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Nitrite therapy prevents chlorine gas toxicity in rabbits

Jaideep Honavar[1], **Stephen Doran**[2,3], **Karina Ricart**[1], **Sadis Matalon**[2,3], and **Rakesh P. Patel**[1,3]

[1]Department of Pathology, University of Alabama at Birmingham, Birmingham AL 35294

[2]Department of Anesthesiology, University of Alabama at Birmingham, Birmingham AL 35294

[3]Center for Free Radical Biology and Lung Injury and Repair Center, University of Alabama at Birmingham, Birmingham AL 35294

Abstract

Chlorine (Cl_2) gas exposure and toxicity remains a concern in military and industrial sectors. While post-Cl_2 exposure damage to the lungs and other tissues has been documented and major underlying mechanisms elucidated, no targeted therapeutics that are effective when administered post-exposure, and which are amenable to mass-casualty scenarios have been developed. Our recent studies show nitrite administered by intramuscular (IM) injection post-Cl_2 exposure is effective in preventing acute lung injury and improving survival in rodent models. Our goal in this study was to develop a rabbit model of Cl_2 toxicity and test whether nitrite affords protection in a non-rodent model. Exposure of New Zealand White rabbits to Cl_2 gas (600ppm, 45min) caused significant increases in protein and neutrophil accumulation in the airways and ~35% mortality over 18h. Nitrite administered 30min post Cl_2 exposure by a single IM injection, at 1mg/Kg or 10mg/Kg, prevented indices of acute lung injury at 6h by up to 50%. Moreover, all rabbits that received nitrite survived over the study period. These data provide further rationale for developing nitrite as post-exposure therapeutic to mitigate against Cl_2 gas exposure injury.

Keywords

halogen; nitric oxide; inflammation

Introduction

Chlorine gas (Cl_2) is used widely in numerous industrial processes world-wide and in most cases requires transport, largely by rail, across long distances and through populated areas. There are several examples of train derailments and accidental exposure of humans to high levels of Cl_2 (6, 9, 26, 36, 38). Moreover, Cl_2 has as long history of use, including evidence in current day conflicts, as a chemical weapon. Recent research efforts have shown that Cl_2 exposure results in extensive airway and systemic toxicity, that occurs both during and importantly post-exposure. Current therapies are limited to treating symptoms observed

Corresponding Author: Rakesh P. Patel, PhD, Department of Pathology, University of Alabama at Birmingham, 901 19th Street South, BMR-2, room 532, Birmingham, AL 35294, Tel: 205 975 9225, Fax: 205 934 7447.

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immediately post-exposure and largely entail respiratory supportive actions. A major limitation of current treatments is the lack of consideration of post-exposure toxicities, and the mechanisms involved. This is important since while little may be done to prevent during exposure injury, understanding post-exposure mechanisms may provide key information to develop novel and targeted therapeutics.

Recent studies using experimental models of Cl_2 exposure have developed a comprehensive understanding of post-Cl_2 exposure injury. This occurs over a time span ranging from hours-days and possibly longer, and afflicts the airways, pulmonary and systemic vasculatures (10, 17, 24, 29, 31, 35, 41). Injury is characterized by hypoxemia, oxidative and inflammatory stress, cellular dysfunction and death. Clinically, this presents initially as acute lung injury and development of acute respiratory distress syndrome and more chronically as reactive airway disease and increased sensitivity to pulmonary infections, as well as dermal injury (5, 10, 13, 18, 23, 33, 35, 40). In addition, extrapulmonary injury has also been documented to the pulmonary and systemic vasculature, and the heart (1, 15, 17, 40).

