Environmental Toxicology and Pharmacology 45 (2016) 340–345

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Thymoquinone effectively alleviates lung fibrosis induced by paraquat herbicide through down-regulation of pro-fibrotic genes and inhibition of oxidative stress

Fatemeh Pourgholamhossein[a,b], Fariba Sharififar[c], Rokhsana Rasooli[a,b], Leyla Pourgholi[b], Fatemeh Nakhaeipour[b], Hojjat Samareh-Fekri[d], Maryam Iranpour[f], Ali Mandegary[b,e,*]

[a] Pharmaceutics Research Center, Neuropharmacology Institute, Kerman University of Medical Sciences, Haft-Bagh Blvd., Kerman 7616911319, Iran
[b] Department of Toxicology & Pharmacology, School of Pharmacy, Kerman University of Medical Sciences, Haft-Bagh Blvd., Kerman 7616911319 , Iran
[c] Department of Pharmacognosy Herbal and Traditional Medicines Research Center, School of Pharmacy, Haft-Bagh Blvd., Kerman 7616911319, Iran
[d] Central Research Laboratory, Deputy of Research, Kerman University of Medical Sciences, Haft-Bagh Blvd., Kerman 7616911319, Iran
[e] Gastroenterology and Hepatology Research Center, Institute of Basic and Clinical Physiology Sciences, Afzalipour's Hospital, Imam Highway, Kerman University of Medical Sciences, Kerman 7616913911, Iran
[f] Pathology and Stem Cell Research Center, Kerman University of Medical Sciences, Kerman 7617939555, Iran

ARTICLE INFO

Article history:
Received 20 January 2016
Received in revised form 15 June 2016
Accepted 17 June 2016
Available online 18 June 2016

Keywords:
Thymoquinone
Pulmonary fibrosis
Paraquat
Oxidative stress
Gene expression

ABSTRACT

The potential preventive and therapeutic effects of thymoquinone (TQ) and its molecular mechanism were evaluated in paraquat (PQ)-induced pulmonary fibrosis in mice. TQ was administered orally at the doses of 20 and 40 mg/kg during the course and after development of fibrosis. Pathological changes, expressions of genes involved in fibrogenesis, hydroxyproline (HP) and oxidative stress parameters were determined in the lung tissues. TQ dose-dependently recovered the pathological changes induced by PQ. TQ decreased hydroxyproline content, lipid peroxidation and restored the antioxidant enzymes to the normal values. In molecular level, expressions of TGF-β1, α-SMA, collagen 1a1 and collagen 4a1 genes were also returned to the control level by TQ. This study indicated that TQ has the preventive and therapeutic potentials for the treatment of lung fibrosis by inhibition of oxidative stress and down-regulation of profibrotic genes.

1. Introduction

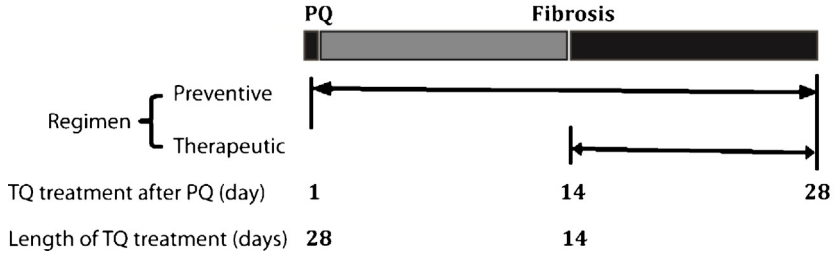
Pulmonary fibrosis (PF) also known as interstitial lung diseases results from accumulation of excess fibrous connective tissue and lung scarring. Exposure to some xenobiotics such as silica, paraquat and bleomycin is one of the causes of PF. However, most cases of pulmonary fibrosis have no known cause is called idiopathic pulmonary fibrosis (IPF). PF is a progressive and often lethal interstitial lung disorder characterized by destruction of alveolar structures with an abnormal formation of fibroblasts or myofibroblasts and exaggerated synthesis and deposition of extracellular matrix (Noble et al., 2012; Todd et al., 2012). Even though the precise mechanisms of PF are uncertain, three main mechanisms of inflammation and immune mechanisms, oxidative stress and oxidative signaling, and procoagulant mechanisms have been the main culprits of PF (Todd et al., 2012).

Paraquat (PQ), a bipyridyl and non-selective quaternary nitrogen herbicide, is a highly toxic herbicide causing fatal pulmonary damage. Because of selective accumulation in the lung, pulmonary effects represent the most lethal and least treatable manifestations of PQ toxicity. PQ-induced lung fibrosis is a well-established animal model of pulmonary fibrosis and can be used to study and evaluate anti-fibrotic and modulating agents (Dong et al., 2016; Tomita et al., 2007). Although the exact mechanism of PQ toxicity has not been fully understood, it is demonstrated that the primary mechanism is related to the chemical cascades leading to the reduction of PQ, the generation of free radicals and lipid peroxidation creating lethal

Abbreviations: TQ, thymoquinone; PQ, paraquat; SOD, superoxide dismutase; MDA, malondialdehyde.

