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Protective effects of trimetazidine against hepatic warm ischemia/reperfusion injury on rats

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is related to insulin resistance, obesity, hyperlipidemia, and oxidative stress. Trimetazidine (TMZ) is an anti-ischemic piperazine derivative. This study aims to investigate the protective effect of the TMZ against warm ischemia-reperfusion (I/R) damage in the liver on an experimental NAFLD model. The blood samples for biochemical analysis and liver tissue for histopathological analysis were collected from rats. A total of 32 *Sprague Dawley* male rats was divided into four groups. Group 1 (controls, n = 6); rats were fed the standard basal diet for eight weeks and underwent no surgery. Group 2 (sham-operated group n = 6); rats were fed a high-fat diet for eight weeks and underwent laparotomy without warm I/R damage. Group 3 (warm I/R group, n=10); rats were fed a high-fat diet for eight weeks, and warm I/R damage was created. Group 4 (warm I/R+TMZ group (n=10); rats were fed a high-fat diet for eight weeks, and the TMZ (10 mg/kg) was given a single dose by the orogastric way 30 minutes before the warm I/R was created. In Group 4, while the TMZ caused to decrease in levels of oxidative stress markers; MPO ($p<0.001$), NOX ($p<0.001$), and Cyt-C ($p<0.001$) increased the activity of the antioxidant enzyme CAT ($p<0.001$) as compared to Group 3. There is no significant difference in levels of other oxidant markers, MDA and 8-OHDG in all groups ($p>0.05$). The hepatocyte cell damage in cell groups related to I/R damage was observed. Against this observation, the TMZ improved dilatation of sinusoids, inflammation, and structural integrity of hepatic plaques in group 4. In conclusion, TMZ could improve the antioxidant defense mechanism upon oxidative damage and increase cells' survival rate by protecting mitochondrial integrity in NAFLD.

Keywords: Warm ischemia, reperfusion injury, non-alcoholic fatty liver disease, oxidative stress, trimetazidine

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a syndrome characterized by steatosis of more than 5% of the liver parenchyma (Serfaty and Lemoine, 2008). Fatty liver is considered a source for transplantation, whereas NAFLD's presence exacerbates liver damage after liver surgery (Kato et al., 2014). NAFLD's presence is a significant factor that increases the risk of morbidity and mortality after liver surgery (Tashiro et al., 2014).

The fat-laden hepatocyte cells face oxidative stress acutely exacerbated during ischemia-reperfusion (I/R) and cause extensive parenchymal damage in NAFLD (Gupta et al., 2014). Besides, the mitochondrial disorder is associated with fatty liver I/R damage. The fatty liver produces higher levels of mitochondrial reactive oxygen species (ROS) in response to low antioxidant levels during I/R damage. The decreased adenosine triphosphate (ATP) levels after reperfusion lead to reductions in healing capacity and an increase in cell death (Prieto and Monsalve, 2017). Increased cytochrome C (Cyt-C) release, inhibition of ATP production from mitochondria, and the severity of inflammation response notably enhance parenchyma damage in fatty liver versus healthy liver (Selzner et al., 2003).

Trimetazidine (TMZ) is an anti-ischemic piperazine derivative (Nadkarni et al., 2015). During ischemia, the TMZ reduces intracellular Ca^{+2} input, prevents intracellular pH levels from falling, and shows protective efficacy on mitochondrial damage by increasing the amount of ATP (Onay-Besikci and Özkan, 2008).

Limited studies investigating the protective efficacy of TMZ against I/R damage caused by liver surgery in NAFLD

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cases. This study aims to examine the protective efficacy of TMZ against liver warm I/R damage in rats with the NAFLD model.

2. Materials and methods

2.1. Ethics statements

All experimental protocols followed the Declaration of Helsinki principles and were carried out by the European Communities Council Directive (86/609/EEC). The study procedure was approved with 11/02/2015 dated and 82678388/16 numbered decision by Experimental Animals Local Ethics Committee.

2.2. Experiment design

In this study, 32 Sprague Dawley male rats with body weights between 185-200 grams were used. The rats were housed at 22 ± 2 °C for 12 hours in darkness and 12 hours in light. The rats were randomly divided into four groups;

Group 1 (control group, n=6): Rats were fed the standard basal diet from the beginning to the end of the experiment (8 weeks) and underwent no surgery. Group 2 (sham group, n=6): Rats were fed a high-fat diet from the beginning to the end of the experiment (8 weeks) and had laparotomy, hilus dissected, and no I/R. Group 3 (warm I/R group, n=10): Rats were fed a high-fat diet from the beginning to the end of the experiment (8 weeks), and I/R was created (n=10). Group 4 (warm I/R+TMZ group, n=10): Rats were fed a high-fat diet from beginning to end (8 weeks), and a single dose of TMZ (10 mg/kg) was given orally by gavage 30 minutes before the I/R was created.

