Protective mechanisms of piperine against acetaminophen-induced hepatotoxicity may be mediated through TGFBRAP1

M.A. MORSY[1,2], N.S. YOUNIS[1,3], A.A.K. EL-SHEIKH[2,4], F.H. AL TURAIFI[1], M. EL-DALY[5], O.M. MOHAFEZ[6,7]

[1]Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Saudi Arabia
[2]Department of Pharmacology, Faculty of Medicine, Minia University, El-Minia, Egypt
[3]Zagazig University Hospitals, Zagazig University, El-Sharkia, Egypt
[4]Basic Health Sciences Department, Faculty of Medicine, Princess Nourah Bint Abdulrahman University, Riyadh, Saudi Arabia
[5]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Minia University, El-Minia, Egypt
[6]Department of Biomedical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Saudi Arabia
[7]Department of Biochemistry, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, Assiut, Egypt

Abstract. – **OBJECTIVE:** To investigate the possible protective mechanisms of piperine against acetaminophen (APAP)-induced hepatotoxicity in mice.

MATERIALS AND METHODS: Mice were given APAP (650 mg/kg i.p. once) with or without pretreatment with piperine (50 mg/kg/day orally for 3 days).

RESULTS: APAP caused liver toxicity as indicated by increased serum alanine aminotransferase and liver microscopic pathology, decreased hepatic superoxide dismutase and glutathione reductase activities, without affecting nuclear factor erythroid 2-related factor 2 (Nrf2) expression. APAP administration induced inflammation and apoptosis manifested as increased NF-κB p65 and dysregulation of caspase 3/Bcl2 expression, respectively. In addition, APAP increased the expression of transforming growth factor-β receptor-associated binding protein 1 (TGFBRAP1). On the other hand, pretreatment with piperine improved liver function and structure, reserved hepatic antioxidative defense, and attenuated inflammatory and apoptotic markers. Interestingly, piperine administration enhanced hepatic TGFBRAP1 expression compared to APAP alone.

CONCLUSIONS: The hepatoprotective effects of piperine against APAP are mediated via its antioxidant, anti-inflammatory, and anti-apoptotic effects, in addition to regulation of TGF-BRAP1.

Introduction

Acetaminophen (APAP; also known as N-acetyl-p-aminophenol or paracetamol) is widely used in the treatment of fever, as well as mild to moderate pain. APAP is available globally in pharmacies and/or non-pharmacy outlets as an over the counter drug[1]. Unfortunately, acute systemic exposure to a high dose of APAP is hepatotoxic and can be fatal. Moreover, APAP overdose is the most frequent clinically encountered cause of acute liver failure[2]. The detoxification of APAP in the liver requires the presence of the endogenous antioxidant molecule reduced glutathione (GSH). However, in the case of overdose, depletion of endogenous GSH causes hepatocellular damage[2]. The standard antidotal therapy involves the administration of N-acetylcysteine, which is a precursor of glutathione. If the patient is treated early enough with the right dose of N-acetylcysteine, a positive prognosis is expected. However, failure to deliver appropriate antidotal therapy at the right time makes the development of hepato-

toxicity inevitable[3]. The resultant oxidative stress may trigger the initiation of a series of events of inflammation, apoptosis, and necrosis that might end up with hepatic failure[4]. Thus, given its widespread use, its well-documented hepatotoxicity, besides the high incidence of overdose, the APAP-induced liver toxicity in experimental animals is regarded as one of the most famous models of drug-induced acute liver injury[5,6].

Transforming growth factor (TGF)-β is a pluripotent cytokine involved in normal cellular functions, such as cell growth, proliferation, differentiation, and tissue repair, as well as in pathological processes, including fibrosis and apoptosis[7]. During APAP-induced hepatotoxicity, TGF-β is increased both in serum and liver tissue[8], as one of the mechanistic pathways involved in the progression of liver damage. The TGF-β receptor-associated protein 1 (TGFBRAP1; *aka* TRAP-1) is a member of the heat shock protein family that is usually present in the cytoplasm, where it mainly regulates TGF-β-mediated signaling by binding inactive TGF-β type 2 receptor and dissociates upon receptor activation[9]. Later, it was illustrated that the dissociated TGFBRAP1 is associated with the TGF-β type 1 receptor and acts as an essential scaffold for phosphorylation of receptor-mediated small mothers against decapentaplegic 4 (Smad4)[10]. Besides, a human analog of TGFBRAP1 also differentially regulates different Smads complex formation and cellular compartmentalization[11]. Activation of both receptor subtypes phosphorylates Smad2 and Smad3, which in turn heterodimer with Smad4[12]. Recently, dysregulation of TGFBRAP1-mediated activation of inhibitory Smad7 was observed in lung fibrosis[13]. However, the role of TGFBRAP1 in the pathogenesis of APAP-induced hepatotoxicity was not previously investigated.

