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Post Chlorine gas exposure administration of nitrite prevents lung injury: effect of administration modality

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Abstract

Cl₂ gas toxicity is complex and occurs during, and post exposure leading to acute lung injury (ALI) and reactive airway syndrome (RAS). Moreover, Cl₂ exposure can occur in diverse situations encompassing mass casualty scenarios underscoring the need for post-exposure therapies that are efficacious and amenable to rapid and easy administration. In this study, we compared the efficacy of a single dose, post (30min) Cl₂ exposure administration of nitrite (1mg/kg) via intraperitoneal (IP) or intramuscular (IM) injection in rats, to decrease ALI. Exposure of rats to Cl₂ gas (400ppm, 30min) significantly increased ALI and caused RAS 6–24h post exposure as indexed by BAL sampling of lung surface protein, PMN and increased airway resistance and elastance prior to and post methacholine challenge. IP nitrite decreased Cl₂ - dependent increases in BAL protein but not PMN. In contrast IM nitrite decreased BAL PMN levels without decreasing BAL protein in a xanthine oxidoreductase independent manner. Histological evaluation of airways 6h post exposure showed significant bronchial epithelium exfoliation and inflammatory injury in Cl₂ exposed rats. Both IP and IM nitrite improved airway histology compared to Cl₂ gas alone, but more coverage of the airway by cuboidal or columnar epithelium was observed with IM compared to IP nitrite. Airways were rendered more sensitive to methacholine induced resistance and elastance after Cl₂ gas exposure. Interestingly, IM nitrite, but not IP nitrite, significantly decreased airway sensitivity to methacholine challenge. Further evaluation and comparison of IM

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and IP therapy showed a two-fold increase in circulating nitrite levels with the former, which was associated with reversal of post-Cl₂ exposure dependent increases in circulating leukocytes. Halving the IM nitrite dose resulted in no effect in PMN accumulation but significant reduction of BAL protein levels indicating distinct nitrite dose dependence for inhibition of Cl₂ dependent lung permeability and inflammation. These data highlight the potential for nitrite as a post-exposure therapeutic for Cl₂ gas induced lung injury and also suggest that administration modality is a key consideration in nitrite therapeutics.

Keywords

inhaled irritants; nitric oxide; inflammation; lung; nitrite

Introduction

Chlorine gas (Cl₂)-induced lung injury is a complex process comprising initial injury during exposure that continues post exposure over hours to days resulting in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)[1, 2]. Typically exposure to higher levels of Cl₂ gas are required to cause extensive lung injury leading to ALI and ARDS, but this remains a concern owing to potential accidental exposure (e.g. derailment of trains that transport vast quantities of Cl₂ through populated centers) and continued usage in chemical warfare [1, 3–7]. In these situations, treatment of primary symptoms of injury (namely hypoxemia and ALI with supplemental oxygen and / or mechanical ventilation) remains the therapeutic approach. To date, no directed post exposure therapy has been developed for clinical use reflecting the lack of knowledge in Cl₂ gas injury mechanisms.

Recent studies have shown that initial injury to the lungs during Cl₂ exposure most likely occurs via reactions between various biomolecules and either Cl₂ and/or Cl₂-derived hypochlorous acid [8]. After cessation of exposure, injury continues for hours – days characterized by increased inflammation and oxidative / nitrosative stress to the lungs and systemic vasculature resulting in ALI, ARDS, dysfunction vascular homeostasis, and over longer times airway remodeling associated with reactive airway syndrome[1, 2, 9–13]. In other words, although the Cl₂ exposure per se may be relatively brief, the long term sequelae are significant underscoring the need for targeted therapies that can be administered in a post exposure manner. Encouragingly, recent studies building on insights that oxidative stress and altered epithelial ion transport plays a role in post exposure injury, show that administration of antioxidants (ascorbate and desferal (an iron chelator) or a catalytically active metalloporphyrin based antioxidant) or β₂-agonists, demonstrate significant protection against indices of lung injury, dysfunction and mortality when administered after Cl₂ gas exposure in rodent models[12, 14–16].

