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Empagliflozin attenuates cyclophosphamide-induced hepatotoxicity *via* targeting Nrf2/HO-1 signaling, oxidative stress, and inflammation

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ABSTRACT

Cyclophosphamide (CYP) is the most commonly used antineoplastic against numerous malignant tumors. Hepatic injury induced by CYP is a pivotal issue in clinical practice that limits its therapeutic use. In this study, CYP induced significant hepatotoxicity that is manifested by functional, biochemical, and histopathological alterations.This was linked with a significant increase of hepatic oxidative/nitrosative stress along with elevated hepatic contents of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), which seems to be among the key biomarkers that modulate the hepatotoxicity of CYP. Besides, inflammatory biomarkers; of tumor necrosis factor-α (TNF-α) and nuclear factor kappa B (NF-κB); significantly increased with significant multiple individual cell deaths and vacuolar degeneration in hepatocytes. Empagliflozin (EMP); a potent and selective SGLT2 inhibitor; possesses anti-inflammatory, anti-fibrotic, and antioxidant effects. EMP's co-administration with CYP in the current study induced a significant restoration of hepatocyte architecture which appears to be primarily mediated via modulation of Nrf2/HO-1 signaling and in turn attenuation of CYP-induced oxidative stress and inflammatory responses. In conclusion; EMP attenuated CYP-induced hepatotoxicity by modulation of Nrf2/HO-1 signaling pathway and consequent inhibition of oxidative stress and inflammation.

Keywords: Cyclophosphamide; Empagliflozin; Nrf2; HO-1; NF-κB; TNF-α

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INTRODUCTION

Cyclophosphamide (CYP) is an immunosuppressive and antineoplastic agent that is commonly utilized for the management of several malignancies as lymphomas and solid tumors and non-malignant disorders as multiple sclerosis and rheumatoid arthritis[1]. Regardless of its efficiency, CYP has been proven to induce hepatotoxicity[2], cardiotoxicity, teratogenicity, mutagenicity, and lung toxicity, which may restrict its therapeutic uses[3]. CYP is metabolized by the liver cytochrome P450 system to phosphoramide mustard and acrolein, a highly reactive metabolite, which initiates the generation of free radicals and can impede the cellular DNA synthesis leading to cell death[4]. In addition, acroleinbinds to the cellular antioxidants like reduced glutathione (GSH) causing the diminution of antioxidant defenses and the augmentation of lipid peroxidation [5].

CYP-induced reactive oxygen species (ROS) generation and oxidative stress are implicated in the triggering of the antioxidant response element (ARE) causing initiation of antioxidant genes to guard hepatocytes against oxidative stress. Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates ARE-driven antioxidant gene expression [6]. Nrf2 also participates vital in maintaining the normal function of hepatocytes as well as the pathogenesis of hepatic fibrosis, inflammatory responses, and carcinogenesis[7]. Under homeostatic conditions, Nrf2 is inhibited in the cytoplasm*as itubiquitinates* by Kelch like-ECH-associated protein 1 (Keap1) and Cullin 3. Upon cell exposure to oxidative stress, Keap1 is oxidized, which initiates the release of Nrf2 from Keap1- Cullin 3 complexes resulting in activation and translocation of Nrf2 into the nucleus. Then Nrf2 binds to the ARE and initiates antioxidant and cytoprotective gene expression[6, 8]. HO-1 is among the enzymes up-regulated following Nrf2 stimulation. HO-1 exhibits antioxidant and anti-inflammatory activities by inhibiting the release of pro-inflammatory mediators such as nuclear factor kappa B (NF-κB) in hepatocytes[9]. Therefore, activation of Nrf2 and its downstream effector HO-1 can augment the antioxidant defenses and frustrate the CYP-induced hepatic oxidative damage.

Empagliflozin (EMP); a selective sodium glucose co-transporter 2 (SGLT2) inhibitor; is a relatively new FDA-approved agent for the therapy of type 2 diabetes [10]. SGLT2 is mostly found in the apical brush border membrane of the S1 segment of the proximal convoluted tubules and controls 90% of the glucose re-absorption from the glomerular filtrate. Therefore, EMP can enhance renal glucose excretion and decrease blood glucose in an insulin-independent way through selective SGLT2 blockade[10]. Besides antihyperglycemic effects, EMP has been found to have pleiotropic activities as anti-inflammatory and antioxidant actions that make it a potential hepatoprotective agent[11]. As inflammation and oxidative stress have been displayed to be a chief mechanism in CYP-induced hepatotoxicity[12], we wanted to test if EMP will affect CYP-induced hepatotoxicity via these mechanisms. EMP has been reported to have anti-inflammatory and anti-fibrotic activities on diabetic nephropathy partly by suppressing ages-receptor axis [13]. Previous studies done in streptozotocin-induced type 1 diabetic rats have revealed that EMP can attenuate oxidative stress in pancreatic β-cells and aortic vessels along with the prevention of endothelial dysfunction in the aortic rings[14]. Also, the hepatoprotective effect of EMP was proven in many animal models[15, 16]. EMP ameliorated the characteristic alterations of hepatic steatosis, lipogenesis, and gluconeogenesis in OLETF rats [15]. While the effect of EMP against CYP-induced hepatotoxicity still to be clarified. Therefore, the aim of the current work was to examine the effect of EMP on CYP-induced hepatotoxicity in rats. Considering the fundamental roles of tumor necrosis factor-α (TNF-α), NF-κB, Nrf2 and HO-1 in the triggering of CYP-induced hepatotoxicity, the present investigation inspects the effect of EMP on the hepatic expression of these mediators as a potential underlying hepatoprotective mechanism.

