Original Article

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Thiol-reducing agents abate cholestasis-induced lung inflammation, oxidative stress, and histopathological alterations

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Abstract

Cholestasis is not only influences the hepatic function but also damages many other organs. Lung injury is a critical secondary organ damage associated with cholestasis/cirrhosis. Pulmonary histopathological alterations, respiratory distress, and hypoxia are related to cholestasis/cirrhosis-induced lung injury. It has been found that oxidative stress plays a crucial role in this complication. The current study was designed to investigate the effect of N-acetyl cysteine (NAC) and dithiothreitol (DTT) as thiol-reducing and antioxidant agents against cholestasis-induced lung injury. Bile duct ligated (BDL) rats were monitored for the presence of inflammatory cells, TNF-α, and IgG levels in their broncho-alveolar fluid (BALF) at scheduled time intervals (3, 7, 14, and 28 days post-BDL surgery). These markers reached their highest level in the BALF of BDL rats on day 28 after the surgery. Therefore, in another set of experiments, the BDL animals were treated with NAC (100 and 300 mg/kg/day, i.p, for 28 consecutive days) and DTT (10 and 20 mg/kg/day, i.p, for 28 consecutive days). Meanwhile, a significant increase in the levels of TNF-α and IgG was detected in the BALF of BDL rats. The BALF level of neutrophils, monocytes, and lymphocytes was also significantly increased in cholestatic animals. A significant increase in lung tissue biomarkers of oxidative stress was detected in the BDL rats. It was found that NAC and DTT could significantly blunt pulmonary damage induced by cholestasis. The effects of these agents on oxidative stress biomarkers and inflammatory response seem to play a pivotal role in their mechanisms of protective properties.

Keywords: Bile acid, Cirrhosis, Cholestasis, Inflammation, Oxidative stress, Pulmonary injury.

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1. Introduction

Cholestasis is a well-known anomaly that obstruction of the bile flow can cause through several liver diseases or xenobiotics (1). Although the liver is the primary organ influenced by cholestasis, we have frequently reported that other organs could also be damaged during this complication (2-7). Prolonged cholestasis could lead to severe hepatic tissue fibrosis and cirrhosis (8-11). The

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lung is an organ seriously damaged by cholestasis/cirrhosis (12-14). Several *in vivo* and *in vitro* experimental and clinical data indicate pulmonary complications of cholestasis/cirrhosis (12-15). Pulmonary damage induced by cholestasis/cirrhosis could lead to a wide range of symptoms, from respiratory distress and disruption of blood gas exchange to more severe complications such as hepato-pulmonary syndrome (12-16). Unfortunately, no specific pharmacological intervention for managing cholestasis-induced pulmonary complications is available.

Several studies have evaluated the mechanisms of lung injury induced by cholestasis/cirrhosis (6, 14, 17-21). The occurrence of oxidative stress in the lung tissue is the primary mechanism known for cholestasis/cirrhosis-induced lung injury (17-22). Reactive oxygen species (ROS) level has been reported to significantly increase in the lung tissue of experimental cholestasis models (17-22). On the other hand, the lung tissue antioxidant system is disrupted, and targets such as biomembrane lipids are damaged (lipid peroxidation) during cholestasis/cirrhosis (18-21).

It has also been found that inflammatory response plays a pivotal role in the mechanism of cholestasis/cirrhosis-induced lung injury (15, 23-25). The infiltration of inflammatory cells, release of cytotoxic cytokines, and various other inflammatory mediators have been documented in the lung tissue in experimental models of cholestasis/cirrhosis (15, 23, 25). It should also be mentioned that the accumulation of inflammatory cells in various organs is directly linked to oxidative stress (26). Several enzymes (e.g., NOX) in inflammatory cells can produce considerable ROS (26).
Thiol-reducing agents such as NAC and DTT are frequently used in experimental models of human diseases (27-29). These chemicals can effectively reduce the oxidized thiol bonds (e.g., in glutathione molecule) and preserve the cellular redox environment (30). The antioxidant effect of thiol-reducing agents has been repeatedly mentioned in various investigations (30-37). Thiol-reducing agents can also effectively protect vital organelles such as mitochondria (31, 38, 39).

Interestingly, some studies also mentioned the robust anti-inflammatory properties of thiol-reducing agents (40-42). Agents such as NAC could significantly mitigate the infiltration of inflammatory cells into tissues and prevent the release of potentially cytotoxic cytokines (40, 41, 43). Hence, these compounds could act as effective anti-inflammatory agents.

As oxidative stress and inflammatory response play a vital role in the pathogenesis of cholestasis/cirrhosis-associated pulmonary complications, the current study was designed to evaluate the potential protective properties of thiol-reducing agents against cholestasis/cirrhosis-induced lung injury.

