Bull. Egypt. Soc. Physiol. Sci. 38(2), 149- 164

Bull. of Egyp. Soc. Physiol. Sci.

(Official Journal of Egyptian Society for Physiological Sciences)

(pISSN: 1110-0842; eISSN: 2356-9514)

Effects of Activation of GHSR1a on Hepatic Fibrosis in Type 2 Diabetic Rats

*** Mohamed Adel**

Department of Medical Physiology, Faculty of Medicine, Mansoura University, Egypt

Received: 25 Oct 2017
Accepted: 25 Feb 2018
Available online: 1 June 2018

Keywords

- GHS-R
- T2DM
- Ghrelin
- oxidative stress.

Abstract

Background: Diabetes is associated with nonalcoholic liver disease, steatohepatitis, and liver cirrhosis with their increased complications. In the current study, ghrelin, the agonist of GHS-R 1a was investigated. **Materials and Methods:** thirty rats were randomly divided into: control negative, control positive (Diabetic) and acylated ghrelin + T2DM groups each has 10 rats. Serum glucose and insulin, and also, triglyceride to high density lipoproteins (TG: HDL) ratio of all rats were measured to confirm the development of T2DM. Measurement of oxidative stress biomarkers in liver homogenate were performed. **Results:** In the diabetic group that received ghrelin, tissue MDA levels were significantly lower than in the diabetic group. Moreover, serum AST and ALT levels were higher in the diabetic group, but there was a significant decrease in the ghrelin-treated group. These results suggested that GHSR-1a can protect the liver of diabetic rats against the oxidative stress effects. **Conclusion:** the antioxidant activity of ghrelin could attenuate diabetic-induced liver fibrosis.

Corresponding author: Mohamed Adel Department of Medical Physiology, Faculty of Medicine, Mansoura University, Egypt.
Email: madel7744@yahoo.com

Introduction

Type 2 diabetes (T2DM) is a metabolic disease characterized by elevated blood glucose level, peripheral insulin resistance (IR), and a defective metabolism (1). Although there are several medications currently available to manage T2DM, there is an increasing need for more effective therapy than those currently available. T2DM is associated with a number of disorders and comorbidities, including diabetic retinopathy, blindness, diabetic nephropathy, lower limb amputation and cardiovascular disease mortality (2). It was recorded that diabetes itself caused 5 million persons to die in 2013 (1). Nearly, the whole list of hepatic diseases is seen in patients with type 2 diabetes. This includes abnormal hepatic enzymes, nonalcoholic fatty liver disease (NAFLD), cirrhosis, hepatocellular carcinoma, and acute hepatic failure. In addition, there is an unexplained association of diabetes with hepatitis C. Finally, the prevalence of diabetes in cirrhosis is 12.3–57% (3). Thus, patients with diabetes have a high prevalence of hepatic diseases and patients with hepatic disease have a high prevalence of diabetes. Expression of GHS-R1a was found primarily in pituitary somatotrophs and hypothalamus (4). Also, in other extra-hypothalamic areas of nervous system, such as hippocampus, substantia nigra, ventral tegmental area, dorsal and medial raphe nuclei, Edinger-Westphal nucleus, pons and medulla oblongata (5). Determination of the GHS-R1a in peripheral tissues gave controversial results. We hypothesize that GHS-R1a regulates diabetic hepatic injury and fibrogenesis. To prove this hypothesis, we investigated the effect of GHS-R1a agonist recombinant ghrelin in diabetic liver injury model. So, the aim of this work is to study the possible protective therapeutic effect of stimulation of GHSR-1Aa on the diabetic- induced liver fibrosis and the underlying mechanism.

Subjects and methods:

Thirty albino Sprague Dawley rats were used in the present study. The animals had free access to food and water and were housed in individual cages with a 12-hour light-dark cycle. The animals were adapted to these conditions for at least 1 week before being used in the experiments and general conditions were monitored throughout the study. All experimental procedures were approved by the ethical committee (IRB) and given the code number R/17. 07.42

Study design

Our study lasts for thirteen weeks, during which rats were randomly divided into 3 groups (10 rats each) divided as follow; a) Control negative: normal rats on normal diet ad libitum (60% CHO/10% fat/30% protein, as a percentage of total kcal). b) Control positive (Diabetic). c) Acylated ghrelin + T2DM: acylated ghrelin is administered by subcutaneous injection at a dose of (0.2 µg) per day for four subsequent days **(**6).

Induction of T2DM in control positive and acylated ghrelin groups

Rats were fed on a high fat diet (HFD) (Table 1) (7) (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) for about 2 month. After 2 month of HFD, 35 mg/kg streptozotocin (STZ) (Sigma Chemical Co., St. Louis, MO, USA)

dissolved in 10 mM citrate buffer (PH = 4.5) was given by intraperitoneal method in the lower right quadrant of the abdomen for induction of T2DM in control positive and acylated ghrelin groups, while the control negative rats were injected with citrate buffer alone (8). After one week of streptozotocin injection, serum glucose, insulin and also triglyceride to high density lipoproteins (TG: HDL) ratio of all rats were measured by retro-orbital plexus sampling to confirm the development of T2DM in diabetic and ghrelin-treated groups (9). Development of T2DM and insulin resistance (IR) was confirmed, plasma glucose level of more than (200 mg/dl) was used as the "cut off value" of hyperglycemia for confirming T2DM (10). Insulin resistance cannot be measured directly, therefore (TG: HDL) ratio was used as an insulin resistance marker. The cut off value of ratio as 1.8 was used to mark insulin resistance (11). (TG: HDL) ratio produces similar results to other methods used to measure insulin resistance such as the QUICKI and HOMA-IR (9). At the end of experimentation, the body weight was measured then the animals were anesthetized with pentobarbital [0.6 ml/ kg] and the blood collected by cardiac puncture and allowed to clot for 30 minutes. Serum was separated by centrifugation at 2500 rpm for 15 minutes and used for biochemical estimations. After that, rats were sacrificed by cervical dislocation and the abdomen of terminated animal was cut open quickly and the liver was immediately removed and washed thoroughly with ice-cold 0.9 % sodium chloride solution (saline) and dried with filter paper and weighted. Then portion of the liver was used to assess biochemical parameters and the other was fixed in 10 % formalin for histological examinations.

