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Ghrelin treatment alleviates appetite-related receptor expressions and oxidative stress in fructose-streptozotocin-induced diabetic rat duodenum

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Ghrelin (GHR), a peptide that regulates appetite and energy balance, is important for type 2 diabetes (T2D). Glucagon-like peptide-1 receptor (GLP-1R) and cannabinoid receptor 1 (CB-1R) are two receptors associated with energy and food metabolism. Understanding the effects of GHR on GLP-1R and CB-1R may help develop treatments for weight regulation in T2D. We investigated the effects of GHR supplementation on both appetite-related receptor expression and oxidative stress in the duodenum of a rat model of diabetes. Rats (n=21) were divided into control (CO), T2D, and T2D+GHR groups. The number and intensity of GLP-1R and CB-1R immunopositive cells were detected by immunohistochemistry. Expression levels of GLP-1R and CB-1R mRNAs were analyzed by qPCR in the duodenum. Oxidative stress parameters were measured in duodenal tissues. During the third and fourth weeks of the experiment, body weight in the T2D+GHR group was significantly reduced compared to the T2D group. The number and intensity of CB-1R and GLP-1R immunopositive cells were significantly lower in the T2D+GHR group than in the T2D group. The results of GLP-1R and CB-1R mRNA expression paralleled the immunohistochemical staining. According to our findings, GHR supplementation may contribute to healing in the duodenum of a diabetic rat model by suppressing appetite-related receptors.

Keywords: Cannabinoid receptor 1, Gastric peptide, Immunohistochemistry, Biochemistry, Type 2 diabetes

Type 2 diabetes (T2D) is a chronic metabolic disorder with increasing prevalence worldwide. It is estimated that 537 million people had diabetes in 2021 and by 2045 the number will be 783 million[1]. It is known that a combined approach of weight loss and lifestyle changes can ameliorate the adverse effects and fatal complications of diabetes[2]. Therefore, understanding the impact of eating behaviors and appetite may be crucial to the success of T2D treatments. In addition, diabetes therapies may be based on improvements in glycemic and weight control, such as incretin-based therapies[3]. It has been suggested that incretins may be related to ghrelin (GHR)[4]. GHR is a gastric peptide expressed in the intestine, pancreas, adipose tissue, and heart that regulates hypothalamic circuits controlling appetite and energy homeostasis by stimulating growth hormone secretion[5]. Researchers have reported that GHR controls body weight and food intake[6]. Therefore, GHR may reduce body weight in obese individuals. It has been suggested that GHR may play a therapeutic role in the treatment and prevention of obesity-induced type 2 diabetes due to its effects on glucose metabolism and lipid homeostasis[7]. There are also reports that GHR is an antioxidant that reduces oxidative stress damage[8].

Endogenous cannabinoids (endocannabinoids) are known to control energy homeostasis, immune and inflammatory responses, and food intake. Endocannabinoids bind to specific receptors, one of which is the cannabinoid receptor 1 (CB-1R). CB-1R is expressed in the brain as well as in other tissues such as the intestine under pathological conditions[9]. It has been reported that endocannabinoids acting through CB-1R are associated with ghrelin release and may partially increase food intake[10].

Glucagon-like peptide-1 (GLP-1) is a gut hormone secreted by intestinal L-cells. The glucagon-like peptide-1 receptor (GLP-1R) participates in the regulation of energy metabolism and food intake[11]. There is an interaction between GHR and both CB-1R and GLP-1[12,13]. There is a strong association between hyperglycemia-induced oxidative stress and the development of T2D[14]. In addition, studies suggest that oxidative stress is related to nutrition. The GHR signaling system plays a role in appetite control with

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the help of antioxidants[15]. We investigated the relationship between GHR supplementation and the gene expression of two appetite-related receptors, CB-1R and GLP-1R, in the duodenum of diabetic rats. We also examined whether GHR supplementation had any effect on oxidative stress, which has been suggested to affect appetite metabolism.

Materials and Methods

Drugs and chemicals

Fructose from Merck (1.04007.1000, Darmstadt, Germany), streptozotocin (STZ) from Sigma-Aldrich (S0130, St. Louis, Missouri, USA), GHR from AnaSpec (24160, Fremont, CA, USA), ketamine-HCl (Ketalar) from Pfizer (NY, USA) and xylazine-HCl (Rompun) from Bayer (Ontario, Canada) were purchased.

