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Araştırma

The Effect of Ghrelin Treatment on Cell Survival and Inflammation in Type 2 Diabetic Rat Liver

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ABSTRACT

Objective: *The aim of the study is to investigate the effect of ghrelin treatment on regulation of cell survival, inflammation and oxidative stress in the rat liver of type-2 diabetes model.*

Materials and Methods: *Twenty-one male Sprague-Dawley rats (8-10 weeks old) were divided into three groups: control, type-2 diabetes (T2D) and T2D+Ghrelin (25 µg/kg with intraperitoneal (i.p) injection for two weeks). Type-2 diabetes was induced by feeding 10% fructose solution in drinking water for 2 weeks and followed by a single i.p injection of streptozotocin (40 mg/kg). Control animals received tap water. The liver samples were obtained from rats at the end of experiment. Glutathione (GSH), lipid peroxidation (LPO) and protein carbonyl (PCO) levels were measured in liver tissue. Matrix metalloproteinases (MMP-2 and -9), nuclear factor kappa B (NF-κB), peroxisome proliferator-activated receptor gamma (PPAR-γ), interleukin 6 (IL-6) and proliferating cell nuclear antigen (PCNA) expressions were determined by immunohistochemical methods.*

Results: *The number of MMP-2, MMP-9, NF-κB, PPAR-γ and IL-6 immunopositive cells increased in the diabetic rat liver as compared to control. The ghrelin treatment significantly reduced the numbers of MMP-2, MMP-9, NF-κB, PPAR-γ and IL-6 immunopositive cells in diabetic rat liver. However, the number of PCNA immunopositive cells increased in diabetic rats treated with ghrelin. Therapeutic effect of ghrelin was not shown in terms of the biochemical parameters including GSH and LPO but PCO levels decreased in the liver.*

Conclusions: *According to our findings, ghrelin treatment could prevent diabetes-induced inflammation in the liver. However, this treatment did not adequately affect oxidative stress in diabetic rats.*

Keywords: *Ghrelin, inflammation, matrix metalloproteinases, oxidative stress, type-2 diabetes*

ÖZ

Tip 2 Diyabetik Sıçan Karaciğerinde Ghrelin Tedavisinin Hücre Sağkalımı ve İnflamasyonu Üzerine Etkileri

Amaç: *Çalışmamızın amacı, tip 2 diyabet modeli sıçan karaciğerlerinde ghrelin uygulamasının hücre sağkalımı, inflamasyon ve oksidatif stres düzenlenmesi üzerine etkilerinin incelenmesidir.*

Gereç ve Yöntemler: *21 adet Sprague-Dawley sıçanlar (8-10 haftalık) üç gruba ayrıldı: Kontrol, tip-2 diyabet (T2D) ve T2D+Ghrelin (25 µg/kg iki hafta boyunca intraperitoneal (i.p.) olarak enjekte edildi). Tip-2 diyabet %10 fruktoz solüsyonunun iki hafta boyunca içme suyuna katılmasını takiben tek doz i.p. streptozotosin (40 mg/kg) enjekte edilmesi ile oluşturuldu. Kontrol hayvanlara çeşme suyu verildi. Deney sonunda karaciğer doku örnekleri alındı. Glutatyon (GSH), lipid peroksidasyonu (LPO) ve protein karbonil (PCO) seviyeleri karaciğer dokusunda ölçüldü. Matriks metalloproteinaz (MMP-2 ve MMP-9), nuklear faktör kappaB (NF-κB), peroksizom proliferatör-aktive reseptör gama (PPAR-γ), interlökin 6 (IL-6), ve prolifere hücre nukleus antijeni (PCNA) ekspresyonları immunohistokimyasal metod ile belirlendi.*