Piperine (1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine) is a natural organic alkaloid abundantly present in black pepper[14], where it is responsible for its known sharp flavor and biting taste, as well as its widespread use as an additive to everyday meals in different cultures. Piperine has a wide spectrum of pharmacological activities, which include being a strong antioxidant that can restore redox balance in antioxidant-depleted conditions, as in isoproterenol-induced myocardial ischemia[15]. Besides, piperine possesses pronounced anti-inflammatory effects, as shown in murine chondrogenic cells treated with lipopolysaccharide *in vitro*[16], as well as protection against inflammation of the stomach of Mongolian gerbils *in vivo* after a *Helicobacter pylori* challenge[17]. In addition, piperine exhibited other pharmacologically beneficial immunomodulatory, antimutagenic, anticancer, anti-asthmatic, anticonvulsant, and antimicrobial activities[14]. In the liver, Panahi et al[18] showed that supplementation of piperine combined with curcuminoids had favorable effects on hepatic functional biomarkers in type 2 diabetic patients. Thus, we hypothesized that piperine will protect against APAP-induced acute hepatic injury.

Sabina et al[19] suggested that piperine, *via* its antioxidant effect, might ameliorate liver toxicity induced by APAP. However, the mechanisms by which piperine exerted these hepatoprotective effects were not well-explored. Therefore, the aim of the current study is to investigate the possible mechanisms by which piperine protects against APAP-induced hepatotoxicity, including the activation of TGFBRAP1.

Materials and Methods

Drugs and Chemicals

Both APAP and piperine were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Alanine aminotransferase (ALT) UV kinetic assay kit was purchased from United Diagnostics Industry (Dammam, Saudi Arabia). Enzyme colorimetric assay kits for the determination of superoxide dismutase (SOD) and glutathione reductase (GR) were purchased from Abcam (Cambridge, UK). Rabbit polyclonal antibody against nuclear factor (NF)-κB p65 subunit was purchased from Abcam (Cambridge, UK), while rabbit polyclonal antibodies against nuclear factor erythroid 2-related factor 2 (Nrf2), caspase 3, B-cell lymphoma 2 (Bcl2), TGFBRAP1, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animal Protocol and Sample Collection

Twenty-four male Swiss mice weighing 25-30 g were left to acclimatize in standard laboratory animal housing for one week before the start of experiments, with food and water available *ad libitum*. The study was approved (356:1/2020) and performed following the Animal Research Ethical Standards of the Research Ethics Committee, Faculty of Medicine, Minia University, Egypt, in compliance with the Research Ethics Committee, King Faisal University, Saudi Arabia. Mice were divided into 3 groups (n=8), where the first group

served as a control. The second group was injected with a single dose of APAP (650 mg/kg i.p.) in a total volume of 7.5 ml/kg at the end of the third day of the experiment to induce hepatotoxicity[20]. The third group was pretreated with oral piperine (10 ml/kg) in a dose of 50 mg/kg/day[21] for 3 consecutive days before APAP injection as done in the second group. Control animals, which did not receive any drugs, were given equivalent volumes of the vehicles [saline (i.p.) for APAP and 1% aqueous solution of carboxymethyl cellulose (oral) for piperine]. Four hours after APAP injection, all animals were euthanized by cervical dislocation. Blood was collected and centrifuged at 3000 g for 10 min, and samples of clear sera were kept at -20°C for further analysis. The liver was rapidly excised, and a slice was taken for histopathological examination. The rest of the liver was homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4) and was kept at -80°C until used.

Liver Histopathological and Biochemical Assessment

Fixation of the liver tissues was performed in neutral buffered 10% formalin. The formalin-fixed samples were then embedded in paraffin, and sections of 5 μm were mounted on clean glass slides and stained with hematoxylin and eosin. Tissue sections were histopathologically examined by light microscopy. Semi-quantitative scoring of the histopathological changes was performed blindly on all sample sections (3 fields/ section). Samples showing normal liver histology were indicated by (-), whereas mild, moderate, and severe histological abnormalities of less than 25, 50, and 75% of the total examined fields were indicated by (+), (++), and (+++), respectively. The serum enzymatic activity of ALT, as a biochemical marker of liver function, was quantified using a UV kinetic assay commercial kit, according to the manufacturer's protocol, and the results were represented as U/l. Liver tissue homogenates were used for the determination of the activity of the antioxidant enzymes SOD and GR using commercially available kits.

Western Blot Analysis

Western blot analyses of target proteins were performed according to a previously described protocol[22]. Briefly, proteins in the supernatants of the liver homogenates were separated via SDS-PAGE gel electrophoresis and transferred to PVDF membranes. The membranes were then blocked for non-specific binding by incubation in Tris-buffered saline (TBS) containing 3% bovine serum albumin for 1 h at room temperature. The membranes were then washed in TBS and further incubated for 2 h at room temperature with one of the primary antibodies (anti-Nrf2, anti-NF-κB p65, anti-caspase 3, anti-Bcl2, or anti-TGFBRAP1) at a dilution of 1:500. The membranes were also simultaneously incubated with antibodies raised against β-actin for normalization of the results against a "housekeeping protein". The membranes were washed 3 times in TBS and then incubated for 1 h with the HRP-conjugated secondary antibody at room temperature. The chemiluminescence produced from luminol reagent was detected with the C-DiGit Blot Scanner (LI-COR Biotechnology, Lincoln, NE, USA).

