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Full Length Research Paper

Effect of obestatin on morphometry of testes and testosterone secretion in male rats

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This study was designed to evaluate the effects of chronic intra peritoneal administration of obestatin on plasma testosterone concentrations and cellular morphometry of the testes in male Sprague Dawly rats. The treatment groups were injected with obestatin (1 nmol/100 µl saline i.p), while the control groups received saline (100 µl i.p) for ten consecutive days. Blood samples were collected at day 1 and 10 during the dose administration and day 5 and 15 after the dose administration. All the samples were collected at 10:00 a.m. Testes were removed after sacrificing the rats on days 5 and 15 after the last injection. Plasma testosterone concentrations were found significantly high ($p < 0.05$) in the obestatin treated groups when compared with the control groups. Testicular histomorphometry revealed that, obestatin treatment caused a significant increase in the primary spermatocytes ($P < 0.0001$), secondary spermatocytes and spermatids ($P < 0.005$) and leydig cells population ($p < 0.0001$) both after 5 and 15 days. These findings indicated that obestatin can be a stimulator of testicular functions.

Key words: Obestatin, male reproduction, testis, rats.

INTRODUCTION

Various peptide hormones are secreted from the gut that enter the circulation and regulates food intake, energy balance and gastric motility (Tache and Perdue, 2004; Young, 2005; Baynes et al., 2006; Cummings and Overduin, 2007). The oxyntic mucosa cells secrete two peptide hormones, ghrelin and obestatin which are considered as functional antagonists of each other. Ghrelin increase food intake, while obestatin reduce food intake (Zhang et al., 2005; Gualillo et al., 2006).

G-protein coupled receptor GHS-R1a is considered as a ghrelin receptor (McKee et al., 1997). Another G-protein coupled receptor GPR39 which belongs to the ghrelin receptor family is considered as a receptor of obestatin (Zhang et al., 2005). In rodents, obestatin expression was reported in various tissues including gastric mucosa, myentric plexus, perinatal pancreas as well as in the leydig cells of the testis (Zhang et al., 2005; Chanonie et al., 2006; Dun et al., 2006). However, some studies indicate that obestatin is not the natural ligand for GPR39 but it is regulated by Zinc ions (Yasuda et al., 2007). It was reported that, obestatin cannot pass the blood brain barrier; however, its expression has been reported in the brain. It was thus postulated that, obestatin might reach the brain through the median eminence (Pan et al., 2006; Jackson et al., 2006).

Obestatin exerts no effect on growth hormone, corticosterone and leptin secretion (Zhang et al., 2005; Bresciani et al., 2006; Nogueiras et al., 2007). However, it was reported that 24 h fasting caused a decrease in obestatin (Zizzari et al., 2007).

Data regarding the involvement of obestatin in the reproductive functions is still lacking however, in a recent study it was found that obestatin might be involved in the regulation of ovarian granulosa cells functions. It was reported that obestatin significantly increase progesterone secretion and induce apoptosis in cultured porcine ovarian granulosa cells (Meszarosova et al., 2008). Our previous study showed that, single i.v injection of obestatin increased testosterone secretion in adult male rats (Jahan et al., 2010). In the back drop of these findings, this study was designed to investigate the chronic effect of obestatin on testosterone production and testicular cell proliferation in male Sprague Dawley rats.

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MATERIALS AND METHODS

Animals

The male Sprague-Dawley rats used for this study were obtained from the rat colony maintained at the animal house in Quaid-i-Azam University Islamabad, Pakistan. The day the litter was born was considered as the day first of postnatal life. All the animals were kept at 14 h light/10 h dark period having free access to pelleted food and tap water was available ad libitum. Animals were restrained for one week prior to sample collection. All the animal handling and scarifications were approved by the Department of Animal Sciences Quaid-i-Azam University, Islamabad, Pakistan.

Experimental design

20 pubertal (45 days old) male rats weighing 105±4.53 g were allocated for the study. Two groups (n = 5 animals/group) of the animals were assigned as the treatment groups and two groups (n = 5 animals/group) served as the control. The treated groups received 1 nmol obestatin/100 µl saline i.p daily in the morning for 10 consecutive days, whereas the control animals received 100 µl of saline.