Liver functions assay

Biochemical analysis were carried out to determine the serum concentrations of total protein, albumin, total bilirubin, cholesterol, triglycerides (TG) and the activities of liver enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Biochemical analysis was done by using commercial kits provided by Biomerieux, Egypt and Uvikon 930 spectrophotometer (Kontron Instrument, Milan, Italy). Aspartate and alanine aminotransferases were determined based on a colorimetric method (12), in addition to alkaline phosphatase (13). The absorbance of the test samples were read at 505 nm for AST and ALT and at 510 nm for ALP. Total protein was determined by the biuret method, albumin by the bromocresol green method (14, 15). Triglycerides (TG) concentration was determined (16) and total cholesterol concentrations were determined (17). The absorbance of the test samples for TG and cholesterol were read at 546 nm against blank. High Density Lipoprotein Cholesterol (HDL-C) concentrations were determined using enzymatic methods as described in the instructions provided with the kits (Analyticon® Biotechnologies AG, Germany). The absorbance of the testes samples were read using spectrophotometer adjusted at 500 nm.

Liver homogenate preparation

Samples of liver tissue were homogenized (1:10, w/v) in chilled phosphate buffer (0.1 M, pH 7.4) containing KCI (1.17%).The homogenate was centrifuged at 8000 rpm for 5 minutes at 4°C to separate the nuclear debris and supernatant was collected. The centrifuged homogenates were stored at -25°C until they were analyzed.

Measurement of liver reduced glutathione (GSH) concentration, Superoxide dismutase (SOD), and catalase (CAT) activity

Liver homogenate was subjected for the assay of free radical metabolizing enzymes by using a spectrophotometer and commercially available colorimetric kits (Bio Diagnostics, Dokki, and Giza, Egypt). Determination of liver reduced glutathione (GSH) concentration (µmol/ gm) was determined (18). Superoxide dismutase (SOD) activity (U/ mg) was measured (19). Liver catalase (CAT) activity (U/ mg) (20). The absorbance of the test samples were read at 412 nm for GSH, 560 nm for SOD and at 240 nm for CAT.

Assay of lipid peroxidations marker malondialdehyde (MDA) and Serum advanced oxidation protein products (AOPP) in liver homogenate

The assay for lipid peroxidation of the liver was carried out (21). It was measured as MDA. Thiobarbituric acid reacts with malondialdehyde to yield a coloured product that can be measured at 535 nm. The results were expressed as nmol MDA formed/hr/g. tissue. Serum advanced oxidation protein products (AOPP) (µmol/ L) was measured in serum (22). The absorbance was measured at 340 nm against blank. Kits used were commercially available colorimetric kits (Bio Diagnostics, Dokki, Giza, Egypt).

Histopathological examination of liver

Liver were dissected out and fixed instantaneously in 10% formal saline for 24 hours. The specimens were washed in tap water, dehydrated in ascending grades of ethanol, cleared in xylene, embedded in paraffin wax at melting point 55-60 °C. Sections of 5 µm thickness were prepared and stained with haematoxylin and eosin (H&E) to examine morphology. Masson's trichrome stain was used to assess fibrosis. All slides were examined under light microscopy at low (X 100) and high (X 400) magnification. Grading and scoring for liver injury was done according to presence and degree of fatty change, hepatocellular ballooning, inflammation, necrosis and fibrosis. The liver pathology was scored [23] as follows: Score 0 = no visible cell damage, Score 1 = focal hepatocyte damage on less than 25 % of the tissue, Score 2 = focal hepatocyte damage on 25-50 % of the tissue, Score 3 = extensive, but focal, hepatocyte lesions, Score 4 = global hepatocyte necrosis. While hepatic fibrosis was graded [24] as the following: Absent (-) = normal liver, Few (+) = increase of collagen without formation of septa, Mild (++) = formation of incomplete septa from portal tract to central vein, Moderate (+++) = complete but thin septa interconnecting with each other.

Statistical analysis

Data were tabulated, coded then analyzed using the computer program SPSS (**Statistical package for social science**) version 17.0. Descriptive statistics were calculated in the form of Mean ± Standard

deviation (SD). Student t-test was used to compare between mean of two groups of numerical (parametric) data. While, ANOVA (analysis of variance):- Used to compare between more than two groups of numerical (parametric) data. P value <0.05 was considered statistically significant.

Table (1): Food composition given to the groups

Normal diet		HFD	
Ingredients	**Diet (g/kg)**	**Ingredients**	**Diet (g/kg)**
Wheat flour	**285**	Casein	254
Wheat brawn	**285**	Cornstarch	169
Salt (common)	**5**	Sucrose	85
Mollasen	**15**	Wheat bran	51
Soybean oil (ml/kg)	**50**	Safflower oil (ml/kg)	339
Fish meat	**150**	Gelatin	19
Vitamins/ minerals	**10**	Salt mix	67
Dried skimmed milk	**200**	Vitamin e acetate (500 iu/g)	0.31
		VITAMIN MIX	13
		DL-Methionine/L-cystine	3

Results

Table (2) shows that STZ administration increased non- fasting glucose serum levels (mg/dl) significantly from 92.2±13.2 to 211.8±15.1 (P < 0.001). Also, Table (2) shows that ghrelin administration increased non- fasting glucose serum levels (mg/dl) significantly from 212.6±21.5 to 220.2±11.5 (P < 0.001).

