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Liposomal Coenzyme Q10 abates inflammation, apoptosis and DNA damage induced by an overdose of paracetamol in rat's liver

Ahlam Alhusaini[a], Laila Fadda[a], Leena Albogamy[b], Noura Alnaim[b], Wedad Sarawi[a], Dareen Mattar[c], Iman Hasan[a]

[a] Department of Pharmacology and Toxicology, Pharmacy College, King Saud University, Riyadh – KSA
[b] Pharmacy College, King Saud University, Riyadh – KSA
[c] Department of Physiology, College of Medicine, Umm AlQura University, Makkah, Saudi Arabia.

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L-CoQ10 counteracts NHPE-induced liver injury

Authors mails:

Dr. Ahlam M. Alhusaini Mail: aelhusaini@ksu.edu.sa

Prof. Laila M Fadda Mail: faddalolo@yahoo.com

Miss. Leena Albogamy Mail: leena21f@gmail.com

Miss. Noura Alnaim Mail: Nerah238@hotmail.com

Dr. Wedad Sarawi Mail: wsarawi@ksu.edu.sa

Dr. Dareen Mattar Mail: dsmattar@uqu.edu.sa

Dr. Iman H. Hasan Mail: ihasan@ksu.edu.sa

Corresponding author

1-Dr. Ahlam M. Alhusaini
Department of Pharmacology and Toxicology
Pharmacy College
King Saud University
Mail: aelhusaini@ksu.edu.sa

Abstract

Objectives: Paracetamol (PCM) is widely used for its pain-relieving and antipyretic properties. However, acute intoxication of PCM remains one of the most common causes of drug-induced hepatic failure. A comparative study is conducted to evaluate the effectiveness of coenzyme Q10 (CoQ10) with that of liposomal CoQ10 (L-CoQ10) against PCM -induced liver injury.

Method: Acute liver injury was induced by a single oral dose of PCM (1000 mg/kg). CoQ10 and L-CoQ10 treatments were given orally (10 mg/kg), 1 and 12 h following PCM administration. Specific oxidative stress, and inflammatory and apoptotic biomarkers were measured and proved by histopathological analysis.

Results: PCM administered rats exhibited a remarkable increase in the levels of serum aminotransferase enzymes, interleukin-6, and C-reactive protein as well as hepatic malondialdehyde (MDA), nitric oxide (NO); whereas reduced glutathione (GSH) level and superoxide dismutase (SOD) activity were decreased. BAX, Nuclear factor-kappa B (NF-κB) and cytochrome C were overexpressed, while BCL-2 was downregulated. Histopathological examination of liver tissue supported the previous biochemical markers. Using either CoQ10 or L-CoQ10 caused a significant reduction of oxidative stress by enhancing GSH and SOD activity levels and diminishing MDA and NO levels. Moreover, both CoQ10 and CoQ10 caused downregulation of pro-inflammatory cytokines and BAX, and upregulation BCL-2, and these effects mostly noticed in L-CoQ10 treated rats. Likewise, rats receiving L-CoQ10 exhibited restoration of normal hepatic architecture.

Conclusion: L-CoQ10 supplement is useful for counteracting the hepatotoxicity induced by PCM overdose, through its antioxidant, anti-inflammatory and anti-apoptotic activities.

Key words: Paracetamol, L-CoQ10, Cytochrome C, BAX, and BCL-2.

1. Introduction

Paracetamol (PCM) is a nonprescription analgesic and antipyretic agent. It has an excellent safety profile; however, PCM toxicity is one of the most common drug toxicity worldwide [1]. PCM overdose can result in acute hepatotoxicity which is characterized by fatal massive hepatocyte necrosis if left untreated [2]. The hepatic injury induced by PCM is attributed to its metabolism, as the main pathway of PCM metabolism is through cytochrome P450-mediated oxidation to produce a metabolite, N-acetyl-p-benzoquinone imine (NAPQI) which is directly toxic to the liver [3]. When PCM is taken in therapeutic doses, this potentially dangerous metabolite is rendered harmless by the endogenous antioxidant, namely reduced glutathione (GSH). Nevertheless, excessive intake of PCM can increase the rate of NAPQI formation, deplete the stored GSH in the liver and eventually lead to liver dysfunction [4].

