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Molecular mechanism of the protective effect of adenosine triphosphate against paracetamol-induced liver toxicity in rats

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Abstract. Toxic doses of paracetamol are also known to be close to therapeutic doses. This study aimed to biochemically investigate the protective effect of ATP against paracetamol-induced oxidative liver injury in rats and to examine the tissues histopathologically. We divided the animals into the paracetamol alone (PCT), ATP + paracetamol (PATP), and healthy control (HG) groups. Liver tissues were examined biochemically and histopathologically. Malondialdehyde level, AST and ALT activity in the PCT group were significantly higher than those in the HG and PATP groups ($p < 0.001$). The glutathione (tGSH) level, superoxide dismutase (SOD) and catalase (CAT) activity in the PCT group was significantly lower than that in the HG and PATP groups ($p < 0.001$), while animal SOD activity was significantly different between the PATP and HG groups ($p < 0.001$). The activity of CAT was almost the same. In the group treated with paracetamol alone, lipid deposition, necrosis, fibrosis, and grade 3 hydropic degeneration were observed. No histopathological damage was observed of the ATP-treated group, except for grade 2 edema. We discovered that ATP reduces the oxidative stress caused by paracetamol ingestion and protects against paracetamol-induced liver injury at the macroscopic and histological levels.

Key words: Paracetamol — Liver toxicity — ATP — Pain

Introduction

Paracetamol is an antipyretic and analgesic from the group of nonsteroidal anti-inflammatory drugs (NSAIDs) (Agrawal and Khazaeni 2022). Although paracetamol has a good safety profile at therapeutic doses, it can cause severe liver damage when taken in large quantities (Ye et al. 2018). The hepatotoxic effect of paracetamol is known to be because of the formation of the metabolite N-acetyl-p-benzoquinoneimine (NAPB) in the liver. It is usually detoxified by endogenous glutathione (GSH). High doses of paracetamol lead to depletion of GSH stores, inadequate detoxification of NAPB, and toxicity (More et al. 2017; Jiang et al. 2020). In the literature, excessive production of reactive oxygen species (ROS) in mitochondria has been blamed for the hepatotoxicity of paracetamol (Yan et al. 2018). Previous studies suggest that ROS, generated during paracetamol metabolism, trigger the lipid peroxidation (LPO) reaction and subsequently lead to oxidative liver damage (Du et al. 2016). Some studies have argued that the

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toxic effects of paracetamol are related to the depletion of adenosine triphosphate (ATP), the energy source of cells (Jiang et al. 2015). As described in the literature, energy is required to synthesize low molecular weight antioxidants (Yi et al. 2010). Superoxide dismutase (SOD) is induced by the upregulation of Cu/Zn- SOD protein expression (Dvořáková et al. 2006). ATP is known to potentiate Cu/Zn-SOD expression (Chen et al. 2006). The hydrogen peroxide (H_2O_2) formed in this process stimulates the production of antioxidants and enzymes that neutralize ROS (Heck et al. 2010), and also a study showed that ATP has catalase-like activity, which enables H_2O_2 consumption (Ying et al. 2019). Under stress, ATP may also be involved in the metabolism of lipid peroxidation. Under these conditions, free radicals are formed and must be scavenged by the enzymatic and non-enzymatic potential of cells to maintain normal cell life. ATP may be required for the production and maintenance of these enzymes and for membrane repair processes (Saquet et al. 2000). Therefore, the supply of exogenous ATP provides sufficient usable energy to maintain and enhance antioxidant defence systems and to maintain membrane integrity by preventing the accumulation of ROS. The energy level can regulate the balance between the ROS production and the antioxidant system by increasing both enzyme activities and the ability to scavenge free radicals (Yi et al. 2010). Since ATP is a charged molecule that cannot freely pass through cell membranes, its protective effects must be achieved in ways other than simply supplying energy to cells. When ATP is released, it is rapidly converted to adenosine by various extracellular enzyme families such as ecto-50-nucleotidase, ectonucleoside triphosphate diphosphohydrolases, ectonucleotide pyrophosphatase/phosphodiesterases, and alkaline phosphatases. ATP is a key molecule for extracellular signal transduction (Yegutkin 2008; Maldonado et al. 2013). One possible mechanism could be the activation of A1 and A3 adenosine receptors, which play a role in triggering a cardioprotective effect against ischemia-reperfusion injury, a phenomenon known as ischemic preconditioning (Kudo et al. 2002; Hochhauser et al. 2007). A recent study reported that ATP protects oral mucosal tissues from oxidative damage by preventing an increase in malondialdehyde (MDA) and decreasing the amount of total glutathione (tGSH) (Yıldırım et al. 2020). This also suggests that ATP may help treat the hepatotoxicity of paracetamol. No studies in the literature have investigated the protective effect of ATP against paracetamol hepatotoxicity. Therefore, our study aimed to biochemically investigate the protective effect of ATP against paracetamol-induced oxidative liver injury in rats and to examine the tissues histopathologically.

