Free Radical Research, March 2010; 44(3): 340–355

informa
healthcare

Acetaminophen induced acute liver failure via oxidative stress and JNK activation: Protective role of taurine by the suppression of cytochrome P450 2E1

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(Received 16 September 2009; In revised from 28 October 2009)

Abstract

The present study was carried out to investigate whether taurine plays any beneficial role in acetaminophen (APAP)-induced acute hepatotoxicity. APAP exposure increased the plasma levels of ALT, AST, LDH, TNF-*α* and NO production. Moreover, APAP treatment reduced the glutathione level and antioxidant enzyme activities, increased lipid peroxidation and caused hepatic DNA fragmentation which ultimately leads to cellular necrosis. Also, incubation of hepatocytes with APAP reduced cell viability, enhanced ROS generation and increased CYP2E1 activity. APAP overdose caused injury in the hepatic tissue and hepatocytes via the upregulation of CYP2E1 and JNK. Taurine treatment was effective in counteracting APAP-induced hepatic damages, oxidative stress and cellular necrosis. Results indicate that APAP overdose caused hepatic injury due to its metabolism to hepatotoxic NAPQI (N-acetyl-p-benzoquinone imine), usually catalysed by CYP2E1, and via the direct activation of JNK-dependent cell death pathway. Taurine possesses prophylactic as well as therapeutic potentials against APAP-induced hepatic injury.

Keywords: *Acetaminophen, hepatic, oxidative stress, reactive oxygen species, necrosis, taurine, antioxidant*

Abbreviations: *ALT, alanine aminotransferase; ALP, alkaline phosphatise; CAT, catalase; FRAP, Ferric Reducing/ Antioxidant Power; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; GPx, glutathione peroxidase; GR, glutathione reductase; GDH, lactate dehydrogenase; MDA, malonaldehyde; NAPQI, N-acetyl-p-benzoquinone imine; MAPK, mitogen-activated protein kinases; ROS, reactive oxygen species; APAP, acetaminophen; SOD, superoxide dismutase; TNF-α, tumour necrosis factor-α.*

Introduction

Acetaminophen is a frequently used effective analgesic and anti-pyretic agent for the relief of mild and moderate pain. However, APAP overdose produces severe liver and kidney damage in humans and animals [1,2]. Therefore, APAP-induced acute toxicity has become an essential model for studying drug-induced liver and kidney failure. The toxicity is initiated by P450-mediated reactions that convert APAP to the reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), causing glutathione depletion and covalent binding to hepatic parenchymal cell proteins and DNA with resultant liver injury [3]. Apart from metabolic activation of APAP and NAPQI binding to target proteins and DNA, generation of reactive oxygen and nitrogen species, lipid peroxidation, mitochondrial dysfunction, disruption of calcium homeostasis and induction of necrosis are also involved in APAP-induced hepatotoxicity [4].

Therefore, it is important to find out protective molecules that would give maximum protection of the liver during APAP-induced hepatic damage and practically very little or no side-effects would be exerted during their function in the body. Taurine is

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2010 Informa UK Ltd. (Informa Healthcare, Taylor & Francis AS)
DOI: 10.3109/10715760903513017

a conditionally essential amino acid and possesses a number of cytoprotective properties as an antioxidant, osmoregulator and intracellular calcium flux regulator [5]. Taurine (end product of L-cysteine metabolism) is the most abundant free amino acid in many tissues. We have also previously reported that taurine could prevent the damage of several body's organ from oxidative stress induced by environmental toxicants and effectively reduced GSH depletion, lipid peroxidation, DNA fragmentation, generation of reactive oxygen species, mitochondrial damage and cell death (both apoptotic and necrotic) [6–10]. However, very little is known about the molecular mechanisms for the protective role of taurine against drug induced (APAP-induced) hepatic damage.

The present study has, therefore, been designed and conducted to investigate the mechanisms underlying the protective action of taurine in APAP-induced hepatic pathophysiology using both *in vivo* and *in vitro* working models. APAP-induced liver damage was assessed by measuring serum enzymes (ALT, ALP and LDH) leakages; nitric oxide (NO) level (as a measure of reactive nitrogen species generation [11]); level of the cytokine, tumour necrosis factor-α (TNF-*a*), known to involve in inflammatory responses [12]; lipid peroxidation, induced by oxidative stress and occuring readily in the tissues rich in highly oxidizable polyunsaturated fatty acids [13]; levels of cellular metabolites such as GSH and GSSG, as NAPQI depletes cytosolic and mitochondrial GSH that triggers the loss of cellular homeostasis leading to liver injury [14]; and *in vivo* antioxidant power and activities of antioxidant enzymes in the hepatic tissue of rats exposed to APAP alone or taurine pre- and post-treated animals. Moreover, the cell viability and reactive oxygen species (ROS) generation were also examined in APAP and APAP+taurine treated hepatocytes.

To investigate the mode of cell death in APAP-induced hepatotoxicity, we used both the DNA fragmentation and flow cytometric analyses. The protective role of taurine against APAP-induced hepatic injury was further investigated by assessing (1) MAPKs (including JNK and p38) signalling pathways, as these could be stimulated by a diverse range of extracellular and intracellular stimuli including reactive oxygen species (ROS), environmental stress, ultraviolet radiation and pro-inflammatory cytokines, heat and osmotic shock [15,16]; ERK has also been found to be involved in cell proliferation and survival [17] and (2) CYP2E1 enzyme activity and protein levels, because the most relevant isoenzyme among all P450 enzymes is CYP2E1 that can metabolize APAP [18].

To further analyse whether apoptosis may co-exist with necrosis in APAP-induced hepatotoxicity, we investigated the possible role of Bcl 2 proteins (Bcl 2 and Bcl Xl) and caspase 3 under this pathophysiological condition.

Materials and methods

Chemicals

Taurine (2-aminoethane sulphonic acid), acetaminophen, anti-JNK, anti-p38, anti-ERK, anti-Bcl 2, anti-Bcl XL, anti-caspase 3 and anti-CYP2E1 antibodies were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

Animals

Swiss albino male rats of 4 weeks weighing ~120–130 g and adult albino mice of Swiss strain, weighing between 20–25 g were purchased from M/S Gosh Enterprises (Kolkota, India). Animals were acclimatized under laboratory conditions for 2 weeks prior to experiments. All the experiments with animals were carried out according to the guidelines of the institutional animal ethical committee.

Experimental design for in vivo *treatments*

Experimental design needed for the present *in vivo* study has been summarized as follows: 6-week old rats were randomly assigned to five groups. Rats in the 'Normal group' received only water as vehicle. Rats in the 'APAP treated group' received acetaminophen (APAP) orally at a single dose of 500 mg/kg body weight in distilled water for 16 h. Rats in the 'Taurine pre-treated group' received taurine orally at a dose of 150 mg/kg body weight in distilled water for 3 days followed by APAP administration once at a dose of 500 mg/kg body weight in distilled water. Rats in the 'Taurine post-treated group' received APAP orally at a dose of 500 mg/kg body weight in distilled water once followed by taurine administration at a dose of 150 mg/kg body weight in distilled for 3 days. Rats in the 'SP600125 pre-treated group' were injected intraperitoneally (IP) with the specific JNK inhibitor, SP600125 (30 mg/kg) 1 h before APAP administration (500 mg/kg body weight in distilled water).

Determination of time-dependent effect of APAP by ALT assay

To find out the time needed for APAP induced acute damage in rat liver, experiments were carried out with seven groups of animals consisting of six animals in each group. The first group received water as vehicle and served as normal control. APAP was administered orally to the other six groups at a single dose of 500 mg/kg body weight for 4, 8, 12, 16, 20 and 24 h, respectively.

At selected times after APAP treatment, all rats were sacrificed. ALT levels were measured using serum of all different experimental rats.

Determination of dose and time-dependent activity of taurine by ALP assay

For this study, rats were randomly distributed into eight groups each consisting of six animals. The first two groups served as normal control (receiving only water as vehicle) and toxin control (receiving APAP at a single dose of 500 mg/kg body weight, orally), respectively. The remaining six groups of animals were treated with six different doses of taurine (25, 50, 75, 100, 150 and 200 mg/kg body weight for 3 days) followed by APAP intoxication (at a single dose of 500 mg/kg body weight, orally).

To determine the time-dependent effects of taurine for pre-treatment in APAP-dependent hepatic disorder, rats were divided into seven groups each consisting of six animals. The first two groups served as normal control (receiving only water as vehicle) and toxin control (treatment with APAP at a single dose of 500 mg/kg body, orally), respectively. The other five groups of animals were treated with taurine orally at a dose of 150 mg/kg body weight, once daily for 1, 2, 3, 4 and 5 days prior to APAP intoxication (at a single dose of 500 mg/kg body weight, orally).