Nitric oxide (NO) is a key mediator of homeostasis in all organ systems and important in regulating inflammation. Our previous studies have shown that NO-formation mechanisms are disrupted by Cl_2 exposure and that endothelial derived NO formation and signaling is inhibited (15, 17). We have suggested that decreased NO-bioavailability may underlie post-Cl_2 exposure inflammation, and that therapeutic repletion of NO-signaling may prevent post-exposure ALI(32). Consistent with this idea, post-exposure administration of nitrite, an anion that is reduced to NO and other NO-containing species in vivo in hypoxic tissues, improved survival and prevented ALI in mice and rats exposed to Cl_2 (15, 33, 39). While these data provide impetus for further development of nitrite as a post-exposure therapeutic, they are limited to demonstration of efficacy in small rodents. A key consideration for further development of nitrite as post-Cl_2 exposure therapeutic is testing and demonstration of efficacy in larger animal models. Rabbits have been used extensively in studies aimed to identify the mechanisms of hyperoxic induced lung injury and develop severe hypoxemia and ALI (25, 30). Like Cl_2, hyperoxia is known to upregulate oxidative stress and inflammation and cause extensive injury to the blood gas barrier. Thus, in this study, we exposed rabbits to Cl_2 (in concentrations likely to be encountered in the vicinity of industrial accidents) and returned them to room air. We then tested whether post-Cl_2 exposure administration of nitrite, by intramuscular injection, could improve survival and limit lung injury,

Materials and Methods

Materials

Unless stated otherwise all reagents were purchased from Sigma (St. Louis, MO, USA). Male (2.5 – 3 Kg) New Zealand White rabbits were purchased from Charles River (Indianapolis, IN, USA) and kept on 12h light-dark cycles with access to standard chow and water ad libitum prior to and post chlorine gas exposure. Rabbits were allowed to acclimate 2–4 days prior to initiating experiments

Methods

Rabbit exposure to chlorine gas

Whole body exposures of male rabbits to Cl_2 were performed as previously described (22, 41). Exposures were performed with one rabbit in the chamber at any one time and all exposures were performed between 8am–12pm. Exposure conditions were 600ppm for 45min using Cl_2 cylinders at this concentration in air. Cylinders were replaced when the pressure dropped to 500psi. In each case, immediately following exposure, rabbits were returned to room air. The rabbits were monitored hourly for 12h and every 6h thereafter for 24h. All experiments involving animals were conducted according to protocols approved by the UAB IACUC.

Previous studies have shown that post Cl_2-exposure administration of the opioid analgesic buprenorphine improved locomotion and decreased immobility post exposure, presumably by decreasing pain (12). However, whether or not buprenorphine administration decreased lung injury and survival, and whether analgesics which may also affect inflammatory responses, affects the therapeutic efficacy of nitrite have not been assessed. Thus in some experiments as indicated, buprenorphine (0.05mg/kg) was administered via sub-cutaneous route in the fold of skin behind the neck 30min prior to chlorine gas exposure. The second group of animals did not receive any buprenorphine prior to Cl_2 gas exposure before being returned to room air.

Intramuscular Nitrite administration

Rabbits received a single injection of PBS (vehicle) or sodium nitrite in PBS (1–10mg/kg final concentration) in the gluteus maximus region 30min post cessation of Cl_2 exposure. Nitrite stocks were prepared daily in sterile PBS with injection volumes of 1ml.

Acute Lung injury

At indicated times, rabbits were sacrificed by lethal dose of ketamine/dexmedetomidine/acepromazine (100/0.5/2mg/Kg) administered by IM injection. An incision was made at the neck to expose the trachea, and an endotracheal cannula (OD 7mm, L 50mm) inserted. Lungs were lavaged with 3 × 30ml of PBS (similar to the total lung capacity (~90ml) of a 2Kg rabbit(14)); ~20ml was recovered. Bronchoalveolar lavage (BAL) protein and cell numbers reported have been adjusted for dilution accordingly. Rabbits were then exsanguinated by cardiac puncture for collection of blood. Lavage fluid recovered was gently mixed with rocking motion and 1ml aliquots of lavage fluid were kept on ice and centrifuged immediately at 300*g* for 10min to pellet cells. Supernatants were removed and stored on ice for protein analysis using the Bio-Rad Protein Assay Reagent Kit compared with BSA standards. Cells were resuspended in 100μl PBS and counted using a Neubauer hemocytometer. Cells were then placed on slides using a cellspin (Tharmac, Drosselweg, Germany) and stained using a two-stain set consisting of eosin Y and a solution of thiazine dyes (Quik-Stain; Siemens, Washington, DC). Differential counts (specifically monocytes, neutrophils, and lymphocytes) were then performed on slides via light microscopy.

