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Chlorine inhalation induces acute chest syndrome in humanized sickle cell mouse model and ameliorated by postexposure hemopexin

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ABSTRACT

Triggering factors of Acute Chest Syndrome (ACS) is a leading cause of death in patients with Sickle Cell Disease (SCD) and targeted therapies are limited. Chlorine (Cl_2) inhalation happens frequently, but its role as a potential trigger of ACS has not been determined. In this study, we hypothesized that Cl_2 exposure resembling that in the vicinity of industrial accidents induces acute hemolysis with acute lung injury, reminiscent of ACS in humanized SCD mice. When exposed to Cl_2 (500 ppm for 30 min), 64% of SCD mice succumbed within 6 h while none of the control mice expressing normal human hemoglobin died ($p<0.01$). Surviving SCD mice had evidence of acute hemolysis, respiratory acidosis, acute lung injury, and high concentrations of chlorinated palmitic and stearic acids ($p<0.05$) in their plasmas and RBCs compared to controls. Treatment with a single intraperitoneal dose of human hemopexin 30 min after Cl_2 inhalation reduced mortality to around 15% ($p<0.01$) with reduced hemolysis (decreased RBCs fragility ($p<0.001$) and returned plasma heme to normal levels ($p<0.0001$)), improved oxygenation ($p<0.0001$) and reduced acute lung injury scores ($p<0.0001$). RBCs from SCD mice had significant levels of carbonylation (which predisposes RBCs to hemolysis) 6 h post-Cl_2 exposure which were absent in RBCs of mice treated with hemopexin. To understand the mechanisms leading to carbonylation, we incubated RBCs from SCD mice with chlorinated lipids and identified sickling and increased hemolysis compared to RBCs obtained from control mice and treated similarly. Our study indicates that Cl_2 inhalation induces ACS in SCD mice via induction of acute hemolysis, and that post exposure administration of hemopexin reduces mortality and lung injury. Our data suggest that SCD patients are vulnerable in Cl_2 exposure incidents and that hemopexin is a potential therapeutic agent.

1. Introduction

Sickle Cell Disease (SCD) affects more than 100,000 Americans (1/365 African Americans) and 20 million people worldwide with reduced life expectancy by more than 20 years when compared to the general population of the United States [1,2]. Acute Chest Syndrome (ACS) is a leading cause of death and intensive care unit admission in patients with SCD and is responsible for 25% of SCD related mortality [3,4]. Outdoor pollution and environmental factors such as ozone, nitrogen dioxide, particulate matter, and carbon monoxide levels are associated with the risk of ACS hospital admissions [5,6]. Tobacco exposure – as an indoor pollutant - is associated with increased ACS hospitalization both in children and adults [7,8]. In the largest ACS study, the triggering factor for 46% of ACS crises could not be determined [4]. The role of other indoor and outdoor exposures in triggering ACS is unknown. It is vital to determine if exposure to household chemicals, occupational exposure, and toxic inhalants during industrial accidents or chemical warfare can trigger this fatal SCD complication. Determining SCD patients as a vulnerable population during such exposures and developing targeted therapy to prevent and treat ACS can be lifesaving.

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Chlorine (Cl_2) is a common agent in household compounds (such as cleaning agents, swimming pool disinfectants, and tobacco smoke), industrial manufacturing (plastic production, waste sanitation, bleaching liquids), and is the most commonly encountered substance in industrial accidents and chemical warfare resulting in large number of fatalities and it might have even higher impact on patients with SCD [9–14]. The Hazardous Substances Emergency Events Surveillance (HSEES) system reported 623 accidents involving Cl_2 in the United States between 1996 and 2001 which means an accident every 2–3 days in average. Victims of Cl_2 exposure accounted for 5.5% of all hazardous substance release victims with the highest odds of causing injuries at 3.5 times compared to other agents [15]. In the Graniteville train accident in 2005, it was determined that Cl_2 levels during a 30 min exposure period were 4428, 550 and 161 ppm at 0.2, 0.5 and 1 km from the epicenter of the accident. People exposed to 550 ppm Cl_2 for 30 min required hospitalization and a number of them developed ARDS with significant increase in hospital admissions for pulmonary causes in the vicinity [16]. The large majority of rodents exposed to 600 ppm Cl_2 for 45 min survive the exposure although they develop bradypnea and signs of respiratory distress [17,18]. Due to the high number of Cl_2 involved accidents and high prevalence of SCD among African American population, patients with SCD are at risk of Cl_2 exposure in work or at their leisure time which can have detrimental consequences in this vulnerable population. Cl_2 is highly reactive and it interacts with the aqueous interface in the pulmonary lining to form mostly hypochlorous acid (HOCl), that in turn interacts with plasmalogens included in the phospholipid layer of cell membranes producing chlorinated lipids (Cl-Lip) that consist of fatty acids and aldehydes [19,20]. The level of Cl-Lip has been shown to increase immediately after Cl_2 exposure in mice with gradual decline over the following 72 h [21]. In addition, the plasma levels of both 16 and 18 chlorinated fatty acids (ClFA) (16ClFA and 18ClFA) increase after accidental exposure to Cl_2 in humans [22]. Therefore, Cl-Lip are considered to be biomarkers as well as mediators of Cl_2 toxicity to Red Blood Cells (RBCs), mitochondria, and cardiac myocytes via their effect on the sarco(endo)plasmic reticulum Ca2+-ATPase [21,23–25]. They also increased the permeability of lung cells resulting in acute lung injury [26].

Exposure to household cleaning compounds has been shown to increase asthma prevalence and severity [27,28]. Asthma diagnosis in SCD doubles the risk of ACS and it increases the risk of early death by 2.4 folds [29,30]. Cl_2 exposure has been shown to significantly increase plasma heme levels in humans following accidental environmental exposures and in mice [31]. A single injection of hemopexin post Cl_2 exposure in mice reversed acute and chronic lung injury and decreased mortality in wild mice [23,32]. Hemolysis triggered by halogen exposure can be exacerbated in patients with SCD given increased RBC fragility. Studies on humanized SCD mice confirmed the role of cell-free heme in triggering ACS and the protective effect of hemopexin against heme induced lung injury [33,34].

We hypothesized that Cl-Lip resulting from Cl_2 exposure cause exaggerated RBCs damage in SCD and trigger ACS like acute lung injury. We tested our hypothesis using humanized SCD mice and compared them to humanized normal hemoglobin mice as control and proved that SCD mice exhibit higher mortality and morbidity when exposed to Cl_2 gas. After establishing that, we attempted to identify the mechanisms responsible. Our data demonstrated that SS mice had higher levels of chlorinated fatty acids in their plasma and RBCs, that their RBCs have higher levels of carbonylation and are more likely to fracture when exposed to mechanical stress as compared to wild type controls at 6 h post Cl_2 exposure. Furthermore, we showed for the first time that a single injection of hemopexin post Cl_2 exposure decreased heme levels to normal and greatly improved mortality and lung injury score.