Table (2): Non- fasting glucose serum levels (mg/dl) all over the period of the experiment in the three groups of rats.

	1st week	**8th week**	**1 week after STZ**	**1 week after AG**	**4 week after AG**
C-ve					
Mean ± SD	100.2 ± 12.7	108.3 ± 11.2	105.4 ±1 2.7	117.5±1 1.1	111.4±16.1
P1	-	< 0.05	0.225	< 0.05	< 0.05
P2			-	< 0.05	< 0.05
C+ve					
Mean ± SD	97.2 ± 13.2	92.2 ± 13.5	211.8 ± 15.1	233.5 ± 24.5	224.9 ± 23.2
P1	-	< 0.05	< 0.001	< 0.001	< 0.001
P2			-	< 0.05	0.087
AG+T2DM					
Mean ± SD	115.3 ± 14.3	111.7 ± 12.7	212.6 ± 21.5	220.2 ± 11.5	224.2 ± 12.7
P1	-	0.177	< 0.001	< 0.001	< 0.001
P2			-	0.213	0.069

P: Probabilty Test used: Student's paired T-test All values have been expressed as Mean ± SD. Control negative group(C-ve; n=10), control positive group (C+ve; n=10), and AG +T2DM group (n=10). P1 ≤ 0.05significant as compared with 1st week, and P2≤ 0.05 significant as compared with 9th week (1 week after STZ). Highly significant: P < 0.001

Table (3) shows that non fasting insulin serum levels (Pmol/L) increased significantly from 63±7 to 155±18 (P < 0.05). Also, table (3) shows that ghrelin administration increased non- fasting insulin serum levels (Pmol/L) from 161±15 to 166±15 (P = 0.344).

Table (3): Non fasting insulin serum levels (Pmol/L) all over the period of the experiment in the three groups of rats.

	1st week	8th week	1 week after STZ	1 week after AG	4 weeks after AG
C-ve					
Mean ± SD	66±5	63±6	63±6	67±4	65±7
P1	-	0.47	0.57	1.00	0.5
P2			-	0.6	0.8
C+ve					
Mean ± SD	63±7	69±9	155±18	159±11	166±14
P1	-	0.57	< 0.05	< 0.05	< 0.05
P2				0.12	< 0.05
AG +T2DM					
Mean ± SD	63±7	71±7	161±15	166±15	172±11
P1	-	0.099	< 0.001	< 0.001	< 0.001
P2			-	0.344	0.2

P: Probabilty Test used: Student's paired T-test. All values have been expressed as Mean ± SD. Control negative group(C-ve; n=10), control positive group (C+ve; n=10), and AG +T2DM group (n=10). P1 ≤ 0.05significant as compared with 1st week, and P2≤ 0.05 significant as compared with 9th week (1 week after STZ). Highly significant: P < 0.001

Table (4) shows that TG: HDL ratio increased significantly from 1.31±0.4 to 2.23±0.7 (P < 0.001). Also, table (4) shows that ghrelin administration decreased TG: HDL ratio from 2.9±0.9 to 2.5±0.8 (P = 0.33).

Table (4): TG: HDL ratio all over the period of the experiment in the three groups of rats.

	1st week	8th week	1 week after STZ	1 week after AG	4 weeks after AG
C-ve					
Mean ± SD	1.27 ± 0.3	1.265 ± 0.3	1.355 ± 0.2	1.35 ± 0.2	1.225 ± 0.3
P1	-	0.97	0.56	0.27	0.78
P2			-	5	5
C+ve					
Mean ± SD	1.31 ± 0.4	1.57 ± 0.2	2.23 ± 0.7	2.25 ± 0.7	2.30 ± 0.5
P1	-	0.117	< 0.001	< 0.001	< 0.001
P2			-	0.87	0.61
AG +T2DM					
Mean ± SD	1.2 ± 0.4	1.30 ± 0.3	2.9 ± 0.9	2.5± 0.8	2.44 ± 0.3
P1	-	0.53	< 0.05	< 0.05	< 0.05
P2			-	0.33	0.19

P: Probabilty Test used: Student's paired T-test. All values have been expressed as Mean ± SD. Control negative group(C-ve; n=10), control positive group (C+ve; n=10), and AG +T2DM group (n=10). P1 ≤ 0.05significant as compared with 1st week, and P2≤ 0.05 significant as compared with 9th week (1 week after STZ). Highly significant: P < 0.001

Table (5) shows that diabetes led to a non-significant decrease in total proteins, it decreased from 12.5± 1.1 to 9.5± 1.22 (g/L) (P =0.14). While ghrelin administration increases total proteins from 9.5± 1.22 to 11.6±1.1. Also, table (5) shows that diabetes led to a significant decrease in serum albumin, it decreased from 1.9± 0.1 to 1.3± 0.22 (g/L) (P <0.05). While ghrelin administration increases albumin from 1.3± 0.22 to 1.33±0.1(g/L). Table (5) shows that diabetes led to a significant increase in TG, it increased from 1.77± 0.4 to 3.36± 1 (P <0.05). While ghrelin administration decreases TG from 3.36± 0.22 to 2.5±1 (mg/dl) (P <0.05). Table (5) shows that diabetes led to a non-significant increase in cholesterol, it increased from 4.4 ± 1.1 to 6.10±2 (P=0.055). While ghrelin administration decreases cholesterol from (6.1± 2) to (5.31±1.14) (mg/dl) (P =0.489). Diabetes led to a highly significant increase in ALP, ALT and AST, it increases these liver enzymes from (19.6± 1.7), (26.71± 1.7) (IU/L) and (47.55±7.7) (IU/L) to (63.5±2.9), (89.87±4.9) and (111.08±12.4) (P < 0.001) respectively. While ghrelin led to a highly significant decrease in ALP and ALT from (63.5±2.9), (89.87±4.9) to (27.2± 1) and (44.9± 1) (P < 0.001).