Materials and Methods

Animals

For study, 18 male albino Wistar rats weighing 280–290 g were obtained from Binali Yıldırım University Medical Experimental Application and Research Center. Before the experiment, the animals were housed at room temperature (22°C) with 12 h of light and 12 h of darkness and fed regular animal food. To allow the animals to acclimate to the environment, they were kept for one week in the laboratory environment where the experiment was to be conducted.

Ethical procedures

The protocols and procedures were approved by the local Animal Experimentation Ethics Committee (Date: 31.03.2022 Meeting No: 2022/03).

Chemicals

The chemicals used were supplied by the specified companies. Thiopental sodium (Pental sodium 1 g/flacon injection form, IE Ulagay, Turkey), paracetamol (Parol 500 mg/tablet oral form, Atabay Drug Company, Turkey), and ATP (ATP 10 mg/ml injection form, Zdorovye Narodu, Ukraine).

Experimental groups

The experimental animals were divided into the paracetamol alone (PCT), ATP + paracetamol (PATP), and healthy control (HG) groups.

The procedure of the experiment

To perform the experiment, 25 mg/kg of ATP was intraperitoneally (ip) injected into the PATP group ($n = 6$). Distilled water as a solvent was injected ip into the PCT ($n = 6$) and HG ($n = 6$) groups. One hour before administering ATP and distilled water, paracetamol was administered orally at a 1000 mg/kg dose to all rats (except the HG group). This dose of paracetamol was shown to cause oxidative liver damage in a previous study (Kisaoglu et al. 2014).

Twenty-four hours after paracetamol administration, all animals were killed with a high dose of anesthesia (50 mg/kg thiopental sodium), and their liver tissues were removed. Before killing the animals, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined in blood samples taken from the animals' tail veins. MDA, tGSH, SOD and CAT measurements were made in the excised liver tissues. In addition, the tissues were examined histopathologically. Biochemical and histopathological re-

sults of the PATP and HG groups of animals were compared with PCT and evaluated.

Biochemical analysis

Preparation of the samples

The tissue samples were placed in Petri dishes after washing with physiological saline. The tissues were ground into powder in the presence of liquid nitrogen. To evaluate SOD activity as well as GSH, TBARS, and protein levels, tissue samples were homogenized. The supernatants were used for SOD, CAT, GSH, MDA, and protein analysis.

MDA analysis

MDA is a naturally occurring product of lipid peroxidation. Measurement of thiobarbituric acid reagents (TBARS) is a well-established method for screening and monitoring lipid peroxidation. For MDA determination, 250 µl of radioimmunoprecipitation assay (RIPA) buffer which contains 50 mM Tris-HCI, pH 7.6, containing 150 mM sodium chloride, 1% Tergitol (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) was used to sonicate tissue samples. The homogenate obtained during sonication was centrifuged at 1,600 × *g* and 4°C for 10 min. The supernatant was used for analysis. Colorimetrically, the MDA-TBA adduct formed by the reaction of MDA and TBA at high temperatures (90–100°C) and acidic conditions was detected at an excitation wavelength of 530–540 nm. MDA levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits for experimental animals (part no. 10009055, Cayman Chemical Company).

