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Investigation of the Effect of Thymoquinone on Kidney Damage in Isoproterenol-Induced Myocardial Infarction in Rats and Cardiorenal Interactions

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Abstract

This study aimed to determine whether thymoquinone has any protective effects on renal tissue after an isoproterenol-induced myocardial infarction (MI). Experimental groups were formed as 4 groups (n=8). Control group (C). Thymoquinone group (THQ), 20 mg/kg THQ was administered intragastrically (i.g.) daily as a single dose for seven days. Isoproterenol group (ISO), ISO was administered 100 mg/kg intraperitoneally in two doses on days seventh and eighth of the experiment. Thymoquinone+Isoproterenol group (THQ+ISO), THQ 20 mg/kg i.g. was administered once a day for seven days. In addition, two doses of ISO 100 mg/kg i.p. were administered on the seventh and eighth days. Kidney tissues were evaluated histopathologically. Tumour necrosis factor alpha (TNF-α) and alpha Smooth Muscle Actin (α-SMA) immunoreactivity density changes were determined by immunohistochemistry.

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Glutathione (GST), Glutathione S-transferases (GSTs) and Interleukin-6 (IL-6) levels were evaluated by ELISA method. Isoproterenol injection caused severe histopathological changes on kidney tissue. Also TNF-α and α-SMA levels were found to be higher in groups where ISO was administered. THQ could be effective on kidney tissue to partially correct these histopathological damages, by decreasing fibrosis and inflammation. This study shows that treatment with THQ is effective in preventing kidney damage caused by ISO-induced MI. In this case, we think that the use of THQ as a supplementary food will be effective in preventing kidney damage.

Key words: *Isoproterenol, Thymoquinone, Inflamation, Fibrosis, Kidney*

Introduction

Isoproterenol is a catecholamine that damages heart tissue by acting as an adrenergic agonist. In experimental animal models, it is also a crucial myocardial infarction inducer (1–3). There are quite comprehensive studies conducted with myocardial infarctions and protective phyto-chemical (4, 5). In addition, other studies have shown that myocardial infarction affects organs such as the brain, liver and kidneys as well as the heart (6). There is a complex interaction between the heart and kidneys due to physiological, biochemical and hormonal relationships. Cardiorenal syndrome is a general term used to refer to the interaction between heart and kidney. Myocardial infarction frequently results in complications including acute renal damage (7, 8). Myocardial infarction is one of the most common life-threatening conditions that can cause kidney disease through inflammation and oxidative stress. Essentially, the heart and kidneys have complex physiological and endocrinological connections; in other words, a heart condition can have an impact on the kidneys, and the reverse is true for a kidney condition. The most significant bioactive component included in Nigella sativa (black cumin) essential oil at a rate of 18.4–24% is thymoquinone (THQ) (9, 10). It also has gastroprotective (11), hepatoprotective (12), neuroprotective (13) and nephroprotective (14) properties. Thus, it was aimed to determine the protective effect of thymoquinone, which may be an alternative compound used against the kidney damage caused by ISO-induced myocardial infarction. Antioxidant enzymes are known to be responsible for the elimination of oxidative damage caused by free radicals (15). Glutathione (GSH) is an endogenous antioxidant. It is a regulator that helps protection of cells from ROS, free radicals, and peroxides (16).

Glutathione transferases (GSTs) are one of the most important detoxifying enzyme families in the nature (17). THQ, induces GST and GSTs expression and/or activity (18, 19). Interleukin-6 (IL-6), is an important mediator of acute phase response activated with inflammation and an effective pro-inflammatory cytokine with anti-inflammatory and protective properties (20). Additionally, tumor necrosis factor alfa (TNF-α) is other candidate pro-inflammatory cytokine that might cause renal fibrosis (21).

In this study, histopathological examinations as well as the demonstration of TNF-α and α-SMA in the tissue for the determination of ISO-induced kidney damage are important in terms of revealing the severity of the damage. Determination of the related fibrotic changes (α-SMA) and controlling the change in cytokines (TNF-α and IL-6) are factors that will help to understand the extent of damage. In addition, evaluation of GSH and GST levels in the blood will give an idea about oxidative damage in terms of systemic effects of ISO and THQ.

THQ is a substance that is well-tolerated, safe, and exhibits beneficial anti-inflammatory and antioxidant activities (22). Previous research has shown that different materials can protect against renal and myocardial infarction (3). However, the mechanisms of action of thymoquinone on renal injury in myocardial infarction are not clearly known. The present study evaluates thymoquinone's protective effects on renal tissue after isoproterenol-induced myocardial infarction.

