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Original Article

Differential Impact of Subcutaneous Neurokinin B Administration on Epididymal Proliferation in Adult New Zealand White Rabbits: A Dose-Dependent Study

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ABSTRACT

Objective: To investigate the potential of variable doses of Neurokinin B on histomorphology of epididymis in adult rabbits.
Study Design: Laboratory-based experimental study.
Place and Duration of Study: Gomal Centre of Biochemistry and Biotechnology, Gomal University, Dera Ismail Khan, Pakistan, from Jun 2019 to Jun 2020.
Methodology: Adult male New Zealand White rabbits with an average weight of 1.5–2 Kg were randomly assigned to three groups. Study animals were administered subcutaneously with Neurokinin B in two variable doses: 1 µg and one ηg. Control rabbits were injected with distilled water in parallel. After 12 days of continuous peptide treatment, animals (n=06) in each group were sacrificed. Epididymis were dissected and processed for light microscopy and sperm count.
Results: Mean epididymis weight (mg) increased significantly (p<0.001) with the treatment of Neurokinin B 1 ηg and Neurokinin B 1 µg as compared to control. With the increase in doses of Neurokinin B (1 ηg, 1 µg), the lumen of caput, corpus and cauda of the epididymis got significantly (p<0.001) dilated dose-dependently in Neurokinin B treated animals. Sperm count increased significantly (p<0.001) in the Neurokinin B-treated rabbits compared to the control. Light microscopy revealed increased luminal diameter compared to control in all Neurokinin B treated groups as compared to control.
Conclusion: Continuous administration of Neurokinin B could benefit the epididymis, as shown by increased luminal diameter and sperm count.

Keywords: Epididymis, Histomorphology, Neurokinin B, Spermatozoa, Tachykinins.

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INTRODUCTION

The epididymis consists of a pair of elongated tubular structures connected to the dorsal surface of the testes, with one epididymis corresponding to each testis. Each epididymis is anatomically divided into three segments: the head (caput), body (corpus), and tail (cauda). Positioned on the posterior aspect of the testis, the caput is at the upper pole, while the cauda is at the lower pole.[1] Neurokinin B, a tachykinin peptide hormone family member, is synthesised from the pre-protachykinin-B gene.[2] The prepropeptide is cleaved enzymatically to pro neurokinin B and then to Neurokinin B.[3] Biological effects of Neurokinin B are mediated through NK3R (Neurokinin 3 receptor), which belongs to the G-protein coupled membrane receptor.[2]

Topaloglu *et al*. (2009) reported that mutations in TAC3 (gene encoding Neurokinin B) or its receptor TACR3 (gene encoding NK3R) resulted in hypogonadotropic hypogonadism.[4] This finding provides the initial evidence of the essential role of Neurokinin B in the reproductive signalling system and the regulation of the gonadotropic axis. Further evidence supporting the crucial role of Neurokinin B in the reproductive axis emerged when deletional and missense mutations in TAC3 and TACRs were found to be associated with hypogonadotropic hypogonadism.[5]

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.[6] In prepubertal male rats, central administration of Senktide (Neurokinin B agonist) increases FSH, whereas adult rats exhibit no FSH or LH responses.[7] However, in adult male mice, central administration of Senktide significantly increased FSH and LH levels. Additionally, Neurokinin B antagonists are potent suppressors of follicular growth and estradiol secretion in females.[9]

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While Neurokinin B is recognised for its significant involvement in the onset of puberty and maintenance of reproductive capability, information regarding its chronic effects on the epididymis is scarce. Consequently, the present study was designed to investigate the effects of variable doses of Neurokinin B on the histomorphology of adult male rabbits' epididymis after subcutaneous administration.

METHODOLOGY

This is Laboratory based experimental study was conducted at the Gomal Centre of Biochemistry and Biotechnology, Gomal University, Dera Ismail Khan, Pakistan, from June 2019 to June 2020 after approval by the Ethics Review Board, Gomal University (No. 118/QEC/GU dated January 29, 2019). Animal handling was also in accordance with the European Union regulations on animal research guidelines.[10]

Inclusion Criteria: Adult male New Zealand white rabbits (Oryctolagus cuniculus) [n=18] weighing 1.5-2 Kg were inlcuded.