Table (5): Liver function tests in different studied groups

Parameters	**Group 1 (Control)**	**Group 2 (Diabetic)**	**Group 3 (AG+ Diabetes)**
Total protein (g/L)	12.5 ±1.1	9.5±1.22	11.6± 1.1
P1			0.79
P2		0.14	0.38
Albumin (g/L)	1.9 ± 0.1	1.3 ±0.22	1.33± 0.1
P1			0.05
P2		<0.05	0.99
TG (mg/dl)	1.77± 0.4	3.36± 1	2.5± 1
P1			0.102
P2		<0.05	<0.05
Cholesterol (mg/dl)	4.4 ± 1.1	6.10±2	5.31±1.14
P1			0.379
P2		0.055	0.489
Total bilirubin (mg/dl)	1.33± 0.5	3.51 ± 1	2± 0.5
P1		0.000	0.092
P2			0.000
ALP (IU/L)			27.2± 1
P1	19.6± 1.7	63.5±2.9	
P2			0.075
		0.000	0.000
ALT (IU/L)	26.71± 1.7	89.87±4.9	44.9± 1
P1			<0.05
P2		0.000	0.000
AST (IU/L)	47.55±7.7	111.08±12.4	67.33±11.5
P1			0.000
P2		0.000	0.000

Test used: ANOVA followed by posthoctukey for multiple comparisons. Values are expressed as mean ± S.D. (n = 10). P <0.05 significant, P <0.001 highly significant P1as compared with control and P2 as compared with diabetic group. Alkaline phosphatase (ALP), alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), triglycerides (TG).

Table (6) shows a significant decrease in hepatic reduced glutathione (GSH) concentration in the diabetic group, it decreased significantly from 8.19± 1.7 to 4.6± 1.9 (µmol/gm) (P <0.001). While ghrelin administration increases, significantly, hepatic reduced glutathione (GSH) concentration from 4.6± 1.9 to 7.57±1.4 (µmol/gm). Also, table (6) shows a significant decrease in superoxide dismutase concentration in the diabetic group, it decreased significantly from (11.55± 2) to (5.28± 1) (µmol/gm) (P <0.001). While ghrelin administration increases, significantly, superoxide dismutase concentration from (5.28± 1) to 9.11±1.3 (µmol/gm). Moreover, table (6) shows a significant decrease in catalase activity in the diabetic group, it decreased significantly from (11.93± 2) to (7.75± 1.3) (U/mg) (P <0.001). While ghrelin administration increases its activity from (7.75± 1.3) to 9.8±1.2 (U/mg) (P <0.05).

Table (6): Hepatic reduced glutathione (GSH) concentration, superoxide dismutase (SOD), and catalase (CAT) activity in different studied groups

Parameters	**Group 1 (Control)**	**Group 2 (Diabetic)**	**Group 3 (AG+ Diabetes)**
GSH (µmol/gm) **P1** **P2**	8.19± 1.7	4.6± 1.9 0.000	7.57±1.4 0.5 0.000
SOD (U/mg) **P1** **P2**	11.55 ± 2	5.28± 1 0.000	9.11±1.3 <0.05 0.000
CAT (U/mg) **P1** **P2**	11.93± 2	7.75±1.3 0.000	9.8± 1.2 <0.05 <0.05

Test used: ANOVA followed by posthoctukey for multiple comparisons. Values are expressed as mean ± S.D. (n = 10). P <0.05 significant, P <0.001 highly significant P1as compared with control and P2 as compared with diabetic group.

Table (7) shows a significant increase in malondialdhyde (MDA) concentration in the diabetic group, it increased significantly from (22.63± 2) to (71.19±1.3) (nmol/h/g) (P <0.001). While ghrelin administration decreases, significantly, malondialdhyde (MDA) concentration from (71.19 ± 1.9) to (30.54±2.5) (nmol/h/g) (P <0.001). Also, table (7) shows a significant increase in advanced oxidation protein products **(**AOPP) concentration in the diabetic group, it increased significantly from (27.82± 2) to (44.68±0.3) (µmol/L) (P <0.001). While ghrelin administration decreases, significantly, AOPP concentration from (44.68±0.3) to 31.50±2.5 (µmol/L) (P <0.001).

Table (7): Hepatic lipid peroxidation (MDA) and serum advanced oxidation protein products (AOPPs) levels in different studied groups

Parameters	Group 1 (Control)	Group 2 (Diabetic)	Group 3 (AG+ Diabetes)
MDA (nmol/h/g)	22.63± 2	71.19±1.3	30.54±2.5
P1			<0.05
P2		0.000	0.000
AOPP (µmol/L)	27.82± 2	44.68±0.3	31.50±2.5
P1			0.13
P2		0.000	0.000

Test used: ANOVA followed by posthoctukey for multiple comparisons. Values are expressed as mean ± S.D. (n = 10). P <0.05 significant, P <0.001 highly significant P1as compared with control and P2 as compared with diabetic group.