Method

Animals

In this study, 32 adult Wistar albino male rats weighing 200-300 g, 3 months/12 weeks old, raised at Erciyes University Experimental and Clinical Research Centre (DEKAM) were used. The rats were kept in cages at 21 ^{0}C and 12 hours of light/dark environment at the normal course of the day, and their water and feed needs were met. Rats were weighed and those with similar weights were brought together to form experimental groups. Experimental groups were formed as follows: Control group (C) (n=8), Thymoquinone group (THQ) (n=8), Isoproterenol group (ISO) (n=8), Thymoquinone + Isoproterenol group (THQ+ISO) (n=8).

Ethical Committee

Ethics committee approval was obtained from Erciyes University Animal Experiments Local Ethics Committee for the tissues used in this study (approval dated 02.06.2021 and numbered 21/129). The study was carried out in accordance with the principles of "Guide for the Care and Use of Laboratory Animals".

Experimental procedure

Group C: (Control) No action was taken for seven days.

Group THQ: Starting from day 1 of the experiment, thymoquinone 20 mg/kg intragastrically was administered as a single daily dose for 8 days.

Group ISO: Two doses of 100 mg/kg intraperitoneal isoproterenol were given on the 7th and 8th days of the experiment.

Group THQ+ISO: Starting from the 1st day of the experiment, 20 mg/kg thymoquinone was administered once a day for 8 days. On the 7th and 8th days, two doses of 100 mg/kg ISO were administered (23). Isoproterenol was dissolved in 1 ml distilled water and thymoquinone was dissolved in 500 µl saline. The experiment was terminated 24 hours after the second ISO dose administered on the 8th day of the experiment. At the end of the experiment, blood samples were collected from the animals under ketamine (75 mg/kg) + xylazine (10 mg/kg) anaesthesia. Afterwards, kidney tissues were rapidly removed and placed in 10% formaldehyde solution.

Histological Analysis

At the end of the experiment, kidney tissues were fixed in 10% formaldehyde solution. After fixation, the kidney tissues were

dehydrated in increasing degrees of alcohol (50%, 70%, 80%, 96%, 3x100). After transparency with xylol, they were embedded in paraffin. Sections of 5 µm thickness taken from paraffin blocks were stained with Hematoxylin-Eosin to evaluate the histological structure. TNF-α and α-SMA immunoreactivity intensity changes were determined by immunohistochemistry.

Hematoxylin-Eosin (H-E) staining protocol

Tissue sections were incubated at 58 ºC and then passed through xylene 3 times for 10 minutes. Then they were passed through decreasing alcohol series (2x100%, 96%, 80%, 80%, 70% and 50%). Sections were kept in hematoxylin solution for 10 minutes and kept in eosin solution for 5 minutes. The sections were passed through increasing alcohol series (70%, 96%, 3x100), kept in xylene, covered with entellan and examined under light microscope (19).

Immunohistochemistry staining protocol

The Avidin-Biotin-Peroxidase Complex (ABC) method and a couple of immunohistochemical methods were used to determine TNF-α and α-SMA immunoreactivities in the kidney. Paraffin section was cut at a thickness of 5 µm on slides in the microtome and kept in an oven at 58°C overnight. Then, they were rehydrated with a series of progressively lower grades of alcohol (100%, 96%, 80%, and 70%) after being deparaffinized with xylene. After rehydration, the sections were washed in distilled water 3 times for 5 minutes. For antigen retrieval, tissues were heated for 15 minutes in 10% citrate buffer in a microwave oven at 600 W. The sections were washed with phosphate buffered saline (PBS) and treated with 3% hydrogen peroxide (H_2O_2) for 12 minutes in order to block endogenous peroxidase. For the next stages, the Large Volume Detection System (Thermo Fisher Scien-tific, Waltham, MA, USA, Catalog no: TP-125-HL) immunochemistry staining kit was used. To prevent background staining, the sections were treated with Ultra V block for 5 minutes. Then, the sections were incubated overnight at 4°C with TNF-α (bs-2081R, Bioss, USA) and α-SMA antibodies (bsm-33188m, Bioss, USA). Negative controls were treated with PBS in place of the primary antibodies. Processes in the other stages mentioned above were carried out in the same way. After primary anti-body incubation, the sections were rinsed. Reverse staining was performed using appropriate biotinylated secondary antibodies (biotinylated goat anti-polyvalent, TP-060-BN, Thermo Scien-tific), Streptavidin-HRP (Horse Radish Peroxi-dase), DAB (3,3'-Diaminobenzidine) chromogen, and Gill's Hematoxylin (24). Finally, the sections were cleared in xylene before being coverslipped with Entellan® (Merck, Kenilworth, NJ, USA). Sections stained with TNF-α and α-SMA were captured under Nikon Ni-U (Nikon, Tokyo, Japan) model light microscope with DS-Ri2 model digital camera.