2. Methods

2.1. Animals

We used adult (8–16 weeks) humanized SCD mice (SS) and humanized normal hemoglobin control mice (AA) to perform the experiments (Supplementary materials).

2.2. Study design

Sickle (SS) and control (AA) mice were randomly assigned to air or Cl_2 exposure at 500 ppm for 30 min. After the exposure, mice were monitored every 30 min to assess survival and well-being. Previous animal studies showed that ACS develops within 2–4 h after noxious exposures in humanized SCD mice [34,35]. After 6 h, the surviving mice were anesthetized using 5 ppm of isoflurane and blood collection from the abdominal aorta was performed. While still under sedation, lungs were harvested with the left lung was flash frozen, while the right lung was inflated with 10% formaldehyde at 25 cmH_2O for histologic evaluation.

The same experiment was repeated using Cl_2 exposure at the same dose followed by either purified human hemopexin IP injection at 10 mg/kg or vehicle with the same volume of PBS 30 min after the Cl_2 exposure. Each experiment was repeated at least twice.

2.3. Chlorine exposure

Mice were exposed to 500ppm of Cl_2 for 30 min in a cylindrical glass chamber (Specialty Glass, Houston, TX; part no. X02AI99C15A57H5) as described before [36]. The choice of Cl_2 exposure dose and timing were based on human epidemiologic data [16] and previously published murine experiments [17,18] as mentioned earlier.

2.4. Determination of survival

After exposure to air or Cl_2, mice were observed every 30 min to assess survival. The death time was recorded and compared to the exposure time to calculate survival time in minutes and Kaplan-Meier survival proportions was performed.

2.5. Heme level measurement

Plasma heme was measured on blood obtained from abdominal aorta using two methods; QuantiChrom heme assay kit (Product No. DIHM-250; BioAssay Systems, Hayward, CA), and then by least square fitting of the spectral deconvolution as reported by our lab before [37].

2.6. RBC fragility measurement

After washing the RBCs to remove free heme, mechanical fragility of RBC was measured using glass beads and rotated at 24 rpm for 2 h as published earlier by our lab (Supplementary materials) [22].

2.7. Plasma and RBC ClFA measurement

Plasma and RBC pellets were flash frozen and shipped in dry ice to Dr. Ford lab at St. Louis University. Free and total (free + esterified) ClFA were measured with liquid chromatography /mass spectrometry as described before [38].

2.8. Acute lung injury score

We used the official report of the American Thoracic Society to score acute lung injury (ALI) utilizing 5 parameters; alveolar space neutrophil count, interstitial space neutrophils, alveolar hyaline membrane, airspace proteinaceous debris, and alveolar septal thickening [39].

2.9. Blood gas measurement

Arterial blood gas from abdominal aorta was obtained from anesthetized mice with Isoflurane at the terminal procedure using Element POC analyzer (Heska, Loveland, CO) as detailed in our previous publication [40].

2.10. Effect of chlorinated liposomes on RBCs ex vivo

RBCs freshly harvested from SS and AA mice were incubated *ex vivo* with chlorinated liposomes or vehicle for 4 h and then evaluated by two methods: RBC mechanical fragility as explained above and Sickle Cell Index (SCI) as reported earlier (Supplementary materials) [41].

2.11. Measurement of carbonyl adducts in the RBCs

We used Oxyblot protein oxidation kit to measure the level of protein carbonyl adducts on the RBC from AA and SS mice after separating them from plasma and hemolyzing them with 20 mM hypotonic Hepes Buffer as described in the published paper from our lab and detailed in the supplementary materials [22].

2.12. Statistical analysis

Values presented as mean ± SEM. We used log-rank (Mantel-Cox) method to compare Kaplan Meir survival proportion curves and then confirmed with Gehan-Breslow-Wilcoxon test. Two-tailed unpaired student test was used to compare two study groups of mice. One-way ANOVA was utilized to compare multiple groups. GraphPad Prism 8 was used to perform statistical analysis and create the graphs. Significance was defined as a p value of less than 0.05.

3. Results

3.1. Exposure to chlorine resulted in a higher mortality rate in the SCD mice compared to the control mice and post-exposure administration of hemopexin reduced mortality

3.1.1. Cl_2 versus air

Of the 14 SCD mice exposed to 500 ppm of Cl_2 for 30 min, only 5 (35.7%) survived 6 h after the exposure; on the contrary, none of the 7 AA mice died ($p<0.01$) (Fig. 1A).

3.1.2. Cl_2+vehicle versus Cl_2+hemopexin

Sickle mice injected with hemopexin at a dose of 10 mg/kg body weight IP 30 min after Cl_2 exposure (n=13) had significantly improved survival when compared to SCD mice exposed to Cl_2 and injected with vehicle (n=14) (84.6% vs 35.7%; $p<0.01$) with a hazard ratio for the hemopexin treatment of 0.175 (95%CI: 0.053-0.577) (Fig. 1B). None of the AA mice exposed to Cl_2 and injected with vehicle or hemopexin died within 6 h of the inhalation.

3.2. Plasma heme level and RBC fragility were higher in SCD mice than in control normal human hemoglobin mice with further increased after Cl_2 exposure, and were reduced by post-exposure injection of hemopexin

3.2.1. Cl_2 versus air

While maintained on room air, SCD mice had higher plasma total cell-free heme level than control mice (24.10 ± 3.03 mg/dL in SS mice vs 12.07 ± 0.77 mg/dL in AA mice; p=0.001) (n-7-8 per group). Heme level further increased in SS mice 6 h after exposure to Cl_2 500 ppm for 30 min (from 24.10 ± 3.03 mg/dL to 38.77 ± 2.08 mg/dL; p=0.002) (Fig. 1C). Six hours after Cl_2 exposure, plasma heme level was significantly higher in SS mice than AA mice with the same exposure ($p<0.0001$). There was no significant increase of heme level in the AA mice 6 h after Cl_2 exposure compared to baseline (p=0.09) (Fig. 1C).

