ROLE OF APELIN IN SPERMATOGENESIS AND STEROIDOGENESIS IN THE MICE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF DOCTOR OF PHILOSOPHY

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MZU REGN NO.: 1904353
Ph.D. REGN NO.: MZU/Ph.D./1410 OF 01.08.2019

DEPARTMENT OF ZOOLOGY
SCHOOL OF LIFE SCIENCES
SEPTEMBER, 2023

ROLE OF APELIN IN SPERMATOGENESIS AND STEROIDOGENESIS IN THE MICE

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In partial fulfillment of the requirement for the degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl.

CERTIFICATE

I certify that the thesis entitled "**Role of Apelin in spermatogenesis and steroidogenesis in the mice**" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **Milirani Das** is a record of research work carried out during the period of 2019-2023 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or institution of higher learning.

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DECLARATION

I, **Milirani Das**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any other University or Institute.

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ACKNOWLEDGMENT

This thesis becomes a reality with the kind support I received from many individuals, and I would to express my sincere thanks to them.

Foremost, I express my heartfelt gratitude to my supervisor, **Dr. Vikas Kumar Roy**, Associate Professor, Department of Zoology, Mizoram University. His scholarly advice, meticulous scrutiny and scientific approach had helped me developed for the better and in the completion of this thesis. I am proud and honored to be his Ph.D. student, for I have learnt a great deal of techniques, innovative ideas, and life philosophy too. His motivation and understandings towards his students must be appreciated for creating a suitable laboratory environment, and lessen our mental stress.

I am grateful to the present and former heads of the Department of Zoology, and **Prof. G. Gurusubramanian** for providing me departmental facilities. Their encouragement and support have helped me throughout my research work.

I am highly obliged to thank teachers of Zoology Department: **Dr. H.T. Lalremsanga, Dr. Amit Kumar Trivedi, Dr. Esther Lalhmingliani, Dr. Zothansiama, Dr. Kiran Ramesh Karat, Dr. Vabeiryureila Mathipi** for their kind suggestions and scientific inputs during my Ph.D. tenure.

I express my deep gratification to my labmates **Dr. Jeremy Malsawmhriatzuala, Dr. Annie Lalranwgbawli**, **Chuckles Ch. Marak, Vanlalrempuia, Anima Borgohain, Nicy Vanrohlu, Rahul Kumar and Vikash Kumar** for their unconditional support, motivation and being my source of inspiration.Last but not the least, I would like to convey sincere thanks to my Parents **Mr. Laxmidhar Das and Smt. Pramila Das** and all the family members for supporting me financially and making this work a successful one.

(MILIRANI DAS)

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ABBREVIATIONS

µl	Microlitre
µm	Micrometre
Akt	Protein Kinase B, PKB
ANOVA	Analysis of Variance
APJ	Apelin receptor
AR	Androgen receptor
BAX	BCL2 Associated X- protein
BCL2	B-cell lymphoma-2
BrdU	Bromodeoxyuridine
Caspase-3 protease-3	Cysteine dependent aspartate directed
DAB- 3,3'	diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's Modified Eagle Medium
DPX	Dibutyl phthalate Polystyrene Xylene
ECL	Electrochemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
ERα and β	Estrogen receptor α and β
g	Gram
GCNA	Germ Cell Nuclear Antigen
h	hour
H&E	Hematoxylin and Eosin
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
IgG	Immunoglobulin G
MDA	Malondialdehyde
mg	Milligram
min	Minute
ml	Millilitre

mm	Milimolar
n	Number
NF-κB	Nuclear factor kappa B
nm	Nanometre
nmol	Nanomole
ML221	Apelin receptor antagonist
NaCl	Sodium Chloride
p-Akt	Phosphorylated Akt
PBS	Phosphate buffer saline
PBST	Phosphate buffered saline tween 20
PCNA	Proliferating Cell Nuclear Antigen
pH	potential of Hydrogen
PND	Postnatal development
PVDF	Polyvinylidene fluoride
RPM	Revolutions Per Minute
SDS-PAGE- Sodium dodecyl sulfate	Polyacrylamide gel electrophoresis
UV	Ultraviolet

General Introduction

Adipose tissue, commonly known as fat tissue, is now identified as an active endocrine organ that secretes various bioactive molecules called adipokines or adipocytokines **(Gyamfi et al., 2019; Liang et al., 2019).** These adipokines play a crucial role in regulating metabolic and reproductive functions in both males and females. Some of the well-known adipokines include leptin, adiponectin, resistin, visfatin, apelin and many others. These adipokines are involved in a complex interplay with the endocrine system, influencing multiple physiological processes **(Kershaw and Flier, 2004)**. In terms of reproduction, adipokines can have significant effects on fertility, menstrual cycle regulation, and sex hormone production in both males and females. For example, adipokines such as leptin and adiponectin have been shown to influence the hypothalamic-pituitary-gonadal (HPG) axis, which is a key regulator of reproductive function. In males, adipokines also regulates reproductive functions. They can affect testosterone production, secretion, sperm quality, and overall fertility **(Elfassy et al., 2018)**.

Furthermore, adipokines are involved in regulating metabolism, insulin sensitivity, inflammation, and energy balance, all of which can indirectly impact reproductive functions. Disorders related to adipokine imbalance, such as obesity, can lead to disruptions in reproductive health in both males and females **(Zorena et al., 2020)**. Adipokines have been linked to adipocyte differentiation, energy metabolism, insulin resistance, inflammation, immunology, cancer, and angiogenesis **(Badman and Flier, 2007)**. It is widely established that an excess or shortage of white adipose tissue affects puberty, sexual development, and fertility in several animals. Furthermore, differences in white adipose tissue quantity alter adipokine expression and concentrations in serum **(Dupont et al., 2012)**. These adipokines and their receptors were also localised and expressed in the testes, suggesting a local synthesis and role of these adipokines animals and humans **(Estienne et al., 2019)**. However, only a small number of studies have investigated the role of adipokines in reproductive activities in the case of ageing, obese, and T2D (type 2 diabetes

mellitus) males **(Liang et al., 2019)**. Despite various adipokines, the role of apelin has not been investigated in details in any mammalian testis.

Apelin and Apelin Receptor

Apelin is a peptide hormone that plays a role in various physiological processes in the human body. It is primarily produced in the heart, lungs, brain, adipose tissue, uterus, ovary and testis. Apelin binds to a specific receptor called the apelin receptor (APJ) to exert its effects. Apelin was isolated and identified as an endogenous ligand for APJ, an orphan G-Protein Coupled Receptor (GPCR). The human apelin (APLN) gene encodes a 77-aa preproprotein. After cleavage of the signal peptide, the 55-aa proprotein forms multiple active fragments, including APLN-36, APLN-17, and APLN-13. APLN-13 is highly active and is responsible for APJ binding and biological functions of mature apelin. Apelin appears to play a function in both peripheral and central food intake control. However, given the contradictory nature of the results gathered so far on the central effects, further research is needed to highlight the mechanism of actions of apelin **(Goazigo et al., 2009)**. Apelin and APJ have been found in pancreatic beta cells from a variety of mammalian species **(Ringström et al., 2010)**. Apelin expression has also been found in human and mouse alpha cells, as well as a subset of pancreatic polypeptide (PP) cells. One of the well-known functions of apelin is its cardiovascular effects. It acts as a vasodilator, meaning it widens the blood vessels, leading to a reduction in blood pressure. This mechanism suggests that apelin may have potential applications in the treatment of hypertension and cardiovascular diseases **(Tatemoto et al., 2001)**.

Apelin is also involved in the regulation of fluid balance and body water homeostasis. It can influence water intake and urine production by acting on certain areas of the brain that control these processes **(Hu et al., 2021)**. Furthermore, apelin has been implicated in the regulation of energy metabolism, insulin sensitivity, and glucose homeostasis, making it a target of interest in research related to obesity and diabetes **(Knauf et al., 2013)**.

Additionally, apelin has been found to have effects on the central nervous system. It plays a role in neuroprotection, neuronal development, and the regulation of

neurotransmitter release. Studies have suggested its involvement in modulating anxiety, stress responses, and the reward system **(Szczepanska-Sadowska et al., 2010)**. The understanding of apelin's role in various systems may lead to the development of novel treatments for cardiovascular diseases, metabolic disorders, and neurological conditions in the future.

Apelin system in male reproduction

There were only two published studies until 2018 that show the influence of apelin on male reproduction. In male rats, apelin-13 infusion substantially decreased LH release compared to vehicle values, while FSH levels did not change significantly across groups **(Sandal et al., 2015; Tekin et al., 2016)**. Furthermore, serum testosterone levels were statistically lower in the apelin-13 group than in the control group; histological examination revealed that apelin-13 infusion significantly reduced the number of Leydig cells, implying that apelin may play a role in central regulation and decrease testosterone release by suppressing LH secretion. Finally, these researchers concluded that the APJ agonist may be a valuable medication in the treatment of male infertility **(Sandal et al., 2015).** Despite above two studies, recently, the work on apelin system in the testicular activity has gained some momentum including our work in the present dissertation (**Trosi et al., 2022; Brzoskwinia et al. 2020; Akkan et al, 2020; Das et al., 2021; 2022; 2023).** Furthermore, apelin and APJ were found in the Leydig cells that suggests its possible role on the testicular steroidogenesis **(Brzoskwinia et al. 2020)**. It has also been shown the presence of the apelin-APJ system in the dog testes as previously reported in the testes of adult rats **(Trosi et al., 2022; Brzoskwinia et al. 2020).** The presence of apelin system in the dog testis suggests potential function of the apelin/APJ system in both normal and pathological conditions in dogs **(Trosi et al., 2022)**. The apelin and APJ expression was found to be higher in control rat testis compared to the varicocoele groups **(Akkan et al, 2020).** It has been suggested that apelin may play a role in the varicocoele etiopathogenesis.

Recently, apelin was measured by Enzyme Linked Immune Sorbent Assay (ELISA) in seminal fluid, spermatozoa, and seminal fluid and testis of human. Apelin was

found in spermatozoa, and its levels were negatively correlated with normal sperm morphology and positively with IL-1β levels. In the fertile males, APJ-immunofluorescence was faint in the sperm tail, but they were strong along the tail, cytoplasmic residues, and post-acrosomal sheath. In the testis, apelin and APJ labels were strong inside the seminiferous tubule and visible in Leydig cells. It has been suggested that the presence of apelin/APJ system in the human spermatozoa and testicular tissue might be important in human fertility **(Moretti et al., 2023).**

Adipokines in postnatal developmental process

Evidence suggests that adipokines play important roles in postnatal development. In mice, leptin plays an important role in the development of hypothalamic feeding circuits during the first three weeks of life by regulating neurogenesis, axon growth, and synaptogenesis **(Bouret et al., 2004; Bouret 2010)**. Additionally, this postnatal leptin surge is required for the production of beige adipocytes during development. Leptin deficiency during development impairs the formation of projections from the arcuate nucleus of the hypothalamus (ARH) to other brain regions involved in energy homeostasis, including the paraventricular nucleus (PVH), dorsomedial hypothalamic nucleus (DMH), and lateral hypothalamic area (LHA). During development, an excess or deficit of leptin changes the production of projections in these circuits **(Bouret et al., 2004; Bouret 2010; Wu et al., 2020)**.

Adiponectin is an adipokine that correlates negatively with adult obesity (**Arita et al., 1999)**. It is also abundant in the cord blood of term infants, with concentrations two to three times higher than in adults **(Weyermann et al., 2006)**. However, investigations on the connection between maternal serum/cord blood adiponectin levels and newborn birth weight have shown widely divergent results, ranging from an adverse association to no correlation to even a positive relationship. Little research examined at the long-term associations between levels of adiponectin and development **(Mantzoros et al., 2009)**.

In context to adipokines and its expression during postnatal testicular development, only a few adipokines have been described in the testis during postnatal developmental stages. It has been shown that leptin, which also control metabolism

and reproduction leptin is expressed in mouse testis in a cell-type and stage-dependent manner using immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) **(Herrid et al., 2008).** Leptin expression was mostly localised to gonocytes in the testes of 5-day-old mice, whereas leptin immunostaining was restricted to spermatogonia in the testes of 10-day-old animals. Leptin and its receptor isoforms, Ob-Ra, Ob-Rb, and Ob-Re, were all expressed in the testes of 5- to 60-day-old mice and it has been shown that leptin primarily affects the hypothalamic-pituitary axis along with its direct effects on the proliferation, differentiation, and regulation of testicular steroidogenesis by autocrine and paracrine pathways **(Herrid et al., 2008)**. Leptin was also proposed to play a direct function in the proliferation and differentiation of germ cells through the phosphorylation of signal transducer and activator of transcription-3, which may help to partially explain the sterility observed in leptin-deficient mice **(El-Hefnawy et al., 2000)**.

Adiponectin receptors expression in the mouse testis has also been shown to be modulated from postnatal and weaning stages and it was demonstrated that adiponectin stimulates spermatogenesis but inhibits the production of testosterone and estradiol in the testes **(Choubey et al., 2019**). The expression of the adiponectin gene in the testes appears to be regulated by developmental signals and hormonal influences. Before puberty, there were modest mRNA levels of adiponectin in the rat testis, which considerably rose following pubertal development **(Caminos et al., 2008)**. Resistin gene expression was shown to occur throughout postnatal development, peaking in adult rat testis. Resistin peptide was immunodetected in interstitial Leydig and Sertoli cells in seminiferous tubules during developmental stage. Resistin's expression in the testis was regulated by pituitary gonadotropin hormones and had stage-specificity, reaching its maximal levels between Stages II and VI of the seminiferous epithelial cycle **(Nogueiras et al., 2004)**. The other adipokines, visfatin, which expression in the testis was developmentally regulated. Visfatin has been found in mouse testis in both Leydig cells and germ cells, which suggests that it has a function in spermatogenesis and testicular steroidogenesis **(Rempuia et al., 2023)**. By using an enzyme immunoassay, plasma visfatin levels,

was shown to be higher in adult male chickens than pre-pubertal chickens. It was suggested that visfatin is involved in testicular activities including spermatogenesis and steroidogenesis **(Ocón-Grove et al., 2010).** However, the postnatal developmental expression of apelin and APJ has not been investigated in the testis of any mammalian species. Throughout postnatal developmental testis requires a number of factors for optimum testicular activity during later stages. Spermatogenesis, the process through which spermatogonial stem cells differentiate into mature spermatozoa, occurs throughout a man's life. This cycle is controlled by a complicated mechanism in the testis that includes endocrine signalling, physical interactions between germ and somatic cells, spermatocyte meiosis, and timely discharge of spermatozoa. It has been shown that Epas1 activation are important components of spermatogenesis and disruption of Epas1 during postnatal stage leads to male infertility as well as diminished testis size and weight **(Gruber et al., 2010).** Spermatogenesis is the process by which sperm is produced from primordial germ cells. When vertebrate primordial germ cells (PGCs) reach the genital ridge of a male embryo, they integrate into the sex cords. When they reach maturity, the sex cords hollow out to produce seminiferous tubules, and the tubules of epithelium develop into Sertoli cells **(Zhao et al., 1996)**.

In the testis of postnatal mice, the first wave of spermatogenesis occurs in synchrony. New germ cell types begin to fill the testis at certain postnatal days; as a result, more developed germ cell types gradually arise until the full spectrum is present **(Russell et al., 1990)**. Sustained spermatogenesis in the adult is dependent on a successful first wave of spermatogenesis and the establishment by apoptosis of suitable germ cell numbers in proportion to the size of the Sertoli cell population and dysregulation of apoptosis can lead to developmental abnormalities or contribute to diseases later in life **(Orth et al., 1988; Russell and Griswold, 1993)**. Thus, the question arises, whether the expression of apelin and APJ are developmenatly regulated in the mice testis? Whether, APJ signalling could be involved in the process of proliferation and apoptosis. To best of our knowledge, no study has been conducted on the postnatal developmental expression of APJ in the mice testis.

It has been reported that acrosomal leptin receptor expression is linked to cholesterol efflux and acrosome response, whereas leptin receptor expression in tail of human sperm may indicate leptin's control of hyperactivated sperm motility. Leptin STAT3 signalling may allow undifferentiated germ cells to proliferate without losing their potency, while also causing late-stage spermatocytes to grow and differentiate **(Phillips et al., 2010)**. Furthermore, leptin influences the nutritional support for spermatogenesis provided by human Sertoli cells (**Alves et al., 2016**). Indeed, a Portuguese study discovered that leptin significantly reduces acetate synthesis by human Sertoli cells, a key molecule for spermatogenesis **(Phillips et al., 2010)**.

Adipokines and steroidogenesis

Steroidogenesis refers to the processes that convert cholesterol into physiologically active steroid hormones. Steroid hormones control a vast range of developmental and physiological processes from infancy through adulthood. Steroidogenesis is best understood as a single process that is repeated in each gland with cell-type-specific variations on a single theme, in contrast to the way that most endocrine texts typically cover adrenal, ovarian, testicular, placental, and other steroidogenic processes. Understanding the biochemistry of the numerous steroidogenic enzymes and cofactors as well as the genes that encode them is therefore essential to comprehending steroidogenesis **(Miller and Auchus, 2011)**. In the testis, Leydig cell mainly synthesizes and secrete male hormone, testosterone.

Leptin and leptin expression in the testis have already been described. It has been shown that it exerts direct regulatory activity at many levels of the male gonadal axis, involving both stimulatory and inhibitory actions **(Tena-Sempere and Barreiro 2002; and Kawwass et al., 2015).** Resistin is expressed specifically in rat testicular Leydig cells throughout the postnatal period, indicating that resistin plays a direct function in testicular steroidogenesis **(Nogueiras et al., 2004).** Basal and human choriogonadotropin-stimulated testosterone secretion was considerably reduced by recombinant adiponectin **(Caminos et al., 2008)**. Chemerin and its receptors are also expressed in human and rat testes, where they decrease Leydig cell steroidogenesis, implying that chemerin has direct anti-gonadal effects on testicular

steroidogenesis **(Li et al. (2014; Singh et al. (2018).** It has been proposed that NUCB2/nesfatin-1 expression in mouse Leydig cells is regulated by the hypothalamic-pituitary-gonadal axis, and that nesfatin-1 expression by Leydig cells may regulate steroidogenesis in autocrine manner **(Ahn et al., 2023 Ranjan et al., 2019;Ranjan, et al., 2020).**

Researchers discovered that administration of apelin (a pro-obesity peptide) decreased blood levels of LH, FSH, and testosterone and had a detrimental influence on reproductive function in the animals studied. The lack of substantial differences in GnRH immunostaining across groups shows that apelin-13 suppressed gonadotropin (LH and FSH) release at the pituitary level **(Tekin et al., 2016)**. It is also reported that intracerebroventricular infusion of apelin-13 induced a substantial decrease in serum LH, indicating that it plays a function in central reproduction control by decreasing LH secretion **(Sandal et al., 2015)**. These two studies on the possible role of apelin in the testicular activity have suggested an inhibitory role of apelin on testicular hormone secretion by infusing apelin 13 peptides. However, the role of endogenous APJ is not clear from these studies. Thus, it would have been interesting to inhibit the endogenous APJ signalling by its inhibitor to unravel its role in the testicular hormone secretion.

Cytokines/adipokine and seminiferous epithelium cycle

The seminiferous epithelium cycle refers to the series of cellular changes that occur within the seminiferous tubules of the testes during the process of spermatogenesis, which is the production of sperm cells. The seminiferous tubules are highly coiled structures within the testes where spermatogenesis takes place. The walls of these tubules are lined with a specialized epithelium consisting of different types of cells, including Sertoli cells and germ cells (spermatogonia, spermatocytes, spermatids, and spermatozoa) **(Clermont 1972)**. The seminiferous epithelium cycle is characterized by a sequence of cellular events that occur in a synchronized and repeating pattern. It can be divided into several stages, each representing a distinct phase of spermatogenesis. The secondary spermatocytes undergo meiosis II, resulting in the formation of haploid spermatids. Spermatids undergo a series of

structural changes, including the development of a flagellum (tail) and the formation of acrosomal vesicles at the head region. This transformation results in the formation of mature spermatozoa, also known as sperm cells.

The duration and number of stages in each spermatogenesis cycle varied amongst mammals **(Berndston 1977)**. Each cycle in adult rats has 14 stages and takes 12.9 days to complete, with the duration of the seminiferous epithelial cycle in rats being 48-53 days, but mice and humans have just 12 and 6 stages, respectively.

In rats or mice, extensive junction remodelling occurs during stage VIII of the seminiferous epithelial cycle. Spermiation occurs at the luminal border of the apical compartment of the epithelium during this stage to discharge elongated spermatids (i.e., spermatozoa) into the lumen of the seminiferous tubule **(Mruk and Cheng 2004)**. Cytokines, specifically tumour necrosis factor (TNF) and transforming growth factor-3 (TGF-3), are thought to be germ cell signals that function in tandem with androgens, such as testosterone, generated by Leydig cells to control these activities **(Michelle et al., 2009)**.

Another cytokine involved in the regulation of junction dynamics during spermatogenesis is interleukin-1 (IL-1). It was shown that treatment to adult rat testes increased BTB permeability **(Sarkar et al., 2008)**. Pachytene spermatocytes in immature rats (Haugen et al., 1994) and Sertoli cells **(Jonsson et al., 1999; Gerard et al., 1991)** expressed IL-1. Its release by Sertoli cells is dependent on the existence of germ cells, since IL-1 was not found in the testis after busfulan-induced azoospermia or after foetal irradiation to knock down germ cells **(Jonsson et al., 1999)**. Except for stage VII, when it was expressed at a very low level; IL-1 was expressed in the seminiferous epithelium at all stages of the epithelial cycle **(Jonsson et al., 1999; Wahab-Wahlgren et al., 2000)**. This contrasts with the high levels of TNF and TGF3 seen throughout stages VII-VIII of the seminiferous epithelial cycle. IL-1 acts biologically by attaching to its receptor, which is found in both Sertoli and germ cells **(Gomez et al., 1997)**. In the mouse testis, on postnatal day 20, the seminiferous epithelium's spermatocytes at stages VII to XII of the cycle showed stage-specific expression of leptin (**Herrid et al., 2008)**. Similarly, resistin also

showed stage specific presence in the testis and had stage-specificity, reaching its maximal levels between Stages II and VI of the seminiferous epithelial cycle **(Nogueiras et al., 2004)**. Based on these evidences from the different expression pattern of factors during seminiferous tubule epithelium cycle, it might be suggested that expression of apelin and APJ in the mice testis could also be stage dependent. However, stage dependent localization of apelin and APJ has not been investigated yet.

Adipokines and diabetes

Adipokines play a significant role in various physiological processes, including energy metabolism, inflammation, and insulin sensitivity **(Kwon et al., 2013)**. They act as signaling molecules, regulating communication between adipose tissue and other organs such as the liver, muscle, and pancreas. In the context of diabetes mellitus, which is a metabolic disorder characterized by impaired insulin function and elevated blood glucose levels; adipokines have been found to influence several aspects of the disease **(Al-Badri et al., 2015).** Diabetes can have various effects on male fertility such as it affects sperm quality, including sperm count, motility, and morphology. High blood sugar levels and oxidative stress associated with diabetes can contribute to these abnormalities. Diabetes can lead to decreased testosterone production, which can affect sperm production and function **(La et al., 2012; Zhong et al., 2021)**. Testosterone is essential for healthy sperm development and maturation. Diabetes-related oxidative stress can cause DNA damage in sperm. This can affect the genetic material carried by sperm, potentially leading to infertility.

