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Hepatoprotective effect of obeticholic acid on acetaminophen induced hepatotoxicity in mice

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Acetaminophen (APAP) is commonly used as analgesic and antipyretic drug for relieving mild and moderate pain, but at high doses produces hepatic necrosis. Though, Obeticholic acid (OCA) has been tested in range of diseases, its therapeutic potential against APAP-induced hepatic injury remains to be elucidated. Thus, in this study, we investigated the preventive effect of OCA along with N-acetylcysteine (NAC) and Silymarin (SIL) against acetaminophen-induced hepatotoxicity in mice. SIL (100 mg/kg, po) and OCA (30 mg/kg, po) were administered continuously for six days prior to APAP administration. After sixth dose, animas were fasted for 12 h and treated with 300 mg/kg APAP and then received SIL (100 mg/kg, po), NAC (500 mg/kg, ip) and OCA (30 mg/kg, po) at 1 h after APAP. Mice were sacrificed 6 h after APAP injection. Analysis of serum Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), liver glutathione (GSH) and histopathology were employed for assessment of hepatotoxicity. APAP group showed a significant increase in ALT, AST, ALP and centriolobular hepatic necrosis with a significant decrease in glutathione in comparison to control group. All these parameters were significantly improved in all the three treated groups when compared to APAP group. In conclusion, Obeticholic acid (OCA), Silymarin (SIL) and N-acetylcysteine (NAC) are suggested to protect against APAP-induced hepatotoxicity in mice by ameliorating liver enzymes, antioxidant effect and decreasing liver necrosis.

Keywords: N-Acetyl cysteine, Liver toxicity, *Silybum marianum*, Silymarin

Acetaminophen (APAP, N-acetyl-p-aminophenol, paracetamol) is one of the most reliable drugs used for analgesic and antipyretic properties and widely used for the treatment of a variety of musculoskeletal pain and in painful disorder like headache, myalgia and neuralgia. APAP use is considered to be safe in therapeutic concentrations, however an overdose may lead to hepatotoxicity and acute liver failure (ALF)[1]. Hepatotoxicity is initiated when phase II metabolizing enzymes are saturated after APAP overdose, excessive accumulation of NAPQI results in depletion of glutathione (GSH) followed by covalent binding with hepatic and renal cellular proteins, especially mitochondrial proteins[2]. This results in mitochondrial dysfunction leading to hepatic[3] and renal injury[4]. Despite recognition of APAP overdose hepatic and renal toxicity, therapeutic options are limited to treat or control its toxic effects.

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Obeticholic acid (OCA) has been tested in range of clinical trials for the treatment of alcoholic hepatitis[5], nonalcoholic steatohepatitis[6,7], nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus[8]. Nevertheless, whether OCA inhibits APAP-induced hepatic toxicity remains to be determined. Currently, N-Acetyl cysteine (NAC), a GSH precursor, is the only clinically proven anti-dote against APAP-induced hepatic toxicity[9]. NAC anti-dote against APAP liver injury was used orally or parenterally[10]. However, both the treatment approaches proved effective only for patients who are admitted to the clinics within hours of an acute overdose and is less effective for late presenting patients[11]. However, despite recognition of NAC to treat APAP-induced blood and hepatotoxicity, limitations associated with NAC treatment such as time bound treatment, toxicity and adverse effects drive us to explore an option of safe and better therapeutic medication.

Silymarin is a natural compound present in *Silybum marianum* (milk thisle) known for its antiproliferative, antiviral, antioxidant and hepatoprotective

properties[12]. It is effectively used for the treatment of chronic liver disease and prevented hepatic injury and liver fibrosis caused by toxins, i.e., ethanol and CCl_4[13]. In the current study, we investigated the protective effect of OCA along with SIL in comparison to the current anti-dote NAC, on APAP-induced liver toxicity in mice.

