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Original Article

Profiling of cell stress proteins reveals decreased expression of enzymatic antioxidants in tracheal epithelial tissue of pigs raised indoors

Jenora T Waterman[1], Chakia J McClendon[1,2*], Rohit S Ranabhat[1,2*], KeYona T Barton[1]

[1]Department of Animal Sciences, [2]Energy and Environmental Systems, North Carolina Agricultural and Technical State University, Greensboro, NC, USA. [*]Equal contributors.

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Abstract: Exposure to indoor swine production facilities (SPF) environments causes airway inflammation and diseases including asthma, chronic bronchitis and chronic obstructive pulmonary disease (COPD) in facility workers. However, less is known about the impact of SPF exposures on the respiratory health of pigs. Respiratory symptoms are associated with repeat exposure to SPF, specifically inhalation of organic dust and other air pollutants therein. A thorough understanding of the molecular pathways regulated by SPF exposure is needed to understand airway inflammation and chronic inflammatory lung disease. The present study measured the expression of proteins associated with oxidative stress and antioxidant defenses in the tracheal epithelial tissues of pigs reared in SPF or on pasture. Proteome profiler cell stress arrays, western blotting and enzyme activity assays were utilized to measure protein expression and activity levels in tracheal epithelial tissue extracts of pigs. It was determined that pigs raised in SPF express significantly less enzymatic antioxidants, including superoxide dismutase (SOD), within their tracheal epithelial tissues compared to pasture raised pigs. Concomitantly, tracheal epithelial tissues of SPF raised pigs had lower SOD and catalase antioxidant activity levels compared to pasture raised pigs. The observations summarized herein provide evidence that exposure to swine production environments influence endogenous enzymatic antioxidant defenses within the tracheal epithelial tissues of pigs. This study offers insight for understanding the effect of continuous exposure to SPF pollutants on endogenous antioxidant defenses in the airway epithelial and may be helpful in understanding human airway responses to swine barn exposures.

Keywords: Swine confinement facility, tracheal epithelium, oxidative stress, antioxidants, pig model

Introduction

Occupational exposure to organic dust in swine production buildings or SPF is associated with development of respiratory diseases in humans [1, 2]. Both SPF workers and pigs experience adverse health effects from exposures within SPF; however, the cellular and molecular impact of these hazards on pig respiratory health is less clear. Herd management practices and a variety of environmental factors can complicate disease susceptibility in pigs. Housing types, specifically conventional (indoor) and pasture (outdoor) swine operations, differ in the environmental exposures such as fresh air and organic dust. Animals and workers within conventional swine buildings are exposed to a myriad of respiratory irritants and pollutants including organic dust, gases, odor and chemicals; however, organic dust has been widely studied for its ability to elicit or exacerbate respiratory disease. Respiratory diseases, such as porcine reproductive and respiratory syndrome (PRRS), swine influenza (flu) and Mycobacterium hyopneumoniae infection (pneumonia), are leading causes of pig mortality in the swine industry. The PRRS virus, the costliest pathogen affecting the US pork industry, accounts for $664 million annually in productivity losses in the breeding and growing pig-herd due to reduced or loss pregnancies, death of young piglets, and poor growth performance [3]. Long-term exposure to organic dust within swine barns causes airway diseases, including asthma, chronic bronchitis and hypersensitivity pneumonitis in facility workers [2].

Organic dust is a complex mixture of chemical substances and biological particles derived from a variety of sources including feed, microbes and their products (endotoxin, peptidoglycan) and animals [4, 5]. All of the aforementioned substances are capable of eliciting barrier and immunological responses by the airway epithelium. It is well established that SPF dust exposure stimulates and contributes to acute and chronic inflammation in the airways of humans [6-8]. However, repeat exposure to organic dust has been associated with dampened inflammation response or 'adaptive response' by human, mice and piglet monocytes following repeat exposure to organic dust extract (DE) or continuous exposure to conventional livestock production environments [9-12]. While swine farmers are less likely to experience acute airway inflammation in response to organic dust exposure compared to naïve subjects, they still exhibit symptoms of chronic inflammation, such as cough, phlegm and wheeze [1, 2, 13]. Chronic exposures in agricultural settings can cause airway remodeling [14], goblet cell metaplasia [15] and ultimately COPD [16] in humans.

Chronic inflammation and airway oxidative stress, an imbalance between oxidants and antioxidant defenses, are hallmarks of COPD, where levels of oxidants are increased and antioxidants are decreased [17]. Endogenous enzymatic antioxidants such as superoxide dismutase (SOD) neutralize the effects of reactive oxygen species (ROS). SOD converts superoxide anion into molecular oxygen and hydrogen peroxide, another type of ROS, which is neutralized by catalase into water and molecular oxygen. Superoxide anion and other ROS have been implicated in the pathophysiology of COPD and asthma in addition to decreased activity of antioxidant expression [18]. Although excessive generation of ROS has been found in chronic inflammatory lung diseases, superoxide anion in particular has been implicated in the pathophysiology of COPD [19]. Superoxide anion levels were higher in asthma patients [20] and the antioxidant enzyme CuZnSOD (SOD1) and total SOD activity is decreased in asthmatic airway epithelium [15]. Superoxide dismutase and other ROS contribute to elevated levels of lipid peroxidation in the lungs and is associated with COPD [21]. Other enzymatic antioxidants such as paraoxonase (PON) family, prevent oxidative modification of lipoproteins and phospholipids. PON1 and PON3 enzymes are specifically responsible for metabolizing oxidized lipid products within the blood, whereas PON2 tends to act at the cellular level [22-25].

Differences in the breathing zones and time spent within production facilities among pigs and farmers indicates that farmer exposure levels are potentially lower for total organic dust and hydrogen sulfide, and higher for ammonia than SPF pig exposure levels [26, 27]. Numerous studies have investigated and reported adverse health effects of swine facility organic dust on humans. However, swine exposed to environmental conditions within SPF develop adverse health effects as well [28]. Few studies have investigated the effect of conventional or pasture production systems on airway epithelial inflammation and oxidative stress in pigs. In the present study, we sought to screen the expression of cell stress proteins within tracheal epithelial tissues of pigs reared continuously in SPF and pasture environments, referred to hereafter as indoor and outdoor pigs, respectively. We report diminished expression of proteins involved in oxidative stress and inflammation including superoxide dismutase (SOD) in the tracheal epithelial tissue of indoor pigs. These findings reveal previously undocumented differences between endogenous enzymatic antioxidant defense capacity of indoor and outdoor pigs from our university farm, and support for future studies to investigate the antioxidant capacity in airway epithelial tissues of commercial breeds.

