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Protective effect of dexamethasone against paraquat-triggered toxicity in A549 cells through inhibiting inflammation, apoptosis and TGF-β1/Smad3 pathway

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Abstract: Dexamethasone is a glucocorticoid that is used for the treatment of interstitial pneumonia and pulmonary fibrosis as it possesses anti-inflammatory and anti-fibrosis properties. In the current study, A549 cells were exposed to paraquat, dexamethasone, or both of them, to investigate the potential effect of dexamethasone against paraquat-triggered poisoning in A549 cells. The inflammatory response was evaluated by measuring tumor necrosis factor-α, interleukin-1β, and interleukin-6 while the degree of fibrosis was assessed by detecting collagen I and fibronectin using enzyme-linked immunosorbent assay. Western blotting was used to assess the protein expression of apoptotic proteins as well as transforming growth factor-β1, Smad 3 and phospho-Smad 3. DNA ladder assay was performed to estimate DNA damage in different groups of the alveolar epithelial cells. Dexamethasone protected against paraquat-induced inflammatory response as shown by the significantly reduced levels of the pro-inflammatory cytokines and it also alleviated paraquat-provoked fibrosis as it substantially diminished collagen I and fibronectin levels. Moreover, dexamethasone remarkably decreased the relative expression levels of transforming growth factor-β1 and phospho-Smad3 that were upregulated upon PQ treatment. Dexamethasone also protected against paraquat-induced genotoxicity and apoptosis. In conclusion, dexamethasone may protect against paraquat-induced inflammation, fibrosis, genotoxicity, and apoptosis via modulating TGF-β1/Smad 3 signaling pathway.

Keywords: Apoptosis, dexamethasone, inflammatory response, paraquat, TGF-β1/Smad3.

INTRODUCTION

Paraquat (PQ) is an herbicide that is extensively used worldwide despite its confirmed toxic effects on human. Multiple organ injury was detected as a result of PQ toxicity. Lungs are among the main target organs for PQ intoxication (Jamalian *et al.*, 2020; Amin *et al.*, 2021).

PQ-induced pulmonary injury was ascribed with oxidative stress-associated cell damage and inflammation (Hoshina *et al.*, 2018; Fathy *et al.*, 2021). However, the molecular mechanism underlying PQ-induced lung damage needs more elucidation (Jeon *et al.*, 2016).

Triggered lung fibrosis was ascribed with DNA damage and cell death with subsequent secretion of inflammatory cytokines. Autophagy and apoptosis were reported following the alveolar epithelial cells' destruction (Du *et al.*, 2019). Progressive inflammation and cell death lead to fibroblasts and myofibroblasts' formation. Subsequently, a huge amount of the extra cellular matrix is released with subsequent secretion of fibrogenic factors and epithelial mesenchymal transformation (EMT) that leads to alveolar damage and fibrosis (Pang *et al.*, 2021). It has been reported that EMT is an essential factor in fibrosis and tumor formation (Pang *et al.*, 2021).

Moreover, the produced inflammatory cytokines were reported to activate the transforming growth factor (TGF-β)/Smad pathway (Pang *et al.*, 2021). Smad3 is one of the Smads lipocalin family revealing immune suppression effect through TGF-β (Millet and Zhang, 2007). It has been reported that PQ treatment elevated serum and pulmonary TGF-β1 levels in rats (Kan *et al.*, 2014). The induced TGF-β/Smad pathway was detected following PQ treatment as shown by elevated TGF-β1 expression and Smad3 phosphorylation (Qianwen *et al.*, 2020). Consequently, targeting TGF-β/Smad3 signaling pathway mitigates PQ-induced pulmonary fibrosis and fibronectin secretion as previously reported (Han *et al.*, 2015; Xie *et al.*, 2016).

Several trials were performed to establish a proper treatment to protect lung tissue and cells from PQ toxicity (Bai *et al.*, 2019; Fathy *et al.*, 2021). Dexamethasone (DXM), a glucocorticoid agent, is used for treating individuals suffering from interstitial pneumonia and pulmonary fibrosis due to its anti-inflammatory and anti-fibrosis outcome (Zhang *et al.*, 2020).

