

The effects of long-term leptin administration on morphometrical changes of mice testicular tissue

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ARTICLE INFO

Article type:

Original article

Article history:

Received: Feb 15, 2015

Accepted: Aug 6, 2015

Keywords:

Leptin

Meiotic index

Sertoli cell

Spermatogenesis

Spermatogonia cell

ABSTRACT

Objective(s): Leptin is a novel and interesting hormone for anyone trying to lose weight, but its effects on male gonad structure in longitudinal study is unknown. The present study was designed to explore morphometrical changes of mouse testicular tissue after long-term administration of leptin.

Materials and Methods: Thirty healthy mature male mice were randomly assigned to either control (n=15) or treatment (n=15) groups. Leptin was intraperitoneally injected to the treatment group (0.1 µg/100 µl of physiological saline) once a day for 30 consecutive days, and control animals received normal saline with the same volume and route. Five mice from each experimental group were sacrificed at 15, 30 and 60 days after the beginning of treatments. Left testes were removed, weighted and then fixed in 10% buffered formalin, and stained with hematoxylin and eosine for morphometrical assays.

Results: Except for sertoli cell nucleus diameter, which was affected from 30th day, evaluation of other morphometrical parameters such as Johnsen's score, meiotic index, spermatogenesis, epithelial height, seminiferous tubules diameter and spermatogonial nucleus diameter revealed significant decrease from 15th day after leptin administration compare to those of the control group ($P<0.05$). Thus, meiotic index and spermatogonial cell nucleus diameter were two parameters that were further disturbed on 30th day compare to the day 15 (3.09 ± 0.03 vs. 3.23 ± 0.03 , $P=0.006$ and 5.50 ± 0.09 vs. 6.08 ± 0.14 , $P=0.007$, respectively).

Conclusion: Our results showed that long-term administration of leptin could disturb testicular tissue structure and delay spermatogenesis process.

► Please cite this article as:

Esmaili-Nejad MR, Babaei H, Kheirandish R. The effects of long-term leptin administration on morphometrical changes of mice testicular tissue. Iran J Basic Med Sci 2015; 18:1176-1182.

Introduction

Obesity is a major health problem in adult population, which is associated with serious comorbidities including diabetes, cardiovascular disease, osteoarthritis and an increased risk of many forms of cancer. New research suggests that ob/ob mouse can slim down without even any exercise trying or fasting with the help of an appetite control hormone called leptin (1), so it was hoped that this new hormone would have a similar effect in common forms of obesity in humans. Leptin, the product of the ob gene, is hypothesized to be a feedback signal in the long-term regulation of energy balance, informing the brain of the size of body fat store (2). The initial therapeutic use for leptin grew directly from its discovery. The administration of leptin to the ob/ob mice had a dramatic effect in inducing weight loss (3-5). This led to the use of leptin to

treat patients with congenital defects in leptin production (6, 7). The second indication for the use of leptin is in another hypoleptinemic state, lipodystrophy (8, 9).

In both male and female mice, it has been reported that leptin has direct effects on fertility (10); in fact, administration of the hormone is able to reverse the infertility of ob/ob mice lacking the leptin gene (11). The biological actions of leptin on body weight, neuroendocrine function, and fertility are carried out through interactions with its specific obese receptor in target tissues (12). Expression of the obese gene results in several alternatively spliced isoforms that participate in the extracellular domain, but differ in the length of their transmembrane and cytoplasmic coding regions (13-15). The long form and several short isoforms are shown in an increasing number of peripheral tissues including liver, kidneys, lungs,

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Table 1. Johnsen's score for spermatogenesis