Materials and methods

Animals and production style

The North Carolina Agricultural and Technical State University Institutional Animal Care and Use Committee reviewed and approved all experimental procedures involving animals. Studies were performed in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching [29]. In this study, 28 indoor (14 adults) and outdoor (14 adults) pigs of either sex-primarily Yorkshire and Landrace crossbreds-were respectively raised in confinement or on pasture with hoop structure access until market weight of ~250 lbs was reached (i.e., for 5 months

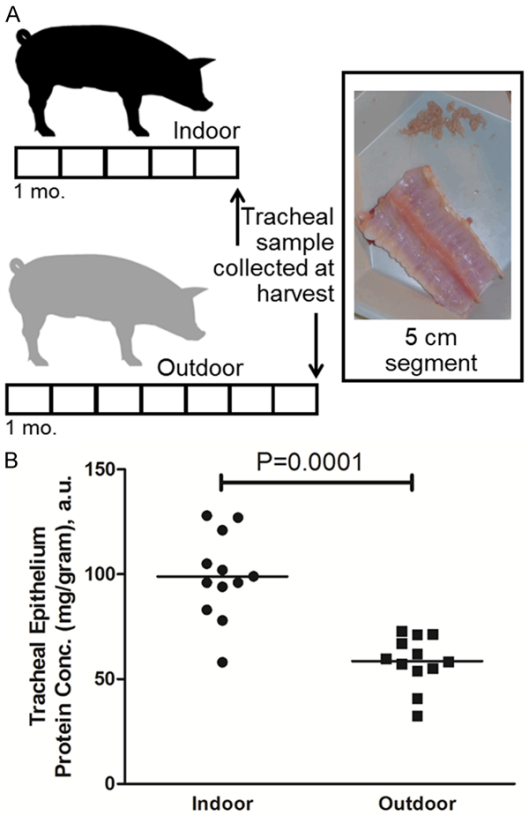

Figure 1. Experimental design and protein levels in tracheal epithelial tissue extracts. A. Schema of experimental design. Pigs were housed for 5-7 months until market weight (~250 lbs) was reached indoors in a confinement building or outside within hoop structures. At harvest, tracheas were collected and the epithelial tissue from the intraluminal surface was removed to make protein extracts. mo, month. B. Porcine epithelial tissue was removed from the intraluminal surface of the 5-cm tracheal sections from indoor and outdoor pigs. One gram of tissue was used to make protein extracts and concentration was determined using the Bradford assay. Results expressed as mean ± SE (n = 12 pigs each).

indoor; 7 months outdoor, **Figure 1A**). Feed and water were provided ad libitum. At the time of processing at a local meat processor, porcine airways were harvested and analyzed as described below.

Porcine tracheal epithelial tissue acquisition

At harvest, the lower respiratory tract was dissected from viscera using a scalpel and tracheal samples were dissected from the larynx (anterior to the first cartilaginous ring of the trachea) and at the carina-directly above the point of tracheal bifurcation into the primary bronchi. Excess connective tissue, lymph nodes, heart and lung tissue were removed from specimens using a scalpel and surgical tools. After dissection, tracheas were placed into zip top plastic bags and kept on wet ice during transportation. In the laboratory, a two-inch (i.e., ~5 cm) segment of each trachea was removed, rinsed with ice cold PBS and stored at -80°C until needed.

Airway epithelial tissue protein extraction and quantitation

Tracheal epithelial tissue protein lysates were prepared by thawing the two-inch tracheal rings on wet ice and removing one gram of the epithelia layer of tissue from the intraluminal surface of the tracheal rings. The tissue was homogenized in an extraction buffer [one Complete Protease Inhibitor Cocktail tablet (11697498001; Roche Applied Science, Indianapolis, IN) in a 5 ml solution of 0.5 g/ml of 1X Radio Immunoprecipitation Assay (RIPA) lysis buffer solution (Millipore, Temecula, CA), 50 µl of phosphatase inhibitor cocktail 1 (Cat. No. #2850, Sigma-Aldrich, St. Louis, MO) and 50 µl of protease inhibitor cocktail 1 (Cat. No. #8340, Sigma-Aldrich ST. Louis, MO)]. Tissue samples were homogenized into smaller fragments with a Brinkman Homogenizer (PT10/35), for 10 seconds on setting #9 (equivalent medium to high speed) interspersed with 1-minute incubations on wet ice to keep samples cold. These steps were repeated three times. The lysates were sonicated for 7 seconds using a Fisherbrand Model 505 Sonic Dismembrator (ThermoFisher Scientific) interspersed with 1-minute incubations on wet ice to keep samples cold. After homogenization and sonication, samples were centrifuged for 30 minutes in 4°C at the speed of 3,220 × *g* with an IEC Centra-7R refrigerated centrifuge (International Equipment Company: Needham, MA, USA). To clarify extracts, the supernatant was recovered and transferred to clean microcentrifuge tubes and centrifuged for another 30 minutes in 4°C at the speed of 20,124 × g with the Beckman Microfuge R Centrifuge (Beckman; model #365626, Palo Alto, CA, USA). The final supernatant was recovered and stored at -80°C as protein extracts until further investigation. Protein concentrations were determined by Bradford Protein Assay (Bio-Rad) according to manufacturer protocol.

Proteome profiler array to detect cell stress proteins in porcine tracheal epithelia

A cell stress array kit (R&D systems Inc.; Minneapolis, MN, USA) was used to evaluate all stress proteins that may be differentially modu-

lated in porcine airways. Equivalent amounts of protein samples (i.e., 100 μg each, total 600 μg) from six animals from the same swine housing type were pooled and applied to a single array membrane. This was repeated for six more animal samples, for a total of two arrays/housing type. The experiment was performed according to the standardized protocol manual provided in the kit. The developed proteome profiler arrays were imaged using a ChemiDoc MP Imaging System (Bio-Rad). Proteome profiler array fold difference densitometry analysis was performed using a method similar to that reported by McKnight and colleagues [30]. Briefly, spot pixel densities were captured using Image Lab version software. Each protein spot was log2-transformed and each spot pair was averaged; the three pairs of reference spot density averages were averaged to provide a single reference spot density average (3 sets of reference spot density averages/3). Each protein spot density average was divided by the single reference spot density average to yield a normalized protein spot density (spot density average/single reference spot density average). Fold differences were determined by dividing the normalized indoor value by outdoor value for each spot. Ratios above 1 or below 0.5 were considered differentially expressed.