In the current study, we aimed to assess the contribution of TGF-β/Smad3 signaling pathway in the cytoprotective action of DXM against PQ toxicity. The cytoprotective effect of DXM against PQ toxicity was first investigated in A549 cells, commonly used as a model of alveolar epithelial cells (Ihara *et al.*, 2020; Cheng *et al.*, 2021; Dehcheshmeh *et al.*, 2021). Next, we sought to examine whether DXM influences TGF-β/Smad3 signaling

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pathway in the presence of PQ. The anti-genotoxic effect of DXM against PQ was also investigated. Moreover, pro-apoptotic and anti-apoptotic protein expression levels were examined.

MATERIALS AND METHODS

Chemicals and antibodies

PQ, DXM and Dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, USA. Dulbecco's Modified Eagle Medium with Ham Nutrient Mixture F-12 (DMEM/F-12), Fetal bovine serum (FBS), penicillin G, and streptomycin, were bought from GIBCO, USA. Primary antibodies to Bax (sc-7480), Bcl2 (sc-7382) and TGF-β1 (sc-130348) were obtained from Santa Cruz Biotechnology, USA. Smad3 (C67H9) and phospho-Smad3 (p-Smad3) (Ser423/425) were obtained from Cell Signaling Technology, USA. β-actin antibody (ab6276) was obtained from Abcam, USA. Mouse anti-rabbit IgG conjugated to Horseradish Peroxidase (HRP) (sc-2357) and mouse IgG kappa binding protein (m-IgGκ BP) conjugated to HRP (sc-516102) were purchased from Santa Cruz Biotechnology, USA. All chemicals and reagents were of analytical grade.

Preparation of PQ and DXM

PQ was dissolved in 0.9% saline solution and DXM powder was dissolved in DMSO. Final concentrations were reached upon dilution in the culture medium.

Cell viability test

Sulphorhodamine-B (SRB) assay was used to determine the cell viability percent at different concentrations of PQ and DXM to assess their cytotoxicities and calculate their IC_{50} values as previously described (Sharaky *et al.*, 2020). Briefly, A549 cells were seeded in 96 well plates in regular medium at a density of 3 x 10^3 cells/well. 24h later, cells were ready to be treated with different concentrations of PQ (125µM, 250µM, 500µM and 1000 µM) or DXM (10^{-4}µM, 10^{-3}µM, 10^{-2}µM and 10^{-1}µM). After treatment for 48 h, cells were fixed using cold 20% trichloroacetic acid, washed in water, and stained with 0.4% SRB dye. The optical density (OD) was then measured for different wells using enzyme-linked immunosorbent assay (ELISA) microplate reader at 570 nm (TECAN sunrise 3, Germany). Cell viability percent was calculated using the following equation; Cell viability percent = Mean OD treatment/Mean OD control ×100%. Subsequently, IC_{50}, the concentration that leads to 50% cell growth inhibition, was calculated using nonlinear regression curve created by GraphPad PRISM 8.4.3 (686).

Cell culture and treatment

Human alveolar epithelial cells (A549) were purchased from Cancer National Institute in Egypt and were cultured in DMEM/F-12 medium supplemented with 10% (v/v) heat-inactivated FBS and 1% (penicillin/streptomycin) at 37°C/5% CO_2. 60 mm cell culture plates were used to seed the cells (4 x 10^5 cells/well) (Jeon *et al.*, 2016). 24h later, different treatments were administrated and the cell cultures were maintained for 48 h before the collection of supernatants for ELISA and cells for Western blot and DNA ladder assay.

ELISA assay

Tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, IL-6, collagen I and fibronectin proteins were assessed using ELISA as previously described (Zheng *et al.,* 2021) following the manufacturer's instructions to evaluate the alleviating effect of DXM against inflammation and fibrosis induced by PQ. After treatment with PQ (200 µM) for 48 h with/without DXM (10^{-5} µM), culture media were collected and centrifuged. The supernatants were used to measure the desired proteins using the following commercial kits as per the manufacturer instructions: Human TNF Alpha ELISA Kit PicoKine™ (Pleasanton, USA), Human IL-1β ELISA Kit (MyBioSource, USA), IL-6 (human) ELISA Kit (Cayman Chemical, USA), Human Collagen Type I ELISA Kit (Novus Biologicals, USA), and Human Fibronectin ELISA Kit (BioVendor, Czech Republic),