Resistin is an adipokine that directly produces insulin resistance in muscles and liver. It stimulates the expression of endothelin-1 messenger RNA in endothelial cells, contributing to endothelial dysfunction. It also raises the expression of the cellular adhesion molecule VCAM-1 and the MCP-1, both of which are involved in the creation of early atherosclerotic lesions **(Steppan et al., 2001)**. Resistin also promotes cardiovascular disease through proinflammatory pathways **(Verma et al., 2003)**. Resistin serum concentrations are higher in obese people **(Degawa-Yamauchi et al., 2003)**, as well as in T2D patients **(Youn et al., 2004)**. Omentin, a

newly discovered adipokine, has been shown to have anti-inflammatory properties, which may give a potential therapeutic target **(Yamawaki et al., 2011)**. Study suggests that omentin directly enhances endothelial function in diabetics and CVD risk factors **(Hayashi et al., 2019)**. This might be a valuable indicator of vascular dysfunction in type 2 diabetes individuals.

Despite above mentioned studies in relation to diabetes and adipokines, the expression of testicular adipokines in diabetic condition still needs further investigation. To best of our knowledge, only a few works has been done in relation to adipokine and diabetic testis. Visfatin expression has been shown to be deregulated in the diabetic rat testis **(Gurusubramanian and Roy 2014).** In the testis of HFD/STZ-induced T2D mice, adiponectin controls glucose and lactate homeostasis and decreases apoptosis and it has been shown that treatment with adiponectin and nesfatin- improves testicular steroidogenesis, metabolism, oxidative stress, and apoptosis in T2D animals **(Choubey et al., 2020; Rajan et al., 2020).** Recently only, the expression of apelin and APJ has been reported in the mouse diabetic testis by our laboratory **(Das et al., 2021).** In addition, apelin system has been shown to be deregulated in human and mice diabetic testis and suggested that targeting apelin system during diabetic-associated pathogenesis of testicular impairment could be used for therapeutic potential **(Song et al., 2022)**.

Therefore, the present dissertation evaluated the postnatal developmental changes of apelin and APJ expression in mice testis along with its possible involvement in steroidogenesis, proliferation and apoptosis at juvenile and adult age. Furthermore, the seminiferous epithelium stage dependent localization of APJ has also been performed. The present dissertation also elucidated the role of apelin in the pathogenesis of diabetic testis in mice model.

CHAPTER 1

Title

To study the age dependent changes in the testicular apelin and its receptor*

*** Journal of Steroid Biochemistry and Molecular Biology 224 (2022) 106178**

Introduction

Spermatogenesis in testis known be initiated at neonatal stages, where quiescent germ start differentiation, which results in appearance of first wave of spermatogenesis at postnatal (PND) age of 6 weeks **(Busada et al., 2016; Busada and Geyer 2015)**. It has been reported that at juvenile stage (PND20), round spermatids appear whereas sperm appears at PND35 in the mice testis **(Bellve et al., 1977)**. It has been emphasized that the first wave of spermatogenesis at early stage is a well-regulated and synchronized processes, where at different stage of postnatal life, germ cells proliferate and differentiate, and this is an important event for maturation of germ cells **(Russell et al., 1990).** The establishment of successful first wave of spermatogenesis is critical for continuous spermatogenesis throughout adulthood **(Orth et al., 1988; Russell and Griswold, 1993)**. Furthermore, this successful wave of spermatogenesis requires appropriate germ, Sertoli cell number, and the number of these cells is established by apoptosis. Apoptosis is important and constant feature of testis during early developmental stages and adulthood as well **(Shaha et al., 2010)**. Germ cell apoptosis during first wave of spermatogenesis is required to eliminate the abnormal germ cells from the testis **(Furuchi et al. 1996; Rodriguez et al., 1997, Russell et al., 2002; Yan et al., 2000).** Therefore, regulation of testicular activity by various endogenous factors in early postnatal period, such as juvenile stage is important to study.

It is believed that endogenous factors, such as hormones, mainly testosterone, estrogen, and follicle-stimulating hormone along with other endocrine and paracrine/autocrine players also regulate testicular activity in relation to germ cell proliferation and apoptosis (**Migrenne, et al., 2012; Catizone et al., 2006).** It has been evidenced that differentiation of Leydig cell occurs after initial differentiation of Sertoli cells **(Habert et al., 2001).** Despite germ cell proliferation, and apoptosis, pre-pubertal testis also synthesizes testosterone by

Leydig cells. Testis of both rat **(Habert and Picon 1984)** and mice **(Livera et al., 2006)** has been shown to synthesize testosterone from 15.5dpc and 14.5 dpc respectively. It has also been shown that treatment of testosterone suppresses the apoptosis during first wave of spermatogenesis **(Rodriguez et al., 1997).** Furthermore, not only testosterone rather estrogen also regulates proliferation and maturation of cells in the testis **(Mahmoud et al., 2015).**

Apelin receptor, (APJ) is a classical heterotrimeric guanine nucleotide binding protein (G-protein) coupled receptor (GPCR), and the physiological function and signal transduction produced by the combination of Gαi protein subtypes **(Chapman et al., 2014)**. It has been shown that APJ signaling is mediated by activation of Gαi2 or Gαi3 through molecular rearrangements, however, APJ signaling activation might also be mediated by Gαo and Gαq through classical dissociation model **(Bai et al., 2014)**.

The expression of apelin and apelin receptor (APJ) has been shown in the testis of rat and mice **(Akkan et al., 2020; Brzoskwinia et al., 2020; Das et al., 2021).** Localization study has shown the presence of Apelin and AP in the Leydig cell as well as in the seminiferous tubules, which suggests a possible role of apelin and APJ in testicular steroidogenesis and germ cell proliferation in the adulthood (**Brzoskwinia et al., 2020; Das et al., 2021).** To best of our knowledge, there is paucity of literatures on role of apelin on the testis. A previous study by **Sandal et al (2015)** has shown that apelin 13, infusion in male rats suppresses testosterone synthesis by decreasing LH secretion. These studies have provided evidence of apelin and APJ in the testis with potential role in adult stage, however, the ontogeny of apelin and APJ in postnatal stage and its function remains unclear in the mouse during early developmental stages. Apelin has been shown to inhibit apoptosis and promotes proliferation in rat granulosa cells as well as in mouse osteoblastic cell line via Akt signaling **(Shuang et al., 2016; Tang et al., 2007)**. Since the first wave spermatogenesis involves, Leydig cell steroidogenesis, germ cell proliferation and germ cell loss by apoptosis, thus question arises, whether apelin can influences these functions during first wave of spermatogenesis or not.

Moreover, it is also important to analyse the developmental expression of apelin and its receptor.

Therefore, the aims of present study were to investigate the developmental expression of apelin and APJ in the mice testis, also to unravel the role of apelin and APJ during first wave of spermatogenesis in relation to hormone secretion, proliferation and apoptosis.

Materials and methods

Animals

Breeding cages were set with healthy adult Swiss albino mice and were maintained in aerated polypropylene cage with *ad libitum* food and water, under standard experimental conditions (12 hrs light-dark cycles, 25±2ºC). All the animal experiment was approved by Mizoram University Institutional Animal Ethical Committee (MZUIAEC-9), Mizoram University, Mizoram, India. Male pups of different postnatal day 1 (PND1), 7 (PND7), 14 (PND14), 21 (PND21) and 42 (PND42) were used for the postnatal study.

Sample collection

Mice at PND1 (n= 10), PND7 (n=10), PND 14 (n= 5), PND 21 (n=5), PND 42 (n= 5), were sacrificed. One side of testis was fixed in Bouin's fluid for immunohistochemistry and contralateral testis was freezed for western blot analysis. The serum sample was collected from blood by centrifugation at 2500 RPM and stored at -20ºC for hormone assays.

In vivo study

To unravel the role of apelin receptor (APJ) in the testis during juvenile stage, the antagonist of APJ (ML221) was given from PND14 to 20. Male mice PND14 were randomly divided into two 1. Control group (CON, n=5) and 2. Treated group (ML221, n=5). ML221 (cat # SML0919, Sigma Aldrich, St Louis, USA) dissolved in DMSO and then diluted with double distilled water. ML221 was given at dose

of 150 µg/kg based on previous study **(Hall et al., 2017)**. The duration was for 7 days, from PND14 to 20, because during PND14 to 20, expression of APJ was up regulated, therefore, this duration was selected for ML221 treatment. Equal amount of vector (DMSO diluted with distilled water) was injected to the control animals. On 21th day the animals were sacrificed and tissues collected and stored in Bouin's fixative solution for histology and immunohistochemistry and at -20ºC for western blotting. Serum samples were collected after centrifugation of the blood for different hormone assays.

***In vitro* Study**

In vitro culture of mice testes was performed to study the direct effect of ML221 on testicular activity. The testes were taken from PND 18 mice and were cleaned of adhering tissue. Testes excised, were cultured in two groups, first were untreated (CON) and second were apelin receptor antagonist (ML221) treated group. Tissues were cut in to equal pieces, and the testes explants were cultured for 24 h according to the method described earlier **(Jeremy et al., 2019)**. briefly, the testes were cut in equal fragments and cultured as control with no treatment and ML221 (50 µM) **(Huarong et al., 2019; Ishimaru et al.,2017)** (cat # SML0919, Sigma Aldrich, St Louis, USA) in a DMEM: Ham'F12 medium (1:1) with penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 at 37 °C for 24 h. The tissue and media were harvested stored at -20°C for western blot analysis and hormone assay.

Histology and Histomorphometric study

The fixed testes were properly dehydrated by gradually ethanol grade, embedded in paraffin for histomorphometry and immunohistochemistry as earlier described **(Jeremy et al., 2019).** Then paraffin block was sectioned at 7 µM thick by Leica rotary microtome (RM2125 RTS). The sections were spreaded on a glass slide coated with poly-*L*-lysine and incubated overnight at 37°C. Hematoxylin and Eosin staining was performed by following deparaffinization, rehydration, staining, dehydration, clearing; mount with DPX **(Bancroft and Gamble, 2008)**.

The mounted slides were observed and seminiferous tubule diameter, germinal epithelial height and lumen diameter were measured by using an ocular micrometer.

Immunolocalization of apelin, apelin receptor (APJ), PCNA and GCNA

Apelin and apelin receptor (APJ) were localized in postnatal developmental groups (PND1, 7,14,21 and 42) and the proliferating markers, PCNA and GCNA were performed in *in vivo* treatment groups (CON and ML 221) as described earlier **(Annie et al., 2020)**. Briefly, the spread sections were deparaffinized in xylene, rehydrated in ethanol and distilled water and blocked by goat serum (1:100, Lot# H2114; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at the room temperature. Then, the tissue sections were incubated with the primary antibodies to apelin (1:50, cat # SAB4301741; Sigma Aldrich, St Louis, USA), APJ (1:100, cat # ABD43; EMD Millipore Corporation, USA) and PCNA (1:100, cat # SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA, USA)for overnight at 4ºC.The primary antibody wash was done with phosphate-buffered saline (PBS), and the tissue sections were incubated with Horseradish peroxidase (HRP) tagged goat anti-rabbit immunoglobulin secondary antibody for 3 h at the room temperature. After secondary incubation the sections was washed in PBS. The GCNA immunolocalization was done by the primary antibody GCNA (1:200, cat # 10D9G11; DSHB, University of Iowa, Iowa, USA) followed by the goat anti-mouse secondary antibody conjugated with horseradish peroxidase. Then the slides were washed in PBS and developed by using 0.05% diaminobenzidine (DAB) solution (3,3'-diaminobenzidine tetrahydrochloride, in Tris-HCl buffer, pH 7.6 and hydrogen peroxide). The apelin and APJ sections were counterstained with hematoxylin, and then the stained sections were dehydrated and mounted by DPX. The negative control slides were run with 1% rabbit non-immune IgG for apelin, APJ to check the specificity of the primary antibody. The slides were observed and photographed by Nikon microscope (E200, Nikon, Japan).

The PCNA and GCNA staining in control and ML221 mice testes were calculated by ImageJ software. The stained area by DAB for PCNA and GCNA in the testis

was acquired by using threshold tool of ImageJ as described previously **(Jensen et al., 2013)**, and the data was presented as percentage area of PCNA and GCNA staining. The percentage area for immunostaining was described earlier **(Annie et al., 2020)**.

Western blot analysis

Western blotting was performed in testis sample by following the standard protocol of **Jeremy et al., 2019.** Briefly, 10% (w/v) tissue homogenate were prepared with lysis buffer, containing 0.01 M Tris-HCl (pH 7.6), 1.0 mM EDTA, (pH 8.0),0.1 M NaCl,100 µg/ml phenylmethylsulfonyl fluoride, and the protein concentrations were estimated by Bradford method **(**Bradford 1976**)**, The equal amount (50 µg) of protein from each group loaded to each well along with molecular weight marker in a 10% SDS-PAGE. The resolved proteins were transferred to PVDF membrane using wet transfer apparatus for 12 hrs, after successful protein transfer the membrane blocked for 30 min at the room temperature with 5% non-fat skimmed milk prepared using PBST(cat # GRM1254-500G; HiMedia Laboratory private limited, Mumbai, India) and then overnight incubated at 4ºC with the primary antibodies to apelin receptor (APJ) (1:2000, cat # ABD43; EMD Millipore Corporation, USA), androgen receptor (1:250, Ref # PA5-16363; Thermo Fisher Scientific, USA), antiapoptotic marker BCL2 (1:2000,cat # SC7382; Elabscience Wuhan, China) and proapoptotic marker – active caspase-3 (1:1000, cat # STJ97448; St. John's Lab London, UK), estrogen receptor α (1:500, cat # P03372, DSHB, University of Iowa, Iowa, USA) and estrogen receptor β (1:500, cat # CWK-F12, DSHB, University of Iowa, Iowa, USA). For phosphorylated Akt protein the membranes were blocked in 1X TBS 1% casein blocker (cat # 161-0782, Bio-Rad Laboratories, USA) for 30 mins and then incubated with p-Akt Ser473(1:500, cat # 4060T, Cell Signaling Technology, USA). Then the primary antibody was washed for 30 min with PBST, and the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase at the room temperature for 2 hrs. After incubation the membranes were washed and developed onto X-ray film by using the

electrochemiluminescence (ECL) (cat # 1705060; Bio-Rad, Hercules, CA, USA) method. The band intensities were quantified by using the ImageJ software (imagej.nih.gov/). Total Akt protein performed in the membrane after p-Akt Ser473 and probed with Akt (1:1000, cat # 9272S, Cell Signaling Technology, USA). The membranes were stripped and reprobed for β-Tubulin (1:4000, cat # E-AB-20033; Elabscience, Houston, Texas, USA) for loading control. Immunoblotting of AR, ER α and β were performed in testis explants by following the same method.

Hormone assay

Hormone concentration such as testosterone and estrogen was estimated in the serum from PND groups, APJ inhibitor *in vivo* study and in media from *in vitro* study by using a commercial human testosterone kit (cat # EIA K209; Xema-Medica Co.Ltd, Moscow, Russia) according to manufacturer's instruction. Sensitivity of the assay was assessed as 0.15nmol/l. The circulatory apelin levels were measured in PND 1 to PND 42 animals by using mouse apelin EIA kit (cat # RAB0018; Sigma-Aldrich Co. LLC, USA) as per instruction manual. Minimum detectable concentration is 5.84 pg/mL. This kit shows no cross reactivity with any of the cytokines.

Statistical analysis

Using GraphPad Prism9, all statistical analyses were performed and all numerical data were expressed as mean±*SEM*. To compare the data from different groups, One-way Analysis of variance (ANOVA) followed by Tukey's test was used for *in vitro* testosterone assay, and the student's *t* test was used for *in vivo* study. The normal distributions of the data were analyzed by the Shapiro-Wilk normality test. The data were considered as significant at the $p<0.05$.

Results

Changes in the circulating apelin, and steroids (Testosterone, estrogen and androstenedione) hormone levels

The circulating apelin levels were minimum at PND1 and maximum at PND42. The levels of apelin at PND7 and PND14 did not show a significant change, whereas, a decline in apelin levels was noticed at PND21 compared to the PND7, 14 and 42 **(Figure 1.1A)**. The circulating testosterone levels showed highest at PND42 compared to the other PND groups **(Figure 1.1B)**. However, the circulating androstenedione didn't show marked variation. On the otherhand estrogen levels showed significant ($p<0.05$) increase at PND14 and 21 compare to other postnatal stages **(Figure 1.1C-D)**.

Expression and localization of apelin receptor (APJ) during postnatal stages

Western blot analysis showed presence of APJ in the testis from PND1 to PND42. The expression of APJ was significantly ($p<0.05$) elevated at PND21 and PND42, compared to the other groups. The expression of testicular APJ from PND1 to PND14 showed a mild elevation, however, it was not significant **(Figure 1.2G)**.

Immunohistochemical localization of testicular APJ also showed the presence from PND1 to PND42. The localization of testicular APJ from PND1 to PND7 **(Figure 1.2A-B)** was mainly confined to the interstitium, with moderate staining in the Leydig cells. At PND14, spermatocytes and Leydig cells showed mild staining **(Figure 1.2C)**. However, at PND21, APJ immunostaining was confined to Leydig cells only **(Figure 1.2D)**. At PND42, a faint immunolocalization of APJ was observed in the Leydig cells. However moderate staining of APJ was observed in round spermatid showed moderate immunostaining **(Figure 1.2E)**.

In order to analyze testicular apelin, immunohistochemistry of apelin was performed. Immunolocalization of apelin showed faint immunostaining in the interstitium from PND1 to PND14 **(Figure 1.3A-C)**, however, at PND21 no immunostaining was observed **(Figure 1.3D).** Apelin at PND42 showed a moderate immunostaining in the Leydig cells **(Figure 1.3E)**.

To gain further insight about the involvement of apelin system in testicular steroidogenesis during postnatal stages, a correlation study was performed between the expression of testicular APJ expression and circulating steroid levels. The result of correlation study showed a significant positive correlation of postnatal testicular APJ expression with circulating apelin (r= 0.4782, P=0.0156), testosterone (r= 0.9388, P<0.0001) and androstenedione levels (r= 0.4438, P=0.0263) **(Figure 1.4A-C)**. However, expression of testicular APJ did not show significant correlation with estrogen levels (r= -0.1038, P=0.6216) **(Figure 1.4D)**.

Effects of APJ inhibition by ML221 on body weight, testis weight and gonad somatic index (GSI)

In order to unravel the role of apelin on testis during early postnatal stages, APJ antagonist, (ML221) was given from PND14-PND20. The treatment of ML221 did not show any significant change in body weight, testis weight and GSI **(Figure 1.5A-C)**.

Effects of APJ inhibition by ML221 on testis histology, seminiferous tubule diameter, germinal epithelium height and lumen diameter

The treatment of APJ antagonist, (ML221) during PND14-PND20 showed observable changes in the testis. The histological section of testis exhibited vacuole (V), sloughing of germ cell in the lumen (arrow) and darkly stained germ cells (*), which could be pyknotic cells **(Figure 1.6D-E)**. The histomorphometrical measurements of seminiferous tubule diameter, germinal epithelium height and lumen diameter was significantly (p<0.05) decreased in the ML221 treated group compared to the control **(Figure 1.6A-C)**.

Effects of APJ inhibition by ML221 on testosterone and estrogen secretion

Furthermore, to unravel the role of apelin on hormone secretion during early postnatal period, testosterone and estrogen were measured after ML221 treatment. The treatment of ML221 significantly (p<0.0) increased the estrogen secretion without significant change in testosterone secretion **(Figure 7A-B)**.

Effects of APJ inhibition by ML221 on testicular proliferation

Since testis is diving organ, therefore, expression markers of two germ cell proliferations, GCNA and PCNA were performed by immunohistochemistry. Immunolocalization of GCNA exhibited abundance in the control testis, whereas in the testis of ML221 mice GCNA immunostaining was very faint. Similarly, the abundance of PCNA was also observed in the control testis compared to the ML221 treated testis **(Figure 1.8A-D)**.

Semi-quantitative analysis of GCNA and PCNA by Image J also showed a significant ($p<0.05$) decrease in the GCNA and PCNA percentage area in ML221 treated group compared to the control testis **(Figure 1.8E-F)**.

Effects of APJ inhibition by ML221 on the expression of BCL2, active caspase3, ERα, ERβ and AR

It is evident that testis undergoes apoptosis during early postnatal period, thus, marker of anti-apoptosis, BCL2 and apoptosis, active caspase 3 was performed by Western blot analysis after ML221 treatment. The expression of BCl2 was unaffected after ML221 treatment, whereas, expression of active caspase3 was significantly ($p<0.05$) up-regulated in ML221 group compared to the control group **(Figure 1.9A-B)**.

It is also well known that testosterone and estrogen regulate testicular activity, thus receptor for androgen, AR and receptors for estrogen ERα, ERβ were also analyzed by western blot analysis. ML221 treatment slightly elevated the expression of ERα, ERβ; moreover, this increase was not statistically significant **(Figure 1.9C-D)**. On the other hand, expression of AR was significantly decreased in ML221 group compared to the control **(Figure 1.9E)**.

Effects of APJ inhibition by ML221 on the Akt phosphorylation Ser473 (p-Akt S473)

It has been shown that apelin exhibit biological effect via Akt phosphorylation; therefore, Akt phosphorylation Ser473 was measured by Western blot analysis after ML221 treatment. The APJ, antagonist, ML221 significantly ($p<0.05$) decreases Akt phosphorylation compared to the control **(Figure 1.10)**.

Effects of APJ inhibition by ML221 on the testosterone, estrogen secretion and on the AR, ERα and ERβ: An in vitro study

In order to strengthen the findings of in vivo study, in vitro study was conducted on PND 18 testis. The treatment of ML221 to the testis fragment, showed no significant change in testosterone and estrogen secretion. Expression of ERs showed a mild elevation in ML221 treated groups compared to the control, however, it was not significant. Expression of AR was significantly ($p<0.05$) down-regulated after ML221 treatment compared to the control **(Figure 1.11A-E)**.

Discussion

The present study has undertaken to analyze the expression and distribution of apelin and its receptor in the testis of mice during postnatal developmental periods. Apelin is one of the important cytokines, which is expressed in the various organs such as brain, ovaries, kidney, pancreas, heart and breast **(O'Carroll et al., 2013)**. The expression of apelin and its receptor has also been shown in the testis of mice and rats **(Das et al., 2021; Akkan et al., 2020; Brzoskwinia et al., 2020)**. Despite its expression in the different organs, including testis, and human Leydig cells, the expression and localization of apelin and apelin receptor has not been investigated in the testis during postnatal periods. To best of our knowledge, this is a first attempt to unravel the expression and distribution of apelin in the testis of mice during postnatal developmental stages. Our results showed that the circulating apelin was minimum at PND1 and levels increased again at PND42. As our aim was also to analyze the expression of APJ in the testis, and western blot analysis showed that expression of APJ was significantly increased in the

testis of PND21, and PND42 compared to the PND1-14. This finding suggests that apelin signaling would be important in the testis from PND14-PND42, most likely during first wave of spermatogenesis. Immunohistochemical study showed presence of APJ in the testis from PND1 to PND42, in the Leydig cells, as well as in germ cells at PND14 and PND42. The immunostaining of apelin was observed only in the Leydig cells of PND42, however, the circulating apelin levels was increased in other age groups compared to the PND1. The correlation study of testicular APJ and circulating apelin showed a positive correlation. Based on these results, it may be suggested that apelin receptor signaling would also be important for testicular growth as well as steroid biosynthesis. Whether, the circulating apelin or locally synthesized apelin could mediate APJ activity in the Leydig cells of postnatal testis, this is not clear from the present study. Similarly, the ligand for APJ on germ cells at PND 42, whether comes from circulation or locally synthesized source, needs to be investigated further. Since we do not have data on whether apelin can cross blood testis barrier or not, thus, emphasizing role of circulatory or local apelin would be very speculative.

