Research Square

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Central MOTS-c Infusion Affects Reproductive Hormones in Obese and Non-Obese Rats

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Abstract

MOTS-c, a mitochondrial-derived peptide, acts as a systemic hormone and MOTS-c level is inversely correlated with markers of obesity. Obesity is a risk factor for male reproductive physiology and is expressed as an important cause of infertility. In this study, we aimed to determine the effects of MOTS-c, which has been proven in the hypothalamus and testicles, on the actors involved in the reproductive axis.

In the study, 80 male Wistar-Albino rats were divided into two main groups, obese and non-obese ($n = 40$). Rats in the first main group were fed with fatty diet feed and obesity was induced. The second main group was fed with normal diet feed. Each main group was divided into 4 small groups (Control, Sham, 10 and 100 μM MOTS-c). The lateral ventricles of the animals in the treatment groups were infused with 10 and 100 μM MOTS-c (solvent in Sham group) for 14 days. At the end of the experiment, hypothalamic Gonadotropin-Releasing Hormone (GnRH) gene expression level, serum testosterone, Luteinizing hormone (LH) and Follicle stimulating hormone (FSH) levels were determined.

MOTS-c infusion caused an increase in GnRH mRNA, protein expression levels and serum testosterone, LH and FSH levels in obese and non-obese rats ($p < 0.05$). MOTS-c administration more significantly upregulated hormone levels in non-obese rats ($p < 0.05$).

Our results reveal that MOTS-c plays a role in the central regulation of reproductive behavior, as well as causes increased LH, FSH and testosterone release. MOTS-c may emerge as a novel regulator for the prevention of obesity-induced infertility.

1. Introduction

The hypothalamic-pituitary-testicular (HPT) axis is a central system that mediates reproduction and has important connections with other systems. The hypothalamus, which is located at the center of the axis, regulates Gonadotropin-Releasing Hormone (GnRH) secretion both in response to neural messages from the central nervous system (CNS) and hormones secreted from the testes in the periphery [1]. GnRH, which is of great importance in the control of reproduction, is released from hypothalamic neurovascular terminals into the portal circulation in a pulsatile manner [2]. GnRH plays a role in the secretion of Luteinizing hormone (LH) and Follicle stimulating hormone (FSH), which are anterior pituitary gonadotropins [3]. LH causes the secretion of testosterone from Leydig cells in the testicels, which mediates spermatogenesis and the emergence of male sex characteristics. FSH stimulates spermatogenesis in the presence of testosterone by activating Sertoli cells. The absence of LH causes Leydig cells to shrink and testosterone production to stop, leading to infertility [4]. In addition to disruptions in the HPT axis, peripheral signaling, chronic diseases such as obesity and diabetes, and many other factors may directly or indirectly support infertility. Therefore, identifying new players in the reproductive axis may enable successful treatment of the causes of infertility.

Mitochondrial-derived peptide (MOTS-c) was defined in 2015 as a peptide encoded by mitochondrial DNA and plays a role in energy homeostasis [5]. Lee et al. reported that MOTS-c leads to AMPK activation by

inhibiting folate cycle and de novo purine biosynthesis. Researchers have shown that MOTS-c administration to mice reduces high-fat diet-induced insulin resistance and prevents diet-induced obesity [5]. It was reported that the MOTS-c analog CB4211 decreased the levels of free fatty acids secreted from cultured adipocytes, causing a decrease in fat mass and body weight of obese mice [6]. In addition to these results, it is reported that MOTS-c can be used in the treatment of type 2 diabetes and may be effective in the prevention of cellular aging due to its metabolism-regulating effects [5, 7]. Published studies are aimed at explaining the relationship between circulating levels of MOTS-c and obesity and diabetes [8, 9]. Du et al. showed that circulating MOTS-c levels are decreased in obese male children and adolescents and associated with insulin resistance and markers of obesity [10]. In contrast, MOTS-c is induced by exercise and may mediate the anti-obesity effects of exercise. Reynolds et al. reported that MOTS-c levels increased after exercise in muscle and plasma samples obtained from healthy young male volunteers before and after exercise. These findings suggest that exercise induces the expression of mitochondrial-encoded regulatory peptides in humans [11].

The fact that both reproductive and nutritional centers are controlled in the same region of the hypothalamus (by different neurons) suggests that the neuroendocrine regulation of energy balance and reproduction are closely related. It is known that energy and fat metabolism have very important effects on the reproductive system [12]. It has been reported that obesity decreases the frequency of ovulation in females and causes a decrease in sperm quality in males and thus may cause infertility in both groups [13, 14].

