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Original Article

Physiologic and molecular changes in the tracheal epithelium of rats following burn injury

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Abstract: Pneumonia is the leading complication in the critical care of burn victims. Airway epithelial dysfunction compromises host defense against pneumonia. The aim of this study is to test the hypothesis that burn injury alters the physiology of the airway epithelium. A rat model of 60% TBSA third degree scald burn was used. At 24 hours after injury, tracheal epithelial ultrastructure was studied using transmission electron microscopy (TEM) and proliferation was measured by Ki67 immunohistochemistry. Mucociliary clearance (MCC) was measured using fluorescent microspheres. The level of malondialdehyde (MDA), an indicator of lipid peroxidation, was also measured. Changes in epithelial mRNA expression were measured using microarray. Burn injury led to a ten-fold reduction in MCC that was statistically significant ($p = 0.007$) 24 hours after injury. No significant change was noted in the morphology of tracheal epithelial cells between groups, although a marginal increase in extracellular space was noted in injured animals. Ki67 nuclear expression was significantly reduced (25%, $p = 0.008$) in injured rats. There was a significant increase in MDA levels in the epithelial lysate of burned animals, $p = 0.001$. Microarray analysis identified 59 genes with significant differences between sham and injured animals. Burn injury altered multiple important functions in rat tracheal epithelium. The decrease in MCC and cell proliferation may be due to oxidative injury. Mechanistic studies to identify physiological processes associated with changes in airway function may help in designing therapeutic agents to reduce burn-induced airway pathogenesis.

Keywords: Burn, airway, gene expression, oxidative stress, lipid peroxidation

Introduction

Although pneumonia is the leading cause of morbidity during the critical care of burn victims [1], very little information is available on how physiological changes due to burn injury may alter the innate defense properties of the airway epithelium. Through mucociliary clearance (MCC) and its antibacterial secretions, functions of the airway epithelium are essential for maintenance of a sterile lung environment. The airway surface layer (ASL), that contains antibacterial peptides, mucus, glucose, electrolytes and water, is tightly regulated and influenced by many physiological processes [2]. Thus conceptually, the profound perturbations caused by burn injury have the potential to alter the physiology of the airway epithelium, which may impair airway innate defense.

Support for the concept that burn injury impairs upper airway function can be seen in autopsy tissue of burn victims. In a review of 62 autopsy cases, distal migration of upper airway mucus into the small airways and parenchyma was evident in all cases [3]. Normal airways continuously move upper airway mucus toward more proximal airways and ultimately into the oropharynx. Interestingly, distal migration of upper airway mucus was seen in multiple victims of scald burns in whom there was no exposure to toxic smoke. Furthermore, in a more recent study using systematically sampled autopsy tissues, extensive obstruction of bronchioles was common in virtually all cases regardless of the presence of smoke inhalation injury, or 36% of the cases showed bacterial invasion into the airway mucosa [4]. These studies identify a significant problem related to burn pathophysiology that has received little investigation, and an area of research that may improve the critical care of burn victims. Therefore, the objective of the current study was to test the hypothesis that scald burn injury can alter the physiology of

the airway epithelium. The concept behind this study was to test the validity of this global hypothesis, not to investigate the underlying mechanisms, which would need to be the subjects of additional research should the hypothesis be confirmed. To test this hypothesis, a rat model of 60% total body surface area (TBSA) scald burn injury was used. This animal injury model has been characterized as producing systemic inflammatory [5], hyperglycemic [6] and hypermetabolic responses [7] similar to those seen in victims of pediatric burns.

Outcome measures explored in this study included structural, proliferative, functional, and genomic changes of the rat tracheal epithelium at 24 hours after burn injury. Structural changes in the tracheal epithelium were assessed using both light and electron microscopy. The percentage of tracheal epithelial cells staining positive for Ki67 was used as an indicator of cell proliferation. Mucociliary clearance (MCC) was measured using fluorescent microspheres. Oxidative tissue injury was assessed by measuring levels of malondialdehyde (MDA), a lipid peroxidation byproduct, in tracheal tissue extracts. Microarray analysis of tracheal epithelial cell specific mRNA was performed to assess changes in gene expression following scald burn injury.