In addition to inflammation, the initial stages of post-Cl₂ gas exposure toxicity is characterized by hypoxemia[17]. The anion, nitrite can be reduced to nitric oxide (NO) in ischemic tissues by oxygen sensitive heme / metalloproteins, which in turn can prevent injury via multiple possible mechanisms that include anti-oxidant, anti-inflammatory and cytoprotective effects[18, 19]. Based on this rationale, we recently tested and showed that post-Cl₂ exposure intraperitoneal (IP) administration of nitrite decreased Cl₂-induced damage to the blood gas barrier as indexed by lower protein levels in bronchoalveolar lavage fluid (BAL)) and prevented Cl₂ induced cell death in the rat lung[17]. As a therapeutic, nitrite is a good candidate owing to its relatively long stability (shelf life), cost-effectiveness, and is already used in cyanide antidote kits. Moreover, recent studies show that the required doses to observe protection against ischemic injury are significantly lower than those that promote hypotension or methemoglobinemia and these doses can be

achieved in a safe and reproducible manner [20–24]. In our previous study, we only tested the effectiveness of multiple injections of IP nitrite. Since, IP injection is not amenable to mass casualty scenarios, we compared IP with intramuscular (IM) nitrite administration as therapeutics to attenuate post-Cl₂ exposure induced lung injury. Data show that both IP and IM nitrite protected against Cl₂ induced ALI, but surprisingly did so by distinct mechanisms.

Materials and Methods

Materials

Unless stated otherwise all reagents were purchased from Sigma (St. Louis, MO, USA) and AbCam (Cambridge, MA, USA). Male Sprague Dawley rats (200–300g) were purchased from Harlan (Indianapolis, IN, USA) and kept on 12h light-dark cycles with access to standard chow and water *ad libitum* prior to and post chlorine exposure.

Methods

Rat exposure to chlorine gas

Whole body exposure of rats to different doses of Cl₂ gas was performed as previously described [12, 25]. Two rats were exposed in the same chamber at any one time and all exposures were performed between 8–10am and were 30min in length followed by return to room air. Age matched controls included rats exposed to air only. All experiments involving animals were conducted according to protocols approved by the UAB IACUC.

IP and IM Nitrite administration

Rats were injected with solution of sodium nitrite in PBS (0.1 - 1mg/kg) either in the gluteus maximus region (intramuscular (IM)) or intraperitoneal area (IP). Injections were made either once, 30 min post-Cl₂ exposure (single dose protocol) or immediately (5–15min) after Cl₂ gas cessation and every two hours after that (four injections in total) in the multiple dose protocol. In experiments where effects of C-PTIO (an NO-scavenger) or allopurinol (inhibitor of xanthine oxidoreductase) were tested (at 1mg/Kg and 100mg/Kg respectively), these compounds were administered by IP injection 15 min post cessation of Cl₂ exposure and 15 min prior to nitrite administration.

Measurement of BAL Protein and cells

6 hours post exposure, rats were euthanized with intraperitoneal ketamine/xylazine and a 3mm endotracheal cannula inserted in their tracheas. Lungs were lavaged with 8 ml of 0.9% NaCl three times as previously described[12]. Recovered aliquots of lavage fluid were kept on ice, centrifuged immediately at 300g for 10 min to pellet cells. Supernatants were removed and stored on ice for protein analysis using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) and comparing to BSA standards. In separate studies, BioRad protein assay was also used with no-significant differences observed. Also, pellets were mixed with 500 µL ACK buffer to lyse RBC and inflammatory cells quantified using a hemocytometer and cell differential analysis performed as described[17].

Immunohistochemistry

For immunohistochemistry, lungs were fixed with 4%paraformaldehyde at 30 cm H₂O of constant pressure through the tracheal cannula. Lung pieces were embedded in paraffin and sectioned at 5 µm. Hot citrate buffer or trypsin were used for antigen retrieval. Endogenous peroxidase was blocked with a 10% peroxide solution and nonspecific binding was blocked with IgG-free bovine serum albumin (Jackson ImmunoResearch Laboratories, West Grove,

PA) for 30 min at room temperature. Sections were immunostained using the manufacturer's suggested procedure with an avidin-biotin-peroxidase ABC kit (Vector Labs, Burlingame, CA). The signal was detected with 3',3'-diaminobenzidine tetrahydrochloride. Controls included the substitution of primary antibody with phosphate buffered saline.

