

## Original Article

# Xanthine oxidase contributes to sustained airway epithelial oxidative stress after scald burn

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**Abstract:** Respiratory tract infections and pneumonia are major causes of morbidity and mortality in burn victims, however, limited studies have examined the effects of burn injury on airway epithelium. The current study examines the effect of scald burn injury on rat tracheal epithelium at 5 days after injury and tests the hypothesis that treatment with febuxostat (FBX), an inhibitor of xanthine oxidase (XO), can be protective of cell homeostasis. Sprague Dawley rats were randomly divided into uninjured (sham), injured (control) and injured and FBX treated groups, n = 8. Control and FBX treated groups received 60% total body surface area scald burn injury. The FBX group received an i. p. dose (1 mg/kg) at 1 hour after injury and every 24 hours. At 5 days after injury, the animals were sacrificed and tracheal epithelial cell lysates were collected. Malondialdehyde (MDA), ATP, and XO activity were measured. Formation of 8-OHdG in tracheal epithelium was determined using immunohistochemistry (IHC) and immunoreactivity was quantitated. MDA levels were significantly increased in injured control animals ( $24.8 \pm 2.3$ ) compared to sham ( $7.93 \pm 1.2$ ,  $p = 0.002$ ). FBX treatment attenuated this response ( $12.6 \pm 2.7$ ,  $p = 0.02$ ). ATP levels were significantly decreased in control ( $0.7 \pm 0.16$ ) compared to sham, ( $2 \pm 0.14$ ,  $p = 0.01$ ). ATP levels were increased with FBX treatment ( $1.8 \pm 0.1$ ,  $p = 0.03$ ) compared to controls. There was a significant increase in XO activity in control animals,  $1.04 \pm 0.06$  compared to sham ( $0.34 \pm 0.05$ ,  $p = 0.03$ ), and this response decreased with FBX treatment  $0.46 \pm 0.07$  ( $p = 0.04$ ). Immunolabeling of 8-OHdG in control animals was significantly increased ( $25.1 \pm 0.7$  compared to the sham group  $5.5 \pm 1.9$  ( $p = 0.01$ )), and was decreased with FBX treatment ( $7.0 \pm 2.3$  compared to control ( $p = 0.03$ )). The current study indicates that lipid peroxidation and ATP depletion persist in tracheal epithelium for 5 days after injury along with increased XO activity and 8-OHdG. These effects were significantly attenuated by FBX treatment, suggesting that reactive oxygen species generated by XO contribute to airway epithelial injury following scald burn.

**Keywords:** Burn, airway, reactive oxygen species, oxidative stress, lipid peroxidation, malondialdehyde, xanthine oxidase, xanthine oxidase, xanthine oxidoreductase, febuxostat, ATP

## Introduction

In the United States alone, 45,000 people are hospitalized per year with a burn injury with worldwide mortality from burns estimated at 265,000 [1, 2]. Key factors contributing to the morbidity and mortality of burn victims are age, total body surface area (TBSA) burn and the presence of an inhalation injury [3]. Major causes of mortality include pneumonia, respiratory failure, sepsis and multi-organ dysfunction [3, 4]. Although significant advances in burn care have occurred over the past three decades, pneumonia remains the leading complication in the critical care of burn victims and accounts for over 40% of deaths [1, 3, 5].

For those that died during critical care at our burn care institution, virtually every autopsy case showed extensive airway pathology. In a review of 64 autopsy cases, all but two cases showed distal migration of upper airway mucus into the bronchioles and parenchyma [6]. In a follow-up study to compare the changes in airway obstruction between burn only patients and those with an inhalation injury using systematically collected tissues at autopsy, we found similar levels of obstruction between the two groups. However, in cases with pneumonia as a primary cause of death, obstruction was 5-fold greater than in those without pneumonia. Thirty eight percent of these cases showed bacterial invasion into the airway mucosa [7]. In a

more recent study of 72 autopsy cases we showed a statistically significant correlation of airway epithelial loss with age and percentage of total body surface area (TBSA) burn. Together, the above studies suggest that burn injury alone impacts the structural and functional properties of the airway epithelium [8]. We recently confirmed this hypothesis in rats at 24 hours after a 60% TBSA scald burn, showing an increase in malondialdehyde (MDA), a marker of lipid peroxidation, a decrease in mucociliary clearance, changes in gene expression, and a decrease in cell proliferation [9]. These studies in autopsy tissues and in the rat model show that burn injury alone has effects on remote tissues and affects airway epithelial structure and function. Further study of this dysfunction may have implications for improving the clinical care of burn patients.