We noticed similar changes in RBC fragility with increased fragility

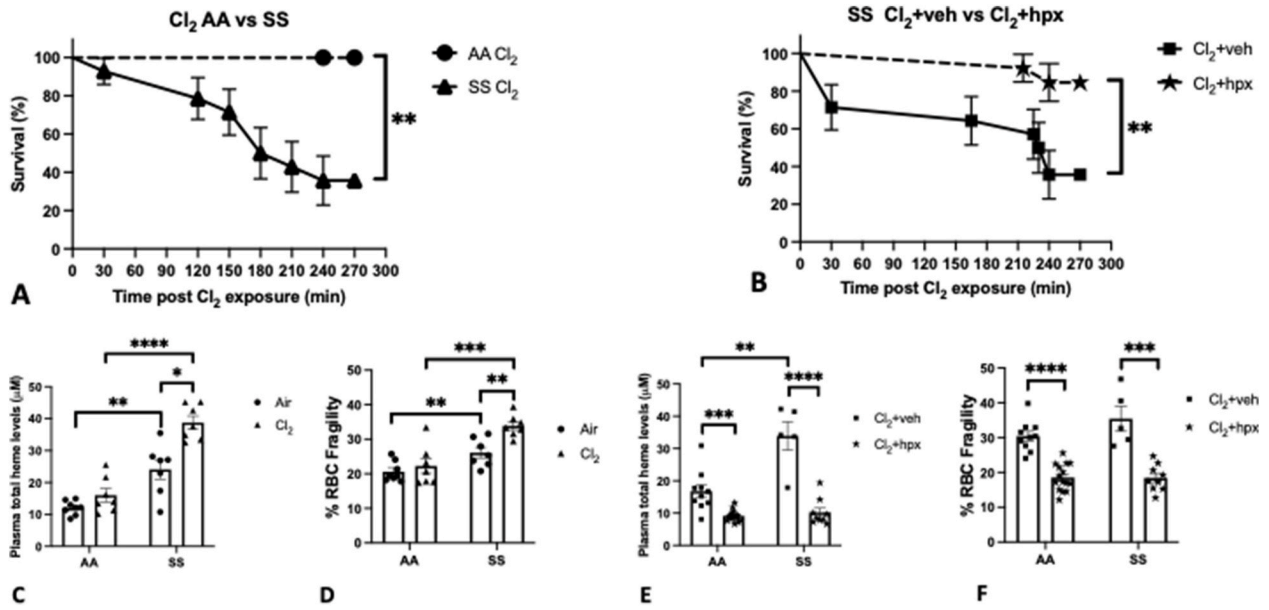

Fig. 1. Survival proportions and hemolysis indicators associated with Cl2 exposure with and without hemopexin treatment. The Kaplan Meir survival proportions with error bars representing standard error compares survival of AA (n=7) to SS (n=14) mice exposed to chlorine at 500 ppm for 30 min in (A) and compares survival of SS mice exposed to Cl_2 with intraperitoneal injection of vehicle (n=14) versus chlorine with intraperitoneal hemopexin injection (n=13) 30 min after the Cl_2 exposure in (B). Bar charts representing the changes in plasma heme level and RBC fragility with exposure to chlorine 500 ppm for 30 min compared to air (C, D) and with exposure to chlorine inhalation 500 ppm for 30 min and vehicle injection IP 30 min after chlorine exposure compared to exposure to chlorine and hemopexin injection at 10 mg/kg IP 30 min after chlorine exposure (E, F). Cl_2: Chlorine; AA: normal human hemoglobin control mice; SS: sickle human hemoglobin mice; Cl_2+veh: chlorine exposure and vehicle injection; Cl_2+hpx: chlorine exposure and hemopexin injection. RBC: red blood cell. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

of RBC in the SCD mice at baseline compared to that of control mice (SS: 26.1 ±1.54% vs AA: 20.6 ± 1.02%; p=0.009). There was further increase in RBC fragility in the SCD mice 6 h after Cl_2 exposure, becoming higher than both RBC fragility in the SCD mice at baseline (from: 26.1 ±1.54% to: 33.9 ± 1.21%; p=0.0018) and RBC fragility of control mice that were exposed to Cl_2 at an identical exposure level (SS + Cl_2: 3.9 ± 1.21% vs AA + Cl_2: 22.3 ± 2.20%; p=0.0006) (Fig. 1D).

Hemopexin injection to SS mice breathing air did not improve the heme level (p=0.30) or RBC fragility (p=0.38)

3.2.2. *Cl_2+vehicle versus Cl_2+hemopexin*

Hemopexin injection 30 min after Cl_2 exposure significantly reduced plasma cell-free heme level - compared to vehicle injection - in both the SCD mice (from 33.88 ± 4.37 mg/dL in the Cl_2+veh (n=5) to 10.19 ± 1.38 mg/dL in the Cl_2+hpx (n=9); p<0.0001), and control mice (from 16.77 ± 1.96 mg/dL in the Cl_2+veh (n=10) to 9.14 ± 0.45 mg/dL in the Cl_2+hpx (n=15); p=0.0001). Cell-free heme level in SS mice with Cl_2+vehicle was higher that of AA mice Cl_2+vehicle 6 h after exposure (p=0.0011); but there was no difference between the two groups when exposed to Cl_2 and given IP hemopexin at the same time point (p=0.39) (Fig. 1E).

Hemopexin treatment given 30 min after exposure to Cl_2 also improved RBC fragility in both the SS mice (from 35.42 ± 3.61% in the Cl_2+veh (n=5) to 18.42 ± 1.28% in the Cl_2+hpx (n=9); p=0.0002), and AA mice (from 30.43 ± 1.391% in the Cl_2+veh (n=10) to 18.58 ± 0.99% in the Cl_2+hpx (n=15); p<0.0001) (Fig. 1F). RBC fragility was comparable in SS and AA mice exposed to Cl_2+vehicle (p=0.139) and when both groups (SS and AA) exposed to Cl_2 and given hemopexin 30 min later (p=0.92) (Fig. 1F).

3.3. *Hematocrit and hemoglobin levels were lower in SCD mice than in control mice with further drop after Cl_2 inhalation*

Hematocrit level was lower in SS mice at baseline when compared to control mice (p=0.001) with further drop after Cl_2 exposure in the SS mice (p=0.04). Hematocrit level has not changed in the AA group after Cl_2 exposure (p=0.74) (Supplementary Fig. 1A). Similar changes were noticed in the hemoglobin level that was lower in the SS group than AA group on room air (p=0.001) with further reduction in the SS mice after Cl_2 inhalation (p=0.04), and no change in hemoglobin level in AA mice after inhalation of Cl_2 (p=0.41) (Supplementary Fig. 1B).

Hemopexin IP administration did not improve hematocrit level after Cl_2 exposure in either groups (SS: p=0.86, and AA: p=0.50) (Supplementary Fig. 1C). Hemoglobin level followed the same trend with no change in hemoglobin level with hemopexin injection (SS: p=0.89, and AA: p=0.66) (Supplementary Fig. 1D). Detailed results are in the supplementary materials.

3.4. *Chlorine exposure increased the levels of chlorinated fatty acids in both the plasma and RBC of SCD and control mice, with more profound increase in the SCD mice*

After Cl_2 exposure, all forms of ClFA increased in both the SCD and control mice (Fig. 2). Compared to control mice, SCD mice exposed to Cl_2 had higher plasma levels of all forms of ClFA (Fig. 2 A–D), while in the RBCs, the level of 16ClFA was higher in the SS mice and the levels of 18ClFA were not significantly different (Fig. 2 E–H).

