Effect of food restriction on reproductive-related genes and reproductive hormones in adult female rats

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Abstract. – OBJECTIVES: A number of factors involved in the control of energy balance and metabolism act as modulators of gonadal axis. Ghrelin, a peptide secreted from the stomach and hypothalamus, has emerged as an orexigenic food intake controlling signal acting upon hypothalamus. Recently, the potential reproductive role of ghrelin has received great attention. This study was designed to investigate the influence of food restriction and consequent metabolic hormone (ghrelin) on the level and gene expression of female reproductive hormones in adult rats.

MATERIALS AND METHODS: To study the effect of chronic food restriction on ghrelin level in adult female rats and its relation to female reproductive hormones, 32 adult female Sprague Dawley rats divided into 4 groups: Group I (control group) comprised 8 rats fed ad libitum for 30 days, Group II, III and IV (food-restricted groups for 10, 20 and 30 days respectively) each consisted of 8 rats fed 50% of ad libitum intake determined by the amount of food consumed by the control group.

RESULTS: Mean body weight of food restricted rats was observed to decrease during the period of the experiment. Food restriction produced significant increase of serum ghrelin with significant decrease of both gastric and hypothalamic ghrelin accompanied with significant increase in its gene expression in stomach and hypothalamus. Estradiol (E2), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels showed significant decrease correlated with down-regulation of gonadotropins, cyclin-dependent kinase (cdc2), cyclin B and kisspeptin (Kiss1) genes in food restricted rats compared with control group.

CONCLUSIONS: Ghrelin could be one of the hormones responsible for the suppression of female reproductive axis in case of negative energy balance. Thus, ghrelin may operate as an autocrine/paracrine regulator of ovarian function. Overall, ghrelin may represent an additional link between body weight homeostasis and reproductive function.

Key Words:
Ghrelin, Food restriction, Kisspeptin, Reproductive hormones, Rats.

Introduction

Ghrelin was discovered at the end of 1999 as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R). Growth hormone secretagogues (GHSs) are a group of synthetic compounds with the ability to induce growth hormone secretion in all species.

Ghrelin is a 28 amino acid peptide hormone possessing an unusual octanoyl group on the serine in position 3, crucial for its biological activity. Human ghrelin gene is localized on chromosome 3 (3p25-26), contains 4 exons and 3 introns. Only exons1 and 2 code for the mature peptide. Ghrelin is predominantly produced by the stomach but also by many other tissues such as pituitary, hypothalamus, lung, heart, pancreas, kidney, ovary and testis. It is a multi-functional peptide hormone that affects a range of processes, including growth hormone and insulin release, appetite regulation, reproduction, and cancer cell proliferation.

In mammals, proper gonadal function critically relies on a complex regulatory network of systemic (endocrine) and locally-produced (paracrine and autocrine) signals. Among others, a number of factors primarily involved in the control of energy balance and metabolism have been proven as putative modulators of the gonadal axis, thus providing the basis for the well-known link between energy homeostasis and fertility. Ghrelin has been demonstrated as a pleiotropic regulator involved in a large array of endocrine and non-endocrine functions, including food intake and energy balance. Thus, it is suggested the involvement of ghrelin in the control of some aspects of gonadal function.

Although the effects of acute administration of ghrelin has suggested a possible role in the control of gonadotropins axis, the effect of chronic food restriction (FR) on ghrelin level and its impact on reproductive function in rats revealed substantial controversy.
The orexigenic gut peptide ghrelin negatively modulates the hypothalamic-pituitary-gonadal (HPG) axis. Hyperghrelinaemia (HGE) results during negative energy balance, a state often associated with delayed puberty and disrupted fertility. Exogenous ghrelin has been found to suppress pulsatile luteinizing hormone (LH) secretion. The recent identification of kisspeptin (Kiss1) and its G protein-coupled receptor (GPR)54 (Kiss1r) as an essential component of the HPG axis controlling gonadotropin secretion raises the possibility that kisspeptin-Kiss1r signalling may play a critical role in the transduction of ghrelin-induced suppression of gonadotropins. Considering the pivotal role for kisspeptin signalling in the activation of the HPG axis, the ability of ghrelin to down-regulate Kiss1 expression in hypothalamus may be a contributing factor in ghrelin-related suppression of pulsatile LH secretion.