Material and Methods

Chemicals and reagents

Acetaminophen (Cat No. A7085) and N-Acetyl L-cysteine (Cat No. A7250) were purchased from Sigma-Aldrich. Obeticholic acid (Cat No. HY-12222) and Silymarin were procured from MedChemExpress and Labex respectively. Alanine transaminase (ALT-121173), Aspartate transaminase (AST-121172) and Alanine phosphatase (ALP-121158) were procured from ERBA Mannheim (TRANSASIA, India). Glutathione kit (Item No. 703002) was purchased from Cayman chemical.

Animals

Male C57BL/6NTac mice (Taconic) of age 12 weeks with weight range 20±5 g were housed in a group of 3 animals in individually ventilated polysulphone cages with autoclaved corn cob bedding. Feed (SAFE® D131-gamma irradiated) and autoclaved reverse osmosis water were provided *ad libitum.* However, mice were fasted before treatment with APAP as indicated in the following. Environmental controls for the animal room were set to maintain a temperature of 22±3°C, humidity of 30-70%, and a 12 h light/dark cycle (light on 7:00 am. to 7:00 pm.). All experimental procedures were conducted in accordance with the guideline of GVK Institutional Animal Ethics Committee (IAEC). Every effort was made to minimize pain or discomfort to the experimental mice.

Experimental design and protocols

Mice were acclimated for 7 days in laboratory conditions before initiating any experimental procedures. Mice were randomly assigned into five experimental groups, each containing six mice as follows: Gr. I: Mice received 1% Tween80+99% methyl cellulose (0.5%) at 10 mL/kg for 7 days through oral gavage and served as vehicle control; Gr. II (APAP): Mice were administered with single dose of acetaminophen (300 mg/kg)[14] formulated in warm 0.9% sodium chloride as 15 mg/mL concentration solution administered through intra-peritoneal route on day 6, served as APAP control; Gr. III (APAP+NAC): Mice were treated with single dose of N-acetyl-L-Cysteine (500 mg/kg)[15], formulated in 0.9% sodium chloride, through intra-peritoneal route on terminal day, 1 h after APAP administration; Gr. IV (APAP + OCA): Mice were pre-treated for 6 times at an interval of 24 h with Obeticholic acid (30 mg/kg)[16] formulated in 1% Tween 80 and 99% methylcellulose (0.5%) through oral gavage once daily and 1 h after APAP administration on terminal day; and Gr. V (APAP + SIL): Mice were pre-treated for 6 days with silymarin (100 mg/kg)[17] formulated in 0.5% methyl cellulose through oral gavage once daily and 1 h after APAP administration on terminal day. All mice were fasted for 12 h before administration of APAP and feed was returned to the cages after dosing.

Measurement of liver enzymes

The mice were sacrificed after completion of the treatment regimen, which was 7 days, and blood was withdrawn from each mouse at the indicated times after treatment (6 h after APAP administration)[18] by cardiac puncture in pathogen free sterile tubes. The collected blood was subjected to centrifugation at 4000 rpm at 4°C for 10 minu. Plasma was separated and stored at –20°C for analysis of biochemical parameters. They were processed for estimation of enzymatic activities of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) with spectrophotometric technique by the ERBA EM-360 clinical chemistry analyzer using ERBA Mannheim (India) diagnostics kits and presented as IU/L.

Measurement of hepatic GSH

After dissection, the liver was excised rinsed with phosphate buffer saline solution to remove any blood clots and total liver weight was recorded. Part of liver left lobe (~100 mg tissue) was snap frozen and processed for GSH estimation based on kit protocol provided by Caymans GSH kit and absorbance was read at 414 nm. Total Glutathione levels were presented as μM per mg liver tissue.

Histopathological assessment of liver specimens

For histopathological study, another part of the left liver lobe was fixed in 10% neutral-buffered formalin solution for at least 24 h, processed routinely and embedded in paraffin. According to standard histological practices, four micrometer-thick sections were prepared and stained with hematoxylin and eosin (H&E) before examinations[19]. Stained sections were examined under light microscope (Leica DM3000

LED) equipped with Leica DMC4500 camera to observe pathological changes. Liver histological scoring was performed by a certified pathologist according to a previous report[20]. In brief, necrosis grade was expressed in score as 0, within normal limit; 1, minimal (1-10% hepatocytes are affected); 2, slight (11-30% hepatocytes are affected); 3, moderate (31-60% hepatocytes are affected); and 4 marked (>60% hepatocytes are affected).