Measurement of interleukin-8

BALF IL-8 levels were measured using ELISA (D800C) according to manufacturer's instructions (R&D Systems, Inc, Minneapolis, MN). Optical densities were read using an synery H4 hybrid multimode microplate reader (Bio-TEK Instruments, Winooski, VT). IL-8 concentration was calculated by polynomial regression analysis. Samples were used undiluted and measured in duplicate per replicate.

Survival Analysis

For each exposure, rabbits were randomly pre-assigned to either be exposed to Cl_2 only, or Cl_2 followed by nitrite therapy. Rabbits were euthanized based on any the following triggers alone or in combination a) body temperature below 90°F, b) >20% weight loss within 24h, c) inability of rabbit to support itself or laying on side.

Statistical analysis

The numbers of replicates are indicated in the figure legends. Survival was assessed using the Log rank (Mantel-Cox) test. Changes in BAL protein and cells were assessed by unpaired t-test or 1-way ANOVA with Tukey post-test as indicated. All analyses were performed using GraphPad Prism. Significance was set at 0.05.

Results

Nitrite therapy prevents Cl_2 dependent ALI in rabbits

Rabbits were exposed to Cl_2 (600ppm, 45min), and then treated with nitrite or vehicle 30min post-exposure. Rabbits were sacrificed at 6h and BAL fluid collected and analyzed for total protein concentration (Fig 1A) and inflammatory cell accumulation (Fig 1B–C). Cl_2 exposure resulted in significant increases in BAL protein (Fig 1A) and cell content (Fig 1B), the increase in latter largely due to increased PMN (Fig 1C). Nitrite at 1 and 10mg/Kg significantly attenuated BAL protein and cell accumulation, with the lower dose being more effective in decreasing BAL protein and the higher dose more effective for decreasing the number of cells; these findings are similar to our previous observations using a rat model of Cl_2 induced ALI(33). The highest dose of nitrite also significantly decreased the proportion of BAL cells that were neutrophils with a parallel increase in the percent of macrophages.

Nitrite therapy improves post-Cl_2 gas exposure survival

Rabbits were exposed to Cl_2 at 400ppm (30min), 500ppm (30min) or 600ppm (45min) and 18h survival assessed. No mortality was observed with 400–500ppm Cl_2 (not shown). Exposure to 600ppm Cl_2 resulted in ~35% mortality over 18h, which was completely reversed with IM nitrite administered 30min post exposure (Fig 2A). For these studies, per IACUC recommendations, exposure protocol was modified to include administration of buprenorphine for pain management. Pilot studies (n=4 per group) showed no differences in Cl_2 (600ppm, 45min) induced mortality in rabbits treated with either vehicle or buprenorphine. Moreover, Fig 2B–C shows BAL protein and inflammatory cell levels in rabbits still alive at 18h, and shows that nitrite treatment significantly decreased these indices by 95% and 50% respectively. For BAL cells, this level of protection afforded by

nitrite was similar to that observed 6h after Cl_2 exposure (Fig 1A). However, for BAL protein levels, the protection was greater 18h after Cl_2 exposure. Nitrite elicited similar effects of the composition of BAL cells observed in Figure 1, with a shift towards more macrophages and fewer neutrophils (Figure 2D). Moreover, Figure 2E shows that nitrite treatment decreased BAL IL-8 levels by >90%.

Discussion

Exposure to high levels of Cl_2 has occurred in the military arena and after accidental release. Unfortunately, the possibility of Cl_2 exposure to civilian populations remains in both settings and at least with accidental exposure, likely to remain given the widespread use of Cl_2 in various industrial processes that require transport of this halogen across populated areas. For example, modelling studies using data from the release of Cl_2 gas after a train derailment in South Carolina, USA, in 2005 indicate that humans 0.5km downwind of the accident were exposed to >500ppm Cl_2 gas for 30–60min(19). The last 10 years has seen many advances in our understanding of the effects and underlying mechanisms by which post-Cl_2 exposure injures the cardiorespiratory system. Acute lung injury is an early feature that readily develops into reactive airway syndrome, characterized by increased permeability, inflammation, fibrosis and airway plugging. Paralleling damage to the lung, are injury to the pulmonary and systemic vasculatures and cardiac system (1, 15, 17, 40). In addition to morbidities, mortality is also evident both during and post-exposure. Thus any therapeutic that could be administered early post-exposure by first responders, which improves acute post-exposure survival, would be of great benefit allowing subsequent transport of exposed individuals to primary care facilities.