Bulgular: *MMP-2, MMP-9, NF-κB, PPAR-γ ve IL-6 immünpozitif hücre sayısı diyabetik sıçan karaciğerinde kontrole göre arttı. Ghrelin tedavisi diyabetik sıçan karaciğerindeki MMP-2, MMP-9, NF-κB, PPAR-γ ve IL-6 immünpozitif hücre sayılarını önemli derecede azalttı. Fakat ghrelin uygulanan diyabetiklerde PCNA immünpozitif hücre sayısı arttı. Sıçan karaciğerinde ghrelinin tedavi edici etkisi GSH ve LPO parametreleri için anlamlı bulunmadı fakat PCO seviyesi azaldı.*

Sonuç: *Bulgularımıza göre, ghrelin tedavisi karaciğerdeki diyabetin neden olduğu inflamasyonu önleyebilir. Fakat bu tedavi diyabetik sıçanlarda oksidatif stresi yeterince etkilemedi.*

Anahtar kelimeler: *Ghrelin, inflamasyon, matriks metalloproteinazlar, oksidatif stres, tip-2 diyabet*

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INTRODUCTION

Diabetes mellitus is one of the most important public health problems in the worldwide. Diabetes is a chronic illness that occurs either when insulin is not produced enough by the pancreas, or the body is unable to use the insulin. The majority of people with diabetes (90-95%) are affected by type 2 diabetes that is known as non-insulin-dependent [1,2]. The liver disease occurs as a result of diabetes. In type-2 diabetes (T2D), an important cause of death is liver diseases [3].

Ghrelin, a gastric peptide hormone, is mostly produced in stomach and less in the small intestine and colon. Ghrelin may also be expressed in many tissues such as pancreas, pituitary, hypothalamus, lung, heart, and several tissues in the periphery [4-6]. Recent studies have suggested that ghrelin has wide physiological actions in the regulation of gastrointestinal, glucose homeostasis, immune functions, cardiovascular functions, antioxidant defense and bone formation [6-8].

The strong evidences have emerged demonstrating a close relationship between metabolic processes and inflammation. The inflammatory reaction plays a role in the pathogenesis of T2D [9,10]. Furthermore, there is an association between oxidative stress and inflammation. Oxidative stress leads the development and progression of inflammation, and thus contributes to the pathophysiology of diabetes [11]. A condition known as oxidative stress is caused by impaired unbalance between free radicals and antioxidants. Free radicals may show adverse effects on lipids, proteins and DNA, which are associated with changes in their structure and functions, and thus cause many disease [12]. Free radicals-induced disruption of cell organelles and membranes leads to hepatocyte injury, the consequences of which cause programmed or necrotic cell death [13].

In the present study, we aimed to clarify the ghrelin treatment ability in regulation of cell survival and inflammation in liver of type 2 diabetic rats. Therefore, the changes of oxidative stress and matrix metalloproteinases (MMP-2 and MMP-9), nuclear factor kappa B (NF-κB), peroxisome proliferator-activated receptor gamma (PPAR-γ), interleukin 6 (IL-6), and proliferating cell nuclear antigen (PCNA) expressions were investigated.

MATERIALS and METHODS

Animal model and treatment

Twenty-one male Sprague-Dawley rats (8-10 weeks old) were housed individually under 12 h light, 12 h dark cycles at a constant temperature and humidity with ad libitum access to food and water. All studies were performed according to the guidelines of Istanbul University, Local Ethics Committee on Animal Research.

The animals were randomly divided into three groups of seven rats as control, type II diabetes and type II diabetes+Ghrelin. Control group drank tap water. Type II diabetes (T2D) was induced by feeding 10% fructose solution (Merck, 104005) in drinking water for 2 weeks and followed by a single intraperitoneal (i.p) injection of streptozotocin (40 mg/kg, Sigma-Aldrich, S0130) [14]. The rats with blood glucose levels 200 mg/dL or more were accepted as diabetic. In type II diabetes+Ghrelin (T2D+Ghr) group, diabetic rats were received 25 µg/kg ghrelin (AnaSpec, 24160) with i.p. injection for two weeks. At the end of the experiment, the liver tissue samples were obtained from rats under ketamine-HCl (50 mg/kg, Ketalar, Pfizer) and xylazine hydrochloride (10 mg/kg, Rompun, Bayer) anesthesia.