3.5. *Human hemopexin injection induces mouse hemopexin in the control mice but not in the SS mice*

Mouse hemopexin level was higher in SS mice than control mice (SS:

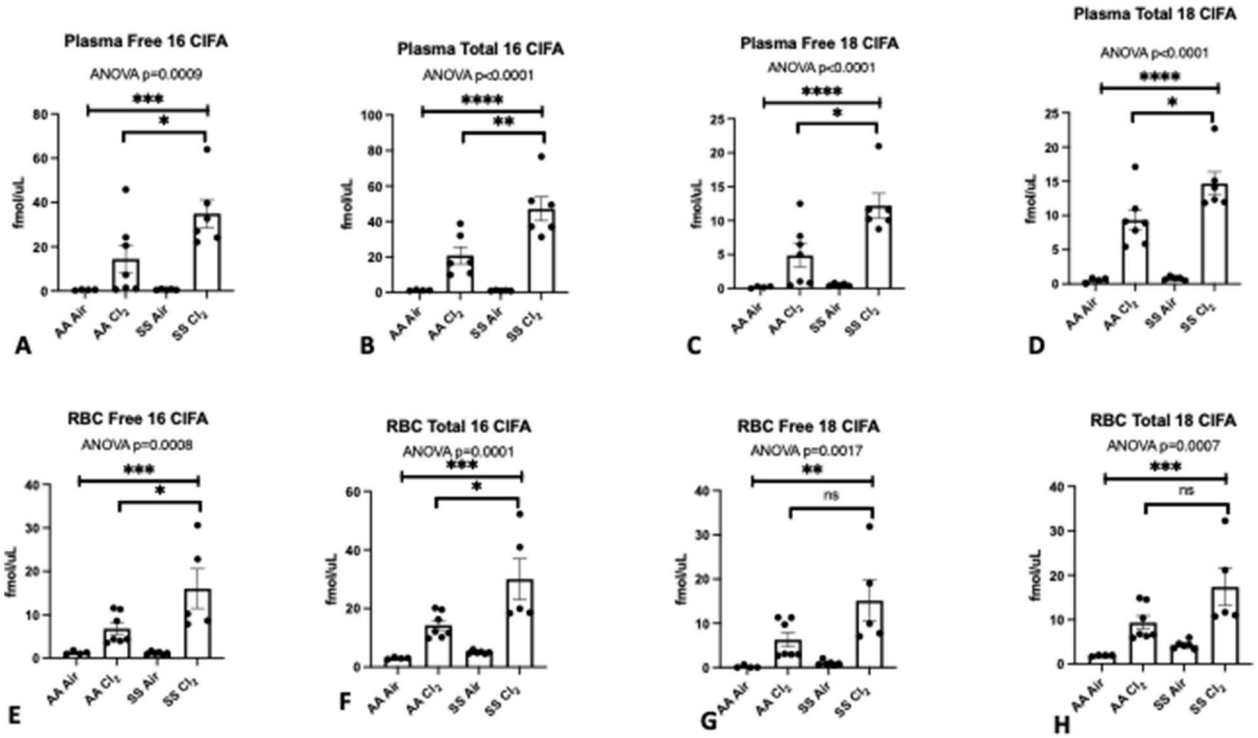

Fig. 2. Chlorinated fatty acids levels in control and sickle mice exposed to air and chlorine (Cl_2). Both 16 carbon chlorinated fatty acids (16 ClFA) and 18 carbon chlorinated fatty acids (18 ClFA) levels in both forms free and total (free + esterified) were measured in the plasma (A–D) and RBCs (E–H) of humanized normal hemoglobin control (AA) mice and humanized sickle (SS) mice. Plasma and RBCs levels of all forms of ClFA increased after Cl_2 exposure at 500 ppm for 30 min when compared to room air exposure with significant one-way ANOVA p values (A–H). Compared to control mice, SS mice exposed to Cl_2 had higher plasma levels of all forms of ClFA (A–D). In the RBCs, while free and total 16 ClFA are higher in the SS mice (E, F), the levels of 18 ClFA both free and total are not significantly different. Values are means ± SEM vs. AA air exposed mice, by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

209 ± 80 μg/ml vs AA:736 ± 120 μg/ml; p<0.01) at baseline while in air. Human hemopexin injection after Cl_2 exposure increased endogenous hemopexin in the control mice (p<0.05), but not in the SS mice (p=0.92) (Fig. 3).

3.6. Acute lung injury score was worse in SCD mice exposed to Cl_2 as compared to control mice with improvement after hemopexin treatment

3.6.1. Cl_2 versus air

Lung harvested from SCD mice at baseline (Air) (n=6) showed evidence of lung injury when compared to the lungs of control mice (n=6) with higher ALI score (SS: 0.344 ± 0.064 vs AA: 0.155 ± 0.016; p=0.0001) with further increase in ALI score 6 h after exposure to Cl_2 in the SS group (n=6) (0.51 ± 0.011 for Cl_2 exposure vs 0.34 ± 0.064 for air; p<0.0001) (Fig. 4A, C). Control mice also showed signs of acute lung injury after exposure to Cl_2 (n=5) (ALI from 0.155 ± 0.016 in Air to 0.222 ± 0.021 after Cl_2 exposure; p=0.03).

3.6.2. Cl_2+vehicle versus Cl_2+hemopexin

To evaluate the effect of hemopexin treatment on SS mice exposed to Cl_2, we calculated the ALI score for the Cl_2+vehicle group and compared it to that of Cl_2+hemopexin group. The ALI score improved significantly in the SS mice after the post-exposure hemopexin treatment (from: 0.53 ± 0.073 for Cl_2 + vehicle (n=5) group to 0.30 ± 0.032 for Cl_2 + hemopexin group (n=6); p <0.0001). Similar to the first experiment, SS mice with Cl_2 + vehicle had a worse ALI compared to AA with the same treatment (n=6) (p=0.02) (Fig. 4B, D).

3.7. Worse oxygenation at baseline in sickle mice, with the development of acidosis after Cl_2 exposure, and improved oxygenation following hemopexin injection

3.7.1. Cl_2 versus air

Sickle mice (n=8) had worse oxygenation while breathing room air when compared to control AA mice (n=7) documented with lower levels of both partial pressure of arterial oxygen (pO_2) (SS: 72 ± 5 mmHg vs AA: 116 ± 10 mmHg; p=0.01), and oxygen saturation (SO_2) (SS: 92.6 ± 0.73% vs AA: 97.9 ± 0.79%; p=0.0003) (Supplementary Fig. 2 A, B). There was no difference between SS and AA mice at baseline while breathing room air in partial pressure of carbon dioxide (pCO_2) (p=0.99) or hydrogen ion concentration (H^+) (p=0.50) (Supplementary Fig. 2 C, D). In SS mice, inhalation of Cl_2 (n=7) worsened pCO_2 level (from: 29 ± 4 to 42 ± 4 mmHg; p=0.042), increased H^+ (from: 44.77 ± 1.54 nM to 55.89 ± 4.32 nM; p=0.0256) but did not have effect on pO2 (p=0.06) or SO_2 (p=0.132). Cl_2 exposure did not have significant effects on those parameters in the AA mice (n=6) (pO_2: p=0.63; SO_2: p=0.328; pCO_2: p=0.34; and H^+: p=0.28) (Supplementary Fig. 2 A-D).