Score	Histological findings
10	Complete spermatogenesis, numerous spermatozoa, germinal epithelium of regular height, tubular lumen of normal diameter
9	Numerous spermatozoa, germinal epithelium disorganized with sequestration of germinal cells, tubular lumen obturated
8	Less than 5-10 spermatozoa per tubular cross-section
7	No spermatozoa, numerous spermatids, spermatocytes and spermatogonia
6	No spermatozoa, 5-20 spermatids, numerous spermatocytes and spermatogonia per cross-section
5	No spermatozoa and spermatids, numerous spermatocytes and spermatogonia
4	No spermatozoa and spermatids, less than five spermatocytes, but numerous spermatogonia per cross-section
3	Only spermatogonia
2	No germinal cells, only sertoli cells
1	No cells at all within the tubules

small intestine, heart, pituitary cells, spleen, pancreas, adrenal gland and adipose tissue (16-19). The obese gene receptor is expressed in rodent testis, but with a different cellular site. The obese gene receptor is also expressed in Leydig cells of rat, but in germ cells of mouse (20, 21), although the long isoforms of leptin receptor have not been recognized on human sperm (22). Leptin and leptin receptor were found in human seminiferous tubules (23), human seminal plasma (24), human and boar sperm (25, 26) and recently was reported its involvement in sperm capacitation and survival (27), sperm count and abnormality (28), motility and kinetic parameters (29). In the present study, we investigated the morphometrical changes of mouse testicular tissue following the long-term administration of leptin.

Materials and Methods

Animals

A total of 30 healthy adult male NMRI mice, six weeks of age and weighting 30 to 35 g were used in this study. The animals were purchased from Neuroscience Research Center of Kerman, Iran, and kept in the Center for Laboratory Animal Care at the Veterinary Faculty of Shahid Bahonar University of Kerman, Iran for one week before treatment. The mice were fed with standard commercial laboratory chow [(pellet form), Javaneh Khorasan Co., Mashhad, Iran] and water *ad libitum* and housed under standard laboratory conditions (12 hr light: 12 hr dark and 22 ± 2 °C) during the experimental period. All the investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals. The experimental protocol was approved by the Ethics Committee of Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Iran.

Study design

Male mice were randomly assigned to either control or treatment groups with fifteen mice in each group. To monitor the effects of leptin on testicular

tissue changes, intraperitoneal (IP) injection of leptin (0.1 µg/100 µl of physiological saline) to the treatment groups (30) or 100 µl physiological saline to the control group was applied once a day for 30 consecutive days. Mouse leptin (Code No., L3772) was purchased from Sigma-Aldrich Co. LLC, USA. At 15, 30 and 60 days after the beginning of the treatments, 5 cases of 15 animals from each experimental group were anaesthetized by diethyl ether (May and Baker Ltd., Dagenham, England) and then sacrificed by cervical dislocation. Their left testes were removed, weighted and then used for histopathological evaluations.

Histopathological procedures

All tissue specimens of testes were fixed in 10% buffered formalin solution, embedded in paraffin wax, sectioned with 5 µm thicknesses, stained with haematoxylin and eosin (H&E) and studied by an ordinary light microscope.

Morphometrical assays

Spermatogenesis (SP) was determined by the semi-quantitative (Johnsen's score, Table 1) method in 100 cross-sections in each case at the same magnification and was summed up as mean Johnsen's score (JS) (31), and quantitative method in which two hundred seminiferous tubules were examined under light microscopy. In quantitative method, the presence of spermatozoa within the seminiferous tubule was considered as the evidence of SP. Lack of the spermatozoa even in the presence of orderly progression of primary and secondary spermatocytes was not considered as the evidence of SP for the purpose of this experimental study (32). The seminiferous tubule diameter (STD) and epithelial height (EH) were measured in each testis for evaluation of morphometrical assays. The ten smallest, roundest tubules were selected for each animal per experimental group, and the epithelium height and diameter of tubules were measured with an ocular micrometer under light microscopy. The average diameter of spermatogonia cells and sertoli

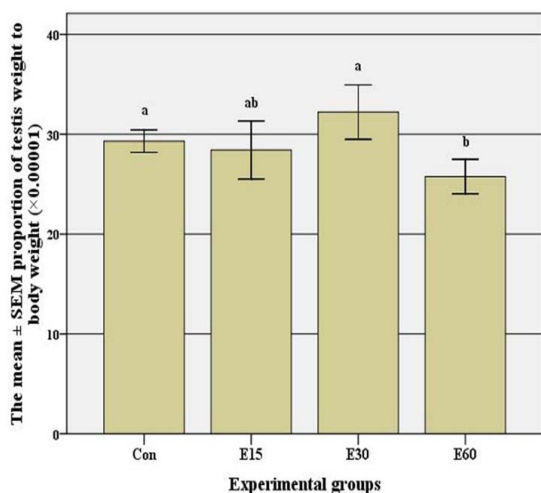


Figure 1. The effects of long-term leptin administration on the proportion of testis weight to body weight. At each column, different alphabets shows significant difference between each experimental groups ($P<0.05$). Con, control; E15, experimental group in day 15; E30, experimental group in day 30; E60, experimental group in day 60

cells nuclei were measured from 10 cells for each testis. The number of round spermatids for each pachytene primary spermatocytes (meiotic index) was also calculated for determination of cell loss percentage during cell division (33).