Western blot analysis

Protein extracts (50 μg) from tissues were combined with 4X Laemmli buffer. After boiling (at 100°C, 5 minutes), protein extracts (50 μg/lane of gel) were fractionated using 10% TGX PAGE (Bio-Rad) at 60 volts for 15 minutes, and 120 volts for 75 minutes. Proteins were transferred to nitrocellulose membrane (0.45 μm) and blocked with 5% non-fat milk in Tris-Buffered Saline-Tween (TBS-T) at room temperature for 1 hour. The membranes were washed with TBS-T three times for 10-15 minutes and probed with the following primary antibodies (1:1000) -anti-COX-2, anti-SOD 1 and anti-SOD2 (Cell Signaling Technology), anti-HSP-60 and anti-HSP-70 (ThermoFisher Scientific, Rockford, IL), anti-PON2 and anti-β-actin (Santa Cruz, Dallas, TX)- dispersed in 0.5% bovine serum albumin (BSA) in TBS-T at 4°C overnight with rocking. The next day membranes were washed as described above. A secondary antibody (anti-rabbit, IgG, 1:2000 (Cell Signaling, Danvers, MA)); (anti-goat, IgG, 1:2000 (Cell Signaling, Danvers, MA)) was used to detect primary antibodies listed above. The membranes were washed and bands of interest detected using enhanced chemiluminescence (ECL) detection reagents (GE Healthcare Life Sciences; Buckinghamshire, HP7 9NA UK). Bands were visualized using a ChemiDoc MP Imager (Image Lab 5.2). Band volume density was used to determine protein abundance/expression levels. Four indoor and four outdoor pig samples were analyzed on the same blot; therefore, three western blots were performed for each protein of interest.

Enzyme activity assays

In order to evaluate SOD and CAT activity in tracheal epithelial tissue from indoor and outdoor pigs, commercially available activity kits were used according to manufacturer's instruction (Cayman Chemical, Ann Arbor, Michigan). One gram of freshly isolated tracheal epithelial tissue from each animal was utilized. The absorbance was read at 540 nm using a microplate reader.

Lipid peroxidation (LPO) assay

Freshly isolated tracheal epithelial tissue (1 gram) from indoor and outdoor pigs was homogenized in nano-pure water and transferred to sterile glass test tubes. Lipid peroxides were extracted into chloroform and quantified using a lipid hydroperoxide assay kit according to manufacturer's protocol (Cayman Chemical). The absorbance was read at 500 nm using a microplate reader.

Statistical analysis

Each pig was considered a biological unit. For analysis, unpaired T-tests with Welch's Correction (for unequal variances) were applied to detect significant differences between means of all parameters measured with tracheal epithelial tissue samples from pigs raised in the indoor and outdoor production environments (GraphPad Prism 7). For all analyses, a two-sided significance level of $P < 0.05$ was used.

Results

Fold difference of cell stress protein in tracheal epithelial tissue of pigs reared indoors and outdoors

We sought to compare the relative expression of cell stress-related proteins in extracts from intraluminal tracheal epithelial tissue of pigs