This study aims to reveal the possible effects of central MOTS-c infusion on GnRH, LH, FSH and testosterone hormones involved in the regulation of reproductive function in obese and non-obese male rats.

2. Material and Method

2.1. Animal Ethics

The study was carried out with the approval of the Local Ethics Committee of Animal Experiments at Inonu University Faculty of Medicine (protocol number 2020/4–5, dated 10.03.2020) in the Experimental Animal Production and Research Center of Inonu University Faculty of Medicine, Department of Physiology, Faculty of Medicine, and Molecular Biology and Genetics Laboratories of Bartın University Faculty of Science.

2.2. Experimental Design

Number of animals to be used in the experiment, type 1 error (α) 0.05 and type 2 error (β) (Power = 0.80), it was determined by Power Analysis that there should be at least 10 animals in each group and at least 80 animals in total. In the study, 80 (10 in each group) male Wistar Albino rats were used.

21-day-old rat pups were weighed after weaning and divided into 2 main groups (n = 40) with similar body weights (Fig. 1). The animals were assigned to the groups according to the determined body weights by simple random assignment method based on a computer algorithm (MedCalc 12.7.0 for Windows). One-way analysis of variance showed that there was no difference between the groups in terms of weight ($p = 0.685$).

For 12 weeks, one of the main groups was fed ad libitum with normal diet feed (NDF) and the other with high fat diet feed (HDF; Research Diets: D12451). During the experimental period, the environment in which the rats were housed was set as single cages with a temperature range of 20–22ºC and a 12-h light/12-h dark period [14]. It was determined that an experimental obesity model was created as a result of Lee index scoring of the groups fed with NDF and HDF (Table 1) [15].

Table 1
Lee Index Values of the Animals in the Groups

	Control	Sham	10μM MOTS-c	100μM MOTS-c
Fed with NDF	0.19 ± 0.03^{a}	0.21 ± 0.01^{a}	0.20 ± 0.02^{a}	0.20 ± 0.04^{a}
Fed with HDF	0.41 ± 0.02^{b}	0.43 ± 0.03^{b}	0.40 ± 0.04^{b}	0.42 ± 0.03^{b}

Data are presented as mean ± standard devation. ($p < 0.05$). $^{a-b}$Results were statistically different within the same parameter assessed.

After the obesity model was established, the main groups were divided into 4 groups (Control, Sham, 10 µM MOTS-c, 100 µM MOTS-c). Rats in the control group were not treated. Sham and MOTS-c group rats were anesthetized intraperitoneally with 70 mg/kg ketamine (Richter Pharma AG, Australia) and 8 mg/kg xylazine (Alfazyne, The Netherlands). The scalp of the rats was shaved and the animals were fixed in a stereotaxic device (Rodent Stereotaxic Instruments, Harvard Apparatus, USA). Right lateral ventricle coordinates (1.40 mm lateral, 0.8 mm posterior and 4.8 mm vertical) were determined with reference to the Bregma point according to the Paxinos & Watson rat brain atlas [16]. The skull of the rats was drilled with a drill at the determined point. The brain infusion kit (BIK) (Alzet brain infusion kit 1, USA) was placed into the right lateral ventricle and fixed to the skull with dental cement [17]. The cannula of the BIC was placed under the nape of the neck of the animals and the incision site was sutured. One week later, the rats were reanesthetized and osmotic mini pumps (Alzet 2ML2) were connected to the BICs and placed in the midscapular area. The treatment groups were infused with 10 µM and 100 µM doses of MOTS-c and the sham group with artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 5.0 mM KCl, 1.2 mM KH2PO4, 2.4 mM CaCl2, 1.3 mM MgSO4, 26 mM NaHCO3, and 30 mM glucose, pH:7.2) for 14 days (5 µl/h) via osmotic mini pumps [18]. At the end of intracerebroventricular (ICV) MOTS-c infusion, rats were decapitated and hypothalamus and blood samples were collected for analysis.

