Targeted Aerosolized Delivery of Ascorbate in the Lungs of Chlorine-Exposed Rats

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Abstract

Background: Chlorine (Cl₂)-induced lung injury is a serious public health threat that may result from industrial and household accidents. Post-Cl₂ administration of aerosolized ascorbate in rodents decreased lung injury and mortality. However, the extent to which aerosolized ascorbate augments depleted ascorbate stores in distal lung compartments has not been assessed.

Methods: We exposed rats to Cl₂ (300 ppm for 30 min) and returned them to room air. Within 15–30 min postexposure, rats breathed aerosolized ascorbate and desferal or vehicle (mean particle size 3.3 μm) through a nose-only exposure system for 60 min and were euthanized. We measured the concentrations of reduced ascorbate in the bronchoalveolar lavage (BAL), plasma, and lung tissues with high-pressure liquid chromatography, protein plasma concentration in the BAL, and the volume of the epithelia lining fluid (ELF).

Results: Cl₂-exposed rats that breathed aerosolized vehicle had lower values of ascorbate in their BAL, ELF, and lung tissues compared to air-breathing rats. Delivery of aerosolized ascorbate increased reduced ascorbate in BAL, ELF, lung tissues, and plasma of both Cl₂ and air-exposed rats without causing lung injury. Based on mean diameter of aerosolized particles and airway sizes we calculated that approximately 5% and 1% of inhaled ascorbate was deposited in distal lung regions of air and Cl₂-exposed rats, respectively. Significantly higher ascorbate levels were present in the BAL of Cl₂-exposed rats when aerosol delivery was initiated 1 h post-Cl₂.

Conclusions: Aerosol administration is an effective, safe, and noninvasive method for the delivery of low molecular weight antioxidants to the lungs of Cl₂-exposed individuals for the purpose of decreasing morbidity and mortality. Delivery is most effective when initiated 1 h postexposure when the effects of Cl₂ on minute ventilation subside.

Key words: epithelial lining fluid, bronchoalveolar lavage, lung injury, HPLC, urea

Introduction

EXPOSURE TO CHLORINE (Cl₂) represents a serious and underestimated threat to public health. The accidental mixing of bleach (sodium hypochlorite) with acidic solutions in household settings results in the generation of Cl₂, which may cause severe bronchoconstriction especially in people with preexisting lung diseases.^(1,2) Children and adults inadvertently exposed to Cl₂ in swimming pool accidents (such as the recent malfunction of Cl₂ delivery systems to a water park near Sacramento, CA, <http://sanfrancisco.cbslocal.com/>

2011/08/15/20-hospitalized-from-chlorine-leak-at-sacramento-water-park/) experienced bronchoconstriction and lung function impairment lasting for several months.⁽³⁾ Furthermore, the accidental release of large amounts of Cl₂ in 30 large cities world-wide during the last 20 years (see, e.g., ref ⁽⁴⁾) and the deliberate release of Cl₂ during acts of terrorism by insurgents in the Iraq conflict,⁽⁵⁾ caused significant mortality and morbidity to humans and animals. In addition to these public incidents, there were about 9000 calls for Cl₂ related injuries to U.S. poison control centers each year from 2000–2005.⁽⁶⁾

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The extent of Cl_2 -induced injury in humans and animals depends on the concentration and duration of exposure. Our theoretical analysis shows that Cl_2 first reacts with antioxidants in the lung epithelial lining fluid (ELF).⁽¹⁾ When antioxidants are depleted, Cl_2 hydrolyzes to form hypochlorous acid (HOCl) and its conjugate base (OCl^-), which react with proteins, components of the extracellular matrix, and unsaturated fatty acids. Products of these reactions (chloramines, lipid hydroperoxides, and low molecular weight hyaluronic acid fragments) are considerably toxic. In previous studies we assessed the onset and development of lung injury in rodents exposed to Cl_2 concentrations likely to be encountered in the vicinity of industrial accidents. A prominent finding, in agreement with our theoretical analysis, was the significant and sustained decrease of lung ascorbate, a key antioxidant in both rodents and humans.^(2,7) This was followed by injury to respiratory and alveolar epithelial cells resulting in compromised ion transport, increased airway resistance and hyperreactivity, surfactant dysfunction, increased levels of protein in the alveolar space, pulmonary edema, and even death from respiratory failure.^(2,5,7-11) In addition to pulmonary toxicity, Cl_2 caused systemic injury and endothelial dysfunction, resulting from the inactivation of endothelial nitric oxide synthase in pulmonary arteries.⁽¹²⁾