Statistical Analysis

Results were represented as the mean ± SEM and were analyzed statistically *via* one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post-analysis test that compares all groups using GraphPad Prism® version 5.00 for Windows (San Diego, CA, USA). The difference between groups was reported as significant when the *p*-value was less than 0.05..

Results

Effect of Piperine on Liver Function and Structure

As a marker of liver function, the activity of ALT enzyme was measured in serum and showed significant elevation in the APAP group compared to control animals (Figure 1A), indicating deterioration of liver function. Pretreatment with piperine in the APAP/piperine group significantly succeeded in decreasing serum ALT compared to the APAP group. Our preliminary experiments showed that piperine alone had no effect on the liver function marker ALT. As expected, histological examination of the liver of the control group showed normal hepatic architecture, normal portal vein, and no sinusoidal congestion (Figure 1B). On the contrary, the APAP group showed deteriorated hepatic histology (Figure 1C), which is in line with the altered liver function measured by serum ALT. These sections showed severely congested portal and sinusoidal vessels, cellu-
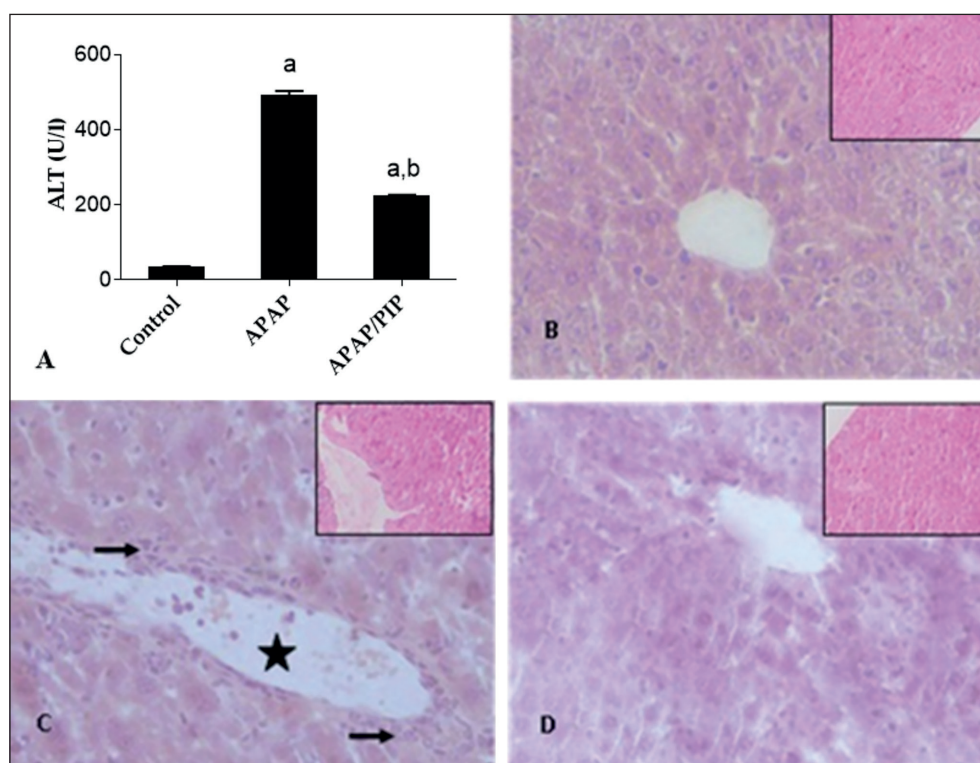
Figure 1. Effect of piperine (PIP) on serum alanine aminotransferase (ALT) and liver histopathological changes in acetaminophen (APAP)-induced hepatotoxicity in mice. **A,** Serum ALT activity expressed as means ± SEM. Results are significantly different if $p<0.05$. "a" significantly different from the control group; "b" significantly different from the APAP group. Mice liver photomicrographs stained by hematoxylin and eosin (×400) of (**B**) control, (**C**) APAP, and (**D**) APAP/PIP groups. Black arrows indicate cellular infiltration and black star indicates central vein vascular congestion. Low magnification (×100) is included in each photomicrograph as an insert. Semi-quantitative scoring of the histopathological changes is summarized in Table I.

lar infiltration, as well as moderate hepatic cellular necrosis, fat deposits, and pyknotic cells, as scored in Table I. On the other hand, animals pretreated with piperine (the APAP/piperine

Table I. Effect of piperine (PIP) on histopathological scoring of microscopic liver picture in acetaminophen (APAP)-induced hepatotoxicity in mice.

