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RESEARCH ARTICLE

Lipid-lowering, anti-inflammatory, and hepatoprotective effects of isorhamnetin on acetaminophen-induced hepatotoxicity in mice

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ABSTRACT

Isorhamnetin is a hepatoprotective flavonoid molecule derived from the leaves and fruits of *Hippophae rhamnoides* L. However, the protective effect of isorhamnetin on acetaminophen (APAP) induced hepatotoxicity is still unknown. Thus, we aimed to investigate the lipid-lowering, anti-inflammatory, and hepatoprotective effects of isorhamnetin on APAP-induced hepatotoxicity in mice. Hepatotoxicity was induced by a single injection of APAP (300 mg/kg, intraperitoneally). Isorhamnetin (50 or 100 mg/kg, orally) and N-acetylcysteine (NAC) (200 mg/kg, orally), or vehicle control, were administered 1 h before the administration of APAP. Total antioxidant status (TAS) and total oxidative status (TOS) of liver tissue and levels of inflammatory factors (TNF-α, IL-1β, and IL-6) were analyzed by ELISA. Lipid profiles and liver function parameters were measured using an autoanalyzer. In addition, liver tissue was examined histopathologically. Isorhamnetin treatment significantly reduced the APAP-induced increase in the liver weight and liver index; it also reduced the APAP-induced increase in serum liver parameters (ALT, AST, ALP, and LDH) ($p < 0.05$). Isorhamnetin significantly reduced APAP-induced oxidative stress and inflammation by increasing TAS levels and decreasing TOS, TNF-α, IL-1β, and IL-6 levels ($p < 0.05$). Moreover, isorhamnetin treatment significantly regulated lipid profiles (TG, T-C, LDL-C, and HDL-C levels) that changed in response to APAP administration ($p < 0.05$). In histopathological examination, liver degeneration observed in the APAP group was significantly reduced in the NAC and isorhamnetin-treated groups ($p < 0.05$). This study suggests that isorhamnetin has a significant protective effect on APAP-induced hepatotoxicity in mice through its lipid-lowering, antioxidant, and anti-inflammatory effects.

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Isorhamnetin; acetaminophen; hepatotoxicity; N-acetylcysteine; mice

1. Introduction

Acetaminophen (APAP) is a widely used antipyretic and analgesic drug (Wang *et al.* 2017). Despite its many beneficial effects, serious complications such as acute liver injury (ALI) and hepatotoxicity have been reported, which can lead to death. Drug-induced liver injury has become prevalent in the community as a major public health issue due to increasing sales of new over-the-counter drugs (Tang *et al.* 2015). Among the causes of liver injury, ALI is the most frequent in many countries (Saeedi Saravi *et al.* 2016, Yoon *et al.* 2016). APAP-induced liver damage is caused by toxic drug properties and its metabolites (Villanueva-Paz *et al.* 2021). At therapeutic doses, a substantial percentage of APAP is transformed into harmless molecules in the liver, via hepatic sulfation and glucuronidation, and these are then eliminated mostly in the urine (Uysal *et al.* 2016). The remaining APAP is converted to N-acetyl-p-benzoquinone imine (NAPQI) by CYP450. NAPQI is conjugated with reduced glutathione to form nontoxic compounds. An overdose of APAP leads to the excessive production of NAPQI in the liver cells, which depletes intracellular GSH stores and has a toxic effect on the cells (Mazraati and Minaiyan 2018). In addition, APAP causes oxidative stress by reducing the antioxidant capacity of cells and increasing reactive oxygen species (ROS)(Cha *et al.* 2018, Mazraati and Minaiyan 2018). APAP has been reported to increase ROS-mediated oxidative stress in the liver through the depletion of GSH (Cha *et al.* 2018). ROS directly damages the lipids, proteins, enzymes, and DNA of the liver cells, and can also promote immune-mediated oxidative damage (Villanueva-Paz *et al.* 2021). It is important to measure the overall status of oxidative stress in the body, for example by characterizing total antioxidant status (TAS) and total oxidant status (TOS). Interestingly, APAP-induced cell necrosis leads to increased production of proinflammatory cytokines that exacerbate inflammation in the liver (McGill and Jaeschke 2014, Saeedi Saravi *et al.* 2016).

For several decades, N-acetylcysteine (NAC), a precursor of GSH, has been the only approved antidote by the Food and Drug Administration (FDA) for APAP-induced toxicity. Therefore, treatment options for APAP-induced hepatotoxicity are limited. NAC works by restoring GSH levels and increasing anti-ROS activity. However, it exerts its effect only about 10 hours after APAP exposure and is only partially effective. Moreover, its adverse effects such as nausea, vomiting, and

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anaphylaxis have prompted researchers to find a safer and-more effective drug for APAP-induced ALI (Cai *et al.* 2015, Mazraati and Minaiyan 2018).

Flavonoids are natural substances found in vegetables, fruits, stems, roots, tea, wine, and flowers and are traditionally used to treat various liver diseases. They have a wide range of biological activities such as antioxidant, anticarcinogenic, anti-inflammatory, and antimutagenic effects, as well as the ability to alter CYP enzymatic activity (Panche *et al.* 2016). Isorhamnetin is a hepatoprotective flavonoid molecule that is a metabolite of quercetin (Kim *et al.* 2013), derived from the leaves and fruit of *Hippophae rhamnoides*. It has been previously shown that isorhamnetin has various biological effects, including antioxidant, anti-inflammatory, anti-apoptotic, and anti-cancer properties (Li *et al.* 2016, Lu *et al.* 2018).