GSH analysis

GSH values in experimental animals were determined using commercially available ELISA kits (part no. 703002, Cayman Chemical Company). For GSH determination, tissue samples were homogenized with 50 mM cold phosphate buffer (pH 6–7) containing 1 mM ethylenediaminetetraacetic acid and centrifuged at 10,000 × *g* and 4°C for 15 min. GSH was then determined using 5,5-dithiobis-(2-nitro benzoic acid) (DTNB) in the supernatant. When the DTNB disulfide sulfhydryl groups are reduced, a yellow compound is formed, and its absorbance at 412 nm is measured spectrophotometrically.

Determination of SOD activity

SOD was measured using ELISA kits (part no. 706002, Cayman Chemical Company). Tissue samples were homogenized in 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, and centrifuged at 1,500 × *g* for 5 min at 4°C. For analysis the supernatant was used. The Cayman SOD assay uses a tetrazolium salt to detect superoxide radicals formed by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme required to dismutate 50% of superoxide radicals. The absorbance was measured spectrophotometrically at 560 nm.

Determination of CAT activity

For CAT analysis, tissue was homogenized in 10 ml of buffer (50 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.2) and centrifuged for 15 min at 10,000 × *g* and 4°C. The supernatant was used to determine the CAT activity. The analysis is based on the reaction of CAT with methanol in the presence of H_2O_2. The resulting formaldehyde is measured colorimetrically with a chromogen called 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole. Upon oxidation, the chromogen turns from corrosive to purple (Aebi 1984).

Protein determination

This measurement is based on detecting dye solutions absorbance at 595 nm due to the binding of the acidic solution of Coomassie Brilliant Blue G-250 to proteins (Bradford 1976).

Determination of AST and ALT activity

To assess blood AST and ALT spectrophotometrically for liver function tests, a Cobas 8000 autoanalyzer (Roche Diagnostics GmBH, Mannheim, Germany) and commercially available kits (Roche Diagnostics) were utilized. Anticoagulant-free venous samples of blood were collected in tubes. After clotting, serum was centrifuged and kept at −80°C before tested.

Histopathological examination

Subject tissues were fixed in 10% formaldehyde solution for 72 h. Tissues were then placed in a cassette and washed with running water for 24 h. They were then dehydrated through an increasing series of alcohols (70, 80, 90, and 100%). Liver tissue purified in xylene was embedded in kerosene blocks, and sections 4–5 µm thick were taken. Using the Olympus DP2-SAL software (Olympus® Inc. Tokyo, Japan), sections were assessed and photographed after being stained with a hematoxylin-eosin double stain. The severity of histopathological damage of each liver tissue section was scored from 0 to 3 (0 – normal, 1 – mild damage, 2 – moderate damage, and 3 – severe damage). For the study groups histopathological evaluation was performed by a blinded histologist.

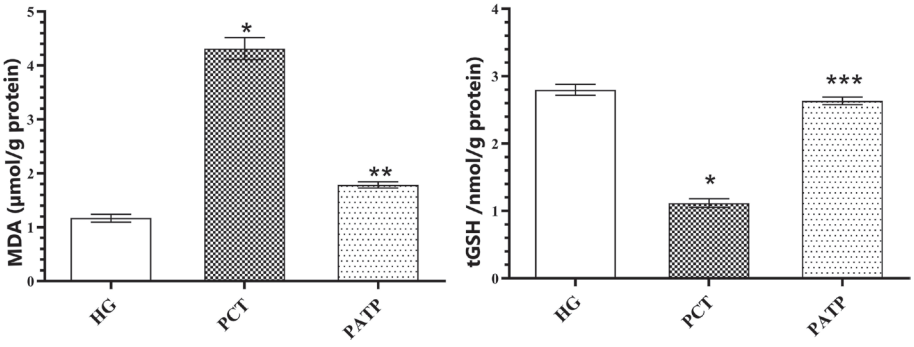

Figure 1. MDA and tGSH determination of study groups. Data are means ± SEM. * $p < 0.001$ *vs.* HG, and PATP; ** $p < 0.001$ *vs.* HG; *** $p = 0.002$ *vs.* HG. MDA, malondialdehyde; tGSH, total glutathione; HG, healthy group; PCT, paracetamol group; PATP, paracetamol + ATP group.

