The preventive effect of exogenous adenosine triphosphate on methanol-induced cardiotoxicity in rats

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Exposure to methanol can cause serious consequences such as permanent visual disturbances and death. The heart tissue is highly vulnerable to ATP deficiency. Our study aimed to investigate whether exogenous ATP administration may alleviate methanol-induced ATP deficiency and subsequent oxidative damage in rat heart tissue. A total of 30 rats were divided into equal five groups; Healthy Group (HG), Methotrexate (MXG), Methanol (MeOH), Methotrexate+Methanol (MXM), and Methotrexate+Methanol+ATP (MMA) groups. We inhibited tetrahydrofolate synthesis by methotrexate to induce methanol toxicity. Methotrexate was administered to MXG, MXM, and MMA group animals for seven days with a catheter directly to the stomach at a 0,3 mg/kg dose per day. At the end of this period, % 20 methanol at a dose of 3 g/kg was administered to MeOH, MMA and MXM group animals. Immediately after methanol application, MMA group animals were injected with ATP at a 4 mg/kg dose intraperitoneally. Blood samples and heart tissues were used for biochemical analysis and histopathological examination. Co-exposure to methanol and methotrexate substantially exacerbated cardiac damage, indicating the potent cardiotoxic effects of methanol. However, the administration of exogenous ATP to MMA group animals brought biochemical oxidative damage parameters and histopathological findings closer to HG.

Keywords: ATP. Cardiotoxicity. Methanol. Methotrexate. Rat.

INTRODUCTION

Methanol is a colorless liquid usually used as an industrial solvent and cleaner (Prabhakaran, Ettler, Mills, 1993). Accidental exposure to methanol causes nonspecific symptoms, such as nausea, vomiting, vertigo, headaches, abdominal pain, respiratory difficulty, and blurred vision. If not treated early, excessive methanol oxidation to toxic metabolites may be followed by serious consequences, such as permanent visual disturbances, neurological sequel, and death (Kuteifan *et al.*, 1998; Liu *et al.*, 1998). Methanol intoxication leads to a deficiency of adenosine triphosphate (ATP), a vital energy source for cell survival. The heart is

a high ATP-demanding organ, and mitochondria primarily provide the energy demands via oxidative phosphorylation reactions (OXPHO) in the form of ATP (Balaban, 1990). OXPHO is the main mechanism for ATP production, and a decrease in the high-energy phosphate source results in oxidative stress and membrane lipid peroxidation (LPO) (Rappaport, Oliviero, Samuel, 1998). Previous studies have shown that the overproduction of reactive oxygen species (ROS) plays an important role in the development of cardiotoxicity (Šimůnek *et al.*, 2009). The low content of antioxidants in heart tissue makes it more vulnerable to the detrimental effects of ROS (Wojtacki, Lewicka-Nowak, Lesniewski-Kmak, 2000). Although the literature reporting the adverse cardiovascular effects of methanol toxicity is lacking, some studies have suggested that mitochondrial dysfunction due to methanol metabolites reduces ATP production (Liesivuori, Savolainen, 1991;

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Tephly, 1991). These results suggest that ATP exogenous replacement can contribute to protecting cardiac tissue from adverse effects of methanol toxicity. In clinical practice, methanol-induced toxicity is related to cardiac adverse events, such as susceptibility to arrhythmias and hemodynamic instability due to contractile impairment (DeFelice, Wilson, Ambre, 1976; Kraut, Kurtz, 2008). A previous study reported that exogenous ATP administration alleviated rat ovary injury in an ischemia/reperfusion-induced oxidative stress model (Kumbasar *et al.*, 2014). Typical treatment of methanol intoxication includes folate administration, co-exposure to ethanol, and hemodialysis (Kruse, 1992; Kuteifan *et al.*, 1998; Wolfson, Singer, 1988). Methotrexate is a folate antimetabolite drug widely used for the treatment of many types of cancer and autoimmune diseases, as well as for experimental methanol intoxication (Abdel-Daim *et al.*, 2017).

