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Research

Research on the Endoplasmic Reticulum Stress-mediated Protective Effect of Melatonin against Cardiotoxicity Following Cisplatin Treatment

Sisplatinin Oluşturacağı Kardiyotoksisiteye Karşı Melatoninin Endoplazmik Retikulum Stresi Aracılı Koruyucu Etkisinin Araştırılması

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ABSTRACT

Objective: Cisplatin (CP) is a chemotherapeutic drug that causes cardiotoxicity. Melatonin (MEL) is secreted by the pineal gland throughout the night. This study aimed to investigate the protective effect of MEL against cardiotoxicity associated with CP exposure.

Methods: Physiological saline was applied to Group 1 (control) throughout the experiment. A single dose of CP (7 mg/kg) was administered to Group 2 on the 5th day of the experiment. Group 3 received MEL (10 mg/kg) for 7 days and CP (7 mg/kg) on day 5. MEL (10 mg/kg) was administered to group 4 for 7 days. On the 8th day of the experiment, the hearts were removed under anesthesia. Sections taken from heart tissue samples were stained with hematoxylin and eosin for histopathological evaluation. Additionally, heart tissue sections were immunohistochemically stained for 78-kDa glucose-regulated protein (GRP-78), growth arrest and DNA damage-inducible gene 153 (GADD 153), and connexins (Cx 43) expression.

Results: CP application caused cellular damage and disrupted heart tissue tissue integrity. At the same time, CP application caused an increase in the expression of GRP-78 and GADD 153, whereas it caused a decrease in the expression of Cx43. MEL application heals cell damage and impaired tissue integrity. However, while reducing GRP-78 and GADD153 expression; CX had an effect of increasing expression.

Conclusion: MEL may have a protective effect against CP-induced cardiotoxicity in rats.

Keywords: Cardiotoxicity, cisplatin, endoplasmic reticulum stress, melatonin, rat.

ÖZ

Amaç: Sisplatin (CP), kardiyotoksisiteye neden olan kemoterapötik bir ilaçtır. Melatonin (MEL) epifiz bezinden gece boyu salgılanan bir moleküldür. Bu çalışmada CP'nin sebep olacağı kardiyotoksisteye karşı MEL'in koruyucu etkisinin araştırılması amaçlandı.

Gereç ve Yöntem: Grup 1'e (kontrol) deney boyunca serum fizyolojik uygulandı. Grup 2'ye deneyin 5. gününde tek doz CP (7 mg/kg) uygulandı. Grup 3'e 7 gün boyunca MEL (10 mg/kg) ve 5. günde CP (7 mg/kg) uygulandı. Grup 4'e 7 gün boyunca MEL (10 mg/kg) uygulandı. Deneyin 8. gününde aneztezi altında sıçanların kalpleri çıkarıldı. Kalp dokularından alınan kesitler histopatolojik değerlendirme için hematoksilen&eosin ile boyandı. Ayrıca kalp dokusu kesitleri GRP 78, GADD 153 ve Cx 43 ekspiresyonlarını değerlendirmek için immünohistokimyasal olarak boyandı.

Bulgular: CP uygulaması kalp dokusunda hücresel hasar ve doku bütünlüğünü bozucu etki göstermiştir. Aynı zamanda CP uygulaması GRP 78 ve GADD 153 ekspireyonunun artmasına sebep olurken Cx 43 ekpiresyonunun azalmasına sebep olmuştur. MEL uygulaması hücre hasarı ve

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ÖZ

bozulmuş doku bütünlüğünü iyileştirici etki göstermiştir. Bununla birlikte GRP 78 ve GADD 153 ekspiresyonunu azaltırken; CX ekspiresyonunu artırıcı etki göstermiştir.

Sonuç: Sonuçlarımıza göre sıçanlarda CP'nin sebep olacağı kardiyotoksisiteye karşı MEL koruyucu etki gösterebilir.

