

ORIGINAL ARTICLE

## Protective Effect of Ghrelin on Isoniazid-induced Liver Injury in Rat

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Ghrelin (GHR) is a peptide that has protective effects on many tissues injury. It has anti-inflammatory and anti-oxidant effects. Isoniazid (INH) a widely used antituberculosis drug, has hepatotoxic side effect. The aim of this study was to evaluate the protective role of ghrelin in liver toxicity due to isoniazid. Eighteen male rats were used in this study and divided in to three groups. Including: control, isoniazid, isoniazid and ghrelin groups. Nitric oxide(NO), prostaglandin E2(PGE2), and hepatic enzymes, ALT(alanine aminotransferase), AST(aspartate aminotransferase), ALK(alkaline phosphatas), were assessed and histologic study of liver were performed as indicators of liver damage following isoniazid toxicity. Ghrelin significantly increased NO metabolites and decreased PGE2 level comparison with INH group, but had no significant change compared to the control group. This study showed that ghrelin administration inhibited liver injury in rats due to isoniazid toxicity. The liver protective role of ghrelin may be mediated at least in part by its anti-inflammatory effect.

*Key words: Ghrelin, Isoniazid(INH), liver injury, nitric oxide(NO), prostaglandin E2(PGE2)*

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**Key words:** Ghrelin, Isoniazid(INH), liver injury, nitric oxide(NO), prostaglandin E2(PGE2)

Isoniazid or isonicotinyl hydrazine (INH) is widely used in the treatment and prophylaxis of tuberculosis throughout the world (Weber and Hein, 1979). Its toxicity is the most common cause of hepatic failure requiring liver transplantation (Nolan *et al.*, 1999).

Isoniazid is metabolized mainly in the liver via acetylation (Weber and Hein, 1979) and the hepatic cytochrome P450 enzyme system (CYP) (Self *et al.*, 1999). INH is thought to be an inhibitor of CYP enzymes. The hepatotoxicity of INH is supposed to be initiated by CYP mediated metabolism of INH to

acetylhydrazine and hydrazine (Sarich, 1999; Jenner and Timbrell, 1994). The acetylhydrazine can covalently bind to liver proteins (Timbrell *et al.*, 1980) and also hydrazine (Timbrell *et al.*, 1980; Vuilleumier *et al.*, 2006; Whitehouse *et al.*, 1983) and cytochrome P450 (Huang *et al.*, 2003; Shen *et al.*, 2006; Yue *et al.*, 2004) can cause hepatotoxicity by production of free radicals (Gonzalez, 2005). Using of isoniazid has been showed to induce hepatotoxicity in some of human beings in normal doses and in experimental animals in toxic doses (Maryam *et al.*, 2010).

Ghrelin is a newly discovered gut hormone, mainly produced in the stomach (Ariyasu *et al.*, 2001), but also identified in endocrine cells of the gastrointestinal tract (Date *et al.*, 2000). Ghrelin might also be synthesized in other organs, where it might have outocrine or paracrine effects (Leite-Moreira and Soares, 2007).

Ghrelin plays a role in a number of different physiological processes. For example, it enhances growth hormone secretion (Shiyya *et al.*, 2002) and increases appetite (Shiyya *et al.*, 2002), regulates cell proliferation (Yoshihara *et al.*, 2002), stimulates prolactin and adrenocorticotrophic hormone (Kojima *et al.*, 2004), promotes slow wave sleep (Weikel *et al.*, 2003) and memory retention (Carlini *et al.*, 2002).