MATERIALS AND METHODS

Animals: Adult male Sprague Dawley rats (200–220 g) were obtained from Vacsera center, Helwan, Egypt. Rats were adapted for 10 days before the experimentation. Rats were housed under typical environmental and nutritional conditions all over the investigational time and allowed free water and food access during the investigational time. All animal experimentations were in agreement with the ethical guidelines of "Research Ethics Committee of Faculty of Pharmacy, Mansoura University, Egypt".

Drugs and Chemicals: Empagliflozin (EMP; Behringer, Ingelheim, Germany) was suspended in DMSO 5% in water for injection (the vehicle) for oral administration. Cyclophosphamide (CYP), Endoxan vials (Baxter Oncology GmbH, Halle, Germany) and 0.9% (w/v) saline served as the vehicle for I.P. injection.

Experimental design: After the acclimatization period, the rats were randomly separated into four groups (n=6) and were treated as follows:

Group I (Normal): The rats were administered DMSO 5% in water for injection, orally for 2 weeks and a single dose of saline interaperitoneally on the 8th day.
Group II (EMP): The rats were treated with empagliflozin (EMP, 10 mg/kg/day, orally) for 2 weeks[17].
Group III (CYP): The rats were injected once with cyclophosphamide (CYP, 200 mg/kg, IP, dissolved insaline) on the 8th day[12].
Group IV (CYP/EMP): The rats were treated with empagliflozin (EMP, 10 mg/kg/day, orally) for 2 weeks[17]. On the 8th day, rats were injected once with cyclophosphamide (CYP, 200 mg/kg, IP, dissolved insaline)[12].

However, the dose of ***EMP*** was chosen depending on our preliminary experiments and prior report [15-17]. In addition, the dose of 10 mg/kg/day was previously reported to be efficient and to correspond to the equivalent active low dose in humans. Twenty four hours after the last EMP treatment, the rats were anaesthetized, and sacrificed by decapitation. The blood samples were collected for the assessment of serum liver enzymes. Liver was removed and washed in ice-cold physiological saline and weighed for calculation of hepatic/body indices and finally was used for biochemical and histopathological assays.

Biochemical assessment of serum biomarkers of hepatic injury: The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin and alkaline phosphatase (ALP) were estimated by means of commercial kits (Spinreact, Spain); as well as serum lactate dehydrogenase (LDH) activity was measured using Human assay kit (Germany).

Preparation of liver homogenate and biochemical assessment of oxidative/anti-oxidative stress biomarkers: Left lobes of livers were homogenized in phosphate buffer saline to prepare 10% tissue homogenate. Hepatic homogenate was centrifuged at 3000 × g for 15 min and stored at -80 °C until further examination. Oxidative/nitrosative stress biomarkers were colorimetrically determined in the homogenate including; malondialdehyde (MDA), total nitric oxide (NO) content and reduced glutathione (GSH) concentration using available Bio-Diagnostic (Giza, Egypt) assay kits as instructed by the manufacturer. In addition, hepatic antioxidant enzyme; superoxide dismutase (SOD); activity was colorimetrically determined in the homogenate using available Bio-Diagnostic (Giza, Egypt) assay kit.

Determination of heme oxygenase-1 (HO-1) and nuclear factor erythroid 2-related factor 2 (Nrf2)in liver tissue homogenates: HO-1and Nrf2 contents were measured in liver homogenates by means of ELISA kits; Code No. **E4525** (Biovision Inc.) and ab207223 (Abcam); respectively.

Determination of Assessment of NF-κB and TNF-α in liver tissue homogenates: Hepatic NF-κB and TNF-α contents were evaluated in tissue homogenates using ELISA kits; Code No. CSB-E13148r (Cusabio Biotech) and ERT2010-1 (Assaypro LLC), respectively.

Histopathological examinations: The right lobes of the liver were fixed at 10% buffered formalin solution. Then, tissues were sliced into two portions; one portion was stained with H&E, the other portion was stained with Masson's Trichrome for inspection of histopathological alterations.

Statistical analysis: Data are expressed as mean ± standard error of the mean (S.E.M.), and n=6. One way analysis of variance (ANOVA) followed by post *hoc*Tukey-Kramer test was used for statistical analyses of parametric data. Significant results were assumed at $p<0.05$. Statistical analyses were carried out using GraphPad Prism software (GraphPad Software Inc. Version 5, San Diego, California, USA).