2. Materials and methods

2.1. Chemicals

5, 5′-dithiol-bis-2-nitrobenzoic acid (DTNB), 4,2 Hydroxyethyl,1-piperazine ethane sulfonic acid (HEPES), fatty acid-free bovine serum albumin (BSA) fraction V, reduced glutathione, sucrose, ethylene glycol-bis (2-aminoethyl ether)-N, N, N′, N′-tetra acetic acid (EGTA), sodium phosphate dibasic (Na2HPO4), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acids (TCA), meta-Phosphoric acid, potassium chloride (KCl), mannitol, sodium chloride (NaCl), trypsin, and hydroxymethyl aminomethane hydrochloride (Tris-HCl) were purchased from Merck (Merck KGaA, Darmstadt, Germany). All salts for preparing buffer solutions were of analytical grade and prepared from Merck (Merck KGaA, Darmstadt, Germany).

2.2. Animals

Mature male Sprague-Dawley (SD) rats (n=48, weighing 250-300 g) were obtained from the laboratory animals breeding center of Shiraz University of Medical Sciences, Shiraz, Iran. Animals were maintained in a standard environment (12:12 light: dark cycle, temperature 23±1 ºC, and ≈ 40 % relative humidity) with free access to tap water and a regular rodent chow diet (Behparvar®, Tehran, Iran). All experimental animal procedures

were approved by the institutional ethics committee of Shiraz University of Medical Sciences, Shiraz, Iran (94-01-36-11083).

2.3. Bile duct ligation surgery and experimental setup

Animals were randomly allotted into sham-operated and BDL groups (n=24/group). In the BDL group, animals were anesthetized (10 mg/kg of xylazine and 70 mg/kg of ketamine, i.p), a midline incision (≈2 cm) was made through the linea alba, and the common bile duct was localized and doubly ligated (44, 45). The sham operation involved laparotomy and bile duct identification and manipulation without ligation (44). Six animals from each group (Sham and BDL) were anesthetized (Thiopental, 80 mg/kg, i.p) at scheduled time intervals (3, 7, 14, and 28 days after BDL surgery). The model animals were exposed (i.p) to 100 and 300 mg/kg/day of NAC and 10 and 20 mg/kg/day of DTT for 28 consecutive days. The doses were selected based on previous publications (5). Serum and tissue samples were collected for further assessment.

2.4. Biochemical measurements

Blood samples (5 mL) were obtained from the abdominal aorta, transported to serum preparation tubes (Improvacuter®; gel and clot activator-coated tubes; Guangzhou, China), and centrifuged (3000 g, 15 min, 4 ºC). Commercial kits (Pars Azmun®, Tehran, Iran) and a Mindray BS-200® autoanalyzer (Guangzhou, China) were employed to assess BALF bilirubin content. Using a fluorometric method, serum and tissue bile acids were measured using the EnzyFluo™ Bile Acid Assay Kit (BioAssay Systems, Hayward, CA 94545, USA). To assess the tissue level of bilirubin and bile acids, 1 mL of the tissue homogenate (10 % w/v) was digested with 100 µL of trichloroacetic acid (TCA; 50 % w/v). Samples were centrifuged (12000 g, 4 ºC, 15 min), and the supernatant was used.

2.5. Broncho-alveolar lavage fluid (BALF) preparation

Animals were anesthetized using thiopental (80 mg/kg, i.p). Animals were placed in a dorsal position, and the trachea was exposed and cannulated using a 20 G catheter. The catheter was stabilized with a cotton thread. Then, 1 ml of ice-cooled saline-EDTA (2.6 mM EDTA in normal saline; 0.9 % w/v NaCl) was injected into the lung, and the chest was gently massaged (10 sec) (46). The solution was re-aspirated and kept on ice. This procedure was repeated (5 times/animal and 1 ml each time). Then, the pooled lavage preparations were centrifuged (5 minutes, 300 g, 4 °C) to pellet cells. The supernatant was collected to analyze TNF-α, IgG, bilirubin, and bile acids (46, 47). Then, 500 µL KCl (0.6 M) and 1.5 ml of ultrapure water were added to the cell pellet for erythrocyte lysis (10 sec). Samples were homogenized by inverting and centrifuged (5 min, 300 g, 4 °C). Finally, the supernatant was discarded, 1 ml of saline-EDTA was added to the cell pellet, and homogenized by inverting. The cell suspension was kept at 4 °C and used for cellular analysis (46).