* Corresponding author at: Department of Toxicology & Pharmacology, School of Pharmacy, Kerman University of Medical Sciences, Haft-Bagh Blvd., Kerman 7616911319, Iran.
E-mail addresses: f.pourgholamhosein@gmail.com (F. Pourgholamhossein), sharififar@yahoo.com (F. Sharififar), rokhsana66@yahoo.com (R. Rasooli), leyla.pourgholi5@gmail.com (L. Pourgholi), 89bahar73@gmail.com (F. Nakhaeipour), hojjatfekri@gmail.com (H. Samareh-Fekri), dr.iranpour.86@gmail.com (M. Iranpour), alimandegary@kmu.ac.ir, alimandegary@yahoo.com (A. Mandegary).

http://dx.doi.org/10.1016/j.etap.2016.06.019
1382-6689/



Scheme 1. Schematic diagram showing TQ treatment schedules.

lesions associated with lung fibrosis and failure (Dinis-Oliveira et al., 2008; Khalighi et al., 2015; Mohammadi-Bardbori and Ghazi-Khansari, 2008).

There are many obstacles in the treatment of PQ poisoning, and there are no broadly accepted procedures for treatment of PQ poisoned patients (Gawarammana and Buckley, 2011). Currently available therapies for the PQ-induced lung fibrosis consist of anti-inflammatory (glucocorticoids) and immunosuppressive/cytotoxic (azathioprine, cyclophosphamide) agents (Dinis-Oliveira et al., 2008; Gawarammana and Buckley, 2011). Treatment protocol is based on the rationale that the inflammation initiates the injury thereby results in scarring of the body. Moreover, many reports show the oxidant/antioxidant balance plays a major role in processes of lung fibrosis (Todd et al., 2012). The fibrosis may be alleviated and/or prevented by targeting the inflammatory and oxidative responses.

Thymoquinone (TQ) the predominant bioactive constituent of *Nigella sativa* seeds is a pharmacologically active quinone that has been found to exhibit strong antioxidant, anti-inflammatory and antiapoptotic properties (Ali and Blunden, 2003; Khader and Eckl, 2014a; Woo et al., 2012). Recently, El-Khouly et al. (2012) have shown the antifibrotic effects of a single dose of TQ in a bleomycin-induced lung fibrosis in rats. There are also reports of antifibrotic activity of TQ in animal models of liver fibrosis (Bai et al., 2014).

In light of the above reports and lack of effective treatment for PQ-induced lung fibrosis, the present study was designed to evaluate the preventive and therapeutic activity of TQ against PQ induced lung fibrosis in mice. Meanwhile, for determining the underlying mechanisms, tissue oxidative stress parameters as well as the expression of profibrotic genes including TGF-β1, α-SMA, collagen 1a1 and collagen 4a1 were investigated in the mice lung tissues.

2. Materials and methods

2.1. Materials

Thymoquinone, hydroxyproline, 4-dimethylaminobenzaldehyde, chloramine T, malondialdehyde (MDA) and paraquat were purchased from Sigma–Aldrich Chemical Co. (USA). TRIzol® RNA isolation reagent and HyperScript™ first strand cDNA synthesis kit were purchased from Invitrogen (Germany). SYBR Green Master Mix was obtained from Takara (Japan).

2.2. Animals

Male NMRI mice weighing 18–25 g were housed in normal laboratory conditions at 21 ± 2 °C under a 12 h/12 h light–dark cycle. All the mice had free access to water and standard laboratory food and were kept in standard cages. All the animals were treated humanely according to the guideline on ethics standard for investigation of experimental pain in animals approved by the Animal Experimentation Ethics Committee of Kerman Neuroscience Research Center (EC/KNRC/90).

2.3. PQ-induced lung fibrosis in mice

Pulmonary fibrosis was induced by intraperitoneal (i.p.) administration of a single dose of 20 mg/kg PQ. Three days after the induction of fibrosis, the animals were divided randomly in four experimental groups each having eight animals. Pulmonary fibrosis developed in 2 weeks (Day14), confirmed by morphological changes in the lungs and excessive accumulation of interstitial collagen (Hubner et al., 2008). The groups receiving TQ during the course of fibrosis development (Days 1–28 after PQ administration) were considered as prophylactic or preventive, and those receiving TQ after fibrosis development (Days 14–28) were considered therapeutic in the present protocol (see Scheme 1).The experiment was performed in the mice divided into four groups: PQ-induced fibrosis rats (PQ group), low-dose TQ treatment (TQ1; 20 mg/kg body weight), high-dose TQ treatment (TQ2; 40 mg/kg body weight), and normal animals receiving vehicle (olive oil) as sham control.

2.4. Sample collection and analytical procedures

At the end of the treatment period (28 days), the mice were anesthetized by injecting intraperitoneally by 20% Ketamine/Xylazine (10 ml/kg body weight). The lungs were promptly removed and divided into two halves. The right lung was stored at −70 °C for analysis of oxidative stress, hydroxyproline content and gene expression. The left lung was immersed in 10% buffered formalin for histological examination.

