THE ROLE OF GHRELIN AGAINST ACUTE CARBON TETRACHLORIDE HEPATOTOXICITY IN RATS

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ABSTRACT

Objective: *Due to its anti-inflammatory and antioxidant effects, the peptide ghrelin has a protective effect in a variety of tissues. Antioxidant properties of ghrelin have been recently reported in various types of oxidative stress. Therefore, we designed this study to explore the antioxidative effects of ghrelin in acute liver injury induced by carbon tetrachloride (CCl_4) in rats.*

Material and Method: *In this experimental study, 24 Spraque-Dawley genus albino rats were equally divided into three groups as follows: control, CCl_4 and CCl_4+ghrelin. 4 ml/kg olive oil was administered intraperitoneally (i.p.) to the control group, 4 ml/kg CCl_4 (1:1 dissolved in olive oil) was administered i.p. to the animals in other two groups. After three and six hours, 80 mcg/kg ghrelin was administered i.p. to the CCl_4+ghrelin group. Twenty four hours after administrating CCl_4, all of the rats were sacrificed. Biochemical assessments were performed using serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), malondialdehyde (MDA), tissue MDA, myeloperoxidase (MPO) and nitric oxide (NO) levels. Histopathological assesments were performed using hematoxilen & eosine staining in light microscope.*

Results: *Serum AST, ALT, MDA and tissue MDA, MPO levels were all increased in rats exposed to CCl_4. These values were significantly decreased in the group subsequently treated with ghrelin. Histopathological data showed a significant decrease in congestion, polymorphonuclear leukocytes, mononuclear leukocytes, vacuolar degeneration of hepatocytes and hepatocellular necrosis in rats treated with ghrelin.*

Conclusion: *No effective therapies are currently available for patients with acute liver injury. Our study indicates that ghrelin protected rats from acute liver injury by reducing oxidative stress and inflammation.*

Key Words: *Liver injury, carbon tetrachloride, ghrelin.* ***Nobel Med 2013; 9(3): 43-48***

KARBONTETRAKLORÜR İLE AKUT KARACİĞER TOKSİSİTESİ GELİŞTİRİLEN RATLARDA GHRELİNİN ROLÜ

ÖZET

Amaç: Ghrelin vücudun birçok dokusu üzerinde koruyucu etkileri olan bir peptittir. Antioksidan ve antiinflamatuar etkileri vardır. Ghrelinin antioksidan özellikleri üzerine son zamanlarda çeşitli çalışmalar bildirilmiştir. Biz de bu nedenle, sıçanlarda karbon tetraklorür (CCl_4) ile oluşturulan akut karaciğer hasarında ghrelinin muhtemel antioksidan etkilerini araştırmak için bu çalışmayı planladık.

Materyal ve Metod: Deneysel olarak yapılan bu çalışmada 24 Spraque-Dawley türü albino sıçan, kontrol, CCl_4, CCl_4+Ghrelin şeklinde eşit sayıda olmak üzere 3 gruba ayrıldı. Kontrol grubuna 4 ml/kg zeytinyağı i.p. (intraperitoneal) olarak verilirken diğer iki gruba 4 ml/kg CCl_4 (1:1 yağda eritilerek) i.p. olarak verildi. Üçüncü ve altıncı saatlerde 80 mcg/kg ghrelin, CCl_4+Ghrelin grubuna i.p. olarak verildi. CCl_4 verilmesinden 24 saat sonra bütün sıçanlar genel anestezi altında dekapite edildi. Biyokimyasal değerlendirme serum aspartat aminotransferaz (AST), alanin aminotransferaz (ALT), malondialdehid (MDA), doku MDA, miyeloperoksidaz (MPO) ve nitrik oksit (NO) düzeylerine bakılarak yapıldı. Hematoksilen-eozin ile boyanmış dokuların ışık mikroskobunda histopatolojik değerlendirmesi yapıldı.

Bulgular: CCl_4 verilen grupta serum AST, ALT, MDA ve doku MDA, MPO değerlerinin hepsi yüksekti, fakat ghrelin verilen grupta (AST, ALT, serum MDA ve doku MDA, MPO düzeylerinde) anlamlı derecede azalma saptanıldı. Histopatolojik değerlendirme sonucunda ghrelin verilen grupta konjesyon, inflamasyon, vakuolar dejenerasyon ve karaciğer hücre nekrozunun daha az oranda olduğu gözlendi.

