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Pathological Study on the Role of Thymoquinone in Experimentally Induced Acute Lung Injury in Rats

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Abstract

Acute lung injury (ALI) is a major cause of morbidity and mortality in humans and animals. In traditional and modern medicines, *Nigella sativa* extract, thymoquinone (TQ) has several benefits. Here, we examined the counter effects of TQ in ALI induced by Lipopolysaccharide (LPS). Tissue sections and serum samples were collected from the following groups of rats: i) none treated control, ii) TQ only, iii) intratracheally (I.T) installed with LPS 200 µg/rat once, iv) TQ protected received intraperitoneally (I.P) 1 mg/rat for one week. Samples were subjected to histopathology, immunohistochemistry, ELISA and electron microscopy. TQ-treated rats revealed reduction in peribronchial, perivascular and interstitial inflammatory edema, thickening of interalveolar septa, inflammatory exudates in the lumens of airways and alveoli, hypertrophied smooth muscles of pulmonary blood vessels and airways and hyperplasia of bronchial associated lymphoid tissue (BALT). Electron microscopy revealed highly activated pneumocyte with vacuolated cytoplasm in TQ-treated group. Immunomodulators, IL1$_\beta$ and TNFα showed lower levels in TQ-treated group. Meanwhile, NF-κB was absent according to immunohistochemistry. It could be concluded that TQ restores lung architecture and reduces inflammatory Immunomodulators in ALI.

Keywords: Lipopolysaccharide, Thymoquinone, Acute lung injury, Rat

Introduction

Acute lung injury (ALI) is a clinical syndrome that involves disruptions of the gas exchange apparatus, therefore, it is considered as a severe complication often observed in intensive care units. Gram negative bacterial endotoxin lipopolysaccharides (LPS) administration has been used as an animal model of ALI and plays a main important role in the development of ALI. It could lead to mortality rates between 25 and 40% in the United States **[**1]. Many experimental protocols applied to achieve LPS-induced ALI via intratracheal [2,3], intranasal [4], atomized inhalation [5] or intraperitoneal route [6] were reported. The lesion was indicated by a notable inflammatory cells infiltration, interstitial and intra-alveolar edema, thickening of the alveolar septa and airways epithelium, hyaline membrane formation and some collapse in alveoli. ALI induced by LPS showed significant increase in the expression of pro-inflammatory cytokines TNFα, IL-1$_\beta$ IL-6, IL-8 [6-8], NF-κB [9,10], reactive oxygen species (ROS), nitric oxide (NO) and prostaglandin E2 (PGE2) [8]. These inflammatory mediators initiate and amplify the inflammatory response which results in the development of lung injury. Tang *et al.* [3] noted that, the I.T instillation of LPS caused pulmonary edema, microvascular protein leakage and cell damage. Moreover, Liu *et al.* [11] mentioned that the ALI is initially characterized by disruption of the interface between capillary/alveoli resulting in leakage of edema fluid into the interstitial and alveolar space, followed by an extensive release of proinflammatory cytokines, chemokines and infiltrations of neutrophil. These events cause reduced gas exchange and systemic inflammation with multi-organs failure. Ultrastructure changes in mice after LPS instillation was followed by severe injury of alveolar epithelium, swollen and fragmented type I and II cells (lamellar bodies in the alveolar space), intact endothelium of capillaries, hyaline membrane formation and neutrophil apoptosis [12]. Yuan *et al.* [13]

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noticed that the LPS-induced alterations in ultrastructures of endothelial cell (increase in the gap of intercellular junctions) which were abated by treatment with Ginsenoside in a rat.

Prophylactic and treatments attampts were applied to ameliorate the ALI induced by LPS through suppression of proinflammatory cytokines after treatment by acanthotic acid [4], andrographolide sulphate [5], mangolol [2], ginger extract or zingerone [14] and TQ [6,15-17]. TQ is a natural product with main constituents of the volatile oil from *Nigella sativa* seeds which exhibits anti-inflammatory and anticancer activities [18]. TQ had been proved to be effective in the treatment of many respiratory manifestations [9,15,19-23]. El Gazzar *et al*. [15] suggested that TQ attenuates the inflammatory response in LPS-stimulated mast cells by modulating nuclear transactivation of NF-κB and TNFα production. Jafri *et al.* [10**]** reported inhibition of LPS induced NF_k-B by prior treatment with TQ in mice. This study aimed to evaluate the effectiveness of TQ on ALI induced by LPS via evaluation of histopatholoical, ultrastructure and histochemical changes in lung tissue beside proinflammatory cytokines in serum.

