Original Article

The Potential Protective Effect of Curcumin and α-Lipoic Acid on N-(4-Hydroxyphenyl) Acetamide-induced Hepatotoxicity Through Downregulation of α-SMA and Collagen III Expression

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Ahlam Alhusain[1], Laila Fadda[1], Wedad Sarawi[1], Hatun Alomar[1], Hanaa Ali[2], Raeesa Mahamad[3], Iman Hasan[1], and Amira Badr[1,4]

Abstract

Background and objectives: N-(4-hydroxyphenyl) acetamide (NHPA) is the most commonly used analgesic and antipyretic agent worldwide; however, it remains the leading cause of drug-induced acute liver failure. This study explored the potential impact of curcumin (Curc) and/or α-lipoic acid (Lip acid) on liver damage induced by NHPA overdose.

Materials and Methods: Male Wistar rats were intoxicated with a single oral dose of NHPA (1000 mg/kg) and treated with Curc (200 mg/kg p. o.) and/or Lip acid (100 mg/kg i. p.). These treatments were given in 2 doses at 2 hours and 10 hours post-NHPA-administration. Animals were sacrificed 24 hours post-NHPA-administration.

Results: Treatment with Curc and/or Lip acid showed effective reduction of NHPA-induced liver injury, demonstrated by reducing serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, as well as hepatic nitric oxide and malondialdehyde. Curc and/or Lip acid treatments counteracted these changes. They also ameliorated NHPA-induced centrilobular hepatocellular necrosis, evidenced by histopathological examination. Moreover, Curc and Lip acid reduced the expression of alpha-smooth muscle actin and collagen III, upregulated by NHPA intoxication in response to oxidative stress and inflammation.

Discussion and Conclusion: Curc and Lip acid can be considered as promising natural therapies against liver injury, induced by NHPA, through their antioxidant and antifibrotic actions.

Keywords

N-(4-hydroxyphenyl) acetamide, α-lipoic acid, curcumin, α-smooth muscle actin and collagen III

Introduction

N-(4-hydroxyphenyl) acetamide (NHPA) is the most prevalently consumed analgesic and antipyretic agent worldwide. When taken excessively, it initiates oxidative stress and generates highly reactive oxygen intermediates, which may represent a core cause of NHPA-induced liver injury.[1] It has been suggested that the generation of reactive oxygen species (ROS) appears to be an earlier event that precedes intracellular glutathione depletion and cell damage. The superoxide formation promotes peroxynitrite generation and protein nitration that may further result in oxidative damage to proteins, DNA, and lipids.[2] Once

[1]Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
[2]Department of Genetics and Cytology, National Research Centre, Dokki, Egypt
[3]Department of Anatomy, College of Medicine, King Saud University, Riyadh, Saudi Arabia
[4]Department of Pharmacology and Toxicology, College of Pharmacy, Ain Shams University, Cairo, Egypt

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Corresponding Author:
Amira Badr, Pharmacology and Toxicology Department, Pharmacy College, King Saud University, Saudi Arabia, Riyadh 11451, Saudi Arabia.
Email: Amibadr@ksu.edu.sa

oxidation begins in one of these macromolecules, it can trigger endless oxidative events in other cellular components due to their complex interactions. In addition, both NHPA and its dangerous metabolite N-acetyl-p-benzoquinone imine (NAPQI) can interact with the mitochondria, thereby inducing depletion of mitochondrial glutathione content, uncoupling of the mitochondrial respiratory chain with electron leakage, decline in ATP content, and induction of DNA fragmentation, which ultimately leads to hepatocyte necrosis.[3]

N-acetylcysteine (NACS), a precursor of glutathione (GSH), is still considered the best antidote for NHPA overdose and is used to prevent depletion of hepatic GSH.[4,5] It has been used in clinical practice for several decades due to its beneficial effects in reducing many pathological events, including NHPA intoxication, respiratory distress syndrome, heavy metal toxicity, chemotherapy-induced toxicity, and psychiatric disorders through its antioxidant action.[6] However, NACS-administration may induce multiple side effects and requires prompt and careful dosing regimens.[7,8] Considering the hazard of drug resistance and the high cost of NACS, natural compounds have attracted researchers' interest in this field.[9]