This study aimed to elucidate the effect of restriction of daily caloric intake and the consequent metabolic hormone i.e., ghrelin, on the reproductive hormones and expression of their related genes in adult female rats.

**Materials and Methods**

**Experimental Design**

Thirty-two sexually mature, healthy female Sprague Dawley rats, with an initial body weight of 130 ± 10 g, were obtained from the Animal House Colony of the National Research Centre (NRC), Giza, Egypt. The animals were caged at the Animal House of NRC under standard conditions (humidity, 60-65%; temperature, 23 ± 2 ºC; automatic ventilation; photoperiod 12L: 12D and free access to the water). The rats were 90 days old at the beginning of the experiment. The control rats were fed once per day with a standard granulated rat food mixture (15 g/animal/day) containing 86% dry matter, 18% crude proteins, 2.5% fat, 5.5% fiber, vitamins and minerals. This meal dosage was empirically calculated as equivalent to the standard daily food intake of adult female rats fed ad libitum. All animal procedures were performed after approval from the Ethics Committee of the National Research Centre and in accordance with the recommendations for the proper care and use of laboratory animals (NIH publication NO. 85-23, revised 1985). Before initiation of feeding manipulation, all animals were adapted for 15 days to receive their daily food ration. Thereafter, at the day of 105 post-partum, twenty-four of the animals were subjected to food restriction (FR). These rats received 50% of daily standard ration (7.5 g/animal/day) for 10 (group II, n=8); 20 (group III, n=8); and 30 (group IV, n=8) days, respectively. One group of rats (8 rats) were fed ad libitum and continued to receive the standard fixed amount of food (15 g/animal/day) until termination of the experiment and served as a control group (group I). Body weight was recorded only twice (at the beginning and end of the experiment). To minimize the potential influence of immediate food and/or water consumption on actual body weights, access to food and water was prevented 12 h before weighing of animals.

After completion of the regimen of FR, the animals were fasted overnight and subjected to diethyl ether anaesthesia and killed by decapitation. Blood samples were collected into glass tubes, serum were separated by centrifugation for 10 min at 4°C and 3000 rpm, and then frozen at −80°C until used for ELISA tests. Tissue samples including hypothalamus, pituitary gland, stomach and ovary were taken immediately after killing rats, frozen in liquid nitrogen and stored at −80°C prior to extraction.

**Biochemical Analysis**

**Preparation of Hypothalamus Homogenate**

Approximately 0.25 g of hypothalamus was weighed, homogenized immediately to give 10% (w/v) homogenate in ice-cold medium containing 50 mM Tris-HCl and 300 mM sucrose, pH: 7.4. The homogenate was centrifuged at 3000 rpm for 10 min in cooling centrifuge at 4°C. The supernatant (10%) was used for ghrelin analysis.

**Preparation of Stomach Homogenate**

Approximately 0.5 g of stomach tissue were removed, diced into < 1 mm³ pieces, boiled for 7 min in a 5-fold volume of water, and then immediately immersed in an ice bath. Glacial acetic acid was then added to bring the resulting solution to a final concentration of 1 M. The mixture was then homogenized using ultrasonic mixer. The mixture was centrifuged at 10,000 rpm for 30 min and then the supernatant was collected and lyophilized.

Circulating concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and estradiol (E₂) were estimated in serum using specific ELISA kits purchased from Dima Gesellschaft für Diagnostika (GmbH, Göttingen,
Germany) according to the methods described by Harris and Naftolin12, Rebar et al13 and Tsang et al14 respectively.

Ghrelin level was assayed in serum samples and tissue homogenates prepared from stomach and hypothalamus using specific ELISA kit purchased from Phoenix Pharmaceuticals, Inc (Saint Joseph, MO, USA) according to the method described by Porstmann and Kiessig15.