The standard feed content includes 15% protein, 2.5% fat, 15% cellulose, 14% clay, 13% water. The high-fat diet was created by adding 100 grams of a fresh tail, a type of animal fat, to the standard diet of 25 grams. Thus, 65% of the total energy was provided by a fat-derived diet (Zhou et al., 1998). TMZ was administered orally with a 10 mg/kg single dose (Vastarel®, Servier, Istanbul) 30 minutes before the I/R operation, while all other groups were administered with the same volume of saline (SF) as TMZ. It was reported that the optimal dosage of TMZ sustains the normal functions of mitochondria isolated from rat liver subjected to I/R is 10 mg/kg (Elimadi et al., 1998). The primary elimination route of TMZ is via the urine, and the elimination half-life is about six hours. In experimental studies, toxicity has not been found even in higher doses comparing therapeutic doses (Ergücük et al., 2020). All animals were weighed and anesthetized with an intramuscular injection of 75 mg/kg ketamine hydrochloride (Ketalar®; Parke-Davis, Istanbul) and 15 mg/kg xylazine (Rompun®, Bayer, Istanbul). Once the surgical area had been cleaned, an abdominal incision has occurred. The total ischemia was applied to the liver by placing a mini-vascular bulldog clamp on the hepatic pedicle with midline laparotomy (Pérez et al., 2016). During the operation, the rats were kept closed to avoid loss of abdominal heat. The Bulldog clamp was opened at 60th minutes of ischemia, and two ccs SF was left to the bat, and it was closed in one plan with 2/0 silk and taken back to its cage. During the operation, the room temperature was kept at 25 °C. At the 6th hour of reperfusion, blood samples were taken by an intracardiac puncture to measure biochemical markers, and the rats were sacrificed by exsanguination. After being kept at room temperature for 30 min, blood samples were centrifuged at 1800 g for 15 min. Serum samples were stored in a deep freezer at -80 °C for three months. The liver tissues of each animal were removed, cleaned, dried, and processed for histopathological evaluation.

Serum catalase (CAT) and myeloperoxidase (MPO) activities were measured by the colorimetric method (respectively, Goth, 1992, Bradley et al., 1982). Serum cytochrome C (Cyt-C), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase, NOX), malondialdehyde (MDA), and 8-hydroxyguanosine (8-OHdG) measurements were performed using rat-specific enzyme immune absorbent assay (ELISA) kits (Elabscience Biotechnology Co., Beijing, PRC). The optic density had been read at a 450 nm wavelength on an ELISA reader (Biotek, ELX 800). The measurement of serum aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) activities were performed by an autoanalyzer (Hitachi PP Moduler) using commercial kits (ARCHITECT).

2.3. Histopathological evaluation

The liver tissue samples obtained for histopathological examination were fixed with 10% formalin. The rats' liver tissue was sectioned and stained with hematoxylin and eosin (HE) and subjected to histopathological evaluation according to the scoring system defined by French et al. (French et al., 2000). The liver tissue sections were examined for degeneration, the structural integrity of hepatic plaques, steatosis, lymphocyte infiltration, and sinusoid dilatation.

2.4. Statistical evaluation

Normal distribution control of the data analyzed by the Kolmogorov-Smirnov test and homogeneity control of groups analyzed by the Levene test. The data for the weight variable levels of one of the two-factor factors were analyzed by repeated-measures variance analysis (Two-way ANOVA with repeated measures on one factor). The remaining variables were evaluated by one-way variance (One-way ANOVA) analysis. Tukey multi-comparison test was performed at a 5% significant level to determine different groups. The results were presented as mean ± standard deviation (SD).

3. Results

The weight values of the groups are shown in Table 1. There were no significant differences among all groups at the beginning of the study ($p>0.05$). At the end of the study, significantly increased average body weight was found in all groups compared to beginning values ($p<0.001$), and the weight averages of the rats in all groups were significantly higher than controls. ($p<0.001$).