Curcumin (Curc), an active component in rhizomes of *Curcuma longa* species, has antioxidative, anticarcinogenic, anti-inflammatory, antihyperlipidemic, and hypoglycemic properties.[10] It can also remarkably attenuate the severity of CCl_4-induced liver fibrosis by inhibiting the transforming growth factor-β (TGF-β1)/α-Smad3 signaling pathway.[11] Furthermore, potent inhibitory activities of Curc have been reported on nuclear factor kappa B (NF-kB) activation[12,13] and expressions of some oncogenes such as phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), mitogen-activated protein kinases, extracellular signal-regulated kinase, and inducible nitric oxide synthase (iNOS).[12]

Alpha-lipoic acid (Lip acid) is a naturally occurring antioxidant compound with recognized beneficial effects on chronic inflammatory diseases such as obesity and diabetes.[14] It can be synthesized in the human body and acts as an essential cofactor for various multienzyme complexes in the mitochondria. It has been described that this cofactor deficiency results in an overall disturbance in the antioxidant defense network, leading to increased inflammation, insulin resistance, and mitochondrial dysfunction.[15] It has a scavenging activity to ROS and a capacity to regenerate endogenous antioxidants such as GSH, and vitamins C and E.[16] It is also useful in preventing hepatic oxidative stress, down-regulating the expression of hepatic pro-inflammatory cytokines, as well as inhibiting NF-κB expression. A recent study revealed the modulatory action of Lip acid on the course of infection through its antioxidant, anti-inflammatory, and antiviral properties.[17]

Intoxication with NHPA, either in high doses or excessive chronic consumption of this drug, even in regular doses, can trigger several catastrophic events in hepatocytes. These damaged cells activate the immune cell infiltration that causes trans-differentiation of hepatocytes into collagen-producing myofibroblast-like cells marked by alpha-smooth muscle actin (α-SMA) expression.[18-20] Progressive accumulation of extracellular matrix (ECM) can destroy a large portion of liver tissue and possibly lead to liver fibrosis. Based on that, this study was designed to determine whether the combination therapy of Lip acid and Curc can ameliorate NHPA hepatotoxicity compared to NACS and also to elucidate the mechanisms of action underlying their actions and their effects on α-SMA and collagen III. This was achieved by measuring oxidative stress, inflammatory markers, and protein expression of α-SMA and collagen III.

Materials and Methods

Materials

N-(4-hydroxyphenyl) acetamide, Curc, NACS, and Lip acid were purchased from Sigma Chemical Co., St Louis, MO, USA. NHPA and all antioxidants were dissolved in 1% carboxymethylcellulose (CMC).

Animals

Thirty-six healthy male Wistar albino rats (150–190 g) were supplied by the Experimental Animal Center, College of Pharmacy, and King Saud University. Animals were kept under standard temperature and humidity and allowed 1 week to acclimatize to the lab environment. Standard chow pellets and water were available *ad libitum*. Animal utilization protocols were performed according to the guidelines of the Animal Care and Use Committee at King Saud University and approved by the King Saud University Ethical Committee; Ethics Reference No. is KSU-SE-20-5.

Experimental Design

One week after acclimation, 36 rats were randomly divided into 6 groups (n = 6) as follows: Group 1—normal control rats that received one dose of 1% CMC; while 30 rats intoxicated with a single oral dose of NHPA 1000 mg/kg[21] were then allocated into Group 2—untreated rats; Group 3—NACS-treated rats were administered 20 mg/kg oral dose of NACS[22]; Group 4—Curc-treated rats that received 200 mg/kg of Curc orally[23]; Group 5—Lip acidtreated rats that received 100 mg/kg of Lip acid intraperitoneally[24]; and Group 6, a combination of Curc with Lip acid-treated rats that received both natural compounds at the previously mentioned doses. Curc and Lip acid treatments were given in 2 doses at 2 and 10 hours post-NHPA administration.

Twenty-four hours after the NHPA dose, rats were euthanized using gradual concentrations of CO_2, and blood samples were collected for serum separation. Some liver sections from each group were kept in 10% formalin for histopathological and immunohistochemical examinations. Other liver sections were stored at −80°C for molecular analysis.

Biochemical Serum Analysis

Serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total protein, and

bilirubin were performed using diagnostic kits obtained from Randox Laboratories (Crumlin, UK).