N-acetylcysteine (NACC) is the mainstay treatment of PCM toxicity used to hinder the rapid depletion of hepatic GSH as one of the main precursors for GSH synthesis thus, NACC supports the detoxification process and prevents the NAPQI accumulation [5]. For these reasons, NACC therapy is still considered the best therapeutic option for patients administered an overdose of PCM [6]. However, NACC can cause common side effects: nausea, vomiting, and diarrhea. Intravenous NACC administration is associated with anaphylactoid reactions; urticaria, pruritus, hypotension, headache, angioedema and bronchospasm [7], [8]. In order to manage these reactions, additional therapies like antihistamines and steroids are required, which add further burden on the liver. Hence, receiving this antidote within hours after PCM ingestion is essential because it is less effective for late-presenting patients [9], [10].

Moreover, prolonged administration of NACC may slow liver regeneration and recovery after acute PCM overdose [10]. An intravenous NACC regimen has a high potential for medication errors, particularly in the frequency, dosing and infusion rate[11]. Therefore, it is crucial to discover therapeutically potent, effective and safe compounds that can prevent PCM overdose-induced hepatotoxicity.

Coenzyme Q10 (CoQ10), ubiquinone, is an endogenous lipid-soluble benzoquinone compound that functions as a diffusible electron carrier in the mitochondrial respiratory chain [12]. In recent years CoQ10 gained significant research attention as a dietary supplement capable of generating cellular bioenergy and counteracting the damaging effects caused by free radicals [13]. The protective effects of CoQ10 have been extensively studied as it lessens the occurrence and progression of metabolic illnesses such as cardiovascular, hepatic, metabolic and neurodegenerative diseases [14]–[16] as CoQ10 deficiency has also been reported in such diseases [17].

Recently, pharmaceutical sciences have developed liposomal drugs to improve drug properties, including drug tissue targeting, biocompatibility, and conjugation with proteins, peptides, and DNA [18]. Liposomes are nano-phospholipid bubbles that can diffuse through cell membranes and deliver their contents into cell cytoplasm [19]. [3] For these reasons, the present study was conducted to evaluate the efficiency of liposomal-CoQ10 (L-CoQ10) to counteract the hepatotoxicity induced by PCM overdose in rats. Additionally, the possible mechanisms underlying this effect were also investigated.

2. Materials and methods

2.1 Drugs

PCM powder was purchased from (Sigma Chemical Co., USA); CoQ10 (Nutra Manufacturing Inc., Greenville, USA) and L-CoQ10 were obtained from Lipolife (Boreham, UK), and it is CoQ10 encapsulated into liposomes with nano-sized vesicle (less than 200 nm) made from a phospholipid to ensure maximum absorption. PCM was prepared in normal saline and stabilized by 1% carboxymethylcellulose (CMC); both CoQ10 and L-CoQ10 were suspended in 1% CMC.

[96] 2.2 Animals and treatments

Wistar albino rats weighing 200 ± 10 g were obtained from the Bio-Resource Unit, College of Pharmacy, King Saud University. [39] The animals were kept in standard conditions (24 ± 1 °C, 45 ± 5% humidity and 12 h light/dark cycle) and fed with standard laboratory

chow and water ad libitum. They left for at least one week before starting the experiments to acclimatize to the lab environment.

The rats were randomly divided into four equal groups (n = 6). The rats in the first group received a single oral dose of 1% CMC and served as a control group. Hepatotoxicity was induced in animals of the second, third and fourth groups by PCM given at a single oral dose of 1000 mg/kg [20]. The third and fourth groups received either CoQ10 or L-CoQ10 (10 mg/kg, orally) at 1 and 12 h following PCM administration (Fouad and Jresat, 2012) [24]. [21].

The experimental protocol was approved by the Animal Care Committee at King Saud University (Ethical number [KSU-SE-20-58]). All experimental procedures were performed in accordance with international guidelines for the care and use of laboratory animals.

2.3 Sample preparation and biochemical studies

Twenty-four hours after PCM administration, the rats were sacrificed under ketamine/xylazine anesthesia (0.1ml/100g rat wt. i.p.). Blood samples were collected and centrifuged to obtain clear sera, which were subsequently stored at −80 °C. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured colorimetrically according to the manufacturer's procedure (Randox Laboratories Ltd., UK).