Quantitative immunohistochemistry

8 slides from each group were evaluated for TNF-α and α-SMA immunoreactivity intensity. Photographs were taken from each slide by selecting the area from 5 cortex, 5 medulla regions. The immunoreactivity of 5 areas in each photograph was measured. In total, 400 areas were evaluated for each group. The density of these areas was measured using the Image-J Program. The data obtained were evaluated statistically.

Biochemical Analysis

Blood samples were taken from the inferior vena cava after anesthesia and serum was

separated. To obtain serum, blood serum samples were taken into empty tubes and centrifuged at 1500 g for 10 minutes. The serum obtained was used to determine the levels of Glutathione (GSH) (Sunred Biological Technology, Cat No: 201-11-5137), Glutathione S-transferases (GSTs) (Sunred Biological Technology, Cat No: 201-11-5110) and Interleukin-6 (IL-6) (Sunred Biological Technology, Cat No: 201-11-0136). Analyses were performed spectrophotometrically using commercial ELISA kits (Enzyme-Linked Immuno Sorbent Assay) according to the kit protocol (96 Wells Elisa Kit; Sun Red Biological Technology Co, Ltd).

Statistical analysis

The data obtained for this investigation were evaluated using the JASP 0.14.1 package program. The normal distribution of the data was evaluated with skewness and kurtosis values. Anova test was performed because the data showed normal distribution. Tukey HSD test was used if variance homogeneity was present (in TNF α immunoreactivity analysis), Dunn test was used to determine significant differences between groups (α-SMA immunoreactivity analysis) after Anova.

RESULTS

Histology, H+E Findings

Histopathological analysis of kidneys in control and THQ groups showed that glomerulus and tubules were in normal structure (Figure 1; A,B,C, D,E,F).

Significant pathological changes were observed in ISO-treated rat kidneys. These were: renal interstitial haemorrhage, focal necrosis, hyperaemia, mononuclear cell infiltration, large vacuolisation of renal tubule epithelium and swelling of renal tubular cells, atrophic tubules filled with eosinophilic material, congestion in blood vessels, atrophy in some glomeruli (Figure 1; G,H,I). Especially in the ISO group, areas of intense haemorrhage in both the cortex and medulla of the whole tissue were quite remarkable.

In THQ+ISO group, swelling of renal tubular cells, renal interstitial haemorrhage and atrophic tubules filled with eosinophilic materials were observed (Figure 1; J,K,L). It was noted that these pathological changes were observed less in the ISO group. Mononuclear cell infiltration and haemorrhage areas in the whole tissue area were also less compared to the ISO group.

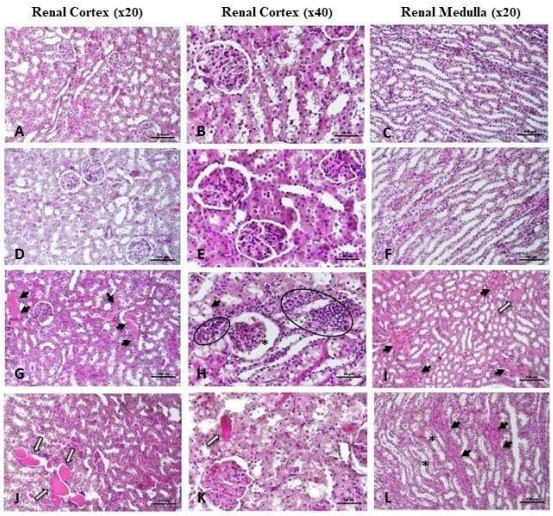

Figure 1: Structure of kidney in different groups of rats. A,B,C; In controls and D,E,F; THQ group rats, normal kidney architecture was observed. G,H,I; ISO groups. G; renal interstitial hemorrhage, hyperemia (arrows); H, enlargement of the renal capsule and atrophy of the glomerulus(*), severe vacuolization of the renal tubule epithelium and cellular swelling of renal tubular cells (arrow), mononuclear cell infiltration (marked areas) I, atrophic tubules filled with eosinophilic secretion (white arrows), renal interstitial hemorrhage (arrows); J,K,L; THQ+ISO groups. J, K, atrophic tubule filled with eosinophilic secretion (white arrow); L, renal interstitial hemorrhage (arrows), tubules with swelling of renal tubular epithelial cells (*); Section were stained with H & E. A, C, D, F, G, I, J, L; Scale bar = 100 µm, B, E, H, K; Scale bar = 50 µm.

