

*Full Length Research Paper*

# Obestatin induces testosterone secretion from rat testis *in vitro*

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**In this study, the effect of obestatin (23 amino acid peptide) on testosterone secretion *in vitro*, in the rat testis was observed. For this purpose, two different doses of obestatin ( $10^{-9}$  M and  $10^{-8}$  M) were used alone and in combination with human chorionic gonadotropin (hCG) in fasting and fed conditions in two age groups. Fasting induced a significant reduction in body weight ( $p < 0.05$ ) and plasma testosterone concentrations (0.001). hCG stimulated testosterone secretions were significantly ( $p < 0.05$ ) high as compared to the basal control testosterone concentrations after 90 min in some groups and 180 min of incubation in all groups. Obestatin at the dose of  $10^{-9}$  M alone and in combination with hCG failed to change testosterone concentrations in all groups; however,  $10^{-8}$  M obestatin significantly ( $p < 0.05$ ) induced hCG stimulated testosterone concentrations in both normally fed pre-pubertal and adult rats. No significant difference was noticed in 48 h fasted groups. This data suggests that, obestatin is a positive modulator of testosterone secretion and its effect depends upon the nutritional status of the body.**

**Key words:** Obestatin, testosterone, rats, *in vitro*.

## INTRODUCTION

Obestatin, isolated from the oxyntic mucosa of the stomach is a 23-amino acid anorexigenic peptide, produced by the enzymatic cleavage of the proghrelin (Kojima et al., 1999; Bednarek et al., 2000). The name 'obestatin' was given because of its appetite-suppressing potential (Zhang et al., 2005).

Experiments using labeled obestatin revealed that obestatin binds jejunum, ileum, stomach, pituitary and hypothalamus with high affinity. There is debate on receptor of obestatin but it is believed that GPR39 is the binding site for it (Zhang et al., 2005). GPR39 belongs to the class A-7 transmembrane (TM) domain G-protein-coupled receptors (GPCRs), which is the ghrelin receptor sub family (Mckee et al., 1997; Kojima et al., 1999). GPR39 is found in various tissues including jejunum, ileum, stomach, pituitary and hypothalamus and testes. GPR39 was tested for its potency to bind obestatin in competition with other hormones ghrelin, motilin, neurotensin and neuromedin U. It was found that; obestatin

as high affinity to bind GPR39 more than the other brain/gut hormones. In addition, it was also found that, obestatin stimulate cyclic adenosine monophosphate (cAMP) formation in the Chinese hamster ovary cells, which has high expression of GPR39 when compared with the other peptides (Zhang et al., 2005).

It was reported that, both ghrelin and obestatin are produced from the same precursor molecule, their role in the body functioning regulation was studied for the reason that, whether these peptides have the same properties or they are opposite in their actions. On the basis of these studies, it was found that obestatin and ghrelin are functional antagonists of each other in the sense that ghrelin facilitate food intake, while obestatin suppress food intake (Guo et al., 2007). Similarly, when these two peptides were administered together in rodents, no effect on GH secretion was observed. Ghrelin increased GH secretion but when co administered with obestatin the ghrelin effect was suppressed (Zizzari et al., 2007). The obestatin immunoreactivity has been found in the gastric mucosa, perinatal pancreas, myentric plexus and Leydig cells of the testis (Zhang et al., 2005; Chanonie et al., 2006; Dun et al., 2006). In the previous experiments in the laboratory, it was observed that i.v. administration of

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obestatin increase testosterone production in adult male Sprague dawley rats (Jahan et al., 2010).

The main objective of the present study was to observe the direct effect of obestatin on testicular tissues in basal and human chorionic gonadotropin (hCG) stimulated testosterone secretion in fed and 48 h fasted pre-pubertal and adult male rats.

## MATERIALS AND METHODS

### Animals

Pre-pubertal (32 to 35 days) and adult (125 to 135 days) male Sprague dawley rats were used in this experiment. The day the litter was born was considered as day one. Animals were caged under standard conditions of light (12 h light/12 h dark) and temperature (22 to 25°C). Animals were provided with laboratory animal feed and tap water *ad libitum*. Body weights of pre-pubertal and adult animals were ranged between 97 to 110 and 265 to 283 g, respectively. Animals were kept in groups in which each group comprised of three animals per cage. Pre-pubertal and adult animals were divided into fasting groups and normally-fed groups. In the fasting animals, the feed was removed at 10:00 a.m. These animals only had access to water, while the normally-fed animals had free access to both laboratory feed and water. Adult group and the pre-pubertal group were equally fasted for 48 h.

### Chemicals

Rat/mice obestatin (MW of 2517.9 g) was purchased from AnaSpec U.S.A. Human chorionic gonadotropin (hCG) (Gonachore) and Pencillin and Streptomycin were purchased locally. DMEM/Ham F12 (1:1 ratio) was purchased from Hiclone, Thermo Scientific. Inc. U.S.A.

