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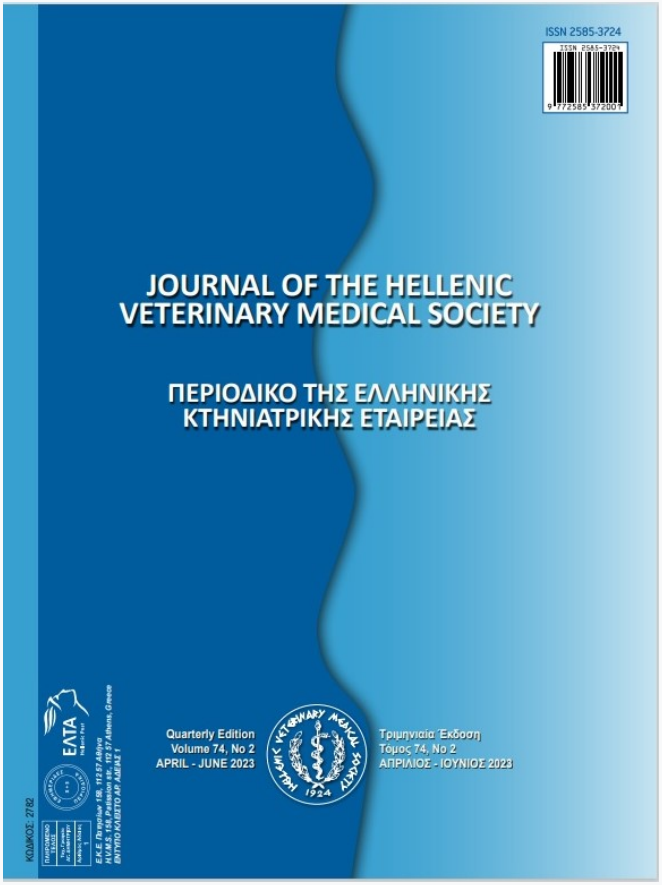

Thymoquinone attenuates doxorubicin-induced lung damage via heat shock proteins, inflammation and endoplasmic reticulum stress

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Research article
Ερευνητικό άρθρο

Thymoquinone attenuates doxorubicin-induced lung damage via heat shock proteins, inflammation and endoplasmic reticulum stress

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ABSTRACT: The aim of this study is to determine possible therapeutic effects of THQ on DOX induced lung damage in rats. Rats were divided into five groups (n = 8): control, THQ, olive oil, DOX (a single dose of 15 mg/kg intraperitoneally (i.p.) on seventh day of the experiment), and DOX+THQ (10 mg/kg THQ per day and 15 mg/kg DOX i.p. on seventh day). Animals were euthanized, and lung tissues were evaluated histopathologically and immunohistochemically. Caspase 3, GRP78, GADD153, HSP90, HSP70, PCNA, COX2 and TNFα immunostaining were performed to determine the expression levels of these proteins among groups. TUNEL method was used for evaluation of apoptotic index. Moreover, total antioxidant status (TAS), total oxidant status (TOS) and IL6 in lung tissue were measured by ELISA assay. The DOX group had histopathological deterioration compared to the control group. There was an increase in apoptotic index, caspase 3, GRP78, GADD153, HSP90, HSP70, PCNA, COX2 and TNFα expressions in the DOX group. While TAS level of the DOX group decreased, IL6 and TOS level increased when compared with the other groups. However, there was improvement in lung tissue in DOX + THQ group compared to the DOX group. There was a decrease in apoptotic index, caspase 3, GRP78, GADD153, HSP90, HSP70, PCNA, COX2, TNFα expressions and TOS, IL6 in DOX+THQ group compared to the DOX group. We suggest that THQ can be used as a protective and therapeutic agent to reduce the toxic effects of DOX.

Keywords: Doxorubicin, lung, rat, thymoquinone.

