

Hepatoprotective Role of α -Lipoic acid and Thymoquinone in Acetaminophen- Induced Liver Injury: Down-Regulation of COX-2 and flt-1 Expression

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ABSTRACT

Acetaminophen (APAP) is a widely-used analgesic, while toxic doses of which induce liver injury. Inducible cyclooxygenase-2 (COX-2) is derived prostaglandins which play an anti-inflammatory role in acetaminophen-induced hepatotoxicity. Selective activation of vascular endothelial growth factor (VEGFR1, flt -1) on endothelial cells increased mRNA levels of hepatocyte mitogens (IL-6) and hepatocyte growth factor leading to prosurvival effects on hepatocytes. The aim of this study was to compare the hepatoprotective effect of N-acetylcysteine (NAC; the antidote for APAP) with that of α -Lipoic acid (ALA) and/or Thymoquinone (THQ) either alone or in combination on liver injury induced by APAP.

APAP administration elevated most of the previously measured parameters and decreased GSH, SOD, and total protein levels compared with the control group. Liver sections of H&E demonstrate liver injury characterized by centrilobular hepatocellular necrosis, COX-2, and flt-1 expressions were also increased. Treatment with all fore mentioned antioxidants ameliorated most of the altered parameters compared to APAP-treated group. Treatment with the combination of ALA and THQ was the most effective therapy in the attenuation of liver injury assessed by a decrease in ALT and ALP activities and down-regulation of COX-2 and flt-1 expression. Section of liver from rat received APAP, ALA and THQ shows a marked improvement of hepatic degeneration which restricted to few hepatocytes with mild vacuolation of their cytoplasm while the nuclei appear normal mimic to control cells. It was concluded that the natural antioxidants such as ALA and THQ, may be considered as a potential antidote in combating liver injury induced by APAP.

Key words: Acetaminophen; α -Lipoic acid; Thymoquinone; cyclooxygenase-2

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INTRODUCTION

Liver damage induced by a drug is a world wide's health problem. It is a major health issue that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies¹. Acetaminophen (N-acetyl-p-aminophenol, APAP; paracetamol) is commonly prescribed analgesic and antipyretic drug producing dose-dependent hepatic- and nephrotoxicity².

The reactive metabolite of APAP is N-acetyl-p-benzoquinone imine (NAPQI), which is formed by cytochrome P-450 (CYP) by a direct two electron oxidation of APAP^{3,4}. The reaction of NAPQI with glutathione occurs by conjugation to form 3-glutathion-S-yl-acetaminophen and by reduction to acetaminophen⁵. Moreover, the reaction could be catalyzed by glutathione transferase, and NAPQI is one of the best substrates ever described for this enzyme⁶.

Treatment of mice with APAP reduced mRNA levels of TNF- α and other cytokines (IL-10, IL-6, COX-2, and IL-18) and there was an increase in liver toxicity assessed by increased serum ALT. Ju and colleagues postulated that these findings suggest alternative roles for Kupffer cells in the toxicity, and suggest that Kupffer cells may counteract inflammation or have a role in liver repair⁷.

Reilly and coworkers showed that COX-2 was induced in livers of APAP-treated mice with COX-2 playing an anti-inflammatory role in APAP-induced hepatotoxicity⁸.

Increased expression of vascular endothelial growth factor, VEGFR1 (flt -1), VEGFR2, and VEGFR3 protein was apparent throughout the time course of APAP administration, with peak expression during 8 to 72 h⁹.

Natural antioxidants are alternatives best ways to minimize liver damage and were used prophylactically and also as antidotes.

ALA is synthesized by the liver and other tissues. It is a natural cofactor in pyruvate dehydrogenase complex where it binds acyl groups and transfers them from one part of the complex to another¹⁰. ALA is a potent antioxidant. Three distinct antioxidant actions of ALA and its reduced form, dihydroLipoic acid, have been observed: reactive oxygen species scavenging activity; capacity to regenerate endogenous antioxidants such as glutathione and vitamins C and E and metal –chelating activity¹⁰.

