# ONLINE SUPPLEMENTARY MATERIAL

## Ascorbate and deferoxamine administration post chlorine exposure decrease mortality and lung injury in mice

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## SUPPLEMENTAL METHODS

**Whole body exposure to $Cl_2$:** A custom made glass chamber (Specialty Glass, Inc. Houston Texas) was used to expose mice to a mixture of air and chlorine ($Cl_2$). Two mass flow controllers (MFC) with Kalrez seals (Scott Specialty Gases, part # 05236A1V5K) and a microprocessor control unit (Scott Specialty Gases, part # 05236E4) were used to control the flow of each gas delivered to the exposure chamber. Communication between each MFC and the control unit were with an RS-232 cable. Cylinders of medical grade air and 1000ppm $Cl_2$ in air were purchased from Airgas, (Birmingham, AL). Stainless steel tubing was used to connect the $Cl_2$ cylinder to the first MFC, and Teflon tubing was used to connect the air cylinder to the second MFC. Teflon tubing was used to exit each MFC. The gases were joined at a junction before introduction into the exposure chamber. Swagelok fittings were used to make all connections. A bubble flow meter was used to validate MFC accuracy on a weekly basis.

Mice were exposed by first opening the air cylinder followed by the $Cl_2$ cylinder. This prevented a high influx of chlorine into the exposure chamber. Air and $Cl_2$ were initially mixed at a three-way junction, and they were further mixed by passing through a diffuser located inside the top lid of the exposure chamber. Gases exited the chamber via two large-bore diameter ports in its bottom half. The exposure chamber was placed inside a chemical fume hood located in a negative-pressure room. A specially designed diffuser located within the lid of the chamber assured uniform gas phase composition throughout the chamber interior.

Six mice were placed inside the cylindrical glass chamber and exposed to 600 ppm $Cl_2$ for 45 min. The $Cl_2$ concentrations in the chamber were monitored with an Interscan Corporation (model # RM34-1000m) $Cl_2$ detector (operating on the principle of electrochemical reduction), connected to a data logger for data storage. The sample was diluted with air at a ratio of 20:1 to avoid corrosion of the detectors sensor (**Figure 1**). At the end of each exposure the chamber was vented with compressed air for 2–3 min, and the mice were removed and returned to their cages, where they breathed room air. Breath rates were measured by observation when mice returned to room air. Food and water were provided *ad libitum*.

**Murine Nose-Only Inhalation Exposure System:** The murine nose-only inhalation exposure system consisted of six components (**Figure E1**): (i) a compressed air source; (ii) an aerosol delivery line; (iii) a radial nose-only inhalation exposure plenum; (iv) an aerosol characterization platform; (v) an air handling station, and (vi) an exhaust platform.

Air was delivered to the exposure system from a cylinder of compressed USP breathing quality air. The aerosol delivery line consisted of a PARI LC Plus nebulizer (PARI Respiratory Equipment, Inc., Midlothian, VA), a radial in-line aerosol mixer (In-Tox Products, LLC; Albuquerque, NM), a filtered air passive dilutor, and a Quad-Trak Diffusion Dryer™ (In-Tox Products, LLC; Albuquerque, NM). The 24-port, radial inhalation exposure plenum (In-Tox Products, LLC; Albuquerque, NM) was fitted with Positive Flow-By™ murine restraint tubes (In-Tox Products, LLC; Albuquerque, NM) and isoaxial sample collection ports which interfaced with the aerosol characterization platform. The aerosol characterization platform included a 47 mm in-line filter holder (In-Tox Products, LLC; Albuquerque, NM) and a seven-stage cascade impactor. The air handling station interfaced with the aerosol delivery line, the aerosol characterization platform, and the exhaust platform and consisted of regulated gas flow controllers (Alicat Scientific, Inc.; Tucson, AZ and Brooks Instruments; Hatfield, PA). The exhaust platform consisted of high-efficiency particulate absorbing (HEPA) filters, a differential pressure magnehelic, and a vacuum pump.