### Tissue incubation

Analysis of the direct effect of obestatin on testosterone secretion of these groups were carried out by using testicular slice incubation which was done as previously described by Tena-sempere et al. (1999) with slight modifications. Briefly, the testicular tissues were obtained from four different groups of animals; Group 1, Pre-pubertal normally fed animals; Group 2, pre-pubertal fasted animals; Group 3, adult normally fed animals; Group 4, adult fasted animals.

After 48 h of fasting, these animals were sacrificed by decapitation and blood was collected for plasma testosterone measurement. Testes were then, immediately removed from scrotal sac and decapsulated by the help of forceps. The whole tissue was cut into equal slices of approximately 100 mg  $\pm$  1.08 for pre-pubertal groups and adult groups. Testicular slices were incubated for 1 h in 1 ml Dulbecco's modified eagle's medium/Ham F12 (DMEM/Ham F12 1:1 ratio) containing 1.2 g/l sodium bicarbonate and supplemented with 50 IU/ml penicillin and 50  $\mu$ g/ml streptomycin in 10 ml culture tubes under 5% CO<sub>2</sub> and 95% air at 32°C. After incubation for 1 h, the media was replaced with fresh medium (acting as control) or medium with obestatin at the dose rate of 10<sup>-9</sup> M and 10<sup>-8</sup> M (acting as treated). In order to evaluate the effect of obestatin on human chorionic gonadotropin (hCG) induced testosterone secretion, the tissues were incubated with 10 IU hCG alone in the medium (acting as hCG control) or in combination with obestatin (10<sup>-9</sup> and 10<sup>-8</sup> M). hCG was used to check the viability of the Leydig cells to secrete testosterone in the culture conditions. All these treatments were

applied on the testicular slices obtained from the testis from each animal in each group. Aliquots of 100  $\mu$ l were then taken from each medium after 90 and 180 min of incubation for testosterone measurement. Aliquots were carefully placed in Eppendorf tubes and stored at -20°C until assay.

The blood obtained after decapitation was immediately centrifuged at 3000 rpm for 10 min at 4°C. Plasma was separated and was stored at -20°C until assay.

### Hormonal analysis

Testosterone was quantitatively determined by using enzymatic immunoassay (EIA) kits purchased from Amgenix.int.inc, USA.

### Statistical analysis

Values were expressed as mean  $\pm$  SEM. Paired t-test was employed to compare body weights and plasma testosterone concentrations of normally fed and 48 h fasted pre-pubertal and adult male rats. ANOVA followed by Tukey's test was employed to the testosterone concentrations in each group. This allows comparing the different dose response in each group when compared with their corresponding control groups. All the values are expressed as ng/ml.100 mg of tissue.

## RESULTS

Fasting induced a significant ( $p < 0.05$ ) reduction in body weight gain ( $p < 0.05$ ) and plasma testosterone concentrations ( $p < 0.01$ ) in both pre-pubertal and adult male Sprague dawley rats (Table 1).

### ***In vitro* effect of obestatin (10<sup>-9</sup> and 10<sup>-8</sup> M) alone or in combination with hCG (10 IU) on testosterone secretion in normally fed pre-pubertal male rats after 90 and 180 min of incubation.**

Testosterone concentrations were not significantly different in obestatin 10<sup>-9</sup> M and 10<sup>-8</sup> M treated groups when compared with the control group (0.01  $\pm$  0.20 versus 1.43  $\pm$  0.16 and 1.54  $\pm$  0.20 ng/ml.100 mg of tissue, respectively). After 180 min of incubation, testosterone concentrations in 10<sup>-9</sup> M obestatin treated group (4.22  $\pm$  0.19 ng/ml.100 mg of tissue) were non significantly high when compared with the control group (2.78  $\pm$  0.21 ng/ml.100 mg of tissue). Testosterone concentrations in 10<sup>-8</sup> M obestatin treated group (3.75  $\pm$  0.55 ng/ml.100 mg of tissue) were also not significantly high after 90 min of incubation when compared with the control group. In the control and hCG (10 IU) treated group, significant difference ( $p < 0.05$ ) was noticed in testosterone concentrations after 90 min (1.01  $\pm$  0.20 versus 1.54  $\pm$  0.20 ng/ml.100 mg of tissue) as well as after 180 min of incubation (2.78  $\pm$  0.21 versus 8.86  $\pm$  0.90 ng/ml.100 mg of tissue). Testosterone concentrations were not significantly different in obestatin (10<sup>-9</sup> M) plus hCG treated group (3.61  $\pm$  0.30 ng/ml.100 mg of

**Table 1.** Mean  $\pm$  SEM body weights and plasma testosterone concentrations in normally fed and 48 h fasted pre-pubertal (35 days old) and adult (125 days old) male Sprague dawley rats.

