Modulation of Pituitary Gonadal Axis by Obestatin in Type 2 Diabetic Male Sprague Dawley Rats

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ABSTRACT

***Objective*:** To demonstrate the effect of Obestatin supplementation on oxidative stress and serum levels of testosterone, follicular stimulating hormone, luteinizing hormone and leptin in type 2 diabetic rats.

***Study Design*:** Laboratory-based experimental study

***Place and Duration of Study*:** Physiology Department, Army Medical College Rawalpindi from Mar-June 2015.

***Methodology*:** Forty-five healthy male Sprague Dawley rats were randomly divided into three groups. Group-I (healthy rats) were fed with regular pellet diet (NPD). Group-II (diabetic rats) and group-III (Obestatin treated diabetic rats) were fed with high-fat diet (HFD) followed by a single IP injection of Streptozotocin in the dose of 35 mg/kg on 15th day. After ten weeks, group-III was treated with intraperitoneal Obestatin (1 nmol/100ml). Blood samples were obtained by terminal intra-cardiac sampling for bioassays of Insulin, testosterone, FSH, LH, MDA and leptin by ELISA.

***Results*:** Obestatin supplementation in diabetic rats resulted in significant increase in FSH (9.4 ± 0.74 ng/dL), LH (3.89 ± 0.10 ng/dL) and testosterone levels (2.01 ± 0.09 ng/dL) in comparison with non-treated diabetic rats (7.04 ± 0.50 ng/dL), (1.70 ± 0.28 ng/dL) and (1.10 ± 0.40 ng/dL) respectively. Whereas leptin (3.91 ± 0.24 ng/dL) and MDA levels (1.75 ± 0.22 ng/dL) were reduced significantly when compared to the healthy rats (6.10 ± 1.29 ng/dL) and (1.96 ± 0.07 ng/dL) respectively.

***Conclusion*:** Testosterone levels are increased by Obestatin supplementation in type 2 diabetic rats by stimulating the pituitary-gonadal axis due to a decrease in circulating leptin levels and oxidative stress.

Keywords: Follicular stimulating hormone (FSH), Leptin, luteinizing hormone (LH), Obestatin.

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INTRODUCTION

Complex neuropathologies associated with obesity and type 2 diabetes mellitus (T2DM) affect the functioning of the hypothalamo-pituitary-gonadal axis in males, resulting in hypogonadotropic hypogonadism (HH).[1] In type 2 diabetes mellitus (T2DM), hypogonadism is closely interlinked with concomitant obesity and metabolic syndrome. This has been associated with decreased testosterone concentration and subnormal FSH and LH concentrations. Insulin resistant states like T2DM and obesity result in the decreased Insulin responsiveness of GnRH neurons, causing decreased levels of pituitary gonadotropins. Incidence of HH is documented to rise upto 50% if T2DM and obesity exist simultaneously.[2,3]

An anorexigenic peptide hormone, Obestatin, is released by gastric parietal cells and has been documented to act through a G protein-coupled receptor *(GPR39)* associated with the ghrelin hormone receptor.[4,5] Obestatin level is negatively correlated with body mass index and leptin levels and it is documented to be decreased in obese individuals. Therefore, it is believed to function as a novel regulator of adipocyte function as it decreases leptin levels and increases the adiponectin level.[6,7] In addition, Obestatin decreases the generation of ROS species in cardiac muscle in type 1 diabetic rats acting via glucagon-like peptide receptor (GLP 1R) on the pancreas. Obestatin inhibits cytokine-induced apoptosis of beta cells of the pancreas, thereby augmenting proliferation and survival of beta cells in islets.[8,9]

Diabetes mellitus induced dysfunction of the male reproductive system, including the serum gonadotropin levels and leptin levels, can be modulated by Obestatin. Therefore, Obestatin was supplemented in diabetic rats in the present study to evaluate its effects on gonadotrophic hormones, oxidative stress and leptin levels on T2DM.