Determination of Hepatic Oxidative Stress and Antioxidant Biomarkers

The degree of lipid peroxidation in hepatic tissues was determined by measuring thiobarbituric acid reactive substances (TBARS)[25] that reflected the level of malondialdehyde (MDA). The absorbance was measured spectrophotometrically at 532 nm. Reduced GSH was determined using Ellman's[26] (1959) method at 412 nm. Total nitrite was measured according to the method described by Moshage (1995) using Griess reagent.[27] Superoxide dismutase (SOD) activity was assayed according to the methods described previously by Marklund, S. and Marklund, G.[28]

Histopathology and Immunohistochemistry

Following sacrifice, samples from the livers of the control and treated groups were fixed in 10% neutral buffered formalin for 24 h. The fixed samples were used to prepare 4-µm thick paraffin sections, which were subsequently stained with hematoxylin and eosin (H and E) to analyze the liver's structure. Other sections from the liver were processed for the immunohistochemical detection of α-SMA (ab124964) and collagen III (ab34712). Briefly, the sections were impeded by immersion in a 3% hydrogen peroxide (H_2O_2) solution for 5 min. After washing in Tris-buffered saline (TBS; pH 7.6) for 10 min, the slides were incubated with protein block (Novocastra, UK) for 5 min to block the non-specific binding of antibodies. The sections were examined with rabbit polyclonal anti-α-SMA (1:1000 dilution) and collagen III (1:200 dilution), washed in TBS 3 times, and afterward probed with biotinylated IgG (Novocastra, UK) for 30 min. After washing in TBS, diaminobenzidine (DAB) substrates were added, and the sections were counterstained with Mayer's hematoxylin. The sections were mounted and examined. Negative control slides were processed through the same steps but without the primary antibody. The slides were analyzed and visualized by a Nikon microscope.

Statistical Analysis

The statistical analysis was performed using Graph-Pad Prism 8 (Graph-Pad Software, San Diego, CA, USA). Data were expressed as mean ± SEM for quantitative measures, and the differences between the groups were determined by one-way analysis of variance (ANOVA), followed by the TukeyKramer multiple comparisons test.

Results

Elevated serum activity of liver enzymes is considered a sign of liver diseases, including liver toxicity. NHPA-administration led to a significant increase in serum ALT, AST, and ALP activity levels compared to those of the control group. The use of NACS, Curc, and/or Lip acid significantly reduced the toxic effects of NHPA on those enzymes, as shown in Figure 1; however, all antioxidants were not able to completely restore the average enzyme level as in the control rats.

Moreover, NHPA overdose caused a significant reduction in the total protein level and a marked increase in the level of total bilirubin (Figure 2). Treatment with NACS or Curc and Lip acid, alone or in combination, mostly restored the normal level of total protein. Total bilirubin was significantly reduced after using Curc, or Lip acid, or their combination. Surprisingly, NACS, the standard treatment, failed to cause a significant decrease in the level of total bilirubin, and showed less beneficial effects than the natural antioxidants. Interestingly, the combined therapy of Curc and Lip acid showed a significant reduction ($P \leq .01$) of the total bilirubin level compared to NACS. Likewise, antioxidants markedly increase the level of total protein (Figure 2).

When assessing the oxidative stress markers, rats exposed to NHPA overdose exhibited a marked reduction in the hepatic SOD activity and GSH levels compared to control rats. Rats

