Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2023;48(3):844-851
DOI: 10.17826/cumj.1314101

RESEARCH

Thymoquinone reduces methotrexate-induced heart damage: a histopathological study in rats

Timokinon metotreksatın neden olduğu kalp hasarını azaltır: sıçanlarda histopatolojik bir çalışma

Ayşegül Burçin Yıldırım[1], Emin Kaymak[2], Tayfun Ceylan[3], Ali Tuğrul Akın[4], Nurhan Kuloğlu[5], Meryem Sayan[6], Necla Değer[6], Esra Önal[6], Derya Karabulut[6]

[1]Gaziantep Islam Science and Technology University, Faculty of Medicine, Department of Histology and Embryology, Gaziantep, Turkey
[2]Yozgat Bozok University, Faculty of Medicine, Department of Histology and Embryology, Yozgat, Turkey
[3]Cappadocia University, Department of Dentistry, Nevşehir, Turkey
[4]İstinye University, Faculty of Medicine, Department of Medical Biology, İstanbul, Turkey
[5]Niğde Ömer Halisdemir University, Health Care Services Department, Niğde, Turkey.
[6]Erciyes University, Faculty of Medicine, Department of Histology and Embryology, Kayseri, Turkey.

Abstract

Purpose: The study aimed to evaluate the effect of thymoquinone on cardiac tissue in MTX-induced cardiac toxicity in rats with various parameters.

Materials and Methods: Group I (n=8) was administered intraperitoneal saline for 10 days. Intraperitoneal olive oil was applied to Group II (n=8) for 10 days. Group III (n=8) received 10 mg/kg Thymoquinone (THQ) intraperitoneally for 10 days. Group IV (n=8) was administered a single dose of 20 mg/kg Methotrexate (MTX), 500 mg/20 ml, intraperitoneally on the 1st day of the experiment. Group V (n=8) MTX: 20 mg/kg single dose intraperitoneally on the 1st day; THQ: 10mg/kg i.p. administered for 10 days. Since Methotrexate was in liquid form, no solvent was used. At the end of the experimental period, the rats were sacrificed for analysis of heart tissue. The structure of heart tissue was evaluated by hematoxylin-eosin staining. Immunohistochemically, Connexin-43, HSP90, and HIF-1α antibodies were stained.

Results: Group IV was found to have histopathological deterioration, which was ameliorated by THQ. In addition to this; Connexin-43 immunoreactivity was the lowest in Group IV compared to other groups: 108.5±7.4. Compared to other groups, HSP90 immunoreactivity was highest in Group IV: 103.6±10.4. Compared to other groups, HIF-1α immunoreactivity was highest in Group IV: 95.2 ±9.1.

Öz

Amaç: Bu çalışmada, sıçanlarda MTX ile indüklenen kardiyak toksisitede timokinonun kardiyak doku üzerindeki etkisinin çeşitli parametrelerle değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: Grup I'e (n=8) 10 gün boyunca intraperitoneal salin uygulandı. Grup II'ye (n=8) 10 gün boyunca intraperitoneal zeytinyağı uygulandı. Grup III (n=8) 10 gün boyunca 10 mg/kg timokinon (THQ) intraperitoneal olarak almıştır. Grup IV'e (n=8) deneyin 1. gününde tek doz 20 mg/kg Metotreksat (MTX), 500 mg/20 ml, intraperitoneal olarak uygulandı. Metotreksat sıvı formda olduğu için herhangi bir çözücü kullanılmamıştır. Grup V (n=8) MTX: 1. gün 20 mg/kg tek doz intraperitoneal; THQ: 10mg/kg i.p. 10 gün boyunca uygulandı. Deney süresinin sonunda sıçanlar kalp dokusu analizi için sakrifiye edilmiştir. Kalp dokusunun yapısı hematoksilen-eozin boyama ile değerlendirildi. İmmünohistokimyasal olarak, konneksin-43, HSP90 ve HIF-1α antikorları ile boyandı.

Bulgular: Grup IV'ün histopatolojisinde bozulmalar olduğu belirlendi, THQ'nun bu bozulmayı iyileştirdiği görüldü. Bunun yanı sıra, diğer gruplara göre; konneksin-43 immunureaktivitesi, Grup IV'de: 108.5±7.4 ile en düşük, HSP90 immunureaktivitesi diğer gruplara göre, Grup IV'de: 103.6±10.4 en yüksek, HIF-1α immunureaktivitesi diğer gruplara göre Grup IV'de: 95.2 ±9.1 en yüksek değerlerdeydi.