Materials and methods

Studies to assess changes in airway epithelium following scald burn injury in rats were approved by the Animal Care and Use Committee of the University of Texas Medical Branch and complied with the guidelines of the American Association for Accreditation of Laboratory Animal Care (AAALAC). A total of 38 female Sprague-Dawley rats weighing 250-300 g were used in our studies.

Burn injury

Prior to study, rats were acclimated to the research facility for 1 week before experiments and housed in wire bottom cages with a 12-hour light-dark cycle. Rats received water *ad libitum* for the entire study period. Burn injury was induced following a standardized protocol [8]. Prior to burn injury, animals were anesthetized with ketamine and xylazine (40 and 5 mg/kg body weight, i.p.) and received buprenorphine as an analgesic (0.05 mg/kg body weight, s.c.) every 12 h. The dorsum of the trunk and the abdomen were shaved and a 60% total body surface area burn was administered by placing the animals in a mold exposing defined areas of the skin, and then in 96°C to 98°C water, scalding the back for 10 sec. and the abdomen for 2 sec. This method delivers a full-thickness cutaneous burn as confirmed by histologic examination. Following injury, the animals were immediately resuscitated by i.p. administration of lactated Ringer solution (40 mL/kg body weight) and transferred to individual cages. Unburned sham control animals received the same anesthesia, handling, shaving and treatment as the burned animals, but were not burned.

Assessment of mucociliary clearance (MCC)

Twelve animals were used to assess MCC using a modification of the method developed by Coote et al [9]. Briefly, 20 hours after burn injury, rats were anesthetized and suspended by their upper incisors on a custom-made intubation stand that allows direct visualization of the glottis. A specially constructed laryngoscope made from an aluminum “mag light” flashlight (Mag Instruments Inc., Ontario, CA) fitted with a fiber-optic illuminator was used under direct vision using a head-mounted jeweler’s magnifier. Fluorescent polystyrene microspheres 1 micron in diameter (Molecular probes, Eugene, OR) suspended in sterile phosphate buffered saline (PBS) at 2.5×10^9/ml were nebulized into rat airways and lung with a microsprayer (Penn-Century, Philadelphia, PA). Four hours after microspraying, the animals were deeply anesthetized with i.p. administration of ketamine (50 mg/kg) and xylazine 10 mg/kg). Broncho-alveolar lavage (BAL) was performed by flushing the trachea three times with 3 ml of lactated Ringer solution.

The animals were then euthanized using i.p. ketamine (100 mg/kg) and xylazine (20 mg/kg) and death was assured by exsanguination. A 4 hour MCC rate was obtained by measuring the number of microspheres in the BAL fluid. Briefly, 200 µl aliquots of BAL fluid were placed in 96 well opaque sided assay plates (Packard Viewplate) in a FluoStar Optima fluorescent plate reader (BMG Lab Tech., Cary, NC) and the fluorescence intensity (FI) was determined using 580 nm excitation and 605 nm emission and adjusted for BAL volume recovery. From

triplicate FI measures, a mean measure for each animal was calculated and converted to number of microspheres in the BAL fluid using a standard curve produced with a known number of microspheres.

Morphological assessment

To assess structural changes in the tracheal epithelium, ten animals were studied, five sham control and five with burn injury. After sacrifice at 24 hours after injury, the trachea was carefully removed and the upper half was placed in formalin for routine sectioning and H & E staining. The lower half was placed in 2% glutaraldeyde in 0.08 M sodium cacodylate buffer and fixed overnight at room temperature for transmission electron microscopy (TEM). Post-fixation was accomplished with 1% osmium tetroxide for 1 hour followed by dehydration and embedding in Spurr epoxy resin. Semi-thin, (0.5 μm thick) sections were cut and stained with toluidine blue and examined with light microscopy for assessment of tissue injury. For TEM, areas of the epithelium were selected to include cross sections oriented perpendicular to the airway axis. Qualitative assessment by TEM was accomplished by three scientists with experience in airway epithelial morphology (SJ, HKH, RAC).