Material and Methods

Materials

Thymoquinone (2-isopropyl-5-methyl-1, 4-benzoquinone), available in a yellow crystalline form was purchased from Sigma (St. Louis, Mo, USA). The dose of TQ was 1 mg/200 µL normal saline per rat dissolved in DMSO after I.P injection according to El Gazzar *et al.* [15]. *Escherichia coli* O55: B5 LPS was purchased from Sigma. LPS (I.T) installed by a dose of 200 µg/rat dissolved in 200 µL normal saline according to Hou *et al.* [7]. Ketamine, xylazine and other chemicals were purchased from Kahira Pharmaceuticals & Chemical Industries Company (4 Abdel-Hamid Eldeeb St. Victoria SQ. Shoubra. Cairo – Egypt). $IL1_\beta$ and TNFα ELISA kit were purchased from Abcam® (332 Cambridge Science Park Milton Road Cambridge, Cambridge Shire UK). Immunohistochemistry primary antibody Rabbit polyclonal IgG to rat NF-κB was purchased from sigma.

Animals

Forty male healthy albino rats weighing 150±50 g were used in this study. Animals were obtained from the animal house of the Faculty of Veterinary Medicine, Zagazig University. They were housed for one week before the experiment for acclimatization. They were fed with a standard pellet ration (El-Nasr Chemical Company, Cairo, Egypt) and get free accesses to water *ad libitum*. All animals were managed according to the recommendations of the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Experimental design

Rats were divided into equal four experimental groups (N=10, each). The first group was considered non-treated control group without any treatment. The second group was I.P injected by TQ 1 mg/rat dissolved in DMSO daily for one week. The third group was I.T installed by LPS 200 µg/rat dissolved in 200 µL normal saline once after anesthetized. Finally, the fourth group was I.P injected by TQ daily for one week before and through the LPS installation. Animals were sacrificed after twelve and twenty-four hours from LPS installation. Serum samples and lung tissue specimens were collected from all forty sacrificed rats to study the different parameters.

LPS-induced acute lung injury

LPS-induced ALI was applied after a modified manner similar to previous reports of Rivera *et al.* [24]. Briefly, rats were anesthetized with ketamine-xylazine at a dose 80 mg/kg and 12 mg/kg, respectively, by I.P injection. The rat was fixed on (our designed) glass board at an angle of 70° in a supine position**.** A volume of 200 µL normal saline or normal saline containing 200 µg LPS was installed into the rat trachea by use of a 3-gauge intravenous needle. After I.T instillation, the rats were placed in a vertical position and rotated for 0.5–1 min to distribute the instillation evenly within the lungs.

Table 1: Lesion scoring according to different criteria

	Criteria	Experiment Groups			
		CON	TQ	LPS	TQ-LPS
Airways	Proliferated epithelium and goblet cells metaplasia	-	-	+++	+
(bronchus, bronchioles)	Injury of smooth muscles	-	-	+++	+
	Hyperplasia of bronchial associated lymphoid tissues (BALT)	-	-	+++	+
Pulmonary blood vessels	Injury of endothelium	-	-	+++	+
	Hyperplasia of smooth muscles	-	+	+++	+
	Perivascular edema	-	+	+++	+
Alveolar tissue (alveoli and air-blood parries)	Hypertrophied and hyperplasia of pneumocytes I & II	-	-	+++	+
	Thickening of alveoli septa	-	+	+++	+
	Atelectasis	-	+	+++	+
	Inflammatory cells Infiltration	-	+	+++	+
	Emphysema	-	+	+++	+

- = Normal, += Mild, ++= Moderate, +++= Severe.

Histopathological techniques

Rats were sacrificed after anesthetized on 12 h and 24 h post LPS installation. Pathological specimens (lungs) were collected and put in 10% neutral buffered formalin fixation. Fixed tissues were processed routinely by the paraffin embedding technique [25]. Preparations were evaluated by a light microscopy by using lesions score. Lesion scoring was represented by - = Normal, += Mild, ++= Moderate and +++= Severe according to Robert [26] and Gibson-Corley *et al.* [27].