Address for Correspondence: Ayşegül BurçinYıldırım, [1]Gaziantep Islam Science and Technology University, Department of Histology and Embryology, Gaziantep, Turkey E-mail: aysegulburcin@gmail.com
Received: 15.06.2023 Accepted: 23.08.2023

Conclusion: Thymoquinone has a positive effect on Connexin-43, one of the proteins providing conduction in intercalary discs, HSP90, one of the chaperones in the cell and HIF-1α expression against MTX toxicity. At the same time, THQ provides a significant improvement in cardiac tissue histopathologically by showing a cardioprotective effect.
Keywords: Connexin-43, HSP90, HIF-1α, methotrexate, thymoquinone

Sonuç: Timokinon, MTX toksisitesine karşı interkalar disklerde iletimi sağlayan proteinlerden biri olan konneksin-43, hücre içindeki şaperonlardan biri olan HSP90 ve HIF-1α ekspresyonu üzerine olumlu etki göstermektedir. Aynı zamanda THQ, kalp dokusunda histopatolojik olarak kardiyoprotektif etki göstererek anlamlı bir iyileşme sağlamaktadır.
Anahtar kelimeler: Konneksin-43, HSP90, HIF-1α, metotreksat, timokinon

INTRODUCTION

Methotrexate (MTX) is a folate antagonist used in the treatment of malignancies. Apart from neoplastic diseases, it is also used as an anti-inflammatory and immunosuppressive drug[1]. MTX is cytotoxic. While destroying tumor cells, it also affects vital organs such as the heart. Studies have emphasised the significance of clinicians being aware that high doses of MTX may cause cardiac disorders and arrhythmias in healthy individuals. MTX chemotherapy has adverse side effects and more experimental research is required in this area[2]. Oxidative stress and inflammation are known to be involved in the development of MTX toxicity[3]. Studies are showing that MTX induces pathological changes in heart tissue in experimental animals[4]. Cardiac tissue, which consists of different cell types, is a very complex structure. In enormous tissues and organs, there are gap junctions between two cells, which are very vital for communication. In the heart, they connect cardiomyocytes and take part in the transmission of impulses. The proteins found in these junctions are called "connexins". Connexins are found in cardiomyocytes as well as in cardiac tissue, it is also expressed in fibroblasts, endothelial cells, and macrophages[5]. Biochemical studies have shown that connexins exhibit a graded distribution. Some are located in the atrioventricular conduction axis[6] some of them are localised in the endocardial area, whereas connexin43 (Cx43), the connexin type we evaluated in our study, is localised in the endo-, mid, and epicardium[7] appears to be dispersed[8].

Heat shock proteins are a family of molecular chaperones that are expressed at low levels in normal conditions but are induced in cellular response in conditions such as heat shock, hypoxiaand toxic agents[9]. Heat shock protein 90 (HSP90), one of these chaperones, is a protein involved in the folding and stabilisation of many proteins such as Hypoxia Inducible Factor (HIF)[10,11]. HIF, which is synthesised under hypoxic conditions, has a signal transduction function in response to hypoxia. In addition, HIF is an important regulator of angiogenesis[12]. In addition to hypoxia, some oncogenic and inflammatory conditions activate HIF-1. HIF is also effective in conditions such as inflammation[13,14]. Thymoquinone is a component of the essential oil of Nigella sativa[15]. It has a wide spectrum of pharmacological effects including anti-inflammatory, immunomodulatory, anti-cancer, hepatoprotective, gastroprotective, and cardioprotective effects[16].

This study aimed to evaluate the effect of thymoquinone on cardiac tissue in Methotrexate-induced cardiac toxicity in rats with various parameters. The structures of heart tissues were evaluated by hematoxylin-eosin staining. Moreover immunohistochemically, the tissues were stained with connexin-43, HSP90, and HIF-1α antibodies. This study provides a different perspective by evaluating the effect of thymoquinone against cardiac damage and some mechanisms that have not been investigated before. In addition, it contributes to the literature in elucidating the pathways of the mechanisms of action of thymoquinone on the cardiac tissue.