Parameter	Pre-pubertal		Adult	
	Fed	Fasted	Fed	Fasted
Body weight (g)	136.7 $\pm$ 4.4	112.7 $\pm$ 4.3*	315.0 $\pm$ 8.5	287.7 $\pm$ 3.9*
Plasma testosterone (ng/ml)	1.6 $\pm$ 0.3	0.3 $\pm$ 0.1**	5.6 $\pm$ 0.6	1.3 $\pm$ 0.4**

\* $p < 0.05$ ; \*\* $p < 0.01$  fasted versus corresponding fed group (paired-t test).

tissue) when compared with the hCG treated group (4.32  $\pm$  0.89 ng/ml.100 mg of tissue) post 90 min of incubation. Testosterone concentrations were significantly ( $p < 0.05$ ) high in the obestatin ( $10^{-8}$  M) plus hCG treated group when compared with hCG treated group (4.32  $\pm$  0.89 ng/ml.100 mg of tissue were in hCG control and 6.14  $\pm$  0.70 ng/ml.100 mg of tissue in obestatin ( $10^{-8}$  M) plus hCG treated group) (Figure 1).

After 180 min of incubation, the testosterone concentration in the control and hCG treated groups were 2.78  $\pm$  0.21 ng/ml.100 mg of tissue and 8.86  $\pm$  0.90 ng/ml 100 mg of tissue. A significant increase in testosterone concentrations ( $p < 0.05$ ) was observed between control and hCG treated group. In obestatin ( $10^{-9}$  M) plus hCG treated group, testosterone concentrations (10.98  $\pm$  1.70 ng/ml.100 mg of tissue) were not significantly different when compared with the hCG treated group (8.86  $\pm$  0.90 ng/ml.100 mg of tissue) after 180 min of incubation. However,  $10^{-8}$  M obestatin plus hCG caused a significant ( $p < 0.05$ ) increase in the testosterone concentrations (15.76  $\pm$  2.19 ng/ml.100 mg of tissue) when compared with hCG treated group (Figure 1).

#### ***In vitro* effect of obestatin ( $10^{-9}$ and $10^{-8}$ M) alone or in combination with hCG (10 IU) on testosterone secretion in 48 h fasted pre-pubertal male rats after 90 and 180 min of incubation.**

There was no significant difference between control (0.76  $\pm$  0.18 ng/ml.100 mg of tissue), obestatin  $10^{-9}$  M (1.43  $\pm$  0.31 ng/ml.100 mg of tissue) treated and  $10^{-8}$  M (0.89  $\pm$  0.55 ng/ml.100 mg of tissue) treated groups after 90 min of incubation. Similarly, no significant change in the testosterone concentrations was observed in obestatin ( $10^{-9}$  M) plus hCG treated group (1.03  $\pm$  0.44 ng/ml.100 mg of tissue) and obestatin ( $10^{-8}$  M) plus hCG treated group (3.34  $\pm$  1.23 ng/ml.100 mg of tissue) when compared with hCG treated group (1.43  $\pm$  0.31 ng/ml.100 mg of tissue) after 90 min of incubation. A non significant increase was noticed in testosterone concentrations between control and hCG treated groups

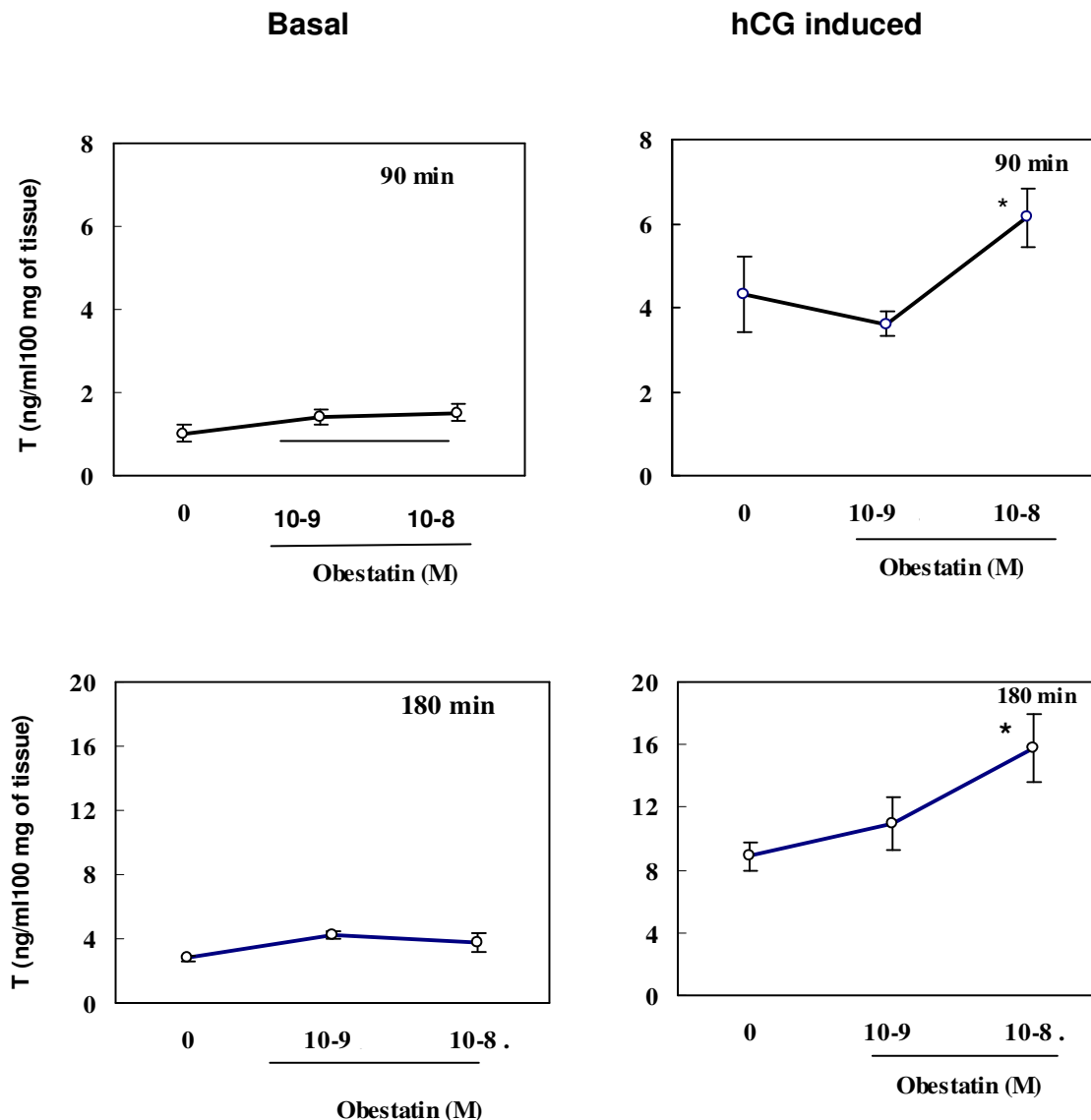
Obestatin on both doses tested ( $10^{-9}$  M and  $10^{-8}$  M) failed to cause any significant change in basal and hCG induced testosterone concentrations after 180 min of incubation, however, a significant difference was found