Folate pathway activity is high in rat livert, therefore, we used methotrexate to create a methanol intoxication model (Rajamani *et al.*, 2006). Previous publications have emphasized that methotrexate can also induce ROS formation (Albasher *et al.*, 2018). Since the cardiotoxicity may occur due to exposure to both agents, we tested the effects of methanol and methotrexate alone or in combination in animals. Our study focused on investigating whether the exogenous ATP administration to rat heart tissue could alleviate methanol-induced cardiotoxicity.

MATERIAL AND METHODS

Animals

Albino Wistar male rats approximately weighing 245-255 grams were obtained from Atatürk University Medical Experimental Application and Research Center. The rats were housed at average room temperature (22°C). The animals had access to feeding and water without restriction all day and night during the experiment. The local Animal Experimentation Ethics Committee approved the protocols and procedures (Date: 27.12.2018, Meeting no: 13/243).

Experiment groups

A total of 30 rats were divided into five groups, six in each. Healthy Group (HG), Methotrexate (MXG), Methanol (MeOH), Methotrexate+Methanol (MXM), and Methotrexate+Methanol+ATP (MMA) groups.

Chemical substances

Thiopental sodium was obtained from IE Ulagay-(Turkey), ATP was obtained from Zdorove Narodu (Ukraine), Methotrexate was obtained from Atafarm (Turkey).

Experimental procedure

Methotrexate was administered to the MXG (n = 6), MXM (n = 6), and MMA (n = 6) animal groups with a catheter connected directly to the stomach at a 0,3 mg/kg dose per day for seven days. The HG (n = 6) and MeOH (n = 6) groups received an equal volume of distilled water as solvent in the same way. At the end of the treatment period, 20% methanol at a 3 g/kg dose was administered to the MeOH, MMA, and MXM groups (Rajamani *et al.*, 2006). Immediately after methanol application, the MMA group animals were intraperitoneally injected with ATP at a dose of 4 mg/kg. Eight hours after ATP administration, all animals were sacrificed with 50 mg/kg thiopental sodium and their hearts were removed. Blood samples and heart tissues were used for biochemical analysis and histopathological examination. Troponin I (TP-I) and creatine kinase MB (CK-MB) levels were determined in blood samples taken from the tail veins before the anesthesia procedure. All group results obtained from the experiment were compared with each other.

Biochemical analysis

Tissue samples weighing 0.2 grams were collected from removed hearts. Heart tissues were homogenized in ice-cold phosphate buffers (pH = 7.5) for tGSH measurement and 1.15% potassium chloride solution for Malondialdehyde (MDA) measurement. The tissue homogenates were centrifuged at 10000 rpm for 15

minutes at 4°C and the supernatant portion was used for analysis.

Malondialdehyde (MDA) levels in heart tissue

The absorbance of the pink-colored complex formed by thiobarbituric acid (TBA) and MDA was measured spectrophotometrically. The serum/tissue-homogenate sample (0.1 mL) was added to a solution containing 0.2 ml of 80 g/L sodium dodecyl sulfate, 1.5 mL of 200 g/L acetic acid, 0.3 mL distilled water and, 1.5 mL of 8 g/L 2-thiobarbiturate. 5mL of n-butanol: pyridine (15:1) was added to the mixture after incubating at 95°C for one hour. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3- tetramethoxypropane (Ohkawa, Ohishi, Yagi, 1979).

Myeloperoxidase (MPO) activity in heart tissue

H_2O_2 containing phosphate buffer (50 mM, pH 6) was used as substrate to determine MPO activity in serum/tissue homogenates. Initially, the test buffer was prepared (7.5 mg of o-dianisidine-HCl, 5 mL of 0.0005% H_2O_2 in 40 mL of phosphate buffer). 280 µL of test buffer was added to 20 µL of serum/tissue homogenate. MPO activity was measured kinetically at 460 nm for 5 min. (Bradley *et al.*, 1982).