Sonuç: Akut karaciğer hasarı çeşitli faktörler sonucu olabilir ve akut karaciğer hasarı gelişen hastalar için halen etkin bir tedavi yoktur. Çalışmamız ghrelinin oksidatif stresi ve inflamasyonu azaltarak akut karaciğer hasarında koruyucu etkinlik gösterdiğini desteklemektedir.

Anahtar Kelimeler: Karaciğer hasarı, karbon tetraklorür, ghrelin. **Nobel Med 2013; 9(3): 43-48**

INTRODUCTION

The liver is commonly affected by toxic agents and drugs due to its anatomic localization and physiologic and biochemical roles. Hepatotoxicity resulting from these drugs and toxins can manifest as acute, chronic or fulminant hepatitis, or in later stages as cirrhosis or carcinoma. Acute liver injury is characterized by deterioration of the normal liver parenchyma by toxic agents or metabolic events, which lead to inflammation and congestion.[1]

Carbon tetrachloride (CCl_4) is a lipid-soluble hepatotoxic agent. Several studies have shown that toxic liver injury induced by CCl_4 in experimental models shares similar physiological and histological features with human disease.[2,3] In addition to functional and morphological changes, changes in antioxidant defense systems are also observed in acute liver injury. These changes increase reactive oxygen species accumulation and lipid peroxidase formation, rendering the liver tissue more sensitive to injury.

In recent years, the use of antioxidants as protective and therapeutic agents in cases of acute liver injury has become more prevalent. The antioxidant properties of ghrelin have been reported recently in various cases of oxidative stress.[4] Ghrelin is a gut hormone intially discovered as a potent growth hormone secretagogue. It also plays a major role in the regulation of food intake. Recently, peripheral effects such as cytoprotection, vasodilatation, regulation of energy balance, and gastrokinesis also have been attributed to ghrelin. Several reports describe the effects of ghrelin on cell proliferation, the cardiovascular system, carbohydrate metabolism, energy metabolism, pancreatic exocrine and endocrine functions, the gastrointestinal system, and liver steatosis.[5] Finally, ghrelin has recently been reported to exert beneficial effects on various oxidative stresses as a result of its antioxidant properties. The primary site of ghrelin synthesis is the stomach, but transcripts have been detected in other organs, including the liver, bowel, pancreas, kidneys and lungs.[6] Most ghrelin actions are mediated by the growth hormone secretagogue receptor (GHS-R), which is mainly expressed in the pituitary gland but also has been reported in the pancreas, spleen, and adrenal glands. Another receptor is believed to exist, based on reports of cells that do not express GHS-R responding to ghrelin stimulation.[7] In light of the antioxidant properties of ghrelin in other systems, we designed this study to explore the effects of this peptide on acute liver injury induced by CCl_4 in rats.

MATERIAL and METHOD

Experimental toxic hepatitis induced by carbon →

tetrachloride is recognized as the most effective experimental model because of similarity of histological and physiologic responses with human beings. All the experimental protocols were approved by the Eskişehir Osmangazi University. These animal studies were approved by the Eskisehir Osmangazi University Medical Faculty Ethical Committee (approval date and number: 21.01.2009/82), and experimental procedures were conducted in compliance with the National Instuties of Health Guide for the Care and Use of Laboratory Animals.

Male and female 3 month old Sprague-Dawley rats weighing 200-250 grams were divided in three groups, control (group 1), CCl_4 (group 2), and CCl_4+ghrelin (group 3), with n=8 for each group.

At the start of the study, CCl_4 (4 mg/kg i.p., Merck KGaA, Darmstadt, Germany) was administered to induce hepatolysis; control rats received vehicle (olive oil). Aft er three and six hours, rats in the CCl_4+ghrelin group were treated with recombinant ghrelin (80 micrograms/kg, i.p. Sigma-Aldrich Chemical Co. St. Louis, MO USA). All other rats received saline injections (2 ml/kg).

Twenty-four hours after the initial treatment with CCl_4, and following 8 hours of fasting, 50 mg/kg sodium pentothal was administered subcutaneously, followed by laparotomy. Approximately 3 ml blood samples were drawn by intracardiac puncture in all rats. Thereafter, animals were sacrificed by decapitation and livers were removed.