RESULTS

Effect on hepatic/body index: CYP injection induced a significant increase in hepatic/body index by 23% compared to the normal group. Hepatic/body index was significantly decreased upon EMP administration in CYP/EMP group by 16.6% compared to the CYP group (figure 1).

Effect on serum indices of liver injury: CYP injection significantly raised serum levels of ALT, AST and ALP by 2.3-, 1.6- and 3-fold; and declined serum albumin level by 30% in comparison with normal group. **EMP** treatment for 2 weeks in CYP/EMP group produced an intense fall in serum levels of ALT, AST and ALP by 1.4-, 1.3- and 2.3-fold with an escalation in serum albumin level by 28% in comparison with the CYP group **(Table 1)**.

Serum LDH activity significantly elevated by 3.6-fold in the CYP group in comparison with the normal group. Significant decrease in serum LDH activity (by 2.4-fold) was observed in CYP/EMP group in comparison with CYP group **(table 1).**

Effect on hepatic oxidative/nitrosative stress biomarkers: Rats intoxicated with CYP in group III demonstrated a significant increase in hepatic content of MDA by 1.8-fold, concomitant with a significant decrease in hepatic content of GSH by

1.8-fold when compared to the normal group. In addition, CYP-administered rats showed significant decrease in hepatic SOD activity by 2.4-fold when compared to the normal group. Nevertheless, intervention with EMP significantly reversed these changes in CYP/EMP group when compared to CYP-treated rats **(table 2).**CYP-treated rats also showed significant impairment of liver nitrosative hemostasis, this was demonstrated by the significant increase in hepatic NO content by approximately 3.8-fold.Upon oral treatment with EMP, liver NO content significantly declined by 2.3-foldin CYP/EMP group **(table 2).**

Effect on hepatic HO-1 and Nrf-2 contents: Hepatic HO-1 and Nrf-2 contents were significantly decreased following CYP administration by 2.4- and 2.5-fold; respectively; when compared to the normal group as shown in **figures2A&2B**. EMP treatment significantly increased Hepatic HO-1 and Nrf-2 contents in group IV by 1.3- and 1.7-fold; respectively; when compared to the CYP group.

Effect on hepatic NF-κB and TNF-α contents: CYP-intoxicated rats showed significant upregulation of NF-**κ**B and TNF-**α** expression in hepatic tissue by 3.2-& 2.3-fold; respectively; when compared to the normal rats **(Figures 3A&3B)**. On the other hand, pretreatment of the CYP-intoxicated rats with EMP significantly down-regulated hepatic NF-κB and TNF-□α□ expression by 1.9-&1.4-fold; respectively; in comparison with the CYP group.

The effect of histopathology alterations in H&E stained liver specimen: H&E stained liver section srevealed normal parenchymain the normal group **(Figures 4A&4B)** and EMP-treated group **(Figures 4C&4D)**; multiple individual cell deaths (thin black arrows) with vacuolar degeneration in hepatocytes (thin red arrows) (**Figures 4E&4F**) with congested hepatic blood vessels in CYP-treated group (thick arrows) (**Figures 4G&4H**). Pretreatment with EMP restored normal liver structure in CYP/EMP-treated group **(Figures 4I&4J)**.

Effect on hepatic histopathology alterations in Masson's Trichrome stained liver specimen: Masson's Trichrome stained liver sections revealed thin blue fibrous tissue around central veins in the normal group (thin arrows) (**figures 5A&5B**), marked increase of the periportal fibrosis (thin arrows) (**Figures 5E&5F**) besides the presence of focal areas of degenerated hepatocytes (thick arrows) **Figures 5G&5H**) in CYP-treated group. Treatment with EMP showed normal liver structure in the EMP-treated group **(Figures 5C&5D)** and the CYP/EMP-treated group **(Figures 5I&5J)**.

DISCUSSION

CYP is an antineoplastic agent active against several malignancies, but the generation of ROS limits its use due to oxidative injury and toxicity predominantly in hepatic tissue[4, 5]. Numerous reports have dedicated that the agents with antioxidant and anti-inflammatory activities can alleviate CYP-induced hepatotoxicity [12, 18].In the current work, CYP-induced hepatotoxicity is manifested by the augmented serum activities of ALT, AST, LDH, and ALP as well as the decreased albumin levels. These data are in accordance with previous reports proving elevated liver functions in serum of CYP-treated rats [19, 20]. The augmented liver functions might be explained by the cell damage triggered by CYP-induced oxidative stress and inflammatory responses[21]. The assessed serum ALT, AST, albumin and other enzymes are indicators of hepatic damage as they originate in the cytosol of hepatocytes and escape into blood subsequent to cell damage [22]. Furthermore, the augmented ALP levels also reveal the damage of hepatocytes [19].