2.3. RT-PCR analysis

Total mRNA isolation from collected hypothalamus tissue was performed using the EZ-10 Spin Column Total RNA Miniprep Kit (BioBasic, Canada). cDNA synthesis from RNA samples was performed using the iScript cDNA Synthesis Kit (BioRad, USA). RT-PCR analysis was performed on the CFX Connect RT-PCR Detection System (Bio-Rad Laboratories) using SYBR Green Master Mix (BioRad Biosystems) in the presence of specific primers. The primers used in the study are as follows: Rattus norvegicus beta-actin (Actb): 5' CTAAGGCCAACCGTGAAAAG 3' (forward) and 5' GCCTGGATGGCTACGTACA 3' (reverse); Rattus norvegicus GnRH1: 5' TCTGCGAGGAGCTCTGG 3' (forward) and 5' GGGCCAGTGCATTACATCTT 3'(reverse). Reaction volumes were set as 10 µl. The 5 µl master mix containing 0.5 µl real time ready mix, 2 µl PCR grade water, and 2.5 µl cDNA was prepared. Samples were run as triplicate. The cycling protocol was set as an initial 10 min denaturation step at 95°C, followed by 55 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 1 s. To determine the change in the GnRH gene expression among the groups, β-actin gene was selected as housekeeping gene; and relative messenger RNA (mRNA) expression levels were calculated according to housekeeping genes using the $2^{-\Delta\Delta Ct}$ method [19].

2.4. Western blot

The hypothalamus tissues of rats were lysed using a bead mill homogenizer (Qiagen tissuelyser lt, Germany) in a lysis buffer [20]. Total protein concentration and the amount of protein were determined using a BCA protein analysis kit. 20 µg of the total protein from each tissue was treated with 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis and then transferred to 0.22 µm PVDF membranes. The membranes were blocked with 5% dry milk prepared with TBS-T buffer and then incubated with GnRH primary antibodies at 4°C overnight. After the incubation, the membranes were washed with TBS-T and left to incubate with secondary antibodies conjugated with HRP for 1 hour at room temperature. Finally, the membranes treated with the ECL solution were displayed in the Fusion FX-7 (Vilber, Germany) system. The blots were analysed in the Image J program. The tissue protein level measured in the experiment was proportioned to β-actin [21].

2.5 Determination of LH, FSH and Testosterone levels by ELISA method

Blood tissue samples in gel sterile tubes were centrifuged at 3500 rpm for 10 minutes. LH (Elabscience, E-EL-R0026), FSH (Elabscience, E ELR1106) and testosterone (Cayman, 582701) were measured from the separated serum samples by ELISA method. The kit protocol was followed in the analyzes.

2.6 Statistical Analysis

IBM SPSS Statistics 24.0 for Windows package program was used for data evaluation. Compliance with normal distribution was examined by Shapiro Wilk test. Kruskal Wallis H test was used for intergroup comparisons of quantitative variables. When significant differences were determined, multiple comparisons were performed with Mann Whitney U test with Bonferroni correction. Quantitative data were summarized with median, minimum and maximum values. In the analysis of differences between two groups, Independent Sample T-Test was used for normally distributed data and Mann-Whitney U test

was used for non-normally distributed data. Values were given as mean ± standard deviation. $p < 0.05$ was considered statistically significant.

3. Results

3.1. MOTS-c Infusion Increased GnRH mRNA and Protein Levels

The effects on GnRH mRNA expression level in hypothalamic tissue of obese and non-obese rats are shown in Fig. 2. 1A-1B. In both groups, MOTS-c concentrations significantly increased the level of GnRH mRNA expression ($p < 0.05$).

Comparison of the effect on GnRH mRNA expression level in hypothalamic tissue in obese and non-obese rats is shown in Fig. 2. 1C. It was determined that GnRH mRNA level was lower in obese rats compared to non-obese rats ($p < 0.05$).

The effects of obese and non-obese rats on hypothalamic GnRH protein expression levels are presented in Fig. 3. 2A-2B. In both groups, MOTS-c concentrations significantly increased GnRH protein expression levels ($p < 0.05$).

Comparison of the effects of MOTS-c concentrations on hypothalamic GnRH protein expression levels in obese and non-obese rats is given in Fig. 3. 2C. The effect on GnRH protein expression levels in obese rats was found to be at a lower level compared to non-obese rats.

3.2. MOTS-c Increased Serum LH, FSH and Testosterone Levels

The effects on serum LH and FSH levels of obese and non-obese rats and comparison of these groups are given in Fig. 4. 3A-3B-3C-4A-4B-4C. Both concentrations of administered MOTS-c increased serum LH and FSH levels compared to Control and Sham groups ($p < 0.05$). There was a significant difference between obese rats and non-obese rats in all groups ($p < 0.05$).

The effects of MOTS-c administered ICV on serum testosterone levels in obese and non-obese rats are shown in Fig. 5. 5A-5B. Accordingly, no significant difference was found between the Control group and Sham group ($p > 0.05$). However, both 10 µM MOTS-c and 100 µM MOTS-c groups had significantly higher testosterone levels and this difference was dose dependent ($p < 0.05$).