The comparison of serum CAT, MPO, NOX, MDA Cyt-C, and 8-OHdG levels obtained from the groups are presented in Table 2. When compared to controls, significantly increased serum MPO (respectively, $p<0.01$, $p<0.001$), NOX ($p<0.001$), and Cyt-C ($p<0.001$) levels, and also decreased CAT activity ($p<0.001$) were found in Groups 2 and 3. TMZ caused a significant decrease in serum NOX ($p<0.001$) and Cyt-C (respectively, $p<0.01$, $p<0.001$) levels and an increase in CAT activity ($p<0.001$) in Group 4 as compared to both Groups 2 and 3. The serum MPO activity significantly decreased in group 4 compared to group 3 ($p<0.001$). Serum MDA and 8-OHdG levels did not differ significantly among the groups ($p>0.05$). The highest AST and ALT activities were obtained in Group 3. Compared to Group 3, significantly decreased AST and ALT activities were obtained in Group 4 (respectively, $p<0.01$, $p<0.001$).

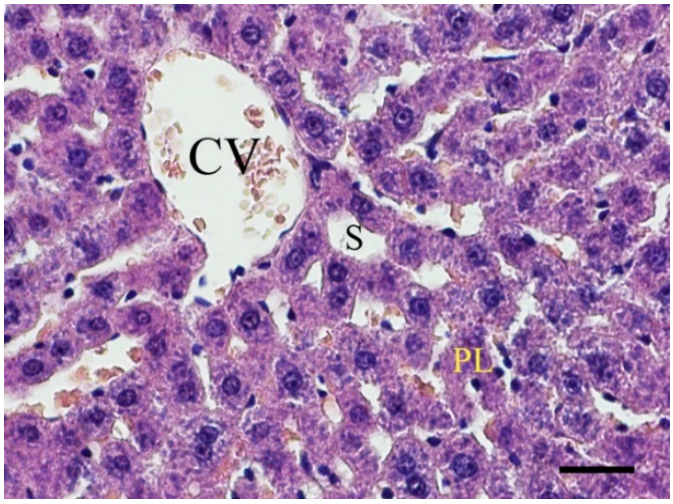

Fig. 1. The histological structure of the control group (Group 1). The liver represented a histological structure of the central vein (CV) and hepatocytes by hematoxylin and eosin (H&E) staining in controls. The hepatic plaque (PL), bar magnification=20 µm

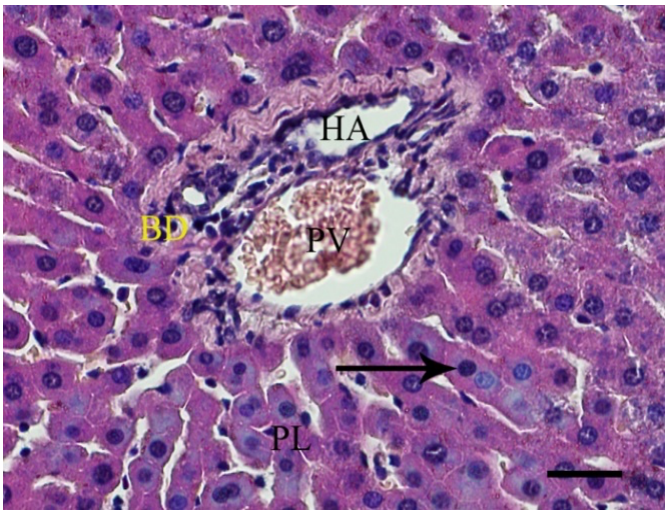

Fig. 2. The hepatocyte cells leading to apoptosis in the sham group (Group 2). The sham group showed apoptotic hepatocyte cells (arrows) with a condensed nucleus and the area with the pale cytoplasm (asterisk) near the portal. The portal vein (PV), hepatic plaques (PL), duct of gall (BD), hepatic arterial (HA). H&E staining, bar magnification=20 µm