Similarly, to determine the time-dependent effects of taurine for post-treatment studies in APAP-dependent hepatic disorder, rats were divided into eight groups each consisting of six animals. The first three groups served as normal control (receiving only water as vehicle), toxin control (treatment with APAP at a single dose of 500 mg/kg body, orally) and recovery (receiving APAP at a single dose of 500 mg/kg body, orally and normal diet for next 5 days), respectively. The other five groups of animals were treated with taurine orally at a dose of 150 mg/kg body weight, once daily for 1, 2, 3, 4 and 5 days after APAP administration (at a single dose of 500 mg/kg body weight, orally).

At selected times after APAP and taurine treatment, all rats were sacrificed. ALP levels were measured using serum of all experimental rats.

Measurement of APAP-induced cytotoxicity

Liver samples were homogenized (1:4, w/v) in ice-cold 0.1 M phosphate buffer (pH 7.4) containing 2 mM EDTA. The homogenate was centrifuged at 10,000 g for 30 min at 4°C. The supernatant was collected and further centrifuged at 105,000 g for 60 min. The resulting microsomal pellets were then suspended in a 0.25 mM sucrose solution containing 1 mM EDTA and stored at –80°C until use. The supernatant was collected and used for the experiments as cytosolic fraction. The protein contents of the cytosolic fraction and microsomal fraction were measured by the method of Bradford [19] using crystalline BSA as standard. Serum was stored at –80°C until use. Specific markers related to hepatic dysfunction, e.g. ALT and ALP levels in the sera, were estimated by using standard kits. LDH activity was determined according to the method of Kornberg [20]. The serum NO level was indirectly assessed by measuring the nitrite levels in plasma using a colourimetric method based on the Griess reaction [21]. The lipid peroxidation was estimated according to the method of Esterbauer and Cheeseman [22]. Cellular metabolites levels (GSH and GSSG), Ferric Reducing/Antioxidant Power (FRAP) and activities of antioxidant enzymes (SOD, CAT, GST, GR, GP_X) in the hepatic tissue were determined following the method described by Das et al. [7] and G6PD activity was measured by the method of Lee [23].

Detection of cell death pathway by flow cytometry and DNA fragmentation assay

Hepatocytes were isolated from APAP and taurine treated rats. The animals were anaesthetized, sacrificed and livers were collected. After collection, the organs were extensively perfused in phosphate buffer saline to get rid of blood and irrigated in a buffer containing Hepes (10 mM), KCl (3 mM), NaCl (130 mM), $NaH_2PO4–H2O$ (1 mM) and glucose (10 mM) pH 7.4 and incubated with a second buffer containing $CaCl_2$ (5 mM), 0.05% collagenase type I mixed with the buffer previously described for ~45 min at 37°C. The liver sample was then passed through a wide bore syringe, filtered, centrifuged and the pellet was suspended in PBS. Cells were washed with PBS, centrifuged at 800 g for 6 min, resuspended in ice-cold 70% ethanol/PBS, centrifuged at 800 g for a further 6 min and resuspended in PBS. Cells were then incubated with propidium iodide (PI) and FITC-labelled Annexin V for 30 min at 37°C. Excess PI and Annexin V were then washed off; cells were fixed and then stained cells were analysed by flow cytometry using FACS Calibur (Becton Dickinson, Mountain View, CA) equipped with 488 nm argon laser light source; 515 nm band pass filter for FITC-fluorescence and 623 nm band pass filter for PI-fluorescence using CellQuest software. A scatter plot of PI-fluorescence (*y*-axis) vs FITC- fluorescence (*x*-axis) has been prepared.

The DNA fragmentation has also been assayed by electrophoresing genomic DNA samples, isolated from normal as well as experimental rat liver, on agarose/EtBr gel by the procedure described by Sellins and Cohen [24].

Isolation of primary mouse hepatocyte

Hepatocytes were isolated from mice liver following the method of Das et al. [25] with some modifications. The animals were anaesthetized, sacrificed and

livers were collected. After collection, the organs were extensively perfused in phosphate buffer saline to get rid of blood and irrigated in a buffer containing Hepes (10 mM), KCl (3 mM), NaCl (130 mM), $NaH_2PO4–H2O$ (1 mM) and glucose (10 mM) pH 7.4 and incubated with a second buffer containing $CaCl_2$ (5 mM), 0.05% collagenase type I mixed with the buffer previously described for ~45 min at 37°C. The liver sample was then passed through a wide bore syringe, filtered, centrifuged and the pellet was suspended in DMEM containing 10% FBS and the suspension was adjusted to obtain ~2×10^6 cells/ml. Hepatocytes were then treated with APAP (8 mM), taurine (6 mM) in combination (either 1 h before or 1 h after) with APAP and SP600125 (30 μM) 15 min before APAP exposure and incubated at 37°C for 6 h for further molecular and biochemical analyses.