Table (8): Hepatic histopathology score in different studied groups

Parameters	Group 1 (Control)	Group 2 (Diabetic)	Group 3 (AG+ Diabetes)
Hepatocyte necrosis	0	0	0
Inflammation	0	4	1
Cell swelling	0	3	2
Fatty degeneration	0	4	2
Fibrosis	-	+++	+

Histopathology results

The photomicrograph of a section in the liver of control negative group stained with Masson's tichrome stain (MT×200) shows normal distribution of collagen (no fibrosis) (Figure 1). While **t**he photomicrograph of a section in the liver of diabetic group (Figure 2) stained with Masson's tichrome stain (MT×200) shows marked perivenular fibrosis. The photomicrograph of a section in the liver of ghrelin-treated group stained with Masson's tichrome stain (MT ×200) shows no evidence of fibrosis (Figure 3). The photomicrograph of a section in the liver of diabetic group (H&E×200) shows minimal steatosis and ballooning (Figure 4). While the photomicrograph of a section in the liver of ghrelin-treated group (H&E×200) shows minimal steatosis and ballooning but inflammatory filtrate is reduced.

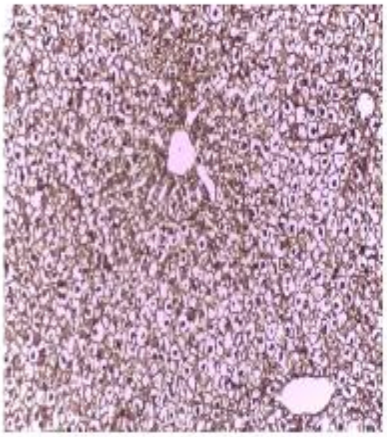

Figure 1: The photomicrograph of a section in the liver of control negative group. Masson's tichrome stain (MT×200) shows normal distribution of collagen (no fibrosis)

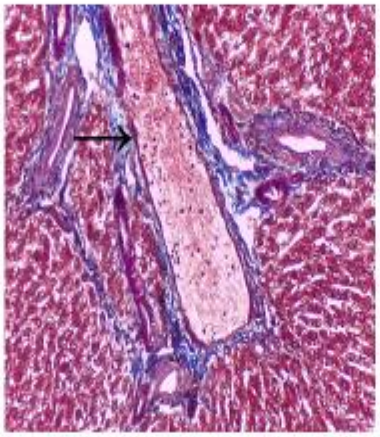

Figure 2: The photomicrograph of a section in the liver of diabetic group. Masson's tichrome stain (MT×200) shows marked perivenular fibrosis

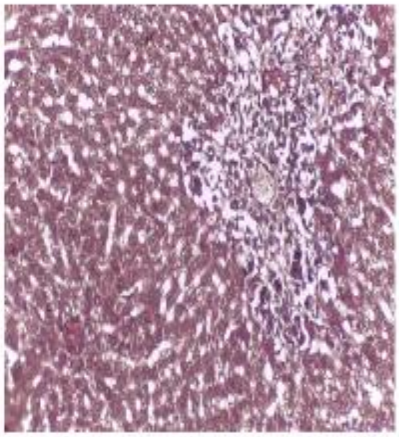

Figure 3: The photomicrograph of a section in the liver of ghrelin-treated group. (Masson's tichrome stain (MT ×200) shows no evidence of fibrosis

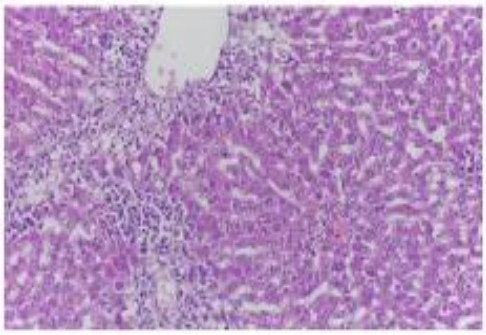

Figure 4: The photomicrograph of a section in the liver of diabetic group. (H&E×200) shows minimal steatosis and ballooning

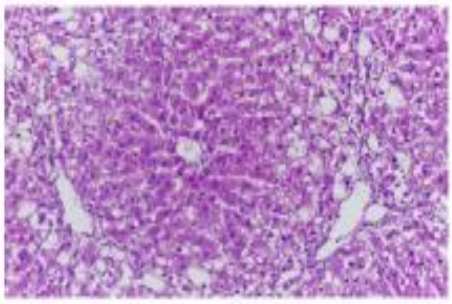

Figure 5: The photomicrograph of a section in the liver of ghrelin-treated group. (H&E×200) shows minimal steatosis and ballooning but inflammatory filtrate is reduced

Discussion

Hepatic functions are vital for the maintenance of glucose, lipid, and cholesterol homeostasis. Frequency of hepatic disorders increases yearly, and experimental studies for treatment of these disorders are performed.

Hepatic fibrosis is the continuous accumulation of extracellular matrix that occurs in various types of chronic hepatic diseases. Now, the only effective treatment for liver fibrosis is to eliminate the leading cause (e.g. suitable antiviral treatment in hepatic patients). For those patients in whom the underlying mechanism cannot be removed (e.g. liver fibrosis associated with diabetes), there are no effective antifibrotic therapy. Diabetes is associated with a spectrum of hepatic disorders including nonalcoholic liver disease, steatohepatitis, and liver cirrhosis with their increased complications (25). In the present study,

we used an experimental approach to characterize a new potential hepatoprotective mechanism via ghrelin (The agonist of GHS-R 1a.) in diabetic rats.

Ghrelin is a gastrointestinal hormone (28 amino acids) firstly discovered as a growth hormone secretagogue. Moreover, it plays a major role in food ingestion control (26). Peripheral effects such as cytoprotection, vasodilatation and control of calories, input and output have been, also, referred to ghrelin (27). The main site of ghrelin synthesis is the gastric mucosa, but ghrelin transcripts have been identified in many other locations, including the liver, intestine, pancreas, renal circulation, and lungs. Most ghrelin effects are mediated by growth hormone secretagogue sensors (GHS-R 1a) (2), which are mainly expressed in the hypophysis cerebri but also in other sites, including the pancreas, spleen, and suprarenal gland (28). Ghrelin has useful actions in different locations and cell types including pancreas, heart, and digestive system (29 and 30).

