

*Full Length Research Paper*

## Protective effect of ghrelin on paracetamol induced acute hepatotoxicity in rats

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Received 3 May, 2014; Accepted 22 August, 2014

In this study we investigated if ghrelin plays a protective role against paracetamol-induced acute hepatotoxicity in rats and the possible mechanism of action. Forty adult male albino rats were divided into four equal groups; control, ghrelin, paracetamol and ghrelin plus paracetamol. Evaluations were made for lipid peroxidation, enzyme activities and biochemical parameters. Liver histopathology was also performed. Paracetamol (PCM) treatment increased plasma and liver tissue malondialdehyde (MDA) content and plasma nitric oxide (NO) level, and decreased erythrocyte and liver tissue superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities when compared to control group. At the same time, PCM treatment increased the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities. By contrast, ghrelin pretreatment reduced plasma and liver MDA content and plasma NO level, and increased erythrocyte, liver tissue SOD, CAT and GPx activities when compared with paracetamol-treated group. The PCM-induced histopathological changes were also reduced by the ghrelin pretreatment. In conclusion, our results proved that ghrelin was found to protect the liver against paracetamol-induced oxidative damage in rats and the hepatoprotective effect may be correlated with its antioxidant effect.

**Key words:** Antioxidant, ghrelin, hepatoprotective, hepatotoxicity, paracetamol.

### INTRODUCTION

Ghrelin, a 28 amino acid peptide, was recognized as the main endogenous ligand for the growth hormone secretagogue receptor (GHSR) (Kojima et al., 1999). Ghrelin is secreted primarily from the stomach, but ghrelin transcripts have been found in many other organs, including the liver, kidney, lung, pancreas, hypothalamus and heart, suggesting an endocrine as well

as extra-endocrine action of ghrelin (Kojima et al., 1999; Gnanapavan et al., 2002). Many studies have indicated that ghrelin has beneficial effects on gastrointestinal, cardiovascular, reproductive, immune and coagulation systems (Eter et al., 2007; Chang et al., 2004; Işeri et al., 2005; Kheradmand et al., 2009; Yada et al., 2006; Arıcı and Çetin, 2010). Other studies have revealed that

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ghrelin may be an antioxidant and anti-inflammatory agent. Kheradmand et al. (2010) reported that ghrelin promotes antioxidant enzyme activity and reduces lipid peroxidation in the rat ovary. In addition, Obay et al. (2008) have shown that ghrelin prevents lipid peroxidation and reduction of antioxidant enzyme activities and glutathione level against pentylenetetrazole-induced oxidative stress in the erythrocytes, liver and brain of rats. Also, Xu et al. (2008) demonstrated that ghrelin significantly reduced the concentration of malondialdehyde and increased the activity of antioxidant enzymes such as superoxide dismutase and catalase in primary cultured cardiomyocytes. In addition, Moreno et al. (2010) showed that ghrelin may attenuate fibrosis by exerting a hepatoprotective effect. Paracetamol, the widely used analgesic antipyretic drug, though considered a safe drug; it produces hepatic necrosis and renal failure when given in high doses (Abraham, 2005; Ahmed and Khater, 2001). Oxidative stress was reported to play a fundamental role in the pathogenesis of PCM-induced liver damage (Srinivasan et al., 2001). The present study aims to investigate the possible protective effect of ghrelin on PCM induced hepatotoxicity by studying its effect on serum liver functions and oxidative stress biomarkers, as well as on hepatic histopathology of the rat.

## MATERIAL AND METHODS

### Animals

Forty male albino rats weighing 150 to 200g were used in this study. They were obtained from the Laboratory Animal Research Unit of College of Agriculture, Zagazig University, Egypt. Rats were housed in a continuously ventilated room at a mean temperature of  $24 \pm 1$  °C with a lighting period of 12h dark and 12h light. The animals had free access to standard pellet rat chow and drinking water. All experiments were performed in accordance with the institutional guidelines for the care and use of animals for scientific purposes and in accordance with the recommendations from Helsinki Declaration. All animal experiments were accepted by ethical committee for animal use.