between basal control and hCG treated groups in 48 h fasted pre-pubertal rats. The testosterone concentrations in control,  $10^{-9}$  M obestatin,  $10^{-8}$  M obestatin, hCG, hCG plus  $10^{-9}$  M obestatin and hCG plus  $10^{-8}$  M obestatin treated groups were 3.01  $\pm$  0.66 ng/ml.100 mg of tissue, 4.50  $\pm$  2.07 ng/ml.100 mg of tissue, 5.98  $\pm$  1.88 ng/ml.100 mg of tissue, 8.18  $\pm$  1.46 ng/ml.100 mg of tissue, 10.01  $\pm$  1.99 ng/ml.100 mg of tissue and 9.77  $\pm$  1.80 ng/ml.100 mg of tissue, respectively (Figure 2).

#### ***In vitro* effect of obestatin ( $10^{-9}$ and $10^{-8}$ M) alone or in combination with hCG (10 IU) on testosterone secretion in normally fed adult male rats after 90 and 180 min of incubation.**

In the normally fed adult rats, obestatin at all doses failed to cause any significant change in both basal and hCG induced testosterone concentrations when compared with their corresponding control groups after 90 min of incubation. Testosterone concentrations in control,  $10^{-9}$  M and  $10^{-8}$  M obestatin treated groups, hCG treated group, hCG plus  $10^{-9}$  M and hCG plus  $10^{-8}$  M obestatin treated group were 1.81  $\pm$  0.20 ng/ml.100 mg of tissue, 2.56  $\pm$  0.54 ng/ml.100 mg of tissue, 3.85  $\pm$  1.05 ng/ml.100 mg of tissue, 2.71  $\pm$  0.32 ng/ml.100 mg of tissue, 3.95  $\pm$  0.50 ng/ml.100 mg of tissue and 3.54  $\pm$  0.54 ng/ml.100 mg of tissue, respectively. After 90 min of incubation no significant increase was noticed in testosterone concentrations between control and hCG treated groups.

After 180 min of incubation, testosterone concentrations in the hCG treated group (5.73  $\pm$  0.06 ng/ml.100 mg of tissue) was significantly high ( $p < 0.05$ ) when compared with the control group (3.56  $\pm$  0.08 ng/ml.100 mg of tissue). Obestatin ( $10^{-9}$  M) failed to cause any significant change in the basal testosterone concentrations after 180 min of incubation (4.61  $\pm$  0.79 versus 3.56  $\pm$  0.08 ng/ml.100 mg of tissue). In  $10^{-8}$  M obestatin treated group testosterone concentrations were 9.32  $\pm$  2.42 ng/ml.100 mg of tissue which was significantly ( $p < 0.05$ ) high when compared with the control group (3.56  $\pm$  0.08 ng/ml.100 mg of tissue).  $10^{-8}$  M obestatin plus hCG caused a significant ( $p < 0.005$ ) increase in testosterone concentrations when compared with hCG treated group (12.09  $\pm$  0.43 versus 5.73  $\pm$  0.06 ng/ml.100 mg of tissue). However, obestatin at the dose