Western blot

Western blot was used according to (Ma *et al.*, 2021), to investigate apoptosis via measuring Bax and Bcl2 protein levels. TGF-β1, Smad3, and p-Smad3 protein expressions were also determined to evaluate the impact of different treatments on the TGF-β1/Smad3 signaling pathway. Cell pellets were used to extract the total protein using the ReadyPrep TM protein extraction kit (Catalog #163-2086) as per the manufacturer's protocol. Protein concentrations were assessed by Bradford Protein Assay Kit (SK3041) according to the manufacturer's instructions. Proteins were separated using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories, USA) at 100 V for 1.30h before transferring on a polyvinylidene fluoride membrane (Bio-Rad Laboratories, USA) at 15 V for 1h. Blocking of the membranes was performed by incubation with tris-buffered saline with Tween 20 (TBST) and 3% bovine serum albumin for 1hr at room temperature. The membranes were first incubated with the primary antibodies overnight at 4°C. After rinsing in TBST for 5 min, the membranes were incubated with HRP-conjugated secondary antibodies. Then, the membranes were rinsed again in TBST for 5 min. Images were acquired using a CCD camera-based imager and analyzed by Image lab™ software (BioRad).

DNA ladder assay

To assess the potential effect of DXM on the genotoxicity induced by PQ, Apoptosis DNA Ladder Assay Kit (Abcam, ab66090) was used as described before (Li *et al.*, 2019), according to the manufacturer's protocol. In Brief,

cells were washed with phosphate buffer saline and lysed with Tris-EDTA lysis buffer. The lysate was first incubated with enzyme A solution for 10 min, followed by incubation with enzyme B solution for 30 min. Ammonium acetate and isopropanol were then added and the samples were kept at -20°C for 10 min. DNA was precipitated by centrifugation and washed with 70% ethanol then left to air-dry. The samples were dissolved in DNA suspension buffer before analysis and visualization by gel electrophoresis.

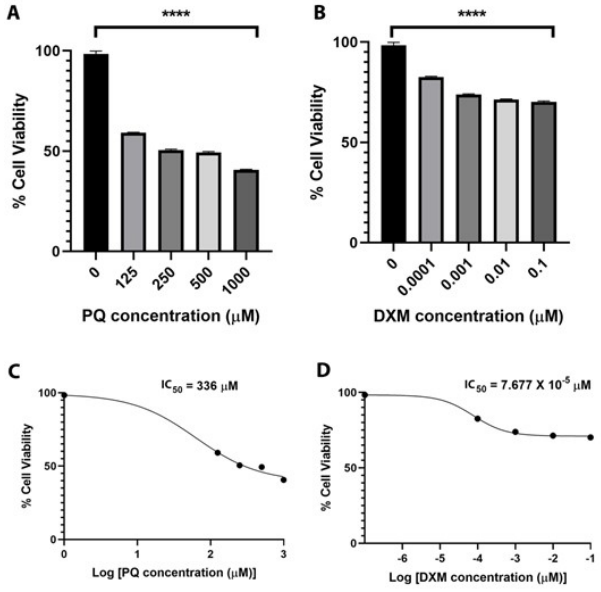
STATISTICAL ANALYSES

All results were collected from three replicate experiments. The statistical investigations were achieved by GraphPad PRISM 8.4.3 (686). Results were examined using one-way ANOVA, and Tukey's multiple comparison test. Data were represented as mean ± SD. Values of $P<0.05$ were represented as statistically significant. Nonlinear regression curve was used to calculate the IC_{50}.