In order to find out a possible relationship between apelin system, and the circulating the steroids hormones, the levels of testosterone, estrogen and androstenedione were also measured. Furthermore, the circulating the circulating testosterone, and androstenedione levels showed a significant positive correlation with expression of APJ. These observations provide evidence for stimulatory role of apelin signaling in steroid biosynthesis during postnatal developmental stages. It has previously been reported that apelin may modulate the testosterone biosynthesis in male rats **(Sandal et al., 2015)**. The short term (7 and 14 days) treatment of apelin to adult male rats, decreases the circulating testosterone levels (**(Sandal et al., 2015; Tekin et al., 2017).** It has been suggested that apelin might a negative impact on reproductive functions (**Sandal et al., 2015; Tekin et al., 2017)**. Our recent study has shown that apelin treatment increases testosterone biosynthesis from diabetic mice testis **(Das et al., 2021)**. Since there is no report on developmental expression of apelin system in any mammalian species, and

moreover, our study showed a progressive increase in the APJ expression from PND1 to PND42 in mice testis, therefore, more study is needed to explore the exact role of apelin system in the testicular physiology. It may be suggested that there may be developmental stage dependent role of apelin in the testis.

In order to unravel the role of apelin in early developmental stages, apelin receptor antagonist (ML221) treatment was given from PND14 to PND20. Since, we have observed a sharp increase in the expression of APJ in the testis from PND14-PND21, therefore, the treatment of ML221 was given during this period. Our findings showed that the treatment of APJ antagonist, ML221 had no effect on the testis and body weight. Interestingly, seminiferous tubule diameter, germinal epithelium height and lumen diameter was in decreased by ML221 treatment. These observations suggest a possible role of apelin signaling in the testis of early developmental stages. To best of our knowledge, this is the first report on role of apelin in testis during early developmental stages. It has been shown that the first wave of spermatogenesis starts in murine testis from a few days later from birth **(Laiho et al., 2013**). It has also been shown that pachytene spermatocytes appear around at PND14, round spermatids appear at PND20 ND sperm appears at PND35 **(Busada et al., 2016)**. Since the first wave of spermatogenesis includes germ cell proliferation, and apoptosis in the rodent testis **(Rodriguez et al., 1997; Jahnukainen et al., 2004).** We have accessed the proliferation in the testis ML221 treatment by immunohistochemistry of two proliferating markers GCNA and PCNA. Our finding revealed that inhibition of apelin signaling by ML221, decreased germ cell proliferation. In other words, apelin seems to play important role in the germ cell proliferation during first wave of spermatogenesis. The direct involvement of apelin in germ cell proliferation has not been investigated. However, it has been shown that apelin is an angiogenic factor, which induces endothelial cell proliferation **(Kurowska et al., 2018)**. The apelin agonist, apelin-13 peptide has been shown to stimulate proliferation in breast cancer line **(Peng et al., 2015)**. Hypoxia induced apelin expression has also been shown to regulate endothelial cell proliferation **(Eyries et al., 2008)**. Thus, exact mechanism is not

very clear from the present study, how apelin regulates germ cell proliferation. Furthermore, ML221 treatment decrease the abundance of p-Akt, which suggests that apelin could be involvement in signaling from PND14-PND20. Previous study has also shown that apelin stimulates muscle cell proliferation via Akt signaling (Liu et al., 2010) and also inhibits apoptosis via Akt pathway (Liu et al., 2017).

Since the proliferation and apoptosis are simultaneous processes, therefore, expression of antiapoptotic protein, Bcl2 and pro-apoptotic protein, active caspase3 was analyzed after ML221 treatment. The expression of Bcl2 was not changed; however, expression of active caspas3 was up-regulated in the ML221 treated testis. These results suggest that apelin receptor antagonist increase apoptosis and decrease germ cell proliferation in the testis of early developmental stages. In other words, apelin increases the proliferation and decreases apoptosis in the testis of early developmental stages. Furthermore, it may be hypothesized that apelin mediated stimulated proliferation and decreased apoptosis might have a regulatory role for quality control of spermatogenesis. It has been previously suggested that early loss of germ cells in the rodent testis is requisite for establishment of functional spermatogenesis **(Rodriguez et al., 1997; Jahnukainen et al., 2004)**. It has also been shown that testis early developmental stages secrets androgen by fetal Leydig cells up to PND25 **(Cooke and Walker 2021; Wen et al., 2016)**. Thus, question arises whether apelin can regulates steroidogenesis in testis of early developmental stages, therefore, the levels of circulating of testosterone and estrogen was measured after ML221 treatment. Our results showed that treatment of apelin receptor antagonist increased the circulating estrogen levels without change in circulating testosterone levels. In order to gain further role apelin on testicular steroidogenesis, the explants of testis were cultured with ML221. The in vitro treatment of ML221 did not show effect on testosterone and estrogen secretion.

It is well known that testosterone and estrogen acts via androgen and estrogen receptors. The expression of AR has been detected in the mice testis first at age of

PND14 in spermatogonia, Sertoli cells, and myoid cells, whereas as Leydig cells express AR at PND21 **(Zhou et al., 1996).** In human testis also, ontogeny of AR is similar to rodents and plays important role in Sertoli cells maturation along with initiation of spermatogenesis at adult **(Rey et al., 2009; Al-Attar et al., 1997)**. Similarly, it has been shown that expression of ER in the mice testis is developmentally regulated **(Mahmoud e al., 2015)**. Therefore, we have analyzed the expression of AR, ERα and ERβ in mice testis after in vivo treatment of apelin receptor antagonist. Our results showed that ML221 treatment down-regulated the AR expression in the testis, whereas, expression of ERs was unchanged. To gain further insight on expression of these receptors after ML221 treatment, in vitro was performed. The in vitro study also showed that treatment of apelin receptor antagonist, ML221 down-regulated expression of AR and expression of ERs were unchanged. These results of in vivo and in vitro study showed that apelin directly regulates the testicular AR expression. However, exact mechanism of apelin mediated regulation of AR expression needs further study. It may also be suggested that apelin mediated AR might be important to regulates the proliferation and apoptosis in early stages of testis. The significance of these events with respect adult spermatogenesis required further study.

In conclusion, this is first study on the developmental expression of apelin and apelin receptor in the testis. Furthermore, the present showed first time role of apelin on early stages of testicular developments. Our study unravels that apelin might promotes germ cell proliferation and suppresses apoptosis during first wave of spermatogenesis. The apelin mediated regulation of proliferation and apoptosis might be largely dependent on androgen signaling by AR.

Summary

The expression of apelin system has been shown in the adult testis of rat and mice. It has also been emphasized that regulation of testicular activity in early stages is important to sustain normal testicular activity in adulthood. Since the expression of apelin receptor (APJ) has been shown in the adult testis, moreover, developmental expression of APJ and its role has not been explored yet. Thus, we have examined the testicular expression of APJ during postnatal stages with special reference to proliferation, apoptosis and hormone secretion in early postnatal stage. Postnatal analysis showed that circulating apelin was lowest at PND1 and maximum at PND42. Among testosterone, estrogen and androstenedione, only circulating testosterone showed a gradual increase from PND1 to PND42. Testicular expression of APJ was also developmenatly regulated from PND1 to PND42, revealing a positive correlation with circulating apelin, testosterone, and androstenedione. Immunohistochemical study showed that APJ was mainly confined to Leydig cells of early postnatal stages, whereas, seminiferous tubules at PND42 showed immunostaining in the round spermatids. APJ inhibition from PND14-PND20 by ML221 suppresed the testicular proliferation, increased apoptosis and increased estrogen secretion. However, expression of AR was down-regulated by ML221 treatment. Furthermore, ML221 decreased the abundance of p-Akt. *In vitro* study also showed that APJ antagonist, ML221 decreased AR expression. These results suggests that apelin signaling during early developmental stages might be required to stimulate the germ cell proliferation, and inhibition of apoptosis. Both *in vivo* and *in vitro* study have shown that expression of AR was regulated by apelin signaling. Since the first wave spermatogenesis involves proliferation and apoptosis, therefore, further study would be required to unravel the exact mechanism of apelin mediated regulation of testicular activity during early postnatal stages. In conclusion, the present results are an indicative of apelin mediated signaling during early postnatal stage for regulation of germ cell proliferation, apoptosis and AR expression.

Figure 1.1 Postnatal changes in the circulating apelin (A), testosterone (B), androstenedione (C) and estrogen levels. The circulating apelin levels showed a significant ($p<0.05$) decrease at PND1 compared to the PND7-42. The circulating testosterone levels did not show significant change between PND1 and PND7, however, testosterone levels showed stage dependent significant ($p<0.05$) increase from PND14 to PND42 compared to the PND1-7. The circulating androstenedione levels did not show stage dependent marked variation. The circulating estrogen levels showed significant ($p<0.05$) increase at PND14-21 compared to the other stages. Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups.

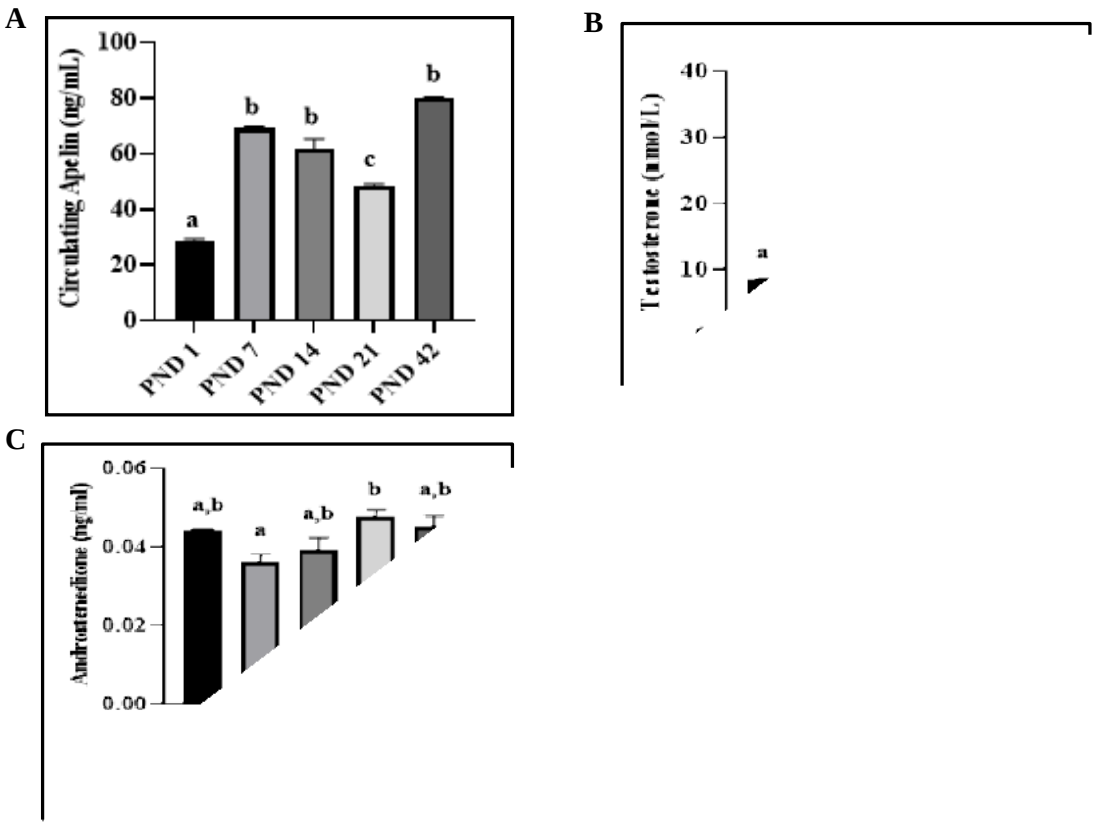
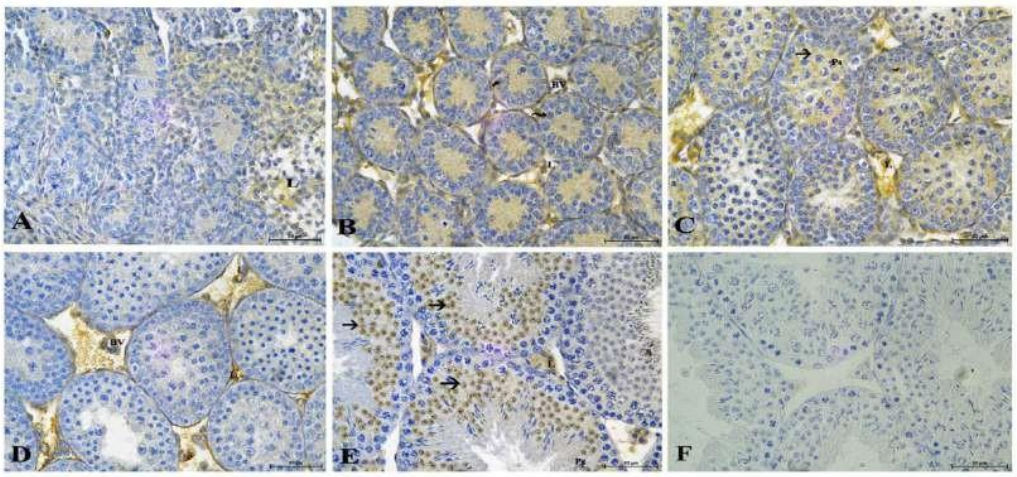
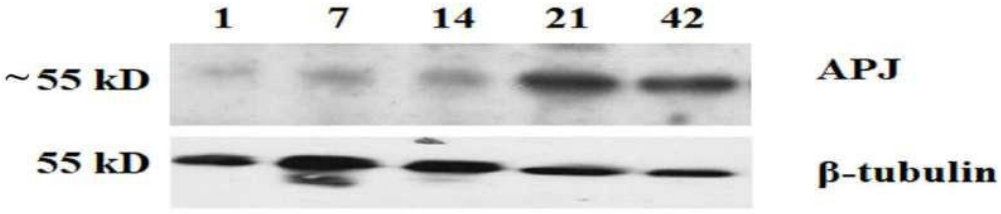

A
Circulating Apelin (ng/mL)
100
80
60
40
20
0
a
b
b
c
b
PND 1
PND 7
PND 14
PND 21
PND 42
B
Testosterone (nmol/L)
40
30
20
10
a
C
Androstenedione (ng/ml)
0.06
0.04
0.02
0.00
a,b
a
a,b
b
a,b

Figure 1.2 Postnatal changes in the expression and localization of testicular apelin receptor (APJ) by Western blot analysis. Histogram represents densitometric analysis of blots. Expression of APJ showed a stage dependent increase, however, from PND1 to PND14, it was not significant. The expression of APJ showed a significant increase in PND21 and PND42 compared to the other groups. At PND42, expression of APJ was significantly highest. Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups (G). Localization of APJ was observed in the Leydig cells (L) from PND1-PND42 with faint immunostaining at PND42 (A-E). Germ cells, mainly spermatocytes also showed mild immunostaining (arrow) at PND14 (C), whereas, at PND21, no immunostaining was seen in seminiferous tubules (D). PND42 testis showed moderate immunostaining in the round spermatids (arrow) (E). The negative control (F) was replaced with 1% non-immune rabbit IgG, showed no immunostaining. L. Leydig cell, Ps. Primary spermatocytes, BV. Blood Vessels.


A
B
C
D
E
F


1
7
14
21
42
~55 kD
APJ
55 kD
β-tubulin

G

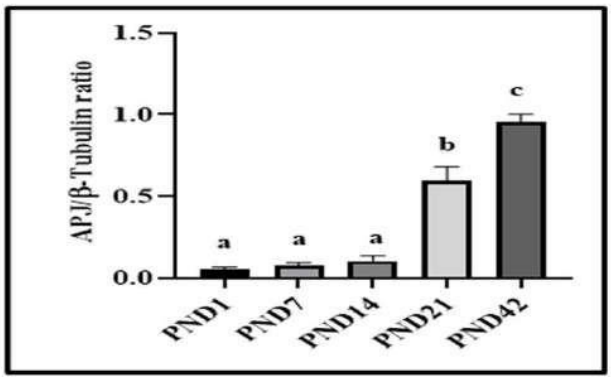

APJ/β-Tubulin ratio
1.5
1.0
0.5
0.0
a
a
a
b
c
PND1
PND7
PND14
PND21
PND42

Figure 1.3 Postnatal day dependent changes in the localization of testicular apelin. Immunolocalization of apelin also showed mild immunostaining in the Leydig cells (L) at PND1, 7, and 14 (A-C). No immunostaining was observed at PND21 (D), whereas, Leydig cells at PND42 showed moderate immunostaining (E). The negative control (F).

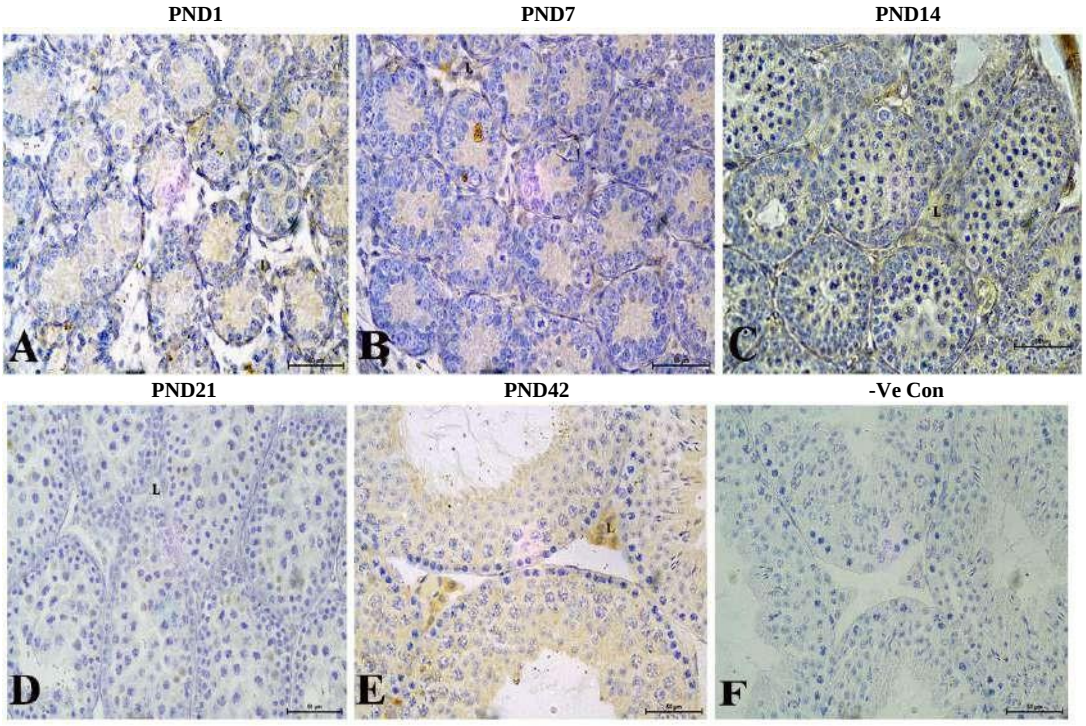

PND1
PND7
PND14
A
B
C
L
PND21
PND42
-Ve Con
D
E
F
L

Figure 1.4 Correlation analysis of circulating hormones, apelin (A), testosterone (B), androstenedione (C) and estrogen levels with APJ expression. Postnatal testicular expression of APJ showed a significant positive correlation with apelin (A) ($r= 0.4782$, $P=0.0156$), testosterone (B) ($r= 0.9388$, $P<0.0001$), androstenedione (C) ($r= 0.4438$, $P=0.0263$), however, APJ showed an insignificant ($r= -0.1038$, $P=0.6216$) negative correlation with estrogen levels (D).

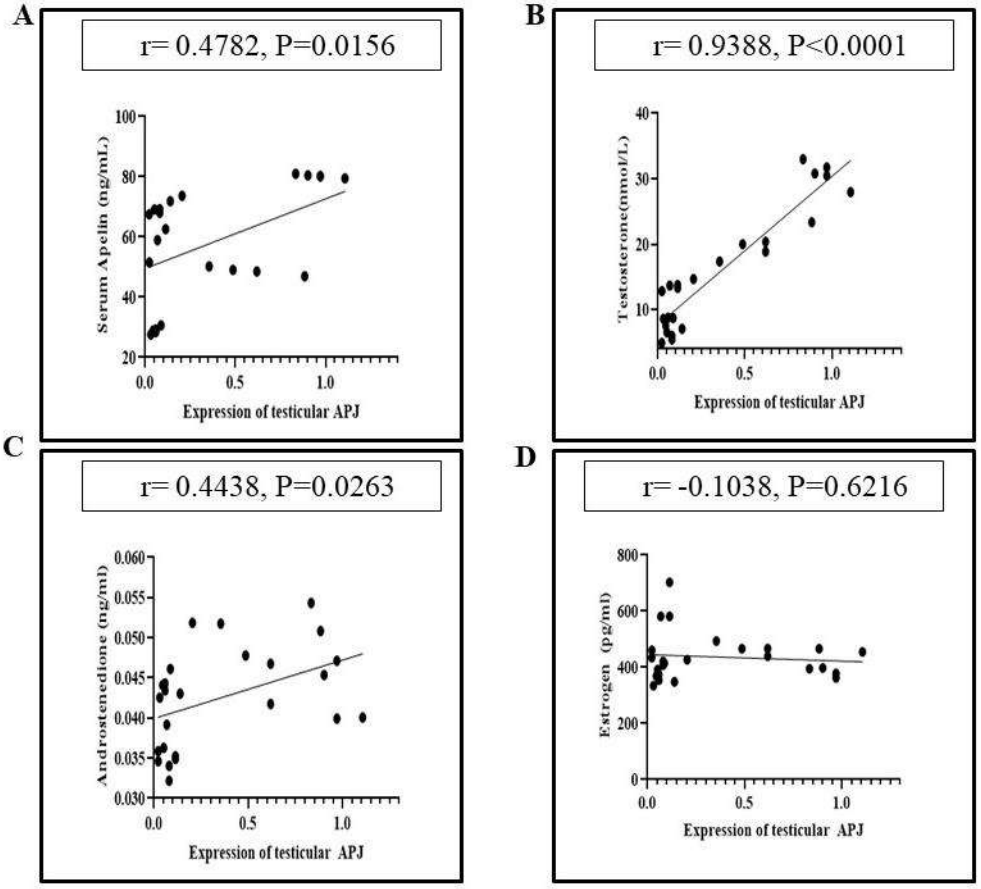

A
r= 0.4782, P=0.0156
Serum Apelin (ng/mL)
Expression of testicular APJ
B
r= 0.9388, P<0.0001
Testosterone(nmol/L)
Expression of testicular APJ
C
r= 0.4438, P=0.0263
Androstenedione (ng/ml)
Expression of testicular APJ
D
r= -0.1038, P=0.6216
Estrogen (pg/ml)
Expression of testicular APJ

Figure 1.5 Effects of APJ antagonist treatment (PND14-PND20) on body weight (A), testis weight (B), gonad somatic index (GSI) (C) and testicular histology. The treatment of ML221 did not show significant ($p>0.05$) change on body weight (A), testis weight (B) and GSI (C). Data are expressed as mean±SEM The control mice testis showed normal histoarchitecture with presence of primary spermatocytes, round spermatid, Sertoli cells Leydig cells (A). The testicular section of ML221 treated mice showed some degenerative changes, vacuole (V), sloughing of germ cell in the lumen (arrow) and darkly stained cells (*) (B).