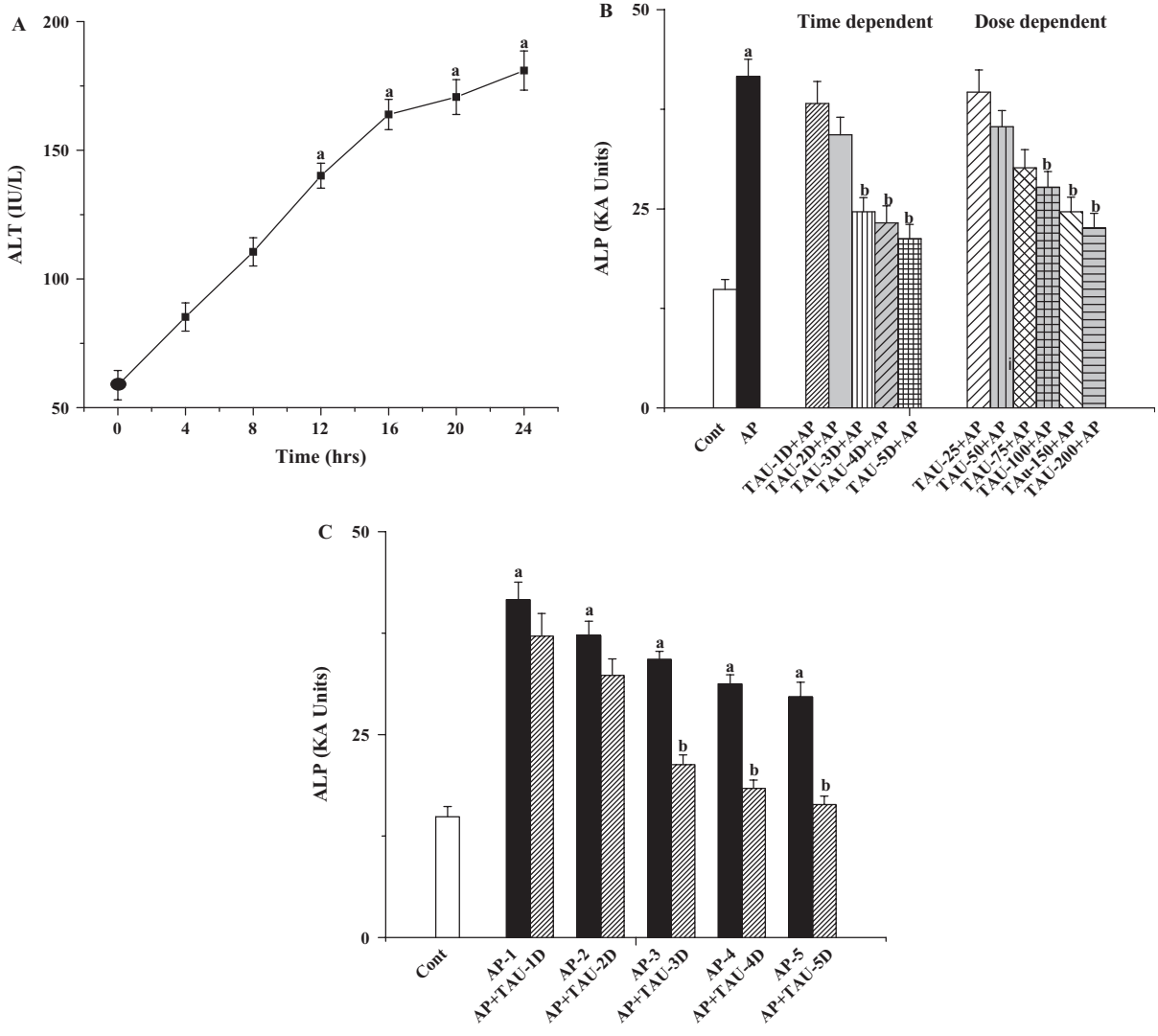


Figure 1. (A) Time-dependent effect of APAP on serum ALT level. Closed circle: ALT level in normal rats, Closed square: ALT level in APAP intoxicated rats (500 mg/kg body weight, orally) for 4, 8, 12, 16, 20 and 24 h, respectively. Each column represents mean ± SD, $n = 6$. [a]The significant difference between the normal control and APAP intoxicated groups ($p^a < 0.05$). (B) Dose and time-dependent effect of taurine on ALP level in APAP induced toxicity in the serum of the experimental rats. Cont: ALP level in normal rats, AP: ALP level in APAP intoxicated rats, TAU-25+AP, TAU-50+AP, TAU-75+AP, TAU-100+AP, TAU-150+AP, TAU-200+AP: ALP level in taurine (TAU) treated rats for 3 days at a dose of 25, 50, 75, 100, 150 and 200 mg/kg body weight prior to APAP administration; TAU-1D+AP, TAU-2D+AP, TAU-3D+AP, TAU-4D+AP, TAU-5D+AP: ALP level in taurine (TAU) treated rats for 1, 2, 3, 4 and 5 days, respectively, at a dose of 150 mg/kg body weight prior to APAP administration. Each column represents mean ± SD, $n = 6$. [a]The significant difference between the normal control and APAP intoxicated groups and [b]the significant difference between the APAP intoxicated and taurine pre-treated groups ($p^a < 0.05$, $p^b < 0.05$). (C) Time-dependent effect of taurine on ALP level against APAP induced toxicity in the serum of the experimental rats. Cont: ALP level in normal rats, AP-1, AP-2, AP-3, AP-4, AP-5: ALP level in APAP intoxicated rats after 1, 2, 3, 4 and 5 days, respectively, AP+TAU-1D, AP+TAU-2D, AP+TAU-3D, AP+TAU-4D, AP+TAU-5D: ALP level in taurine (TAU) treated rats for 1, 2, 3, 4 and 5 days, respectively, at a dose of 150 mg/kg body weight after APAP administration. Each column represents mean ± SD, $n = 6$. [a]The significant difference between the normal control and APAP intoxicated groups and [b]the significant difference between taurine post-treated and recovery groups ($p^a < 0.05$, $p^b < 0.05$).

Table I. Effect of APAP and taurine on the levels of the serum markers related to hepatic dysfunction in the experimental rats.

Name of the parameters	Normal Control	APAP	TAU+APAP	APAP+TAU	SP600125+APAP
ALT (IU/L)	58.76 ± 1.91	163.91 ± 7.86[a]	116.46 ± 4.21[b]	113.05 ± 4.23[b]	127.05 ± 4.43[b]
ALP (KA units)	14.89 ± 0.52	41.62 ± 1.75[a]	24.64 ± 1.03[b]	21.29 ± 0.87[b]	28.23 ± 0.97[b]
LDH (U/L)	235.14 ± 8.78	1381.66 ± 30.69[a]	578.13 ± 14.98[b]	546.57 ± 15.69[b]	613.53 ± 15.29[b]
NO production (mM)	46.14 ± 1.68	157.37 ± 6.21[a]	92.64 ± 3.28[b]	89.37 ± 3.63[b]	108.31 ± 3.53[b]

Values are expressed as mean ± SD, for six animals in each groups. [a]values differs significantly from normal control ($p^a < 0.05$); [b]values indicate the significant difference between APAP and either TAU or SP600125 treated group ($p^b < 0.05$).

Cell viability assessment

About 2×10^6 hepatocytes isolated from mice liver were incubated with APAP either alone or after taurine administration. After incubation, the media was removed and the hepatocytes were washed twice with phosphate buffered saline. Cell viability was assessed by MTT assay following the method of Madesh and Balasubramanian [26].

Measurement of intracellular ROS production

Briefly, hepatocytes isolated from mice liver were incubated with DCF-DA (10 mM) for 1 h at 37°C in the dark. After treatment, the cells were immediately washed and resuspended in PBS. Intracellular ROS production was detected using the fluorescent intensity of the oxidant sensitive probe 2,7dichlorodihydrofluorescein diacetate (H_2DCFDA) in a fluorescence microscope.

Measurement of CYP2E1 activity

CYP2E1 activity was determined according to the method of Ito et al. [27]. In brief, hepatocytes isolated from mice liver were treated with taurine (6 mM), APAP (8 mM) and taurine (6 mM) in combination (1 h before) with APAP and incubated at 37°C for 6 h. After incubation, hepatocytes were washed twice, again incubated with 0.5 mM *p*-nitrophenol in Krebs-Henseleit buffer containing 200 mg/L glucose at 37°C for 60 min and the reaction was terminated by adding trichloroacetic acid to a final concentration of 5% (w/v). Cells were centrifuged at 10,000 *g* for 10 min and the supernatants were assayed for 4-nitrocatechol by adding 10 M NaOH (1:10) and immediately determining the absorbance at 546 nm.

Immunoblotting

For immunoblotting, cytosolic samples containing 50 μg proteins and microsomal fraction containing 10 μg protein were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked at room temperature for 2 h in blocking buffer containing 5% non-fat dry milk to prevent non-specific binding and then incubated with anti-p38 (1:1000 dilution), anti-JNK (1:1000 dilution), anti-ERK (1:1000 dilution), anti-Bcl 2(1:1000 dilution), anti-Bcl xL (1:1000 dilution) and caspase 3 (1:1000 dilution) antibodies for the cytosolic proteins and anti-CYP2E1 (1:1000 dilution) primary antibody for the microsomal proteins obtained from liver tissues separately at 4°C overnight. The membranes were washed in TBST (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) for 30 min and incubated with appropriate HRP conjugated secondary antibody (1:2000 dilution) for 2 h at room temperature and developed by the HRP substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB) system (Bangalore, India).

Determination of serum TNF-α

TNF-α levels were determined from sera of normal and experimental animals using sandwich ELISA following the method of Ausubel et al. [28].

Table II. Effect of APAP and taurine on lipid peroxidation, status of the thiol based antioxidants and Ferric reducing antioxidant power in the experimental rats.

Name of the parameters	Normal Control	APAP	TAU+APAP	APAP+TAU
MDA (nmol/mg protein)	2.41 ± 0.08	6.02 ± 0.21[a]	2.92 ± 0.09[b]	2.84 ± 0.11[b]
GSH (nmol/mg protein)	24.64 ± 1.03	13.82 ± 0.43[a]	20.59 ± 0.82[b]	21.96 ± 0.86[b]
Redox ratio (GSH/GSSG)	31.18 ± 1.12	7.19 ± 0.31	25.58 ± 0.97[b]	26.84 ± 0.94[b]
Ferric reducing antioxidant power (% over control)	100 ± 3.52	47.35 ± 2.02[a]	81.26 ± 3.15[b]	85.32 ± 3.21[b]

Values are expressed as mean ± SD, for six animals in each groups. [a]values differs significantly from normal control ($p^a < 0.05$); [b]values indicate the significant difference between APAP and TAU-treated group ($p^b < 0.05$).

Table III. Effect of APAP and taurine on the activities of the antioxidant enzymes.

Name of antioxidant enzymes	Normal Control	APAP	TAU+APAP	APAP+TAU
SOD (Unit/mg protein)	179.71 ± 5.89	130.29 ± 6.23[a]	164.49 ± 7.38[b]	172.49 ± 7.45[b]
CAT (μmol/min/mg protein)	131.11 ± 4.55	63.89 ± 2.89[a]	115.98 ± 4.49[b]	118.48 ± 4.52[b]
GST (μmol/min/mgprotein)	3.19 ± 0.09	1.13 ± 0.05[a]	2.27 ± 0.06[b]	2.42 ± 0.07[b]
GR (nmol/min/mgprotein)	118.19 ± 4.53	58.42 ± 2.68[a]	98.29 ± 4.08[b]	100.94 ± 4.15[b]
GPx (nmol/min/mg protein)	127.95 ± 5.83	59.02 ± 2.29[a]	115.49 ± 4.13[b]	118.03 ± 4.14[b]
G6PD (nmol/min/mg protein)	252.71 ± 8.28	127.86 ± 6.04[a]	234.66 ± 9.37[b]	240.28 ± 9.41[b]

Values are expressed as mean ± SD, for six animals in each groups. [a]values differs significantly from normal control ($p^a < 0.05$); [b]values indicate the significant difference between APAP and TAU-treated group ($p^b < 0.05$).

Hepatic taurine level detection

Hepatic taurine level was measured according to the method of Ferreira et al. [29]. In brief, sulphosalicylic acid solution was added to the homogenate and allowed to stand for 10 min. Then it was filtered through W42 paper and derivatized with o-phthalaldehyde and 2-mercaptcethanol. The derivative was then analysed by HPLC using an UV absorbance detector at 350 nm.

Histological studies

Livers from the normal and experimental rats were fixed in 10% buffered formalin and were processed for paraffin sectioning. Sections of ~5 μm thickness were stained with haematoxylin and eosin to evaluate under a light microscope.

Statistical analysis

All the values are expressed as mean ± SD ($n=6$). Significant differences between the groups were determined with SPSS 10.0 software (SPSS Inc., Chicago, IL) for Windows using one-way analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). A difference was considered significant at the $p < 0.05$ level.

Results

Time-dependent effect of APAP by ALT assay

To determine the time-dependent APAP-induced hepatic damage, we carried out a time-dependent study using ALT assay as an index of that damage. As evidenced from Figure 1A, in APAP treated animals (at a single dose of 500 mg/kg body weight), maximum ALT activity in plasma was reached after 16 h with no significant change up to 24 h. This time was, therefore, chosen as for APAP-induced hepatic damage throughout the study.