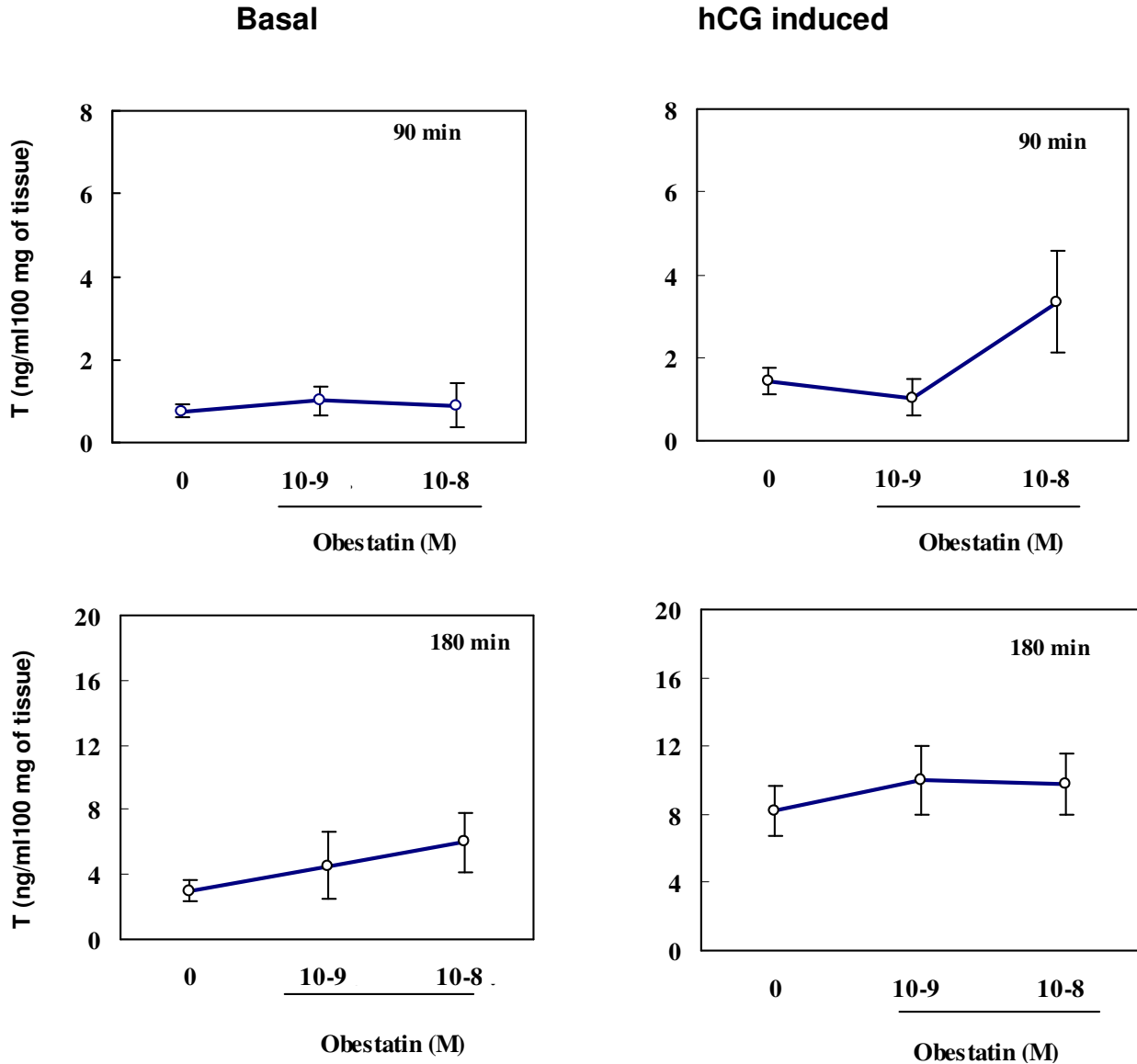
**Figure 1.** *In vitro* effects of obestatin in control (left panels) and in hCG treated (right panels) testosterone (T) secretion in normally fed pre-pubertal male rats. Testes from normally-fed males were challenged with increasing concentrations ( $10^{-9}$  and  $10^{-8}$  M) of obestatin alone (left panels) or obestatin plus 10 IU hCG (right panels) and testosterone release to the incubation medium was assessed after 90 and 180 min. Testes incubated in the presence of medium alone (0) served as controls. Values are given as means  $\pm$  S.E.M (n = 5). \*p < 0.05 versus values from corresponding control (ANOVA followed by Tukey's test).

of  $10^{-9}$  M in combination with hCG failed to cause any significant change in testosterone concentrations when compared with hCG treated group ( $10.71 \pm 2.05$  versus  $5.73 \pm 0.06$  ng/ml.100 mg of tissue) (Figure 3).