Assessment of proliferation

To determine whether burn injury alters the rate of proliferation of the airway lining epithelium, formalin fixed, paraffin embedded tracheal tissues were used. Immunolocalization of Ki67, a marker of cell proliferation [10, 11], was accomplished following an established protocol [12]. From each study animal, 4 μm sections were deparaffinized and placed in epitope retrieval buffer (Biogenex, San Ramon, CA) and maintained near boiling in a steamer for 20 min. and then allowed to cool for 20 min to improve detection of the antigen. Sections were pretreated with 0.06% hydrogen peroxide in 100% methanol to reduce endogenous peroxidase activity and incubated for 1 hour in normal horse serum to reduce nonspecific labeling. Sections were then incubated with 1:1000 diluted monoclonal antibody against human Ki67 antigen, (Biogenex, San Ramon, CA) overnight at 4°C. Following primary antibody incubation, sections were incubated with biotinylated horse anti-mouse antibody and avidin-biotin-peroxidase complex for 1 hour each. Visualization of the bound antibody complex was achieved using diaminobenzidine (DAB) as a chromogen (Vector Laboratories, Burlingame, CA) and sections were counterstained with hematoxylin. Quantitation of epithelial cell proliferation was accomplished on masked slides. For each slide, the percentage of cells positive for nuclear localization of Ki67 was determined by examining the first 500 cells starting at the 12 o'clock position of the tracheal ring and moving clockwise. Following identification of the extent of Ki67 nuclear staining, slides were unmasked and the percentage of cells with positive nuclear staining for each animal was tabulated into groups for statistical analysis.

Quantitation of lipid peroxidation

For assessment of levels of malondialdehyde (MDA), ten animals were studied, five sham controls and five with burn injury. At 24 hours after injury the animals were sacrificed by exsanguination and their tracheas were carefully removed without contamination with blood. Holding the isolated trachea vertically over a micro-centrifuge tube, 100 μl of protein extraction reagent (T-PER, Thermo Scientific, Rockford, IL) was passed through the trachea 20 times. Preliminary studies using light microscopic analysis of the tracheal epithelium showed optimal removal of the tracheal epithelium without disruption of the underlying mucosal stroma. To avoid auto-oxidation, 10 μl 0.5 M butylated hydroxytoluene prepared in pure acetonitrile was added per ml of tissue lysate. The protein concentrations within the lysates were determined by the method of Bradford, using bovine serum albumin as the standard [13]. Formation of the lipid peroxidation product MDA was determined using a kit (LPO-586) supplied by Oxis Research Inc. (Portland, OR) [14]. Briefly, airway tissue lysates prepared as described above were centrifuged at 1000 g for 15 min and the supernatant was used. Absorbance was measured at 586 nm on a GENios microplate reader (Tecan, Morrisville, NC). A sample blank was made by adding 650 μl of 75% acetonitrile and 25% methanol and used for background correction. Levels of MDA in the lysates were quantitated using a standard curve of authentic MDA and expressed as nmol/mg protein.

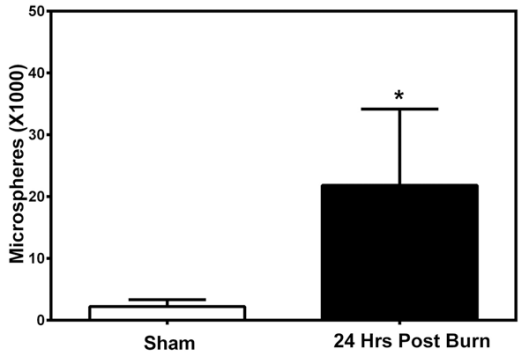

Figure 1. Graph showing significantly reduced mucociliary clearance ($p = 0.007$) in rats at 24 hours after burn injury compared to those without injury (*sham animals*).