2.5. Histopathological analysis

After embedding the fixed lung tissues into liquid paraffin, 5 μm thick tissue sections were prepared. The sections were stained by hematoxylin and eosin (H & E) and Masson's trichrome staining methods as follows: Lung specimens were fixed in 10% formalin solution and embedded in paraffin wax blocks. Afterwards, 3 μm sections were stained with haematoxylin and eosin (H&E) and Masson's trichrome for collagen fibers. Masson's trichrome staining was done by deparaffinization and rehydration of slides through 100%, 95% and 70% alcohols, washing in distilled water, rinsing in running tap water for 5–10 min, staining in Weigert's iron hematoxylin working solution for 10 min, rinsing in running warm tap water for 10 min, washing in distilled water, staining in Biebrich scarlet-acid fuchsin solution for 10–15 min, differentiating in phosphomolybdic-phosphotungstic acid solution for 10–15 min, transferring the sections directly to aniline blue solution and staining for 5–10 min, rinsing briefly in distilled water and differentiation in 1% acetic acid solution for 2–5 min, washing in distilled water, dehydration through 95% and absolute ethyl alcohol and clearing in xylene, respectively. The presence and grading of inflammation and fibrosis were evaluated in the stained sections

based on the scoring indicated in previous studies (Hubner et al., 2008).

2.6. Oxidative stress determination

The right lung tissues of the control and experimental groups were homogenized with 0.1 M Tris–HCl buffer (pH 7.4) at 4 °C using a tissue homogenizer. The resulting tissue homogenate was used for biochemical measurements. The lipid peroxidation level was measured on the basis of reaction of malondialdehyde and other lipid peroxides in the lung tissue with 2-thiobarbituric acid (TBA) in the acidic and hot conditions, thereby the resulting product was measured at 532 nm (Mandegary et al., 2012) The activities of two important antioxidant enzymes, SOD and CAT, were determined in the lung homogenate according to the standard colorimetric methods.

2.7. Collagen measurement

Hydroxyproline, as an index of collagen deposited in the lung tissue and fibrosis, was measured using method of Reddy & Enwemeka (Reddy and Enwemeka, 1996) with minor modifications (Ghazi-Khansari et al., 2007). Briefly, hydroxyproline in the lung tissue homogenate was hydrolyzed with 1 M acetate buffer and oxidized with 1.4% chloramine T; then a reddish purple complex was formed using 1 M Ehrlich's reagent (4-dimethylaminobenzaldehyde). Finally, the mixture was incubated for 20 min at 65° C resulting in formation of chromophore which measured at 550 nm.

2.8. Determination of fibrotic genes expression by real-time RT-PCR

The total RNA was extracted from pulmonary tissues using TRIzol® reagent according to the manufacturer's protocol. The samples (2 μg RNA) were reverse-transcribed using a HyperScript™ first strand cDNA synthesis kit. The synthesized cDNA was used in real-time RT-PCR (lightcycler® 96 Roche, Germany) experiments using SYBR GREEN Supermix and analyzed with lightcycler® 96 Software. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, β-actin cDNA was used as an internal standard. The primers used were TGF-β1: forward- 5′-CGC CAT CTA TGA GAA AAC C-3′, reverse-5′-GTA ACG CCA GGA ATT GT-3′ (Ruiz et al., 2005); alpha smooth muscle actin (α-SMA): forward-5′-TGAC GCT GAA GTA TCC GAT AGA-3′, reverse-5′- CGA AGC TCG TTA TAG AAA GAG TGG-3′ (Li et al., 2011); collagen type 1 (Col 1a1): forward-5′-CTG CTG GCA AAG ATG GAG A-3′, reverse-5′-ACC AGG AAG ACC CTG GAA TC-3′(Li et al., 2011); collagen type 4 (Col 4a1): forward-5′-AGC TGC TAA AGG TGA CAT TCC T-3′, reverse-5′-GGA GGC CCA GGT ACT CCT-3′ (Li et al., 2011);and β-actin: forward-5′-CCA ACC GTG AAA AGA TGA CC-3′, reverse-5′-CCA GAG GCA TAC AGG GAC AG-3′ (Li et al., 2011).

2.9. Statistical analysis

Data were presented as mean ± standard deviation (SD). The differences among the means were analyzed using one-way analysis of variance (ANOVA) followed with Tukey HSD post-hoc test. For all the experiments, $p < 0.05$ was considered as significance level. The data were analyzed using SPSS 18.0.

