# ONLINE SUPPLEMENTARY MATERIAL

## Post exposure antioxidant treatment decreases airway hyperplasia and hyperreactivity due to chlorine inhalation in rats

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**SUPPLEMENTAL METHODS**

***Animal experiments and experimental protocols***

All animal experiments were in full compliance with regulations issued by the University of Alabama at Birmingham animal resources program and were approved by the Institutional Animal Care and Use Committee. For all experiments, adult male Sprague Dawley rats (200-250 g bw; Harlan Inc., Indianapolis, IN) were used and housed under standard conditions. Food [Purina rodent chow (Dyets Inc., Bethlehem, PA)] and autoclaved water was provided *ad libitum*.

***Chlorine exposure***

Rats were exposed to 0ppm or 400ppm chlorine in air for 30 minutes in a customized glass chamber (Specialty Glass Inc., Houston, TX) within a chemical fume hood (Labonco Inc., Kansas City, MO) as previously described (E1, E2). Briefly, two mass flow controllers (MFC; Scott Specialty Gases, Plumsteadville, PA) and a microprocessor control unit (Scott Specialty Gases, Plumsteadville, PA) were used to control the volume of medical grade air and 1000ppm chlorine (Airgas South Inc., Kennesaw, GA) delivered to the exposure chamber. Mixing was achieved at a T-junction followed by a specially designed diffuser located in the lid of the glass chamber. Flow rates for each gas were confirmed with a bubble flow meter (Supelco Inc., Bellefonte, PA). The volume of the glass exposure chamber was 4L while the total flow rate for all exposures was 5L/min for a turnover rate of 1.25 volume changes per minute.

The chlorine concentration in the chamber was monitored with an Interscan Corporation (model# RM34-1000m) chlorine detector, connected to a data logger, as previously described (E2). Rats were weighed, placed inside the cylindrical glass chamber two at a time and exposed to 400ppm chlorine for 30 minutes. At the end of each exposure the chamber was vented with compressed air for 2–3 min, and the rats were removed and returned to their cages, where they breathed room air.

***Antioxidant administration and surgical procedures***

Peripheral oxygen saturations were measured on the rat tails with a MouseOx Small Animal Oxymeter (STARR Life Sciences, Allison Park, PA) connected to a personal computer equipped with analysis software (E3). Respiratory rates were measured by observation prior to and following the chlorine exposure and prior to and following aerosol administration. Saline, or a combination of ascorbic acid (20mg/rat) and deferoxamine mesylate (15mg/kg), 0.2ml total volume, were administered via tail vein injection within five min following chlorine exposure. To avoid oxidation of ascorbate, solutions were prepared freshly for each experiment.

At 1 hour and 15 hours post chlorine exposure, rats were administered an aerosol of saline, or a combination of ascorbic acid (150 mg/mL) and deferoxamine mesylate (0.357mg/mL) by nose-only inhalation for 1 hour. The aerosol was generated with a Retec®nebulizer (In-Tox Products LLC, Albuquerque, NM) as previously described (E2). The aerosol was conditioned with medical grade air using a radial inline mixer (In-Tox Products LLC, Albuquerque, NM). It was dried with passive dilution air to the desired particle size and directed through the aerosol delivery line into a radial nose-only exposure plenum (In-Tox Products LLC). The six port exposure plenum was equipped with two customized rat exposure tubes and an oxygen probe (Hudson RCI; Durham, NC). A cascade impactor (In-Tox Products LLC) was used to determine particle size distributions, and a filter housing (In-Tox Products LLC) with filter (Millipore, Billerica, MA) was used to for the gravimetric determination of delivered aerosol. The sixth port was plugged due to the fact that it was not being utilized. All compressed air and vacuum air flows were measured with pressure controllers and mass flow controllers (Alicat Scientific, Inc.; Tucson, AZ). The entire exposure system, with the exception of the medical grade air cylinders and vacuum pumps, was housed inside a Class II Biosafety Cabinet (The Baker Company; Sanford, ME). The system was considered to be stable and the aerosol was considered to be characterized after three consecutive runs with similar results. The particle size distribution and the mean particle size were determined using a cascade impactor (In-Tox Products LLC). The ascorbate delivered was determined by