Statistical analysis

The results were subjected to analysis by SPSS17.0 (SPSS Inc., Chicago, IL, USA) package. Evaluation of significant difference between the means of different experimental groups was performed with using one-way analysis of variance (one-way ANOVA) followed by Tukey's test as *post hoc* for multiple comparisons. Values were expressed as mean \pm SEM. The significance considered level was $P<0.05$.

Results

Testis weight

The testis weight was corrected according to the body weight for obtaining reliable results. The mean proportion of testis weight to body weight among the control and leptin treated animals at different days was compared and presented in Figure 1. It was observed that administration of leptin caused a significant decrease in the mean proportion of testis weight to body weight following 60 days compared to the control group ($P=0.016$). Instead, there was no significant difference after 15 and 30 days of leptin injection with the control group ($P>0.05$).

Morphometrical evaluations

The mean of some morphometrical parameters including SP, meiotic index (MI), EH, STD, sertoli cell nucleus diameter (SeND) and spermatogonia cell

nucleus diameter (SpND), which are explained below were evaluated.

Spermatogenesis (semi-quantitative and quantitative methods)

Estimation of spermatogenesis by application of semi-quantitative method of the JS revealed the impairment of spermatozoa in the treatment group at the three different evaluated days in comparison with the control group ($P<0.05$). Similar finding was observed in quantitative method in which the percentage of spermatozoa was evaluated in seminiferous tubules. Significant difference was observed between the percentage of spermatogenesis of the control group and leptin treated animals at different days (15, 30 and 60 days), and long-term administration of leptin could not have impact on the percentage of spermatogenesis among the evaluated time intervals (88 ± 0.86 , 87 ± 0.54 and 86 ± 0.81 , $P>0.05$).

Meiotic index

During the first 15 days following leptin administration, a significant decrease was observed in the MI in comparison with the control group (3.23 ± 0.03 vs. 3.53 ± 0.01 , $P=0.0001$). Although there was not any significant difference between 30th and 60th day after administration of leptin (3.09 ± 0.03 and 3.01 ± 0.02 , $P=0.297$); nevertheless, both of the above mentioned days had significant difference with day 15.

Epithelial height and seminiferous tubules diameter

Leptin administration caused a significant reduction in STD after 15 days compared to the control group (219.8 ± 2.29 vs. 272.8 ± 2.85 , $P=0.0001$), but it did not have any more deleterious effect on STD at 30 and 60 days. Likewise, a similar findings was observed for the EH after 15 days of leptin administration compared to the control group (75.10 ± 0.94 vs. 92.17 ± 0.83 , $P=0.0001$). Also, long-term administration of leptin caused a significant decrease in the EH at the 60th day in comparison with the days 15 and 30 (69 ± 1.17 vs. 75.10 ± 0.94 , $P=0.0001$ and 74.40 ± 0.75 , $P=0.002$).

Sertoli and spermatogonia cell nucleus diameter

A significant decrease in SeND was observed after 30 days of leptin administration in comparison with the control group (10.39 ± 0.39 vs. 12.09 ± 0.19 , $P=0.0001$), which did not have difference with day 60 (10.39 ± 0.23 vs. 9.8 ± 0.26 , $P=0.683$). SpND significantly decrease after 15 days of leptin administration in comparison with the control group (6.08 ± 0.14 vs. 6.49 ± 0.09 , $P=0.041$). Also further damage was observed at 30th day compare to the day 15 following leptin administration, which did not have any significant difference with day 60 after administration of leptin.