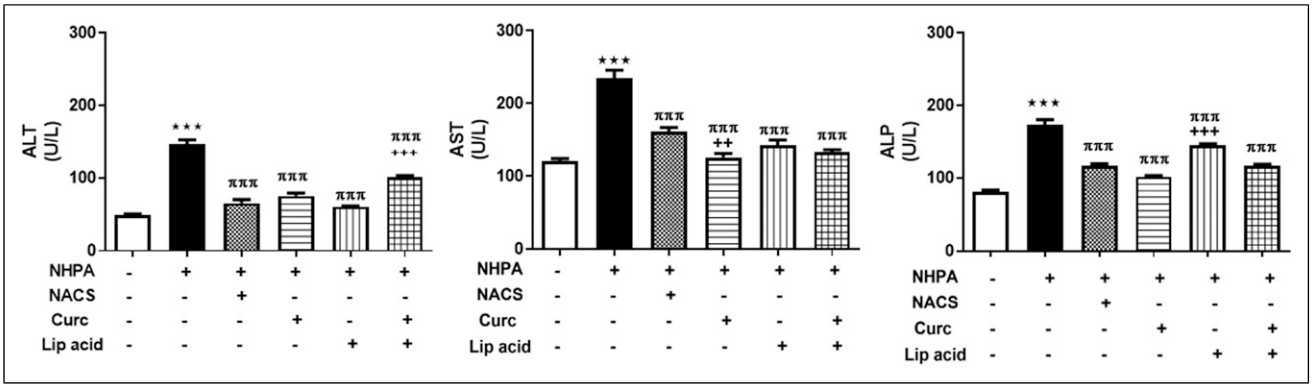

Figure 1. Effect of N-acetylcysteine, Curc, and/or Lip acid on serum transaminases (alanine aminotransferase and aspartate aminotransferase) and alkaline phosphatase in N-(4-hydroxyphenyl) acetamide-intoxicated rats. Data are mean ± SEM (n = 6). $^{\star\star\star}P \leq .001$ vs control, $^{\pi\pi\pi}P \leq .001$ vs the N-(4-hydroxyphenyl) acetamide group, and $^{+++}P \leq .001$ and $^{++}P \leq .01$ vs N-acetylcysteine group.

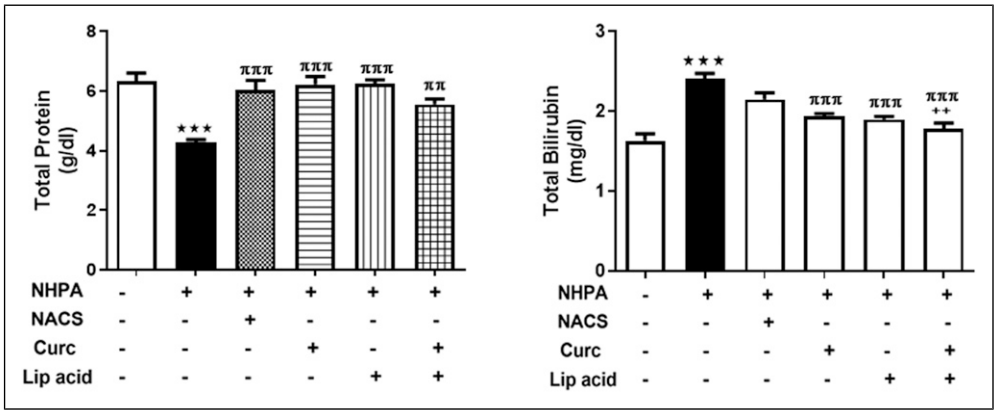

Figure 2. Effect of N-acetylcysteine, Curc, and Lip acid, alone or in combination, on total protein and total bilirubin after N-(4-hydroxyphenyl) acetamide intoxication. Data are mean ± SEM (n = 6). $^{\star\star\star}P \le .001$ vs control, $^{\pi\pi\pi}P \le .001$ and $^{\pi\pi}P \le .01$ vs the N-(4-hydroxyphenyl) acetamide group, and $^{++}P \le .01$ vs the N-acetylcysteine group.

Table 1. Hepatic Levels of GSH, SOD, NO and MDA in Control, NHPA-Administered Group, and all Treated Groups.

Group	GSH (μ/g wet tissue)	SOD (U/mg protein)	NO (nmol/100 mg)	MDA (U/mg protein)
Control	.45 ± .021	167.24 ± 1.9	.8 ± .035	84.75 ± 1.5
NHPA	.29 ± .007***	112 ± 1.12***	1.3 ± .077***	175 ± 4.6***
NACS	.38 ± .016 $^{\pi}$	131 ± 1.08 $^{\pi\pi\pi}$	.97 ± .032 $^{\pi\pi\pi}$	127.1 ± 1.5 $^{\pi\pi\pi}$
Curc	.36 ± .019	142 ± .76 $^{\pi\pi\pi+}$	.77 ± .03 $^{\pi\pi\pi+}$	107.1 ± 1.54 $^{\pi\pi\pi++}$
Lip acid	.33 ± .016	141 ± 1.3$^{\pi\pi\pi+++}$	1.1 ± .043 $^{\pi\pi\pi++}$	138.6 ± 3.4 $^{\pi\pi\pi}$
Curc+Lip acid	.36 ± .024	153 ± 1.7 $^{\pi\pi\pi+++}$	.89 ± .028 $^{\pi\pi\pi}$	91.5 ± 2.8 $^{\pi\pi\pi+++}$