Dose and time-dependent study of taurine by ALP assay

ALP assay was used to determine the optimum dose and time necessary for taurine for the protection of rat liver against APAP-induced oxidative damages. Experimental results suggest that APAP exposure increased the ALP level and that could be prevented by the pre-treatment with taurine linearly up to a dose of 150 mg/kg body weight for 3 days (Figure 1B). Similarly, post-treatment with taurine at a dose of 150 mg/kg body weight for 3 days after APAP administration also decreased the elevated level of ALP (Figure 1C). This dose and time were, therefore, chosen as the optimum dose and time for taurine treatment throughout the study.

Cytotoxic effect of APAP and protection by taurine

As expected, a single oral dose of APAP (500 mg/kg body weight) showed significant hepatotoxicity, as evidenced by a dramatic elevation of ALT, ALP and LDH leakage (Table I). NO level was also increased in APAP-treated animals (Table I). Pre-treatment with taurine significantly decreased the serum ALT, ALP, LDH and NO levels compared to those of the APAP administered groups. Furthermore, significant reduction in serum ALT, ALP, LDH and NO levels were also observed in taurine post-treated groups. We have also observed that treatment with JNK inhibitor (SP600125) could reduce all these serum markers related to hepatic dysfunction.

Lipid peroxidation indicates cellular injury mediated by reactive oxygen intermediates with the resultant destruction of membrane lipids and production of lipid peroxides. The present study showed that APAP significantly enhanced hepatic lipid peroxidation in the rats, whereas taurine administration before and after acetaminophen exposure significantly inhibited hepatic lipid peroxidation (Table II).

GSH plays an important role in detoxification of the acetaminophen metabolite NAPQI. We found that APAP administration resulted in rapid depletion of hepatic GSH, therefore decreasing the GSH/GSSG ratio (Table II). However, the redox ratio was significantly increased in both taurine pre-treated and post-treated groups.

APAP caused a significant reduction in FRAP value (Table II) and activities of antioxidant enzymes (CAT, GST, GPx, GR, SOD and G6PD) (Table III). Treatment with taurine both prior to and post-APAP

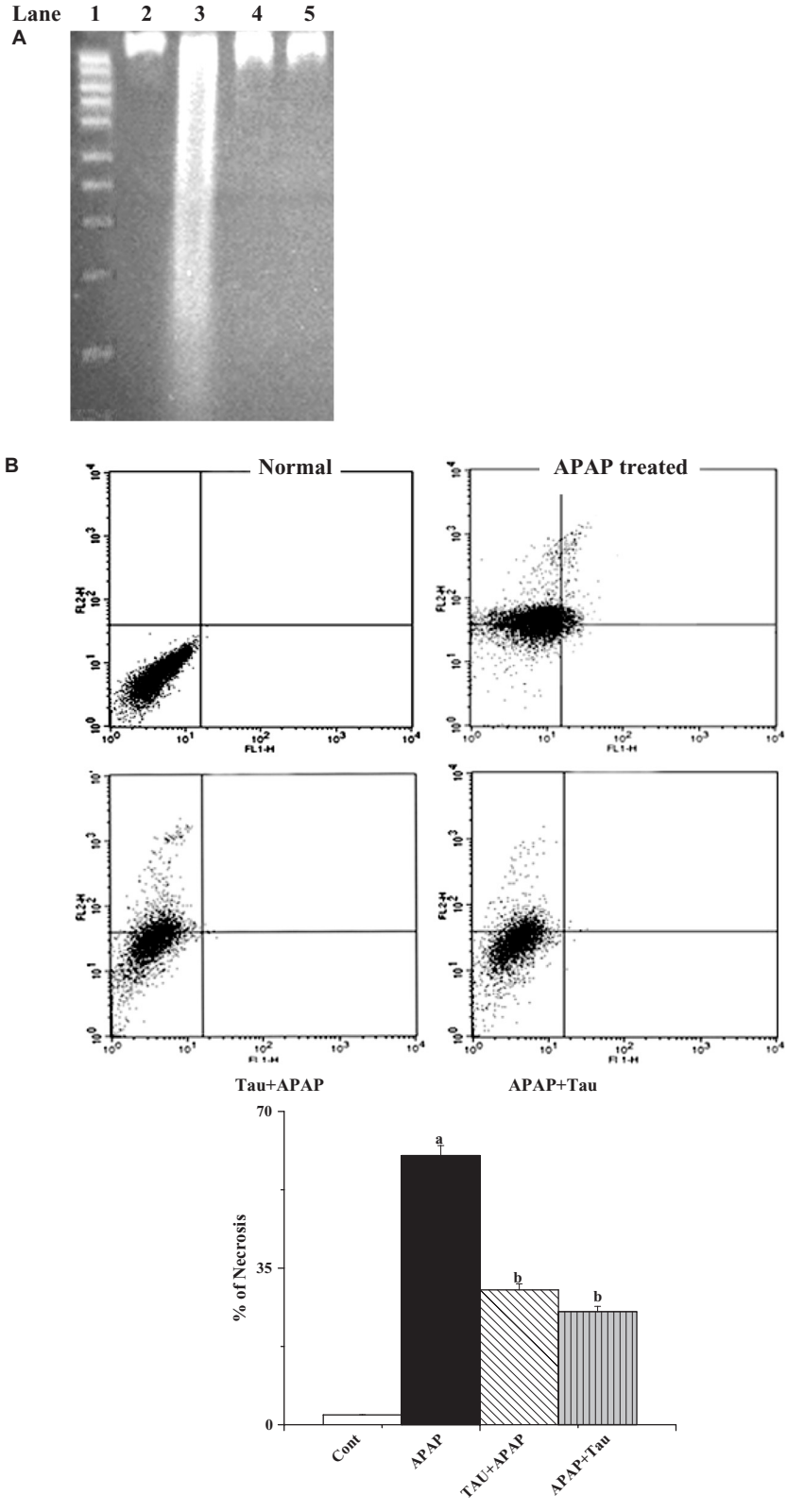


Figure 2. Detection of the mode of cell death in APAP intoxicated rats in the absence (APAP) and presence of taurine (TAU+APAP and APAP+TAU). (A) DNA fragmentation on agarose/EtBr gel. DNA isolated from experimental liver tissues was loaded onto 1% (w/v) agarose gels. Lane 1: Marker (1 kb DNA ladder); Lane 2: DNA isolated from normal liver; Lane 3: DNA isolated from APAP intoxicated liver; Lane 4: DNA isolated from taurine pre-treated liver; Lane 5: DNA isolated from taurine post-treated liver samples. (B) Percentage distribution of apoptotic and necrotic cells. Cell distribution analysed using Annexin V binding and PI uptake. The FITC and PI fluorescence measured using flow cytometry with FL-1 and FL-2 filters, respectively. Results expressed as scatter plot representing as one of the six independent experiments. The measurements were made six times. The bottom panel represents the corresponding bar diagram of percentage distribution of necrotic cells.

administration increased the antioxidant power and the activities of antioxidant enzymes.

APAP-induced necrosis of hepatocytes: Modulation by taurine

To investigate the mode of hepatocytes death during APAP overdose, we used fluorescence-activated cell-sorting (FACS) and DNA gel electrophoresis techniques to detect the occurrences of necrosis and apoptosis in the liver. DNA isolated from APAP-treated rats showed large amounts of smearing (a hallmark of necrosis) on the agarose gel (Figure 2A). Similarly, from flow-cytometric data we found that, in comparison to control untreated hepatocytes, hepatocytes isolated from APAP-treated rats showed maximum PI staining (60%), but very little Annexin V-FITC-binding, indicating the majority of cells underwent necrosis (Figure 2B). However, administration of taurine prior to and after APAP administration showed a considerable improvement in hepatocytes viability.

Effect of taurine on APAP-exposed primary mouse hepatocytes

The present study showed that APAP caused loss in cell (hepatocytes) viability. In order to investigate whether this loss in cell (hepatocytes) viability could be prevented by taurine-treatment, MTT assay has been performed. Figure 3A shows that with increasing APAP concentration, cell viability decreased. Optimum reduction in cell viability was observed at a concentration of 8 mM, after that cell viability did not change much even with increasing APAP concentration. APAP (alone) treated hepatocytes had a viability of only 48%; however, taurine treatment gradually increased the viability in a dose-dependent manner and when treated with 6 mM (optimum concentration) (Figure 3B), cell viability was increased to 86%.

In the present study we measured CYP2E1 activity in terms of catalytic activity of *p*-nitrophenol in APAP-treated hepatocytes because CYP2E1 is the most active among the CYP450 enzymes in catalysing the metabolism of acetaminophen to hepatotoxic NAPQI. Figures 3C and D show that APAP-exposure increased the CYP2E1 activity in hepatocytes in both dose- and time-dependent manner. Taurine treatment, however, could effectively reduce the CYP2E1 activity in APAP-exposed hepatocytes.