This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (http://www.nap.edu/catalog/5140.html). Ethics committee approval was obtained.

Biochemical Evaluations

Alanine aminotransferase (ALT), aspartate amino-transferase (AST) and malondialdehyde (MDA) levels were compared between baseline and final blood samples. Serum ALT and AST levels were measured by Roche Modular System automated analyzer using original kits from Roche Diagnostics. Serum MDA levels were determined according to the method of Ohkawa et al.[8] Liver tissue samples were homogenized in 10x 0.1 M phosphate buffer (pH 7.4) using a T18 basic homogenizer (IKA Laboratory Equipment, Wilmington NC, USA). The supernatant was used for tissue MDA, MPO and NO level and protein analyses. Tissue MDA was assayed spectrophotometrically by the method of Ohkawa et al. with some modifications.[8] 1,1,3,3'-tetraethoxypropan was used as external standard. The MPO levels were determined by the method of Suzuki et al.[9] The oxidation of catalyzed by MPO was measured at 655 nm in a spectrophotometer (Shimadzu UV-1601, Japan). Tissue NO levels were determined using the kinetic cadmium reduction method. Briefly, nitrite and nitrate in homogenates were reacted with copper-coated cadmium granules and the absorption of the reaction was monitored at 545 nm in a spectrophotometer.[10]

Protein concentrations in tissue homogenates were analyzed using a commercial Lowry protein kit purchased from Sigma-Aldrich (St, Louis, MO, USA).

Histopathological Evaluations

Liver samples were fixed in neutral formalin for 24 hours and processed for histology by a tissue processor.→

Table 1: Biochemical findings

Biochemical Findings	Groups	Median (25^{th}-75^{th})	Multiple Comparison Results		
			CCl_4	CCl_4+Ghrelin	Control
AST serum (U/L)	CCl_4	2587[a,b] (1204-8913)		*	**
	CCl_4+Ghrelin	1255[b,a] (50-5357)	*		**
	Control	260.00[c,a] (183-336)	**	**	
	H=11.384 DF=2				
ALT serum (U/L)	CCl_4	1792[a,b] (420-543)		*	**
	CCl_4+Ghrelin	634,[b,a] (309-1575)	*		**
	Control	228[c,a] (50-118)	**	**	
	H=11.425 DF=2				
MPO tissue (nmol/g protein)	CCl_4	6.587[a,b] (3.20-7.66)		*	**
	CCl_4 +Ghrelin	3.179[b,a] (1.89-3.76)	*		**
	Control	2.07[c,a] (1.02-3.85)	**	**	
	H=11.282 DF=2				
MDA serum (pg/ml)	CCl_4	12.67[a,b] (10.8-26.7)		*	**
	CCl_4 Ghrelin	7.92[b,a] (6.98-8.34)	*		**
	Control	6.78[c,a] (5.2-11.9)	**	**	
	H=12.128 DF=2				
MDA tissue (nmol/g protein)	CCl_4	53.82[a,b] (43.8-75.6)		*	**
	CCl_4 +Ghrelin	36.70[b,a] (28.6-42.2)	*		**
	Control	10.14[c,a] (10.2-11.9)	**	**	
	H=11.752 DF=2				

*: $p < 0.05$,**: $p < 0.001$, KRUSKAL-WALLIS H, a: CCl4+Ghrelin vs Control $p < 0.001$, b: CCl4+Ghrelin vs CCl4, $p < 0.05$, c: CCl4 vs Control, $p < 0.001$, MPO: Miyeloperoksidaz, MDA: Malondialdehid, ALT: Alanin aminotransferaz, AST: Aspartat aminotransferaz

Table 2: Biochemical findings

Biochemical findings	Groups	Mean±SD	Multiple Comparison Results		
			CCl_4	CCl_4+Ghrelin	Control
NO tissue (nmol/g protein)	CCl_4	0.288 ± 0.28			
	CCl_4+ Ghrelin	0.321 ± 0.43	ns		ns
	Control	0.402±0.37	ns	ns	
	F(2:21)=2.409 p=0.114				

ns: $p > 0.05$, ANOVA (Analysis of variance), NO: Nitric Oxide, CCl_4: Carbon tetrachloride