In the stomach, ghrelin affects gastric acid secretion and motility, and exhibits gastroprotective effect (Brzozowski *et al.*, 2004). In mammals, ghrelin plays an important role in the immune system (Yada *et al.*, 2006). Previous findings showed ghrelin administration inhibited liver damage in rats due to acetaminophen toxicity (Golestan Jahromi *et al.*, 2010). Ghrelin significantly increasing nitric oxide bioactivity (Tesauro *et al.*, 2005) and mucosal prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (Sibilia

*et al.*, 2003) and a decrease in cytokine production (TNF- $\alpha$ -NF- $\kappa$ B) (Konturek *et al.*, 2006). The anti-oxidant and anti-inflammatory effects of ghrelin were previously investigated (El Eter *et al.*, 2007). As the main mechanism of INH toxicity is via inflammation and generation of free radicals, the present study was investigated to assessment the possible protective effect of ghrelin in liver damage secondary to INH toxicity in rats.

## MATERIALS AND METHODS

### Materials

Isoniazid (INH) and Ghrelin were purchased from Sigma-Aldrich (Sigma Chemical Company, St. Louis, MO, USA).

### Animals and experimental protocols

Eighteen male Wistar rats (150-200 g) were used. All animals were kept in an animal room in a controlled temperature and 12:12 h light/dark cycle with free access to food and water. All procedures were approved by the ethical committee of Tehran University of Medical Sciences.

The animals were equally divided into three groups (n=6) including: (A) control group; animals were given saline solution as vehicle, (B) INH group; rats were given once a day INH (200 mg/kg B.W i.p) for five consecutive days (Nahrevanian *et al.*, 2009) and (C) INH+ghrelin group; rats were treated with ghrelin (10 nmol/kg single dose i.p) 1 hour after INH injection in days 4 and 5 (Golestan Jahromi *et al.*, 2010).

Three hours after the last injection of gherlin, rats were anesthetized with i.p injection of ketamine-zylaxine (50 and 8 mg/kg, respectively) (Golestan Jahromi *et al.*, 2010) and the abdomen was opened medially. Then, whole blood samples (1 ml) harvested from the heart. Blood samples were centrifuged (4000 g, 15 min) and serum was

collected for serological tests including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALK). Also, liver middle lobe tissue was fixed in a buffered-formaldehyde solution (10%) for histological studies. Other lobes immediately frozen and kept at -70 °C for measurement of PGE2 and NO by ELISA and Griess methods, respectively.

#### *Liver tissues homogenate preparation for determination of PGE2 and NO*

Weighed samples of liver tissue (0.5 g) were placed in 1.5 ml microfuge tubes and homogenized using an electrical homogenizer (Model RS541-242, RS Components, Corby, UK) (Golestan Jahromi *et al.*, 2010). Then homogenates tested for PGE2 using a high sensitivity PGE2 Chemiluminescence Enzyme Immunoassay (CEI) Kit (Assay Designs Inc., MI, USA) and NO metabolites with the Griess Micro Assay method (Nahrevanian *et al.*, 2009).

#### *Histology and microscopic structural examinations*

The liver middle lobe tissue from the specimens were fixed in 10% formaldehyde, passaged and embedded in paraffin. The paraffin blocks were sectioned by 3-5 µm thickness and stained with Hematoxylin and Eosin (H & E) and Masson Trichrome stains to study the general structure under microscopic observations.

The grade of histological study was semi quantitatively scored using the following parameters (Ishak *et al.*, 1995):

##### A\* Piecemeal necrosis:

- 0- Absent,
- 1- Mild (focal, few portal areas),
- 2- Mild/moderate (focal, most portal areas),
- 3- Moderate (continuous around <50% of tracts or septa),

4- Severe (continuous around >50% of tracts or septa).

##### B\* Confluent necrosis:

- 0- Absent,
- 1- Focal confluent necrosis,
- 2- Centrilobular necrosis in some areas,
- 3- Centrilobular necrosis in most areas,
- 4- Centrilobular necrosis + occasional portal-central (P-C) bridging,
- 5- Centrilobular necrosis + multiple P-C bridging,
- 6- Panlobular or multilobular necrosis.

C\* Focal (Spotty) lytic necrosis, apoptosis, and focal inflammation:

- Absent,
- One focus or less per 10× objective,
- One to four foci per 10× objective,
- Five to 10 foci per 10× objective,
- More than 10 foci per 10× objective.

