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Dose Dependent Proliferation of Testicular Tissue Subsequent to Exogenous Neurokinin B Administration in Adult New Zealand White Rabbits

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ABSTRACT

Background: Neurokinin B is a regulator of puberty and reproduction, however its chronic effects on gonads in adult male mammals remain elusive.

Objectives: To investigate potentials of variable doses of Neurokinin B on histomorphology of testicular tissue and serum testosterone levels in adult rabbits.

Material and Methods: This Laboratory based experimental study was conducted at Gomal Centre of Biochemistry and Biotechnology, Gomal University, Dera Ismail Khan, Pakistan from June-2019 to June-2020. Adult male New Zealand white rabbits (n-18) with an average weight of 1.52kg were randomly assigned to three groups. Study animals were administered subcutaneously with Neurokinin B in two variable doses: 1 µg and 1 ηg. Control rabbits were injected with distilled water. After 12 days of continuous peptide treatment, animals (n=06) in each group were sacrificed. Testicular tissues were dissected and processed for light microscopy. Blood collection was performed directly from left ventricle, serum was separated and assayed for testosterone by enzyme linked immunosorbent assay.

Results: Neurokinin B did not alter the body weight of experimental rabbits as compared to control. No significant change on testicular weight, testicular volume and gonadosomatic index was observed at any dose of Neurokinin B. Seminiferous tubular diameter (µm) increased non-significantly while seminiferous epithelial height (µm) increased significantly in high dose group as compared to control group. Serum testosterone concentration increased significantly (P?0.01) at both low (1ηg) and high (1µg) doses of Neurokinin B as compared to control.

Conclusion: Continuous administration of Neurokinin B effect positively on serum testosterone and testicular morphology.

Keywords: Histomorphology, Neurokinin B, Tachykinins, Testes, Testosterone

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INTRODUCTION

The onset of puberty and reproductive capacity in an organism hinges on intricate interactions among hypothalamic nuclei, the anterior pituitary gland, reproductive organs, and target cells affected by gonadotropins and sex steroid hormones.[1] These interactions triggers sexual maturation in the hypothalamicpituitarygonadal (HPG) axis, culminating in the development of secondary sexual characteristics and fertility.[1] The HPG axis is primarily regulated by intermittent gonadotropin-releasing hormone (GnRH) release from the neurosecretory cells of the arcuate nucleus in the hypothalamus, the secretion of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) by gonadotropic cells in the anterior pituitary gland, and the presence of gonadotropin-responsive elements within the reproductive organs.[1,2]

Neurokinin B, member of tachykinin peptide hormones family, is synthesized from preprotachykinin-B gene.[3] The prepropeptide is cleaved enzymatically to proneurokinin B and then to Neurokinin B.[4] Biological effects of Neurokinin B are mediated through NK3R (Neurokinin 3 receptor), which belongs to the G-protein coupled membrane receptor.[3]

Topaloglu et al, (2009) reported that mutations in TAC3 (gene encoding Neurokinin B) or its receptor TACR3 (gene encoding NK3R) resulted in hypogonadotropic hypogonadism.[5] This finding provides the initial evidence of the essential role of Neurokinin B in the reproductive signaling system and the regulation of gonadotropic axis. Further evidence supporting the crucial role of Neurokinin B in reproductive axis was emerged when deletional and missense mutations in TAC3 and TACRs were found to be associated with hypogonadotropic hypogonadism.[6,7]

Pharmacologic administration of Neurokinin B or its agonists and antagonists has resulted in disparate effects on GnRH and LH secretion. The simultaneous administration of Neurokinin B and Kisspeptin amplifies the stimulatory effects of Kisspeptin while suppressing its positive impact on GnRH release in male rodents.[8] In prepubertal male rats, central administration of Senktide (Neurokinin B agonist) increases FSH, whereas adult rats exhibit no FSH or LH responses.[9] However, in adult male mice, central administration of Senktide significantly increased FSH and LH levels.[10] Additionally, Neurokinin B antagonists are potent suppressors of the follicular growth and estradiol secretion in females.[11-13]

While Neurokinin B is recognized for its significant involvement in the onset of the puberty and maintenance of the reproductive capability, information regarding its chronic effects on the testicular tissue is

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scarce. Consequently, the present study was designed to investigate the effects of variable doses of Neurokinin B, in adult male rabbits, on histomorphology of testes subsequent to its subcutaneous administration.