sampling from the exposure plenum through a filter at a given flow rate. In order to estimate the ascorbate concentration in the aerosol, ascorbate collected on filters under carefully controlled conditions, was extracted into a 1mM phosphoric acid solution, a solution in which it is very soluble (E4). The absorbance was recorded at 243 nm, near the maximal absorbance of the conjugate acid of ascorbate. The phosphoric acid solution was selected in order to ensure that ascorbate [p$K_a$ = 4.25 (E4)] will be present predominantly as the more stable conjugate acid, and to simplify the spectrophotometric analysis since the conjugate acid and base forms of ascorbate display different electronic spectra. When necessary, the extracts were diluted further and their absorbances were recorded and directly compared to a standard curve for ascorbic acid in 1mM phosphoric acid that covered the absorbance range from 0 to 1. Based on these measurements (weight of solute on the filter and amount of ascorbate present) we calculated the amount of ascorbate delivered to each rat/min by the following formula: Delivered ascorbate/min= Increase of filter weight/min x Fraction of ascorbate on filter. The estimated inhaled dose was then calculated by multiplying the delivered dose times the minute ventilation.

At 24 hours or 7 days post chlorine exposure, rats were deeply anesthetized via IP injection of 0.4mL diazepam (5mg/mL) (Hospira Inc., Lake Forest, IL) and 0.4mL ketamine HCl (5mg/mL) (IVX Animal Health Inc., St. Joseph). Rat lungs were evaluated for either biochemical analysis or for histology. For biochemical analysis, the rib cage was completely removed and 1.5mL blood was drawn from the left ventricle of the heart with 0.1mL heparin (American Pharmaceutical Partners Inc, Schaumburg, IL) in the syringe to prevent agglutination and immediately placed on ice. The lungs were flushed with 20 ml ice-cold saline via the right ventricle; the lungs were removed, weighed, and homogenized in an equal volume (mL) to tissue weight (g) of 10% metaphosphoric acid (MPA) and stored at -80°C for determination of ascorbate and reduced glutathione (E1). Blood was centrifuged at 700g for 10 minutes at 4°C and the plasma was stabilized with an equal volume of 10% MPA in deionized water, stored at -

80°C for measurements of antioxidants.

***Determination of ascorbate by HPLC***

All procedures were as previously described (E1). BALF was spun down at 16,000 g for 4 minutes at 4°C and the supernatant was separated from the pellet. The plasma samples were further diluted to a ratio of 1:4 (total dilution 1:8 when considering the sample preparation step) with 50 mM phosphate buffer 2.2. The diluted plasma samples were spun down at 16,000g for 4 minutes at 4°C and the supernatant was saved. A small volume of the tissue homogenate was further diluted with 50mM phosphate buffer 2.2 at a ratio of 1:20 (total dilution 1:40 when considering the sample preparation step) and then spun down at 16,000g for 4 minutes at 4°C. All supernatants were filtered through a 0.22µm filter with a syringe into HPLC vials for analysis. All samples were kept on ice until transferred to the HPLC.

The HPLC mobile phase consisted of a mixture containing 50mM phosphate buffer pH 3.3, 100µM sodium octylsulfonate and $CH_3CN$ (ratio 98.25:1.75) (Sigma Aldrich, Saint Louis, MO) at a flow rate of 0.6mL/minute. A Phenomenex Luna reversed phase column C18 (2) 250 x 4.60mm (Phenomenex Inc., Torrance, CA) and a Phenomenex guard column (ODS, 4mm L x 3.0mm ID) were used. An LC-2010CHT Shimadzu HPLC (Shimadzu Scientific Instruments, Columbia, MD) equipped with a Peltier temperature control autosampler set to 4°C was used to isocratically separate sample analytes. Analyte detection was made possible by way of an ESA CoulArray electro chemical detector model 5600A (ESA Laboratories Inc., Chelmsford, MI). Additionally, a pre-detector peak filter was used. All samples and standards were run at least in duplicate and the results were averaged. The data was analyzed using ESA CoulArray®software and Microsoft Excel. Standard curves were prepared with ascorbic acid, glutathione (GSH), oxidized glutathione (GSSG) and uric acid (UA) (Sigma Aldrich, Saint Louis, MO).