However, there is currently insufficient evidence in the literature on the anti-inflammatory, lipid-lowering, and hepatoprotective effects of isorhamnetin. In this study, we investigated the protective effect of isorhamnetin on lipid profile, inflammation, and liver function in a mouse model of APAP-induced hepatotoxicity.

2. Materials and methods

2.1. Chemicals

APAP (Parol®, Atabay, Istanbul, Turkey) and NAC (NAC®, Neutec, Sakarya, Turkey) were purchased from the local pharmacy. Isorhamnetin was purchased from Sigma-Aldrich (Saint. Louis, MO, USA).

2.2. Animals and experimental design

Balb/C mice (25–30 g) were provided by the Laboratory of Experimental Animals, Faculty of Medicine, Sivas Cumhuriyet University (Sivas, Turkey). Mice were housed under standard laboratory conditions (at 25 °C ± 1 room temperature, 55 ± 1 relative humidity, 12-h light cycle) with food and water *ad libitum*. Animal experiments were approved by the Local Ethics Committee of the Animal Experiments of Sivas Cumhuriyet University (HADYEK, Registration Number: 65202830–050.04.04–444, Sivas, Turkey) and carried out in line with EU Directive 2010/63/EU. Mice were randomly allocated into six equal groups (6 animals per group) as follows: control group, Isor100 group, APAP group, APAP + Isor50 group, APAP + Isor100 group, and APAP + NAC group. Isorhamnetin was dissolved in 2% DMSO. The control group received vehicles only. Isorhamnetin (50 or 100 mg/kg) and NAC (200 mg/kg) were administered by gavage daily, once a day for 3 days (Kim *et al.* 2013, Papackova *et al.* 2018). The mice were given APAP (300 mg/kg, intraperitoneally) on the fourth day. Isorhamnetin and NAC were also administered orally 1 h before the injection of APAP. The mice were euthanized 24 h after APAP injection.

2.3. Body weight and tissue index

Body weight was monitored before and after the study. The liver of the mice was removed after euthanasia, washed with cold saline, and weighed. The liver index was calculated using the formula: liver weight/body weight x 100.

2.4. Detection of liver function-related indicators and lipid profiles

Blood samples were taken from the heart under anesthesia and centrifuged for 10 minutes at 3500 rpm (Nüve NF 800 R, Ankara, Turkey). Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total cholesterol (T-C), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) were measured using an autoanalyzer (Mindray BS 200, China).

2.5. Detection of inflammatory factors in the liver tissue

TNF-α, IL-1β, and IL-6 levels were determined using the commercial ELISA kit (Bioassay Technology Laboratory, Shanghai, China). Tissue homogenates were prepared according to the manufacturer's instructions and absorbances were determined with a microplate reader at a wavelength of 450 nm, and results were expressed as pg/g tissue (Thermo Multiskan Go Microplate Reader, Waltham, MA).

2.6. Measurement of TAS and TOS

TAS and TOS in the liver were determined using ELISA kits (Sun Red, Shanghai, China).

In brief, tissues were homogenized in phosphate-buffered saline (PBS) with a beat beater device (HT 24 Bead Beating Homogenizer, OPS Diagnostics, USA) for analysis of both parameters. The homogenates were then centrifuged at 4000 rpm for 10 minutes at 4 °C. Results were obtained at 450 nm in accordance with the procedure with the coated sandwich ELISA kit from the supernatants obtained. The results were calculated as U/g tissue for TAS and nmol/g tissue for TOS.

2.7. Histopathological examination

Liver samples were collected from mice euthanized under anesthesia by cervical dislocation. Tissues were fixed in 10% buffered formaldehyde solution for 24 hours, then embedded in paraffin and cut into 5 μ thick slices. The liver samples were examined blindly under the microscope (Olympus BX-51, Tokyo, Japan) after staining with hematoxylin-eosin. In the microscopic examination, the previously reported method based on the lesion score averages in ten different regions at 20x magnification was used. The findings were scored as follows: score 0, no histopathological change; 1 (mild), mild hepatocellular swelling due to hydropic degeneration in the centrilobular region; 2 (moderate), moderate degeneration and hepatocellular swelling in the centrilobular and mid-

zonal region; 3 (severe), severe ballooning degeneration and centrilobular necrosis; 4 (very severe), diffuse, severe ballooning degeneration and necrosis (panlobular) (Ates and Ortatatli 2021).

2.8. Statistical analysis

Data were statistically analyzed using SPSS 25.0. One-way ANOVA and Tukey's *post hoc* test were used to analyze normally distributed data. Statistical analysis of the histopathologic score was performed using a post-hoc Bonferroni-corrected Mann–Whitney *U*-test following the Kruskal–Wallis test. Significance was set at $p < 0.05$. Data are shown as mean ± standard error (mean ± S.E.M.).