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INTRODUCTION

A hundred years ago, cancer was not so prevalent; but, since the last couple of decades, its incidence has been rising importantly. Cancer is one of the most terrible diseases of the 20th century and spreading further with continuance and increasing incidence in the 21st century (Roy PS & Saikia BJ, 2016). Chemotherapy is an substatial method in vital diseases, such as cancer, however chemotherapy drugs generally cause notable toxicity. Chemotherapy drugs are used for treatment of several cancer types. Doxorubicin (DOX), an anthracycline antibiotic, is one of them. Several studies have reported that the mechanism of anticancer activity is inhibition of DNA replication by inhibiting DNA topoisomerase II activities, RNA and DNA polymerase. These limitations cause the apoptosis in most cell types. In addition, its utilization is limited due to detrimental side effects on several organs (Ahmed et al., 2013). Especially, studies have been reported to be cellular lipid peroxidation, oxidative stress and apoptosis (Jo et al., 2018). Herbal medicines play a important role for treatment of many disease in recent years. Thymoquinone (THQ), an antioxidant and anti-inflammatory, is a component of Nigella sativa seed oil. Its high biological activity and low systemic toxicity make it a promising alternative to traditional therapeutic drugs (Darakhshan et al., 2015).

Heat shock proteins (HSPs), have cell protective properties, are one of the intracellular chaperones. Overexpression of HSPs is induced by many types of cell stress, such as toxic compounds, inflammation and oxidative stress (Ciocca & Calderwood, 2005). HSP90 and HSP70 heat shock proteins are among HSPs. HSP90, which functions together with HSP70, has been demonstrated to be include in cell-cycle regulation, apoptotic pathways and signal transduction (Öztürk et al., 2020). Also, impaired protein synthesis in the cell leads to endoplasmic reticulum stress (ERS), which is regulated by the some proteins. The key protein of endoplasmic reticulum stress is glucose regulatory protein 78 (GRP78). If ERS doesn't improve, C/EBP Homologous Protein (CHOP, GADD153) induces apoptosis (So, 2018). Apoptosis has a significant role in most defense mechanism such as immune reaction triggered when cells are injured by hazardous agents or a disease (Norbury & Hickson, 2001). In this study, we planned to investigate the relationship among inflammation, heat shock proteins, endoplasmic reticulum stress and apoptosis in DOX-induced lung injury. We studied the proteins of these mechanisms immunohistochemically to examine the effect of THQ on them. We also evaluated histopathologically and examined antioxidant and oxidant levels biochemically.

MATERIALS AND METHODS

The study protocol was accepted by the Erciyes University's Experimental Animal and Local Ethics Committee with number 19/084/2019. In this study, all the animals received human care according to standard guidelines. In this study, 40 male wistar albino rats (10 weeks old, weighing 220-240) were gotten from Experimental and Clinic Research Center, Erciyes University, Kayseri, Turkey. Rats were allowed ad libitum Access to food and water and kept at a 12-h light:dark cycle at room temperature (20-24 C). Firstly rats were randomly divided into five groups as follows: The control group (n=8) were untreated rats, the olive oil (n=8) group administered olive oil intraperitoneally (i.p.) (100 microliter/rat), the THQ (n=8) group given 10 mg/kg THQ i.p. (274666-5G, purity 98%, Sigma–Aldrich Co., St Louis, MO, USA) per day (dissolved in olive oil) throughout the experiment. The DOX (n=8) group injected with a single dose of 15 mg/kg i.p DOX on seventh day of the experiment and the DOX+THQ (n=8) group administered 10 mg/ kg THQ per day and 15 mg/kg DOX i.p. on seventh day of the experiment. Experiment continued for 14 days. Animals were anesthetized with 4 mg/kg xylazine and 30 mg/kg ketamine at the end of the experiment. Then, blood samples were taken for serum isolation and lung tissues were taken and preserved in formaldehyde solution. Collected serum samples were centrifuged at 3000 r/min for 10 minutes. Serum samples were kept at -80°C for further biochemical testing.

Histopathological evaluation: The lung tissues were fixed in %4 formaldehyde fixative for histopathological evaluation. Following dehydration (50%, 70%, 80%, 96% and three times absolute alcohol) and clearing (xylene) embedded in paraffin. 5-µm-thick sections were stained with Hematoxylen-Eosin (H-E). Photographs were taken with a light microscope (Olympus BX51, Center Valley, PA, USA) (Karabulut et al., 2020).