Statistical analysis

Data are presented as mean±SEM. The data was analysed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-hoc test using the statistics software, GraphPad Prism 5. Statistical differences between groups were considered significant at the level of P <0.05. Comparison of body weight between different groups was carried out by two-way ANOVA test and Bonferroni's multiple comparison test.

Results

Effect of OCA on body weight and relative liver weight

Daily body weight was recorded before treatment and percentage change in body weight was calculated against day 0. Terminal day body weight was recorded after 12 h of fasting and no significant difference was observed in percentage change in body weight between the treated groups (Fig. 1A).The relative liver weight (liver wt./body wt.) was calculated for all the groups and no significant difference in relative liver weight was observed among the groups (Fig. 1B).

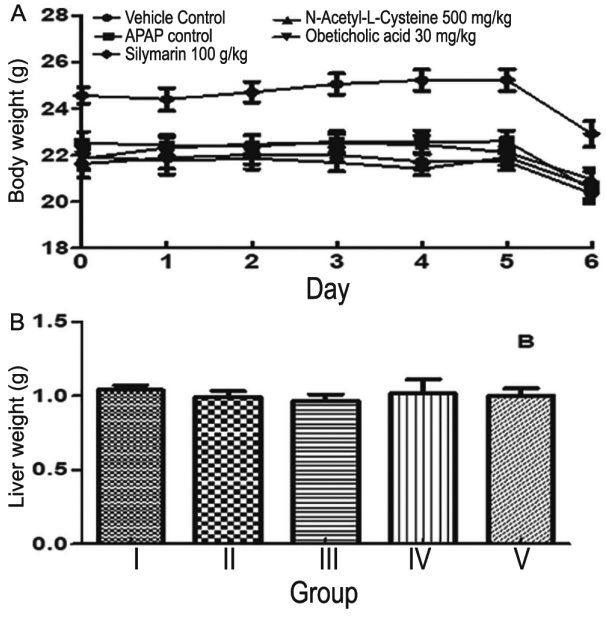

Fig. 1 — Effect of N acetylcysteine (NAC), Obeticholic acid (OCA) and Silymarin (SIL) against N-acetyl-p-aminophenol, paracetamol (APAP) induced liver toxicity on (A) body weight change (in %); and (B) relative liver weight.

Effect of OCA on liver enzyme

Table 1 shows the results of the AST, ALT and ALP analysis. APAP overdose can alter the liver enzymes activities. Serum ALT, AST and ALP levels indicate a measure of hepatic function. APAP administration resulted into significant increase in ALT (P <0.0001), AST (P <0.0001) and ALP (P <0.05) when compared with normal control mice (Fig. 2 A-C). APAP administration increased serum ALT, AST and ALP activity by 13623.64, 4032.16 and 24.47%, respectively. Pretreatment with OCA, SIL and post treatment with NAC significantly prevented APAP-induced increase in ALT (87.92, 45.31 and 85.50%, respectively), AST (89.06, 40.40 and 64.35%, respectively) and ALP (27.12, 31.07 and 29.94%, respectively).

Effect of OCA on liver tissue GSH

GSH plays an important role in detoxifying the reactive intermediate of APAP[21]. As depicted in (Fig. 2D), hepatic GSH content of APAP (1.65±0.43) group mice decreased by 58% of the vehicle control animals (3.88±0.17). However, GSH depletion was restored after pretreatment with OCA, SIL and post treatment with NAC. GSH levels increased significantly with OCA (4.17±0.35, P<0.0001), SIL (3.14±0.50, P <0.05) and NAC (3.36±0.42, P <0.05) compared to APAP group.