3. Results

3.1. Effects of isorhamnetin on body weight and liver index

The body weights of the mice were recorded before and after the experimental protocol. Table 1 shows the changes in body weight and liver index for all experimental groups. There was no significant difference in the final body weight of animals between the treatment groups ($p > 0.05$). However, liver weight and liver index noticeably increased in the APAP treated group when compared to the control group ($p < 0.001$). By contrast, these parameters were significantly decreased in APAP-treated animals pretreated with isorhamnetin or NAC as compared to the APAP group ($p < 0.001$).

3.2. Effect of isorhamnetin on serum LDH and liver biomarkers

Table 2 shows the serum levels of liver-related markers and LDH in each group. The blood levels of ALT, AST, ALP, and LDH were significantly higher in the APAP group than in the control group ($p < 0.001$). APAP-treated animals pretreated with isorhamnetin and NAC had significantly lower ALT, AST, and ALP serum levels than the APAP-treated group ($p < 0.01$). The level of serum LDH in the APAP + Isor100 group and APAP + NAC group was also found to be decreased when compared with the APAP group ($p < 0.001$). Interestingly, significant differences in LDH levels were not detected in the APAP + Isor50 group or the APAP + Isor100 group when compared with the APAP + NAC group ($p < 0.05$).

3.3. Effect of isorhamnetin on serum lipid profiles

Table 3 shows the changes in serum lipid profile for all experimental groups. APAP-treated mice significantly increased TG, T-C, and LDL-C ($p < 0.001$), while it notably and,

Table 1. Changes in body weight, liver weight, and liver index of mice after liver injury by APAP administration.

	Initial body weight (g)	Final body weight (g)	Liver weight (g)	Liver index
Control	28.66 ± 0.43	28.90 ± 0.44	1.33 ± 0.01	4.59 ± 0.05
$Isor_{100}$	27.50 ± 0.84	27.82 ± 0.85	1.23 ± 0.05	4.41 ± 0.08
APAP	28.42 ± 0.25	28.56 ± 0.25	$1.54 \pm 0.01^{***}$	$5.41 \pm 0.05^{***}$
APAP + $Isor_{50}$	27.56 ± 0.21	27.80 ± 0.21	$1.27 \pm 0.02^{\#\#\#,ns}$	$4.60 \pm 0.06^{\#\#\#,ns}$
APAP + $Isor_{100}$	27.38 ± 0.28	27.70 ± 0.30	$1.23 \pm 0.01^{\#\#\#,ns}$	$4.43 \pm 0.07^{\#\#\#,ns}$
APAP + NAC	27.18 ± 0.31	27.48 ± 0.28	$1.26 \pm 0.02^{\#\#\#}$	$4.59 \pm 0.06^{\#\#\#}$

APAP: Acetaminophen; Isor: Isorhamnetin; NAC: N-acetylcysteine; ns: not significant.
$^{***}p < 0.001$denotes significant difference vs. control group, $^{\#\#\#}p < 0.001$denotes significant difference vs. APAP group, and ns denotes no significant difference vs. NAC group.

Table 2. Levels of serum LDH and liver biomarkers in each group of mice.

	ALT (U/L)	AST (U/L)	ALP (U/L)	LDH (U/L)
Control	28.80 ± 0.82	72.20 ± 3.76	79.08 ± 3.44	285.32 ± 12.60
$Isor_{100}$	26.00 ± 2.30	85.94 ± 4.73	85.94 ± 4.73	298.10 ± 9.48
APAP	$84.16 \pm 2.68^{***}$	$245.66 \pm 6.86^{***}$	$142.20 \pm 9.18^{***}$	$512.44 \pm 37.39^{***}$
APAP + $Isor_{50}$	$53.80 \pm 9.18^{\#\#\#,ns}$	$176.00 \pm 4.59^{\#\#\#,ns}$	$110.22 \pm 3.37^{\#\#,ns}$	469.20 ± 11.46^{ns}
APAP + $Isor_{100}$	$30.20 \pm 2.97^{\#\#\#,ns}$	$88.80 \pm 5.96^{\#\#\#,ns}$	$92.18 \pm 3.50^{\#\#\#,ns}$	$359.84 \pm 15.35^{\#\#\#,ns}$
APAP + NAC	$36.60 \pm 3.24^{\#\#\#}$	$84.20 \pm 4.84^{\#\#\#}$	$88.02 \pm 2.49^{\#\#\#}$	$314.57 \pm 8.85^{\#\#\#}$

APAP: Acetaminophen; Isor: Isorhamnetin; NAC: N-acetylcysteine; ns: not significant.
$^{***}p < 0.001$ denotes significant difference vs. control group, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ denotes significant difference vs. APAP group, and ns denotes no significant difference vs. NAC group.

Table 3. Levels of serum lipid profiles in each group of mice.