### Experimental design

Ghrelin (Sigma Chemical, St. Louis, MO) was dissolved in 0.9% physiological saline and injected subcutaneously at a dose of 10 ng/kg body weight (Işeri et al., 2008). PCM (Paracetamol) was purchased from EIPICO, 10th of Ramadan City, Egypt. PCM was suspended in pathogen-free normal distilled water before use. All the diagnostic kits assaying hepatic function tests, the levels of lipid peroxidation and antioxidants were obtained from Bio-Diagnostic Co., Giza, Egypt. Acute liver injury was induced in rats through a single oral administration of PCM (3 g/kg) (Madkour and Abdel-Daim, 2013). The rats were randomly divided into four groups containing ten rats each and were treated as follows; the control group was given 1ml of physiological saline, subcutaneously, daily for 5 days. The PCM group was treated with a single oral administration of PCM (3 g/kg). The ghrelin group was injected subcutaneously with ghrelin daily for 5 days. The ghrelin plus PCM group was pretreated with ghrelin daily for 5 days prior to treatment with a single dose of PCM.

### Collection and processing of blood and tissue samples

Animals were anesthetized with diethyl ether 24h after the last injection and blood was collected by cardiac puncture. The blood samples were collected into heparinized and non-heparinized tubes, and then heparinized tubes were centrifuged at 5000rpm for 10min for the separation of plasma and sera. The lower erythrocyte layer in the heparinized tubes was washed three times with phosphate buffered saline (pH:7.4) and diluted with an equal volume of the indicated solution. Next the erythrocytes were hemolysed with ice-cold distilled water (1:5) (Winterbourn et al., 1975). Immediately after blood collection, the animals were sacrificed by cervical dislocation. The liver of the animals was excised and used to determine the level of lipid peroxidation and antioxidant enzyme activities and for further histopathological study. The excised tissues were washed with distilled water for the removal of blood. Homogenization was performed in a phosphate buffer solution with a pH value adjusted to 7.4, and later the supernatant was separated by means of centrifugation at 20,000rpm for 1h. The supernatant and hemolysate obtained were used for the analyses of SOD, GPx and CAT. Furthermore, erythrocyte hemoglobin, plasma MDA and NO levels were measured. The serum obtained was used for the analyses of certain biochemical parameters (AST, ALP and ALT activities).

### Measurement of oxidative stress markers and serum biochemical parameters

Plasma MDA level was estimated by the method of Yoshioka et al. (1979). Tissue MDA level was assessed according to the method of Ohkawa et al. (1978). The measurements were performed using a spectrophotometer. Plasma NO level was determined in accordance with the Archer. (1993). Tissue and erythrocyte superoxide dismutase activity was measured by the method described by Sun et al. (1988), catalase activity was determined as described by Luck (1965) and glutathione peroxidase activity was measured according to Paglia and Valentine (1967). AST, ALT and ALP activities were measured with a spectrophotometer (Shimatzu UV-1700) using commercial assay kits (Bio-Diagnostic Co.) according to the manufacturer's direction. Histopathological examination was made in liver tissues. For light microscopic examinations, liver samples were fixed in 10% neutral buffered formalin solution. The tissues were embedded in paraffin. The paraffin blocks were cut in 5µm thick. The sections were stained with Hematoxylin-Eosin (H&E).

### Statistical analysis

Statistical analyses were carried out using SPSS 17.0 for Windows statistical package (SPSS). Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test.  $p \leq 0.05$  was considered statistically significant. All values were expressed as mean values  $\pm$  standard deviation (SD).

## RESULTS

### Effect of ghrelin on oxidative stress markers in plasma and liver tissue

The plasma MDA and NO content and tissue MDA level were significantly increased ( $p < 0.05$ ) in the liver tissue of animals receiving a single oral toxic dose of PCM compared to controls. Ghrelin pretreatment prior to PCM significantly prevented the elevations (Tables 1 and 2).