The comparison of the effect of MOTS-c on serum testosterone levels in obese and non-obese rats is shown in Fig. 5. 5C. There was a significant difference between obese and non-obese rats in all groups ($p < 0.05$).

4. Discussion

The findings of this study show that MOTS-c administered to obese and non-obese rats causes an increase in hypothalamic GnRH mRNA and protein levels and serum LH, FSH and testosterone levels. These effects of MOTS-c on the HPT axis suggest that the peptide may be closely related to reproductive physiology.

The literature mentions the positive effects of MOTS-c on energy metabolism in general. Studies show that MOTS-c reduces obesity and insulin resistance [5, 7] and regulates metabolism [7, 9, 22]. In addition, the decrease in MOTS-c levels in brain and muscle tissue due to starvation indicates that this peptide has important physiological roles in nutrition and energy balance [23]. Few studies are insufficient to explain the effects of MOTS-c on other systems.

The reproductive system works not only in its own right but also in close relationship with other systems and processes. The relationship between obesity and reproduction is a current topic and it is generally believed that increasing obesity is among the leading causes of infertility. In addition to hormonal changes in the hypothalamic-pituitary-testicular (HPT) axis, obesity appears to affect men's reproductive potential through various mechanisms such as changes in spermatogenesis, sperm quality and/or sexual health [24]. It has been reported that obesity causes low GnRH expression [25], while severely obese men (BMI > 40) have low LH and free testosterone levels [26]. The capacity of the human testis to secrete testosterone is recognized to decrease in aging men [27] and this has been shown to be due to a decrease in the responsiveness of the aging testis to intravenous LH pulses [28]. Studies have shown that in obese men, low LH levels accompany a decrease in testosterone [9, 29].

The few studies on the association of mitochondrial-derived peptides (MDPs) with reproduction have focused on the overall tissue distribution of these peptides [30, 31]. Humanin, a mitochondrial peptide, is expressed in the testes and its levels in human seminal plasma and spermatozoa are associated with sperm quality [32]. Studies on other MDPs and their effects on reproductive physiology are very limited. Considering the regulatory roles of MOTS-c in energy metabolism, the question of whether this peptide is involved in obesity-induced infertility is an important puzzle waiting to be answered. Although a few studies cannot fully answer this question, they share important findings with us. Lu et al. [33] report that MOTS-c treatment prevents ovariectomy-induced obesity and insulin resistance in mice.

In this study, researchers reported that in addition to low estrogen levels, there was an excessive increase in fat mass in mice after ovariectomy and insulin resistance developed by disrupting normal fat function. It was found that brown fat activation increased and overiectomy-induced fat accumulation decreased after MOTS-c treatment [33]. In another study, it was reported that circulating MOTS-c level was low in obese men [10].

In our study, we showed that MOTS-c stimulates hormones on the HPT axis. Hypothalamic GnRH mRNA and protein levels were increased in MOTS-c treated groups. This increase was accompanied by increases in the expression of LH, FSH and GnRH, which are involved in the HPT axis. In addition, the

levels of testosterone, which is located at the last step of the axis, also increased after MOTS-c administration. These changes were common in both obese and non-obese groups. The decreased expression of hormones on the HPT axis in obese groups compared to non-obese groups suggests that obesity may cause infertility as stated. The fact that MOTS-c caused an increase in the expression of hormones on the HPT axis in both groups (obese and non-obese) indicates that this peptide may have therapeutic effects for infertility.

5. Conclusion

In this study, the changes in reproductive hormones caused by centrally administered MOTS-c in relation to obesity were defined. These data are important as the first contribution to the literature showing the effect of MOTS-c on reproductive physiology. We anticipate that further studies on the effect of MOTS-c on reproductive metabolism will provide important contributions on issues such as mechanism, modeling and infertility. In this context, the results of our study have a guiding feature in MOTS-c-based obesity and infertility research.

6. Results

GnRH mRNA and protein expression was low in obesity-induced rats compared to non-obese rats (Fig. 1C-2C). After 14 days of ICV MOTS-C administration, hypothalamic GnRH mRNA and protein expression was significantly increased in obese and non-obese animals compared to control and sham groups rats (Fig. 1C-2C). The administered doses of MOTS-c similarly caused changes in GNRH gene expressions. Low and high doses of MOTS-c increased GnRH gene expression approximately twofold. This indicates that MOTS-c upregulates GnRH expression in the hypothalamus.