	TG (mg/dl)	T-C (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
Control	62.84 ± 2.45	90.02 ± 4.75	97.50 ± 3.97	6.08 ± 0.66
$Isor_{100}$	68.86 ± 6.18	93.96 ± 3.37	92.02 ± 2.71	9.38 ± 2.66
APAP	$96.17 \pm 2.25^{***}$	$139.58 \pm 3.22^{***}$	$59.74 \pm 2.58^{***}$	$31.11 \pm 2.18^{***}$
APAP + $Isor_{50}$	86.45 ± 6.18^{ns}	$116.44 \pm 3.99^{\#\#,ns}$	$88.97 \pm 4.00^{\#\#\#,ns}$	$21.14 \pm 0.96^{\#\#,ns}$
APAP + $Isor_{100}$	$77.78 \pm 4.00^{\#,ns}$	$115.02 \pm 4.52^{\#\#,ns}$	$87.50 \pm 3.82^{\#\#\#,ns}$	$19.94 \pm 1.37^{\#\#,ns}$
APAP + NAC	$75.34 \pm 2.40^{\#}$	$95.84 \pm 2.79^{\#\#\#}$	$89.43 \pm 3.02^{\#\#\#}$	$8.38 \pm 1.65^{\#\#\#}$

APAP: Acetaminophen; Isor: Isorhamnetin; NAC: N-acetylcysteine; ns: not significant.
$^{***}p < 0.001$ denotes significant difference vs. control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ denotes significant difference vs. APAP group, and ns denotes no significant difference vs. NAC group.

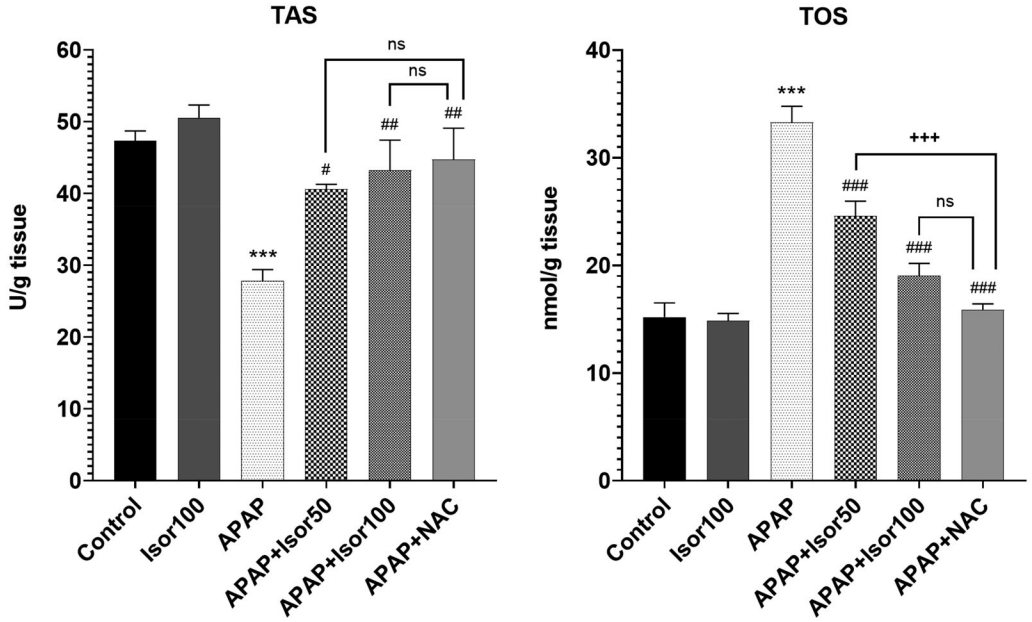

Figure 1. Changes in TAS and TOS levels of mice after liver injury by APAP administration. Abbreviations: APAP: Acetaminophen; Isor: Isorhamnetin; NAC: N-acetylcysteine; ns: not significant. $^{***}p < 0.001$ denotes significant difference vs. control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ denotes significant difference vs. APAP group, $+++p < 0.001$ denotes significant difference vs. NAC group, and ns denotes no significant difference vs. NAC group.

significantly decreased HDL-C levels as compared to controls ($p < 0.001$). Treatment with isorhamnetin (100 mg/kg) or NAC significantly improved the APAP-induced impairment in lipid ($p < 0.05$). While the decrease in TG levels in the APAP + Isor50 group was not statistically significant when compared to that of the APAP group, there was no significant difference between these levels in the APAP + Isor50 group and the APAP + NAC group ($p > 0.05$).

3.4. Effect of isorhamnetin on liver tissue total oxidant and antioxidant status

The administration of APAP dramatically decreased TAS levels while significantly increasing TOS levels ($p < 0.001$). Treatment with NAC and isorhamnetin dramatically improved the levels of TAS and TOS in the liver tissue of APAP-treated mice ($p < 0.05$, $p < 0.001$, respectively). Interestingly, the TOS level was significantly higher in the APAP + Isor50 group when compared to the APAP + NAC group ($p < 0.001$) (Figure 1).

3.5. Effect of isorhamnetin on liver tissue levels of inflammatory cytokines

TNF-α, IL-1β, and IL-6 levels were elevated in the liver after administration of APAP, whereas treatment with NAC and isorhamnetin decreased the level of these proinflammatory cytokines ($p < 0.001$). Interestingly, the levels of TNF-α, IL-1β, and IL-6 were notably higher in the APAP + Isor50 group than in the APAP + NAC group ($p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively) (Figure 2).