Statistical analysis

The results obtained from the experiments were expressed as "mean value ± standard error of mean" (x ± SEM). A Shapiro-Wilk test was used to test for the normality of the distribution for continuous variables. The significance of the difference between groups was determined by using a one-way ANOVA test. Then, Fisher's LSD (least significant differences) was made as a *post-hoc* test. All statistical operations were performed in the SPSS for Windows, 25.0 (Armonk, NY: IBM Corp.) and the $p < 0.05$ value was accepted significant.

Results

Biochemical results

As seen in Figure 1, MDA levels in the PCT group were significantly higher than those in the HG and PATP groups ($p < 0.001$). Similarly, a significant difference between the PATP and HG groups was calculated ($p < 0.001$). In addition, the tGSH content in the liver tissue of the animals in the PCT group was significantly lower than that in the HG and PATP groups ($p < 0.001$). Although the tGSH content was close to that of HG and PATP, a significant difference was found between them ($p < 0.002$).

As shown in Figure 2, SOD and CAT activity in the liver tissues of animals in the PCT group were significantly lower than those in the HG and PATP groups ($p < 0.001$). While animal SOD activity was significantly different between the PATP and HG groups ($p < 0.001$), the activity CAT was almost the same.

The blood activities of AST and ALT were significantly higher in the PCT group than in the HG and PATP groups ($p < 0.001$) (Fig. 3). However, animal blood ALT activities were close between the PATP and HG groups ($p > 0.05$), and the AST activities were significantly different ($p < 0.001$).

Histopathological findings

As shown in Figure 4A, no histopathological damage was observed in the liver tissue of the healthy group. In the group treated with paracetamol alone, lipid deposition, necrosis, fibrosis, and grade 3 hydropic degeneration were observed (Fig. 4B). No histopathological damage was observed in the ATP-treated group, except for grade 2 edema (Fig. 4C).

Discussion

In this study, we investigated the effects of ATP on preventing paracetamol-induced liver injury, which had not been previously investigated in the literature, and found that ATP was of great benefit in inhibiting the increase in paracetamol-

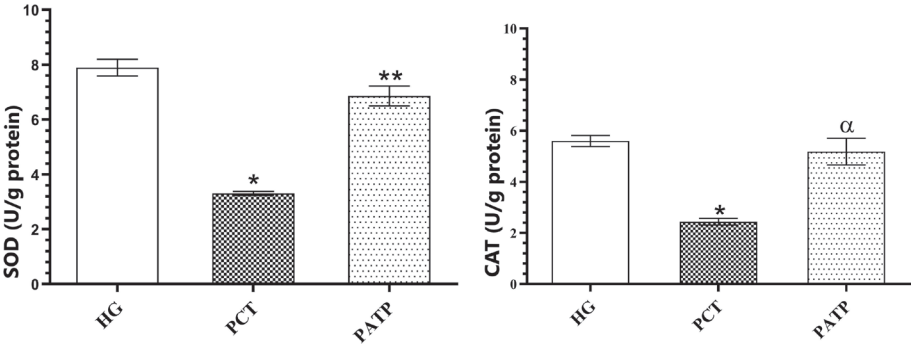

Figure 2. SOD and CAT activity of study groups. Data are means ± SEM. * $p < 0.001$ *vs.* HG, and PATP; ** $p < 0.001$ *vs.* HG; $^{\alpha}$ $p > 0.05$ *vs.* HG. SOD; superoxide dismutase, CAT; catalase. For more abbreviations, see Figure 1.