**Table 1.** Show Plasma MDA and NO levels and erythrocyte SOD, CAT and GSH-Px activities.

Group	MDA (nmol/ml)	NO (nmol/ml)	SOD (U/mgHb)	CAT (k/gHb)	GPx (μmol)
Control G	10.50 ± 0.60	215.22 ± 36.06	1.18 ± 0.14	70.30 ± 13.07	32.17 ± 5.67
Ghrelin G	10.98 ± 0.80	207.00 ± 26.58	1.21 ± 0.11	79.49 ± 11.12	31.19 ± 3.13
Paracetamol G	17.69 ± 1.84*	297.16 ± 35.35*	0.72 ± 0.14*	22.71 ± 6.70*	18.15 ± 2.52*
Ghrelin + Paracetamol G	11.62 ± 1.87**	221.79 ± 37.18**	1.25 ± 0.09**	67.27 ± 7.79**	35.89 ± 6.88**

\*Significantly different from the control group ( $p < 0.05$ ). \*\* Significantly different from the Paracetamol group ( $p < 0.05$ ). MDA: malondialdehyde. NO: nitric oxide. SOD: superoxide dismutase. CAT: catalase. GPx: glutathione peroxidase.

**Table 2.** Show Liver MDA level and SOD, CAT and GPx activities.

Group	MDA (nmol/mg-protein)	SOD (U/mg-protein)	CAT (k/g-protein)	GPx (μmol NADPH+H+)
Control G	0.48 ± 0.09	0.37 ± 0.10	67.84 ± 20.08	53.60 ± 10.24
Ghrelin G	0.50 ± 0.11	0.32 ± 0.07	65.55 ± 10.13	52.71 ± 13.14
Paracetamol G	0.91 ± 0.14*	0.18 ± 0.12*	38.55 ± 12.44*	28.89 ± 7.13*
Ghrelin+ Paracetamol G	0.56 ± 0.12**	0.44 ± 0.16**	69.77 ± 14.05**	47.00 ± 7.49**

\*Significantly different from the control group ( $p < 0.05$ ). \*\*Significantly different from the Paracetamol group ( $p < 0.05$ ).

### Effect of ghrelin on antioxidant enzyme activities in erythrocyte and liver tissue

When compared with the control, the activities of SOD, CAT and GPx were significantly decreased ( $p < 0.05$ ) in the erythrocyte and liver tissue of PCM group. Pretreatment of rats with ghrelin significantly prevented ( $p < 0.05$ ) the decrease in SOD, CAT and GPx activities in the erythrocyte and liver tissue when compared with the PCM group (Tables 1 and 2)

### Effect of ghrelin on PCM-induced acute hepatotoxicity

The effects of pretreatment with ghrelin on the PCM-induced elevation of serum AST, ALT and ALP activities are shown in Table 3. When compared with the control, AST, ALT and ALP activities were significantly elevated in the PCM group. Pretreatment of animals with ghrelin significantly reduced ( $p < 0.05$ ) serum AST, ALT and ALP activities when compared with PCM group.

### Histopathology

Histopathological studies showed that PCM, when compared to the control (Figure 1) induced inflammation, hemorrhage, broad infiltration of lymphocytes and extensive hepatocellular necrosis (Figure 2). Microscopic examinations showed that the severe hepatic lesions induced by PCM were markedly reduced by the pretreatment with ghrelin (Figure 3). Ghrelin treatment alone did not cause any change in the liver histology (Figure 4)

(H&E x 200).

