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Effects of adenosine triphosphate, thiamine pyrophosphate, melatonin, and liv-52 on subacute pyrazinamide proliferation hepatotoxicity in rats

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Abstract

Background Hepatotoxicity of pyrazinamide, an antituberculosis drug, limits its therapeutic use and oxidative stress has been implicated in this toxicity. This study investigated the protective effects of adenosine triphosphate (ATP), thiamine pyrophosphate (TPP), melatonin, and Liv-52, which have previously been shown antioxidant activities, on pyrazinamide-induced hepatotoxicity.

Methods 36 albino Wistar male rats were divided into randomized six groups; healthy (HG), pyrazinamide (PZG), ATP + pyrazinamide (APZG), TPP + pyrazinamide (TPZG), melatonin + pyrazinamide (MPZG) and Liv-52 + pyrazinamide (LPZG) groups. ATP 4 mg/kg and TPP 25 mg/kg were administered intraperitoneally (IP). Melatonin 10 mg/kg and Liv-52 20 mg/kg were given orally. One hour after administration of ATP, TPP, melatonin, and Liv-52, 250 mg/kg pyrazinamide was applied orally to all rats except HG group. The treatment was repeated (1 × 1) for 4 weeks. Then, blood samples were taken for determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Immediately after, the rats were euthanized with thiopental sodium (50 mg/kg, IP), and the livers were removed. The tissues were analyzed for malondialdehyde (MDA), total glutathione (tGSH), superoxide dismutase (SOD), and catalase (CAT) also hydropic degeneration, necrosis, and apoptosis (caspase 3) were examined.One-Way ANOVA was used in biochemical analyses and Tukey test was used as post-hoc. For histopathological and immunohistochemical analysis, the Kruskal-Wallis test was used and Dunn's test as a post-hoc.

Results Pyrazinamide increased MDA land decreased tGSH, SOD, and CAT levels in liver tissues ($p < 0.001$). It also increased serum ALT and AST activities and caused severe hydropic degeneration and necrosis in liver tissue ($p < 0.001$). ATP, TPP, melatonin, and Liv-52 significantly prevented the biochemical and histopathological changes induced by pyrazinamide ($p < 0.05$). On the other hand, Liv-52 was more successful than other potential protectors in protecting liver tissue from pyrazinamide damage ($p < 0.05$).

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Conclusions ATP, TPP, melatonin, and Liv-52 can be used to protect liver tissue from pyrazinamide-induced hepatotoxicity in rats.

Keywords Pyrazinamide, Liver toxicity, ATP, Thiamine pyrophosphate, Liv-52, Antioxidant effect

Introduction

First synthesized in 1936, pyrazinamide is an essential element of tuberculosis combination therapy and continues to be used as a first-line treatment drug [1]. Including pyrazinamide in the tuberculosis drug regimen has reduced the duration of treatment from 12 to 6 months [2]. The mechanism of action of pyrazinamide is incompletely known. However, it has been observed to penetrate Mycobacterium tuberculosis and to produce its effect by being converted to its active form, pyrazinoic acid, by pyrazinamidase [3]. Pyrazinamide is an anti-inflammatory drug that explicitly reduces inflammatory cytokine signaling and lesion activity in tuberculosis patients [4]. Although pyrazinamide was initially reported to be safe, recent studies have shown that it is more hepatotoxic than isoniazid and rifampin and even causes liver failure [1]. Pyrazinamide is a well-known cause of elevated serum aminotransferase levels and liver injury [5]. The high hepatotoxicity of pyrazinamide, a first-line antituberculosis drug, prevents its widespread use for therapeutic purposes. It has been suggested that the increase in reactive oxygen species (ROS) during the biotransformation of pyrazinamide leads to oxidative liver damage [6]. In hepatotoxicity of pyrazinamide, a decrease in total antioxidant capacity, reduced total glutathione (tGSH), and superoxide dismutase (SOD) levels and an increase in malondialdehyde (MDA) levels were found and the role of oxidative stress in the pathogenesis of this hepatotoxicity was drawn attention [7]. Oxidative stress is widely implicated in the development of many acute and chronic diseases. On the other hand, molecules with antioxidant activity can inhibit oxidative stress with excellent ROS scavenging activity in a minimal or nontoxic manner [8]. These molecules can be produced endogenously or taken exogenously from the outside [8, 9]. It has been reported that the antimycobacterial effect of pyrazinamide may be through the depletion of cellular adenosine triphosphate (ATP) reserves [3]. In addition, it was determined that ATP was significantly decreased and energy deficit occurred in female rats receiving pyrazinamide [10]. As is known, there is a link between the production of ROS and the depletion of cellular ATP levels [11]. ATP, a nucleoside triphosphate composed of adenine, a ribose sugar, and three phosphate groups [12], has been shown to participate in the production of ROS scavengers and antioxidants and to be an energy source for the synthesis of antioxidants [13, 14]. Eksogen ATP has also been reported to protect organs and tissues from oxidative damage and normalize their functions by preventing the overproduction of MDA and the depletion of tGSH [15]. Thiamine pyrophosphate (TPP), the effect of which we investigated against the possible hepatotoxicity of pyrazinamide in our study, is the active metabolite of thiamine, known as vitamin B_1 [16]. TPP previously decreased oxidant load and increased antioxidants in oxidative stress-induced hepatotoxicity. Consequently, it prevented liver dysfunction and exhibited hepatoprotective activity [17]. There is information in the literature that TPP protects eye tissue from oxidative damage caused by ethambutol [18]. In this study, melatonin was another agent tested against the possible hepatotoxicity of pyrazinamide. As is known, melatonin is a methoxyindole synthesized and secreted by the pineal gland [19]. Melatonin is among the most powerful antioxidants that protect the liver [20]. One melatonin molecule can scavenge up to 10 ROS. It also maintains the integrity of mitochondria, promotes ATP synthesis, and limits mitochondrial ROS production. It performs its antioxidant function directly by neutralizing ROS and indirectly by stimulating antioxidant enzymes [20]. Melatonin and its metabolites exhibit antioxidant capacity and therefore its antioxidant activity against oxidative stress persists after metabolism [21]. Melatonin has been previously shown to provide cytoprotection in various models of experimental acute liver injury [20]. Another product tested for the effect of pyrazinamide against oxidative liver damage in this study is Liv-52. Liv.52, a multi-herbal formulation, consists of eight ingredients including seven herbs; Capparis spinosa (65 mg), Solanum nigrum (32 mg), Cichorium intybus (65 mg), Terminalia Arjuna (32 mg), Achillea millefolium (16 mg), Tamarix gallica (16 mg), Cassia occidentalis (16 mg) and Mandur Bhasma (33 mg). These potent components of Liv.52 are reported to possess a broad spectrum of hepatoprotective properties [22, 23]. This hepatoprotective property has been demonstrated in clinical and preclinical studies [24]. It has been reported to alleviate pyrazinamide-induced hepatic dysfunction in patients previously treated with pyrazinamide [23]. Liv-52 has been reported to prevent cytotoxicity resulting from increased lipid peroxidation (LPO) and decreased GSH, SOD, and catalase (CAT) content [25].