The livers were excised carefully, washed with ice-cold saline, stored at −80 °C, and then homogenized in phosphate buffer (0.05 M, pH 7.4). The homogenates were centrifuged at 3000 rpm for 10 min at 4 °C, and the resulting supernatants were used to determine malondialdehyde (MDA), GSH and nitric oxide (NO) levels, and superoxide dismutase (SOD) activity. The levels of interleukin-6 (IL-6) and C-reactive protein (CRP) in liver homogenates were determined by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's guidelines (R&D Systems, USA).

2.4 Determination of hepatic MDA, GSH, SOD and NO

Hepatic MDA was assayed as previously described [R]. Briefly, the liver tissue homogenate was mixed with TBA, SDS, and acetate buffer, then heated for one hour. Then, n-butanol was was added to the mixture, centrifuged, and the absorbance of the organic

layer was measured at 532 nm.[20] GSH was determined based on the method of Ellman [R]. In breif, GSH reacts with 5,50-dithio-bis (2-nitrobenzoic acid) to give yellow-colored product that reads at 412 nm.[51] SOD activity was determined in accordance with the method of Marklund and Marklund [R], in which the pyrogallol autoxidation was examined in the presence of EDTA in PH ranging from 7.9 to 10.6,[7] and the activity of SOD was assayed based on its ability to inhibit autoxidation of pyrogallol.

2.5[7] Western blot analysis for nuclear factor kappa B (NF-κB) and cytochrome C

Western blotting was utilized to determine the protein expression of NF-κB (ab16502) and cytochrome C (ab133504). The liver sample was homogenized in RIPA buffer accompanied by proteinase/phosphatase inhibitors, centrifuged, and the supernatant was collected. Protein concentration was assayed using Bradford protein assay kit (BioBasic, Markham, Canada), and 60 µg protein was subjected to 10% SDS/PAGE and electrotransferred to nitrocellulose membranes which subsequently blocked by 5% milk. The membranes were probed with primary antibodies against NF-κB, cytochrome C, and β-actin overnight at 4 ◦C. The membranes were washed multiple times with TBST, then probed with secondary antibodies, washed again, and developed using Clarity™ Western ECL Substrate from BIO-RAD (Hercules, CA, USA).[11] Protein bands were visualized using the ECL-Plus detection system (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions.[11] Positive immunoreactive bands were quantified densitometrically by ImageJ and compared with control.

2.6 BAX and BCL-2 mRNA expression

Changes in the gene expression of BAX and BCL-2 were determined by RT-PCR. Briefly, RNA was isolated from the frozen liver tissues using TRIzol (ThermoFisher Scientific, USA). Following treatment with RNase-free DNase (Qiagen, Hilden, Germany), RNA was quantified using a nanodrop. RNA with OD260/OD280 nm ratio of ≥ 1.8 were reverse transcribed into cDNA. The produced cDNA was improved using PCR master mix (Qiagen, Hilden, Germany) and the primer pairs listed in Table 1.

The PCR products were loaded in 1.5% agarose gel, electrophoresed, and the bands were visualized using UV transilluminator. The images were analyzed by ImageJ, and the values were normalized to β-actin.

2.7 DNA fragmentation

DNA samples were fragmented by ultrasound using Covaris S220 (Covaris, Woburn, MA, USA) in Snap-Cap micro TUBEs with sample (130 µl). The settings used for targeted fragmentation were set up according to the original manufacturer's protocol. Briefly, the tissue samples were lysed and centrifuged to produce fragmented DNA (supernatant) and intact chromatin (pellet). The samples were treated with diphenylamine, and the absorbance of the developed color was measured at 600 nm.

2.8 Histopathological examination of liver tissue

Liver tissue samples from each animal were fixed in 4% formalin, dehydrated, and embedded in paraffin wax. Sections were cut at 4 µm, stained with hematoxylin and eosin (H&E) and visualized under a light microscope.

2.9 Statistical analysis

All values were expressed as mean ± SEM. The results were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test for multiple comparisons using SPSS 11.0. Differences were considered significant at p 0.05.

3. Results

3.1 CoQ10 and L-CoQ10 improved liver functions in PCM-induced hepatotoxicity

The current work revealed that rats who received PCM overdose showed liver injury manifested by the cellular leakage and elevation in serum AST and ALT (Fig. 1). However, concomitant administration of CoQ10 or L-CoQ10 significantly lowered liver enzymes, particularly the ALT, to a level close to controls.