Electron microscopic techniques

The lung specimens were rapidly sectioned in thin sections and fixed directly in 2.5% glutaraldehyde at pH 7.2 (at ambient temperature) for 4 h and in 1.33% osmium tetroxide buffered overnight at 4 degrees (refrigerator) and embedded in epoxy resin (plastic media). Ultra-thin sections were obtained by ultra-microtome according to Cheville and Stasko [28]. They were evaluated for detection of lesions before the tissue upload on the grid and stained by uranium and lead citrate. Finally, the ultra-sections were evaluated by transmission electron microscopy (JEOL JEM-1230).

Immunohistochemical detection of activated NF-κB

Paraffin-embedded tissue sections of 3 µ thickness were deparaffinized in xylene and then rehydrated in descending graded alcohol. The sections were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 2 h. The sections were immunostained with primary antibody (Rabbit polyclonal IgG to rat NF-κB) at a concentration of 1 g/mL containing 5% BSA in TBS and incubated overnight at 4°C. Post incubation, the slides were washed by TBS, goat anti-rabbit secondary antibodies were added to the slides and then incubated. Sections were washed by TBS then incubated for 5–10 min in 0.02% diaminobenzidine containing 0.01% hydrogen peroxide. Counter staining was applied by using hematoxylin, the slides were visualized under a light microscope.