Serum LH, FSH and testosterone levels were lower in obese rats compared to non-obese rats (Fig. 3C-4C-5C; respectively). After MOTS-c infusion, serum LH, FSH and testosterone levels were significantly higher in both obese and non-obese groups compared to control and sham groups. Moreover, MOTS-c increased testosterone levels in a dose-dependent manner in both groups (obese and non-obese). These results suggest that MOTS-c upregulates the expression of genes involved in the HPT axis and may play a role in preventing the negative effects of obesity on the reproductive axis.

Declarations

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Conflict of Interests

The authors declare that they have no competing interests.

Author Contributions

All authors contributed to the study conception and design. Dilara ALTAY OZTURK and Suat TEKIN designed the experiments. Yavuz ERDEN contributed to laboratory studies and manuscript preparation. Suat TEKIN supervised the study, and provided support in the data interpretation and manuscript preparation.

Ethics Approval

This study is approved by Inonu University Faculty of Medicine, Local Ethics Committee (10.03.2020 with the protocol number 2020/4-5).

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Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

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Figures

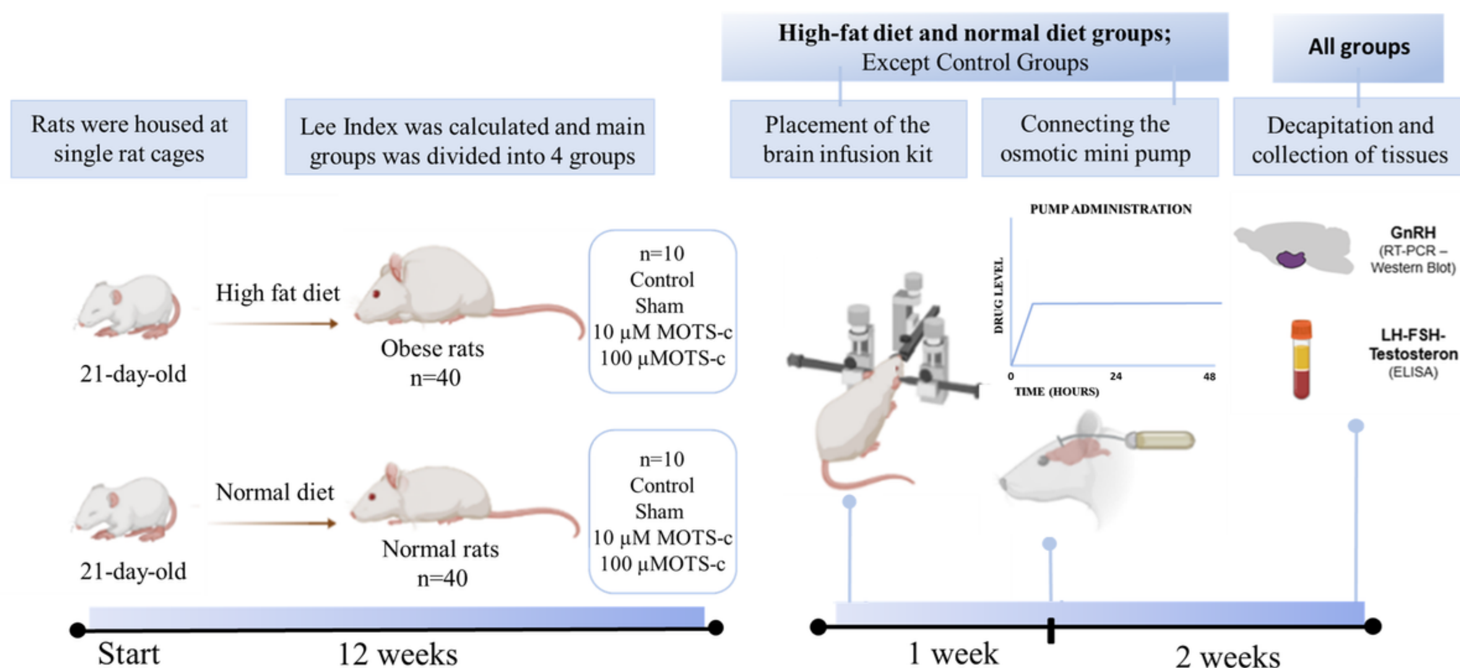

Figure 1

Experimental plan of the study. For 12 weeks, one of the main groups was fed with normal diet feed (NDF) and the other group was fed with high fat diet feed. Obesity model was created in the group fed with HFD. During the study period, the control group did not undergo any treatment or surgical operation. A brain infusion kit was placed in the sham and treatment groups. After 1 week, osmotic mini pumps were inserted and the groups were infused with yBOS, 10 µM and 100 µM MOTS-c. At the end of 2 weeks, animals were decapitated and tissues were collected.