METHODOLOGY

This laboratory-based animal study was conducted at the Department of Physiology, Army Medical College from March to June 2015. Forty-five male Sprague Dawley rats were randomly recruited from the

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National Institute of Health Sciences (NIH), Islamabad. The study was approved by the Ethical Review Committee (ERC/ID/116) of the Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College, Rawalpindi.

Inclusion Criteria: Healthy Sprague-Dawley male rats with weight 250 ± 50 grams and age range of 70-80 days were included in the study.

Exclusion Criteria: Diabetic rats or rats with any obvious injury were excluded from the study.

Study rats were assigned into three groups (15 rats in each group). NPD was given to control rats (group-1) ad libitum for three weeks. Tail vein sampling (2mL) was conducted after an overnight 12 hours fast to determine the Insulin resistance by Homeostatic Model assessment for Insulin (HOMA-IR) and detailed measurement of fasting Insulin and plasma glucose level.[10,11] NPD was continued for the next eight weeks. Intra-peritoneal (IP) injections of normal Saline (100uL) were administered at the end of the study and continued daily for ten days.

For two weeks, a high-fat diet was given to the diabetic rats (group-2), followed by an intra-peritoneal administration of Streptozotocin with the dose of 35 mg/kg on the 15th day.[12] HFD was administered to these rats for one more week, followed by tail vein sampling after 12 hours overnight fast for HOMA-IR determination to ensure the inclusion criteria for the development of T2DM. After eight more weeks of HFD, IP administration of normal Saline (100uL) was conducted daily for ten days**.**

Obestatin-administered diabetic rats (group-3) were treated similarly to group-2, but at the end of the study, Obestatin (1ml/100uL) were daily administered intra-peritoneally for ten days.[13]

Euthanisation of rats was carried out with an overdose of Ether at the end of the study. An intra-cardiac blood sample was drawn and four milliliters of blood was collected in serum gel separator tubes for serum hormone assay, while one-milliliter blood was collected in Sodium fluoride tubes for plasma glucose estimation. The blood in the Sodium fluoride tubes and serum gel separator tubes was centrifuged at 4000 rpm for 15 minutes in the cold centrifuge at 40°C. Obtained-serum was stored at -80°C to estimate the serum testosterone, Insulin, FSH, LH, MDA and leptin by enzyme-linked immunosorbent assay (ELISA).

Statistical Package for Social Sciences (SPSS) version 23 was used for the data analysis. Quantitative variables were expressed in mean and standard deviation (Mean ± SD). One-way analysis of variance (ANOVA) was applied to find out the mean differences among the groups. Post Hoc Tukey's test was applied for further inter-group comparisons. The *p*-value of ≤0.05 was considered statistically significant.

RESULTS

Forty-five male Sprague Dawley rats with the mean weight of 250 ± 5.3 gm and fasting plasma glucose of 88.13 ± 5.95 mg/dl were randomly inducted into the study. Serum FSH, LH, testosterone, leptin and MDA were measured in all the groups. Diabetic rats exhibited frank hyperglycemia (468.2 ± 6.11 mg/dl), which was higher than the cut off value of 200 mg/dl to establish hyperglycemia and the development of T2DM.

Levels of testosterone, FSH, LH, leptin, and MDA were different in all the three groups (Table-I).

Table-I: Group-wise comparison of testosterone, leptin, FSH, LH and MDA levels (ANOVA).

Serum Parameters	Group-1 (Control) (n=15)	Group-2 (Diabetic) (n=15)	Group-3 (Obestatin treated diabetic) (n=15)	*p*-value
FSH (ng/ml)	10.59 ± .29	7.04 ± 0.50	9.4 ± 0.74	0.045
LH (ng/ml)	4.18 ± 0.81	1.70 ± 0.28	3.89 ± 0.10	0.042
Testosterone (ng/ml)	2.37 ± 0.11	1.10 ± 0.40	2.01 ± 0.09	0.029
Leptin (ng/ml)	2.82 ± 0.31	6.10 ± 1.29	3.91 ± 0.24	0.019
MDA (ng/ml)	1.45 ± 0.03	1.96 ± 0.07	1.75 ± 0.22	0.015