Data are mean ± SEM (n = 6). $^{\star\star\star}P \le .001$ vs control, $^{\pi\pi}P \le .05$, and $^{\pi\pi\pi}P \le .001$ vs NHPA group, $^{+}P \le .05$, $^{++}P \le .01$, and $^{+++}P \le .001$ vs NACS group.

treated with NACS exhibited a significant elevation ($P \le .05$) of GSH level vs NHPA group, while administration of Curc and Lip acid, either alone or in combination did not produce a significant change in the GSH level (Table 1). It was noted that the Lip acid, alone or when combined with Curc, showed a highly significant increase in SOD level compared to NACS-treated rats ($P \le .05$, $P \le .01$). On the other hand, the hepatic levels of NO and MDA were significantly increased in the NHPA-administered group compared to those of the control group; NACS, Curc, and Lip acid treatments ameliorated the increased levels of NO and MDA compared to those of the NHPA-treated group ($P \le .001$). The combination therapy showed an apparent reduction in MDA level more than other treatments ($P \le .001$) (Table 1).

The histopathological examination further confirmed the hepatotoxic effect of NHPA. While the control rats showed normal liver histology after H&E staining (Figure 3A). Liver sections of NHPA-intoxicated rats showed hepatic degeneration and pyknotic nuclei besides other pathological features in comparison to normal liver sections, as shown in (Figure 3B). NACS, Curc, and Lip acid, either alone or in combination, clearly prevented hepatic damage as shown in (Figures 3C-3F).

Immunostaining of α-SMA antibody showed that the NHPA-treated rats expressed a significant immunoreactivity signal in the cytoplasm of hepatic cells after exposure to NHPA in comparison to the untreated group (Figures 4A and 4B). The rats treated with NACS, Curc, Lip acid, or combination showed various degrees of a moderate reduction in α-SMA expression, whereas the Lip acid treated group demonstrated the lowest α-SMA immunoreactivity signals (Figures 4C-4F). On the other hand, collagen III deposition significantly increased in the liver of NHPA-treated rats (Figures 5A and 5B) relative to controls. This deposition markedly decreased after treating rats with NACS, Curc, Lip acid, or combination (Figures 5C-5F). Interestingly, concurrent treatment with Lip acid and Curc expressed a comparable reduction in collagen III depositions relative to those observed among NACS-treated rats.

Discussion

The current study assessed the hepatoprotective effects of Curc and Lip acid, either singly or concurrently, on the rat model of acute NHPA-induced liver toxicity. The toxicity of NHPA is strongly associated with its metabolism inside the body. Typically, it metabolizes to yield NAPQI, which has direct toxic effects on the liver and kidney.[29] NAPQI eventually depletes the endogenous glutathione due to the