These reports obtained from the literature implicate that ATP, TPP, melatonin, and Liv-52 may have protective effects against pyrazinamide hepatotoxicity. There is no information in the literature investigating the protective effects of ATP, TPP, melatonin, and Liv-52 against pyrazinamide hepatotoxicity. Therefore, the objective of

this trial was to investigate the effects of pyrazinamide on the liver in rats and to examine the protective effects of ATP, TPP, melatonin, and Liv-52 against possible hepatotoxicity.

Materials and methods

Animals

In the experiments, 36 albino Wistar male rats (280–290 g) were used. The rats were obtained from Erzincan Binali Yıldırım University Experimental Animals Application and Research Centre. Before the experiment, the animals were divided into six groups and housed under appropriate conditions (21 ± 2 ℃, 12-hour light-dark cycle) and fed ad libitum. Before and during the experiment, the animals were fed with normal tap water and animal feed. All experiments were carried out by European Parliament Directive 2010/63/EU (Approval Number 2016-24-199) and ARRIVE guidelines.

Chemical substances

Thiopental sodium used in this study was supplied by IE Ulagay (Istanbul, Turkey). ATP was supplied by Zdorove Narodu (Kharkiv, Ukraine). Thiamine pyrophosphate (50 mg of solution powder) was obtained from Bio-Pharma (Moscow, Russia). Melatonin was supplied by Przedsiebiorstwo Farmaceutczne LekAm (Zakroczym, Poland). Liv-52 was provided by Himalaya drug (Bengaluru, India) and Pirazinamid was supplied by Koçak Pharma (Tekirdağ, Turkey).

Experimental groups

Rats were separated into six different groups ($n = 6$ / each group): healthy group (HG), pyrazinamide group (PZG), ATP + pyrazinamide group (APZG), TPP + pyrazinamide group (TPZG), melatonin + pyrazinamide group (MPZG) and Liv-52 + pyrazinamide group (LPZG).

Experimental procedure

In this study, 4 mg/kg ATP was injected into APZG group [15], and 25 mg/kg TPP was injected intraperitoneally (IP) into TPZG group [26]. MPZG received 10 mg/kg melatonin [27], and LPZG received 20 mg/kg Liv-52 orally by gavage [24]. One hour after ATP, TPP, melatonin, and Liv-52 treatments, pyrazinamide (250 mg/kg) was given orally to all groups except HG [28]. This protocol was replicated once daily (1 × 1) for four weeks. At the end of four weeks, rats were euthanized with 50 mg/kg thiopental sodium and livers were collected. MDA, tGSH, SOD, and CAT levels were analyzed in the liver tissues. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined in blood samples taken from tail veins. Tissues were also examined histo-pathologically and immunohistochemically.

Biochemical analyses

Determination of MDA, GSH, SOD, CAT and protein in liver tissue

Liver tissue samples were frozen in liquid nitrogen and pulverized. The supernatant separated after centrifugation was used for analysis. Determination of MDA, GSH, and SOD in liver tissues was performed using enzyme-linked immunosorbent assay (ELISA) kits available for rats purchased from Cayman Chemical Company (Ann Arbour, Michigan, USA) and each assay was performed following the kit instructions (product numbers 706002, 703002, and 10009055, respectively). CAT activity was assessed according to the method described by Goth [29]. The tissue protein amount was analyzed according to the Bradford method [30].

Serum ALT and AST measurement

Venous blood samples were collected in anticoagulant-free tubes. Following clotting, serum was separated by centrifugation and stored at -80 ℃ until analysis. Venous blood samples were collected in anticoagulant-free tubes. Following clotting, serum was separated by centrifugation and stored at -80 ℃ until analysis. Using a Cobas 8000 autoanalyzer (Roche Diagnostics GmBH, Mannheim, Germany) serum AST and ALT activities were measured spectrophotometrically.

Histopathological method

Liver tissues taken from the rats at necropsy were fixed in 10% neutral formalin. The tissues were embedded in paraffin blocks after routine alcohol-xylol processing. The 4 µ sections taken from the slides were stained with hematoxylin-eosin and the hydropic degeneration and necrosis in the hepatocytes and the mononuclear cell infiltration in the periportal areas were graded semi-quantitatively as none (0), mild (1), moderate (2) and severe (3).

Immunohistochemical method

5 µm liver sections taken on polylysine slides were treated with xylol and alcohol. They were washed with phosphate-buffered saline, and endogenous peroxidase (PBS) was inactivated by keeping in 3% hydrogen peroxide for 10 min. Then, it was treated with antigen retrieval solution at 500 watts for 2 × 5 min. After the protein block, the tissues were washed with PBS and incubated with Caspase 3 (Elabscience, Catalogue no. E-AB-30004) primary antibody at a dilution ratio of 1/200 at +4°C overnight. As a secondary; Large Volume Detection System: anti-polyvalent, HRP (Thermofischer, Catalogue no: TP-125-HL) was used as recommended by the manufacturer. DAB (3,3'-Diaminobenzidine) was used as the chromogen. After counterstaining with Mayer's hematoxylin, the slides were covered with an aqueous mounting medium and examined under a light microscope.