Western Blotting

Protein was isolated from homogenized lungs in RIPA buffer containing complete mini protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Quantification was done by Bradford protein assay. 50µg of total protein was run on a 10% polyacrylamide gel and then transferred to nitrocellulose membrane. The membranes were blotted overnight with rabbit anti-rat XOR (1:1000 BD Transduction Laboratories, 610296) and 60min with anti-rat β -actin (1: 20,000, Cell Signaling, 4967), as a loading control. HRP conjugated secondary antibodies (1:4000) and Pierce West Dura Chemiluminescence substrate was used for detection

Lung histology and morphology

Following sacrifice and opening of the chest, rat lungs were filled *in situ* very slowly with 10% formalin to 25 cm of H₂O; they were then removed *en bloc* and immersed in 10% formalin for 48 h. The left lung was sliced perpendicular to the main airway axis into three pieces, and sections from the middle block processed for paraffin sectioning and staining with hematoxylin and eosin. 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).

Airway Hyperresponsiveness

Rats were mechanically ventilated and challenged with increasing concentrations of aerosolized methacholine 24h after Cl₂ gas exposure. Rats were sedated with xylazine (10 mg/kg i.p.) and anaesthetized with sodium pentobarbital (40 mg/kg i.p.). After the trachea was cannulated with a 16G cannula, rats were connected to ventilator (FlexiVent, Scireq, Montreal, PQ, Canada) and paralyzed using pancuronium chloride (1mg/kg i.p.). Rats were ventilated at a rate of 90 breaths per minute with a positive end-expiratory pressure of 3cm H₂O. Increasing concentrations of methacholine (0–20 mg/ml) were administered via aerosolization. From 20 seconds up to 3 minutes after each aerosol challenge, resistance (R cmH₂O.s/mL) and elastance (E cmH₂O/mL) were recorded continuously as previously described[26].

Lung cytokines / chemokine measurements

3h post Cl₂ exposure the right lung was excised, chopped and then homogenized in 1 ml of PBS supplemented with protease inhibitors (Roche). Lung homogenate was clarified by centrifugation (14,000 g, 10 min) and supernatant collected and stored at –80°C prior to analysis. Chemokines and cytokines were measured using the 23-plex Multiplex rat inflammation and immunology cytokine kit from EMD Millipore.

Nitrite pharmacokinetics and measurement

Rats were exposed to Cl₂ gas (400ppm, 30min), and then 30 min thereafter nitrite (1mg/Kg) administered by IP or IM injection. Pharmacokinetics were determined by tail vein sampling of whole blood immediately before, or at indicated times post nitrite administration. Immediately upon collection, ice-cold methanol (300µl) was added to 150µl whole blood, followed by vortex mixing and centrifugation (20000 × g, 5min). Nitrite and nitrate were measured in supernatants by the Griess reaction coupled to HPLC separation using the

ENO-20 (EiCom, Japan). Nitrite and nitrate levels were calculated by comparison to standard curves and adjustment for extraction efficiency.

Statistical analysis

Data were analyzed by one-way or two-way analysis of variance (ANOVA) with Tukey or Bonferroni post test, or t-test as indicated using GraphPad Prism.

Results

Effects of Nitrite administration modality on protective effects against Cl₂ induced ALI

Our previous studies showed that post-Cl₂ exposure administration of nitrite (1mg/Kg once every 2h for 6h) by IP injection, decreased accumulation of protein in the BAL, but did not decrease the number of inflammatory cells in the BAL [17]. A similar profile of protection was observed using a single dose protocol, with nitrite (1mg/Kg) being administered once by IP injection 30min post-Cl₂ exposure (Fig 1A and B). Cl₂ gas increased both BAL protein concentration and number of inflammatory cells 6h post exposure, with only protein levels being decreased in rats treated with IP nitrite. In contrast to IP nitrite, IM injection of nitrite at 1mg/Kg nitrite significantly decreased the number of cells recovered by BAL, but had no effect on Cl₂ induced protein accumulation. Similar effects of IP vs. IM nitrite were observed when a multiple administration protocol was used (every 2h for 6h, not shown). Cell differential analysis showed that in air treated rats, macrophages were the predominant cell type in the BAL (96.6%). Consistent with our previous studies [17] Cl₂ exposure increased the percent of PMN (55%) and monocytes (22%) in the recovered BAL, with no change in lymphocytes. IM nitrite did not change monocyte numbers, but significantly decreased Cl₂ induced increased in BAL PMN (Fig 2).