Total glutathione (tGSH) analysis in heart tissue

The mechanism was defined by Sedlak and Lindsay (1968). 5,5'-dithiobis [2- nitrobenzoic acid] disulfate, which is reduced by sulfhydryl groups, is chromogenic in the medium. The yellow color was generated and measured by spectrophotometry at 412 nm to provide reduction. A cocktail solution (5.85 mL 100 mM Na-phosphate buffer, 2.8 mL 1 mM DTNB 3.75 mL 1 mM NADPH, and 80 µL 625 U/L Glutathione reductase) was prepared for the measurement. 0.1 mL meta-phosphoric acid was added to 0.1 mL serum/tissue-homogenate and was centrifuged for 2 min at 2000 rpm for deproteinization before the measurement. The 0.15 mL cocktail solution was added to 50 µL of the supernatant and the standard curve was obtained by using GSSG (tGSH) (Sedlak, Lindsay, 1968).

Troponin I (Trop-I) assay

Troponin I levels were measured in the VIDAS Troponin I Ultra kit by utilizing the ELFA (Enzyme-Linked Fluorescent Assay) technique. The sample was transferred to anti-cardiac troponin I antibodies which were marked with alkaline phosphatase. Troponin I conjugated after the mixture was released into the solid phase supplier. Composed conjugate bound to the inner wall of the antigen. Unbound content washed and removed. The conjugate enzyme catalyzes 4-methylumbelliferyl phosphate and the generated product (4-methylumbelliferone) is measured at 450 nm.

Creatine kinase-MB (CK-MB) analysis

CK-MB, which was acquired from rats' plasma, was measured by Roche/Hitachi Cobas c 701. The test was carried out by immunological UV test according to the procedure. CK-MB isoenzyme has two active sites called CK-M and CK-B. The catalytic activity of the CK-M subunits was inhibited to 99,6% in the sample with the help of the CK-M specific antibodies without affecting the CK-B subunits. The remainder of the CK-B activity, corresponding to half of the CK-MB activity, was analyzed by the total CK method.

Histopathological examination

10% formalin solution was used to fix the heart tissues for 24 hours. 4 micron thick sections were procured from the paraffin blocks. These sections were stained with Hematoxylin&Eosin and examined under a light microscope (Olympus BX 52, Tokyo, Japan) by two pathologists.

Statistical analysis

The results were expressed as "mean value ± standard deviation" (x ± SD). Normality assumption was confirmed with the Shapiro-Wilk test. One-way

analysis of variance (ANOVA) was used to determine differences between groups. After the ANOVA Tukey post hoc test was performed. IBM SPSS Statistics 21 (Armonk, NY: IBM Corp.) was used to analyze the obtained data and p values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Biochemical results

MDA, MPO, and tGSH heart tissue analyses

The MDA levels and MPO activities of the MXG group animals increased (p < 0.001) compared to the HG, MeOH, and MMA groups. However, MDA levels and MPO activities of the MXM group animals were significantly higher (p < 0.0001) than those of the other groups. The difference in the MDA levels and MPO activities between HG and MMA was statistically insignificant (p > 0.05). In the MeOH group, these biochemical parameters were statistically insignificant (p > 0.05) compared to the HG and MMA groups (Figure 1A and B). MXG group tGSH levels (p < 0.001) were lower than MeOH, MMA, and HG groups. However, the tGSH levels of the MXM group animals were significantly lower (p < 0.0001) than those of the other groups. The differences in tGSH levels between the HG and MMA groups were statistically insignificant (p > 0.05). Further, the tGSH levels of the MeOH group were found to be close to those of the HG and MMA groups (p > 0.05) (Figure 1C).