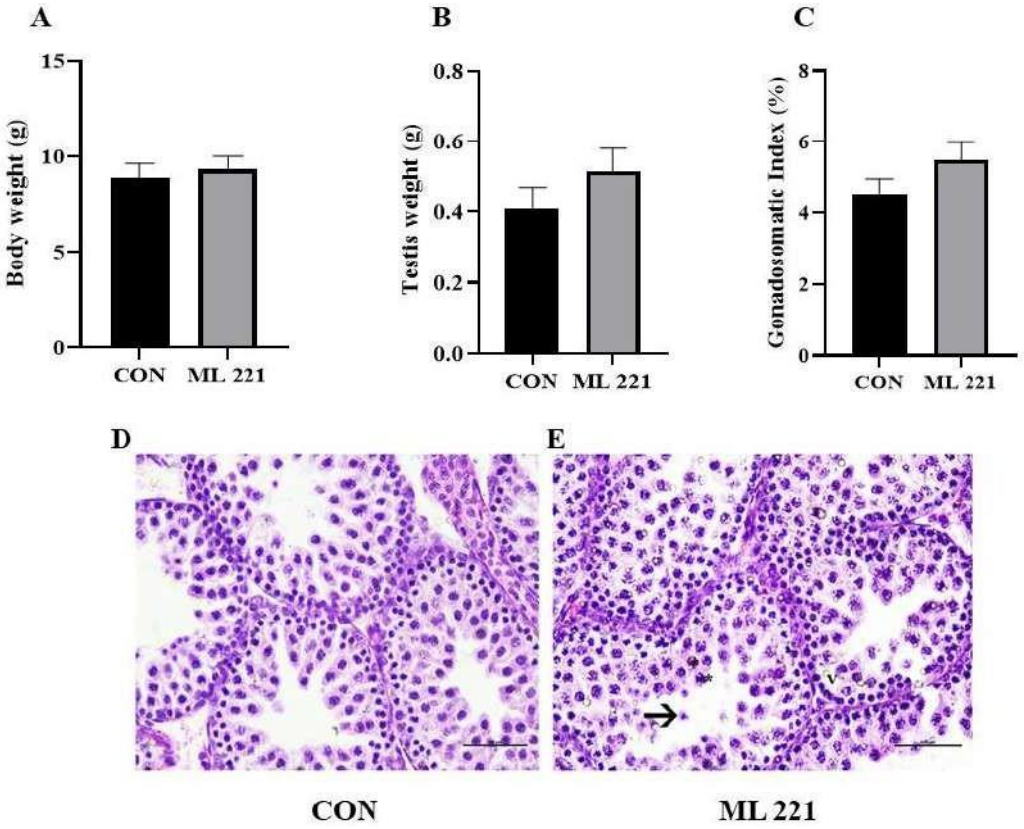

A
Body weight (g)
15
10
5
0
CON
ML 221
B
Testis weight (g)
0.8
0.6
0.4
0.2
0.0
CON
ML 221
C
Gonadosomatic Index (%)
8
6
4
2
0
CON
ML 221
D
E
CON
ML 221

Figure 1.6 Effects of APJ antagonist treatment (PND14-PND20) on testicular histomorphometric parameters. APJ antagonist, ML221 significantly decreased seminiferous tubule diameter **(A)**, germinal epithelium height **(B)** and luminal diameter **(C)**. Data are expressed as mean±SEM. (*, $p<0.05$ vs CON).

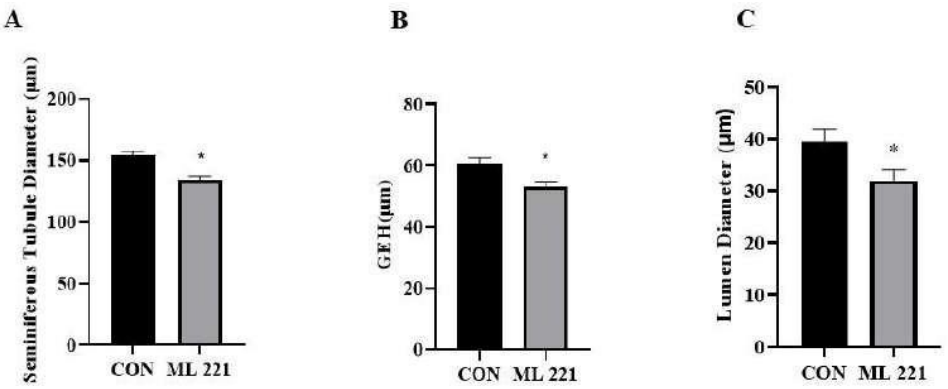

A
Seminiferous Tubule Diameter (µm)
200
150
100
50
0
*
CON
ML 221
B
GEH(µm)
80
60
40
20
0
*
CON
ML 221
C
Lumen Diameter (µm)
50
40
30
20
10
0
*
CON
ML 221

Figure 1.7 Effects of APJ antagonist treatment (PND14-PND20) on testosterone (A) and estrogen secretion (B). APJ antagonist, ML221 significantly increased estrogen secretion; however, testosterone secretion did not change. Data are expressed as mean±SEM. (*, $p<0.05$ vs CON).

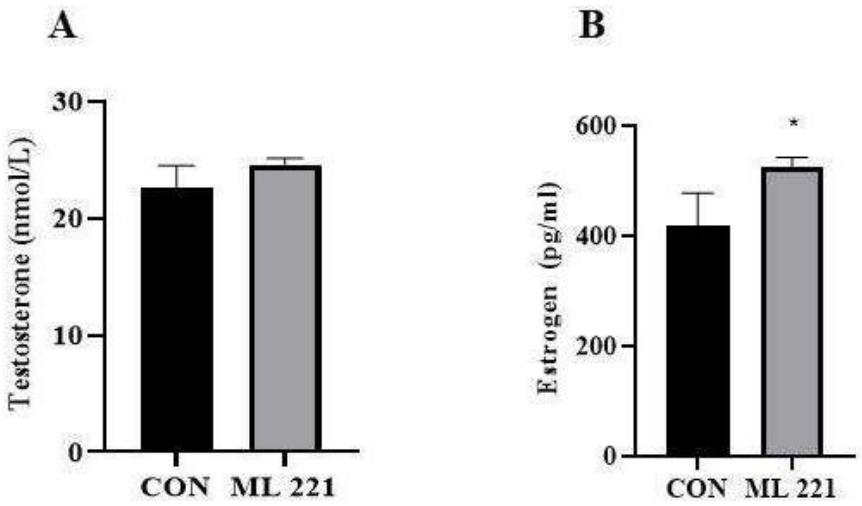

A
Testosterone (nmol/L)
30
20
10
0
CON
ML 221
B
Estrogen (pg/ml)
600
400
200
0
*
CON
ML 221

Figure 1.8 Effects of APJ antagonist treatment (PND14-PND20) on GCNA (A-B) and PCNA (C-D) expression. APJ antagonist, ML221 treatment showed mild immunostaining of PCNA in the testis (D), whereas GCNA showed faint immunostaining (B). The semi quantitative measurement of GCNA (E) and PCNA (F) stained area showed a significant decrease in ML221 treated group compared to the control. Data are expressed as mean±SEM. (*, $p<0.05$ vs CON).

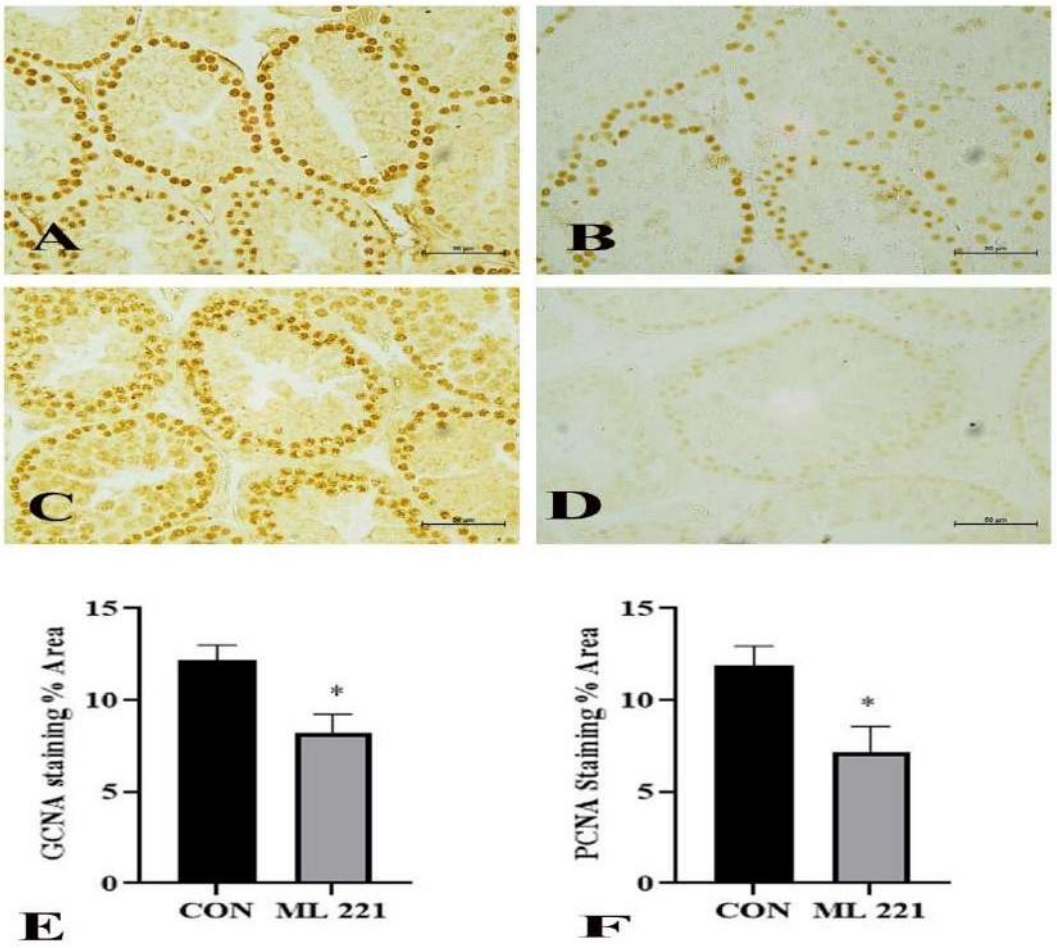

A
B
C
D
GCNA staining % Area
15
10
5
0
*
CON
ML 221
E
PCNA Staining % Area
15
10
5
0
*
CON
ML 221
F

Figure 1.9 Effects of APJ antagonist treatment (PND14-PND20) on the expression of BCl2(A), active caspase3 (B), ERα (C), ERβ and AR (D) by Western blot analysis. Histogram represents densitometric analysis of blots. Expression of Bcl2, ERα, and ERβ did not show significant change. Expression of active caspase3 was significantly up-regulated (B) and expression of AR was significantly down-regulated (E) by APJ antagonist. Data are expressed as mean±SEM. (*, $p<0.05$ vs CON).

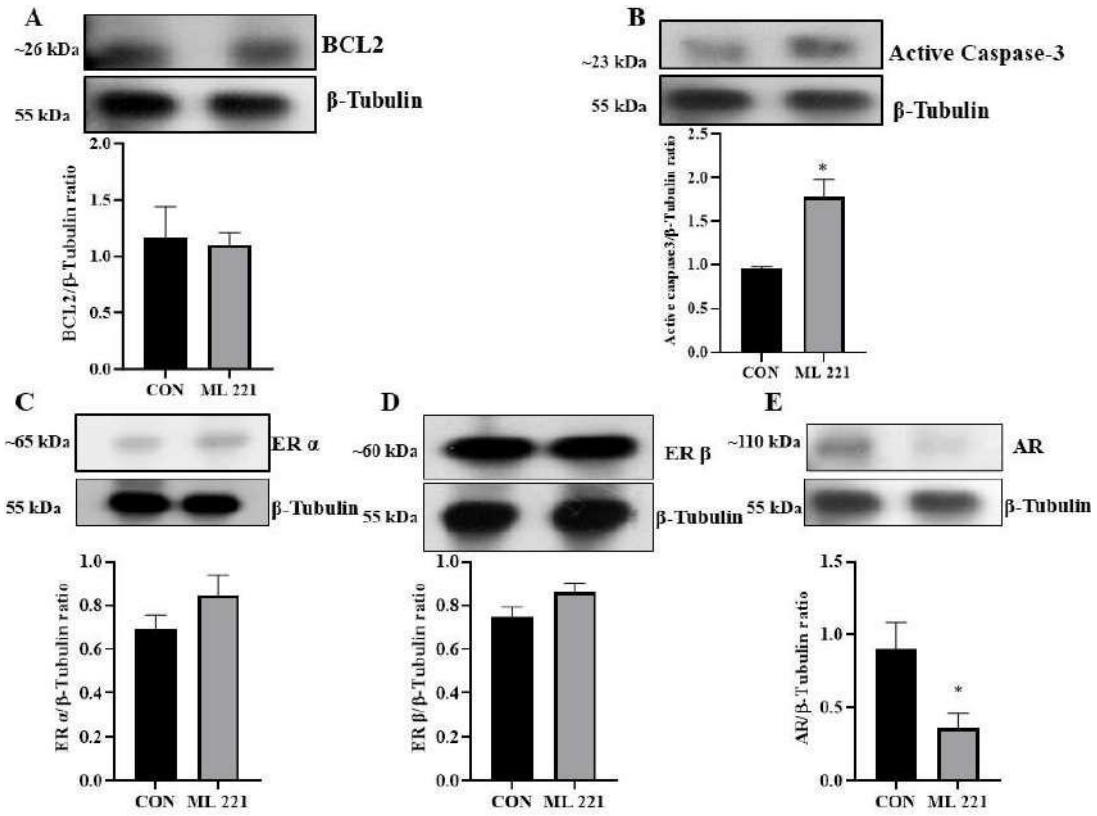

A
~26 kDa
BCL2
55 kDa
β-Tubulin
BCL2/β-Tubulin ratio
2.0
1.5
1.0
0.5
0.0
CON
ML 221
B
~23 kDa
Active Caspase-3
55 kDa
β-Tubulin
Active caspase3/β-Tubulin ratio
2.5
2.0
1.5
1.0
0.5
0.0
*
CON
ML 221
C
~65 kDa
ER α
55 kDa
β-Tubulin
ER α/β-Tubulin ratio
1.0
0.8
0.6
0.4
0.2
0.0
CON
ML 221
D
~60 kDa
ER β
55 kDa
β-Tubulin
ER β/β-Tubulin ratio
1.0
0.8
0.6
0.4
0.2
0.0
CON
ML 221
E
~110 kDa
AR
55 kDa
β-Tubulin
AR/β-Tubulin ratio
1.5
1.0
0.5
0.0
*
CON
ML 221

Figure 1.10 Effects of APJ antagonist treatment (PND14-PND20) on Akt phosphorylation Ser473 (p-Akt S473) by Western blot analysis. Histogram represents densitometric analysis of blots. ML221 treatment significantly decrease the abundance of p-Akt S473 compared to the control. Data are expressed as mean±SEM. (*, $p<0.05$ vs CON).

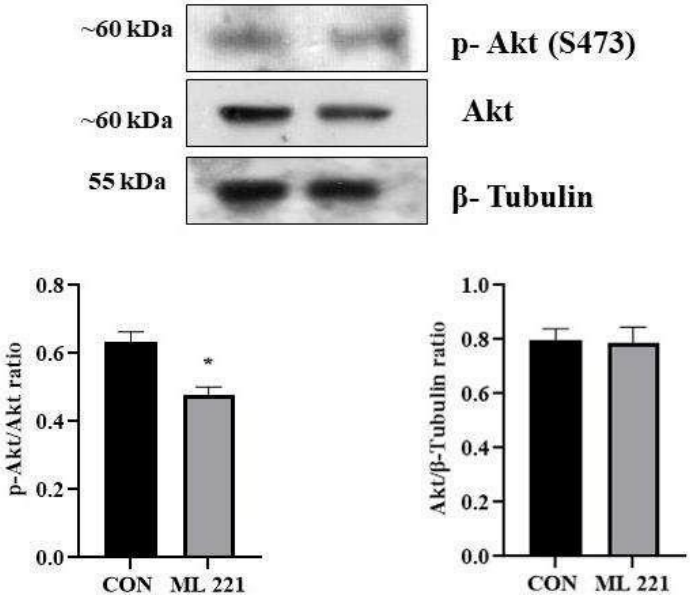
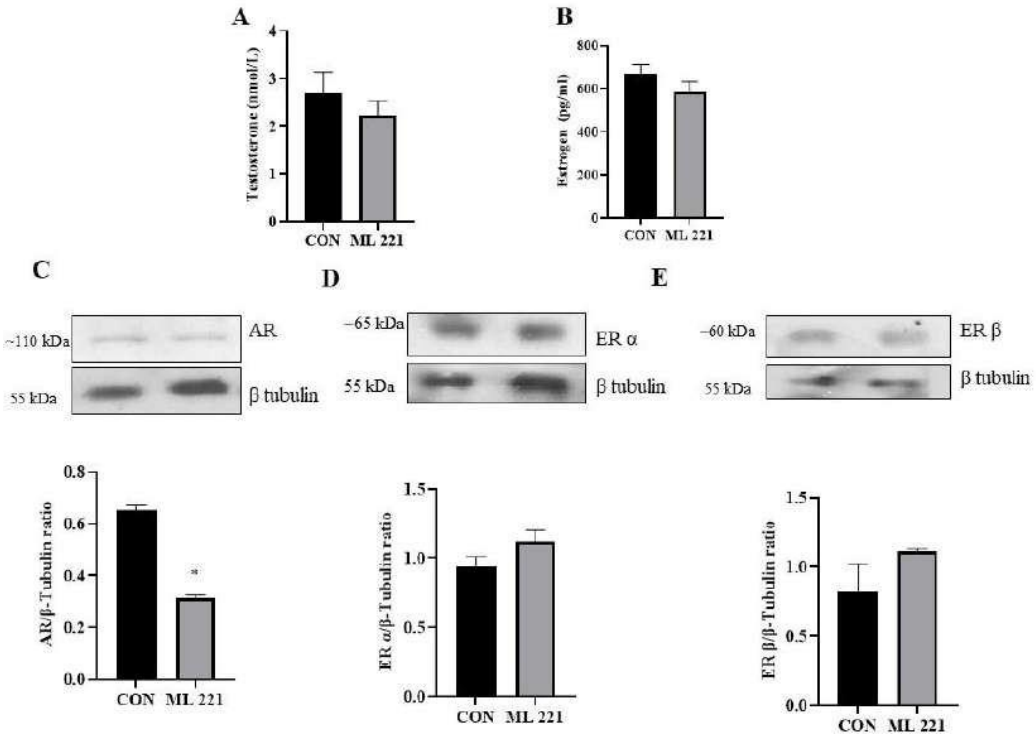

~60 kDa
p- Akt (S473)
~60 kDa
Akt
55 kDa
β- Tubulin
p-Akt/Akt ratio
0.8
0.6
0.4
0.2
0.0
*
CON
ML 221
Akt/β-Tubulin ratio
1.0
0.8
0.6
0.4
0.2
0.0
CON
ML 221

Figure 1.11 In vitro effects of ML221 on the testosterone (A), estrogen (B) secretion and on AR (C), ERα (D) and ERβ (E) expression. The *in vitro* treatment of ML221 did not affect testosterone and estrogen secretion. Expression of AR was significantly down-regulated by ML221 and expression of ERs did not significant change. Data are expressed as mean±SEM. (*, $p<0.05$ vs CON).


A
Testosterone (nmol/L)
CON ML 221
B
Estrogen (pg/ml)
CON ML 221
C
~110 kDa
AR
55 kDa
β tubulin
AR/β-Tubulin ratio
CON ML 221
D
~65 kDa
ER α
55 kDa
β tubulin
ER α/β-Tubulin ratio
CON ML 221
E
~60 kDa
ER β
55 kDa
β tubulin
ER β/β-Tubulin ratio
CON ML 221

CHAPTER 2

Title

To study the role of apelin in spermatogenesis and steroidogenesis of adult mice*

***Neuropeptides (2023)102354**

Introduction

The various regulatory factors for testicular activity in males have been reported, like gonadotropin releasing hormone (GnRH) from the hypothalamus, gonadotropin from the pituitary and steroid hormones, testosterone and estrogen by the testis itself. Despite this, regulatory control of testis by GnRH and gonadotropin from hypothalamus, and pituitary respectively, and testis also express a variety of cytokines such as leptin, adiponectin, resistin, visfatin and nesfatin, which regulates testicular functions in an autocrine and paracrine manner. Apelin is an endogenous ligand for a G-protein coupled receptor (APJ), which was first reported in bovine stomach extracts **(Tatemoto et al., 1998)**. Apelin, which is made up of sections and fragments with varying numbers of amino acids (such as apelin-13, apelin-17, and apelin-36), is derived from a preproapelin with 77 amino acids **(Chen et al., 2023, Zhou et al., 2021)**. Apelin mRNA and proteins have also been shown in the white adipose tissue and are called adipocytokines (**Czarzasta et al., 2019)**

It has been shown that apelin can be synthesized in the hypothalamus, pituitary, ovary and testis in several species **(Kurowska et al., 2018)**. The presence of apelin in the hypothalamic area like the supraoptic and the paraventricular nuclei suggested it as a neuropeptide factor (**De Mota et al., 2004).** Not only apelin, but rather mRNA expression of APJ has also been shown in the hypothalamic area of rat brains **(Czarzasta et al., 2014; De Mota et al., 2000; O'Carroll et al., 2013; Mohseni et al., 2021)**. It has been suggested that the presence of apelin and APJ in the hypothalamus, and anterior pituitary might have a regulatory role in the FSH and LH secretion from the anterior pituitary **(De Mota et al., 2000; Sandel et al., 2015)**. The direct evidence of apelin-mediated LH and FSH release from the pituitary has been shown by **Sandel et al (2015),** where an intracerebroventricular infusion of apelin-13 peptide was given and it was shown that apelin 13 decreased LH secretion. It has also been shown that intraperitoneal injection of apelin 13 decreased testosterone and

gonadotropin and suggested that the apelin system might have an inhibitory role in the testicular activity **(Tekin et al., 2017)**. It has also been shown that the apelin system gets elevated in diabetic-mediated testicular impairment in rodents and humans **(Das et al., 2021; Song et al., 2022)**. The role of apelin in apoptosis, insulin secretion, oxidative stress and angiogenesis has been involved in the pathogenesis of diabetic complications **(Li et al., 2022).**

Recently, from our laboratory as well as from other laboratories, the presence of apelin and APJ have been shown in the testis of rat, mice, and dogs **(Das et al., 2021;2022; Brzoskwinia et al., 2020; Troisi et al., 2022)**. Our recent study has shown that expression of testicular apelin and APJ is developmentally regulated in the mice testis and regulates the testicular steroidogenesis, proliferation and apoptosis in juvenile mice **(Das et al., 2022)**. It should be noted that the presence of apelin and APJ in the testis in different compartments suggested some regulatory role of the apelin system in steroidogenesis and spermatogenesis. However, to the best of our knowledge, no study has been conducted to decipher the role of the endogenous apelin system in testicular activity. The previous studies **(Sandel et al., 2015; Tekin et al., 2017)** showed the effect of exogenous apelin-13 peptides on testicular activity, although the role of the endogenous apelin system in the testicular activity is yet to be investigated in the adult.

Therefore, the present study investigated the role of the endogenous apelin system on the testicular activity in adult mice by using the APJ antagonist, ML221.

Materials and methods

Animals

Adult male Swiss albino mice were used in this study. Mice were handled according to the protocol (MZUIAEC-9), approved by Mizoram University Institutional Animal Ethical Committee (MZUIAEC), Mizoram University, Mizoram, India and all animal

experiments was complied with the ARRIVE guidelines. The mice were maintained in an aerated polypropylene cage under standard experimental conditions (12 hrs light-dark cycles, temperature 25±2ºC) with *ad libitum* food and water.

Experimental design

This study investigated the role of apelin in spermatogenesis and steroidogenesis by blocking the apelin receptor (APJ) with the antagonist (ML221) according to the previous study **(Hall et al., 2017)**. Mice were randomly divided into 3 groups, Group-1: Vehicle control (Con, n=8), Group-2: ML221 15 µg/kg (ML-15, n=8) and Group 3: ML221 150 µg/kg (ML-150, n=8). The APJ antagonist ML221 (cat # SML0919, Sigma Aldrich, St Louis, USA) was prepared by dissolving in DMSO and then diluted with double distilled water. The treatment was given for one spermatogenic cycle (35 days) intraperitoneally. The treated mice were received ML221 in 15 µg/kg body weight and 150 µg/kg body weight in 100 µl volume and equal volume of vehicle (DMSO diluted with distilled water) was injected intraperitoneally to the control mice for 35 days.