We hypothesize that GHSR-1a regulates diabetic hepatic fibrogenesis. To confirm this hypothesis, we investigated the effect of recombinant ghrelin on liver fibrosis induced by diabetes. We confirmed this evidence that recombinant ghrelin exerts protective and antifibrotic effects in the fibrosed liver. This is in accordance with the findings that ghrelin knockout (Ghrl–/–) rats are more prone to carbon tetrachloride induced liver fibrosis than wild type (Ghrl+/+) mice. Moreover, there is an evidence that ghrelin is locally produced in the human hepatic tissue. It has been demonstrated that lipid peroxidation is one of the key factors in acute hepatic toxic injury. Free oxygen radicals trigger lipid peroxidation by receiving a hydrogen atom from polyunsaturated fatty acids, and, ultimately, hydroperoxides are synthesized. As a result of these reactions, cell membranes lose their viscosity, and membrane integrity decreases, leading to release of cell fractions to the surroundings, and resulting in cell death. Moreover, these released subcellular structures promote inflammation and exaggerate the damage (31, 32 and 33). There are several methods used for indicating lipid peroxidation in tissue, the most common of which is measuring the malondialdehyde (MDA) (34).

The role of ghrelin in diabetic-induced hepatic injury was demonstrated in many previous studies and in different animal models. Administration of ghrelin might improve inflammation, oxidative stress, and apoptosis during and after non-alcoholic fatty liver disease development (NAFLD) (35), which is considered as a result and as a mechanism for insulin resistance (36). The relation of the three ghrelin gene components (Ghrelin, DAG, and obestatin) and their involvement in those metabolic and inflammatory pathways that are linked with the development of NAFLD was confirmed (37). Ghrelin and obestatin concentrations positively correlated with fibrosis stage. Apparently, products of the ghrelin gene may be essential for the genesis of NASH and fibrosis (37). Moreover, serum ghrelin levels significantly correlated with liver function indices (38). Histopathological examination of the hepatic tissues of diabetic group shows an extensive liver steatosis, inflammation and extensive perivenular fibrosis while the treatment with acylated ghrelin

reduces these histopathological changes. This antifibrotic effect of ghrelin is in agreement with the findings that ghrelin therapy stops neutrophil infiltration and decreases the amount of myofibroblast present in the injured hepatic tissue (39, 40). So, ghrelin downregulates the expression of collagen-α1 and TGF-β1 in the hepatic stellate cells, the principal fibrogenic cells in liver (39), leading to a decreased collagen formation (39, 40). Also, the results of this study in agreement with this work which found that ghrelin has anti-inflammatory and antifibrotic effects also in TAA-induced hepatic injury in rats where it decreases hepatic injury and collagen deposition through inhibition of hepatic cell apoptosis and antioxidative activity, in a way partially performed by the stimulation of nitric oxide (41).

Moreover, serum ghrelin concentrations are correlated with a low risk of developing NAFLD (42). The present study showed similar results, we demonstrated that MDA concentration, which is a product of lipid peroxidase and an indicator of oxidative stress, was significantly elevated in hepatic tissue of the diabetic group (Table 7), suggesting that there was a toxic injury in the tissue. In the diabetic group that received ghrelin, tissue MDA levels were significantly lower than in the diabetic group (Table 7). Table **(7)** shows a significant increase in malondialdehyde (MDA) concentration in the diabetic group, it increased significantly from (22.63± 2) to (71.19±1.3) (nmol/h/g) (P <0.001). While ghrelin administration decreases, significantly, malondialdhyde (MDA) concentration from (71.19 ± 1.9) to (30.54±2.5) (nmol/h/g) (P <0.001). Also, table (7) shows a significant increase in advanced oxidation protein products **(**AOPP) concentration in the diabetic group, it increased significantly from (27.82± 2) to (44.68±0.3) (µmol/L) (P <0.001). While ghrelin administration decreases, significantly, AOPP concentration from (44.68±0.3) to 31.50±2.5 (µmol/L) (P <0.001).

Moreover, in the present study, table (5) shows that diabetes led to a non-significant decrease in total proteins, it decreased from 12.5± 1.1 to 9.5± 1.22 (g/L) (P =0.14). While ghrelin administration increases total proteins from 9.5± 1.22 to 11.6±1.1. Also, table (5) shows that diabetes led to a significant decrease in serum albumin, it decreased from 1.9± 0.1 to 1.3± 0.22 (g/L) (P <0.05). While ghrelin administration increases albumin from 1.3± 0.22 to 1.33±0.1(g/L). Table (5) shows that diabetes led to a significant increase in TG, it increased from 1.77± 0.4 to 3.36± 1 (P <0.05). While ghrelin administration decreases TG from 3.36± 0.22 to 2.5±1 (mg/dl) (P <0.05). Table (5) shows that diabetes led to a non-significant increase in cholesterol, it increased from 4.4 ± 1.1 to 6.10±2 (P=0.055). While ghrelin administration decreases cholesterol from (6.1± 2) to (5.31±1.14) (mg/dl) (P =0.489). Diabetes led to a highly significant increase in ALP, ALT and AST, it increases these liver enzymes from (19.6± 1.7), (26.71± 1.7) (IU/L) and (47.55±7.7) (IU/L) to (63.5±2.9), (89.87±4.9) and (111.08±12.4) (P < 0.001) respectively. While ghrelin led to a highly significant decrease in ALP and ALT from (63.5±2.9), (89.87±4.9) to (27.2± 1) and (44.9± 1) (P < 0.001). All of these findings can be explained by the effect of ghrelin in decreasing oxidative stress and, thus, lipid peroxidation. These findings