Increased oxidative stress, inflammation and dysfunction in endogenous repair processes and are all underlying mechanisms for post Cl_2 toxicity and this information is being used to develop and test a host of targeted therapies (4, 7, 8, 10, 13, 17, 20, 22, 24, 27–29, 31, 34, 39, 41). Indeed post exposure administration of Vitamin C (an antioxidant) and desferal (an iron chelator) have been shown to improve survival and enhance lung epithelial repair(11). Our recent focus has been on NO-repletion therapeutics. The rationale for this being that Cl_2 exposure results in endothelial dysfunction characterized by a loss of NO-bioavailability (15, 17) which would also predispose to inflammation and oxidative stress. NO-repletion therapies exist; the two most appreciated being inhaled NO administration or use of PDE-5 inhibitors (21, 37). However, the efficacy of these towards Cl_2 toxicity is unclear and with inhaled NO certainly, logistic and price constraints would likely preclude its administration in the field, in a mass casualty situation. We have tested nitrite therapy on the basis that circulating nitrite levels are decreased post-Cl_2 exposure (17) and that nitrite is a relatively inexpensive therapeutic amenable to storage for long periods and administration by IM injection. Also, our previous studies have shown nitrite administered within 30–60min post exposure to mice or rats post-Cl_2 exposure, prevents leak and inflammatory injury to the lungs, prevents development of reactive airways and improves post-exposure survival, especially in the early phases post exposure (15, 33, 39). Moreover, nitrite is an active agent in cyanide antidote kits and has an established safety profile for use in humans.

This therapeutic profile provided rationale for further testing of nitrite as a counter-measure for Cl_2 toxicity. In this context, a key and required element of developing nitrite-therapeutics is to test efficacy in a different non-rodent animal model. We utilized a rabbit model and first established Cl_2 exposure conditions and then tested whether nitrite could prevent ALI improve 18h survival, a time over which significant protection is observed in mice. At both 1mg/Kg and 10mg/Kg, doses shown to protect in mouse and rat modes, nitrite administered by IM injection 30min post Cl_2 exposure, prevented accumulation of protein and inflammatory cells in the airways. Consistent with our previous data with mice, lower doses of nitrite were more effective and preventing BAL protein, whereas the higher doses are more effective at inhibiting BAL neutrophil accumulation. We do not have an explanation for this dose-selectivity of nitrite towards permeability and inflammation component of ALI and underscore the important point that both these measures were decreased supporting protective effects of nitrite. Notable also was that nitrite therapy not only decreased total inflammatory cell accumulation but at longer times post-Cl_2 exposure altered the cell composition with less neutrophils and more macrophages. This is consistent with our previous data with mice showing that nitrite-dependent protection was largely mediated by limiting neutrophil-dependent ALI(16). We also show that a likely mechanism for this effect is reduction in IL-8 levels, a pro-neutrophilic chemokine and consistent with our prior studies suggesting limiting neutrophil trafficking to the lungs is an important mechanism underlying nitrite therapy. Exposure of humans and animals to Cl_2 causes pain presumably because of the stimulation of transient receptor potential ankyrin 1 (TRPA1) channels in airway sensory neurons and tissue damage(2, 3). Pain in turn may cause inflammation which may aggravate lung injury. However, in our studies, nitrite was protective even in rabbits treated with buprenorphine. This is important and suggests future experimental studies evaluating nitrite will not be affected by possible requirement to use pain-relieving medication. We also show that nitrite acutely improves survival after Cl_2 exposure in rabbits. This is an important result which in addition to documenting efficacy in a non-rodent model, further supports the proposal that nitrite will be an effective therapeutic to be administered by first responders to improve immediate / short-term survival after Cl_2 exposure that will allow for administration of other targeted therapies that can be administered in a more controlled primary care setting type environment.