Blood collection

Blood samples (0.5 ml) were collected at different day intervals that is, the 1st and 10th day during the dose administration and on the 5th and 15th day after the last injection of obestatin. Blood samples on the 1st day and 10th day were withdrawn from the tail vein through cannula (Omaye et al., 1987; Staszyk et al., 2002), while on the 5th and 15th day of the treatment blood was collected at the time of scarifications in heparinized eppendorf tubes. Blood was immediately centrifuged at 3000 rpm for 10 min at 4°C. Plasma was separated and stored at -20°C until assay.

Reagents

These included heparin (Sinochem Ningbo Imp and Export.Corp Ningbo People's Republic of China) diethyl ether (Sigma-Aldrich, USA) and rat obestatin (5 mg, catalog no: PGH-3891-P1, molecular weight: 2517.9 a.m.u AnaSpec, USA).

Testosterone assay

Plasma testosterone was quantitatively determined using enzyme immuno assay kits (Amgenix International. Inc. USA) according to the manufacturer's instructions.

Animal sacrification

Animals were weighed and then killed by diethyl ether anesthezia on the 5th and 15th day after they received the last injection. Testes were surgically removed by incising the scrotal sac. The collected whole testes were weighed. Tissues were then immersed in fixative sera. Following dehydration in the descending and ascending grades of ethyl alcohol, tissues were clarified in cedar wood oil and then were embedded in paraffin. The 5 µm thick sections were cut out of paraffin block by using Reichert Microtome. Sections were then affixed to pre-cleaned albuminized glass slides and stretched at 60°C on fisher slide warmer.

Histology and cytometry

Hematoxylin and Eosin (H and E) staining was carried out and the slides were then examined under a Nikon optishot research microscope equipped with an automatic micro photographic system. Histomorphometry was carried out by counting 25 seminiferous tubules in each slide and the mean number and nuclear diameter of different cell types in one tubule in the control and the treated groups was reported.

Statistical analysis

For each animal five slides and in each slide, 25 seminiferous tubules were observed under optiphot research microscope equipped with an automatic microphotographic system (Leica, Germany). All values were presented as mean ± SEM. Student's "t" test was employed to compare the treatment groups with the corresponding control groups. Values were considered to be statistically significant at $p < 0.05$.

RESULTS

Body weight

The mean ± SEM body weights of the control and treated groups were 152.2 ± 4.83 and 154.0 ± 5.57 g on day 5, and were 213.0 ± 6.92 and 210 ± 6.97 on day 15 after the last treatment, respectively. No significant difference in the body weight of the control and the treated groups of animals was noticed.

Plasma testosterone concentrations

Mean ± SEM plasma testosterone concentrations of both the control and treated groups are presented in Figure 1. In the control and treated groups animals, no significant difference in plasma testosterone concentrations was observed before the obestatin administration. The plasma testosterone concentrations were 0.35 ± 0.07 and 0.47 ± 0.06 ng/ml in the control and in the treated groups' animals, respectively. In the treated group, after ten days of obestatin treatment, the testosterone concentrations (1.04 ± 0.1 ng/ml) were significantly high ($p < 0.05$) when compared with the control groups (0.78 ± 0.09 ng/ml). The mean testosterone concentrations on the 5th day of the treatment were 2.93 ± 0.26 ng/ml in the obestatin treated group and 2.08 ± 0.21 ng/ml in the saline treated group. On the 15th day after the last dose was administered, the mean testosterone concentrations in the obestatin treated group (3.27 ± 0.15 ng/ml) were significantly high ($p < 0.05$) when compared with the control group (1.97 ± 0.32 ng/ml).