There is published evidence of burn injury induced physiological alterations and damage to distal organs mediated in part through the production of reactive oxygen species (ROS) [10-13]. Following burns, ROS may attack cellular components and damage proteins, lipids, and DNA, thereby altering cell function and homeostasis. Animal models have identified damage to the kidney, lung, liver, intestine, and heart after burn [14-21]. An enzyme system that has been implicated in causing distal organ injury following burn is xanthine oxidase (XO), which has been associated with vascular leakage in the lungs [22] and cardiac cell injury [19, 23]. Xanthine oxidoreductase (XOR) is a dimeric metalloflavoprotein that oxidizes hypoxanthine, xanthine and other compounds via enzymatic activity, E.C. 1.1.1.204. Reversible cysteine oxidation or irreversible proteolysis converts this enzyme to xanthine oxidase (XO), which catalyzes a different oxidative reaction, E.C. 1.1.3.22, that generates hydrogen peroxide and/or superoxide. Xanthine oxidase is ubiquitously expressed in rat tracheal (personal observations) and bronchial epithelium [24].

The primary objective of the current study was to examine the tracheal epithelium of rats at five days after a scald burn injury to determine whether oxidative stress is sustained in this cell population and to determine whether burn injury alters the production of ATP in these cells. A second objective was to test the hypothesis that XO activity causes increased oxidative

stress in the airway epithelium following scald burn. To explore the contribution of XO to tracheal epithelial oxidative stress after burn, we used the selective inhibitor Febuxostat (FBX), which is a non-purine XO inhibitor with the empiric chemical formula [2-[3-cyano-4-(2-methylpropoxy)-phenyl]-4-methylthiazole-5-carboxylic acid] [25]. Treatment of humans with FBX produces sustained reductions in serum uric acid level and does not interfere with other enzymes in purine or pyrimidine metabolism [26]. Previous studies have indicated significantly greater beneficial effects of FBX compared to allopurinol in attenuating tissue injury scores in organs including lung, liver and intestine after ischemia reperfusion injury to rats [27]. To our knowledge, no previous research has assessed the capacity of FBX to protect against distal organ injury following burn injury.

### Materials and methods

The studies described 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).

#### *Burn injury*

Male Sprague Dawley rats (200-250 g) were used in the studies. Rats were acclimated to the research facility for 1 week before experiments with a 12-hour light-dark cycle. Rats received water and food *ad libitum* for the entire study period. Prior to burn injury, animals were anesthetized with ketamine and xylazine (40 and 5 mg/kg body weight, i.p.) and received Buprenorphine-SR as an analgesic (1 mg/kg body weight, s.c.) every 24 hours. Animals were randomly divided into uninjured (sham), injured but untreated (control) and injured and FBX treated groups, n = 8. All animals received the same procedures and anesthetic and analgesic treatment. Control and FBX treated groups received a standardized 60% total body surface area scald burn injury. The FBX group received an i. p. dose of FBX (1 mg/kg) dissolved in ethanol (0.01% v/v) at 1 hour after injury and every 24 hours for 5 days. 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

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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.

### *Quantitation of lipid peroxidation*

At 5 days 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 buffer (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 following this procedure. To avoid auto-oxidation, 5 µl butylated hydroxytoluene (100X) prepared in pure acetonitrile was immediately added per ml of tissue lysate. The protein concentrations in the lysates were determined using bovine serum albumin as the standard, as previously described [27]. Formation of the lipid peroxidation product MDA was determined using a kit obtained from Abcam (Cambridge, MA). Briefly, the epithelial lysates described above were centrifuged at 13,000 g for 10 minutes at 4°C and the supernatant was collected. A total of 240 µg protein (approximately 160 µl) of sample supernatant was reacted with 480 µl of thiobarbituric acid (TBA) to generate the MDA-TBA adduct. The reaction mixture was incubated at 95°C for 1 hour and cooled in ice for 10 minutes. By adding 200 µl (80 µg protein) of reaction mixture to triplicate microplate wells, absorbance was measured on a GENios microplate reader (Tecan, Morrisville, NC) at 532 nm. A sample blank was made by replacing the sample with 160 µl assay buffer in the above incubation system and was 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.

