Influence Of Combined High-Fat-High-Carbohydrate Diet On Ghrelin In Female And Male Rats

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Abstract: Ghrelin is secreted from stomach and plays a key role in appetite and glucose regulation.

Aim: The aim of our work was to study the influence of high-fat-high-carbohydrate diet (HFHCD) on ghrelin expression in female and male rats.

Material and methods: Two-month-old Wistar rats were divided into four groups: control - female (FC) and male (MC), and dietary-manipulated - female (FD) and male (MD). The dietary-manipulated animals were fed HFHCD for 16 weeks, the control groups - standard rat chew. Weight, BMI and waist circumference were measured in the course and at the end of the experiment. After the sacrifice of the animals, serum concentrations of glucose, insulin and ghrelin were measured. The stomach of each rat was examined immunohistochemically for ghrelin expression.

Results: FD and MD compared with the controls had higher values of body weight, BMI, waist circumferences, glucose, insulin and HOMA-IR and developed insulin resistance. Their serum ghrelin concentrations were lower, with gender differences – lower in FD than MD. Stronger immunohistochemical reaction was detected in the stomach of the dietary-manipulated rats.

Conclusion: HFHCD induces changes in ghrelin expression and makes it a risk factor for the development of obesity and metabolic syndrome.

Keywords: high-fat-carbohydrate-diet, ghrelin, rats

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I. Introduction

Ghrelin is a gastrointestinal hormone secreted mainly by gastric mucosa. [1] It is the basic peripheral humoral signal to the central nervous system during hunger and is referred to as ‘hunger hormone’. [2] Changes in ghrelin secretion are adaptive reactions which stimulate or suppress appetite depending on the current energy imbalance. [3] Ghrelin regulates the exocrine pancreatic function and is related to insulin secretion. [4] It influences glucose and lipid metabolism, and energy homeostasis. [5] The various metabolic effects of ghrelin make it a potential risk factor for atherosclerosis, hypertension, diabetes type 2, obesity. [6,7] Serum ghrelin levels in obesity fall below normal values. [8,9]

The chief factors which regulate the secretory activity of ghrelin-producing cells and ghrelin blood level respectively are the contents of the food. All nutrient types given orally are capable of inhibiting ghrelin secretion.[10] The high protein content induces long-term reduction of appetite and body weight despite the compensatory changes in the daily plasma concentrations of ghrelin. [11] In patients with diabetes type 2 a five-week high-protein diet does not influence the ghrelin level in blood. [12] The high fructose level of food in the form of corn syrup increases ghrelin secretion. [13] Peroral and intravenous glucose administration decreases plasma ghrelin concentration in rodents [14,15] and man [16,17] Fat-rich diet in rats results in an increase in body weight and a significant reduction in ghrelin levels. [18,19,20] Despite the numerous researches, molecular mechanisms of the effect of basic nutrients on ghrelin-producing cells and ghrelin release remain unclear. In available literature there aren’t any studies on ghrelin expression in the course of dietary induction of obesity and metabolic syndrome with a high-fat-high-carbohydrate diet.
II. Aim

The aim of our work was to study the influence of a combined high-fat-high-carbohydrate diet (HFHCD) on the ghrelin serum levels and its stomach immunohistochemical expression in female and male rats.

III. Material And Methods

In order to follow the recommendations of the National Institute of Health (NIH, 1993) to conduct parallel investigations in both sexes, male and female animals were used in this experiment. An equal number of female (n=16) and male (n=16) Wistar rats were used, with an initial body mass of 160-180 g. They were kept in individual metabolic cages at 20 ± 2 °C, controlled humidity and 12:12 h light-dark period. The experiment was approved by the Commission on Ethical Treatment of Animals of the Bulgarian Food Safety Agency (№ 119, 18.06.2015). In the preparatory period all rats received a standard rat chew for two weeks while adapting to the conditions in metabolic cages. At the end of the preparatory period, the rats were divided into four groups: 2 control groups – male (MC) and female (FC) and 2 dietary-manipulated – male (MD) and female (FD). The rats from MD and FD groups were subjected to HFHCD and that from MC and FC received standard rat chew for 16 weeks. The energy content of the food of MC and FC groups was 2908 kcal kg⁻¹ and of groups MD and FD - 4298 kcal kg⁻¹. HFHCD was prepared by adding lard and sucrose to standard pellets.