BrdU incorporation

To investigate the effect of ML221 on germ cell proliferation BrdU labelling was done. Before 12 hrs of the sacrifice, three mice from each group were intraperitoneally injected with BrdU (200 mg/kg) **(Balu et al., 2009)** dissolved in phosphate buffer saline (PBS).

Sample collection

After 24 hrs of the last treatment mice's body weight was measured and sacrificed, and the testes were weighed. The sperm parameters were performed immediately. The testes were collected and fixed in Bouin's solution for immunohistochemical studies

and stored at -20ºC for western blot analysis. Serum was collected for the hormone assays.

Sperm parameters

The caudal epididymis was collected and minced in PBS with a fine scissor for the sperm analysis. In brief, the caudal portion of epididymis was dissected out and minced in 250µl PBS (10mM PBS, pH 7.4) maintained at 37℃ and a drop of it was immediately placed in clean slide covering with the cover slip and observed the sperm motility within 5 minutes. The stock solution was further diluted in 200µl PBS and a drop of it was observed in Neubauer chamber for analysis of sperm concentration as per the standard protocol of WHO laboratory manual (1999). Sperm motility and concentration was calculated by using the following formula

Motility (%) = (Number of motile spermatozoa/ Total number of spermatozoa) × 100
Concentration of sperm= Average number of spermatozoa counted (N) X multiplication factor (10000) X dilution factor (20)

Hormone assays

Circulating and intra-testicular testosterone, and estrogen was estimated by using the human testosterone (cat # EIA K209; Xema-Medica Co. Ltd, Moscow, Russia) and estrogen (cat # EIA K206; Xema-Medica Co. Ltd, Moscow, Russia) kit as per manufacturer instruction. Circulating LH and FSH were estimated using Mouse LH (cat # E-EL-M3053, Elabscience,) and Mouse FSH (cat #E-EL-M0511, Elabscience) ELISA kit.

Histology and Histomorphological studies

Testis tissue embedded in paraffin and sectioned at 7μM by following the protocol described earlier (John and Gamble 2008). The sections were stained with Hematoxylin and Eosin stain by following the steps of deparaffinization, rehydration, staining, dehydration, clearing and finally DPX mount. Seminiferous tubule diameter and germinal epithelial height were measured and the seminiferous epithelial cycle stage was counted **(Das et al., 2022)** by using a microscope (E200, Nikon, Japan).

Immunohistochemical analysis

Immunolocalization of proliferation markers such as BrdU, GCNA, PCNA and steroidogenic marker 3β HSD and androgen receptor (AR) was performed following the protocol described earlier (Jeremy et al 2019). Briefly, the sections were deparaffinized, rehydrated and blocked with goat serum (1:100, Lot# H2114; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 mins. Sections were incubated with primary antibodies GCNA (1:200, cat # 10D9G11; DSHB, University of Iowa, Iowa, USA), PCNA (1:100, cat # SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 3β HSD conjugated with Horseradish Peroxidase (HRP) and AR (1:25, Ref # PA5-16363; Thermo Fisher Scientific, USA), and for BrdU the sections were treated with 2N HCl at 37 ºC and 0.1 M borate buffer after rehydration with grade ethanol and distilled water. Primary antibody of BrdU (1:50, cat # DSHB, University of Iowa, Iowa, USA) incubated at 4ºC overnight. The primary wash was done with PBS and for PCNA sections were incubated with goat anti-rabbit and for BrdU and GCNA goat anti-mouse HRP- Conjugated secondary antibody for 3 hrs. at room temperature. Then the slides were washed in PBS and developed by using 0.05% diaminobenzidine (DAB) solution (3,3'-diaminobenzidine tetrahydrochloride, in Tris-HCl buffer, pH 7.6 and hydrogen peroxide). After dehydration, the slides were mounted with DPX, observed and photographed using a Euromax microscope. 3β-HSD, BrdU, PCNA, GCNA and AR staining were measured, the stained area by DAB in the testis was

acquired by using the threshold tool of ImageJ as described previously **(Annie et al 2019)**, and the data was presented as a percentage area of staining.

Western blot analysis

Western blotting was performed in the testis sample by following the standard protocol of **Jeremy et al., 2019**. Briefly, 10% (w/v) tissue homogenate was prepared with lysis buffer, containing 0.01 M Tris-HCl (pH 7.6), 1.0 mM EDTA, (pH 8.0),0.1 M NaCl,100 µg/ml phenylmethylsulfonyl fluoride, and the protein concentrations were estimated by Bradford method **(Bradford 1976)**, The equal amount (50 µg) of protein from each group loaded to each well along with molecular weight marker in a 12% SDS-PAGE. The resolved proteins were transferred to the PVDF membrane using wet transfer apparatus for 12 hrs, after successful protein transfer the membrane was blocked for 30 min at room temperature with 5% non-fat skimmed milk prepared using PBST and then overnight incubated at 4ºC with the primary antibodies to the androgen receptor (1:250, Ref # PA5-16363; Thermo Fisher Scientific, USA), antiapoptotic marker BCL2 (1:2000, cat # SC7382; Santa Cruz Biotechnology Inc. Dallas, USA) and proapoptotic marker – active caspase-3 (1:1000, cat #; ElabSciences), BAX (1:100, cat #), NFκB (1:1000, cat #), estrogen receptor α (1:500, cat # P03372, DSHB, University of Iowa, Iowa, USA) and estrogen receptor β (1:500, cat # CWK-F12, DSHB, University of Iowa, Iowa, USA). Then the primary antibody was washed with PBST, and the membranes were incubated with the secondary antibody conjugated with horsera at room temperature for 3 hrs. After incubation, the membranes were washed and developed onto X-ray film by using the electrochemiluminescence (ECL) (cat # 1705060; BioRad, Hercules, CA, USA) method. The band intensities were quantified by using the ImageJ software (imagej.nih.gov/). The membranes were stripped and reprobed for β-Tubulin (1:4000, cat # E-AB-20033; Elabscience, Houston, Texas, USA) for loading control.

Oxidative stress and enzyme assays

Lipid Peroxidation

The oxidative stress marker was measured by estimating malondialdehyde (MDA) levels in the testis by following the previously described method **(Ohkawa et al. 1979, Aboul-Soud et al. 2011).** The testis tissue was 10% homogenized in PBS. 15 % trichloroacetic acid (TCA) and 0.375 % thiobarbituric acid (TBA) were prepared in 0.25N HCl for this experiment. The TCA and TBA solutions were added in a ratio of 1:1:1 with the tissue homogenate and the mixture solution was incubated at 37ºC for 15 mins, cooled and centrifuged at 5000 RPM for 10 min. Then the supernatant absorbance was read at the wavelength 532 nm by using a spectrophotometer (Eppendorf, BioSpectrophotometer). The MDA levels were expressed as nmol/mg of protein.

Catalase activity

The catalase enzyme assay was performed according to the previously described method **(Hadwan, 2018)**. According to the method, 10% tissue homogenate was prepared and incubated with H_2O_2 for 2 minutes at 37℃. After this, the reagent working solution (Cobalt (II) solution, sodium hexametaphosphate solution and sodium bicarbonate solution) was added and incubated at room temperature for 10 minutes in the dark chamber. The absorbance of colour intensity was read at 440nm. The reagent mixture was taken as blank. Catalase activity was calculated by the following equation.

Catalase activity of sample kU =2.303/Time × log (standard absorbance/ sample absorbance) Where k= rate constant of a first-order reaction. The final concentration of catalase was expressed according to the protein concentration as kU/ mg protein.

Glutathione peroxidase (GPx)

GPx assay was done according to the previously described method **(Nicy et al., 2022)**. The mixture containing 0.8mM of EDTA, 10 mM sodium azide, 4 mM reduced GSH and 2.5mM H_2O_2 of 20µl each was mixed with 10µl of 10% tissue homogenate and water along with 40µl of PBS. The solution was incubated at 37℃ for 10 minutes and 50µl 10% Trichloro acetic acid was added and centrifuged. From the supernatant, 50µl was pipette out and to it, 300µl of disodium hydrogen phosphate and 100µg DNTB were added. The chromophore absorbance intensity was read at 412nm. The mixture devoid of tissue homogenate was used as the standard. The concentration of GPx was expressed as µmol GSH oxidized/ min/mg protein.

GPx activity = (changes in Absorbance/time × GSH standard × Total reaction volume)/ (Standard absorbance × molecular weight of GSH (307.3235 g/mol) × vol. of sample × sample protein concentration in mg). GSH standard was prepared by dissolving 20 mg of GSH in 100ml of distilled water (0.2 mg/ml).

Statistical analysis

Using GraphPad Prism9, all statistical analyses were performed and all numerical data were expressed as mean±*SEM*. To compare the data from different groups, a One-way Analysis of variance (ANOVA) followed by Tukey's test was used. The normal distributions of the data were analyzed by the Shapiro-Wilk normality test. The data were considered significant at $p<0.05$

Results

Changes in the body weight, testis weight, gonado-somatic index (GSI) and sperm count

Treatment of ML221 (APJ antagonist) with two doses (15 and 150 µg/kg) significantly decreased the percentage body weight increase ($p<0.05$) in a dose-dependent manner **(Figure 2.1A)**. The testis weight did not show a significant change between control and ML221 treated groups **(Figure 2.1B)**. ML221 treatment at 150 µg/kg dose significantly ($p<0.05$) increased GSI compared to the control and 15 µg/kg dose groups **(Figure 2.1C)**.

Sperm concentration also showed a significant increase in ML221 treated groups compared to control and exhibited a dose-dependent increase in sperm concentration by ML221 treatment **(Figure 2.1D)**.

Testis histology and morphometric analysis

To analyze the effect of APJ inhibition on the testis; histology and morphometric analysis were also performed. The histological sections of the control and ML221-treated mice testis did not show any gross observable changes **(Figure 2.2AC).** The morphometrical parameters, seminiferous tubule diameter and germinal epithelium height did not show any change **(Figure 2.2D, E).** However, the number of VII/VIII stages showed a significant increase ($p<0.05$) in 150 µg/kg ML221 group compared to the other groups **(Figure 2.2F)**.

Changes in the circulating testosterone, estrogen, LH, FSH levels and intra-testicular testosterone and the estrogen concentration

The circulating testosterone levels were found to be significantly ($p<0.05$) elevated in the ML221 treated groups (15 and 150 µg/kg) compared to the control **(Figure 2.3A)**. However, circulating estrogen was found to be significantly ($p<0.05$) decreased at 150

µg/kg dose compared to the control and 15 µg/kg groups **(Figure 2.3B)**. To find out the effect of APJ signaling on the pituitary function, we have measured the gonadotropin, LH and FSH levels. Both doses of ML221 (15 and 150 µg/kg) significantly ($P<0.05$) increased the levels of LH compared to the control and also exhibited a dose-dependent increase ($P<0.05$) in ML221 groups **(Figure 2.3C)**. The levels of FSH were significantly elevated at 150 µg/kg dose group compared to the control and 15 µg/kg groups **(Figure 2.3D).** The intra-testicular testosterone concentration showed a significant increase in ML221 treated groups to the control **(Figure 2.3E).** The intra-testicular estrogen concentration was significantly ($p<0.05$) elevated at 150 µg/kg dose compared to the control and 15 µg/kg groups **(Figure 2.3F).**

Changes in the oxidative stress (MDA levels) and antioxidant enzymes, GPx and catalase

To unravel the relationship between the apelin system and oxidative stress in the adult testis, we have measured the marker of oxidative stress (malondialdehyde levels) and enzyme activity of two, antioxidant enzymes, GPx and catalase. The treatment of ML221 at both doses, (5 and 150 µg/kg), significantly decreased the MDA levels compared to the control **(Figure 2.4A).** However, the enzymes activity of GPx and catalase were significantly ($p<0.05$) elevated at both the doses, (5 and 150 µg/kg) of ML221 compared to the control **(Figure 2.4B, C)**.

Immunolocalization of 3βHSD

To find out the direct role of the APJ system on the testicular steroid markers, we have accessed the abundance of 3βHSD by immunohistochemistry. The treatment of ML221 showed an increased abundance of 3βHSD proteins in the Leydig cells, whereas moderate staining of 3βHSD was also observed in the Leydig cells of the

control testis **(Figure 2.5A-C)**. The staining areas were significantly ($p<0.05$) higher in 150 µg/kg group than control and 15 µg/kg groups **(Figure 2.5D)**.

Changes in the BrdU labelling and PCNA and GCNA localization

Since the testis is proliferating organ and the apelin system has been shown to regulate cell proliferation, therefore, we have measured the markers of cell proliferation, BrdU, PCNA and GCNA. ML221 treatment showed more BrdU **(Figure 2.6A-C)**, PCNA **(Figure 2.6D-F)** and GCNA (**Figure 2.6G-I)** positive cells in the testis than control testis. The BrdU, PCNA and GCNA staining areas were significantly ($p<0.05$) higher in 150 µg/kg dose compared to the control and 15 µg/kg groups **(Figure 2.6J-L)**.

Immunolocalization of AR after ML221 treatment

Immunohistochemistry of AR was performed in control and ML221 treated groups. The intense immunostaining was of AR found in the spermatogonia, primary spermatocytes and Leydig cells of 150 µg/kg dose group **(Figure 2.7A-C)**. The staining areas were significantly ($p<0.05$) higher in 150 µg/kg dose group than control and ML-15 **(Figure 2.7D)**.

Changes in the expression of BCL2, BAX, Active Caspase3 and NF-κB

To unravel the role of APJ in testicular apoptosis, we have measured the expression of BCL2, BAX and active caspase 3. The expression of BCL2 was significantly ($p<0.05$) up-regulated by ML221 at a dose of 150 µg/kg compared to the control and 15 µg/kg groups **(Figure 2.8A)**. The expression of active caspase 3, BAX and NF-κB were significantly ($p<0.05$) down-regulated in 150 µg/kg ML treated groups compared to the control and 15 µg/kg groups **(Figure 2.8B-D)**.

Changes in the expression of AR, ERα and ERβ

To unravel the effect of APJ signaling on the steroid receptors' expression, we have measured the expression of AR, ERα and ERβ. Expression of AR was significantly ($p<0.05$) up-regulated at both doses of ML221 (15 and 150 µg/kg) compared to the control **(Figure 2.9A)**. However, expression of ERα showed a significant ($p<0.05$) decline in a dose-dependent manner **(Figure 2.9B)**. The expression of ERβ was unchanged in all the groups **(Figure 2.9C)**.

Discussion

The present study has investigated the effects of APJ antagonist (ML221) treatment on the various testicular. It has been well documented that ML221, a potent APJ antagonist, has widely been used to unravel the biological role of apelin **(Hall et al., 2017**). The present study is the first attempt to unravel the role of the endogenous apelin system on testicular activity by ML221 treatment on the testicular proliferation, apoptosis, antioxidant system and testicular steroidogenesis as well. It has been documented that testis expresses apelin and its receptor, APJ in the various cell types **(Das et al., 2020: 2022)**, thus suggesting its possible role in the regulation of testicular functions. The treatment of ML221 decreased body weight in a dose-dependent manner. This result suggests that inhibition of APJ might have decreased food consumption. Our results are in agreement with the previous report, which showed that apelin 13 peptide treatment increases food consumption and body weight in a rat model **(Tekin et al., 2017)**. The presence of apelin and APJ in the hypothalamic nuclei have been suggested to regulate energy utilization and reproductive behaviour **(Pope et al., 2012; Malagon and Vaudry 2013).**

Since we aimed to unravel the role of endogenous apelin on the testicular activity, therefore, we measured various parameters and it was found that testis weight was

unaffected by the ML221 treatment, whereas GSI and sperm concentration showed a significant increase in the ML221 treated groups. This observation indicated that endogenous apelin might have an inhibitory role in testicular activity. Earlier studies by **Sandal et al., (2015)** and **Tekin et al., (2017)** have also shown that apelin 13 peptide treatments in the rat inhibit reproductive functions. It should be noted that these studies have accessed the role of apelin by infusing exogenous apelin 13 peptides; however, our study has shown the probable role of endogenous apelin on testicular activities. The inhibition of apelin action by ML221 increased the levels of circulating and intra-testicular testosterone and estrogen levels. However, circulating estrogen levels showed a decline. Thus, the inhibition of APJ by ML221 has a stimulatory role in testicular steroid biosynthesis. The testicular steroidogenesis and spermatogenesis are regulated by the pituitary gonadotropin, FSH and LH, and the levels of both FSH and LH increased by the ML221 treatment. These results suggest that apelin acts along the hypothalamic-pituitary-testicular axis and regulate testicular functions. Since we have not estimated the GnRH levels, thus this is the speculative point in the present study. Moreover, it was suggested that reduced testosterone levels by apelin 13 in rats were due to the inhibitory effects of apelin-13 on gonadotropin (LH and FSH) secretion from the anterior pituitary gland **(Tekin et al., 2017).** It has also been suggested that the hypothalamic-pituitary-gonad (HPG) axis expresses apelin/APJ and might act in an autocrine and/or paracrine manner in the brain, pituitary, ovaries, and testes **(Kurowska et al., 2018)**. To the best of our knowledge, there are two published data which showed the effect of apelin on male reproduction **(Tekin et al., 2017; Sandal et al., 2015).** Moreover, the expression of apelin and APJ is well-known in the testis of mice, rats and dogs **(Das et al., 2022; Brzoskwinia et al., 2020; Troisi et al., 2022)**. It has also been reported that apelin-mediated signals in the testis of rodents and humans might be deregulated in the pathological condition such as diabetes and inhibition of apelin signalling by ML221 improves the testicular activity **(Das et al., 2021: Song et al., 2022).** Our results also showed that the abundance of 3βHSD was

elevated in the Leydig cells of ML221-treated testis. This finding further strengthens the idea of the local role of apelin signalling in testosterone biosynthesis. Previous studies have also shown that Leydig cells express APJ (**Das et al., 2022; Brzoskwinia et al., 2020; Troisi et al., 2022).**

The above-mentioned facts on the role of apelin in the testis hinted that apelin has a regulatory rather inhibitory role in hormone secretion. Thus, the question arises, if apelin has an inhibitory role on the testis, then it might also be regulating the germ cell proliferation in the testis. Our histological analysis did not show the observable changes in the testis; however, number of VII/VIII stages were more in the ML221 treated group along with increased sperm concentration at higher dose. These results suggest that ML221 might have some role in germ cell proliferation, thereby increases the sperm concentration. It has been shown that apelin/APJ is a regulator of cell proliferation, apoptosis, pro-inflammatory activity, and revascularization in the liver along with cell proliferation, and apoptosis in the gonads **(Lv et al., 2017; Kurowska et al., 2018).** Thus, we have accessed the germ cell proliferation by PCNA GCNA and BrdU labelling. Our results showed these markers of cell proliferation were up-regulated after ML221 treatment. These results suggest that apelin might be suppressing germ cell proliferation in the adult testis. To the best of our knowledge, there is no report on the direct role of apelin in testicular germ cell proliferation. Our recent study has shown that apelin stimulates germ cell proliferation and decreases apoptosis in juvenile mice testis **(Das et al., 2022).** It was also noted in the present study that ML221 treatment increased the expression of BCL2 and decreased the expression of BAX and active caspase 3, suggesting apelin-promoting apoptosis in the adult mice testis. The results of our recent (Das et al., 2022) and present study showed that apelin has an age-dependent role in testicular proliferation and apoptosis. Apelin has been shown to inhibit the proliferation in rat pulmonary arterial smooth muscle cells **(Zhang et al., 2014).** It has also been shown that the apelin/APJ system may

inhibit or stimulate apoptosis under various conditions (**Liu et al., 2017).** Since the testis undergoes continuous cell proliferation and apoptosis, thus, it might be suggested that endogenous apelin signaling under normal conditions in the adult male testis could be responsible for the disposal of damaged germ cells. However, this statement is only indicative and requires further study to support this statement.

The balance of oxidative stress and the antioxidant system in the testis is critical for spermatogenesis and testicular functions (**Aitken, and Roman, 2008; Asadi et al., 2017).** It has been shown that oxidative stress and apelin/APJ are closely related (**Zhou et al., 2016).** Apelin can reduce oxidative stress in cardiomyocytes (**Than et al., 2014)** and also reduces the synthesis and release of ROS in adipocytes (**Foussal et al., 2010)**. However, it has also been shown that apelin-13 can stimulate the production of ROS in vascular smooth muscle cells (**Li et al., 2011**). Our results also showed that treatment with ML221 increased the testicular antioxidant enzymes, catalase and GPx and decreases the oxidative stress. These findings suggest that apelin-mediated signaling might be stimulating testicular oxidative stress and apoptosis. We have also measured the expression of NF-κB after ML221 treatment and results showed that APJ inhibition by ML221 decreases the expression of NF-κB. The decreased expression of NF-κB coincides with decreased apoptosis and increased antioxidant and germ cell proliferation in the adult testis. It has been shown that NF-κB regulates germ cell apoptosis in the testis and is suggested to have a pro-apoptotic role **(Pentikainen et al., 2002; Rasoulpour et al., 2007).** It has also been shown that expression of the apelin-13/APJ/NF-κB pathway increased in various tissues under the induction of oxidative stress and also suggested that increased APJ signalling and NF-κB expression could be responsible for increased oxidative stress **(Bakary et al., 2021).** The present study has also investigated the expression of AR, ERα and β after ML221 treatment. Inhibition of APJ increased the expression of AR and decreased the expression of ERα, however, ERβ was unaffected. Since both AR and ERs are

important regulators of spermatogenesis, however, in the present study, it seems that apelin-mediated suppression of AR might be associated with increased apoptosis, oxidative stress, and suppressed germ cell proliferation in the adult testis. Our immunolocalization study showed that ML221 treatment increase the abundance of AR in the spermatogonia, primary spermatocytes and Leydig cells of 150 µg/kg dose group. These findings could suggest cell specific role of apelin in the testis. Our recent report showed that apelin signaling up-regulates the expression of AR in the juvenile mice testis. These findings provide evidence of the stage-dependent role of endogenous apelin signaling in testicular functions.

In conclusion, this is the first study on the role of endogenous apelin signaling in adult mice testis. Our study is the first attempt to unravel the role of the endogenous apelin system on steroidogenesis, proliferation, apoptosis and oxidative stress. Our study showed that apelin might suppress germ cell proliferation and increases apoptosis in adult testis. The apelin-mediated apoptosis could be a disposal mechanism for damaged germ cells and the overall role of apelin signaling in adult testis seems to be inhibitory via down-regulation of AR expression.

Summary

The expression of apelin and its receptor (APJ) has been shown in the hypothalamus-pituitary-testicular axis. It has also been suggested apelin and APJ are neuropeptide factors. The presence of apelin and APJ in the seminiferous tubules and interstitium might be predicted to act as a local regulator of testicular activity, although the function is not well known in the mice testis. In the present study, we have investigated the effects of APJ, antagonist, ML221 on the gonadotropin levels, testicular steroidogenesis, proliferation, apoptosis and antioxidant system. Our results showed that inhibition of APJ by ML221 increased the sperm concentration, circulating testosterone, FSH, LH levels and intra-testicular testosterone concentration. Furthermore, ML221 treatment stimulates the germ cell proliferation and antioxidant system in the testis. The expression of BCL2, AR was up-regulated whereas, the expression of BAX and active caspase3 was down-regulated after ML221 treatment. Immunohistocehmical analysis of AR also showed increase abundance in the spermatogonia, primary spermatocytes and Leydig cells of 150 µg/kg dose group. These findings suggest that in adult testis, the apelin system might have an inhibitory role in germ cell proliferation and a stimulatory role in apoptosis. It might also be suggested that the apelin system could be involved in the disposal mechanism for damaged germ cells during spermatogenesis via the down-regulation of AR.