Animals and GHR supplementation

Twenty-one male Sprague-Dawley rats (2-2.5 months old) were obtained from the Aziz Sancar Institute of Experimental Medicine, Istanbul University. Rats were housed under standard conditions with a 12 h light and 12 h dark cycle and ±21°C room temperature. All animals were given food and tap water *ad libitum*. Animal experiments were performed according to protocols approved by the Local Ethical Committee for Animal Research, Istanbul University.

Rats were divided into three groups: (I) control (CO, n=7) group, (II) type-2 diabetes (T2D, n=7) group, and (III) type-2 diabetic rats supplemented with ghrelin 25 μg/kg (T2D+GHR, n=7). The CO group was provided standard pellets and tap water *ad libitum*. The T2D group was fed with 10% fructose in drinking water and standard pellet for two weeks, and then a single dose of STZ (40 mg/kg) was injected intraperitoneally. The animals fasted overnight, and blood glucose was measured with a glucometer (Roche Diagnostics, Mannheim, Germany) in the morning. All rats in the T2D group had a blood glucose level ≥ 200 mg/dL or more and were considered diabetic. In the T2D+GHR group, the type 2 diabetic rats were treated with 25 μg/kg GHR (dissolved in saline) intraperitoneally for two weeks. The body weight of animals was measured weekly. At the end of the experiment, the animals were anesthetized with ketamine-HCl (50 mg/kg) and xylazine-HCl (10 mg/kg). Intracardiac blood was taken from the anesthetized animals, which then were sacrificed by exsanguination. Duodenum samples were collected immediately from the rats, washed with saline, and placed in either liquid nitrogen for subsequent biochemistry or 10% neutral buffered formalin for subsequent immunostaining (Fig. 1).

RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

The weight of the duodenum samples ranged from 10 to 15 mg. Total RNA was isolated from the tissues using the High Pure RNA Tissue Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. This RNA was used for cDNA synthesis using the First Strand cDNA Synthesis kit (Fermantas, Hanover, MD). Each qPCR reaction was set up to contain 10 μL of the Lightcycler® TaqMan® Master Kit (Roche Diagnostics, Mannheim, Germany), 1 μL of each primer (final concentration of 400 nM), and 4 μL of PCR-grade water; 5 μL of template DNA was added to a total reaction volume of 20 μL. According to the manufacturer's recommended protocol, the qPCR reactions were performed on a Lightcycler® 480 real-time PCR system (Roche Diagnostics, Mannheim, Germany). Each reaction was run for 40 cycles, followed by a high-resolution dissociation (melting) analysis. The program included 95°C for 10 min, 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 1 second, and 40°C for 30 seconds. β-Actin was used as a housekeeping gene. qPCR data were obtained using the Pfaffl method[16]. Primers used are listed in Table 1.

Immunohistochemistry

Duodenum samples were fixed in 10% neutral buffered formalin for 16-18 h at +4 °C. Formalin-fixed duodenum specimens were processed routinely. Tissue samples were embedded in paraffin and sectioned at 4 μm using a microtome. Immunohistochemical staining was used to evaluate the localization and intensity of CB-1R and GLP-1R in duodenal tissue sections. For antigen retrieval, tissue sections were boiled in citrate buffer (10 mM, *p*H 6.0) for 15 mins. Endogenous peroxidase activity was blocked with 3% H_2O_2 dissolved in methanol. A blocking solution (Invitrogen, CA, USA) was applied to tissue sections. They were incubated overnight in a humidity chamber at 4ºC with 1:50 rabbit anti-CB-1R (Cayman Chemicals, MC, USA) or 1:50 rabbit anti-GLP-1R (Novus, CO, USA). After rinsing in phosphate-buffered saline, the slides were reacted with biotinylated secondary antibody for 15 min followed by PBS wash (Histostain Plus Broad Spectrum Kit, Invitrogen, CA, USA, cat no: 859043).