RESULTS

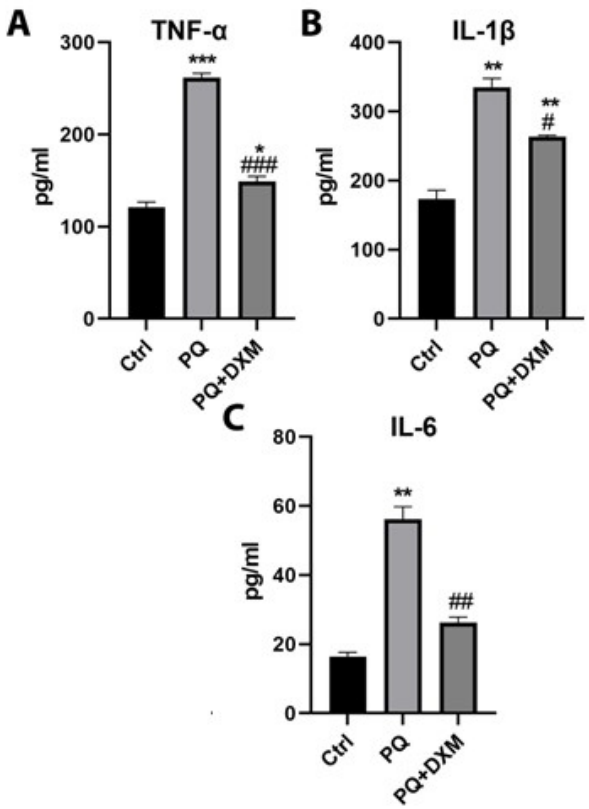
Cell viability test

A549 cell viability percent was assessed after receiving different concentrations of PQ and DXM, fig. 1 (A, B). From these results, the IC_{50} was calculated using nonlinear regression curve. The IC_{50} was found to be 336 µM and 7.677 x 10^{-5} µM for PQ and DXM, respectively, fig. 1 (C, D). Accordingly, we used PQ (200µM) and DXM (10^{-5} µM) in later experiments.



Cell viability percent of A549 cells treated with different concentrations of PQ (A) and DXM (B), assessed by SRB assay, along with their nonlinear regression curves revealing the IC50 of PQ (C) and DXM (D). Data are expressed as mean ± SD, n = 3. ****: P<0.0001.

Fig. 1: Cytotoxicity of paraquat (PQ) and dexamethasone (DXM) on A549 cell line



The levels of TNF-α (A), IL-1β (B) and IL-6 (C) in Ctrl; control cells, PQ; cells treated with PQ (200µM) and PQ+DXM; cells treated with PQ (200µM) and DXM (10^{-5}µM). Data are expressed as mean ± SD, n=3.*: P<0.05, **: P<0.01 and ***: P <0.001 compared with control cells; #: P<0.05, ##: P<0.01, and ###: P<0.001 compared with PQ treated cells.

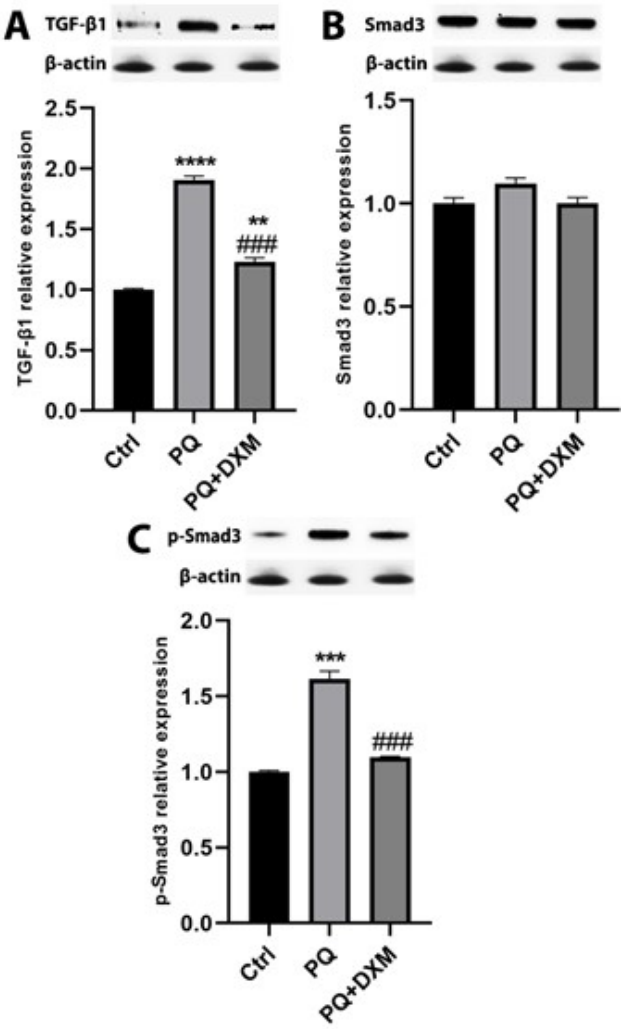
Fig. 2: Effect of dexamethasone (DXM) on paraquat (PQ)-induced increase of the inflammatory cytokines in A549 cells

DXM attenuates PQ-induced inflammatory response

PQ (200µM) elevated inflammatory cytokine release in A549 cells. This was represented by evident increase in the protein level of TNF-α (P<0.001), IL-1β (P<0.01) and IL-6 (P<0.01) in relation to the non-treated cells. Co-treatment of A549 cells with DXM (10^{-5}µM) significantly reduced these responses at values of (P<0.001), (P<0.05), and (P<0.01), respectively, when compared to PQ-treated cells (fig. 2).