Immunopositivity was evaluated semiquantitatively as absent (0), mild (1), moderate (2), and severe (3).

Statistical analyses

All statistical procedures were performed with 'SPSS for Windows 22.0' statistical program and $p < 0.05$ was considered significant. The suitability of the numerical data for normal distribution was evaluated by the Kolmogorov Smirnov test and the test results showed that the data were normally distributed. Therefore, one-way ANOVA was used for statistical analysis. Levene's test showed that the variances were homogeneous. Therefore, the Tukey test was preferred as a post hoc test. Biochemical results were expressed as mean value ± standard deviation (X ± SD). Since histopathological and immunohistochemical data were semiquantitative, the Kruskal-Wallis test was used for analysis and Dunn's test was preferred as a post-hoc test. Histopathological and immunohistochemical data were presented as median (quartile 1-quartile 3) and X ± SD.

Biochemical findings

Results of MDA, tGSH, SOD, and CAT analyses in liver tissues

As seen in Fig. 1A, the tissue MDA level of the PZDG (6.31 ± 0.27) was significantly ($p < 0.001$) higher than the healthy group (3.45 ± 0.21). ATP (4.31 ± 0.20),

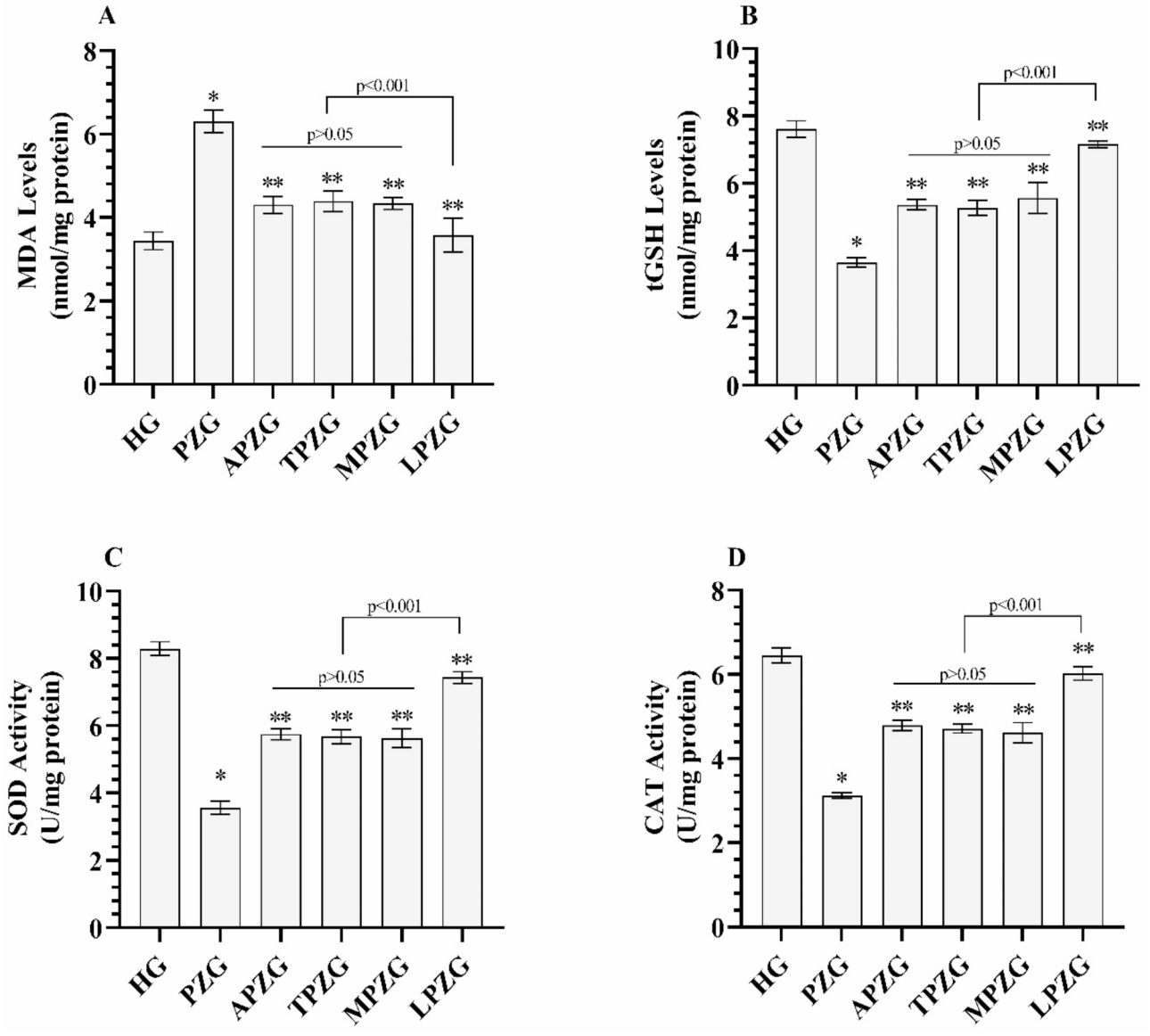

Fig. 1 (A-D) Evaluation of oxidant and antioxidant amounts in liver tissues of experimental groups. Bars shows mean ± dtandard deviation, $n = 6$. *; $p < 0.001$ vs. HG, **; $p < 0.001$ vs. PZG. MDA; malondialdehyde, tGSH; total glutathione, SOD; superoxide dismutase, CAT; catalase, HG; healthy group, PZG; pyrazinamide group, APZG; adenosine triphosphate + pyrazinamide group, TPZG; thiamine pyrophosphate + pyrazinamide group, MPZG; melatonin + pyrazinamide group, LPZG; Liv-52 + pyrazinamide group

TPP (4.40 ± 0.24), melatonin (4.34 ± 0.14), and Liv-52 (3.58 ± 0.41) significantly ($p < 0.001$) suppressed the pyrazinamide-induced elevation in MDA amounts. The difference between the MDA levels in the APZG, TPZG, and MPZG groups was calculated to be insignificant ($p > 0.05$). Liv-52 was the best inhibitor of increased MDA in the liver tissue ($p < 0.001$). The difference between the MDA levels in the Liv-52 and healthy groups was statistically insignificant ($p = 0.937$).