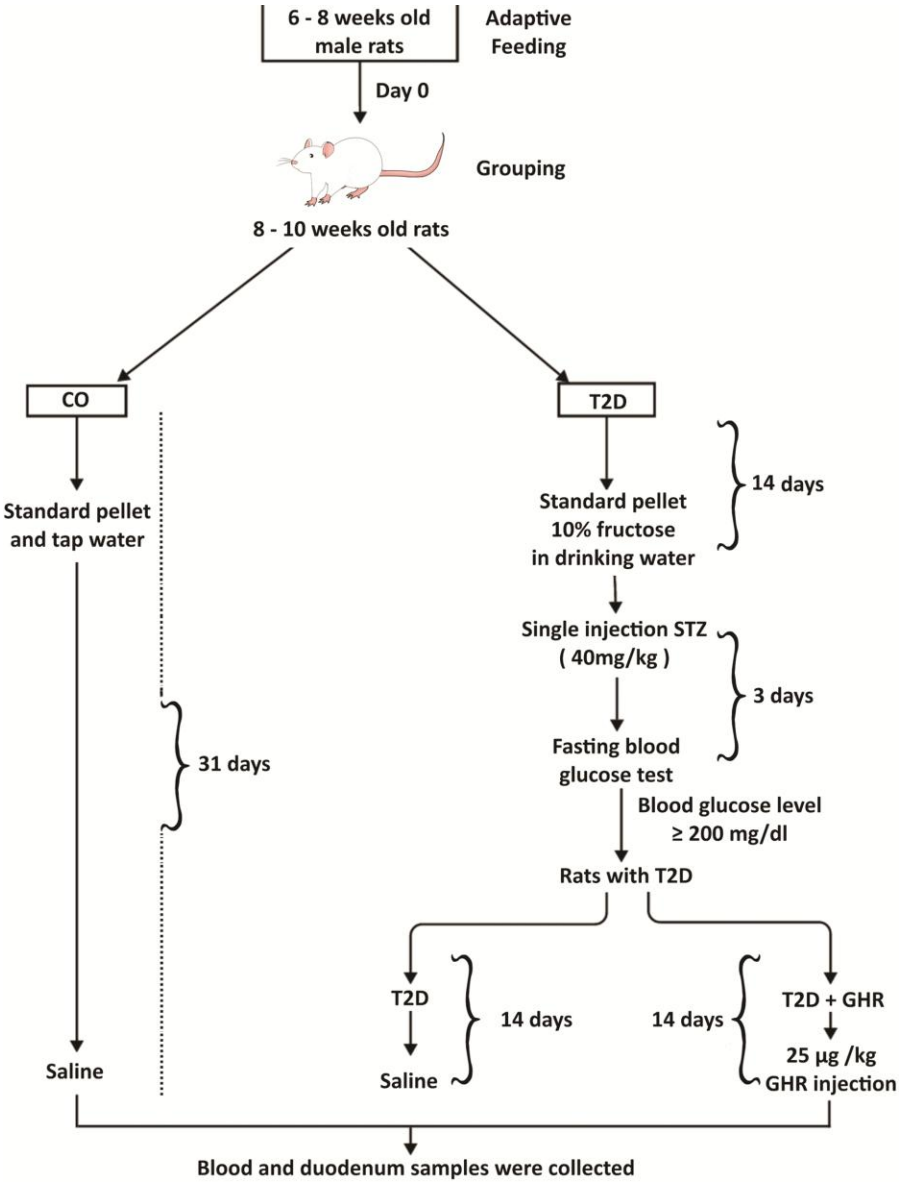

Fig. 1 — Flow chart of animal experiments. CO, control; T2D, type 2 diabetes; GHR, ghrelin; STZ, streptozotocin.

Table 1 — Primer sequences used for quantitative real-time polymerase chain reaction (qPCR)

Genes	Forward Primer Sequence (5' 3') →	Reverse Primer Sequence (5' 3') →
CB-1R	GGACATGGAGTGCTTTATGATTC	GAGGGACAGTACAGCGATGG
GLP-1R	CACTTCCTCCAGGGCTTT	CGAAACTCCATCTGGACCTC
β-actin	CCCGCGAGTACAACCTTCT	CGTCATCCATGGCGAACT

Signals were detected with streptavidin-peroxidase kits (Invitrogen, CA, USA, cat no: 00-2007) using a 3-amino-9-ethyl-carbazole (AEC) substrate kit (Invitrogen, CA, USA) as a chromogen. Sections were counterstained with Mayer's hematoxylin. Microscopic analysis was performed using the ×40 objective and ×10 ocular systems of the Nikon Eclipse 80i light microscope (Nikon, Melville, NY, USA), and the image was transferred through the NIS-Elements-D 3.1 microscope imaging software program (Nikon, Melville, NY, USA). Immunopositive cells and the intensity of the cells were counted in the CO (n = 7), T2D (n = 7), and T2D+GHR (n = 7) groups. In each slide, 10 randomly selected areas in the 166 × 133 μm^2 computer screen image were evaluated for each rat, and

immunopositive cells were counted. The staining intensity of immunopositive cells was scored as follows: (-), negative; (+), weakly positive; (++), positive; (+++), strongly positive. Specificity controls were performed. For negative control sections, phosphate-buffered saline was used without the primary antibodies. Brain tissue was used for the CB-1R positive control and pancreatic tissue was used for the GLP-1R positive control.