Immunohistochemistry

To determine the differences in expression of Caspase-3 (Cas3) (ab4051, anti-caspase3 antibody, abcam), Cyclooxygenase-2 (COX2) (E- AB- 30999, Elabsciens, China), Tumor necrosis factor alpha (TNFα), HSP70 Heat Shock Protein 70 (HSP70) (sc-33575, Santa Cruz Biotechnology, Santa Cruz, CA), Heat Shock Protein 90 (HSP90) (PB9635; Boster Biolog-

ical Technology, Pleasanton, CA), : proliferating cell nuclear antigens (PCNA) (sc-56, Santa Cruz Biotechnology, USA), C/EBP Homologous Protein (CHOP, GADD153) (sc-56107, Santa Cruz Biotechnology, USA) and Glucose Regulated Protein (GRP78) (bs-1219R; Bioss) in lung tissue, streptavidin-biotin-peroxidase method was used for marking. . The procedure was explained in our previous study (8). Under the light microscope (Olympus BX51, Center Valley, PA, USA) and images were obtained Caspase3, COX2, PCNA, TNFα, HSP70, HSP90, GADD153 and GRP78 immunoreactivity were measured with image J programme.

Apoptosis (TUNEL)

The terminal deoxynucleotidyl transferase 20 -deoxyuridine, 50 -triphosphate nick-end labeling (TUNEL) method was used to demonstrate apoptosis of lung tissue, as previously described. (8). An in situ Cell Death Detection Kit Fluorescein Kit (11684795910; Roche, Mannheim, Germany) was utilized. After casing the tissues with a solution containing glycerol, they were all examined with the Olympus BX51 fluorescence microscope at 450-500 nm wavelength. Cells were considered to be apoptotic when the cell nuclei demonstrated positive TUNEL staining. For quantification of TUNEL positive cells, 10 fields per section were analyzed and counted at 400 fold magnification.

Elisa assay

We centrifuged blood samples taken from rats at 10,000 g at 4C for 15 min. Total antioxidant status (TAS) (DZE201112672, Sunred Biological Technology Co., Ltd., 96 wells ELISA kit, Shanghai, China), Total oxidant status (TOS) (DZE201111669, Sunred Biological Technology Co., Ltd., 96 wells ELISA kit, Shanghai, China), Interleukin 6 (IL6) (Cat. No: 201-11-0136, Sun Red) were measured in lung tissue. The ELISA procedure was done according to the protocol recommended by the manufacturers.

Ethical Committee: 19/084/2019 of Erciyes University Experimental Animals

Statistical analysis: All statistical analyses were carried out using SPSS statistical software (SPSS for Windows, version 24.0, SPSS Inc., Chicago, Illinois, USA) and graphs were drawn using GraphPad Prism 8.0 software. The Kolmogorov–Smirnov test was used to identify normal distribution of the data. In case of normal distribution, quantitative variables were compared using one-way analysis of variance and Tukey's post hoc test. The data were presented as the mean of normalized data + standard deviation of mean. The value of $P<0.05$ was considered as statistically significant.

RESULTS

Histopathological findings

The lung tissues of control, olive oil and THQ groups exhibited normal histomorphology. DOX induced venous congestion, hemorrhage, bronchiole bleeding and loss of epithelial cells in rats. DOX+THQ group had normal histologic appearance (Figure 1).