Anahtar Kelimeler: Endoplazmik retikulum stresi, kardiyotoksisite, melatonin, sıçan, sisplatin

INTRODUCTION

Cisplatin (CP) (cis-diamminodichloroplatinum II) is an antitumor agent used against various types of cancer (1). CP is effective against various tumors, such as head and neck, testicular, ovarian, cervix, bladder, and lung cancers. Despite its antitumoral effects, side effects, such as dose-related nephrotoxicity, hepatotoxicity, spermotoxicity, and cardiotoxicity, limit its clinical application (1-3). It is believed that the cytotoxic effect of CP occurs through the formation of covalent adducts between the platin compound in its structure and DNA bases (4). Studies have reported that acute and cumulative cardiovascular complications, a common side effect of CP, affect quality of life after treatment. These complications include arrhythmias, myocardial ischemia, heart failure, and ventricular hypertrophy (4, 5). These changes, which are difficult to reverse, may directly or indirectly cause patient death. Therefore, studies on the molecular mechanisms reducing CP-induced cardiotoxicity are necessary.

It is known that endoplasmic reticulum (ER) stress induces CP-induced cardiotoxicity (6). ER stress initiates myocardial cell damage associated with protein accumulation in the cell. This event is mediated by the ER chaperone 78-kDa glucose-regulated protein (GRP-78). Cells sequester GRP-78 from membrane receptors to regulate protein folding and accumulation. GRP7-8 aims to restore normal ER function by activating the unfolded protein response. If ER stress does not persist or ER homeostasis is disrupted, apoptotic cell death may occur. Apoptotic death caused by ER stress is involved in the pathophysiology of many cardiovascular diseases, as well as Huntington's disease and Alzheimer's disease (7, 8). One ER stress-mediated apoptotic pathway component is growth arrest and DNA damage-inducible gene 153 (GADD 153) (9). GADD 153, also known as Chop, is expressed at low levels under normal conditions. However, GADD 153, a leucine zipper transcription factor, is strongly expressed in response to stress. Agents that stop cell growth or damage DNA disrupt ER homeostasis by inducing the expression of GADD 153 (10). Treatment with CP increases GADD 153 expression (11). Gap junctions allow electrical connections between adjacent cardiomyocytes. These structures are formed by connexins (CX) (12). CX43 is by far the most abundant CX isoform and is widely expressed among atrial and ventricular myocyte (13).

Melatonin (MEL) was discovered by Aaron Lerner in 1958 by isolating it from the bovine pineal gland. It is chemically named 5-methoxy-N-acetyltryptamine (14). MEL is secreted at night under normal physiological conditions. MEL is an indoleamine molecule produced by the pineal gland by activation of the suprachiasmatic nucleus of the hypothalamus. It has sleep-inducing effects. It also regulates seasonal and circadian rhythms. MEL functions as a chronobiotic or endogenous synchronizer. Moreover, it induces numerous biological activities with potent antioxidant, anti-stimulant, anti-inflammatory, immunomodulatory, vasomotor, and metabolic properties. It is known that MEL plays therapeutic and protective roles against human health and diseases. These findings have made MEL an important research topic in cardiovascular research (15-17).

It has been shown in many studies that CP causes cardiotoxicity. However, studies on the protective effects of this damage are insufficient. In our study, we aimed to observe the protective effect of MEL against cardiotoxicity using histopathological and immunohistochemical methods, unlike other studies. The results of our study showed that MEL had a protective effect against CP-induced cardiotoxicity.

METHODS

Animals and Drug Administration

The experimental procedure was approved by Erciyes University Animal Experiments Local Ethics Committee (decision no: 23/259). The rats used in the study were obtained from Erciyes University Experimental Animal Laboratory. Forty adult male Wistar albino rats were used as subjects in the study. The weight of the rats was between 150 and 220 g, and their age was between 8 and 10 weeks. Rats were housed at 20°C–22°C under a 12:12 light/dark photoperiod and fed pellet-type feed.

Experimental procedure

Control group (n=10): Daily intraperitoneal (i.p) isotonic solution (0.1 mg/kg).