Immunohistochemistry

TNF-α immunoreactivity was presented in Figure 2 for all groups. Results of statistical analysis were also presented in Table 1. According to results of statistical analysis, there was significant difference between all groups. There were no significant differences only between C and THQ groups (p =0.001), (Table 1).

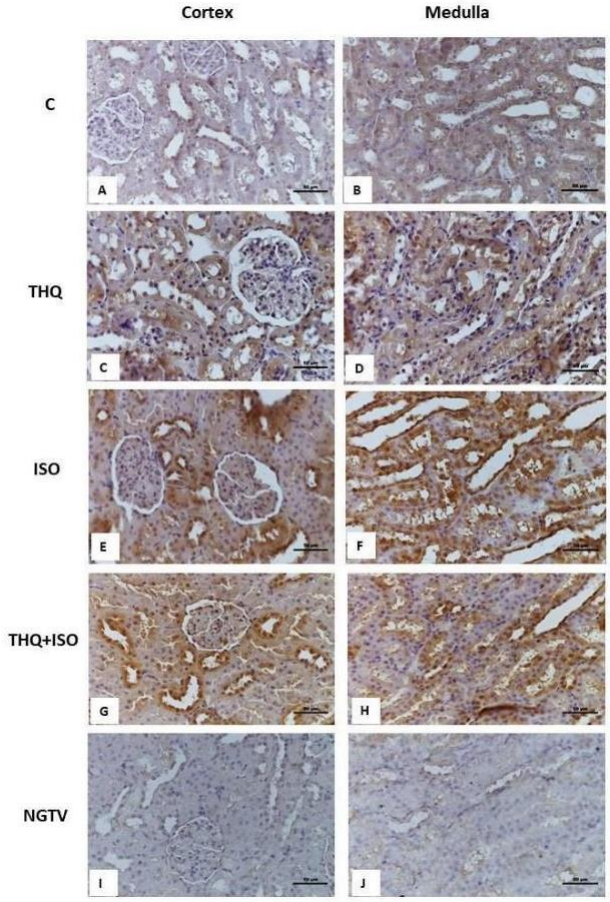

Figure 2: Immunohistochemical localization of TNF-α expression of the kidney tissue are seen in the all groups (A,B) Group C; (C,D) Group THQ; (E,F) Group ISO; (G,H) Group THQ+ISO and (I,J) Negative controls, Bar:50µm.

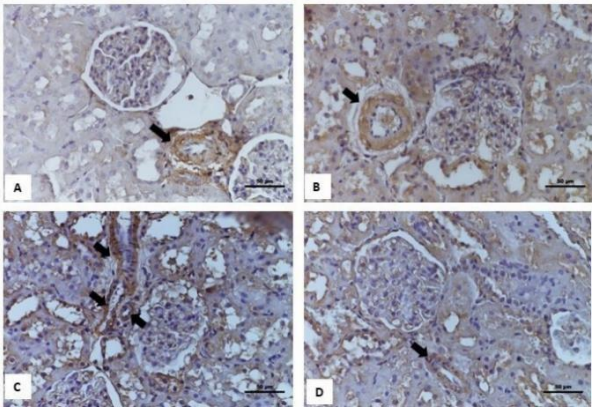

Figure 3: Immunohistochemical localization of α-SMA expression of the kidney tissue are seen in the all groups (A) Group C; (B) Group THQ; (C) Group ISO; (D) Group THQ+ISO. A,B,C,DX40, Bar:50µm.

α-SMA immunoreactivity was presented in Figure 3 for all groups. Also results of statistical analysis were also presented in Table 1. According to results of statistical analysis there was significant difference between C-ISO groups and THQ-ISO groups (p =0.001), (Table 1).

Biochemistry

When GSH and GSTS data were evaluated, no statistically significant difference was found between the groups (Table 1). However, it was remarkable that GSH was higher in the ISO group compared to the other groups and GSTS was higher in the ISO group. IL-6, one of the pro-inflammatory cytokines, did not show a significant difference between the groups (Table 2).