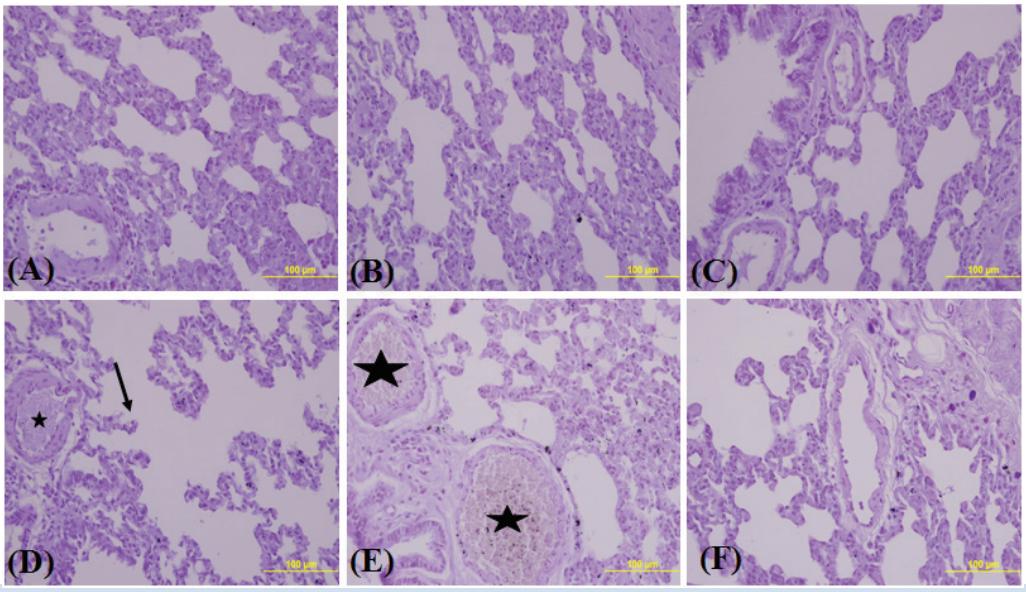

Figure 1. H-E staining of lung tissue. (A-C) In control, Olive oil and THQ groups, normal lung appearance were shown. In DOX group (D, E), venous congestion, hemorrhage (star), bronchiole bleeding and loss of epithelial cells in rats (black arrow). (F) In DOX+THQ group, normal lung architecture was exhibited.

Immunohistochemical findings

Immunohistochemical staining was performed using the avidin–biotin method to determine the lung tissue expressions of ERS, inflammation, heat shock protein and apoptosis markers. Expressions of Caspase 3, GRP78, CHOP, HSP90, HSP70, COX2, PCNA and TNFα were observed in the alveoli in DOX group. The Caspase 3, GRP78, CHOP, HSP90, HSP70, COX2, PCNA and TNFα expressions in the lung of olive oil and THQ groups were similar to those in the control group.

Caspase 3, GRP78, CHOP, HSP90, HSP70, COX2 and TNFα expressions of DOX+THQ were significantly less compared to those in the DOX group and PCNA expression was statistically higher in the DOX+THQ group compared to the DOX group. (Table1, Figure 2,3 and 4).

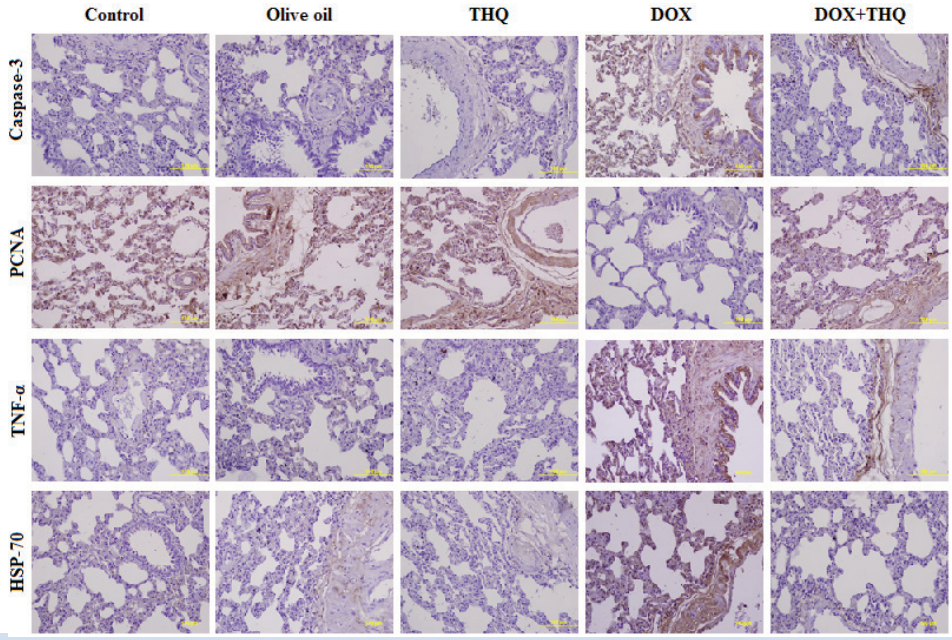

Figure 2. Caspase-3, PCNA, TNF-α and HSP-70 immunohistochemistry staining of lung tissue in experimental groups. Control group, Oive oil, THQ, DOX and DOX+THQ group. DOX group stained immunohistochemistry out of PCNA. Abbreviations: THQ: Thymoquinone; DOX: Doxorubicin. Scale bar 100 μm.