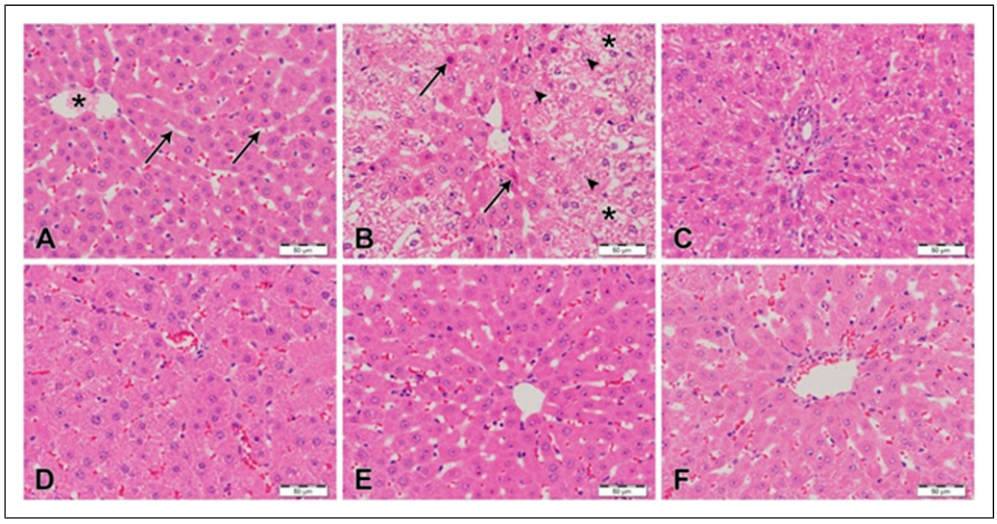

Figure 3. Representative photomicrographs of H and E-stained liver sections. (A) Sections from the control rat showed normal structure of liver and hepatocytes (arrows). (B) Sections of liver from rats that received NHPA exhibited massive diffuse necrosis of hepatocytes, loss of normal hepatic architecture with pyknosis or karyolysis of nuclei (arrows and arrowheads), and the cytoplasm of these cells revealed marked vacuolization (asterisks). Treatment with N-acetylcysteine (C), Curc (D), Lip acid (E), or combination of Curc and Lip acid (F) markedly ameliorated almost all histopathological lesions that occurred post N-(4-hydroxyphenyl) acetamide overdose. Scale bar, 50 μm.

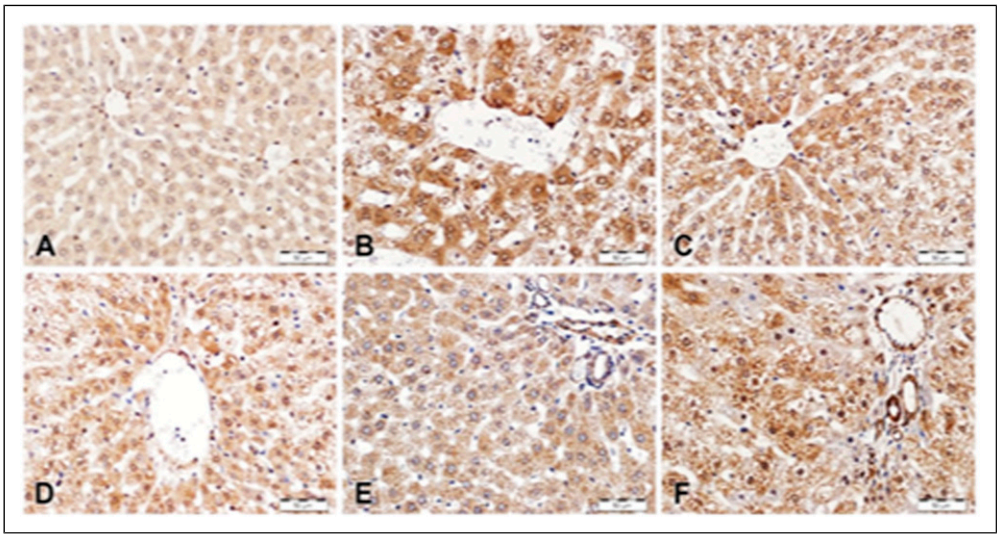

Figure 4. Immunoreactive signal of alpha-smooth muscle actin in rat liver. (A) Control rats showed negative immunostaining. (B) N-(4-hydroxyphenyl) acetamide-treated rats revealed strong immunoreaction in both the cytoplasm and nuclei of almost all hepatocytes. Treatment with N-acetylcysteine (C), Curc (D), Lip acid (E), or combination of Curc and Lip acid (F) caused a reduction in alpha-smooth muscle actin immunoreactivity in hepatocytes. Scale bar, 50 μm.

increasing oxidative stress and generation of reactive species. These alterations facilitate the opening of the mitochondrial membrane transition pore (MTP), leading to cellular dysfunction, hepatic toxicity, and consequent cell death.[30]

Concerning liver function, the current study revealed acute hepatocellular damage and inflammation after NHPA overdose as it markedly induced the serum level of liver function parameters including ALT, AST, and ALP. A high level of these enzymes indicates loss of hepatocellular integrity and leakage of cell contents into the bloodstream. It has been shown that the supratherapeutic dose of NHPA significantly induced elevation of ALT and AST levels.[31] Whereas, using NACS or Curc and Lip acid alone or in combination attenuated the increase in those parameters without any significant differences between single and combined therapies.