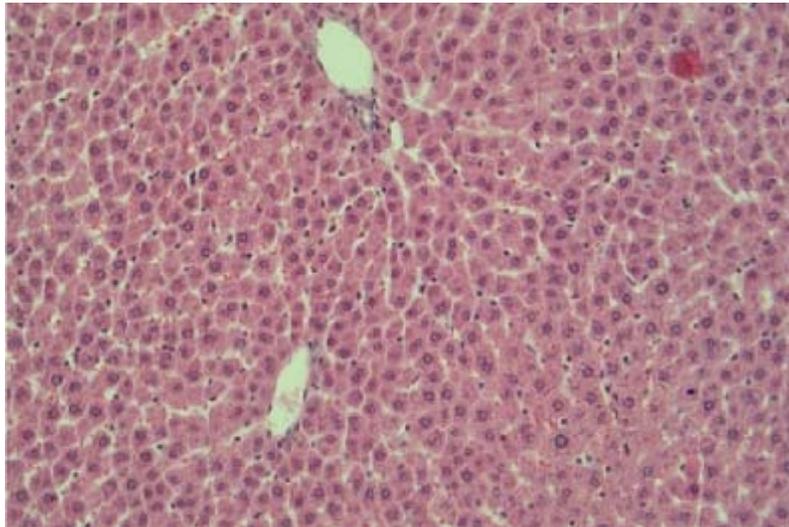
### DISCUSSION

The hepatotoxicity of PCM in experimental animals has been studied extensively and used widely as a model agent for inducing free radical damage. The toxicity of PCM probably depends on an accidental over dosage administration of PCM as an antipyretic drug that can result in hepatic damage (Raj Kapoor et al., 2008). Overdosage of PCM will result in accumulation of *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which will bind to glutathione (GSH) to form conjugates that will lead to the oxidation and conversion of GSH to glutathione disulfide (GSSG) resulting in the reduced level of blood and liver GSH (Yanpallewar et al., 2003). Depletion of GSH level in blood and liver due to this process can result in mitochondrial dysfunction, increase of lipid peroxidation and development of acute hepatic necrosis. From the results obtained in the present study, PCM, in a toxic dose of 3.g/kg, showed significant elevation of serum level of hepatic enzymes, ALT and AST. Histopathological observations provide evidence of reducing number of viable cells with massive necrotic cells around the centrilobular zone extending to parenchymal zone. The development of PCM-induced hepatotoxicity seems to depend partly on the existence of free radicals and oxidative processes. For that reason, it is hypothesized that compounds possessing free radical scavenging and/or antioxidant activities could also demonstrate hepatoprotective activity against the PCM toxic effect. This is supported by claim that the combination of hepatoprotective

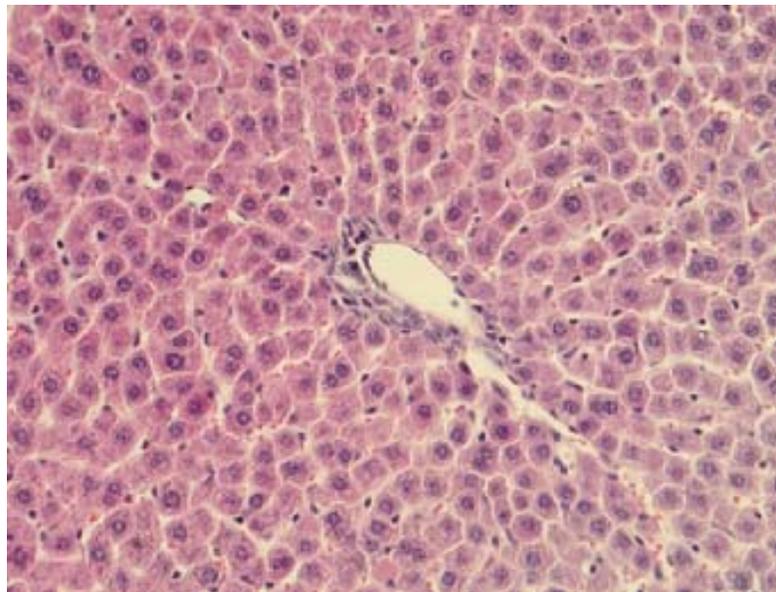
**Table 3.** Serum AST, ALP and ALT activities.