CP group (n=10): On day 5 single dose i.p. CP (7 mg/kg) (1).

CP+MEL group (n=10): Daily i.p. MEL (10 mg/kg) + on the 5th day single dose i.p. CP (7 mg/kg).

MEL group (n=10): Daily i.p. MEL (10 mg/kg) (17).

Animals were sacrificed under anesthesia on the 8th day. Heart tissue samples were collected for histological and immunohistochemical examination.

Hematoxylin&eosin (H&E) staining

Routine histological tissue monitoring was performed for histopathological evaluation. Tissues were embedded in paraffin blocks. 5 µm sections were obtained from the paraffin blocks. Sections were spread on slides. Paraffin was removed from the slides using xylol. The slides were then diluted by passing them through a series of gradually decreasing alcohol solutions. Slides were prepared using H&E staining. After staining, the slides were passed through a series of gradually increasing alcohol. Then, xylene was added. After applying the occlusion medium, the preparations were examined under a microscope (Olympus BX51, Tokyo, Japan) (18). H&E dyes were purchased from Nanotek Lab (Kayseri, Türkiye).

Immunohistochemical staining

Immunohistochemistry was used to investigate GRP78 (bs-1219R, Bioss), GADD153 (sc-56107, SantaCruz Biotechnology, USA), and Cx43 (E-AB-30999, Elapscience, USA) immunoreactivities in heart tissue. 5 µm sections were obtained from paraffin blocks and placed on lysine slides. The sections were placed in an oven at 60 °C to remove the paraffin and were then kept. Paraffin was removed from the sections using xylol. The sections were then diluted by passing them through a series of gradually decreasing alcohol solutions. Sections were placed in sterile urine cups containing 0.01 M citrate buffer and heated in a microwave oven at 350 W for antigen retrieval. The sections were kept in phosphate buffered saline (PBS) (repeated 3 times). Sections were maintained in 3% (wt/vol) H_2O_2. Thus, endogenous peroxidase activity was blocked. The sample was washed with PBS. Ultra-V block solution was added to the tissues. Then, GRP-78, GADD 153, and Cx43 antibodies were added to the tissues and incubated at 4°C overnight. Tissues were washed again 3 times with PBS. The secondary antibody (TA-125-HDX, Thermo Fisher Scientific, Waltham, MA, USA) was applied at room temperature. The sample was then washed with PBS. The immune reaction was enhanced by the streptavidin-avidin-peroxidase complex. Sections were visualized using 3,30-β-diaminobenzidine tetrahydrochloride (TA-060-HDX, Thermo Fisher Scientific, Waltham, MA, USA). Gill hematoxylin was then applied. Finally, the Sections were passed through a series of gradually increasing alcohol. Then, xylene was added. After applying the occlusion medium. Images were obtained using a light microscope during preparation. The ImageJ program was used to evaluate antibody expression in the obtained images (19, 20).

Statistical Analysis

All quantitative data were statistically analyzed using GraphPad Prism v8.0 for MacOS (GraphPad Software, La Jolla, California, USA). To determine the data's normal distribution, the D'Agostino-Pearson omnibus test was performed. The quantitative variables were compared using Kruskal-Wallis and Tukey's post hoc test. $P<0.05$ was used to determine statistically significant differences.

RESULTS

Microscopic Examination

In our study, histological evaluations of the heart tissue of the experimental groups were performed using H&E-stained preparations. Normally structured tissue sections are observed in the heart tissue sections of the control group. In the heart tissue sections in the group in which we applied CP, intense eosinophilic staining of cardiomyocytes, vacuolization-like cytoplasmic spaces and disorganization of muscle bundles were observed ($p<0.0001$). In the heart tissues of the group in which we applied CP and MEL, eosinophilic staining of cardiomyocytes, vacuolization-like cytoplasmic spaces, and disorganization in muscle bundles were significantly reduced ($p<0.0001$). Heart tissue sections in the group in which only MEL was applied had a normal appearance, similar to the control group. H&E-stained sections of heart tissue and histopathological scoring chart are shown in Figure 1.