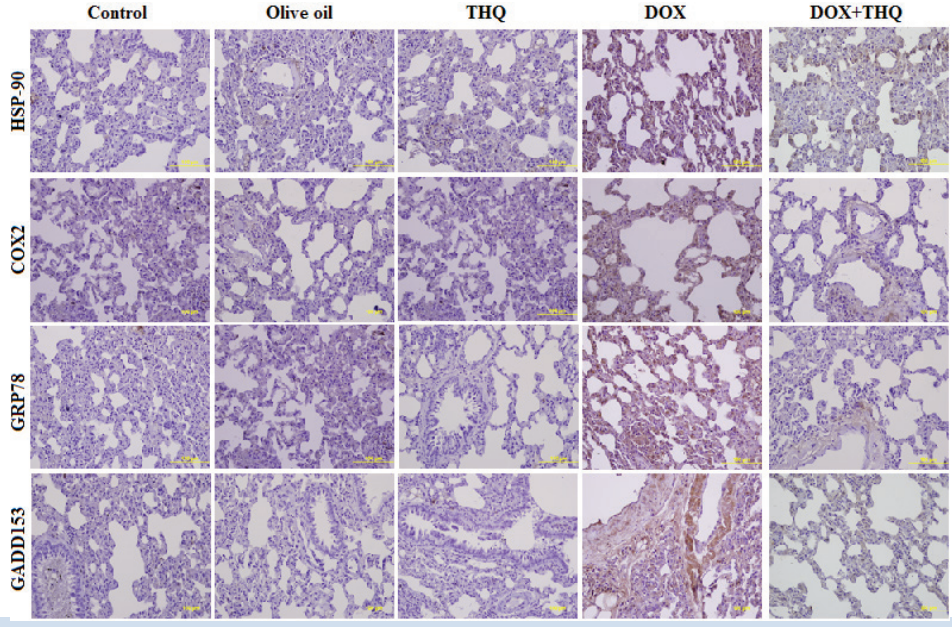

Figure 3. Caspase-3, PCNA, TNF-α and HSP-70 immunohistochemistry staining of lung tissue in experimental groups. Control group, Oive oil, THQ, DOX and DOX+THQ group. DOX group stained immunohistochemistry out of PCNA. Abbreviations: THQ: Thymoquinone; DOX: Doxorubicin. Scale bar 100 μm.

Table 1. Immunohistochemistry results of experimental groups.

Groups	Group I	Goup II	Group III	Group IV	Group V	P
Caspase-3 immunoreactivity	84.60±3.0^{6}a	84.44±3.16^{a}	85.22±3.16^{a}	106.85±9.67^{b}	89.95±5.31^{c}	0.0001
PCNA positive cell counts	66.30±15.8^{3}a	67.38±14.88^{a}	67.12±16.94^{a}	7.24±4.51^{b}	50.04±14.09^{c}	0.0001
TNF-α immunoreactivity	84.61±2.77^{a}	84.49±2.88^{a}	84.50±2.76^{a}	102.00±4.91^{b}	91.49±2.33^{c}	0.0001
HSP-70 immunoreactivity	83.97±2.41^{a}	83.77±2.2^{4}a	83.90±2.63^{a}	104.98±3.97^{b}	91.42±3.92^{c}	0.0001
HSP-90 immunoreactivity	83.88±2.50^{a}	83.28±3.26^{a}	83.88±3.47^{a}	103.09±2.02^{b}	92.70±2.68^{c}	0.0001
COX2 immunoreactivity	84.67±2.54^{a}	84.57±2.54^{a}	85.79±2.69^{a}	103.57±3.08^{b}	92.90±4.17^{c}	0.0001
GRP78 immunoreactivity	83.90±3.22^{a}	83.87±3.35^{a}	84.37±2.86^{a}	102.04±5.22^{b}	92.18±3.25^{c}	0.0001
GADD153(CHOP) immunoreactivity	85.17±3.54^{a}	85.23±3.54^{a}	85.09±3.03^{a}	100.89±9.44^{b}	92.33±3.16^{c}	0.0001

Goup I: Control. Goup II: Olive oil, Group III: Thymoquinone, Group IV: Doxorubicin, Group V: Doxorubicin+thymoquinone. The data are expressed as mean + SEM. P<0.05 was accepted as significant. There were no significant differences between the groups expressed with the same letter (a-b-c).