Reactive oxygen species (ROS) mediates oxidative damage and can attack lipid membranes, proteins and DNA, which in turn disrupt cellular function and integrity. Therefore, we measured the degree of ROS production in hepatocytes and found that incubation of the cells with APAP significantly increased the ROS production (Figure 3E), although both taurine and SP600125 treatment could prevent it.

Effect of taurine on APAP-induced phosphorylation of JNK, p38 and activation of CYP2E1

The most thoroughly studied signal transduction pathways, the mitogen-activated protein kinase (MAPK) pathways, have been shown to participate in a diverse array of cellular programmes, including cell differentiation, movement, proliferation and death [30]. To investigate the mechanism for APAP-induced necrotic hepatocytes death, we therefore studied the MAP kinases (JNK, p38 MAP kinase and ERK) involved in early signal transduction in both the hepatic tissues and cells (hepatocytes). Western blot analyses showed a significant enhancement in the expression of phospho-JNK, with a slight activation of phospho-p38 (Figure 4), whereas no significant changes were found in the expression of phospho ERK1/2 (data not shown). Furthermore, we investigated the effect of APAP on CYP2E1 protein level by Western blot analyses in both hepatic tissues and hepatocytes. We found that CYP2E1 protein level was increased in a time- (in hepatic tissues, the protein level was maximum at 16–24 h) and dose-dependent manner (in hepatocytes, protein level was maximum at 8–12 mM APAP concentration). However, taurine treatment (pre- as well as post) could normalize all these changes and maintains the normal physiology of both the organs and cells.

Role of apoptosis in APAP-induced hepatic damage

To investigate whether apoptosis may coexist with necrosis in APAP-induced hepatic pathophysiology, we have carried out immunoblotting analysis of Bcl 2 proteins (Bcl 2 and Bcl xL) and caspase 3. We have observed a significant up-regulation in phospho Bcl 2 and phospho Bcl xL expression in both APAP-exposed rats and hepatocytes (Figure 5). However, treatment with taurine and SP600125 effectively reduced these levels. On the other hand, APAP-exposure neither increased caspase 3 nor cleaved-caspase 3 expression (Figure 5). From the above results it has been concluded that apoptotic response initiated due to the blocking of anti-apototic proteins Bcl 2 and Bcl xL failed to go to full completion as the execution caspase 3 was not activated.

Effects on serum TNF-a

TNF-α signalling appears to play an important role in many forms of liver injury, including ischemia/reperfusion and fulminant hepatic failure [31]. To assess whether TNF-α concentrations in the serum are influenced by APAP, we measured TNF-α concentration in serum following APAP administration. Our experimental data suggest that APAP exposure caused increases in serum TNF-α level (Figure 6). However, taurine treatment both before and after APAP administration significantly decreased the

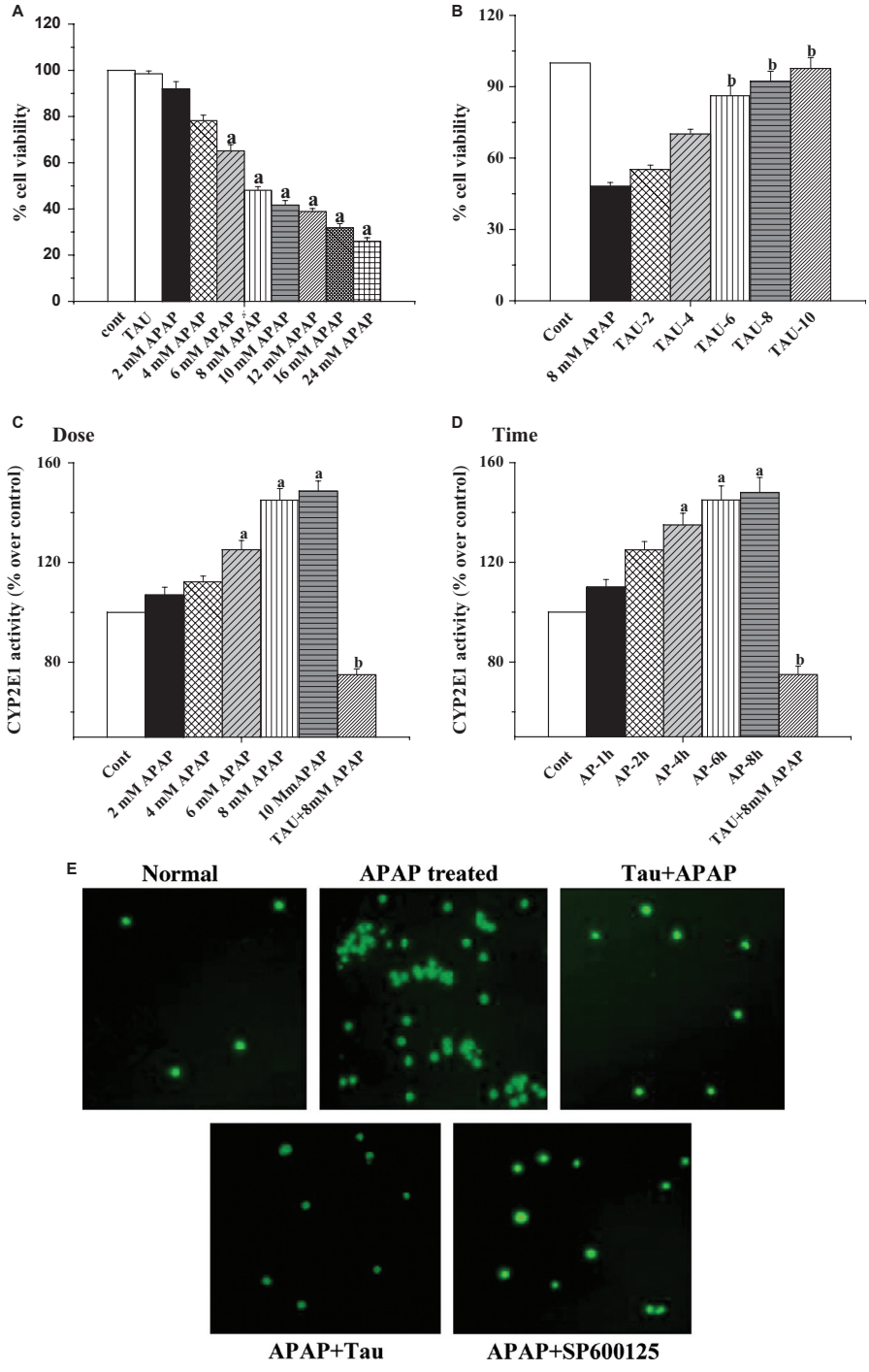
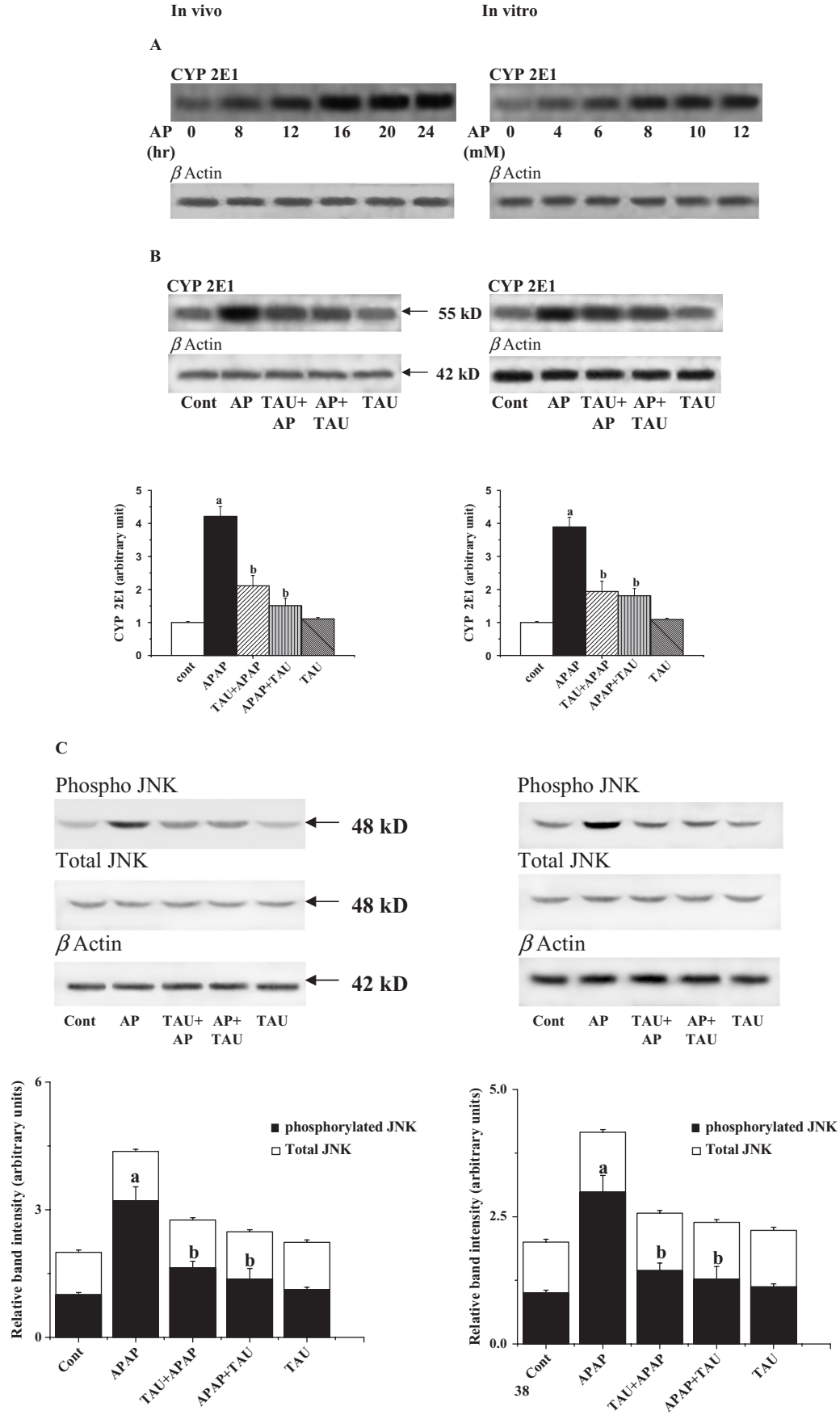