Histologic evaluation of IP and IM nitrite dependent protect against Cl₂ induced ALI

Figure 3 shows representative H&E photomicrographs of airways 6h after air, Cl₂ and / or IP or IM nitrite administration. Air exposed airways displayed normal morphology with an intact ciliated, columnar epithelial lining (Fig 3A). Cl₂ gas caused extensive airway injury indicated by loss of columnar epithelial cells (Fig 3B). Consistent with our previous study using multiple IP nitrite injections[17], one single IP nitrite injection partially protected against Cl₂ induced injury with focal areas of ciliated epithelia evident (Fig 3D). Similarly, IM nitrite also resulted in the presence of focal areas of ciliated cells and attached epithelium. However, this was more variable indicated by Fig 3C and 3E which show representative images reflecting this variability within an airway from one animal. Fig 3F shows that the epithelial thickness was decreased by Cl₂ gas, which was not significantly reversed by IP or IM nitrite. Finally, upon comparing IP nitrite with IM nitrite, it was noticed that there appeared to more epithelial coverage of airways in the IM nitrite group. To quantify this, the percent of images (4–5 images taken per animal) on which attached epithelium (cuboidal or columnar) was observed was determined and showed that IM nitrite increased epithelial coverage by ~2-fold (Fig 3G).

IM and IP nitrite therapy differentially affect post Cl₂ gas airway responsiveness

Previous studies have shown that brief Cl₂ gas exposure in mice leads to reactive airways syndrome that starts as early as 1h post exposure and lasting for 7 days[13, 26]. This was confirmed with rats using flexivent to measure basal and methacholine induced airway resistance and elastance 24h post Cl₂ gas exposure (Fig 4). After exposure to Cl₂ methacholine induced resistance and elastance reached maximal levels of 2–3 cm H₂O/ml/s and ~20 cm H₂O/ml respectively with 5mg/ml methacholine (Fig 4A-B). Interestingly these responses are lower in magnitude and methacholine sensitivity compared to previous studies

using identical Cl₂ gas exposure protocols with mice [26]. Figure 4C-D show that basal resistance (i.e. in the absence of methacholine) is also increased in Cl₂ compared to air exposed rats. Neither IM nor IP nitrite affected basal resistance (Fig 4C), however Cl₂ induced changes in airway elastance were reversed only by IM nitrite (Fig 4D). A differential effect of IP vs. IM nitrite was further indicated in methacholine dependent changes (Fig 4A-B), with only IM nitrite attenuating Cl₂ dependent exacerbation of resistance and elastance. Since methacholine dose dependent changes saturated at 5–10mg/ml, the change in resistance and elastance between 0–5mg/ml methacholine was calculated and shown in Fig 4E-F respectively. Cl₂ increased both these parameters ~2–3 fold which were not affected by IP nitrite but significantly reversed by IM nitrite.

Differential effects of IM and IP nitrite on circulating leukocytes

We also tested whether nitrite therapy affected circulating leukocyte levels post Cl₂ exposure. Leukocytes were increased at both 6h and 24h post Cl₂ exposure (Fig 5A and 5B). In rats that received IP or IM nitrite therapy, no significant effect was observed by 1-way ANOVA analysis at 6h, although a trend towards a decrease in leukocytes was observed with IM nitrite ($P = 0.06$), but not IP nitrite ($P = 0.32$) compared to the Cl₂ alone group (Fig 5A). At 24h however, IM nitrite therapy completely attenuated Cl₂-dependent increases in leukocytes, whereas IP nitrite had no effect (Fig 5B). Chemokines and cytokines play key roles in orchestrating inflammatory responses. To test if IP or IM nitrite differentially affected these mediators, a panel of 23 chemokines / cytokines were measured in lung tissue 3h post Cl₂ exposure. 1-way ANOVA analysis showed significant increases only in IL1 α after Cl₂ exposure, although trends for increases in MIP1 α , IL6 and KcGro are noted. Neither IP nor IM nitrite significantly affected IL1 α levels (supplementary data Table 1).