3.6. Effect of isorhamnetin on liver histopathology

Histopathological examination of the liver showed that the hepatocytes of some animals in the control group had mild hepatocellular swelling due to hydropic degeneration in the centrilobular zone. Panlobular necrosis and severe balloon-like degeneration were observed in the APAP group (Figure 3). In addition, the administration of APAP led to the enlargement of sinusoids, congestion of blood vessels, and inflammatory cell infiltration in the portal areas. On the other hand, in animals treated with NAC and isorhamnetin, it was observed that APAP-induced liver damage regressed at different rates, and lesions were confined to the centrilobular zone. The liver sections showed lesions that had progressed from centrilobular degeneration to balloon-like degeneration and necrosis. Statistical analysis of the liver lesion scores showed significant liver damage in the APAP group compared to the control ($p < 0.01$). Our data also revealed that treatment with isorhamnetin or NAC dramatically reduced APAP-induced liver injury ($p < 0.01$) (Table 4).

4. Discussion

Mouse models with APAP-induced liver damage are widely used for the development of hepatoprotective medications and the investigation of oxidative stress-induced liver damage (Jaeschke *et al.* 2011). Previous studies have shown that APAP-induced hepatotoxicity is linked to excessive ROS production and changes in the activities of hepatic antioxidant defense system enzymes (Yan *et al.* 2018, Wu *et al.* 2019, Zhou *et al.* 2021). NAPQI, a highly reactive intermediate, and other free radicals, such as hydrogen peroxide (H_2O_2), superoxide anion radicals (O2•−), and hydroxyl radicals (•OH), are produced during the metabolism of APAP by CYP450 in hepatocytes (Verma *et al.* 2016).

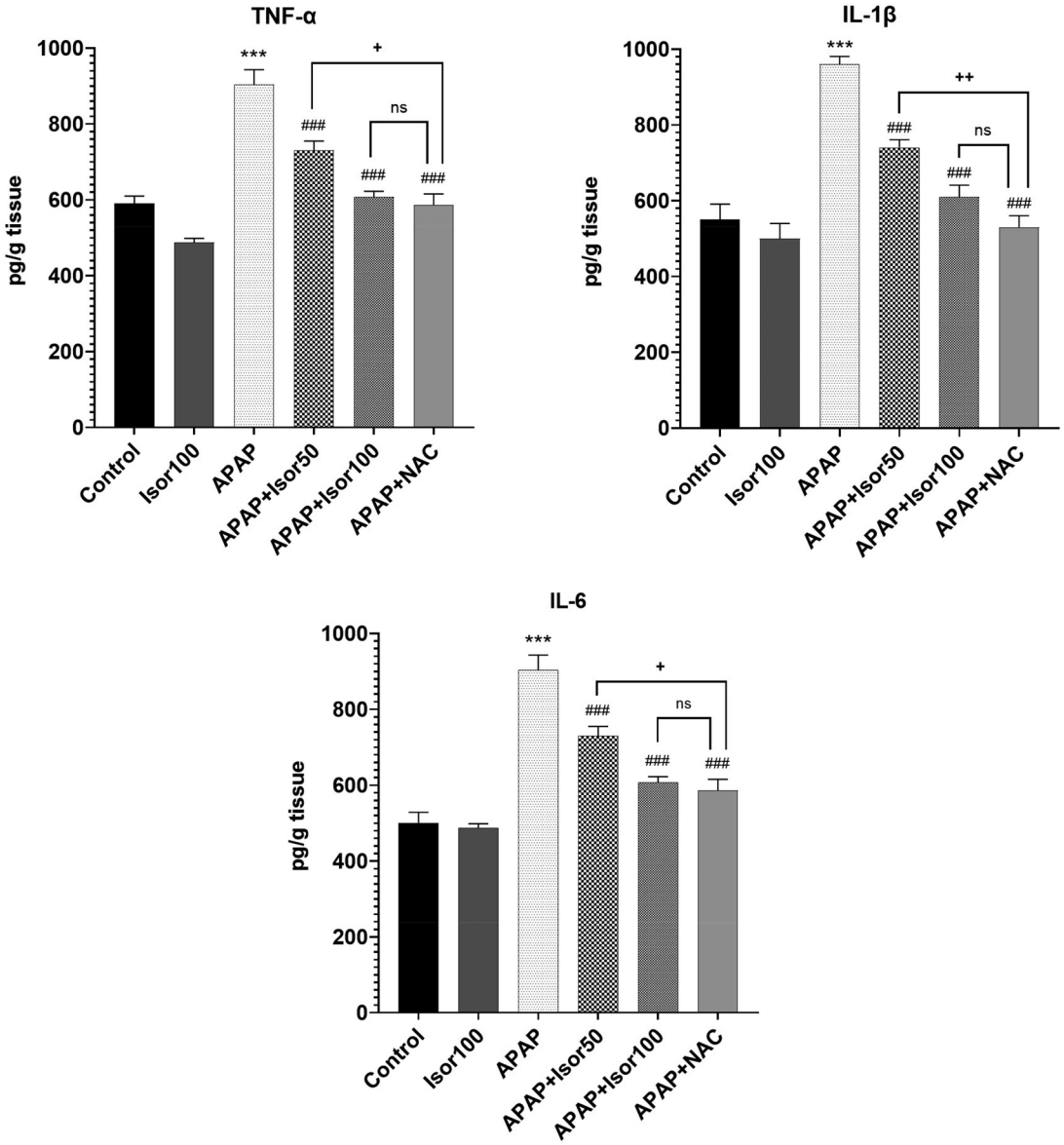

Figure 2. Changes in TNF-α, IL-1β, and IL-6 levels of mice after liver injury by APAP administration. Abbreviations: APAP: Acetaminophen; Isor: Isorhamnetin; NAC: N-acetylcysteine; ns: not significant. $^{***}p < 0.001$ denotes significant difference vs. control group, $^{###}p < 0.001$ denotes significant difference vs. APAP group, $+p < 0.05$, $++p < 0.01$ denotes significant difference vs. NAC group, and ns denotes no significant difference vs. NAC group.

