ARAŞTIRMA / RESEARCH

Evaluation of the cytoprotective effects of thymoquinone on isoproterenol-induced rat aorta

Timokinonun izoproterenol ile indüklenen sıçan aortu üzerindeki sitoprotektif etkilerinin değerlendirilmesi

Rifat Özmen[1] , Necla Değer[2] , Derya Karabulut[2]

[1]Erciyes University Faculty of Medicine, Department of Cardiovascular Surgery, [2]Department of Histology-Embryology, Kayseri, Turkey.

Abstract

Purpose: The aim of this study was to evaluate histologically and immunohistochemically the cytoprotective effects of thymoquinone (THQ) against isoproterenol (ISO)-induced aortic tissue damage.

Materials and Methods: Rats were divided into four groups (n=8). Control group (Control); were untreated rats, Thymoquinone group (THQ); 20 mg / kg intragastrically (ig) THQ at 24 hour intervals for 8 days, Isoproterenol group (ISO); on the 7th and 8th day of the experiment, 100 mg/kg subcutaneous (sc) ISO (dissolved in 1 ml sterile distilled water) was given at 24 hour intervals. Thymoquinone + Isoproterenol group (THQ+ISO); THQ was administered ig at 20 mg/kg for 8 days, and 100 mg/kg ISO was administered on day 7 and day 8 of the experiment. Aortic tissues and blood were collected from rats. Tissues were stained by hematoxylin-eosin and immunohistochemically by interleukin-6 (IL-6) and interleukin-17 (IL-17). TNF-α, ELISA was examined in blood sera.

Results: Aortic wall thickness was found to be increased in the ISO group compared to the control and THQ groups. In addition, IL-6 and IL-17 immunoreactivity increased in this group. IL-17 height was statistically significant. THQ corrected both the increase in wall thickness and the expression levels of IL-6 and IL-17. TNF-α was found to be decreased in the ISO group, but no statistically significant difference was observed between the groups.

Conclusion: THQ serves as a cytoprotective agent against ISO-induced aortic tissue damage.

Keywords:. Isoproterenol, timokinon, aort.

Öz

Amaç: Çalışmada isoproterenol (ISO) kaynaklı aort doku hasarına karşı timokinonun (THQ) sitoprotektif etkilerinin histolojik ve immunohistokimyasal olarak değerlendirilmesi amaçlanmıştır

Gereç ve Yöntem: Sıçanlar dört gruba ayrıldı. (n=8). Kontrol grubu (Kontrol); tedavi uygulanmadı, Timokinon grubu (THQ); 20 mg / kg intragastrik (ig) 8 gün boyunca 24 saat aralıklarla THQ, Isoproterenol grubu (ISO); deneyin 7. ve 8. gününde 100 mg / kg subkutan (sc) ISO (1 ml steril distile suda çözüldü) 24 saat aralıklarla uygulandı. Timokinon+Isoproterenol grubu (THQ+ISO); THQ ig 20 mg/kg 8 gün boyunca ve 100 mg/kg ISO deneyin 7. ve 8. gününde uygulandı. Sıçanların aort dokuları ve kanları alındı. Dokular hematoksilen eozin ve immunohistokimayasal olarak interlökin-6 (IL-6) ve interlökin-17 (IL-17) boyandı. TNF-α, kan serumlarında ELISA ile incelendi.

Bulgular: ISO grubunda aort duvar kalınlığı kontrol ve THQ grubuna kıyasla arttı. Ayrıca bu grupta IL-6 ve IL-17 immunoreaktivitesi de artış gösterdi. IL-17 istatistiksel olarak anlamlıydı. THQ hem duvar kalınlığını hem de IL-6 ve IL-17 ekspresyon seviyelerini düzeltti. TNF-α, ISO grubunda azaldı, fakat gruplar arasında istatistiksel bir farklılık gözlenmedi.

Sonuç: ISO kaynaklı aort doku hasarına karşı THQ sitoprotektif bir ajan olarak hizmet eder.

Anahtar kelimeler: Isoproterenol, thymoquinone, aorta.