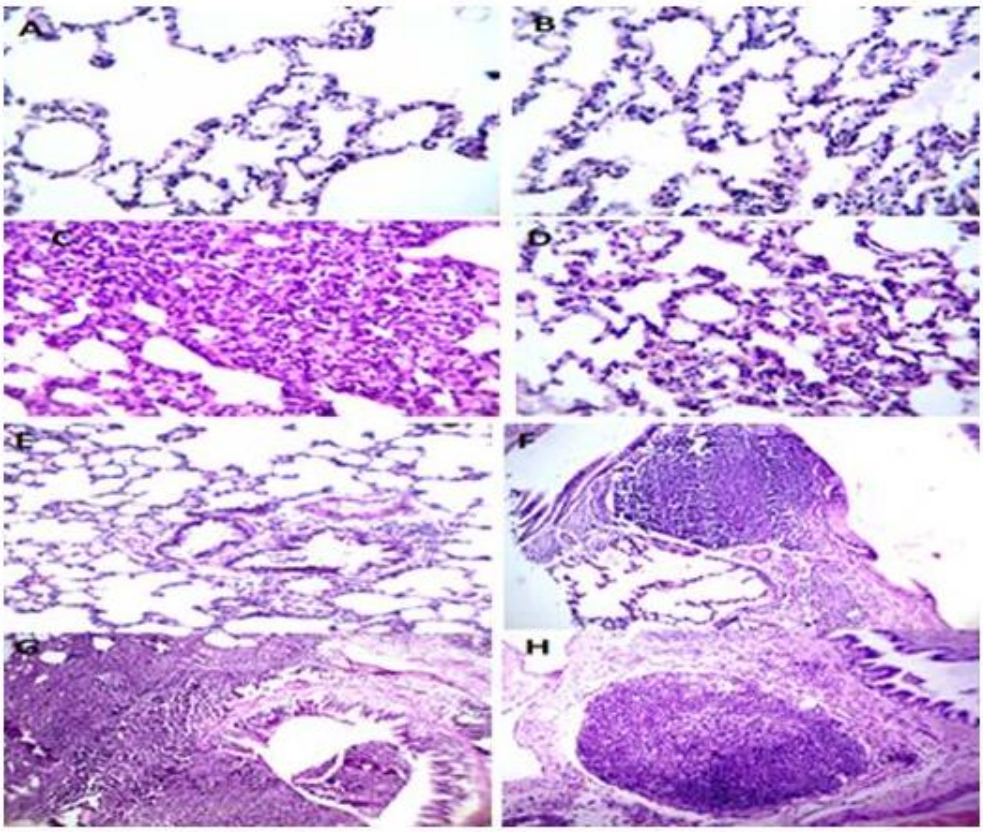

Figure 1: Photomicrograph of rat lung 24 hours post LPS installation showing alveolar normal septa in group A (Control) and group B (TQ only). Thickening of septa with focal replacement by edema and inflammatory cells infiltrations were noticed in group C (LPS) and restore of pulmonary architecture were common in group D (TQ+LPS). H&E X 400. Bronchial tree appeared normal in group E (Control) and mild hyperplastic of BALT in group F (TQ only). Severe thickening of bronchial wall with exudate are common in group G (LPS). Meanwhile, mild hyperplasia of BALT and bronchial epithelial were seen in group H (TQ+LPS) H&E X 200.

Enzyme-Linked Immunosorbent Assay (ELISA)

Sera levels of IL1$_\beta$ and TNFα were measured by using ELISA kit, according to Chehl *et al*. [29]. The sensitivity of the assay was 5.1 pg/mL. The samples were examined in duplicate and the experiment was repeated at least three times.

Results

Histopathological changes

Rats in group i (control) and group ii (TQ only) revealed alveolar septa and lung tissue was within the normal morphohistological structures. Meanwhile, rats instilled I.T with 200 µg LPS and sacrificed 12 and 24 h post installation revealed variable degrees of lesions represented by perivascular, peribronchial and intra-alveolar serous or serofibrinous edema admixed with inflammatory cells mainly lymphocytes, granulocytes, plasma cells and few macrophages. Thickening of alveolar septa was noticed due to congestion and hemorrhage and inflammatory cell infiltrations. Hypertrophy and hyperplasia of pneumocytes type I and type II were detected. The wall of alveoli showed hyalinized and eosinophilic membrane with partial destruction of their lining epithelium and intraluminal infiltration of leukocytes and erythrocytes. Some alveoli showed focal hypertrophic or hyperplastic changes in pneumonia type I and type II. Focal compensatory alveolar emphysema was also seen. Rats in group four which treated with TQ (I.P) prior LPS (I.T)**,** returned back to the normal alveolar septal morphohistological structures, however, mild thickening of alveolar septa by congestion and infiltration of dead and living neutrophils were seen in some cases (Figure 1: A,B,C,D).

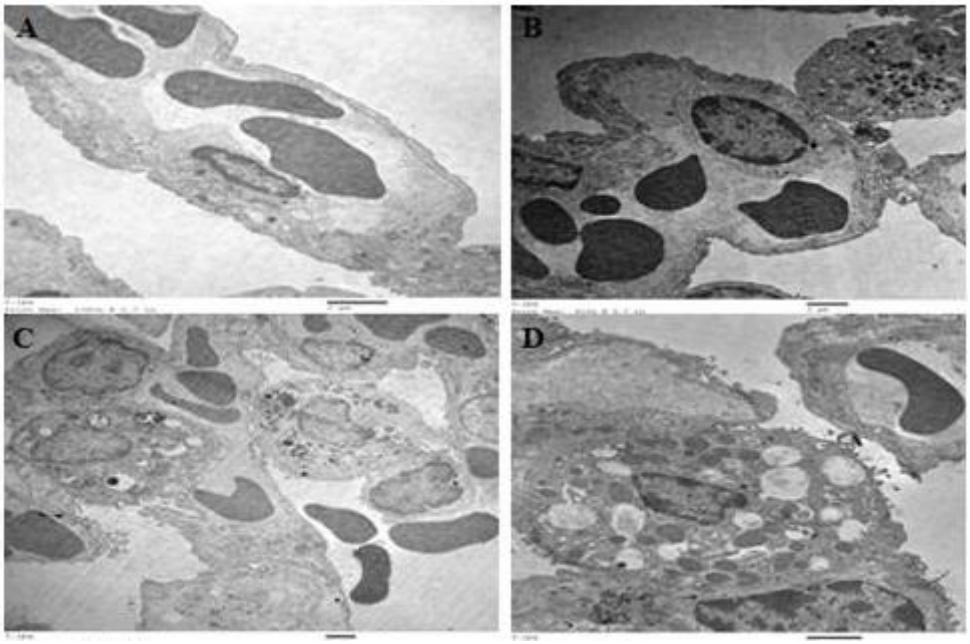

Figure 2: A: Electron micrograph of the ultrastructure of capillaries which appeared blood-air barrier (intact capillary wall) within normal morphology seen in group A (control). Dilated capillaries contain active monocyte and pneumocytes contain dense bodies following by excess surfactant in intercellular gaps were seen in group B (TQ only). Exude pneumocytes contain lamellar bodies and erythrocytes with the alveolar lumen group C (LPS). Highly activated pneumocyte with vacuolated cytoplasm was seen in group D (TQ+LPS). Uranium and lead citrate stain. Magnification A) X 2000, B) X 1500, C) X 1000, D) X 2000.