Immunohistochemical Examination

In our study, the immunoreactivity of GRP-78, GADD 153, and CX43 antibodies in sections obtained from cardiac tissues was measured. The obtained data were statistically compared. When the CP group was compared with the control group; CX43 ($p<0.01$) immunoreactivity decreased. However, GRP78 ($p<0.01$) and GADD 153 ($p<0.0001$) immunoreactivity increased. When the CP+MEL group was compared with the CP group; CX43 ($p>0.05$) immunoreactivity increased. However, GRP78 ($p<0.01$) and GADD 153 ($p<0.0001$) immunoreactivity decreased. There were no significant differences in the immunoreactivities of GRP-78, GADD 153, and CX43 between the control and MEL groups. Immunohistochemical staining showing GRP-78, GADD 153, and CX43 expression is shown in Figure 2. GRP-78, GADD 153, and CX43 immunoreactivity measurements are presented in Figure 3.

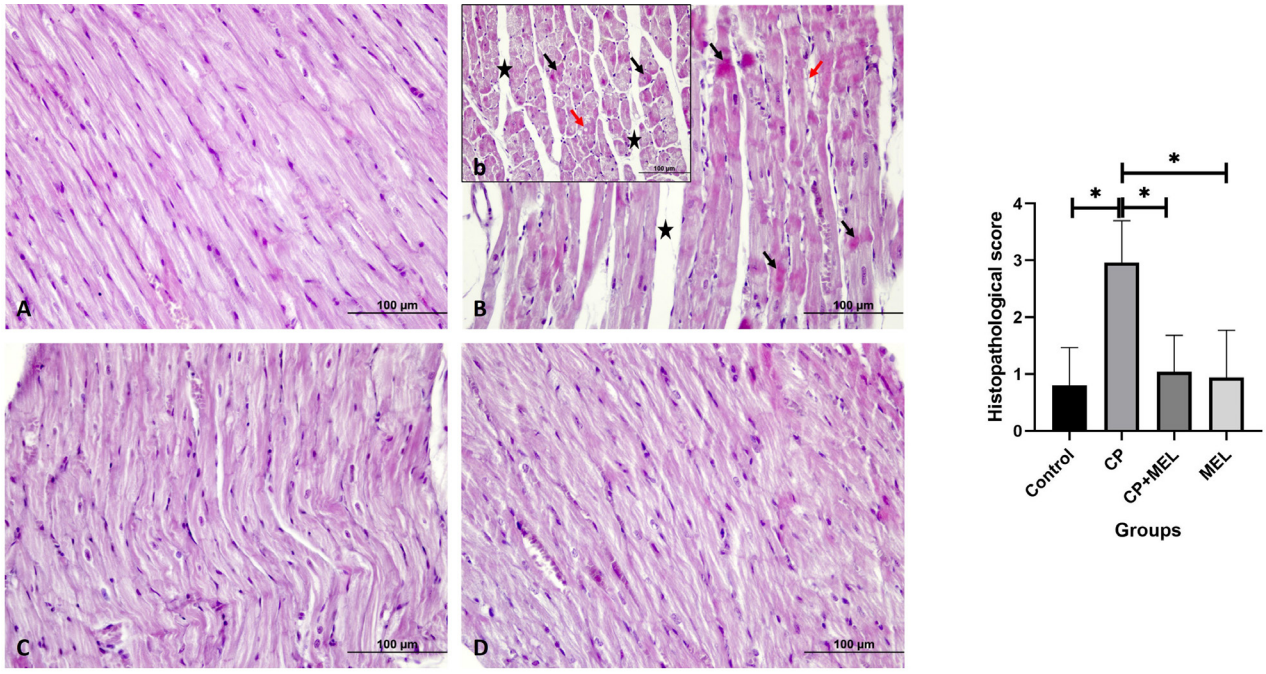

Figure 1: Light microscopic images taken from the hearts of the experimental groups and histopathological scoring graph. Regularly located cardiomyocytes were observed in the Control, CP+MEL, and MEL groups. In the CP group, intense eosinophilic staining of cardiomyocytes (black arrow), vacuolization-like cytoplasmic spaces (red arrow) and disorganization of muscle bundles (star) are observed. Inset photo (b) shows a cross-section of a different area belonging to the CP group. **A:** Control; **B:** CP; **C:** CP + MEL; **D:** MEL. (Hematoxylin&Eosin Staining) Olympus microscope, 400x. *p<0.0001