INTRODUCTION

Isoproterenol is mainly a beta-1 and beta-2 adrenergic receptor agonist. These adrenergic receptors exert their effects through a G-alpha stimulating second messenger system. While beta-1 adrenergic receptors are mainly concentrated in heart tissue, beta-2 adrenergic receptors cause inactivation of myosin light chain kinase (MLCK) leading to smooth muscle relaxation[1]. Isoproterenol, as a synthetic catecholamine, causes severe stress in the heart tissue and infarction-like necrosis of the myocardium[2]. It has been reported in various studies that it shows this effect by increasing the production of ISO-induced free radicals and causes metabolic disorders in the heart tissue[3,4]. Although it is known that ISO therapy causes myocardial oxidative stress in heart tissue, information on its effects on the vascular system is limited. ISO causes intracellular cyclic adenosine monophosphate (cAMP) to increase by G-protein coupled receptor activation. Thus, protein kinase A (PKA) is activated and phosphorylates MLCK, which is responsible for the phosphorylation of myosin in these smooth muscle cells. Myosin-actin cross-bridge formation allows the muscle to contract. It may cause peripheral and bronchial dilatation, gastrointestinal and uterine smooth muscle relaxation by causing MLCK inactivation via ISO beta-2 receptor agonist[1,5].

In addition to the known cardiac effect of ISO, its effects on the aorta, which is an indispensable part of the vascular system, should also be revealed. Endothelial dysfunction due to vascular oxidative stress has been shown in the aortic tissue of rats treated with ISO[6]. In general, it has been reported that increased nitric oxide synthase activity (NOS) is important in maintaining vascular tone and that various cytokines such as interleukins are associated with endothelial dysfunction[7,8]. Therefore, it is important to investigate the effects of ISO-induced vascular oxidative stress on cytokine release and to develop related treatment options.

Herbal medicines have always been of great importance from the past to the present and have been used by the public as a material in the treatment of various diseases. Thymoquinone (THQ) is the most abundant component of the essential oil of *Nigella sativa* seeds (black seed or black cumin). THQ has been reported to have great potential as an antioxidant and anti-inflammatory agent[9]. It also prevents DNA damage by clearing free radicals caused by oxidative stress, which play a role in the pathogenesis of different cancer types[10]. THQ acts on the pathways involved in the proliferation of tumor cells by inhibiting interleukin-6 (IL-6)[11]. It also reduces inflammation by reducing tumor necrosis factor-alpha (TNF-α) and IL-6 in blood and tissues[9,12].

ISO causes an increase in the level of cAMP in smooth muscles, resulting in activation of adenylate cyclase and thus relaxation[13]. It also affects inflammatory cytokine release due to increased vascular oxidative stress[14]. Therefore, our hypothesis is the possible antihypertensive effect of THQ in ISO-induced vascular damage to the aorta. This will be evaluated based on the presence of IL-6 and IL-17 cytokines. In addition, the cytoprotective effect of THQ in the changes caused by ISO in the aorta will be revealed through histological and cytokines. With this study, the possible antihypertensive effects of THQ will be brought to the literature.

MATERIALS AND METHODS

Animals

Thirty-two Wistar albino rats in this study were used obtained from the Hakan Çetinsaya Experimental and Clinic Research Center, Erciyes University, Kayseri, Turkey. Rats were housed in plastic cages in a well-ventilated rat house and allowed ad libitum access to food and water and kept at a 12-h light: dark cycle. All the animals received humane care according to the standard guidelines. Ethical approval for the study was obtained from date 04.03.2020, number 03 and decision no 20/053 (for aorta 21/177) with the consent of all participants by Erciyes University Animal Research Local Ethics Committee.

Chemicals

Isoproterenol hydrochloride (Sigma-Aldrich I6504-1G, USA) was used as a toxic agent. Rats were given subcutaneous (sc) 100 mg/kg depending on their body weight[15]. Thymoquinone (274666-5G, Purity ≥98%, Sigma–Aldrich Co. USA) 20 mg/kg intragastric (ig) was administered by dissolving in olive oil[16]. At the end of the experimental period, the animals were killed by decapitation under intraperitoneal ketamine (75 mg/kg) + xylazine (10 mg/kg) anesthesia.