Pyrazinamide caused a decrease in tissue tGSH levels. When the tGSH levels in the pyrazinamide (3.65 ± 0.15) and healthy (7.61 ± 0.25) groups were compared, the difference between them was significant ($p < 0.001$). ATP (5.36 ± 0.15), TPP (5.27 ± 0.22), melatonin (5.40 ± 0.10), and Liv-52 (7.16 ± 0.11) suppressed the pyrazinamide-induced decrease in tGSH levels ($p < 0.001$). While ATP, TPP, and melatonin provided similar levels of success in maintaining tGSH levels ($p < 0.001$), it was observed that tGSH levels were higher in rats receiving Liv-52 ($p < 0.001$) (Fig. 1B).

As presented in Fig. 1(C-D), SOD and CAT activities decreased in the liver tissues in the pyrazinamide group (3.56 ± 0.20, 3.12 ± 0.07, respectively) than healthy group (8.29 ± 0.21, 6.46 ± 0.18, respectively) ($p < 0.001$). Pyrazinamide-induced decrease in SOD and CAT activities was significantly inhibited in rats receiving ATP (5.75 ± 0.16, 4.79 ± 0.12, respectively), TPP (5.68 ± 0.21, 4.72 ± 0.11, respectively), melatonin (4.72 ± 0.11, 4.62 ± 0.24, respectively) and Liv-52 ($p < 0.001$). Liv-52 (7.43 ± 0.17, 6.03 ± 0.16, respectively) was more effective than ATP, TPP, and melatonin in maintaining SOD and CAT activities ($p < 0.001$).

Results of ALT and AST analyses in serum

Serum AST and ALT activities were higher in PZG rats (167.33 ± 5.01, 277.83 ± 9.15, respectively) than in HG rats (35.50 ± 3.73, 29.33 ± 4.80, respectively) ($p < 0.001$). ATP (96.00 ± 7.59, 128.67 ± 12.26, respectively), TPP (99.83 ± 10.83, 123.33 ± 9.37, respectively), melatonin (94.17 ± 7.99, 127.17 ± 10.69, respectively), and Liv-52 (89.72 ± 44.10, 51.17 ± 6.37, respectively) treatments significantly prevented pyrazinamide-induced increase in ALT and AST activities ($p < 0.001$). ALT and AST activities were similar in the APZG, TPZG, and MPZG groups, but lower in the LPZG group ($p < 0.001$). The data of HG and LPZG groups were similar for ALT activity ($p = 0.198$) (Fig. 2A-B).

Histopathological findings

Histopathological examinations revealed statistically significant differences between the groups (Table 1). As seen in Fig. 3A, the livers of the HG-control group rats had a normal histological appearance. Grade-3 hydropic degeneration and necrosis were observed in hepatocytes in the PZDG group (Fig. 3B). Histopathological damage findings were found to be grade-2 in the APZG, TPZG, and MPZG groups treated with ATP, TPP, and melatonin (Fig. 3C-E). In the LPZG group treated with Liv-52, the severity of hydropic degeneration and necrosis was detected in grade 1 (Fig. 3F).

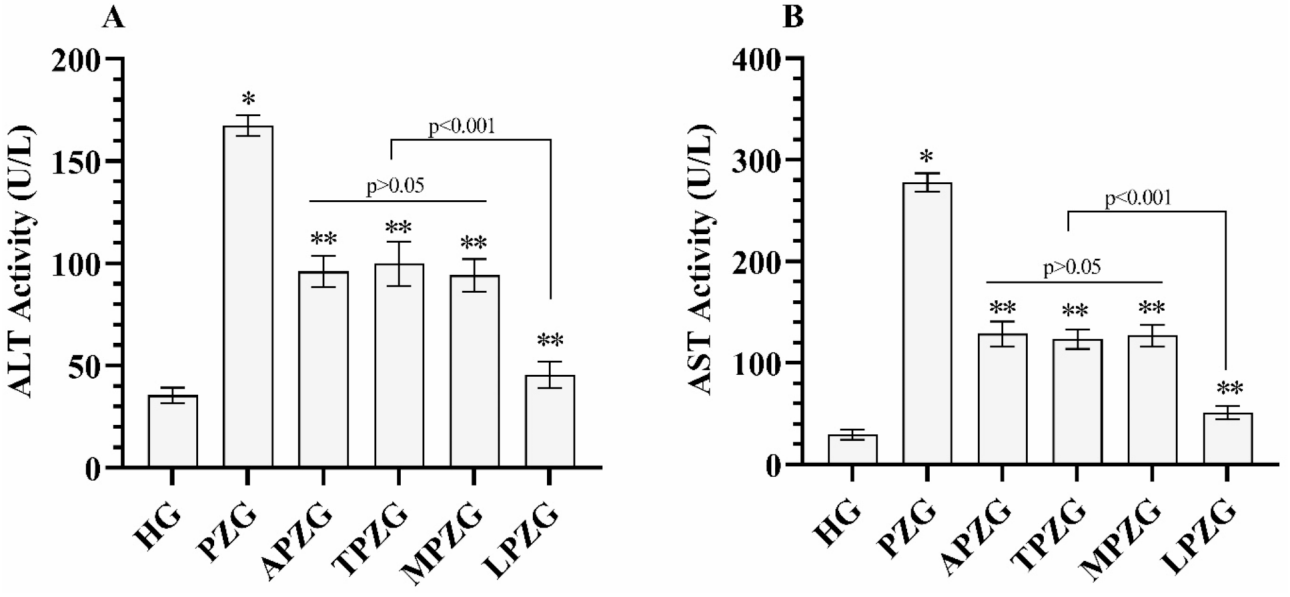

Fig. 2 **(A-B)** Evaluation of liver function tests in the serum of the experimental groups. Bars shows mean ± dtandard deviation, $n = 6$. *; $p < 0.001$ vs. HG, **; $p < 0.001$ vs. PZG. ALT; alanine aminotransferase, AST; aspartate aminotransferase, HG; healthy group, PZG; pyrazinamide group, APZG; adenosine triphosphate + pyrazinamide group, TPZG; thiamine pyrophosphate + pyrazinamide group, MPZG; melatonin + pyrazinamide group, LPZG; Liv-52 + pyrazinamide group