The aerosol delivery line, inhalation exposure plenum, and aerosol characterization platform were placed inside a Class II Type A Biosafety Cabinet (Forma Scientific, Inc., Marietta, OH). The inhalation exposure plenum was operated at a slightly negative pressure relative to the biosafety cabinet. The biosafety cabinet was operated at a slightly negative pressure with respect to the inhalation laboratory. The temperature inside the Class II biosafety cabinet was monitored using a laboratory thermometer. Inhalation exposure plenum oxygen levels were monitored continuously with a Model 5800 Intelligent Oxygen Monitor (Hudson RCI; Durham, NC). A schematic of the inhalation exposure system is presented in **Figure E1**.

**Murine Nose-Only Inhalation Exposure Procedure:** Prior to inhalation exposure, mice were trained/acclimated to the nose-only restraint tubes. Mice were placed in nose-only restraint tubes for approximately 30 minutes and monitored to confirm proper orientation and respiration. For inhalation exposure, mice were placed in nose-only restraint tubes and connected to the plenum. The 47 mm filter holder was prepared by inserting a pre-weighed 47 mm filter (Pallflex® Fiberfilm™; Pall Corporation, Ann Arbor, MI) and connected to the plenum. The PARI LC Plus nebulizer was filled with either USP water for injection for vehicle control exposures or a solution containing 0.3577 mg/mL deferoxamine (Hospira Inc.; Lakeforest, IL), and 150 mg/ml of ascorbic acid (American Regent, Inc, Shirley, NY) in sterile water (Braun, Irvine, CA) containing. The plenum exhaust and radial mixer gas flow controllers were

set at flow rates of twenty and five liters per minute, respectively. The start of the exposure period (T = 0) began once the nebulizer gas flow controller was activated. All mice were exposed to vehicle or ascorbic acid and deferoxamine aerosol for 20 minutes. The aerosol vehicle (sterile) water was removed by a diffusion dryer in the aerosol delivery line prior to reaching the mice. Thus the amount of water delivered to the mice was small. In additional studies we found that delivery of aerosolized ascorbate and deferoxamine under similar conditions did not increase the volume of the epithelial ling fluid of lung water in normal lungs.

To assess the aerosol concentration of ascorbate and deferoxamine delivered to each animal, a filter was inserted into one of the plenum ports during aerosol delivery. Two five minute filter samples were collected during each exposure at approximately T=5 minutes and T= 15 minutes. The filters were dried overnight in a covered container inside a Class II Type A2 biosafety cabinet and weighed using a Mettler MX5 analytical microbalance (Mettler Toledo, Inc., Columbus, OH). Aerosol concentration was determined from the differential weight of the filter samples and reported as milligrams per liter of air (mg/L). Because the concentration of ascorbate was forty times higher than deferoxamine, the weight of the filter mainly reflected the amount of ascorbate deposited. However, sodium hydroxide and sodium bicarbonate which are included in the ascorbate solution also contributed to the weight of filter. Thus this gravimentric method probably overestimated the amount of delivered ascorbate by 15-25%.

To assess the particle size distribution of aerosolized ascorbate and deferoxamine, a single cascade impactor sample was collected from the plenum at approximately ten minutes from the initiation of aerosol delivery. The aerosol particle size distribution, reported as mass median aerodynamic diameter (MMAD), and geometric standard deviation, GSD, both in microns, were determined by linear probit and non-linear histogram plot reduction analyses of the cumulative mass collected. The inhaled dose (ID) of ascorbic acid was estimated using the modified Inhalation Dose Exposure Equation (E1), $ID = C_T \times V_m \times T$, where $C_T$ = aerosol Concentration (mg/L), $V_m$ = murine minute ventilation (L/min), and T = exposure time (min). Murine minute ventilation was estimated using data given by Flandre *et al*. (E2). **Experimental protocols**: Mice were euthanized with an intraperitoneal injection of ketamine/xylazine (100 and 10 mg/kg body weight respectively) 72 h after exposure to $Cl_2$. The chest cavity was opened while care was taken not to puncture the lungs, the trachea was exposed and the lungs were lavaged. Specifically, one ml of normal saline was instilled and withdrawn slowly three times. Approximately 80% of the instilled fluid was recovered in both groups. The samples were centrifuged at 300 *g* for 10 min at 4°C to pellet cells and cellular debris. The cell-free supernatant was split into one-ml aliquots and placed on ice. 143 µL of 40% metaphosphoric acid (MPA) in water were added to the sample that would be used for HPLC analysis. The lung tissue was harvested, homogenized with 10% MPA double their weight in volume for subsequent HPLC analysis and stored along with BALF samples at -80°C as previously