Isorhamnetin has been shown to have multiple pharmacological effects, including hepatoprotective effects (Lu *et al.* 2018, Tian *et al.* 2021). Although the antioxidant effect of isorhamnetin has been studied in APAP-induced liver damage in mice, the mechanisms that contribute to its hepatoprotective effect in APAP-induced hepatotoxicity are unclear. In the present study, we determined that isorhamnetin exhibited a protective effect on APAP-induced hepatotoxicity, similar to that seen for NAC.

NAC has several functions, including inhibiting lipid peroxidation, scavenging ROS, preventing DNA damage, regulating cell metabolic activity, inhibiting the production of inflammatory mediators, and providing mitochondrial protection (Hendrickson 2019, Tan *et al.* 2019). NAC is the only antidote approved by the FDA for APAP toxicity. Hence, in this study, NAC was used as the positive control drug.

Previous studies have shown that APAP overdose administration results in excessive ROS production in hepatocytes, which can oxidize macromolecules such as lipids, proteins, and DNA; this can lead to cellular dysfunction and, eventually, cell death (Nelson 1990, Verma *et al.* 2016). The decrease in TAS levels in the liver may contribute to hepatotoxicity caused by free radicals (Gündüz *et al.* 2015, Verma *et al.* 2016, Feng *et al.* 2019).

This study showed that an increase in TOS levels, which indicates ROS scavenging capabilities, and a decrease in TAS, which indicates total oxidant levels, were seen in the livers of APAP-exposed mice. These changes in enzyme activity levels result in a tendency toward increased oxidation reactions in terms of redox balance (Erel 2004, 2005, Maciejczyk *et al.* 2018). Recent studies have found that isorhamnetin decreased the expression of inducible nitric oxide synthase