Table 1 Analysis of histopathological and immunohistochemical analyses results in liver tissues

Experimental groups	Histopathological analyses		Immunohistochemical analysis
	Hydropic degeneration	Necrosis	Caspase 3
	Median (Quartile$_1$ - Quartile$_3$) Mean ± Standart deviation		
HG	0 (0–0) 0 ± 0	0 (0–0) 0 ± 0	0 (0–0) 0.17 ± 0.38
PZG	3 (3–3)* 2.83 ± 0.38	3 (3–3)* 2.56 ± 0.50	3 (2–3)* 2.67 ± 0.48
APZG	2 (2–2)$^{**,\#}$ 1.64 ± 0.49	2 (1–2)$^{**,\#}$ 1.64 ± 0.49	2 (1–2)** 1.75 ± 0.55
TPZG	2 (2–2)$^{**,\#}$ 2.00 ± 0.34	2 (2–2)$^{**,\#}$ 1.83 ± 0.38	2 (2–2)$^{**,\#}$ 2.00 ± 0.48
MPZG	2 (1–2)** 1.56 ± 0.50	2 (1.25-2)$^{**,\#}$ 1.75 ± 0.44	2 (2–2)** 1.86 ± 0.49
LPZG	1 (1–1)** 1.00 ± 0.41	1 (1–1)** 0.83 ± 0.38	1 (1–2)** 1.36 ± 0.54

Grading (0–3): 0; absent, 1; mild, 2; moderate, 3; severe. *; $p < 0.001$ vs. HG, **; $p < 0.05$ vs. PZG, #; $p < 0.05$ vs. LPZG. HG; healthy group, PZG; pyrazinamide group, APZG; adenosine triphosphate + pyrazinamide group, TPZG; thiamine pyrophosphate + pyrazinamide group, MPZG; melatonin + pyrazinamide group, LPZG; Liv-52 + pyrazinamide group. Kruskal-Wallis test was used for statistical analysis, followed by post hoc Dunn's test

Immunohistochemical findings

Statistically significant differences were found between the groups in immunohistochemical staining with Caspase 3 (Table 1). While nonsignificant immunopositivity was observed in the HG-Control group (Fig. 4A), different levels of immunopositivity were detected in the other treatment groups. While severe immunopositivity was observed in the PZDG group (Fig. 4B), moderate immunopositivity was observed in the APZG, TPZG, and MPZG groups (Fig. 4C-E) and mild immunopositivity was observed in the LPZG group (Fig. 4F).

Discussion

In this study, the protective effects of ATP, TPP, melatonin, and Liv-52 against possible liver damage caused by pyrazinamide were investigated. The most important factor limiting the use of pyrazinamide, which plays an effective role in shortening the treatment period in tuberculosis, is severe hepatotoxicity [1, 6]. Pyrazinamide-induced hepatotoxicity has been confirmed by both preclinical and human studies [5, 31–33]. In the study of Yew et al., it was reported that the basis of hepatotoxicity of antituberculosis drugs is an increase in ROS and suppression of the antioxidant defense system [34]. In addition, in individuals with liver disease, high levels of ROS and LPO products were observed, while decreased expression of antioxidants such as SOD, CAT, and GSH was found [5, 33]. As is known, an increase in ROS leads to mitochondrial dysfunction and decreased ATP production, causing an intracellular energy crisis. This results in the loss of hepatocytes by apoptosis or necrosis [35]. In this process, the accumulation of LPO products, such as MDA and membrane damage, is particularly striking [36]. In this study, it was observed that MDA levels were significantly increased in the group that was only administered pyrazinamide compared to the healthy group. In addition, a significant decrease was detected in the levels of tGSH, SOD, and CAT in the liver tissue. These findings are consistent with previous studies demonstrating pyrazinamide-induced oxidative stress [32, 37, 38]. In a recent investigation of pyrazinamide-induced liver damage, it was reported that MDA levels increased due to LPO, and antioxidant enzyme activities such as SOD and CAT decreased [38].

The literature emphasizes the potential benefits of antioxidant therapy in reducing the risk of hepatotoxicity. These treatment approaches suppress ROS and MDA levels while increasing the levels of endogenous antioxidants such as GSH, SOD, and CAT and alleviate the severity of oxidative stress [26, 39–41]. According to the biochemical findings of our study, the increase in oxidant markers in the pyrazinamide group was suppressed by ATP, TPP, melatonin, and Liv-52 administration. In addition, the decreases in endogenous antioxidant levels were significantly prevented by ATP, TPP, melatonin, and Liv-52. It is known that pyrazinoic acid, the toxic metabolite released due to the metabolism of pyrazinamide in the liver, exhibits its mycobactericidal activity by depleting cellular ATP [42]. ATP and its hydrolytic products (ADP and adenosine) serve as extracellular signals to regulate various physiological and pathophysiological processes in hepatocytes [43]. In addition, disruption of the proton gradient that directs most of the ATP production in mitochondria leads to necrotic or apoptotic cell death in the liver [28, 44]. In addition, it has been reported that ATP is an energy source for the production of antioxidants and that external ATP supplementation protects organs against oxidative damage [9, 15, 45]. In our study, it was observed that increased MDA levels in the liver tissue of rats to which ATP was added to pyrazinamide