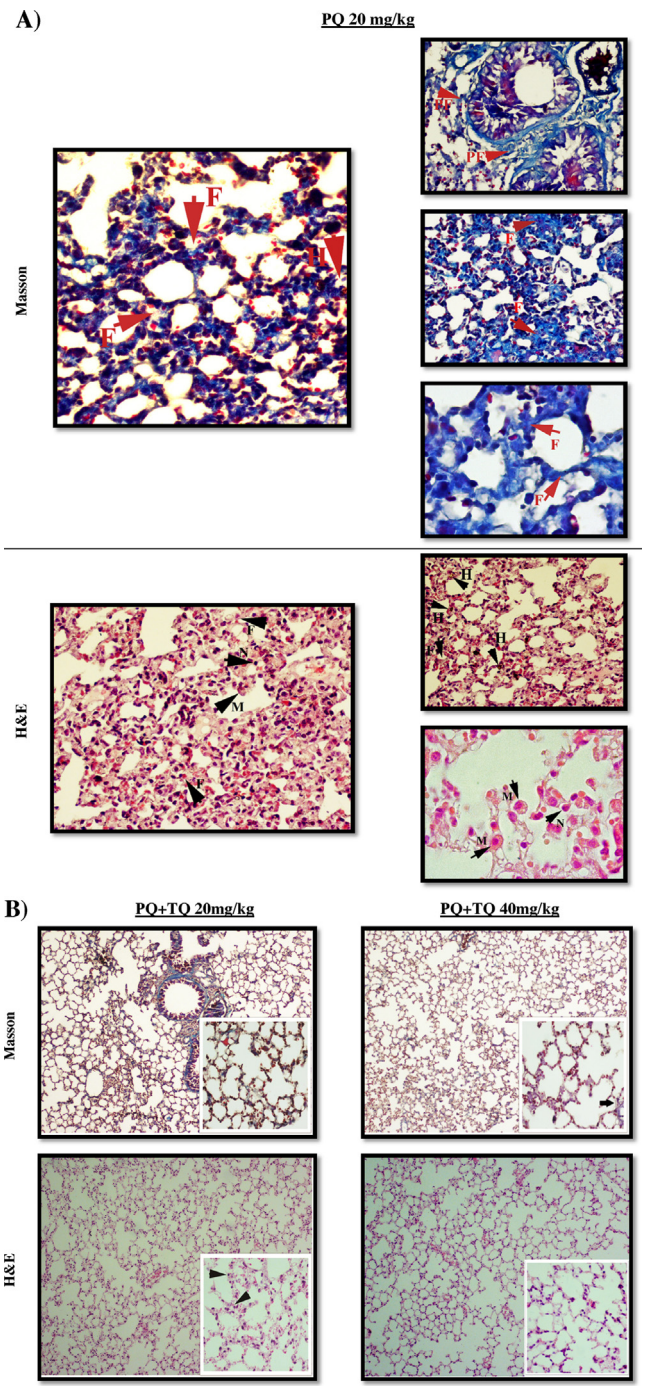


Fig. 1. Histopathological changes in the lungs of the mice. A: Lung fibrosis was induced in the lungs of the mice by injection of PQ (20 mg/kg, i.p.). B: Mice were then treated with olive oil, TQ 20 mg/kg/day, and TQ 40 mg/kg/day for 28 days. The morphopathological changes in the lung tissues were analyzed by hematoxylin and eosin (H&E), and Masson's trichrome staining. PQ: paraquat; TQ: Thymoquinone; F: Fibrosis, PF: Peribronchial fibrosis, H: Hemosiderin deposit, M: Alveolar macrophages, N: Interstitial inflammation (Neutrophil).

3. Results

3.1. Histopathology

The histopathological changes in the test and control groups are shown in Fig. 1 and Table 1. The histological evaluation of lung sections four weeks after the PQ injection revealed evidence of obvious interstitial inflammation, macrophages infiltration, local alveolar

Table 1
Effect of TQ on PQ-induced lung fibrosis on histological lesions.

	Sham	PQ	PQ + 14d TQ 20	PQ + 28d TQ 20	PQ + 14d TQ 40	PQ + 28d TQ 40
Alveolar hemorrhage	absent	focal	mild	absent	mild	absent
Hemosiderin deposit	absent	present	absent	absent	absent	absent
Alveolar macrophages	absent	present	mild	absent	absent	absent
Interstitial inflammation	absent	moderate	mild	mild	mild	absent
Peribronchial fibrosis	absent	present	mild	absent	absent	absent

Mice were treated with 20 and 40 mg/kg TQ for 14 days (Therapeutic) and 28 days (Preventive) after i.p. injection of single dose of PQ.
PQ: Paraquat 20 mg/kg; TQ: Thymoquinone.

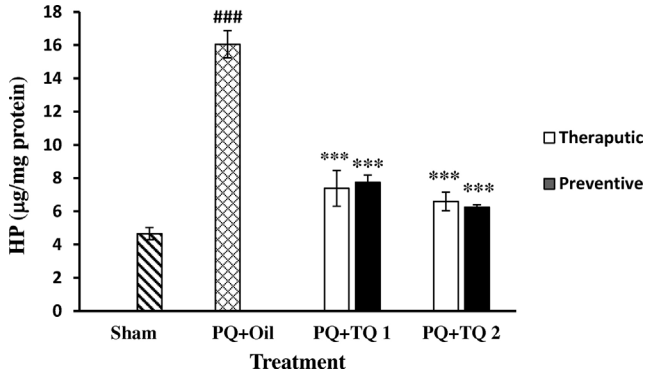


Fig. 2. Effect of TQ on the lung collagen contents. The doses of 20 and 40 mg/kg of TQ were administered orally to the mice for 14 days (therapeutic) and 28 days (preventive) after i.p. injection of PQ (20 mg/kg). The content of hydroxyproline, as the marker of collagen accumulation, was determined in lung tissues of the treated mice at day 28. The values are the means of 8 replicates ± SD.
Sham: (no treatment); Negative control: PQ + Olive Oil; TQ 1: Thymoquinone 20 mg/kg; TQ 2: Thymoquinone 40 mg/kg; HP: Hydroxyproline.
***p < 0.001 in comparison with the negative control group.
###p < 0.001 in comparison with the sham group.