DXM modulates TGF-β1/Smad3 signaling cascade

The protein expression of TGF-β1, p-Smad3 and Smad3 were measured in A549 cells from different groups to understand the mechanism behind the protecting effect of DXM (10^{-5}µM) against PQ-induced toxicity. At odds with Smad3 expression, TGF- β1 and p-Smad3 protein expressions substantially increased in response to PQ (200µM) administration (P<0.0001 and P<0.001, respectively). Nevertheless, DXM (10^{-5}µM) had an evident modulating effect against these changes (P<0.001), fig. 3.



Relative protein expressions of TGF- β1 (A), Smad3 (B), and p-Smad3 (C) in Ctrl; control cells, PQ; cells treated with PQ (200 µM), and PQ+DXM; cells treated with PQ (200 µM) and DXM (10^{-5}µM). Data are expressed as mean ± SD, n=3. **: P<0.01, ***: P<0.001 and ****: P <0.0001 compared with control cells; ###: P<0.001 compared with PQ treated cells.

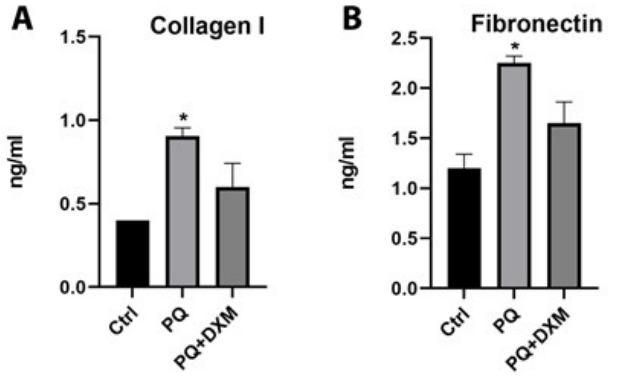
Fig. 3: Effect of paraquat (PQ) with/without dexamethasone (DXM) on TGF-β1/Smad3 signaling pathway

DXM alleviates PQ-induced fibrosis

A549 tendency towards fibrosis in response to PQ (200 µM) administration was revealed by the increase in collagen I and fibronectin protein levels (P<0.05), whereas, DXM (10^{-5}µM) was noticed to reduce the production of these proteins to values similar to that of the control cells (fig. 4).

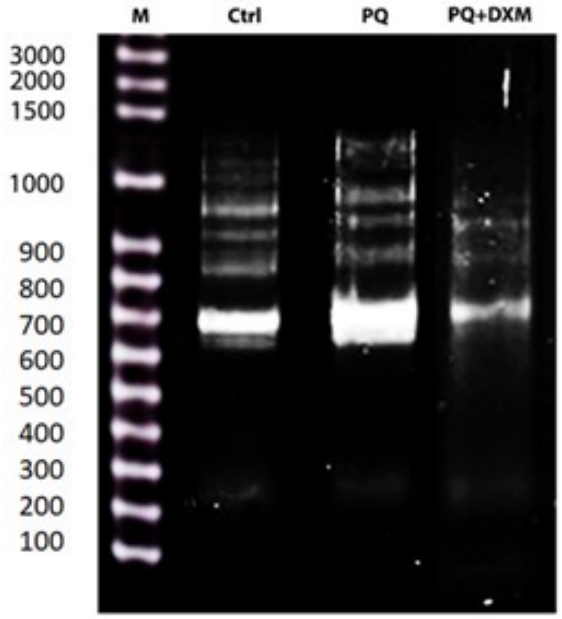
DXM protects against PQ-induced genotoxicity

DNA ladder assay showed a laddering pattern of the extracted DNA from alveolar epithelial cell line treated with PQ (200 µM). However, a more typical pattern was displayed by the genomic DNA taken out from the cells treated with both PQ (200µM) and DXM (10^{-5}µM), fig. 5.



Collagen I (A) and fibronectin (B) levels in A549 cells, assessed by ELISA in Ctrl; control cells, PQ; cells treated with PQ (200µM) and PQ+DXM; cells treated with PQ (200 µM) and DXM (10^{-5}µM). Data are expressed as mean ± SD, n = 3. *: P<0.05 compared with control cells.