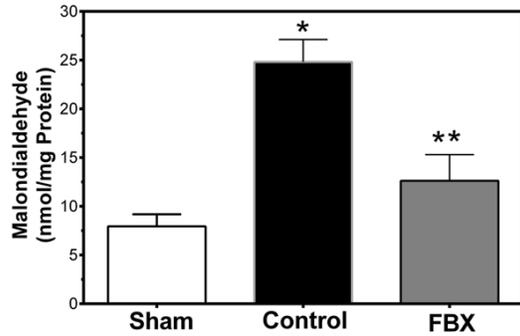
### *Determination of ATP*

To measure ATP levels in the tracheal epithelial lysates a rat kit by MyBiosource Inc (San Diego, CA) was used. This kit is designed to detect ATP colorimetrically, using a monoclonal ATP-HRP conjugation. Briefly, tracheal cell lysates were incubated with ATP-HRP conjugate in a pre-coated microplate for 1 hour and then with substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. The optical density (O.D.) was measured spectrophotometrically at 450 nm in a microplate reader. The color intensity is inversely proportional to the ATP concentration since ATP from samples competes with the ATP-HRP conjugate for the anti-ATP antibody binding site. A standard curve was plotted relating the intensity of the color (O.D.) to the concentration of standards and the ATP concentrations in the samples were interpolated from this standard curve and expressed as nmol/min/mg protein. The sensitivity of the assay is approximately 1 ng/ml.

### *Immunohistochemistry of 8-OHdG*

To assess burn induced oxidative stress in tracheal lining epithelium, immunohistochemistry for 8-OHdG was performed in formalin fixed, paraffin embedded tracheal tissues as described previously [9]. Briefly, from each study animal, 4 µm sections were deparaffinized and placed in antigen retrieval buffer (Biogenex, San Ramon, CA) and maintained near boiling in a steamer for 20 minutes and then allowed to cool for 20 minutes to improve detection of the antigen. Sections were pre-treated 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 mouse monoclonal antibody against human 8-OHdG (Catalogue #ab626623) antigen, (Abcam, Cambridge, MA) overnight at 4°C. Following primary antibody incubation and rinsing, 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,

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**Figure 1.** Graph depicting the levels of MDA, the product of lipid peroxidation, in rat tracheal epithelium at 5 days after scald burn injury as compared to sham animals. Note the statistically significant ( $*p = 0.0023$ ) increase in MDA in injured rats as compared to sham and its significant attenuation by FBX treatment ( $**p = 0.015$ ) as compared to control.

CA) and the sections were counterstained with hematoxylin.

### Quantitation of 8-OHdG

Immunoreactivity was visually determined before applying software based image analysis. For each slide, the percentage of cells positive for localization of 8-OHdG was determined by examining under the microscope at 20 $\times$  magnification and counting the first 500 cells starting at the 12 o'clock position of the tracheal ring and moving clockwise. Immunoreactivity was further quantified using Image-Pro Premier software (version 9.2, Media Cybernetics) as described by Shi et al. [28]. Briefly, IHC slides were masked and 20 $\times$  digital images were acquired from each slide using a Nikon Optiphot microscope equipped with a digital camera (DFC 290, Leica). Images were acquired at the same time with a constant set of imaging parameters on the microscope and imaging software. The images were then analyzed by the Image-Pro Premier DAB application software. For each image, the airway epithelium was selected as an area of interest (AOI). DAB intensity range selection was based on a software-generated histogram, where the brown chromagen color was selected. The analysis was completed on 5 non-overlapping fields from each slide/animal. The integrated optical density of the AOI [integrated optical density (IOD)/unit area] was determined by the software. Following identification of the extent of 8-OHdG staining, slides were unmasked and

the mean integrated optical density/ $\mu\text{m}^2$  area for each animal was tabulated into groups for statistical analysis.