hemorrhage, hemosiderin deposit, and peribronchial fibrosis. Pretreatment and treatment with both concentrations of TQ caused a noticeable less lung damage and improvement in pathological changes produced by PQ. The sections of the sham group showed structural integrity without inflammation or fibrosis development. There were no observable differences in the pathological changes between the high (40 mg/kg) and low (20 mg/kg) concentrations of TQ.

3.2. Effect of TQ on the lung collagen contents in PQ-induced lung fibrosis

As shown in Fig. 2, the hydroxyproline content in the lung of PQ-treated mice significantly increased compared to the sham group. Administration of TQ (20 and 40 mg/kg) dose-dependently reduced the content of hydroxyproline in the lung tissue ($p < 0.001$). These findings were consistent with the histological results.

3.3. Effect of TQ on the oxidative stress parameters in PQ-induced lung fibrosis

The levels of lipid peroxidation and the antioxidant activities of SOD and CAT were determined in the lung tissue of the control and experimental mice (Fig. 3). A significant rise in the levels of lipid peroxidation in the lung tissue was observed in the PQ treated animals ($p < 0.05$). This effect was followed by a decrease in the enzymatic activities of SOD and CAT. The administration of TQ significantly decreased lipid peroxidation in a dose dependent manner and restored the SOD activity to near normal ($p < 0.05$). There were no remarkable differences in catalase activity between the TQ treated groups and the PQ group.

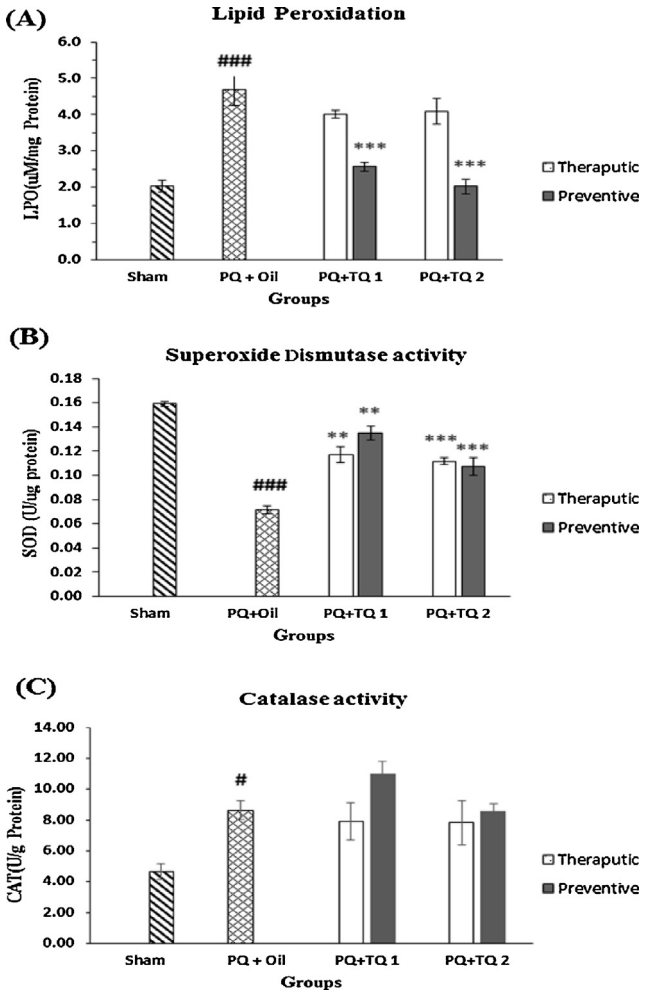


Fig. 3. Effect of TQ on the oxidative stress parameters in the lung. The doses of 20 and 40 mg/kg of TQ were administered orally to the mice for 14 days (therapeutic) and 28 days (preventive) after i.p. injection of PQ (20 mg/kg). The oxidative stress parameters including MDA levels (A), SOD (B) and CAT (C) activities were determined in the lung tissues of the treated mice at day 28. Values are the means of 8 replicates ± SD.
Sham: (no treatment); Negative control: PQ + Olive Oil; TQ 1: Thymoquinone 20 mg/kg; TQ 2: Thymoquinone 40 mg/kg.
***p < 0.001 and **p < 0.01 in comparison with the negative control group.
#p < 0.05 and ### p < 0.001 in comparison with the sham group.