MATERIALS AND METHODS

Animals

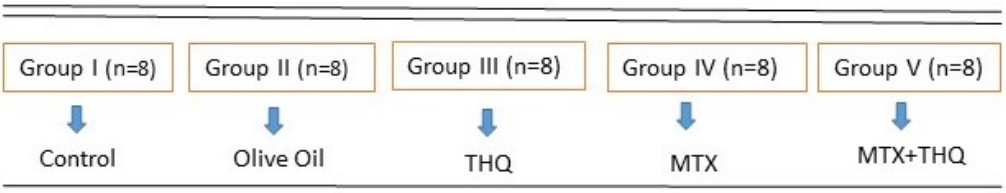
This study was conducted at the Erciyes University, Hakan Çetinsaya Experimental and Clinic Research Center. All animals in the facility are housed in accordance with the "Regulation on the welfare and protection of animals used for experimental and other scientific purposes". The experimental procedure was followed by researchers with a PhD in Histology-Embriyology department and they have the certificate of Experimental Animal Use. Forty adults twenty-week-old Wistar albino rats were used in this study. The rats were placed in a well-ventilated plastic cage rat house, and kept on a 12-h light and dark cycle, while the feed and water were provided ad libitum. All animals were given good care according

to the standard guidelines. Ethical approval from the Erciyes University, Animal Research Ethics Committee was obtained for the study (no:23/051; date:02.03.2023). Ethical regulations were strictly followed according to the national and institutional guidelines. The rats were assigned randomly to five groups of eight rats per group.

Experimental procedure

Group I (control) (n=8) was administered intraperitoneal saline for 10 days. Intraperitoneal olive oil was applied to Group II (n=8) for 10 days. Group III (n=8) received 10 mg/kg Thymoquinone (THQ), 274666-5G, purity 98%, Sigma-Aldrich Co., St Louis, MO, USA, intraperitoneally for 10 days. Group IV (n=8) was administered a single dose of 20 mg/kg Methotrexate (MTX) [17], 500 mg/20 ml, Koçak Farma, Turkey, intraperitoneally on the 1st day of the experiment. Since Methotrexate was in liquid form, no solvent was used. Group V (n=8) MTX: 20 mg/kg single dose intraperitoneally on the 1st day; THQ: 10mg/kg i.p. applied for 10 days. Since THQ is dissolved in olive oil, Group II was formed, which was given olive oil. The rats were killed under high-dose ketamine + xylazine anesthesia 2 hours after the last injection.

Table 1. Experimental procedure chart



Histological analysis

At the end of the experiment heart tissues in a 10% formaldehyde solution were determined. After determination heart tissues that went through increasing degrees of alcohol (50%, 70%, 80%, 96%, 3x100%) were dehydrated. After getting cleared with Xylol, they were embedded in paraffin. 5 µm thick sections from paraffin blocks were stained with Hematoxylin-Eosin to assess the histological structure. Connexin-43, HIF-1α, and HSP90 immunoreactivity intensity change were determined with the immunohistochemical method.

Hematoxylin-Eosin (H-E) staining protocol

Tissue sections were incubated at 58 ºC after which they were kept in xylene 3 times for 10 minutes. The sections that went through decreasing alcohol series (2x100%, 96%, 80%, 70%, and 50%) to bring in water to tissue and were washed in tap water. Sections that were washed in tap water were kept in hematoxylin solution at room temperature for 10 minutes. The sections were once again washed in tap water after which they were kept in eosin solution for 5 minutes and they were washed again in tap water. Sections that went through increasing alcohol series (70%, 96%, 3x100%) were kept in xylene after which they were closed with entellan and studied under a light microscope.

Immunohistochemistry staining protocol: Avidin-Biotin-Peroxidase Complex (ABC) method was used for immunohistochemical analysis. Connexin-43 (Anti Connexin 43 Antibody, Bioss, USA), HIF-lα (Anti HIF-1α Antibody, Bioss, USA), and HSP90 (Anti HSP90 alpha antibody, Bioss, USA), immunoreactivities were determined in heart tissue with this method. Sections were cut from 5 µm thick paraffin blocks onto slides coated with poly-(L-lysine). It was kept in an oven at 58°C overnight. Xylene was taken for deparaffinization and rehydrated by passing through decreasing grade alcohol (100%, 96%, 80%, 70%) series, respectively. 3% hydrogen peroxide (H_2O_2) was used for endogenous peroxidase blockade. Citrate buffer was used for antigen retrieval. Washes were done with phosphate-buffered saline. Other steps were performed according to the procedure of Thermo Fisher Scientific, Waltham, MA, USA, Catalog no: TP-125-HL immunochemistry staining kit. In the staining of the sections, DAB (3,3'-

Diaminobenzidine) was used as chromogen first and then stained with Gill I hematoxylin[18]. Finally, the sections were cleared in xylene before being coverslipped with Entellan® (Merck, Kenilworth, NJ, USA). Sections stained with connexin-43, HIF-1α, and HSP90 were captured under Nikon Ni-U (Nikon, Tokyo, Japan) model light microscope with a DS-Ri2 model digital camera.