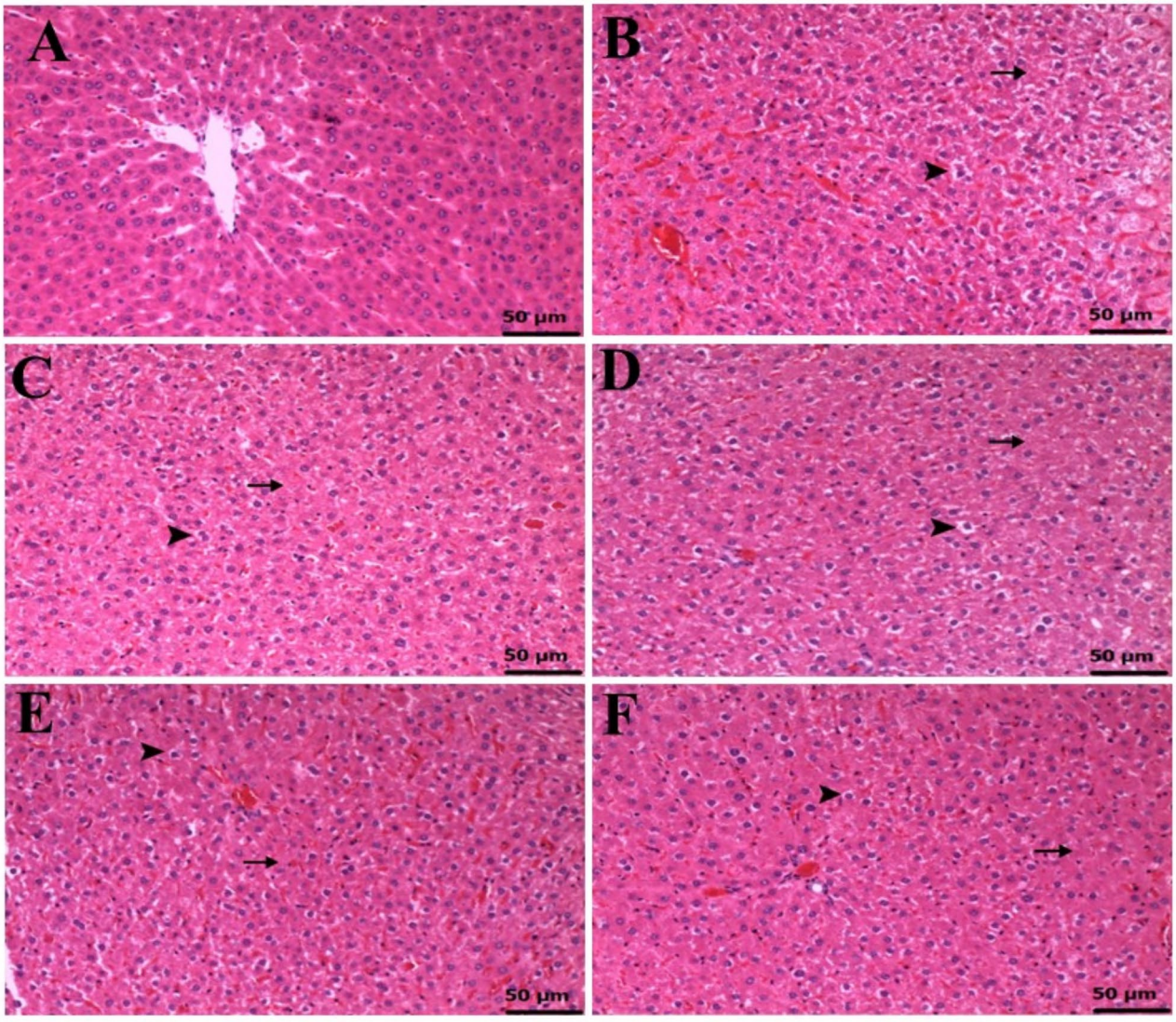

Fig. 3 **(A-F)** Histological appearances of liver tissues of HG (**A**), PZG (**B**), APZG (**C**), TPZG (**D**), MPZG (**E**), and LPZG (**F**) groups (Hematoxylin-eosin staining). **A**: Normal histological appearance of liver tissue. **B**: Severe hydropic degeneration (arrowhead) and necrosis (arrow) in liver tissue. HG; healthy group, PZG; pyrazinamide group. **C**: Moderate hydropic degeneration (arrowhead) and necrosis (arrow) appearance in liver tissue. **D**: Moderate hydropic degeneration (arrowhead) and necrosis (arrow) appearance in liver tissue. **E**: Moderate hydropic degeneration (arrowhead) and necrosis (arrow) appearance in liver tissue. **F**: Mild hydropic degeneration (arrowhead) and necrosis (arrow) appearance in liver tissue. HG; healthy group, PZG; pyrazinamide group, APZG; adenosine triphosphate + pyrazinamide group, TPZG; thiamine pyrophosphate + pyrazinamide group, MPZG; melatonin + pyrazinamide group, LPZG; Liv-52 + pyrazinamide group

treatment were suppressed, and tGSH, SOD, and CAT levels were significantly preserved. These findings are consistent with previous literature demonstrating the protective effect of ATP against hepatotoxicity [46, 47].

Another molecule we evaluated against pyrazinamide hepatotoxicity, TPP, is a basic cofactor required in the general metabolism of all organisms. TPP is the active metabolite of thiamine formed by the thiamine pyrophosphokinase enzyme in the liver [48]. In addition, TPP is required for a transketolase enzyme that plays an important role in mitochondrial ATP synthesis and maintenance of cellular redox status [49]. Although the antioxidant effect mechanism of TPP has not been fully elucidated, recent studies have revealed the protective effects of TPP against oxidative damage in different tissues [12, 17, 18, 50, 51]. In particular, the hepatoprotective effects of TPP against drug-induced liver damage have been supported by other studies [26, 52, 53]. It has been shown that TPP has a similar protective effect to the hepatoprotective agent N-acetyl cysteine against acetaminophen liver damage in rats [53].