MATERIAL AND METHODS

Animals Handling and Housing

This Laboratory based experimental study was conducted at Gomal Centre of Biochemistry and Biotechnology, Gomal University, Dera Ismail Khan, Pakistan from 5th June 2019 to 4th June 2020. Healthy adult male New Zealand white rabbits (Oryctolagus cuniculus) [n-18] weighing 1.5-2 kg were purchased from the National Institute of Health, Islamabad, Pakistan. Adult male rabbits already on drug therapy, used in any study/experiment within the last 6 months, or currently on medication for any disease within the last month were excluded. They were retained in animal house facility of Gomal University, Dera Ismail Khan, Pakistan during trial period. Animals were acclimatized for 10 days before the start of experiment. They were maintained at 12 hour Light:Dark cycle, temperature of 25±2°C, food and water ad libitum.

All animal protocols were conducted as per guidelines of ethics review board of Gomal University, Dera Ismail Khan. Animal handling was also in accordance with the European Union regulations on animal research guidelines.[12] Approval of the study was granted by the Ethics Review Board, Gomal University, Dera Ismail Khan (study approval No. 118/QEC/GU dated January 29, 2019).

Dosage and Treatments

Neurokinin B (Catalogue # N4143-1MG) in lyophilized form was purchased from the Sigma-Aldrich (Saint Louis, USA). Neurokinin B was dissolved in 1 ml of dimethyl sulfoxide (DMSO) (VWR, USA) to obtain the stock solution. Stock solution was then further diluted with distilled water to be injected subcutaneously. Animals were assigned randomly (by lottery method) into 3 groups (n=6 in each group). Group-I rabbits constituted the control and were injected subcutaneously with distilled water. Group-II rabbits received 1 µg (1.2 µmol) Neurokinin B, Group-III rabbits received 1 ηg (1.2 ηmol) Neurokinin B subcutaneously twice daily, after every 12 hours, for 12 days consecutively. After 12 days of treatments the animals in each group were sacrificed and testicular tissue was dissected to investigate the effects administration of variable doses of Neurokinin B.

Collection of blood samples

To determine the serum testosterone concentration, blood collection was performed at the end of the experiment directly from the left ventricle of the heart after anesthetizing. The obtained blood samples were immediately transferred to the gel vacutainers (yellow top vacutainers) kept on ice. Serum was separated by centrifugation at 3000 rpm for 10 minutes and the obtained serum was stored at 80 °C until assayed for testosterone.

Collection of Tissue Samples

To investigate the effect of peptide treatments, animals were anesthetized by sodium pentobarbital (60mg/kg body weight subcutaneously) three hours after the last dose of Neurokinin B. Animals were dissected; testis was identified and dissected. Excised tissue was weighed and rinsed in phosphate buffered saline (P4417, Sigma-Aldrich, Saint Louis, USA). The excised tissue was then processed for light microscopy.

Gross and light microscopic examination

The excised testicular tissues were examined for gross morphology and weighed. Gonadosomatic Index (GSI) in percentage was calculated by using the below mentioned formula:

$$GSI\ (\%) = \frac{Weight\ of\ Rat\ Testis}{Weight\ of\ Rat\ Body} \times 100$$

Dimensions (length and width) of the both the testicular tissues were calculated by using a digital Vernier calipers. Testicular volume (mm^3) was determined by using the equation of ellipsoid as mentioned by Pochron and Wright.[13] The left and right testicular tissue volumes were then averaged.

$$Testicular\ Volume\ (mm^3) = \frac{4}{3} \times \pi \times L \times W^2$$

Where L = Length of the testis, W = Width of the testis

For histologic examination, testicular tissue was fixed in Bouin's solution (Sigma-Aldrich, Germany; HT10132) for 48 hours. For light microscopy tissue samples were dehydrated using increasing concentrations of alcohol, cleared by xylene and embedded in paraffin wax. Tissue sections of 5µm were cut using a microtome (Sakura Accu-Cut SRM 200, USA), stained with Harris's Hematoxylin and eosin and mounted in DPX Dibutylphthalate Polystyrene Xylene (Merck, Germany; 100579).