It was not our goal to use the rabbit model to discern long-term effects of Cl_2 gas exposure and test effects of nitrite-therapy in this regard, but to focus on toxicity occurring acutely after exposure. Over this small time period, it is unlikely that major improvements in lung histology will be observed; data from rodent studies demonstrate that the majority of the airway injury characterized by de-epithelialization occurs during Cl_2 exposure(10). Our previous studies did demonstrate a modest improvement in airway injury and cell death in rats exposed to Cl_2 and nitrite however (39). Further studies evaluating the therapeutic effects of nitrite that focus on longer-term assessment of airway histology in rodent and rabbit models is warranted. The goal of this study was to test if nitrite therapy administered by IM injection post exposure could protect against primary morbidity and mortality observed after Cl_2 exposure in a rabbit model. Our data suggest that nitrite is protective in this model and that this is similar to prior studies using mice and rats. Collectively, these data provide rationale for further development of nitrite as counter-measure therapy that can

be administered in mass-casualty situations to improve immediate survival and limit ALI that occurs after Cl_2 exposure

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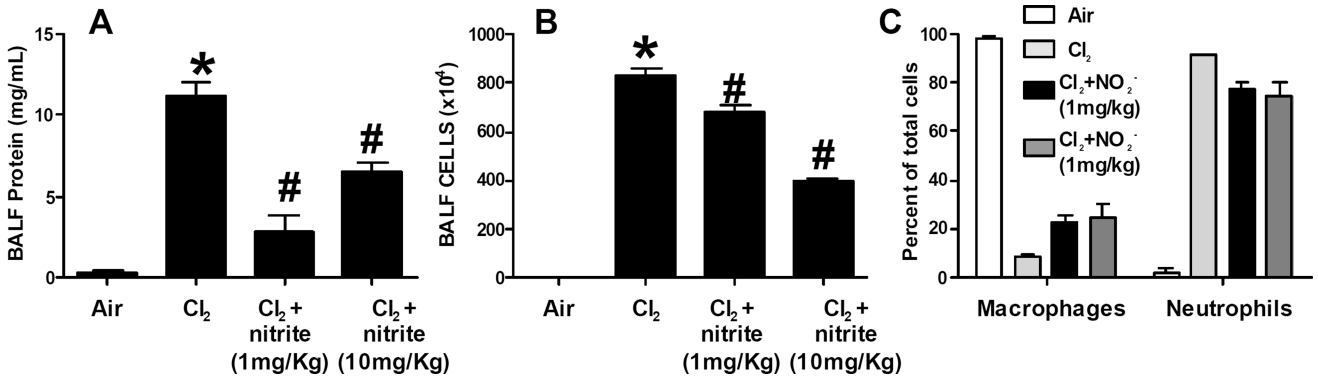

Figure 1. Effects of nitrite on Cl_2 dependent acute lung injury

Panel A–C: New Zealand white male rabbits were exposed to Cl_2 (600ppm, 45min) and then returned to room air. Nitrite was administered by a single IM injection at the indicated doses 30min post-exposure. 6h post-exposure, rabbits were euthanized and BALF levels of protein (Panel A), inflammatory cells (Panel B) and cell differentials (Panel C) measured. For panels A-B, data shown mean ± SEM (n=3) *P<0.05 relative to air, $^{\#}$P<0.05 relative to Cl_2 by 1-way ANOVA with Tukey post-test. For panel C, *P<0.05 relative to Cl_2 by 1-way ANOVA with Tukey post-test.