Quantitative immunohistochemistry

5 slides from each group were evaluated for connexin-43, HIF-1α, and HSP90 immunoreactivity intensity. Five photos were taken from each slide. The immunoreactivity of 5 areas in each photograph was measured. In total, 250 areas were evaluated for each group. The density of these areas was measured using the Image-J Program. The data obtained were evaluated statistically.

Statistical analysis

JASP 0.14.1 package program was used to evaluate the immunohistochemical data. Whether the data showed a normal distribution or not was evaluated by looking at the skewness and kurtosis values. After these evaluations, the Anova test was performed. When ANOVA was performed, continuous variables were connexin-43, HIF-1α, and HSP90 immunoreactivity, while group variables were group 1-2-3-4-5. Tukey HSD test was used if variance homogeneity was present (in connexin-43, HIF-1α, and HSP90 immunoreactivity analysis) after ANOVA. With a 98% confidence interval and 97% test power, the number of subjects to be included in the study was determined. The sample size for the one-way ANOVA study was obtained from five groups whose means were to be compared. With an alternative of equal means (PASS11 software), a sample of 40 subjects was obtained using a F test with a level of significance of 0.05.

RESULTS

In groups I, II, and III, the oval nucleus in myocardial muscle fibers were located in the middle of the cell, and the muscle fibers showed a regular arrangement. Irregularly arranged muscle fibers were seen in Group IV sections. An increase in connective tissue and a fibrotic appearance were observed between these myocardial fibers. Intense eosinophilia was seen in the cytoplasm of some myocardial cells. Congestion was also observed between myocardial fibers. In group V, decreased myocardial muscle fiber irregularity was observed. The connective tissue between the muscle fibers was also significantly reduced. Muscle fibers showed a more regular arrangement than in group IV. Figure 1 shows the pictures of all groups.

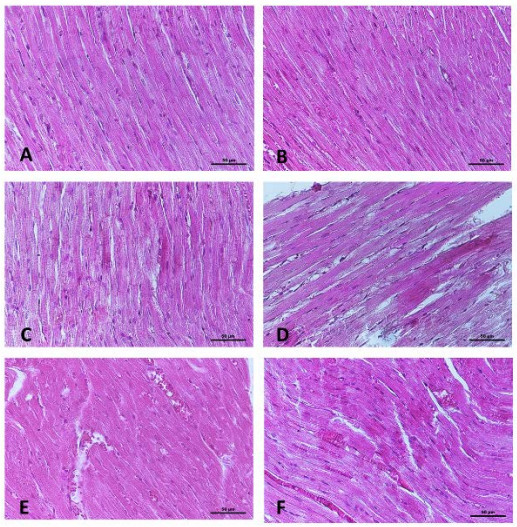


Figure 1. HE-stained histological images of myocardial cells in all groups. (A) Group I, (B) Group II, (C) Group III normal heart muscle fibers are seen. (D) Group IV eosinophilic areas, connective tissue increase, and myofibril irregularity (E) Group IV bleeding areas are seen, (F) Group V, decrease in bleeding areas. 40X objective, Bar: 50 µm. HE, Hematoxylin-eozin.

Immunohistochemistry Connexin-43 Immunoreactivity

Connexin-43 was immunolocalised in the intercalary discs between cardiomyocytes. Immunreactivity intensity mean and standard deviation values were as follows: Group I:113.2±7.3, Group II:114.3±7.5, Group III: 111.2±8.0, Group IV: 108.5±7.4; Group V: 116.4±6.9. The lowest connexin-43 expression was in Group IV, the MTX-treated group. According to the results of statistical analysis, there was a difference between Group IV and all groups (Figure 2). There was a significant difference between Group V and Group I and Group III (Figure 2). In conclusion, MTX decreased the protein expression in the intercalated discs of cardiomyocytes and THQ showed its therapeutic effect on connexin-43 proteins.