Hormonal analysis

Serum testosterone was determined quantitatively using the enzyme linked immunosorbent assay (ELISA). Testosterone ELISA kits (Catalogue # 3725-300) were purchased from Monobind Inc. (Lake Forest, CA 92630, USA). Assays were done according the manufacturer instructions and the optical densities were calculated at 450ηm using microplate reader.

Statistical analysis

Results obtained were organized in the Microsoft Excel 2013 and then transferred to Statistical Package for Social Sciences (SPSS, version 26, IBM Inc. Chicago, Illinois, USA). Normality of the data was confirmed by Kolmogorov-Smirnov test. Obtained data were expressed as Mean±SEM (standard error of mean).

Statistical significance was tested using the one way ANOVA followed by post hoc Tukey's test and P<0.05 was considered statistically significant.

RESULTS

Effect on Body Weight and testicular parameters

Neurokinin B did not alter the body weight of experimental rabbits as compared to control. No significant change on testicular weight, testicular volume and gonadosomatic index was observed at any dose of Neurokinin B (Table 01). Non-significant increase was observed in the seminiferous tubular diameter (µm) while the seminiferous epithelial height (µm) increased significantly at high dose as compared to control group (Table 01).

Effect on Testosterone Concentration

Plasma testosterone concentration increased significantly (P< 0.01) at both low (1 ηg) and high (1 µg) doses of Neurokinin B as compared to control (Figure 01).

Effect on Histomorphology of Seminiferous Tubules

Light microscopic examination of testicular sections from saline treated control rabbits showed normal seminiferous tubules with intact germinal epithelium. Spermatogonia and primary spermatocytes were present towards the line of basement membrane. Secondary spermatocytes with small stained nucleus were present downward from primary spermatocytes. Round and elongated spermatids were present as characterized by acrosome, towards the lumen. Spermatozoa were present with a darkly stained head and lightly stained tail towards the lumen. Sertoli cells were irregular shaped, lightly stained and were evenly spaced. Leydig cells were present in the interstitial tissue, with a round, darkly stained nucleus (Figure 02).

At 1 ηg Neurokinin B dose lumen size was increased as compared to control group. An increase in the number of Leydig cells was evident. Multinucleated giant cells were also present. The number of spermatids was higher as compared to control (Figure 02).

At 1 µg Neurokinin B dose, Leydig cells were increased and loss contact with seminiferous tubules. A marked increase in lumen size was also observed as compared to control and low dose. It was filled with necrotic tissues. Intraepithelial vacuolization was evident in the lumen. Increased number of elongated spermatids was also observed (Figure 02).

Table 01. Effect of Neurokinin B on the body weight and testicular parameters in various study groups of adult male rabbits

	Control	Neurokinin B (1 µg)	Neurokinin B (1 ηg)
Body Weight (gm)	2091.32±113.12	2189.73±121.25	2130.71±132.41
Testicular Weight (mg)	1889±33.45	1930±29.25	1905±31.19
Testicular Volume (mm3)	9.83±0.75	9.85±0.78	9.78±0.84
Gonadosomatic Index (%)	0.53±0.02	0.54±0.03	0.53±0.02
Seminiferous Tubular Diameter (µm)	238.81±17.46	245.13±16.23	243.57±18.67
Seminiferous Epithelial Height (µm)	61.45±5.48	68.85±6.24**	64.28±5.89