Oxidative stress has been implicated in the pathology of hepatic injury following excessive doses of NHPA. Herein, NHPA induced significant depletion in hepatic GSH content

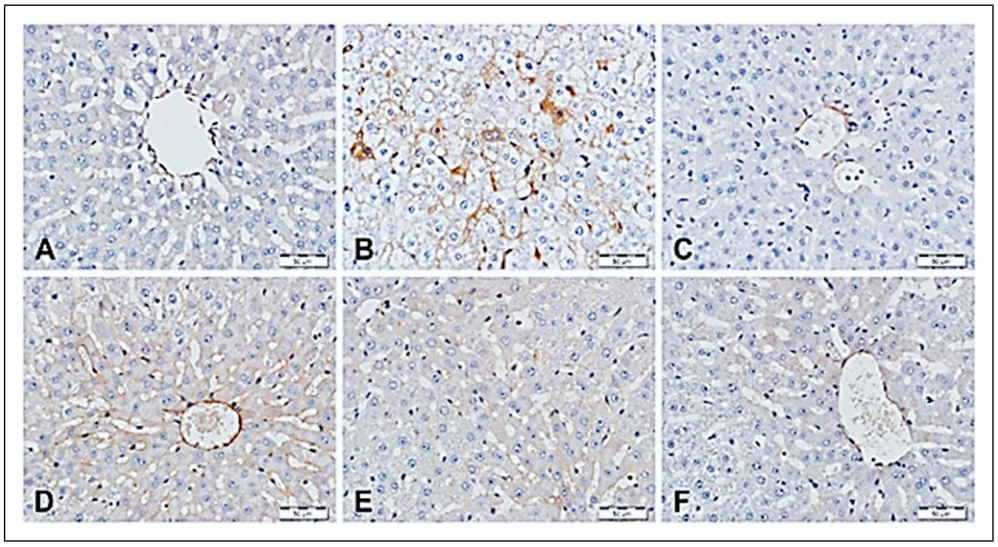

Figure 5. Immunoreactive signal of collagen III in rat liver. Sections of liver from (A) control rats, immunostained to demonstrate collagen III fibers, showed slight immunopositive fibers in the central vein wall. (B) N-(4-hydroxyphenyl) acetamide-treated rats showed a marked increase of immunopositive collagen III fibers in between hepatocytes. (C, D, E, and F) treated with N-acetylcysteine, Curc, Lip acid, or combination of Curc and Lip acid showed a reduction of immunopositive collagen III fibers. Scale bar, 50 μm.

and SOD activity compared with the control, while it caused an increase in hepatic NO and MDA levels. Combined treatments with Curc and Lip acid showed more reduction of MDA in comparison to a single treatment. Our results were parallel with the results obtained by Mamdouh and colleagues, which revealed a marked reduction in the hepatic GSH level and SOD activity in NHPA-intoxicated rats.[32] GSH depletion has been linked to a decline in cellular defense mechanisms against ROS-induced injury, leading to necrotic cell death.[32] Moreover, Aycan et al[33] (2015) reported that NO and MDA levels were higher in the renal tissues of the NHPA group than controls. In parallel with our results, Roy et al's[34] (2015) study reported that NHPA administration downregulated SOD activity and GSH level, whereas MDA levels were elevated. Treatment with Lip acid and/or Curc successfully opposed the changes in the levels of the previously measured parameters and restored the oxidant/antioxidant balance, but without further superiority of combined treatments.

Furthermore, a single high dose of NHPA is associated with a significant elevation in α-SMA production in the cytoplasm of hepatic cells. α-SMA is a reliable marker of hepatic stellate cell activation that precedes fibrous tissue deposition, and it can be used to identify the earliest stage of hepatic fibrosis and monitor the efficacy of the therapy.[35] According to the literature, increase in this type of actin is associated with activation of ECM synthesis that results in liver fibrosis.[36] Treating animals with the glutathione precursor (NACS) or antioxidants (NAC, Curc, Lip acid, or combination of Curc and Lip acid) post-NHPA exposure caused a moderate reduction in the synthesis of α-SMA in various responses, especially among the Lip acid-treated group. These antioxidants exhibit moderate hepatoprotective properties against activated myofibroblast-like hepatocytes induced by a single high dose of NHPA via reduction of the synthesis of α-SMA.