These studies led to the exciting and novel concept that replenishment of lung ascorbate post Cl_2 exposure decreases the extent of lung injury. In order for antioxidants to work efficiently, they must be delivered in such a fashion as to replenish antioxidants stores in the lung epithelial lining fluid and tissues rapidly. Aerosols are an effective and noninvasive means of delivering antioxidants to large airways and distal lung spaces. However, bronchoconstriction as well as local edema may limit the effectiveness of aerosolized antioxidants. Herein, we exposed rats to sublethal concentrations of Cl_2 , returned them to room air, and assessed the efficiency of aerosolized ascorbate to deplete depleted ascorbate levels in lung epithelial lining fluid, lung tissues, and plasma if administered shortly after return to room air. Furthermore, we measured levels of H_2O_2 in the bronchoalveolar lavage (BAL) of rats following administration of ascorbate to test the possibility that ascorbate may act as a pro-oxidant by generating hydrogen peroxide (H_2O_2).⁽¹³⁻¹⁶⁾ Our results demonstrate that inhalation of aerosolized ascorbate via nose-only inhalation is an effective means of delivering ascorbate to distal lung regions of Cl_2 -exposed rats without causing significant damage to the airway and alveolar epithelia.

Materials and Methods

Reagents

Ascorbic acid for injection USP 500 mg/mL without preservative (American Regent Inc., Shirley, NY) and deferoxamine mesylate for injection USP 500 mg/vial lyophilized (Hospira Inc., Lake Forest, IL) were used in the preparation of the solutions to be administered. All solutions were prepared in a Class II biosafety cabinet (Labonco Inc., Kansas City, MO). Only pyrogen free consumables were used. All glassware was heat treated accordingly. Dilutions of the ascorbic acid stock solution and the lyophilized deferoxamine were done with sterile and pyrogen free saline 0.9% 308 mOsmol/liter (Hospira Inc.). Solutions to be aerosolized were prepared with sterile deionized water (Hospira Inc.).

Animals

Male Sprague-Dawley rats (Harlan Inc., Indianapolis, IN), with a body weight between 200 and 250 g, were used for all experiments. All rats were housed in the University of Alabama at Birmingham Animal Facility under standard conditions. Food (Purina rodent chow, Dyets Inc., Bethlehem, PA) and autoclaved water were provided *ad libitum*. All animal experiments were approved by the UAB Institutional Animal Care and Use Committee.

Exposure of rats to Cl_2

Rats were exposed to a mixture of 300 ppm Cl_2 in air for 30 min in environmental chambers and returned to room air as previously described.^(7,8) Within 15–30 min from the end of exposure, rats breathed a mixture of aerosolized ascorbic acid and deferoxamine (desferal) mesylate by nose-only inhalation for 1 h. The nebulizer concentrations were 150 mg/mL for ascorbate and 0.357 mg/mL for deferoxamine. We opted to use a combination of ascorbate and desferal because at high concentrations ascorbate may act as a pro-oxidant by reducing transition metals, thus initiating a variety of radical reactions, including the formation of hydroxyl radicals through Fenton chemistry.⁽¹³⁾ Deferoxamine decreases the pro-oxidant activity of ascorbate by chelating ferric ions and at the same time maintaining ascorbate in the reduced state and thereby ensuring that ascorbate is not oxidized during the aerosol administration. In another set of experiments, air- or Cl_2 -exposed rats were connected to the aerosol delivery system and breathed vehicle (water) under identical conditions. The aerosol delivery system has been described previously,⁽⁸⁾ and is shown in Figure 1. Peripheral oxygen saturations were measured with a MouseOx Small Animal Oxymeter (STARR Life Sciences, Allison Park, PA) connected to a computer equipped with MouseOx software.⁽¹¹⁾ The sensor was placed on the rat tail.

