Original Article

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Dexamethasone Blunts Lung Inflammation in Cholestatic Mice

Hossein Niknahad[1,2];PhD, Narges Abdoli[3];PhD, Mohammad Mehdi Ommati[1,4,5];PhD, Asma Najibi[2];PhD, Ardeshir Zare[2,6];MSc, Zahra Mazloomi[2,6];MSc, Issa Sadeghian[1,7];PhD, Khadijeh Mousavi[2];PhD, Negar Azarpira[8];PhD, Reza Heidari[1*];PhD

[1]Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Department of Pharmacology and Toxicology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Food and Drug Administration, Iran Ministry of Health and Medical Education, Tehran, Iran
[4]Department of Life Sciences, Shanxi Agricultural University, Shanxi, Taigu, China
[5]Henan Key Laboratory of Environmental and Animal Product Safety College of Animal Science and Technology Henan University of Science and Technology Luoyang 471000 Henan, China
[6]Students Research Committee, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran
[7]Biotechnology Incubator, Shiraz University of Medical Sciences, Shiraz, Iran
[8]Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Cholestasis/cirrhosis is a multifaceted clinical complication that influences many organs, including the liver, kidney, heart, skeletal muscle, and lung. Cirrhosis-associated lung injury could lead to severe and lethal consequences, including acute respiratory syndrome and patient dearth. Unfortunately, there is no specific pharmacological intervention to manage cholestasis-induced lung injury. It has been revealed that severe inflammation and its associated complications, such as oxidative stress, are involved in the pathogenesis of cholestasis-associated pulmonary damage. The current study was designed to evaluate the role of dexamethasone (DXM) on lung inflammation in cholestatic mice. For this purpose, bile duct ligated (BDL) mice received DXM (1 and 2.5 mg/kg, i.p, 2 times/week) for 14 days. On day 15, the bronchoalveolar lavage fluid (BALF) was prepared. Several markers, including inflammatory cell infiltration, TNF-α, and IgG, were assessed in the BALF of BDL animals. Significant infiltration of inflammatory cells along with increased TNF-α and IgG were detected in the BALF of BDL mice (14 days after surgery). Moreover, significant ROS formation, glutathione depletion, lipid peroxidation, and protein carbonylation were evident in the lung tissue of the BDL group. It was found that DXM (1 and 2.5 mg/kg) significantly blunted inflammation and oxidative stress in the lung of cholestatic mice. Moreover, lung tissue histopathological changes, including inflammatory cell infiltration, were significantly mitigated in DXM-treated mice. These data offer the potential therapeutic effects of DXM against cholestasis-related complications. Therefore, patients with cholestasis-induced lung injury might benefit from repurposing DXM in clinical settings.

Keywords: Bile acid, Inflammation, Lung injury, Oxidative stress, Pulmonary disease

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1. Introduction

Cholestasis is the stoppage of bile flow which could be induced by various diseases or xenobiotics (1, 2). It is well-known that cholestasis influences liver function and could also significantly affect other organs, including the brain, kidneys, heart, skeletal muscle, and lung (3-8). Meanwhile, cholestasis-induced lung injury is a serious complication that could lead to severe respiratory problems if not appropriately managed (9-11). In clinical settings, considerable evidence indicates

Corresponding Author: Reza Heidari, Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. Email: rezaheidari@hotmail.com; rheidari@sums.ac.ir

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that respiratory complication, especially respiratory distress syndrome in neonates, is directly associated with cholestasis (9-11). Respiratory complications occur in adults with cholestasis, and its signs and severances could be more complicated in neonates born with intrahepatic cholestasis of pregnancy (12). Unfortunately, there is no specific pharmacological intervention to alleviate cholestasis-induced pulmonary damage despite supportive care and ventilators. Cholestasis-induced lung injury is named bile acid pneumonia (12).

Many investigations indicate that inflammatory response and the release of various cytokines play a critical role in the pathogenesis of cholestasis-induced lung injury (12-15). On the other hand, it is well-known that inflammation is directly connected to the occurrence of oxidative stress (16, 17). There is also evidence of severe oxidative stress in the lung tissue in experimental cholestasis models (9, 10, 18). Recently, we also found a connection between cholestasis/cirrhosis and increased biomarkers of oxidative stress in the lung tissue in the bile duct ligated animal model of cholestasis (18). Based on these data, anti-inflammatory drugs could potentially be therapeutic agents against cholestasis-induced lung injury.