Group	AST (U/L)	ALP (U/L)	ALT (U/L)
Control G	167.2± 25.8	278.5 ± 37.6	42.9±18.4
Ghrelin G	151.0 ± 21.4	246.4 ± 32.4	41 .0 ± 7.9
Paracetamol G	324.1 ± 36.5*	386.0 ± 44.9*	119.4 ± 38.6*
Ghrelin+ Paracetamol G	180.3± 28.7**	293.2±35.0**	46.4± 21.9**

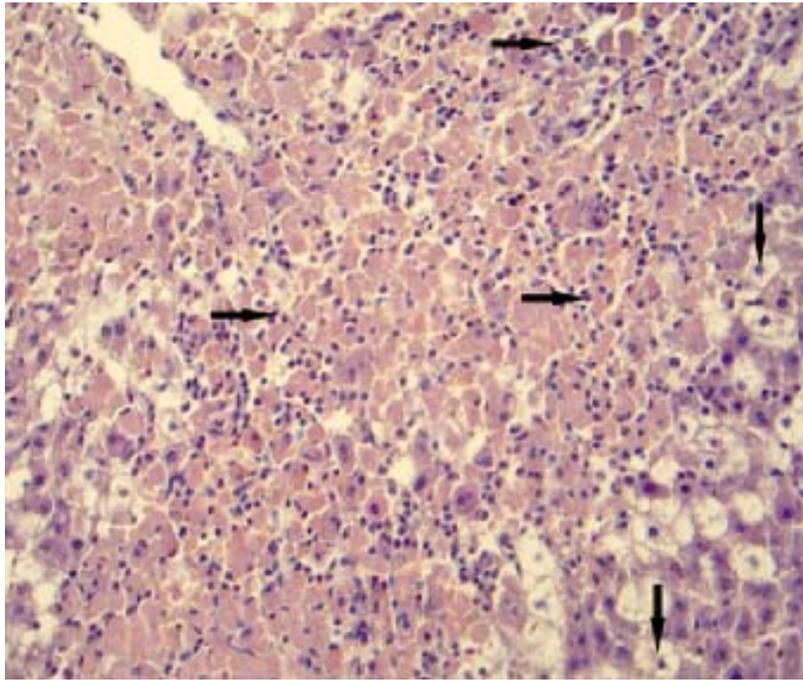
\*Significantly different from the control group ( $p < 0.05$ ). \*\*Significantly different from the Paracetamol group ( $p < 0.05$ ). AST: aspartate aminotransferase. ALT: alanine aminotransferase. ALP: alkaline phosphatase.



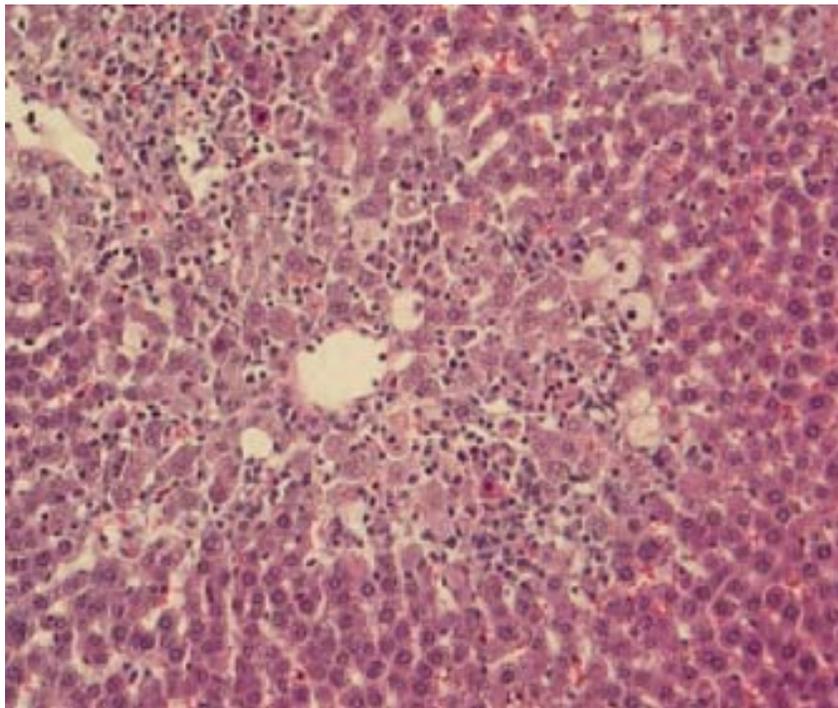
**Figure 1.** Control rats, no signs of liver damage (H&E ×200).