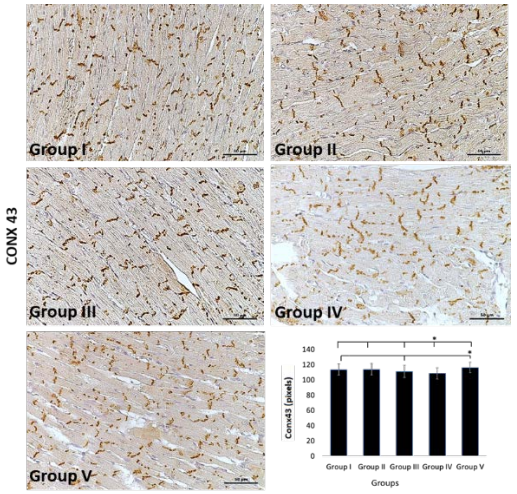


Figure 2. Connexin-43 immunohistochemistry staining of all groups and graph showing the difference between groups. Scala Bar: 50µm, Objective: X40. Data are reported as means±SD. *P<0.001 was accepted as significant (one-way ANOVA, Tukey). SD: Standart deviation.

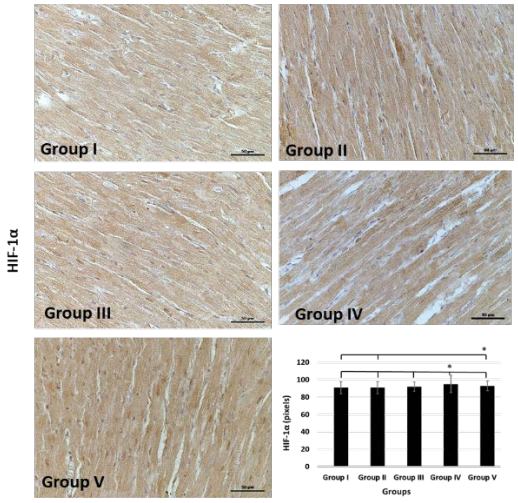


Figure 3. HIF-1α immunohistochemistry staining of all groups and graph showing the difference between groups. Scala Bar: 50µm, Objective: X40.Data are reported as means±SD. *P<0.05 was accepted as significant (one-way ANOVA, Tukey). HIF-1α: Hypoxia Inducible Factor 1α. SD: Standart deviation.

HIF-1α Immunoreactivity

HIF-1α was immunolocalised in both cytoplasm and nuclei of cardiomyocytes. Immunreactivity intensity mean and standard deviation (means±SD) values were as follows: Group I: 92.6±7.8, Group II: 91.1 ±7.7, Group III: 92.2± 6.5, Group IV: 95.2 ±9.1; Group V: 93.1 ±6.4. According to the results of statistical analysis, there was a significant difference between Group IV and Groups I, II, and III, V (Figure 3). There was also a significant difference between Group V and Group I and Group II (Figure 3). MTX activated HIF-1α expression in cardiomyocytes, whereas HIF-lα expression decreased in Group V treated with MTX+THQ similar to the other groups.

HSP90 Immunoreactivity

HSP90 was immunolocalised in the cytoplasm of cardiomyocytes. Immunreactivity intensity mean and standard deviation values were as follows: Group I: 96.3±4.6, group II: 97.0±4.6, Group III: 96.1±6.4, Group IV: 103.6±10.4; Group V: 96.7 ±7.0. There was a significant difference between group IV and all groups. Its expression increased in group IV, that is, in the group given MTX. Its expression decreased in the group given MTX+THQ.

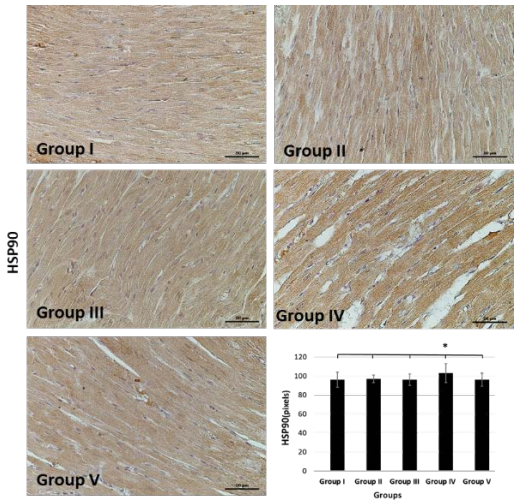


Figure 4. HSP90 immunohistochemistry staining of all groups and graph showing the difference between groups. Scala Bar: 50µm, Objective: X40. Data are reported as means±SD. *P<0.001 was accepted as significant (one-way ANOVA, Tukey). HSP90: Heat shock protein 90. SD: Standart deviation.