Histopathological analysis

Liver sections of normal vehicle control mice stained with H&E showed a lobular architecture and hepatocyte normal structure (Fig. 3A). In contrast, APAP administered mice livers exhibited large areas

Table 1 — Effect of NAC, OCA and SIL on serum ALT, AST and ALP levels in mice treated with hepatotoxic dose of acetaminophen

Groups\ Parameters	ALT (U/L)	AST (U/L)	ALP (U/L)
vehicle control	39.83±1.14	153.35±15.10	118.50±12.54
APAP control	5466.58±165.47$^{###}$	6336.67±735.29$^{###}$	147.50±2.50$^{##}$
APAP+NAC	792.58±43.14***	2259.00±208.76***	103.33±8.03**
APAP+OCA	660.33±381.04***	693.17±350.59***	107.50±13.09*
APAP+SIL	2989±882.88**	3776.75±1150.78*	101.67±6.41**

[Data are expressed as mean of 6 mice ± SEM. $^{###}P$ <0.0001, compared to vehicle control group. **P <0.001, ***P <0.0001, compared to acetaminophen-intoxicated group (ANOVA followed by Dunnett's multiple comparison post-hoc test). NAC, N acetylcysteine; OCA, Obeticholic acid; SIL, Silymarin; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ALP, Alkaline phosphatase; SEM, Standard error of mean; and ANOVA, Analysis of variance]

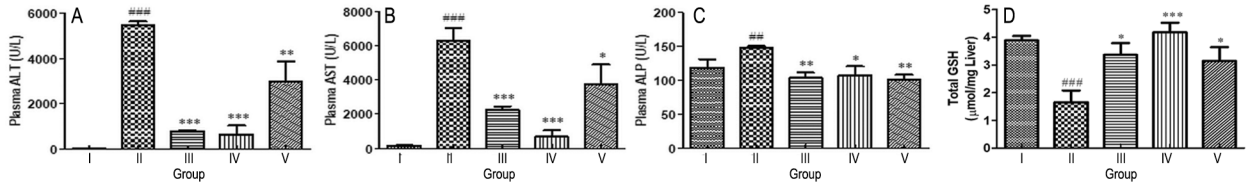

Fig. 2 — Effect of NAC, OCA and SIL against APAP induced liver toxicity in biomarkers of hepatic damage. (A) Serum Alanine aminotransferase (ALT); (B) Aspartate aminotransferase (AST); and (C) Alkaline phosphatase (ALP); and (D) Glutathione enzyme levels. [Data are expressed as mean of 6 mice±SEM. $^{\#\#}P$ <0.001, $^{\#\#\#}P$ <0.0001, compared to vehicle control group. **P* <0.01, ***P* <0.001, ****P* <0.0001, compared to acetaminophen-intoxicated group (ANOVA followed by Dunnett's multiple comparison post-hoc test)]