Anti-inflammatory effects of IM nitrite are mediated by Xanthine oxidoreductase

As mentioned earlier, amenability to mass casualty scenarios is an important consideration in developing a post-Cl₂ exposure therapeutic. IM injection is clearly a better option than IP injection in this context and was chosen therefore to further characterize and investigate mechanisms of protection. One proposed mechanism for nitrite dependent protection especially in the lung is xanthine oxidoreductase (XOR) mediated one electron reduction to NO[27]. Fig 6A shows that XOR protein expression increased in lungs post Cl₂ gas exposure. Nitrite administration to air or Cl₂ gas exposed animals had no effect on XOR expression (not shown). Fig 6B shows that the XOR inhibitor, allopurinol had no effect on Cl₂ dependent changes on PMN levels in BAL, but did prevent nitrite dependent decreases in PMN accumulation. We could not test the effects of the NO-scavenger CPTIO as this compound alone significantly attenuated BAL PMN accumulation after Cl₂ exposure (not shown).

Nitrite pharmacokinetics after IM or IP administration

Fig 7A-B show whole blood nitrite and nitrate concentrations in Cl₂ exposed rats after IM or IP nitrite administration. Area under the curve analysis showed that nitrite peaked at similar times (~5min) for both IP and IM administration, but that peak nitrite concentrations were ~3-fold higher with IM nitrite, and total circulating nitrite concentrations were ~2-fold higher with IM administration. Nitrate increased at a faster rate after IM nitrite administration over the initial 20min, but then slowed leading to similar nitrate levels after 40 mins with IM and IP administration modalities (Fig 7B). To gain insights into NO-formation, nitrosylhemoglobin (HbNO) was measured 40min post IP or IM nitrite administration. No differences in HbNO levels were observed ($9.7 \pm 3.5\mu\text{M}$ and $10.6 \pm 1.8\mu\text{M}$ for IP and IM respectively). At the 40min time point, lungs were harvested and nitrite measured. No differences in lung nitrite between these conditions were observed (not shown). To test if two-fold higher total nitrite levels associated with IM administration

accounted for the differential effects of IP vs IM nitrite on Cl₂ dependent BAL protein and cell accumulation, the effects of IM nitrite at 0.5mg/Kg was tested. Figure 1 shows that administration of IM nitrite at 0.5mg/Kg significantly decreased BAL protein levels but had no significant effect on the number of inflammatory cells. Furthermore, 0.1mg/Kg IM nitrite did not alter Cl₂ dependent increases in BAL protein nor number of inflammatory cells (Fig 1).

Discussion

Cl₂ gas toxicity has been documented in a range of scenarios from accidental exposure (e.g. train derailments) to intentional release as a chemical warfare agent[28]. In either case, there is potential for relatively large scale exposures and mass casualty scenarios. Recent insights have demonstrated that pathogenesis of Cl₂ gas toxicity is not restricted to during exposure only phase, but is characterized by a post exposure period (hours – weeks) that is associated with hypoxemia, increased pulmonary permeability and inflammation that results in ALI/ARDS, RAS and systemic vascular toxicity[10]. The precise mechanisms are still under investigation and the lack of targeted post-Cl₂ gas exposure therapies, that can protect against the above mentioned pathologies, and which are also amenable for treatment paradigms required for mass casualty situations remains an active area of investigation [29].

The rationale for testing nitrite in this study stems from observations that this anion protects various tissues against toxicity in which ischemia, reperfusion and inflammation, are key elements in the disease pathogenesis[18, 24], with the primary proposed mechanism being reduction of nitrite to NO by an ever increasing list of hypoxia regulated nitrite reductases [30]. Our previous studies [17] showed that post-Cl₂ gas administration of nitrite by IP injection protected against development of edema, a key hallmark of ALI. However, no effect on inflammatory cell accumulation in the lung, also an important element of ALI pathogenesis was observed. The apparent lack of effect towards the inflammatory component of ALI, coupled with IP injection not being a clinically convenient administration modality led us to test and compare IM nitrite administration with IP injection. IM nitrite (1mg/kg) protected against ALI, but surprisingly did so by distinct mechanisms compared to the same dose of IP nitrite. Consistent with our previous study, IP nitrite only decreased BAL protein levels and not inflammatory cells. In contrast, IM nitrite decreased only PMN levels on the airway surface. Differences in protective mechanisms were also indicated by airway morphology and importantly airway function also. Significantly more intact airway epithelial cells were apparent in IM nitrite treated rats. Whether this is a result of an inhibition of post-Cl₂ gas exposure mediated loss of epithelia and / or accelerated repair is not clear. Previous studies have shown that one role for PMN infiltration into the lung after exposure to inhaled irritants is to clear necrotic epithelial cells[31]. The lack of PMN accumulation in IM nitrite treated rats may account for increased epithelial cells therefore. Although decreased PMN emigration into the lung in this context could hinder repair, PMN infiltration is also causally linked to promoting inflammatory tissue injury[32]. This is underscored by data showing that only IM nitrite reversed Cl₂ dependent basal elastance and elevated methacholine dependent airway resistance and elastance suggesting that PMN infiltration rather than increased permeability per se, plays an important role in mediating post- Cl₂ exposure airway hyperresponsiveness.