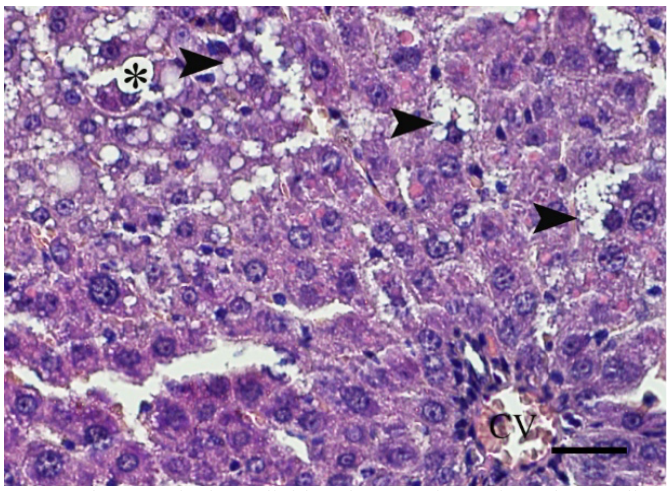

Fig. 3. The vesicular lipid degeneration in fatty liver ischemia/reperfusion group (Group 3). The ischemia/reperfusion group showed the hepatocyte size increase, foamy appearance, and micro vesicles in the cytoplasm because of the degeneration of lipids. In the ischemia group pointed out the disruption in the structural integrity of sinusoids and hepatocytes. H&E staining, bar magnification=20 µm

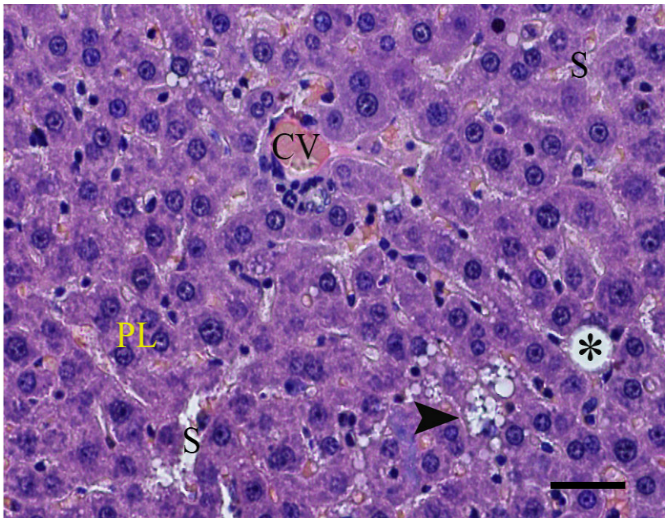

Fig. 4. The macro vesicular steatosis in TMZ treatment group (Group 4). The group treated with TMZ showed the micro vesicular (arrowheads) and macro vesicular steatosis (asterisks) in the hepatocytes. The central vein (CV) and the hepatic plaques (PL). H&E staining, bar magnification=20 µm

The light microscope images of the groups are presented in Figures 1-4. In group 1; histological structures of hepatocytes, central vein, sinusoid, and portal vein were typical and scored as 0. In group 2; sinusoids showed severe dilatation. Hepatocyte cells with cloudy cytoplasm due to lipid storage were at the onset of degeneration, and their nucleus was condensed. It was observed that the hepatocyte cells undergo apoptosis, and hepatocyte damage scored as 1. Macro vesicular and micro vesicular steatohepatitis observed in group 3. In macro vesicular steatosis, it was observed that one or more round oil droplets caused the hepatocyte nucleus to displace towards the periphery of the cell. In microvascular steatosis, it was observed that many small oil droplets surrounding the hepatocyte nucleus gave the hepatocyte a foamy and vesicular appearance. Sinusoid dilatation, deterioration in the structural integrity of hepatocytes, and

hepatic plaques were also noted. Lymphocyte infiltration was observed in the central vein and peripheric portal area. Widespread hepatocyte damage observed in the cell and cell groups and scored as 4. In group 4, a small number of hepatocytes showing micro vesicular and macro vesicular steatosis were seen, and the severity of steatosis scored as score 2. As compared to Group 3, enlargement of sinusoids and inflammation were decreased, and the structural integrity of hepatic plaques improved.

Table 1. The comparison of initial and final weights of rats in the groups

	Group 1 X ± SD	Group 2 X ± SD	Group 3 X ± SD	Group 4 X ± SD
Initial weight (gr)	191.50± 5.68	194.00± 7.04	193.60± 9.11	195.40 ± 8.83
Final weight (gr)	242.00± 6.98 a*	320.33±15.68 a*b*	305.70±16.88 a*b*	313.20±14.01 a*b*

compared to: a; the initial weight of each group, b; final weight of group 1. *p<0.05