FSH levels of diabetic rats (7.04 ± 0.50 ng/ml) were significantly lower (*p*=0.039) than healthy rats (10.59 ± 0.29 ng/ml). It was significantly higher in Obestatin treated diabetic rats (9.4 ± 0.74) (*p*-value= 0.013) when compared to non-treated diabetic rats. The non-treated diabetic (1.70 ± 0.28 ng/ml) rats revealed significantly lower (*p*= 0.041) levels of LH compared to the healthy rats (4.18 ± 0.81 ng/ml); however, the levels of LH were significantly increased in the Obestatin treated rats (3.89 ± 0.10) (*p*-value=0.01) when compared to diabetic rats (Table-II).

On the comparison of testosterone levels among groups, it was revealed that diabetic rats (1.10 ± 0.40 ng/ml) displayed significantly lower (*p*=0.032) levels as compared to the healthy rats (2.37 ± 0.11 ng/ml). In contrast, the testosterone levels were significantly

raised in the Obestatin treated group (2.01 ± 0.09) (p=0.01) compared to the diabetic rats.

Table-II: Inter-group comparison of testosterone, leptin, FSH, LH and MDA levels (Post hoc Tukey's test).

Inter-group Comparison	Serum FSH	Serum LH	Serum Testosterone	Serum Leptin	Serum MDA
Group-1 Vs. Group-2 (p-value)	0.039	0.041	0.032	0.005	0.034
Group-1 Vs. Group-3 (p-value)	0.048	0.04	0.021	0.025	0.024
Group-2 Vs. Group-3 (p-value)	0.015	0. 03	0.03	0.029	0.043

Serum leptin levels in diabetic (6.10 ± 1.29 ng/ml) groups were significantly raised when compared with the healthy rats (2.82 ± 0.31 ng/ml) (p=0.005) due to their increased body weight; however, the Obestatin treated diabetic group displayed significantly decreased leptin levels (3.91 ± 0.24) (p=0.029) on comparison with the diabetic rats.

Serum MDA levels were significantly raised (p=0.034) in diabetic (2.67 ± 0.18 ng/ml) groups when compared with healthy rats (1.45 ± 0.03 ng/ml). It manifested the enhanced oxidative stress in the diabetic group. However, the levels of MDA have significantly lowered in Obestatin treated rats (1.75 ± 0.22) (p-value= 0.043) compared to diabetic rats.

DISCUSSION

Obestatin increases the testosterone output by increasing the LH and FSH release from the anterior pituitary and decreasing leptin and MDA levels in obese type 2 diabetic rats. The diabetic group of rats exhibited significantly decreased FSH, LH and testosterone levels compared to healthy rats, which was similar to the observations documented by Ayuob *et al,* in which induction of diabetes mellitus through low dose STZ and HFD resulted in decreased testosterone levels.[11] The decline in pituitary gonadotropins and testosterone was associated with Insulin deficiency at the hypothalamic-pituitary level and Leydig cell. Moreover, the pituitary response to exogenous GnRH supplementation is blunted in diabetic rats resulting in diminished GnRH induced FSH and LH output.[12]