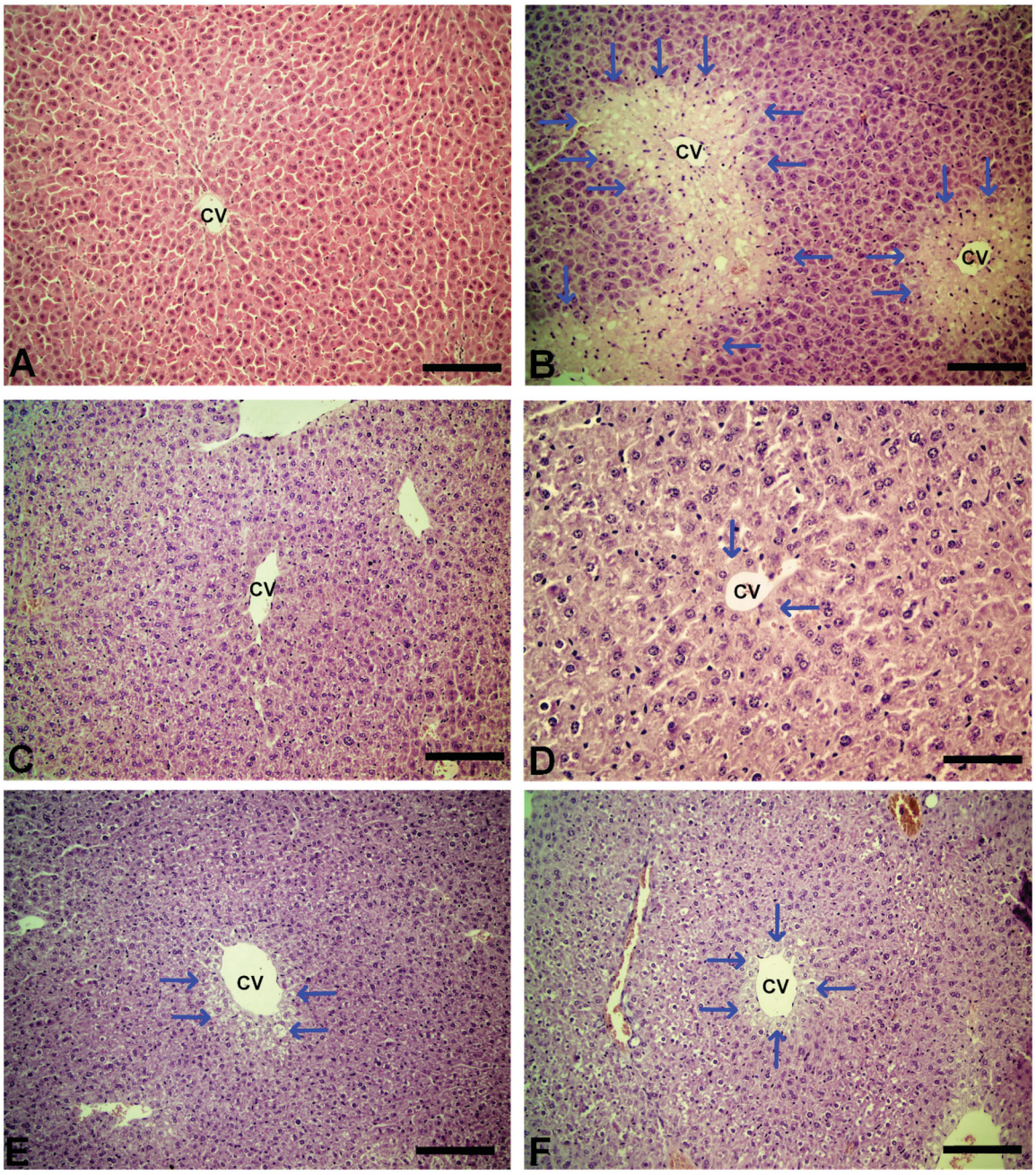

Figure 3. Histopathological changes of the liver tissue after APAP administration. (A) Normal appearance of liver, control group; (B) severe panlobular necrosis (arrows), APAP group; (C) normal appearance of liver, ISOR100 group; (D) mild hepatocellular swelling (arrows) in centrilobular region, ISOR50 + APAP group; (E) centrilobular region moderate balloon-like degeneration (arrows), ISOR100 + APAP; (F) moderate degeneration and hepatocellular swelling in centrilobular region (arrows), NAC + APAP, 20X, HE, Scale bar: 100 μm.

Table 4. Effect of isorhamnetin on histopathological score in liver tissue.

Control	$Isor_{100}$	APAP	APAP + $Isor_{50}$	APAP + $Isor_{100}$	APAP + NAC
			Mean (Median) ± SEM		
0.66 (1.00) ± 0.21	1.17 (1.00) ± 0.17	3.33 (3.00) ± 0.21**	1.83 (2.00) ± $0.17^{\#\#,ns}$	1.67 (2.00) ± $0.21^{\#\#,ns}$	1.83 (2.00) ± $0.17^{\#\#}$

APAP: Acetaminophen; Isor: Isorhamnetin; NAC: N-acetylcysteine; ns: not significant.
**$p < 0.01$ denotes significant difference vs. control group, $^{\#\#}p < 0.01$ denotes significant difference vs. APAP group, and ns denotes no significant difference vs. NAC group.

(iNOS) and the formation of nitric oxide (Seo *et al.* 2014); thus, intracellular GSH levels increased, and consequently, protection against oxidative stress-induced liver damage was enhanced (Seo *et al.* 2014, Yang *et al.* 2014). Küpeli *et al.* showed that isorhamnetin restored liver enzymes, increased GSH content in the liver, and inhibited lipid peroxidation in the plasma and livers of mice models with APAP-induced hepatotoxicity (Küpeli *et al.* 2006). In our study, isorhamnetin treatment reversed the APAP-induced decrease in liver TAS levels and decreased the elevated TOS levels. Thus, data have

shown that isorhamnetin can restore the oxidant/antioxidant imbalance induced in the liver by APAP treatment.

In the current study, serum TC, TG, and LDL-C concentrations increased significantly in the APAP-exposed mice, while HDL-C concentrations dramatically decreased. These changes in the serum lipid profile indicated that the lipid metabolism was impaired in the APAP-exposed mice. The increase in serum TG concentration might be attributed to the failure of normal lipid uptake, conjugation, and excretion caused by the injured liver parenchyma. TG accumulation due to APAP-treatment may result from the inhibition or disturbance of the TG secreting mechanism (Kosanić and Ranković 2011). Hypercholesterolemia, typically associated with biliary obstruction, is a common symptom of APAP-induced toxicity (Zhao and Hu 2013).

Flavonoids have been reported to inhibit oxidation processes, which contribute to the development of various diseases, while also lowering LDL-C levels and increasing HDL-C concentrations in hepatotoxicity (Madi Almajwal and Farouk Elsadek 2015). In the present study, the isorhamnetin treatment decreased the serum levels of TG, TC, and LDL-C and increased HDL-C levels in mice exposed to APAP. Consistent with our data, it was reported that TG decreased with isorhamnetin treatment (50 mg/kg, orally) in a mouse model of nonalcoholic steatohepatitis (NASH) (Ganbold *et al.* 2019). Therefore, our data suggest that isorhamnetin may be effective in alleviating the abnormal lipid profile in APAP-induced hepatotoxicity.