Table 2. The comparison of the mean CAT, MPO, NOX, MDA Cyt-C, 8-OHdG, AST, and ALT levels of the groups. (CAT; catalase, MPO; myeloperoxidase, NOX; nicotinamide adenine dinucleotide phosphate-oxidase, MDA; malondialdehyde, Cyt-C; cytochrome C, 8-OHDG; 8-hydroxyguanosine, AST; Aspartate aminotransaminase, ALT; alanine aminotransaminase)

	Group 1 Mean ± SD	**Group 2 Mean ± SD**	**Group 3 Mean ± SD**	**Group 4 Mean ± SD**
CAT (kU/L)	119.80± 8.18	89.33± 4.53^{a***}	77.80± 7.96^{a***}	110.40± 2.14^{b***c***}
MPO (U/L)	52.00± 3.46	148.43± 42.53^{a**}	189.21± 54.15^{a***}	84.44± 34.50^{c***}
NOX (ng/mL)	5.49± 1.30	17.66± 1.31^{a***}	19.15± 0.99^{a***}	6.33± 0.76^{b***c***}
MDA (ng/mL)	413.28± 112.89	583.08± 127.12	596.63± 163.53	555.53± 153.30
Cyt-C (ng/mL)	2.28 ± 0.24	5.10 ± 0.52^{a***}	5.94 ± 0.79^{a***}	3.92 ± 0.45^{b**c***}
8-OHdG (ng/mL)	37.625 ±10.47	48.55 ± 23.56	52.075 ± 22.04	41.398± 17.896
AST (U/L)	64.83±7.16	140.66±3.20^{a**}	226.90±49.14^{a***b**}	163.60±33.45^{a***c**}
ALT (U/L)	31.66±6.02	100.50±3.01^{a**}	348.50±28.08^{a***b**}	241.10±36.58^{a***c***}

compared to: a; group 1, b; group 2, c; group 3. *p<0.05, **p<0.01, ***p<0.001

4. Discussion

NAFLD prevalence is increasing rapidly and emerging as the leading cause of chronic liver disease and liver transplantation worldwide (Kato et al., 2014; Tashiro et al., 2014). Since there is no standardized pharmaco-therapeutic agent for NAFLD treatment, clinical trials of many drugs are ongoing. The acetyl-CoA carboxylase inhibitors, glucagon-like peptide 1 agonists, farnesoid X receptor agonists, peroxisome proliferator-activated receptor alpha/delta agonist, and chemokine receptor 2 and 5 antagonists are some current pharmaco-therapeutics agents under development in phase II/III trials (Yoo et al., 2019). The present experimental study aimed to investigate the effect of TMZ against liver warm I/R damage on NAFLD induced by a high-fat diet.

The NOX is a multimeric transmembrane enzyme complex that contributes to superoxide production and hydrogen peroxide from molecular oxygen. This complex enzyme system normally functions for cell defense, but in I/R-induced damage, it could cause more harmful effects to the cell than a defense mechanism (Kapoor et al., 2018). Our study results revealed that liver I/R damage causes an increase in NOX enzyme activity due to increased oxidative stress, but TMZ contributes to cell healing by reducing NOX activity. Similar to our study, it was showed that TMZ reduces myocardial fibrosis in mice by inhibiting NOX-mediated ROT activity (Liu et al., 2016). It was also reported that Apocynin, a selective NOX inhibitor, significantly reduced I/R damage in the fatty liver (Kimura et al., 2016). We believe that TMZ might have a protective effect on the cell in ischemic conditions by reducing NOX activity. The MPO is an enzyme that catalyzes the formation of hypochlorous ($HOCl^-$) acid from hydrogen peroxide (H_2O_2) and is thus involved in the body's natural immune system antimicrobial agent. In this study, TMZ caused a decrease in MPO activity, increasing with I/R damage. Studies suggest that MPO contributes to the progression of NAFLD (Tao et al., 2016). It was reported that MPO activity started to increase at the 3rd hour after reperfusion and peaked at the 6th hour (Liu et al., 2015). Besides, MPO-deficient mice have been reported to reduce the severity of non-alcoholic steatohepatitis by reducing hepatic inflammation and fibrosis (Alshammari et al., 2018). In this study, the significant decrease in MPO activity in the TMZ applied group suggests that it may contribute to cell healing by reducing possible oxidative stress in cells against uncontrolled oxygenation during the reperfusion phase. In this study, while the liver warm I/R injury leads to a decrease in CAT activity due to increased oxidative stress response, the TMZ significantly increased CAT activity. The excessive ROS production and decreased antioxidant defense system due to either high-fat diet and I/R damage might negatively affect the cell integrity. It was reported that both increased oxidative stress and reduced antioxidant enzyme levels contribute to NAFLD progression and its complications (Park et al., 2019). The results of this study suggest that increased CAT activity in the TMZ-treated group compared to the fatty liver I/R group