Several studies revealed that dexamethasone (DXM) as a glucocorticoid could significantly prevent cytokine release and blunt pulmonary inflammation in a various experimental models of lung injury (19, 20). DXM is also widely used in inflammatory and allergic disorders and nausea (e.g., induced by chemotherapeutic agents) (19, 20). The inhibition of the nuclear factor-kappa B (NFkB) as the major cell signaling involved in the inflammatory response is a critical mechanism of the anti-inflammatory action of DXM (21). NFkB inhibition prevents the gene transcription and synthesis of a wide range of pro-inflammatory cytokines (21). On the other hand, glucocorticoids such as DXM could directly inhibit the activity of inflammatory cells such as neutrophils and T-cells (21). Based on these data, DXM seems to be an appropriate candidate against cholestasis-induced pulmonary inflammation and its associated complications.

As mentioned, in the BDL model of cholestasis, the plasma level of potentially cytotoxic molecules (e.g., bile acids) is dramatically increased. These cytotoxic molecules could finally accumulate in various organs. Oxidative stress and inflammatory response are the characteristic fractures of hydrophobic bile acids accumulated in the lung. Therefore, it is reasonable that anti-inflammatory agents such as DXM could suppress cholestasis-induced lung injury. The data obtained from the current study could help a potential safe pharmaceutical agent with potential clinical application in patients suffering from cholestasis-associated respiratory complications.

2. Materials and methods

2.1. Chemicals and reagents

4,2 Hydroxyethyl,1-piperazine ethane sulfonic acid, hexadecyl-trimethyl-ammonium bromide (HTAB), reduced glutathione (GSH), 2′,7′-dichlorofluorescein diacetate, 2,4,6-tripyridyl-s-triazine, methanol, and ethylenediaminetetraacetic acid (EDTA), trichloroacetic acids, m-phosphoric acid, hydroxymethyl aminomethane hydrochloride (Tris-HCl), O-dianisidine hydrochloride, potassium chloride, sodium chloride, hydrochloric acid, ferric chloride, hydrogen peroxide, sucrose, and 2,4-dinitrophenyl hydrazine (DNPH) were purchased from Merck (Merck KGaA, Darmstadt, Germany). Dexamethasone was purchased from Darou Pakhsh (Tehran-Iran). Kits for assessing immunoglobulin and cytokine in BALF were purchased from Shanghai Jianglai Biology® (China). BALF level of bile acids was measured using an EnzyFluo™ bile acids assay kit (BioAssay® Systems, USA).

2.2. Animals

Male C57BLC/6J mice (n=28, 20±25 g) were obtained from Shiraz University of Medical Sciences, Shiraz, Iran. Animals were maintained in a standard environment (12 h photo schedule, ≈45±5% relative humidity, and temperature 24±1 ºC) with free access to tap water and a regular rodents diet (RoyanFeed®, Isfahan, Iran). The institutional ethics committee of Shiraz University of Medical Sciences approved laboratory animal care and use (Approval code: IR.SUMS.REC.1399.1344).

2.3. Bile duct ligation operation and treatments

Animals were randomly allotted into sham-operated and bile duct ligated (BDL) groups. For BDL surgery, animals were anesthetized (10 mg/kg of xylazine and 80 mg/kg of ketamine, i.p), and a midline incision (≈2 cm) was made through the linea alba. The common bile duct was ligated (#04 silk suture) and cut between ligatures (6, 22-26). Animals were recovered in separate cages under IR light and had free access to easy food and tap water during experiments. The sham operation involved laparotomy without bile duct ligation (27). The treatments were as follow (n=7/group): 1) Sham-operated (Control), 2) BDL, 3) BDL + DXM (1 mg/kg, i.p, 2 times/week), and 4) BDL + DXM (2.5 mg/kg, i.p, 2 times/week). Animals were assessed 14 days after the BDL operation (28).