3.2 CoQ10 and L-CoQ10 attenuated oxidative, inflammatory and apoptotic damage in PCM-induced hepatotoxicity

Further assessment of the hepatic protective effects of CoQ10 was conducted by measuring the levels of oxidative, inflammatory and apoptotic markers.

3.2.a. Oxidative markers

Both MDA and NO were significantly increased ($P \leq 0.001$), whereas GSH level and SOD activity were decreased following PCM intoxication, thus indicating high oxidative damage. The ingestion of CoQ10 or L-CoQ10 ameliorated the toxic effects on PCM on the previous parameters (Fig. 2). L-CoQ10 showed a better antioxidant response than CoQ10.

3.2.b. Inflammatory markers

The expressions of the inflammatory biomarkers, IL-6 and CRP, were upregulated following PCM overdose but reduced in CoQ10 treated groups apparently with the liposomal form (Fig. 3). Moreover, acute intoxication with PCM in rats significantly induced the hepatic phospho-NF-κB and cytochrome C expression ($P \leq 0.001$) relative to control rats. On the contrary, rats who received the antioxidants in question with PCM revealed a reduction in the expression of phospho-NF-κB and cytochrome C, notably in L-CoQ10 treated rats (Fig. 4).

3.2.c. Apoptotic markers

In parallel with the previous findings, PCM overdose provoked apoptosis by causing a significant increase in BAX but decreased Bcl-2 gene expression in the liver of intoxicated rats (p 0.001). Nevertheless, the use of CoQ10 and L-CoQ10 showed a remarkable reduction in apoptosis by downregulating BAX and upregulating Bcl-2 gene expression (Fig. 5). Further analysis of the anti-apoptotic efficacy of CoQ10 and L-CoQ10 was determined by assessing the DNA fragmentation. Upon visualization of the electrophoretic pattern under UV light, a smeared DNA signal was detected in the liver of PCM intoxicated rats, indicating damaged cells, while intact genomic DNA from viable cells was detected at the top of the gel in the controls and after antioxidant treatment (Fig. 6).

3.3 CoQ10 and L-CoQ10 prevented the histopathological changes after PCM overdose

The hepatoprotective effect of CoQ10 and L-CoQ10 was confirmed by the histological examination of liver sections stained with H&E. PCM -intoxicated rats showed congested central vein with eosinophilic plasma surrounded by degenerated and vacuolated hepatocytes; however, the treated rats with CoQ10 apparently showed normal central vein that surrounded by normal hepatocytes however few hepatocytes still showed cytoplasmic degeneration. While liver sections from rats receiving L-CoQ10 restored normal architecture of hepatic lobules and central vein, almost all hepatocytes appear of normal histological features (Fig. 7).

4. Discussion

CoQ10 represents one of the most consumed dietary supplements, which gained a growing interest in the global healthcare trend due to its potent antioxidant activity. Therefore, we predict that CoQ10 is a valuable candidate for preventing drug-induced liver damage. The current study demonstrated that CoQ10 and L-CoQ10 at a dose of 10 mg/kg significantly reverse the acute liver failure induced by PCM toxicity. The liver has a complex and vital role in carbohydrate, protein and fat metabolism. It detoxifies many metabolic wastes, toxins and drugs to less harmful products or excretes them into bile. Additionally, it has a central role in albumin production and immunological responses. Injured hepatocytes leak certain chemicals in a range higher than average amounts, including liver enzymes. PCM overdose exhibited a marked increase in serum AST and ALT activities that indicated liver injury. It has been shown that elevated liver enzymes above the upper range of the normal are associated with increased liver-related mortality [22], [23]. The current study revealed that treatment with either CoQ10 or L-CoQ10 retained the serum levels of these enzymes to normal after acute PCM hepatotoxicity. As previously reported, intraperitoneal administration of CoQ10 markedly protected rat liver against acetaminophen-induced liver toxicity [24]. CoQ10 also normalizes the hepatic enzymes after thioacetamide hepatotoxicity [25]. In a double-blind placebo-controlled

trial, the daily use of CoQ10 supplement significantly declined the serum level of aminotransferases and CRP in non-alcoholic fatty liver disease (NAFLD) patients [26].