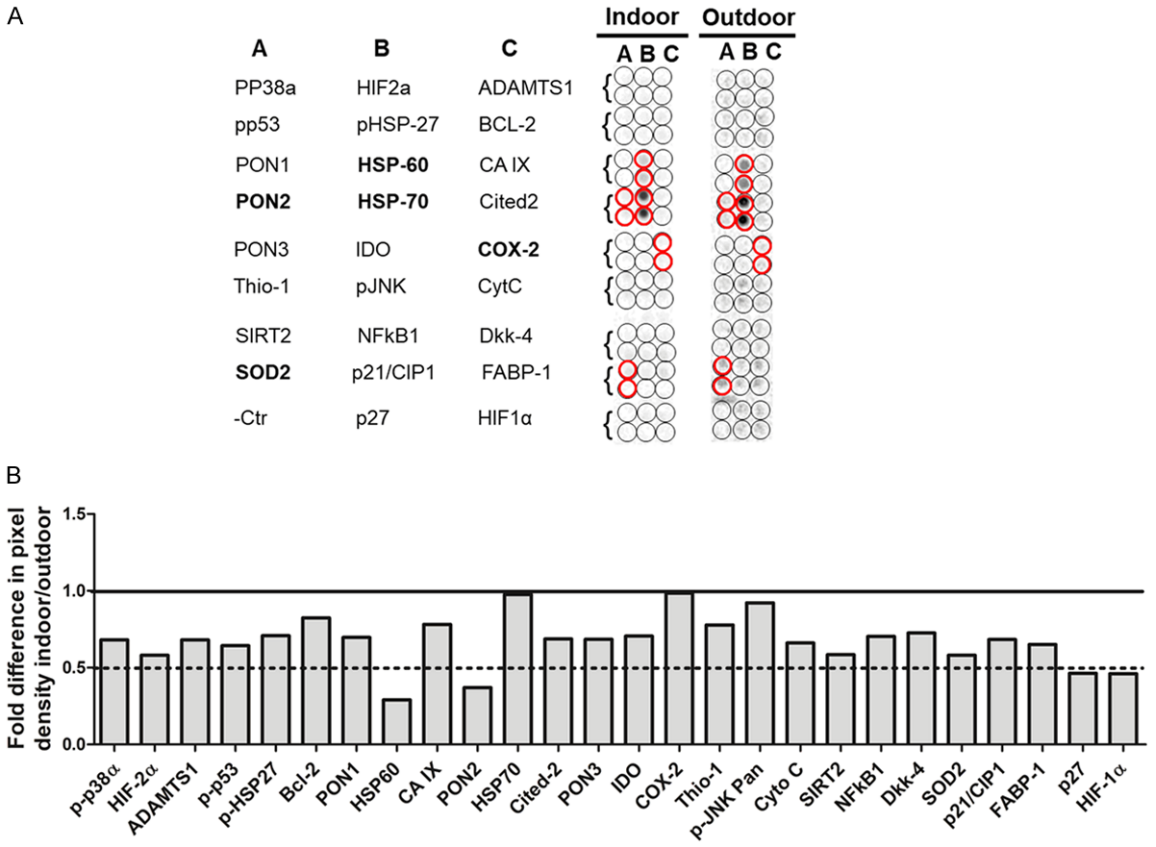

Figure 2. Profiling of cell stress proteins in porcine tracheal epithelium. A. Equal amounts of tracheal epithelia protein from pigs raised indoors (n = 6) and pigs raised outdoors (n = 6) were pooled, separately, and used for cell stress proteins array analysis. Proteins in bold and spots with red circles were validated by western blot. B. Fold difference densitometry analysis of cell stress protein from porcine tracheal epithelial. The intensity of each spot was measured and fold difference was determined as described in "Materials and Methods". The solid and dotted lines represent the 1-fold and 0.5-fold difference cut-offs, respectively. Representative image of two arrays. pp38α, phospho-p38 alpha (T181/Y185); HIF2α, hypoxia inducible factor 2 alpha; ADAMTS1, a disintegrin and metalloproteinase with thrombospondin motifs 1; pp53, phospho-p53 (S46); pHSP-27, phospho-heat shock protein-27; BCL-2, B cell lymphoma-2; PON1, paraoxonase 1; HSP-60, heat shock protein-60; CA IX, carbonic anhydrase IX; PON2, paraoxonase 2; HSP-70, heat shock protein-70; Cited2, Cbp/p300-interacting transactivator; PON3, paraoxonase 3; IDO, indoleamine 2,3-dioxygenase; COX-2, cyclooxygenase-2; Thio-1, thioredoxin-1; pJNK, phospho c-Jun n-terminal kinase (T183/Y185); CytC, cytochrome C; SIRT2, sirtuin 2; NFkB1, nuclear factor kappa B1; Dkk-4, dickkopf-4; SOD2, superoxide dismutase 2; p21/CIP1, cyclin-dependent kinase inhibitor 1A; FABP-1, fatty acid binding protein-1; -Ctr, negative control; p27, cyclin-dependent kinase inhibitor 1B; HIF1α, hypoxia inducible factor 1 alpha.

reared, separately, in confinement and pasture-based housing. Evaluation of protein concentration in one gram of tracheal epithelial tissue showed that indoor pigs had a significantly higher protein-to-tissue ratio compared to outdoor pigs (**Figure 1B**). Proteome profiling of tracheal epithelial tissue of pigs raised indoors and outdoors revealed differential expression of cell stress-related proteins as detected on a 26-protein array membrane (**Figure 2A**). Depicted in **Figure 2B** are fold differences determined by dividing normalized indoor densities by their respective outdoor spot from **Figure 2A**. Expression levels of 1-fold or greater (represented by solid line) was considered higher expression in indoor animals as depicted for two proteins from indoor pig samples (HSP70, COX-2). A 0.5-fold or lesser value was used to represent proteins with reduced expression in indoor pigs compared to outdoor pigs (dotted line). Four proteins (HSP60, PON2, p27, HIF1α) from indoor samples had densities ≤ 0.5 compared to outdoor. There were 20 cell stress-related proteins (p-p38α, HIF2α, ADAMTS1, p-p53, pHSP-27, BCL-2, PON1, CA IX, Cited-2, PON3, IDO, Thio-1, p-JNK Pan, Cyto C, SIRT2,

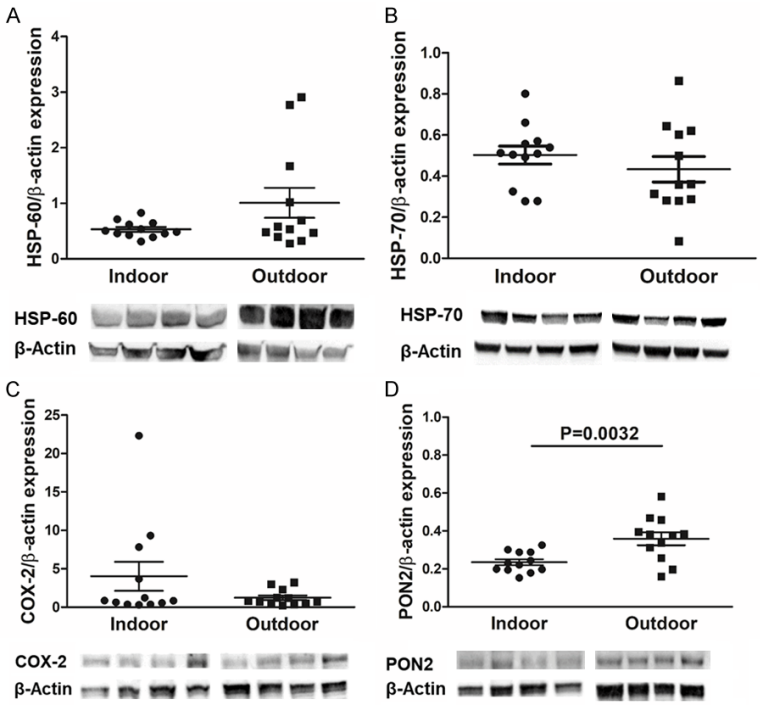

Figure 3. Western blot analysis of protein expression in tracheal tissue of pigs. Protein extracts from indoor (n = 12) and outdoor (n = 12) reared pigs were subjected to western blot analysis against (A) HSP60, (B) HSP70, (C) COX-2 and (D) PON2. Blots were stripped and reprobed to detect β-actin as a loading control. Normalized data are plotted as mean ± SEM. The blots are representative of three blots.

NFκB1, Dkk-4, SOD2, p21/CIP1, FABP-1) with expression levels greater than 0.5, but less than 1-fold difference.

Validation of protein expression and activity

Western blotting was carried out to validate expression of five proteins (HSP60, HSP70, COX-2, PON2 and SOD2) captured by the proteome profiler antibody array that met the criteria for differential expression. **Figure 3A-D** shows protein expression levels of HSP60, HSP70, COX-2 and PON2. The results revealed a trend toward increased HSP-60 in outdoor pigs compared to indoor pigs; although, these levels did not reach formal significance (P = 0.0936, **Figure 3A**). Likewise, there was no difference in HSP70, but there seemed to be a trend toward higher levels present in tracheal tissues of indoor pigs compared to outdoor pigs (**Figure 3B**). COX-2 expression (**Figure 3C**) was detected in both indoor and outdoor groups; however, no significant differences were found amongst the tissue samples (P = 0.1706). The expression of antioxidant PON2 was significantly higher in porcine tracheal epithelial tissue of outdoor pigs compared to those raised indoors (P = 0.0032, **Figure 3D**). Overall, the western blotting results for these proteins seem to be consistent with the findings of the proteome profiler array.

Another cell stress protein expressed at markedly lower levels in tracheal epithelial tissue of indoor pigs is SOD2, a major endogenous enzymatic antioxidant. Since there are two SOD isoforms localized within the cell, SOD1 (cytoplasmic) and SOD2 (mitochondrial), we performed western blot analysis of both proteins. The western blot results validated the outcome of the array studies, showing significantly less SOD1 (P = 0.0170, **Figure 4A**) and SOD2 (P = 0.0142, **Figure 4B**) proteins within the tracheal epithelial tissues of indoor pigs compared to outdoor pigs. Based on the results of the arrays and western blotting studies, we evaluated enzymatic activity levels of total SOD within tracheal epithelial tissue extracts of pigs raised indoors and outdoors. Tracheal epithelial tissue of outdoor pigs was found to have significantly higher SOD activity than indoor pigs (P = 0.0001, **Figure 5A**). SOD relieves oxidative stress by converting superoxide anion to hydrogen peroxide and molecular oxygen. A second major enzymatic antioxidant, catalase, converts reactive H_2O_2 molecules to molecular oxygen and water. Given the connection between these two antioxidants, we assessed catalase activity in the tracheal epithelial tissues of indoor and outdoor pigs. Compared to outdoor pigs, catalase activity within tracheal epithelial protein extracts was lower in indoor pigs (P = 0.0001, **Figure 5B**). Together, the results shown in **Figures 3-5** reveal a trend toward lower expression/activity of antioxidants and environmental sensing proteins in indoor pigs compared to outdoor pigs.