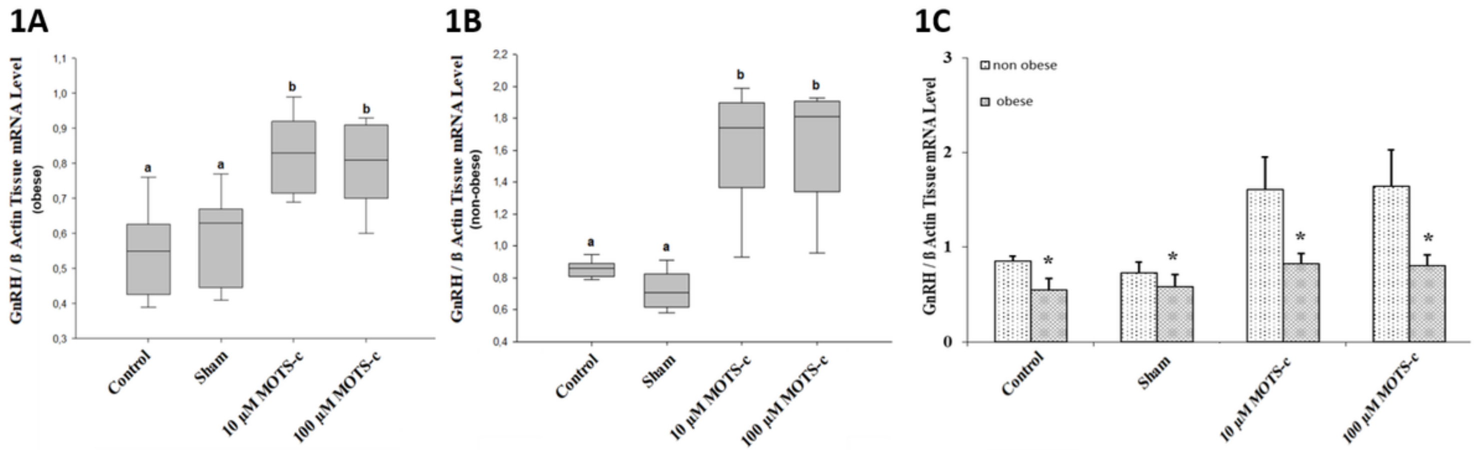

Figure 2

Effect of ICV MOTS-c administration to obese and non-obese rats on GnRH mRNA expression in the hypothalamus (1A-1B) (Data were analyzed using Kruskal Wallis H test. Multiple comparisons were evaluated by Mann Whitney U test with Bonferroni correction. Values were expressed as median (minimum-maximum). Different letters indicate the difference between the groups ([a,b]$p<0.05$). Comparison of these values in obese and non-obese groups (1C) (In the analysis of the differences between groups, Independent Sample T-Test was used for normally distributed data and Mann-Whitney U test was used for non-normally distributed data. Values were given as mean ± standard deviation (*$p<0.05$).