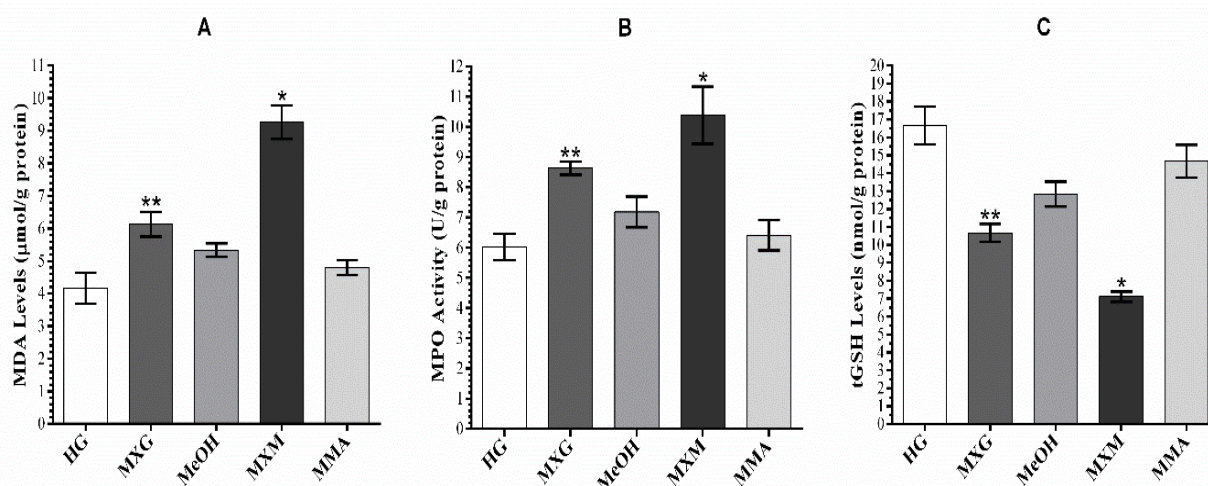**FIGURE 1 -** A; MDA levels in the heart tissue of study groups (n=6). MMA and HG groups compared with MXG, MeOH and MXM groups. *=p<0.0001 according to HG and MMA groups. **=p<0.001 according to HG and MMA groups. B; MPO levels in the heart tissue of study groups (n=6). MMA and HG groups compared with MXG, MeOH and MXM groups. *=p<0.0001 according to HG and MMA groups. **=p<0.001 according to HG and MMA groups. C; tGSH levels in the heart tissue of study groups (n=6). MMA and HG groups compared with MXG, MeOH and MXM groups. *=p<0.0001 according to HG and MMA groups. **=p<0.001 according to HG and MMA groups.

Abbreviation definition: MDA (Malondialdehyde), MPO (Myeloperoxidase), HG (Healthy group), tGSH (total glutathione), MXG (Methotrexate group), MeOH (Methanol group), MXM (Methotrexate+Methanol group), MMA (Methotrexate+Methanol+ATP group).

TP-I and CK-MB blood serum results

TP-I and CK-MB levels were found to be (p < 0.0001) increased in the MXG group blood compared to the MeOH, MMA, and HG groups. However, TP-I and CK-MB levels were significantly higher in the MXM group (p < 0.0001) than in the other groups. The differences in TP-I and CK-MB levels between the HG

and MMA groups were insignificant ($p > 0.05$). Blood serum TP-I and CK-MB levels of the MeOH group were found to be statistically insignificant ($p > 0.05$) compared to those of the HG and MMA groups (Figure 2A, B).
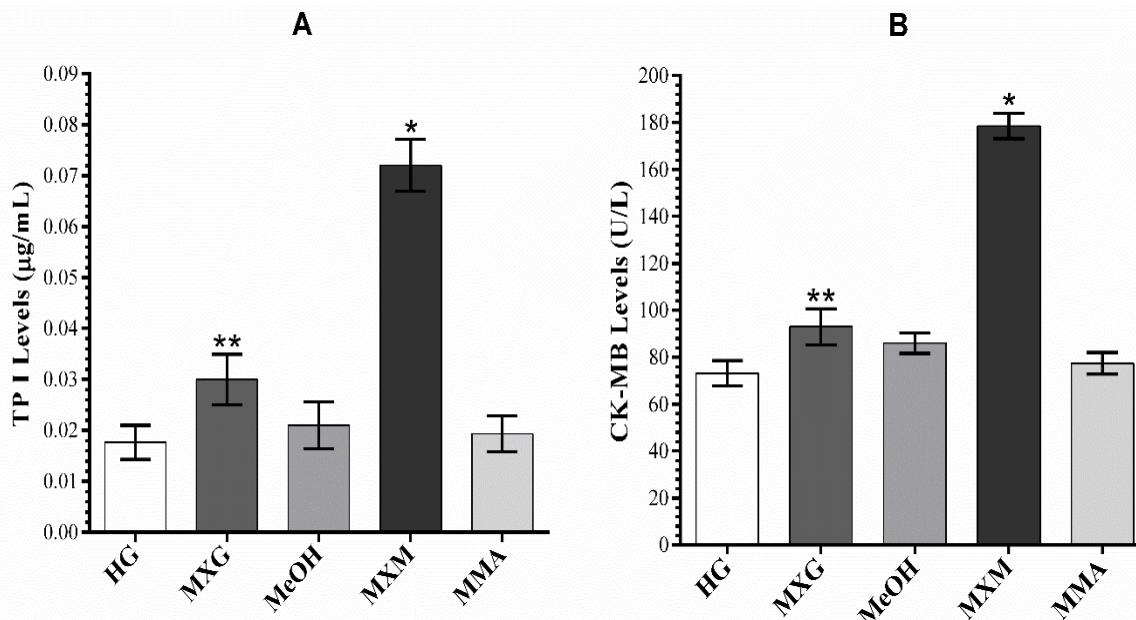