The CYP-induced hepatotoxicity was further supported by the histological alterations, including multiple cell deaths, vacuolar degeneration in hepatocytes with congested hepatic blood vessels and periportal fibrosis. Pretreatment of the CYP-intoxicated rats with EMP significantly recovered serum levels of liver functions and restored the normal hepatocyte structure, ascertaining the hepatoprotective and membrane stabilizing abilities of EMP. In view of that, studies have established that EMP decreased liver functions and preserve the normal liver architecture in a mouse model of the spontaneous atherosclerosis model [16] and in the *OLETF* rat model of type 2 diabetes [15].According to prior reports, oxidative stress represents the key cornerstone of CYP-induced hepatotoxicity [19, 20]. In the present research, CYP treatment has increased hepatic lipid peroxidation, MDA, and NO levels. These findings are in harmony with previous studies that proven involvement of lipid peroxidation and upregulation of iNOS leading to increase in MDA and NO levels; respectively; in CYP-induced hepatotoxicity [19, 20]. The released NO could interact with superoxide anions to yield the effective oxidant peroxynitrite, and initiate the synthesis of inflammatory cytokines *via* the NF-κB activation in Kupffer cells [23]. We also demonstrated that CYP administration produced depletion of GSH in the liver that could be caused by the direct conjugation of CYP and its toxic metabolites to GSH [24]. GSH has a vital function in preserving the cellular homeostasis, by neutralizing the hydroxyl radicals, or as a substrate for glutathione peroxidase [25]. The exhaustion of intracellular GSH content caused

by CYP represents amodification in the cellular redox state proposing that cells could be more responsive to reactive metabolites resulting in a decrease in the effectiveness of antioxidant defense[25]. For that reason, avoidance of GSH exhaustion could be a reasonable justification of the hepatoprotective effect of EMP. Pretreatment of CYP-intoxicated rats with EMP revealed a boost in the GSH content probably because of the antioxidant- and free radical-scavenging capacities of EMP.

Accordingly, CYP intoxication showed significant reduction in the hepatic SOD activity. SOD plays a vital role in protecting the body against the damaging effects of ROS and free radicals [26]. In the present study, pretreatment of CYP-intoxicated rats with EMP significantly restored the hepatic contents of the GSH, MDA and NO as well as hepatic SOD activity, which reflected that the hepatoprotective effect of EMP on CYP-induced hepatic injury may be based on its antioxidant properties. Ojima *et al.* (2015) specified that EMP diminished oxidative damage through decreasing AGE production as well as inhibiting the AGEs/RAGE (receptor for advanced glycation end products) axis [13]. As well, EMP was confirmed to reduce oxidative stress biomarkers in both the circulation and the aorta of diabetic mice [27]besides in the mitochondria of cardiac endothelial cells [28].

The escalation in activity of the antioxidant defense enzyme; SOD; and hepatic GSH level by EMP in the present investigation could be well interconnected to upregulation of the transcription factor Nrf2.Nrf2 functions as a sensor for oxidative stress and controls the induction of protective HO-1 gene expression that could protect the hepatocytes against the harmful effects of oxidative stress and encourage the hepatocyte survival [29]. CYP has been reported to down-regulate the Nrf2/ARE antioxidants signaling by targeting the activities of its downstream genes, NQO1 and HO-1 as a result of excessive production of ROS generated by CYP metabolism [9].Consequently, we speculated that this signal pathway may participate in the hepatoprotection of EMP against CYP-induced liver injury. Our results support previous studies where Nrf2 and HO-1 exhaustion was detected in CYP-intoxicated rats in comparison with normal rats. EMP significantly augmented the hepatic Nrf2 and HO-1 expression in CYP-intoxicated rats. Consequently, activation of Nrf2/HO-1 signaling pathway shows a crucial role in the hepatoprotective effect of EMP. Li *et al.*, (2019) have reported that EMP mitigates oxidative stress in the myocardium by stimulating Nrf2 to translocate to the nucleus and up-regulates Nrf2/HO-1signaling in a mouse model of type 2 diabetes mellitus [30]. Our work is the first to display up-regulation of Nrf2/HO-1 signaling by EMP in the hepatic tissues of CYP-intoxicated rats. Oxidative stress was also linked to inflammatory cytokine production and leukocyte infiltration after CYP administration [31]. The TNF-α is an inflammatory cytokine released mainly from macrophages and monocytes [32] and CYP intoxication was reported to markedly increase hepatic TNF-α gene expression and protein [9, 31] and this supported the results of the current study. The produced ROS could trigger NF-κB inflammatory pathway and therefore promote the hepatic TNF-α expression[33]. Nrf2 has been documented to antagonize NF-κB pathway [34] and Nrf2-deficient mice revealed great expression of NF-κB in response to inflammatory stimulation [35]. Our findings showed a significant rise in the hepatic expression of NF-κB and consequently TNF-α in CYP-intoxicated rats, indicating that EMP could counteract the CYP-induced hepatotoxicity as a result of attenuation of inflammatory mediator's expression. Increased Nrf2 and HO-1 expression might also intermediate EMP-induced inhibition of NF-kB and TNF-α as well as prevention of inflammation in the liver of CYP-treated rats. This suppression of NF-κB translocation to the nucleus may result from the stimulation/nuclear translocation of Nrf2. The Keap1/Nrf2/ARE signaling pathway has been displayed to be included in inhibition of NF-κB and its downstream target genes [36]. The current study proposes that EMP may protect against CYP-induced hepatotoxicity through modulation of the crosstalk between NF-κB and Nrf2.In support of our findings, EMP possessed anti-inflammatory activity *via* suppression of inflammatory mediators, including TNF-α, interleukin (IL) 6 and 1β in heart failure rats [37] and in ApoE−/− mice fed a western diet[16].