Accumulation of lipid hydroperoxides in tracheal epithelial tissue of indoor pigs

Given the results of the western blots and activity assays, we were interested in knowing whether low levels of enzymatic antioxidants

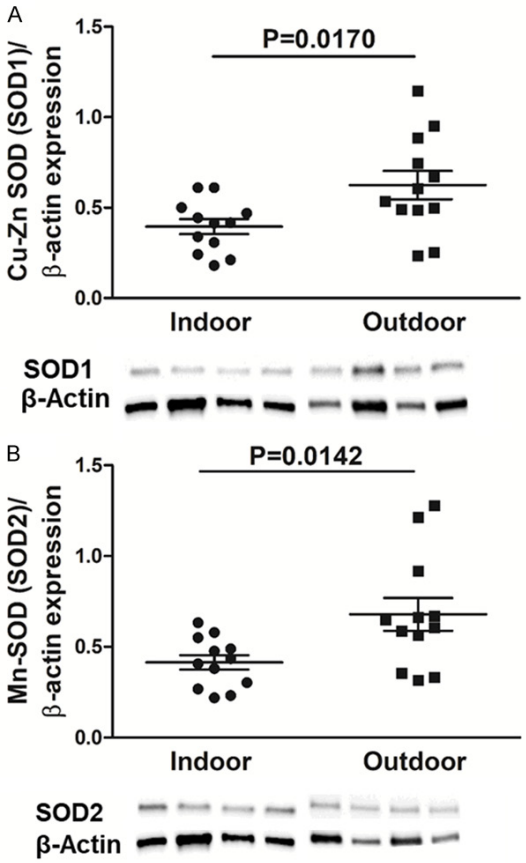

Figure 4. Superoxide dismutase 1 and 2 protein expression in tracheal tissue of pigs. Protein extracts from indoor and outdoor reared pigs were subjected to western blot analysis using antibodies to detect Cu-Zn SOD 1 (A) and Mn-SOD 2 (B). Blot were stripped and reprobed to detect β-actin as a loading control for normalization. The blots are representative of three blots. Results expressed as mean ± SE (n = 12 pigs).

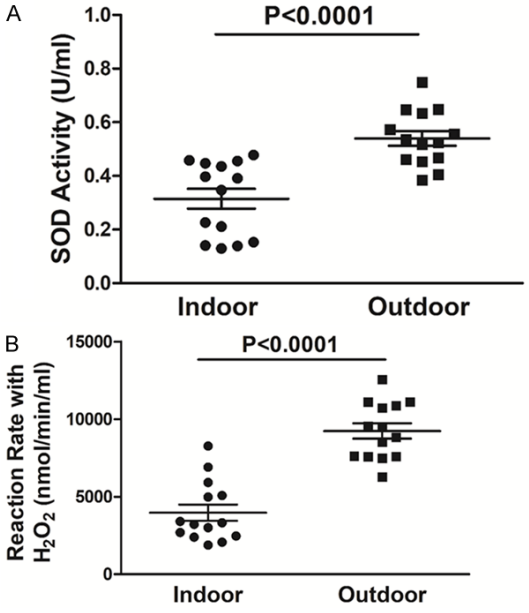

Figure 5. Assessment of superoxide dismutase and catalase enzymatic activity in tracheal epithelial tissue of pigs. Antioxidant activity of SOD (A) and catalase (B) enzymes in tracheal epithelial tissue extracts of pigs raised in indoor and outdoor environments. Results expressed as mean ± SE (n = 14 pigs each group).

are associated with oxidant accumulation in tracheal epithelium of indoor pigs. Oxidative stress can lead to peroxidation of membrane lipids, which can be assessed by measuring levels of lipid hydroperoxides (LPO) in tissue homogenates. We quantified the levels of lipid hydroperoxides in tracheal epithelial extracts and observed significantly higher levels of LPO in the tracheal epithelial tissues of indoor pigs compared to outdoor pigs (P = 0.0001, **Figure 6**), and thus, higher oxidative stress in tracheal epithelial tissue of indoor pigs.

Discussion

Inhalation of organic dust, gases and chemicals from swine production buildings is associated with chronic inflammatory lung disease in exposed people [1, 31]. Indoor pigs are continuously exposed to the same air pollutants; however, less is known about the effects of continuous exposures on swine respiratory health. In the present study, we provide evidence that continuous exposure to the indoor production environment is associated with reduced endogenous antioxidant enzyme expression and activity levels and accumulation of oxidants within tracheal epithelial tissue of SPF pigs. Protein profiling of tracheal epithelial tissue obtained from indoor and outdoor pigs from our university farm showed that indoor pigs had a trend toward lower levels of environmental stress sensing protein, HSP60, and significantly less endogenous antioxidant proteins PON2 and SOD1/2. The present findings indicate a critical role for continuous exposure to the indoor SPF environment in antioxidant defenses within the airway epithelium of indoor pigs.

Members of the HSP family, including HSP60 and HSP70, are known to be key environmental stress sensors and regulators of proteotoxic effects. Their primary responsibilities within the cell include chaperoning proteins during synthesis, folding, assembly and degradation.

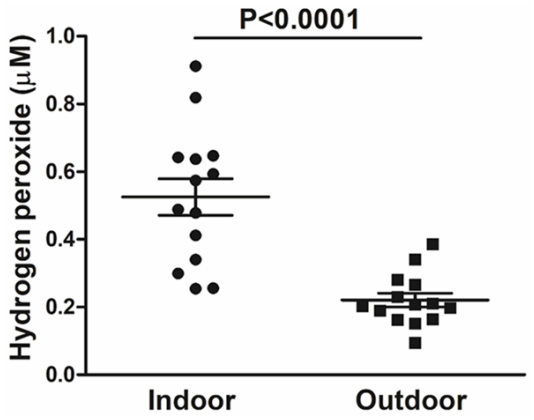

Figure 6. Evaluation of oxidative stress in tracheal epithelial tissue. Accumulation of lipid hydroperoxides in fresh tracheal epithelial tissues of indoor (n = 14) and outdoor (n = 14) pigs was measured using a chloroform extraction technique as described in the methods section. Mean ± SE.