In the current study, there was a decline in FSH, LH and testosterone in diabetic rats as compared to the healthy rats due to the development of HFD diet-induced Insulin resistance with a concomitant decline in Insulin levels in diabetic rats. Ballester *et al,* conducted a study to highlight the role of Insulin in the secretion of male reproductive hormones. They showed that Insulin mimics a significant positive correlation with FSH. Decreased testosterone secretion in Insulin-dependent diabetes had been associated with the loss of stimulatory effect of insulin on Leydig cells, while LH output was decreased due to the decrease in insulin-dependent FSH output, which in turn caused the decrease in LH.[13] Obestatin levels have been documented to decrease in type 2 diabetic patients.[14] The diabetic rats were also documented to manifest a decline in Obestatin levels compared to normal rats.[15] A study conducted by Granata *et al,* showed that the Insulin secretion, beta-cell genesis and survival were up-regulated by Obestatin at genetic level.[16] Administration of Obestatin in diabetic rats resulted in a significant increase in FSH, LH, and testosterone compared to diabetic rats. Michal Szlis demonstrated that intra-cerebroventricular administration of Obestatin resulted in an increase in LH release from anterior pituitary cells by modulating protein synthesis, accumulation and release. FSH expression and accumulation was also increased in pituicytes following Obestatin administration.[17] Central Obestatin administration at the hypothalamic level suggested a possible modulation of GnRH mRNA and protein expression.[18] Study conducted by Jahan revealed that intra-peritoneal and intravenous administration of Obestatin in healthy rats resulted in a significant elevation in testosterone levels when compared to non-treated rats.[10]

In the present study, oxidative stress was developed in the diabetic group depicted by the rise in serum MDA levels. Increased lipid peroxidation in Leydig cells might have resulted in a decline in testosterone output in diabetic rats. Reactive oxygen species (ROS) have been documented to directly damage the critical components of the steroidogenic pathway to compromise testicular steroidogenesis. In enhanced oxidative stress states, increased nitric oxide production in testes would result in accelerated production of peroxynitrates which can subsequently cause a decline in testosterone secretion.[19] MDA levels were significantly reduced by Obestatin administration in diabetic rats compared to untreated diabetic rats. Obestatin has been documented to reverse the rise in MDA and TNF by enhancing the antioxidant enzymes and increasing caspase 3 and caspase 8 activities in the testicular reperfusion injury in rats.[20]

Testosterone concentration has been negatively correlated with Insulin resistance states such as T2DM and obesity. It has been documented that testicular

steroidogenesis is enhanced by Insulin via Insulin receptors under normal physiological conditions. However, in the case of the development of Insulin resistance, this stimulatory action of Insulin is lost, resulting in impaired steroidogenesis in Leydig cell, culminating in low testosterone output from testes.[21] Leptin resistance has been documented to develop if the HFD intake exceeds 8 weeks' duration and is observed to inhibit the stimulatory action of leptin on the hypothalamopituitary gonadal axis. Leydig cells exhibit leptin receptors and in vitro studies have demonstrated an inhibitory action of elevated leptin levels in Insulin-resistant states on hCG stimulated testosterone secretion from Leydig cells in rats.[22] The negative correlation between leptin and testosterone concentration has been demonstrated in our study. Obestatin supplemented diabetic rats showed significantly lower leptin levels when compared to the diabetic rats. The body mass index and leptin levels are inversely correlated with Obestatin levels. At a cellular level on pre-adipocytes, the phosphorylation of AMP-activated protein kinase is increased by Obestatin, which modulates leptin expression.[23]

Our study highlighted the beneficial effects of Obestatin on the pituitary gonadotrophic axis through modulation of leptin and oxidative stress in diabetic rats. This hormone can be exogenously used as an adjunct therapy in treating T2DM induced cases of male infertility.

LIMITATIONS OF STUDY

The mechanism of action of obestatin on the pituitary hypogonadal axis in male diabetic rats could be explored in depth by immunohistochemistry of obestatin receptor on Leydig cells *in vivo*. However, that was not undertaken in this study due to the lack of external funding sources.

CONCLUSION

Testosterone levels are increased by Obestatin supplementation in type 2 diabetic rats by stimulating the pituitary-gonadal axis due to a decrease in circulating leptin levels and oxidative stress.

Conflict of Interest: None.

Authors' Contribution

NL: Principal investigator, conceptualisation and manuscript writing, article submission after review, AR: Contributed in finalising of core concept, data collection and SPSS analysis, SA: Has made substantial contribution in Data analysis and interpretation. Ensured that every aspect of work is appropriately investigated and resolved, FM: Has made substantial contribution in manuscript writing, SBA: Critical analysis of of important intellectual content and proof reading, AF: Has made substantial contribution in Manuscript writing.

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