2.4. Bronchoalveolar lavage fluid (BALF) sample

Animals were anesthetized using thiopental (80 mg/kg, i.p) and were placed in a dorsal position. The trachea was revealed and cannulated using a 20 G catheter. The catheter was stabilized with a cotton thread (04 silk). Then, 1 ml of ice-cooled saline-EDTA (2.6 mM EDTA in 0.9% NaCl) was injected into the lung, and the chest was gently massaged (10 sec) (29). The solution was re-aspirated and kept on ice (4 °C). This procedure was repeated (at least six times/animal; 1 mL each time). Afterward, 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 (29, 30). Then, 500 µL of 600 mM KCl 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, and 1000 µl of saline-EDTA solution (2.6 mM EDTA in 0.9% NaCl) was added. The cell pellets were homogenized by inverting, and the cell suspension was kept at 4 °C for further analysis (29).

2.5. BALF cellular analysis, cytokine, and immunoglobulin levels

Commercial kits were employed to assess IgG and TNF-α in BALF (Shanghai Jianglai Biology®, China). BALF level of bile acids was analyzed by an EnzyFluo™ Bile Acids Assay Kit (BioAssay® Systems, USA). A Prokan® automatic blood cell counter was used for the differential inflammatory cell count of BALF.

2.6. Reactive oxygen species in the lung tissue of BDL mice

The level of reactive oxygen species (ROS) formation in the pulmonary tissue was assessed based on the fluorescent intensity of 2′, 7′ dichlorofluorescein diacetate (DCF-DA) (22, 31-36). 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 resulted tissue homogenate was added to 1 ml of Tris-HCl buffer (40 mM, pH=7.4) containing 10 µM of DCF-DA (37-42) and incubated in the dark (10 min, 37 ºC incubator). Finally, the fluorescence intensity was assessed using a FLUOstar Omega® fluorimeter (λ excit=485 nm and λ emiss=525 nm) (43-50).

2.7. Lung tissue lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) test was used to assess lipid peroxidation in the pulmonary tissue of BDL mice (43). Briefly, 500 µL of the lung tissue homogenate (10 % w: v in 40 mM Tris-HCl buffer, pH=7.4) was treated with 1 mL of TBARS assay reagent (a mixture of of 0.375 % w: v thiobarbituric acid and 50 % w: v of trichloroacetic acid; pH=2) (43). 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 (16000 g, 10 min). Finally, the absorbance of the upper phase was measured (λ=532 nm, EPOCH® plate reader, USA) (43).

2.8. The total antioxidant capacity of the lung tissue

The pulmonary tissue's ferric reducing antioxidant power (FRAP) was measured as an index of pulmonary total antioxidant capacity (43). Briefly, 100 µL of the lung tissue homogenate was added to 1 mL of a freshly-prepared working FRAP mixture (composed of ten parts of 300 mmol/L acetate buffer with one part of 10 mmol/L

of 2, 4, 6-tripyridyl-s-triazine, and one part of 20 mmol/L ferric chlorides). Samples were incubated at 37 ºC (5 min, in the dark). Finally, the absorbance was assessed at λ=593 nm (EPOCH plate reader, USA) (43).

2.9. Protein carbonylation

The protein carbonylation of the pulmonary tissue of cholestatic animals was evaluated based on the dinitrophenyl hydrazine (DNPH) test (51-53). Briefly, the tissue sample (200 mg) was homogenized in 2 mL of Tris-HCl buffer containing 0.1% v: v of triton-x 100. Then, the tissue homogenate was centrifuged (700 g, 10 min, 4 ºC), and the resulting supernatant was treated with 500 µL of 10 mM DNPH (dissolved in HCl). Samples were then incubated at room temperature (25 °C) for 60 minutes (vortexing every 15 min) (54, 55). Then, 500 µL trichloroacetic acid (20% w: v) was added, samples were centrifuged (17000 g, 5 min, 4 ºC), and the supernatant was removed. The pellet was washed (at least four times) using ethanol: ethyl acetate (1 mL of 1:1 v:v). Finally, the residue was dissolved in 1mL of 6 M guanidine chloride solution (pH=2.3), and the absorbance was measured at λ=370 nm (EPOCH® plate reader, USA) (56, 57).