2.6. Bronchoalveolar lavage fluid (BALF) cellular analysis

Kits for determining IgG and TNF-α in BALF were purchased from Shanghai Jianglai Biology® (China). BALF level of bile acids was analyzed by an EnzyFluo™ Bile Acids Assay Kit (BioAssay® Systems, USA). BALF total bilirubin was assessed using a Parsazmoon® kit (Tehran, Iran). A Prokan® automatic blood cell counter was used for BALF's differential inflammatory cell count.

2.7. Reactive oxygen species in the lung of BDL rats

The level of reactive oxygen species (ROS) formation in the lung was estimated using 2′, 7′ dichlorofluorescein diacetate (DCF-DA) as a fluorescent probe (44, 48-51). For this purpose, 400 mg of the lung tissue was homogenized in 4 mL of ice-cooled Tris-HCl buffer (40 mM, pH=7.4). Then, 100 µL of the resulting tissue homogenate was added to 1 ml of Tris-HCl buffer (40 mM, pH = 7.4) containing 10 µM of DCF-DA (52, 53) and incubated in the dark (10 min, 37 ºC incubator). Finally, the fluorescence intensity was assessed using a FLUOstar Omega® multifunctional fluorimeter (λ_{excit} =485 nm and λ_{emiss}=525 nm)

(32, 50, 54, 55).

2.8. Lung tissue lipid peroxidation

Lipid peroxidation in the lung tissue was assessed using the thiobarbituric acid reactive substances (TBARS) test (54, 56-59). Briefly, 500 µL of the lung tissue homogenate (10 % w/v in 40 mM Tris-HCl buffer, pH = 7.4) was treated with 2 mL of TBARS assay reagent (a mixture of 1 mL of thiobarbituric acid 0.375 % w/v, 1 mL of 50 % w/v of trichloroacetic acid, pH = 2) (54, 60, 61). Samples were vortexed well (1 min) and heated (100 °C water bath, 45 min). Afterward, 2 mL of n-butanol was added, and samples were mixed and centrifuged (10000 g, 20 min, 4 °C). Finally, the absorbance of the n-butanol phase was measured (λ = 532 nm, EPOCH® plate reader, USA) (58, 62-65).

2.9. The total antioxidant capacity of the lung tissue

The pulmonary tissue's ferric-reducing antioxidant power (FRAP) was measured based on a previously reported procedure (64, 66, 67). Briefly, a working FRAP mixture was freshly prepared by mixing ten parts of 300 mmol/L acetate buffer (pH = 3.6) with one part of 10 mmol/L of 2, 4, 6-tripyridyl-s-triazine (dissolved in 40 mmol/L hydrochloric acids), and with one part of 20 mmol/L ferric chlorides. Tissue samples were homogenized in Tris-HCl buffer (40 mM; pH = 7.4; 4 ºC), containing five millimolar of dithiothreitol and 0.2 M sucrose (56, 68-70). Then, 1.5 mL FRAP reagent and 200 µL deionized water were added to 100 µL tissue homogenate and incubated at 37 ºC for 5 minutes (in the dark). Finally, the absorbance was assessed at λ=593 nm (EPOCH plate reader, USA) (66, 68, 71, 72).

2.10. Lung tissue gluthatione content in cholestatic rats

The Ellman's reagent (5, 5′-dithiol-bis-2-nitrobenzoic acid; DTNB) was used for assessing lung GSH content based on a previously reported protocol (73-77). Briefly, 1 mL of the lung tissue homogenate (10 % w/v in 40 mM Tris-HCl buffer, 4 °C) was added to 1 mL of deionized water (4 °C) and 100 µL of trichloroacetic acid (50 %; w/v). The mixture was vortexed and centrifuged (10000 g, 4 °C, 20 minutes). Then, the supernatant was mixed with 1 mL of Tris-HCl buffer and 100 µL of 10 mM DTNB solution (dissolved in methanol) (52, 61). Finally, the absorbance was measured at λ=412 nm (EPOCH® plate reader, BioTek®, USA).

2.11. Lung tissue histopathology

Lung tissue samples were fixed in a 10% v/v buffered formalin solution. Then, samples were embedded in paraffin blocks, and a 5-µm-thick slice of each sample was prepared by a microtome and stained with hematoxylin and eosin (H&E) according to the literature (78, 79). A pathologist blindly analyzed tissue slides.

2.12. Statistical analysis

Data are represented as mean±SD. Data comparison was performed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparison test as the post hoc. Pulmonary histopathological change data were analyzed by Kruskal-Wallis as a non-parametric test followed by the Mann-Whitney U test. Values of $P < 0.05$ were considered statistically significant.