3.4. Expression of collagen 1a1, collagen 4a1, α-SMA and TGF-β1 genes

The mRNA expression of collagen1a1, collagen 4a1 and α-SMA were significantly increased in the PQ group compared to sham group. In a dose and time dependent manner, the mRNA expression of collagen1a1, collagen 4a1 and α-SMA were decreased in TQ treated mice compared to PQ group which suggested that TQ could inhibit the activation of profibrotic genes and ECM deposi-

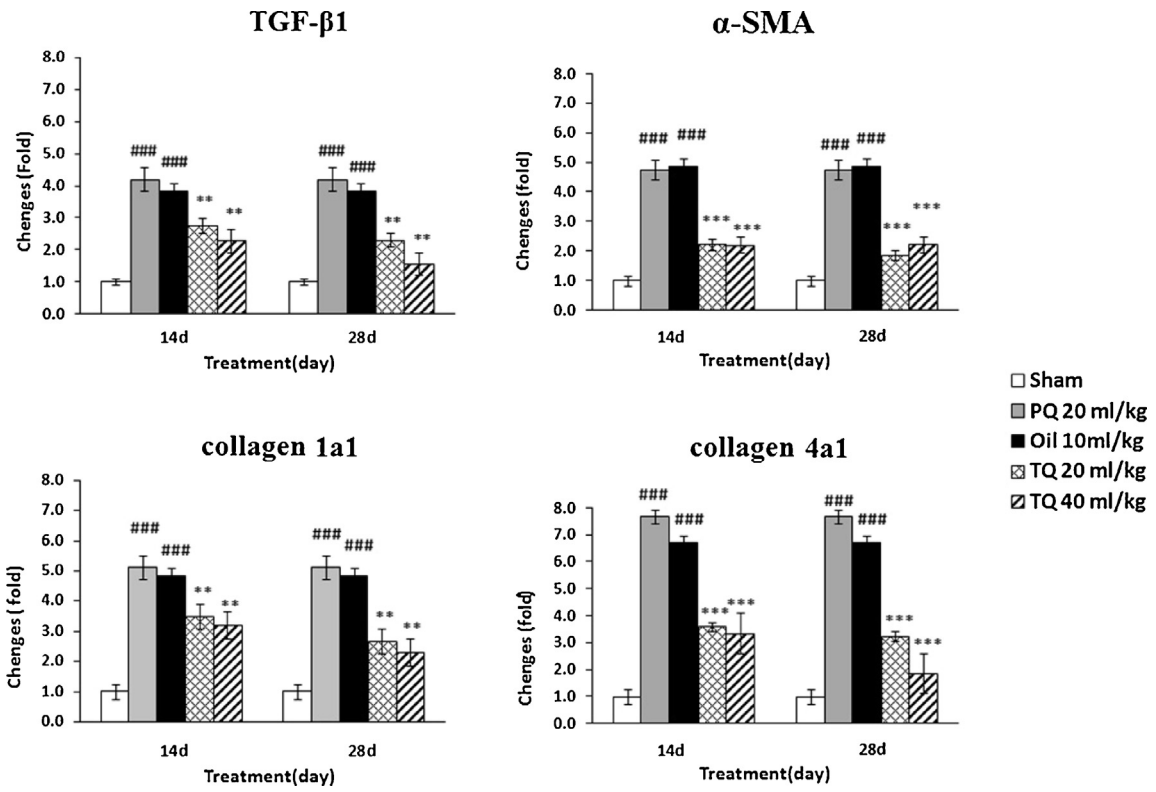


Fig. 4. The effect of TQ on the expression of fibrotic genes in PQ-induced lung fibrosis. The doses of 20 and 40 mg/kg of TQ were administered orally to the mice for 14 days (therapeutic) and 28 days (preventive) after i.p. injection of PQ (20 mg/kg). The expressions of α-SMA, TGF-β1, collagen 1a1, and collagen 4a1 mRNA were determined in the lung of the mice by real-time RT-PCR. The data are means ± SD (two replicates in each assay) for 8 mice.
***$p < 0.001$ and **$p < 0.01$ in comparison with the negative control group (Olive oil).
###$p < 0.001$ in comparison with the sham group.

tion (Fig. 4). Meanwhile, TQ treatment in a dose-dependent manner inhibited the mRNA expression of TGFβ-1 in contrast to PQ group.

4. Discussion

PQ either in respiratory or systemic exposure makes an extensive and irreversible lung damage followed by fibrosis which resembles the toxicity of some other pulmonary toxicants like bleomycin (Dong et al., 2016). In the PQ toxicity, the production of free radicals and depletion of antioxidants both contribute to the oxidative stress in the pulmonary cells, especially the alveolar type I and type II epithelial cells, and their damage and destruction (Destructive Phase). When the overproduction of reactive oxygen species exceeds antioxidant capacity of the lung, oxidative stress results in cell and tissue damage. Free radicals accumulation leads to lipid peroxidation and production of oxidation markers such as MDA. SOD and CAT are two important enzymatic defense complexes that protect cells against oxidative damage, and their activities can indirectly represent the levels of lipid peroxidation. Destroying the pulmonary cells induces extensive alveolitis and pulmonary edema which lead to the widespread fibrosis in the lung (Proliferative Phase) (Dinis-Oliveira et al., 2008). Our results showed the prominent fibrogenetic effect of PQ in the lung tissue of the mice and a significant increase in the hydroxyproline content after PQ intoxication. Meanwhile, the oxidative stress parameters including an increase in the lipid peroxidation level and decrease in the SOD activity were seen in the PQ treated group.