	Control	APAP	APAP/PIP
Vascular congestion	-	+++	+
Cellular infiltration	-	+++	+
Hepatocellular necrosis	-	++	+
Fat deposits	-	++	-
Pyknosis	-	++	-

Normal liver histological picture was considered (-). Mild, moderate, and severe histological abnormalities were designated by (+), (++), and (+++), indicating deformities of less than 25, 50, and 75% of total fields examined, respectively. Results are of liver tissue sections from each mouse (n=8), 3 fields/section.

group) showed marked improvements in the hepatic histopathological parameters after APAP challenge when compared to the APAP alone group (Figure 1D).

Effect of Piperine on Liver Tissue Oxidative Stress Markers

To examine whether the antioxidant potential of piperine plays a role in its demonstrated hepatoprotection against APAP-induced toxicity, the activities of the antioxidant enzymes SOD and GR in liver tissues were assessed (Figures 2A and 2B, respectively). Mice treated with APAP alone to induce hepatotoxicity showed significantly reduced hepatic levels of SOD and GR in comparison with the normal control animals. On the other hand, pretreatment with piperine before the APAP challenge caused significant increases in the hepatic activities of both SOD and GR in comparison with the APAP group. Further, the protein expression of the upstream regulatory
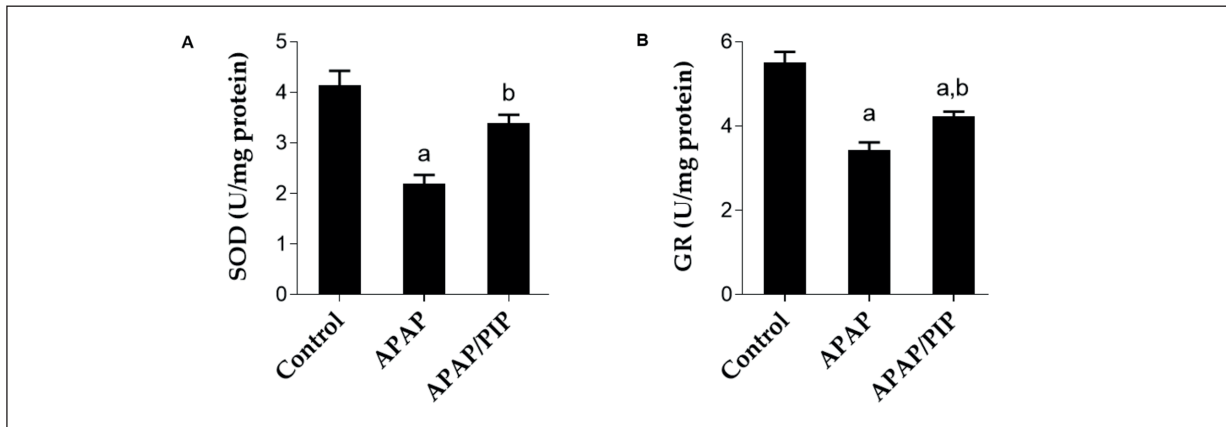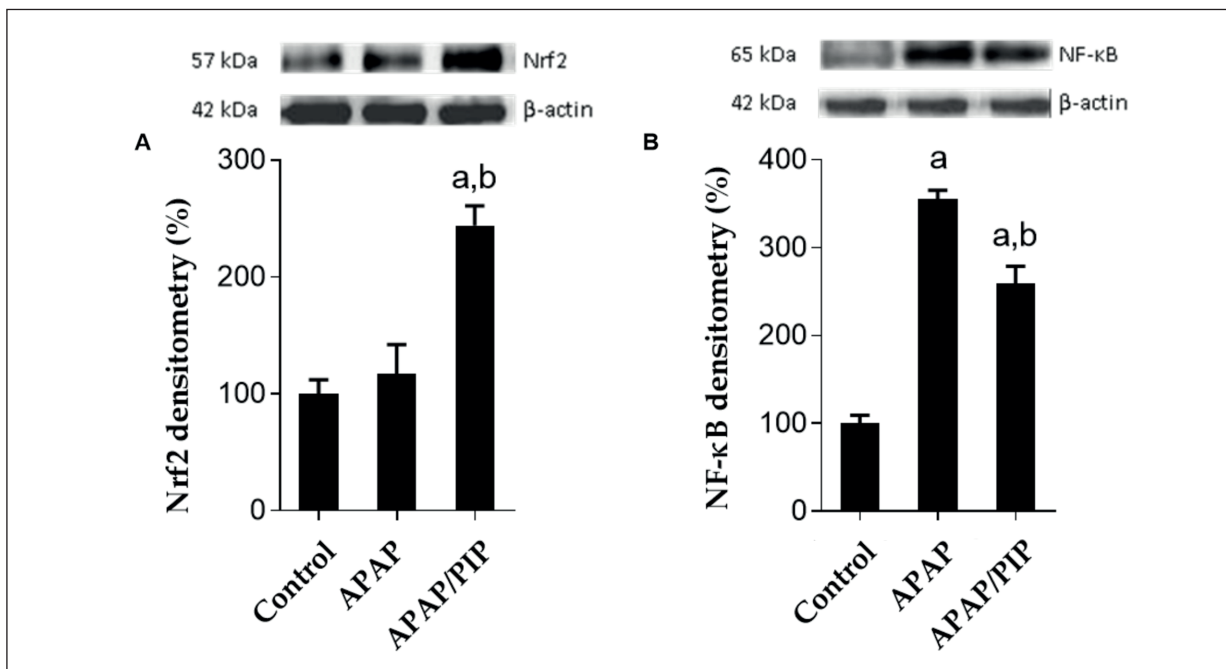
Figure 2. Effect of piperine (PIP) on the hepatic enzymatic activity of superoxide dismutase (SOD) and glutathione reductase (GR) in acetaminophen (APAP)-induced hepatotoxicity in mice. Results are a representation of means ± SEM. Results are significantly different if $p<0.05$. "a" significantly different from the control group; "b" significantly different from the APAP group.