CP: Cisplatin, MEL: Melatonin

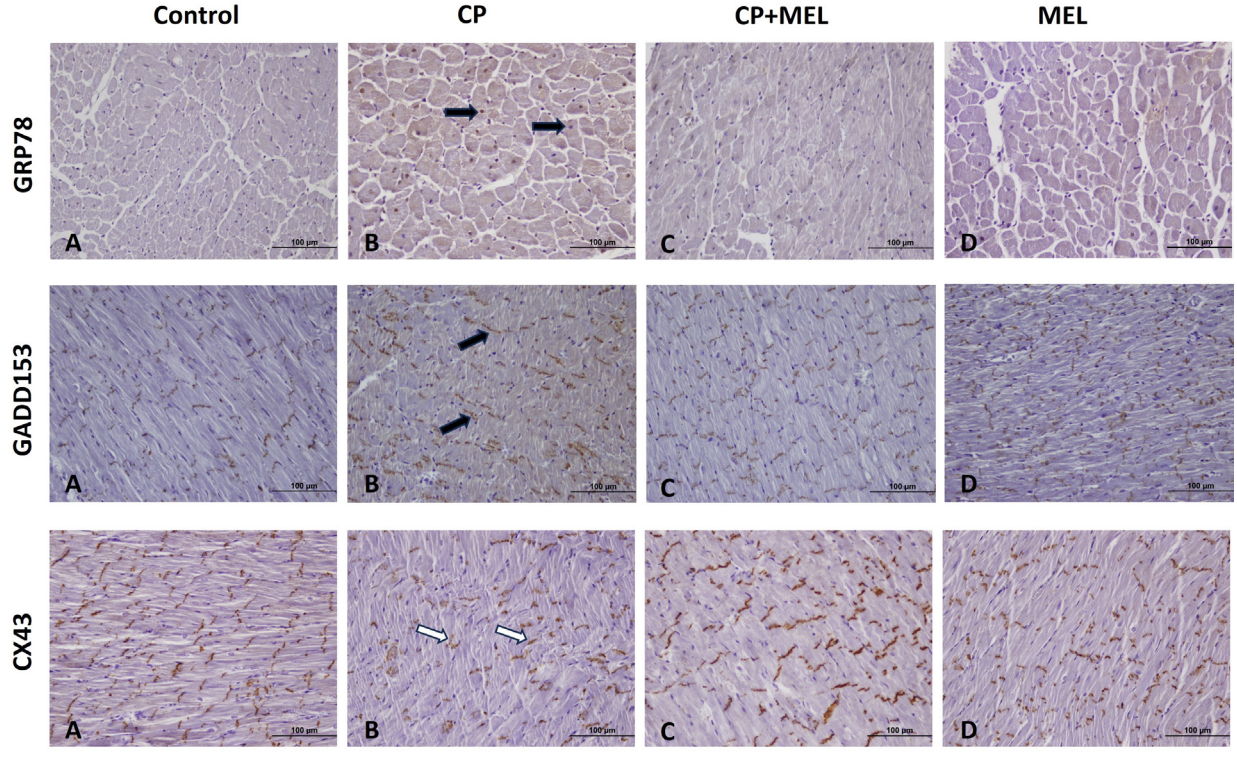

Figure 2: Immunohistochemical staining of GRP-78, GADD 153, and CX43 in experimental groups. The black arrow indicates immunohistochemically stained structures. The white arrows indicate structures that are not or are understained immunohistochemically. **A:** Control; **B:** CP; **C:** CP + MEL; **D:** MEL. Immunohistochemical staining, Olympus microscope, ×400.