Fig. 4: Effect of dexamethasone (DXM) on paraquat (PQ)-induced raising of fibrosis marker proteins

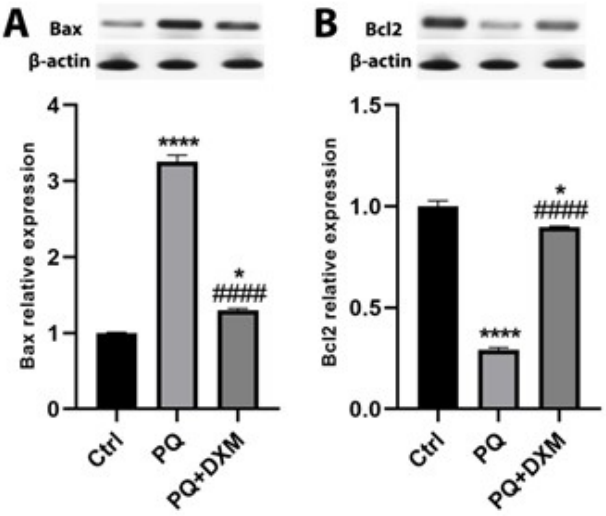


M lane; Marker, Ctrl lane; DNA from control cells, PQ lane; DNA from cells treated with PQ (200µM), (PQ+DXM) lane; DNA from cells treated with PQ (200µM) and DXM (10^{-5}µM).

Fig. 5: Effect of dexamethasone (DXM) on paraquat (PQ)-induced genotoxicity as demonstrated by DNA ladder assay.

DXM mitigates PQ-induced apoptosis

Apoptosis was demonstrated in A549 cells treated with PQ (200µM) through the obvious elevation of the pro-apoptotic Bax protein (P<0.0001) and reduction in anti-apoptotic Bcl2 protein (P<0.0001). These changes were markedly reversed in the presence of DXM (10^{-5} µM), (P<0.0001), fig. 6.



Relative expressions of Bax (A) and Bcl2 (B) in Ctrl; control cells, PQ; cells treated with PQ (200μM) and PQ+DXM; cells treated with PQ (200 μM) and DXM (10^5μM). Data are expressed as mean ± SD, n=3*: P<0.05 and ****P<0.0001 compared with control cells and ####: P < 0.0001 compared with PQ treated cells.

Fig. 6: Effect of dexamethasone (DXM) on PQ-induced apoptosis

DISCUSSION

PQ poisoning is associated with multiple organ injuries with the lung as an essential target organ since the alveolar epithelium was recorded to absorb PQ actively (SreeHarsha, 2020). After exposure to PQ, acute pulmonary injury with inflammatory response and oxidative damage propagates (Shen *et al.*, 2017). Meanwhile, chronic lung fibrosis due to PQ toxicity can be life-threatening as it leads to the failure of respiratory function (Bai *et al.*, 2019).

The current article demonstrated that DXM attenuated toxicity in PQ-treated cells via modulating TGF-β1/Smad3 signaling cascade. It was observed that TGF-β1 is a crucial inducer for fibrosis formation and triggering factor of EMT mediated via Smad-dependent and Smad independent pathways (Yu *et al.*, 2018). DXM mitigating effect in bleomycin-induced lung fibrosis was suggested to be through reducing TGF-β1 expression level in rats (Guo *et al.*, 2013). Moreover, the present work revealed increased secretion of IL-6, IL-1β, and TNF-α in PQ supplemented cells which was reversed upon treatment with DXM. It has been reported that PQ-induced acute pulmonary injury is attributed to inflammatory cell recruitment and pro-inflammatory cytokine secretion (Zheng *et al.*, 2021). TNF-α and IL-1β were recorded to be the essential parameters involved in the inflammatory response propagation (Zheng *et al.*, 2021). Various reports also elucidated the synergetic effect of TNF-α and IL-1β with TGF-β1 released from epithelial cells for EMT formation and collagen accumulation (Pérez *et al.*, 2017; Wang *et al.*, 2017).

DXM interfered with the release of the pro-inflammatory cytokines that was triggered by PQ. This finding is in line with (Zhang *et al.*, 2020) who suggested that the anti-fibrosis effect of DXM was attributed to the modulation of epithelial cytokine secretion. We also found that DXM effectively inhibited the phosphorylation of Smad3, suggesting that DXM may prevent PQ-induced toxicity in A459 cell line by targeting TGF-β1/Smad3 pathway and the secreted pro-inflammatory cytokines from alveolar A459 cells.