Figure 2.1 Effect of ML221 on the body weight (A), testis weight (B), gonado-somatic index (GSI) (C) and sperm count (D)

Treatment of ML221 (APJ antagonist) with two doses (15 and 150 µg/kg), the percentage of body weight increased significantly decreased ($p<0.05$) compared to the control and two doses of ML221 (A). The testis weight did not show a significant change between the control and ML221-treated groups (B). ML221 treatment at 150 µg/kg dose significantly ($p<0.05$) increased GSI compared to the control and 15 µg/kg dose groups (C). Sperm concentration also showed a significant increase in ML221 treated groups compared to control (D). Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 µg/kg (ML221)-ML-15, 150 µg/kg (ML221)-ML-150.

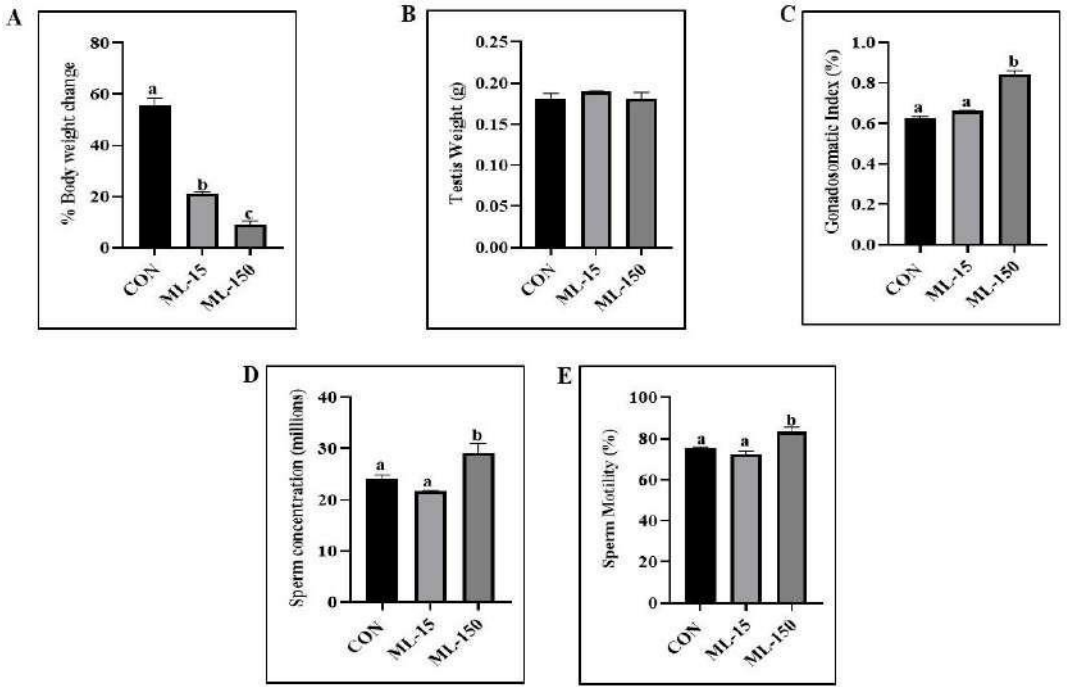

A
% Body weight change
80
60
40
20
0
a
b
c
CON
ML-15
ML-150
B
Testis Weight (g)
0.25
0.20
0.15
0.10
0.05
0.00
CON
ML-15
ML-150
C
Gonadosomatic Index (%)
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
CON
ML-15
ML-150
D
Sperm concentration (millions)
40
30
20
10
0
a
a
b
CON
ML-15
ML-150
E
Sperm Motility (%)
100
80
60
40
20
0
a
a
b
CON
ML-15
ML-150

Figure 2.2 Change in testicular histoarchitecture (A-C), histomorphometry and seminiferous epithelial cycle stage count

Treatment of ML221 did not show any gross observable changes in histology (A-C). The seminiferous tubule diameter (D) and germinal epithelium (E) height did not show any change. ML221 treatment at 150 µg/kg dose significantly ($p<0.05$) increased the no. of VII-VIII seminiferous epithelial cycle stage compared to control and 15 µg/kg dose groups (F). Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups.

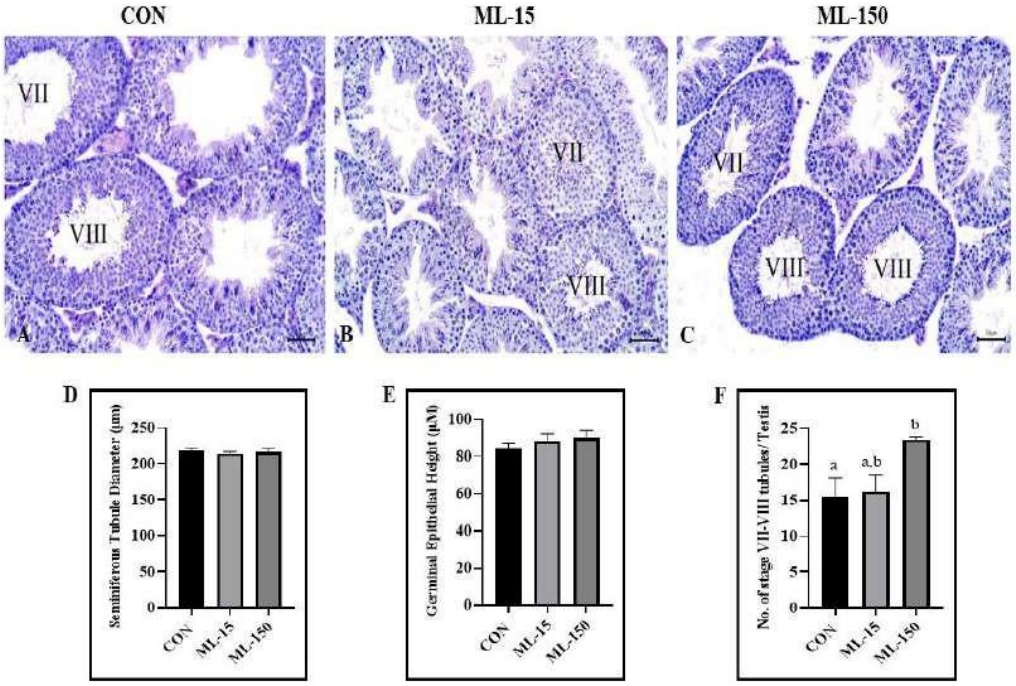

CON
ML-15
ML-150
VII
VIII
VII
VIII
VII
VIII
VIII
A
B
C
D
Seminiferous Tubule Diameter (µm)
250
200
150
100
50
0
CON
ML-15
ML-150
E
Germinal Epithelial Height (µM)
100
80
60
40
20
0
CON
ML-15
ML-150
F
No. of stage VII-VIII tubules/Testis
25
20
15
10
5
0
a
a,b
b
CON
ML-15
ML-150

Figure 2.3 Treatment of ML221 on circulating testosterone (A), estrogen (B), LH (C), FSH (D) levels and intra-testicular testosterone (C) and estrogen concentration (D)

The circulating testosterone levels significantly ($p<0.05$) elevated in the ML221 treated groups 15 and 150 µg/kg compared to the control (A). However, circulating estrogen was found to be significantly ($p<0.05$) decreased at 150 µg/kg dose compared to the control and 15 µg/kg groups (B). Both doses of ML221 15 and 150 µg/kg significantly ($P<0.05$) increased the levels of LH compared to the control groups(C). The levels of FSH were significantly ($p<0.05$) elevated at the 150 µg/kg dose group compared to the control and 15 µg/kg groups (D). The intra-testicular testosterone concentration showed a significant increase in ML221 treated groups to the control (E). The intra-testicular estrogen concentration was significantly ($p<0.05$) elevated at 150 µg/kg dose compared to the control and 15 µg/kg groups (F). Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups.

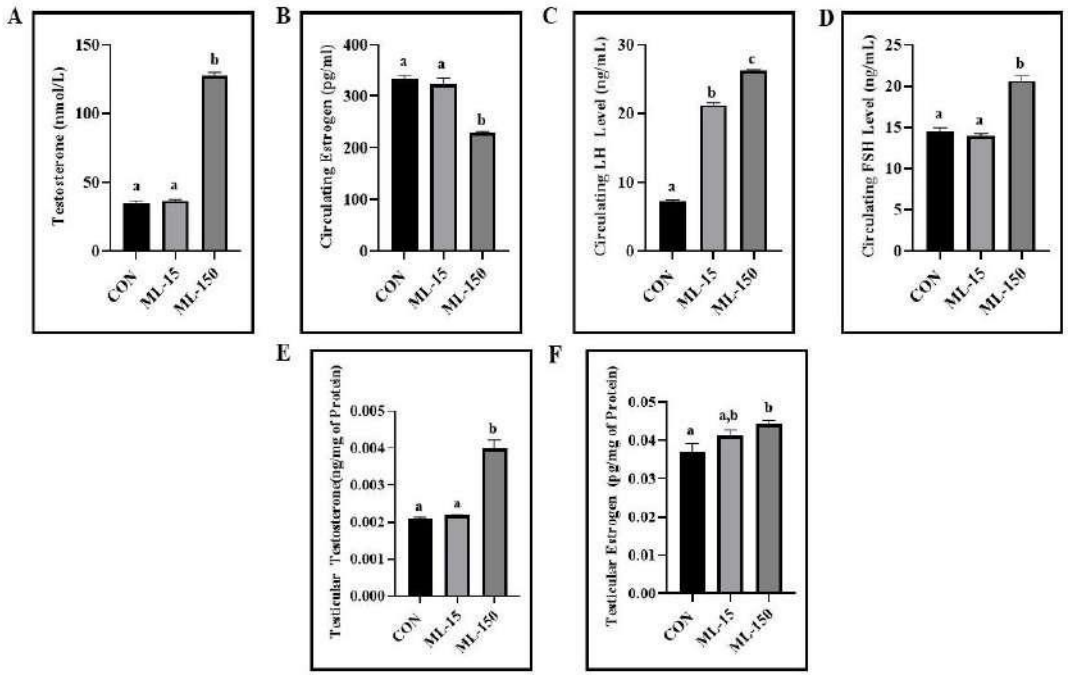

A
Testosterone (nmol/L)
150
100
50
0
a
a
b
CON
ML-15
ML-150
B
Circulating Estrogen (pg/ml)
400
300
200
100
0
a
a
b
CON
ML-15
ML-150
C
Circulating LH Level (ng/mL)
30
20
10
0
a
b
c
CON
ML-15
ML-150
D
Circulating FSH Level (ng/mL)
25
20
15
10
5
0
a
a
b
CON
ML-15
ML-150
E
Testicular Testosterone(ng/mg of Protein)
0.005
0.004
0.003
0.002
0.001
0.000
a
a
b
CON
ML-15
ML-150
F
Testicular Estrogen (pg/mg of Protein)
0.05
0.04
0.03
0.02
0.01
0.00
a
a,b
b
CON
ML-15
ML-150

Figure 2.4 Effect of ML221 on oxidative stress marker MDA levels (A) and antioxidant enzymes, GPx (B) and catalase (C)

The treatment of ML221 at both doses 15 and 150 µg/kg, significantly decreased the MDA levels compared to the control (A). However, the enzyme activity of GPx and catalase were significantly ($p<0.05$) elevated at both the doses, 15 and 150 µg/kg of ML221 compared to the control (B, C). Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups.

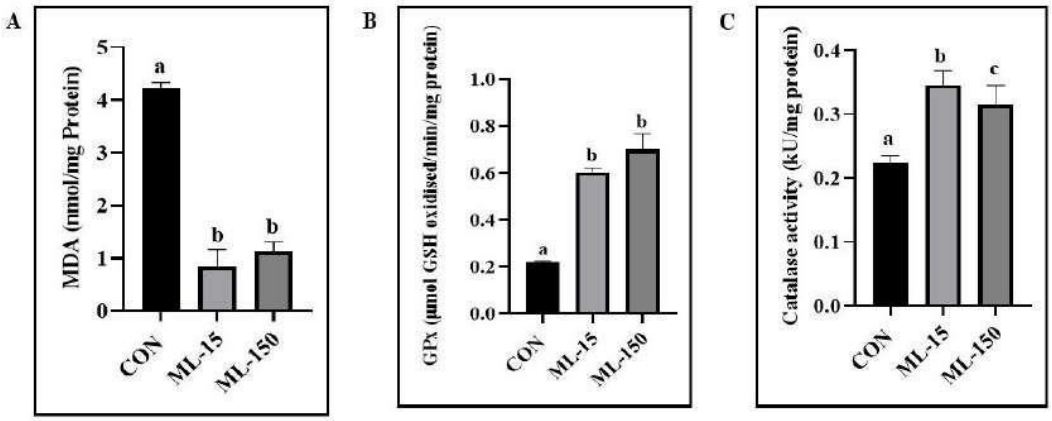

A
MDA (nmol/mg Protein)
5
4
3
2
1
0
a
b
b
CON
ML-15
ML-150
B
GPx (µmol GSH oxidised/min/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
CON
ML-15
ML-150
C
Catalase activity (kU/mg protein)
0.4
0.3
0.2
0.1
0.0
a
b
c
CON
ML-15
ML-150

Figure 2.5 Change in immunolocalization of 3βHSD after ML221 treatment

The treatment of ML221 showed an increased abundance of 3βHSD proteins in the Leydig cells, whereas moderate staining of 3βHSD was also observed in the Leydig cells (L) of control testis (A-C). The staining areas were significantly ($p<0.05$) higher in 150 µg/kg dose compared to the control and 15 µg/kg groups. Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 µg/kg (ML221)-ML-15, 150 µg/kg (ML221) -ML-150.

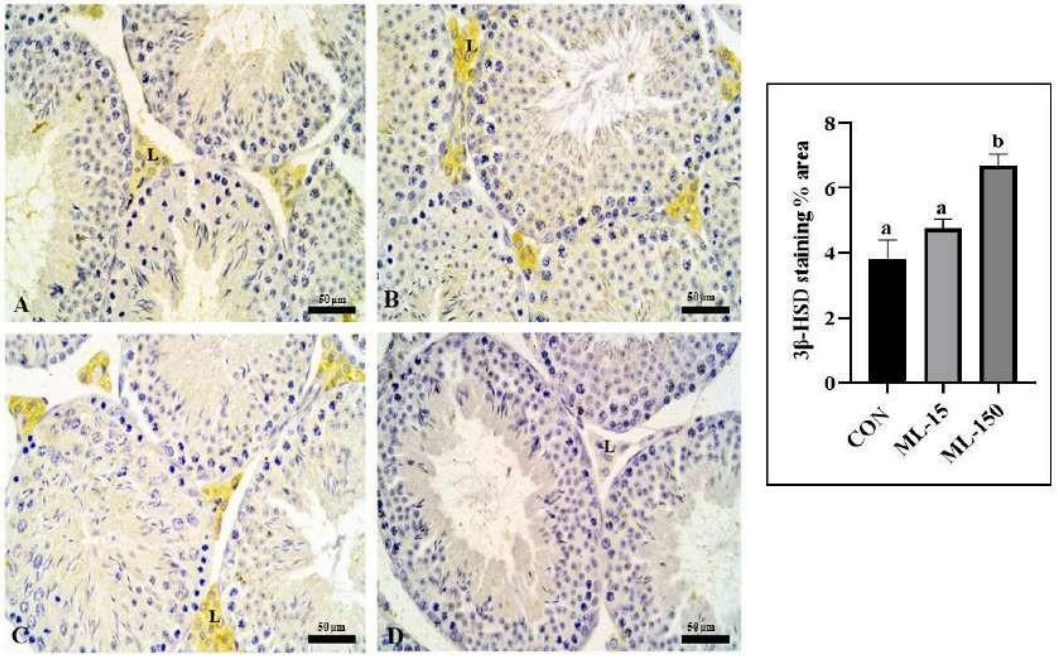

A
B
C
D
L
50 µm
3β-HSD staining % area
8
6
4
2
0
a
a
b
CON
ML-15
ML-150

Figure 2.6 Changes in the BrdU labelling and PCNA and GCNA by immunohistochemistry

ML221 treatment showed more BrdU (A-C), PCNA (D-F) and GCNA (G-I) positive cells in the testis than control testis. The BrdU, PCNA and GCNA staining areas were significantly ($p<0.05$) higher in 150 µg/kg dose compared to the control and 15 µg/kg groups (J-L). Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups.

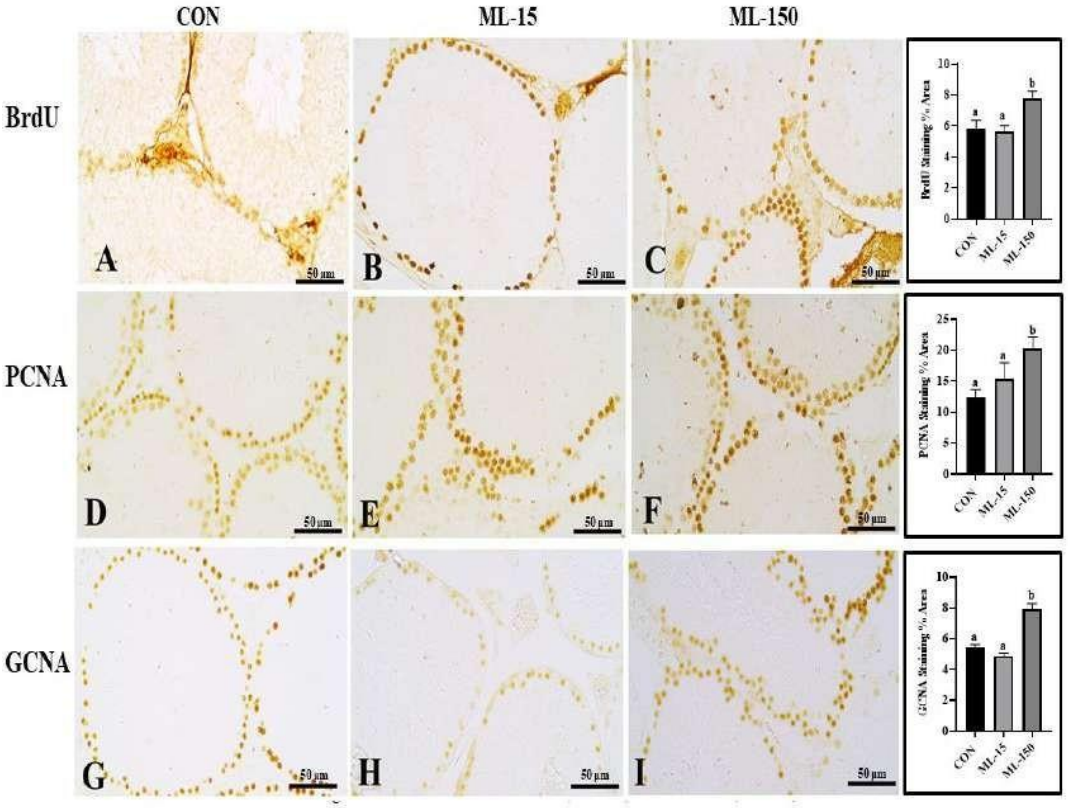

CON
ML-15
ML-150
BrdU
PCNA
GCNA
A
B
C
D
E
F
G
H
I
50 μm
BrdU Staining % Area
PCNA Staining % Area
GCNA Staining % Area
a
a
b
CON
ML-15
ML-150

Figure 2.7 Changes in the immunolocalization of AR after ML221 treatment

Immunostaining of AR showed increase abundance in 150 µg/kg dose group than the other two groups (A-C). The staining areas were significantly ($p<0.05$) higher in 150 µg/kg dose than control and 15 µg/kg groups (D). Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 µg/kg (ML221)-ML-15, 150 µg/kg (ML221) -ML-150.

L: Leydig cell; Spg: Spermatogonia; Ps: Primary spermatocytes.

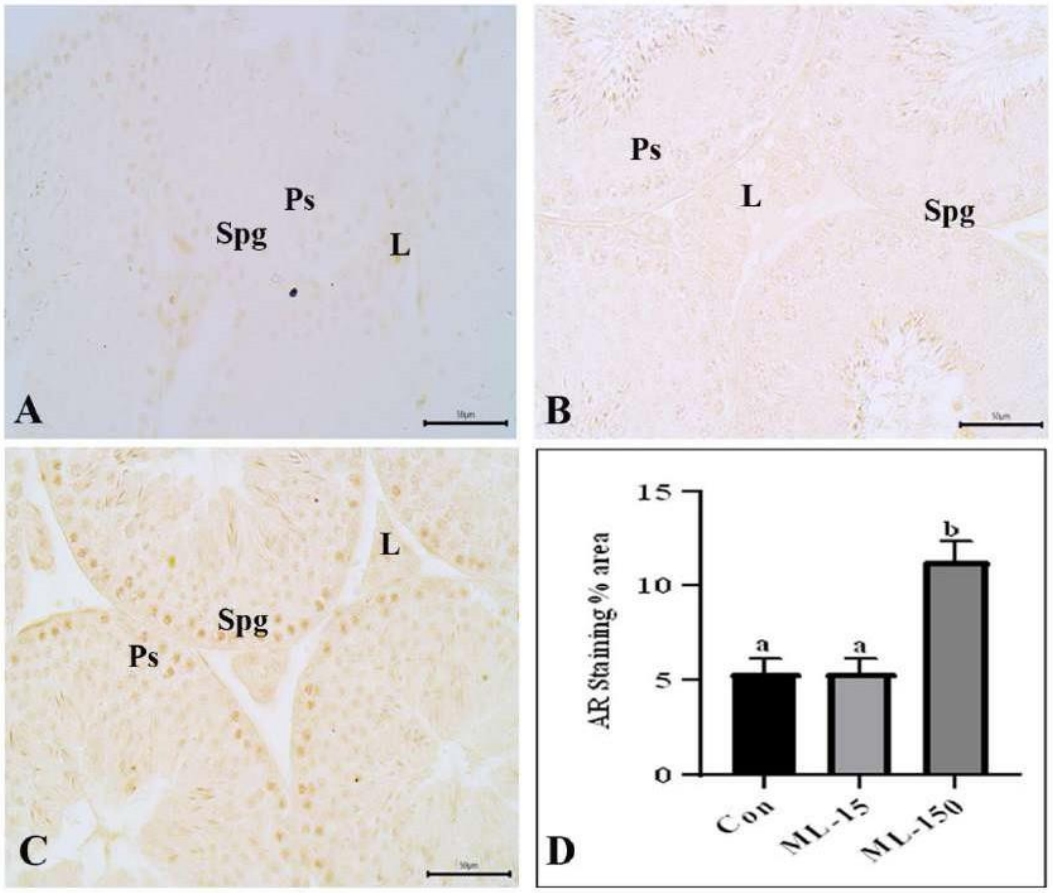

Ps
Spg
L
A
Ps
L
Spg
B
L
Spg
Ps
C
AR Staining % area
15
10
5
0
a
a
b
Con
ML-15
ML-150
D

Figure 2.8 Changes in the expression of BCL2, BAX, Active Caspase3 and NF-κB after ML221 treatment

The expression of BCL2 was significantly ($p<0.05$) up-regulated by ML221 at the dose of 150 µg/kg compared to the control and 15 µg/kg groups (A). The expression of active caspase 3, BAX and NF-κB were significantly ($p<0.05$) down-regulated in 150 µg/kg ML221 treated groups compared to the control and 15 µg/kg groups (B-D). Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups.