CP: Cisplatin, MEL: Melatonin, GRP-78: 78-kDa glucose-regulated protein, GADD 153: Growth arrest and DNA damage-inducible gene 153

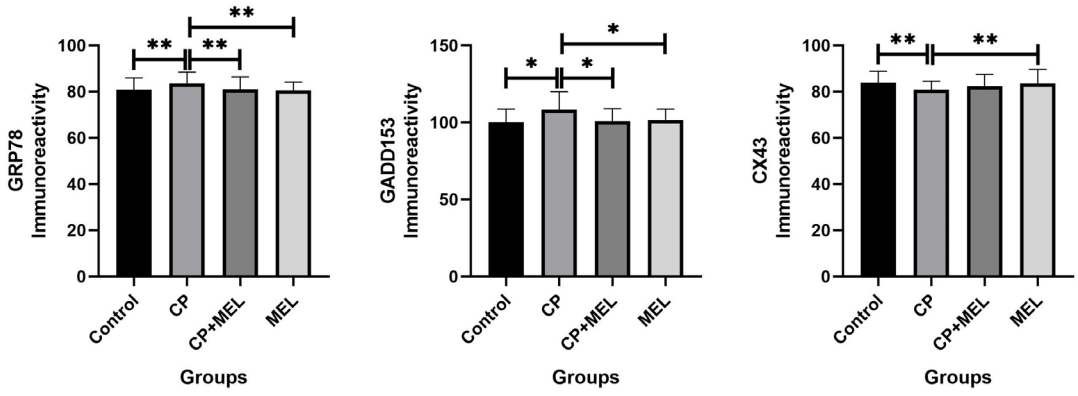

Figure 3: Statistical analysis of the immunoreactivity of GRP-78, GADD 153, and CX43 in the experimental groups. *p<0.0001 **p<0.01. GRP-78: 78-kDa glucose-regulated protein, GADD 153: Growth arrest and DNA damage-inducible gene 153

DISCUSSION

Cardiac toxicity is a significant problem and a burden in patients with cancer. Cardiotoxicity induced by CP, which is used in various cancer treatments, occurs by triggering ER stress and causing damage to CXs. These changes may also disrupt the functionality of the heart by triggering apoptosis (7, 21). The aim of this study was to investigate the potential protective role of MEL against CP-induced cardiac toxicity in rats, focusing on the pathophysiological pathways. For this purpose, in our study, we performed histopathological and immunohistochemical evaluations to investigate the protective effect of MEL against CP-induced cardiotoxicity.

Various studies have shown that CP has a damaging effect on cardiomyocytes. In a study conducted on rat experimental models with a single dose of CP, acidophilic stained sarcoplasm and degenerated and separated muscle fibers were observed (21). In a study in which CP was applied chronically, muscle fibers were shown to degenerate and separate. Additionally, in the same study, cardiomyocytes had irregular or shrunken nuclei and cytoplasmic vacuoles were formed in some areas (6). Studies have revealed that CP application causes cardiomyocyte damage and muscle fiber separation. Similar to other studies, in our study, intense eosinophilic staining of cardiomyocytes was observed in the heart sections of CP-treated rats. In addition, vacuolization-like cytoplasmic spaces and disorganization of muscle bundles are observed in heart sections. These damages were significantly reduced in the heart tissue sections of CP-treated rats treated with MEL. These results show that MEL protects against histopathological damage caused by CP in the heart. At the same time, the mechanism underlying this protective effect needs to be elucidated. For this purpose, we also evaluated the immunoreactivities of GRP-78, GADD 153, and CX43 in our study.