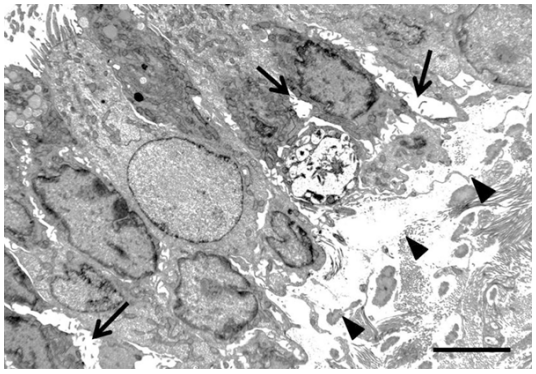

Figure 2. Transmission electron micrograph (TEM) showing a marginal increase in intercellular gaps in rats with burn injury. Note the extracellular space between basal cells and the columnar epithelial cells (*arrow heads*), and intercellular space between columnar epithelial cells (*long arrows*), in the injured animals. Scale bar approximately 4 μm.

RNA isolation and gene expression

To determine the genome-wide changes in mRNA expression in rat airway epithelium after burn injury, eight rats were used. Four received burn injury and four served as sham controls. Twenty-four hours after injury, the animals were anesthetized and their tracheas were removed and held vertically over an RNAase free microcentrifuge tube, and four aliquots of 100 μl of RNA lysis buffer were flushed through the tracheal lumen and captured in the tube. Light microscopic assessment of the tracheal tissue after RNA extraction showed cytoplasmic disruption with epithelial cell nuclei still attached to the airway surface, but no detectable disruption of the lamina propria or deeper structures. RNA was quantitated using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Approximately 175 ng/μl total RNA per animal was obtained by this method. Confirmation of the purified RNA quality was assessed by visualization of 18S and 28S RNA bands using an Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA). Changes in gene expression were measured using the Affymetrix RG 230 2.0 Array (Affymetrix, Santa Clara, CA). Data analysis was performed by the Molecular Genomics Core of the University of Texas Medical Branch, using iReport™ (Ingenuity® Systems, Qiagen, Valencia, CA) with the following filtering parameters; fold change (FC) > 1.5, p-value < 0.05, with further analysis including adjustments for false discovery rate (FDR).

The functional roles of the genes identified as significantly different between the two study groups were identified using the rat genome database (RGD), (http://rgd.mcw.edu/rgdweb/search/genes).

Statistical analysis

Statistical analysis of the data was accomplished with Sigma Plot software (Systat Software Inc, San Jose, CA). For analysis of data (excluding gene microarray data), normality and variance were determined. When criteria for normality and equal variance were met, t-tests were used to assess differences between study groups. In the absence of normality or equal variance, nonparametric rank sum tests were used. A p value of less than 0.05 was used to define statistically significant differences. Mean and standard error of the mean (SEM) are used in graphical presentation of the data. In tables, the mean ± standard deviation (SD) was used.

Results

Mucociliary clearance

Mucociliary clearance (MCC) was measured at 24 hours after burn injury. Data indicated a significant ($p = 0.007$) increase in the number of microspheres collected in the BAL fluid of burned animals compared to sham treated animals. In sham animals the mean number of microspheres measured was 2.2×10^3 compared to 21.8×10^3 in rats subjected to scald