Abdel-Zaher et al.¹¹ reported that pretreatment of rats with ALA (100mg/kg) protected against hepatotoxicity and nephrotoxicity induced by an acute oral toxic dose of APAP (2.5 g/kg) as assessed by biochemical measurements and by histopathological examination.

Thymoquinone (THQ) is the most potent component of *Nigella sativa*. Protective effects of THQ were established in doxorubicin, carbon tetrachloride, cisplatin, ethanol and aflatoxin-induced oxidative damage. In addition, the anti-inflammatory, anti-tumoral, anti-microbial, anti-histaminic and immuno-modulatory effects of THQ have been reported. Moreover, it has been suggested that THQ may act as an antioxidant agent and prevent the membrane lipid peroxidation in hepatocytes¹². So the present investigation was carried out to study the hepatoprotective effects of THQ and ALA either alone or in combination compared with that of N-acetylcysteine (NAC) in APAP- induced toxicity model.

MATERIALS AND METHODS

Experimental Animals:

Thirty-six healthy male albino rats (120– 160 g) were supplied by the Experimental Animal Center, College of Pharmacy, King Saud University. Animals were kept in special cages and maintained on a constant 12-h light/12-h dark cycle with air

conditioning and a controlled temperature of 20–22 °C and humidity of 60%. Rats were fed a standard rat pellet chow with free access to tap water ad libitum for 1 week before the experiment for acclimatization. Animal utilization protocols were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the College of Pharmacy, King Saud University.

Chemicals:

All chemicals used were of high analytical grade, a product of Sigma and Merck companies. APAP, NAC, THQ and ALA were purchased from Sigma Chemical Co. (Sigma, St. Louis, MO, USA while.

Experimental design

One week after acclimation, the rats fasted overnight before treatment and randomly divided into six groups, each of six rats as follows, Group 1: a control group which was administered saline. Group 2: rats were administered in a single oral dose of APAP 750 mg/kg¹³.

The fore mentioned antioxidants were administered in oral three doses of NAC 20mg/kg¹⁴, ALA 200 mg/kg¹⁵ and/or THQ 15mg/kg¹⁶ either alone or in combination (G3, G4 ,G5 ,G6), respectively. The first dose was 24 h before and the second was two hours after a single dose of 750 mg/kg of APAP, then a third dose of the antioxidants were taken after 12h from APAP administration. One day after APAP administration, all animals were sacrificed; blood samples were withdrawn from and serum were separated by centrifugation at 3000 rpm for 20 min and used for biochemical serum analysis. After blood collection, the livers were collected, washed using chilled saline solution. The livers were minced and homogenized in ice-cold bi-distilled water to yield 20% homogenates. The homogenates were centrifuged for 20 min at 3000 rpm at 5 °C, and the supernatants were used for biochemical tissue analysis. Three livers from each group were kept in 4% formalin for histopathological examination and immunohistochemistry examinations.

Biochemical analysis

Serum Biochemical Assays

Serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Lactate dehydrogenase (LDH), Alkaline Phosphatase (ALP), total bilirubin and total protein levels were measured using commercial diagnostic kits from Randox Company (UK) following the manufacturer's instructions.

Determination of lipid peroxidation (MDA) in liver tissues

The degree of lipid peroxidation in hepatic tissues was determined by measuring thiobarbituric acid reactive substances (TBARS) in the liver homogenate¹⁷. The absorbance was measured spectrophotometrically at 532 nm.

Determination of hepatic glutathione (GSH)

Reduced glutathione (GSH) was determined using the method of Ellman GL¹⁸ based on its reaction with 5,5'-dithiobis (2-nitrobenzoic acid) to yield the yellow chromophore, 5-thio-2-nitrobenzoic acid at 412 nm.

Determination of hepatic total nitrite concentration

Total nitrite was measured according to the method described Moshage et al.¹⁹ using Griess reagent (sulfanilamide and N-1-naphthylethylenediamine dihydrochloride) in acidic medium.