***In vitro* effect of obestatin ( $10^{-9}$  and  $10^{-8}$  M) alone or in combination with hCG (10 IU) on testosterone secretion in 48 h fasted adult male rats after 90 and 180 min of incubation.**

In the 48 h fasted adult male rats, obestatin at all doses

tested failed to cause any significant change in both basal and hCG induced testosterone concentrations after 90 min of incubation. Testosterone concentrations were  $0.98 \pm 0.29$  ng/ml.100 mg of tissue in control,  $1.80 \pm 0.56$  ng/ml.100 mg of tissue in obestatin  $10^{-9}$  M treated group,  $1.56 \pm 0.53$  ng/ml.100 mg of tissue in obestatin  $10^{-8}$  M treated group,  $1.84 \pm 0.14$  ng/ml.100 mg of tissue in hCG treated group,  $2.22 \pm 0.44$  ng/ml.100 mg of tissue in hCG plus obestatin  $10^{-9}$  M treated group and  $2.91 \pm 0.81$  ng/ml.100 mg of tissue in hCG plus obestatin  $10^{-8}$  M obestatin treated group, respectively. No significant difference was found between control and hCG treated



**Figure 2.** *In vitro* effects of obestatin in control (left panels) and in hCG treated (right panels) testosterone (T) secretion in 48 h fasted pre-pubertal male rats. Testes from 48 h fasted males were challenged with increasing concentrations ( $10^{-9}$  and  $10^{-8}$  M) of obestatin alone (left panels) or obestatin plus 10 IU hCG (right panels) and testosterone release to the incubation medium was assessed after 90 and 180 min. Testes incubated in the presence of medium alone (0) served as controls. Values are given as means  $\pm$  S.E.M ( $n = 5$ ). \* $p < 0.05$  versus values from corresponding control (ANOVA followed by Tukey's test).

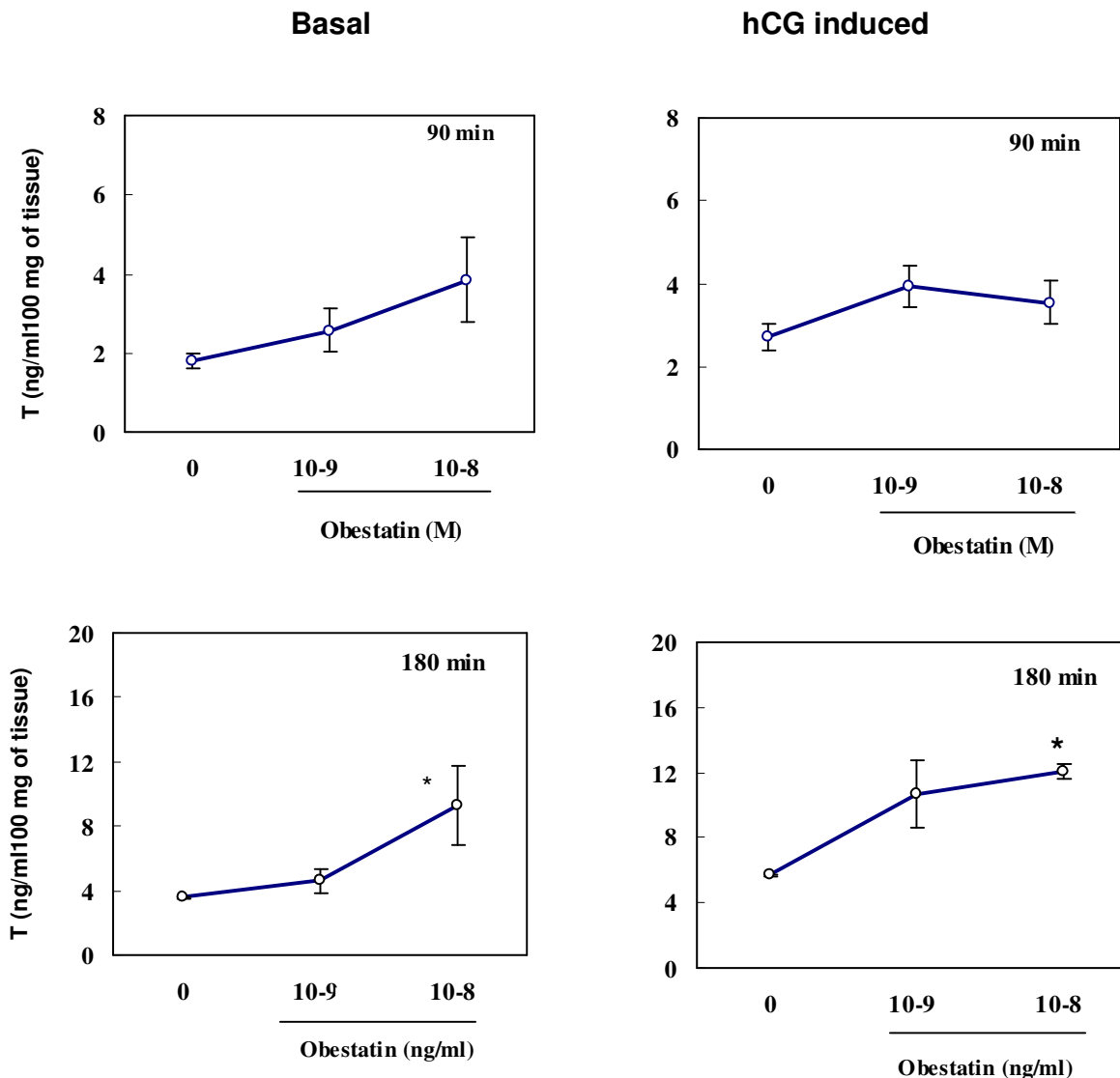
groups.