3. Resuls

To evaluate the appropriate induction of inflammatory response and lung damage, first, a set of animals underwent the BDL operation, and their BALF was monitored at scheduled time intervals (3, 7, 14, and 28 days after post-BDL surgery). As shown in Figures 1 and 2, inflammatory cells such as lymphocytes, neutrophils, and monocytes reached their maximum levels on day 28 post-BDL operation (Figures 1 and 2). No significant changes in the BALF level of eosinophils were detected in the current model among all exposed groups compared to the control group (Figures 1 and 2). On the other hand, it was found that BALF levels of IgG and TNF-α were significantly increased, with the maximum level on day 28 post-BDL surgery in a time-dependent manner (Figure 3). Based on these data, the BDL animals were treated with the investigated thiol-reducing agents for 28 consecutive days, and biomarkers of inflammation, oxidative stress, and lung histopath-

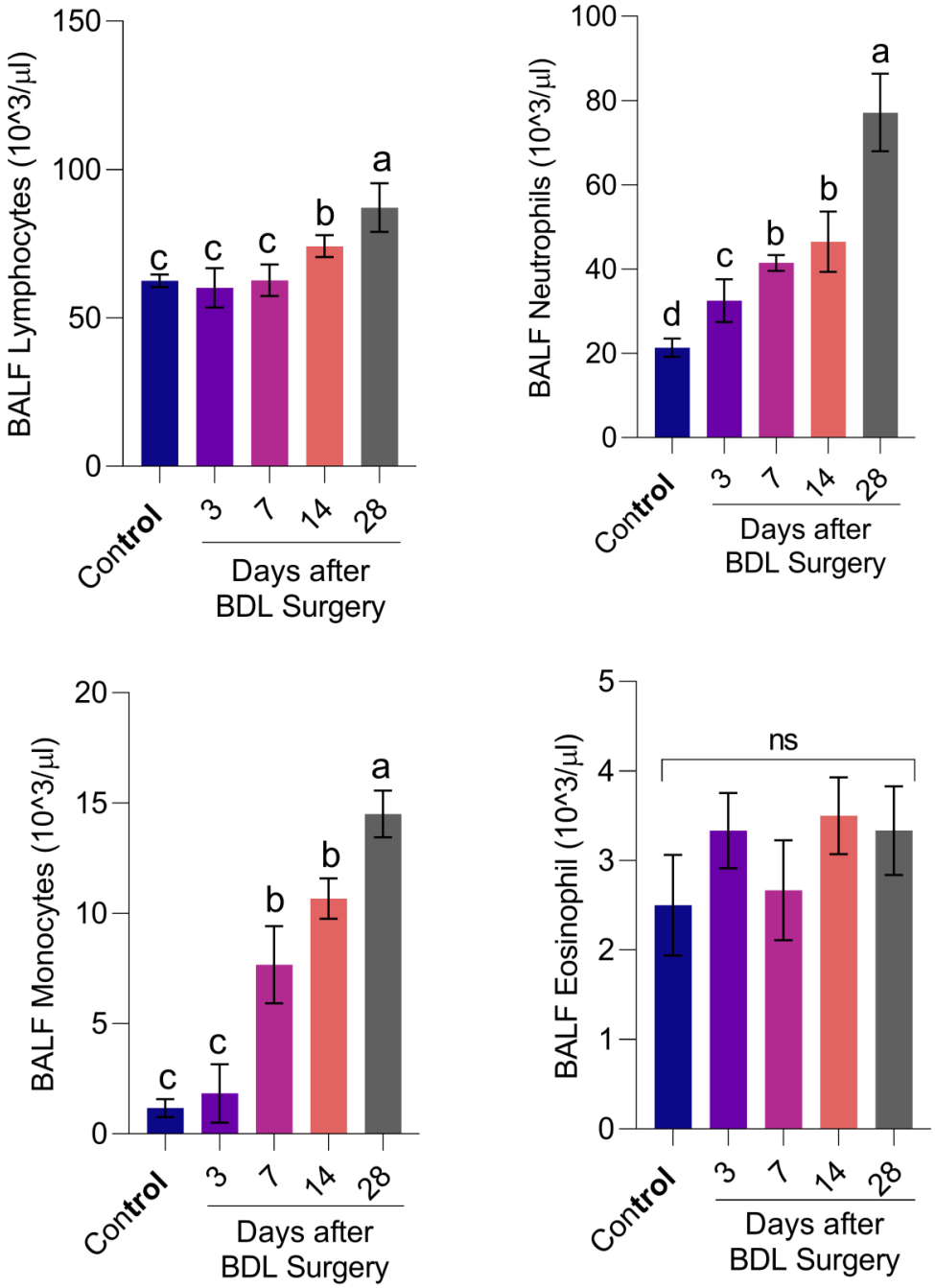

Figure 1. The level of inflammatory cells in the broncho-alveolar lavage fluid (BALF) of bile duct ligated (BDL) rats at different time points after surgery.
Data are represented as mean ± SD (n=6).
Data sets with various alphabetical superscripts are significantly different ($P<0.05$).

ological changes were assessed as an endpoint of cholestasis-induced lung injury.