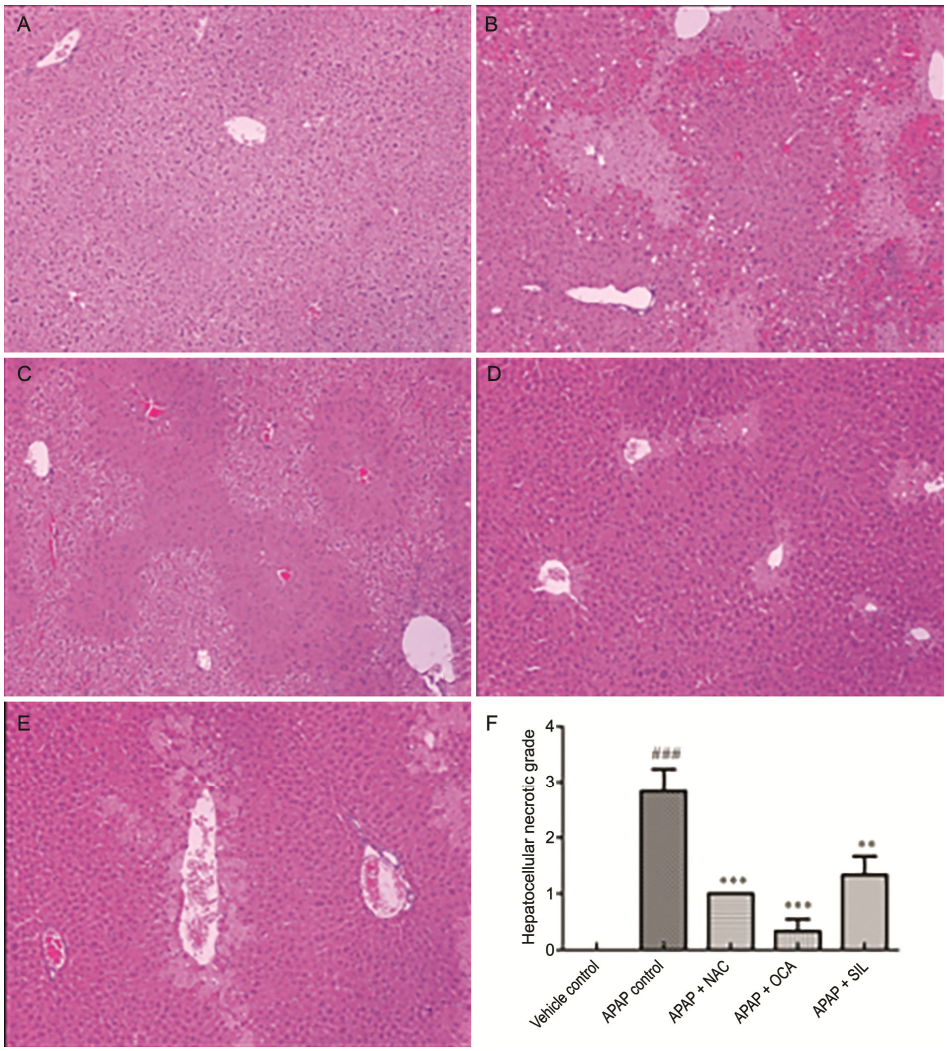

Fig. 3 — NAC, OCA and SIL treatment showed significant liver protective effect produced by APAP over dose. H&E stained liver sections of (A) normal saline treated group; liver showing normal architecture; (B) Acetaminophen-induced liver toxicity group; liver exhibited severe centilobular hepatic necrosis, central vein congestion, inflammatory cell infiltration and ballooning degeneration; (C) Acetaminophen + N-acetlycysteine group, normal hepatocyte with slightly congested central vein, moderate fatty change with infiltrating inflammatory cells and a remarkable reduction in centrilobular necrosis; (D) Acetaminophen + Obeticholic acid group, normal hepatocyte with slightly congested central vein, very few inflammatory cells and notable decrease in centrilobular necrosis; (E) Acetaminophen + silymarin group, normal hepatocyte with moderate congested central vein, mild fatty liver changes and pronounced reduction in centrilobular necrosis; and (F) Results of histopathological scores. Original magnification 100X. [Arrows indicate necrotic area. Data are presented as mean of 6 mice ± SEM. $^{\#\#\#}$P<0.0001, compared to vehicle control group. ***P* <0.001, ****P* <0.0001 compared to acetaminophen-intoxicated group (ANOVA followed by Dunnett's multiple comparison post-hoc test)]

of necrosis with centrilobular vein congestion, polymorphonuclear infiltration and vacuolization of hepatocytes as shown in (Fig. 3B). APAP administered animals exhibited markedly high centrilobular hepatocellular necrotic grades (2.83±0.40, P <0.0001) when compared with vehicle control mice.