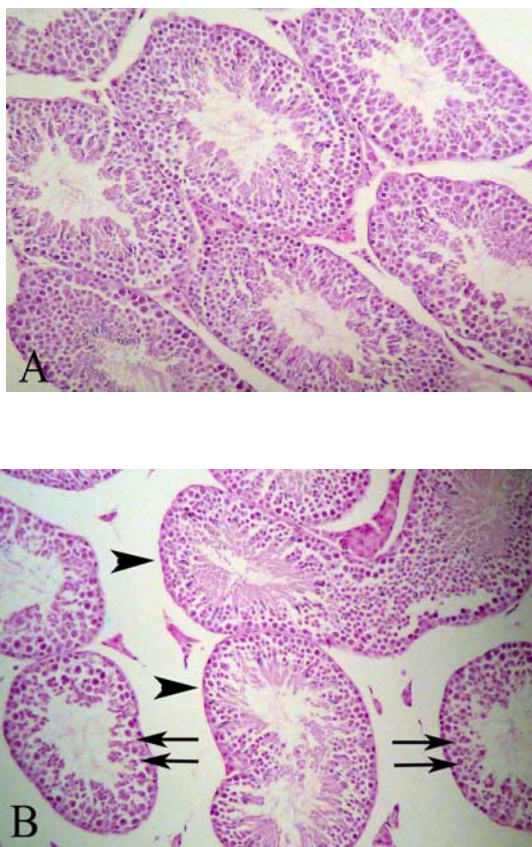


Figure 2. Testicular section stained with haematoxylin and eosin ($\times 100$). A: Control group. Photomicrograph showing normal seminiferous tubules morphology. B: The seminiferous tubules epithelium at 60th day of the beginning of leptin administration (E60 group). Photomicrograph showing two degenerated (black arrows) and two normal (black arrow heads) seminiferous tubules

Discussion

Leptin is a relative newcomer on the field of hormones, and it is one of the most interesting hormones that can assist weight loss. It is a 16 kDa adipocyte-derived hormone that suppresses food intake, stimulates energy expenditure, elevates metabolic rate, and eventually leads to loss of body fat (34, 35). Usually, researches on administration of leptin to obese subjects for weight reduction is over a long period of time (36, 37), meanwhile we have investigated the effects of leptin administration on testes of mice in a long-term experimental research. The results of our experiment demonstrated that long-term administration of leptin is adversely able to affect mouse testicular structure. Our observation is in agreement with the previous researches (28, 38), in which they reported a decrease in sperm count and increase in the fraction of abnormal spermatozoa in adult rats. Although, leptin was originally thought to act indirectly via the central nervous system, recent studies have revealed that leptin exerts its effect through direct actions on target tissues. Expression of the obese receptor gene

results in several alternatively spliced isoforms that take part in the extracellular domain but vary in the length of their transmembrane and cytoplasmic coding regions (15, 39). The hypothalamus is supposed the only tissue expressing predominantly the full length of obese gene receptor. However, the long form and several short isoforms are observed in the number of peripheral tissues including liver, heart, kidneys, lungs, spleen, small intestine, and pancreas (19). Moreover, based on the characterization of leptin receptor distribution and expression on *in vitro* systems, additional sites for leptin action including the male gonad have been suggested (40, 41). Indeed, evidences indicate that leptin is able to inhibit testosterone secretion at the testicular level (42, 43). Hence, a previous study in mice indicated that testicular function and synthesis of testosterone increase after administration of leptin (44). Also, leptin is able to cross the testis-blood barrier suggesting that both testicular and peripheral sources of leptin may participate in reproduction (45). Thus, as serum leptin levels increase, intratesticular leptin levels would be expected to increase with leptin action limited by receptor expression in the testis. Together, these results strongly demonstrate that leptin directly modulates testicular functions (11).