The administration of NAC (100 and 300 mg/kg) and DTT (10 and 20 mg/kg) significantly decreased the BALF level of lymphocytes, neutrophils, and monocytes as assessed 28 days after the BDL surgery (Figure 2). The effect of thiol-reducing agents on the BALF level of inflammatory

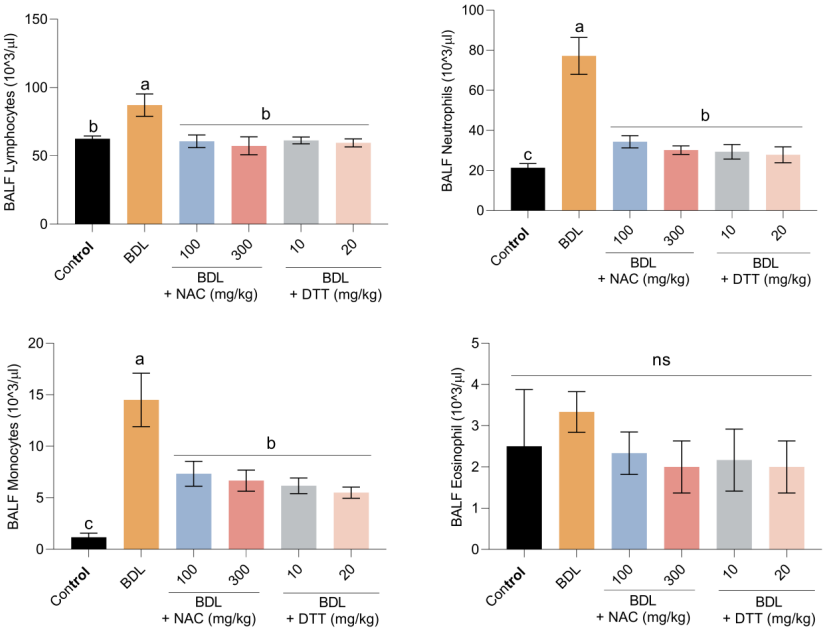

Figure 2. The effect of thiol-reducing agents on the level of inflammatory cells in the broncho-alveolar lavage fluid (BALF) of bile duct ligated (BDL) rats (28 days after the BDL surgery).
Data are represented as mean ± SD (n = 6).
Data sets with different alphabetical superscripts are significantly different ($P < 0.05$).

cells was not dose-dependent in the current study (Figure 3). The present study detected no changes in the eosinophil levels of BALF in the BDL animals who were also co-exposed to the thiol-reducing agents (Figures 1 and 2).

NAC (100 and 300 mg/kg) and DTT (10 and 20 mg/kg) administration also significantly decreased the level of TNF-α and IgG in the BALF

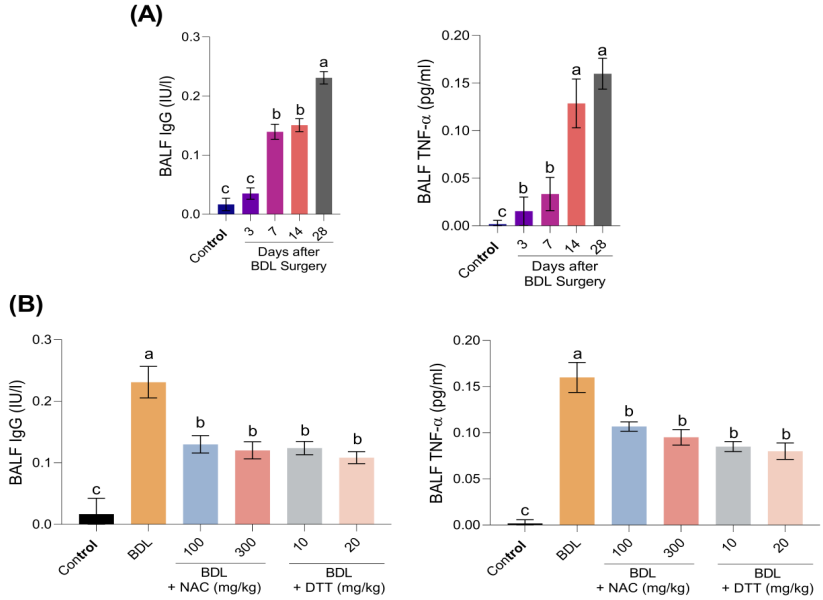

Figure 3. BALF levels of TNF-α and IgG at different time intervals in cholestatic rats. Administration of thiol-reducing agents significantly decreased TNF-α and IgG in the BALF of cholestatic rats (28 days post-BDL surgery).
Data are represented as mean ± SD (n = 6).
Data sets with different alphabetical superscripts are significantly different ($P < 0.05$).

