Effect of calcium hypochlorite as a source of hypochlorous acid in ameliorating cyclophosphamide-induced pulmonary and cardiac injury in mice

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Abstract:

Acute respiratory distress syndrome (ARDS) is recognized to be accompanied by severe lung and heart complications. This study aimed to demonstrate the efficacy of Calcium hypochlorite in cyclophosphamide (CP)-induced pulmonary and cardiac injury in mice. The CP-induced toxicity in the lung and heart is like that caused by ARDS. Calcium hypochlorite significantly attenuated doth lung and heart functionality and significantly reduced the CP-induced perivascular inflammation, congestion of blood vessels, and severe morphological changes of the alveolar wall. It also exerts anti-inflammatory and antioxidant effects on CP-induced pulmonary and heart toxicity. Given these results, calcium hypochlorite successfully ameliorates ARDS symptoms rendering calcium hypochlorite to be a promising agent to be tested in models of pneumonia caused by bacterial or viral infections, including those of acute respiratory distress syndrome (ARDS).

Keywords:

Acute respiratory distress syndrome (ARDS), hypochlorous acid (HOCl), inflammatory response, oxidative stress biomarkers.
1. Introduction

Acute respiratory distress syndrome (ARDS) is an acute deterioration of the exchange of gases associated with alveolar inflammation, elevated vascular pulmonary permeability, and pulmonary edema due to normal cardiac activity [1]. Approximately 29-42% of Coronavirus disease 2019 (COVID-19) patients developed ARDS and 15-52% of COVID-19 ARDS cases resulted in mortality [2–4].

Hypochlorous acid (HOCl) is a weak acid that arises after chlorine dissolves in water, producing hypochlorite which partly dissociates itself, and chlorine solutions are the essential disinfection agents [5]. It is produced in the body by neutrophils which are the first recruited immune cells at the site of infection and they play an important role to phagocyte the pathogen, ingest and destroy it [6]. After phagocytosis, neutrophils excrete many oxidizing agents to destroy the pathogen. Interestingly, HOCl is the major end-product of the neutrophil respiratory burst. Moreover, HOCl is documented to have a powerful antimicrobial nature in that it has been reported in vitro activity against microorganisms in many studies [7–10]. This proves its strong effect and antiviral and antibacterial action, as it is one of the immune defense mechanisms used by neutrophils in the process of phagocytosis [6]. Calcium hypochlorite is used as an HOCl source [11].

Cyclophosphamide (CP) is an alkylating agent used as an anticancer treatment. On the other hand, it is also reported to cause many organ damages as acute lung, kidney, and heart injury [12]. So, it is used in this study on a mouse model to induce respiratory and cardiac malfunction similar to that caused by acute respiratory distress syndrome (ARDS).

Herein, in the current study, we aimed to evaluate calcium hypochlorite treatment activity against CP-induced pulmonary and cardiac toxicity.

It is very similar to acute respiratory distress syndrome (ARDS), allowing us to evaluate the effect of calcium hypochlorite against lung infections that lead to inflammation or acute respiratory distress syndrome.

2. Material and methods

2.1. Animals:

Thirty adult male Swiss albino mice; 8 weeks of age weighing about 25 ± 5 g, were purchased from the "Experimental Research Centre of Nephrology and Urology Center", Mansoura University. Mice were randomly divided into three groups (10 mice/group) and were maintained under standard nutritional and environmental conditions. The experimental protocol obeys the ethical guidelines and the principles of keeping, using, and handling experimental animals approved by "The research Ethics Committee", Faculty of Pharmacy, Mansoura University. Egypt by "Principles of Laboratory Animals Care" [13].

2.2. Drugs and chemicals

Calcium hypochlorite: was purchased from (Xiamen Kerda trade CO., LTD, Xiamen City, China) and it was dissolved in distilled water for oral administration.

Cyclophosphamide: Cytoxan vials, powder for injection (Bristol-Myers Squibb, New Jersey, USA), and it was dissolved in 0.9% w/v saline for intraperitoneal (I.P.) injection.

2.3. Experimental protocol

Acute lung and heart injuries were induced by single Cyclophosphamide (CP) injection (75 mg/kg, I.P.) as reported by [12].

Thirty adult male Swiss albino mice were randomly allocated to 3 groups as follows:

a. Normal control (10 mice): mice received the vehicle (5 ml/kg, distilled water, orally) once daily for 7 days after single I.P. saline.

b. CP control (10 mice): mice received a single dose of CP (75 mg/kg, I.P.) and distilled water
(5 ml/kg, distilled water, orally) once daily for the following 7 days.

c. **Calcium hypochlorite and CP group (10 mice):**
mice received calcium hypochlorite (125 mg/kg, orally) once daily for 7 days after a single I.P. CP injection (75 mg/kg).