**P<0.01 compared to control

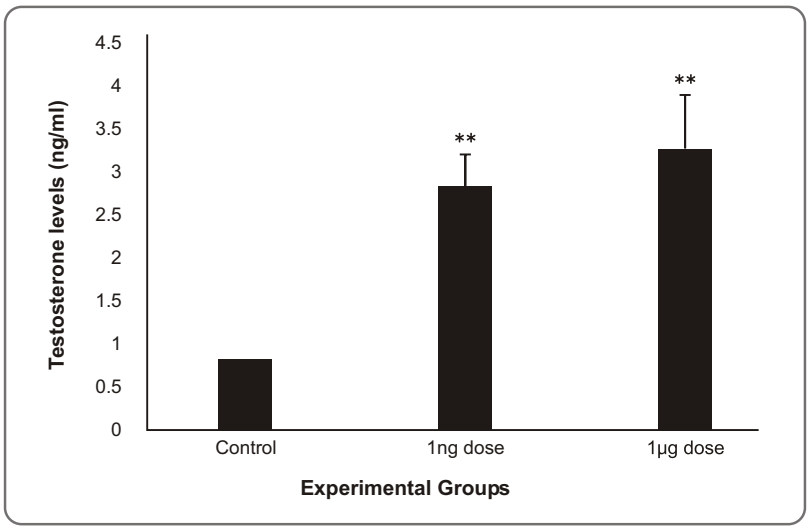


Figure 01. Neurokinin B significantly increased testosterone levels (P<0.01 compared to control)**