Histological Analysis

Liver specimens were excised and stored in 4% paraformaldehyde in phosphate buffer overnight, embedded in paraffin wax at 57°C. Serial sections were cut at 4 µm using a Spencer 820 microtome. These sections were used for histopathological examination using H&E stains were performed.

Immunohistochemistry study of COX-2 and I κ B

Immunostaining of hepatic paraffin sections for detection of the abnormal immune reaction of different primary antibodies (COX-2 and I κ B) was performed using streptavidin-biotinylated horseradish peroxidase (S-ABC) method (Novolink Max Polymer detection system, Novocastra). The procedure involved the following steps: endogenous peroxidase activity was inhibited by 3% H₂O₂ in distilled water for 5 minutes, and then the sections were washed in Tris-buffered saline (TBS) (Sigma, T 5030-100 TAB, PH 7.6) for 10 minutes. Non-specific binding of antibodies was blocked by incubation with protein block for 5 minutes (Novocastra). Sections were incubated with rabbit polyclonal or mouse anti-rat primary antibodies diluted 1:500 for 1 hour at room temperature. Sections were washed in Tris buffer for 3 times each for 3 minutes, then incubated with biotinylated anti-rabbit IgG (Novocastra) for 30 minutes. This was followed by washing in Tris buffer for 3 times, each for 3 minutes, and then incubated with Novolink polymer (Novocastra) for 30 minutes. Then sections were washed in Tris buffer for 3 times, each for 3 minutes. Peroxidase was detected with working solution of Diaminobenzidine (DAB) substrate (Novocastra) for 10 minutes. Finally, sections were washed in distilled water for 10 minutes, nuclei were stained with Mayer's hematoxylin and sections were mounted in DPX. For negative control sections, the same procedure was followed with the omission of incubation in primary antibodies²⁰.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, Calif., USA), and all statistical comparisons were made by means of the one-way analysis of variance test followed by Tukey's test post hoc analysis. Results were expressed as mean \pm SE of the mean, and a *p*-value ≤ 0.01 was considered significant.

RESULTS

Effect of ALA & THQ either alone or combination on APAP liver toxicity

APAP administration exhibited a significant increase in Serum ALT, AST, LDH, ALP and total bilirubin levels ($P \leq 0.01$) compared to normal control values (Fig. 1), with a significant decrease in serum total protein level at $P \leq 0.01$. NAC alone, ALA and/or THQ either alone or in combination successively alleviated the changes in previously mentioned serum biochemical parameters (Fig. 2).

Data represented in Fig. 1 shows the effect of APAP on oxidative stress and antioxidant biomarkers, there were an increase in NO and MDA levels and decrease SOD and GSH levels compared to control rats ($P \leq 0.01$). Oral supplementation of NAC along with the APAP ameliorated the altered oxidative stress parameters. ALA and THQ combination treatment revealed the best results compared with NAC group or to each one alone.

Histopathological changes

Microscopic investigation of H&E-stained liver sections from the control rats demonstrated normal liver histoarchitecture. Liver sections of the APAP-intoxicated rats show multiple large foci of degenerated hepatocytes with vacuolated cytoplasm and pyknotic nuclei, it showed several widened areas of inflammation surrounding degenerated and hepatic cell necrosis.

NAC treatment markedly decreased the damage in rat liver and inflammatory cellular infiltration when compared with The APAP control rats. Liver section from rat received APAP and ALA shows also marked improvement of hepatic cells degeneration with few cells with minimal cytoplasmic degeneration, liver section from rat received APAP and THQ shows small foci of hepatic cells with moderate cellular degeneration. Section of liver from rat received APAP, ALA and THQ shows a marked improvement of hepatic degeneration which restricted to few hepatocytes with mild vacuolation of their cytoplasm while the nuclei appear normal mimic to control cells (Fig. 3A-F).

