necrosis (Fig. 3F vs. 3C), and some survival of hair follicle and sebaceous gland epithelial cell (Fig 3E vs. 3B). The epithelial necrosis score of 2.7 ± 0.2 in the LF lotion-treated group was significantly lower than that of the burn sites in the untreated burn group (3.0 ± 0.0) (Table 1, $p < 0.05$). The scores of endothelial necrosis and vessel blockade (Table 1) of the LF-treated burn sites were also significantly ($p < 0.05$) lower than their corresponding scores in the burn sites of untreated animals.

Masson trichrome staining showed that in the control rat skin (Fig. 4A) the skeletal muscle was red and the dermal and hypodermal collagen was blue. In the burn sites, however, the collagen turned red (indicating denaturation) and the skeletal muscle was discolored and destructed (Fig. 4B). Interestingly, vessel blockade with denatured clots was found to be surrounded by relatively intact collagen (blue) (Fig. 4B). The LF lotion-treated burn sites showed substantially less collagen discoloration and less vessel blockade as well (Fig. 4C vs. 4B). The score of the microscopic depth of collagen discoloration in the LF lotion-treated burn sites was significantly lower than that of the untreated burn sites (2.1 ± 0.4 vs. 2.8 ± 0.1 , $p < 0.01$, Table 2).

The unburned interspaces

The untreated interspaces showed a different histology profile from that of untreated burn sites: 1) A certain length of untreated interspaces survived with normal epidermal histology (Fig 5A-B), but the length of survived epidermis was significantly shorter compared to the burn site of either side (Fig. 6B); 2) the middle of the intact interspaces showed no obviously tissue necrosis (Fig. 5B) but the part of the interspace adjacent to burn sites showed epidermal necrosis (Fig. 7B); 3) the middle of the interspaces showed no vessel blockade with denatured clots but exhibited dilation and congestion of the vessels in the capillary loops and subpapillary plexus (Fig 5A-C); 4) the middle of interspaces showed muscle destruction and discoloration (Fig 5A, 6B, 7B) even though its extent of damage was less severe than that in the burn sites (Fig. 5A vs. 3A); 5) the middle of interspaces showed severe inflammatory cell infiltration around vessels and in interstitial spaces in hypodermal and sub-muscular areas comparing to that in the burn sites (Fig 5A-C vs. 3A-C); 6) the whole area of the interspace showed certain degree of collagen discoloration (Fig. 7B).

When compared to the untreated interspaces, the LF lotion-treated interspaces showed a marked decrease in skeletal muscle destruction and discoloration (Fig. 5D vs. 5A), vascular dilation and congestion in the capillary loops and subpapillary plexus (Fig. 5D-F vs. 5A-C), and inflammatory cell infiltration around dilated and congested vessels in the hypodermal regions (Fig. 5F vs. 5C). The LF lotion-treated interspaces also showed obvious less collagen discoloration in dermis and hypodermis area marked decrease in skeletal muscle destruction and discoloration (Fig. 7C vs. 7B). LF lotion-treated interspaces also showed longer length of survived epidermis (Fig. 6C) when compared to that of the burn alone sections (Fig 6B). The measured microscopic length of survived interspace epidermis in the burned wound was 3.76 ± 1.46 mm, while in the Burn + LF it was 5.78 ± 1.05 mm- an over fifty percent improvement in the depth of the burn wound with LF lotion treatment ($p < 0.05$) (Table 1). This data indicates the protective effect that the LF lotion had on mitigating the burn injury progression into the unburned interspaces.

As shown in Fig 8A, skin sections from control animals showed moderate protein-HNE immunoactivity in epidermal, hair follicle and sebaceous gland epithelial cells. We also observed HNE immunoreactivity in the mesenchymal cells of the skeletal muscles (not shown). Light immunoactivity was also found in the vascular endothelial cells of the patent vessels in the hypodermis (Fig. 8D) and below. The center of the burn sites with or without topical LF lotion application showed minimal immunoactivity of protein-HNE in all cellular structures, and revealed no difference among the two groups (image not shown). In comparison with the control sections, the middle of untreated interspaces revealed more intensive immunoactivity in the epidermal, hair follicle and sebaceous gland epithelial cells (Fig. 8B) and in the vascular endothelial cells (Fig. 8B, 8E). Vessels with enhanced endothelial protein-HNE staining include dilated capillaries (Δ) and dilated and congested veins ($\blacktriangle$), which were found from dermis (Fig. 8B) to hypodermis (Fig. 8E). The LF lotion-treated interspaces displayed much less protein-HNE immunoactivity in the epidermal, hair follicle, sebaceous gland epithelial cells (Fig. 8C vs. 8B), and in the vascular endothelial cells (Fig. 8F vs. 8E), indicating an antioxidative effect of LF lotion in burn injury prevention.