Experimental design

Rats were divided into four groups. The experiment was planned for eight days. All groups: Control group (Control) (n=8); were untreated rats, Thymoquinone group (THQ) (n=8); 20 mg/kg ig THQ at 24-hour intervals for 8 days, Isoproterenol group (ISO) (n=8); on the 7th and 8th day of the experiment, 100 mg/kg sc ISO (dissolved in 1 ml sterile distilled water) was given at 24-hour intervals. Thymoquinone + Isoproterenol group (THQ+ISO) (n=8); THQ was administered ig at 20 mg/kg for 8 days, and 100 mg/kg ISO was administered on day 7 and day 8 of the experiment. The experimental procedure of the study was performed by all authors.

Histological examination

Aortic tissues were removed rapidly and fixed in 4% formaldehyde fixative for histological examination. It was embedded in paraffin after dehydration and cleaning. 5 mm sections were taken from the paraffin blocks and spread on slides. Slides were stained with hematoxylin-eosin (HE), photographs taken with a light microscope (Olympus BX51, Center Valley, PA, USA). Vessel wall thickness was measured from 5 different regions in the aortic tissue of all subjects. This measurement was carried out to cover the tunica intima and tunica media. All histological analyzes were performed by a histologist.

Hematoxylin and Eosin staining

Sections were taken on slides. Paraffin was removed with xylol and passed through graded alcohol series. Sections were stained with HE to see the general histological structure[17]. Sections were examined after passing through increasing alcohol series and xylene. The slides were then examined under an Olympus BX51 microscope.

Immunohistochemistry

Immunohistochemistry was applied to sections of aortic tissues to determine IL-6 (Anti-IL-6, ab9324, Abcam, Cambridge, UK) and IL-17 (bs-1183R, Bioss, USA) immunoreactivity. The paraffin removed sections were first made transparent and then rehydrated. Citrate buffer was applied to the sections for antigen recovery. Then it was held at H_2O_2. Phosphate buffer saline (PBS) was used as a washing solution. For the next steps, the staining kit (TP-125-HL, Thermo Scientific, Lab Vision, UltraVision, CA, USA) was applied according to the manufacturer's

directives. 3,30-p-diaminobenzidine tetrahydrochloride (DAB) (TA-060-HDX, Thermo Fisher Scientific, Waltham, MA, USA) was applied to identify antibody-dependent regions in the tissue. Counterstaining was done with Gill-hematoxylin. Tissues passed through the alcohol and xylene series were sealed with entellan[18]. Images were taken using a light microscope. Image J program was used to evaluate antibody expressions.

Biochemical analysis

The level of tumor necrosis factor-alpha (TNF-α) (E-EL-R0019, 96 Wells kit, Elabscience Biotechnology Co., Ltd.) was examined in blood serum samples. The ELISA procedure was done according to the protocol recommended by the manufacturers[19].

Statistical analysis

The number of subjects to be included in the study was determined by a statistician with a power test. Sample sizes of 8, 8, 8, and 8 were obtained from the four groups whose averages were to be compared. An F test with a significance level of 0.05 was used (PASS11 software). Statistical analysis of histological and biochemical results in the study was performed with GraphPad Prism version 7.00 for Mac, GraphPad Software, La Jolla, California, USA. D'Agostino Pearson omnibus test was used to identify the normal distribution of the data. In the case of normal distribution, quantitative variables were compared using One-Way analysis of variance and Tukey's posthoc test. The data were expressed as the mean of normalized data±standard deviation of the mean. $p<0.05$ was considered as statistically significant.