Effect of ALA & THQ either alone or combined on flt-1 and COX-2

The immunohistochemical staining of the liver with anti flt-1 and anti COX-2 primary antibody revealed normal weak immune staining of the cytoplasm of few hepatic cells and cells in the endomysium of liver from normal control (Fig. 4&5 A). APAP-administered rats showed the diffuse strong positive immune reaction of the nuclei of the inflammatory cells in the endomysium and the cytoplasm of degenerated liver cells (Fig. 4&5 B). Co-administration of APAP with NAC, ALA, THQ and combined group showed a marked decrease of the immune reaction, especially the hepatic cells cytoplasm (Fig. 4&5 C-F).

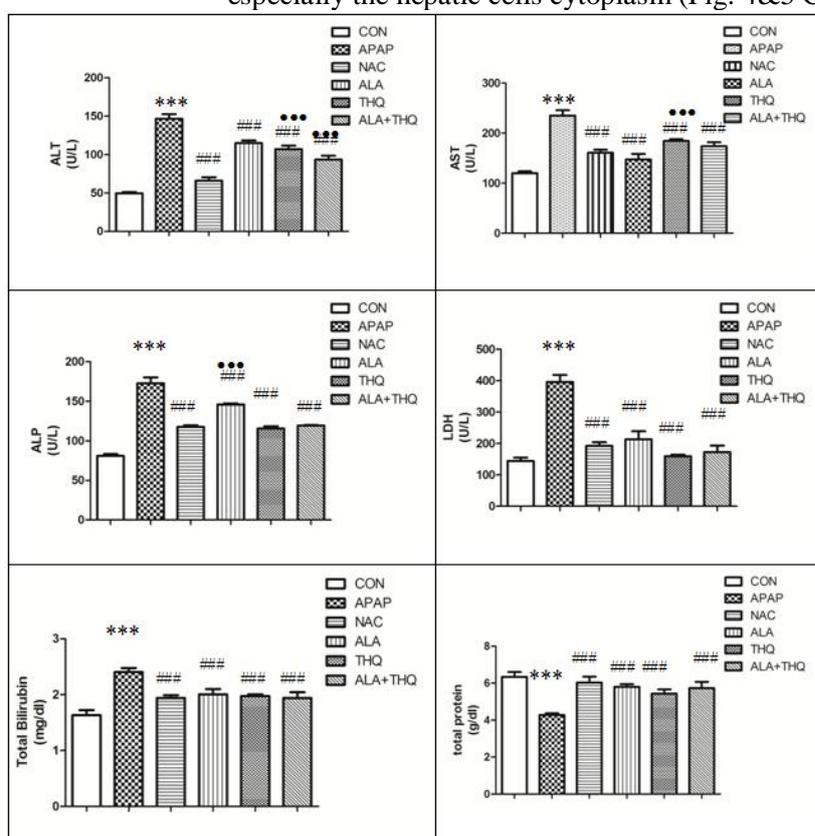


Fig. 1 Effect of NAC, ALA and THQ on serum biochemical parameters levels in APAP intoxicated rats.

Notes: Data are mean \pm SEM (n=6). *** P <0.01 vs control, ### P <0.01 vs APAP group and ● vs NAC group.