Despite nitrite being an anion at physiologic pH (pK~3–4) presumably necessitating controlled transport across membranes [23], re-distribution of exogenously added nitrite across all tissues is surprisingly rapid after IP injection [33]. We note that due to the focus of this study being nitrite therapy, nitrite pharmacokinetic measurements were performed only in rats that had been pre-exposed to inhaled Cl₂ gas. Notwithstanding this issue, similar rapid (over 5min) increases in blood nitrite were observed with both IM and IP

administration as reported with control (non stressed) mice [33]. Interestingly however, nitrite and its oxidation product nitrate, increased to a greater extent after IM compared to IP administration suggesting significant consumption of nitrite in the latter context, presumably due to first pass hepatic metabolism[34]. This could reflect a species effect also, as comparison of oral vs. IV nitrite administration in healthy (unstressed) humans showed little difference in pharmacokinetics suggesting first pass effects in the liver are low[35]. In this context, we acknowledge limitations in the pharmacokinetic studies presented herein as changes in nitrite concentrations in specific tissue and / or subcellular compartments [21, 36] may have occurred that are not reflected in whole blood levels although no differences in lung nitrite levels were observed 40min post nitrite administration in the IP vs. IM groups. To test if differential effects of IP and IM nitrite were due to 2-fold greater circulating levels with IM administration, the effects of IM nitrite 0.5mg/Kg, a dose which should be equivalent to total circulating nitrite doses achieved by IP 1mg/Kg, was tested. Interestingly, at this lower dose, the protective mechanisms of IM nitrite shifted to resemble IP nitrite at 1mg/Kg from preventing cell accumulation in the BAL to inhibiting protein accumulation. This suggests that the anti-inflammatory and permeability protective properties of nitrite occur with distinct dose-dependencies with the difference between IM and IP being the attained circulating nitrite concentrations with each administration modality. An additional consideration is that the therapeutic effects of nitrite against IR injury have demonstrated a 'U' shaped dependence on nitrite dose [37]. Similarly, the protective effect of IM nitrite was 'U' shaped since no effects on BAL protein were seen with 0.1mg.Kg. This therapeutic window between 0.1 and 1mg/Kg is narrower compared to previous studies which show at least a 40- fold range in doses over which nitrite can either protect against IR injury in the liver or stimulate NO-dependent angiogenesis in vivo [37, 38]. The loss of protection at higher nitrite doses is likely due to oxidative reactions of nitrite that lead to production of reactive nitrogen species or heme oxidation [30]. Whether this reflects differential organ sensitivities to nitrite therapy or unique to Cl₂ -induced injury, is not clear. Additional and more comprehensive nitrite dose-dependent studies evaluating multiple indices of ALI are required but it is important to stress that a therapeutic window is present and that nitrite can be administered without causing additional toxicity supporting its further development as a post exposure therapeutic.

A limitation of the current study is the lack of insights into how different doses of circulating nitrite may differ in their ability to respectively, target either the permeability or inflammatory components of ALI. We note that our previous studies with IP nitrite demonstrated that protection against BAL protein accumulation was associated with decreased cell death in the airway compartment[17]. Our focus for this study was to evaluate therapeutic efficacy with different nitrite administration modalities, a concept that has received little attention despite precedent. Previous studies have shown that neither IV nor IP nitrite protected in models of renal IR injury, whereas topical administration onto renal tubules directly did afford protection [39, 40]. In the context of the anti-inflammatory effects, a possible role in modulating PMN trafficking is suggested by decreased circulating PMN. Whether this reflects effects of nitrite on leukocyte tissue adhesion, transmigration or release and/or recycling from the bone marrow remains to be determined. Cytokine / chemokine analysis did not reveal any significant effects of nitrite, although limitations of these studies include use of whole lung homogenates precluding assessment of compartmentalized differences that may be present in the BAL and assessment of only one time point post Cl₂ exposure (3h). Future studies evaluating time dependent changes in the circulation, lung homogenates and BAL are required to fully assess if the effects of differential circulating nitrite concentrations on PMN trafficking are mediated by specific effects on chemokine and cytokine profiles. Interestingly recent studies have shown that nitrite (and nitrate) therapy may attenuate inflammatory injury in the intestine and kidney via inhibiting the ability of leukocytes to adhere to the inflamed tissues [41, 42].