Immunohistochemistry

Liver samples were fixed with 10% neutral formalin for 24 h. After graded alcohol dehydration, the tissue samples were embedded with paraffin, and then cut in 4-µm sections for immunostaining of MMP-2, MMP-9, NF-κB, PPAR-γ, IL-6, and PCNA by using streptavidin-biotin-peroxidase technique.

For immunohistochemistry staining, tissue sections were dewaxed and rehydrated, and then the antigens were unmasked in 0.01 M citrate buffer (pH 6.0) by microwave and the sections were treated with hydrogen peroxide to quench the endogenous peroxidase activity for labeling of all antibodies. Histostain Plus Broad Spectrum Kit (Invitrogen, 859043) and MMP-2 antibody (Santa Cruz sc-13595; 1:40 dilution, overnight at room temperature), MMP-9 antibody (Santa Cruz sc-21733,1:40 dilution, overnight at room temperature), PCNA antibody (Thermo MS-106-p, 1:400

dilution, for 30 min at 25°C and then overnight at +4°C), IL-6 antibody (Santa Cruz sc-1265-R; 1:50 dilution, for 120 min at 25°C and then 48 h at +4°C), NF-κB antibody (Santa Cruz sc-8414; 1:40 dilution, for 120 min at 30°C and then 48 h at +4°C) and PPAR γ antibody (Santa Cruz sc-7273; 1:40 dilution, 120 min at 30°C and then 48 h at +4°C) were used for labeling. Finally, the signals were visualized by a 3-amino-9-ethyl carbazole substrate kit (Invitrogen, 00-2007). The sections were counterstained in Mayer's hematoxylin.

Slides were imaged with a Nikon Eclipse 80i light microscope equipped with a digital camera (DS-U2, Nikon). In each slide, ten randomly selected areas were evaluated and the numbers of the immunopositive cells and staining intensities within these areas were determined. The intensity of immunostaining was semiquantitatively evaluated by the following categories: 0 (negative), + (weakly positive), ++ (positive), +++ (strongly positive), and ++++ (very strongly positive).

Biochemical assays

For biochemical analysis, liver tissue samples were frozen in liquid nitrogen and then stored at -80°C until the day of experiment. The liver tissues were homogenized in cold 0.9% NaCl and made up to 10% homogenate. The homogenates were centrifuged, and the clear supernatants were used for determination of protein, glutathione (GSH), lipid peroxidation (LPO) and protein carbonyl (PCO) levels.

GSH levels were assayed in liver tissues by the method of Beutler [15]. LPO levels in homogenates were determined by Ledwozyw's method [16]. PCO levels were estimated by the method of Reznick and Packer [17]. The protein content in the supernatants was determined according to Lowry's method [18].