Another molecule that we investigated against the possible hepatotoxicity of pyrazinamide is melatonin. Melatonin, synthesized from tryptophan and produced in

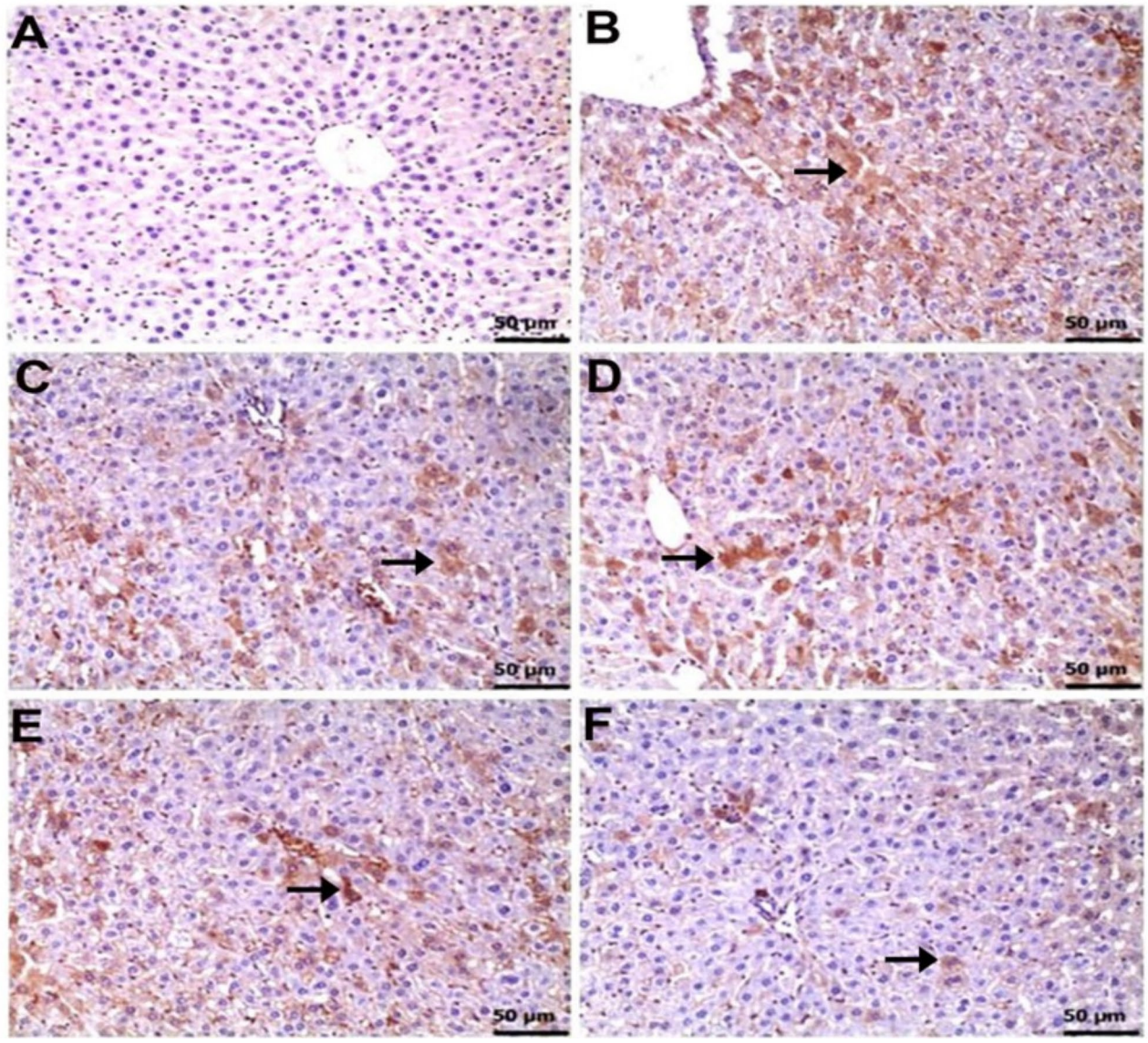

Fig. 4 **(A-F)** Caspase 3 immunopositivity in liver tissue of HG (**A**), PZG (**B**), ATPG (**C**), TPZG (**D**), MPZG (**E**), and LPZG (**F**) groups (Immunohistochemistry). **A**: Immun negativity B: Severe immunopositivity (arrow). **C**: Moderate immunopositivity (arrow). **D**: Moderate immunopositivity (arrow). **E**: Moderate immunopositivity (arrow). **F**: Mild immunopositivity (arrow). HG; healthy group, PZG; pyrazinamide group, APZG; adenosine triphosphate + pyrazinamide group, TPZG; thiamine pyrophosphate + pyrazinamide group, MPZG; melatonin + pyrazinamide group, LPZG; Liv-52 + pyrazinamide group

many tissues, especially the pineal gland, is a powerful antioxidant and anti-inflammatory agent [19]. The aromatic indole ring of melatonin acts as an effective buffer in ROS scavenging. In addition, it induces other enzymatic endogenous antioxidant enzymes such as SOD and CAT [54]. The effect of melatonin is not limited to oxidative stress alone but has the potential to regulate different physiological processes [55]. Therefore, the therapeutic effects of melatonin on drug- or alcohol-induced liver damage have been comprehensively addressed in other studies [56]. Melatonin has also been proven to prevent carbon tetrachloride-induced liver oxidative damage in rats by histopathological findings [27]. In another study, the protective effect of melatonin against hepatotoxicity induced by the combination of isoniazid and rifampin was demonstrated histopathologically and immunohistochemically [57]. In our research, the fact that melatonin prevented the changes in MDA, tGSH, SOD, and CAT levels by pyrazinamide is consistent with the previous literature demonstrating its antioxidant properties [27, 57].