The control skin exhibited very little protein-MDA immunoreactivity in the hair follicle epithelial cells and vascular endothelial cells, and had light staining in the epidermal and sebaceous gland epithelial and muscle cells (Fig. 9A). The center of the burn sites of untreated animals were negative for protein-MDA staining (image not shown). The untreated interspaces (Fig. 9B), however, showed a significant increase in the protein-MDA immunoreactivity present in epidermal, hair follicle and sebaceous gland epithelial cells, as well as in skeletal muscles, but not in the endothelial cells (not shown). The center of the LF lotion-treated interspaces had a similar protein-MDA immunohistochemistry profile to that of the control skin (Fig. 9C vs. 9A). Immunohistochemical staining for protein-ACR revealed similar results implying that the lotion prevented oxidative damage associated with the burn injury.

Local IHC expression of ALDH1 and ALDH2

Control animal skin showed slight immunoreactivity of ALDH1 in the cytoplasm of sebaceous gland. In contrast, there was much more ALDH1 immunoreactivity in the hair follicle epithelial cells, and specially in the hair bulbs and shafts (Fig. 10A). There was no detectable ALDH1 immunoreactivity in the epidermal layer, the vascular endothelial cells of the dermis, the hypodermal areas, and the skeletal muscles. The burn sites of the untreated burn wound showed no detectable ALDH1 immunoreactivity in hair follicle cells and all other cellular components (data not shown). The untreated interspaces exhibited the similar immunoreactivity in sebaceous gland cells but very little activity in hair follicle cells (Fig. 10B) when compared to that in the control skin group (Fig. 10A). LF-treated interspaces, however, retained almost the same amount of ALDH1 immunoreactivity in the hair follicle cells as those in the control sections (Fig. 10C vs. 9A). Immunoreactivities in some of the epidermal cells was also present (Fig. 10C).

ALDH2 IHC staining in control sections showed moderate to intense immune activities in all cell types in the rat skin including epidermal, sebaceous gland and hair follicle epithelial cells (Fig. 10D). This is also similar to ALDH1 activity in all of the mesenchymal cells, including skeletal muscle and endothelial cells (images not shown). This is different from that of ALDH1A1 staining in controls. Untreated burn sites showed little or no ALDH2 activity in all of the cellular structures (not shown). The untreated interspaces exhibited a slightly reduced ALDH2 immunoactivity in SG and hair follicle cells (Fig. 10E) when compared to that in control skin. LF-treated interspaces show recovery of ALDH2 immunostaining in the hair follicle cells when compared with the activity in the untreated interspaces (Fig. 10F vs. 10E).

Discussion

The role of oxidative stress in burn injury progression has been extensively recognized [3-5] and for the last two decades efforts have been made to investigate and establish interventions aimed at modulating oxidative stress-induced secondary damages to burn wound.. Some of these studies are focused on lipid peroxidation inhibition [31], platelet activating factor antagonism [32], xanthine-oxidase inhibition [8, 33, 34], and treatment with hydroxyl radical scavengers and anti-oxidant enzymes themselves or the enzyme stimulator (melatonin) [33, 35, 36]. Meanwhile, some other studies reported treatment with rosiglitazone, a agonist of PPAR- γ (peroxisome proliferator-activated receptor gamma) [37], pretreatment of curcumin with antioxidant and antiapoptotic properties [38], and supplement of esterified glutathione (GSH) [39], vitamin C [40, 41] or α -tocopherol with vitamin E activity [42]. Our current study further focused on therapeutic intervention aiming to block the formation of free radicals with a metal chelator. Redox active metals such as iron (Fe), copper (Cu), chromium (Cr), cobalt (Co) and others have been proposed as important players in the processes of producing reactive radicals by undergoing redox cycling reactions [43]. Among them, iron, the most studied redox active metal is no doubt an effective catalyst in the process of lipid peroxidation (LA) although the mechanism by which iron initiates LA is not defined entirely [16]. Iron chelators such as EDTA and deferoxamine (DFO) are able to bind both Fe(II) and Fe(III) [44], making EDTA as one of the choices for iron chelation therapy. Although EDTA along with a permeability enhancer has been locally applied on rat eyes to prevent diabetic cataract [19], the current report is the first attempt to use an EDTA-containing lotion (LF lotion) on burn wound to evaluate the capacity of this metal chelator in preventing or reducing burn injury progression. The lotion is a combination of disodium EDTA and MSM (methyl sulfonyl methane) as a permeability enhancer that facilitates the delivery of EDTA (18-20). Our results demonstrate that local application of LF lotion to the brass comb - burn area improved the pathological changes in the burn sites that