3.7.2. Cl_2+vehicle versus Cl_2+hemopexin

Compared to SS mice exposed to Cl_2 and survived for 6 h (n=5), SS mice received hemopexin injection 30 min after Cl_2 inhalation (n=6) had improved oxygenation measured with both indicators, pO_2 (from: 72 ± 2 mmHg to 89 ± 4 mmHg; p=0.0002), and SO_2 (from: 91.7 ± 1.16% to 95.75 ± 0.36%; p=0.0023) (Supplementary Fig. 2 E, F). No improvement in pCO_2 (p=0.77) or H^+ level (p=0.18) was noticed after injection of hemopexin compared to vehicle in SS mice (Supplementary Fig. 2 G, H).

Hemopexin injection did not have effect on blood gas in SCD exposed to air (n=5) when compared to SCD mice exposed to air and received vehicle (n=12) in all blood gas parameters including pH (p=0.85), pO2 (p= 0.75), pCO2 (p=0.92), or O2 saturation (p=0.21).

3.8. Chlorinated liposomes induced ex vivo hemolysis and sickling of the SCD mice RBCs with increased carbonyl adducts in the RBCs

Blood obtained from SS mice had increased Sickle Cell Index (SCI) at baseline when compared to AA mice (SS: 19.35 ± 2.07%; AA: 10.34 ± 1.67%; p=0.015) with significant increase after exposure to chlorinated

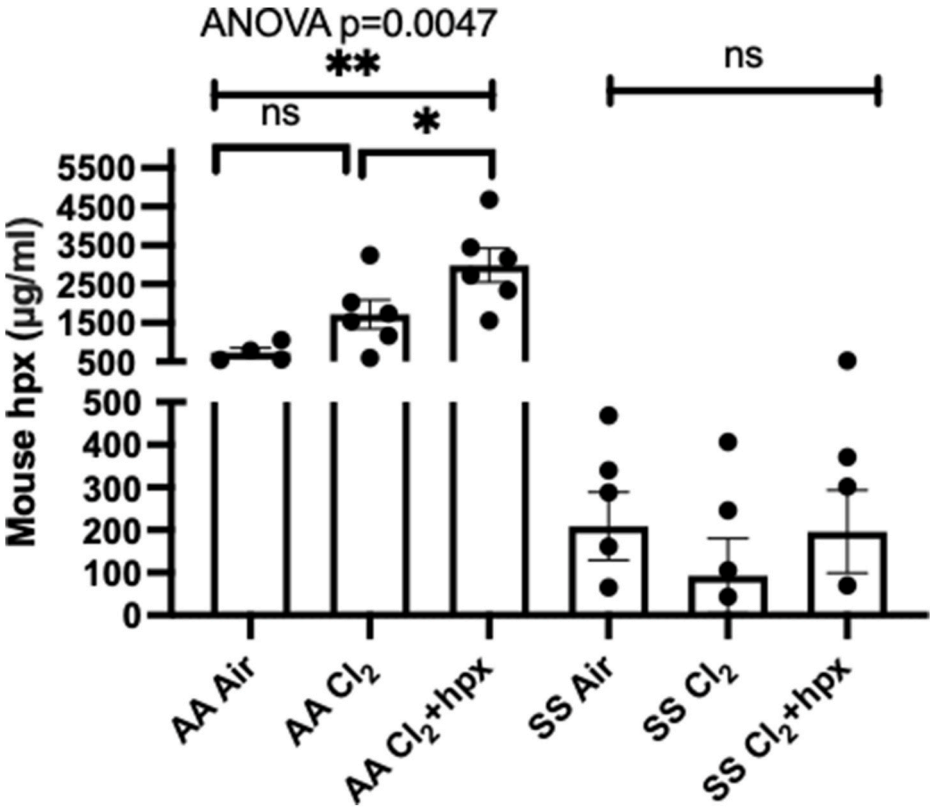

Fig. 3. Bar chart representing mouse plasma hemopexin levels in control versus sickle mice. The mouse hpx level was measure in humanized normal hemoglobin control (AA) mice and humanized sickle (SS) mice exposed to air, chlorine (Cl_2) at 500 ppm for 30 min or exposed to Cl_2 followed by intraperitoneal injection of 10 mg/kg of purified human hemopexin 30 min after exposure. There were significant changes in the hemopexin level in the control mice but not the SS mice. There was increase in the endogenous hemopexin level after the human hemopexin injection in the AA mice with no change in the level in the SS mice. Values are means ± SEM vs. AA air exposed mice, by one-way ANOVA. *p<0.05, **p<0.01.