Bronchial tree in group i (control) appeared within the normal histomorphological architectures. Mild hyperplastic of BALT was observed in group ii (TQ only). Rats instilled LPS revealed acute bronchitis, and bronchiolitis. Their luminae contained few amount of mucus with desquamated epithelium and inflammatory cells. Proliferated epithelia and goblet cells metaplasia were detected with or without intraepithelial inflammatory cells, together with edematous and hypertrophied vascular smooth muscles. Bronchial associated lymphoid tissues (BALT) were hyperplastic and had reactive germinal centers. Rats in group four (TQ+LPS) revealed enhanced curative effects of TQ as represented by mild to moderate acute bronchitis include bronchial epithelium desquamation and infiltration of the bronchial wall by inflammatory cells. Mild hyperplasia of BALT could be seen. Some of the examined sections of the same rats appeared as apparently healthy alveolar and bronchial tissue (Figure 1: E,F,G,H). Other blood vessels revealed vascular endothelial hyperplasia (endotheliosis), subintimal and medial hyalinization and vacuolation in group iii (LPS). Semi-quantitative analyses of lung injury scores for the experimental groups were demonstrated (Table 1).

Ultra-structure changes

Electron microscopic examination of LPS installated rats revealed many ultrastructure changes, mainly thickening of air-blood barriers by activated pneumocytes type II which contained a large amount of lamellar bodies (surfactant). While, some alveolar luminae contained exuded pneumocytes, inflammatory cells and erythrocytes. TQ treated rats showed mild electron microscopically changes as represented by the presence of some lamellar bodies within activated pneumocytes type II and presences of interalveolar degenerated inflammatory cells (Figure 2).

Immunohistochemical changes of NF-κB expression in lung tissue

The concentration of NF-κB was determined by immune-histology. The control and TQ only groups were not stained, while, the TQ+LPS group has obviously decreased positivity rates than the LPS only group (Figure 3).

Enzyme-Linked Immunosorbent Assay for serum levels of IL1$_\beta$ and TNFα

The levels of IL1$_\beta$ and TNFα measured by ELISA technique showed normal levels in rats subjected to TQ only and control (79.05, 82.7 and 175.6, 173 pg/mL) respectively. Rats received LPS had high levels of IL1$_\beta$ and TNFα (182.5 and 231.5 pg/mL), respectively. Meanwhile, rats treated with TQ+LPS revealed a significant reduction in levels of IL1$_\beta$ and TNFα (162.4 and 192 pg/mL), respectively (Figure 4).