HSP60, for example, is known to be a 'danger signal' in the innate immune system due to its ability to modulate expression of adhesion molecules by endothelial cells and release of potent proinflammatory cytokines including tumor necrosis factor alpha and interleukin-6 by human and mouse macrophages and smooth muscle cells *in vitro* [32, 33]. HSP60 has been shown to be a ligand for toll like receptor 4 with similar effects as lipopolysaccharide, a bacterial endotoxin and potent stimulator of the immune system [34]. In the present study indoor pigs expressed less HSP60 than outdoor pigs. This finding suggests that indoor pigs may be suppressing innate immune response within the airways. It is, however, noteworthy that indoor pigs do not have lower levels of HSP70 protein. In mucosal tissues, HSP70 is important for secretion of mucin- the protein component of mucus-into the airways for trapping and removal of inhaled particles via mucociliary clearance [35]. We speculate that the higher levels of protein we measured in indoor pig tracheal epithelial samples are partially attributed to mucin content. In a separate study, we observed higher levels of mucin proteins in bronchoalveolar lavage of indoor pigs compared to outdoor pigs (unpublished results).

Oxidants are highly reactive molecules, which can bind to and damage macromolecule such as DNA, proteins and membrane phospholipids. At physiological conditions, oxidants are balanced by antioxidants, which, for example, can neutralize oxidants by converting them into water and molecular oxygen [36]. Oxidant-antioxidant imbalance in the body has been linked to various diseases including chronic inflammatory lung diseases. The PON family (PON1, PON2 and PON3) are potent antioxidants and are involved in defense mechanisms against oxidative stress within blood and cells of the vasculature [23-25]. PON1 and PON3 are known to be associated with high-density lipoprotein (HDL) in blood due to their ability to hydrolyze oxidized lipids. Whereas PON2, which is expressed in all tissues, is reported to regulate oxidative stress at the cellular level [22, 37]. Decreased serum PON activity was noted in children with asthma when oxidant status, including lipid hydroperoxide levels, increased [38]. In the present study we detected less PON2 protein in tracheal epithelium of indoor pigs compared to their outdoor counterparts suggesting that indoor pigs may be susceptible to accumulation of oxidized lipids or cellular oxidative stress.

Aberrations in antioxidant defenses within the airway epithelium contributes to progression and exacerbation of a myriad of respiratory diseases including asthma and chronic obstructive pulmonary disease (COPD) in which diminished SOD levels and elevated ROS are observed in asthma and COPD patients [39, 40]. Observance of lower levels of SOD protein expression in tracheal epithelial tissue of indoor pigs is likely attributable to continuous exposure to organic dust and other substances within the SPF. Repeated inhalation of particulates and noxious substances can deactivate enzymatic antioxidants as observed in COPD patients and cigarette smokers when compared to non-smokers, who have higher levels of SOD, CAT and glutathione activity [39]. Accumulation of ROS can damage macromolecules including lipids. High levels of lipid hydroperoxides in airway epithelial tissue extracts of indoor pigs in the present study was associated with decreased CAT expression and activity. Thus, continuous exposure to conditions within the indoor swine production environment diminishes SOD and CAT abundance and activity within the airway epithelial tissues of pigs. Therefore, exposure to bioaerosols-comprised of organic dust, microorganisms, bacterial endotoxins and chemical toxicants-mediates oxidative stress events in porcine tracheal epithe-

lial tissue. In addition to exogenous sources of oxidants, inflammatory cells including macrophages and neutrophils can contribute to the milieu of ROS that can damage the airway epithelium. Chronic exposure to organic dust causes dampened inflammation responses by respiratory epithelial and immune cells of humans, mice and pigs *in vitro* [9-12] and *in vivo* [11]. This dampened inflammatory response has been referred to as the "chronic inflammation adaptive response" (as reviewed by [9, 41]). Findings of the present study are consistent with those reports and thus, it stands to reason that tracheal mucosa of indoor pigs may have adapted to continuous production environment exposures by developing a barrier that is better able to deal with the CAFO-style facility.

We observed signs of oxidative stress in the airways of indoor pigs. Namely, concentrated lipid hydroperoxides levels in tracheal epithelia were associated with diminished endogenous antioxidants in indoor pigs. Lipid peroxides form when oxygen radicals such as superoxide anion bind to free and membrane-bound lipids to produce lipid peroxides. Lipid peroxidation, a chain reaction of lipid deterioration, is a major contributor to chronic inflammatory lung diseases including chronic obstructive pulmonary disease. Accumulation of lipid hydroperoxides within the tracheal epithelium of indoor pigs could be explained by the diminished levels of SOD. Given the importance of SOD in the pathophysiology of COPD, it is plausible that diminished levels of SOD protein and activity creates oxidative stress permissive conditions as well. Low antioxidant defenses within the airways of indoor pigs could make them more susceptible to infection. A significant number of clinical and laboratory studies have demonstrated that oxidative stress increases susceptibility to infections with influenza (A and B) and respiratory syncytial virus (RSV); viruses which together account for approximately 64% of COPD exacerbations requiring hospitalization [33, 42-46].

There are several important limitations to consider for this study. First, the pigs on study were from a university farm rather than a large-scale commercial facility. It is expected that levels of organic dust and other air pollutants will vary from facility to facility. Animal stocking density within commercial indoor facilities differ from one facility to the next. Commercial facilities can house several hundred to more than a thousand head of hogs and environmental conditions will vary depending on management practices. Whereas our indoor swine facility has a capacity of 250 hogs and housed approximately 150 hogs at the time of this study. Although on a smaller scale, our university swine facility is modeled after conventional commercial facilities within the state of N.C., which ranks 2nd nationally for pork production and contains the No. 1 hog producing county in the nation. While the conditions within the university swine facility is sufficient to pilot effects of commercial facilities in general, there is a need to investigate the effect of continuous exposure to high density conventional (indoor) and pasture (outdoor) production environments on the airway epithelium of commercial pigs. Studies that will investigate the sources of oxidants and signaling pathways that contribute to reduced endogenous antioxidants and high levels of oxidants within the porcine airway epithelium are needed. Second, differences in airway protein between indoor and outdoor pigs was not investigated as part of this study. We figure mucin content partly explains this difference; however, studies are planned to identify components of the airway epithelial proteome of indoor and outdoor pigs. Third, the mechanism for lower levels of enzymatic antioxidants in indoor pigs was not explored here. The association between chronic inflammatory lung disease and oxidative stress is clear; however, the underlying mechanisms are poorly understood. In some cases, antioxidant levels have been reported as elevated [47], lower [48] or a mixture [17] in COPD patients. The explanation for these discrepancies is complex and is due to multiple reasons including variances in cigarette smoking status, clinical definitions, dietary intake of antioxidants and comorbidities [49-52]. In spite of antioxidant status, oxidant burden is typically increased in COPD.