***Lung histology and morphology.***

For histology, lungs were inflated via a tracheal cannula with 10% formalin at 25cm $H_2O$

pressure for 1 hour, removed *en bloc* and immersed in 10% formalin for an additional 48 hours. The left lung was microdiseccted to ensure that proximal airways were between intrapulmonary airway generations 2-3, cut perpendicular to the long axis, and three sections were processed for paraffin sectioning and staining with hematoxylin and eosin (H&E) or Alcian blue/period acid-Schiff (AB/PAS) stain (E5) to identify epithelial mucous cells. Images were recorded on an Olympus BX41 microscope with a Q-Color 3 digital camera attached to a personal computer using QCapture software (QImaging, Surrey,BC, Canada), and composed in Adobe® Photoshop®. Morphometric analysis was performed on midlevel airway epithelium (intrapulmonary generations 2 and 3) and on terminal bronchioles using standard procedures (E6). All measurements were made using a 40X objective and 5-micron thick sections. The following cell types were evaluated in H&E sections: (1) nonciliated cells (identified by the lack of cilia and nuclei located away from the basal lamina); (2) ciliated cells (identified by cilia on the luminal sides and nuclei located near the basal lamina); and (3) basal cells (identified by triangular shape, nuclei near the basal lamina with cytoplasm that did not reach the lumen). Cells that were not identifiable as one of the three defined cells types were defined as "other". The cell volumes, as measured by volume ($\mu m^3$) of airway epithelial cells per unit area ($\mu m^2$) of basement membrane (Vs), were estimated from point and intercept counts using a 136-point cycloid grid by the equation: $Vs=(Po \times 2)/(\pi \times Int)$; where Po represents the points counted for each object of interest and INT represents the number of intercepts if the basal lamina. Thickness of the epithelium is calculated as the total volume of all cell types present. In AB/PAS stained sections, epithelium was identified as AB/PAS positive mucosubstances or non AB/PAS epithelium (E6). The volume of the stored mucosubstances present in the airway epithelium was also measured by point and intercept counts and reported as nanoliters of intraepithelial mucosubstances per $mm^2$ of basal lamina (E5).

**Respiratory system mechanics:** Rats were mechanically ventilated and challenged with increasing concentrations of methacholine as described previously (E7). Briefly, rats were anesthetized with

diazepam (17.5mg/kg) and ketamine (450mg/kg), intubated, connected to a ventilator (FlexiVent; SCIREQ, Montreal, PQ, Canada) and ventilated at a rate of 160 breaths per minute at a tidal volume of 0.2mL with a positive end-expiratory pressure of 3cm $H_2O$. Total respiratory system resistance (R) and elastance (E) were recorded continuously as previously described (E8). Baseline was set via deep inhalation. Increasing concentrations of methacholine chloride (0–50 mg/ml, Sigma-Aldrich, St Louis, MS) were administered via aerosolization within an administration time of 10 seconds. Airway responsiveness was recorded every 15 seconds for 3 minutes after each aerosol challenge. Broadband perturbation was used and impedance was analyzed via constant phase model.

***Statistics***

Data presented as mean ± standard error (biochemical assays) or as mean ± standard deviation (morphometric data). Statistical analyses were performed with Graph Pad InStat software (GraphPad Software Inc., San Diego, CA). The level of significance was determined using one way analysis of Variances (ANOVA) and Bonferroni multiple comparisons test.

**Supplemental References**

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