2.10. Myeloperoxidase activity in the lung tissue

The MPO activity in the liver and kidney tissue was assessed as an index of tissue inflammatory cell infiltration (27, 32). For this purpose, tissue specimens (200 mg) were homogenized in 2 mL of hexadecyl trimethyl-ammonium bromide (HTAB) solution (0.5 % w: v; dissolved in 50 mM potassium phosphate buffer; pH=6, 4, 4 °C) and centrifuged (3000 g, 20 min, 4 °C). Then, 100 µL of the supernatant was added to 1 mL of potassium phosphate buffer (50 mM; pH=6; containing 16.7 mg/100 mL of O-dianisidine hydrochloride and 0.0005 % v: v of hydrogen peroxide). After the incubation period (5 min, 25 °C), the reaction was stopped by adding 100 µL of HCl (1.2 N). Finally, the absorbance was assessed at λ=400 nm (EPOCH® plate reader; USA) (58, 59).

2.11. Lung tissue gluthatione (GSH) content in cholestatic mice

The Ellman's (5, 5-dithio-bis-2-nitrobenzoic acid; DTNB) assessed lung GSH content based on a previously reported protocol (60-66). Briefly, 1000 µL of the lung tissue homogenate (10% w: v in 40 mM Tris-HCl buffer, 4 °C) was added to 1000 µL of deionized water (4 °C) and 100 µL of trichloroacetic acid (50%; w: v). The mixture was mixed well and centrifuged (10000 g, 4 °C, 20 min). Then, the supernatant was mixed with 1 mL of Tris-HCl buffer and 100 µL of DTNB solution (10 mM, dissolved in methanol) (27, 37, 67-68). Finally, the absorbance was read at λ=412 nm (EPOCH® plate reader, USA).

2.12. Lung Nitrite (NO_2^-) and nitrate (NO_3^-) in the lung tissue

NO_2^-/NO_3^- production, as the index of nitric oxide (NO) synthesis, was measured in the lung homogenate with a NO assay kit (Nanjing Jiancheng Corp. China) following the manufacturer's instruction (69). Briefly, lung tissue homogenate (2 mL of 10% w: v in Tris-HCl buffer) was centrifuged (1000 g, 15 min, 4 °C), and the supernatant was used to assess NO_2^-/NO_3^- production. The absorbance of samples was evaluated at λ=540 nm using a microplate reader (EPOCH® plate reader, USA).

2.13. Lung tissue histopathology

Lung tissue samples were fixed in a buffered formalin solution (10% v: v). Then, samples were embedded in paraffin blocks, and a 5-µm-thick slice of samples was stained with hematoxylin and eosin (H&E). A pathologist analyzed tissue slides blindly.

2.14. Statistics

Data are given as mean ± SD (n=7/group). Data comparison was performed by the one-way analysis of variance (ANOVA) and the Tukey's multiple comparison test as the *post hoc*. A $P<0.05$ was considered statistically significant.

3. Results

The BALF level of inflammatory cells, TNF-α, and IgG were assessed 14 days after the