Morphometrical analysis

The different cell counts are presented in Table 1. In the

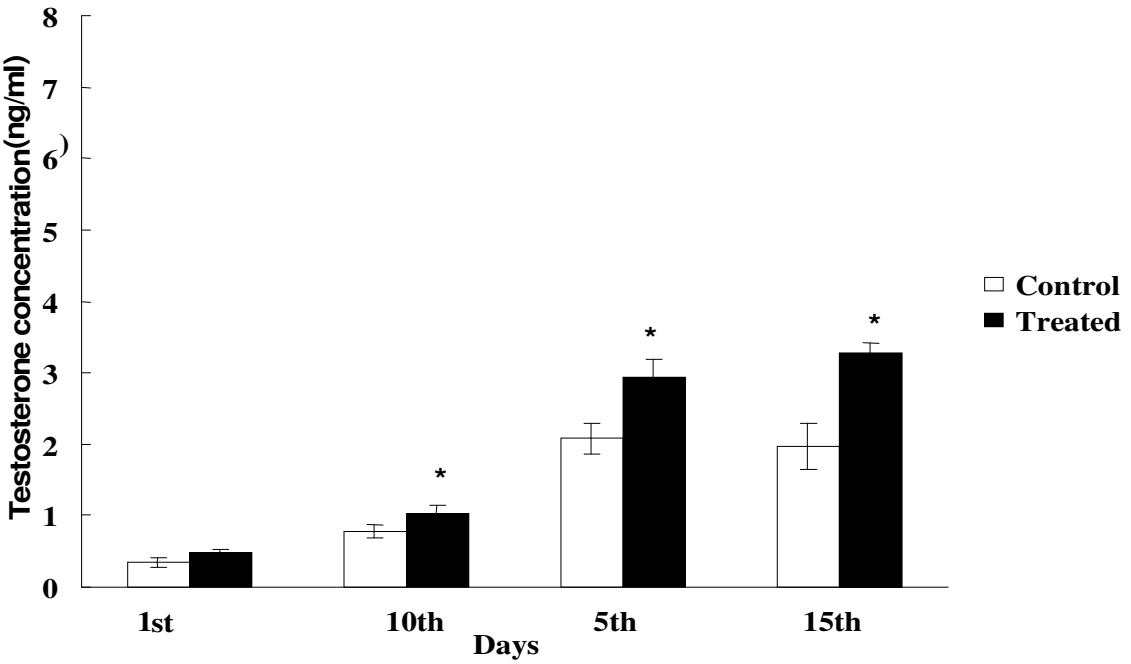

Figure 1. Mean ± SEM testosterone concentrations in the control and treated animals on different time intervals.* p < 0.05 value versus corresponding control group (student's t test).

Table 1. Numbers of different cell types in the testicular sections of each seminiferous tubule per field (mm^2) in the treated and control animals (Mean ± S.E.M).

Cell type	Day 5		Day 15	
	Control (n=5)	Treated (n=5)	Control (n=5)	Treated (n=5)
Spermatogonia	41.7 ± 1.4	43.4 ± 1.2	35.3 ± 0.9	38.0 ± 1.6
P. Spermatocytes	31.3 ± 1.4	39.6 ± 1.3***	28.3 ± 1.4	37.2 ± 1.1***
S. Spermatocytes	17.9 ± 0.8	22.5 ± 1.0**	17.6 ± 0.9	21.1 ± 1.0**
Spermatids	32 ± 1.6	45.3 ± 2.7***	30.6 ± 1.6	41.5 ± 2.5**
Sertoli cells	26.3 ± 1.7	28.3 ± 1.9	25.9 ± 1.7	27.0 ± 1.4
Leydig cells	7.6 ± 0.4	11.2 ± 0.3***	7.2 ± 0.3	10.4 ± 0.2***

Values (Mean ± SEM). *p<0.05; **p<0.005; ***p<0.0001 (student's t test).

obestatin treated animals, the mean number of spermatogonia was higher when compared with the control animals, but this increase was not statistically significant. However, the mean number of primary spermatocytes was significantly higher at different experimental days in the obestatin treated animals when compared with the control animals (P < 0.0001). The mean number of secondary spermatocytes was also significantly high in the obestatin treated animals on both the 5th and 15th day (P < 0.005). Mean number of round and elongated spermatids present in the seminiferous tubule were significantly (P < 0.005) higher in both the treatment groups dissected on different experimental days when compared with the corresponding control groups. Similarly, the mean number of leydig cells were significantly (p < 0.0001) higher in the treated groups when compared with the control groups, but the mean number of Sertoli cells present in the seminiferous tubule was not significantly high in the different treated groups when compared with the control groups on the different experimental days (Figures 2 and 3).