Analysis of oxidative stress markers

Duodenum samples were homogenized in saline on ice. Briefly, duodenal tissue homogenates were centrifuged, and the clear supernatants were used for analyses. Glutathione (GSH) levels were determined using the Beutler method[17]. Malondialdehyde (MDA) and protein carbonyl (PCO) levels in the tissue homogenates were determined by the methods of Ledwozyw *et al.* and Reznick & Packer, respectively[18,19]. Superoxide dismutase (SOD) enzyme activity was quantified by the method of Sun[20]. Total protein concentration in tissue homogenates was evaluated by the Bradford method[21]. GSH, MDA, and PCO results were expressed in nmol per mg protein (nmol/mg). SOD results were expressed in units per mg protein (U/mg).

Statistical analysis

Data are mean ± SEM. Nonparametric Mann-Whitney and Kruskal-Wallis tests were used to compare between groups. GraphPad Prism software version 5 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. The significance level was set at $P \leq 0.05$.

Results

Body Weight

Body weights are shown in Fig. 2. During weeks 1 and 2, rats in the T2D and T2D+GHR groups received fructose only in their water. The body weight of the T2D group increased significantly compared to the CO group during weeks 1 and 2 ($P < 0.001$ and $P < 0.01$, respectively). The body weight of the T2D group did not change after STZ injection compared to the CO group ($P > 0.05$). In contrast, a decrease in body weight was observed in diabetic rats receiving supplemental GHR, compared to the CO and T2D groups ($P < 0.05$ and $P < 0.01$, respectively).

Determination of CB-1R and GLP-1R gene expressions

Expression of both CB-1R and GLP-1R mRNA was lower in the T2D group than for the CO group

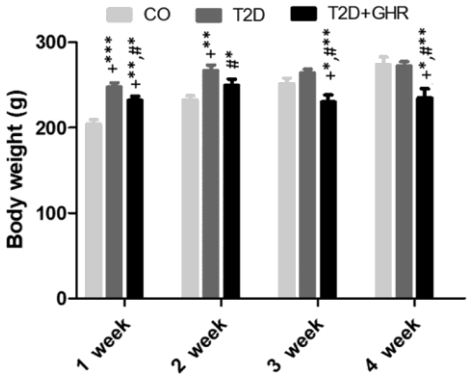

Fig. 2 — Effect of ghrelin (GHR) administration on body weight (g) of the experimental groups at different time intervals. Data are mean ± SEM. CO, control; T2D, type 2 diabetes. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, + significant difference versus CO group, and # significant difference versus T2D group.

($P < 0.05$ for all comparisons) **(**Fig. 3 & 4**)**. Ghrelin supplementation significantly reduced CB-1R and GLP-1R mRNA expression in the T2D+GHR group compared to the T2D group ($P < 0.05$ for all comparisons). Similarly, the number of CB-1R and GLP-1R immunostained cells was significantly reduced in the T2D group compared to the CO group ($P < 0.01$ and $P < 0.001$, respectively). This reduction was further reduced by GHR supplementation and paralleled our mRNA findings. The number of CB-1R and GLP-1R immunostained cells was lowest in the T2D+GHR group compared to the T2D group ($P < 0.001$ and $P < 0.05$, respectively) and the control group ($P < 0.001$ for all comparisons) (Fig. 3 & 4). The intensity of immunostaining was directly proportional to the number of immunostained cells **(**Fig. 3 & 4**)**. The intensity of CB-1R and GLP-1R immunostained cells was lowest in the T2D+GHR group compared to the T2D ($P < 0.001$ and $P < 0.05$, respectively) and CO groups ($P < 0.001$ for all comparisons).