gene; Nrf2, which is involved in the control of redox homeostasis, was assessed by Western blotting (Figure 3A). Induction of liver toxicity by APAP caused no significant effect on the expression of hepatic Nrf2 compared to control animals. However, the administration of piperine for 3 days prior to APAP (APAP/piperine group)

caused a statistically significant upregulation of hepatic Nrf2 expression in comparison with the APAP group.

Effect of Piperine on Liver Inflammation

The protein expression of the p65 subunit of the inflammatory marker NF-κB was assessed

Figure 3. Effect of piperine (PIP) on the hepatic expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor (NF)-κB p65 in acetaminophen (APAP)-induced hepatotoxicity in mice. Upper panels; Western blot bands of (**A**) Nrf2 and (**B**) NF-κB p65, lower panels show their densitometric protein expression as a ratio of their corresponding β-actin, represented as a percentage of control. Results are a representation of means ± SEM. Results are significantly different if $p<0.05$. "a" significantly different from the control group; "b" significantly different from the APAP group.

using Western blotting. The APAP-treated mice displayed significantly increased hepatic NF-κB p65 expression when compared to the control animals (Figure 3B). On the other hand, the piperine pretreatment significantly decreased the level of NF-κB p65 protein in the livers of the APAP/piperine group when compared to the APAP group.

Effect of Piperine on Liver Apoptosis

Assessment of the liver cell apoptosis was carried out by measuring the pro-apoptotic marker caspase 3 and the anti-apoptotic maker Bcl2 (Figures 4A and 4B, respectively). The results show that the induction of acute hepatotoxicity in the APAP group significantly enhanced the level of caspase 3 protein in comparison with the control group. On the other hand, the APAP/piperine group showed significantly lower expression of caspase 3 compared to the APAP group. Interestingly, APAP *per se* had no significant effect on hepatic Bcl2 expression, whereas pretreatment with piperine before the APAP challenge caused a significant increase in the level of this protein when compared to either the control or the APAP groups (Figure 4).

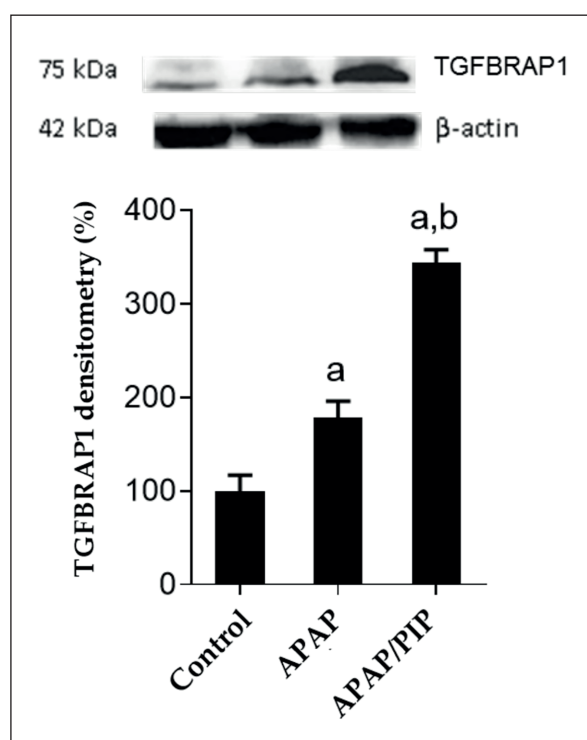### Effect of Piperine on Liver Expression of TGFBRAP1

Hepatic expression of TGFBRAP1, a regulator of the cellular stress response, was evaluated using Western blotting (Figure 5). Samples from the APAP group showed significantly increased protein levels of TGFBRAP1 compared to the control animals. Interestingly, pretreatment with piperine before induction of hepatotoxicity by APAP caused a further increase in liver TGF-BRAP1 protein expression, which was significantly higher than the effect of APAP alone.