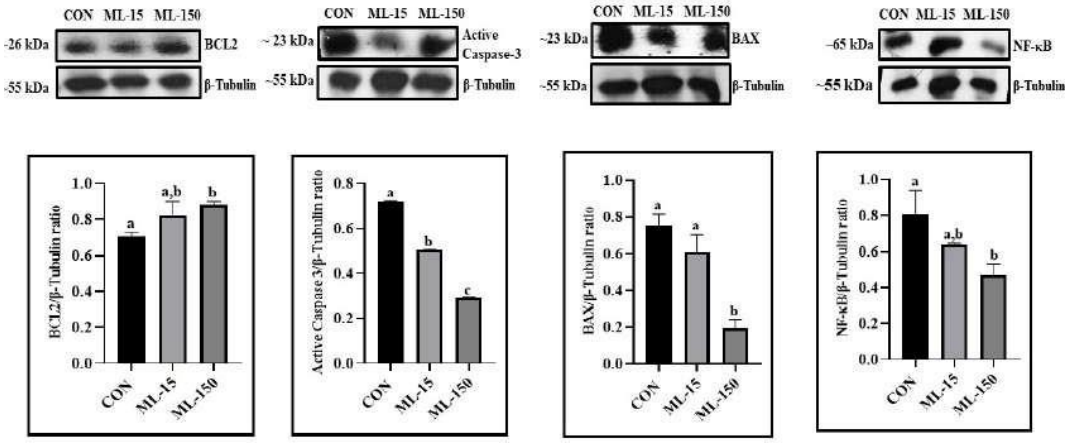
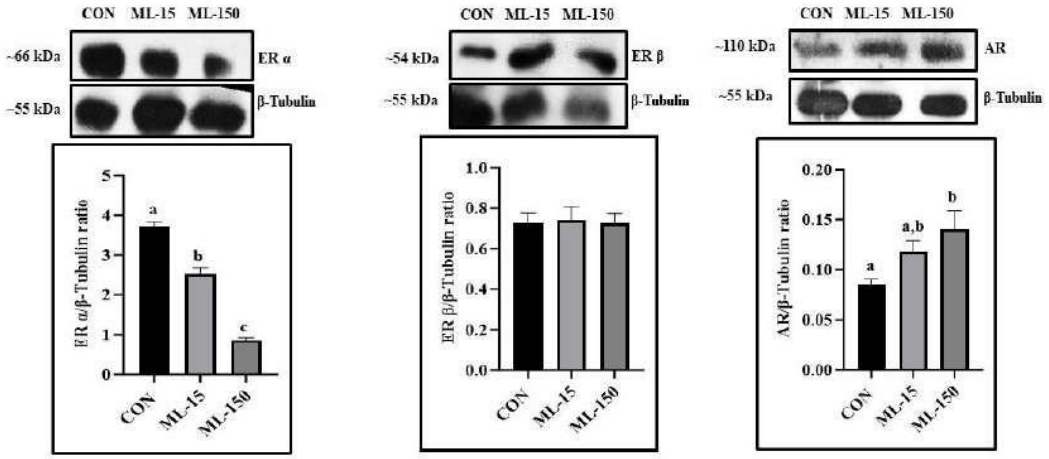

CON ML-15 ML-150
~26 kDa
BCL2
~55 kDa
β-Tubulin
CON ML-15 ML-150
~ 23 kDa
Active Caspase-3
~55 kDa
β-Tubulin
CON ML-15 ML-150
~23 kDa
BAX
~55 kDa
β-Tubulin
CON ML-15 ML-150
~65 kDa
NF-κB
~55 kDa
β-Tubulin
BCL2/β-Tubulin ratio
a
a,b
b
CON
ML-15
ML-150
Active Caspase 3/β-Tubulin ratio
a
b
c
CON
ML-15
ML-150
BAX/β-Tubulin ratio
a
a
b
CON
ML-15
ML-150
NF-κB/β-Tubulin ratio
a
a,b
b
CON
ML-15
ML-150

Figure 2.9 Changes in the expression of AR, ERα and Erβ after ML221 treatment

Expression of AR was significantly ($p<0.05$) up-regulated at both doses of ML221 15 and 150 μg/kg compared to the control (A). However, expression of ERα showed a significant ($p<0.05$) decline in a dose-dependent manner (B). The expression of ERβ was unchanged in all the groups (C). Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups.


CON ML-15 ML-150
~66 kDa
ER α
~55 kDa
β-Tubulin
ER α/β-Tubulin ratio
a
b
c
CON
ML-15
ML-150
CON ML-15 ML-150
~54 kDa
ER β
~55 kDa
β-Tubulin
ER β/β-Tubulin ratio
CON ML-15 ML-150
~110 kDa
AR
~55 kDa
β-Tubulin
AR/β-Tubulin ratio
a
a,b
b

CHAPTER 3

Title

To study the role of apelin in seminiferous epithelium cycle

Introduction

Seminiferous tubules are the site of the highly controlled process known as spermatogenesis, which produces differentiated sperm as a result of intricate morphologic changes. Spermatogenesis may be split into three primary stages: spermatogonial proliferation, spermatocyte meiosis, and haploid spermatid spermiogenesis. The seminiferous tubules include distinct groupings of spermatogenic cells known as stages **(Oakberg 1959)**, which may be recognized by the morphological characteristics of spermatids divided into steps 1 through 16 in the mouse **(Russell et al., 1990).**

Undifferentiated type A spermatogonia divide mitotically to produce intermediate and type B spermatogonia during spermatogonial proliferation. Preleptotene spermatocytes at stages IV–VIII are produced in the seminiferous tubules following the last mitosis of type B spermatogonia, where they begin meiosis and give birth to leptotene and zygotene spermatocytes at stages IX–XII. At stages I-X and XI, respectively, these cells develop into pachytene and diplotene spermatocytes. At stages XII and I, haploid step 1 spermatids are produced. 16 levels of morphological classification are used for haploid spermatids **(Ventelä et al., 2002; Xu et al., 2021)**. Numerous different stage-specific factors have been found that are very likely required in the local regulation of spermatogenesis **(Parvinen 1993**). It has been shown that expression of claudin protein depends on the stage of seminiferous epithelium and might have role in blood-testis barrier function **(Morrow et al., 2009; 2010).** Transcriptional factor EB promote cell migration across the blood testis barrier and transport along the seminiferous epithelium **(Liu et al., 2018)**. It has also been shown that notch component shows a specific cell-type and time-window expression pattern in the mouse testis and might regulates the spermatogenic cycle **(Murta et al., 2013).** It has been suggested that specific spatiotemporal expression of differentiation markers mighty be unique to spermatogonia, spermatocytes, and round spermatids ensures its completion. The round spermatid specific Acrv1 gene, which makes acrosomal protein SP-10, could be involved in the round spermatid-specific

transcription regulation **(Reddi et al., 2023).** The various markers (ZBTB16, GDNF, Connexin43) have been indentified which changes during the cycle of the seminiferous epithelium **(Grasso et al., 2012; Batias et al., 2000)**. In the mouse and rat, several testis-specific genes whose expression is begun in round spermatids have been identified **(Penttila et al., 1995).**

Despites above mentioned factors, there could be a large number of other intra-testicular factors which expression might be stage specific in the testis. In context with various intra-testicular factors, the adipokines such as leptin, apelin, resisitin and adiponectin have been shown in the testis **(Barreiro et al., 2003)**. These adipokines are known to regulate energy metabolism and might be a link between metabolism and reproduction. The expression of adiponectin and their receptors have been shown in the rat testis and their levels changes across the seminiferous epithelium cycle **(Caminos et al., 2008)**. Similarly, the expression of leptin receptor has also been shown to be stage-specific in mouse testis **(El-Hefnawy et al., 2000)**. The expression of apelin receptor (APJ) has also been shown in the testis of mice, rat and dogs **(Das et al., 2021; Estienne et al. 2019; Troisi et al., 2022).** However, the stage wise localization of APJ has not been investigated in testis of any animals. Therefore, the aim of present study was to investigate the stage specific localization of APJ in the mouse testis.

Materials and methods

Animals and tissue collection

Swiss albino mice were maintained in aerated polypropylene cage with *ad libitum* food and water, under standard experimental conditions (12 hrs light-dark cycles, 25±2ºC). Adult mice were chosen for this study. All the animal experiment was approved by Mizoram University Institutional Animal Ethical Committee (MZUIAEC-9), Mizoram University, Mizoram, India. Adult (n=5) mice were sacrificed and the testes tissue was collected. The testes sample was fixed in Bouin’s solution for immunohistochemical studies.

Intra-testicular administration of APJ antagonist, ML221

To unravel the role of apelin in seminiferous epithelium cycle of adult mouse model, the mouse was given a single intra-testicular administration of apelin receptor antagonist (ML221) at two doses according to the previous study **(Hall et al., 2017)** and the control mice received distilled water (vehicle). Mice were randomly divided into three groups, group 1: Control (Con, n=5), group 2: ML221 at doses 15 µg/kg (ML-15, n=5) and group 3: 150 µg/kg (ML-150, n=5). Both the testis injected with the ML221. On the 8^{th} after treatment mice were scarified and testis was collected for histological analysis.

Immunohistochemistry of apelin receptor (APJ)

Immunolocalization of apelin receptor was performed in testes section of adult mice following the protocol by **Jeremy et al., 2019**. Briefly, the spread sections were deparaffinized in xylene followed by rehydrated in ethanol and distilled water. After rehydration, sections were blocked by goat serum (1:100, Lot# H2114; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at the room temperature. Then, the tissue sections were incubated with the primary antibody to APJ (1:100, cat # ABD43; EMD Millipore Corporation, USA) for overnight at 4^{o}C followed by the incubation with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase. Then the slides were washed in PBS and developed by using 0.05% diaminobenzidine (DAB) solution (3,3'-diaminobenzidine tetrahydrochloride, in Tris-HCl buffer, pH 7.6 and hydrogen peroxide). The sections were counterstained with Hematoxylin, and then the stained sections were dehydrated and mounted by DPX. The sections were observed and photographed using Euromax microscope (iScope series). Photography was done by using 40X and 60X objective lens.

Results

Seminiferous epithelium cycle stage dependent changes in the immunolocalization of apelin receptor (APJ)

Two generation of spermatids are found in stage I-VIII, the round (step 1-8) and elongate (step 13-16) spermatids. The immunostaining of APJ was observed in the

newly formed round spermatid step 1 at stage I to round spermatid step 6 (stage I-VI). Older generation of spermatids (step 13-16) did not exhibit immunostaining of APJ **(Figure 3.1A-C)**. The immunoreactivity of APJ in step 7-8 spermatids was faint than other round spermatids **(Figure 3.1C)**. Only single generation of spermatids were found in the stage IX-XII. The round spermatids begin to be flattened and elongated throughout the stages. Immunostaining of APJ was seen in the entire length of spermatid heads in the steps 9-12. The head of Step-12 spermatid appeared longest in stage XII **(Figure 3.1G)**. The Leydig cells and myoid cells also showed moderate immunostaining of APJ in adult mice testis **(Figure 3.1A-G).** The primary spermatocytes also showed immunostaining of APJ in adult mice testis in all the stages **(Figure 3.1A-G)**.

Staining of APJ in the different Stages at a glance

The epithelium from each stage has been shown at higher-magnification (60X) images to describe immunostaining of APJ in the spermatid **(Figure 3.2)**. In brief the expression of APJ starts with the new generation spermatids Step 1-6 and in spermiation stage (stage VII-VIII) it was found to be less. Again, the staining was seen in the elongated spermatids in the Step 9-12.

Testicular Histology after intratesticular administration of ML221

As the immunolocalization study of APJ showed stage dependent staining in the spermatid, thus we hypothesized that APJ could be important for spermiogenesis process. To unravel the role of APJ in the spermiogenesis, the intra-testicular administration of ML221 was performed at two doses 15 and 150 μg/kg. Our results showed the conspicuous changes in the seminiferous tubules. The control testis showed the regular arrangement of germ cell in the seminiferous tubules **(Figure 3.3 A, D, E)**, however, testis of mice treated at 15 μg/kg dose showed scant round spermatid in some tubules **(Figure 3.3G)**. Some of seminiferous tubules of mice testis at 150 μg/kg dose exhibited complete absence of round spermatid, however, only elongated spermatid was present **(Figure 3.3I)**. Testis of both groups also showed normal tubules with regular arrangement germ cells **(Figure 3.3F and H).**

Discussion

The present study unraveled the stage wise localization of APJ within the mouse seminiferous epithelium cycle. The immunostaining of APJ showed its presence in the interstitium as well as in the seminiferous tubules. In the interstitium, APJ was localized in the Leydig cells and this suggests role apelin signaling in the testicular steroidogenesis. Our previous studies **(Das et al., 2021; 2022)** also showed that apelin signaling is modulating testicular steroidogenesis. The Peritubular cells (myoid cells) also showed a mild staining of APJ. The exact role of apelin signaling is yet to be investigated. APJ have been detected in the testis of human and laboratory animals such as rats, mice and dogs **(Medhurst et al. 2003; Estienne et al. 2019; Kawamata et al. 2001; Pope et al. 2012; Troisi et al., 2022)**.

The immunolocalization of APJ within seminiferous tubules showed its presence in the different cell types. Our results showed the localization of APJ in the primary spermatocytes, round spermatids, elongated spermatids and sperm. The expression of APJ in the Sertoli cell was not detected. In a recent study, however, the expression of APJ has been shown in the mice Sertoli cells and it has been suggested that apelin mediated signaling might have several effects on the testicular activity like on blood testis barrier and testosterone biosynthesis **(Song et al., 2022)**. Since we could not detect the expression of APJ on Sertoli cells, it could be due to different strain of mice and antibodies used, however, our results does not ruled out the presence of apelin system in Sertoli cells. Despite the presence of APJ in different cell types of mice testis, the localization of APJ showed a remarkable change in the round and elongated spermatids. Therefore, we have investigated the localization of APJ in different stages of mouse seminiferous epithelium cycle.

The round spermatids from step 1 to 4 at stages I-IV showed moderate immunostaining of APJ. The immunostaining of APJ in spermatid steps 5-6 at stage V-VI showed mild staining, however, 7-8 spermatids showed very faint immunostaining of APJ. The immunostaing of APJ again showed a mild staining in the steps 9 spermatids followed a moderate immunostaining of APJ in 10-12 steps of spermatids (stage X-XII). The spermatids steps from 13 to 16 showed either faint or no immunostaining of APJ.

These results suggest that expression of APJ within the seminiferous tubules could be stage- and cell-specific within the seminiferous epithelium of mice. It has been suggested that the numerous stage-specific marker that might be required for the local control of spermatogenesis **(Parvinen 1993)**. It has been shown that based on the spermatid development, 12 stages (I-XII) of seminiferous epithelium can be distinguished **(Ahmed and de Rooij, 2009; Meistrich and Hess, 2013)**. Previous studies by **Osuru et al., (2017; 2014)** have shown that the expression of specific proteins like acrosomal protein SP-10 and TAR DNA-binding protein of 43 kD exhibits stage and cell specific expression with respect to the different stages of seminiferous epithelium cycle in mice testis. These studies **(Osuru et al., 2014; 2017)** have been the genesis of our present work. Furthermore, conspicuous changes in the immunolocalization of APJ was in spermatids, thus it might be suggested that apelin mediated signaling could be important for spermiogenesis, although, this is only a speculative statement, unless explored for further analysis. It has also been shown that apelin signaling facilitates endothelial and osteogenic cell differentiation **(Hang et al., 2019; Masoud et al., 2020)**.

It should be noted that immunostaining was less in the spermatid 7-8 along with 16 step spermatid at stage VII-VIII. These stages (VII-VIII) coincide with the process of spermiation. However, the exact role of apelin signaling in spermiation requires further investigations. As it has been stated that from steps 1 to steps 6, the immunostaining of APJ was declining, thus it can also be suggested that APJ signaling could be also a regulatory control for acrosomal formation, because during these stages acrosomal formation occurs **(Osuru et al., 2014).** The immunostaining of APJ showed increase in APJ abundance from 9 to 12 steps spermatids. It has been shown that from spermatid steps 8, the round spermatid begins to elongate length wise and attained maximum elongation at steps 12 **(Osuru et al., 2014).** Whether APJ signaling is required for chromatin remodeling for round spermatid, is yet to be investigated. Since the increased APJ abundance coincides with elongation of round spermatid, thus it might be suggested that apelin signaling could be important for this transformation. There could be several spermiogenic processes where apelin signaling might have important role. It has been shown that manchette is a temporary, skirt-like structure

that forms in the expanding spermatid head along with the spermatid nucleus' extension and condensation as well as the expansion of the centrosome-derived axoneme **(Kierszenbaum 2002)**. Microtubules, actin filaments, and the corresponding motor protein (such as myosin), together, create the fundamental framework of the manchette (**Lehti and Sironen 2016)**. It has also been shown that apelin induces the phosphorylation of myosin light chain and helps in vasoconstriction **(Hashimoto et al., 2006)**. Apelin has also been shown to improve cardiac contractility by stimulating expression of alpha and beta myosin chain **(Shahrivar et al., 2016)**. Thus, it could be suggested that APJ signaling might be involved in the formation of manchette and could also be important marker for spermatid specific functions. It has also been suggested that spermatid specific factors are required for sperm physiology **(Chen et al., 2016)**. To unravel the role of APJ on the spermatid, we have injected testis with APJ antagonist (ML221) at dose of 15 and 150 µg/kg and testis was examined after 8 days. Our results showed that at both doses of ML221, testis exhibited noticeable changes. Some seminiferous tubules were devoid of round and elongated spermatids at low dose. At higher doses some seminiferous tubules were devoid of round spermatid and showed only sperm. At both the doses, it was shown that round spermatid was mainly affected. As the expression of APJ was shown in the round spermatids (step1), thus, it might be suggested that ML221 treatment could inhibit the further differentiation of spermatids; therefore, some of the tubules could be devoid of round and elongated spermatids. Our recent study showed treatment of ML221 has a stimulatory effect on the germ cell proliferation and steroidogenesis in adult mice **(Das et al., 2023)** whereas present study suggests that apelin could also be required for spermatid differentiation and elongation to spermatozoa. Based on this observation it can be suggested that apelin might have differential role in the testis. However, further study would be required to unravel this discrepancy of stimulatory and inhibitory role of apelin in the testis.

In conclusion, APJ is expressed by both seminiferous tubules and interstitium of the mouse testis. The marked changes in localization of APJ in spermatids seem to be stage specific during spermatogenesis. Furthermore, apelin signaling might be important for in transformation of round spermatid to elongated spermatid. However,

the exact role of APJ during spermiogenesis requires further investigation, because only on the basis of localization of APJ, suggesting other physiological roles will be speculative only.

Summary

The apelin receptor (APJ) belongs to the member of the G protein-coupled receptor family, and expression of APJ has been reported in the different cell type of testis. The seminiferous tubules in the testis can be identified as different stages (I-XII). It has been also suggested that different factors could be expressed in stage and cell specific manner in the seminiferous tubules. Recently, we also shown that expression of APJ is developmenatly regulated in the testis from PND1 to PND42. Therefore, we analysed the expression of APJ in the testis of adult mice by immunohistochemistry. Immunohistochemistry showed that the APJ was highly specific for the round and elongated spermatids with stage dependent changes. The seminiferous tubules at stages I-VII showed APJ immunostaining in the spermatid steps 1 to 8, not steps of 13-16. The seminiferous tubules at stages IX to XII showed APJ immunostaining in the spermatid steps 9 to 12. These results suggested the possible role of APJ in the spermiogenesis process. The intra-testicular administration of APJ antagonist, ML221 showed the a few round spermatids in the seminiferous tubules and some of tubules with complete absence of round spermatid. Overall, we present evidence that APJ expression in spermatid is dependent on the stages of seminiferous epithelium cycle and APJ could be involved in the differentiation of round spermatid to elongated spermatid.

Figure 3.1 Immunolocalization of apelin receptor (APJ) in seminiferous epithelial cycle of adult mouse model (Stage I-XII)

Immunostaining of APJ is present in step 1-3 spermatids and absent in step 13-15 of stage I-III **(A)**, and step 4-6 spermatids of stage IV-VI **(B)**. The immunoreactivity of APJ in step 7-8 spermatids was faint and absent in step 16 than other generation of spermatids in stage VII-VIII **(C)**, Leydig cells and primary spermatocytes in adult mice testis. The immunostaining of APJ was found in head of the step 9-12 spermatids in stage IX **(D)**, X **(E)**, XI **(F)**, and XII **(G)**. The Leydig cells and myoid cells also showed moderate immunostaining of APJ in adult mice testis **(A-G)**. The primary spermatocytes also showed immunostaining of APJ in adult mice testis in all the stages **(A-G)**. No staining was observed in negative control.

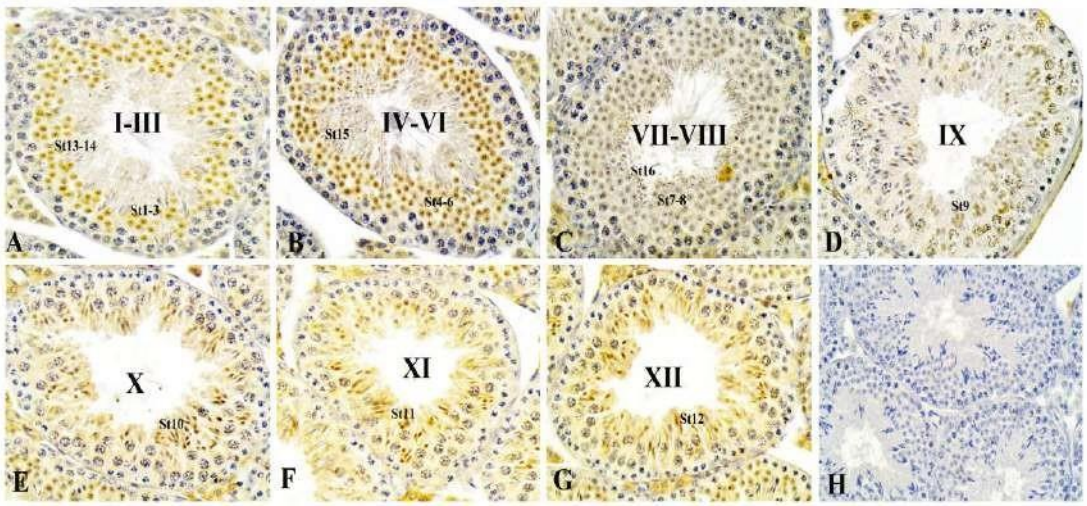

I-III
St13-14
St1-3
A
IV-VI
St15
St4-6
B
VII-VIII
St16
St7-8
C
IX
St9
D
X
St10
E
XI
St11
F
XII
St12
G
H

Figure 3.2 Staining of APJ in the different Stages at a glance

The epithelium from all 12 stages has been shown at higher-magnification (60X) images to describe immunostaining of APJ in the spermatid. The seminiferous epithelial cycle stages are marked with Roman numerals on the top. The round spermatids are referred to as St1-8 and the elongated spermatids as St9-12.