FIGURE 2 - A; TP-I levels in the blood serum of study groups (n=6). MMA and HG groups compared with MXG, MeOH and MXM groups. *=$p<0.0001$ according to HG and MMA groups. **=$p<0.001$ according to HG and MMA groups. B; CK-MB levels in the blood serum of study groups (n=6). MMA and HG groups compared with MXG, MeOH and MXM groups. *=$p<0.0001$ according to HG and MMA groups. **=$p<0.001$ according to HG and MMA groups.

Abbreviation definition: TP-I (Troponin I), CK-MB (Creatinine kinase MB), HG (Healthy group), tGSH (total glutathione), MXG (Methotrexate group), MeOH (Methanol group), MXM (Methotrexate+Methanol group), MMA (Methotrexate+Methanol+ATP group).

Histopathological results

Normal endocardium, myocardium, epicardium, and blood vessels were observed in HG animals (Figure 3A). In the MXG group animals, moderate edema, loss of myofibrillar transverse striations, and mild necrosis in the myocardial layer were observed (Figure 3 B).

In the MXM group, we observed more severe signs of destruction, including extensive edema in all layers of the heart tissue, migration of inflammatory cells, and substantial necrosis in the myocardial layers (Figure 3C). However, in the MMA group, only slightly dilated and congested blood vessels with minimal edema were observed (Figure 3D).
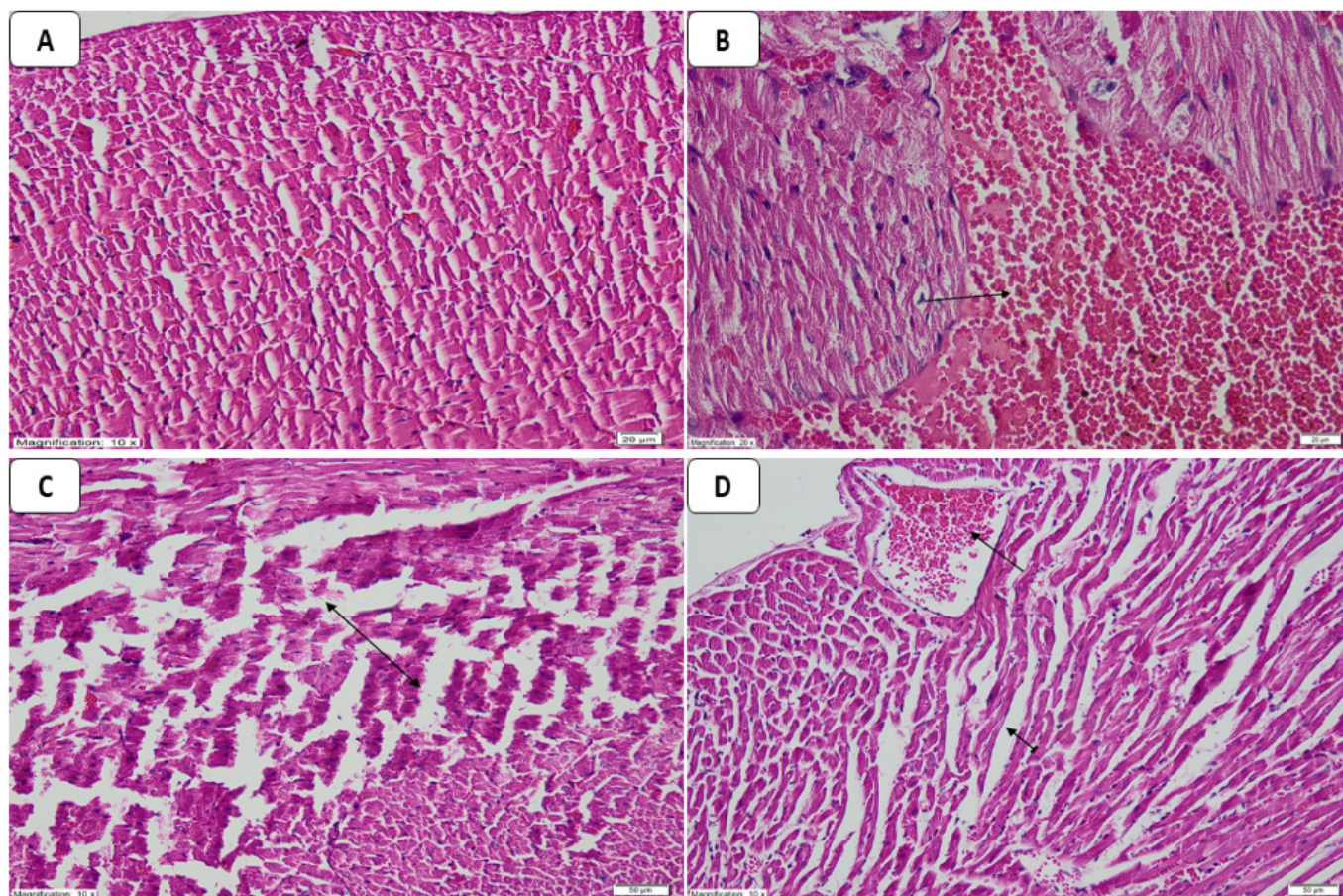
FIGURE 3 - A; Healthy heart tissue fibers (H&E, x200). B; Moderately destructed heart tissue layers with dilated and congested blood vessels in a methotrexate alone administered animal (straight arrow), (H&E x400). C; Severe destruction evidence in rat administered methanol and methotrexate in combination. Extensive edema in all layers of heart tissue. Loss of myofibrillar transverse striations and substantial necrosis in myocardial layer (double arrow), (H&E x200). D; Animal treated with ATP (adenosine triphosphate), heart tissue layers are close to healthy animals tissue (striped arrow) except for slightly dilated and congested blood vessels and minimal edema (straight arrow).