##### D\* Portal inflammation:

- 0- None,
- 1- Mild, some or all portal areas,
- 2- Moderate, some or all portal areas,
- 3- Moderate/marked, all portal areas,
- 4- Marked, all portal areas.

##### \*\* Fibrosis:

- 0- No fibrosis,
- 1- Fibrous expansion of some portal areas, with or without short fibrous septa,
- 2- Fibrous expansion of most portal areas, with or without short fibrous septa,

- 3- Fibrous expansion of most portal areas with occasional portal-portal (P-P) bridging,
- 4- Fibrous expansion of portal areas with marked bridging (portal-portal (P-P) as well as portal central (P-C)),
- 5- Marked bridging (P-P and/or P-C) with occasional noduls (incomplete cirrhosis),
- 6- Cirrhosis, probable or definite.

*Statistical analysis*

All values were expressed as Mean±S.E. Statistical analysis performed with analysis of variances (ANOVA) and post hoc Tukey tests using version SPSS 17.0. For the comparison of nonparametric data using Kruskal-Wallis test. P<0.05 was considered statistically significant.

**RESULTS**

*Liver enzyme measurement*

In INH and INH+GHR groups ALT, AST and ALK showed no significant change compared to the control group (Table 1).

*NO metabolites measurement*

Our finding showed that levels of NO metabolites of liver tissue in INH group (group B) had significant change compared to the control group (group A) (76.73±11.17, 141.29±24.21 μM/gr.Wet weight, respectively) (p<0.05) (Figure 1), it significantly increased by using ghrelin after INH administration (125.75±22.19 μM/gr.Wet

weight) (p<0.05) (Figure 1). Also, our data showed that levels of NO of liver tissue in group that received ghrelin after INH (group C) had no significant change compared to the control group (P<0.3) (Figure 1). Therefore, ghrelin elevated it to the control group.

*PGE2 measurement*

PGE2 level of liver tissue in INH group was significantly higher than control group (23.17±2.68, 11.58±1.29 Pg/gr Wet weight, respectively) (p<0.05) (Figure 2), but it significantly decreased by using ghrelin after INH administration (8.25±1.05 Pg/gr Wet weight) (p<0.01) (Figure 2). Also, our data showed that levels of PGE2 of liver tissue in group that received ghrelin after INH (group C) had no significant change compared to the control group (P<0.07) (Figure 2). Therefore, ghrelin decreased it to the control group.

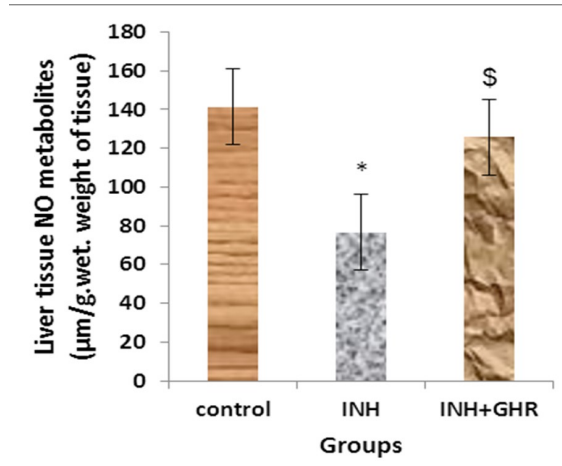
*Histological study*

The microscopic study showed infinitesimal damage in rat liver tissue (0.83±0.3), but did not show any fibrosis in control group. Morphological changes following INH injected (5.6±0.49) and areas of confluent fibrosis (2±0.36) were significantly increased than to the control group (p<0.001, p<0.001, respectively). Treatment with ghrelin has significantly reduced liver injury and fibrosis (1.6±0.49, 0.16±0.16, respectively) compared to the INH group (p<0.001, p<0.001, respectively) (Table2, Figure 3).