Measurement of oxidative stress markers

Measurements of GSH, MDA, PCO levels, and SOD enzyme activity are summarized in Fig. 5. Although no significant difference was observed among the groups, GSH levels and SOD activity were highest in the T2D+GHR group. Malondialdehyde and PCO levels were higher in the T2D group than in the CO group. We found that GHR supplementation slightly reduced the increased diabetes-induced MDA and PCO levels.

Discussion

We investigated the effects of GHR on the CB-1R and GLP-1R gene expression and oxidative stress in

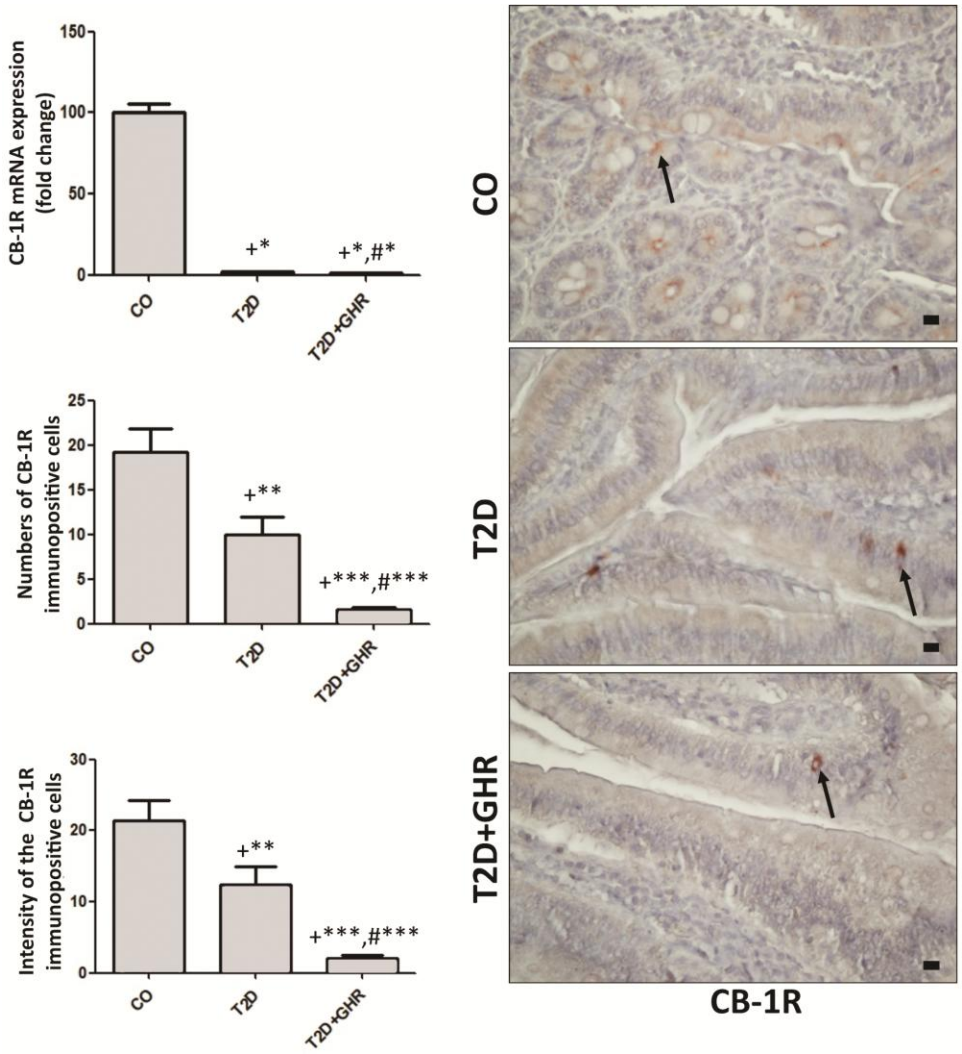

Fig. 3 — Cannabinoid receptor 1 (CB-1R) gene expression in the duodenum of the experimental groups. Representative photomicrographs showing immunostained cells (arrows) expressing CB-1R in the duodenum of experimental groups. Data are mean ± SEM. CO, control; T2D, type 2 diabetes; GHR, ghrelin. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, + significant difference versus CO group, and # significant difference versus T2D group. Scale bars = 20 µm.