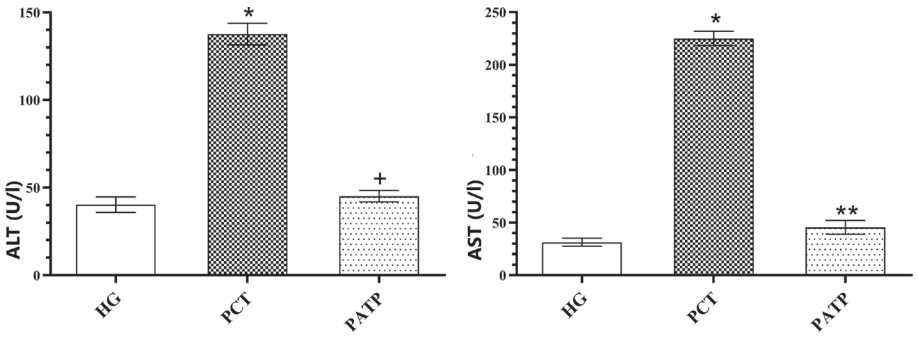

Figure 3. ALT and AST activity in the blood in study groups. Data are means ± SEM. * $p < 0.001$ *vs.* HG, and PATP; ** $p < 0.001$ *vs.* HG; $^{+}p > 0.05$ *vs.* HG; ** $p = 0.002$ *vs.* HG. ALT, alanine aminotransferase; AST, aspartate aminotransferase. For more abbreviations, see Figure 1.

induced oxidative stress. By macroscopic and histological examination of liver tissue, we found that ATP reduced liver damage associated with paracetamol ingestion.

Paracetamol is probably the most commonly used and best-tolerated analgesic (Ramachandran and Jaeschke 2019). It is commonly used regardless of sex, age, or geographic location. Even the US FDA states that paracetamol is available over the counter, alone, or in combination with other substances (FDA 2022). The wide availability of this potent drug often leads to unintentional overdoses or abuse. Because of its ready availability and concomitant abuse, its name has become synonymous with hepatotoxicity and sudden liver failure (Moore and Scheiman 2018). 46% of cases of acute liver failure in the United States, 40% in the United Kingdom, and 70% of all cases in Europe are caused by paracetamol. The problem is compounded because in the United States alone, paracetamol is responsible for around 500 deaths each year, as well as 100,000 calls to poison control centers, 50,000 emergency department visits, and 10,000 hospitalizations (Lee 2017). This problem has been addressed at two US FDA Advisory Committee meetings in the past 15 years, which adds to the concern.

Since the liver is the organ that plays a central role in the detoxification of paracetamol and hepatocytes are directly damaged at high doses, it is not surprising that toxicity is so common. Although liver cells can self-renew after injury, extensive cell death can trigger the inflammatory response. One way to prevent potential liver damage is to limit inflammation in the liver. At this stage, ATP plays an important role (Amaral et al. 2013).

Although N-acetyl cysteine, which has been shown to restore cellular GSH reserves in the liver, is now used to treat paracetamol-induced liver injury. When oxidative stress occurs, free radicals are formed in the cell (McCord 2000). These chemicals cause changes in the cell, such as protein degradation and deterioration of DNA structure, leading to apoptosis and tissue destruction (Raha and Robinson 2001). Glutathione, an endogenous tripeptide that exists in two forms, GSH and oxidized glutathione (GSSG), plays