Figure 3. Impact of APAP and taurine on (A, B) isolated hepatocytes *(in vitro)* viability, (C, D) enzymatic activity of CYP2E1 in isolated hepatocytes (*in vitro*) and (E) intracellular ROS production in isolated hepatocytes (*in vitro*). (A) Dose-dependent effect of APAP on cell viability; (B) dose-dependent effect of taurine on APAP exposed hepatocytes; Cont: cell viability in normal hepacytes; TAU: cell viability of hepacytes treated with taurine alone; 2 mM APAP, 4 mM APAP, 6 mM APAP, 8 mM APAP, 10 mM APAP, 12 mM APAP, 16 mM APAP and 24 mM APAP: cell viability in APAP exposed hepatocytes for 6 h at a dose of 2, 4, 6, 8, 10, 12, 16 and 24 mM; TAU-2, TAU-4, TAU-6, TAU-8, and TAU-10: cell viability level in hepatocytes treated with taurine (1 h prior to APAP addition) and APAP for 6 h at a dose of 2, 4, 6, 8 and 10 mM. (C) Dose-dependent effect of APAP on CYP2E1 enzyme activity in hepatocytes; (D) time-dependent effect of APAP on CYP2E1 enzyme activity in hepatocytes; 2 mM APAP, 4 mM APAP, 6 mM APAP, 8 mM APAP and 10 mM APAP: CYP2E1 enzyme activity in APAP exposed hepatocytes for 6 h at a dose of 2, 4, 6, 8 and 10 mM; AP-1h, AP-2h, AP-4h, AP-6h, AP-8h: CYP2E1 enzyme activity in hepatocytes treated with APAP (8 mM) for 1, 2, 4, 6 and 8 h, respectively, TAU+8 mM APAP: CYP2E1 enzyme activity in hepatocytes treated with taurine (1 h prior to APAP addition) and APAP (8 mM) for 6 h. ^{a}The significant difference between the normal control and APAP intoxicated cells, ^{b}the significant difference between APAP intoxicated and taurine-treated cells. Each column represents mean ± SD, $n = 6$ ($p^{a} < 0.05$, $p^{b} < 0.05$). (E) The intracellular ROS production was detected by DCF-DA method in APAP intoxicated hepatocytes in absence (APAP) and presence of taurine (TAU+APAP and APAP+TAU). SP600125+APAP: intracellular ROS production in hepatocytes treated with SP600125 followed by APAP exposure. We have taken equal number of cells in each set as observed in microscope under bright field. The measurements were made six times.



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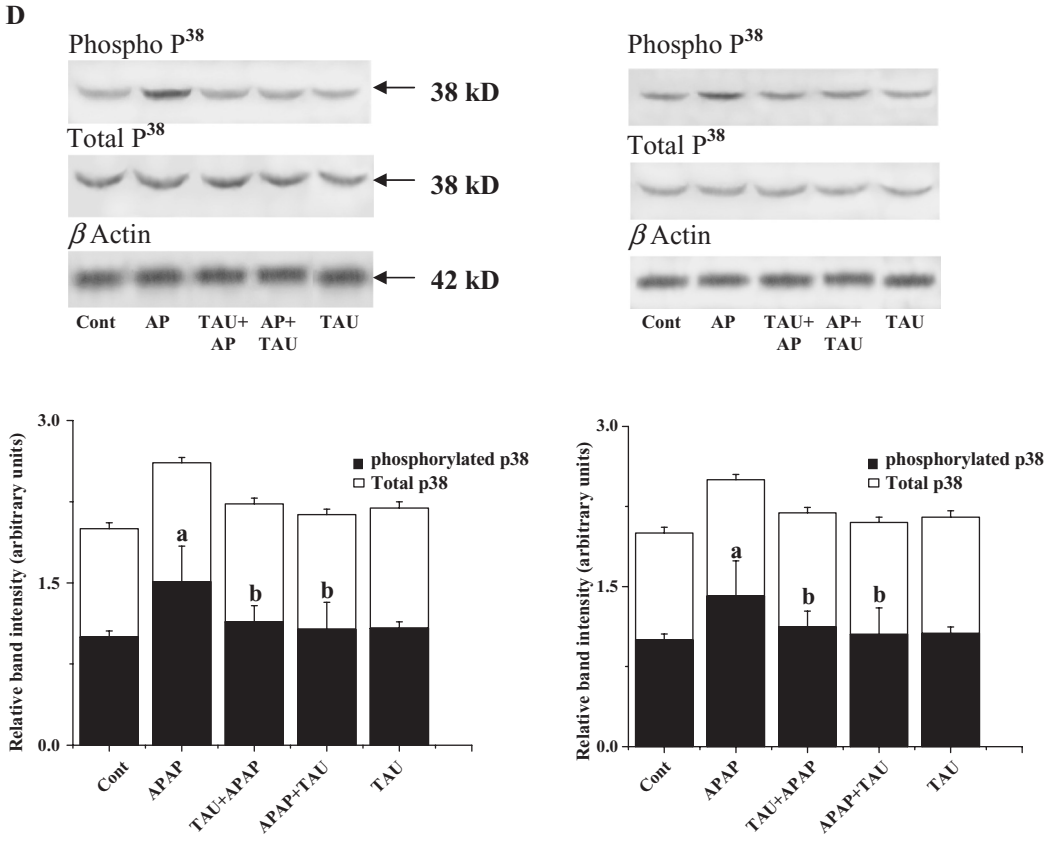


Figure 4. Western blot analysis of MAPkinase family proteins and CYP2E1 both *in vivo* and *in vitro* in the absence (AP) and presence of taurine (TAU+AP and AP+TAU). TAU: effect of taurine alone; (A) CYP2E1 protein content in both time-dependent (*in vivo* after 8, 12, 16, 20, 24 h of APAP administration) and dose-dependent manner (*in vitro* at 4, 6, 8, 10, 12 mM of APAP), (B) CYP2E1 protein content, (C) phospho and total-JNK level, (D) phospho and total-p38 level. Data represent the average ± SD of six separate experiments in each group. [a] The significant difference between the normal control and APAP intoxicated groups, [b] the significant difference between the APAP intoxicated and taurine treated groups (TAU+ APAP and APAP+TAU) ($p^a < 0.05$, $p^b < 0.05$).

serum TNF-α levels. Similarly, treatment with SP600125 also effectively reduced this level, indicating that JNK activation could induce TNF-α expression.

Effect of APAP on hepatic taurine level

Table IV showed the level of taurine in the liver tissue of APAP-exposed animals. APAP administration caused a significant reduction in the taurine level in hepatic tissues and that could be significantly prevented by taurine pre-treatment. Post-treatment with taurine also significantly increased taurine level in the hepatic tissue. On the other hand, taurine levels were less in both taurine pre-treated and post-treated animals compared to the animals treated with taurine alone.