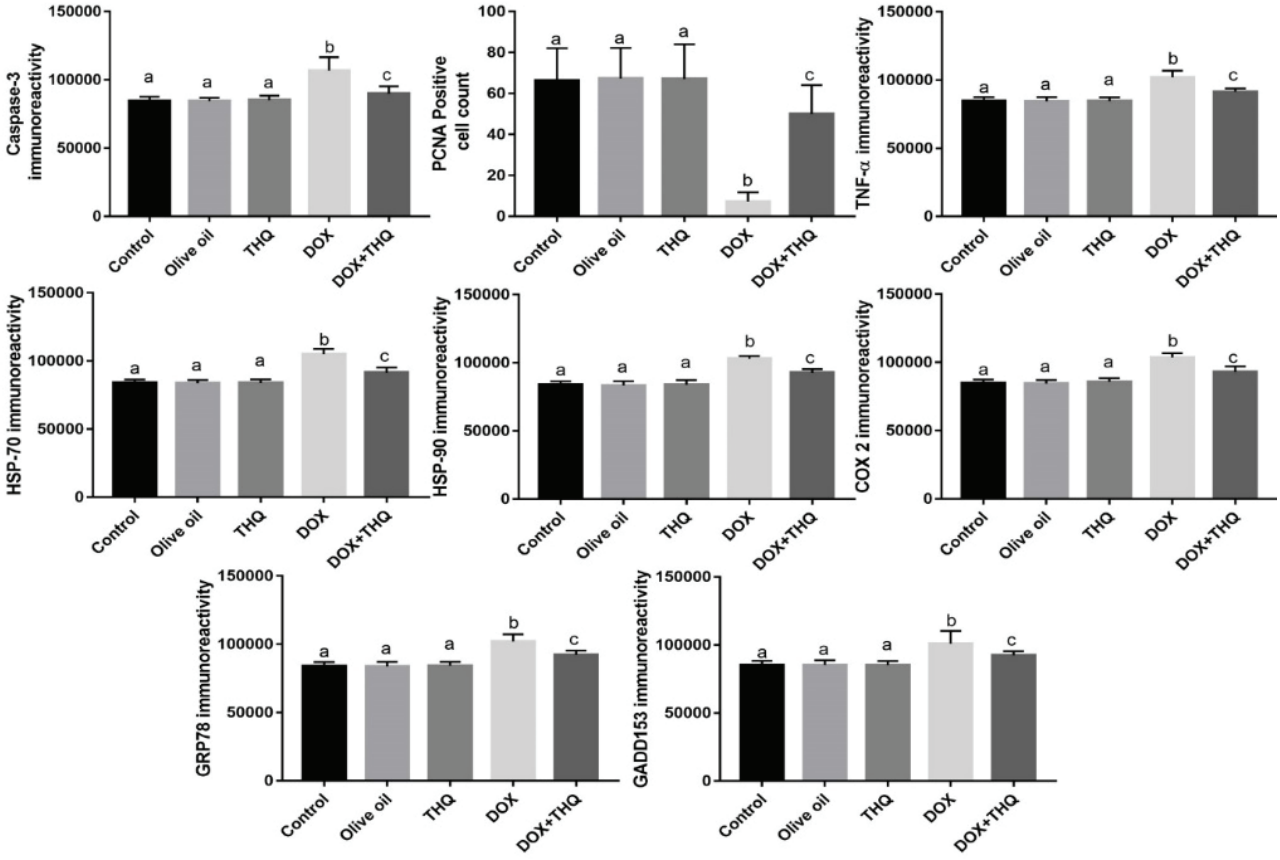

Figure 4. Results of caspase-3, TNF-α, HSP-70, HSP-90, COX2, GRP78 and GADD153 immunoreactivity measurements, PCNA positive cell count and statistical analysis of them in experimental groups. Graphs show that this marker expressions are statistically significant in the DOX group when compared to control, olive oil, and THQ groups. Abbreviations: DOX: doxorubicin; THQ: thymoquinone; PCNA: proliferating cell nuclear antigens; TNF-α: tumor necrosis factor-alpha; HSP-70: heat shock protein-70; HSP-90: heat shock protein-90; COX2: cyclooxygenase-2; GRP78: glucose regulated protein78; GADD153: growth arrest and DNA damage- inducible gene 153.

Biochemical findings

TAS level was lower in the DOX group compared to the other groups. However, TOS and IL6 levels were importantly higher in the DOX group than in the other groups. There was no statistically importance in TAS, TOS and IL6 levels between the other groups excluding DOX group (P>0,05). (Table 2).

Apoptotic findings

TUNEL staining was performed to determine apoptotic cells in lung tissue. The apoptotic cells in the lungs of control, olive oil and THQ groups were found 0.23±0.44, 0.38±0.54 and 0.37±0.52 respectively. There was no statistically significance among this groups. The increase in the apoptotic cell number in DOX group was found 3.89±1.17 and was statistically significant when compared to the control group (P<0.0001). In DOX+THQ group, there was a decrease in TUNEL-positive cells and the apoptotic cell number (0.84±0.83). The decrease in the apoptotic cell number was statistically different in DOX+THQ when compared to the DOX group (P<0.0001). (Table 3, Figure 5).