Table 1: Immunoreactivity intensity values of TNF-α and α-SMA in kidney tissue

	Groups				
	Control	**THQ**	**ISO**	**THQ+ ISO**	**p**
TNF-α (n=400)	123.18±7.3[a]	122.15±9.83[a]	129.99±9.96[b]	126.49±9.5[c]	0.001
α-SMA(n=400)	118.78±8.09*	120.32±8.75	121.87 ±9.22	119.47 ±8.99[*]	0.001

All data are expressed as the mean±SD. p <0.05 was considered as significant. * p < 0.05 in comparison with group ISO.
The same letters indicate that there is no significant difference between the groups, and diffrent letters indicate that there is a significant difference between the groups. Abbreviations: **THQ**, thymoquinone; **ISO**, isoproterenol.

Table 2: GSH, GSTS and IL-6 serum ELISA values

	Groups					
	Control	**THQ**	**ISO**	**THQ+ ISO**	**p**	**n**
GSH (ng/L)	518.11±68.74	481.27±114.44	409.52±79.87	497.23±117.85	0.272	6
GSTS(ng/ml)	67.67±23.20	66.50±19.04	78.82 ±29.29	61.31 ±10.97	0.560	6
IL-6 (pg/ml)	139.85±69.57	147.74±42.78	118.96±51.96	169.84±68.09	0.366	7

All data are expressed as the mean±SD. p <0.05 was considered as significant.
Abbreviations: **THQ**, thymoquinone; **ISO**, isoproterenol; **GSH**, glutatyon; **GSTS**, Glutatyon-S-transferaz; **IL-6**, interleukin 6.

Discussion

In this study investigated protective effects of thymoquinone on kidney damage caused by myocardial infarction induced by isoproterenol. According to the results, histopathological changes in tubule epithelial cells and renal damage such as significant renal interstitial haemorrhage occurred in renal tissue. In addition, TNF-α and α-SMA levels were higher in isoproterenol-treated groups. Thymoquinone may act on renal tissue to partially correct these histopathological damages by reducing fibrosis and inflammation.

There is an interaction between heart and kidney tissue called cardiorenal syndrome. Clinical studies show that heart disease is associated with decreased renal function. Patients with myocardial infarction have been shown to develop secondary organ failure in the kidney, liver, brain or blood (6). Acute kidney injury has been observed in approximately 20% of patients hospitalised with myocardial infarction (25). Considering this finding, prevention of kidney damage in myocardial infarction seems to be extremely important. Mechanisms such as reduction of oxidative stress, inflammation and fibrosis may be effective in the prevention of kidney damage (7). Decreased inflammation,

decreased TNF-α and α-SMA immunoreactivity and decreased renal damage in THQ+ISO group increase the possibility of thymoquinone protecting the kidney.

Thymoquinone is thought to be effective on oxidative stress. In a study with ISO, it was found that cellular antioxidants, catalase activity and glutathione concentrations decreased in ISO-induced rats, while the levels of oxidative stress markers increased significantly. Inflammatory cell infiltration and fibrosis in the kidneys were found to increase in the ISO group.

THQ is a powerful antioxidant and it was found to protect against oxidative damage directly by degrading H_2O_2 to water and indirectly by raising GSH levels (26–28). In line with previous reports, current findings suggest that THQ has strong antioxidant activity against oxidative stress caused by ISO in kidney (29, 30) . This suggests that GSH level decreases to overcome oxidative stress (30). Differences in GSH levels with TMQ administration indicate antioxidant and free radical scavenging activities similar to other studies (29, 31). In a study with ISO administration, it was also noted that GSH concentration in kidney homogenates was not significantly different between groups (3). According to the findings of this study, there was no significant difference between the groups in terms of GSH (endogenous antioxidant) and GSTs levels, while GSH levels were lower in the ISO group and higher in the THQ+ISO group, which is consistent with other studies. At the same time, it is important that GSTs level increased in the ISO group and decreased to levels close to the control group in the THQ+ISO group. Therefore, these results suggest that the antioxidant property of thymoquinone may be effective in ameliorating tissue damage. Also cellular damage with free radical intermediate also develops inflammatory response in tissues (32). ISO treatment also causes a major inflammatory cell infiltration wave in heart and kidney tissues compared to control rats. Inflammatory cells usually contribute to extracellular matrix (ECM) accumulation in tissues and start fibrosis (33, 34).