Shortly (within 5 min) after the completion of aerosol delivery, rats were anesthetized via intraperitoneal (i.p.) injection of 0.4 mL diazepam (5 mg/mL) (Hospira Inc.) and 0.4 mL ketamine HCl (5 mg/mL) (IVX Animal Health Inc., St. Joseph, MO). Depth of anesthesia was verified by lack of toe pinch reflex. The chest was opened and a 1.5 mL blood sample was drawn from the left ventricle in a heparinized syringe. The lungs were then perfused with ice-cold saline until clear of blood, and lavaged with 8 mL ice-cold saline, which was instilled and withdrawn twice. The BAL fluid was centrifuged at $200 \times g$ to pellet cells and debris and stored in ice. The lungs were then removed and large airways as well as non-pulmonary tissue were trimmed. Plasma, BAL and lung tissue were then processed for measurements of reduced ascorbate with high-pressure liquid chromatography using electrochemical detection, as described previously.^(7,8) Most of the ascorbate is in the reduced form. The concentrations of total proteins in the BAL were measured with the Bradford method using the Bio Rad Quick StartTM protein assay kit. Standard curves were generated using bovine serum albumin (Bio Rad Laboratories, Hercules, CA) for each experiment.

Measurement of epithelial lining fluid volume

We calculated the volume of the ELF by measuring the concentrations of urea in both the plasma and recovered

FIG. 1. The aerosol generating and delivery system inside a Biosafety II hood. Arrows point to the components listed below: 1. nose only exposure restraint tubes for rats (shown) or mice; 2. cascade impactor for the analysis of the aerodynamic particle size distribution; 3. filter housing for gravimetric determination of the aerosol concentration; 4. magnahelic pressure gauge; 5. exhaust line; 6. aerosol delivery line/transfer line; 7. six-port radial nose only aerosol inhalation exposure plenum; 8. oxygen saturation probe (on the backside of the plenum); 9. filter housing for gravimetric determination of the aerosol concentration; 10. filters for gravimetric determination of aerosol concentration; 11. brass radial mixer with air supply line; 12. compressed air jet nebulizers (Retec nebulizer); 13. syringes with nebulizer solution and supply line to the nebulizer; 14. oxygen saturation monitor; 15. timer; 16. mass flow controllers connected to air supply lines to regulate flows; 17. vacuum gate valves; 18. compressed air gate valves.

pooled BAL fluid as previously described.^(2,17) Briefly, $\text{ELF} = (\text{BAL}) \times [\text{urea}]_{\text{BAL}} / ([\text{urea}]_{\text{plasma}} - [\text{urea}]_{\text{BAL}})$, where ELF and BAL are the volumes of the ELF and of the BAL used for lavage (8 mL) and $[\text{urea}]_{\text{BAL}}$ and $[\text{urea}]_{\text{plasma}}$ are the concentrations of urea in the BAL and plasma, respectively. We then calculated the concentrations of ascorbate and total protein in the ELF as follows:

$$[\text{Z}]_{\text{ELF}} = ((\text{BAL} + \text{ELF}) \times [\text{Z}]_{\text{BAL}}) / \text{ELF}$$

where $[\text{Z}]_{\text{ELF}}$ is the concentration of ascorbate or protein in the ELF, BAL, and ELF are the volumes of BAL and EKL and $[\text{Z}]_{\text{BAL}}$ is the concentration of ascorbate or protein in the ELF.⁽²⁾

Measurement of hydrogen peroxide in BAL fluid

The concentration of hydrogen peroxide in BAL fluid was determined by using an Amplex Red hydrogen peroxide assay kit (Invitrogen Corp., Carlsbad, CA). The amount of hydrogen peroxide is quantified by the production of resorufin, which has a fluorescence emission maximum at 571 nm. Quantification was done with at 540 nm excitation and 590 nm emission wavelength with a fluorescence plate reader (BMG Labtechnologies, Inc., Durham, NC 27703).