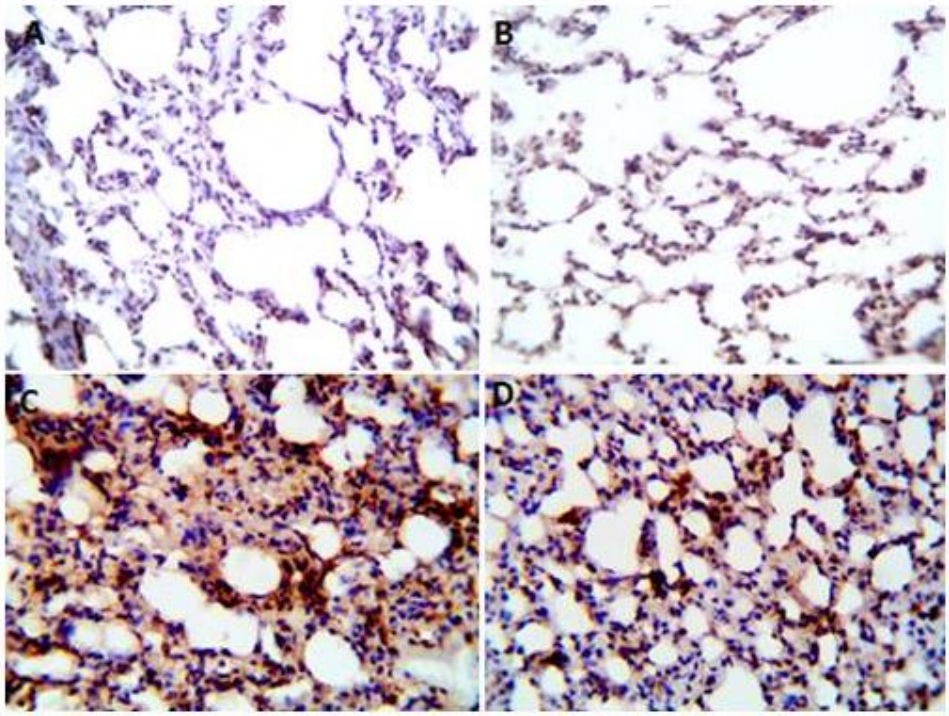

Figure 3: Photomicrograph lung of rat (immunohistochemistry of NF-kB) showing normal expression in both groups A (Control) and B (TQ only). Intense expression of NF-kB was seen in group C (LPS). Mildly immune stain reactivity of NF-kB was noticed in group D (TQ+LPS) X400.

Discussion

TQ has antioxidant and anti-inflammatory activities both *in vitro* and *in vivo* since its extraction from *Nigella sativa* in the 1960s [30]. In the present study, we used rats as an experimental model of LPS-induced acute lung injury to investigate the potential anti-inflammatory effect of TQ. In addition, the underlying mechanisms of TQ were assessed by studying its effect on different parameters mainly pulmonary lesions, ultra-structural changes and proinflammatory cytokines IL1$_\beta$, TNFα as well as the expression of NF-κB in activated form. This investigation declared that LPS could induce a significant lung injury represented by peribronchial, periarteriolar inflammatory edema and thickening of interalveolar wall, hypertrophied and hyperplastic pneumocytes. The vascular tunica had a different degree of angiopathy mainly endotheliosis and hypertrophy of vascular smooth muscle was common. These results coordinate with the results reported by Tang *et al.* [3] and Liu *et al.* [11].

The airways revealed mucous exudates within the lumen admixed by inflammatory cells and desquamated epithelial sheets. Moreover, bronchiolar epithelium showed proliferation with invasiveness by inflammatory cells. Hypertrophied and hyperplasia of BALT were pronounced. These findings were consistent with previous reports [31,32]. Furthermore, emphysema due to the presence of mucus in bronchial lumina was detected, similar finding was also reported [33,34].