Gene Expression Analysis

According to El-Makawy et al16 and Ali et al17 serial analysis of gene expression via semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) were used for mRNA analysis to determine the expression of the selected genes.

First-strand cDNA synthesize from extracted rat RNA

Tissue samples, which stored at −80°C prior to extraction, were used to extract total RNA. The total RNA was isolated from 50 to 100 mg of tissues by the standard TRIzol extraction method (Invitrogen, Paisley, UK) and recovered in 100 µl molecular biology grade water. In order to remove any possible genomic DNA contamination, the total RNA samples were pre-treated using DNA-free™ DNase treatment and removal reagents kit (Ambion, Austin, TX, USA) following the manufacturer’s protocol. The RNA concentration was determined by spectrophotometric absorption (Jasco, Co, Ishikawa-cho, Hachioji, Tokyo, Japan) at 260 nm.

To synthesize the first-strand cDNA, 5 µg of the complete Poly(A)+ RNA isolated from rat samples was reverse transcribed into cDNA in a total volume of 20 µl using 1 µl oligo [poly(deoxythymidine)] primer16. The composition of the reaction mixture consisted of 50 mM MgCl₂, 10x reverse transcription (RT) buffer (50 mM KCl; 20 mM Tris-HCl; pH 8.3), 200 U/µl taq polymerase, and autoclaved water. The PCR products were then loaded onto 2.0% agarose gel, with PCR products derived from β-actin of the different rat samples. Each reaction of the RT-PCR was repeated with 8 rats, generating new cDNA products at least 8 times per each group.

Statistical Analysis

The data were analyzed using version 11.0 of the computer-based statistical product and service solutions (SPSS, 2001, Chicago, IL, USA). All the data are expressed as Mean ± standard error of mean. Analysis of the data was done using one way Anova to detect the significant difference among the studied groups. A level of p < 0.05 was defined as statistically significant.

Results

Effect of Food Restriction on Body Weight of Adult Female Rats

The results depicted in Table I showed the effect of food restriction (FR) on the body weight of adult female rats. The results indicate that there was a significant decrease in the body weight in groups II, III and IV as compared with the control group. The decline in the body weight in these groups were –13.1%, –7.4% and –2.9% from the control group respectively.

Effect of food Restriction on Ghrelin Level in Serum, Stomach and Hypothalamus of Adult Female Rats

The data in Table II showed the effect of food restriction (FR) on the level of ghrelin in the serum, stomach and hypothalamus of the adult female rats. The results revealed that there was a significant decrease in the body weight in groups II, III and IV as compared with the control group. The decline in the body weight in these groups were –13.1%, –7.4% and –2.9% from the control group respectively.

Effect of Food Restriction on Ghrelin Level in Serum, Stomach and Hypothalamus of Adult Female Rats

The data in Table II showed the effect of FR on the level of ghrelin in the serum, stomach and hypothalamus of the adult female rats. The results revealed that there was a significant increase in the serum ghrelin level in groups II, III and IV as compared with the control group. The elevation in the levels of circulating ghrelin in groups II, III and IV were 12.6%, 31.7% and 111.7% respectively, from the control group. Conversely, the level of gastric ghrelin showed a significant decrease in groups III and IV as compared with the control group. The decrease in the gastric ghrelin levels in these groups were –36.4% and –49.1% from the control group.
Food restriction on reproductive-related genes and reproductive hormones in adult female rats

Table I. Percent represents the variation in percentage between the initial and the final weight of the animals studied.

<table>
<thead>
<tr>
<th>Groups</th>
<th>%</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>–</td>
<td>136.8 ± 0.91</td>
<td>146.8 ± 1.31</td>
</tr>
<tr>
<td>Group II</td>
<td>-13.1%</td>
<td>146.8 ± 0.91</td>
<td>127.5 ± 1.88*</td>
</tr>
<tr>
<td>Group III</td>
<td>-7.4%</td>
<td>157.5 ± 0.94</td>
<td>135.8 ± 2.64*</td>
</tr>
<tr>
<td>Group IV</td>
<td>-2.9%</td>
<td>169.3 ± 1.75</td>
<td>151.2 ± 2.95*</td>
</tr>
</tbody>
</table>

Data are represented as Mean ± SE of 8 rats/group; group I: control rats; group II: rats subjected to food restriction for 10 days; group III: rats subjected to food restriction for 20 days; group IV: rats subjected to food restriction for 30 days. *Significant difference from the control group at p < 0.05. %: Percent of change from control group.