occurred 3 days later as shown in Figures 2-7 and summarized in Table 1. The improvement included decreases in the depth of endothelial and epithelial cell necrosis, collagen denaturation, vessel blockade and skeletal muscle damages as compared to that of the untreated burn sites. More impressively, our data further showed obvious morphological improvements in the unburned interspaces. Compared to the untreated interspaces, the lotion-treated unburned interspaces showed a decrease in the degree of skeletal muscle destruction and discoloration, number of vascular dilation and congestion in the capillary loops and subpapillary plexus and inflammatory cells around dilated and congested vessels in the hypodermal regions. Especially, the treated interspaces showed longer measured microscopic length of survived interspace epidermis. These results demonstrate that early topical application of the LF lotion not only reduced burn injury progression down to deeper layers in the burn sites, but also limited the expansion of burn the injury from burn sites to unburned interspaces.

As one of the featured findings, the current study provides evidence for the first time that thermal injury produced excessive amount of protein adducts of the three reactive aldehydes tested, -HNE, MDA and ACR in the interspaces of the burnt injury. These adducts appeared lightly stained in the epithelial cells (epidermal, hair follicle and sebaceous gland) of the control specimen but moderately or extensively labeled in the epithelia of untreated interspaces (Figures 8-10). Thermal injury in this model also demonstrated marked increase in immunological expression of these protein-aldehyde adducts in hypodermal vascular endothelial cells in the untreated interspaces as compared to that of controls. This finding is a novel addition to the literature of causal relationship of oxidative stress and burn injury [45-48]. More importantly, the current study reports for the first time that early topical application of the metal chelator markedly decreased burn-induced expression of the protein-aldehyde adducts in all cellular structures examined of the unburned interspaces. (Figs 8-10). The underlying mechanism must be that iron or metal

chelation directly blocked or limited the formation of potent free radicals through Fenton reaction and Harbor-Weiss reaction [16]. Accordingly, iron chelation with EDTA indirectly reduced or blocked the production of the reactive aldehydes at the initiation step of both classic (autooxidation) and alternative paradigms of lipid peroxidation [11]. Interestingly, the lotion-treated unburned interspaces showed lower immunoactivities of protein-HNE, -MDA and -ACR adducts in epithelial cells than that in control epithelial cells, indicating that iron chelation with EDTA not only blocked injury-induced production of reactive aldehydes but also reduced normal formation of these aldehydes. This result demonstrates the effectiveness of early topical application of EDTA as an iron chelator. It also suggests that early topical application of EDTA could be an alternative approach to reduce or prevent injury progression in burn wounds.

The reactive lipid aldehydes, as compared to free radicals, are metastable end products of lipid peroxidation, that are able to travel and form adducts with various cellular molecules such as proteins, DNA and lipids far from their site of origin. HNE, for example, has reigned in the field of reactive aldehydes research since its discovery as a peroxidation product of *n*-6 PUFSs [49]. This lipid aldehyde is electrophilic and lipophilic, and contains three functional groups including an α,β -unsaturated carbonyl bond, that can undergo several reactions including Michael addition and Schiff base formation with biomolecules containing amino and thiol group to form adducts [50]. In most cases, the adduction of a protein by HNE impairs its function. Targeted HNE adduction has been reported to be able to inhibit or inactivate various enzymes (oxidoreductases, transferases, hydrolases, lyases, and isomerases), membrane proteins (carriers, receptors, ion channels, transporters) and cytoskeletal proteins [50]. Among the various signaling targets of HNE, are cell cycle regulators such as, c-Jun NH₂-terminal kinase (JNK), p38 mitogen-activated protein kinases (p38MAPK), , protein kinase C- β and - δ (PKC- β and PKC- δ) [49]. Oxidative stress/HNE can inactivate and down regulate ALDH and induce toxicity; conversely activation and