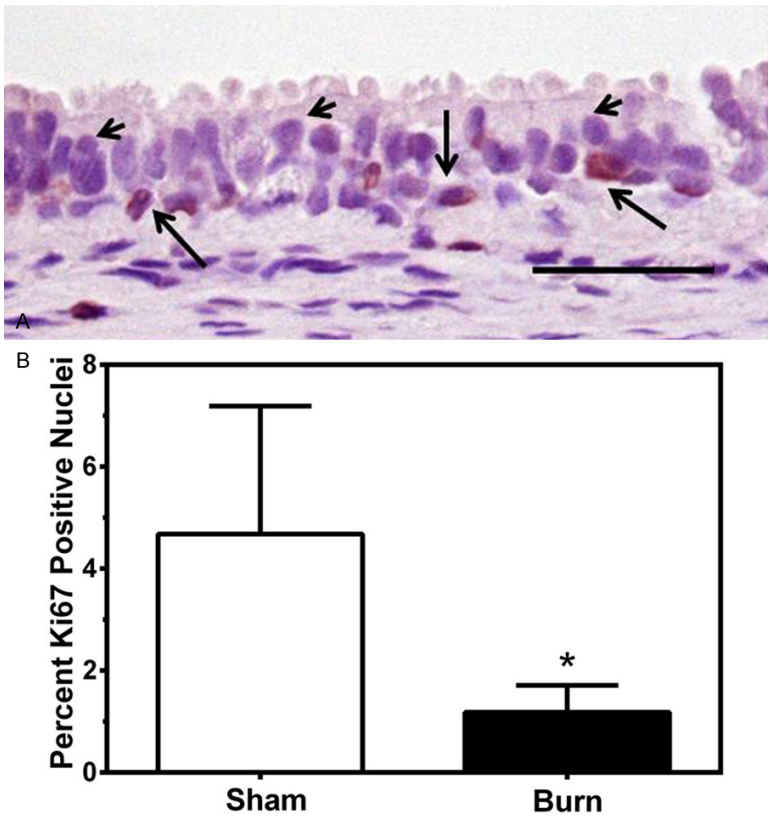

Figure 3. A. Micrograph showing IHC labeling of Ki67 in a rat with burn injury. Long arrows indicate positively stained nuclei and small arrows show nuclei without Ki67 staining. B. Graph depicting the percentage (mean ± SE) of airway epithelial cells exhibiting Ki67 nuclear staining in sham and injured rats at 24 hours after burn injury. A statistically significant decrease (p = 0.008) in the mean degree of Ki67 expression in rats from burn injury was observed as compared to the mean of the uninjured sham animals.

burn injury (**Figure 1**). The data indicate that MCC in the scald burn injured rats was 10 times less effective at 24 hours compared to sham controls.

Morphological assessment of tracheal epithelium

Morphological assessment was conducted using light microscopic slides from both the paraffin and plastic embedded tissues. There was no apparent morphological difference between tracheal epithelial cells of the injured and sham animals. In the plastic embedded sections, higher resolution imaging showed an increase in the extracellular space immediately below the epithelium in 4 of the 5 injured animals and in one focus in one of the 5 sham control animals. Epithelial areas for thin sectioning were selected to show the plane of section that was most nearly perpendicular to the tracheal axis. Ultrastructural images were taken without overlap at 2600 or 3300X magnification starting at one end of the section. The pattern of increased extracellular space beneath the epithelium seen by light microscopy was also evident in the TEM images (**Figure 2**). The edematous space was beneath the lamina densa. The extracellular space between basal cells and the columnar epithelium, and the space between columnar epithelial cells, were both only marginally higher in injured animals compared to sham controls. No difference in the integrity of the apical junctions was seen between the two study groups.

Proliferation

Immunohistochemical detection of nuclear Ki67 was most common in basal cells in sham animals, but occasional columnar cells had Ki67 nuclear staining. A representative micrograph of Ki67 immunoreactivity from an injured rat is shown (**Figure 3A**). Results of quantitation of Ki67 stained nuclei in the epithelium of sham animals ranged from 2.0 to 8.1 percent, with a mean and SEM of 4.7 ± 2.5%. The range of proliferation scores in the animals with burn injury was 0.6 to 1.8, with a mean and SEM of 1.2 ± 0.5% (**Figure 3B**) respectively. A nonparametric rank sum test indicated a statistically significant difference in the mean proliferation index between the two groups, p = 0.008.

Levels of lipid peroxidation products

Malondialdehyde, a major product of lipid peroxidation, was measured in rat airway epithelial lysates in sham and burn injured rats. The data indicate a statistically significant increase in MDA formation in the tracheal epithelium of rats with burn injury compared to sham animals. The mean level of MDA in sham animals was 0.90 ± 0.12 compared to 1.70 ± 0.06

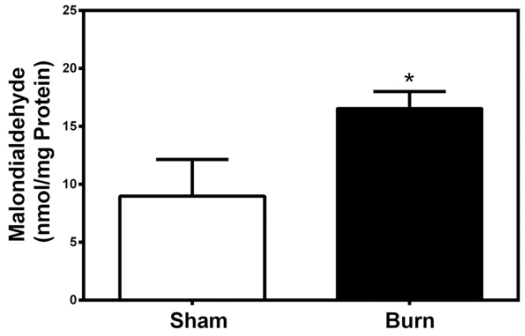

Figure 4. Graph depicting the concentration of MDA, the product of lipid peroxidation, in tracheal epithelial cells of burned rats as compared to that in sham control animals. Note the statistically significant (p = 0.0012) increase in the concentration of MDA in airway cells of injured rats.

nmol/mg protein in the injured animals, p = 0.0012. These data are shown graphically in **Figure 4**.