Effect of Food Restriction on Ghrelin Gene Expression in Several Tissues of Adult Female Rats

Table III, Figures 1 and 2 showed the effect of food restriction on gastric and hypothalamic ghrelin gene expression in adult female rats. Groups II, III and IV showed a significant increase in the gene expression of gastric ghrelin as compared with the control group. The hypothalamic ghrelin gene expression in the groups III and IV also showed a significant increase as compared to the control group.

Table II. Effect of food restriction on ghrelin level in serum, stomach and hypothalamus of adult female rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum ghrelin (ng/ml)</th>
<th>Gastric ghrelin (ng/g tissue)</th>
<th>Hypothalamic-ghrelin (ng/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>0.63 ± 0.05</td>
<td>7.08 ± 0.018</td>
<td>8.5 ± 0.19</td>
</tr>
<tr>
<td>Group II</td>
<td>0.71 ± 0.03* (12.6%)</td>
<td>6.5 ± 0.010 (-8.2%)</td>
<td>5.5 ± 0.23* (-35.2%)</td>
</tr>
<tr>
<td>Group III</td>
<td>0.83 ± 0.06* (31.7%)</td>
<td>4.5 ± 0.019* (-36.4%)</td>
<td>4.72 ± 0.04* (-44.4%)</td>
</tr>
<tr>
<td>Group IV</td>
<td>1.37 ± 0.08* (111.7%)</td>
<td>3.6 ± 0.05* (-49.15%)</td>
<td>1.52 ± 0.18* (-82.1%)</td>
</tr>
</tbody>
</table>

Data are represented as Mean ± SEM of 8 rats/group. See: Table I. *Significant difference from the control group at p < 0.05.

Effect of Hyperghrelinemia on Estradiol (E2), Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) Levels of Adult Female Rats

The results depicted in Table IV, showed the effect of FR on the serum levels of estradiol (E2), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) of the adult female rats. Groups II, III and IV showed a significant decrease in serum estradiol level as compared to the control group. The decrease in the level of estradiol in these groups were −18.96%, −18.96% and −44.4%, respectively. Similarly, the level of hypothalamic ghrelin showed significant decrease in groups II, III and IV as compared with the control group. The decrease in the hypothalamic ghrelin in these groups was −35.2%, −44.4% and −82.1% from the control group respectively.

and luteinizing hormone (LH-β) gene expression in the pituitary tissue of the adult female rats. Follicle-stimulating hormone (FSH-β) gene expression in the pituitary tissue of groups III and IV was over expressed as compared with the control group. Groups III and IV showed a significant down-regulation in the gene expression of LH-β in the pituitary tissue as compared with the control group.

Also, the results in Table V and Figure 5 represented cyclin B and cdc2 gene expression in the ovary tissues of the adult female rats under FR. Cyclin B gene expression of group IV showed a significant decrease as compared with the control group. Similarly, groups II, III and IV showed a significant decrease in the gene expression of cdc2 as compared with the control group.

Effect of Hyperghrelinemia on Female Reproductive Related Genes (FSH-β, LH-β, Cyclin B and cdc2) of Adult Female Rats

Table V, Figures 3 and 4 showed the effect of FR on the follicle-stimulating hormone (FSH-β) and luteinizing hormone (LH-β) gene expression in the pituitary tissue of the adult female rats. Follicle-stimulating hormone (FSH-β) gene expression in the pituitary tissue of groups III and IV was over expressed as compared with the control group. Groups III and IV showed a significant down-regulation in the gene expression of LH-β in the pituitary tissue as compared with the control group.