**Figure 2.** Rats treated with ghrelin (10 ng/kg of bw) alone show no signs of liver damage (H&E ×200).



**Figure 3.** Rats treated with paracetamol. The arrow heads in the right side showed severe and then PCM show decreased signs of liver damage fatty degeneration (H&E  $\times$  200).



**Figure 4.** Rats pretreated with ghrelin (10 ng/kg of bw). In the center and left side show (H&E  $\times$ 200) broad infiltration of lymphocytes and extensive necrosis.

effect and antioxidant activity synergistically prevents the process of initiation and progress of hepatocellular damage (Gupta et al., 2006). The inflammatory processes are thought to be responsible for producing various mediators, which are involved in the production of reactive oxygen species (ROS) and NO that can affect liver damage or repair. Therefore, it is also possible to postulate that compounds -like ghrelin- possessing anti-inflammatory activity might also exhibit hepatoprotective activity.

Mechanisms of hepatoprotection that could take place include prevention of the process of lipid peroxidation (Mujeeb et al., 2009). Increased lipid peroxidation and impaired antioxidant enzyme function in the liver tissue are characteristic observations in PCM-treated rats (Weber et al., 2003). In the present study, MDA concentration significantly increased ( $p < 0.05$ ) 24hr after PCM treatment alone as demonstrated in other studies (Ohta et al., 1997; Chen et al., 2005). This elevated level of MDA in plasma or liver tissue caused by PCM treatment was significantly depressed by the pretreatment with ghrelin. This was attributed to the decreased bioactivation of PCM caused by the ghrelin pretreatment. This result was supported by previous studies showing that ghrelin induced reduction of the increased level of MDA in pre-adipocyte cell culture (Zwirnska-Korczala et al., 2007) testis and ovary of rats (Kheradmand et al., 2009; Kheradmand et al., 2010). Also, our study in agreement with Obay et al. (2008) who demonstrated that pretreatment of rats with different doses of ghrelin prevented pentylenetetrazole-induced elevation in lipid peroxidation. In addition, Işeri et al. (2005) showed that ghrelin administration significantly decreased MDA level in the alendronate-induced gastric tissue injury in rats. In this study, the PCM treatment alone significantly increased ( $p < 0.05$ ) the plasma NO level. The inflammatory processes in the liver after PCM treatment may be responsible for the increase in NO level. Our findings are in agreement with reports that nitric oxide is produced in the liver following acute administration of CCl<sub>4</sub> to rats (Chamulitrat et al., 1994). In the present study, pretreatment with ghrelin prior to the administration of PCM significantly inhibited the generation of NO compared to the rats treated with PCM alone. This effect of ghrelin may be related to inhibition of inducible nitric oxide synthase (iNOS), which causes large and continuous release of NO. This result is supported by the findings of Sibilia et al. (2003) who found that ghrelin decreased iNOS mRNA expression under conditions of ethanol-induced gastric lesions, suggesting that the reduction of NO-derived from iNOS contributes to ghrelin gastroprotection. It has also been demonstrated that ghrelin suppressed iNOS mRNA expression in gastric mucosa of rats subjected to ethanol or water stress (Brzozowski et al., 2004). NO reduction by ghrelin is very important because the primary product of the interaction between NO and superoxide is peroxynitrite (ONOO<sup>-</sup>), a highly reactive radical that can

damage a variety of cell molecules (Radi et al., 1991). Peroxynitrite can induce sulfhydryl oxidation (Radi et al., 1991) and lipid peroxidation (Rodenas et al., 2000). On the other hand, NO may act as a scavenger for reactive oxygen species (Cottart et al., 1999) and terminate lipid peroxidation (Zhu and Fung P.C 2000). The functions of nitric oxide in liver injury are complex, the production of small amounts of NO may exert a cytoprotective effect (Kawachi et al., 2000), while overproduction of NO may damage liver function (Liu et al., 2000). The decrease in the production of MDA and NO induced by ghrelin pretreatment show that anti-lipid peroxidation potency of ghrelin is involved in liver protection.