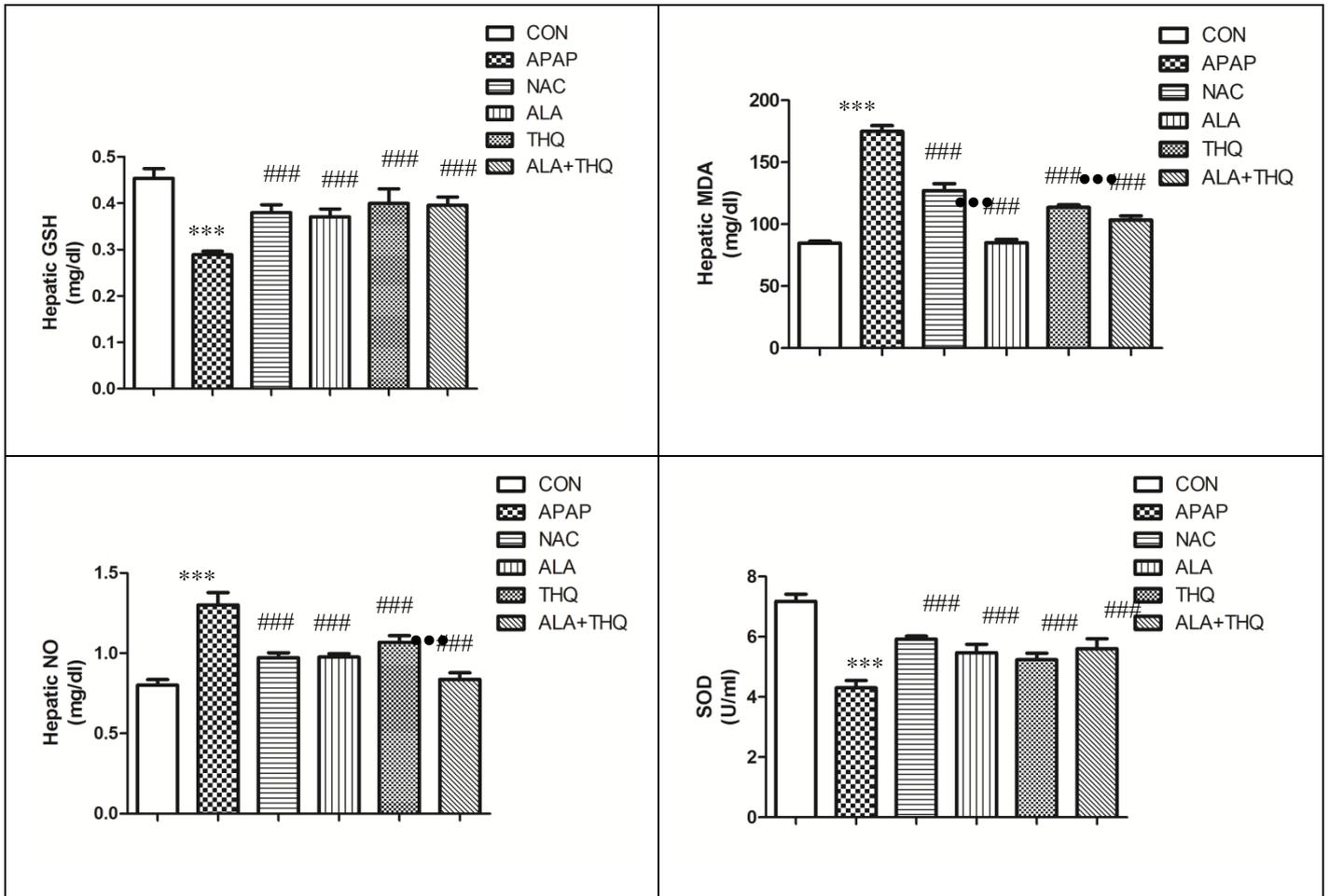


Fig. 2 Effect of NAC, ALA, and THQ on hepatic biochemical parameters levels in APAP intoxicated rats. **Notes:** Data are mean \pm SEM (n=6). *** $P < 0.01$ vs control and ### $P < 0.01$ vs APAP group.