Effect of Food Restriction on Kiss1 Gene Expression in Hypothalamus of Adult Female Rats

Table VI and Figure 6 showed the effect of FR on Kiss1 gene expression in the hypothalamus tissue of female rats subjected to food restriction. RT-PCR was performed with total RNAs isolated from hypothalamus tissues. Lane 1 represents DNA ladder. Lane 2 represents group I, lane 3 represents group II, lane 4 represents group III, lane 5 represents group IV, lanes 6 to 9 represent β-actin gene in groups I to IV, respectively. All samples were normalized on the basis of β-actin expression.

### Table IV

<table>
<thead>
<tr>
<th>Groups</th>
<th>E2 (Pg/ml)</th>
<th>LH (mlu/ml)</th>
<th>FSH (mlu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>26.37 ± 1.19</td>
<td>0.62 ± 0.009</td>
<td>0.98 ± 0.002</td>
</tr>
<tr>
<td>Group II</td>
<td>21.37 ± 1.45* (-18.96%)</td>
<td>0.48 ± 0.005 (-22.5%)</td>
<td>0.97 ± 0.002 (-1.02%)</td>
</tr>
<tr>
<td>Group III</td>
<td>17.37 ± 1.63* (-34.13%)</td>
<td>0.38 ± 0.01* (-38.7%)</td>
<td>0.96 ± 0.001* (-2.04%)</td>
</tr>
<tr>
<td>Group IV</td>
<td>15.75 ± 1.09* (-40.27%)</td>
<td>0.37 ± 0.01* (-40.3%)</td>
<td>0.94 ± 0.003* (-4.08%)</td>
</tr>
</tbody>
</table>

Data are represented as Mean ± SEM of 8 rats/group. See Table II. *Significant difference from the control group at p < 0.05. %: Represent percent of change from control group.
Food restriction on reproductive-related genes and reproductive hormones in adult female rats

Table V. Effect of hyperghrelinemia on female reproductive related genes (FSH-β, LH-β, cyclin B and cdc2) of adult female rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>FSH-β gene (pituitary)</th>
<th>LH-β gene (pituitary)</th>
<th>Cyclin B gene (ovary)</th>
<th>Cdc2 gene (ovary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>1.02 ± 0.045</td>
<td>1.18 ± 0.043</td>
<td>1.25 ± 0.028</td>
<td>1.4 ± 0.040</td>
</tr>
<tr>
<td>Group II</td>
<td>1.04 ± 0.038</td>
<td>1.04 ± 0.024</td>
<td>1.11 ± 0.025</td>
<td>1.20 ± 0.060*</td>
</tr>
<tr>
<td>Group III</td>
<td>1.20 ± 0.022*</td>
<td>0.95 ± 0.025*</td>
<td>1.07 ± 0.038</td>
<td>1.05 ± 0.064*</td>
</tr>
<tr>
<td>Group IV</td>
<td>1.35 ± 0.028*</td>
<td>0.92 ± 0.086*</td>
<td>1.05 ± 0.05*</td>
<td>0.85 ± 0.025*</td>
</tr>
</tbody>
</table>

Data are represented as Mean ± SEM of 8 rats/group. See Table I. *Significant difference from the control group at $p < 0.05$.

Discussion

The results of this study indicate that there was a significant decrease in the body weight of groups II, III and IV as compared with the control group. Our findings agree with the results of Sirotkin et al who found that 50% restriction in daily food intake for 20 days significantly reduced body weight. The significant gradual decrease in body weight of adult rats in the present study was due to the FR in a gradual manner of time.