Moreover, the results of our study demonstrated that treatment with TQ protected the mice lung against fibrosis induced by PQ. The results also indicated that treatment with TQ significantly decreased MDA level and increased SOD activity. TQ is a potent free radical and superoxide radical scavenger while preserving the activity of various antioxidant enzymes (Woo et al., 2012). Our results are in agreement with the recent studies that reported the protective effect of TQ against lung toxicity induced by other agents like bleomycin and toluene (El-Khouly et al., 2012; Kanter, 2011).

Several studies suggest that TGF-β1, a multifunctional cytokine, is the main cytokine involved in the process of fibrosis via the conversion of fibroblasts to myofibroblasts and collagen synthesis (Meng et al., 2016). It is demonstrated that the elevated production of TGF-β1 in human and laboratory animals is associated with PQ-induced lung fibrosis and other chronic inflammatory and fibrotic diseases (Cheresh et al., 2013). Much of the recent mechanistic work regarding oxidative mechanisms in pulmonary fibrosis has been based on the two-way interplay between TGF-β1 and ROS mediated processes (Cheresh et al., 2013). ROS has been shown to activate latent TGF-β1, and TGF-β1 increases the production of ROS in human lung fibroblasts. Exposure of epithelial cells and fibroblasts to TGF-β1 decreases the levels of the antioxidant enzymes and increases cell cytotoxicity mediated by oxidative stress as well as collagen production. In other words, the emergence of fibroblasts is associated with increased TGF-β1, which stimulate α-SMA actin expression in isolated fibroblasts (Zhang et al., 1996). Fibroblasts exist in a collagen-rich lattice *in vivo*. The interaction of these cells with the surrounding collagen fibrils results in a more dense and compact organization of the matrix. Ultra structural and histochemical studies have shown that the number of myofibroblasts, characterized by α-SMA actin expression, increases progressively in the lung fibrosis. These cell types are responsible for the increase in lung type collagen expression which plays an important role in the pathogenesis of pulmonary fibro-

sis. Their presence may contribute to the increased extracellular matrix deposition and contractility of lung tissue in this disease. Our findings seems to show that administration of TQ blocked TGF-β1-augmented collagen gel contraction, expression of α-SMA and collagen. As anticipated, the data were consistent with the results of the hydroxyproline content and observation under light microscope. In line of our results, Bai et al. (2014) showed the inhibitory role of TQ in hepatic fibroblast proliferation and myofibroblast formation by reducing α-SMA and collagen expression. TQ is also reported to alleviate bleomycin- induced pulmonary fibrosis (El-Khouly et al., 2012). It is interesting to know that TQ not only down-regulates profibrotic factors such as TGF-β1, α-SMA and collagen, as observed in our study, but also suppresses nuclear factor kappa-B (El-Khouly et al., 2012) and inflammatory cytokines (TNF-a, IL-4 and IFN-γ) (Keyhanmanesh et al., 2010; Khader and Eckl, 2014b). Such a wide range of activities warrants further investigation for the potential development of TQ as a treatment agent for lung fibrosis.

5. Conclusion

In conclusion, our present study demonstrates that administration of TQ can reduce lung fibrosis induced by PQ herbicide. The anti-fibrotic effect of TQ may be due to the down regulation of profibrotic genes and inhibition of oxidative stress.

Competing interests

There is no competing interest for the authors.

Authors' contributions

FP carried out all the experiments, analyzed data and was involved in drafting the manuscript. FSh conceived the study and participated in its design. RR also conceived the study and wrote the manuscript. SSM set-up the oxidative stress experiments and was involved in the animal studies. SSM, LP and HSF coordinated the experiments, carried out the Real-time RT-PCR, and analyzed the gene expression data. MI evaluated the pathologic injuries. AM designed the study, was involved in the data analysis and interpretation, and revised the manuscript.

Acknowledgements

We are thankful to Professor Sh. Dabiri for interpreting the histopathological data. The authors also appreciate Dr. M. Ghazi-Khansari and Mr. Mehrabi as the English editor of the article.

References

Ali, B.H., Blunden, G., 2003. Pharmacological and toxicological properties of Nigella sativa. Phytother. Res. 17, 299–305.

Bai, T., Yang, Y., Wu, Y.L., Jiang, S., Lee, J.J., Lian, L.H., Nan, J.X., 2014. Thymoquinone alleviates thioacetamide-induced hepatic fibrosis and inflammation by activating LKB1-AMPK signaling pathway in mice. Int. Immunopharmacol. 19, 351–357.

Cheresh, P., Kim, S.J., Tulasiram, S., Kamp, D.W., 2013. Oxidative stress and pulmonary fibrosis. Biochim. Biophys. Acta 1832, 1028–1040.

Dinis-Oliveira, R.J., Duarte, J.A., Sanchez-Navarro, A., Remiao, F., Bastos, M.L., Carvalho, F., 2008. Paraquat poisonings: mechanisms of lung toxicity, clinical features, and treatment. Crit. Rev. Toxicol. 38, 13–71.