could result from the positive effects of TMZ on the antioxidant system. In this study, serum Cyt-C level increased due to the damage caused by possible I/R damage to the mitochondria, and TMZ reversed this increase. We think that the reduction of Cyt-C levels by TMZ may contribute to the protective effect on mitochondrial cell membrane integrity. TMZ can be a protective agent against I/R damage by preserving mitochondrial integrity and rearranging ATP synthesis (Mahfoudh-Boussaid et al., 2012). Additionally, TMZ prevents mitochondrial swelling caused by increased calcium ions in the cell cytoplasm due to prooxidant increase (Onay-Besikci and Özkan, 2008; Ergücük et al., 2020). In this study, TMZ administration reduced the increased Cyt-C levels against liver warm I/R injury, suggesting that TMZ could be a promising agent in NAFLD treatment.

The MDA is a biochemical marker indicated that an increase in lipid peroxidation. Increased oxidative stress negatively affects many biomolecules in cells (Abdel-Salam et al., 2011). The DNA molecule, one of the most essential building blocks of the cell, is also affected by this damage. Many biochemical markers show DNA damage due to oxidative stress. However, nowadays, 8-OHdG is more preferred because it can pass through the cell membrane and be measured in urine and serum (Li et al., 2014). The 8-OHdG and MDA levels between the groups did not differ significantly in our study. Similar to our study, the results of previous studies show that TMZ does not have a lowering effect on 8-OHdG levels (Li et al., 2014; Ayyıldız et al., 2016). It has been reported that adding TMZ to the wash solution lowers elevated MDA levels because of I/R damage in the steatotic liver (Ben Mosbah et al., 2006). However, some studies do not fully support this view. Indeed, in a study investigating the protective effect of TMZ against oxidative kidney damage, it was reported that intraperitoneal administration of TMZ (3 mg/kg) to rats caused a decrease in MPO activity and had no effect on MDA levels (Yalçın et al., 2012). In an experimental study, mice were administered three different TMZ doses (1.8 mg/kg, 3.6 1.8 mg/kg, and 7.2 1.8 mg/kg) orally for two days, which low-dose TMZ administration did not have any reducing effect on brain tissue MDA levels, but high dose TMZ has been reported to reduce MDA levels (Abdel-Salam et al., 2011). In studies investigating the effect of TMZ on MDA level in ischemia damage, it is suggested that obtaining different results may be related to the dose and administration of the drug. In our study, a single dose (10 mg/kg) of TMZ was administered by oral gavage 30 minutes before the liver I/R injury was created, and no washing solution was used. While the TMZ decreased oxidant enzyme activities such as MPO and NOX, it did not cause a significant change MDA and 8OHdG levels. Therefore, we think that further studies should be conducted to investigate the effect of TMZ on MDA and 8OHdG levels by applying TMZ at different doses and durations in I/R damage models experimentally.

In this study, the protective effect of TMZ against I/R damage was proved as histopathological by reducing dilatation and inflammation in sinusoids and preserving the structural integrity of hepatocyte cells. It had been reported that TMZ protects the hepatocyte cell integrity against liver I/R injury by providing ischemic preconditioning (Casillas et al., 2006). It has been reported that stress on the ER after liver transplantation is reduced by applying TMZ (Zaouali et alet al.7). The molecular mechanisms that ensure the protective activity of TMZ against I/R damage have not been fully elucidated. In our study, the histological and biochemical demonstration of the protective effect of TMZ treatment on hepatocyte cell integrity at the cellular level strongly supports the protective effect of TMZ on cell integrity. As a result, it has been shown that a single dose of Trimetazidine (10 mg/kg) administration 30 minutes before the occurrence of warm I/R damage positively contributes to the antioxidant defense mechanism against oxidative damage, which is an essential factor that causes cell destruction against I/R damage in fatty liver. It has also been shown to support cells' survival by preserving mitochondria integrity, which is a significant factor in protecting cellular integrity. However, our study's inability to obtain a significant change in lipid peroxidation and DNA damage markers suggests that our study needs to be supported with Trimetazidine applications by performing different doses and durations.

Conflict of Interest

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

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