These results revealed that serum ghrelin level significantly increased in food restricted groups as compared to control group fed ad libitum. In accordance with our data Yang et al reported following chronic dietary restriction an increase in desacyl ghrelin and in total ghrelin in restricted versus ad libitum fed mice. Also Reimer et al reported a marked elevation in circulating levels of total ghrelin following dietary restriction. The possible explanation for hyperghrelinemia was the interplay between ghrelin and the long-term energy balance signals, leptin and insulin. In fact, in FR it has been observed that the circulating leptin negatively regulates plasma ghrelin concentrations. The reduced plasma concentration of leptin seen with dietary restriction triggers an inverse adaptive change in circulating ghrelin. Likewise, insulin may have a role in increasing serum ghrelin level since insulin is down regulated in response to fasting and it has been reported to negatively regulate the

![Figure 3. RT-PCR confirmation of FSH-β gene expressed in pituitary tissue of female rats subjected to food restriction. RT-PCR was performed with total RNAs isolated from pituitary tissue. Lane 1 represents DNA ladder. Lane 2 represents group I, lane 3 represents group II, lane 4 represents group III, lane 5 represents group IV, lanes 6, 7 represent β-actin gene. All samples were normalized on the basis of β-actin expression.](image)

![Figure 4. RT-PCR confirmation of LH-β gene expressed in pituitary tissue of female rats subjected to food restriction. RT-PCR was performed with total RNAs isolated from pituitary tissue. Lane 1 represents DNA ladder. Lane 2 represents group I, lane 3 represents group II, lane 4 represents group III, lane 5 represents group IV, lanes 6, 7 represent β-actin gene. All samples were normalized on the basis of β-actin expression.](image)
synthesis and release of ghrelin from the stomach into the bloodstream. Therefore, in conditions associated with negative energy balance such as fasting or energy restriction, insulin and leptin signaling are reduced leading to increase in serum ghrelin level.

Our current data showed that gastric ghrelin content significantly decreased, while gastric ghrelin mRNA expression significantly increased in FR groups as compared to control group fed ad libitum. These findings agree with Toshinai et al. who reported that the concentration of ghrelin peptide in the stomach tissue decreased after fasting and increased after refeeding, while ghrelin mRNA expression in the stomach is up-regulated upon fasting.

The diminished concentration of ghrelin peptide level in the stomach tissue after fasting could be due to an inverse pattern of ghrelin levels in the stomach tissue and plasma as a result of an increased secretion of ghrelin out of the stomach tissue in response to fasting leading, therefore, to the decreased content of gastric ghrelin.

The possible explanation for up-regulation of ghrelin expression in stomach tissue is correlated with Krüppel-like factor 4 (KLF4). In fact, treatment with butyrate, an inducer of KLF4 expression, has been found to stimulate ghrelin expression. Fasting induced ghrelin expression has been demonstrated also to increase KLF4 expression, suggesting, therefore, that ghrelin expression is associated with KLF4.

Hypothalamic ghrelin content significantly decreased in FR groups while hypothalamic ghrelin mRNA expression significantly increased as

### Table VI. Effect of hyperghrelinemia on kisspeptin (kiss1) gene expression of adult female rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Kiss1 (hypothalamus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>1.29 ± 0.08</td>
</tr>
<tr>
<td>Group II</td>
<td>1.21 ± 0.047</td>
</tr>
<tr>
<td>Group III</td>
<td>1.09 ± 0.028*</td>
</tr>
<tr>
<td>Group IV</td>
<td>0.93 ± 0.040*</td>
</tr>
</tbody>
</table>

Data are represented as Mean ± SEM of 8 rats/group. See Table I. *Significant difference from the control group at p < 0.05. %: Represent percent of change from control group.

![Image](image.png)
compared to control group fed ad libitum. The decrease in ghrelin content in the hypothalamic tissue of food restricted groups is in agreement with Bowers\textsuperscript{25} who reported that hypothalamic ghrelin content decreased during fasting. This decreased ghrelin content in the hypothalamic tissue could be due to fasting that inducing an excessive secretion of ghrelin from the stomach into the blood decreases the ghrelin peptide content in the stomach. Consequently, fasting stimulates the ghrelin release from the hypothalamus, resulting in a decrease in hypothalamic ghrelin concentrations\textsuperscript{26}. It was also found that 2-deoxy-d-glucose (2-DG), selective blocker of carbohydrate metabolism, reduced hypothalamic ghrelin concentrations\textsuperscript{27}. Therefore, glucoprivic states in hypothalamus, such as fasting or 2-DG treatment, promote hypothalamic ghrelin secretion out of the hypothalamic tissue.