Dong, J., Yu, X., Porter, D.W., Battelli, L.A., Kashon, M.L., Ma, Q., 2016. Common and distinct mechanisms of induced pulmonary fibrosis by particulate and soluble chemical fibrogenic agents. Arch. Toxicol. 90, 385–402.

El-Khouly, D., El-Bakly, W.M., Awad, A.S., El-Mesallamy, H.O., El-Demerdash, E., 2012. Thymoquinone blocks lung injury and fibrosis by attenuating bleomycin-induced oxidative stress and activation of nuclear factor Kappa-B in rats. Toxicology 302, 106–113.

Gawarammana, I.B., Buckley, N.A., 2011. Medical management of paraquat ingestion. Br. J. Clin. Pharmacol. 72, 745–757.

Ghazi-Khansari, M., Mohammadi-Karakani, A., Sotoudeh, M., Mokhtary, P., Pour-Esmaeil, E., Maghsoud, S., 2007. Antifibrotic effect of captopril and enalapril on paraquat-induced lung fibrosis in rats. J. Appl. Toxicol. 27, 342–349.

Hubner, R.H., Gitter, W., El Mokhtari, N.E., Mathiak, M., Both, M., Bolte, H., Freitag-Wolf, S., Bewig, B., 2008. Standardized quantification of pulmonary fibrosis in histological samples. BioTechniques 44, 507–511 (514-517).

Kanter, M., 2011. Thymoquinone attenuates lung injury induced by chronic toluene exposure in rats. Toxicol. Ind. Health 27, 387–395.

Keyhanmanesh, R., Boskabady, M.H., Khamneh, S., Doostar, Y., 2010. Effect of thymoquinone on the lung pathology and cytokine levels of ovalbumin-sensitized guinea pigs. Pharmacol. Rep. 62, 910–916.

Khader, M., Eckl, P.M., 2014a. Thymoquinone: an emerging natural drug with a wide range of medical applications. Iran. J. Basic Med. Sci. 17, 950–957.

Khader, M., Eckl, P.M., 2014b. Thymoquinone: an emerging natural drug with a wide range of medical applications. Iran. J. Basic Med. Sci. 17, 950.

Khalighi, Z., Rahmani, A., Cheraghi, J., Ahmadi, M.R., Soleimannejad, K., Asadollahi, R., Asadollahi, K., 2015. Perfluorocarbon attenuates inflammatory cytokines, oxidative stress and histopathologic changes in paraquat-induced acute lung injury in rats. Environ. Toxicol. Pharmacol. 42, 9–15.

Li, M., Krishnaveni, M.S., Li, C., Zhou, B., Xing, Y., Banfalvi, A., Li, A., Lombardi, V., Akbari, O., Borok, Z., Minoo, P., 2011. Epithelium-specific deletion of TGF-beta receptor type II protects mice from bleomycin-induced pulmonary fibrosis. J. Clin. Invest. 121, 277–287.

Mandegary, A., Sezavar, M., Saeedi, A., Amirheidari, B., Naghibi, B., 2012. Oxidative stress induced in the workers of natural gas refineries, no role for GSTM1 and GSTT1 polymorphisms. Hum. Exp. Toxicol. 31, 1271–1279.

Meng, X.M., Nikolic-Paterson, D.J., Lan, H.Y., 2016. TGF-beta: the master regulator of fibrosis. Nat. Rev. Nephrol. 12, 325–338.

Mohammadi-Bardbori, A., Ghazi-Khansari, M., 2008. Alternative electron acceptors: proposed mechanism of paraquat mitochondrial toxicity. Environ. Toxicol. Pharmacol. 26, 1–5.

Noble, P.W., Barkauskas, C.E., Jiang, D., 2012. Pulmonary fibrosis: patterns and perpetrators. J. Clin. Invest. 122, 2756.

Reddy, G.K., Enwemeka, C.S., 1996. A simplified method for the analysis of hydroxyproline in biological tissues. Clin. Biochem. 29, 225–229.

Ruiz, P.A., Shkoda, A., Kim, S.C., Sartor, R.B., Haller, D., 2005. IL-10 gene-deficient mice lack TGF-beta/Smad signaling and fail to inhibit proinflammatory gene expression in intestinal epithelial cells after the colonization with colitogenic Enterococcus faecalis. J. Immunol. 174, 2990–2999.

Todd, N.W., Luzina, I.G., Atamas, S.P., 2012. Molecular and cellular mechanisms of pulmonary fibrosis. Fibrogenesis Tissue Repair 5, 11.

Tomita, M., Okuyama, T., Katsuyama, H., Miura, Y., Nishimura, Y., Hidaka, K., Otsuki, T., Ishikawa, T., 2007. Mouse model of paraquat-poisoned lungs and its gene expression profile. Toxicology 231, 200–209.

Woo, C.C., Kumar, A.P., Sethi, G., Tan, K.H., 2012. Thymoquinone: potential cure for inflammatory disorders and cancer. Biochem. Pharmacol. 83, 443–451.

Zhang, H.Y., Gharaee-Kermani, M., Zhang, K., Karmiol, S., Phan, S.H., 1996. Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis. Am. J. Pathol. 148, 527–537.