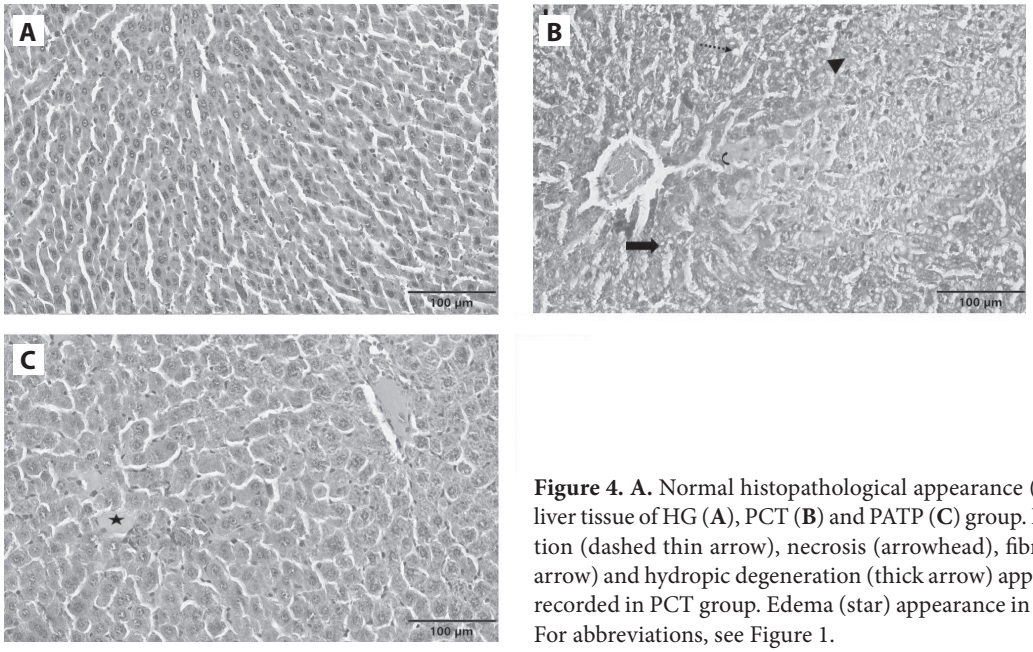

Figure 4. A. Normal histopathological appearance (H&E) of the liver tissue of HG (**A**), PCT (**B**) and PATP (**C**) group. Lipid deposition (dashed thin arrow), necrosis (arrowhead), fibrosis (curved arrow) and hydropic degeneration (thick arrow) appearance were recorded in PCT group. Edema (star) appearance in PATP group. For abbreviations, see Figure 1.

an essential role in removing these radicals (Meister and Anderson 1983). Excessive paracetamol is thought to induce lipid peroxidation *via* GSH depletion. The NAPB metabolite of paracetamol may act as a mediator for hepatotoxic effect. ATP hydrolysis is required for glutathione synthesis. If the metabolism of acetaminophen is examined, 50% is conjugated to glutathione, 40% is conjugated to sulfate, and 5% is oxidized to NAPB. With toxic exposure to acetaminophen, excess acetaminophen is metabolized to NAPB. It is a toxic substance that is reduced by glutathione to nontoxic mercaptan and cysteine compounds and then excreted by the kidneys. When an overdose of acetaminophen depletes glutathione stores and reaches less than 30% of normal, NAPB levels increase and bind to hepatic macromolecules, causing liver necrosis and rendering the condition irreversible (Kuffner et al. 2001, 2007). Endogenous GSH detoxifies this harmful toxin. However, when paracetamol is taken in dangerous amounts, an excess of NAPB is produced that cannot be adequately detoxified by GSH. The synthesis of NAPB to an extent that exceeds the detoxification capacity of GSH leads to liver damage (Xu et al. 2018).

To maintain oxidative balance, the liver uses a variety of antioxidant molecules, including GSH. When the amount of oxidizing free radicals in the cell increases, the enzyme glutathione reductase catalyzes the conversion of GSSG to GSH, which is needed to reduce these radicals (Xu et al. 2018). These toxic reactions are mainly seen in hepatocytes. Hepatic ATP depletion is thought to occur with acetaminophen poisoning, and mitochondrial membrane damage is thought to be the leading cause of decreased ATP production. These findings from the literature are consistent with our own. In the current study, GSH levels in the PCT group were significantly lower than in the HG and PATP groups. The high glutathione levels in the PATP group are thought to be because of ATP, which is the energy donor in the synthesis of antioxidants.

The amount of MDA was lower in the PATP group than in the PCT group. Research has shown that MDA levels increase with oxidative damage to the liver (Demiryilmaz et al. 2012). An increase in the level of MDA in a tissue indicates an increase in oxygen free radicals in that tissue. Lipid peroxidation is accelerated by an increase in free oxygen radicals. MDA is a byproduct of lipid peroxidation that causes cross-linking of cell membrane molecules, resulting in cell damage (Karihtala and Soini 2007; Valko et al. 2007).