Discussion

Drug-induced acute liver failure, of which APAP is the most frequent culprit, is one of the major health hazards whose prevention possesses a challenge. The present study aimed to test the protective effects of piperine against APAP-induced acute liver injury. In the current study, APAP overdose induced deterioration of the liver function, which was manifested as increased serum ALT activity, as well as deteriorated liver histological architecture. Besides, APAP
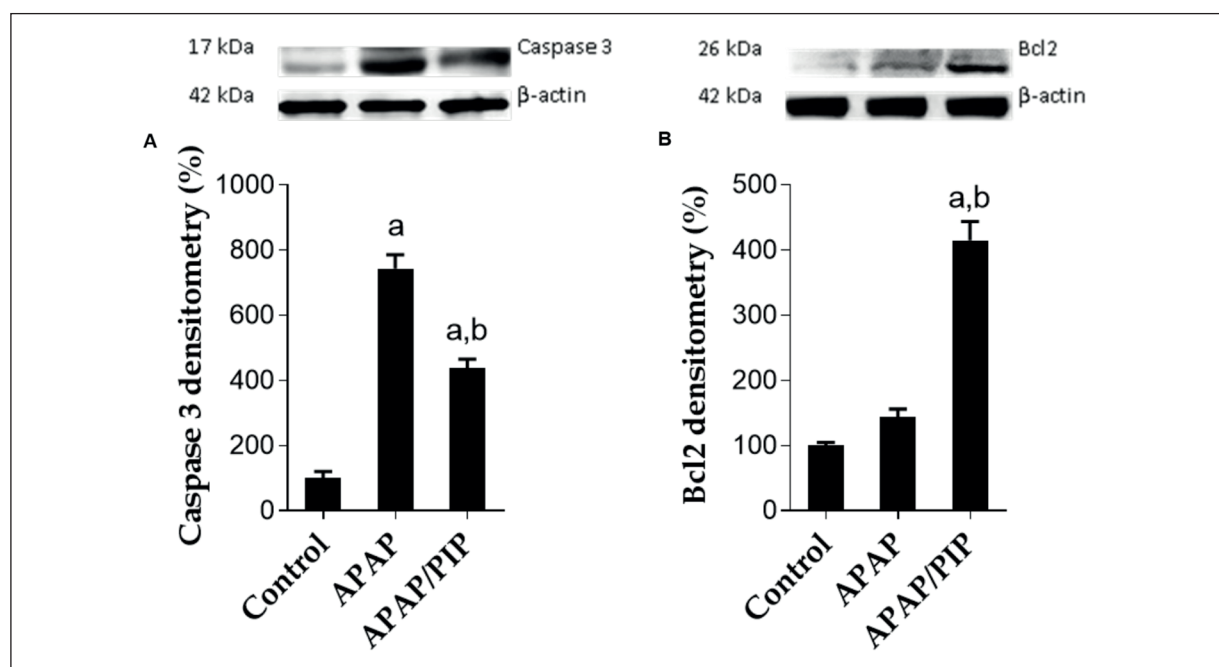
Figure 4. Effect of piperine (PIP) on the hepatic expression of caspase 3 and B-cell lymphoma 2 (Bcl2) in acetaminophen (APAP)-induced hepatotoxicity in mice. Upper panels; Western blot bands of (**A**) caspase 3 and (**B**) Bcl2, lower panels show their densitometric protein expression as a ratio of their corresponding β-actin, represented as a percentage of control. Results are a representation of means ± SEM. Results are significantly different if *p*<0.05. "a" significantly different from the control group; "b" significantly different from the APAP group.

Figure 5. Effect of piperine (PIP) on the hepatic expression of transforming growth factor-β receptor-associated binding protein 1 (TGFBRAP1) in acetaminophen (APAP)-induced hepatotoxicity in mice. Upper panel; Western blot bands of TGFBRAP1, lower panel shows the densitometric protein expression as a ratio of the corresponding β-actin, represented as a percentage of control. Results are a representation of means ± SEM. Results are significantly different if $p < 0.05$. "a" significantly different from the control group; "b" significantly different from the APAP group.

attenuated the endogenous antioxidant defense mechanisms and augmented the hepatic tissue inflammation; both factors may contribute to the observed increase in apoptosis-related signaling and cell death. On the other hand, the results of this study showed that piperine-treated animals were protected against APAP-induced liver toxicity.

Our results showed improved liver function in the piperine pretreatment group in response to APAP-induced toxicity, which is in line with previous studies[19,23]. Piperine successfully restored liver function parameters in hyperlipidemia-induced hepatic steatosis in rats[23], as well as in APAP-induced toxicity[19]. Similarly, the hepatic morphological injury as induced by APAP overdose was almost completely normalized in mice receiving piperine, with decreased vascular congestion, cellular infiltration, as well as amelioration of hepatocellular necrosis, fat deposition,

and pyknosis. Similar improvements of hepatic histopathology were noted in a previous report on APAP-induced liver injury[19], as well as a cholesterol-induced model of steatosis and gallbladder stone formation[24].