DXM treatment reduced the elevated levels of collagen type I, an indicator of EMT process, and fibronectin, a phenotypic marker of fibroblasts, due to PQ toxicity. It has been shown that EMT progression in alveolar epithelial cells was induced by TGF-β1 (Hisatomi *et al.*, 2012). TGF-β1 showed the capability to provoke proliferation and differentiation of pulmonary fibroblasts, and trigger the extensive accumulation of proteins of the extracellular matrix such as collagen in the alveolar cells, with the development of lung fibrosis (Gao *et al.*, 2019). Antibodies used to block TGF-β1 reduced the proliferation of fibroblast and the augmented collagen production (Ohnishi *et al.*, 2016). Subsequently, the anti-fibrosis effect of DXM in the current article might be established via interfering with TGF-β1 release in PQ treated A549 cells (Hisatomi *et al.*, 2012). In line with the previous outcome, pirfenidone treatment reduced EMT development and fibrosis by decreasing collagen type I and fibronectin expressions that were induced by TGF-β1 in A549 cells (Hisatomi *et al.*, 2012). Noteworthy, DXM inhibited bleomycin-triggered lung fibrosis via decreasing TGF-β1 level in rats (Guo *et al.*, 2013).

In addition, we observed that DXM mitigated PQ-induced DNA damage and apoptosis in A549 lung epithelial cells. PQ-stimulated DNA damage was demonstrated by (Tajai *et al.*, 2021). It was suggested that PQ radical resulted from PQ alteration by NADPH, generates superoxide anion via the reaction with molecular oxygen. O^{2-} is transformed into H_2O_2 by superoxide dismutase (SOD), and H_2O_2 reacts with Fe^2 generating hydroxyl radical ($OH^{\bullet}$) which triggers DNA damage (Charão *et al.*, 2015). On the other hand, Radiotherapy-induced DNA damage and fibrosis in breast adipose tissue were mitigated by DXM (Meng *et al.*, 2020), and DNA fragmentation was also inhibited in A549 cells by DXM (Fukazawa *et al.*, 2009). It has been reported that genotoxic stress and DNA damage induce apoptosis (Pistritto *et al.*, 2016; Aluko, 2018).

The ratio of Bax to Bcl2 can be used to predict cell apoptosis (Chen *et al.*, 2018). A higher Bax to Bcl2 ratio

stimulates apoptosis, whereas a lower Bax to Bcl2 ratio retains survival (Arthur *et al.*, 2017). PQ-induced apoptosis was confirmed in the present study by the increased expression of Bax and the reduced expression of Bcl2. Similarly, apoptosis was activated in PQ-treated lymphocytes via modulating the Bax and Bcl2 relative expressions *in vitro* (Ahmadian *et al.*, 2020). Noteworthy, PQ-provoked apoptosis of the alveolar epithelial cells exhibits an essential role in the acute lung damage propagation and subsequent development of pulmonary interstitial fibrosis (Sun and Chen, 2016; Sun *et al.*, 2020). DXM anti-apoptotic effect noticed in this study indicates that it may exert an antifibrosis role via suppressing of apoptosis pathway (Pang *et al.*, 2021). Noteworthy, DXM was recorded to be a strong inhibitor of interferon (IFN)-γ and IFN-γ plus anti-Fas-stimulated apoptosis (Wen *et al.*, 1997). Moreover, epithelial cell apoptosis suppression by corticosteroids was suggested to be one mechanism by which the inflammatory response was reduced (Wen *et al.*, 1997).

Furthermore, it has been found that TGF-β1 is a potent inducer of apoptosis in osteoclasts through the activation of different pathways such as Smad-dependent pathway (Houde *et al.*, 2009). Similarly, PQ-induced apoptosis observed here can be attributed to TGF-β1/Smad3 pathway activation and consequently, the anti-apoptotic effect of DXM was achieved through inhibition of TGF-β1/Smad3 pathway.

CONCLUSION

Anti-inflammatory, anti-fibrosis, antigenotoxic and anti-apoptotic effects of DXM against PQ-induced poisoning in A459 cells may be achieved by targeting TGF-β1/Smad3 pathway.

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