As is known, Liv-52 is a polyherbal ayurvedic formulation known for its strong hepatoprotective effects against hepatotoxicity induced by chemical substances [23]. Its antioxidant properties, widely reported in the literature, stand out as one of the basic mechanisms of this protective effect [22]. Liv-52 is highly valuable because

it reduces MDA levels and prevents GSH depletion by inhibiting mitochondrial beta-oxidation of fatty acids [25]. Antituberculosis drugs can have a hepatotoxic effect alone; however, when used in combination, this toxicity can become cumulative [40]. In the literature, the impact of Liv-52, especially in preventing liver damage due to antituberculosis drugs, has been extensively discussed [58]. In a six-month randomized study, Reshu et al. (2023) showed that tuberculosis patients had better liver enzyme levels with Liv-52 treatment [59]. Çimen et al. (2020) reported histopathologically that Liv-52 significantly improved oxidative stress in liver tissue after ischemia/reperfusion [24]. According to our experimental results, Liv-52 was the protective agent that kept MDA, tGSH, SOD, and CAT levels closest to normal.

To evaluate pyrazinamide-induced liver damage, ALT and AST levels were measured in serum samples taken from the tail vein of animals. The increase in serum levels of these enzymes is considered a biochemical indicator of damage to liver cells [52]. Studies report that pyrazinamide causes an increase in serum ALT and AST levels by inducing LPO and disrupting the cell membrane structure [38, 60, 61]. Wang et al. (2022) presented a case report in which ALT and AST levels increased in a male patient receiving long-term pyrazinamide treatment [5]. This finding confirms the potential effects of pyrazinamide on liver damage in humans. Our study confirmed that ALT and AST levels increased in rats treated with pyrazinamide serum. However, ATP, TPP, melatonin, and Liv-52 significantly inhibited the increase in these enzyme activities. In particular, Liv-52 prevented the increase in ALT and AST levels with pyrazinamide more significantly than ATP, TPP, and melatonin.

Caspase 3 which is an immunohistochemical marker was examined to evaluate apoptosis in the liver tissue of the animals. Caspase-3 is a proteolytic enzyme that plays an important role in apoptosis and inflammation control [62]. Weakening of the antioxidant defense system may render hepatocytes susceptible to TNF-α-induced apoptosis [63]. Cellular oxidative stress may cause cytochrome c to be transported from mitochondria to the cytosol by activating caspase-3 and initiating the apoptotic pathway [64]. The harmful effects of pyrazinamide on the liver are not limited to oxidative stress and inflammation; it may also induce apoptosis, the naturally occurring programmed cell death mechanism in cells [38]. Pyrazinamide is known to aggravate hepatotoxicity by triggering the release of caspase-3, which acts as a key enzyme in apoptosis [61]. In this study, severe caspase 3 immunopositivity was detected in the pyrazinamide group following the literature. On the other hand, caspase 3 activation in TPZG, APZG, MPZG, and LPZG groups was lower than that in the pyrazinamide group.

Our histopathological findings in the liver tissue are parallel to the biochemical findings. In the group given pyrazinamide alone, grade-3 hydropic degeneration and widespread necrosis were observed in hepatocytes. It has been reported in the literature that pyrazinamide causes similar pathological changes in the liver tissue. Taziki et al. (2018) detected widespread necrosis and inflammatory cell infiltration in liver parenchymal cells after pyrazinamide administration [37]. Ali et al. (2022) reported that pyrazinamide caused histopathological changes such as excessive vacuolation, periportal edema, and thickening of bile duct walls in the liver tissue of rats [32]. In the groups treated with ATP, TPP, and melatonin, the severity of damage in the liver tissue decreased, and these histopathological findings regressed to the grade-2 level. This shows that these molecules provide partial protective effects on the liver. However, in the Liv-52-treated group, histopathological damage was significantly reduced, and only mild hydropic degeneration and necrosis were observed (grade 0–1).

The results of this study show that protection from pyrazinamide-induced hepatic damage is parallel with antioxidative activity. This view suggests that different agents such as carotenoids and curcumin may be hepatoprotective in pyrazinamide hepatotoxicity through their antioxidative and antiapoptotic effects [65, 66].

In conclusion, in this study, we observed that pyrazinamide administration in rats caused severe oxidative stress in liver tissue. It was determined that pyrazinamide significantly increased the levels of oxidant biomarkers and significantly decreased the antioxidant levels. Pyrazinamide caused oxidative stress in liver tissue and increased serum AST and ALT levels. In addition, our histopathological analyses confirmed the biochemical findings of pyrazinamide-induced hepatotoxicity. In our study, it was also observed that ATP, TPP, melatonin, and Liv-52 provided positive ameliorative effects on pyrazinamide-related biochemical, histopathological, and immunohistochemical changes. Liv-52 especially stood out as the most potent hepatoprotective compared to other treatment groups. These experimental results suggest that ATP, TPP, melatonin, and Liv-52 may have therapeutic potential in humans for similar conditions. Comparison of these experimental results with clinical findings may reveal the potential of these molecules for human treatment more clearly.

Limitations: (i) Investigation of hepatoprotective effects of ATP, TPP, and melatonin combinations. (ii) Testing at different doses. (iii) Investigation at the molecular level.

Author contributions

S.C.: Conceptualization, writing original draft, writing review, and editing. S.C.: Software, supervision, writing original draft, writing review, and editing. D.A.: Investigation, methodology, project administration, resources, writing original draft, writing review and editing. G.H.: Methodology, resources, writing

review, and editing. N.Y.: Writing original draft, writing review, and editing. A.S.M.: Formal analysis, writing review, and editing. C.S.: Formal analysis, writing review, and editing. H.S.: Conceptualization, data curation, methodology, project administration, writing original draft, writing review and editing. S.B.: Conceptualization, investigation, methodology, writing original draft, writing review, and editing. All authors reviewed the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The Erzincan Binali Yıldırım University, local Animal Experimentation Ethics Committee approved the procedures (Date: 29.08.2024, meeting no: 08/31).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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