upregulation of ALDH prevents oxidation-/HNE-associated damage in cells and tissues [18-19,21-23,25,26,52-55] Our current study, for the first time, demonstrates that thermal injury induced a long lasting decrease in the expression of two of ALDH1A1 and ALDH2 in the untreated unburned interspaces. The decreased expression of these enzymes may be a result of direct effect of heat energy itself or other inflammatory factors released after thermal injury. However, it is reasonable to propose that the decrease of ALDH1A1 and ALDH2 were the direct result from thermal injury-induced excessive production of LA because topical application of LF lotion rescued expression of these enzymes while blocked formation of the LA. These results also suggest that ALDHs might be the targets of LA although it is not explored in this study how thermal injury-produced excessive LA decrease the expression of these enzymes. The human ALDH superfamily consists of 19 putatively functional genes with distinct chromosomal locations and the ALDH enzymes catalyze the NAD(P)⁺-dependent irreversible oxidation of a wide spectrum of endogenous and exogenous aldehydes including LA [51]. Among the ALDHs, ALDH1A1, ALDH2 and ALDH3A1 all catalyze LA reactive aldehydes, HNE, MDA and ACR. A rapid decrease of ALDH3A1 in the corneal at both the RNA and protein levels was recorded after alkali burn of the corneal surface [52]. Activation of ALDH2 with an allosteric agonist, Alda-1 was reported to significantly reduce protein- HNE adducts accumulation in radiation-induced dermatitis [53], and reduced ischemia heart damage in a rodent model [54]. In the present study, the burn-induced decrease of ALDH1A1 and ALDH2 immunoactivities was accompanied with an increased accumulation of LA, suggesting a failure of the defense mechanism in response to LA /oxidative stress. LF lotion-treated interspaces showed recovery of the immunoactivities and indicating a function of iron chelation by LF lotion in restoring the defense mechanism against LA /oxidative stress following burn injury.

In summary, the current study exhibited for the first time that thermal injury induced a marked increase in the expression of protein adducts of reactive aldehydes including HNE,

MDA and ACR and significant decrease in the expression of ALDH1A1 and ALDH2 in the unburned interspaces of a brass comb rat model as examined 72 hours postburn. Early topical application of LF lotion improved thermal injury-induced histology changes in the burn sites and especially the unburned interspaces. It is proposed that early iron chelation blocked the formation of free radicals and in turn prevented the production of reactive aldehydes. It is the reduced production and increased detoxification of the reactive aldehydes that limited the destruction of ALDHs and led to the histology improvement in the unburned interspaces. These initial results suggest that LF lotion could be a new therapeutic intervention to manage acute burn wound. Further studies are warranted.

Table 1 Measurements and microscopic scoring of the pathology of interest in burn sites

Measurements	Control (n=3)	Burn alone (n=3)	Burn plus LF Lotion (n=3)
Length of survived interspace epidermis ^a (mm)	N/A	3.76±0.84	5.78±0.6*
Depth of collagen discoloration ^{b,c}	0±0	2.8±0.1	2.1±0.4**
Depth of vessel blockade ^{b,d}	0±0	4.9±0.1	3.9±0.7*
Depth of epithelial necrosis ^{b,d}	N/A	3.0±0.0	2.7±0.2*
Depth of endothelial necrosis ^{b,d}	N/A	5.0±0.0	4.5±0.3*

Data are presented as the mean ± SD. N/A, not measured.

^a, The interspaces of all four skin segments from each animal were measured and averaged into one value to represent the animal.

^b, All eight burn sites from four skin segments from each animal were measured and averaged into one value to represent the animal.

^c, Scoring microscopic depth of collagen discoloration: 0, no discoloration at all; 1, discoloration limited within the first half of dermis; 2, discoloration extended down to the level of the bottom of hair follicles; 3, discoloration extended down through 25% of the hypodermis; 4, discoloration extended down to 50% of the hypodermis; 5, lesion extended down through 100% of the hypodermis.

^d, Scoring microscopic depth of vessel blockade and necrosis of epithelial and endothelial cells: 0, no lesion at all; 1, lesions limited at or above epidermis; 2, lesions extended down to the dermis but above the bottom of the hair follicles; 3, lesions extended down to the hypodermis but above skeletal muscle; 4, lesions extended down to the skeletal muscle; 5, lesions extended down below the skeletal muscle.

*, p < 0.05; ** p < 0.01, unpaired t-test (two-tailed)

Figure Legends

Fig 1. *A*, Brass comb probe consisting of three (3) 10-mm teeth separated by two (2) 10-mm notches modified from the previous Regas and Ehrlich model (1992). *B*, Diagram of the bottom view of the modified brass comb consisting of three (3) 10x19 mm rectangles of burn sites separated by two (2) 10x19 mm rectangles of interspaces. The second diagram is of the tissue sampling in each comb burn wound; two tissue blocks (9 x 30 mm each) were harvested.