Table 3: Histological findings

	Groups	Median (25^{th} -75^{th})	Multiple Comparison Results		
			CCl_4	CCl_4+Ghrelin	Control
Necrosis	CCl_4	3.000[c] (1.5-3.0)		ns	*
	CCl_4 + Ghrelin	1.500[a, b] (1.0-2.0)[a]	ns		*
	Control	0.00[c,a] (0.0-0.0)	*	*	
	H =13.506 DF=2 p=0.003				
Hemorrhage + congestion	CCl_4	2.500[c] (1.5-3.0)		ns	*
	CCl_4 + Ghrelin	1.000[a,b] (1.0-2.0)	ns		*
	Control	0.000[c,a] (0.0-0.0)	*	*	
	H=13.855 DF=2 p<0.001				
Vacuolar Degeneration	CCl_4	3.000[c] (3.0-3.0)		ns	*
	CCl_4 + Ghrelin	2.000[a,b] (2.0-3.0)	ns		*
	Control	0.00[c,a] (0.0-0.0)	*	*	
	H=14.82 DF=2 p<0.001				
PMNL	CCl_4	3.000[c] (3.0-3.0)		ns	*
	CCl_4 + Ghrelin	2.000[a, b] (2.0-2.0)	ns		*
	Control	0.000[c,a] (0.0-0.0)	*	*	
	H=14.82 DF=2 p<0.001				
MNL	CCl_4	1.000[c] (0.5-1.0)		ns	*
	CCl_4 + Ghrelin	1.000[a,b] (0.5-1.0)	ns		*
	Control	0.000[c,a] (0.0-0.0)	*	*	
	H=14.82 DF=2 p<0.001				

ns : p>0.05, *: p<0.05 KRUSKAL-WALLIS H, Histological findings: Absent (0 degree), mild (1 degree), moderate (2 degree) or severe (3 degree). a: CCl_4+Ghrelin vs Control p< 0.05, b: CCl4+Ghrelin vs CCl4 p>0.05, c: CCl_4 vs Control p< 0.05, MNL:Mononuclear leukocyte, PMN: Polymorphonuclear leukocyte

Paraffin-embedded tissues were sliced in 4 mm serial sections and mounted on poly-L-lysine-coated glass slides. Slides were stained with hematoxylin and eosin (H&E) to reveal general structural features of the livers. Hepatic injury was scored as absent (0 degree), mild (1 degree), moderate (2 degree) or severe (3 degree). Evaluation of hepatic injury was performed by scoring the presence of focal necrosis in the parenchymal portal area and sinusoids, hemorrhage and congestion, vacuolar degeneration in hepatocytes and the location, PMNL and MNL infiltration.

Statistical Analyses

Data were evaluated with Sigma Stat 3.5 and SPSS 15 Shapiro Wilk normality test was applied for all variables. One-way analysis of variance (ANOVA) was performed for normally distributed variables. The post hoc Holm-SIDAK method was applied as a multiple comparison test. Non-normally distributed variables were analyzed by using Kruskal-Wallis H test, with the nonparametric post-hoc Tukey HSD as a multiple comparison test.

Mean±standard deviation (SD) and median (quartiles) were given as the descriptive statistics. p values less than 0.05 were considered statistically significant.

RESULTS

Biochemical results

The results of biochemical analyses of serum ALT, AST, and MDA, as well as tissue MDA, and MPO are shown in Table 1. A significant difference was observed in serum ALT and AST levels between the CCl_4 and CCl_4+ghrelin groups ($p<0.05$). In addition, there was a significant difference between the control and the two treatment groups ($p<0.001$).

Ghrelin also altered serum MDA, which increased from 6.78±0.35 pg/ml in the control group to 12.67±1.77 pg/ml in rats treated with CCl_4, but was 7.92±0.26 pg/ml in the CCl_4+ghrelin group. There was a significant difference between the CCl_4 and CCl_4+ghrelin groups ($p<0.05$), and between the control and treatment groups ($p<0.001$).