Exclusion Criteria: Adult male rabbits already on drug therapy, used in any study/experiment within the last six months, or currently on medication for any disease within the last month were excluded.

Rabbits were purchased from the National Institute of Health, Islamabad, Pakistan. During the study period, they were retained in the animal house facility of Gomal University, Dera Ismail Khan, Pakistan. Animals were acclimatised for ten days before the start of the experiment. They were maintained at a 12-hour Light: Dark cycle, the temperature of 25±2°C, and food and water ad libitum.

Neurokinin B (Catalogue # N4143-1MG) was purchased from Sigma-Aldrich (Saint Louis, USA) in lyophilised form. Neurokinin B was dissolved in 1 ml of Dimethyl Sulfoxide (DMSO) (VWR, USA) to obtain the stock solution. The stock solution was then diluted with distilled water to be injected subcutaneously. Animals were assigned randomly into three groups (n=6 in each group). Group-I rabbits constituted the control and were injected subcutaneously with distilled water. Group-II rabbits received one µg (1.2 µmol) Neurokinin B, Group-III rabbits received one 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 epididymis was dissected to investigate the effects of administering variable doses of Neurokinin B.

To investigate the effect of peptide treatments, animals were anaesthetised by sodium pentobarbital (60mg/kg body weight subcutaneously) three hours after the last dose of Neurokinin B. Animals were dissected; epididymis were identified and dissected. The three regions (Caput, Carpus, and Cauda) of each epididymis were separated by using scalpel blades. 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.

For histologic examination, epididymis was fixed in freshly prepared 4% paraformaldehyde (pH 7.2) (16005, Sigma-Aldrich, Saint Louis, USA) for 6 hours. Tissue samples were dehydrated using increasing concentrations of alcohol, cleared by xylene and embedded in paraffin wax. Tissue sections of 5 µm 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).

To quantify the sperms, the epididymis were snap-frozen using liquid nitrogen and stored at -80°C until they were assayed. Each epididymis was transferred to a petri dish containing phosphate buffer and placed on a preheated slide warmer (35°C–37°C) for 30 seconds. The caudal region of each epididymis was removed by surgical blade and transferred to a petri dish containing preheated buffer. The cauda regions were opened through a surgical blade to release its content. Tissue-containing petri dishes were vortexed and kept at 37°C in an incubator for 15 minutes. After incubation, the contents of the Petri dishes were mixed again using a micropipette and 500 µl was transferred to Eppendorf tubes that already contained 1000 µl of phosphate buffer and vortexed. Eppendorf tubes were incubated in a water bath at ~60°C for 60 seconds. Eppendorf tubes were allowed to cool and mixed again, and ten µl of the solution was loaded at each side of the improved Neubauer chamber. The loaded Neubauer chamber was placed in a petri dish containing moist tissue paper for some time to fix the sperm. After that, a loaded Neubauer chamber was observed under the light microscope. Sperms were counted by using a hand tally counter. The mathematical calculation of sperm count was done through the following formula.

$$Total\ Sperm = Mean\ Count \times Dilution\ Factor$$

$$\text{Mean Count} = \frac{(Count\ 1 + Count\ 2)}{2}$$

$$\text{Dilution factor} = \frac{\text{Total PBS in petri dish}}{\text{Transfered vol.}} \times \frac{\text{Total vol.test tube}}{\text{vol.secondary square} \times \text{No.squares}}$$

Tissue sections for the light microscopy were examined and photographed using Optika B-510BF (Optika, Italy) with a camera attached (C-B10, Optika, Italy). The tubular diameter of the epididymis was measured using the camera software Optika Proview (Optika, Italy) after calibration with the calibration slide at 4x, 10x and 40x.

Results were organised in Microsoft Excel 2013 and then transferred to Statistical Package for Social Sciences (SPSS, version 26, IBM Inc. Chicago, Illinois, USA). The normality of the data was confirmed by the Kolmogorov-Smirnov test. Organ weight and epithelial heights were expressed as Mean±SEM (standard error of the mean). Statistical significance was tested using the one-way ANOVA followed by post hoc Tukey's test, and $p \leq 0.05$ was considered statistically significant.