Finally, albeit higher levels of lipid hydroperoxides were noted in indoor pigs, the types or sources of ROS were not evaluated. It is well established that low to moderate levels of ROS are required for physiological processes including defense against invading pathogens and cell signaling [53]. However, excessive levels can cause cellular damage. More studies are needed to fully understand threshold levels

and effects of oxidants in porcine airways. All things considered, this study provides an important initial look at the impact of continuous exposure to SPF environment on oxidant-antioxidant imbalance within the tracheal epithelium of pigs.

To summarize, antioxidants play an important role in keeping the lungs healthy and disease free. Chronic stress to airway epithelial cells such as with continuous or repeated exposure to indoor swine facilities can alter the effectiveness of antioxidants. The present study showed that continuous exposure to indoor swine production facility causes decreased expression and activity of endogenous antioxidants and accumulation of lipid hydroperoxides in tracheal epithelial tissues of pigs. More studies are needed to elucidate the role of antioxidants and the airway epithelial proteome on signaling cascades that regulate responses to repeat or continuous exposure to swine production environments.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jenora T Waterman, Department of Animal Sciences, North Carolina Agricultural and Technical State University, 1601 East Market Street, Greensboro, NC 27411-0002, USA. Tel: 336-285-4815; Fax: 336-334-7288; E-mail: jdwaterm@ncat.edu

References

[1] Eduard W, Pearce N and Douwes J. Chronic bronchitis, COPD, and lung function in farmers: the role of biological agents. Chest 2009; 136: 716-725.

[2] May S, Romberger DJ and Poole JA. Respiratory health effects of large animal farming environments. J Toxicol Environ Health B Crit Rev 2012; 15: 524-541.

[3] Neumann EJ, Kliebenstein JB, Johnson CD, Mabry JW, Bush EJ, Seitzinger AH, Green AL, Zimmerman JJ. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on U.S. pork producers. J Am Vet Med Assoc 2005; 227: 385-92.

[4] Gerald CL, McPherson C, McDaniel T, Xu Z, Holmes B, Whitley N, Williams L and Waterman JT. A biophysiochemical analysis of settled livestock and poultry housing dusts. Am J Agric Biol 2014; 9: 153-166.

[5] Masclaux FG, Sakwinska O, Charriere N, Semaani E and Oppliger A. Concentration of airborne staphylococcus aureus (MRSA and MSSA), total bacteria, and endotoxins in pig farms. Ann Occup Hyg 2013; 57: 550-557.

[6] Vogelzang PF, van der Gulden JW, Folgering H, Heederik D, Tielen MJ and van Schayck CP. Longitudinal changes in bronchial responsiveness associated with swine confinement dust exposure. Chest 2000; 117: 1488-1495.

[7] Palmberg L, Larssson BM, Malmberg P and Larsson K. Airway responses of healthy farmers and nonfarmers to exposure in a swine confinement building. Scand J Work Environ Health 2002; 28: 256-263.

[8] Mathisen T, Von Essen SG, Wyatt TA and Romberger DJ. Hog barn dust extract augments lymphocyte adhesion to human airway epithelial cells. J Appl Physiol (1985) 2004; 96: 1738-1744.

[9] Sahlander K, Larsson K and Palmberg L. Daily exposure to dust alters innate immunity. PLoS One 2012; 7: e31646.

[10] Poole JA, Alexis NE, Parks C, MacInnes AK, Gentry-Nielsen MJ, Fey PD, Larsson L, Allen-Gipson D, Von Essen SG and Romberger DJ. Repetitive organic dust exposure in vitro impairs macrophage differentiation and function. J Allergy Clin Immunol 2008; 122: 375-382, e371-374.

[11] Poole JA, Thiele GM, Alexis NE, Burrell AM, Parks C and Romberger DJ. Organic dust exposure alters monocyte-derived dendritic cell differentiation and maturation. Am J Physiol Lung Cell Mol Physiol 2009; 297: L767-776.

[12] Knetter SM, Tuggle CK, Wannemuehler MJ and Ramer-Tait AE. Organic barn dust extract exposure impairs porcine macrophage function in vitro: implications for respiratory health. Vet Immunol Immunopathol 2014; 157: 20-30.

[13] Hoppin JA, Umbach DM, Long S, Rinsky JL, Henneberger PK, Salo PM, Zeldin DC, London SJ, Alavanja MC, Blair A, Beane Freeman LE