described (E3). Air control animals were euthanized and samples were collected under the same procedures.

After BALF centrifugation and storage of the supernatant, cell pellets were incubated with of red blood cell lysis solution and centrifuged at 300 x *g* for 10 minutes. Cells were re-suspended in 100 µl of 2% bovine serum albumin (BSA) saline solution and cells were counted using a Neubauer hemocytometer. Cytoslides were prepared and stained with hematoxylin and eosin (Hema 3 System, Fisher HealthCare, Houston, TX). Differential counts of BAL inflammatory (monocytes, neutrophils, lymphocytes) and epithelial cells were determined via light microscopy. Data from cytoslides were used only when more that 300 cells per slide could be counted.

**Western blots.** Protein in equal volumes of BAL (40 µl) from different groups were separated by denaturing SDS-PAGE (10%) gel and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then blotted for IgM and albumin using anti-IgM (cat. no. GTX77095, GeneTex, San Antonio, TX) and anti-albumin (cat. no. GTX77024, GeneTex, San Antonio, TX) antibodies. Secondary antibodies were used in conjunction with donkey antigoat IgG-horseradish peroxidase (HRP). Protein bands were revealed by enhanced chemiluminescence using HRP substrate (Pierce Biotechnology, Rockford, IL) and X-ray films. Densitometry was performed using Adobe Photoshop Elements 8.

## SUPPLEMENTAL RESULTS

### Breath rates

Breath rates were recorded (observational) immediately after exposure in both groups of exposed animals (saline and ascorbate plus deferoxamine) in order to ensure that they had equal respiratory distress as an index of lung injury. The two groups did not differ significantly (91.5±8.1 in saline group vs. 120.4±10.4 in Asc/Def group; **Figure E2**). The approximate normal breathing rate in C57BL/6 mice is around 310 breaths/min (E4). After performing one sample t-test for each group both breathing rates were found to be significantly lower than normal ($p<0.0001$ in both cases).