CONCLUSION

EMP *via* modulation of the Nrf2/ HO-1 signaling and the associated oxidative stress and inflammation-induced hepatic damage could protect against CYP-induced hepatotoxicity. Thus, EMP can be suggested to be an effective therapy to mitigate CYP-induced hepatotoxicity and maximize its associated therapeutic outcomes.

Conflict of interest: The author declares that there are no conflicts of interest

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Table 1:Effect of daily oral administration of EMP (10 mg/kg, orally) on CYP-induced changes in serum parameters of liver injury.

Groups	*Normal*	*EMP*	*CYP*	*CYP/EMP*
ALT (U/L)	45.2 ± 2.7	47.8 ± 3.3	102.3 ± 4 $	73.8 ± 3.2 $#
AST (U/L)	111.2 ± 2.7	117 ± 3.7	182.5 ± 3.8 $	135 ± 3.5 $#
ALP (U/L)	120 ± 3.9	120.2 ± 6.4	370 ± 29 $	161 ± 9.8 #
Albumin (g/dl)	3.8 ± 0.13	3.8 ± 0.06	2.7 ± 0.09 $	3.467 ± 0.04 $#
LDH (U/L)	1097 ± 82	1148 ± 79	4007 ± 245$	1688 ± 98 $#

Hepatic injury was induced by single injection of CYP (200 mg/kg, IP). EMP was administered (10 mg/kg, orally) for 2 weeks; 1 week priors and 1 week post CYP injection

Data are presented as mean ± SE. Statistical analysis was done using (ANOVA) followed by Tukey-Kramer test

$ Significantly different Vs normal (n=6; $p<0.05$)

Significantly different Vs CYP (n=6; $p<0.05$)

Table 2:Effect of daily oral administration of EMP (10 mg/kg, orally) on CYP-induced changes in hepatic oxidative/nitrosative parameters.

Groups	*Normal*	*EMP*	*CYP*	*CYP/EMP*
MDA content (nmol/g tissue)	35.19 ± 2	32.77 ± 3.04	94.85 ± 6.4$	57.24 ± 4.2$#
GSH activity (µmol/g tissue)	4.78 ± 0.1	4.65 ± 0.2	2.7± 0.07 $	3.75 ± 0.1$#
SOD activity (U/mg tissue)	52 ± 1.2	51 ± 2.3	22 ± 1.9 $	37 ± 1.5 $#
NO Content (nmol/g tissue)	337.6 ± 27	355.6 ± 35	1292 ± 103$	556.7± 13 #

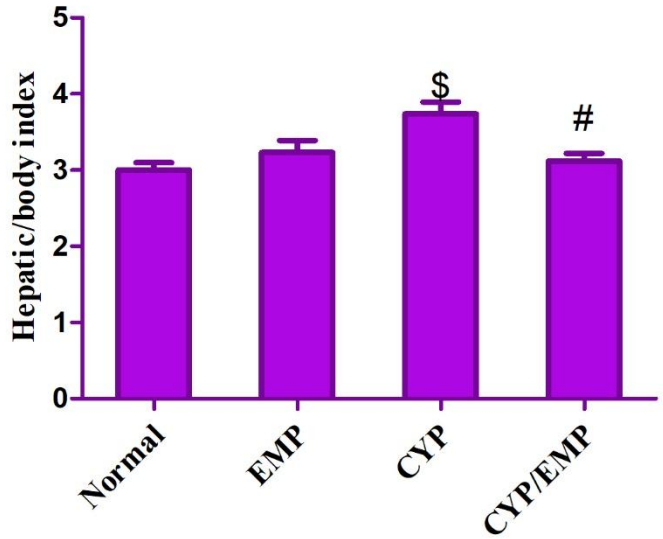
Hepatic injury was induced by single injection of CYP (200 mg/kg, IP). EMP was administered (10 mg/kg, orally) for 2 weeks; 1 week priors and 1 week post CYP injection

Data are presented as mean ± SE. Statistical analysis was done using (ANOVA) followed by Tukey-Kramer test

$ Significantly different Vs normal (n=6; $p<0.05$)

Significantly different Vs CYP (n=6; $p<0.05$)

Figure 1: Effect of daily oral administration of EMP (10 mg/kg, orally) on CYP-induced increase in hepatic/body index