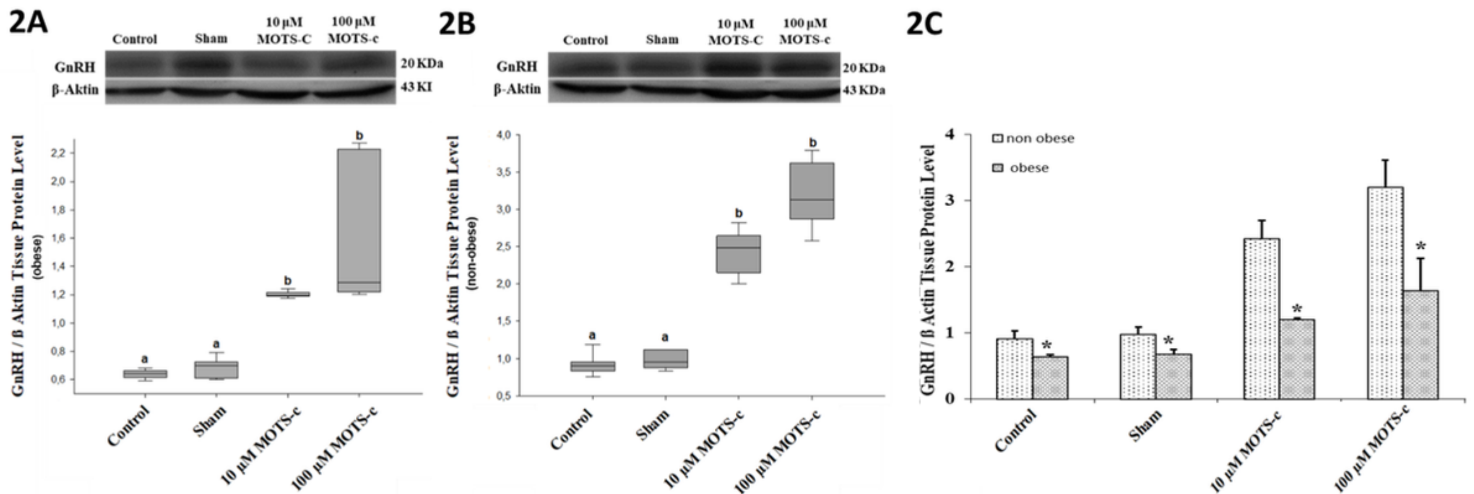

Figure 3

Western blot gel image and densimetric analysis of bands (2A-2B) of the effect of ICV MOTS-c administration on hypothalamic GnRH protein ratio in obese and non-obese rats (Data were analyzed using Kruskal Wallis H test. Multiple comparisons were evaluated by Mann Whitney U test with Bonferroni correction. Values were expressed as median (minimum-maximum). Different letters indicate the

difference between the groups ([a,b]$p<0.05$). Comparison of these values in obese and non-obese groups (2C) (In the analysis of the differences between groups, Independent Sample T-Test was used for normally distributed data and Mann-Whitney U test was used for non-normally distributed data. Values were expressed as mean ± standard deviation (*$p<0.05$).