and Sandler DP. Respiratory disease in United States farmers. Occup Environ Med 2014; 71: 484-491.
[14] Brass DM, Savov JD, Gavett SH, Haykal-Coates N and Schwartz DA. Subchronic endotoxin inhalation causes persistent airway disease. Am J Physiol Lung Cell Mol Physiol 2003; 285: L755-761.
[15] Smith LJ, Shamsuddin M, Sporn PH, Denenberg M and Anderson J. Reduced superoxide dismutase in lung cells of patients with asthma. Free Radic Biol Med 1997; 22: 1301-1307.
[16] Harting JR, Gleason A, Romberger DJ, Von Essen SG, Qiu F, Alexis N and Poole JA. Chronic obstructive pulmonary disease patients have greater systemic responsiveness to ex vivo stimulation with swine dust extract and its components versus healthy volunteers. J Toxicol Environ Health A 2012; 75: 1456-1470.
[17] Nadeem A, Raj HG and Chhabra SK. Increased oxidative stress and altered levels of antioxidants in chronic obstructive pulmonary disease. Inflammation 2005; 29: 23-32.
[18] Oberley-Deegan RE, Regan EA, Kinnula VL and Crapo JD. Extracellular superoxide dismutase and risk of COPD. COPD 2009; 6: 307-312.
[19] Rahman I, Biswas SK and Kode A. Oxidant and antioxidant balance in the airways and airway diseases. Eur J Pharmacol 2006; 533: 222-239.
[20] Jarjour NN, Busse WW and Calhoun WJ. Enhanced production of oxygen radicals in nocturnal asthma. Am Rev Respir Dis 1992; 146: 905-911.
[21] Paredi P, Kharitonov SA, Leak D, Ward S, Cramer D and Barnes PJ. Exhaled ethane, a marker of lipid peroxidation, is elevated in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2000; 162: 369-373.
[22] Ng CJ, Wadleigh DJ, Gangopadhyay A, Hama S, Grijalva VR, Navab M, Fogelman AM and Reddy ST. Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. J Biol Chem 2001; 276: 44444-44449.
[23] Ng CJ, Shih DM, Hama SY, Villa N, Navab M and Reddy ST. The paraoxonase gene family and atherosclerosis. Free Radic Biol Med 2005; 38: 153-163.
[24] Ng CJ, Bourquard N, Grijalva V, Hama S, Shih DM, Navab M, Fogelman AM, Lusis AJ, Young S and Reddy ST. Paraoxonase-2 deficiency aggravates atherosclerosis in mice despite lower apolipoprotein-B-containing lipoproteins: anti-atherogenic role for paraoxonase-2. J Biol Chem 2006; 281: 29491-29500.
[25] Aviram M and Rosenblat M. Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development. Free Radic Biol Med 2004; 37: 1304-1316.
[26] Kim KY, Ko HJ, Kim HT, Kim YS, Roh YM, Lee CM and Kim CN. Monitoring of aerial pollutants emitted from Swine houses in Korea. Environ Monit Assess 2007; 133: 255-266.
[27] Kim KY, Ko HJ, Kim YS and Kim CN. Assessment of Korean farmer's exposure level to dust in pig buildings. Ann Agric Environ Med 2008; 15: 51-58.
[28] Donham KJ. Association of environmental air contaminants with disease and productivity in swine. Am J Vet Res 1991; 52: 1723-1730.
[29] Vaughn SE. FASS guide for the care and use of agricultural animals in research and teaching. Champaign IL: Federation of Animal Science Societies, 2010.
[30] McKnight M, Allen J, Waterman JD, Hurley SL, Idassi and Minor RC. Moringa tea blocks acute lung inflammation induced by swine confinement dust through a mechanism involving TNF-α expression, c-Jun N-terminal Kinase activation and neutrophil regulation. American Journal of Immunology 2014; 10: 73-87.
[31] Millner PD. Bioaerosols associated with animal production operations. Bioresour Technol 2009; 100: 5379-5385.
[32] Kol A, Bourcier T, Lichtman AH and Libby P. Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. J Clin Invest 1999; 103: 571-577.
[33] Chen W, Syldath U, Bellmann K, Burkart V and Kolb H. Human 60-kDa heat-shock protein: a danger signal to the innate immune system. J Immunol 1999; 162: 3212-3219.
[34] Précourt LP, Marcil V, Ntimbane T, Taha R, Lavoie JC, Delvin E, Seidman EG, Beaulieu JF, Levy E. Antioxidative properties of paraoxonase 2 in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 2012; 303: G623-34.
[35] Fang S, Crews AL, Chen W, Park J, Yin Q, Ren XR and Adler KB. MARCKS and HSP70 interactions regulate mucin secretion by human airway epithelial cells in vitro. Am J Physiol Lung Cell Mol Physiol 2013; 304: L511-8.
[36] Wang J, Zhang H, Zhang T, Zhang R, Liu R and Chen Y. Molecular mechanism on cadmium-induced activity changes of catalase and superoxide dismutase. Int J Biol Macromol 2015; 77: 59-67.
[37] Horke S, Witte I, Wilgenbus P, Kruger M, Strand D and Forstermann U. Paraoxonase-2 reduces oxidative stress in vascular cells and decreases endoplasmic reticulum stress-induced caspase activation. Circulation 2007; 115: 2055-2064.

[38] Cakmak A, Zeyrek D, Atas A, Selek S and Erel O. Oxidative status and paraoxonase activity in children with asthma. Clin Invest Med 2009; 32: E327-34.
[39] Al-Afaleg NO, Al-Senaidy A and El-Ansary A. Oxidative stress and antioxidant status in Saudi asthmatic patients. Clin Biochem 2011; 44: 612-617.
[40] Barreiro E, Fermoselle C, Mateu-Jimenez M, Sanchez-Font A, Pijuan L, Gea J and Curull V. Oxidative stress and inflammation in the normal airways and blood of patients with lung cancer and COPD. Free Radic Biol Med 2013; 65: 859-871.
[41] Poole JA and Romberger DJ. Immunological and inflammatory responses to organic dust in agriculture. Curr Opin Allergy Clin Immunol 2012; 12: 126-132.
[42] Case AJ, McGill JL, Tygrett LT, Shirasawa T, Spitz DR, Waldschmidt TJ, Legge KL and Domann FE. Elevated mitochondrial superoxide disrupts normal T cell development, impairing adaptive immune ressponses to an influenza challenge. Free Radic Biol Med 2011; 50: 448-458.
[43] Jaspers I, Ciencewicki JM, Zhang W, Brighton LE, Carson JL, Beck MA and Madden MC. Diesel exhaust enhances influenza virus infections in respiratory epithelial cells. Toxicol Sci 2005; 85: 990-1002.
[44] Akaike T, Ando M, Oda T, Doi T, Ijiri S, Araki S and Maeda H. Dependence on O2-generation by xanthine oxidase of pathogenesis of influenza virus infection in mice. J Clin Invest 1990; 85: 739-745.
[45] Cho HY, Imani F, Miller-DeGraff L, Walters D, Melendi GA, Yamamoto M, Polack FP and Kleeberger SR. Antiviral activity of Nrf2 in a murine model of respiratory syncytial virus disease. Am J Respir Crit Care Med 2009; 179: 138-150.
[46] Seemungal TA, Donaldson GC, Bhowmik A, Jeffries DJ and Wedzicha JA. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2000; 161: 1608-1613.
[47] Stanojkovic I, Kotur-Stevuljevic J, Milenkovic B, Spasic S, Vujic T, Stefanovic A, Llic A and Ivanisevic J. Pulmonary function, oxidative stress and inflammatory markers in severe COPD exacerbation. Respir Med 2011; 105 Suppl 1: S31-37.
[48] Singh S, Verma SK, Kumar S, Ahmad MK, Nischal A, Singh SK and Dixit RK. Evaluation of oxidative stress and antioxidant status in chronic obstructive pulmonary disease. Scand J Immunol 2017; 85: 130-137.
[49] Burlakova EB, Zhizhina GP, Gurevich SM, Fatkullina LD, Kozachenko AI, Nagler LG, Zavarykina TM and Kashcheev VV. Biomarkers of oxidative stress and smoking in cancer patients. J Cancer Res Ther 2010; 6: 47-53.
[50] Wickenden JA, Clarke MC, Rossi AG, Rahman I, Faux SP, Donaldson K and MacNee W. Cigarette smoke prevents apoptosis through inhibition of caspase activation and induces necrosis. Am J Respir Cell Mol Biol 2003; 29: 562-570.
[51] Cantin AM, North SL, Hubbard RC and Crystal RG. Normal alveolar epithelial lining fluid contains high levels of glutathione. J Appl Physiol (1985) 1987; 63: 152-157.
[52] Skyba P, Kluchova Z, Joppa P, Petrasova D and Tkacova R. Nutritional status in relation to respiratory impairment and systemic inflammation in patients with acute exacerbations of COPD. Med Sci Monit 2009; 15: CR528-533.
[53] Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M and Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; 39: 44-84.