Fibrosis leads to organ damage and failure. Previous studies have shown that renal fibrosis is strongly associated with the development of chronic kidney disease (35, 36). Renal fibrosis causes renal failure, hypertension, anaemia and electrolyte deficiency. Therefore, anti-fibrosis may be a target therapy for cardiovascular diseases in the future. Cardiac and renal fibrosis develops as a consequence of various cardiovascular diseases. In contrast, cardio-renal fibrosis causes progression of heart and liver diseases (36, 37).

Previous studies have reported that cardio-renal interactions may be associated with renal fibrosis and endothelial-mesenchymal transition (EndMT) in rats with isoproterenol-induced heart failure. In this experimental study, it was reported that relaxin, which is thought to be effective in cardiac and renal fibrosis, may improve renal fibrosis in rats with ISO-induced heart failure. It was shown that relaxin decreased renal collagen deposition, α-SMA and TGF-β expression, thus possibly inhibiting renal EndMT in the kidneys (38).

The expression of α-SMA, which is an indicator of smooth muscle cells and myofibroblasts, was found only in and around blood cells, but not in glomerular, cortical and medullary epithelial cells. In our study, the highest expression level was found in the ISO group, whereas its expression

decreased in the THQ+ISO group. Therefore, THQ may have a partial protective effect on renal fibrotic changes caused by myofibroblast increase.

Studies show that THQ has a protective effect on the kidneys in various pathogenic conditions. THQ partially decreased oxidative stress and inflammation and provided healing of kidney damages caused by various toxic substances by having a protective effect (10).

Tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are potent pro-inflammatory cytokines with anti-inflammatory and protective properties (20, 39). They play an important role in the development of cardiovascular and renal diseases (40, 41). Reduction of oxidative stress and inflammation may contribute to the prevention of post-infarction renal injury (42). One of the aims of this study was to evaluate the changes in TNF-α and IL-6 levels and to investigate the protective effects of THQ on myocardial infarction-induced kidney injury. IL-6 levels did not differ significantly between the groups. However, in a study evaluating the cytoprotective effect of THQ in ISO-induced aortic tissue damage, it was concluded that ISO causes an increase in the expression of vascular inflammatory cytokines IL-6 and IL-17 in the aortic endothelium and wall, and THQ may regulate these levels (23). ISO was found to increase TNF-α in renal tissue, whereas treatment with THQ increased TNF-α immunoreactivity closer to control group values. This suggests that THQ may have reno-protective effects due to its anti-inflammatory properties.

According to studies, experimental animals are susceptible to kidney damage brought on by exposure to chemotherapeutic drugs, heavy metals, pesticides, and other environmental toxins. This kidney damage can be reduced by giving the animals black cumin and THQ. According to the available data, black cumin/THQ-mediated renoprotective actions are caused by molecular processes involving the NF-kB, caspase, and TGF- signaling pathways (42, 43). In addition to these parameters, important pathways such as TNF-α/IL-6, GSH, GSTs and α-SMA should be investigated. There are limited studies on the effect of THQ against renal damage in myocardial infarction. Our study is important because it investigated the protective effect of THQ against kidney injury through these parameters.

In addition to this, the protective effects of THQ on renal histopathological changes were demonstrated by light microscopic analysis. Similar to other studies (3), significant pathological changes were found in light microscopic analysis of the kidney following ISO administration. Especially intense renal intestinal haemorrhage, atrophic tubules filled with eosinophilic secretion, mononuclear cell infiltration and swelling of tubule cells were important pathological findings following ISO administration. In THQ+ISO group, atrophic tubules filled with eosinophilic secretion were observed as in ISO groups. However, haemorrhage in the whole tissue and swelling of epithelial cells in some tubules decreased. This situation shows the protective effects of THQ.

In this study, THQ treatment was started before ISO administration and the experiment was completed 2 days after ISO administration. It is considered very important to continue THQ treatment for a period of time after ISO is given to determine

the therapeutic effects on damage. This situation is accepted as the limitation of the article.

Conclusions

In the light of these findings, this study demonstrates that treatment with THQ is effective in preventing kidney injury caused by ISO-induced MI. We think that THQ as a dietary supplement would be effective to prevent kidney damage in patients with MI. It is also important to evaluate the kidney in the treatment of MI and to try alternative therapies in addition to drug therapy. Further studies for early diagnosis and treatment of cardiorenal interactions will help to understand the pathophysiological mechanisms.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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