The overdose of APAP is the leading cause of acute liver failure in clinical practice[2]. Moreover, induction of oxidative stress and increased reactive oxygen species formation, after reduced glutathione depletion and diminished endogenous antioxidation mechanisms represent the mainstay of APAP-induced liver injury[4]. As expected, the results of the current study showed that APAP-treated mice suffered increased levels of hepatic tissue oxidative stress, as depicted by the decrease in the activity of two major antioxidant enzymes; SOD and GR. This effect was reversed by the administration of piperine before the induction of hepatotoxicity. The antioxidant effects of piperine are well-established in different models of cellular injury either *in vitro* or *in vivo*, including models of hepatic injury[21,25,26]. The upregulation of antioxidant enzymes was suggested as a mechanism delineating the hepatoprotective effects of piperine. The alleviation of the hepatotoxic effects of APAP[19], microcystin[25], high-fat feeding[23,26] as well as cholesterol-induced gallstone formation[27] by piperine treatment was mediated, at least in part, *via* increasing the hepatic antioxidant enzyme capacity, notably the activity of SOD and GR.

To further identify the mechanisms by which piperine would increase the expression of antioxidant enzymes such as SOD and GR, we tested its effect on the expression of Nrf2 in liver tissue. Nrf2 is a major redox switch and a transcriptional activator of genes encoding endogenous antioxidant enzymes, which regulate hepatocellular responses to increased oxidative stress[28,29]. Not surprisingly, Nrf2 is currently considered an important target for the management of redox- and inflammation-related disease conditions[30]. Interestingly, the APAP-treated mice did not show appreciable changes in hepatic Nrf2 protein expression. Importantly, cytoplasmic Nrf2 is required to translocate into the nucleus to interact with its target promotor region, known as the antioxidant response element[30]. In contrast with the results of the current work, Goldring et al[31] concluded that APAP treatment both dose- and time-dependently increased Nrf2 expression, as well as its nuclear translocation. However, Lv et al[32] showed that although APAP-treatment induces some enhancement in cytoplasmic Nrf2 levels,

its nuclear translocation is diminished. Goldring et al[31] illustrated that the peak induction of Nrf2 is 1 h after APAP administration. However, in the current study, the samples were collected 4 h after APAP treatment, which might explain our different results. Furthermore, Feng et al[33] have reported significantly less than normal levels of cytoplasmic, as well as nuclear Nrf2 in APAP-treated mice. Importantly, Chen et al[34] revealed that APAP-induced activation of c-Jun NH_2-terminal kinase (JNK), which in turn phosphorylates Nrf2 leading to its ubiquitination and proteasomal degradation, is an important mechanism of APAP-induced acute liver injury. Taken together, these results might explain the relatively unchanged levels of expression of Nrf2 in our study after APAP treatment, contrary to previous reports[31,32]. On the other hand, piperine pretreatment in the current study significantly enhanced the expression Nrf2. Evidence illustrating the positive impact of increased cellular expression of Nrf2 against cellular toxicity and inflammation from previous studies[35-37] provides further support to the findings of the current study. In particular, the piperine-mediated increase in hepatic Nrf2 expression in our study is supported by previous studies demonstrating the enhanced expression of Nrf2 *in vitro* in lipopolysaccharide-induced inflammation in BV2 microglia[35] or RAW264.7 cells[36], and in cisplatin-challenged auditory cells[37], as well as in an *in vivo* model of lysolecithin-induced hippocampal demyelination[38].

In the present study, APAP-induced hepatotoxicity was accompanied by an enhanced inflammatory response as illustrated by the increased expression of the NF-κB p65 subunit, which was attenuated by piperine pretreatment. To our best knowledge, the effect of piperine on hepatic NF-κB p65 expression has not been previously investigated. Zhai et al[39] illustrated that piperine administration downregulates of NF-κB p65, which was supported by inhibition of NF-κB dependent signaling pathways in a model of endometritis. Furthermore, Verma et al[40] explained such inhibition by molecular docking studies showing that piperine inhibits NF-κB p65 by binding to its active site, which prevents its activation and nuclear translocation.

Administration of a high dose of APAP in the current study induced apoptotic changes in liver cells as indicated by the imbalance between the expression of the pro- and the anti-apoptotic proteins caspase 3 and Bcl2, respectively, which is supported by the findings of a previous study[32].