are in line with the observation that MDA level decreases by ghrelin treatment on tenth day (43). When free oxygen radicals begin to accumulate after toxic injury to the liver, cells create a defense mechanism by using multiple antioxidant enzymes. In the present study, we demonstrated that ghrelin protects against diabetic-induced liver fibrosis and we suggest that this could be in response to its antioxidant activity. Table (6) shows a significant decrease in hepatic reduced glutathione (GSH) concentration in the diabetic group, it decreased significantly from 8.19± 1.7 to 4.6± 1.9 (µmol/gm) (P <0.001). While ghrelin administration increases, significantly, hepatic reduced glutathione (GSH) concentration from 4.6± 1.9 to 7.57±1.4 (µmol/gm). Also, table (6) shows a significant decrease in superoxide dismutase concentration in the diabetic group, it decreased significantly from (11.55± 2) to (5.28± 1) (µmol/gm) (P <0.001). While ghrelin administration increases, significantly, superoxide dismutase concentration from (5.28± 1) to 9.11±1.3 (µmol/gm). Moreover, table (6) shows a significant decrease in catalase activity in the diabetic group, it decreased significantly from (11.93± 2) to (7.75± 1.3) (U/mg) (P <0.001). While ghrelin administration increases its activity from (7.75± 1.3) to 9.8±1.2 (U/mg) (P <0.05). Increasing the antioxidants hepatic GSH concentration, SOD, and CAT activity in ghrelin-treated group proved the hypothesis that ghrelin protect against diabetic-induced liver fibrosis mainly due to its antioxidant activity.

Conclusion

The results of the present study confirmed the protective action of GHSR-1a and its agonist acylated ghrelin in diabetic-induced hepatic fibrosis. These effects might be explained on basis of its antioxidants activity. Thus, we used an experimental approach to characterize a new potential antifibrotic substance (Ghrelin, the agonist of GHS-R 1a.) and its hepatoprotective effect in diabetic rats

REFERENCES

1. International Diabetes Federation. Diabetes Atlas. 6th ed. Brussels, Belgium: International Diabetes Federation, 2013.

2. Klein R . Hyperglycemia and microvascular and macrovascular disease in diabetes. *Diabetes Care* 18(2):258-68, 1995.

3. Trombetta M, Spiazzi G, Zoppini G, Muggeo M: Review article: type 2 diabetes and chronic liver disease in the Verona diabetes study. Aliment Pharmacol Ther 22 (Suppl. 2):24 –27, 2005.

4. Howard AD, Feighner SD, Cully DF, and Arena JP. A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273:974-977. 1996

5. Muccioli G, Tschop M, Papotti M, Deghenghi R, Heiman M and Ghigo E. Neuroendocrine and peripheral activities of ghrelin: implications in metabolism and obesity. Eur J Pharmacol 440:235-254. 2002

6. Shoaib Bin Aleem, Muhammad Mazhar Hussain. Levo-carnitine supplementation normalizes skeletal muscle functions in type 2 diabetic rats. Int J Diabetes & Metab 21:1-8, 2012.

7. Srinivasan K, Viswanad B, Asrat L, Kaul CL, Ramarao P. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: A model

for type 2 diabetes and pharmacological screening. Pharmacol Res 52:313-20, 2005**.**

8. Olson K, Hendricks B, Murdock D. The Triglyceride to HDL Ratio and Its Relationship to Insulin Resistance in pre- and postpubertal children: observation from the Wausau School Project. Cholesterol. 2012.

9. Yassin MM, and Mwafy SN. Protective potential of glimepiride and nerium oleander extract on lipid profile, body growth rate, and renal function in streptozotocin-induced diabetic rats. *Turk J Biol* 31:95-102, 2007.

10. Mc Laugin T, Abbasi F, Cheal K, Chu J, Lamendola C, Reaven G. Use of metabolic markers to identify overweight individuals who are insulin resistant. *Ann Intern Med* **139**: 802-809, 2003.

11. Barazzoni R, Bosutti A, Stebel M and Cattin MR, Ghrelin regulates mitochondrial-lipid metabolism gene expression and tissue fat distribution in liver and skeletal muscle. Am J Physiol Endocrinol Metab 288:E228-235. 2005

12. Reitman, S. and Frankel, S. Determination of glutamic-oxaloacetic transaminase. America Journal of Clinical Pathology, 28:56-59. 1957

13. Babson, L.A. Estimation of Alkaline phosphatase activity. Clinical Chemistry, 11:789. 1965

14. Doumas, B.T., Watson, W.A. and Briggs, A.G. (1971). Albumin Estimation. Clinica Chimica Acta, 31:87-96. 1971

15. Jendrassik, L. and Grof, V. Estimation of direct and total Bilirubin. Biochemische Zeitschrift, 297:81-89. 1938

16. Mc Gowan, M. W., Artiss, J. D., Strandbergh, D. R. & Zak, B. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. Clin. Chem. 29: 538–542. 1983

17. Richmond, W. Preparation and properties of a cholesterol oxidase from Nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. Clin. Chem. 19: 1350–1356. 1973

18. Hao, J., Shen, W., Tian, C., Liu, Z., Ren, J., Luo, C., Long, J., Sharman, E., & Liu, J. Mitochondrial nutrients improve immune dysfunction in the type 2 diabetic Goto–Kakizaki rats. Journal of Cellular and Molecular Medicine, 12:150-156. 2008