The severity of hepatic injury has reduced with all the treatments. Animals pre-treated with OCA and SIL exhibited mild injuries with minimal hepatic centrilobular necrosis, slightly congested central vein and necro-inflammatory activity. Similar results were observed with NAC post treatment (Fig. 3 C-E). Though, all the test items showed significant reduction in centrilobular hepatocellular necrosis when compared with APAP administered mice, OCA (0.33±0.21, P <0.0001) pretreatment has shown more prominent effect than NAC (1.00±0.00, P <0.0001) and SIL (1.33±0.33, P <0.01).

Discussion

Drug-induced hepatotoxicity is a main cause of liver disease in clinic and the incidence of drug-induced hepatotoxicity augmented with an increase in the number of new drugs available[22]. Among the incidences of drug-induced hepatotoxicity, APAP overdose is responsible for almost half of all acute liver failures in the US and many other western countries[2,23]. APAP at therapeutic doses is an effective and safe analgesic and anti-pyretic drug which is well tolerated by adults and children, however, an overdose can lead to extensive liver injury culminating to acute liver failure[1,24]. APAP over dose results in overexpression of CYP (P450) enzymes, depletion of hepatic GSH level and repression of glucuronidation which leads to elevated levels of APAP metabolite[25]. GSH depletion after APAP overdose triggers excessive accumulation of NAPQI which causes mitochondrial oxidative stress leading to hepatic necrosis[26,27]. APAP overdose damage structural integrity of hepatic cellular membrane resulting in the aminotransferases enzymes leakage into the blood stream and increase in serum activity levels of ALT and AST[28]. Thus, measuring serum levels of specific liver enzymes like ALT, AST and ALP are most commonly used markers in liver toxicity studies and plays an important role in evaluating the extent of liver injury[29].

NAC is the only antidote available in case of APAP overdose in clinical practice. However, NAC treatment proved to be protective against APAP overdose if therapy started within 8 h of APAP ingestion. It has been observed that delayed NAC treatment after APAP liver toxicity has significantly increased mortality incidences and still has some side effects, including low fever, nausea, runny nose, drowsiness and tissue irritation[30]. Thus, it has become an attention point to discover more effective and less toxic novel drug for liver injury.

There are several medicinal plants having therapeutic targets in prevention and treatment of liver disease signs and symptoms, milk thisle is well known among them for their heapto-healing properties. Silymarin, obtained from seeds of *Silybum marianum* is composed of complex flavonoids with silibinin as main active ingredient[31]. Several clinical and preclinical studies have reported hepatoprotective role of silymarin against partial hepatectomy and toxic models in animals using APAP, CCl_4, ethanol, D-galactosamine and *Amanita phalloides* toxin[32]. Hepatoprotective effects of silymarin are mainly attributable to its anti-inflammatory, anti-fibrotic, anti-oxidant and free radical scavenging properties[33].

APAP is known to downregulate hepatic FXR expression and activation of FXR and provide protection against APAP-induced hepatic toxicity[34]. Obeticholic acid, a selective and potent FXR agonistis being currently investigated to treat nonalcoholic steatohepatitis (NASH)[35]. Several previous reports have explained the protective effect of OCA against estrogen-induced cholestasis[36], portal hypertension in cirrhotic rats[37], CCl_4-induced liver toxicity[38], insulin resistance and lipid abnormalities in Zucker (fa/fa) obese rats[39]. Despite of OCA's protective role against various liver toxicity agents, there are contradictory findings regarding protective role of FXR upregulation in hepatic disorders. van Golen *et al*.[40] explained that using OCA in reversible bile duct ligated rats, is associated with biliary injury aggravated[40]. Similarly, silymarin and methanol milk thistle seed extract markedly increase hepatic FXR gene expression and provide protection against APAP-induced hepatic toxicity[41,42].

Our results show that APAP overdose elevated the plasma levels of ALT, AST and ALP, suggesting liver toxicity. However, on treatment with OCA, silymarin and NAC significantly restored these liver biomarkers to normal status compared to APAP over dosed animals. OCA administration showed similar improvement in ALT and AST levels in CCl_4-induced

liver toxicity. In the recent past, studies have reported marked reduction in plasma ALT, AST and ALP levels by silymarin and NAC treatment[43,44].