DISCUSSION

When chemotherapeutic agents are used for therapeutic purposes, complications in the heart tissue should not be ignored. Therefore, it is important to evaluate patients in terms of cardiovascular risk. In our study, we found that MTX has a toxic effect on heart tissue and thymoquinone reduces this effect. Many degenerative changes were seen in a study of mice receiving MTX. These changes; cardiomyocyte necrosis, increased congestion, intermuscular edema and bleeding were observed [4]. Similarly, in our histopathological study, mostly muscle fibre irregularity and loss of muscle fibre myofibrils in some regions, fibrous tissue accumulation due to increased connective tissue between impaired myocardial fibres, and congestion between myocardial fibres were observed. Thymoquinone (THQ) showed an ameliorative effect against histopathological deterioration induced by MTX on cardiomyocytes. These effects are mediated by various mechanisms. In addition, the effects of THQ on Cx43, HIF-1α, and HSP90 expressions against MTX-induced cardiac damage were evaluated in our study.

The fact that cardiomyocytes express connexin-43 (Cx43) is important for the continuity of these cells with each other and the synchronisation of contraction[19]. Cx43 is a protein that forms gap junctions in the myocardium and is essential for intercellular transmission and cell survival. Cx43 is expressed in the sarcolemma as well as in the mitochondria of cardiomyocytes[20, 21, 22]. When the expression of Cx43, a protein found in the intercalary discs providing communication between cardiomyocytes, was evaluated, it was observed that there was a decrease in Cx43 expression in Group IV, whereas there was an improvement in Cx43 expression in the THQ-treated group. In this respect, it can be said that THQ is an important compound in connecting cardiomyocytes with each other.

Studies have shown that mitochondrial uptake of Cx43 mediated by heat shock protein 90 (HSP90) is important in cardioprotection. The primary role of chaperones in the cell is to facilitate the stabilization of proteins during folding and to regulate their access to their active structures[23]. Protein production can be affected by non-physiological high temperatures and the presence of various chemicals[24]. In the cell, HSP90 is activated as protective molecules under cellular stress conditions. Studies have also shown that it is critical for cardioprotection [25, 22]. When we evaluated HSP90 expression, it was shown that expression increased in Group IV and reached levels close to the control group when treated with THQ. Since THQ provides an improvement in HSP90 expression, it is also important in this sense in terms of cardiomyocytes.

Hypoxia occurs when tissues do not receive sufficient oxygen. It is not clear whether methotrexate causes hypoxia in the heart, but chronic hypoxia is known to affect cardiac metabolism and function. Hypoxia triggers the activation of transcription factors called hypoxia-inducible factors (HIF)[26, 27]. Several approaches to chemically target the cellular processes that regulate HIF-1α expression have been experimentally investigated. One study has shown that HIF-lα can be destabilised when HSP90 binding is inhibited[28,14]. Studies have shown that HIF-lα plays a role in the protection of cardiomyocytes against ischemic damage[27]. In recent studies, HIF-lα is thought to constitute an important link between cardiovascular development and physiology[29]. Whether sustained HIF-1α up-regulation is beneficial or detrimental for the heart is still debated and further research is needed to better understand the complex mechanisms involved in this process. It is thought that there may be a cut-off point at which HIF signaling becomes detrimental to cardiac function[30, 31]. In a study in H9c2 (embryonic rat ventricular cardiomyoblast) cell line, HIF-1α levels were found to be increased in MTX-treated cells compared to control cells[32]. In our study, MTX induced hypoxia in the heart. There was a significant difference between group IV and all groups. Thus, THQ down-regulated the HIF-1α level induced by MTX.

The limitation of the study is that the histopathological results of the study could not be supported by molecular analyses such as western and PCR (Polymerase Chain Reaction). In addition, different biomarkers of cardiac damage could also have been analysed. In further studies, it would be useful to investigate the expression of other types of HSPs and other proteins in the intercalated discs between cardiomyocytes.