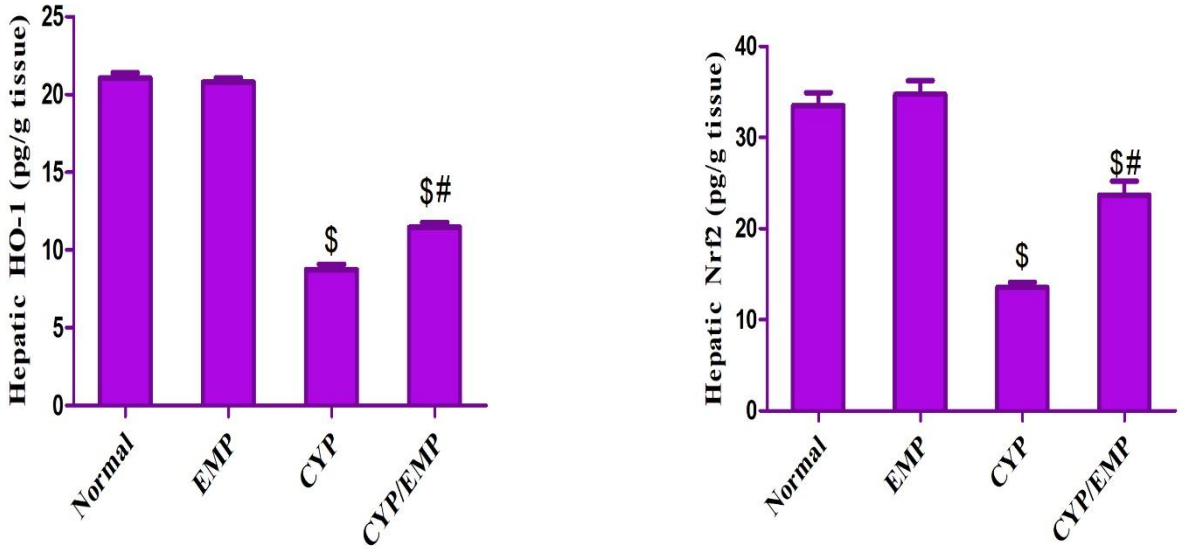
Hepatic injury was induced by single injection of CYP (200 mg/kg, IP). EMP was administered (10 mg/kg, orally) for 2 weeks; 1 week priors and 1 week post CYP injection.

Data are presented as mean ± SE. Statistical analysis was done using (ANOVA) followed by Tukey-Kramer test

$ Significantly different Vs normal (n=6; $p<0.05$)

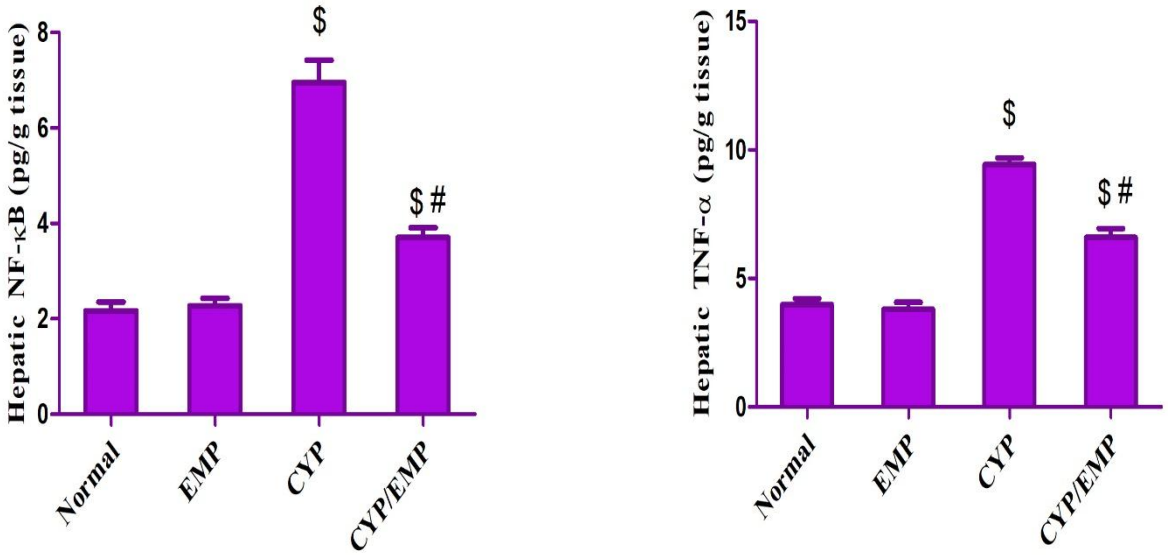
Significantly different Vs CYP (n=6; $p<0.05$)

Figure 2: Effect of daily oral administration of EMP (10 mg/kg, orally) on CYP-induced decrease in hepatic A) HO-1 and B) Nrf2 contents