After 180 min of incubation, testosterone concentrations were  $2.99 \pm 0.49$  ng/ml.100 mg of tissue in control,  $3.93 \pm 0.83$  ng/ml.100 mg of tissue in the  $10^{-9}$  M obestatin treated group,  $3.07 \pm 0.56$  ng/ml.100 mg of tissue in  $10^{-8}$  M obestatin treated group,  $4.06 \pm 0.29$  ng/ml.100 mg of tissue in hCG treated group,  $4.75 \pm 0.35$  ng/ml.100 mg of tissue in the hCG plus  $10^{-9}$  M obestatin treated groups and  $4.37 \pm 0.46$  ng/ml.100 mg of tissue in hCG plus  $10^{-9}$  M obestatin treated groups. No significant difference in testosterone concentrations was noticed in

the treated groups when compared with their corresponding control groups, however, testosterone concentrations were significantly high ( $P < 0.05$ ) in hCG treated group when compared with the control group after 180 min of incubation in 48 h fasted adult rats (Figure 4).

## DISCUSSION

The present study was designed to evaluate *in vitro* effect of obestatin on the testicular testosterone secretion with

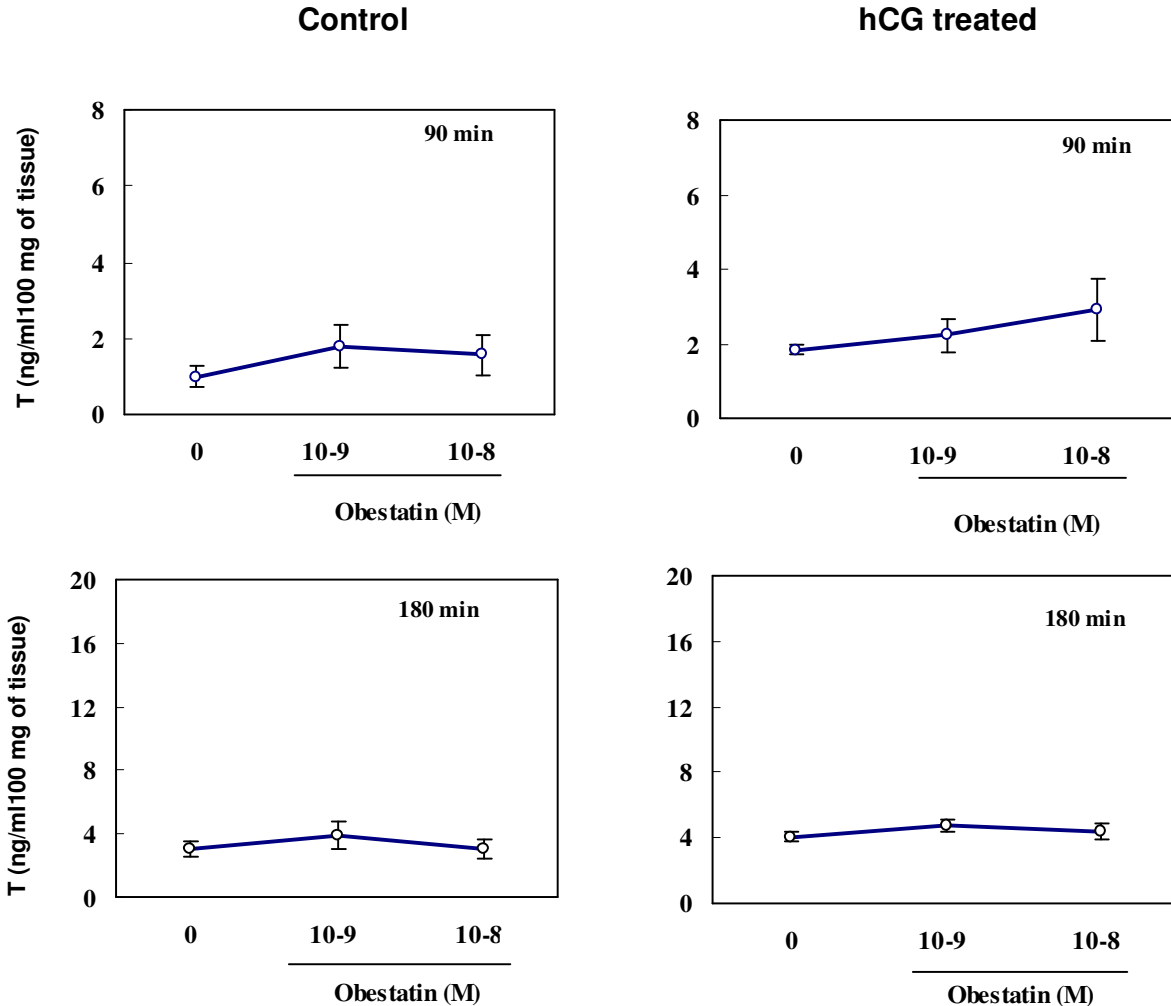


**Figure 3.** *In vitro* effects of obestatin in control (left panels) and in hCG treated (right panels) testosterone (T) secretion in normally fed adult male rats. Testes from normally-fed males were challenged with increasing concentrations ( $10^{-9}$  and  $10^{-8}$  M) of obestatin alone (left panels) or obestatin plus 10 IU hCG (right panels) and testosterone release to the incubation medium was assessed after 90 and 180 min. Testes incubated in the presence of medium alone (0) served as controls. Values are given as means  $\pm$  S.E.M (n = 5). \*p < 0.05 versus values from corresponding control (ANOVA followed by Tukey's test).