the duodenum of T2D rats. Fructose feeding induces metabolic changes that risk the development of diseases such as diabetes[22]. In our study, fructose feeding caused weight gain in weeks 1st and 2nd, and weight gain in animals slowed after GHR injection during weeks 3rd and 4th; the latter may be due to the progression of T2D. Symptoms of T2D include frequent urination, gradually increasing thirst and hunger, weight loss, blurred vision, slow wound healing, and fatigue[23]. GHR signaling is associated with alterations in the regulation of feeding behavior[24]. Plasma GHR levels were lower in patients with T2D than in the control group. In contrast, body weight is higher in type 2 diabetic patients than in controls[25]. Studies have reported that GHR stimulates gastric emptying, weight gain, appetite, and food intake, and growth hormone secretion[26]. In contrast, we found that GHR supplementation reduced body weight in T2D rats. It has been reported that the orexigenic effects of GHR require CB-1R signaling[27]. Researchers have suggested that the distribution of GHR cells is similar to that of the CB1-positive cells in the stomach[28].

According to these studies, gastric GHR secretion and food intake are dependent on gastric CB-1R. On the other hand, gastric GHR secretion was found to be greater in the fasting state than after *ad libitum* feeding. Although a cannabinoid blocker, rimonabant,

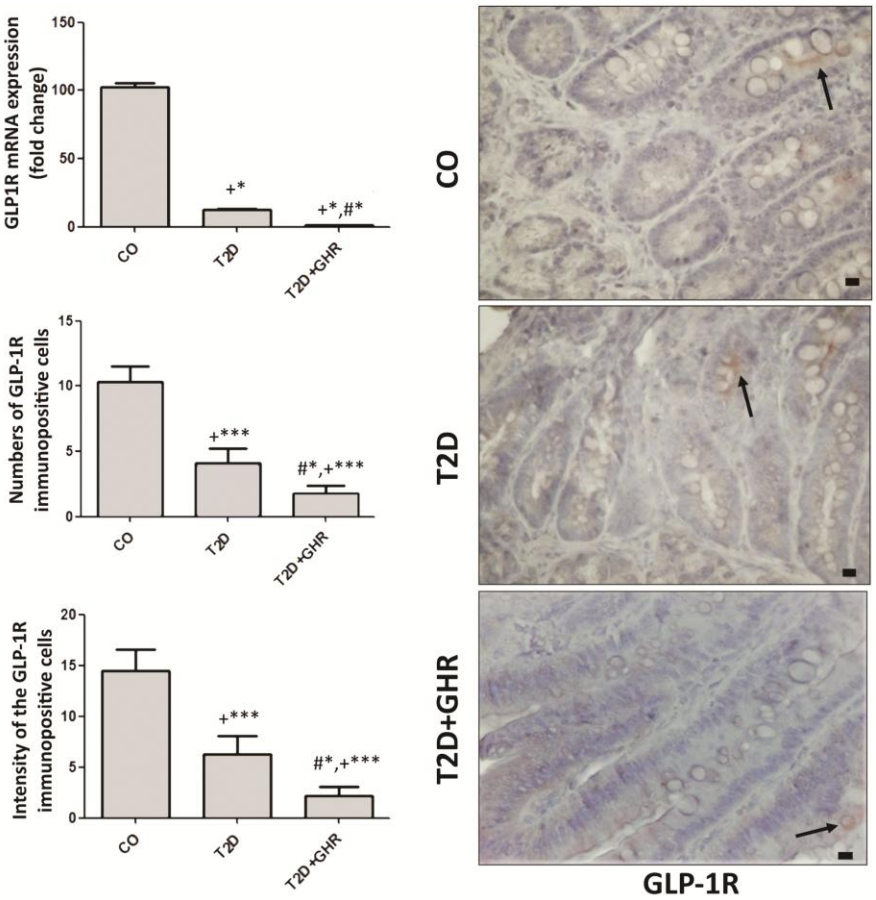

Fig. 4 — Glucagon-like peptide-1 receptor (GLP-1R) gene expression in the duodenum of experimental groups. Representative micrographs illustrating immunostained cells (arrows) expressing GLP-1R in the duodenum of experimental groups. Data are mean ± SEM. CO, control; T2D, type 2 diabetes; GHR, ghrelin. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001, + significant difference versus CO group, and # significant difference versus T2D group. Scale bars = 20 µm.