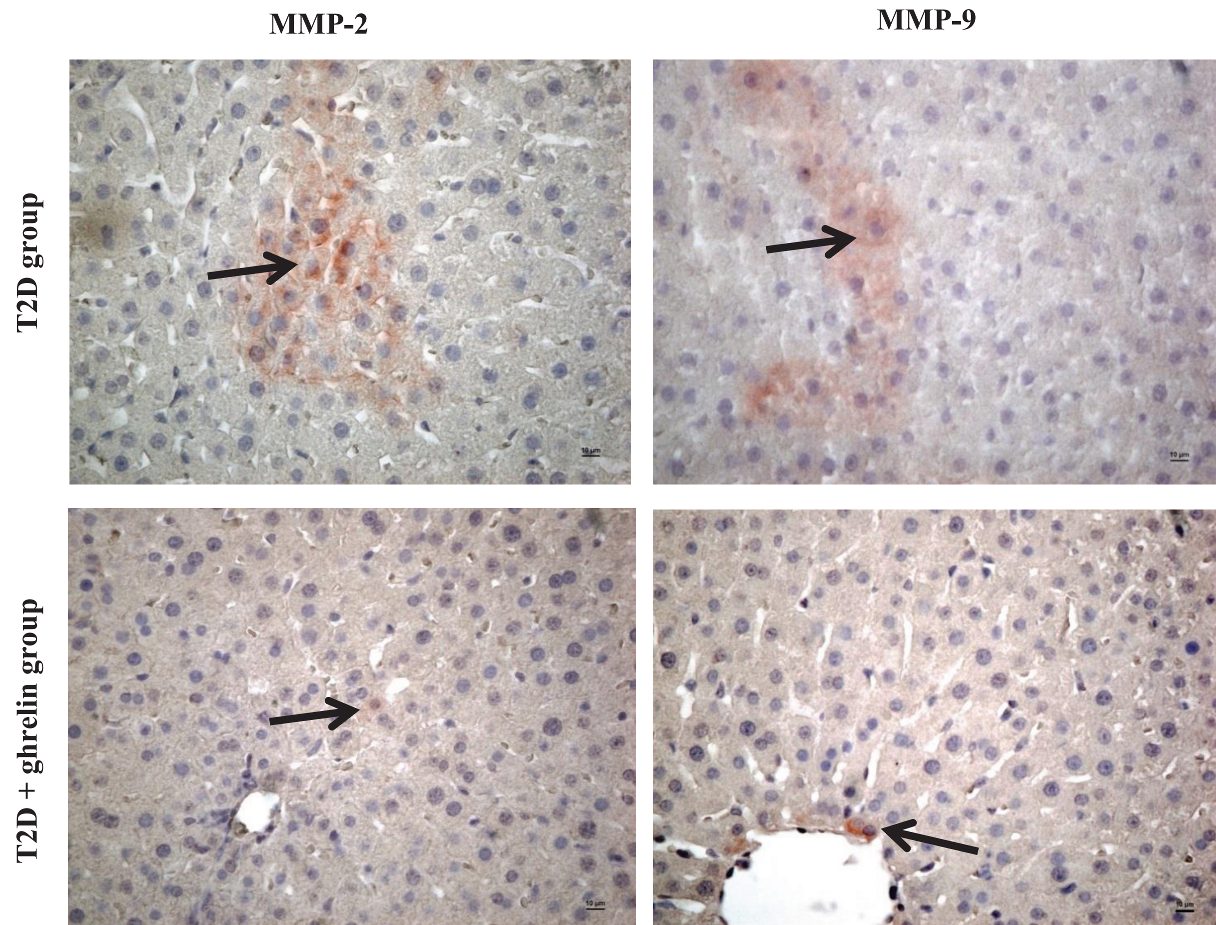

Figure 1. Immunopositive cells (arrow) for matrix metalloproteinase(MMP) -2 and MMP-9 in livers of T2D group and T2D+ghrelin group. Streptavidin-biotin-peroxidase technique, counterstain hematoxylin. Scale bar = 10 µm.

Statistical analysis

Statistical calculations were carried out using SPSS software (version 21.0, SPSS). Experimental data were expressed as the mean ± standard error of the mean (SEM). The statistical analysis was performed for statistical significance using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. The differences were considered significant when the P value was <0.05.

RESULTS

The immunoreactivity of liver cells against MMP-2, MMP-9, NF-κB, PPAR-γ, IL-6, and PCNA peptides are depicted in Figures 1, 2 and 3. The number of immunopositive cells and the staining intensity of the antibodies are presented in Table 1 and 2, respectively.

The number of MMP-2 and MMP-9 immunopositive cells increased in T2D group comparison to the control group (P<0.001). Ghrelin treatment reduced the number of MMP-2 and MMP-9 immunopositive cells in diabetic rats (P<0.001 and P<0.01). Similar findings were also observed in immunopositive cell numbers of NF-κB, PPAR-γ, and IL-6. Type-2 diabetes increased the positive cell numbers of NF-κB, PPAR-γ, and IL-6 (P<0.001, P<0.001, and P<0.01). However, the increased positive cell numbers of NF-κB, PPAR-γ, and IL-6 in diabetic animals were decreased with ghrelin treatment (P<0.001, P<0.001, and P<0.01). The proliferation-associated peptide PCNA was mainly localized in cell nucleus but it rarely found in cytoplasm. The number of PCNA immunopositive cells showed an increase in diabetic rats treated with ghrelin as compared to T2D animals (P<0.05) (Table 1).