Twenty-four hours following the last dose of calcium hypochlorite, mice were humanely sacrificed by an overdose of thiopental sodium (40 mg/kg). Blood samples were collected from the animals by puncture of the retro-orbital venous plexus. The samples could stand for 15 min to clot at room temperature and then centrifuged (3,000 rpm for 10 min) to separate serum. The heart and both lungs were collected and weighed for calculation of lung (mg)/ body weight (g) and heart (mg) /body weight (g) indices.

### 2.4. Assessment of lactate dehydrogenase (LDH) and creatine kinase myocardial band (CK-MB) levels:

The serum was used immediately for the determination of LDH and CK-MB using kinetic methods. LDH activity in the sample is calculated by measuring the per-time absorbance decrease at 340 nm. CK-MB test procedures were carried out according to the supplied manufacturer's instructions.

### 2.5. Preparation of lung and heart homogenates for assessment of lung and heart malondialdehyde (MDA) and total antioxidant capacity (TAC):

The isolated left lobes of the lungs and heart tissue were rinsed in chilled 1.15 % KCl (pH 7.4) and weighed quickly. Homogenization was carried out in ice-cold KCl to yield 10% w/v tissue homogenate [14–16], the homogenate was used for estimation of lung and heart content of MDA, and TAC activity using Biodiagnostic assay kits (Giza, Egypt). Test procedures were carried out according to the supplied manufacturer's instructions.

### 2.6. Histopathology of lung tissue:

The right lobes of the lungs from all the animals were harvested and rinsed in ice-cold saline solution, the heart was cut lengthwise. All specimens were fixed in 10% neutral buffered formalin solution, embedded in paraffin, sectioned (4 to 5 μm) by a specialized histopathological technician, and stained with hematoxylin and eosin (H&E). At least two different sections were examined per sample. The prepared tissue slides were examined under a microscope in random order to assess histopathological changes due to CP injection and Calcium hypochlorite administration and the histopathologist was blinded to the experimental groups.

### 2.7. Statistical analysis

All data are presented as the mean ± the standard error of the mean (SEM), and significance was calculated at $p<0.01$. One-way analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparisons test for statistical comparison between parametric data was used. Statistical calculations were carried out using the Instat-3 computer program (GraphPad Software Inc. V2.04, San Diego, CA, USA).

### 3. Results

#### 3.1. Effect of calcium hypochlorite on lung and heart body weight indices, serum LDH serum CK-MB, tissue MDA content, and tissue TAC content:

Single CP injection (75 mg/kg) significantly increased lung (mg)/body weight (g) and heart (mg) /body weight (g) indices by approximately 15% and 25% respectively, in comparison to the normal control group. On the other hand, there is a significant improvement in both indices in the calcium hypochlorite & CP group when compared to the CP control group (Figure 1 A & B).

Serum LDH (figure 1 C) and CK-MB (figure 1 D)
significantly increased following single I.P. CP (75 mg/kg) by approximately 21 and 2.4 folds in comparison to normal control (n=10; p<0.001). Calcium hypochlorite administration significantly decreased the elevated serum LDH and CK-MB levels, by approximately 63% and 49%, in comparison to the CP control, (n=10; p<0.001).

Lung and heart MDA content significantly increased following a single CP injection, by approximately 2.4 and 2.6 folds respectively in comparison to normal control (n=10; p<0.001). Calcium hypochlorite significantly decreased the elevated lung and heart MDA contents, by approximately 77% and 56% respectively, in comparison to the CP control group, (n=10; p<0.001) (figure 1 E & F).

TAC contents, in the lung and heart, were significantly reduced in the CP control, by approximately 25% and 21% respectively, in comparison to the normal control (n=10; p<0.001). Calcium hypochlorite significantly increased the reduced lung and heart TAC contents, by approximately 20% and 18%, in comparison to the CP control, (n=10; p<0.001), (figure 1 G & H).