The tissue MDA results showed similar effects of both CCl_4 and ghrelin. Baseline tissue MDA was 10.14 nmol per gram protein (nmol/g.pr). This value was increased to 53.82±3.67 nmol/g.pr in the CCl_4 group, but decreased to 36.70±0.40 nmol/g.pr in the presence of ghrelin ($p<0.05$). Tissue MPO level was 2.07 nmol/g.pr in control animals, increased to 6.587±12.6 nmol/g.pr in the CCl_4-treated group, and decreased to 2.793±2.38 nmol/g.pr in animals treated with ghrelin. Again, there was a significant difference, between the CCl_4 and CCl_4+ghrelin groups ($p<0.05$), and between the control and treatment groups ($p<0.001$).

In the control group, tissue NO was 0.402 nmol/g.pr. It was decreased to 0.288 nmol/g.pr in the CCl_4 group and this was increased to 0.321 nmol/g.pr with the addition of ghrelin. However, these values were not significantly different between any of the groups (Table 2).

Histological Findings

The control group did not show any signs of congestion, hemorrhage, vacuolar degeneration, focal necrosis sites, or leukocyte infiltration (Figure 1). In the CCl_4-exposed group, severe hemorrhage was observed in the central vein, portal area and sinusoids. In addition, vacuolar degeneration of hepatocytes was severe in the centrilobular area and moderate along the mid-zonal line. Focal necrosis was present in the parenchyma, and PMNL infiltration was severe. By contrast, MNL infiltration was mild in the central vein, severe in portal area, and moderate in the sinusoids (Figure 2).

Vacuolar degeneration, hemorrhage, congestion, and leukocyte infiltration were all moderate in →

the CCl_4+ghrelin group, and mild focal necrosis was present (Figure 3). Compared to the CCl_4 group, sinusoidal congestion and hemorrhage were decreased in the rats treated with CCl_4 and ghrelin. Similarly, vacuolar degeneration was present only in the centrilobular zone in the CCl_4+ghrelin group, in contrast to more wide-spread damage in the CCl_4 group. Decreased PMNL and MNL infiltration were also observed in the CCl_4+ghrelin group (Figure 3). Histological findings are summarized in Table 3.

DISCUSSION

CCl_4 is a xenobiotic that is commonly used to induce experimental liver injury; in addition to acute liver failure, it can lead to severe pathological conditions, such as fibrosis, hepatitis and cirrhosis of the liver.[11] Previous studies have shown that CCl_4 causes chemical hepatotoxic effects; increases AST levels and increases the lipid peroxidation indicator, MDA, in rats.[12] We used these biochemical and histopathological features of acute liver failure in rats as a model for human disease. As seen in other experimental liver injury models, the serum transaminases indicate hepatocyte injury with high sensitivity.[13] In our study, serum AST and ALT levels were greater than the controls in all CCl_4-treated animals, despite a modulation of this increase in the ghrelin-treated group. These findings are similar to results previously published by other researchers.[14]

Oxygen free radicals generated as a result of CCl_4 toxicity initiate lipid peroxidation by receiving a hydrogen atom from polyunsaturated fatty acids and by generating lipid peroxides, which lead to acute liver injury.[15] As a result of lipid peroxidation, the cell membrane loses viscosity and membrane integrity deteriorates. This leads to the release of cell contents into the environment and cell death. The release of subcellular structures also promotes inflammation, which aggravates the injury.[16] Thus, lipid peroxidation can be used to monitor hepatic damage. Several methods have been used to indicate lipid peroxidation in tissues. The most common method measures the diene conjugate malondialdehyde.[17-19] In our study, the MDA concentration was significantly increased in liver tissue of CCl_4-exposed rats, suggesting toxic injury to the tissue. In animals treated with ghrelin, both serum and tissue MDA levels were decreased, compared to the CCl_4 group. This indicates that ghrelin decreased oxidative stress and thus lipid peroxidation. A previous study reported similar effects of ghrelin treatment, compared to control rats.[20-22]

Hepatic cells produce several antioxidant enzymes, such as catalase, myeloperoxidase, glutathione peroxidase, and glutathione reductase, in response to oxygen free radical accumulation and toxic injury to the liver. Previous studies have shown that ghrelin enhances antioxidant activity.[23] In our study, serum and tissue myeloperoxidase levels were lower in rats treated with CCl_4 and ghrelin, compared to CCl_4 alone, supporting this antioxidant effect of ghrelin. Experimental studies have also shown that NO levels are decreased in acute →