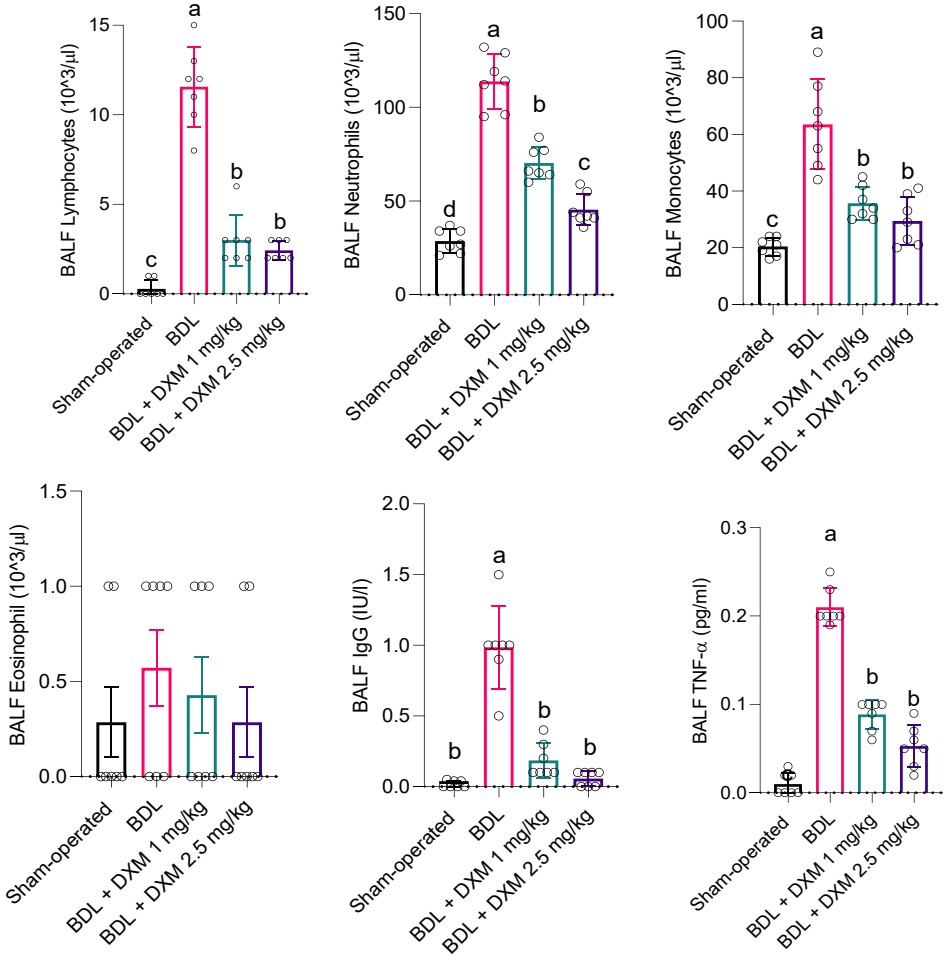

Figure 1. Effects of dexamethasone (DXM) on inflammatory cells infiltration, TNF-α, and IgG in the bronchoalveolar fluid (BALF) of bile duct ligated (BDL) mice (14 days after BDL).
Data are represented as mean±SD (n=7).
Data sets with different alphabetical superscripts are statistically different (P<0.05).

BDL operation (Figure 1). These markers were significantly higher in the BALF of BDL animals than in the sham-operated group (Figure 1). On the other hand, a significant increase in the BALF level of lymphocytes, neutrophils, and monocytes was evident in BDL mice (Figure 1). It was found that the BALF level of eosinophils was not significantly changed in comparison with the control group (Figure 1). Significant increases in BALF levels of TNF-α and IgG were also detected in BDL animals (Figure 1). It was found that DXM (1 and 2.5 mg/kg, i.p, 2 times/week) significantly suppressed the BALF level of inflammatory cells as well as TNF-α and IgG in cholestatic animals (Figure 1). The effect of DXM on BALF level of cytokine and inflammatory cells was not dose-dependent in the current model (Figure 1).

A significant increase in BALF levels of bilirubin and bile acids was also detected in the cholestatic animals compared to the control group (Figure 2). It was found that DXM had no significant effect on lung tissue level of bilirubin and bile acids in the current study (data not shown).

Biomarkers of oxidative stress were assessed in the lung tissue of cholestatic mice (Figure 3). Significant ROS formation, lipid peroxidation, and protein carbonylation were detected in the lung of BDL mice (Figure 3). Moreover, lung GSH and antioxidant capacity were significantly suppressed in cholestatic animals (Figure 3). It was found that DXM (1 and 2.5 mg/kg) significantly blunted oxidative stress in the lung tissue of cholestatic mice (Figure 3). The effect of DXM on oxidative stress biomarkers was not dose-de-

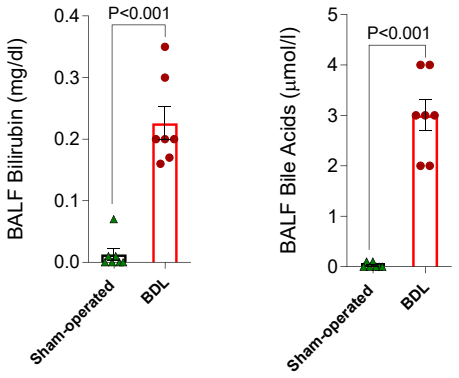

Figure 2. BALF level of bilirubin and bile acids in bile duct ligated (BDL) mice (14 days after BDL surgery). Bile acids and bilirubin levels were significantly higher in BDL animals than in the sham-operated group. Dexamethasone administration had no significant effect on lung tissue level of bilirubin and bile acids in the current study (data not shown).
Data are represented as mean±SD (n = 7).

pendent in most biomarkers assessed in this study (Figure 3).