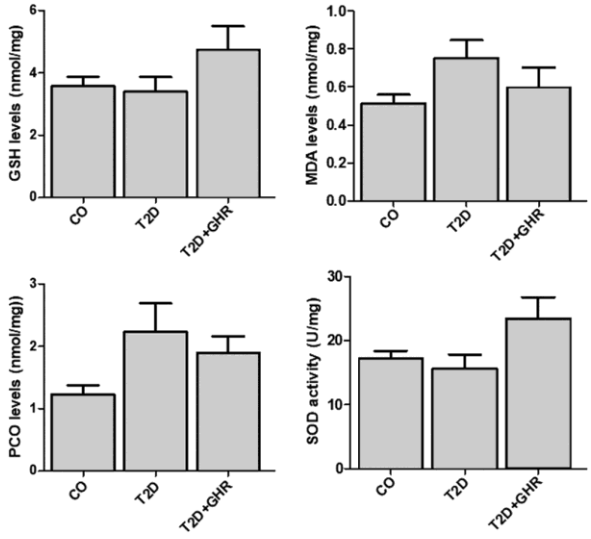

Fig. 5 — Effects of ghrelin (GHR) on the levels of oxidative stress markers in the duodenum of the experimental groups. GSH, glutathione; MDA, malondialdehyde; PCO, protein carbonyl; SOD, superoxide dismutase.

did not affect gastric GHR secretion in *ad libitum* fed animals, it increased gastric GHR secretion in the fasted animals[28]. A study showed that CB-1R negatively regulates GLP-1R in pancreatic islet cells[29]. These investigators also suggested that CB1 inhibits incretin secretion in the fasting state. Altered endocannabinoid levels may affect incretin levels in obesity[30]. According to the study, loss of GLP-1R signaling had little effect on body weight and fat mass in mice fed a high-fat or low-fat diet[31]. Exogenous GLP-1 suppressed increased GHR secretion[32]. GHR treatment has been shown to induce GLP-1 secretion in obese rats[33]. On the other hand, GHR had no direct effect on GLP-1 secretion[34]. According to a review, GHR and GLP-1 exert opposing effects on eating behavior via both independent and overlapping actions[35]. We found that administration of exogenous GHR reduced the expression of both CB-1R and GLP-1R in the duodenum of diabetic rats. The density

of immunopositive cells was also reduced. This may be due to the progression of diabetes affecting the function of the receptors. Interestingly, GHR treatment inhibited the expression levels of both CB-1R and GLP-1R. This may be due to GHR's suppressive effect on these receptors by affecting appetite, energy, or glucose metabolism. We can say that GHR has different effects depending on whether the individual is fasting or eating normally and whether the individual is healthy or diabetic. Consistent with previous reports, we suggest that GHR release should be evaluated according to the nutritional and/or health status of the individual.

Oxidative stress is defined as an imbalance between the oxidative defense system and the production of free radicals. Oxidative stress accompanies T2D; high-fat and high-sugar diets can cause oxidative damage[36]. GHR treatment reduces elevated reactive oxygen species in the A549 human lung cancer cell line. GHR also decreases the MDA levels and increases the GSH levels[37]. It has been reported that antioxidant and anti-inflammatory effects of GHR treatment were observed after colon anastomosis in rats. After the surgical trauma, increased levels of reactive oxygen species were reduced by the administration of GHR[38]. Authors reported that 40 µg/mL GHR, which they applied for 7 days to the traumatic brain injury model they created in rats, showed anti-inflammatory, antioxidant, and neuroprotective effects. They also found that GHR treatment after the traumatic brain injury significantly increased serum GSH, catalase (CAT), glutathione peroxidase, and brain GSH and CAT levels[8]. Similar to the study[37], it was observed that GHR treatment increased the GSH level on day 14[38]. In the present study, PCO and MDA levels were slightly increased in T2D while GSH levels and SOD activity were decreased. Although not significant, GHR supplementation slightly reduced MDA and PCO levels and conversely increased GSH levels and SOD activity. Therefore, oxidative stress markers may be modified by prolonging the application period of GHR.

Conclusion

Our results demonstrate that GHR, which acts as an antioxidant, may have an effect on appetite metabolism in diabetic rats via CB-1R and GLP-1R. Although our findings provide new clues to understand the complex mechanisms involved in regulating the relationship between GHR and appetite in diabetic individuals, further studies are needed to investigate these mechanisms in more detail.

Ethical statement

All animal procedures conform to the Principles of Laboratory Animal Care approved by the Istanbul University Local Ethics Committee on Animal Research.

Funding statement

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose. The author states that there is no conflict of interest in this study.

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