Concerning with up regulation of ghrelin expression in the hypothalamic tissue of FR groups, Amole and Unniappan\textsuperscript{28} reported that there is a significant increase in preproghrelin (precursor of ghrelin) mRNA expression in the brain of Zebrasfish fasted for 3, 5 and 7 days when compared to the expression in ad libitum fed fish. The increase in preproghrelin mRNA expression is a reflection of the increased circulating levels of ghrelin during fasting. The possible factor responsible for the increased ghrelin expression in the hypothalamic tissue may be insulin, since it is down regulated in response to fasting and up regulated in states of obesity. Kamengai et al\textsuperscript{21} reported that insulin negatively stimulates the synthesis and release of ghrelin from the stomach. Insulin, thus, could have also a direct inhibitory effect on the hypothalamic ghrelin expression\textsuperscript{29}. Moreover, insulin has direct effect on preproghrelin gene expression stimulating protein kinase B and extracellular signal-regulated kinase 1 and 2 phosphorylation and leading to repression of preproghrelin gene expression. Therefore, insulin has a direct inhibitory effect on the hypothalamic neurons to decrease preproghrelin gene expression through the classic insulin pathways\textsuperscript{30}. The reversed effect has been suggested in the presence of less insulin as in the case of food deprivation.

Kisspeptin (Kiss1) gene expression in hypothalamus down regulated in adult female rats subjected to 50% FR for 20 and 30 days. In accordance with our data, Forbes et al\textsuperscript{4} also reported the ability of ghrelin to down regulate Kiss1 gene expression. In female rats, it was found that the expression of Kiss1 was positively regulated by estradiol\textsuperscript{31,32}. Moreover, it was found that the expression of Kiss1 was dramatically decreased in the ovariectomized (OVX) female rats. The estrogen supplementation reversed the effects of OVX\textsuperscript{33}. ER\textsubscript{α}, an estrogen-inducible transcription factor, directly binds to a specific DNA sequence defined as an estrogen response element (ERE) through its conserved DNA-binding domain. Alternatively, ER\textsubscript{α} interacts with activator protein 1 (AP1) or specificity protein (Sp) proteins and, thus, indirectly associates with target DNA elements and induces a transcriptional activation.

Another factor for the down regulation of Kiss1 gene expression in female rats is insulin-like growth factor-1 (IGF-1) that may induce Kiss1 gene expression in the hypothalamus\textsuperscript{35}. IGF-1 induction of Kiss1 gene expression is dependent on adequate circulating levels of estradiol (E\textsubscript{2})\textsuperscript{36}. IGF-1 and E\textsubscript{2} work together to regulate the hypothalamic neuronal development, plasticity and neuroendocrine function\textsuperscript{36,37}. Since both IGF-1 and E\textsubscript{2} can independently and synergistically activate the Akt (major signaling component involved in IGF-1 stimulation of the Kiss1 gene) signaling pathway in the adult hypothalamus\textsuperscript{38} and serum E\textsubscript{2} levels were suppressed by the hyperghrelinemia, as in the present study. However, the possibility that the IGF-1 induced the phosphorylation of Akt protein is not achieved. This is due to the fact that during the nutritional deprivation, the first observed change is an increase in IGF-binding protein-1 (IGFBP-1), an in vivo inhibitor of IGF-I action\textsuperscript{39}.

Luteinizing hormone and FSH serum levels showed significant decrease in adult female rats subjected to 50% FR for 20 and 30 days. In accordance with our findings Lorenzi et al\textsuperscript{40} stated that in rats the excess of ghrelin inhibits the gonadotropins secretion in males and females. The decreased LH, FSH serum levels observed in the current study could be due to hypothalamic Kisspeptin (Kiss1) gene expression. Hyperghrelinemia during negative energy balance may affect the reproductive capacity via impacting hypothalamic Kiss1 mRNA expression\textsuperscript{4}. The kisspeptin action on the gonadotropic axis can activate GnRH neurons and elicit GnRH secretions.
which trigger the gonadotropin secretion.