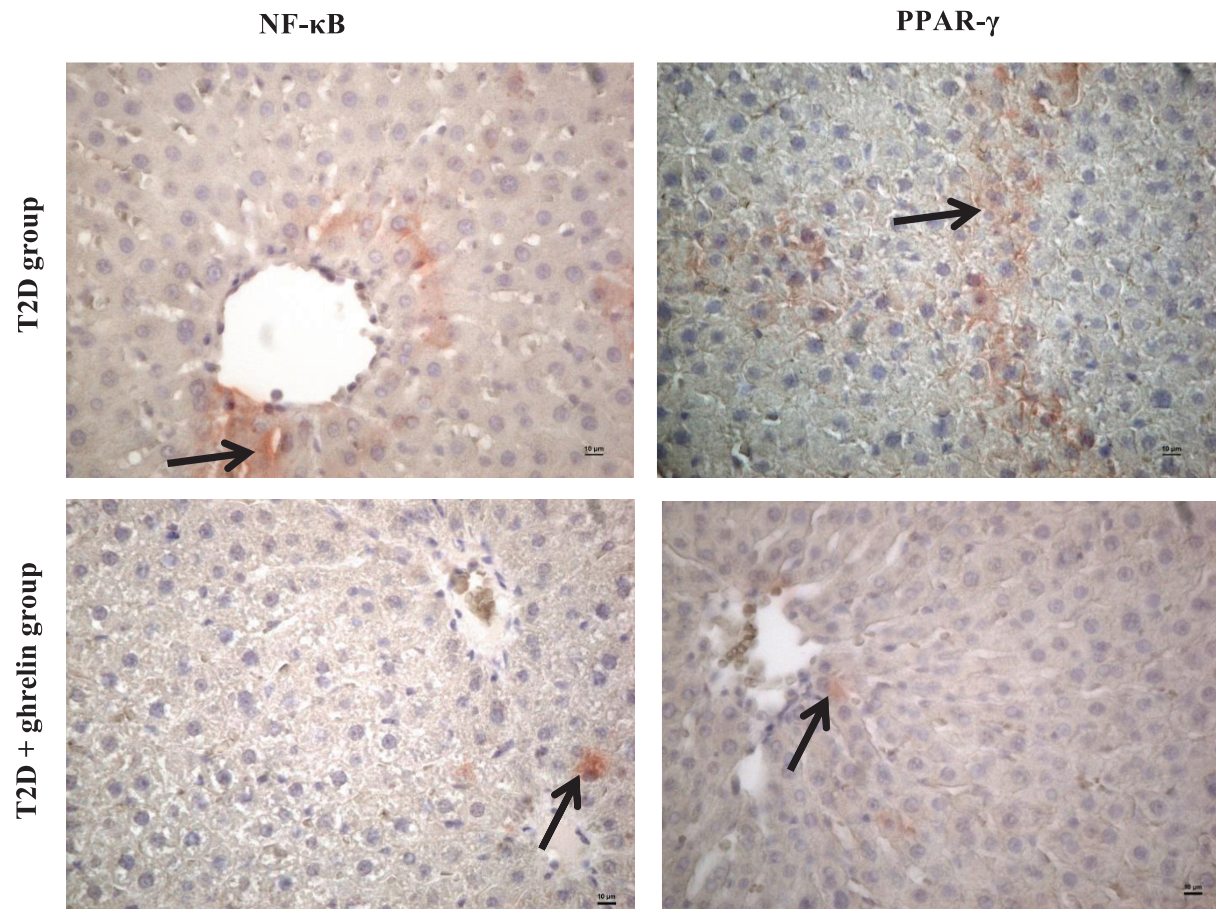

Figure 2. Nuclear factor kappa B (NF-κB) and peroxisome proliferator-activated receptor gamma (PPAR-γ) immunopositive cells (arrow) are seen in liver. Streptavidin-biotin-peroxidase technique, counterstain hematoxylin. Scale bar = 10 µm.