The present study evaluated the effects of exogenous ATP on methanol-induced oxidative heart damage in rats biochemically and histopathologically. Current study results demonstrated that exogenous ATP replacement therapy has a protective effect on oxidative heart damage by compensating for the ATP deficiency that occurs during methanol toxicity. Methanol causes toxic effects on almost all organ systems, but the liver, central nervous, cardiovascular, and respiratory systems are more highly affected by methanol toxicity (Tephly, 1991). Exposure to toxic vapors, ingestion, or cutaneous contact with methanol-containing products can cause damage to many tissues (Jahan, Mahmood, Fahim, 2015).

In humans, methanol is initially oxidized to toxic products to formaldehyde and subsequently to formic acid in liver cells; the latter metabolite is mainly responsible for the harmful adverse effects of methanol, such as metabolic acidosis and blindness (Goodman, Tephly, 1968). Low rates of tetrahydrofolate in human liver cells hinder the ultimate conversion of formic acid to carbon dioxide (Tephly, 1991). However, in rats, methanol is mainly metabolized by the catalase system, particularly by the tetrahydrofolate-dependent pathway, which is extremely located in the rat liver. Hence, we used methotrexate to induce methanol toxicity by inhibiting tetrahydrofolate synthesis in rat heart tissue (Schalinske, Steele, 1996).

Methotrexate can trigger oxidative stress in several ways. Due to its antimetabolite property, it can deactivate the cell via apoptosis, accelerate the expression of inducible nitric oxide synthase (iNOS), and induce the release of

proinflammatory cytokines, such as IL-1b and TNF-a. Methotrexate can also induce oxidative stress by inhibiting NADP synthesis, which plays an active role in maintaining reduced state GSH (AlBasher *et al.*, 2018). Overexpression of iNOS is associated with increasing NO, which interacts with H_2O_2 and generates peroxynitrite anion known to cause dilated cardiomyopathy (Abushouk *et al.*, 2017).