Oxidative stress is the primary event of PCM-induced liver damage in which the scavenging of free radicals, e.g., reactive oxygen species (ROS), is reduced due to impairment in cellular energy metabolism in mitochondria, transition metals such as iron or copper, calcium homeostasis and adenosine triphosphate depletion [2], [24]. Oxidation of one cellular component can initiate endless oxidative events; for example, lipid peroxidation products, like MDA, could disrupt the phospholipid membrane leading to oxidation of other cellular components, including proteins, glucose and DNA, causing abnormalities in their structures and functions. PCM also activates Kupffer cells to release several inflammatory cytokines and signaling molecules, including NO, which is involved in the pathogenesis of PCM hepatotoxicity [27]. Excessive NO production causes peroxynitrite radical formation depletes intracellular GSH and extends the oxidative damage to cell macromolecules[3]. In this work, PCM significantly reduced the endogenous GSH level and SOD activity but increased MDA and NO levels reflecting oxidative stress. Fortunately, treatment with CoQ10 and L-CoQ10 effectively modulated the oxidative stress by increasing the hepatic levels of GSH and SOD and reducing MDA and NO levels. CoQ10 acts as a powerful antioxidant that scavenges free radicals and prevents the initiation and propagation of lipid peroxidation in cellular biomembranes [28], [29]. Moreover, treatment with CoQ10 considerably suppressed lipid peroxidation and prevented the reduction of GSH and catalase activity [24].

To further investigate the mechanisms beyond PCM hepatotoxicity, some important inflammatory cytokines and apoptotic markers were measured. Liver inflammation is another pathological consequence of PCM overdose. This effect was evidenced by PCM-induced IL-6, CRP and phospho-NF-κB levels. It was previously reported that PCM administration could activate Kupffer cells to increase IL-6, TNF-α and CRP levels in mice[30]. In addition, PCM overdose causes NF-κB activation with subsequent inflammatory reactions responsible for hepatic injury [31]. NF-κB is a pleiotropic transcription factor that controls the release of pro-inflammatory cytokines in response to oxidative stress and has a role in cell growth and apoptosis. Elevated TNF-α is

known to be an essential step for activation of the NF-κB signaling pathway[1]. TNF-α is also linked to increased oxidative stress, ROS and reactive nitrogen species and is known to activate other inflammatory cells [32][3]. Blazka and coworkers showed remarkable increases in serum levels of TNF-α and IL-1α in mice treated with PCM [33][5].

Using of CoQ10 and L-CoQ10 significantly attenuated the expression of IL-6, CRP and phospho- NF-κB after the acute exposure to PCM overdose. This reduction was more profound in L-CoQ10 treated group than CoQ10 group. CoQ10 has anti-inflammatory properties as it decreases the production of pro-inflammatory cytokines [34][2]. A meta-analysis gave evidence about the effectiveness of CoQ10 on the plasma levels of CRP, IL-6 and TNF-α in patients suffering from inflammatory diseases including metabolic, autoimmune and cardiovascular diseases[2]. The hepatoprotective effect of CoQ10 can be attributed to its ability to inhibit the activation of the NF-κB signaling pathway that promotes the transcription of TNF-α and iNOS genes [32].

The mitochondrial apoptotic response is another mechanism of PCM-induced liver damage. Acute drug toxicity increased the expression of cytochrome C and BAX but reduced Bcl-2. BAX and Bcl-2 encode some proteins that act as pro- and anti-apoptotic regulators. The imbalance between BAX and Bcl-2 or high BAX/ Bcl-2 ratio is considered an adverse prognostic marker for liver damage [35]. Oxidative stress and inflammation can trigger activation of Bax, which increases mitochondrial membrane permeabilization, cytochrome C release and caspase-3 activation [28][0]. Concurrent treatment with CoQ10 and L-CoQ10 suppressed the expression of cytochrome C and restored the BAX and Bcl-2 balance which reduced cell death and improved the liver recovery after PCM overdose.[5]

Additionally, DNA fragmentation is a subsequent event for apoptosis that has been implicated in PCM-induced hepatotoxicity in which the rate of DNA fragmentation is linked to the development of hepatotoxicity [36]. By using in situ labeling assay, El-Hassan and others detected chromatin condensation and DNA fragmentation in mouse liver following PCM intoxication CoQ10 protected against DNA fragmentation induced by PCM overdose [37].