Our data also suggests that nitrite-dependent effects on PMN accumulation in the BAL are dependent on XOR. Nitrite reduction to NO by XOR has been documented previously [27] and NO is a potent anti-adhesive effector for PMN's providing one plausible mechanism for the protective effects of nitrite. Unfortunately, C-PTIO had effects on Cl₂ induced injury without nitrite, precluding testing of the effects of an NO-scavenger on nitrite dependent protection (not shown). In addition, we note that IM nitrite inhibited post Cl₂ exposure increases in circulating white blood cells. Emerging data highlight the complex interplay between different white blood cells that comprise the effectors of innate and adaptive immune responses (PMN, T-cells etc) in regulating the balance between exacerbating or resolving the inflammatory component of ALI/ARDS as well as affecting airway reactivity [32, 43–45]. Indeed, gamma delta T-cells have been implicated in development of reactive airways post Cl₂ gas exposure [46]. An additional insight gained from these studies is that the same degree of protection is afforded by a single or multiple (every 2h for 6h post Cl₂ exposure) injections of nitrite indicating that the target for nitrite therapy is an early aspect of post Cl₂ induced toxicity, which as speculated above could be leukocyte recruitment from the circulation. Further elucidation of how nitrite therapy affects immune cell function may provide insights into the protective effects observed.

In addition to efficacy, additional beneficial properties of nitrite include its cost effectiveness, relative chemical stability which allows for a long shelf life (allowing stockpiling), its amenability to be administered via multiple routes, and documented safety profile as evidenced by recent studies showing that doses of 267µg/Kg/hr (by IV infusion) can be tolerated in humans [20, 21]. Moreover nitrite is used as an ingredient in cyanide antidote kits and at doses (~300mg IV administration for adults) that are significantly higher than those currently being evaluated for nitrite-based therapies for ischemic diseases. These properties are favorable and similar to other recently tested therapies for Cl₂ toxicity that also show efficacy including ascorbate and β₂-agonists [25, 26]. Indeed, whether IM nitrite therapy could be combined with these, or other recently described therapeutics [14, 47, 48] to prevent both Cl₂ induced increased lung permeability and inflammation is an intriguing possibility that is the subject of ongoing studies.

In summary, we show nitrite administered post Cl₂ injury can prevent select aspects of ALI namely permeability and inflammation respectively depending on the amount of nitrite achieved in the circulation which in turn is dependent on the route of administration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Chlorine causes post exposure acute lung injury (ALI) and airway hyperreactivity
- IP or IM nitrite therapy attenuates Cl₂ gas induced ALI but with distinct dose dependencies
- Route of nitrite administration affects protective mechanisms against ALI

Figure 1. Effects of nitrite administration modality on protective mechanisms against Cl₂ induced lung accumulation of BAL protein and cells

Rats were exposed to Cl₂ gas (400ppm, 30min) and returned to room air. 30min after cessation of Cl₂ gas, saline or nitrite was administered by IP or IM injection, values in parentheses indicate nitrite dose in mg / Kg. 6h after Cl₂ gas administration, rats were sacrificed and BAL protein (panel A) and cells (panel B) measured as described in methods. Data show mean $\pm$ SEM (n= 4 – 19). *P < 0.01 relative to air alone, # P < 0.05 relative to respective Cl₂ + saline by t-test. No significant effect was observed for air + IP or IM nitrite vs. air alone.

Figure 2. Effects of IM nitrite on Cl₂ dependent increases in BAL levels of PMN

Rats were exposed to Cl₂ gas (400ppm, 30min) and returned to room air. 30min after cessation of Cl₂ gas, nitrite (1mg/Kg) was administered IM injection. 6h after Cl₂ gas administration, rats were sacrificed and BAL cells collected and differential analysis performed for PMN. Data show mean $\pm$ SEM (n= 5–13). *P < 0.05 relative to air, #P<0.05 relative to Cl₂ + saline as indicated by 1-way ANOVA with Tukey's post –test.