Mean ± SEM testicular tunica albuginea thickness, seminiferous tubule epithelial height and seminiferous tubule diameter of the control and obestatin treated groups for the different experimental days are presented in Table 2. There was no significant difference found in the testicular tunica albuginea thickness, seminiferous tubule epithelial height and seminiferous tubule diameter of the treated groups when compared with the control groups.

Mean ± SEM nuclear diameter of spermatogonia, primary spermatocytes, secondary spermatocytes, Sertoli

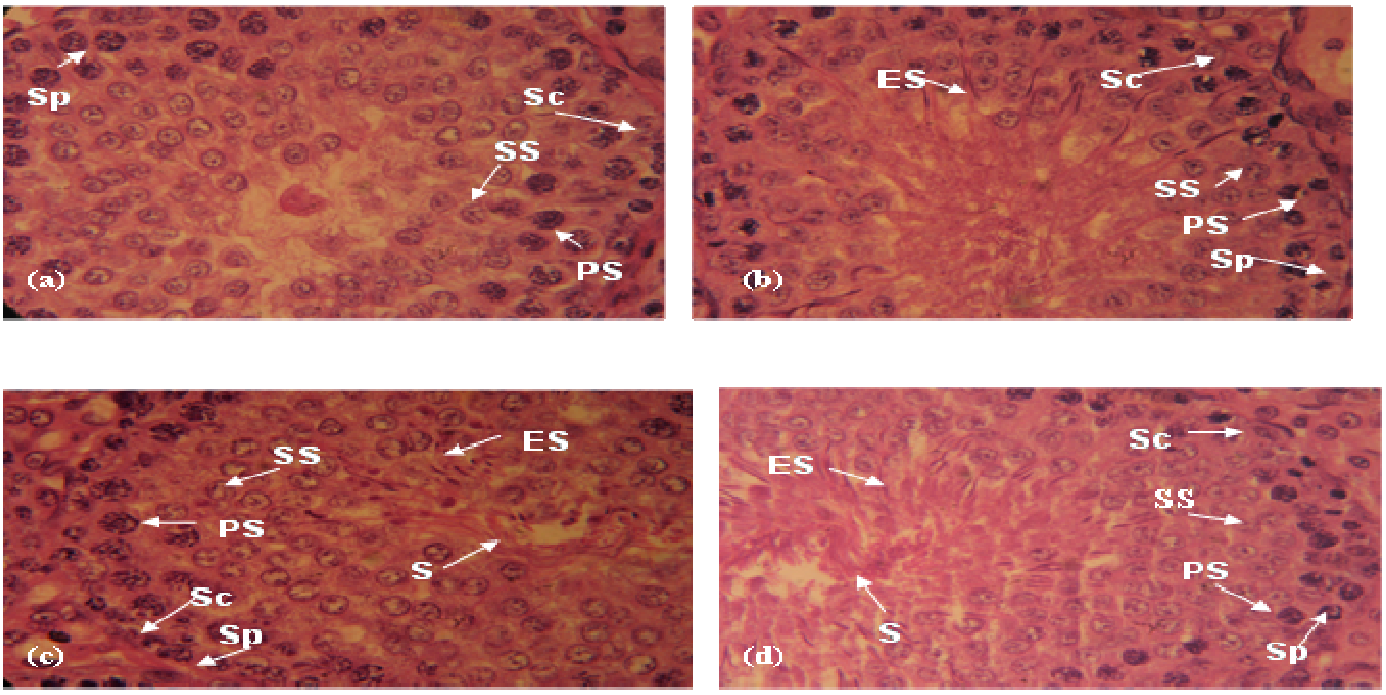

Figure 2. Photomicrographs of testes from the control group. (a) Control (day 5); (b) treated (day 5); (c) control (day 15); (d) treated (day 15). Sp, Spermatogonium; PS, primary spermatocytes; SS, secondary spermatocytes; ES, elongated spermatids; S, spermatozoon; Sc, sertoli cells. 100X, H and E.