Body weight of each animal was measured once a week. An hour before, the food was temporarily deprived and returned to the metabolic cages after determining the body mass. BMI was calculated using the formula:

\[ BMI = \frac{\text{body weight (g)}}{\text{length (cm)}^2} \]

Experimental animals’ waist circumference was measured with a meter in its widest part once a month. At the end of the trial, the experimental animals were decapitated under narcosis with Thiopental 30 mg kg⁻¹ i.p. and mixed blood was collected. Then it was centrifuged in order to measure the serum levels of ghrelin, glucose and insulin in blood serum. Serum concentration of glucose was assayed by analyzer Konelab 60 i (Thermophisher Scientific, USA). Ghrelin and insulin serum levels were measured by the sandwich ELISA method with Ghrelin Rat ELISA Kit (BioVendor Inc.) and Rat Insulin HS ELISA Kit (BioVendor Inc.), respectively. Doubled samples were elaborated on Sirius S microplate reader (SEAC, Italy) and the average of the two measurements was used as a representative for each rat. The homeostatic model for insulin resistance (HOMA-IR), based on measured insulin and fasting blood glucose, was determined. HOMA-IR was calculated by the formula:

\[ \text{Glucose (mmol/L)} \times \text{Insulin (mU/L)} / 22.5 \]

Stomach specimens were taken from each animal after the decapitation, fixed in 10% formalin and after paraffin inclusion were studied immunohistochemically with a primary antibody for ghrelin (goat polyclonal ghrelin antibody: sc-10368 - Santa Cruz Biotechnology USA) by the ABC method with ABC Staining System (Santa Cruz Biotechnology, USA). The ghrelin antibody was diluted in PBS in 1:100 ratio. Positive reaction for ghrelin was reported in the presence of black granules in the cell cytoplasm. The specificity of immunohistochemical reactions was confirmed by negative controls in which the specific antibodies were substituted with a buffer (PBS) or normal non-immune serum. There was no product of the respective reaction in them. Observation and photo documentation of the microscopic preparations were performed with digital photo microscopic camera of a light microscope “Olympus BX51”.

The results are expressed as means ± SEM. In order to assess the two main effects (gender and diet) and their interaction, the results of all tests, which were repeated several times during the experiment (body weight, BMI and waist circumference), were assessed by two-way ANOVA for repeated measures. The results obtained only at the end of the experiment (serum concentration of ghrelin, insulin, glucose, HOMA-IR) were analyzed with two-way ANOVA. The level of significance was set at P<0.05.

IV. Results

1. Body weight

At the beginning of the experiment the body weights of the experimental animals in the four experimental groups were almost equal - male controls (182.13 ± 9.64 g), female controls (172.00 ± 16.49 g), male dietary-manipulated (184.75 ± 13.66 g), female dietary-manipulated (176.25 ± 14.39 g) rats (P<0.05). Until the 11th week HFHCD diet had no effect on the weight of the test animals (P>0.05). From the 12th week until the end of the experiment the administered HFHCD had a significant main effect on the body weight, as dietary-manipulated animals had higher weight than the controls (P<0.05). At the end of the experiment the dietary-manipulated rats had higher weight compared with the controls (P<0.05) (Fig. 1.).
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Fig. 1. Body weight (g) of dietary-manipulated and control rats from 12th week until the end of the experiment. *P<0.05 dietary-manipulated vs control rats.

The gender had a significant main effect on the body mass from the 2nd week until the end of the experiment - male animals had higher body weight than female (P<0.05) (Fig. 2.).

Fig. 2. Body weight (g) of female and male rats during the experiment (*P<0.05).

At the end of the experiment, male rats increased their body weight with 53.17%, female rats - with 44.95%. Analyzing the obtained data of changes in the body mass during the experiment, we did not detect statistically significant two-way interaction (P>0.05).

Body Mass Index (BMI)

At the beginning of the study there was no difference in the BMI of the experimental rats - male controls (0.45 ± 0.05 g·cm⁻²), female controls (0.50 ± 0.05 g·cm⁻²), male dietary-manipulated (0.46 ± 0.05 g·cm⁻²), female dietary-manipulated (0.47 ± 0.04 g·cm⁻²) rats (P>0.05). A significant main effect of HFHCD on BMI was found at the end of the experiment (16th week), as dietary-manipulated rats had higher body mass index compared to the controls (P<0.01) (Fig. 3.).

Fig. 3. Body mass index (g·cm⁻²) of dietary-manipulated and control animals in the course of the experiment. *P<0.01 dietary-manipulated vs control rats.
At the end of the 8th week we found a significant main effect of the gender on BMI (P<0.01). The male rats had higher BMI than female and this effect was maintained until the end of the experiment (P<0.01) (Fig. 4.). No significant interaction between the gender and HFHCD on BMI values of the experimental animals was found (P>0.05).