Gene analysis

Assessment of mRNA expression in sham and injured animals found that fifty-nine genes were differentially regulated between sham and burn injured animals, of which six were up-regulated and 53 were down-regulated (**Table 1**). The functional roles associated with the translated products of these genes included DNA repair, metabolism of exogenous agents, cell cycle regulation, solute and ion transport, protection from oxidative stress, glycoprotein synthesis and RNA degradation.

Discussion

The airway lining epithelium comprises numerous cell types that include ciliated, secretory and basal cells. Airway epithelium plays a vital role in maintaining a sterile lung environment and respiratory health. It seems reasonable to suspect that changes in physiological processes after burn injury may modify the functions of the airway epithelium. However, to our knowledge, studies of this nature have not been reported. The objective of this study was to test the hypothesis that burn injury modifies the physiology of the airway epithelium. Our studies utilized the well-established 60% TBSA scald burn injury model in rats, which has hypermetabolic [5], hyperglycemic [6] and systemic inflammatory responses [7] similar to those seen in burn victims.

Light microscopic assessment of tracheal tissues showed no abnormalities or inflammatory cell influx into the epithelium or mucosa. Ultrastructural assessment of the epithelium of both the sham and injured animals also found no evidence of cell injury, although a marginal increase in extracellular space was noted between adjacent columnar cells and between columnar and basal cells (**Figure 2**). Additionally, no detectable difference was observed in cytoplasmic vacuolation. These findings contrast with the previously described alveolar epithelial and endothelial cell injury seen early (2 hours post injury) in the rat model of scald injury believed to result from neutrophil accumulation in the lung [15]. One possible explanation for the difference between our findings and those of Arbak et al. is that epithelial cells of the alveoli are within microns of the pulmonary circulation, in contrast to the airway epithelium, where capillaries are less frequent and are distributed deeper within the mucosa.

Although distinct morphological changes were not detected in the airway epithelial cells of burn animals, our analysis of mucociliary clearance (MCC) with fluorescent microspheres indicated a statistically significant decrease (10 fold, p = 0.007) in their ability to clear microspheres from the lung compared to sham animals (**Figure 1**). The reduced and/or delayed removal of microspheres from the airways represents a compromise in MCC. Mucociliary clearance is a complex and highly regulated process. Many physiological processes are known to be involved in MCC, including generation of ATP within the airway epithelial cells. Additionally, multiple processes including neural, endocrine and metabolic signaling are involved in the hydration and rheological properties of the airway surface layer that effect MCC. More extensive studies are needed to identify the time course of the MCC compromise found with burn injury and the mechanistic processes associated with this dysfunction. Our selection of this method of MCC analysis was based on the limited inflammatory cell influx observed with microsphere nebulization [9] in contrast to the strong inflammatory reaction that would be produced using bacterial challenge.

In our assessment of cell proliferation, animals with burn injury showed a significant reduction in nuclear Ki67 expression compared to sham