The data showed elevated levels of AST, ALT, ALP, and LDH in the serum, which are biomarkers of liver injury, suggesting that the structural integrity of the hepatocellular membrane is damaged in APAP-induced hepatotoxicity (Jo *et al.* 2019). Increases in liver enzymes suggest that APAP treatment leads to severe liver injury, which is characterized by membrane damage in hepatocytes. This resulted in the leakage of cell components and hepatocyte enzymes into the bloodstream (Rashid *et al.* 2016, Ezhilarasan and Raghunandhakumar 2021). Previous studies support the view that serum transaminase levels return to normal with the healing of the liver parenchyma and the formation of hepatocytes (Shehab *et al.* 2015, Rashid *et al.* 2016). In our study, the increased serum AST, ALT, ALP, and LDH levels caused by APAP decreased with the administration of isorhamnetin and NAC. Interestingly, in primary cultures of rat hepatocytes, Rajaraman and coworkers showed that *Ginkgo biloba* extract, which contains isorhamnetin, did not alter the extent of LDH leakage induced by APAP and that isorhamnetin did not influence the extent of APAP-induced hepatotoxicity (Rajaraman *et al.* 2006, Subramanya *et al.* 2018). However, this result is in contrast to our observations in this *in vivo* study. The results of the present studies suggest that isorhamnetin may have a membrane-stabilizing potential *in vivo*, as it prevented the elevation of serum liver enzyme levels induced by APAP hepatotoxicity (Qadir *et al.* 2014, Khan *et al.* 2017).

The production of various cytokines increases following the onset of APAP toxicity, which is consistent with their role in hepatic reorganization and increased oxidative stress (Hinson *et al.* 2010). Following APAP exposure, proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, have been found to increase in liver tissue (McGill *et al.* 2012, Saeedi Saravi *et al.* 2016). Consistent with previous studies, in our study, the hepatic levels of pro-inflammatory response markers TNF-α, IL-1β and IL-6 increased in the APAP-treated mice (McGill *et al.* 2012, Saeedi Saravi *et al.* 2016). However, isorhamnetin treatment (100 mg/kg) considerably decreased the serum levels of TNF-α, IL-1β and IL-6 in the APAP-treated mice and had a similar effect to NAC treatment. The data suggest that the anti-inflammatory effect of isorhamnetin may be associated with increasing the activation of nuclear factor-erythroid-2-related factor 2 (Nrf2) in a dose- and time-dependent manner (Chirumbolo 2014). Hence, the present study shows that isorhamnetin has anti-inflammatory effects that can inhibit the production of pro-inflammatory cytokines in liver tissue. The study data also suggest that isorhamnetin has an anti-inflammatory effect through antioxidant activity. Nrf2 and other related mechanisms may contribute to the hepatoprotective activity of isorhamnetin (Yang *et al.* 2014, Chirumbolo 2014, Cai *et al.* 2015, Qi *et al.* 2018).

The reactive metabolite NAPQI formed by CYP450 results in mitochondrial dysfunction and, eventually, hepatic necrosis (Butler 2018). Since cytochrome P450 enzymes are found in centrilobular liver cells, necrosis usually begins in the centrilobular region, and the first indication of this is balloon degeneration (Lefkowitch 2016). Most centrilobular hepatocytes in the livers of mice exposed to APAP are swollen, with significant cytoplasmic vacuolation and dense nuclei (Tan *et al.* 2019). The histopathological findings of the current study showed the lesions changing from centrilobular degeneration to ballooning degeneration, as well as necrosis in the liver of the APAP-exposed mice. In addition, it is noteworthy that these ballooning regions contained inflammatory cells such as neutrophils. The data suggest that excessive ROS production may contribute to the centrilobular hepatic necrosis induced by APAP exposure (Chirumbolo 2014, Li *et al.* 2020). In addition, increases in liver enzymes suggest that mitochondrial damage may be associated with this cell necrosis (McGill and Jaeschke 2014, Ding *et al.* 2016, Saeedi Saravi *et al.* 2016, Wu *et al.* 2017).

Isorhamnetin has previously been shown to reduce TG content in the liver and collagen deposition as well as liver weight in an experimental mouse model of liver injury (Ganbold *et al.* 2019). On the other hand, it has been previously reported that NAC treatment alleviates APAP-induced cytoplasmic vacuolization and hepatocyte ballooning in rats (Gündüz *et al.* 2015). In our study, the histopathological evaluation revealed that liver damage in the mice exposed to APAP was alleviated by isorhamnetin and NAC treatments (Figure 3). Our study showed that decreased histological findings, such as panlobular necrosis and balloon-like degeneration, may be associated with normalized liver enzymes, the serum levels of lipid profiles, oxidative stress, and inflammation in mice treated with isorhamnetin and NAC compared to mice exposed to APAP. Thus, the administration of isorhamnetin protects liver tissue from the toxic effects of APAP as much as NAC does and can strengthen both the structural and functional integrity of the liver.

In summary, this study determined that APAP treatment caused liver damage, resulting in increased serum levels of liver marker enzymes and histopathological changes (Mohamad *et al.* 2018). Furthermore, elevated serum levels, which indicate impaired liver fat metabolism, may contribute to the liver damage induced by APAP (Table 3) (Basu *et al.* 2012, Mohamad *et al.* 2018). Similar to the effects of NAC, isorhamnetin was able to reduce oxidative stress, the serum levels of liver marker enzymes, and pro-inflammatory cytokines, and to correct serum lipid profiles, indicating that APAP-induced liver injury was ameliorated by isorhamnetin treatment.

5. Conclusion

The current study found that isorhamnetin has a protective effect on APAP-induced hepatotoxicity in mice by reducing inflammation, oxidative stress, histopathological damage, and improving lipid metabolism, and liver function. Taken together, the data suggest that the hepatoprotective effect of isorhamnetin is similar to that of NAC, a potent hepatoprotective agent. However further research is needed.

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