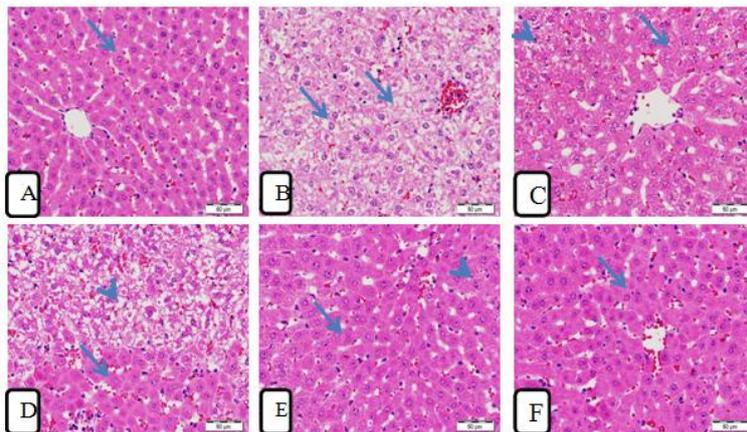


Fig. 3: light photomicrograph of a liver section stained with H&E. Scale bar 50 μ m. (A) liver section from control rat showing normal hepatic architecture with normal hepatocytes (arrow) and blood sinusoids. (B) Liver section from rat received APAP shows massive cytoplasmic degeneration of the hepatocytes that appeared vacuolated (arrows) with the loss of hepatic lobular architecture. (C) Liver from rat received APAP and NAC shows a marked improvement of hepatocellular degeneration (arrow) with the restoration of normal lobular architecture. There are few mildly degenerated hepatocytes (arrowhead). (D) Liver section from rat received APAP and THQ shows also improvement of most of the degenerated hepatocytes (arrow) and lobular architecture, but still there are small areas of degenerated hepatocytes (arrowhead). (E) Liver section from rat received APAP and ALA

shows a marked renewal of the degenerated hepatocytes (arrow) with few mildly degenerated cells (arrowhead). (F) Liver section from rat received APAP and combination of THQ and ALA shows normal hepatic lobular architecture and hepatocytes mimic to control section (arrow).

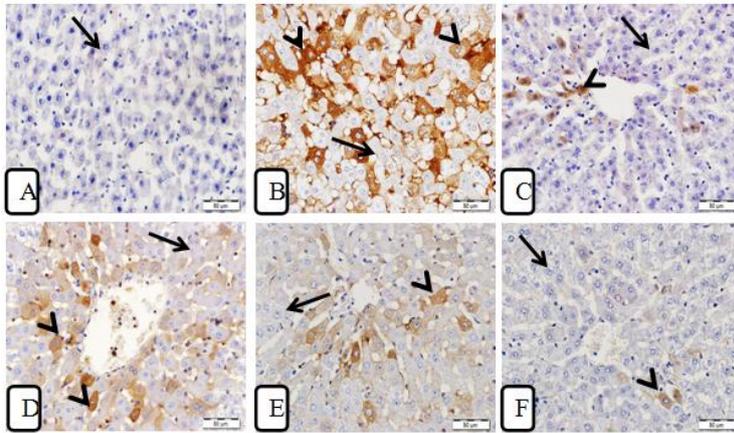


Fig. 4: light photomicrograph of liver sections Immuno-stained with the anti flt-1 primary antibody. Scale bar 50 μ m. (A) liver section from control rat shows the normal absence of immune reactivity (arrow). (B) liver section from rat received APAP shows a marked increase of the strongly immunostained cells ‘ cell membrane and cytoplasm’ (arrowheads) between few unstained hepatocytes (arrow). (C) liver section from rat received APAP and NAC show few immuno-positive hepatocytes (arrowhead) between unstained normal cells (arrow), (D) liver section from rat received APAP and THQ shows a moderate decrease of the density and number of immunostained cells in the center-lobular zone (arrowheads) between unstained cells (arrow). (E) Liver section from rat received APAP and ALA showing many hepatocytes with immuno-negative reaction (arrow) while immune positive cells are less with weak positivity (arrowhead). (E) Represent liver section from rat received APAP and combination of THQ and ALA shows apparently normal unstained hepatocytes (arrow) with few scattered weak immuno-stained cells (arrowhead).