Pretreatment of the mice for 3 days with piperine in the current study mitigated the APAP-induced increase of hepatic caspase 3 expression and inhibition of Bcl2. To the best of our knowledge, these anti-apoptotic effects of piperine on hepatic caspase 3 and Bcl2 have not been previously addressed. However, in other tissues, the anti-apoptotic effects of piperine on caspase 3 and/or Bcl2 were illustrated[41-44]. In conditions characterized by increased oxidative stress, inflammation, and apoptosis, such as experimentally-induced Parkinson's disease[44], ischemia/reperfusion injury[41,43], and epilepsy[42], investigations demonstrated the ability of piperine to inhibit apoptosis in rat brain cells *via* activation of Bcl2 and downregulation of caspase 3. Moreover, the ability of piperine to suppress UV radiation-induced apoptosis in human keratinocytes, which was mediated through inhibition of NF-κB signaling and suppression of cellular apoptosis, provides further support to our results. Interestingly, in contrast to the results presented here, piperine treatment in cancer cell culture systems *in vitro* proved to be pro-apoptotic in ovarian[45,46] and cervical[47] cancer cells *via* activation of different caspases, including caspase 3, as well as other pro-apoptotic signals. Similar effects were observed in human oral squamous cell carcinoma[48] and melanoma[49] cells, where both caspase 3 and Bcl2 were involved. Although these findings are contradictory to the results of the present work, the differences in the experimental settings, as well as the duration of treatment, could both be important factors behind these discrepancies, not to mention that all the above studies were carried out using cancer cell line models with different signaling milieu. For example, most of these studies were carried out either *in vitro*[45-49] or the piperine administration *in vivo* was continued for a much longer duration[49], as opposed to the *in vivo* settings or the relatively shorter exposure time in the current study, respectively.

The results of the current research showed, for the first time, that APAP treatment increased the hepatic expression of TGFBRAP1, probably as a feedback mechanism. Moreover, the pretreatment by piperine of the APAP-challenged mice further increased the liver expression of TGFBRAP1 to nearly 2 folds compared to APAP-treated mice, suggesting a role of this protein in piperine-mediated hepatoprotection. TGFBRAP1 regulates TGF-β-mediated signaling[9-11]. Importantly, TGFBRAP1 is critical for early endosome formation and activation of degradative pathways[50],

which in turn regulates autophagy[51]. Meanwhile, autophagy plays a critical role in counteracting inflammation- and oxidative stress-induced cellular apoptosis/death during APAP-induced liver toxicity[5,52]. Inhibition of autophagy using 3-methyladenine or chloroquine as pharmacological tools resulted in exaggerated hepatotoxicity after the APAP challenge, while activation of this mechanism *via* mTOR inhibition was protective[52]. Thus, increased autophagy might play a role in mitigating APAP-induced acute liver injury. Moreover, humans suffering from non-alcoholic fatty liver disease, a condition characterized by inhibition of autophagy[53], are more susceptible to APAP-induced hepatotoxicity[54]. On the other hand, the flavonoid chrysin was shown to protect against APAP-induced toxicity *via* mechanisms involving the induction of autophagy and the inhibition of apoptosis[6]. Nevertheless, piperine was able to induce autophagy in different systems[55,56]. Thus, induction of autophagy, which might involve activation of TGFBRAP1, could indeed be involved in piperine-mediated hepatoprotection observed in the current work. However, further studies are warranted to unravel the dose- and time-dependent effects of piperine on the expression and function of this protein, as well as exploring other mechanistic parameters involved in the APAP/TGFβ/TGFBRAP1 signaling axis, and its modulation by piperine administration. The results of such investigations should further clarify the causal-effect relationship among these signaling pathways in piperine-mediated hepatoprotection against APAP-induced hepatotoxicity.

The major liver microsomal enzyme involved in APAP-induced toxicity is CYP2E1. Interestingly, the effect of piperine on the activity of CYP2E1 is controversial[54]. Kang et al[57] suggested that piperine inhibits CYP2E1 in the rat liver. Besides, Bedada and Boga[58] attributed the piperine-induced enhancement of chlorzoxazone bioavailability in human volunteers to its CYP2E1 inhibitory effects. Notwithstanding, the results of an *in vitro* study showed that piperine selectively inhibited CYP3A4 (IC_{50} = 5.5 μM), while it was a much less potent inhibitor of CYP2E1 (IC_{50} > 50 μM)[59]. The same research group later showed that piperine did not clinically alter APAP pharmacokinetics in healthy volunteers[60]. Thus, based on these studies, whether the hepatoprotective effects of piperine are mediated via modulation of APAP pharmacokinetics cannot be ruled out.

Conclusions

Summarily, piperine pretreatment conferred protection to the liver against APAP-induced toxicity. This study showed for the first time that piperine confers hepatic anti-inflammatory, as well as anti-apoptotic effects, *via* decreasing the expression of the NF-κB p65 and modulation of hepatic caspase 3 and Bcl2, respectively. Importantly, the results of the current study introduced TGFBRAP1 as a novel potential target for the management of APAP-induced hepatotoxicity. In conclusion, the hepatoprotective effects of piperine against APAP-induced acute liver injury include the induction of endogenous antioxidant pathways, mitigation of inflammation and apoptosis, and possibly the regulation of TGFBRAP1.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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