reference to previous findings in which the expression of GPR39 and obestatin were found in Leydig cells of the testis (Dun et al., 2006; Dong et al., 2009). In this experiment, two nutritional statuses were used because obestatin concentration decreases during fasting and also under nutrition and restricted food intake is negative modulators of reproduction. During childhood and pre-pubertal period, restricted food intake has been shown to significantly delay or prevent the reproductive awakening of the reproductive axis in rat (Schenck et al., 1980; Zizzari et al., 2007). Similarly, fasting induced a significant decrease in the LH receptor expression in the rat testis and decreases testosterone production (Shan

and Hardy, 1992).

Two groups of animals (pre-pubertal and adult) were used in this study because testosterone concentrations are high at birth but then decreased rapidly till the fifth week of the birth and then, from fifth week onwards the testosterone concentration increased in rats (Corpechot et al., 1981). It was also reported that, both LH and androgen receptor (AR) levels were lower in the Leydig cells of 21 day old rats. However, the LH and AR levels were found much higher in the adult male rats (Guezennec et al., 1982; Shan and Hardy 1992). Different doses of obestatin ( $10^{-9}$  M and  $10^{-8}$  M) were used because previously, approximately the same doses



**Figure 4.** *In vitro* effects of obestatin in control (left panels) and in hCG treated (right panels) testosterone (T) secretion in 48 h fasted adult male rats. Testes from 48 h fasted males were challenged with increasing concentrations ( $10^{-9}$  and  $10^{-8}$  M) of obestatin alone (left panels) or obestatin plus 10 IU hCG (right panels) and testosterone release to the incubation medium was assessed after 90 and 180 min. Testes incubated in the presence of medium alone (0) served as controls. Values are given as means  $\pm$  S.E.M (n = 5). \*p < 0.05 versus values from corresponding control (ANOVA followed by Tukey's test).

were used in an experiment on porcine ovarian granulosa cells in which it was found that obestatin is able to increase progesterone secretion from cultured ovarian granulosa cells (Meszarosova et al., 2008). Instead of Leydig cells culturing, the testicular slices were used in this experiment as previously done. This system of slice culturing was found best to check the direct short term effect of chemicals on testosterone production (Tena-Sempere et al., 1999, 2002).

Forty eight hours fasting induced a significant reduction in the body weight and plasma testosterone concentrations in both pre-pubertal and adult male rats. These findings are in accordance with the previous findings in which it was reported that 48 h fasting caused a significant decrease in body weight and plasma testosterone concentrations (Chen et al., 2005; Guezennec et

al., 1982).

The study data provides evidence for the direct stimulatory role of obestatin on testosterone secretion in the rat testis. It was found that, obestatin at the dose of  $10^{-9}$  M alone failed to cause any significant change in both basal and hCG induced testosterone secretion in normally fed and 48 fasted pre-pubertal and adult rat testes both after 90 and 180 min of incubation. This data is in accordance with the previous findings of Meszarosova et al. (2008), in which it was reported that this dose is less effective to cause a change in the reproductive hormones secretion *in vitro* from porcine granulosa cells.

Obestatin at the dose of  $10^{-8}$  M in combination with hCG, significantly increased testosterone secretions in the normally fed pre-pubertal and adult male rats sugges-

ting that, this dose is effective to increase reproductive hormones secretions as previously reported (Meszarosova et al., 2008). However, obestatin at all the doses tested failed to cause any change in both basal and hCG induced testosterone secretions from both 48 h fasted, fed pre-pubertal and adult rats testes after 90 and 180 min of incubation. This data suggest that, obestatin can enhance testosterone secretion from pre-pubertal and adult male rat testes depending upon their body nutritional status. The testosterone concentrations in the treatment groups were high but not significantly different when compared with their corresponding control groups. These findings may be supported by the previous findings that fasting results in the decrease in the LH and AR, results in the decrease in testosterone production (Shan and Hardy 1992). hCG induced a significant increase in the testosterone concentrations from all the groups after 180 min of incubation when compared with the basal control groups suggesting that the tissues were responsive to hCG.

In conclusion, this study reveals that the effect of obestatin on testicular testosterone production is nutritional status dependent. This data suggest that the relationship of obestatin with nutrition, metabolism and reproduction could provide new approaches for treatment of reproductive disorders. However, further studies are required to sort out whether obestatin increase testosterone production, acting through the steroidogenic pathway or only increase its secretion.

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