RESULTS

In the control and THQ group aortic sections, the endothelium and membrane elastic internal layer located in the tunica intima had a normal histological appearance. The distribution of smooth muscle and elastic lamina was regular in the tunica media layer. Tunica adventitia preserved its connective tissue structure. In the aortic wall of the ISO group, the tunica intima had a normal appearance in general. However, some gaps were observed in the tunica media layer. It was observed that elastin membranes were shorter in length in some areas. In the THQ + ISO group, the aorta generally maintained its regular histological structure. No obvious histopathology
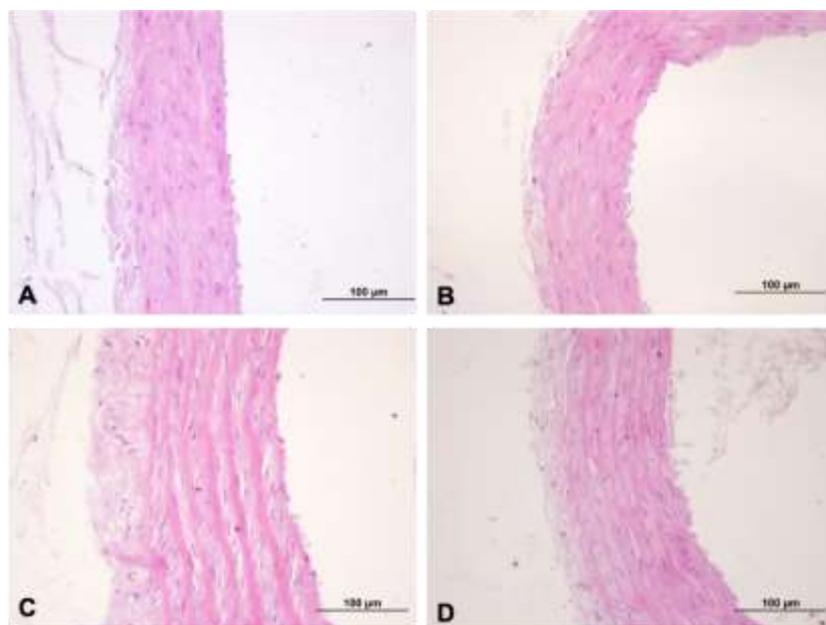
was observed. According to the vessel wall thickness measurement results, no statistically significant difference was observed between the control and THQ groups. There was a statistically significant increase only in the ISO group compared to the control group. Some reduction in vessel wall thickness was observed in the THQ + ISO group compared to the ISO group, but this decrease was not statistically significant. Vessel wall thickness results are shown in Table.1. HE staining images of all experimental groups are shown in Figure.1.

Table.1. Aortic wall thickness of all experimental groups is given as (μm).

Groups	Control	THQ	ISO	THQ+ISO
Aortic wall thickness (μm)	91.15±8.39[a]	90.22±14.5[a]	99.06±16.7[b]	97.73±10.4[a]

Data are shown as ± standard deviation. p <0.05 was considered significant. There was no significant difference between the groups containing the same letter (a, b and c). Abbreviations: ISO: isoproterenol, THQ: thymoquinone.

Figure 1. HE staining of aortic sections belonging to all groups. A- Control group, B- THQ group, C- ISO group, D- THQ+ISO group. Scale bar 100μm. Abbreviations; HE; Hematoxylin-Eosin, THQ; Thymoquinone, ISO; Isoproterenol.

IL-6 and IL-17 immunoreactivity was observed in the aortic wall of all experimental groups, both in the endothelium and in the tunica media layer. There was no statistically significant difference in IL-6 immunoreactivity between the experimental groups. There was no statistical difference in IL-17 immunoreactivity between the control and THQ groups. There was a statistically significant increase in both the ISO group and the THQ + ISO group compared to the control and THQ groups. However, no statistical difference was observed between ISO and THQ + ISO groups. IL-6 and IL-17 immunoreactivity results are shown in Table 2. IL-6 and IL-17 immunoreactivity of all experimental groups are shown in Figure 2.

Table.2. Immunoreactivity results.

Groups	Control	THQ	ISO	THQ+ISO
IL-6 immunoreactivity	103.2±12.45	95.71±15.93	106.3±20.16	104.01±16.62
IL-17 immunoreactivity	92.67±9.7[a]	82.56±2.7[a]	102.17±11.9[b]	106.67±2.24[b]