Our data in the present study showed that JS and MI, which show the characteristic of spermatogenesis process were significantly disturbed following 15 days of leptin administration. In contrast, previous reports elucidated that leptin acts as a positive regulator of spermatogenesis. Leptin signaling via STAT3 suggests a role in the proliferation of undifferentiated germ cells (20). Leptin STAT3 signaling may enable undifferentiated germ cells to replicate without loss of potency, while stimulating later-stage spermatocytes to sustain development and differentiation (20, 46). Probable reason for controversial observations between previous reports and our study may be due to down-regulation of the leptin receptor during long-term exposure against exogenous leptin. In obese males, serum leptin levels are elevated, which may lead to down-regulation of testicular leptin receptors (47). Down-regulation of leptin receptor expression would disturb autocrine/paracrine testicular signaling and possibly spermatogenesis. Moreover, leptin receptors have been found in human and rodent Sertoli cells, Leydig cells, seminiferous tubules and germ cells (46, 48), which their down-regulation may disturb physiological actions of these cells similar to above mentioned phenomenon about spermatogenesis. This hypothesis could explain our observation about deleterious effects of leptin on STD, SeND and SpND following 15 days of administration compared to the control group (Table 2).

Table 2. The Mean±SEM of johnsen's score, mieotic index, spermatogenesis, epithelial height, seminiferous tubules diameter, sertoli cell nucleus diameter and spermatogonia cell nucleus diameter following administration of leptin for 60 days in mice

Parameters (Scale)	Con (n=15)	Leptin (n=15)		
		E15	E30	E60
JS (Count)	8.56 ± 0.08 ^a	7.28 ± 0.16 ^b	7.26 ± 0.15 ^b	6.78 ± 0.13 ^b
MI (Index)	3.53 ± 0.01 ^a	3.23 ± 0.03 ^b	3.09 ± 0.03 ^c	3.01 ± 0.02 ^c
SP (%)	91.33 ± 0.66 ^a	88.00 ± 0.86 ^b	87.00 ± 0.54 ^b	86.00 ± 0.81 ^b
EH (µm)	92.17 ± 0.83 ^a	75.10 ± 0.94 ^b	74.40 ± 0.75 ^b	69.00 ± 1.17 ^c
STD (µm)	272.8 ± 2.85 ^a	219.8 ± 2.29 ^b	227 ± 2.87 ^b	227.6 ± 2.61 ^b
SeND (µm)	12.09 ± 0.19 ^a	11.44 ± 0.26 ^a	10.39 ± 0.23 ^b	9.80 ± 0.26 ^b
SpND (µm)	6.49 ± 0.09 ^a	6.08 ± 0.14 ^b	5.50 ± 0.09 ^c	5.14 ± 0.11 ^c

JS, johnsen score (Testicular biopsy score count); MI, mieotic index; SP, spermatogenesis; EH, epithelial height; STD, seminiferous tubules diameter; SeND, sertoli cell nucleus diameter; SpND, spermatogonia cell nucleus diameter; Con, control; E, Experiment

^{a,b,c} Different alphabetic letters, show significant difference ($P < 0.05$) between the experimental groups for each individual day (15, 30 and 60)

The other explanation about the adverse effects of long-term administration of leptin observed in the present study could be the negative effects of leptin on regulation of testicular steroidogenesis (49). In response to luteinizing hormone (LH), Leydig cells activate protein kinase A (PKA)-dependent gene expression, which triggers the production of testosterone (44, 50). Testosterone is a key hormone for sertoli cells and maintain spermatogenic function (51). Previous reports have shown that leptin prevents division of prepubertal Leydig cells (52) and suppresses human chorionic gonadotropin (hCG)-stimulated testosterone secretion in rat Leydig cell culture; therefore, supports a role for leptin in the negative regulation of steroidogenesis (40, 42). Thus, gonadotropins and sex-steroid hormones are low in obese mice, and this is consistent with the described role for leptin in the regulation of the hypothalamic-pituitary-testis axis (11, 53). Indirectly, leptin regulates the secretion of gonadotropin from hypothalamus by adjusting kisspeptins in the arcuate nucleus (54). Kisspeptins are proteins encoded by the *Kiss 1* gene and transcribed as *KiSS1* mRNA that exert their role via a G-protein-coupled receptor to stimulate GnRH release, thereby triggering the gonadotropin cascade (55).

Conclusion

Our results indicate that long-term administration of leptin is able to decrease male fertility by affecting germ cells and thereby spermatogenesis process.

Acknowledgment

The results described in this paper were part of student thesis. This study was financially supported by a grant for research works by Vice Chancellor of Research of Shahid Bahonar University of Kerman, Kerman, Iran.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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