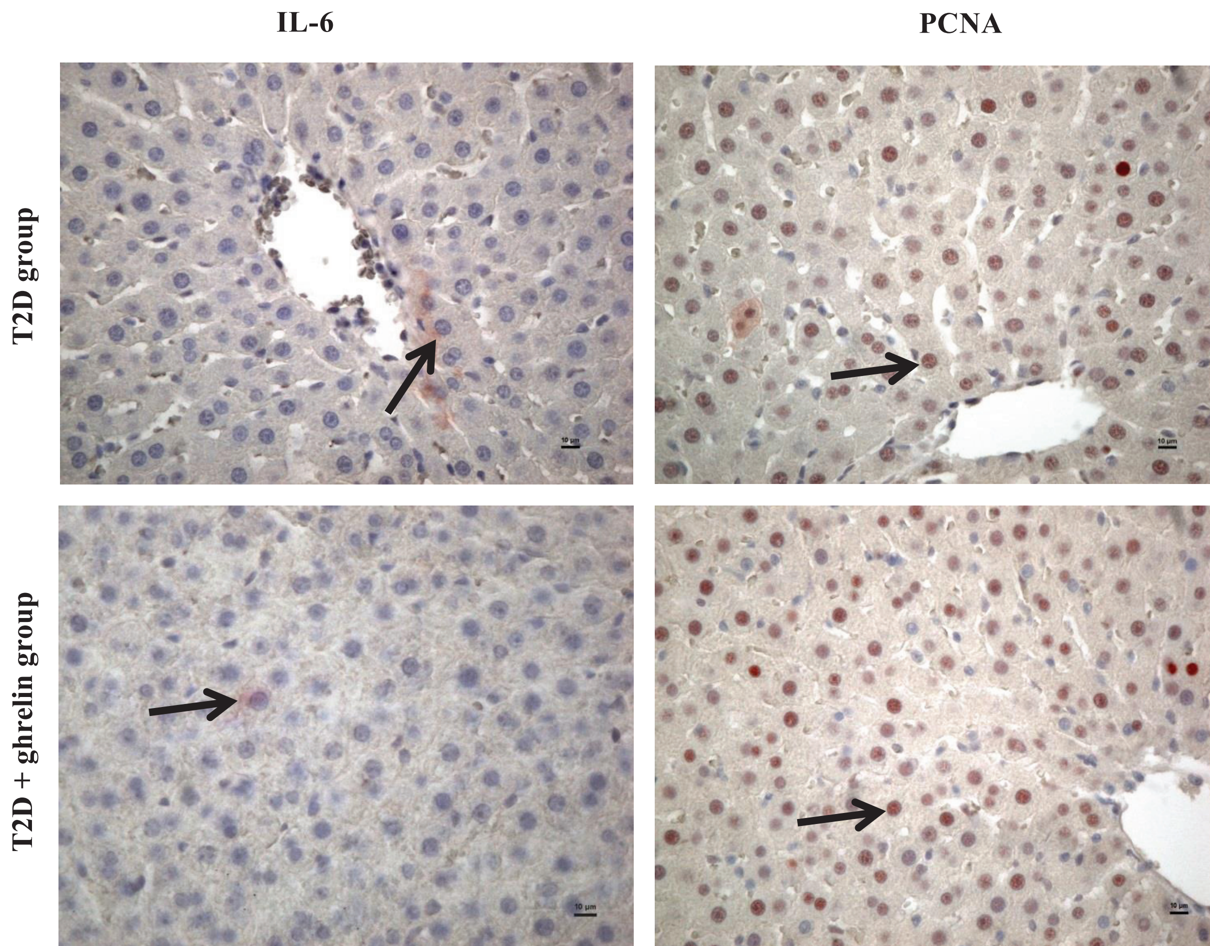

Figure 3. Immunopositive cells for interleukin 6 (IL-6), and proliferating cell nuclear antigen (PCNA) in rat livers. Streptavidin-biotin-peroxidase technique, counterstain hematoxylin. Scale bar = 10 µm.