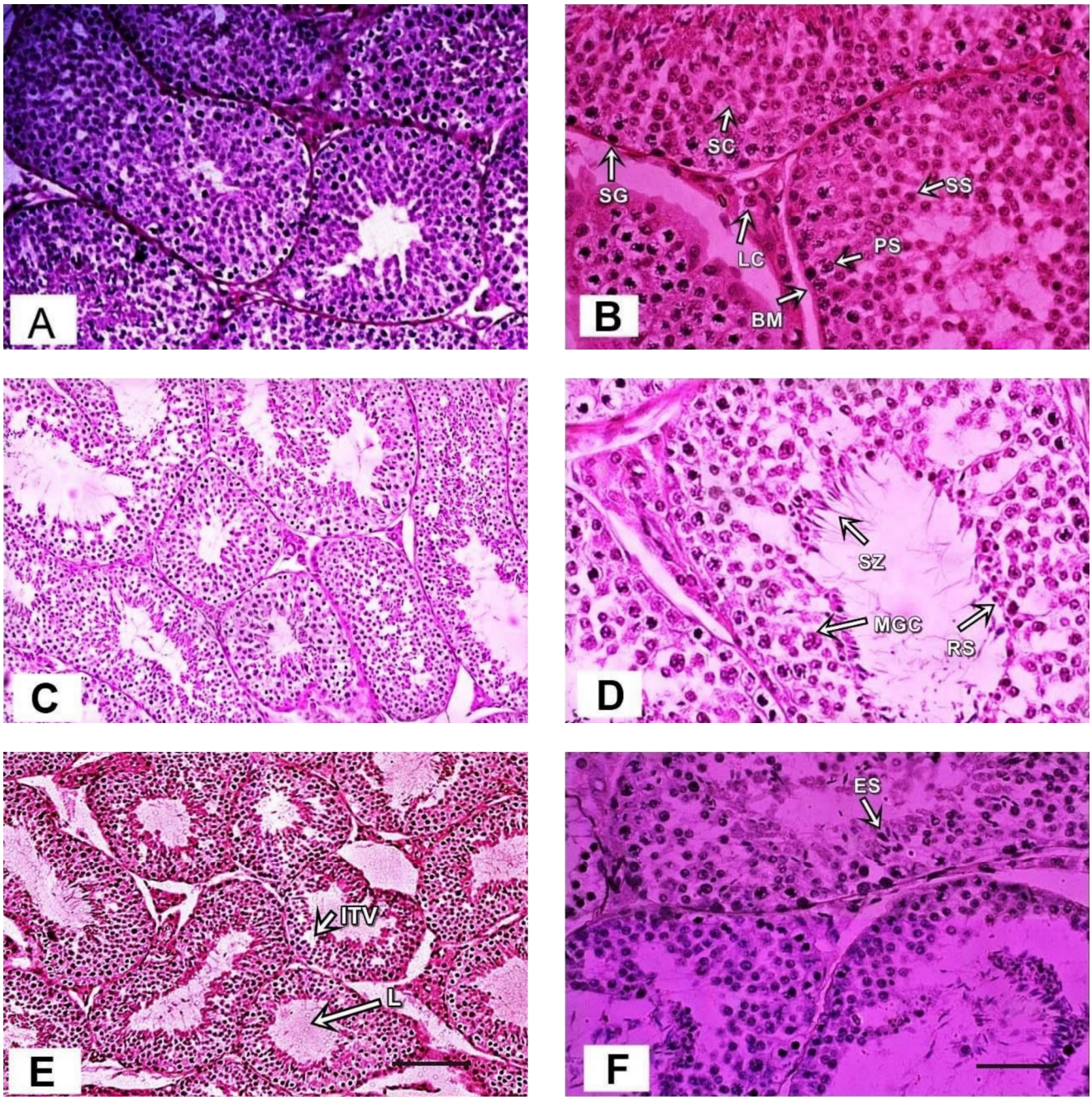


Figure 02: Light microscopy of stained slides of testicular tissue, treated with low (1 ηg) and high dose (1 µg) of Neurokinin B. A-B = control, C-D = low dose (1 ηg), E-F = high dose (1 µg). Abbreviations: SG = spermatogonia, SC = Sertoli cells, LC = Leydig cells, BM = basement membrane, PS = primary spermatocytes, SS = secondary spermatocytes, RS = round spermatids, ES = elongated spermatids, SZ = spermatozoa, MGC = multinucleated giant cells, ITV = intratubular vacuolization, L = Lumen. Scale bar: left panel 50um, right panel 20um

DISCUSSION

No effect on body weight, testicular weight, testicular volume and GSI was observed at any peptide administration. Seminiferous tubular diameter and seminiferous epithelial height increased with Neurokinin B (Table 01). Histological analysis of testicular sections revealed an increase in seminiferous tubular diameter and epithelial height with Neurokinin B treatment (Table 01). A concomitant increase in serum testosterone (Figure 01) concentrations was observed with all Neurokinin B treatments.

The stimulatory effects of acute administration of Neurokinin B and its agonists on reproductive axis has been demonstrated previously in mice and rats.[8-10] Pharmacologic manipulation of reproductive axis is of substantial therapeutic importance in pathologic

conditions, especially in puberty and fertility related disorders and treatment of neoplasms of reproductive origin.[14] GnRH analogs, agonists or antagonists, are routinely used in treatment of these pathologies. Therefore, optimization of hormonal intervention protocols on reproductive axis is of great interest. To this context, recent studies identified that KNDy (Kisspeptin Neurokinin B Dynorphin) neurons in arcuate nucleus of hypothalamus are the main regulators of reproductive axis and are conserved among species. This makes KNDy pathway as possible target for therapeutic intervention of reproductive axis. It has been postulated that, pulsatile secretion of Kisspeptins is regulated by stimulatory effect of Neurokinin B and inhibited by Dynorphin. Kisspeptin in turn stimulates secretion of GnRH and gonadotropins.[14]

The present study provides a contrasting evidence to that of continuous Kisspeptin administration which resulted in degenerative changes in testicular tissue and accessory sex glands in adult and prepubertal male rats. Studies showed that, continuous Kisspeptin administration resulted in desensitization of Kisspeptins potent activity to stimulate GnRH and gonadotropins secretion which resulted in degenerative changes in testicular tissue morphology and testosterone in adult and prepubertal rats.[15-17] Here, we report a stimulatory effect of Neurokinin B on reproductive axis (testis and testosterone) in adult male rabbits. Although, Kisspeptin and Neurokinin B neurons coexist in hypothalamus, their effect on GnRH and LH secretion is different, with Kisspeptin showing a more pronounced stimulatory effect on GnRH and LH compared to Neurokinin B.[8] These stimulatory effects of continuous Neurokinin B and Senktide administration may be due to a different pathway followed by Neurokinin B in pubertal animals and its effect on secretion of GnRH and gonadotropins. Peripheral and central acute administration of Kisspeptin in adult mice and rats causes a significant five-fold increase in GnRH and LH, while there is no significant increase in secretion of GnRH and LH with Neurokinin B alone. However, co-administration of Neurokinin B and Kisspeptin causes a more robust increase in GnRH and LH compared to control and Kisspeptin.[8] It was demonstrated that deficiency of Neurokinin B resulted in pubertal delay and sexual maturation however, it was not as severe as observed in mice with Kisspeptin deficiencies.[18-20] Studies from mice and primates suggested that Neurokinin B acts at a higher level than Kisspeptin as both pharmacological blockage and desensitization of Kisspeptin receptor also blocks stimulatory effect of Neurokinin B on GnRH.[21, 22] In contrast to this pathway, there is also evidence that Neurokinin B directly stimulates release of GnRH as its receptor is found on GnRH neuron terminals.[23] Garcia et al., (2018) demonstrated that Senktide stimulated release of Kisspeptin and GnRH in prepubertal and pubertal male primates. They described that Neurokinin B signaling to GnRH neurons is mediated through Kisspeptins in prepubertal and pubertal primates. However, Kisspeptin signaling to GnRH neurons was mediated through Neurokinin B neurons is limited to prepubertal stage only.[24] Irrespective of the action site, deficiency of Neurokinin B or its receptor resulted in pubertal delay in animals, indicating that Neurokinin B is crucial for normal functioning of reproductive axis.[24]