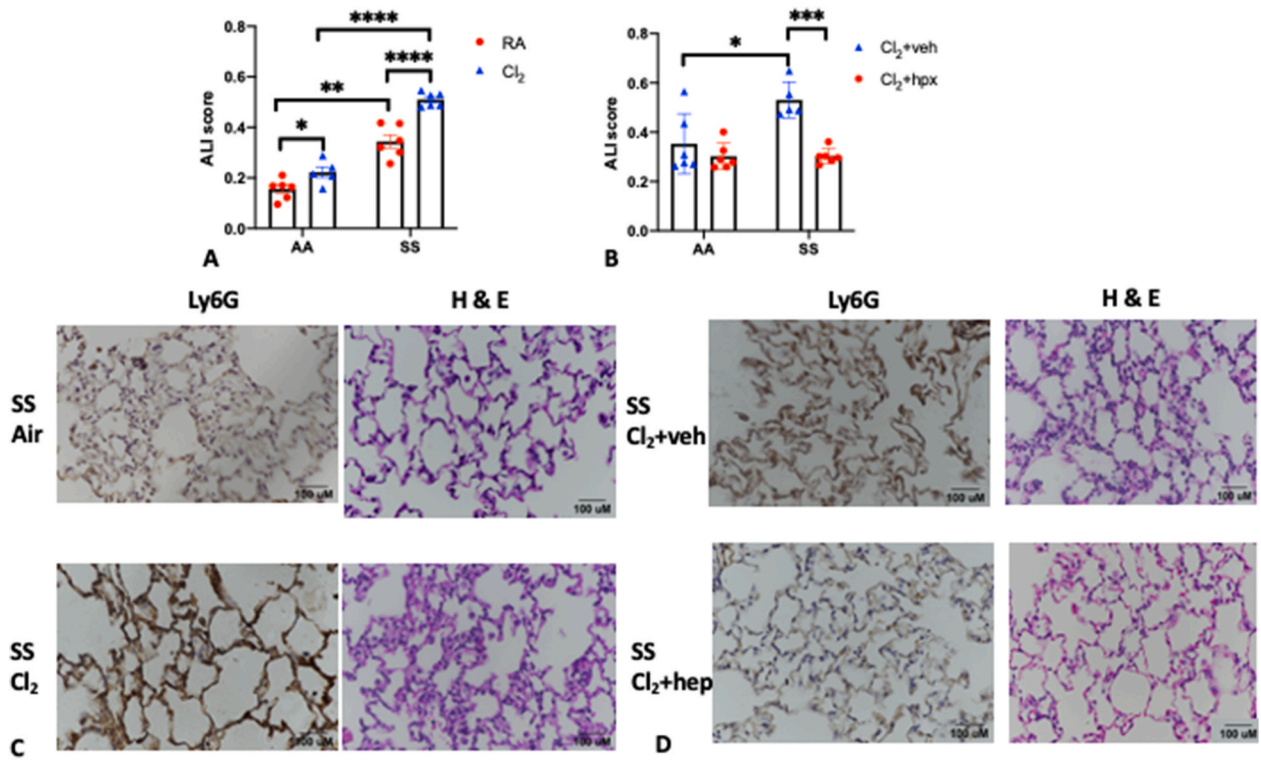

Fig. 4. Acute lung injury (ALI) markers. (A) Bar chart of ALI score comparing AA and SS mice exposed to chlorine 500 ppm for 30 min or air; and (B) AA and SS mice exposed to chlorine 500 ppm for 30 min and vehicle injection IP 30 min after chlorine exposure compared to exposure to chlorine and hemopexin injection at 10 mg/kg IP 30 min after chlorine exposure. (C)Histopathologic changes of the lungs obtained from SS mice exposed to Air versus Cl_2 and (D) Cl_2 + vehicle vs Cl_2 + hemopexin showing increased neutrophil infiltration of the interstitial tissues and alveolar spaces using Ly6g stain and alveolar injury with increased hyaline membranes, protein debris, and interalveolar septum thickness using H&E stain after exposure to Cl_2 with improved acute lung injury markers following treatment with hemopexin (magnification ×400, scale bar x100 μm). Cl_2: Chlorine; AA: normal human hemoglobin control mice; SS: sickle human hemoglobin mice; Cl_2+veh: chlorine exposure and vehicle injection; Cl_2+hpx: chlorine exposure and hemopexin injection. RBC: red blood cell. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

liposomes for 4 h in the SS group (from: 19.35 ± 2.07% to: 67.41 ± 3.36%; p <0.0001) (n=4 of each group). RBCs from AA mice had increased SCI after exposure to chlorinated liposomes as well (from: 10.34 ± 1.67% to: 27.15 ± 0.94%; p=0.0001) but was still lower than SS RBC with the same exposure (p <0.0001) (Fig. 5. A, E). The increased SCI in the AA group was explained by the increased abnormal cells (p=0.0001), with a similar trend of increased abnormal cells in the SS group (p=0.0005) (Fig. 5. B). However, in the SS group, there was significant spike in the number of sickled cells (p=0.0002) with no change in the AA group (p=0.3) (Fig. 5. C, E).

Functionally, RBCs exposed to chlorinated liposomes (10 μM each of chlorinated palmitic and stearic acids and their corresponding aldehydes encapsulated in liposomes) developed increased fragility both in the SS group (RBC fragility increased from: 20.76 ± 1.25% to: 36.38 ± 1.83%; p <0.0001) and AA group (RBC fragility increased from: 14.45 ± 0.61% to: 27.93 ± 1.15%; p <0.0001). RBC fragility was higher in the blood obtained from the SCD mice compared to normal hemoglobin mice both when treated with vehicle (liposomes) (p=0.0007) and chlorinated liposomes (p <0.0001) (Fig. 5. D).

RBCs from sickle mice exposed to Cl_2 have significantly increased carbonyl adducts in their membrane when compared to RBC from SS mice exposed to air (1.67-fold increase from SS Air; p<0.05). The carbonylation decreased after hemopexin treatment to level close to baseline (SS air) (0.84-fold of SS air; p<0.05) (Fig. 6 A, B). No significant difference noticed in the carbonylation of RBC membrane between RBCs obtained from AA and SS mice breathing air (p=0.39) (Fig. 6 C, D).

4. Discussion

This study demonstrates for the first time that exposure of SCD mice to Cl_2 leads to acute lung injury consistent with ACS phenotype. Critically, exposure to Cl_2 increased mortality in the humanized SCD mice as compared to the control mice with humanized normal hemoglobin. Our data is consistent with the hypothesis that Cl_2 action is mediated through acute hemolysis with a resultant increase in plasma cell-free heme level leading to acute pulmonary injury. Treatment with a single dose of hemopexin after Cl_2 inhalation significantly reduced plasma cell-heme level, improved RBC fragility, and ameliorated acute lung injury leading, ultimately, to significantly improved survival rate. *Ex vivo* studies showed that chlorinated liposomes lead to increased carbonylation of the RBCs resulting in enhanced mechanical fragility and sickling of SS RBCs predisposing them to hemolysis. Considering large number of chlorine related accidents these findings have important implications to public health.

Exposure to Cl_2 increased death within 6 h after inhalation in the SCD mice with no mortality among the control mice with humanized normal hemoglobin. This indicates an increased susceptibility of the humanized SCD mice to Cl_2 inhalation with detrimental effects soon after the Cl_2 exposure. This suggests that SCD patients may be more vulnerable to death when exposed to Cl_2 than the general population and they may require particular attention and expedited management in case of industrial accidents or chemical warfare incidents involving Cl_2. In such situation, there is no currently available, validated, and specific countermeasure for Cl_2 toxicity other than removal from the site of exposure, decontamination, and supportive care according the CDC [42]. Hemopexin injection 30 min after Cl_2 exposure significantly