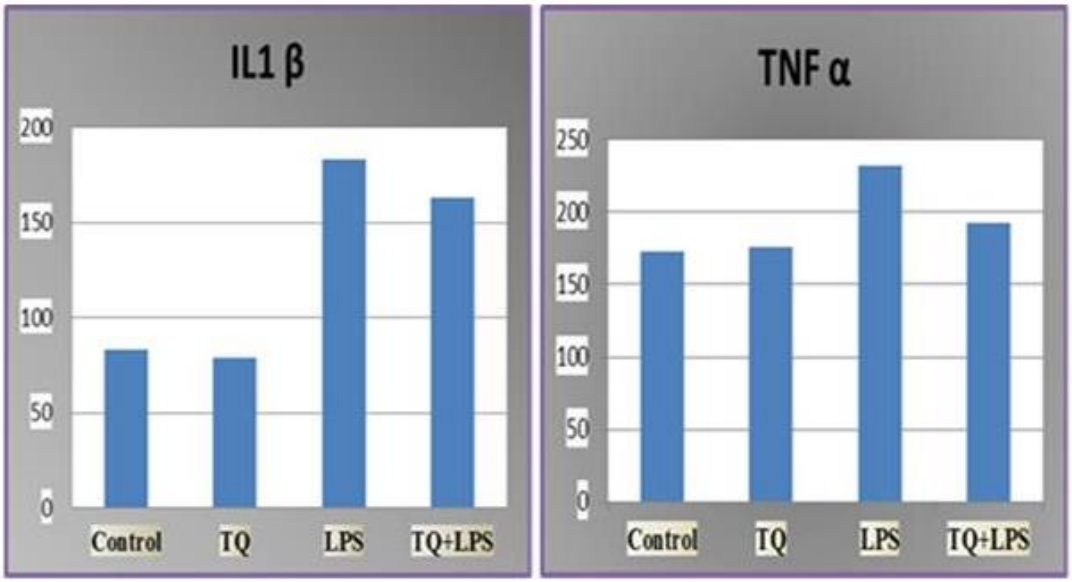

Figure 4: Graphs of serum levels of IL1$_\beta$ and TNFα (pg/ml) in different experiment groups

Moreover, our results revealed a significant increase in sera levels of both IL1$_\beta$ and TNFα. However, NF-κB showed intense reactivity in LPS significant as the previous report [16]. Concurrent treatment of rats with TQ significantly counteracted the inflammatory effect of LPS and moderated all the injury markers up to the normal levels [6,15, 17,35,36]. At the same context, expression of the activated NF-kB revealed a mild degree in rats treated with TQ, a finding consistent with the existence of constitutive NF-κB levels in alveolar macrophages. Recent studies on the anticancer activity of TQ noticed inhibition of NF-κB with regression of epithelial mammary tumor [37,38].

One of the widely mechanisms for LPS-induced ALI declared that LPS is one of the important pathogen-associated molecular patterns (PAMP) and thereby a powerful stimulator of the host inflammatory reaction. LPS elicit this response by binding to the receptor on the surface of cells known as 'pattern recognition receptors' or PRRs which composed of at least three distinct proteins: CD14, Toll-like receptor-4 (TLR4), and Lymphocyte antigen 96. LPS inflammation followed by many inflammatory mediators, including tumor necrosis factor TNF-α and IL1$_\beta$ which play an important role in the cascade of inflammatory events [39].

Conclusion

It is concluded that TQ has ameliorative effects on the ALI via remodeling of the proinflammatory cytokines.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

دراسة باثولوجية عن دور الثيموكينون في اصابات الرئه الحاده المستحدثه تجريبيا في الفئران

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إصابة الرئة الحادة (ALI) سبب رئيسي للوفيات والامراضيه في البشر و الحيوانات. في الأدوية التقليدية والحديثة مستخلص حبة البركه و الثيموكينون (TQ) له العديد من الفوائد. هنا، قمنا بفحص التأثير المضاد للـ TQ علي ALI المستحدث بـ عديد السكريات الدهني (LPS). تم جمع الأنسجة وعينات المصل من المجموعات التالية من الفئران: i) مجموعة التحكم الغير معالجه ii) مجموعه TQ فقط iii) مجموعه الحقن بالقصبة الهوائيه (i.t) بـ LPS ٢٠٠مايكروجرام مرة واحدة iv) المجموعه المحمية بـ TQ حقن في البريتون (i.p) 1ملج لمدة أسبوع واحد. خضعت العينات لفحوصات الهستوباثولوجي، كيمياء الانسجه المناعيه، الاليزا والمجهر الإلكتروني. كشفت الفئران المعالجة بـ TQ تناقص الوذمة الالتهابيه في محيط القصبات الهوائيه و حول الأوعية الدمويه وبين النسج الخلاليه، سماكة من الحاجز بين الحويصلات الرئويه، الافرازات التهابات في تجويف الشعب الهوائية والحويصلات الهوائية و تضخم في العظلات الملساء لجدار الاوعيه الدمويه والقصبات الهوائيه وتضخم الأنسجة اللمفاوية المرتبطة ب الشعب الهوائية (BALT). كشف المجهر الإلكتروني المجهري تنشيطا شديدا للخلايا الرؤيه مع فجوات السيتوبلازم في مجموعة العلاج بـ TQ. مناعيا، $IL1_{\beta}$ و $TNF\alpha$ اظهر مستويات أقل في مجموعة علاج TQ. وفي الوقت نفسه الـ NF-kB كان غائبا وفقا لـ كيمياء الانسجه المناعيه. ويمكن استنتاج أن TQ استعادة بنية الرئة والحد من مناعة التهابات في ALI.