Inhibition of any step the mitochondrial cytochrome chain reaction causes histotoxic hypoxia (Siggaard-Andersen, Ulrich, Gothgen, 1995). Formic acid, a potent toxic metabolite of methanol, inhibits cytochrome-c oxidase, impairs aerobic respiration chain reaction, which may further block ATP production and subsequently cause lactate and ROS overproduction (Mika, Weissmannova-Dolezalova, Fiserova, 2014). The main energy demand of the heart is met in the form of ATP, which is crucial for maintaining heart function. A decrease in the ATP levels causes ATPase pump inhibition, increases intracellular Ca^{2+} ion concentration and initiates oxidative stress in cells (Green *et al.*, 1989).

The heart is a highly ATP-demanding organ; thus, histotoxic hypoxia initiated by formic acid in heart tissue decreases ATP production and makes it susceptible to oxidative damage (Williamson, 1979). Excessive ROS production changes the oxidant–antioxidant balance in favor of oxidants and accelerates lipid peroxidation (LPO) (Jakubczyk *et al.*, 2020).

In the present study, MDA tissue levels in the animal group administered methotrexate alone (MXG) were higher than those of the HG and MMA groups. In this study, ROS overproduction and subsequent oxidative heart damage in the MXG group could be attributed to the robust cytotoxic side effects of methotrexate (Perazella, 2009). However, MDA levels increased significantly higher in the methotrexate and methanol co-exposure group (MXM) compared to the MXG and other groups, indicating significant damage to the heart by methanol. MDA is a cytotoxic end product formed by ROS via the peroxidation of cell membrane lipids. The cross-binding of this end product to membrane components may worsen cell damage. (Esterbauer et al., 1992). In the MMA group, MDA levels were close to the HG group, suggesting a protective effect of exogenous ATP against methanol-induced oxidative damage."

ROS, such as superoxide ions, are often converted into hydrogen peroxide (H_2O_2) by spontaneous dismutation. H_2O_2 is reduced to hypochlorous acid through the MPO enzyme in the presence of chloride ions. Hypochlorous acid is a strong oxidant that can easily react with many biological molecules (Bergendi *et al.*, 1999). High tissue MPO activity in the MXG and MXM groups suggested oxidative damage in the rat heart tissues. Conversely, in the MMA group, MPO activity close to that of the HG group suggested a protective effect of exogenous ATP against methanol-induced oxidative damage.

The reason for the development of more significant heart tissue damage in the MXM group animals than in the MXG group animals can be explained by the fact that the tissues were exposed to more toxic effects with methanol after inhibiting the liver tetrahydrofolate pathway with methotrexate. These results are consistent with those of a study that investigated the effect of ATP on methanol-induced optic nerve damage (Ahiskali *et al.*, 2019). Evidence of biochemical and histopathological damage was insignificant in the MeOH control group compared to the MXM and MXG groups, possibly due to the active tetrahydrofolate pathway in the rat liver.

GSH has an essential role in maintaining cell membrane integrity by neutralizing ROS products (Parthasarathy *et al.*, 2006). GSH exhibits antioxidant properties by reacting with organic peroxides, such as H_2O_2. GSH also has an active role in neutralizing formaldehyde generated during methanol metabolism (Koivula, Koivusalo, 1975). As a result, ROS such as H_2O_2 are removed from the cells; however, these reactions cause a rapid decrease in GSH tissue levels (Spitz *et al.*, 1991). The results of the present study indicate that methanol and methotrexate alone or in combination may suppress the antioxidant defense system; however, exogenous ATP administration significantly prevented the decrease in GSH levels, suggesting its protective effect. Consistent with our study, the inhibitory effect of exogenous ATP on oxidative damage in rats has been demonstrated recently, possibly by inhibiting excessive ROS production (Aldemir *et al.,* 2020).