Table 1. Immunopositive cell numbers of matrix metalloproteinase (MMP) -2 and -9, nuclear factor kappa B (NF-κB), peroxisome proliferator-activated receptor gamma (PPAR-γ), interleukin 6 (IL-6) and proliferating cell nuclear antigen (PCNA).

	MMP-2*	MMP-9*	PPARγ*	NF-κB*	IL-6*	PCNA*
Control	0.04±0.03	0.26±0.20	0.14±0.01	0.19±0.09	0.19±0.12	10.56±1.74
T2D	9.86±1.37[a]	4.49±0.92[a]	4.03±0.74[a]	6.51±1.05[a]	1.31±0.40[d]	30.48±3.06[a]
T2D+Ghr	0.54±0.20[b]	1.43±0.49[c]	0.71±0.24[b]	1.73±0.40[b]	0.07±0.03[c]	40.47±3.28[a,e]
P_{ANOVA}	<0.001	<0.001	<0.001	<0.001	<0.01	< 0.001

**Mean ± SEM.*
[a]P<0.001 vs. control group, [b]P<0.001 vs. T2D group, [c]P<0.01 vs. T2D group, [d]P<0.01 vs. control group, [e]P<0.05 vs. T2D group

The immunostaining intensities of MMP-2, MMP-9, NF-κB, and PPAR-γ were very strongly positive in diabetic group. However, ghrelin administration reduced the immunostaining intensities of MMP-2, MMP-9, NF-κB, and PPAR-γ. The immunostaining intensities of MMP-9 and NF-κB changed as positive and MMP-2 and PPAR-γ were weakly positive in T2D+Ghr group. The immunostaining intensity of IL-6 was positive, and PCNA intensity was strongly positive in T2D animals. While ghrelin treatment decreased IL-6 intensity as weakly positive, it increased PCNA intensity as very strongly positive (Table 2).

A significant alteration in liver GSH and LPO levels was not shown. On the other hand, PCO level of liver showed a decrease in diabetes treated with ghrelin when compared to T2D group (Table 3).

Table 2. Intensities of immunostaining were shown and scored for each group.

	MMP-2	MMP-9	PPARγ	NF-κB	IL-6	PCNA
Control	+	+	+	+	+	++
T2D	++++	++++	++++	++++	++	+++
T2D+Ghr	+	++	+	++	+	++++

Table 3. Glutathione (GSH), lipid peroxidation (LPO) and protein carbonyl (PCO) levels in liver.

	GSH (nmol/mg protein)*	LPO (nmol/mg protein)*	PCO (nmol/mg protein)*
Control	2.14±0.35	0.39±0.02	0.80±0.09
T2D	4.34±1.11	0.43±0.03	0.65±0.09
T2D+Ghr	5.99±1.40	0.41±0.06	0.33±0.05[a,b]
PANOVA	>0.05	>0.05	<0.01

Mean ± SEM.
[a]P<0.01 vs. control group, [b]P<0.05 vs. T2D group

DISCUSSION

The dysregulation of hepatic glucose release contributes significantly to the pathophysiology of diabetes [19]. Type-2 diabetes has also been recognized as a risk factor for the development of liver injury [3]. Furthermore, the evidences have shown that a chronic, sub-acute inflammatory state is associated with metabolic disease such as T2D, obesity and metabolic syndrome [20].

Many members of MMP family are not expressed in healthy tissues. On the other hand, MMPs are expressed in tissue injury and disease development. Excessive MMPs expressions are related with degenerative and inflammatory diseases such as liver injury and cancer. Many MMPs are regulated by inflammatory cytokines during tissue injury [21,22]. Bruschi et al. [23] suggested that MMP-2 and MMP-9 can be considered as inflammatory markers in their study on infected mice. Furthermore, MMP-2 and MMP-9 may trigger the caspase-mediated cell death [24]. In the studies on diabetes, while MMP-2 and MMP-9 levels did not show a significant difference in plasma, their expression levels increased in cultured cells [25,26]. In the present study, MMP-2 and MMP-9 expressions significantly increased in liver cells with diabetes. The excessive expression of MMP-2 and MMP-9 in liver cells with diabetes may be caused by progression of diabetes-induced inflammation. Ghrelin treatment contributes a significant reduction of MMP-2 and MMP-9 expressions in the liver of type 2 diabetic rats.