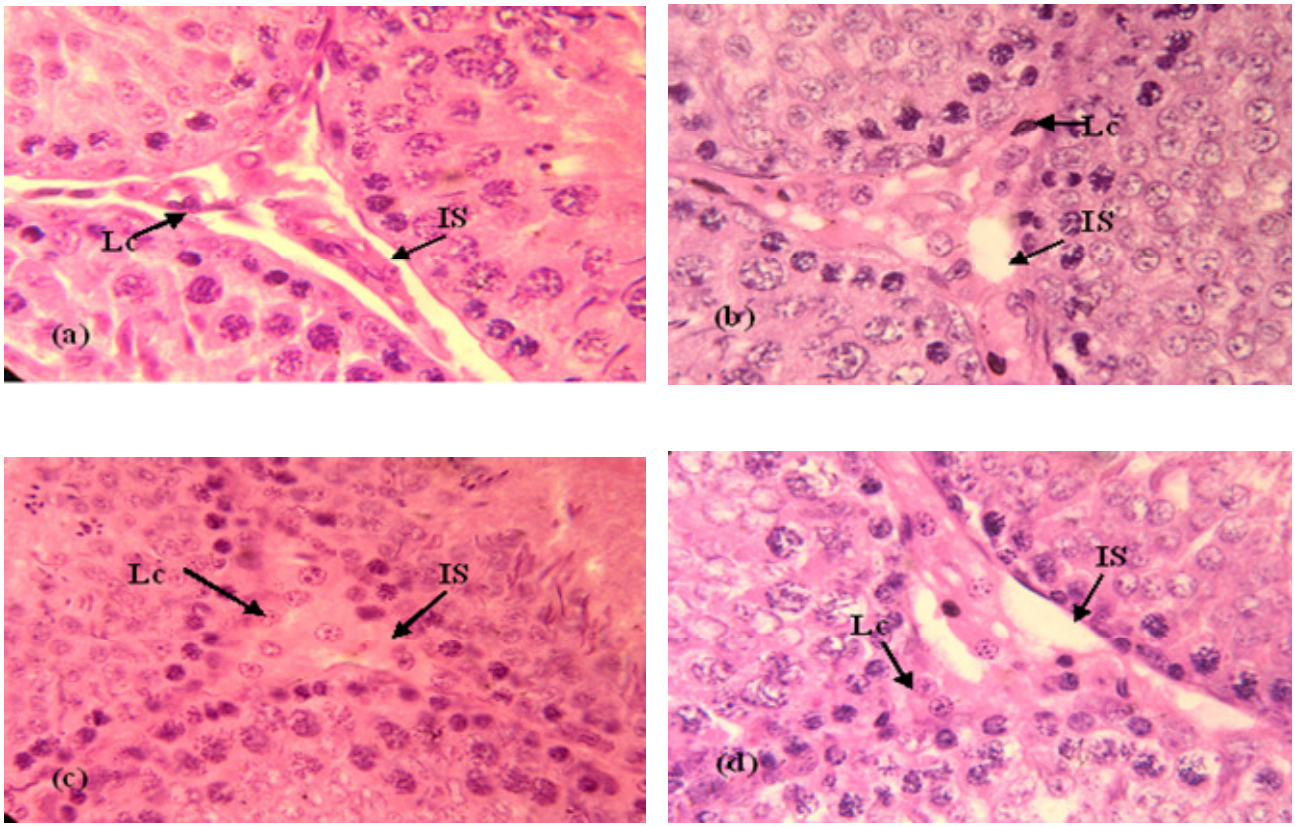

Figure 3. Photomicrographs of adult rats testes showing the interstitial spaces (IS) between seminiferous tubules and leydig cells (Lc). (a) Control (day 5); (b) treated (day 5); (c) control (day 15); (d) treated (day 15) groups; X100, H and E.

Table 2. Tunica albugenia albuginea thickness, seminiferous tubule height and diameter (μm) in the treated and control animals (Mean ± S.E.M).

Parameter	Day 5		Day 15	
	Control (n=5)	Treated (n=5)	Control (n=5)	Treated (n=5)
Tunica albugenia thickness	29.9 ± 0.8	29.9 ± 1.0	30.8 ± 0.9	30.9 ± 0.9
Seminiferous epithelial height	63.5 ± 1.4	63.4 ± 1.3	69.2 ± 2.2	69.1 ± 1.7
Seminiferous tubule diameter	190.2 ± 2.6	190.3 ± 2.8	192.0 ± 1.9	192.3 ± 1.8

Table 3. Nuclear diameters (μm) of the different cell types in the seminiferous tubules in the treated and control animals (Mean ± S.E.M).