Maintaining the balance between Reactive oxygen species (ROS) and antioxidant enzymes is crucial, and could serve as a major mechanism in preventing damage by oxidative stress (Taniguchi et al., 2004). In the present study, we found a decrease in the activities of SOD, CAT and GPx in liver and erythrocyte-induced by acute administration of PCM. This decrease may be attributed to oxidative inactivation of enzyme protein by excess ROS generation. These results are supported by other studies (Abraham 2005; Taniguchi et al., 2004; Lee et al., 2007). Pretreatment with ghrelin showed an improved effect on the activities of antioxidant enzymes examined as compared to PCM-treated only group. Our results were supported by previous data concerning the antioxidative properties of ghrelin in other tissue (Obay et al., 2008; Xu et al., 2008; Işeri et al., 2008; Zwirnska-Korczala et al., 2007). Our findings demonstrated that ghrelin exerts a potent protective effect against PCM-induced acute liver injury by increasing SOD, CAT and GPx activities. In addition, the protective effect of ghrelin against PCM-induced acute liver injury may be depending on the inducible effect of it to hemoxygenase-1 (HO-1) that provides efficient cytoprotection against oxidative stress in a variety of experimental models, such as liver ischemia/reperfusion secondary to transplantation or hemorrhage/resuscitation (Bauer and Bauer 2002). HO-1 induction has been shown to confer protection in CCl<sub>4</sub>-induced hepatotoxicity, as assessed by the measurements of liver transaminase levels and cytological examination of liver histology (Nakahira et al., 2003). It is thought that the induction of HO-1 is regulated by nuclear-factor erythroid 2-related factor 2 (Nrf2) (Alam et al., 1999). Nrf2 has been shown to play a role in protecting liver against acetaminophen-induced hepatotoxicity (Chan et al., 2001) as well as increased levels of lipid peroxidation in Nrf2 livers (Li et al., 2004). Nrf2 plays an important role in the cellular antioxidant defense system against oxidative stress (Chan et al., 2001; Li et al., 2004). In our study, the increase in SOD, CAT and GPx activities in the ghrelin plus PCM group may be also associated with the induction of Nrf2 by ghrelin. The present study indicated that treatment with PCM alone significantly increased ( $p < 0.05$ ) serum AST, ALT and ALP activities which has been

supported by other studies (Chandan et al., 2007). Our results showed that pretreatment of rats with ghrelin effectively protected the animals against PCM-induced hepatic damage, as evidenced by decreased serum AST, ALT and ALP activities. This result is in agreement with the findings that ghrelin induced reduction of the increased activity of AST and ALT in plasma of rats treated with acetaminophen (Jahromi et al., 2010). Our results were also supported by (Moreno et al., 2010) who reported that ghrelin pretreatment caused a decrease in AST activity of rats treated with CCl<sub>4</sub>. Our findings were also confirmed by histological observation. The fatty degenerations, broad infiltration of lymphocytes and extensive hepatocellular necrosis were displayed in PCM hepatotoxicity. Pretreatment with ghrelin markedly reduced these histopathological changes.

## CONCLUSION

The present study demonstrated that ghrelin exerts a potent protective effect against PCM-induced acute hepatotoxicity by decreasing MDA and NO levels, and increasing SOD, CAT and GPx activities. The protective effect of ghrelin in the prevention or treatment of hepatic disease should be investigated in human volunteers to assess its ability as a new drug in clinical medicine.

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