In cell death pathway, the activation of nuclear factor kappa B (NF-κB) is triggered following TNF receptor activation. So, NF-κB goes to the nucleus and then, NF-κB activates the genes, which act to block TNF-induced apoptosis. In resting cells, NF-κB is an inactive form in the cytoplasm [19,27]. TNF-induced NF-κB activation regulates the expression of anti-apoptotic proteins, such as Bcl-2 family members and prevents TNF-induced apoptosis [28]. In addition, the NF-κB pathway is a pro-inflammatory signaling pathway and based on the NF-κB activation. NF-κB may be considered as a target for anti-inflammatory drugs [29]. Cai et al. [20] suggested that NF-κB is a regulator of inflammation and controls the production of inflammation, cytokines, such as IL-6 and TNF. IL-6 is a member of the pro-inflammatory cytokine family and has a potential role in development liver injury [30]. IL-6 concentration in blood increases in type 2 diabetes [31]. According to study on rats, IL-6 mRNA expression of pancreas is higher in STZ-induced diabetes as compared to healthy rats [32]. PPAR-γ, a transcription factor, is a member of the nuclear receptor superfamily. PPAR-γ has been known as anti-diabetic drug marker. PPAR-γ has a role in the immune response due to leading inhibition of inflammatory cytokines expression [33]. We observed that NF-κB expression was higher in diabetic liver, largely is found in the cell cytoplasm. Therefore, it is considered that NF-κB may be a significant target especially in the regulation of inflammation. Furthermore, the NF-κB expression in the diabetic liver was decreased with ghrelin treatment. Similarly, the increased expression of IL-6 and PPAR-γ was decreased with ghrelin administration in the liver cells of diabetic rats. The ghrelin may act as an anti-inflammatory agent in the liver with type 2 diabetes.

It is known that necrotic deaths are associated with inflammation and enhanced pro-inflammatory responses but not apoptotic deaths. Apoptotic death is a physiological cell death and associated with anti-inflammatory signaling activity [34]. Increased inflammation markers including MMP-2, MMP-9, NF-κB, PPAR-γ, and IL-6 may indicate the necrotic cell death in the liver with type-2 diabetes. PCNA has a wide range of function in the cell nucleus. It is also found in the cytoplasm. The Naryzny and Lee suggested that PCNA in cytoplasm has been associated with cytoplasmic oncoproteins [35]. An increase of PCNA

expression by immunohistochemical staining was determined in the diabetic rat liver. PCNA expression may increase due to DNA repair in damaged hepatocytes during liver injury by diabetes. Ghrelin treatment may accelerate DNA repair mechanism.

Oxidative stress is characterized by increased reactive oxygen species (ROS) level in tissues and reduction in their antioxidant defense. There is a positive relationship between oxidative stress and hyperglycemia. The oxidative unbalance in hepatocytes can cause the development of the liver disease in diabetes [36]. GSH can inhibit free radical-mediated injury by eliminating reactive oxygen species. Oxidative stress increases lipid peroxidation and modification of cellular proteins [37]. Picazo et al. [38] showed that oxidative stress parameters are higher in type 2 diabetic patients. In our previous study, we observed that GSH level decreased and LPO level increased in STZ+NAD-induced diabetic rat pancreas [39]. In present study, a significant change did not show in GSH and LPO levels of diabetic rat liver. However, a reduction in PCO levels was determined in liver with T2D. In addition, ghrelin treatment further reduced liver PCO levels in diabetic rats. Ghrelin is able to regulate PCO levels in liver of fructose+STZ-induced rats.

Taken together, the findings from our study demonstrate that diabetes causes highly inflammation in hepatocytes. Ghrelin administration may prevent increased inflammation and, reduced the complication of diabetes in rats. Furthermore, ghrelin may promote cell survival in type 2 diabetes-induced liver injury.

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