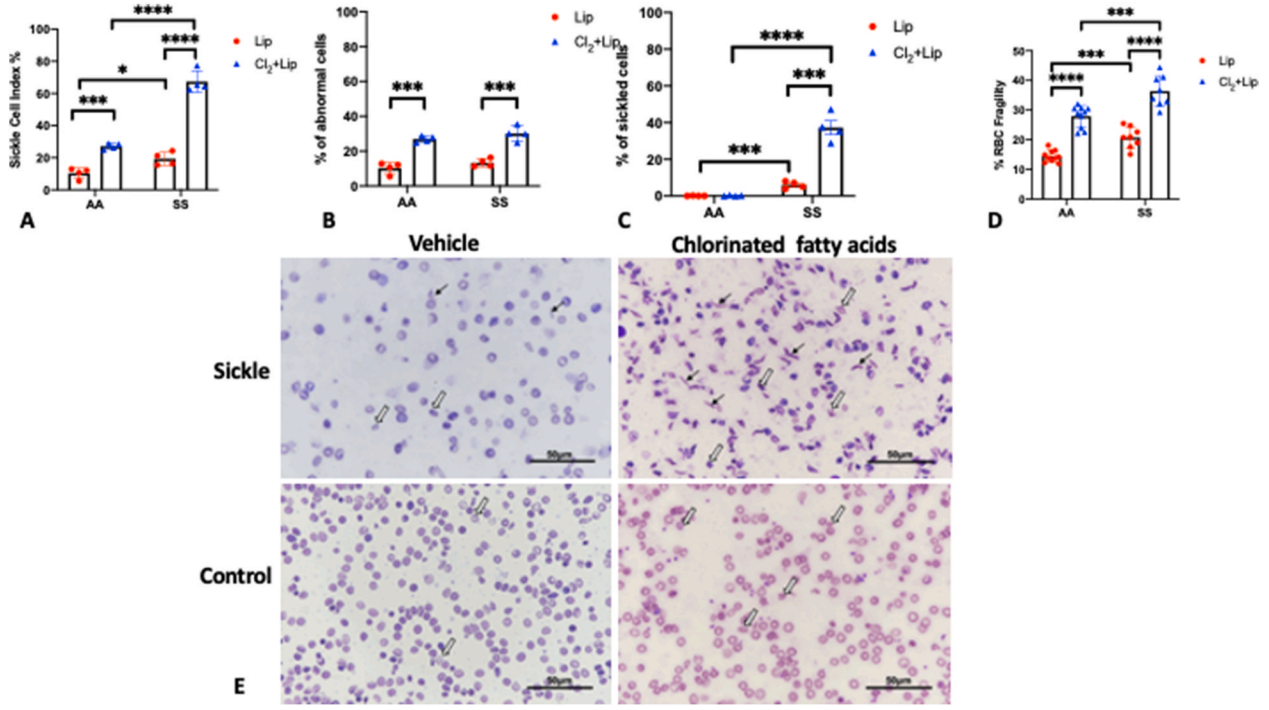

Fig. 5. RBC changes after exposure to chlorinated liposomes or vehicle. (A–C) Bar charts comparing percentage of abnormal and sickled RBCs obtained from SCD and control mice and treated with liposomes alone or chlorinated liposomes showing (A) increased percentage of sickle cell index (which is the sum of abnormal and sickled cells) in SCD mice compared to control mice and (B) the increase in abnormal cells explains the difference within each genotype while (C) the difference in sickled cells is a character of SCD mice. (D) Bar chart shows that those changes resulted in increased RBC fragility in RBC exposed to chlorinated liposomes especially in RBCs taken from SCD mice. (E) Blood smear micrographs showing few sickled (black arrows) and abnormal (white arrows) RBCs in blood obtained from SCD mice and mixed with liposomes with significant increase when mixed with chlorinated liposomes. While control RBC revealed few abnormal cells when mixed with vehicle with significant increase when mixed with chlorinated liposomes (Wright-Giemsa, oil immersion X600). Lip: liposomes; Cl_2+Lip: Chlorinated liposomes; AA: normal human hemoglobin control mice; SS: sickle human hemoglobin mice; RBC: red blood cell. *p<0.05, ***p<0.001, ****p<0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

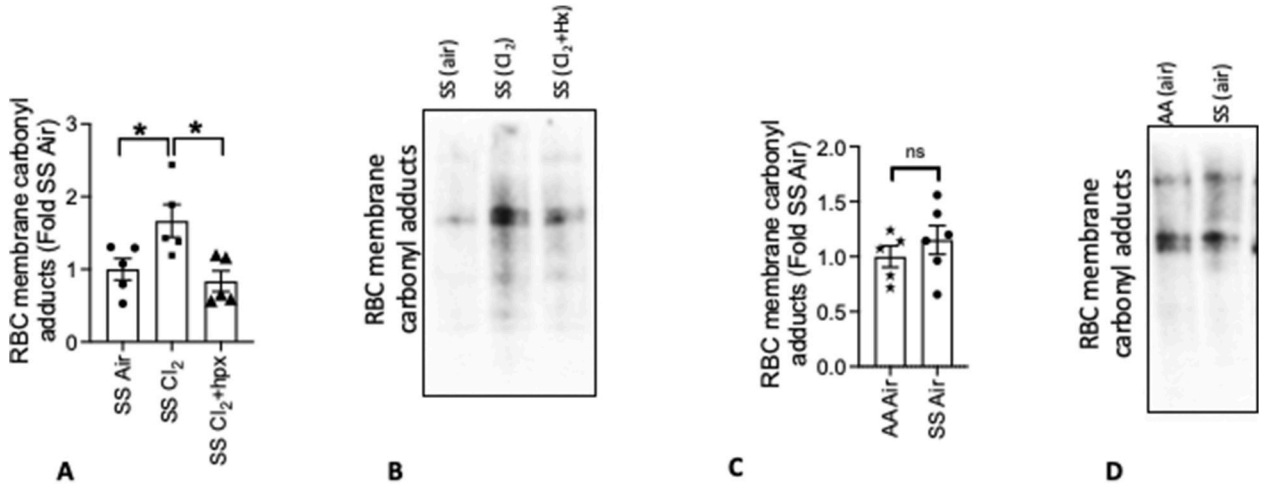

Fig. 6. Bar chart and micrographs reflecting the changes in carbonyl adducts with chlorine (Cl_2) exposure. (A, B) Carbonyl adducts increased in the RBC membrane of SS mice after chlorine exposure (p<0.05), but hemopexin injection 30 min after Cl_2 exposure had protective effect with maintained levels of carbonyl adducts compared to SS mice exposed to Cl_2 without hemopexin treatment. (C, D) Compared to the AA mice, carbonyl adducts are no different in the SS mice at baseline. *p<0.05.

reduced mortality in SCD mice. The current study is the first one to confirm the therapeutic benefit of hemopexin in reducing the mortality from ACS triggered by environmental exposure and specifically as a prospective countermeasure to Cl_2 exposure in patients with SCD.

To investigate the cause of increased death in SCD mice exposed to Cl_2, we measured markers of hemolysis in both humanized sickle and normal hemoglobin mice. At baseline, sickle mice had increased heme level and RBC fragility, together with reduced hematocrit and hemoglobin levels when compared to control mice as reported before [43]. After exposure to Cl_2, SS mice had further increase in heme level and RBC fragility, with further drop of the hematocrit and hemoglobin levels compared to room air confirming acute hemolysis that is accentuated as

compared to Cl_2 exposed humanized normal hemoglobin control mice, wild-type C57BL/6 mice as well as in humans exposed to Cl_2 [31]. The role of cell-free heme in acute lung injury similar to ACS like acute respiratory distress syndrome has been confirmed in multiple studies [44,45]. Proposed mechanisms of heme-mediated injury include nitric oxide consumption with vasoconstriction, oxidative stress of the pulmonary epithelial and endothelial cells, inflammatory pathway activations, and immunosuppression among others [46–48]. To test this hypothesis, we injected our mice with hemopexin, which binds to heme with high affinity, intraperitoneally half an hour after the Cl_2 exposure and reevaluated the outcomes. Hemopexin injection improved the RBC fragility and reduced heme levels in SCD mice and in control mice. The lack of short-term improvement of hematocrit and hemoglobin levels is expected given the longer time needed for the hematocrit and hemoglobin to recover.