**Waist circumferences**

From the 12th week until the end of the experiment, HFHCD had a significant main effect on the values of the waist circumferences of the experimental animals. At the end of the experiment dietary-manipulated rats had larger waists compared with the controls (P<0.001) (Fig. 5.).

**Fig. 4.** Body mass index (g·cm⁻²) of female and male rats in the course of the experiment. *P<0.01 male vs female rats.

**Fig. 5.** Waist circumferences (cm) of dietary-manipulated and control animals in the course of the experiment. *P<0.001, dietary-manipulated vs control rats.

The gender had a significant main effect on the waist circumference from the 4th week until the end of the experiment. At the end of the experiment the male rats had higher values than the female (P<0.001) (Fig. 6.). No significant two-way interaction on the waist circumference values of the experimental animals was found (P>0.05).

**Fig. 6.** Waist circumferences (cm) of male and female animals in the course of the experiment. *P<0.001, male vs female rats.
**Glucose**

We found that HFHCD had a significant main effect on serum fasting blood sugar concentration. The dietary-manipulated animals had higher glucose concentrations compared to the controls (10.10 ± 0.17 mmol·l⁻¹ vs 7.53 ± 0.17 mmol·l⁻¹, P<0.001). There was a significant main effect of the gender on the serum glucose concentration. At the end of the experiment male rats had higher fasting blood glucose concentrations compared with the female rats (8.45 ± 0.27 mmol·l⁻¹ vs 7.53 ± 0.27 mmol·l⁻¹, P<0.05). No significant interaction between the gender and HFHCD was found on the blood glucose concentration (P> 0.05).

**Insulin**

A significant main effect of HFHCD on the insulin levels was found in both male and female rats. Serum insulin concentration in dietary-manipulated animals was higher than the same in the controls (58.12 ± 17.29 mU·l⁻¹ vs 5.76 ± 17.29 mU·l⁻¹, P<0.05). No significant main effect of the gender (P>0.05) and no significant interaction between the gender and HFHCD on insulin concentration (P>0.05) were found.

**HOMA-IR**

Statistical analysis of the obtained data showed presence of insulin resistance at the 16th week of HFHCD administration in both male and female rats. We found a significant main effect of the diet on HOMA-IR. In the dietary-manipulated rats it was significantly higher than in the controls (30.79 ± 9.76 vs 1.82 ± 9.76, P<0.05). No significant main effect of the gender (P> 0.05) and two-way interaction (P>0.05) on HOMA-IR were found.

**Serum ghrelin concentrations**

The 16-week HFHCD administration had a significant main effect on the serum ghrelin concentration, as the dietary-manipulated rats had lower values compared with the controls (3.18 ± 1.09 ng·ml⁻¹ vs 16.18 ± 1.09 ng·ml⁻¹, P<0.001). The gender also had a significant main effect, as female rats had lower serum ghrelin concentrations compared with male animals (7.25 ± 1.09 ng·ml⁻¹ vs 12.11 ± 1.09 ng·ml⁻¹, P<0.001). We found no significant interaction between the gender and HFHCD on the serum ghrelin concentrations of the experimental animals (P>0.05).

**Immunohistochemical expression of ghrelin in the stomach**

In the control groups (MC and FC) we established presence of some ghrelin-producing cells scattered in the body of separate fundus glands of the gastric corpus (Fig. 7. A, B.; Fig. 8. A, B.). The cells with positive ghrelin immunohistochemical reaction were single, with conical shape. The secretory product was evenly scattered in the cytoplasm. The intensity of the reaction was moderate. No difference was found in the ghrelin immunohistochemical expression in male and female rats.

**Fig. 7. A male rat from the control group. Single ghrelin-positive cells in the stomach fundus glands. A. Magn. x 200. B. Magn. x 400.**
In the animals fed HFHCD we established a great number of ghrelin-producing cells in extensive areas of the gastric corpus. They were gathered into cell clusters situated in the body and base of the fundic glands. The large number of ghrelin positive cells demonstrated strong immunohistochemical reaction. The rats from the dietary-manipulated groups had more ghrelin positive cells with stronger reaction than that from the control groups. No difference was found in the ghrelin immunohistochemical expression between the male (MD) (Fig. 9. A., B.) and female (FD) rats (Fig. 10. A., B.).
V. Discussion

In our work we used HFHCD, which is a combination of high level of carbohydrates and fats specifically selected to meet the feeding habits of the contemporary man. At the end of the experiment dietary-manipulated rats had higher values of body weight, BMI, waist circumferences, glucose, insulin and HOMA-IR and developed insulin resistance. Thus, by inducing obesity and metabolic disorders in both male and female rats, we mimic what happens in humans. Our study for the first time gives an idea about the changes in ghrelin production and expression as a result of the 16-week application of HFHCD in both genders and reveals the role of this hormone in the pathogenesis of metabolic syndrome.