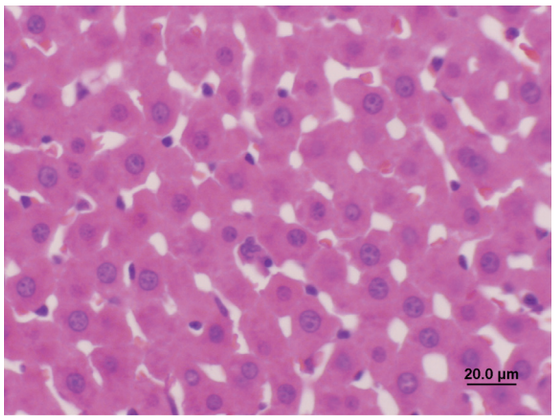

Figure 1: Representative image of hematoxylin and eosin staining in livers from control groups rats (magnification x400)

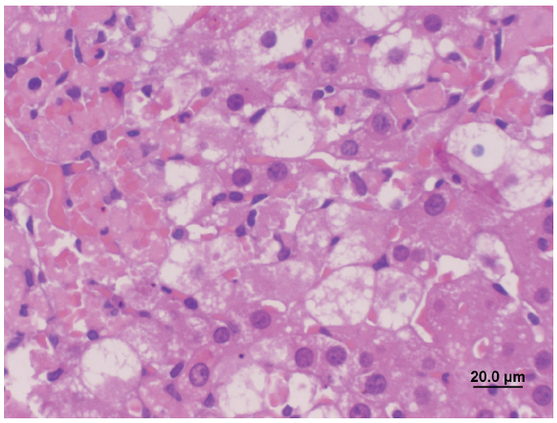

Figure 2: Representative image of hematoxylin and eosin staining in livers from CCl_4 injured rats (magnification x400)

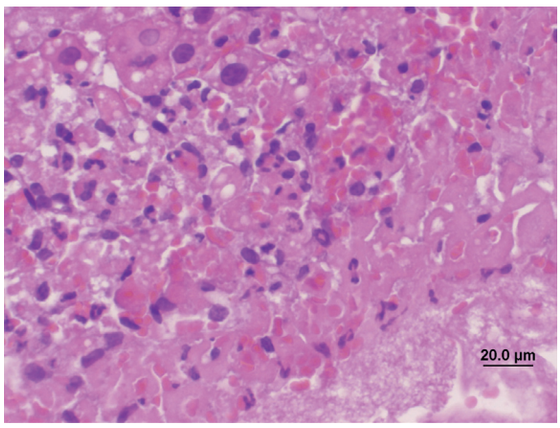

Figure 3: Representative image of hematoxylin and eosin staining in livers from CCl_4 injured rats treated with Ghrelin (magnification x400).

liver injury, and that antioxidant agents can increase NO levels. Similarly, our findings demonstrated higher NO levels in the CCl_4+ghrelin group than the CCl_4 group, suggesting that ghrelin increases antioxidant enzymes.[24-26]

This study shows that acute liver injury induced by CCl_4 in rats causes increased serum ALT, AST, and MDA, as well as increased tissue MDA and MPO. The antioxidant effects of ghrelin, however, decreased these indicators of liver toxicity. Moreover, animals treated with ghrelin show modulation of NO reduction, further indicating limited hepatotoxicity.

Microscopic evaluation of liver tissue revealed moderate congestion, vacuolar degeneration, and leukocyte infiltration in the CCl_4 group compared to hepatocytes of the control group. In rats treated with CCl_4 and ghrelin, congestion in the central and portal vein regions was not improved. However, both degree and extent of sinusoidal congestion, MNL infiltration, and vacuolar degeneration were decreased in this group. Other experimental studies have also demonstrated that histopathological analysis reveals less severe acute liver injury induced by CCl_4 when ghrelin is co-administered.[27] In our study, histopathological comparison of the two groups showed a decrease in necrosis, hemorrhage, congestion, polymorphonuclear leukocyte infiltration, and hepatocyte vacuolar degeneration. Altogether, these results suggest that ghrelin reverses toxic damage induced by CCl_4.

CONCLUSION

No effective therapies currently exist for patients with acute liver injury. This study indicates the antioxidant effects of ghrelin, and suggesting that ghrelin may be a promising therapeutic agent for the treatment of acute liver failure.[28]

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