Likewise, histopathological examination supported these results showing congestion in the central vein with eosinophilic plasma surrounded by degenerated and

vacuolated hepatocytes in rats exposed to high dose PCM. In line with the previous study [24], the histopathological results revealed that CoQ10 treatment restored the normal architecture observed in the livers of control rats.

Taken together, this study demonstrates that L-CoQ10 has the ability to counteract the hepatotoxicity induced by PCM which is encountered by modulating the levels of serum IL-6 and CRP, hepatic cytochrome C and NF-κB proteins and hepatic gene expression of BAX. Furthermore, L-CoQ10 has superior actions over its native drug due to the ability of the liposomes to adhere to the cell membrane and release its content inside the cell, which makes it a candidate drug to be used for PCM intoxicated patients.

Author contributions

AA contributed to the experimental design and writing; LF contributed to the experimental design, experimental work, and writing; LA contributed to the experimental work; NA contributed to the experimental design and writing; WS contributed to revising the manuscript; DM contributed to the experimental design; and IH contributed to the experimental design, experimental work, statistical analysis, and writing.

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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Figure legands

Figure (1): Effects of CoQ10 and L-CoQ10 on serum liver function enzymes in PCM-induced hepatotoxicity in rats.

Notes: Data are presented as mean ± SEM (N=6). $^{***}P\leq 0.001$ vs control, $^{\pi\pi}P\leq 0.01$, $^{\pi\pi\pi}P\leq 0.001$ vs PCM-intoxicated group.

Figure (2): Effects of CoQ10 and L-CoQ10 on the hepatic oxidative stress markers in PCM-induced hepatotoxicity in rats.

Notes: Data are presented as mean ± SEM (N=6). $^{***}P\leq 0.001$ vs control, $^{\pi\pi}P\leq 0.01$, $^{\pi\pi\pi}P\leq 0.001$ vs PCM-intoxicated group and $^{+}P\leq 0.05$ vs to CoQ10 treated group.

Figure (3): CoQ10 and L-CoQ10 reduced hepatic levels of IL-6 and CRP after PCM -induced hepatotoxicity.

Notes: Data are presented as mean ± SEM (N=6). $^{***}P\leq 0.001$ vs control, $^{\pi\pi\pi}P\leq 0.001$ vs PCM -intoxicated group and $^{+++}P\leq 0.001$ vs to CoQ10 treated group.

Figure (4): CoQ10 and L-CoQ10 attenuated cytochrome C and NF-κB overexpression in the liver of PCM-intoxicated rats.

Notes: Data are presented as mean ± SEM (N=6). ***$P\leq 0.001$ vs control, $\pi\pi\pi P\leq 0.001$ vs PCM-intoxicated group, and $^{+}P\leq 0.05$, $^{+++}P\leq 0.001$ vs to CoQ10 treated group.

Figure (5): COQ10 and L-COQ10 modulated the BAX and Bcl-2 gene expression in the liver of PCM-intoxicated rats.

Notes: Data are presented as mean ± SEM (N=6). $^{***}P\leq 0.001$ vs control, $^{\pi\pi\pi}P\leq 0.001$ vs PCM-intoxicated group and $^{+}P\leq 0.05$, $^{+++}P\leq 0.001$ vs to CoQ10 treated group.

Figure (6): Qualitative and quantitative analysis of DNA fragmentation levels in control, PCM-intoxicated and all treated groups.

Notes: Data are presented as mean ± SEM (N=6). $^{***}P\leq 0.001$ vs control, $^{\pi\pi\pi}P\leq 0.001$ vs PCM-intoxicated group.

Figure (7): Representative photomicrographs of liver sections stained with H&E (x400) of control, PCM-intoxicated and treated rats. (A) Liver sections from control rats showing normal architecture of classic hepatic lobule with normal central vein (arrowhead) and normal hepatocytes (arrows). (B) Liver sections from rats received a toxic dose of PCM displays congested central vein with eosinophilic plasma (arrowhead) and degenerated and vacuolated hepatocytes (arrows). (C) Sections from rat treated with CoQ10 shows normal central vein (arrowhead) that surrounded by many normal hepatocytes except presence of few hepatocytes with cytoplasmic degeneration (arrow). (D) L-CoQ10 restored normal architecture of hepatic lobules and central vein (arrowhead), and almost all of hepatocytes appear of normal histological features (arrows).