The used by us HFHCD has a negative effect on serum ghrelin level which corresponds with other works. Previous studies were carried out by loading with only one of the nutrients. Peral oral and intravenous glucose administration decreases plasma ghrelin concentration in rodents. [14,15] The inhibitory effect of glucose on ghrelin is also observed in man. [16] Serum ghrelin levels were decreased in 5-week-old male Wistar rats with fructose-induced metabolic syndrome. [21] Significantly reduced plasma levels of ghrelin and increased body weight were detected in Zucker rats after 8-weeks administration of high-fat diet. [18] Fat-rich diet for 11-weeks resulted in an increase in body weight and a significant reduction in ghrelin levels. [19] Decreased ghrelin levels were found in gastrointestinal tract and serum of rats with obesity induced by fat-rich diet. [20] We found decreased serum ghrelin concentration after the application of HFHCD in both genders, more pronounced in female rats. In available literature there is no evidence for such a gender comparison concerning rats. As for humans, there is a report on gender differentiation of the acetylated ghrelin plasma levels - higher in healthy women than men. [22] We can explain this dissociation in our and other authors’ results with the fact that they concern different species.

Our results revealed a correlation between the low serum ghrelin concentrations and the obesity achieved as a result of the diet. The same negative correlation was proved for ghrelin levels and obesity in people. [23] Ghrelin levels were reduced in obesity and increased after weight loss. [24] We found low ghrelin levels to be in association with the other changes as a result of the diet like insulin resistance and abdominal circumferences. Other works also reported reduced ghrelin levels in correlation with obesity [6,14] and insulin resistance [25], which are components of metabolic syndrome. Significantly lower ghrelin concentration was found in metabolic syndrome in association with fasting insulin and insulin resistance. [26] Both forms of ghrelin were decreased in individuals with metabolic syndrome in connected with insulin resistance. [27] Strong correlation between ghrelin levels and metabolic syndrome was reported in rats where the decreased ghrelin levels were considered a compensatory mechanism against elevated insulin and glucose levels. [21] We also found a negative correlation between serum ghrelin concentration and abdominal circumference which make us accept the idea that it could predict the development of metabolic syndrome. [25]

In contrast to the serum levels of ghrelin, we established increased immunohistochemical ghrelin expression in the gastric mucosa in the dietary-manipulated groups. Similar to our results are those concerning obese patients. Ghrelin producing cells were detected most dense in the proximal stomach of patients with morbid obesity. [28] More ghrelin positive cells with stronger reaction were found in the stomach fundus glands of patients with obesity in comparison to healthy people. [29] We accept that ghrelin is involved in the pathophysiology of obesity [30] and the development of other metabolic disorders, like hyperglycemia, insulin resistance, dyslipidemia and metabolic syndrome as a whole. Increased ghrelin production is probably an adaptive reaction which stimulates growth hormone secretion [31], thus interfering indirectly in the regulation of metabolic processes. Some studies suggest that ghrelin may affect the center of hunger not only by the blood stream, crossing the blood-brain barrier, but also by activation of intramural vagal afferent fibers. With this mechanism, there is no need for high plasma levels of ghrelin to activate the feeling of hunger and obesity. [32]

VI. Conclusion

This is the first study on the effect of HFHCD on ghrelin expression in female and male rats. HFHCD leads to obesity and metabolic syndrome, accompanied by proliferation of a large number of ghrelin-producing cells in the gastric glands and decreased serum ghrelin concentrations. Low blood ghrelin level is an indicator and risk factor of metabolic syndrome.

References
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**Waist circumference**

![Chart showing waist circumference over weeks for male and female rats with dietary manipulation.](chart1.png)

![Chart showing waist circumference over weeks for control and dietary-manipulated groups.](chart2.png)

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![Body mass index graph for male (M) and female (F) rats.

![Body mass index graph for controls (C) and dietary-manipulated (D) groups.

![Body weight graph for male (M) and female (F) rats.

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