Data are shown as ± standard deviation. p <0.05 was considered significant. There was no significant difference between the groups containing the same letter (a, b and c). Abbreviations: ISO: isoproterenol, THQ: thymoquinone.
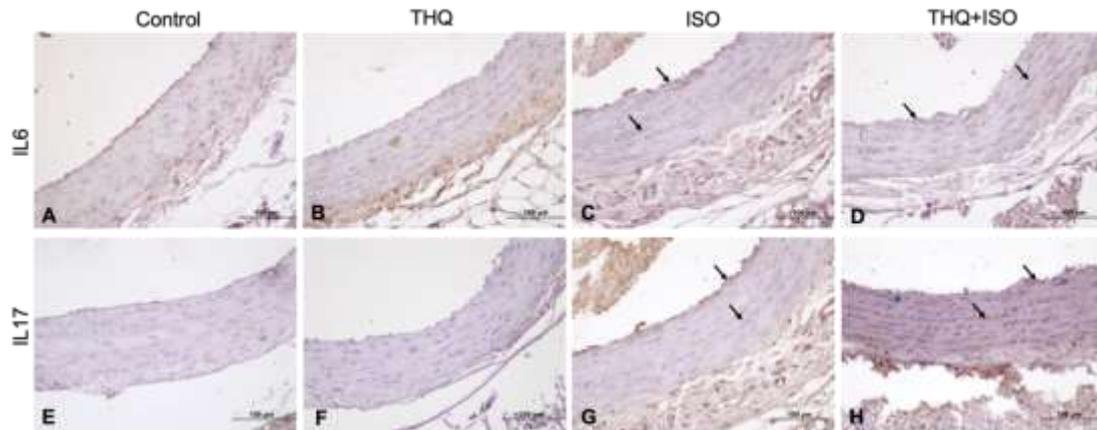
Figure 2. IL-6 and IL-17 immunohistochemical staining. The expressions intensities of the proteins in the groups are indicated by arrows. Scale bar 100 μm. Abbreviations; IL; Interleukin, THQ; Thymoquinone, ISO; Isoproterenol.

Table.3. Statistical analysis of TNF-α levels between groups.

Groups	Control	THQ	ISO	THQ+ISO
TNF-α ng/L	200.5 ± 119.2	173.8 ± 71.87	75.49 ± 29.9	149.2 ± 98.62

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. Abbreviations: ISO: isoproterenol, THQ: thymoquinone, TNF-α: tumor necrosis factor alpha.

The TNF-α level was found to be decreased in the ISO group compared to the control group and the THQ group. This decrease was not statistically significant. It was found that there was a slight increase in the THQ + ISO group. There was no statistically significant difference between the experimental groups.

DISCUSSION

The purpose of this study is to evaluate the cytoprotective effect of THQ in ISO-induced aortic tissue damage histologically and through cytokine release. In this study, it was concluded that ISO in the aortic endothelium and its wall caused an increase in the expression of IL-6 and IL-17, which are vascular inflammatory cytokines, and THQ can also regulate these levels.

It is important to preserve both the endothelial and wall connective tissue structure in the aorta in terms of its role. Histological and physiological changes that will occur in these structures may cause aortic function not to be performed properly. The irregular function of the aorta can affect both the aorta itself and the primary organs with which it interacts directly, and all other tissues and organs related to the vascular system indirectly. ISO causes smooth muscle relaxation by activating various molecules in the cell via adrenergic receptors. This effect is associated with increased oxidative stress and adrenergic stimulation. Because the inflammatory process caused by oxidative stress is a trigger of endothelial dysfunction[20]. Cardiovascular disorders associated with aortic stiffness and smooth muscle relaxation may be caused by endothelial dysfunction[21]. In large vessels such as the aorta, elastin, collagen fiber and smooth muscle cells, as well as changes in