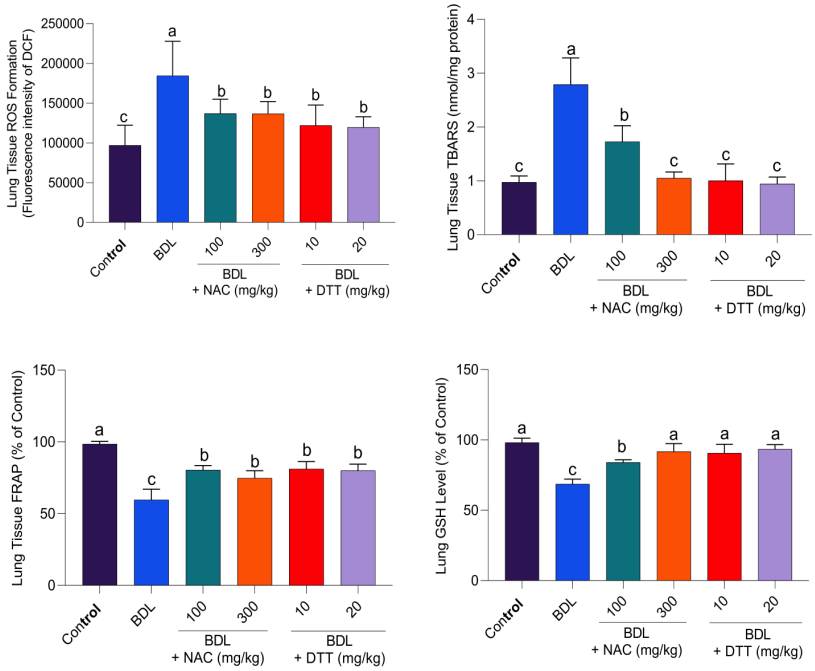

Figure 4. Oxidative stress biomarkers in the pulmonary tissue of cholestatic rats (28 days after BDL surgery). Data are represented as mean ± SD (n=6).
Data sets with different alphabetical superscripts are significantly different (P<0.05).

of cholestatic animals (Figure 3). The effects of thiol-reducing agents on these parameters were not dose-dependent in the current investigation (Figure 3).

Biomarkers of oxidative stress, including increased ROS formation, lipid peroxidation, decreased antioxidant capacity, and depleted glutathione reservoirs, were evident in rats who underwent the BDL surgery (28 days after the BDL operation) (Figure 4). It was found that thiol-reducing agents significantly blunted oxidative stress markers in the lung tissue of BDL rats (Figure 4). The effects of thiol-reducing agents were not dose-dependent in this study (Figure 4).

Bile acids and bilirubin levels were also assessed in the BALF of BDL rats (28 days after the BDL surgery) (Figure 5). It was found that the BALF level of bilirubin and bile acids was dramatically increased in the BDL group compared to those in the control groups (Figure 5). The administration of thiol-reducing agents evaluated in the current study did not significantly change the

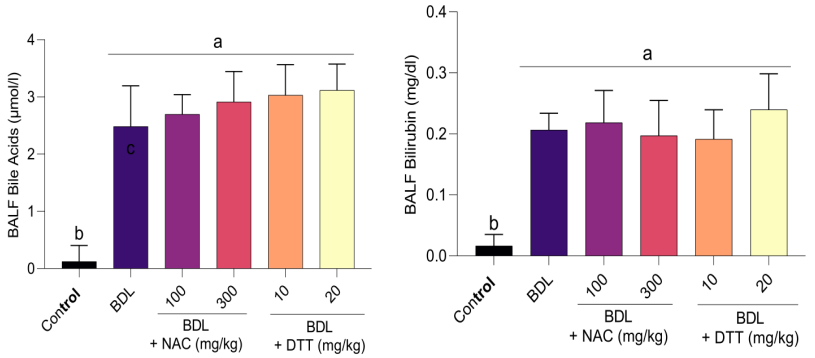

Figure 5. The level of bile acids and bilirubin in the broncho-alveolar fluid (BALF) of bile duct ligated rats (28 days after the BDL operation). Data are represented as mean ± SD (n=6).
Data sets with different alphabetical superscripts are significantly different (P<0.05).