### Determination of xanthine oxidase activity

Xanthine oxidase activity was measured in tracheal lysates at 25 $^{\circ}\text{C}$  by a sensitive fluorometric assay using an XO kit (Sigma-Aldrich, San Louis, MO). Lysates from sham, control and FBX groups were prepared as described earlier. Briefly, assay reaction mixture containing assay buffer, substrate mix, and fluorescent peroxidase mix was added in triplicate to wells of fluorescence microplates. The reaction was started by adding 50  $\mu\text{l}$  lysates and the initial reading was obtained using excitation at 535 nm and emission at 587 nm on a Bio-Rad fluorescent microplate reader (Life Sciences, Hercules, CA). The microplate was incubated at room temperature in the dark and measurements were taken every 5 minutes until the most active sample reached the end of the linear range of the standard curve. XO activity was determined by the rate of change in fluorescence with time using an authentic standard curve of hydrogen peroxide formation and calculated as described in the kit as nmol/min/ml, where one unit of XO is the amount of enzyme that catalyzes the oxidation of xanthine yielding 1.0  $\mu\text{mol}$  of uric acid and hydrogen peroxide per minute.

### Statistical analysis

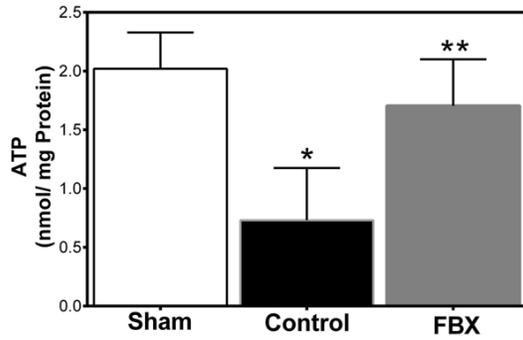
One Way ANOVA and Bartlett's multiple comparison test were used to determine significant differences between group means using software by GraphPad Prizm (La Jolla, CA). In all analyses,  $p < 0.05$  was considered statistically significant. Mean and standard error of the mean (SEM) are used in graphical presentation of the data.

## Results

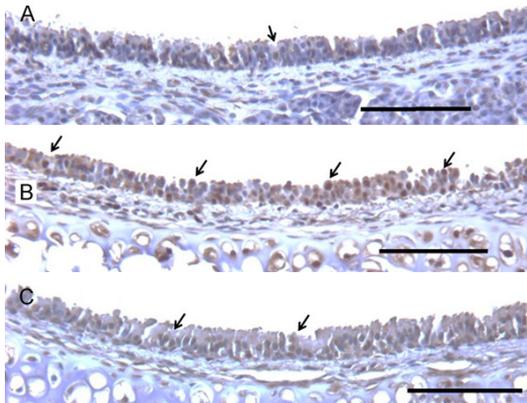
### Levels of lipid peroxidation product

Malondialdehyde (MDA), a major product of lipid peroxidation, was measured in airway epithelial lysates in sham, control, and FBX treated rats. The data indicate a statistically significant increase in MDA formation in the tracheal epithelium of rats with burn injury compared to sham animals (**Figure 1**). The mean level of MDA in sham animals was  $7.9 \pm 1.2$  compared

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**Figure 2.** Graph showing significantly decreased ATP (\* $p = 0.012$ ) in rats at 5 days after burn injury compared to sham animals. FBX treatment increased levels of ATP compared to the injured control animals (\*\* $p = 0.034$ ).

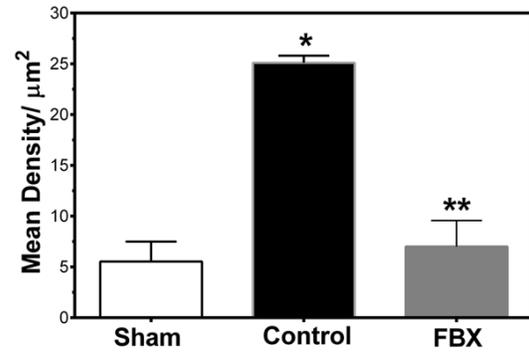


**Figure 3.** Micrographs showing IHC labeling of 8-OHdG in sham animals (A), injured control at day 5 after burn injury (B), and injured controls treated with FBX (C). Arrows indicate positively stained nuclei (brown) for 8-OHdG. Scale bar approximately 80  $\mu\text{m}$ .

to  $24.8 \pm 2.3$  nmol/mg protein in the injured control animals,  $p = 0.015$ . In FBX treated animals, the mean level of MDA was  $12.6 \pm 2.7$  nmol/mg, significantly decreased compared to injured control animals,  $p = 0.002$ . There was a significant, 50% decrease in MDA in FBX treated animals.