Statistics

Statistical significance of multiple group means was assessed by ANOVA followed by the Bonferroni modification of the *t*-test. Group differences were considered to be statistically significant when $p \leq 0.05$. Data are represented as

mean $\pm$ standard error (SE) of the mean. The presence of normal distribution was tested using the Kolmogorov-Smirnov method and equal standard deviation between groups was tested using Bartlett's test. In cases where there was a significant difference of standard deviations between the tested groups, data was normalized by calculating their $\log_{10}$ values. Statistical differences based on calculations with normalized data are labeled. Significant changes in arterial oxygen saturation of aerosol administered animals were determined with two-tailed paired *t*-test.

Results

All rats survived the exposure to Cl_2 . Exposure to Cl_2 resulted in significant decreases of peripheral oxygen saturations (Fig. 2), which returned toward baseline in rats receiving either antioxidants (AA) or vehicle. Aerosol administration caused a small but nonsignificant decrease of oxygen saturations in air-breathing rats.

Characterization of aerosolized ascorbate

Typical particle size distribution data for aerosolized AA are shown in Figure 3. The mean mass median aerodynamic diameter (MMAD), and mean geometric standard deviation (GSD) were $3.3 \pm 0.1 \mu\text{m}$ and 1.9 ± 0.2 , respectively, consistent with our previous studies employing aerosol administration.⁽⁸⁾

Delivery of ascorbate to normal and Cl_2 -injured lungs

In all studies, we measured reduced ascorbate only. In Cl_2 -exposed rats, a fraction of ascorbate in the BAL, ELF, lung

FIG. 2. Peripheral oxygen saturations in mice prior to and following exposure to Cl_2 . Oxygen saturations were measured in conscious rats as described in Materials and Methods at the indicated times. Values are means $\pm$ 1 SE for $n \geq 5$. * $p < 0.05$ compared to the corresponding air values at the same interval.

tissue, and plasma may have been oxidized by Cl_2 , HOCl, and their reactive intermediates to dehydroascorbate. It would be difficult to measure dehydroascorbate because its half-life in the ELF is less than 10 min and its steady-state concentration is insignificant compared to ascorbate. The ascorbate that is oxidized to dehydroascorbate decomposes to various other products that by definition do not contribute to "total ascorbate" (ascorbate + dehydroascorbate). Ascorbate may also be transported from the surface of the lung to various tissues. Thus, measurements of ascorbate in Cl_2 -exposed rats may underestimate the total ascorbate delivered.

FIG. 3. Particle size distribution of the anti-oxidant aerosols determined for the indicated nebulizer concentrations. Cascade impactor samples were collected from the inhalation exposure plenum and reduced using SigmaPlot software. Particle size distribution is presented as mass median aerodynamic diameter (MMAD, μm) and geometric standard deviation (GSD). Y-axis: $dM/M_O d \log d_{AE}$. dM/M_O is mass fraction of particles collected on each stage of the impactor. $d \log d_{AE}$ is the aerodynamic equivalent diameter of the particles collected. Results of typical experiment; similar data were collected during all experiments.

FIG. 4. Epithelial lining fluid volumes (ELF) of air and Cl_2 exposed rats. Rats breathed either air or Cl_2 (300 ppm for 30 min) in environmental chambers and returned to room air. Within 15–30 min later they breathed either aerosolized vehicle (Veh.) or ascorbate and desferal (AA) through a nose-only exposure system for 60 min. Another group of rats breathed air but not aerosols (Untr.). All rats were sacrifice 60 min later and their lungs lavaged. ELF volumes were measured from measurements of urea concentrations in plasma and BAL as described in the text. Values are means $\pm$ 1 SE (in μL). $N=6$ for each group except for the Cl_2 AA ($n=4$). * $p < 0.05$; ** $p < 0.01$ compared with their corresponding air values, respectively.

FIG. 5. Protein amounts in the ELF of air and Cl_2 -exposed rats. Rats breathed either air or Cl_2 (300 ppm for 30 min) in environmental chambers and returned to room air. Within 15–30 min later they breathed either aerosolized vehicle (Veh.) or ascorbate and desferal (AA) through a nose-only exposure system for 60 min. Another group of rats breathed air but not aerosols (Untr.). All rats were sacrifice 60 min later and their lungs were lavaged. Total protein values in the ELF were calculated as shown in the Materials and Methods. Values are means $\pm$ 1 SE in μg . $N=6$ for each group except for the Cl_2 AA ($n=4$). ** $p < 0.01$ compared with their corresponding air values.