RESULTS

Epididymis weights (mg) increased significantly at variable doses of Neurokinin B 1µg (1.92±0.28; p<0.001) and Neurokinin B 1ηg (1.58±0.22; p<0.05) when compared to control (1.14±0.29).

Normal non-peptide treated epididymis ducts consist of smooth muscular walls with columnar to cuboidal epithelium having microvilli. The epithelium consists of principal cells, characterised by their tall columnar shape with apical stereocilia, responsible for secretory and excretory functions. Additionally, smaller basal cells are located peripherally, contributing to detoxification; halo cells serve as immune cells and tall clear cells with absorptive functions. In the head, the tubules exhibit the smallest luminal diameter and the tallest epithelium, featuring a pseudostratified columnar structure with nuclei at the base. Conversely, in the tail, the tubules have a larger luminal diameter, and the epithelium is shorter, displaying a transition from cuboidal to simple columnar, accompanied by round to flattened nuclei (Figures 1– 3).

With the increase in doses of Neurokinin B (1ηg, 1µg), the lumen of caput got a significantly (p<0.001) dilated dose-dependently in Neurokinin B-treated animals. The lumen of the corpus got significantly

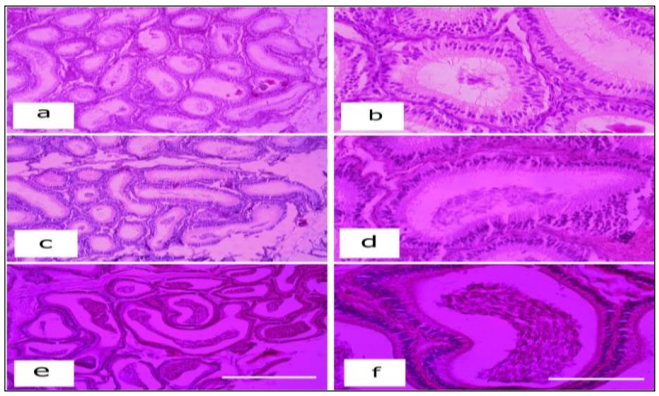

Figure-1: Photomicrographs of caput region of the epididymis of control Oryctolagus cuniculus and administered with 1ηg and 1µg of Neurokinin B. (a-b). Control caput of rabbit's epididymis, showing normal lumen. (c-d). The caput of rabbit's epididymis was administered with 1ηg Neurokinin B, showing lumen dilation. (e-f). Caput of rabbit's epididymis administered with 1µg Neurokinin B. A marked dilatation of the caput lumen was observed as compared to low dose and control. Magnification:- Scale bar, left panel 50µm, right panel20µm

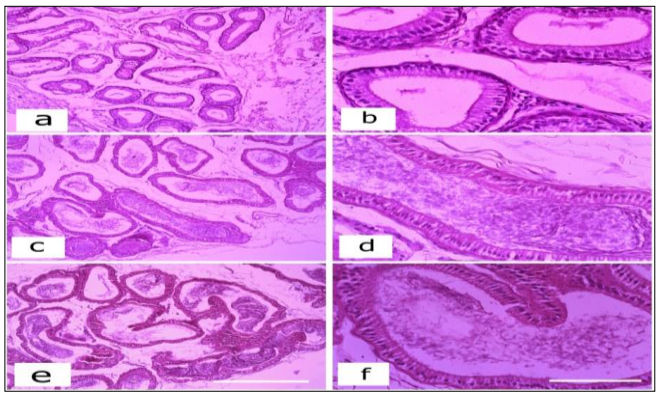

Figure-2: Photomicrographs of corpus region of the epididymis of control Oryctolagus cuniculus and administered with 1ηg and 1µg of Neurokinin B. (a-b). Control corpus of rabbit's epididymis, showing normal lumen. (c-d). Corpus of rabbit's epididymis administered with 1ηg Neurokinin B, showing lumen dilation. (e-f). Corpus of rabbit's epididymis administered with 1µg Neurokinin B, showing marked dilated lumen as compared to control and low dose. Magnification:- Scale bar, left panel 50µm, right panel 20µm

(p<0.001) dilated as the dose of Neurokinin B increased (1ηg,1µg). A dilation in the lumen of cauda of epididymis was observed significantly (p<0.001) after administration of Neurokinin B as compared to control (Figure-4). Sperm count increased significantly (p<0.001) in the Neurokinin B-treated rabbits as compared to control (Figure-5).