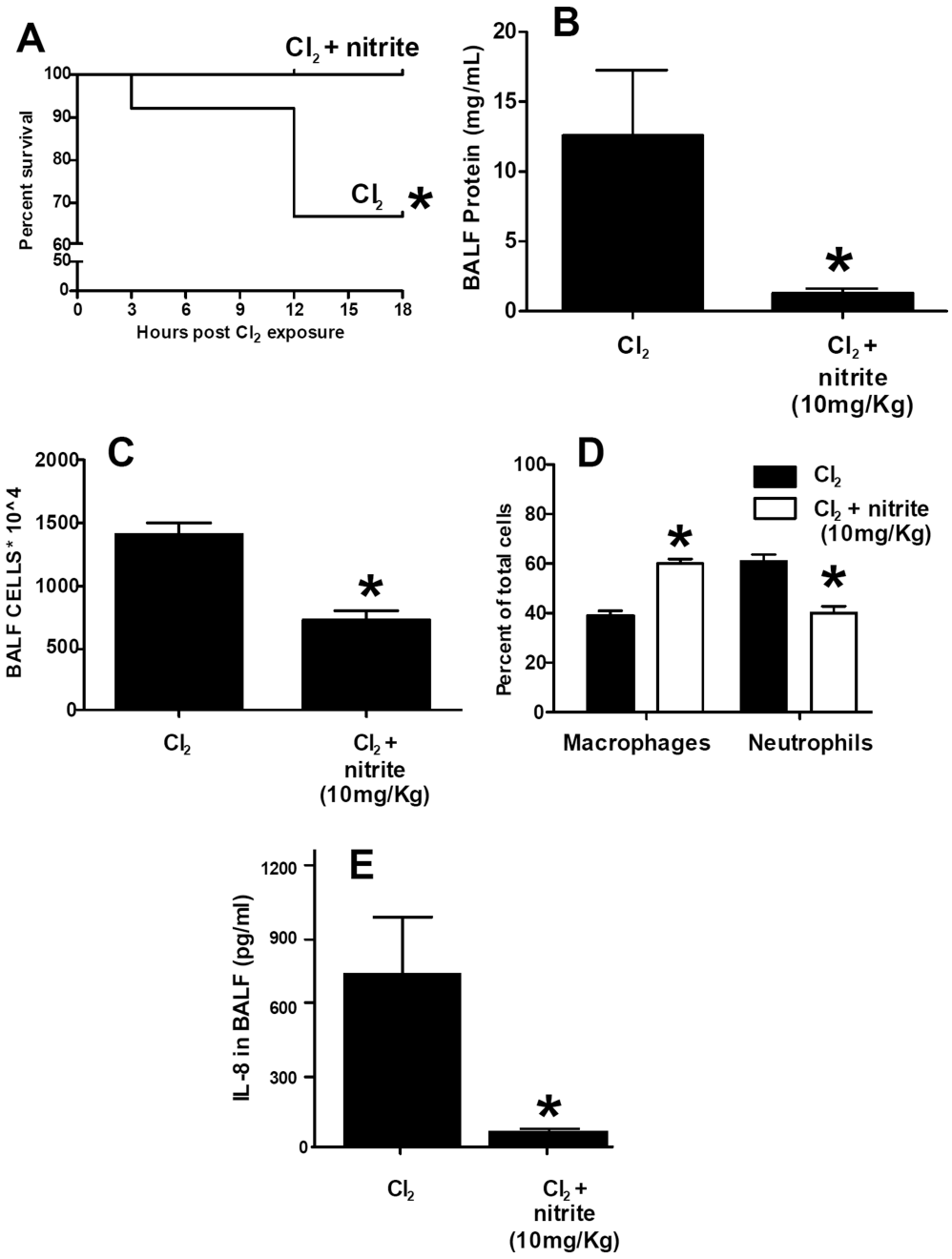

Figure 2. Effects of nitrite on Cl2 gas dependent mortality

New Zealand white male rabbits were administered buprenorphine (0.05mg/Kg) 30min prior to Cl_2 exposure (600ppm, 45min) and then returned to room air. Nitrite (10mg/Kg) was administered by a single IM injection 30min post-exposure. Mortality was assessed over 18h. Data show Kaplan-Meier survival curves, *P<0.04 between groups determined by the Log-rank test (n=12 per group). Panel B-E: BAL protein, inflammatory cells, differential analyses and IL-8 respectively. Data shown mean ± SEM. *P<0.05 by unpaired t-test.