As shown in Figure 4, air-breathing rats not receiving aerosols had mean ELF volumes of $112 \pm 22 \mu\text{L}$; ELF values of air-breathing rats receiving aerosolized vehicle (water) or AA for 60 min and sacrificed shortly after were not statistically different from this value. Similarly, aerosolized AA did not increase the concentrations of proteins in the ELF (Fig. 5); rats exposed to Cl_2 had significantly higher ELF volume and protein values, which were unchanged by aerosolized delivery of AA (Figs. 4 and 5). These findings indicate that aerosolized AA did not cause significant damage to the airway and alveolar epithelial of normal and Cl_2 -exposed rats.

As shown in Figures 6, 7, and 8, aerosol delivery of AA in air breathing rats resulted in significant augmentation of ascorbate levels in BAL, ELF, and lung tissue. Aerosol delivery 15–30 min postexposure also increased the corresponding variables in Cl_2 -exposed rat lungs, albeit at much lower levels, most likely due to depressed ventilation and bronchoconstriction. However, aerosolized AA restored depleted values of ascorbate to their normal controls. In addition, aerosol administration of AA increased ascorbate in the plasma (Fig. 9).

Fraction of inhaled AA reaching the rat distal lung regions

The inhaled ascorbate dose (ID) was calculated by the following equation as described in the online supplement of ref. (8): $\text{ID} = C_T \times V_E \times T$, where C_T = aerosol concentration in the plenum, that is, inhaled concentration (mg/L),

FIG. 6. Reduced ascorbate concentrations in the BAL of air and Cl_2 -exposed rats. Rats breathed either air or Cl_2 (300 ppm for 30 min) in environmental chambers and returned to room air. Within 15–30 min later they breathed either aerosolized vehicle (Veh.) or ascorbate and desferal (AA) through a nose-only exposure system for 60 min. Another group of rats breathed air but not aerosols (Untr.). They were then sacrificed 60 min postinhalation and their lungs were lavaged. Reduced ascorbate was measured by HPLC as described in the Materials and Methods. Values are means ± 1 SE in μM . $N=6$ for each group except for the Cl_2 AA ($n=4$). * $p < 0.05$ compared with their corresponding air values; # $p < 0.01$ compared to the corresponding Veh. value in the same group.

FIG. 7. Reduced ascorbate amounts in the ELF of air and Cl_2 -exposed rats. Rats breathed either air or Cl_2 (300 ppm for 30 min) in environmental chambers and returned to room air. Within 15–30 min later they breathed either aerosolized vehicle (Veh.) or ascorbate and desferal (AA) through a nose-only exposure system for 60 min. Another group of rats breathed air but not aerosols (Untr.). They were then sacrificed 60 min postinhalation and their lungs were lavaged. Reduced ascorbate was measured by HPLC as described in the Materials and Methods. Values are means ± 1 SE in nmol. $N=6$ for each group except for the Cl_2 AA ($n=4$). ** $p < 0.01$ compared with their corresponding air values; ## $p < 0.01$ compared to their corresponding Veh. values in the same group.

FIG. 8. Reduced ascorbate amounts in the lung tissue of air and Cl_2 -exposed rats. Rats breathed either air or Cl_2 (300 ppm for 30 min) in environmental chambers and returned to room air. Within 15–30 min later they breathed either aerosolized vehicle (Veh.) or ascorbate and desferal (AA) through a nose-only exposure system for 60 min. Another group of rats breathed air but not aerosols (Untr.). They were then sacrificed 60 min postinhalation and their lungs were lavaged. Reduced ascorbate was measured by HPLC as described in the Materials and Methods. Values are means ± 1 SE in nmol. $N=6$ for each group except for the Cl_2 AA ($n=4$). * $p < 0.05$ compared with the corresponding air value; # $p < 0.05$; ## $p < 0.01$ compared to the corresponding value in the same group, respectively.