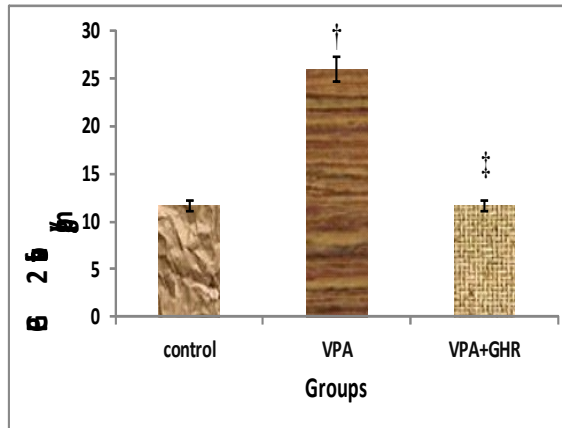
**Table 1:** Effects of ghrelin and isoniazid on levels of hepatic enzymes.

Groups	ALT (U/L)	AST(U/L)	ALK(U/L)
Control	65.5±3.86	170.8±28.23	565.8±44.05
INH	58.5±3.18	182±28.49	554±43.32
INH+GHR	53.8±3.92	186.6±29.15	548.6±42.39

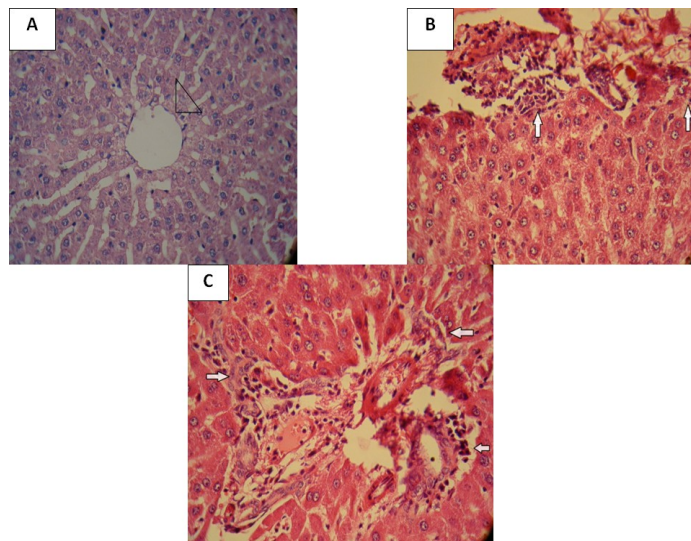
Data were expressed as Mean±SE. n=6, ALT, AST and ALK were similar in all the groups.



**Figure 1:** The level of liver tissue NO metabolites in different experimental groups (Mean±SEM, n=6). \*p<0.05, liver tissue NO in INH group compared to control group, <sup>§</sup>p<0.05, liver tissue NO in INH+GHR group compared to INH group.



**Figure 2:** The level of liver tissue PGE2 in different experimental groups (Mean±SEM, n=6). \*p<0.05, PGE2 level in INH group compared to control group, <sup>§</sup>p<0.01, PGE2 level in INH+GHR group compared to INH group.



**Figure 3:** Liver histopathology. Representative slides from corresponding groups are shown (A: control group, Normal liver tissue. Arrowhead shows central vein (H&E ×40), B: isoniazid group, Aportal tract with moderate inflammination and piecemeal necrosis (arrows) (H&E ×40), C: ghrelin group, Aportal tract with mild inflammination and focal piecemeal necrosis (arrows) (H&E ×40).

**Table 2.** Histological activity index (HAI) was assessed based on the degree of microscopic lesions (Piecerneal necrosis, Confluent necrosis, Focal (Spotty litic necrosis, apoptosis, focal inflamination), Portal inflamination, Fibrosis). Data of each group were reported as Means±SEM based on sum of the scores histological criteria (overall score) using Kruskal-Wallis test (n=6).