### ATP depletion

ATP was determined in rat airway epithelial cell lysates using a kit. The data indicate a statistically significant decrease in ATP levels in injured animals as compared to sham (**Figure 2**). ATP levels (nmol/mg protein) were  $2.12 \pm 0.15$  in the sham group and  $0.73 \pm 0.16$  in control animals,  $p = 0.012$ . Levels of ATP in FBX treated animals were  $1.78 \pm 0.09$  nmol/mg showing



**Figure 4.** Graph showing the increased immunoreactivity (mean integrated density/ $\mu\text{m}^2$ ) of 8-OHdG in sham vs injured control groups of rats after burn injury (\* $p = 0.014$ ) and a significant decrease in immunoreactivity in FBX vs control (\*\* $p = 0.03$ ).

significantly more ATP compared to control animals ( $p = 0.034$ ).

### Levels of 8-OHdG in tracheal epithelium

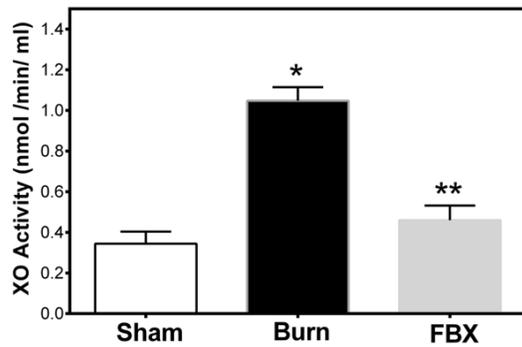
To assess scald burn injury induced oxidative damage to DNA, immunohistochemical labeling for 8-OHdG was conducted using slides of rat trachea obtained from sham, control, and FBX groups. As shown in the micrographs, there was a strong increase in cytoplasmic and nuclear labeling of 8-OHdG in tracheal epithelial cells of injured animals compared to sham (**Figure 3A, 3B**). In injured animals treated with FBX there was less nuclear staining compared to injured animals (**Figure 3C**).

Computer assisted image analysis using the ImagePro application was conducted to assess the intensity of 8-OHdG labeling in tracheal epithelial cells as described above and expressed as integrated optical density/ $\mu\text{m}^2$  (**Figure 4**). The data indicate a significant increase in 8-OHdG labeling in tracheal epithelial cells of injured control rats compared to sham ( $25.1 \pm 0.7$  vs  $5.53 \pm 1.9$  respectively,  $p = 0.014$ ). In injured animals treated with FBX, there was a significant decrease ( $p = 0.03$ ) in the labeling compared to control animals. The mean for 8-OHdG staining in FBX treated animals was  $7.0 \pm 2.3$ .

### Xanthine oxidase activity

XO activity in the injured control group was significantly greater than in the sham group,  $1.04 \pm 0.06$  vs  $0.34 \pm 0.05$  nmol/min/ml, respectively,  $p = 0.03$ . In burned animals treated with

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**Figure 5.** Graph showing a significant (\* $p = 0.032$ ) increase in XO activity (nmol/min/ml) in injured control animals (dark bar) as compared to sham (open bar). Treatment with FBX significantly (\*\* $p = 0.042$ ) attenuated the increase in XO activity (gray bar).

FBX, XO activity was  $0.46 \pm 0.07$  nmol/min/ml, indicating a 2-fold, statistically significant decrease compared to burn, nontreated animals, ( $p = 0.04$ ) (Figure 5).

### Discussion

The airway lining epithelium consists of ciliated, secretory and basal cells that perform significant functions of innate defense through mucociliary clearance (MCC) and production of antibacterial peptides that remove and kill inhaled pathogens. As presented earlier, studies of rats 24 hours after scald burn injury showed a decrease in the ability of burned animals to clear nebulized microspheres, as well as tracheal epithelial cells showing an increase in lipid peroxidation, decrease in cell proliferation and changes in gene expression [9]. The primary goal of this study was to examine tracheal epithelial cells in a more clinically relevant time frame of 5 days after injury. The results of the current study confirm that oxidative stress persists in these cells at 5 days after a 60% TBSA scald burn injury, as indicated by significantly increased MDA formation (Figure 1). Additionally, a disruption in cell hemostasis was identified, as evidenced by a significant decrease in ATP levels in tracheal epithelial lysate in burned animals compared to sham controls (Figure 2). Burn induced oxidative stress in tracheal epithelial cells was further substantiated by significantly increased levels of 8-OHdG, a marker for oxidative DNA damage (Figures 3 and 4). To shed some light on possible mechanisms of oxidative stress in airway epithelium after burn injury, we treated animals with febuxostat