FIG. 9. Reduced ascorbate concentrations in the plasma of air and Cl₂-exposed rats. Rats breathed either air or Cl₂ (300 ppm for 30 min) in environmental chambers and returned to room air. Within 15–30 min later they breathed either aerosolized vehicle (Veh.) or ascorbate and desferal (AA) through a nose-only exposure system for 60 min. Another group of rats breathed air but not aerosols (Untr.). They were then sacrificed 60 min postinhalation and their lungs were lavaged. Reduced ascorbate was measured by HPLC as described in the Materials and Methods. Values are means $\pm$ 1 SE in μ M. $N=6$ for each group except for the Cl₂ AA ($n=4$). ## $p < 0.01$ compared to the corresponding value in the same group.

V_E = minute ventilation (L/min), and T = aerosol delivery time (min). The concentration of inhaled ascorbate in the plenum was calculated by the weight gain of a filter placed in an empty plenum port during aerosol delivery (corrected for the weight of other solutes (NaCl and preservatives) in the solution⁽¹⁸⁾), averaged 2.8 mg/L. Measured values for V_E for normal, conscious, spontaneously breathing rats vary from 60 to 45 mL/min/100 g body weight,^(19,20) which agree well with the predicted values for V_E based on body weight ($V_E = 2.1 \times BW_g^{0.75}$).⁽²¹⁾ Thus, assuming a mean body weight of 220 g and a mean V_E of 0.130 L/min, we calculated an average ID of 22 mg, which corresponds to approximately 124,000 nmol of inhaled ascorbate (based on a MW of 176). The amounts of ascorbate present in the ELF and lung tissues of air breathing rats (i.e., the difference values among air breathing rats receiving AA and vehicle) shortly after the completion of aerosol deliveries were 5136 and 1065 nmol, respectively. Thus, the total amount of ascorbate delivered in distal lung tissues (6201 nmol) was about 5% of the inhaled dose. This value is an underestimate, because plasma ascorbate increased by 1000 nmol (based on a rat plasma volume of about 10 mL) as well, due to the diffusion of ascorbate from the lungs into plasma and eventually into systemic tissues (Fig. 9). Based on the mean MMAD of our aerosolized particles (3.3 μ m)⁽⁸⁾ and existing information in the literature,⁽²²⁾ we calculated that approximately 8% of the inhaled ascorbate should be deposited in the distal lung regions of rats, which agrees well with our experimental findings.

In Cl₂-exposed rats, assuming a 50% drop in V_E based on measurements of respiratory frequency shortly after return

to room air,⁽¹⁸⁾ the corresponding values are: ID=11 mg (62,000 nmol); ELF=200 nmol; lung tissue=400 nmol; % delivered in the distal lung=1%. This was sufficient to normalize depleted ascorbate levels in the ELF and lung tissues. The remaining inhaled ascorbate was most likely deposited in the external nares and upper airways. As mentioned above, this measurement is an underestimate, because plasma ascorbate increased by 1000 nmol as well, reflecting diffusion of ascorbate from the lungs into plasma and eventually into systemic tissues. In addition, in Cl₂-exposed rats, a fraction of ascorbate was probably oxidized to dehydroascorbate and was not detected by our method. In the next series of experiments we tested the hypothesis that delivery to the distal lungs can be improved by delaying the onset of aerosol delivery to 1 h postexposure. These rats also breathed aerosolized ascorbate and desferal through a nose only exposure for 60 min, euthanized shortly thereafter and their lungs lavaged in the same fashion. As shown in Figure 10, the ascorbate concentration the BAL was 10-fold higher when compared to the corresponding value when aerosol administration was initiated within 15–30 min postexposure. This was most likely due to increased ventilation, decreased bronchoconstriction, and partial resolution of nasal congestion as well as less oxidation of ascorbate.

H₂O₂ in the BAL of rats postaerosolization

The concentrations of H₂O₂ in Cl₂-exposed rats that breathed vehicle or ascorbate starting at 15–30 min postexposure for 60 min were 33 $\pm$ 19 and 122 $\pm$ 20 pM (mean $\pm$ 1 SE; $n=6$ and 4, respectively; $p < 0.05$). Only background levels of

FIG. 10. Reduced ascorbate concentrations in the BAL of Cl₂-exposed rats when aerosolized ascorbate was administered either at 15 or 60 min postexposure. Rats breathed either air or Cl₂ (300 ppm for 30 min) in environmental chambers and returned to room air. At 15–30 or 60 min later they breathed aerosolized ascorbate and desferal (AA) through a nose-only exposure system for 60 min. They were then sacrificed 60 min postinhalation and their lungs were lavaged. Reduced ascorbate was measured by HPLC as described in the Materials and Methods. Box-whisker plots showing the individual points, the box and mean values.