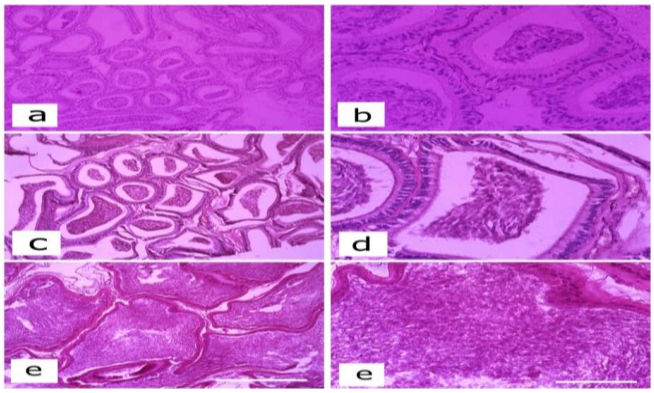

Figure-3: Photomicrographs of the cauda region of the epididymis of control Oryctolagus cuniculus administered with 1ηg and 1µg of Neurokinin B. (a-b). Control cauda of rabbit's epididymis, showing normal lumen. (c-d). Cauda of rabbit's epididymis administered with 1ηg Neurokinin B. Showing lumen dilation. (e-f). Cauda of rabbit's epididymis administered with 1µg Neurokinin B, showing marked dilated lumen as compared to control and low dose. Magnification:- Scale bar, left panel 50µm, right panel 20µm

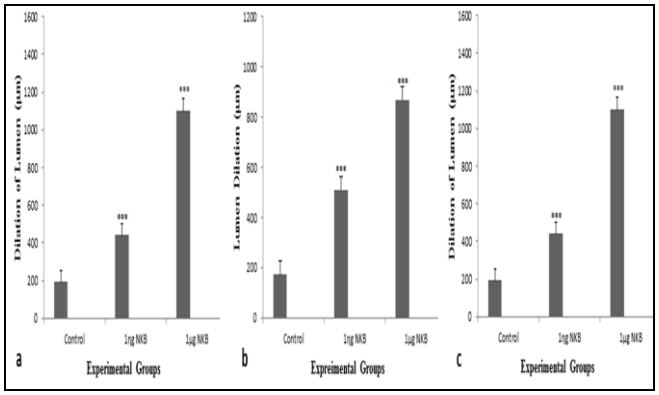

Figure-4: Lumen of caput (a), corpus (b) and cauda (c) regions of epididymis dilated at different doses of Neurokinin B as compared to control (*p<0.001)**

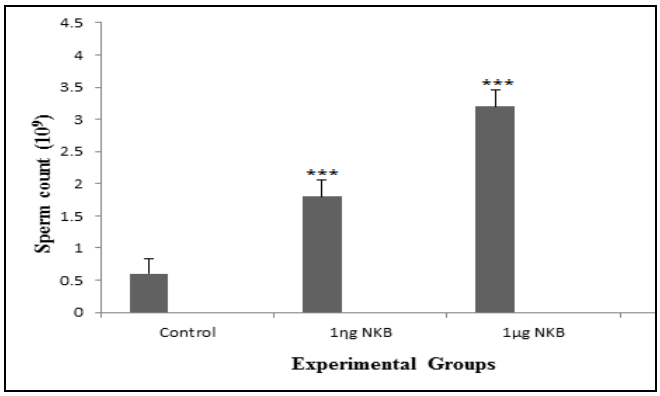

Figure-5: Sperm count (109) increased in Neurokinin B treated groups dose-dependently as compared to the control group (*p<0.001)**

DISCUSSION

Neurokinin B has been recently identified as a key player in regulating the reproductive axis. Effects of acute central and peripheral administration of Neurokinin B, six its agonist (Senktide),[7,8] and antagonist (SB22220),[9] on the reproductive axis have been explored previously. However, the effects of long-term (chronic) administration of these peptides on epididymis have yet to be investigated thoroughly. To this end, Neurokinin B at 02 different doses (1 µg, one ηg) was administered subcutaneously twice daily to adult male rabbits for 12 days continuously to investigate the effect of peptide administration.