Table 2. Biochemical results of experimental groups. TAS, TOS and IL-6 levels of lung tissue obtained by ELİSA assay among groups.

Groups	Group I	Goup II	Group III	Group IV	Group V	P
TAS (U/ml)	10.56±0.75[a]	10.54±0.75[a]	10.53±0.69[a]	6.92±0.40[b]	9.35±0.91[a]	0.001
TOS (nmol/ml)	1.26±0.60[a]	1.26±0.34[a]	1.26±0.69[a]	4.85±0.62[b]	1.97±0.73[a]	0.001
IL-6	28.34±3.71[a]	29.60±2.33[a]	28.95±5.29[a]	47.34±5.26[b]	32.10±4.06[a]	0.001

Goup I: Control. Goup II: Olive oil, Group III: Thymoquinone, Group IV: Doxorubicin, Group V: Doxorubicin+thymoquinone. All data are expressed as the mean±SD (n=8). There is no significant difference among groups with same letters (a, b, c).

Table 3. Statistical analysis of TUNEL positive cell count.

Groups	Group I	Goup II	Group III	Group IV	Group V	P
TUNELpositive cell counts	0.23±0.44[a]	0.38±0.54[a]	0.37±0.52[a]	3.89±1.17[b]	0.84±0.83[c]	0.0001

Goup I: Control. Goup II: Olive oil, Group III: Thymoquinone, Group IV: Doxorubicin, Group V: Doxorubicin+thymoquinone. The data are expressed as mean + standard deviation. There is no significant difference between groups containing the same letter (a, b and c). P <0.05 was considered significant.

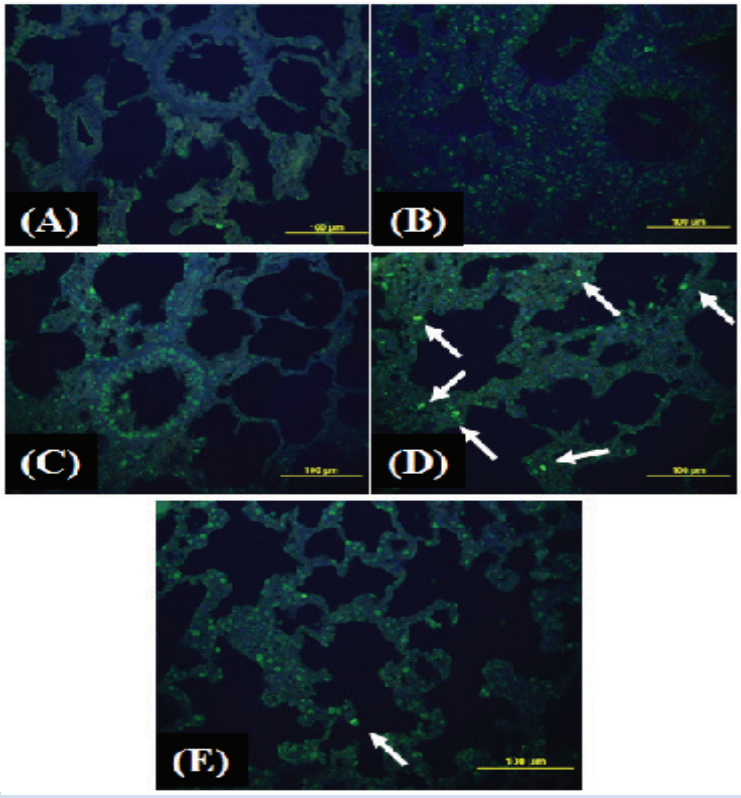

Figure 5. TUNEL staining of lung tissue. A, Group I: Control; B, Group II: Olive oil; C, Group III: THQ; D, Group IV: DOX; Group V: DOX+THQ. The white arrow indicates TUNEL-positive cells. Scale bar = 100 µm. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

DISCUSSION

Doxorubicin, has an influential anticancer activity widely used as a chemotherapeutic drug, is a member of the anthracycline family. However, its usage is limited by side effects, especially on vital organs (Zhang et al., 2017). There are only a few studies investigating the effect of doxorubicin on the lung. we found that 15 mg/kg DOX had a toxic effect on lung tissue and 10 mg/kg THQ reduced this effect. In this study, DOX induced Venous congestion, hemorrhage, bronchiole bleeding and loss of epithelial cells in rats. There are very few studies done so far that show that doxorubicin causes lung damage either alone or with another chemotherapy drug (Suddek et al., 2013). In this study, we demonstrated the reducing effect of thymoquinone on lung injury caused by doxorubicin.