3.2. Effect of Calcium hypochlorite on lung histopathological features:

Microscopic pictures of H&E-stained lung sections showing normal bronchioles, alveoli, and interstitial tissue in normal control (C) (figure 2 A & 2 B). Lung sections from the CP control (figure 2 C to 2 G) showing congested blood vessels (red arrows), perivascular inflammation (yellow arrows), perivascular deposition of adipocytes (blue arrows), and higher magnification show marked narrowing of the alveolar lumen (black arrows) due to thickening of the alveolar wall with congested capillaries (red arrows) and infiltrated inflammatory cells (yellow arrows). Lung sections from the Calcium hypochlorite & CP group (figure 2 H & 2 I) show mildly congested blood vessels (red arrows) and higher magnification shows very mild thickening of the alveolar wall with congested capillaries (red arrows) and infiltrated inflammatory cells (yellow arrows) (figure 2).
Figure 1. Effect of Calcium hypochlorite on lung and heart body weight indices, serum LDH serum CK-MB, tissue MDA content, and tissue TAC content in male mice.

Values are the mean ± SEM of 10 mice per group. Statistical analysis of mean values was done using ANOVA followed by Tukey–Kramer’s test.

*, P < 0.001 normal control group Vs diseased control group.
†, P < 0.001 diseased control group Vs Calcium hypochlorite & CP Group.
Figure 2. Effect of Calcium hypochlorite (125 mg/kg, orally) on histopathological changes in lung specimens in CP injected mice.

Note: C, normal control group, CP, diseased control, and CH, calcium hypochlorite treated group.
Low magnification X:100 bar 100 and high magnification X:400 bar 50
4. Discussion:

The present study renders evidence of the treatment efficacy of calcium hypochlorite against CP-induced pulmonary injury and cardiotoxicity. CP-induced cellular injuries are like the pulmonary and cardiac complications that happened in ARDS. First, we observed that calcium hypochlorite successfully reduced lung (mg)/body weight (g) and heart (mg)/body weight (g) and attenuates the CP-resulted acute cellular damage in the lung and heart.

A possible instructive fact is that redox homeostasis, which depends on the gentle balance between enzymatic cascade reactions, serves a crucial role in adaptive responses under stress conditions [17]. However, the excessive production and accumulation of reactive oxygen species (ROS), known as oxidative stress, occurs during tissue damage and impaired cell function. It is well demonstrated that the use of calcium hypochlorite significantly decreased MDA content in both lung and heart tissue homogenates, this confirms that it decreased lipid peroxidation and diminished the overexpressed ROS. Consequently, its increased TAC content, a marker of antioxidant defense, in both lung and heart tissues. Such improvement in oxidative stress biomarkers is accompanied by a significant reduction in serum LDH which suggests the cytoprotective efficacy of calcium hypochlorite.

More importantly, the increased serum CK-MB, which reflects cardiac injury, was significantly decreased after the administration of calcium hypochlorite suggesting a positive influence on the heart functionality indicating attenuated cardiac tissue damage. Furthermore, on the level of the pulmonary histopathological examination, Calcium hypochlorite remitted morphological changes induced using CP on the lung. It successfully mitigated the congestion of blood vessels and cause a significant decrease in the alveolar wall thickness with mild recruitment of inflammatory cells. In contrast, the CP control mice group showed extensive morphological changes such as areas of congested blood vessels, perivascular inflammation, perivascular deposition of adipocytes, marked narrowing of alveolar lumen due to thickening of the alveolar wall with congested capillaries, and infiltrated inflammatory cells.

Interestingly, the previous studies proved that the prolonged use of calcium hypochlorite for long period with a high dose (750 mg/kg for six weeks) can induce severe hepatic damage via inducing oxidative stress and diminishing the antioxidant defense system in Nigerian commercial cockerels [18]. On the other hand, the current study implies that the use of calcium hypochlorite with a smaller dose (125 mg/kg for 7 days only) can successfully improve the antioxidant ability level and ameliorate the inflammatory response and oxidative stress in mice.

5. Conclusion:

In conclusion, calcium hypochlorite ameliorates inflammatory response and oxidative stress and attenuates the antioxidant defense system in CP-induced lung and cardiac injuries.

Which makes it a promising factor to be tested against pulmonary infections, whether bacterial or viral, that lead to acute respiratory distress syndrome.

Future perspectives

ARDS is one of the most common clinical manifestations of Coronavirus disease -2019 (COVID-19) patients which is caused by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) and the prevention or treatment of ARDS in COVID-19 patients is a big challenge [19,20]. HOCl is documented to have a powerful anti-inflammatory and antimicrobial. Calcium hypochlorite can be used as an HOCl source to be evaluated against COVID-19 manifestations models.

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Statements and Declarations

The authors declare that there are no conflicts of interest.

6. References