Present data disclosed that, continuous administration of Neurokinin B to adult male rabbits significantly increased testosterone concentrations (Figure 01). Increased testosterone concentrations in Neurokinin B groups was quite likely due to increased production or suppression of gonadal testosterone concentrations. This effect might have been due to testicular changes as indicated by histomorphological in respective groups (Figure 02).

Presently, morphological analysis of seminiferous tubules at stage VII of seminiferous cycle fully support Neurokinin B mediated expression of testosterone. McLachlan et al., (1994) demonstrated that withdrawal of LH results in suppression of spermatogenesis and significant reduction in number of spermatogonia, spermatocytes and spermatids.[25] Similarly acute removal of testosterone resulted in degeneration of spermatogenic cells. Spermatocytes and spermatids have shown degenerative changes and maturation arrest, when testosterone was removed for a period of few days.[25,26] Administration of Neurokinin B antagonist (AZD 4901) resulted in suppressed follicular growth and decrease estradiol secretion in healthy women which is in agreement to results of present study.[11]

Enhanced expression of Neurokinin B and tachykinin receptors were reported in rat and human testis.[3,27,28] Therefore a direct testicular effect may also be speculated for local regulation of reproduction at gonadal level in addition to HPG axis with functional Neurokinin B and its receptors. It has been reported that effects of GnRH agonists and antagonists may have a direct extra-pituitary inhibitory and excitatory effect on gonads.[29,30] It has been recently shown that a single injection of Neurokinin B or Senktide caused positive effects while Neurokinin B antagonists caused degenerative changes on testicular tissue and HPG axis.[31,32] Leydig cells are the only source of testosterone production in testis, it might be quite possible that Neurokinin B treatment modulated Leydig cell function and resulted in increased testosterone production. Presently, Neurokinin B treatment contributed towards increased testosterone production (Figure 01). This possibility is further strengthened by observations of the present study that Neurokinin B treatment resulted in increased spermatogenesis in adult rats (Table 01 and Figure 02). LH receptors are present on the Leydig cells and their stimulation resulted in increased testosterone production[33] and both, LH and testosterone, are responsible for normal spermatogenesis in rats.[34,35]

To the best of our knowledge, this is the first study to investigate the effects of continuous administration of variable doses of Neurokinin B on histomorphology of testicular tissue and serum testosterone concentrations, therefore, exact comparison with other studies is not possible at present.

CONCLUSION

Present study concluded that continuous administration of Neurokinin B could have sensitizing effect on the histomorphology of testis and serum testosterone levels.

Ethical Approval

Approval of the study was granted by the Ethics Review Board, Gomal University, Dera Ismail Khan, Peshawar (study approval No. 118/QEC/GU dated January 29, 2019).

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Conflict of Interest

Authors declared no conflict of interest

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DATA SHARING STATEMENT: The data that support the findings of this study are available on request from the corresponding author. The data is not publicly available due to privacy or ethical restrictions.

AUTHORS' CONTRIBUTION

The following authors fullfill authorship criteria as per ICMJE guidelines;

Ramzan F:	Substantial contribution to conception and design, drafting the manuscript, final approval, agreed to be accountable for all the work.
Gul S:	Substantial contribution to conception and design, data acquisition, drafting the manuscript, final approval, agreed to be accountable for all the work.
Ramzan MH:	Analysis and interpretation of data, drafting the manuscript, final approval, agreed to be accountable for all the work.

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