LH-β gene expression in pituitary tissue was downregulated in adult female rats subjected to 50% FR for 20 and 30 days. The down regulation of LH-β gene may be explained since the subunit genes of gonadotropins are differentially regulated by GnRH pulse frequency. The fast frequencies favor α and LH-β and the slow frequencies favor FSH-β. Furthermore, a great importance acquire the gonadal steroids which act both at the hypothalamus to alter GnRH pulsatility and/or directly on the pituitary gonadotropins. Expression of the gonadotropin genes is tightly regulated at both the biosynthetic and secretory levels through the complex interaction of hypothalamic, pituitary and gonadally derived factors. The gonadotropin-releasing hormone (GnRH), has been widely recognized to alter gonadotropin biosynthesis via activation of both the protein kinase C (PKC) and calcium pathways. GnRH may also stimulate the cAMP protein kinase A (PKA) signaling system. Thus, the down regulation of kisspeptin gene expression in the hypothalamus, with a consequent decrease of GnRH in case of food deprivation, might lead to the inhibition of cAMP/PKA system which, finally, results in the down regulation of pituitary LH-β gene expression, as in the present study.

Follicle-stimulating hormone gene expression was over expressed in the adult female rats subjected to 50% FR for 20 and 30 days. In case of low estradiol levels, the acute increases in FSH-β mRNA largely reflect the loss of circulating inhibin, which led to decrease in FSH-β transcription.

Serum estradiol level significantly decreased in FR groups as compared to control group fed ad libitum. A possible explanation is that the granulosa cells serve as the predominant site of ovarian IGF-I production, reception, and action. In addition to its growth-promoting activity, IGF-I is able to synergize with gonadotropins and to amplify their steroidogenic output (i.e., to promote progesterone and estradiol synthesis). IGF-I can also enhance StAR expression, responsible for the transfer of cholesterol into the mitochondria for steroidogenesis in granulosa-lutein cells. IGF-I system is extremely sensitive to metabolic alterations, and changes within it play a key role in the processes that link the nutrition and the growth. The nutritional deprivation, the first observed change is an increase in IGF-binding protein-1 (IGFBP-1), an inhibitor of IGF-1 action. Through these pathways, hyperghrelinemia could lead to suppression of E2 serum level in adult female rats subjected to food deprivation, as in our study.

Cdc2 gene expression in ovarian tissues showed a significant decrease in the adult female rats subjected to 50% FR for 20 and 30 days. Also, the gene expression of cyclin B showed a significant decrease. The possible factor for down regulation of cyclin B gene expression in ovarian tissues may be due to the low level of GnRH caused by hyperghrelinemia where it was found that cyclin B gene expression was maintained in fish treated with GnRH to induce spawning.

The factor for the down regulation of Cdc2 and cyclin B gene expression in the ovarian tissues may be the thrombopoietin (TPO), an hormone/growth factor/cytokine, that plays an important role in stimulating the differentiation of the hemopoetic stem cells, megakariocytes and the platelet production. The presence and turnover of the mRNA for TPO has been described in human and hamster ovarian cells. TPO may be a potent regulator of ovarian function e.g. proliferation, apoptosis and the secretion of peptide hormones, steroids, growth factors and growth factor-binding protein, as well as of the expression of some intracellular messengers. Moreover, TPO induced increase in expression of Cdc2 and cyclin B through the protein kinase A (PKA)-dependent pathway. It has been demonstrated that GnRH stimulates the protein kinase A (PKA) signaling system. Thus, the down regulation of Kiss 1 gene in the hypothalamus with consequent decrease of GnRH in case of hyperghrelinemia might lead to the inhibition of PKA system which regulates the down regulation of the ovarian Cdc2 and cyclin B gene expression.

In conclusion, ghrelin may be one of the hormones responsible for the suppression of female reproductive axis in case of negative energy balance. Ghrelin can operate as an autocrine paracrine regulator of the ovarian function and represents an important additional link between the body weight homeostasis and the reproductive function.

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