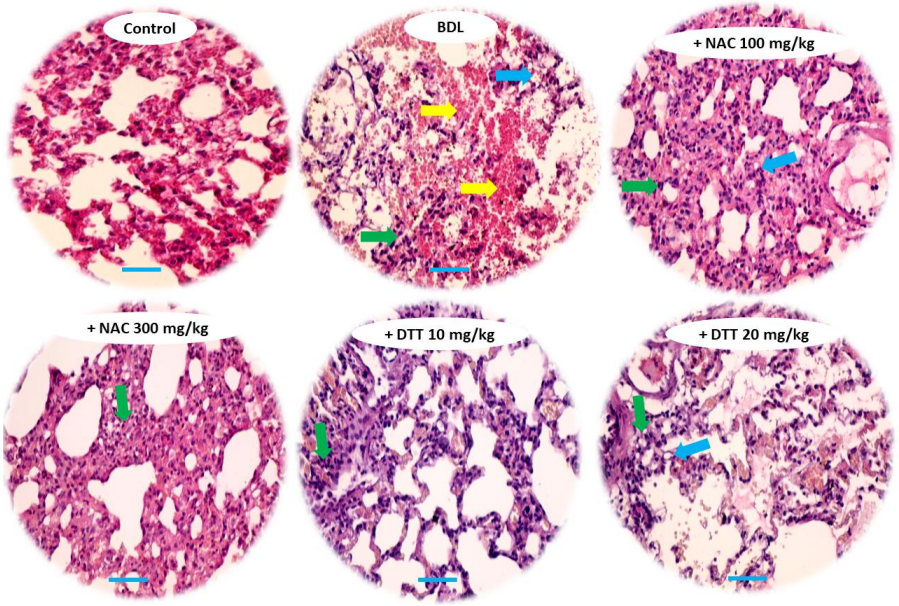

Figure 6. Pulmonary histopathology of bile duct ligated (BDL) mice 28 days after the cholestasis induction included inflammatory cell infiltration, hemorrhage, and necrosis. The administration of NAC and DTT significantly amended cholestasis-induced lung injury. Scores of lung tissue pathological alterations and their statistical analysis are given in Table 1.

BALF levels of bile acids and bilirubin (Figure 5).

Lung tissue histopathological alterations included inflammatory cell infiltration, hemorrhage (probably by disruption of alveolar structure), and necrosis in BDL rats (28 days after the BDL operation) (Figure 6 and Table 1). The effect of thiol-reducing agents on lung tissue histopathology revealed significant alleviation of hemorrhage, necrosis, and inflammation in the BDL animals (Figure 6 and Table 1).

4. Discussion

The stoppage of bile flow induced by diseases or xenobiotics could lead to cholestasis (1). Hepatic tissue is mostly injured by cholestasis (80). Prolonged and untreated cholestasis could lead to fulminant hepatic fibrosis and cirrhosis (80). However, it is well known that the liver is not the only organ influenced by cholestasis (6, 7, 54, 81-83). Other organs, such as the brain, kidneys, skeletal muscle, or even reproductive organs, are damaged by cholestasis/cirrhosis (6, 7, 54, 82-84). Pulmonary complications of cholestasis/cirrhosis are also well-described (85-88). It is well-known that the air ventilation in the lung of cirrhotic patients is disrupted (89). Cirrhosis-induced lung injury could vary from respiratory distress to complete dependence of patients on the ventilator (89). The hepato-pulmonary syndrome is a clinical cirrhosis complication requiring restrictive interventions (85). Unfortunately, there is no specific pharmacological intervention for cholestasis/cirrhosis-

Table 1. Lung tissue histopathological changes in the bile duct ligated rats (28 days after the surgery).

Treatments	Inflammation	Hemorrhage	Necrosis
Control	0 (0, 0)	0 (0, 0)	0 (0, 0)
BDL	4 (2, 2)^{a}	3 (1, 2)^{a}	1 (1, 1)^{b}
BDL + NAC 100 mg/kg	1 (0, 1)^{b}	1 (0, 1)^{b}	0 (0, 0)^{c}
BDL + NAC 300 mg/kg	1 (0, 1)^{b}	(0, 0)^{c}	0 (0. 0)^{c}
BDL + DTT 10 mg/kg	1 (0, 1)^{b}	0 (0, 0)^{c}	0 (0, 0)^{c}
BDL + DTT 20 mg/kg	1 (0, 1)^{b}	0 (0, 0)^{c}	0 (0, 0)^{c}

Data are represented as median and quartile for six histopathological pictures per group. Data sets with various alphabetical superscripts are significantly different.
Data sets with various alphabetical superscripts differ significantly ($P<0.05$).

induced lung injury so far. The current study found that administration of NAC (100 and 300 mg/kg) and DTT (10 and 20 mg/kg) significantly blunted cirrhosis-induced lung injury through mitigation of oxidative stress-triggered inflammatory-related routes. Hence, the effects of thiol-reducing agents on oxidative stress biomarkers and the inflammatory response seem to play a significant role in their mechanism of protective properties observed in the current study.