The endoplasmic reticulum (ER) is an organelle containing chaperone proteins and enzymes responsible for protein folding. As the amount of incorrectly folded or unfolded protein increases in the endoplasmic reticulum, stress occurs in the cell, and this condition is called endoplasmic reticulum stress (Bhandary et al., 2012). Sustained ERS causes apoptosis (Iurlaro & Muñoz-Pinedo, 2014). Apoptosis has an important role in the development of DOX damage in many tissues (Wang et al., 2018). In the study, it was observed that the expression of endoplasmic reticulum stress proteins GRP78 and endoplasmic reticulum stress-related apoptosis marker CHOP (GADD153) increased in doxorubicin group compared to other groups (Chen et al., 2015). Some studies have reported an increase in the expression of endoplasmic reticulum stress proteins in doxorubicin organ damage (Schopf et al., 2017).

Heat shock proteins 90 (HSP90) and 70 (HSP70) are two families of highly conserved ATP-dependent molecular chaperones that fold and remodel proteins. Both are important components of the cellular machinery involved in protein homeostasis and participate in nearly every cellular process. Although HSP90 and HSP70 each carry out some chaperone activities independently, they collaborate in other cellular remodeling reactions (Radli & Rüdiger, 2018). HSP90, which functions together with HSP70, has been demonstrated to be include in cell-cycle regulation, signal transduction, and apoptotic pathways (Gao et al., 2019). In this study, we found that DOX group showed a significant increase in HSP90 and HSP70 expressions compared to the other groups. Until now, there are no studies that examined heat shock proteins in the lungs with doxorubicin. There are studies showing an increase in heat shock protein expression in the damage caused by doxorubicin in other organs (Pecoraro et al., 2015).

TNFα is a lower molecular weight protein originated by macrophages contained in inflammatory reactions which are concerned to apoptosis (Hinz & Brune, 2002). Recent studies reported that COX-2 may also play a major role in multiple physiological processes. The expression of COX-2 is arranged by a wide spectrum of mediators involved in inflammation (Molehin et al., 2019). In studies conducted so far, it has been reported that there is an increase in inflammation protein expressions in organs caused by doxorubicin (Kelleni et al., 2015). In this study, we showed that there was an increase in the expression of inflammation markers TNF-α and COX-2 via immunohistochemically in lung damage caused by doxorubicin. Also, there was an increase in the level of IL6 biochemically in dox group. THQ, with its antioxidant, anti-inflammatory and anti-carcinogenic properties, is used as a antioxidant in many organ damages (Darakhshan et al., 2015).

Studies on lung injury of doxorubicin are few. Take et al. reported that doxorubicin causes lung damage together with paclitaxel in their study (Take et al., 2008). In another study, they showed that fullerenol C60(OH)24 reduced the damage to lung damage caused by doxorubicin in rats, thanks to its antioxidant property (Srdjenovic et al., 2010). There are studies showing that thymoquinone has a therapeutic and protective effect on lung damage (Al-Gabri et al., 2019).. In their study, Yetkin et al. showed the protective effect of thymoquinone on lung damage caused by smoking (Yetkin et al., 2020). In the study of Boskabady et al., they reported that thymoquinone reduced inflammation and pathological changes caused by Lipopolysaccharide in the lung (Boskabady et al., 2021) In another study, they showed that benzo(a) pyrene administered to rats caused lung damage and thymoquinone had a protective role (Alzohairy et al., 2021).

Our study showed that THQ clearly inhibited histopathological damage caused by DOX in the lung tissue. THQ is a protective agent that reduces the negative effects of DOX. It is known as antioxidant and antiapoptotic agent without negative effects. In the present study, we found that THQ decreases oxidative stress, ERS, apoptosis and inflammation caused by DOX.

ACKNOWLEDGEMENTS

This study was conducted as a research project and has not been presented as a paper anywhere.

ETHICAL STATEMENT

The study protocol was accepted by the Erciyes University's Experimental Animal and Local Ethics Committee with number 19/084/2019.

CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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