Figure 3. Histopathological effects of chlorine on proximal airway epithelium 6 hours post-exposure

Representative light micrographs of proximal airway epithelium from rats exposed to 0 ppm chlorine (A), 400 ppm chlorine (B); 400 ppm chlorine plus post-exposure IM nitrite (C, E), or 400 ppm chlorine plus post-exposure IP nitrite (D). Tissues collected 6 hours post chlorine exposure, embedded in paraffin and stained with hematoxylin and eosin. Variability in the amount of intact airway epithelium with the IM nitrite treatment is shown in C and E. Thin arrows indicate squamous cells, CC = ciliated cells, NC = nonciliated cells, * indicates inflammatory cell. Magnification bar = 30 microns. Panels F and G show epithelial thickness and percent of images with attached epithelia as described in results. Data show mean $\pm$ SEM (n = 4–6 rats) (4–5 sections from each rat were analyzed). *P<0.001 by 1-way ANOVA with Bonferroni post test for panel F and **P<0.02 by t-test for panel G.

Figure 4. Effects of IP or IM nitrite on post Cl₂ exposure airway reactivity

Rats were exposed to air or Cl₂ (400ppm, 30min) and then returned to room air and IP or IM nitrite (both 1mg/Kg) administered 30min thereafter. 24h after initiating Cl₂ gas exposure, airway reactivity under basal and then increasing methacholine doses was determined by flexivent. Panels A, B show airway resistance and elastance respectively (□ = air, Δ = Cl₂ gas, ● = Cl₂ + IP nitrite, ▼ = Cl₂ + IM nitrite). Panels C, D show basal (pre methacholine) resistance and elastance respectively. Panels E, F show the methacholine dependence (between 0–5mg/ml) on increases in resistance and elastance respectively as calculated by linear regression fits of methacholine dependent changes between 0–5mg/ml. Data show mean ± SEM (n=3–6). P<0.0001 for air vs Cl₂ and P<0.02 for Cl₂ vs. Cl₂ + IM nitrite by

2-way ANOVA for panel A and panel B. No significant differences between Cl_2 and Cl_2 + IP nitrite were observed. * $P < 0.05$ for Cl_2 vs. air and ** $P < 0.01$ for Cl_2 vs Cl_2 + IM nitrite for panels C-F.

Figure 5. Effects of IP or IM nitrite on post Cl₂ exposure dependent changes in circulating leukocytes

Rats were exposed to air or Cl₂ (400ppm, 30min) and then returned to room air and IP or IM nitrite (both 1mg/Kg) administered 30min thereafter. 6h (Panel A) and 24h (Panel B) after initiating Cl₂ gas exposure, whole blood was collected and leukocyte counted. Data show mean $\pm$ SEM (n = 3–6). P = 0.05 by 1-way ANOVA with *P<0.05 relative to air by Tukeys post test for Panel A. P< 0.001 by 1-way ANOVA with *P<0.001 relative to air and **P<0.001 relative to Cl₂ by Tukeys post test for Panel B

Figure 6. Effects of allopurinol on IM nitrite dependent protection against PMN accumulation in the BAL

A) Rats were exposed to air or Cl_2 gas (400ppm, 30min) and then lung levels of XOR determined at 6h by western blotting. Data shown XOR normalized to β -actin and are mean $\pm$ SEM (n=4-5), * $p < 0.05$ by t-test. Shown representative gel images were acquired from the same western blot. B) Rats were exposed to Cl_2 gas (400ppm, 30min) and then vehicle, allopurinol or IM nitrite (1mg/Kg) administered at 15 min (for allopurinol) and 30min (for nitrite) post Cl_2 gas exposure. Cells in the BAL were measured 6h thereafter. Data show mean $\pm$ SEM, n=5-6. * $P < 0.03$ relative to Cl_2 by t-test.

Figure 7. Nitrite and Nitrate Pharmacokinetics after IP or IM nitrite administration

Whole blood levels of nitrite (panel A) and nitrate (panel B) were measured after Cl₂ gas exposure and after IM (■) or IP (●) nitrite (1mg / Kg) administered 30min post cessation of Cl₂ gas. Data show mean ± SEM (n = 5–6) with time 0 indicating the time immediately prior to nitrite administration. *P < 0.05 by 2-way ANOVA with Bonferroni's post test.