According to our results, THQ has a positive effect on Connexin-43, one of the proteins providing conduction in intercalary discs, HSP90, one of the chaperones in the cell and HIF-1α expression against MTX toxicity. At the same time, THQ provides a significant improvement in cardiac tissue

histopathologically by showing a cardioprotective effect. It is known that MTX may cause the development of heart diseases. Therefore, patients taking MTX should take care of their heart health and regular contact with their doctor is important. Furthermore, it is important to take into account THQ's cardioprotective effects in MTX-treated patients.

Author Contributions: Concept/Design : ABY, EK, DK, NK; Data acquisition: MS, ND, EÖ, TC; Data analysis and interpretation: ABY, ATA, ND, EÖ, TC; Drafting manuscript: ABY, ATA; Critical revision of manuscript: DK, MS, TC, NK; Final approval and accountability: ABY, EK, TC, TA, NK, MS, ND, EÖ, DK; Technical or material support: MS, ND, EÖ, NK; Supervision: NK, MS, ND, EÖ; Securing funding (if available): n/a.
Ethical Approval: Ethical approval was obtained from the Local Ethics Committee of Animal Experiments of Erciyes University with the decision dated 02.03.2023 and numbered 23/051.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
Financial Disclosure: This study was supported by Yozgat Bozok University Scientific Research Projects (BAP) Coordinatorship with project number 21/036.

REFERENCES

1. Genestier L, Paillot R, Quemeneur L, Izeradjene K, Revillard JP. Mechanisms of action of methotrexate. Immunopharmacology. 2000;47:247-57.
2. Perez-Verdia A, Angulo F, Hardwicke FL, Nugent KM. Acute cardiac toxicity associated with high-dose intravenous methotrexate therapy: case report and review of the literature. Pharmacother J Hum Pharmacol Drug Ther. 2005;25:1271-6.
3. Al-Taher AY, Morsy MA, Rifaai RA, Zenhom NM, Abdel-Gaber SA. Paeonol attenuates methotrexate-induced cardiac toxicity in rats by inhibiting oxidative stress and suppressing TLR4-induced NF-κB inflammatory pathway. Mediators Inflamm. 2020;2020:8641026.
4. Abdel-Daim MM, Khalifa HA, Abushouk AI, Dkhil MA, Al-Quraishy SA. Diosmin attenuates methotrexate-induced hepatic, renal, and cardiac injury: a biochemical and histopathological study in mice. Oxid Med Cell Longev. 2017;2017:3281670.
5. Johnson RD, Camelliti P. Role of non-myocyte gap junctions and connexin hemichannels in cardiovascular health and disease: novel therapeutic targets? Int J Mol Sci. 2018;19:866.
6. Severs NJ, Bruce AF, Dupont E, Rothery S. Remodelling of gap junctions and connexin expression in diseased myocardium. Cardiovasc Res. 2008;80:9-19.
7. Ko YS, Yeh HI, Ko YL, Hsu YC, Chen CF, Wu S et al. Three-dimensional reconstruction of the rabbit atrioventricular conduction axis by combining histological, desmin, and connexin mapping data. Circulation. 2004;109:1172-9.
8. Desplantez T. Cardiac Cx43, Cx40 and Cx45 co-assembling: Involvement of connexins epitopes in formation of hemichannels and Gap junction channels. BMC Cell Biol. 2017;18:1-13.
9. Yu X, Mao M, Liu X, Shen T, Li T, Yu H et al. A cytosolic heat shock protein 90 and co-chaperone p23 complex activates RIPK3/MLKL during necroptosis of endothelial cells in acute respiratory distress syndrome. J Mol Med. 2020;98:569-83.
10. Minet E, Mottet D, Michel G, Roland I, Raes M, Remacle J et al. Hypoxia-induced activation of HIF-1: role of HIF-1α-Hsp90 interaction. FEBS Lett. 1999;460:251-6.
11. Isaacs JS, Jung YJ, Mimnaugh EG, Martinez A, Cuttitta F, Neckers LM. Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1α-degradative pathway. J Biol Chem. 2002;277:29936-44.
12. Park SY, Jang WJ, Yi EY, Jang JY, Jung Y, Jeong JW et al. Melatonin suppresses tumor angiogenesis by inhibiting HIF-1α stabilization under hypoxia. J Pineal Res. 2010;48:178-84.
13. Schmid T, Zhou J, Brüne B. HIF-1 and p53: communication of transcription factors under hypoxia. J Cell Mol Med. 2004;8:423-31.
14. Yeo EJ, Chun YS, Park JW. New anticancer strategies targeting HIF-1. Biochem Pharmacol. 2004;68:1061-9.
15. Alrashedi M. The protective role of thymoquinone against drugs toxicity : a review. 2018;24:1-11.
16. Farooq J, Sultana R, Taj T, Asdaq SMB, Alsalman AJ, Mohaini MA et al. Insights into the protective effects of thymoquinone against toxicities induced by chemotherapeutic agents. Molecules. 2022;27:226.
17. Sonmez MF, Cılenk KT, Karabulut D, Unalmıs S, Deligonul E, Ozturk I et al. Protective effects of propolis on methotrexate-induced testis injury in rat. Biomed Pharmacother. 2016;79: 44-51.
18. Yildirim AB, Karabulut D, Dundar M, Ulusoy HB, Sonmez MF. Expression of Ghrelin and GHSR-1a in Long Term Diabetic Rat's Kidney. Braz Arch Biol Technol. 2016;59:150312.
19. Michela P, Velia V, Aldo P, Ada P. Role of connexin 43 in cardiovascular diseases. Eur J Pharmacol. 2015;768:71-6.
20. Boengler K, Dodoni G, Rodriguez-Sinovas A, Cabestrero A, Ruiz-Meana M, Gres P et al. Connexin 43 in cardiomyocyte mitochondria and its increase by ischemic preconditioning. Cardiovasc Res. 2005;67:234-44.
21. Denuc A, Nunez E, Calvo E, Loureiro M, Miro-Casas E, Guarás A et al. New protein–protein interactions of mitochondrial connexin 43 in mouse heart. J Cell Mol Med. 2016;20:794-803.
22. Tu RH, Li QJ, Huang Z, He Y, Meng JJ, Zheng HL et al. Novel functional role of heat shock protein 90 in mitochondrial connexin 43-mediated hypoxic