19. Kakkar, P., Das, B., & Viswanathan, P.N. A modified spectrophotometric assay of superoxide dismutase. Indian J. Biochem. Biophys., 21:130-132. 1984

20. Aebi, h.E.. Catalase in vitro. Methods Enzymol., 105:121. 1984

21. Iqbal, M., Sharma, M.D., Zadeh, H.R., Hasan, N., Abdulla, M., & Athar, M. Glutathione metabolizing enzymes and oxidative stress in ferric nitrilotriacetate (Fe-NTA) mediated hepatic injury. Redox Report, 2:385-391. 1996

22. Witko-Sarsat VM, Friendlander VM, Capeillere C, Nguyen-Khoa AT, Zingraff J, Jungers P., Deschamps Latscha B. Advanced oxidation protein products as a novel marker of oxidative stress in uraemia. Kidney Int., (49): 1304–1313. 1996

23. French SW, Miyamoto K, Ohta Y, Geoffrion Y. Pathogenesis of experimental alcoholic liver disease in the rat. Methods Achiev Exp Pathol; 13:181-207. 2000

24. Ruwart MJ, Wilkinson KF, Rush BD, The integrated value of serum procollagen III peptide over time predict hepatic hydroxproline content and stainable collagen in a model of dietary cirrhosis in the rat. Hepatology 10: 801-806. 1989

25. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest;115:209-218. 2005

26. Kojima M, Kangawa K. Ghrelin: structure and function. Physiol Rev;85:495-522. 2005

27. Van der Lely AJ, Tschop M, Heiman ML, Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev ;25: 426-457. 2004

28. Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, et al. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metab ;87:2988. 2002

29. Gonzalez-Rey E, Chorny A, Delgado M. Therapeutic action of ghrelin in a mouse model of colitis. Gastroenterology;130:1707-1720. 2006

30. Li L, Zhang LK, Pang YZ, Pan CS, Qi YF, Chen L, et al. Cardioprotective effects of ghrelin and des-octanoyl ghrelin on myocardial injury induced by isoproterenol in rats. Acta Pharmacol Sin;27:527-535. 2006

31. Kucharská J, Ulicná O, Gvozdjáková A et al: Regeneration of coenzyme Q9 redox state and inhibition of oxidative stress by Rooibos tea (Aspalathus linearis) administrationin carbon tetrachloride liver damage. Physiol Res; 53(5): 515–21. 2004

32. Li L, Zhang LK, Pang YZ et al: Cardioprotective effects of ghrelin and des-octanoyl ghrelin on myocardial injury induced by isoproterenol in rats. Acta Pharmacol Sin; 27: 527–35. 2006

33. Jaeschke H, Smith CW, Clemens MG et al: Mechanism of inflammatory liver injury: adhesion molecules and cytotoxicity of neutrophils. Toxicol Appl Pharmacol; 139: 213–14. 1996

34. Kheradmand A, Alirezaei M, Asadian P et al: Antioxidant enzyme activity and MDA level in the rat testis following chronic administration of ghrelin. Andrologia; 41(6): 335–40. 2009

35. J.H. Yan Li, L. Lake, C. Xuehui, P.Hua, C. Meng, Z. Qinggui, Administration of ghrelin improves inflammation, oxidative stress, and apoptosis during and after non-alcoholic fatty liver disease development. Endocrine.. doi:10.1007/s12020-012-9761-5. 2012

36. T. Takamura, H. Misu, T. Ota, S. Kaneko, Fatty liver as a consequence and cause of insulin resistance: lessons from type 2 diabetic liver [Review]. Endocr. J. (2012)

37. M. Estep, M. Abawi, M. Jarrar, L. Wang, M. Stepanova, H. Elariny, A. Moazez, Z. Goodman, V. Chandhoke, A. Baranova, Z.M. Younossi, Association of obestatin, ghrelin, and inflammatory cytokines in obese patients with non-alcoholic fatty liver disease. Obes. Surg. 21, 1750–1757 (2011)

38. Y. Okamatsu, K. Matsuda, I. Hiramoto, H. Tani, K. Kimura, Y. Yada, T. Kakuma, S.

Higuchi, M. Kojima, T. Matsuishi, Ghrelin and leptin modulate immunity and liver function in overweight children. Pediatr. Int. 51, 9–13 (2009)

39. M. Moreno, J. F. Chaves, P. Sancho-Bru et al., "Ghrelin attenuates hepatocellular injury and liver fibrogenesis in rodents and influences fibrosis progression in humans," Hepatology, vol. 51, no. 3, pp. 974–985, 2010.

40. S. Ö. Işeri, G. Şener, B. Saglam, F. Ercan, N. Gedik, and B. Ç. Yeğen, "Ghrelin alleviates biliary obstruction-induced chronic hepatic injury in rats," Regulatory Peptides, vol. 146, no. 1–3, pp. 73–79, 2008.

41. N. N. Kabil, H. A. Seddiek, N. A. Yassin, and M. M. Gamal-Eldin, "Effect of ghrelin on chronic liver injury and fibrogenesis in male rats: possible role of nitric oxide," Peptides, vol. 52, pp. 90–97, 2014

42. Y. Gutierrez-Grobe, I. Villalobos-Blasquez, K. Sanchez-Lara, A.R. Villa, G. Ponciano-Rodriguez, M.H. Ramos, N.C. Chavez-Tapia, M. Uribe, N. Mendez-Sanchez, High ghrelin and obestatin levels and low risk of developing fatty liver. Ann. Hepatol. 9, 52–57 (2010)

43. Obay BD, Taşdemir E, Tümer C et al: Dose dependent effects of Ghrelin on pentylenetetrazole – induced oxidative stress in a rat seizure model. Peptides, 29: 448–51. 2008