A significant increase in lung tissue level of myeloperoxidase (MPO) activity was detected in the BDL group (Figure 3). Moreover, a significant elevation in the lung tissue of nitric oxide (NO) was detected in cholestatic mice (Figure 3). It was found that DXM significantly suppressed MPO activity and decreased NO levels in the pulmonary tissue of cholestatic mice (Figure 3).

Lung tissue histopathological assessments revealed severe inflammatory cell infiltration,

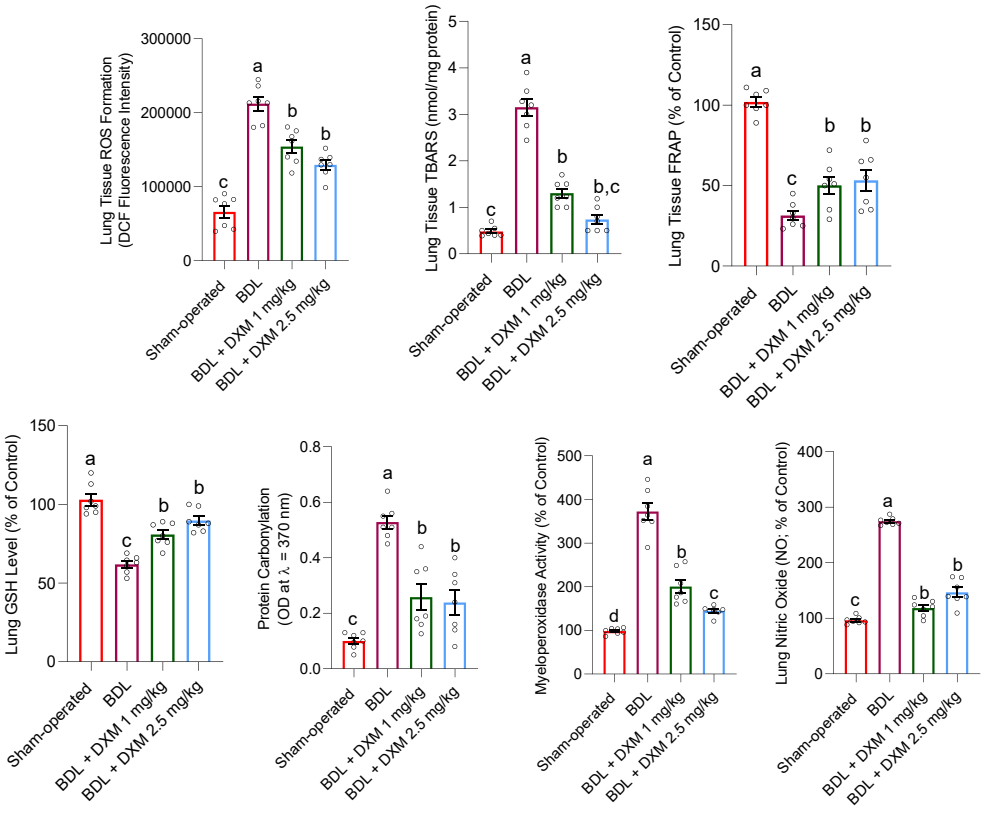

Figure 3. Effect of dexamethasone (DXM) on biomarkers of oxidative stress in the lung tissue of bile duct ligated (BDL) mice.
Data are represented as mean±SD (n=7).
Data sets with different alphabetical superscripts are statistically different (P<0.05).