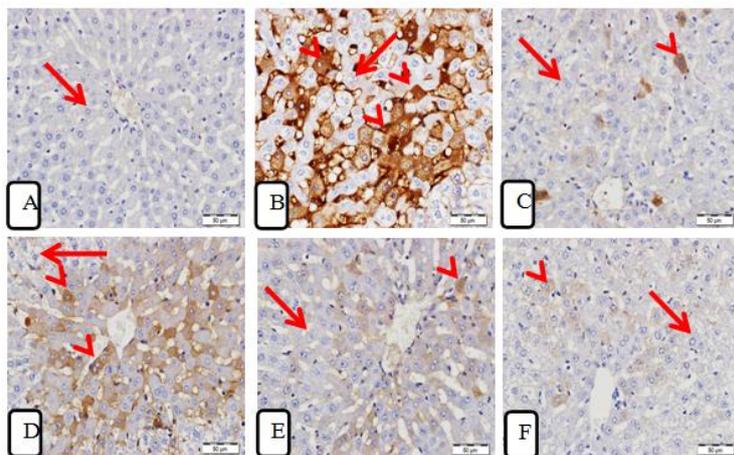


Fig. 5: light photomicrograph of liver sections Immuno-stained with the anti COX-2 primary antibody. Scale bar 50 μ m. (A) liver section from control rat shows the normal absence of immune reactivity (arrow). (B) liver section from rat received APAP shows a marked increase of the strongly immunostained hepatocytes (arrowheads) between few unstained hepatocytes (arrow). (C) liver section from rat received APAP and NAC show few immuno-positive hepatocytes (arrowhead) between many unstained normal cells (arrow), (D) liver section from rat received APAP and THQ shows a moderate decrease of the density and number of immunostained cells in the center-lobular zone (arrowheads) between unstained cells (arrow). (E) Liver section from rat received APAP and ALA shows many hepatocytes with immuno-negative reaction (arrow) while immune positive cells are less with weak positivity (arrowhead). (E) Represent liver section from rat received APAP and combination of THQ and ALA shows apparently normal unstained hepatocytes (arrow) with few scattered very weak immuno-stained cells (arrowhead).

DISCUSSION

Although APAP is considered safe at therapeutic doses, at higher doses, APAP produces a centrilobular hepatic necrosis that can be fatal. Today APAP poisoning accounts for approximately one-half of all cases of acute liver failure in the United States and Great Britain^{21,22}. APAP hepatotoxicity occurs by a complex mechanistic sequence. The reactive metabolite NAPQI which depletes glutathione by a conjugation reaction and covalently binds to proteins; loss of glutathione causes an increased oxidative stress response (decreased detoxification of reactive oxygen and nitrogen species); increased oxidative stress, possibly associated with alterations in calcium metabolism²³. Then initiation of signal transduction responses and mitochondrial permeability transition; which occurs with an even larger increase in oxidative stress, loss of mitochondrial membrane potential, however, the loss of ability of the hepatocyte mitochondria to produce ATP may be the most important event causing necrosis²⁴. In addition, there are a number of modulators of inflammatory responses that can alter the severity of liver injury following the initiation of toxicity^{25,26}. The interactions of these mediators with each other and the interplay of the immune cells that produce them will help to elucidate the significance of their roles in APAP toxicity²³. Apoptotic responses occur in conjunction with these inflammatory events^{27,28}. Finally, liver has a very great capacity to regenerate and various cytokines and growth factors are the major initiators of this process.

Gardner et al.²⁹ reported that toxic doses of APAP to rats induced hepatic-inducible nitric oxide synthase (iNOS) in the centrilobular hepatocytes. The development of toxicity is correlated with the expression of iNOS.

APAP-administration induced profound elevation of nitric oxide (NO) production and oxidative stress, as evidenced by increasing of lipid peroxidation level, reducing of SOD activity and depleting of intracellular GSH level in liver and kidney¹¹.