The epididymis weight increased significantly with the Neurokinin B treatment compared to control. Histological analysis of epididymis sections revealed increased tubular diameter and epithelial height with Neurokinin B treatment.

The stimulatory effects of acute administration of Neurokinin B and its agonists on the reproductive axis have been demonstrated previously in mice and rats.[10] Pharmacologic manipulation of the reproductive axis is of substantial therapeutic importance in pathologic conditions, especially in puberty and fertility-related disorders and treatment of neoplasms of reproductive origin.[11]

The present study provides contrasting evidence to continuous Kisspeptin administration, which resulted in degenerative changes in testicular tissue and accessory sex glands in adult and prepubertal male rats.[12,13] Here, we report a stimulatory effect of Neurokinin B on epididymis in adult male rabbits. It was demonstrated that a deficiency of Neurokinin B resulted in pubertal delay and sexual maturation.[14] Garcia *et al*. demonstrated that Senktide (Neurokinin B agonist) stimulated the release of Kisspeptin and GnRH in prepubertal and pubertal male primates.[15]

In the present study, a significant increase was observed in the luminal diameter of the epididymis and sperm count in Neurokinin B-treated groups. Histomorphological examination of epididymis supported this phenomenon. Rea *et al.* categorised the Rattus. They demonstrated that gonadotropins and testosterone levels were decreased with a reduction of body weight and sperm count when the rats were injected with an antagonist of GnRH.[16] Histomorphological examination of different regions (caput, corpus, cauda) of the epididymis in the present study presents that exogenous Neurokinin B positively affects epididymis by increasing the luminal size and

sperm count. The diameter of the lumen of each region of the epididymis got dilated, and this was parallel to the study of Hamzeh *et al*. (2009), which stated that testosterone has a vital role in sustaining the structure and activity of the epididymis. Testosterone dilates the regressed epididymis after testosterone replacement therapy in orchidectmized Rattus, and the luminal diameter increased significantly in contrast to the control group.[17] McLachlan *et al*. demonstrated that withdrawal of LH results in the suppression of spermatogenesis and a significant reduction in the number of spermatogonia, spermatocytes and spermatids.[18] Presently, the increased luminal diameter of the epididymis and sperm count in Neurokinin B treated groups may be due to the increased production of gonadotropins and sex steroids.

Enhanced expression of Neurokinin B and tachykinin receptors were reported in rat and human testis.[2,19] Therefore, a direct effect may also be speculated for local regulation of reproduction at a 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.[20,21] It has been recently shown that a single injection of Neurokinin B or Senktide caused positive effects. In contrast, Neurokinin B antagonists caused degenerative changes in testicular tissue and the HPG axis.[22,23] Leydig cells are the only source of testosterone production in the testis; it is possible that Neurokinin B treatment modulated Leydig cell function and increased testosterone production. Observations of the present study further strengthen this possibility that Neurokinin B treatment resulted in increased sperm count in adult rabbits (Figure 5). LH receptors are present on the Leydig cells, and their stimulation resulted in increased testosterone production,[24] and both LH and testosterone are responsible for normal spermatogenesis in rats.[25]

This is the first study to investigate the effects of continuous administration of variable doses of Neurokinin B on the histomorphology of epididymis after their subcutaneous administration. Therefore, an exact comparison with other studies is not possible at present.

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CONCLUSION

The present study concluded that continuous administration of Neurokinin B could have a sensitising effect on the histomorphology of epididymis, characterised by increased luminal diameter and sperm count.

Conflic of Interest: None.

Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

FR & RZ: Data acquisition, data analysis, critical review, approval of the final version to be published.

MHR: Concept, Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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