Groups	Piecerneal necrosis	Confluent necrosis	Focal (Spotty litic necrosis, apoptosis, focal inflamination)	Portal inflamination	Overall score	Fibrosis
Control	0	0	0.66±0.21	0.16±0.16	0.83±0.3	0
INH	1.5±0.34	0.16±0.16	2.3±0.21	1.6±0.21	5.6±0.49 <sup>§</sup>	2±0.36 <sup>^</sup>
INH+GHR	0.33±0.21	0	0.16±0.16	0.5±0.22	1.6±0.49 <sup>*</sup>	0.16±0.16 <sup>*</sup>

<sup>§</sup> P<0.001, compared to control group, <sup>\*</sup> P<0.001, compared to INH group, <sup>^</sup> P<0.001, compared to control group, <sup>\*</sup> p<0.001, compared to INH group.

## DISCUSSION

In the present study, hepatotoxicity by acute administration of isoniazid was investigated; it could indeed be ameliorated by treatment with ghrelin.

Gut hormones play a major role in the regulation of fibrogenic processes and inflammatory in a variety of tissues. Ghrelin is a gut hormone that is also produced by extraintestinal tissue and it has protective effects in different organs including the liver, pancreas, heart and gastrointestinal tract (Golestan Jahromi *et al.*, 2010; Moreno *et al.*, 2010).

In the previous study, INH-induced hepatotoxicity reported that serum ALT and AST, the diagnostic marker of liver damage, were elevated by isoniazid after 21 days of treatment (Yue *et al.*, 2004; Yue *et al.*, 2009). Also Golestan Jahromi *et al.* have shown that treatment of rats with ghrelin attenuated liver toxicity and reduced the level of these enzyme (Golestan Jahromi *et al.*, 2010). But in this study, we showed that in INH and INH+ghrelin groups ALT, AST and ALK no significant changes compared to the control group, this discrepancy of finding may be due to duration and/or dosage of drug (INH).

Kupffer cells are the phagocytic macrophages of the liver. When activated, kupffer cells release numerous signaling molecules, including hydrolytic enzymes, eicosanoids, NO and superoxide (Jaeschke *et al.*, 2002; James *et al.*, 2003). They may also release a number of inflammatory cytokines, including TNF- $\alpha$ , interleukins, prostaglandins and oxygen radical are released in liver toxicity (Martinez *et al.*, 1992).

The present study demonstrates isoniazid significantly decreases NO level in the liver tissue, but ghrelin elevated it to the normal level. In addition to in our study, indicate INH significantly increase PGE2 level in the liver tissue, but ghrelin reduced it even lower than control group.

But we did not show any research about INH-induced hepatotoxicity that measurement NO and PGE2 in the liver tissue. Therefore, it seems in our study ghrelin can improve hepatotoxicity by increasing of NO and decreasing of PGE2 level.

Ghrelin also improves endothelial function by inhibiting basal and tumour necrosis factor (TNF- $\alpha$ )-induced production of chemotactic cytokines, increasing nitric oxide (NO) bioactivity (Li *et al.*, 2004; Tesauro *et al.*, 2005).

Recently, Golestan Jahromi *et al.* showed that

ghrelin reduced pro-inflammatory cytokines (e.g. TNF- $\alpha$ ) in liver toxicity (Golestan Jahromi *et al.*, 2010). Also, it is probable, in present study, ghrelin decrease PGE2 and improve liver damage.

INH-induced hepatotoxicity and the pathogenesis of hepatocellular disintegration and vacuolation in the centrilobular region (Young *et al.*, 2008) and kupffer cell hyperplasia with congestion of hepatocytes and microvesicular fatty infiltration with special stains have been observed by Khedun *et al* (Khedun *et al.*, 1993). We also observed piecemeal necrosis, confluent necrosis, spotty lytic necrosis, apoptosis, focal inflammation, portal inflammation and fibrosis in the liver tissue due to isoniazid administration. Treatment with ghrelin improved these histological changes in the liver.

In conclusion, this study showed that ghrelin administration inhibited liver injury in rats due to isoniazid toxicity. The liver protective role of ghrelin may be mediated at least in part by its anti-inflammatory effect.

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