The antioxidant understudy decreased collagen III deposition and fibrotic tissue formation in the liver of NHPA-treated rats. This effect was more pronounced among the group treated with the combination of Curc and Lip acid, in which the result of the reduction in collagen III deposition was comparable to that observed among the group that received the standard treatment of NHPA toxicity (NACS). Collagen types I, III, IV, and V are the predominant collagens detected in the liver; collagen types I, III, and V are mostly interstitial ECM proteins in the portal and central regions, whereas collagen IV is highly detected in basement membranes.[37] Increased collagen III deposition in the hepatocytes and the degree of fibrosis are directly related to the myofibroblast activation in addition to the persistence of hepatocytes necrosis.[38,39]

As the liver is responsible for synthesizing many proteins such as albumin and globulin, which play vital roles in blood homeostasis, immunity, and substance transportation, an NHPA overdose can cause a reduction in the total protein levels, thus increasing the total bilirubin. Similarly, other conditions such as inflammation, infection, or acute damage can also decrease total protein levels, which explains, at least in part, the link between NHPA toxicity, inflammation, and the deterioration of liver function. Treatment with NACS, or Curc and Lip, acid alone or in combination, restored the normal levels of total protein and bilirubin. Curc and Lip acid were superior to NACS in controlling bilirubin elevation. Curc can alter various proteins such as albumin, growth factor receptors, and other important biomolecules.[40] It also possesses anti-inflammatory action and inhibitory effects against reactive oxygen-generating enzymes such as xanthine

dehydrogenase/oxidase, lipoxygenase/cyclooxygenase, and iNOS.[41] It has been shown that Curc directly interacted with several molecular proteins, including inflammatory molecules, cell survival proteins, protein kinases, and reductases, glyoxalase I, and DNA methyltransferases-1.[42]

Lip acid is a natural component that can be synthesized in the human body and acts as an essential cofactor for various multienzyme complexes in the mitochondria. It has been revealed that Lip acid can maintain high vitamin C levels, and participate in vitamin E-recycling, thus complementing some of the functions of GSH.[37] The protective effect of Lip acid can be partially attributed to its mitochondrial protective effects through its antioxidant activity, which could decrease the level of ROS. Additionally, Lip acid may also increase cellular cysteine levels by enhancing cystine uptake from the plasma. In a previous study on NHPA-induced renal dysfunction,[43] it has been revealed that NHPA toxicity significantly elevated serum inflammatory biomarkers including tumor necrosis factor (TNF-α) and interleukin-1 β (IL-1β); and the anti-inflammatory effects of Curc and Lip acid were confirmed by their decreasing expression levels.

Conclusion

Our study reveals that Cur and Lip acid, alone or in combination, represent promising hepatoprotective effects that can aid clinically in cases of hepatotoxicity, especially toxicity with NHPA. The combination is more recommended as based on the results of this study, the combination of Curc and Lip acid shows greater protection as compared to individual drugs. Future studies are still needed to confirm their efficacy clinically. We also recommend examining the combination therapeutic value in experimental models of liver fibrosis based on their inhibitory effect on α-SMA and collagen III.

Author Contributions

Conceptualization: L.F. and A.A.; methodology: I.H.H., A.A., R.M., and W.S.; validation: A.A., L.F., H.A. A.B., and I.H.H; data analysis: I.H.H.; writing original draft preparation: A.A., L.F., and H.M.A.; writing review and editing: A.A., H.A., W.S., and A.B.; and supervision: A.A. and L.F.

Declaration of Conflicting Interests

The author (s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Availability of Data and Materials

The data supporting the findings of the article is available with Dr Ahlam Alhusaini.

ORCID iDs

Ahlam Alhusain https://orcid.org/0000-0003-2905-8882
Laila Fadda https://orcid.org/0000-0003-3910-6462
Hatun Alomar https://orcid.org/0000-0001-7495-0592
Hanaa Ali https://orcid.org/0000-0002-6870-7585
Amira Badr https://orcid.org/0000-0003-3983-868X

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