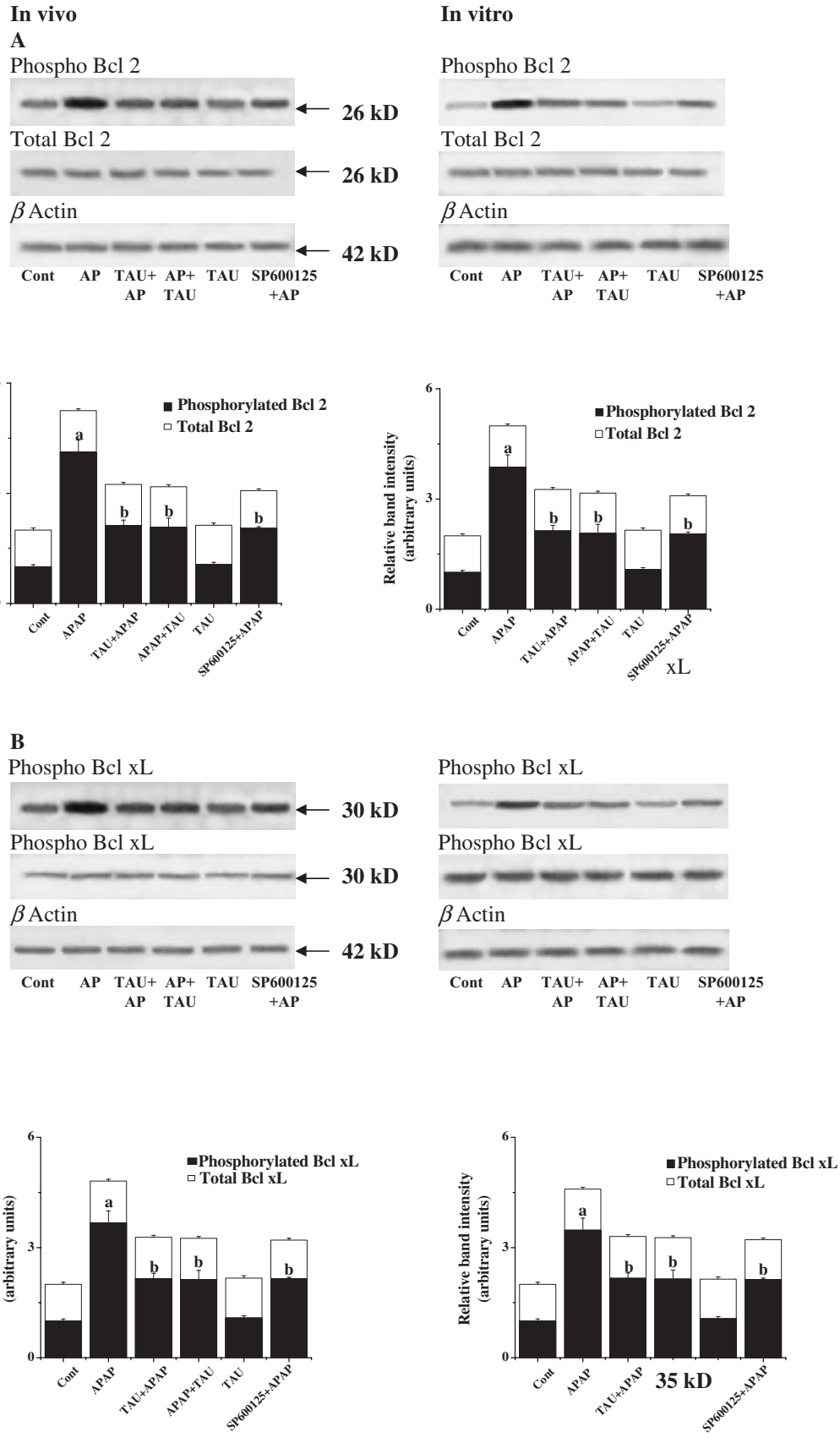
Histological assessment

Histological assessments of different liver segments of the normal and experimental animals have been presented in Figure 7. APAP overdose induced necrosis along the central vein and disorganized the normal radiating pattern of cell plates around it. Taurine treatment both prior to and post-APAP administration showed a considerable improvement in liver morphology.

Discussion

Acetaminophen (APAP) is an effective and safe analgesic when used at therapeutic levels. However, its intake in large doses or chronic use is normally associated with hepatotoxicity and nephrotoxicity in humans and animals [32]. The hepatotoxic effect of APAP is highly dependent on its dose. In the present study we found that APAP overdose induced cytotoxicity *in vitro* (hepatocytes) and hepatotoxicity *in vivo* (rats), whereas the liver conditions have been improved upon taurine treatment. Therefore, we set the aim of the present study as: to investigate the signalling mechanisms involved in the hepatoprotective effect of taurine in APAP-induced liver pathophysiology.

Transport function and membrane permeability are altered due to the hepatocytes injury, leading to leakage of enzymes from these cells. Therefore, the



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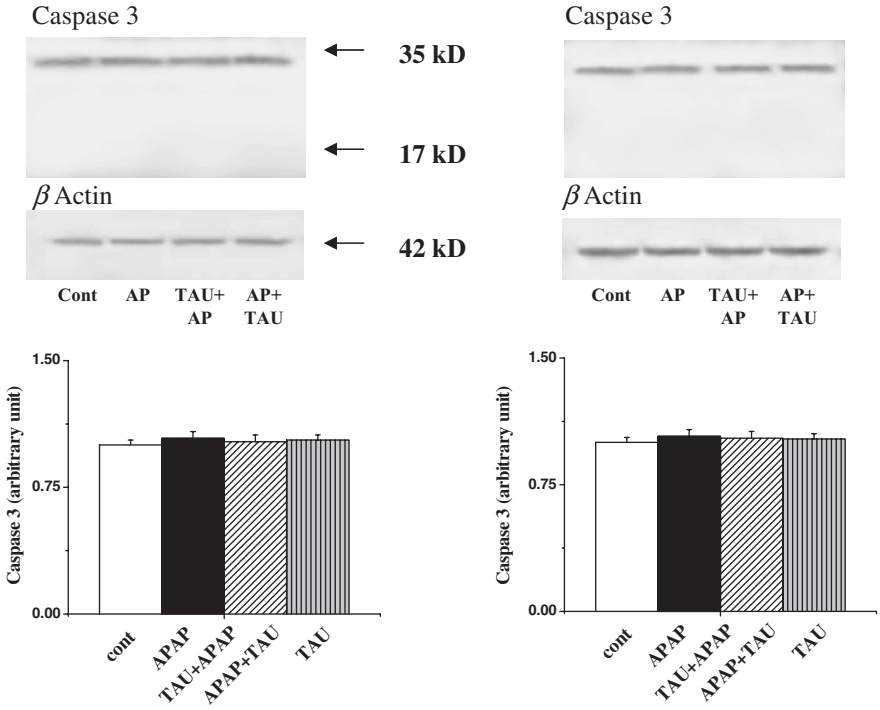

Figure 5. Western blot analysis of Bcl 2 family proteins and Caspase 3 both *in vivo* and *in vitro* in the absence (AP) and presence of taurine (TAU+AP and AP+TAU). TAU: effect of taurine alone; SP600125+AP: Effect of SP600125 treatment in rats and hepatocytes followed by APAP exposure; (A) phospho and total-Bcl 2 protein level, (B) phospho and total-Bcl xL protein level, (C) Caspase 3 protein level. Data represent the average ± SD of six separate experiments in each group. [a]The significant difference between the normal control and APAP intoxicated groups, [b]the significant difference between the APAP intoxicated and either taurine (TAU+APAP and APAP+TAU) or SP600125 (SP600125+APAP) treated groups ($p^a < 0.05$, $p^b < 0.05$).

marked increased levels of serum ALT, ALP and LDH indicate severe damage to hepatic tissue membranes during toxin and drug-induced hepatotoxicity. The increased levels of ALT, ALP and LDH in the present study were thereby indicating the severity of hepatocellular damage induced by APAP. In line with the previous reports, the present study also showed that APAP increased serum levels of nitrate plus nitrite, a marker of NO synthesis [33]. Increased NO formation occurs through up-regulation of iNOS [34]. Increased NO production induced by acetaminophen is responsible for both cell apoptosis and necrosis when NO interacts with superoxide anion to form peroxynitrite [35]. However, both pre- and post-treatment with taurine effectively reduced these alterations in APAP-induced hepatic pathophysiology.

At first APAP is metabolized by sulphation and glucuronidation to unreactive metabolites and then bioactivated by the cytochrome P-450 (CYP2E1) system to result in the formation of N-acetyl-p-benzoquinone imine (NAPQI) [36]. NAPQI is primarily detoxified by conjugation with GSH to form mercapturic acid, leading to its depletion [37]. When the rate of NAPQI formation exceeds the rate of its detoxification by GSH, NAPQI oxidizes tissue macromolecules, such as lipids or protein thiols, and alters the homeostasis of calcium. Disruption of calcium homeostasis is associated with hepatic DNA damage accompanied by cell apoptosis and necrosis [38]. A number of evidences implicate a role of reactive oxygen species (ROS) in oxidative stress produced by APAP [39,40]. Therefore, we measured the intracellular ROS production, GSH and GSSG contents, lipid peroxidation, activities of the antioxidant enzymes (SOD, CAT, GST, GPx and GR) in APAP- and taurine-treated hepatic tissues and CYP2E1 enzyme activity in APAP- and taurine-treated hepatocytes. APAP intoxication significantly increased intracellular ROS production and lipid peroxidation and decreased the GSH/GSSG ratio. APAP intoxication also decreased the activities of antioxidant enzymes SOD and CAT along with GSH dependent enzymes GST, GR and GPx and increased the enzyme activity of CYP2E1. However, both pre- and post-treatment with taurine normalized all the levels as compared to the respective controls.