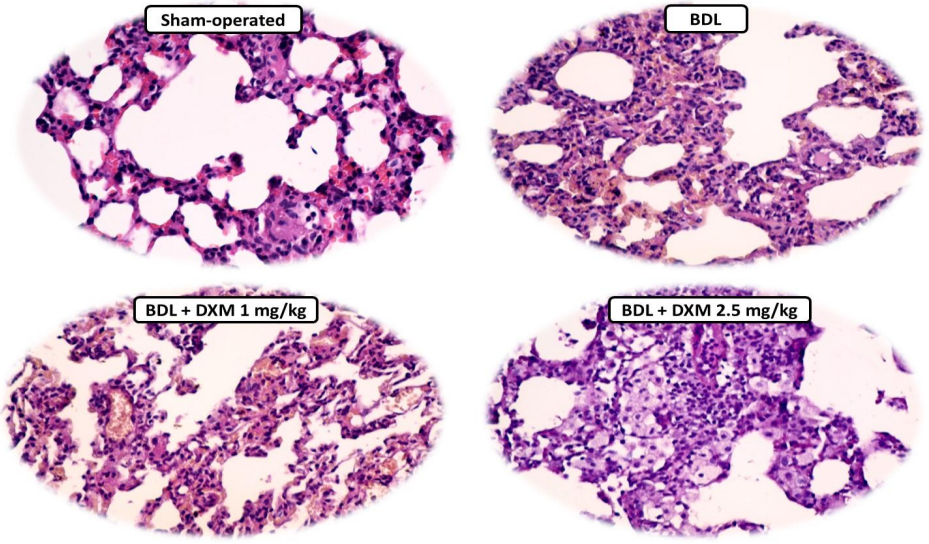

Figure 4. Lung tissue histopathological alterations in bile duct ligated (BDL) animals. Significant inflammatory cell infiltration, hemorrhage, and necrosis were evident in the lung of cholestatic animals (14 days after BDL surgery). It was found that dexamethasone (DXM) significantly prevented lung histopathological changes. Scores of lung tissue pathological conditions are given in Table 1.

mild necrosis, and hemorrhage in BDL animals (14 days after the BDL operation; Figure 4 and Table 1). DXM (1 and 2.5 mg/kg, i.p) was found to ameliorate cholestasis-induced lung histopathological changes (Figure 4 and Table 1).

4. Discussion

Cholestasis is a multifaceted clinical complication that could affect several organs (18, 43, 70-75). Although the liver is the primary organ injured by cholestasis, there is a plethora of evidence of cholestasis-induced renal, brain, and heart injury (18, 75-78). The lung is also a target organ significantly influenced by cholestasis (9-11, 18). It has been found that cholestasis-induced pulmonary system injury could entail serious respiratory complications (9-11). Cholestasis-induced lung injury could also lead to more severe complications such as lung infection or hepato-pulmonary syndrome (9-11). Therefore, it is essential to identify the mechanisms involved in cholestasis-induced lung injury and develop therapeutic options against this complication. The data obtained from this study revealed that dexamethasone (DXM) significantly ameliorated cholestasis-induced lung injury by alleviation of the inflammatory response, decreasing MPO activity, and mitigating oxidative/nitrosative stress.

Cholestasis-induced lung injury could lead to blood oxygen desaturation and cyanosis (12, 79). If not appropriately managed, cholestasis-associated lung injury could develop into more severe complications such as hepato-pulmonary syndrome (79). In clinical settings, infants born with cholestasis usually need axillary apparatus such as ventilators to survive (12, 80). The accumulation of cytotoxic molecules such as hydrophobic bile acids and supraphysiological bilirubin levels in the lung tissue is involved in the pathogenesis of cholestasis-induced lung injury (13). In the current

Table 1. The score of lung tissue histopathological alterations in bile duct ligated mice.

Treatments	Inflammation	Hemorrhage	Necrosis
Control	-	-	-
BDL	+++	++	+
BDL + DXM 1 mg/kg	+	+	-
BDL + DXM 2.5 mg/kg	+	-	-

+ and +++ indicate mild and severe histopathological alterations, respectively.
BDL: Bile Duct Ligation; DXM Dexamethasone.

study, we found a high level of bile acids and bilirubin in the BALF of BDL mice (Figure 2). Most bile acids are hydrophobic compounds that could act as surfactant molecules (81). Therefore, these agents could interrupt alveolar biomembranes and consequently disrupt their normal function.