Moreover Prescott³⁰ reported that a dramatic increase in serum ALT and AST levels, mild hyperbilirubinemia, and increased prothrombin time are resulted from the biochemical changes after APAP administration. Moreover, the formation of superoxide and nitric oxide that react together to produce peroxynitrite, which again exhibits hydroxyl radical-like activity causing hepatotoxicity which is contributed by hepatic macrophages via different mechanisms³¹. These results are in accordance with those of the current work as APAP administration induced an elevation of liver biomarker enzymes while treatment with NAC and ALA and/or THQ either alone or in combination attenuate all the altered parameters. Our results are documented by those of Abdel-Zaher et al.¹¹ who reported that ALA downregulated the elevation of NO and oxidative stress, as evidenced by decreasing of lipid peroxidation level, reducing of glutathione peroxidase (GSH-Px) activity and increasing the depletion of intracellular GSH level in liver and kidney. These results provide evidence that the inhibition of NO overproduction and maintenance of intracellular antioxidant status may play a pivotal role in the protective effects of ALA against APAP-induced hepatic and renal damage. Moreover, our results are in accordance with that of Aycan et al.¹² who reported that co-administration of APAP with THQ serum ALT, AST levels were increased, whereas serum and tissue MDA levels were found to be lower compared to that of the APAP treated group. Histopathological analysis revealed a significant liver necrosis and toxicity with a high dose of APAP where THQ treatment significantly lowered liver injury scores.

Another anti-inflammatory mechanism that has been reported to play a role in APAP toxicity is cyclooxygenase-2 (COX-2)⁸. COX-2 is inducible and plays a role in anti-inflammatory processes. COX-2 generates prostaglandins are recognized for their critical role in female reproduction, bone resorption, renal function, and mucosal defense. Prostaglandins have been reported to play a protective role in various hepatotoxicities³². Reilly and coworkers⁸ showed that COX-2 was induced in livers of APAP-treated mice; it plays an anti-inflammatory role in APAP-induced hepatotoxicity⁸. In the present work immune stain with anti COX-2 revealed that APAP exhibits strong immune response while treatment with NAC and ALA and/or THQ either alone or in combination downregulates COX-2 expression. Mansour³³ reported that inflammation is also mediated by enzyme cyclooxygenase (COX) enzyme COX generates prostaglandins (PGEs) and thromboxane from arachidonic acid. Therefore, both PGEs and LTs function as the main mediators of allergy and inflammation. THQ inhibited both COX and lipoxygenase (LO) pathways of arachidonate metabolism in rats. Thus, inhibition of both COX and LO pathways is a key factor mediating the anti-inflammatory effects of THQ. Also Hegazy³⁴ supported our work and reported that ALA was effective in minimizing age-related oxidative burden. ALA reduced the levels of the pro-inflammatory cytokines IL-1, IL-6, and TNF- α , in addition to inhibiting the activity of COX-2.

VEGF induces the expression of anti-apoptotic proteins in human endothelial cells, suggesting that it promotes the survival of these cells. VEGF mRNA transcripts and protein are expressed by other cell types as well, including hepatocytes³⁵. VEGF has two primary receptors, VEGFR1 (flt-1) and VEGFR2. Data suggest that VEGFR1 may have probiotic effects. Activation of VEGFR1 in vitro resulted in significant increases in the release of mitogens by endothelial cells³⁶. These mitogens included the known hepatocyte mitogens: hepatocyte growth factor (HGF) and IL-6. Hepatic VEGFR1 (flt-1) level was increased 30-fold in mice treated with APAP and followed the onset of toxicity⁹. Moreover, treatment with ALA inhibits VEGF expression by blocking the superoxide anion, causing a decrease in vascular leakage and tissue edema reduction. Taken together, edema formation is the result of synergism between various inflammatory mediators that increase vascular permeability and/or the mediators that increase blood flow³⁷.

Paarakh³⁸ reported that THQ downregulate the expression of VEGFR1. The present results revealed that administration of NAC and ALA and/or THQ either alone or in combination along with APAP downregulates flt-1 expression.

Further studies are needed to examine the effect of inflammatory mediators on oxidative stress and/or signal transduction responses in APAP toxicity. Also, we can conclude that the administration of a combination of ALA and THQ is considered as a safe antidote for APAP toxicity.

CONCLUSION

It was concluded that the natural antioxidants such as ALA and THQ, may be considered as a potential antidote in combating liver injury induced by APAP.

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