Hepatic injury was induced by single injection of CYP (200 mg/kg, IP). EMP was administered (10 mg/kg, orally) for 2 weeks; 1 week priors and 1 week post CYP injection
Data are presented as mean ± SE. Statistical analysis was done using (ANOVA) followed by Tukey-Kramer test
$ Significantly different Vs normal (n=6; $p<0.05$)
# Significantly different Vs CYP (n=6; $p<0.05$)

Figure 3: Effect of daily oral administration of EMP (10 mg/kg, orally) on CYP-induced increase in hepatic A) NF-κB and B) TNF-α contents



Hepatic injury was induced by single injection of CYP (200 mg/kg, IP). EMP was administered (10 mg/kg, orally) for 2 weeks; 1 week priors and 1 week post CYP injection
Data are presented as mean ± SE. Statistical analysis was done using (ANOVA) followed by Tukey-Kramer test
$ Significantly different Vs normal (n=6; $p<0.05$)
# Significantly different Vs CYP (n=6; $p<0.05$)

Figure 4: Effect of daily oral administration of EMP (10 mg/kg, orally) on CYP-induced alterations in hepatic histopathology using H&E stain.

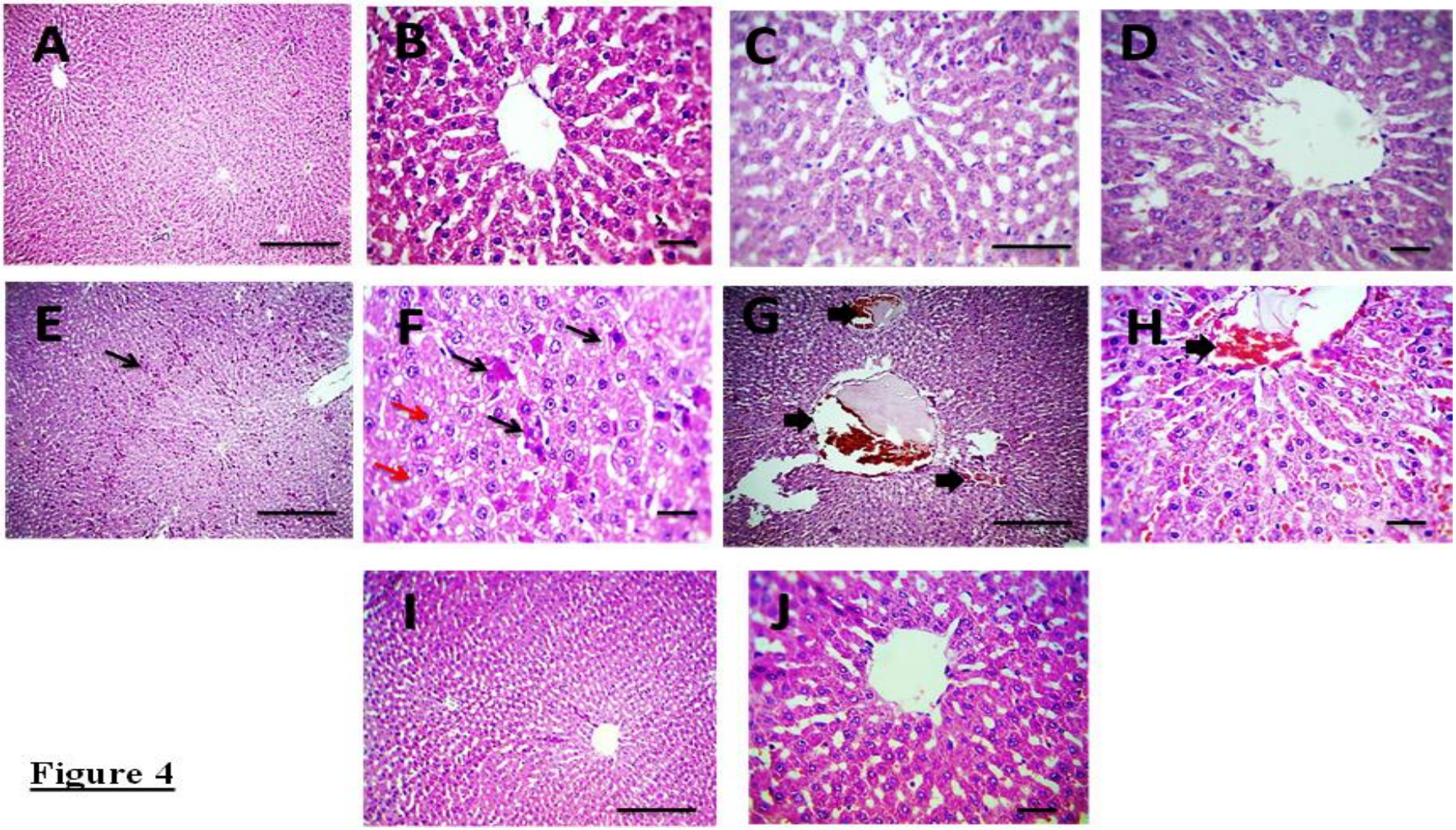


Figure 4

Microscopic pictures of liver showing normal parenchyma in the control group (A&B), CYP group and CYP/EMP group (I&J), multiple individual cell deaths (thin black arrows) with vacuolar degeneration in hepatocytes (thin red arrows) (E&F) with congested hepatic blood vessels in CYP group (thick arrows) (G&H). H&E, X: 100 bar 100 (A, C, E, G, I) and X: 400 bar 50 (B, D, F, H, J).