It has been found that the accumulation of cytotoxic molecules in the lung of cholestatic animals is usually accompanied by a severe inflammatory response and the release of cytokines in the lung tissue (9, 10, 12-14, 18). There are also reports of inflammatory cell infiltration in human cases of cholestasis-induced lung injury (13). In the current study, we found that inflammatory cells such as neutrophils, monocytes, and lymphocytes were significantly higher than their basal level in the BALF of cholestatic mice (Figure 1). Moreover, the BALF level of cytokines such as TNF-α was significantly elevated in BDL animals (Figure 1). The inflammatory cell infiltration was also evident in lung histopathological findings from BDL mice (Figure 4). These data indicate the primary role of inflammatory response in the pathogenesis of cholestasis-induced lung injury. DXM is widely used to treat lung inflammation with different etiologies (82-87). In this study, we found that low-dose DXM could readily blunt cholestasis-induced lung inflammation (Figure 1).

There is a firm connection between inflammatory cell accumulation and the occurrence of oxidative stress (88). It is well-known that the enzymes such as NADPH-oxidase and myeloperoxidase (MPO) located in the inflammatory cells are crucial sources of ROS (69). It has also been found that cytokines (e.g., IL-6) could increase the expression of ROS-forming enzymes such as NADPH-oxidase (89). On the other hand, oxidative stress is another critical factor identified as a pathogenic mechanism of cholestasis-induced lung injury (9, 10, 12-14, 18). ROS formation, lipid peroxidation, and suppressed level of lung tissue antioxidant defense mechanisms have been documented in cholestasis-induced lung injury (9, 10, 12-14, 18). In the current study, we found that biomarkers of oxidative stress such as ROS formation, lipid peroxidation, and protein carbonylation were significantly high in the lung of cholestatic mice (Figure 3). Moreover, lung tissue GSH content was depleted, and tissue antioxidant capacity was suppressed in BDL animals (Figure 3). We found that DXM could mitigate cholestasis-induced oxidative stress in the lung tissue (Figure 3). The effects of DXM on oxidative stress biomarkers could be connected to the anti-inflammatory properties of this drug.

At cellular levels, an essential mechanism for the anti-inflammatory effects of glucocorticoid drugs such as DXM is its effect on NFkB signaling as the primary mechanism of the cellular inflammatory response (21). Activation of NFkB is responsible for gene transcription and synthesis of many cytokines as inflammatory mediators (21). Therefore, suppression of the transcription of NFkB by DXM inhibits the synthesis of a wide range of cytokines (21, 90). DXM could also directly inhibit inflammatory cells' activity, such as neutrophils and T-cells (21). In the current study, we also found that the BALF level of TNF-α was significantly suppressed by DXM (Figure 1). Hence, the suppression of cytokine synthesis also plays a critical role in preventing lung injury induced by cholestasis. Other effects rather than the direct inhibitory properties of glucocorticoids such as DXM also could play a role in its protective effects on the lung tissue during cholestasis (91). For example, the effects of these drugs om the immunosuppression (e.g., T-cells and thymocytes cells apoptosis) (91). Moreover, glucocorticoids enhance lung development as well as significantly decrease the synthesis of molecules such as collagenases (91). Other mechanisms of drugs such as DXM on enzymes involved in energy metabolism might also preserve pulmonary tissue in a healthier state (91) Another possible mechanism might be associated with the effects of glucocorticoids in increasing beta 2-adrenergic receptor transcription in the lung (92). The effects of glucocorticoids on beta 2-adrenergic receptors might has a role in modulating factors such as TGF-β as a pro-fibrotic factor (93).

In exciting point that should be mentioned here, is the general anti-inflammatory effects of DXM in whole body. We know that DXM could provide anti-inflammatory properties in lung as well as the kidneys when it is systemically administered to a human subject. In the current study we

investigated the anti-inflammatory effects of DXM in the lung of cholestatic animals. On the other hand, inflammatory response is also crucially occurring in organ tissues of cholestatic/cirrhotic animals. Therefore, this drug might ameliorate inflammation-associated complications in these tissues (e.g., liver, heart, and kidney), thus, some effects of DXM in preserving cholestatic mice in a healthier state could be associated with its effects on the systemic inflammation.

5. Conclusion

The data obtained from this study indicate the significant therapeutic potential of DXM against cholestasis-associated lung injury. The role of DXM in alleviating inflammation and mitigating oxidative stress seem to play a primary role in its protective mechanisms in the lung of cholestatic animals. More investigations are warranted to confirm the effectiveness of DXM in cholestatic subjects and, finally, its clinical application.

Acknowledgments

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Conflict of Interest

None declared.

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