extracellular matrix components, cause changes in the mechanical properties of the vessel, forming the infrastructure of vascular diseases. In our study, it can be concluded that the increase in the thickness of the aortic vessel wall belonging to the ISO group in general and the observation of gaps in the tunica media layer affected the mechanical properties of the vessel. It has been reported that many vascular diseases occur as a result of changes in the mechanical properties of the aortic wall, and diseases such as abdominal aortic aneurysms are caused by increased stiffness and decreased strain in the aorta[22]. In addition, it was emphasized that the thoracic aortic wall hardens with aging and this is due to wall stress or changes in its composition[23]. For this reason, preserving the chemical and molecular properties of the components that make up the vessel wall structure is important in preventing loss of function. The underlying reasons for the possible increased wall thickness caused by ISO should be clarified. We evaluated the ISO-induced inflammatory response in the study. For this, we examined IL-6 and IL-17 immunohistochemical TNF-α by ELISA method. According to our results, ISO caused an increase in inflammatory response on both the endothelium and wall structure components in the aorta. It can cause deterioration of vasoconstriction by causing ISO adrenergic receptors and increased inflammatory response. It is known that ISO administration increases the level of cardiac IL-6 Mrna[24]. Besides IL-6, inflammatory cytokines such as TNF-α are caused by excessive production of free radicals and oxidant mediators[25]. In the experimental heart failure model, it was observed that IL-6 and IL-17 increased after ISO application[26]. IL-17 plays an important role in hypertension and vascular dysfunction by synergizing with cytokines such as TNF-α to modulate the inflammatory response[27]. It is known that cytokines such as TNF-α increase in ISO-induced tissue damage and may cause myocardial damage[28]. However, it is also known that ISO reduces the level of TNF-α mRNA through intracellular cAMP induced ERK inactivation in cultured astrocytes with β-adrenergic effect[29]. β-adrenergic agonists show their effects by increasing the concentration of cAMP in the cell. It has been reported that ISO decreases lipopolysaccharide-induced TNF-α response by causing an increase in Camp[25]. Increased TNF-α expression, which is widely known in ISO's heart damage, has been mostly shown in the tissue[30,31]. Because the myocardium expresses various cytokines such as TNF-α and interleukin in response to different stimuli. In addition to studies reporting that

TNF-α increases and decreases in ISO-induced cardiac tissue damage, it has also been reported that it does not alter TNF-α mRNA expression in aortic tissue[14]. Therefore, information about the relationship between TNF-α, aorta and ISO is insufficient, as well as its expression in heart tissue. In our study, we found that the blood serum TNF-α level was reduced by ISO, but this decrease was not statistically significant. According to the aortic tissue immunohistochemistry results, IL-6 and IL-17 expression was increased. If we also evaluated the level of TNF-α in the tissue immunohistochemically, it might have increased. We think that the increase in IL-6 and IL-17 in the tissue, by acting on smooth muscles for a short time, changes this cytokine expression, but biochemically it does not cause any change in the level of TNF-α in the blood. We believe this may be due to the lack of time to change the blood level by removing tissue from rats shortly after two doses of ISO. In addition, the fact that TNF-α level was not determined in aortic tissue homogenate in the study constitutes a limitation of the study.

In the study, we used THQ to evaluate the cytoprotective and vascular effects of ISO against its harmful effect. THQ has recently been shown to have protective effects on heart tissue from circulatory system organs[32,33]. THQ is effective in sepsis-induced aortic dysfunction in rats[34], showing strong antioxidant activity and correcting pyrogallol-induced endothelial dysfunction[35]. It performs its protective feature as an antioxidant by cleaning molecules that occur in oxidative conditions such as nitric oxide. In this study, we found that THQ effectively reduced the expression of ISO-induced increased IL-6 and IL-17 inflammatory cytokines in aortic tissue. In addition, the blood serum TNF-α level also showed a similar result close to the control. THQ was also found to be effective in histological thickness with the ISO group. It has been emphasized that THQ is a potential candidate against vascular occlusion diseases such as arterial hypertension and restenosis by suppressing molecules such as reactive oxygen species and cytokines that occur after oxidative stress in the secretion of extracellular matrix proteins on vascular smooth muscle cells[36]. It has been reported that THQ extract may have antihypertensive effects on contracted aortic rings by causing vasocontraction or vasorelaxation[37]. When both the improvement in wall thickness and its effect on cytokines were evaluated in the study; It was concluded that THQ protects the tissue against ISO-induced oxidative stress in aortic

vascular smooth muscle cells and endothelial structure. In our previous study, we showed that THQ is an important substance against chemotherapeutic agent toxicity and the improvements it provides in heart tissue[38]. After finding that THQ causes very good results, in this study, we aimed to determine the effects of ISO on the aortic tissue and the role of THQ on these effects, considering the effects of ISO on the vascular system.

This study is aimed to evaluate the aortic damage caused by ISO through histologically and cytokines and to investigate the cytoprotective effect on THQ. ISO proved to be an effective β-adrenergic agonist on the aorta which cannot be ignored. It can be concluded that THQ may show an antihypertensive effect, as well as antioxidant properties on the aortic wall and this, should be investigated in detail. Therefore, it has been concluded that THQ is a component with protective and therapeutic properties against aortic diseases.

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