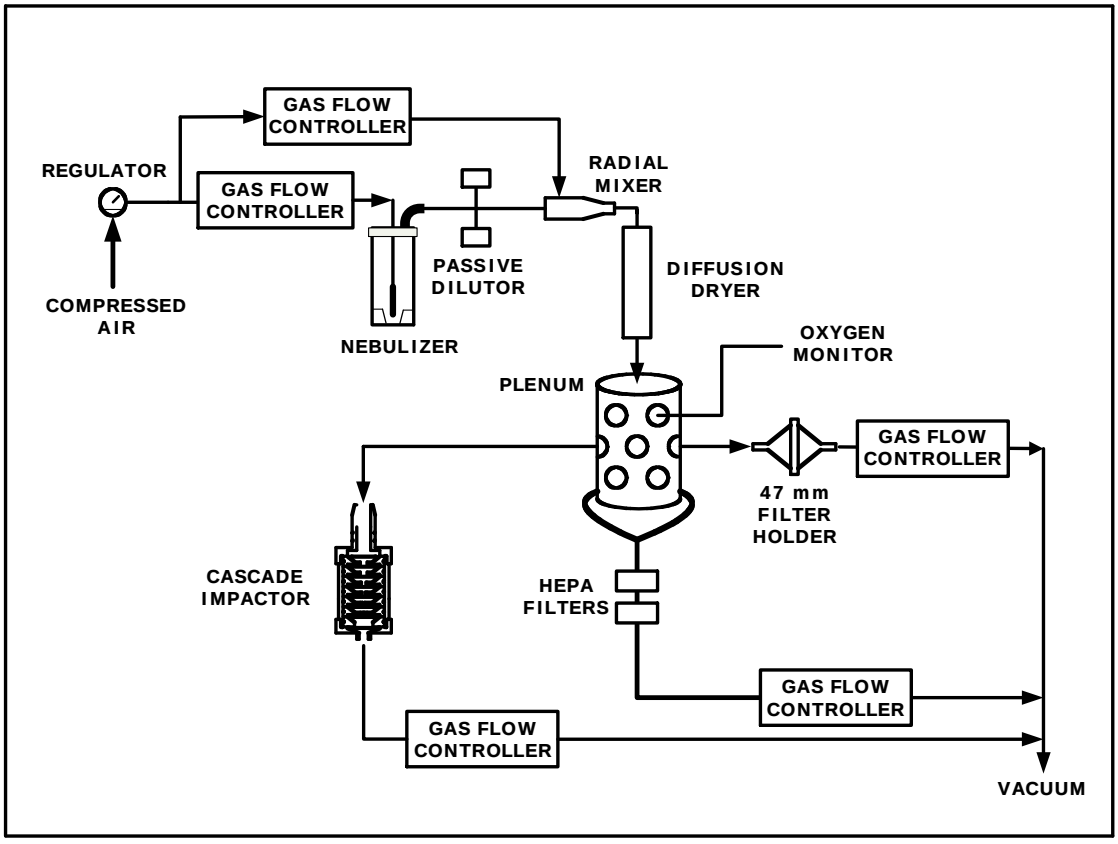


**Figure E1.** Murine Ascorbic Acid Nose-Only Inhalation Exposure System.

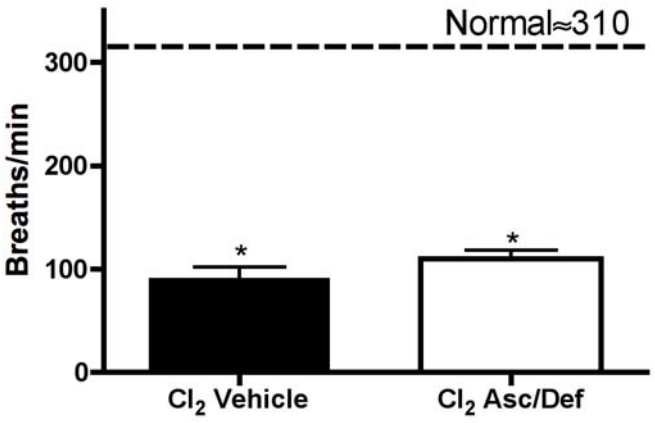


**Figure E2.** Breath rates of mice immediately after $Cl_2$ exposure. Breath rates were recorded by manual counting. Data were analyzed by one sample t-test compared to the normal value of breath rates in C57Bl/6 mice. *$p$<0.0001; n=18 in each group.

## References


E1. ATSDR Public Health Assessment Guidance Manual, Appendix G, Revised January 2005.

E2. Flandre, TD, Leroy PL, Desmecht DJ. Effect of somatic growth, strain, and sex on double chamber plethysmographic respiratory function values in healthy mice. *J Appl Physiol* 2003; 94: 1129-1136.

E3. Leustik M, Doran S, Bracher A, Williams S, Squadrito GL, Schoeb TR, Postlethwait E, Matalon S. Mitigation of Chlorine-Induced Lung Injury by Low-Molecular-Weight Antioxidants. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L733-L743.

E4. Bessac BF, Sivula M, von Hehn CA, Escalera J, Cohn L, Jordt SE. TRPA1 is a major oxidant sensor in murine airway sensory neurons. *J Clin Invest* 2008; 118**:** 1899-1910.