Figure 5: Effect of daily oral administration of EMP (10 mg/kg, orally) on CYP-induced alterations in hepatic histopathology using Masson's trichrome stain.

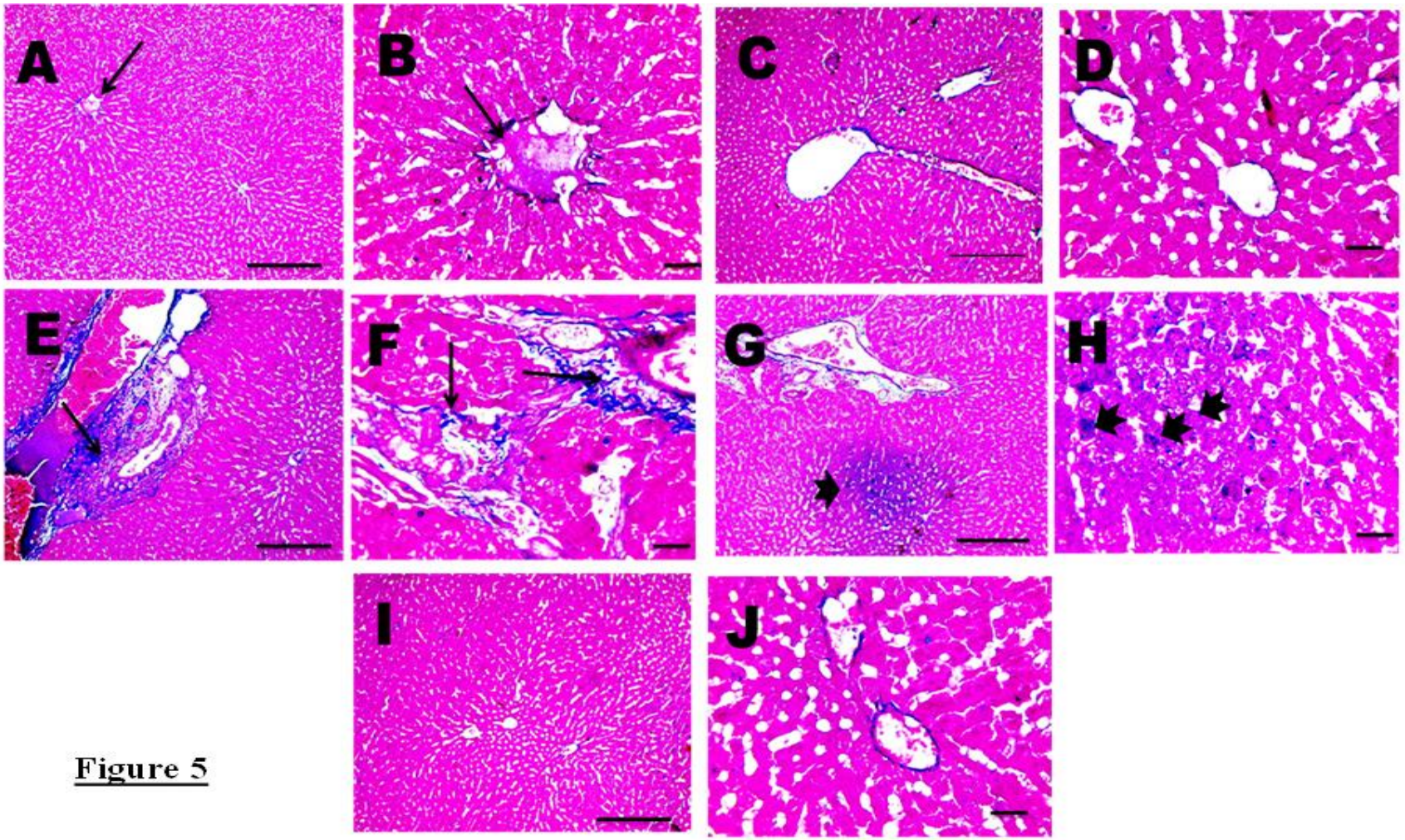


Figure 5

Microscopic picture of liver sections showing thin blue fibrous tissue around central veins in control group (thin arrows) (A&B), marked increase of the periportal fibrosis (thin arrows) (E&F) besides the presence of focal area of degenerated hepatocytes (thick arrows) G&H) in CYP treated group, normal appearance as in EMP-treated group (C&D) and CYP/EMP-treated group (I&J) (Masson's Trichrome A,C,E,G,I :× 100 bar 100 and B,D,F,H,J:x400 bar 50)

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