Oxidative stress plays a key role in APAP-induced hepatotoxicity. Excessive accumulation of ROS results in thiol oxidation that as a result decreases cellular GSH levels[45]. Hepatocellular GSH plays an important role in intracellular defence against ROS and prevents oxidative stress in cells. APAP intoxication resulted in significant depletion of cellular GSH content compared to the control group. These results re-confirm the previous findings which have reported significant decrease in GSH level after APAP administration[35,46]. APAP-induced hepatic injury can be ameliorated via antioxidant activity or through inhibition of free radicals generation. Furthermore, pre-treatment with OCA against APAP-induced hepatotoxicity indicates that OCA precludes liver injury via augmentation of hepatic GSH levels. These results implied the protection of OCA against APAP-induced liver oxidative stress injury. Clen *et al*.[47] have also shown that OCA pre-treatment inhibites GSH depletion in placenta and fetal liver in gestational cholestasis mice model[47]. It has been proved that silymarin can improve oxidative stress and enhance lipid peroxidation scavenging ability and antioxidant defence ability[48]. In a recent study, it has been explained that silymarin possibly restrict APAP-induced GSH depletion by promotion of cysteine by flavonoids to participate in GSH synthesis[49]. NAC, a cysteine prodrug replenishes intracellular GSH levels and it has been successfully used to treat GSH deficiency caused by APAP administration. NAC has been reported to treat GSH deficiency in a wide range of infections, metabolic disorders, genetic defects, COPD and APAP overdose[50,51].

APAP is known to induce liver injuries like central vein congestion, infiltration of inflammatory cells, necrosis, severe stage of parenchymatous degeneration and vacuolization of hepatocytes[52]. We have also observed similar liver pathological changes following APAP administration in the current study. Regeneration and replacement of necrotic hepatocytes post APAP-induced hepatotoxicity are the key hallmark of recovery[53]. Liver tissues morphology and histopathology findings confirmed the protective activity of OCA against APAP-induced hepatotoxicity. It is evident by decreased centrilobular necrosis, less central vein congestion and reduced necro-inflammatory changes after OCA pretreatment. These findings are in line with our results of biochemical parameters and hepatocellular GSH levels, reported above. Similarly, in many other studies, OCA has been seen to protect liver from diet-induced non-alcoholic steatohepatitis[54]. It has been already demonstrated that silymarin partially protects from APAP-induced hepatonecrosis by decreasing group of proinflmmatory cytokines and RIP-3 expression[55]. Moreover, administration of silymarin in liver disease with various etiologies, led to significant improvement in liver histopathological and structural abnormality[43]. It has been well established that APAP overdose trigger hepatic necrosis by mitochondrial dysfunction and NAC protects APAP-induced liver injury by stabilizing mitochondrial dysfunctions which led to regeneration and replacement of necrotic hepatocytes[56].

The present study, investigating the effect of pre-treatment with OCA on APAP-induced acute liver injury, has certain limitations. It did not investigate the effect of OCA on acute liver injury in other animal models and also explored the post-treatment effect of OCA on APAP-induced hepatic injury. Further studies are required as indicated above for detailed understanding.

Conclusion

In summary, the present study suggested that OCA, a semi-synthetic bile acid analogue could significantly lower APAP-induced hepatotoxicity similar to NAC and better than silymarin treatment via modulating plasma ALT, AST, ALP and hepatic GSH as liver function markers. Besides, OCA could mitigate oxidative stress by inhibiting APAP-induced GSH depletion. In addition, we have also demonstrated that pre-treatment with OCA resulted in decreased centrilobular hepatocellular changes triggered by APAP overdose. Moreover, OCA pretreatment had similar and/or better effectiveness than NAC and silymarin in combating APAP-induced heapato-toxicity. Finding of the present study implied that the OCA pretreatment may be taken into account as valuable substitute to adjust APAP-induced liver injury.

Conflicts of interest

Authors declare no competing interests.

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