Most energy-consuming processes during liver regeneration, including cell mitotic and biosynthetic activity, requires energy in the form of ATP. Protein and lipid production, growth factor production, signal transduction, and mitosis during regeneration require ATP. In the regeneration zone, decreased blood flow and oxygenation lead to ischemia that can disrupt the balance between energy production and consumption. It can lead to depletion of energy-rich phosphate ATP, which limits the energy supply to cells and potentially impairs healing (Chien 2010). Paracetamol toxicity has been shown to lead to mitochondrial ATP reduction in hepatocytes (Masubuchi et al. 2005; Shi et al. 2018). Injection of ATP into rabbits' ischemic ear wounds induced early angiogenesis in the wound bed, resulting in faster wound closure and increased formation and reepithelialization than in untreated rabbits (Wang et al. 2009). A recent study concluded that administration of exogenous ATP elicits a protective effect on ischemic skeletal muscle (Maldonado et al. 2013). Administration of exogenous ATP counteracts ischemia and reduces necrosis in tissues (Dvoriantchikova et al. 2010). These data suggest that intracellular ATP distribution promotes wound healing in normal and diabetic wounds and provides a potential therapeutic approach for chronically nonhealing wounds. Although the methods by which intracellular ATP distribution supports wound healing are not fully explained, ATP is an essential component of the healing process.

Endogenous antioxidants, such as CAT and SOD, are enzymes that reduce the rate of lipid peroxidation. SOD protects cells from the damaging effects of superoxide radicals, while CAT directly neutralizes H_2O_2 produced during oxidative stress (Niwa et al. 1990). It is more effective in situations with high H_2O_2 concentrations (Halliwell 1974). GSH concentration, SOD, and CAT activities were higher, and MDA levels were lower in HG and PATP than in PCT. Significant oxidative stress occurs in the livers of rats given paracetamol, according to the literature and our experimental results. AST and ALT were much higher in PCT blood samples than in HG and PATP blood samples. AST and ALT are the most commonly used diagnostic methods to evaluate liver function. When the liver is damaged, then activities of these enzymes are increased in the blood (Yabe et al. 2001). This is caused on by an increase in cell membrane permeability or aminotransferase levels (Giannini et al. 2005). ATP attenuates the increase in ALT, AST, and oxidative stress parameters caused by paracetamol in liver injury (Demiryilmaz et al. 2012).

Cell proliferation, intracellular and transmembrane ion flow, immunomodulation, and thrombosis are just a few of the many cellular and tissue processes that are influenced by purinergic receptors for extracellular nucleotides and nucleosides (Burnstock et al. 1970). The purinergic receptor system in mammals is made up of G protein-coupled P1 receptors A1, A2A, A2B, and A3 for extracellular adenosine, ATP-gated ion channel P2X1–7 receptors, and G protein-coupled P2Y1,2,4,6,11,12,13 and 14 receptors for extracellular ATP (eATP) (Burnstock 2018; Woods et al. 2021). ATP and adenosine are key modulators of the immune system. While ATP is an immunostimulant, adenosine has an immunosuppressive effect. Therefore, the balance between the two is crucial for the proper functioning of the immune system. Extracellular signals provided by ATP and adenosine are detected and transmitted by P2 and P1 receptors, respectively, found on all types of immune cells, thus purinergic

signaling affects all aspects of immunity and inflammation (Cekic and Linden 2016; Richa 2022). An *in vitro* study found that during hepatic necrosis, hypersensitivity to extracellular ATP occurs due to purinergic receptor expression, which increases necrosis (Amaral et al. 2013).

Conclusion

The results reported here suggest that ATP, an energy donor in antioxidant synthesis, may aid in the treatment of liver toxicities in which oxidative stress plays an important pathogenic role. We discovered that ATP reduces the oxidative stress by increasing GSH levels, SOD activities, CAT activities, decreasing MDA levels and protects against paracetamol-induced liver injury at the macroscopic and histological levels. We expect this study to provide more helpful information for therapeutic trials to reduce paracetamol-induced liver injury.

Conflict of interest. We have no conflict of interest to declare.

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