H₂O₂ (<10 pM) were detected in the BAL of rats breathing vehicle.

Discussion

The severity of Cl₂-induced lung injury varies with the level and duration of exposure.^(1,2,23) People and animals that inhale Cl₂ at concentrations less than 50 ppm developed reversible increased mucous production and airway resistance and a decreased respiratory rate most likely due to the activation of Transient Receptor Potential Ankyrin 1 (TRPA1) ion channels, located in a subset of airway sensory neurons.^(24,25) At higher concentrations Cl₂ penetrates into distal lung regions⁽²⁶⁾ and interacts with critical components of the pulmonary surfactant system, as well as with alveolar epithelial cells resulting in severe hypoxemia, pulmonary edema, and even death from respiratory failure.^(5,7,9,10,27–29)

Precise measurements of Cl₂ concentrations at the accident scene are not possible. In the Graniteville, SC, accidents,⁽⁴⁾ average Cl₂ concentrations during a 30-min exposure period were estimated to be 4428, 550, and 161 ppm at 0.2, 0.5, and 1 km downwind from the epicenter of the Cl₂ release (Dr. Eric Svendsen, Tulane University; personal communication). Most people and animals exposed within 0.5 km of the epicenter developed acute lung injury but eventually recovered. Thus, to mimic sublethal injury, in our previous work we exposed rats and mice to Cl₂ concentrations of 187–400 ppm for 30 min and returned them to room air. Rats and mice developed hypoxemia, increased alveolar permeability to plasma proteins, loss of surface and tissue ascorbate, decreased ability to clear fluid, functional surfactant deficiency, pulmonary edema, and reactive airway disease syndrome starting at 1 h postexposure.^(7,11,30,31) More than 90% were alive at 7 days post-Cl₂ exposure and exhibited normal behavior; however, physiological and quantitative morphological measurements revealed the presence of severe airway hyperreactivity,⁽³⁰⁾ mucous metaplasia and airway thickening⁽¹⁸⁾ up to 7 days postexposure.

On the other hand, mice exposed to 600 ppm Cl₂ for 45 min developed symptoms consistent with the presence of adult respiratory distress syndrome starting at 2 h post-Cl₂ exposure. About 75% of the mice died within 36 h postexposure. Surviving mice had considerable injury to their alveolar epithelium.⁽⁸⁾ Humans exposed to equivalent Cl₂ concentrations in Graniteville, Iraq, and in other accidents developed similar symptoms with considerably mortality.^(4,5,32) A recent study also pointed out that the physiological sequelae of Cl₂ exposure deviate from the Haber's law postulate, necessitating that that evaluation of countermeasures against chlorine-induced lung injury should be performed in multiple types of exposure scenarios.⁽³³⁾

Currently, there is no treatment specific for Cl₂-exposed persons. Supplemental oxygen (to alleviate hypoxemia)⁽²³⁾ and administration of β_2 agonists and agents that increase cAMP (to decrease bronchoconstriction and inflammation and enhance alveolar fluid clearance)^(30,31,34) have been used to treat both animals and humans exposed to Cl₂. In addition, agents that inhibit TRPA1 channels prevent the Cl₂-induced decrease of respiration in mice after low concentration exposures.⁽³⁵⁾