postconditioning. Cell Physiol Biochem. 2017;44:982-97.
23. Roberts RJ, Hallee L, Lam CK. The potential of Hsp90 in targeting pathological pathways in cardiac diseases. J Pers Med. 2021;11:1373.
24. Walter S, Buchner J. Molecular chaperones-cellular machines for protein folding. Angew Chem Int Ed. 2002;41:1098-113.
25. Zhong GQ, Tu RH, Zeng ZY, Li Q jie, He Y, Li S et al. Novel functional role of heat shock protein 90 in protein kinase C-mediated ischemic postconditioning. J Surg Res. 2014;189:198-206.
26. Yu J, Bao E, Yan J, Lei L. Expression and localization of Hsps in the heart and blood vessel of heat-stressed broilers. Cell Stress Chaperones. 2008;13:327-35.
27. Hölscher M, Schäfer K, Krull S, Farhat K, Hesse A, Silter M et al. Unfavourable consequences of chronic cardiac HIF-1α stabilization. Cardiovasc Res. 2012;94:77-86.
28. Mabjeesh NJ, Post DE, Willard MT, Kaur B, Van Meir EG, Simons JW et al. Geldanamycin induces degradation of hypoxia-inducible factor 1α protein via the proteosome pathway in prostate cancer cells. Cancer Res. 2002;62:2478-82.
29. Semenza GL, Agani F, Iyer N, Kotch L, Laughner E, Leung S et al. Regulation of cardiovascular development and physiology by hypoxia-inducible factor 1α. Ann N Y Acad Sci. 1999;874:262-8.
30. Fialho M da LS, Abd Jamil AH, Stannard GA, Heather LC. Hypoxia-inducible factor 1 signalling, metabolism and its therapeutic potential in cardiovascular disease. Biochim Biophys Acta BBA-Mol Basis Dis. 2019;1865:831-43.
31. Hashmi S, Al-Salam S. Hypoxia-inducible factor-1 alpha in the heart: a double agent? Cardiol Rev. 2012;20:268-73.
32. Dogan Z, Durmus S, Ergun DD, Gelisgen R, Uzun H. Ranolazine exhibits anti-inflammatory and antioxidant activities in H9c2 cardiomyocytes. Eur Rev Med Pharmacol Sci. 2023;27:2953-63.