Table 1. Gene changes in rats with scald burn injury vs sham animals

	Gene Symbol	Gene Title	RefSeq ID	FC
1	Ggt5	Gamma-glutamyltransferase 5	NM_019235	1.9
2	Hsd17b6	Hydroxysteroid (17-beta) dehydrogenase 6	NM_173305	3.2
3	B3galnt1	Beta-1,3-N-acetylgalactosaminyltransferase 1	NM_001013158	1.9
4	Lig1	Ligase I, DNA, ATP-dependent	NM_001024268	-1.9
5	Trim59	Tripartite motif-containing 59	NM_001108945	-1.7
6	Srgn	Serglycin	NM_020074	-2.7
7	Gmnn	Geminin	NM_001106112	-2.1
8	Laptm5	Lysosomal protein transmembrane 5	NM_053538	-1.9
9	Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	NM_013154	1.8
10	Odc1	Ornithine decarboxylase 1	NM_012615	1.5
11	Dpysl2	Dihydropyrimidinase-like 2	NM_001105717	-1.5
12	Acsl3	Acyl-CoA synthetase long-chain family member 3	NM_057107	-1.6
13	Slc9a6	Solute carrier-9 (sodium/hydrogen exchanger)	XM_001053956	-1.6
14	Dbi	Diazepam binding inhibitor (GABA receptor)	NM_031853	-1.6
15	Slc26a4	Solute carrier family 26, member 4	NM_019214	-1.7
16	Cotl1	Coactosin-like 1 (Dictyostelium)	NM_001108452	-1.7
17	Aif1l	Allograft inflammatory factor 1-like	NM_001108578	-1.8
18	Ect2	Epithelial cell transforming sequence 2 oncogene	NM_001108547	-1.9
19	Efemp1	EGF-containing fibulin-like extracellular protein 1	NM_001012039	-1.9
20	Etv1	ETS variant 1	NM_001108709	-2.0
21	DOCK10	Similar to Dedicator of cytokinesis protein 10	XR_085916	-2.0
22	Arl11	ADP-ribosylation factor-like 11	NM_001013433	-2.1
23	Fam26f	Family with sequence similarity 26, member F	NM_001024976	-2.1
24	Fam65b	Family with sequence similarity 65, member B	NM_001014009	-2.2
25	Arhgap1a	Rho GTPase activating protein 11A	NM_001168524	-2.3
26	Igfbp5	Insulin-like growth factor binding protein 5	NM_012817	-2.4
27	Sash3	SAM and SH3 domain containing 3	NM_001134992	-2.6
28	Capn13	Calpain 13	NM_001025133	-2.7
29	Nusap1	Nucleolar and spindle associated protein 1	NM_001107762	-2.8
30	Cald1	Caldesmon 1	NM_013146	-2.9
31	Spn	Sialophorin	XM_001080140	-2.9
32	Cd5	Cd5 molecule	NM_019295	-3.3
33	Car3	Carbonic anhydrase 3	NM_019292	-3.3
34	Nkg7	Natural killer cell group 7 sequence	NM_133540	-3.7
35	Tcf7	Transcription factor 7, T-cell specific	XM_001073458	-3.7
36	Mki67	Antigen for monoclonal antibody Ki-67	XM_001056221	-4.4
37	Cpa3	Carboxypeptidase A3, mast cell	NM_019300	-5.0
38	Rnase1	Ribonuclease, RNase A family, 1	NM_001029904	-5.2
39	Cyp1b1	Cytochrome P450, family 1, subfamily b	NM_012940	-6.7
40	Retnla	Resistin like alpha	NM_053333	-13.7
41	Mcpt1	Mast cell protease 1	NM_017145	-17.1
42	Mcpt 8	Mast cell protease	NM_001135010	-35.1
43	Lcn2	Lipocalin 2	NM_130741	6.17
44	CTSL2	Cathepsin L2 family gene	NM_001333	2.0
45	C15	Chromosome 15 open reading	NP_001123482	-1.9
46	CELF5	CUGBP, Elav-like family 5	NC_000019	-2.0
47	VSNL1	Visinin-like gene	NM_012686	-2.6

48	TRBL	T-cell receptor beta locus	NG_001333	-2.7
49	EG667604	Pre-coded gene 8721 (no description	EG669604	-3.5
50	*Angptl4*	*Angiopoietin-like 4*	*NM_199115*	2.6
51	*Tnfrsf25*	*TNF- superfamily, member 25*	NM_001137644	-1.7
52	*Cybb*	*Cytochrome b-245, beta polypeptide*	*NM_023965*	-1.8
53	*Coro1a*	*Coronin, actin binding protein 1A*	*NM_130411*	-1.9
54	*Il2rg*	*Interleukin 2 receptor, gamma*	*NM_080889*	*-2.0*
55	*Gata2*	*GATA binding protein 2*	*NM_033442*	*-2.2*
56	*Lat*	*Linker for activation of T cells*	*NM_030853*	*-3.1*
57	*Fcer1a*	*Fc fragment of IgE, receptor*	*NM_012724*	*-3.3*
58	*S1pr1*	*Sphingosine-1-phosphate receptor 1*	*NM_017301*	*-3.4*
59	*Ptprc*	*Protein tyrosine phosphatase, receptor C*	*NM_001109887*	*-2.0*