The higher ALI score in the SCD mice exposed to Cl_2 as compared to control mice confirms that the defective hemoglobin in SCD mice amplifies the damaging effect of Cl_2 inhalation on their lungs. Together with the underlying hypoxemia independent of Cl_2 exposure, Cl_2 inhalation induced hypercapnic respiratory failure seems to be a major factor in the lethality of Cl_2 in SCD. The improvement of the ALI score after the introduction of hemopexin corroborated our physiologic explanation that improved survival in SCD mice is due to neutralizing the effects of heme [34]. Higher dose of purified human hemopexin was used in this experiment (10 μg/g body weight) compared to the dose used on wild animals in previous publication from our lab (4 μg/g body weight) due to the higher baseline levels of plasma free heme [23]. Therefore, patients with SCD exposed to Cl_2 may require higher dose of hemopexin than the general population as a counteract measure. In addition to the heme scavenging effect, hemopexin has other functions that may contribute to improved lung injury such as its anti-inflammatory effects in both infectious and non-infectious conditions [49,50]. The lower level of hemopexin in the SS mice confirms previous reports of low plasma levels of hemopexin in SCD patients and mouse models with SCD [51,52]. The injection of hemopexin resulted in increased the endogenous production of mouse hemopexin after Cl_2 exposure in the control mice as shown before, but not in the SCD mice [23]. This is likely due to the high load of cell free heme in the SCD mice that depletes the endogenously produced hemopexin. The low level of hemopexin at baseline and blunted response to hemopexin injection predisposes SCD mice to heme induced injury.

To investigate the mechanism of Cl_2 inhalation effect on the RBCs, we found that the levels of total and free ClFA increased both in the plasma and RBCs following Cl_2 exposure confirming previous results that Cl_2 effect is mediated through increased ClFA [21]. The higher levels of ClFA in the SS mice may be due to increased respiratory rate in response to hypoxemia and anemia resulting in inhalation of higher Cl_2 volume per time. The higher plasms level of 18 ClFA than that in the RBC levels is likely due to the time lag of the incorporation of 18 ClFA into the RBC after being released into the plasma 6 h after the exposure. The difference between the 16 and 18 ClFA is possibly due to the difference in their affinity to the RBCs.

Up to the authors knowledge, this is the first report of lower oxygenation at baseline in SCD mice compared to control mice of 2–4 months of age. Such hypoxemia is likely due to the underlying pulmonary disease shown in histology and it is consistent with clinical data of lower oxygenation in patients with SCD [53]. Most of the previous reports using the same mouse model found no difference in oxygenation at baseline, while other studies showed a trend toward lower SO2 and pO2 in sickle mice compared to sickle trait mice rather than normal hemoglobin mice, but the difference was not statistically significant [34,54]. Hypoxemia is a significant contributing factor to increased morbidity and mortality in SCD given its role in triggering RBC sickling [55]. Hypoxia/reoxygenation injury triggers a cascade of pathways such as endothelial dysfunction, induction of inflammation, enhanced *trans*--endothelial inflammatory cell migration, nuclear factor-kappa B activation and others resulting in multiorgan damage [56–60]. This worse oxygenation in the sickle mice is indicative of chronic pulmonary insufficiency secondary to the hemoglobinopathy. The vicious cycle of hypoxia induced lung injury that will lead to further worsening of hypoxia can have deleterious effects with time. Hemopexin injection to air breathing mice did not improve their oxygenation status indicating the chronic nature of pulmonary dysfunction. Sickle mice exposed to Cl_2 developed hypercapnia and acidosis likely due to the acute lung injury and tissue ischemia associated with sickling. Hemopexin significantly improved oxygenation but did not have an effect on pCO2 level which is likely due to blunting the effect of hypoxia induced hyperventilation [61].

To investigate if Cl-Lip adducts induces hemolysis primarily, we treated RBCs obtained from SCD and control mice with liposomes loaded with Cl-Lip and compared them with RBCs treated with vehicle in the form of liposomes loaded with unmodified lipids (Lip). The SCD RBCs treated with Cl-Lip showed increased sickling, abnormal cells, and fragility after the treatment both compared to SCD RBCs treated with Lip and control RBCs treated with Cl-Lip. This indicates that Cl-Lip induces sickling and results in exaggerated hemolysis of the SCD RBCs. Cl-Lip results in abnormal RBC-s in control RBCs, explaining that detected hemolysis in control RBCs. Regarding the mechanistic effects of ClFA on the RBC fragility, induction of carbonyl adducts by ClFA has been shown to increase the fragility of the RBC cytoskeleton, Spectrin, predisposing the RBC to hemolysis [22]. We revealed a significant increase in carbonyl adducts in the SS RBCs after exposure to Cl_2 with subsequent reduction to baseline after hemopexin treatment. This further explains increased fragility of RBC with Cl-Lip treatment and the beneficial effect of hemopexin after Cl_2 inhalation.

The study used high exposure levels of Cl_2 to simulate the effects of exposure to industrial and chemical terror incidents on the SCD population. This dose is higher than Cl_2 exposure during day to day domestic and vocational activities. The role of lower doses chronic Cl_2 exposures from household cleaning agents, pool decontaminators, and occupational exposures on pulmonary function and structure needs to be investigated further. Although it was not one of the objectives of our study, but Cl_2 exposure likely affects other vital organs in SCD mice such as kidneys, brain, and immune system and may explain part of the increased mortality and improvement after the hemopexin treatment as well. We did not find gender difference in response to Cl_2 exposure, but we did not power our study to detect such difference. We also studied young adult mice (2–4 months) corresponding to humans at higher risk of Cl_2 exposure, but we did not study the effect of age on mortality and lung injury related to Cl_2.

In conclusion, our data indicates that exposure to Cl_2 results in a lethal ACS like phenotype in humanized SCD mice. We showed that the action of Cl_2 is mediated through cell-free heme induced acute lung injury following acute hemolysis. Cl_2 has a direct effect on the RBCs mediated through the Cl-Lip that induce carbonylation of the RBC membrane increasing their fragility. We also proved that hemopexin ameliorated the effects of Cl_2 exposure and prevented most of the mortality and morbidity. Therefore, hemopexin has the potential to be used as a lifesaving therapy for accidental or intentional Cl_2 induced ACS in SCD. Future studies need to look at the effects of chronic lower dose exposure to Cl_2 in the daily activities (bleaching agents and swimming pools disinfectants) and occupational exposure on the induction of ACS and lung health of SCD patients.

Authorship contributions

A.S.A., T.J., and S.M. designed the experiments, A.S.A drafted the manuscript; A.S.A, T.J., I.A., K.M., M.S., N.A.A., A.M., S.D., C.J.A., and S. A. performed the laboratory experiments and revised the manuscript, A. S.A., M.S., S.A., and T.J. performed the statistical analyses; A.S.A., T.J., N.A., D.F., and S.M. obtained funding and supervised the study. All authors acquired and analyzed the data, and all authors approved the

final version of the paper.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102009.

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