CP-induced cardiotoxicity is associated with ER stress. ER stress induces apoptotic cell death. Excessive GRP78 and GADD153 expression triggers ER stress and ultimately causes apoptotic cell death (6, 7, 9). Saleh et al. (7), in their study in which they applied a single dose of CP, stated that GRP78 activation increased and therefore the cells would tend to undergo apoptosis. Chowdhury et al. (22) reported that single-dose CP treatment increased GRP78 expression in the heart and apoptotic cell death occurred through ER stress. Various studies have reported that CP induces ER stress-mediated apoptotic cell death in the heart. In our study, it was shown immunohistochemically that GRP-78 expression increased with CP application. The decrease in GRP-78 activation in rats treated with MEL as a protective agent indicates that ER stress will decrease. These findings indicate that the tendency of cells to undergo apoptosis will also decrease.

Another apoptotic pathway component that disrupts ER stress is GADD-153. GADD-153, a DNA damage susceptibility gene, can be highly induced by genotoxic agents. The levels of GADD-153, which is also a transcription factor, increase in response to stress (10, 23). An in vitro study showed that cardiomyocytes tend to undergo apoptosis under mechanical stress, and GADD-153 expression increases in these cells (24). According to D'Abrosca et al., protein folding can be significantly disrupted under certain physiological and pathological conditions, leading to ER stress. GADD-153 activation increased ER stress and induced apoptosis (25). In our study, we immunohistochemically determined the expression of the transcription factor GADD-153, which causes the initiation

of apoptosis in cardiomyocytes. We found that GADD-153 expression increased in the heart tissue of rats treated with CP and that MEL treatment reduced this expression level. These findings show that MEL exerts a protective effect against GADD-153- mediated apoptosis caused by CP.

As mentioned above, preventing CP-mediated apoptotic cell death is an important treatment approach for heart tissue. In addition, gap junction connections are required to ensure interaction between cardiomyocytes and to preserve tissue integrity. The structure of these channels that transmit action potentials consists of CX proteins. The most common isoform in the heart is CX43. Damage to CX43 may cause a decrease in the conduction velocity (26). In fact, Cx43 abnormalities are common in all cardiovascular diseases with impaired rhythms and conduction (27). This abnormality in cardiovascular diseases encouraged us to investigate whether there is a change in CX43 expression with CP application. In this study, we demonstrated that CX43 expression in heart tissue decreased with CP application. In diseased heart tissue, CX43 is downregulated and impairs heart function (13). CP-induced damage to CX43, which is common in atrial and ventricular myocyte, also indicates that cardiac functions and electrical stability will be impaired (21). The MEL dose used in our study improved CX43-related damage to a certain extent. However, different doses of MEL to be used in future studies may lead to a more effective improvement of CX43.

In conclusion, previous studies have shown that MEL has therapeutic effects in many tissues (28-30). In this study, MEL was applied to prevent CP from damaging rat heart tissue. There was improvement in the cardiomyocytes in the heart tissue of rats administered MEL together with CP and in the disorganization of muscle bundles. In addition, MEL prevented ER stress, a mediator that causes apoptosis. However, it can also protect against damage that may occur at gap junctions.

As a result, we determined that CP affects many organs and causes serious histopathological damage to the heart tissue. It has been shown that MEL, given for preventive purposes to prevent this damage, may protect the heart. Some mechanisms of this protective effect were revealed in our study. More research is needed to uncover the exact mechanism of MEL's protective effect on the heart.

ETHICS

Ethics Committee Approval: The experimental procedure was approved by Erciyes University Animal Experiments Local Ethics Committee (decision no: 23/259, date: 07.12.2023).

Informed Consent: Since this research was conducted on animals, patient consent was not required.

FOOTNOTES

Authorship Contributions

Surgical and Medical Practices: T.C., M.Ü., E.K., A.T.A., N.K., D.K., Concept: T.C., M.Ü., B.Y., Design: T.C., M.Ü., B.Y., Data Collection or Processing: T.C., E.K., A.T.A., N.K., Analysis or Interpretation: T.C., M.Ü., E.K., A.T.A., N.K., D.K., B.Y., Literature Search: T.C., M.Ü., D.K., B.Y., Writing: T.C., M.Ü., D.K., B.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

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