Exposure of rats to Cl₂ resulted in significant depletion of ascorbate in their epithelial lining fluid and lung tissues^(8,18)

and increased levels of F2-isoprostanes in their lung, a reliable index of lipid peroxidation,⁽¹¹⁾ indicating the presence of reactive intermediates after the cessation of Cl₂ breathing. Similarly, increased levels of reactive intermediates were detected in alveolar epithelial type II cells exposed to Cl₂ *in vitro* (as evidenced by Electron Paramagnetic Resonance and the oxidation of redox-sensitive dyes).⁽³⁶⁾ Thus, postexposure administration of ascorbate, an effective hydrophilic antioxidant that also reduces Vitamin E radicals (generated during reduction of lipid hydroperoxides or peroxy radicals^(1,37)) and decreased the generation of reactive intermediates by inhibiting endothelial cell NADPH oxidase,⁽³⁸⁾ and prevented tumor necrosis factor- α (TNF- α) challenge-related apoptosis of endothelial cells *in vitro*,⁽³⁹⁾ seems a viable strategy for decreasing Cl₂ induced morbidity and mortality. Furthermore, because the primary sites of Cl₂ injury are the airway and alveolar epithelia, inhalation of aerosolized antioxidants appeared an effective and noninvasive means of delivering ascorbate to the Cl₂-exposed lungs. Although a large fraction of inhaled ascorbate was probably trapped in the external nares, sufficient levels reached the upper airways and distal lung regions to restore depleted levels of ascorbate in both the epithelial lining fluid and lung tissue. Furthermore, our data clearly demonstrate that significantly higher levels of ascorbate may be delivered to distal lungs if aerosol administration is delayed to 1 h postexposure, a likely scenario in mass casualty situations.

Ascorbate may also act as a pro-oxidant.⁽¹³⁾ Our data clearly demonstrate the presence of small amounts of H₂O₂ (<100 pM) in Cl₂-exposed rats following ascorbate administration. H₂O₂ becomes a cosubstrate for specific peroxidases and thereby generates reactive centers that in turn react with critical protein thiols forming disulfide linkages in key signaling elements. Fe⁺³, likely to be present in the epithelial lining due to Cl₂-induced cell injury, in conjunction with excess H₂O₂, may result in the formation of hydroxyl radicals, which can cause extensive tissue injury.⁽⁴⁰⁾ Hydrogen peroxide may also trigger various signaling pathways.⁽⁴⁰⁾ Thus, we opted to coadminister desferal with ascorbate, which will scavenge trivalent ions, and minimize any pro-oxidant effects of ascorbate while at the same time prevent hydroxyl radical production via Fenton reactions.

Based on measurements of ELF volume and protein content, we concluded that aerosolized antioxidants did not damage the distal lung epithelium of normal and Cl₂-exposed rats at 1 h postexposure, attesting to the safety of the procedure. In previous studies⁽¹⁸⁾ using quantitative morphological techniques, we found that administration of aerosolized ascorbate and desferal to air breathing rats did not alter the number of ciliated cells or epithelial thickness in large airways up to 7 days postexposure. Intravenous injection of up to 110 g of ascorbate a day in patients with severe burns decreased the extent of lung injury and caused no adverse effect. Others have reported that doses of 5 g/kg body weight of ascorbate are safe and well tolerated by patients with severe burns.⁽⁴¹⁾ However, the safety of combined aerosolized administration of ascorbate and desferal in humans has yet to be demonstrated. Although each agent is FDA approved for human use, the combination of the two via aerosols is not. Previously, we have shown that aerosolized ascorbate did not increase airway resistance in response to

methacholine challenge up to 7 days postexposure.⁽¹⁸⁾ Additional studies are needed to show safety in humans.

In conclusion, data presented here show that aerosolized administration of ascorbate in rats post-Cl₂ exposure increases ascorbate levels in the ELF, lung tissue, and plasma without damaging airway and alveolar epithelia. Based on the results of our studies, we concluded that administration of aerosolized ascorbate is most effective when initiated at least 1 h postexposure. In humans, because of the larger diameter of their airways, about 60% of inhaled ascorbate will reach the distal lung spaces.⁽²²⁾ Because of the higher deposition fraction of inhaled ascorbate and the larger minute ventilation of humans (about 6 L/min), considerable shorter inhalation periods (10–20 min) are needed to deliver sufficient levels to replete depleted ascorbate levels in distal lung spaces of human following exposure to Cl₂ regiments likely to be encountered within 0.5–1 mile from the epicenters of industrial accidents (200–600 ppm for 30 min). Thus, aerosolized antioxidant delivery, especially coupled with β_2 agonists to decrease airway resistance,⁽³⁰⁾ may be a viable strategy for the decrease of lung injury in persons exposed to Cl₂.

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Author Disclosure Statement

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