Several studies investigated the mechanisms involved in the pathogenesis of cirrhosis-induced lung injury (18-21). The accumulation of cytotoxic molecules routinely excreted through the bile (e.g., bile acids and bilirubin) are proposed to be involved in organ injury in experimental models of cholestasis/cirrhosis (6, 14). Hydrophobic bile acids are well-known for their detergent activities (90). These molecules could tremendously disrupt biological membranes and ultimately damage cells (90). Hydrophobic bile acids also damage vital organelles such as mitochondria (91, 92). Supraphysiological levels of bilirubin are also cytotoxic (93, 94). Bilirubin could interact with various intracellular targets, including mitochondrial function (93). The current study found that the BALF level of bile acids and bilirubin were dramatically increased in BDL rats (Figure 5). Hence, this could act as a pathogenic factor in cirrhosis-associated pulmonary damage.

Previous studies have repeatedly mentioned the central role of oxidative stress in the pathogenesis of cholestasis/cirrhosis-related lung injury (18-21). Significant increases in oxidative stress biomarkers and depleted antioxidant capacity of lung tissue have been mentioned in experimental models of cholestasis/cirrhosis (18-21). In the current study, we found that induction of cirrhosis in rats caused increased ROS levels, lipid peroxidation, depletion of GSH stores, and decreased antioxidant capacity in the lung tissue (Figure 4). On the other hand, the administration of thiol-reducing agents significantly blunted oxidative stress in BDL rats (Figure 4). Therefore, the effects of these agents on oxidative stress could play a vital role in their mechanism of protection in the current model.

The elevated level of inflammatory biomarkers in the lung tissue is another crucial factor in the pathogenesis of cholestasis/cirrhosis-induced lung injury (95-98). It is well-known that inflammatory cells infiltrate the lung during cholestasis/cirrhosis (95, 96, 98). In the current study, we also found that the population of inflammatory cells, including neutrophils, monocytes, and lymphocytes, drastically increased in the lung tissue (28 days post-BDL surgery) (Figure 1). The level of markers such as TNF-α as a pro-inflammatory cytokine was also increased in the BALF of cirrhotic animals (Figure 2). We found that the administration of thiol-reducing agents significantly blunted the inflammation biomarkers in the lung of BDL rats (Figures 2 and 3).

Interestingly, the anti-inflammatory properties of thiol-reducing agents such as NAC have been repeatedly mentioned in various experimental models (40-42). These agents can significantly blunt inflammatory cell infiltration into tissues and/or inhibit the release of cytotoxic cytokines (40, 41, 43). Based on these data, the anti-inflammatory effects of the thiol-reducing agents could also play a key role in their protective properties against cirrhosis-associated lung injury.

An important point that should be mentioned here is the tight connection between inflammatory response and oxidative stress. It has long been known that inflammatory cells could generate ROS (26). For instance, the activity of enzymes such as NADPH oxidase (NOX) or myeloperoxidase (MPO) is associated with the generation of a high ROS level (26). This high level of ROS could damage various intracellular targets (26). Therefore, a big part of ROS formation and oxidative stress in the lung of cirrhotic models could be related to the activity of these enzymes because of the infiltration and accumulation of inflammatory cells in the lung tissue. Although not investigated in the current study, previous investigations reported the inhibitory effects of thiol-reducing agents (e.g., NAC) on the activity of enzymes such as NADPH oxidase (99). Therefore, in addition to the direct interaction of thiol-reducing agents with free radicals or their effects on the expression of antioxidant enzymes, the effects of these molecules on ROS formation induced by inflammatory cells is a mechanism that deserves further investigation.

5. Conclusion

Conclusively, our data revealed that thiol-reducing agents such as NAC and DTT could effectively blunt cirrhosis-induced lung injury. The effect of these agents on oxidative stress and inflammatory markers plays a crucial role in their mechanism of protective properties. As the safety profile of these agents is well-established, they could undergo clinical trials to manage cirrhosis-related complications such as cirrhosis-induced pulmonary damage.

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Conflict of Interest

None declared.

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