Taurine usually functions as a direct antioxidant that scavenges or quenches oxygen free radicals and inhibits lipid peroxidation and as an indirect antioxidant it prevents the increase in membrane permeability resulting from oxidative insult in many tissues including liver. The possible mechanism as a result of which taurine prevents acetaminophen-induced hepatotoxicity is associated with its antioxidant property. Taurine supplementation causes improvement in GSH levels by directing cysteine into the GSH synthesis pathway, because cysteine is a common precursor of both taurine and GSH [41]. Besides, taurine

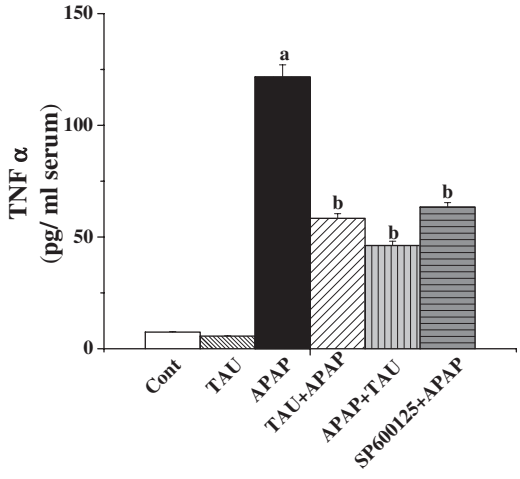

Figure 6. Serum TNF *α* level in absence (APAP) and presence of taurine (TAU+APAP and APAP+TAU) in the experimental rats. TAU: effect of taurine alone; SP600125+APAP: TNF *α* level in SP600125 treated rats followed by APAP exposure; Each column represents mean ± SD, $n = 6$. [a]The significant difference between the normal control and APAP intoxicated groups, [b]the significant difference between the APAP intoxicated and either taurine (TAU+APAP and APAP+TAU) or SP600125 (SP600125+APAP) treated groups ($p^a < 0.05$, $p^b < 0.05$).

can prevent hepatic necrosis after acetaminophen overdose by the regulation of calcium homeostasis. Since CYP2E1 is usually assumed to be the most active CYP450 in catalysing the metabolism of APAP to hepatotoxic NAPQI, inhibitors of CYP2E1 are therefore useful in the treatment of acetaminophen-induced liver disease. It is well known that taurine, by conjugation with cholic acid, forms taurocholic acid, which has been shown to be a potent inhibitor of CYP2E1 [42].

In order to determine the molecular mechanism of the protective effect of taurine against APAP-induced hepatotoxicity, we investigated the MAPKs signalling pathways and observed that APAP administration largely enhanced the expression of phospho-JNK with a little activation of phospho-p38; whereas no significant changes were found in the expression of phospho ERK1/2. Stressful conditions and several toxic agents increase JNK and p38 MAP kinase activities, often in a coordinated fashion. So the absence of p38 MAP kinase activation by APAP is of great interest. In the present study, we also observed that CYP2E1 protein expression in the liver was distinctly increased in APAP intoxicated rats. An earlier report of Bae et al. [43] showed that YH439, a well known inhibitor of CYP2E1, significantly suppressed both CYP2E1 (protein content and its catalytic activity) and JNK activity in APAP-exposed C6 glioma cells. Therefore, we could say that CYP2E1 might directly activate the JNK pathway without affecting p38 MAP kinase. However, taurine treatment both before and after APAP administration attenuated the increased levels of CYP2E1 protein and activation of phospho-JNK.

TNF-*α*, an inflammatory cytokine, plays a significant pathogenic role in acetaminophen-induced hepatotoxicity [44]. It has also been reported that JNK activation could induce TNF-*α* expression and JNK inhibition significantly lowered hepatic TNF-*α* production in APAP-induced hepatotoxicity [15]. Results of our present study showed that APAP significantly increased serum TNF-*α* level, although treatment with taurine before and after APAP administration could normalize it. We also observed that treatment with JNK inhibitor SP600125 significantly reduced the TNF-*α* level compared to that in APAP-exposed animals. Therefore, JNK activation increased the TNF-*α* production, indicating a link between TNF-*α* increase and the MAKP pathway.

We then examined the mode of cell death induced by APAP using DNA fragmentation as well as flow cytometric analyses and checked whether taurine treatment would counteract it. In our study APAP induced random fragmentation of genomic DNA, leading to the formation of a DNA smear on agarose gel, suggesting APAP-induced cell damage occurs via the necrotic pathway. Similarly, flow cytometric analysis also showed that APAP mainly damage the hepatocytes via the necrotic pathway. Both pre- and post-treatment with taurine, however, decreased the degree of necrotic cell death induced by APAP overdose.

Next, we examined whether apoptosis contributes to the induction of acute liver failure caused by APAP. For this, we have investigated the roles of Bcl 2 proteins (Bcl 2 and Bcl xL) and caspase 3. It has been reported that JNK phosphorylates (and thus inactivates) Bcl-XL and Bcl-2, two anti-apoptotic proteins of the Bcl-2 family; as a result, shifting of balance occurs in favour of the pro-apoptotic proteins, which promotes mitochondrial permeability transition [45]. Therefore, in our study we measured the expression of phosphorylated Bcl-XL and Bcl-2 after APAP administration. APAP increased the levels of both

Table IV. Taurine concentration in the liver tissues of experimental animals.

Name of the parameter	Normal Control	APAP	TAU+APAP	APAP+TAU	Control+TAU
Taurine (nmol/g tissue)	1094.2 ± 23.68	736.89 ± 30.28[a]	932.26 ± 36.29[b]	946.25 ± 37.28[b]	1421.01 ± 39.59[c]

Values are expressed as mean ± SD, for six animals in each groups. [a]values differs significantly from normal control ($p^a < 0.05$); [b]values indicate the significant difference between APAP and TAU-treated group ($p^b < 0.05$); [c] values indicate the significant difference between normal and TAU alone-treated group ($p^c < 0.05$).

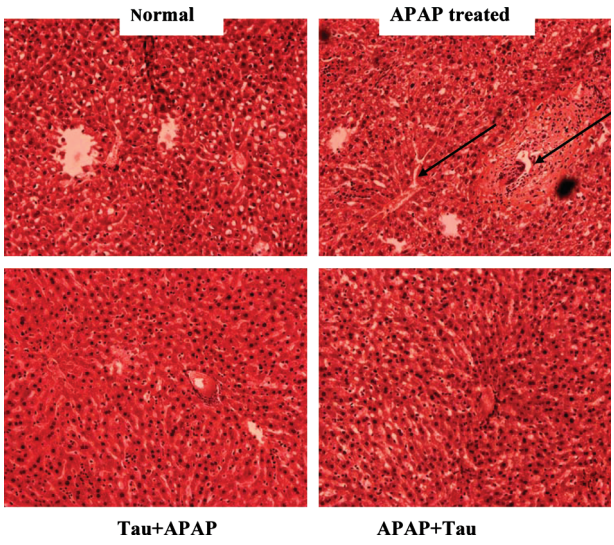

Figure 7. Haematoxylin and eosin stained liver section of (A) normal rats liver (×100), (B) APAP intoxicated liver section (×100), (C) taurine pre-treated liver section (×100) and (D) taurine post-treated liver section (×100). Arrows indicate centrilobular necrosis in the liver tissue compared to the normal liver section.

p-Bcl-XL and p-Bcl-2, likely as a consequence of increased P-JNK levels, although treatment with JNK inhibitor (SP600125) could reduce all these changes. It has also been reported that caspase inhibitors protect liver from APAP-induced injury, suggesting that apoptosis plays a vital role in initiating the events that lead to hepatic damage, but APAP does not activate execution caspases 3 and 7 [46]. Then we investigated the role of caspase 3 after APAP administration and also found that caspase 3 was not activated in our experiments. All these observations suggest that the apoptotic response initiated by APAP failed to go to full completion and degenerated into necrosis.

Finally, results of taurine content measurements in liver homogenate showed that animals that received APAP significantly decreased the taurine levels compared to normal animals. Since it is well known that taurine plays an important role in the normal antioxidant defense system, disruption in its level may be assumed to shift the balance toward a pro-oxidant environment in the hepatic tissues and contribute to oxidative damage that occurs during the APAP overdose. On the other hand, both pre- and post-treatment with taurine significantly increased its level in liver tissue compared to APAP-treated animals, although these levels are markedly less compared to that present in the hepatic tissue of the animals treated with taurine alone. These results indicate a quick intake of exogenous taurine by the hepatic cells under pathophysiological conditions.

In conclusion, the results of the present study demonstrate that taurine supplementation has a prophylactic as well as a therapeutic role in preventing acetaminophen-induced hepatotoxicity, probably through its unique cytoprotective properties like antioxidant activity, modulation of calcium homeostasis and inhibition of nitric oxide production. Most importantly taurine can reduce the activity and protein level of CYP2E1 enzyme, inhibiting the activation of JNK and TNF-α production. All these findings indicate that taurine deserves further consideration as a potential alternative for preventing liver injury caused by acetaminophen-overdose.

Acknowledgement

The authors are grateful to Mr Prasanta Pal for excellent technical assistance for the study.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 26 January 2010.