Cell type	Day 5		Day 15	
	Control (n=5)	Treated (n=5)	Control (n=5)	Treated (n=5)
Spermatogonia	5.0 ± 0.1	5.3 ± 0.15	4.0 ± 0.1	5.7 ± 0.1
P. Spermatocytes	6.8 ± 0.1	6.7 ± 0.1	7.0 ± 0.2	7.2 ± 0.2
S. Spermatocytes	5.8 ± 0.2	5.8 ± 0.2	6.6 ± 0.4	6.7 ± 0.4
Spermatids	6.4 ± 0.2	6.6 ± 0.2	7.3 ± 0.2	7.4 ± 0.1
Sertoli cells	5.6 ± 0.3	5.7 ± 0.4	6.0 ± 0.5	6.0 ± 0.2
Leydig cells	5.4 ± 0.2	5.4 ± 0.1	5.7 ± 0.1	5.8 ± 0.2

Values (Mean ± SEM).

cells and leydig cells of the control and obestatin treated groups on the different experimental days are presented in Table 3. No significant changes in mean ± SEM spermatogonial, primary spermatocyte, secondary spermatocyte, sertoli cells and leydig cells of nuclear diameters of testicular cells were observed in the treated groups when compared with the control groups both at day five and day 15 (Figure 2).

DISCUSSION

Obestatin is a recently identified gut peptide which binds to a G-protein coupled receptor known as GPR39. Obestatin is also the functional antagonist of ghrelin (Zhang et al., 2005; Nogueiras et al., 2007). Obestatin expression has been reported in the leydig cells of testes (Dun et al., 2006); however, the specific role of obestatin in the regulation of reproductive functions is still unknown.

The testis is a complex endocrine organ where different cell types interplay to ensure male fertility, under the control of a plethora of endocrine, paracrine and autocrine regulatory signals (Saez, 1994). In recent years, it has become evident that different factors with key roles in the growth axis (GHRH and IGF-I) and body weight homeostasis (leptin) are potentially involved in the regulation of testicular function (Ciampani et al., 1992; Baker et al., 1996; Tena-Sempere et al., 1999, 2001).

In the study, 45-days-old male rats were allocated for the experiment because this age is considered as the normal timing of puberty occurrence in the male rats (Ojeda and Urbanski, 1994). Infusions (i.p) of obestatin for 10 days was started when the rats were 45 days old and were dissected on the day 5th (60-days-old) and 15th (70-days-old) day after treatment. The first spermatozoon is usually seen in the lumen of semniferous tubules at 45th day of age (Clermont and Perey, 1957) and mature spermatozoon is seen in the vas deferens on the 13th to 14th day later at the age of about 60 days (Clegj et al., 1960). The proliferation rate of the testicular cells is higher in this particular time, so for this reason, the curent experiment was conducted on this phase of development in the male rat.

In this study, chronic infusion (i.p) of obestatin during the pubertal stage, significantly increased the testosterone level in the adult male rats and that elevated testosterone level might have directly enhanced the spermatogenesis as early as observed in majority of the seminiferous tubule in the treated animals when compared with the control animals. Obestatin treatment also caused marked increase in the number of various testicular cells in the seminiferous tubules. This finding shows that, obestatin had a stimulatory role in the modulation of cellular proliferation during the peripubertal period in male rats. This finding also suggests a potential opposing mode of action of obestatin when compared with ghrelin, as it was also previously reported that, ghrelin is a negative modulator of the male reproductive system (Kheradmand et al., 2009). Obestatin treatment for 10 consecutive days failed to cause any significant increase in the number of spermatogonia and

sertoli cell in the seminiferous tubule of the male rats. Yet no data regarding the effect of obestatin on testicular level was reported.

The observations provided evidence for an involvement of obestatin in enhancing the testosterone production from leydig cells. It is either that this increase in the testosterone secretion is the direct result of the binding of obestatin to receptor (GPR 39) which is present in testes (Yammamoto et al., 2007) or it is that obestatin could have enhanced the responsiveness of the leydig cells towards pituitary LH. Further studies are needed to sort out the exact mechanism through which obestatin enhance the testicular testosterone synthesis and the way through which it induce spermatogenesis in male rats testes.

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