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Nitrite therapy prevents chlorine gas toxicity in rabbits

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HIGHLIGHTS

- Chlorine gas induces acute lung injury and death in rabbits.
- Post-chlorine exposure administration of IM nitrite improves 18 h survival.
- Post-chlorine exposure administration of IM nitrite prevents lung leak and airway accumulation of neutrophils.

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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 (600 ppm, 45 min) caused significant increases in protein and neutrophil accumulation in the airways and ~35% mortality over 18 h. Nitrite administered 30 min post Cl_2 exposure by a single IM injection, at 1 mg/kg or 10 mg/kg, prevented indices of acute lung injury at 6 h 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.

1. 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 (Cevik et al., 2009; Evans, 2005; Matalon and Maull, 2010; Van Sickle et al., 2009; Wenck et al., 2007). 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 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 (Fanucchi et al., 2012a; Honavar et al., 2011; Martin et al., 2003; Musah et al., 2012; O'Koren et al., 2013; Tuck et al., 2008; Zarogiannis et al., 2011). Injury is characterized by hypoxemia, oxidative and inflammatory stress, cellular

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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 (Carlisle et al., 2016; Fanucchi et al., 2012a; Gessner et al., 2013; Hoyle, 2010; Li et al., 2013; Samal et al., 2012; Tuck et al., 2008; Zaky et al., 2015). In addition, extrapulmonary injury has also been documented to the pulmonary and systemic vasculature, and the heart (Ahmad et al., 2015; Honavar et al., 2014a, 2011; Zaky et al., 2015).

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 (Honavar et al., 2014a, 2011). 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 (Samal et al., 2010). 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 (Honavar et al., 2014a; Samal et al., 2012; Yadav et al., 2011). 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 (Matalon and Egan, 1981; Nickerson et al., 1981). 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,

2. Materials and methods

2.1. 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 12 h 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

2.2. Methods

2.2.1. Rabbit exposure to chlorine gas

Whole body exposures of male rabbits to Cl_2 were performed as previously described (Leustik et al., 2008; Zarogiannis et al., 2011). Exposures were performed with one rabbit in the chamber at any one time and all exposures were performed between 8 a.m.–12 p.m. Exposure conditions were 600 ppm for 45 min 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 12 h and every 6 h thereafter for 24 h. 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 (Filippidis et al., 2012). 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 (0.05 mg/kg) was administered via sub-cutaneous route in the fold of skin behind the neck 30 min 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.

2.2.2. Intramuscular nitrite administration

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

2.2.3. Acute lung injury

At indicated times, rabbits were sacrificed by lethal dose of ketamine/dexmedetomidine/acepromazine (100/0.5/2 mg/kg) administered by IM injection. An incision was made at the neck to expose the trachea, and an endotracheal cannula (OD 7 mm, L 50 mm) inserted. Lungs were lavaged with 3×30 ml of PBS (similar to the total lung capacity (~90 ml) of a 2 kg rabbit (Holm et al., 1985)); ~20 ml 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 1 ml aliquots of lavage fluid were kept on ice and centrifuged immediately at 300*g* for 10 min 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 100 μl 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.

2.2.4. 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.

2.2.5. 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 24 h, (c) inability of rabbit to support itself or laying on side.

2.2.6. 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