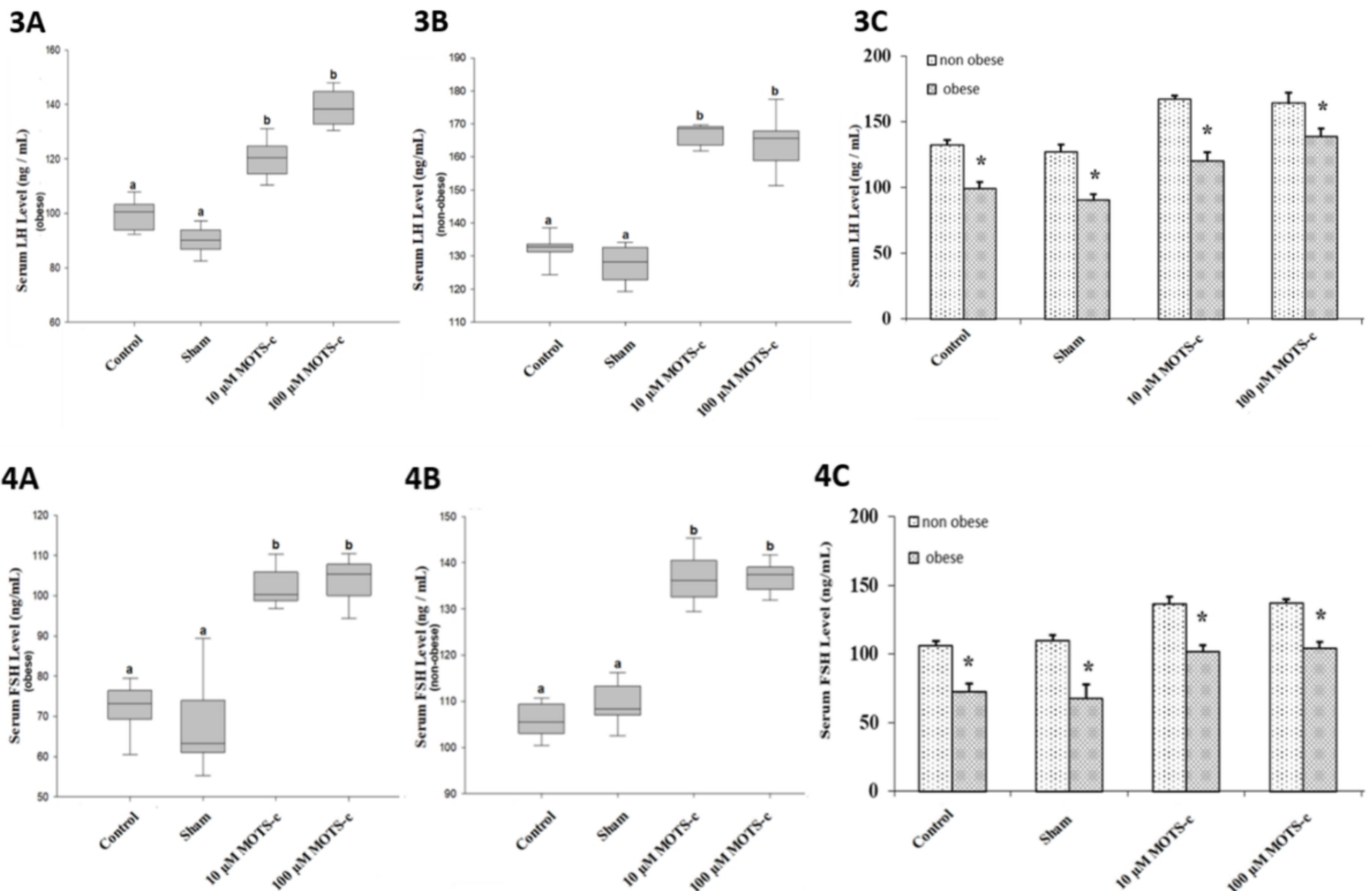

Figure 4

Effect of ICV MOTS-c administration on serum LH (3A-3B) and FSH (4A-4B) levels in obese and non-obese rats (Data were analyzed using Kruskal Wallis H test. Multiple comparisons were evaluated by Mann Whitney U test with Bonferroni correction. Values were expressed as median (minimum-maximum). Different letters indicate the difference between the groups ([a,b]$p<0.05$). Comparison of these values in obese and non-obese groups (3C-4C) (In the analysis of the differences between groups, Independent Sample T-Test was used for normally distributed data and Mann-Whitney U test was used for non-normally distributed data. Values were expressed as mean ± standard deviation (*$p<0.05$).

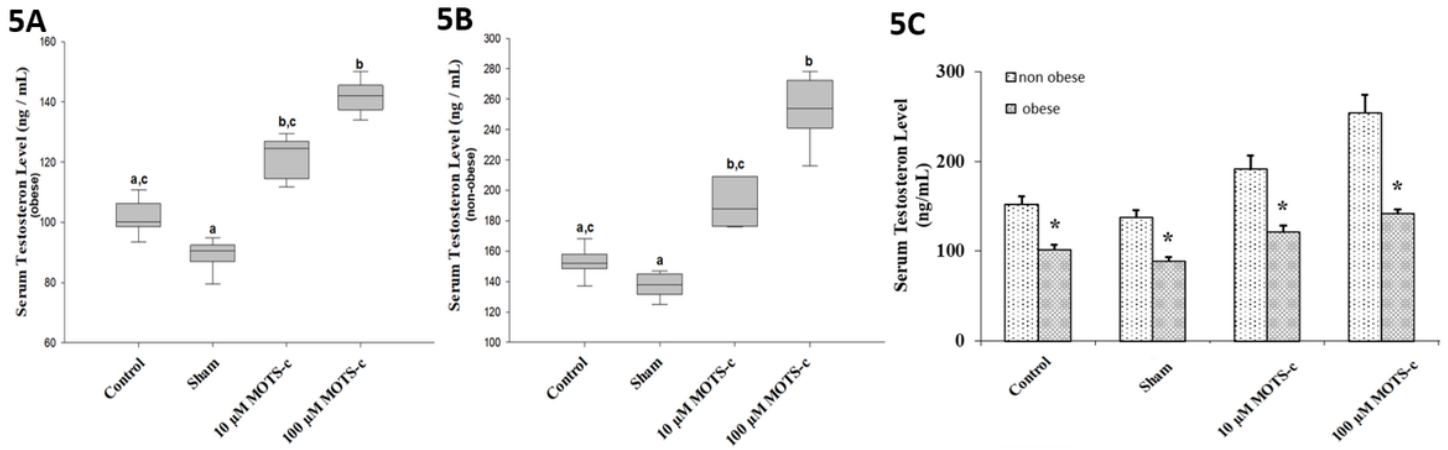

Figure 5

Effect of ICV MOTS-c administration on serum testosterone (5A-5B) levels in obese and non-obese rats (Data were analyzed using Kruskal Wallis H test. Multiple comparisons were evaluated by Mann Whitney U test with Bonferroni correction. Values were expressed as median (minimum-maximum). Different letters indicate the difference between the groups ($^{a,b}p<0.05$). Comparison of these values in obese and non-obese groups (5C) (In the analysis of the differences between groups, Independent Sample T-Test was used for normally distributed data and Mann-Whitney U test was used for non-normally distributed data. Values were expressed as mean ± standard deviation (*$p<0.05$).