(FBX), an inhibitor of both XDH and XO enzymatic activity, and found that treatment decreased lipid peroxidation and restored ATP concentration in tracheal cell lysates to levels seen in sham animals, suggesting a role of this enzyme system in airway epithelial dysfunction following burn injury. Limitations of this study include the lack of a dose response with FBX treatment, comparison to other XO inhibitors and measurement of plasma levels of XO. Since the inhibitor was administered systemically, it cannot be determined from these experiments whether its more important action was to inhibit airway XO activities or those in other parts of the body.

Our initial focus on XO as a contributor to airway epithelial cell dysfunction after burn injury was based on previous studies showing that inhibition of this enzyme could provide protection against remote organ injury and preliminary studies showing XO immunoreactivity in the apical portion of rat tracheal epithelium (data not shown). Previous studies in rats with burn injury have shown increased plasma levels of XO and also that treatment with allopurinol, a nonspecific inhibitor of XO, decreased cardiac dysfunction [19], in addition to decreasing lung and dermal vascular permeability [22, 29]. Other studies in rats have shown a decrease in renal, gastric and intestinal cell injury with allopurinol treatment after burn injury [29-31]. Furthermore, in sheep with burn injury, treatment with allopurinol decreased systemic and lung inflammation and lipid peroxidation [32]. In clinical studies, antioxidant levels associated with an increase in activity of macrophages and neutrophils were found to be significantly decreased in non-surviving burn patients compared to survivors [33]. In other studies related to oxidative stress in burn victims, plasma levels of antioxidants have been shown to be decreased, regardless of the degree of burn injury, as well as increased lipid peroxidation in red blood cells up to 21 days after burn and an association between elevated plasma levels of XO and a poor prognosis [34-36].

The metalloflavoprotein xanthine oxidoreductase (XOR) can exist in two interconvertible forms catalyzing two distinct oxidative reactions; XO and xanthine dehydrogenase (XDH) [37]. Most mammalian XOR is synthesized in the XDH form, however, based on the molecular

environment, oxidation of cysteine residues within the XDH causes its conversion to the XO form. Limited proteolysis also can cause conversion of XDH to XO. XDH is an essential enzyme in ATP catabolism whereby hypoxanthine is converted to xanthine and xanthine to uric acid. The conversion of hypoxanthine to xanthine, and xanthine to uric acid by XDH, utilizes NAD<sup>+</sup> as an electron acceptor to form NADH and the downstream metabolite. Both steps in the metabolism of hypoxanthine to uric acid can also be catalyzed by XO with molecular oxygen as the electron acceptor, producing reactive oxygen species [38]. Increased XO activity has been demonstrated to cause tissue injury in gout, especially in models of ischemia/reperfusion injury. Numerous reviews related to XO and its contribution to cell injury and disease states are available [39-41].

Even though blocking XO activity with allopurinol has been shown to be beneficial for burn injury, no clinical trials have been conducted with this drug. In contrast, studies with allopurinol treatment in animals have strongly supported the concept of antioxidant therapy in burn care [42, 43]. With the advent of more selective XO inhibitors with less adverse side effects, treatment of burn victims might become feasible. In order to understand specific pathways and mechanisms related to airway epithelial cell injury due to burn injury, use of specific inhibitors and new animal models will be required.

In conclusion, our studies showed continued disruption in airway epithelial cell homeostasis five days after a 60% TBSA scald burn injury. Febuxostat treatment provided protection of airway epithelial homeostasis, suggesting a role of xanthine oxidoreductase in causing cell injury. We believe these studies provide a first step in understanding airway epithelial dysfunction after burn injury and lay the foundation for further mechanistic studies. Such studies should explore how post burn physiology and treatments alter not only cell homeostasis but also how therapies may impact the complex processes required for airway innate defense. This knowledge is essential to enable future advances in burn care that can lead to direct monitoring and manipulation of the airway innate defense system to reduce the risk of lung infection.

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### Disclosure of conflict of interest

None.

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