In clinical practice, TP-I and CK-MB are widely used markers of coronary artery disease (Thygesen *et al.*, 2018). ROS damage to myocardial cells may also lead to a release of these markers into circulation (Cordwell *et*

al., 2012). Šálek, Humpolíček and Ponížil (2014) showed that methanol exposure causes damage to myocardial cells in humans. Similarly, in the present study, oxidative damage induced by methanol and methotrexate led to the release of cardiac biomarkers in the MXG and MXM groups, respectively. In the MXM group, co-exposure to methotrexate and methanol caused significantly higher TP-I and CK-MB increases than methotrexate alone administered MXG group, suggesting that methanol substantially induces cardiotoxicity. However, in the MMA group, we detected blood serum TP-I and CK-MB levels that were close to those of the HG, suggesting that exogenous ATP prevented leakage of cardiac biomarkers and maintained the integrity of myocardial cells. The insignificant increase in biomarkers in the methanol-alone administered group compared to the HG group was probably due to the highly active tetrahydrofolate pathway in the rat liver. Insufficient ATP production may reduce cardiac contractility and stroke volume, trigger tachycardia as a compensatory response to offset hypotension, and further cause heart failure and pulmonary edema (DeFelice, Wilson, Ambre, 1976). Therefore, it is necessary to act quickly to avoid such serious side effects of methanol intoxication, particularly in exposures to high doses. In severe cases, correction of acidosis with sodium bicarbonate and folic acid infusion constitutes the initial treatment strategy (Kuteifan *et al.*, 1998). The main treatment strategy is hemodialysis, which may effectively remove formic acid from the blood; however, initiating this complex procedure requires time (Wolfson, Singer, 1988). Unfortunately, formic acid measuarable serum concentration appears with in the first hours of exposure to methanol (Kraut, Kurtz, 2008; Tephly, 1991). Considering the time taken to start hemodialysis, immediate exogenous parenteral ATP administration may be a useful therapeutic option to avoid toxic consequences as much as possible. In acute methanol toxicity, replacing rapidly diminishing ATP with exogenous ATP in highly active, energy-demanding myocardial tissue may prevent cardiac complications.

A widely accepted treatment option to reduce the production of toxic metabolites is ethanol, which takes advantage of its greater affinity to alcohol dehydrogenase than methanol (Kruse, 1992). However, ethanol administration should be given under strict medical supervision and might not be suitable in all cases due to the likelihood of worsening cardiac contractility, leading to the deterioration of hemodynamic status (Thomas *et al.*, 1994). Further, the pro-arrhythmogenic properties of alcohols require continuous monitoring of blood concentration and may provoke an already susceptible cardiac conduction system (Kraut, Kurtz, 2008; White, Carlson, 1981). Therefore, parenteral exogenous ATP might be considered with sodium bicarbonate and folic acid as an initial emergency response (Kruse, 1992). Easy and fast application of the drug may be an advantage in clinical practice to avoid cardiac side effects.

Our histopathological examination showed clear evidence of the protective effect of ATP on methanol-induced cardiac damage in rats. In the MXG animals, moderate destruction signs suggested methotrexate-induced oxidative damage. However, in the MXM group, severe signs of destruction, such as apparent edema in endocardial and myocardial layers, substantial necrosis in the myocardial layer, and inflammatory cell migration, suggested methanol-induced cardiac damage. Conversely, in the MMA group, evidence of tissue destruction was not observed except for slightly dilated and congested blood vessels and minimal edema. Consistent with the present study, methanol has been shown to cause severe histopathological damage to the rat optic nerve (Taşlı *et al.*, 2018). The present study results showed that ATP administration was able to maintain the cell structural integrity of heart tissue damage induced by methanol and methotrexate.

CONCLUSION

Exposure to methotrexate alone induced biochemical and histopathological oxidative damage in rat heart tissues. Co-exposure to methanol and methotrexate resulted in more severe oxidative damage, indicating the potent cardiotoxic effects of methanol. By contrast, exogenous ATP replacement exerted a protective effect on oxidative heart damage, possibly by meeting cardiac ATP demand due to the consequent ATP deficiency during methanol toxicity and preventing related ROS overproduction. Therefore, application facility of ATP may be considered an adjuvant treatment

option in emergency clinical practice to avoid cardiac side effects.

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