Note: FC = Fold Change. All values are $p < 0.05$ when comparing sham vs. burn injured rats. Values are $p < 0.05$ when comparing sham vs burn injured rats.

animals (**Figure 3**). As with airway epithelial function, the alterations in cellular physiology responsible for this deficit may be complex, and identification of mediators would require further investigation. However, the observed change in proliferation provides an endpoint on which to base future studies. Although speculative, the inability to maintain normal epithelial cell turnover rates may impair the integrity of the airway epithelium and predispose burn victims to increasing levels of bacterial contamination and respiratory tract infection.

Assessment of tissue injury by measuring MDA showed a significant increase (1.8 fold, $p = 0.0012$) in MDA levels in the injured animals compared to the sham animals (**Figure 4**). Numerous reports have shown that burn injury is associated with generation of reactive oxygen species and lipid peroxidation. Using an ovine model of burn injury, Demling et al. showed that lipid peroxidation persisted for days after the initial burn injury [16]. Others have shown that burn injury results in distal organ injury [17-20]. Pathways associated with increased lipid peroxidation include loss of plasma and tissue antioxidants [18, 19]. Thus, therapy with antioxidants has long been advocated for use after burn injury [18-20]. High levels of oxidative stress and lipid peroxidation can decrease cell proliferation [21, 22]. Thus, the increase in MDA we observed in the burned animals may be related to the decrease in cell proliferation seen with Ki67 staining. Future studies that focus specifically on mechanisms of oxidative injury and antioxidant therapies after burn injury could provide more evidence regarding the basis of these findings.

Although our studies with gene microarray analysis were preliminary and statistically underpowered to fully define the changes in genomic expression in airway epithelial cells, the results show that gene expression in tracheal epithelial cells is altered by burn injury. To our knowledge, studies of this nature have not been performed in tracheal epithelium following burn trauma. Analysis identified differential expression of 59 genes between the sham and burn injured animals.

Of the 59 genes shown to have altered expression, most (87%) were down- regulated. Among those down-regulated genes were epithelial cell transforming sequence 2 oncogene (#18, ECT2, -2.0 fold) and Ki67 (#36, MKi67, -4.35 fold). The ECT2 gene encodes a protein that regulates the cell cycle [23]. Ki-67 antigen can be exclusively detected within the cell nucleus during all active phases of the cell cycle, but is absent from resting cells [10, 11]. Therefore the observed downregulation of these two genes is consistent with the reduction in epithelial cell proliferation observed with Ki67 immunostaining. Other genes found to be downregulated include those related to DNA repair, solute and ion transport, and RNA degradation. Genes that were upregulated included lipocalin-2 (#43, 6.0 fold) and gamma-glutamyltransferase 5 (#1, GGT-5, 2 fold). The GGT-5 enzyme plays a key role in the synthesis and degradation of glutathione [24, 25]. Lipocalin-2 is considered as an acute phase response protein with a protective function against lipid peroxidation [26, 27]. Importantly, lipocalin-2 has antibacterial properties as a consequence of its binding of iron [28, 29].

Therefore, with respect to these two genes, the epithelium in burned rats may be in the process of recovering from oxidative stress and improving its antibacterial properties. However, to fully identify the effects of burn injury on airway epithelium, more extensive studies are needed, including validation of differences in expression of specific RNA sequences and related protein expression.

In conclusion, this study demonstrates that a scald burn injury can impact airway epithelial homeostasis and function. Animals with burn injury showed decreases in MCC and cell proliferation, changes in gene expression and an increase in epithelial lipid peroxidation compared to unburned sham treated animals. Future research is needed to investigate the dynamics of epithelial change with increasing time after injury, along with mechanistic research to identify how post burn physiological conditions contribute to dysregulation in airway innate defense. Improved understanding of how trauma related pathophysiology alters the airway epithelium is essential to improving the critical care of burn victims and for the development of therapeutic interventions to prevent dysfunction or enhance the normal functions of this vital system.

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Disclosure of conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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