Fig 2. Representative photographs of the burn wounds: *A* (5 min after injury) and *B* (72 hrs after injury). *C* (5 min after injury) and *D* (72 hrs after injury) with LF lotion treatment. Note that at 72 hrs the LF lotion-treated wound (*D*) showed a size of unburned interspace similar to that of the same wound 5 min after burn injury (*C*), while the untreated burn wound (*B*) had a significantly smaller interspace when compared to that of the same wound 5 min after burn (*A*).

Fig 3. Representative H&E stained microphotographs of the burn sites: Middle of burn sites showing microscopic characteristics of burn wound without (*A-C*) or with (*D-F*) LF lotion treatment started 5 min post injury. *B* and *C*, higher power of the upper and lower insert boxes in *A*, respectively. *E* and *F*, higher power of the upper and lower insert boxes in *D*, respectively. SkM, skeletal muscle; SG, sebaceous gland; HF, hair follicles; $\triangle$, dilation and congestion of capillaries, venules and arterioles; $\blacktriangle$, blocked vessels filled with denatured clots; *, inflammatory cell infiltration. $\blacklozenge$, Scale bar = 200 μ m in *A* & *D*; 50 μ m in *B*, *C*, *E*, *F*

Fig 4. Representative microphotographs of Masson's trichrome staining of burn sites, 72 hrs post injury: *A*, Control, without burn; *B*, the middle of a burn site, burn alone; *C*, the middle of a burn site, burn plus LF lotion treatment post injury. SkM, skeletal muscle; SG, sebaceous gland; HF, hair follicles; ▲, blocked vessels filled with denatured clots; *, areas with inflammatory cell infiltration. Scale bar = 200 µm in *A-C*

Fig 5. Representative H&E stained microphotographs of the interspaces: Middle of interspaces showing microscopic characteristics without (*A-C*) or with (*D-F*) LF lotion treatment. *A-C*, 72 hrs post injury; *D-F*, 72 hrs post injury plus LF lotion treatment. *B* and *C*, higher power of the upper and lower insert boxes in *A*, respectively. *E* and *F*, higher power of the upper and lower insert boxes in *D*, respectively. SkM, skeletal muscle; SG, sebaceous gland; HF, hair follicles; △, dilation and congestion of capillaries, venules or arterioles; *, inflammatory cell infiltration. Scale bar = 200 µm in *A & D*, 50 µm in *B,C, E,F*

Fig 6. Representative H&E microphotographs of survived interspace epidermis: *A*, control without burn; *B*, 72 hrs post injury; *C*, 72 hrs post injury plus LF lotion treatment. Yellow arrow line marks the length of survived interspace epidermis. SkM, skeletal muscle; SG, sebaceous gland; HF, hair follicles; ▲, blocked vessels filled with denatured clots; ↓, necrotic epidermis. Scale bar = 500 µm in *A-C*

Fig 7. Representative Masson's trichrome staining of the half interspace (4 mm from its middle line). *A*, control without burn; *B*, 72 hrs after burn; *C*, 72 hrs after burn plus LF lotion treatment post injury. SkM, skeletal muscle; SG, sebaceous gland; HF, hair follicles; ▲, blocked vessels filled with denatured clots; ↓, necrotic epidermis. Scale bar = 500 µm in *A-C*

Fig 8. Representative protein-HNE IHC microphotographs of interspaces : The methyl green counterstained IHC microphotographs show epidermis and dermis (A-C) and hypodermis (D-F). A & D, control, without burn; B & E, 72 hrs post burn; C & F, 72 hrs after a burn plus LF lotion treatment post injury; Ep, epidermis; SG, sebaceous gland; HF, hair follicles; ↓, patent vessels (D, F); △, dilated capillary in dermis (B); ▲, dilated and blocked (E) vessels in hypodermis. Scale bar = 100 µm (A-F)

Fig 9. Representative protein-MDA IHC microphotographs of interspaces: The methyl green counterstained IHC microphotographs show protein-MDA staining in the interspaces of A, control, without burn; B, , 72 hrs after burn; C, 72 hrs after burn plus LF lotion treatment post injury. Ep, epidermis; SG, sebaceous gland; HF, hair follicle. Scale bar = 50 µm (A-C)

Fig 10 Representative ALDH1A1 and ALDH2 IHC microphotographs of interspaces: The methyl green counterstained IHC microphotographs show immunostaining of ALDH1A1 (A-C) and ALDH2 (D-F) in sebaceous gland (SG) and hair follicle (HF) epithelial cells. A & D, Control, without burn; B & E, 72 hrs after burn; C & F, 72 hrs after burn plus LF lotion treatment post injury. Ep, epidermis; SG, sebaceous gland; HF, hair follicle. Scale bar, 100 µm (A-F)

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