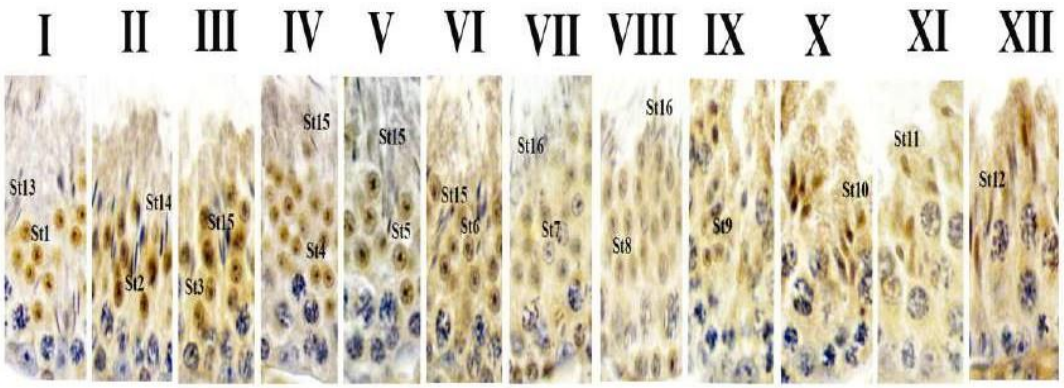

I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
St13
St1
St14
St2
St15
St3
St15
St4
St15
St5
St15
St6
St16
St7
St16
St8
St9
St10
St11
St12

Figure 3.3 Histoarchitecture of mice testis after intratesticular treatment of ML221

The control testis showed the regular arrangement of germ cell in the seminiferous tubules **(A, D, E)**, however, testis of mice treated at 15 µg/kg dose showed scant round spermatid in some tubules **(G)**. Some of seminiferous tubules of mice testis at 150 µg/kg dose exhibited complete absence of round spermatid, however, only elongated spermatid was present **(I)**. Testis of both groups also showed normal tubules with regular arrangement germ cells **(F, H).**

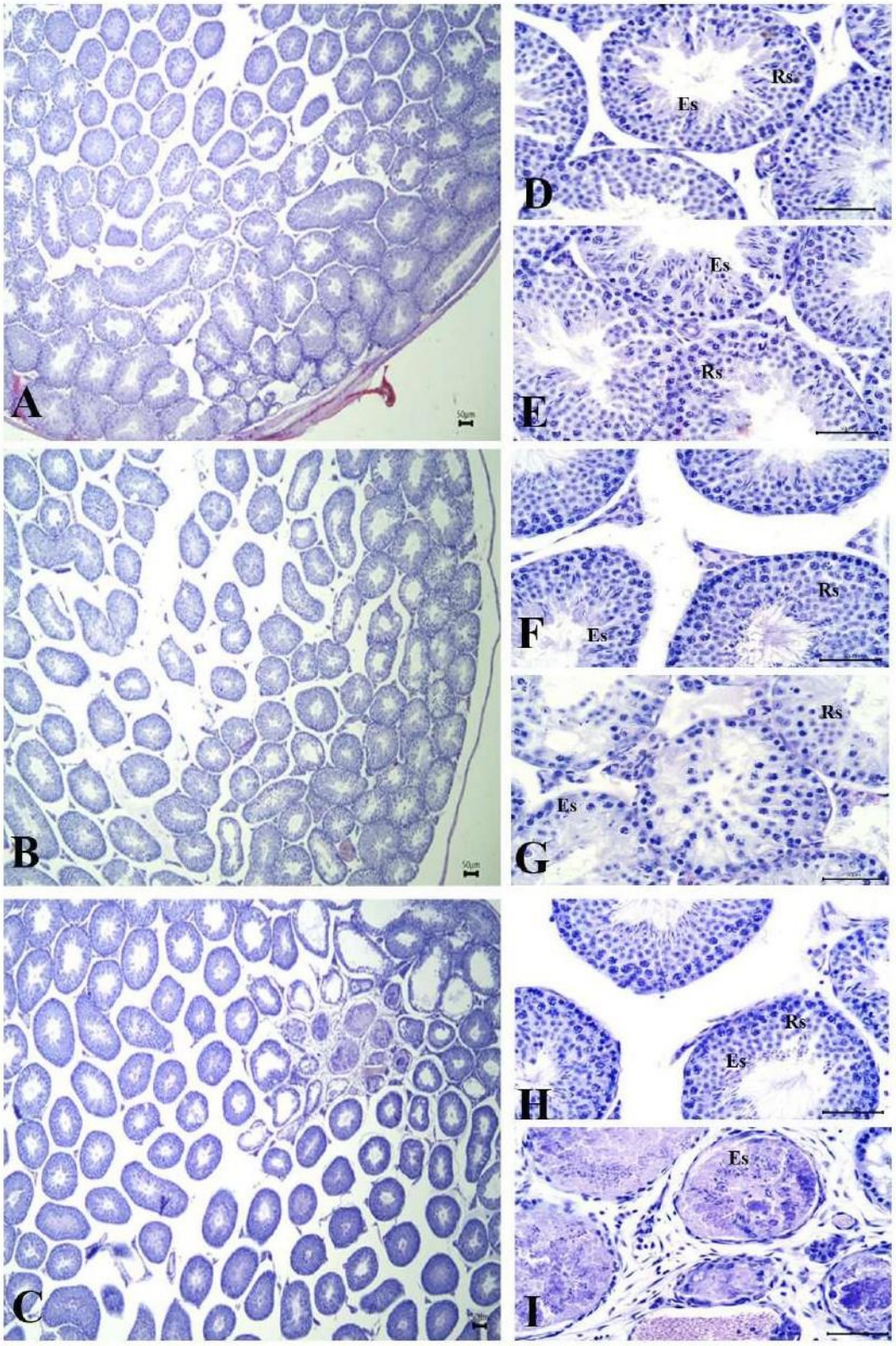
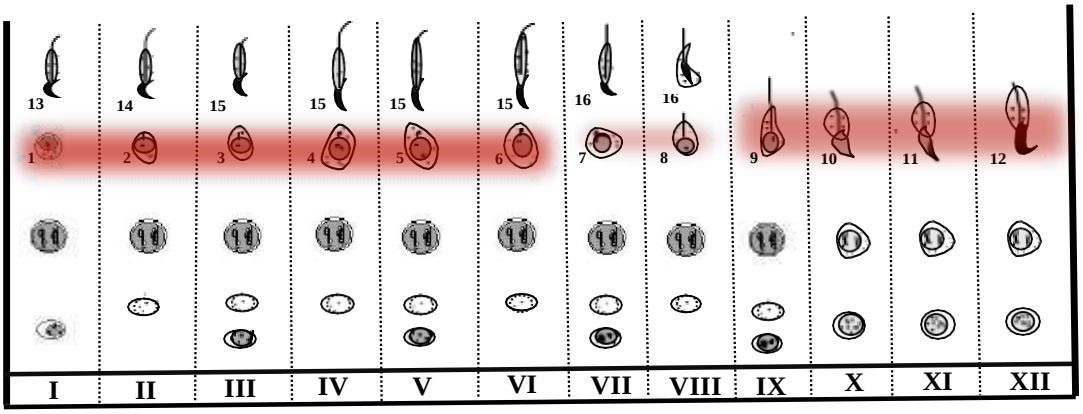

A
B
C
D
Es
Rs
E
Es
Rs
F
Es
Rs
G
Es
Rs
H
Es
Rs
I
Es

Figure 3.4 Schematic representation of APJ staining

The immunolocalization of APJ staining in seminiferous epithelium cycle represented in schematic diagram. Darker staining shows the abundance of APJ protein in mouse testis.


13
14
15
15
15
15
16
16
1
2
3
4
5
6
7
8
9
10
11
12
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII

CHAPTER 4

Title

To study the role of apelin in testicular steroidogenesis of diabetic mice*

***Cytokine 144 (2021) 155554**

Introduction

Cytokines are small secreted proteins from immune cells that have an important role in cell communications **(Zhang and An, 2007)**. Like immune cells, the adipose tissue also secretes a variety of cytokines such as leptin, visfatin, adiponectin, resistin, apelin and chemerin, commonly referred to as adipokines or adipocytokines **(Estienne et al., 2019)**. In humans and animals, these adipokines/cytokines regulate metabolic processes (**Zhang et al., 2017; Meier and Gressner 2004; Bertrand et al., 2015**) and are involved in the control of a wide range of physiological functions, including reproduction **(Dupont et al., 2015; Estienne et al., 2019)**. Since these adipokines regulate the male and female gametogenesis and steroidogenesis, modulating the activity of the hypothalamic-pituitary-gonadal (HPG) axis in both normal and pathological conditions **(Tsatsanis et al., 2015; Reverchon et al., 2014; Campos et al., 2008; Estienne et al., 2019),** they are considered as the important regulators of reproductive functions. There is evidence that adipokines, such as leptin, visfatin, adiponectin, and chemerin, as well as some components of their signaling cascades are expressed in the testes **(Tena-Sempere et al., 2002; Jeremy et al., 2017; Ocon-Grove et al., 2008; Li et al., 2014, Akkan et al., 2020)**. Along with other adipokines, peptides of the apelin family, which are encoded by the proapelin gene *APLN*, were found in the testes of humans and rats **(Tatemoto et al., 1998; Habata et al., 1999; Medhurst et al., 2003; Akkan et al, 2020; Brzoskwinia et al., 2020)**. However, the data on the expression of the apelin receptor (APJ receptor) encoded by the *APLNR* gene in the testes are contradictory (**Medhurst et al., 2003; Pope et al., 2012; Akkan et al., 2020; Brzoskwinia et al., 2020**). Therefore, the question of whether the testes are a direct target for apelin has not been fully resolved. It is important to note that there are no data on the localization of apelin and its receptor in the testes of mice, despite the fact that mice, including transgenic ones, are one of the most frequently used experimental animals for studying reproductive functions.

Type 1 diabetes mellitus (T1DM) is a metabolic disorder characterized by acute hyperglycemia and insulin deficiency. In human and experimental animals, T1DM not

only causes dysfunctions of the cardiovascular, renal and nervous systems (**Sullivan, and Forbes, 2019; Grundy et al., 1999**), but also leads to the reproductive dysfunctions, including impaired testicular steroidogenesis and spermatogenesis **(Jangir and Jain, 2014; Ding et al., 2015; Maresch et al., 2017; Roy et al., 2015, Annie et al., 2020; Derkach et al., 2020).** In diabetic conditions, the expression of adipokines changes significantly, including in the testes **(Yazıcı et al., 2012; Gurusubramanian and Roy, 2014; Derkach et al., 2020; Annie et al., 2020).** We have previously shown that in the testes of rats and mice with T1DM, the levels of visfatin, leptin and leptin receptor were reduced **(Gurusubramanian and Roy, 2014; Annie et al., 2020)**, while in the testes of rats with T2DM, the level of leptin was increased and the expression of leptin receptor was decreased, which was due to leptin resistance **(Derkach et al., 2020).** The treatment of diabetic rats and mice with metformin led to normalization of leptin system in the testes and improvement of the testicular functions **(Derkach et al., 2020; Annie et al., 2020).** Other authors have shown that leptin treatment of diabetic mice with leptin deficiency improves testicular function **[30] (Schoeller et al., 2014).** There are studies that show a decrease in the expression of adiponectin and its receptor in the testes in T1DM **(Wang et al., 2015**), and the restoration of testicular functions after treatment with adiponectin (**Choubey et al., 2020; Shi et al., 2020**). However, there is no information available on the expression of apelin and its receptor in the diabetic testes, as well as on the possible regulatory effects of apelin receptor ligands on testicular functions. We hypothesized that the expression and levels of apelin and APJ receptor in the testes of mice with T1DM can be altered, and such changes may indicate the involvement of the intratesticular apelin system in the etiology and pathogenesis of male reproductive disorders in diabetic pathology.

Therefore, the aim of the present work was to investigate the localization and expression of apelin and its receptor, APJ, in the testes of mice with streptozotocin (STZ)-induced T1DM and to study the effects of agonist (apelin-13) and antagonist (ML221) of APJ receptor on the testosterone production by the cultured explants isolated from the diabetic testes.

Materials and Methods

Animals

The adult Swiss albino male mice were selected for this study. The animals were maintained in aerated polypropylene cage with *ad libitum* food and water, under standard experimental conditions (12 hrs light-dark cycles, 25±2ºC). The animals were handled according to the protocol (MZUIAEC-8) approved by Mizoram University Institutional Animal Ethical Committee (MZUIAEC), Mizoram University, Mizoram, India.

Diabetes induction in mice model and sample collection

Healthy adult mice were weighed and randomly divided into two groups: control (CON)) and diabetes (DM). After 24 hr fasting blood glucose was measured before diabetes induction using glucometer (BG-03, Dr. Morpen). The mice of DM group was injected with four consecutive doses of streptozotocin (STZ) (75 mg/Kg) **[26, 27] (Annie et al., 2020; Derkach et al., 2020)** which was dissolved in 0.1 M sodium citrate buffer (pH 4.4). Two weeks after STZ injection, the mice with >15 mM blood glucose were considered as diabetic. The control animals were given vehicle only (sodium citrate buffer). At the end of experiment, the mice were sacrificed (CON, n=5; DM, n=5), and the tissues were collected and stored at -20ºC and fixed in Bouin's Fluid. The serum sample extracted from blood by centrifugation and stored at -20ºC for further analysis.

In vitro study

To study the effects of apelin-13 and APJ inhibitor (ML221) on testosterone secretion, the second set of experiment was performed. The diabetic group included mice that were treated with STZ four times, similar to that described above, and which had blood glucose levels >15 mM. The mice were sacrificed after mild anesthesia, and the testes from the control (n=4) and diabetic mice (n=4) were taken out and cleaned of adhering tissue. The testes were cut in to equal pieces and testes explants cultured were

performed from control and diabetic mice. Further, the testes explants were cultured for 24 hrs according to the method described earlier **(Jeremy et al., 2019)**. In brief, the testes were cut in equal fragments and cultured in the presence of apelin-13 (1 µg/ml) or ML221 (1 µM) (cat # SML0919, Sigma Aldrich, St Louis, USA) in a DMEM: Ham'F12 medium (1:1) with penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 for 24 h at 37 °C. The fragments of diabetic testes were cultured in the presence of apelin-13, and the dose of 1 µg/ml was selected from previous study **(Li, et al., 2018)**, or in the presence of ML221, APJ antagonist, and in this case the dose of 1 µM was also selected from previous studies **(Ishimaru et al., 2017; Lou et al., 2021)**. After successful cultivation, the tissue and media were harvested for further study.

Immunohistochemistry of PCNA, GCNA, apelin and apelin receptor and quantification by ImageJ

The excised testis was embedded in paraffin for immunohistochemistry as described earlier **(Jeremy et al., 2019)** and sectioned at 7 µM thick by Leica rotary microtome (RM2125 RTS). The testis sections were spread on a glass slide previously coated with poly-*L*-lysine. The slides were processed for immunolocalization of PCNA, GCNA, apelin and apelin receptor (APJ). PCNA, apelin and APJ receptor were developed by ABC staining kit (Lot# sc 2018; Santa Cruz Biotechnology, CA, USA). Briefly, the sections were deparaffinised and rehydrated by following the earlier method, and then the tissue sections were blocked with goat serum (Lot# H2114; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at the room temperature. After blocking, the tissue sections were incubated with the primary antibodies to apelin (1:50, cat # SAB4301741; Sigma Aldrich, St Louis, USA), APJ (1:100, cat # ABD43; EMD Millipore Corporation, USA) and PCNA (1:100, cat # SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA, USA), for overnight at 4ºC in humidified chamber, and then the primary antibody was washed with phosphate-buffered saline (PBS), and the tissue sections were incubated with biotinylated goat anti-rabbit immunoglobulin secondary antibody for 30 min at the room temperature. After rinsing in PBS, the

slides were incubated with horseradish avidin-peroxidase conjugate for 30 min at the room temperature, and then the slides were washed. The GCNA immunolocalization was performed by the primary antibody to GCNA (1:200, cat # 10D9G11; DSHB, University of Iowa, Iowa, USA) followed by the goat anti-mouse secondary antibody conjugated with horseradish peroxidase. After incubation the slides were washed and developed by using 0.05% diaminobenzidine (DAB) solution (3,3'-diaminobenzidine tetrahydrochloride, in Tris-HCl buffer, pH 7.6). For immunohistochemical determination of apelin and APJ, the tissue sections were counterstained with hematoxylin, and then the obtained sections were dehydrated and mounted by DPX. The negative control slide was also run with 1% mouse non-immune IgG for GCNA and 1% rabbit non-immune IgG for PCNA, apelin or APJ to check the specificity of the primary antibody. The slides were observed and photographed by Nikon microscope (E200, Nikon, Japan).

The semi-quantification of PCNA and GCNA staining in the testes of the control and diabetic mice was executed by ImageJ software. The stained area by DAB for PCNA and GCNA in the testis was acquired by using threshold tool of ImageJ as described previously (**Jensen, 2013**), and the data was presented as percentage area of PCNA and GCNA staining. The percentage area for immunostaining was described earlier for the other tissues (**Annie et al., 2020**). The quantification of apelin was done same way after using a color deconvolution tool (**Croweand and Yue, 2019**).

Western blot analysis

Western blotting of testis tissue was performed by following the standard protocol of **Jeremy et al, 2019.** Briefly, 10% (w/v) tissue homogenate were prepared with lysis buffer which contains 0.01 M Tris-HCl (pH 7.6), 1.0 mM EDTA, (pH 8.0),0.1 M NaCl, 1 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride, and the protein concentrations were estimated by Bradford method **(Bradford, 1976)**, The equal amount (50 µg) of protein from each group loaded to each well along with molecular weight marker in a 10% SDS-PAGE. The resolved proteins were transferred to PVDF membrane using wet transfer apparatus for 12 hrs, after successful protein transfer the

membrane blocked for 30 min at the room temperature with 5% non-fat skimmed milk prepared using PBST(cat # GRM1254-500G; HiMedia Laboratory private limited, Mumbai, India) and then overnight incubated at 4ºC with the primary antibodies to apelin receptor (APJ) (1:2000, cat # ABD43; EMD Millipore Corporation, USA), antiapoptotic marker BCL2 (1:2000,cat # SC7382; Elabscience Wuhan, China) and proapoptotic marker – active form of caspase-3 (1:1000, cat # STJ97448; St. John's Lab London, UK). Then the primary antibody was washed for 30 min with PBST, and the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase at the room temperature for 2 hrs. After incubation the membranes were washed and developed onto X-ray film by using the electrochemiluminescence (ECL) (cat # 1705060; BioRad, Hercules, CA, USA) method. The band intensities were quantified by using the ImageJ software (imagej.nih.gov/). The membranes were stripped and reprobed for β-Tubulin (1:4000, cat # E-AB-20033; Elabscience, Houston, Texas, USA) for loading control. Immunoblotting of the cultured explants isolated from the diabetic testes were performed following above protocol.

Hormone assay

Testosterone concentration was estimated in the serum from *in vivo* study and in media from *in vitro* study by using a commercial human testosterone kit (cat # EIA K209; Xema-Medica Co.Ltd, Moscow, Russia) according to manufacturer's instruction. The apelin levels in the testes of control and diabetic animals were measured by using mouse apelin EIA kit (cat # RAB0018; Sigma-Aldrich Co. LLC, USA) as per instruction manual.

Statistical analysis

Using GraphPad Prism7, all statistical analyses were performed and all numerical data were expressed as mean±*SEM*. The normal distributions of the data were analyzed by the Shapiro-Wilk normality test. To compare the data from different groups, One-way Analysis of variance (ANOVA) followed by Tukey's test was used for *in vitro*

testosterone assay, and the Student's *t* test was used for *in vivo* study. The data were considered as significant at the $p<0.05$.

Results

Changes in the body weight, testis weight, blood glucose and circulating testosterone levels

The diabetic mice exhibited a significant decline in the body ($p=0.0013$) and testis weight ($p=0.0035$) compared to the control mice **(Figure 4.1A-B)**. The blood glucose levels ($p=0.0022$) and circulating testosterone concentration ($p=0.0001$) in diabetic mice also showed a significant elevation compared to the control animals **(Figure 4.1C-D)**.

Changes in the localization pattern and intratesticular apelin concentration

In order to find out the distribution of apelin in the diabetic testis, immunolocalization of apelin was performed. The immunolocalization of apelin showed moderate staining in the Leydig cells and faint staining in the sperm of control testis, whereas in the diabetic testis, intense immunostaining in the Leydig cells, primary spermatocytes, round spermatid and sperm was detected **(Figure 4.2A-B)**. The semi quantitative analysis showed an increase in the stained area in the diabetic testis compared to the control testis ($p=0.0175$) **(Figure 4.2C)**. Furthermore, the quantitative analysis also showed elevated concentration of apelin in the diabetic testis compared to the control testis ($p=0.0004$) **(Figure 4.2D)**.

Changes in the localization pattern and expression of apelin receptor (APJ)

To gain further insight to the testicular apelin system in the diabetes, the expression and localization of APJ was also performed. Immunohistochemical study showed the presence of APJ in the Leydig cells, primary spermatocytes, elongated spermatid and sperm in the control **(Figure 4.3A-B)** and diabetic testis **(Figure 4.3C-D)**. The immunostaining of APJ was found to be more pronounced in the different cell types of diabetic testis than in the control testis. The severely damaged tubules of diabetic

testis showed intense immunostaining in the multinucleate cells. In the diabetic testis APJ immunostaining was very strong in the covering of seminiferous tubules.

The expression of APJ was found be significantly higher (t=2.437, df=6, p=0.0408) in the diabetic testis compared to the control testis, as measured by western blot analysis **(Figure 4.3F)**.

Changes in the proliferating markers, PCNA and GCNA

The immunolocalization of two proliferation markers, PCNA (p<0.0001) **(Figure 4.4A-B)** and GCNA (p<0.0001) **(Figure 4.4D-E)** showed a significant decrease in their expression in the diabetic testis compared to the control testis.

Changes in the anti-apoptotic (BCL2) and pro-apoptotic (active caspase3) protein

The expression of BCL2 was down regulated (p<0.0001) **(Figure 4.5A)** and active form of caspase-3 (p<0.0001) **(Figure 4.5B)** was up regulated in the diabetic testis, as compared to the control.

Effect of apelin-13 and ML221 on the testosterone secretion- an *in vitro* study

The untreated diabetic testis explants showed a significant (p<0.05) decline in testosterone secretion compared to the control testis explants. The treatment of the diabetic testis explants with apelin-13 (1 µg/ml) did not show changes in testosterone secretion compared to the untreated diabetic and control testis explants. The treatment of the diabetic testis explants with 1 µM of APJ antagonist (ML221**,** 5-[(4-nitrobenzoyl)oxy]-2-[(2-pyrimidinylthio)methyl]-4H-pyran-4-one) significantly stimulated the testosterone secretion compared to control testis explants and to untreated and apelin-13-treated explants isolated from diabetic mice (p<0.05) **(Figure 4.6)**.

Discussion

In the present study, we first demonstrated the comparative immunolocalization of apelin and its receptor (APJ) in the testes of control and diabetic mice and assessed the intratesticular levels of apelin and APJ, and then compared these levels with testosterone secretion and the proliferative activity of spermatogenic cells. In order to study the role of the apelin system in the functions of Leydig cells, the effect of apelin-13, the most active form of peptides of the apelin family, and ML221, a low molecular weight antagonist of APJ receptor, on testosterone production in the testis explants obtained from the diabetic mice was studied in the *in vitro* conditions.

We have shown that streptozotocin-induced T1DM leads to hyperglycemia, disorganization of the testicular architecture, and a decrease in testosterone secretion in mice, which is fully consistent with the results of previous studies on diabetic patients and animals with experimental models of diabetes **(Jangir et al., 2014; Roy et al., 2015; Maresch et al. 2017, Annie et al., 2020; Derkach et al., 2020)**. At the same time, the levels of apelin and the expression of APJ receptor in the testes of diabetic mice were significantly higher than in the control group, and this is the first report of an increase in the expression of apelin signaling system components in the testes in the conditions of metabolic disorders. Earlier, an increase in the level of apelin and its receptor was shown in the adipose tissue of rats with streptozotocin-induced diabetes **(Kohan et al., 2020)**.

Using immunohistochemical methods, a moderate increase in the content of apelin and APJ in testicular cells of diabetic mice was shown compared to control animals. Along with this, it was shown that apelin is localized within the germ cells of the control and diabetic testes. Earlier, the expression of APJ was found in the brain, ovaries, kidney, pancreas, heart and breast, and is stimulated by insulin, estrogens, glucocorticoids, cAMP and some transcriptional factors **(O'Carroll et al., 2006, 2013)**. Recently, there have been reports on the distribution of apelin and APJ in the testes of rats **(Akkan et al, 2020; Brzoskwinia et al., 2020)**, but such information was not available for mice prior to our study. In addition, despite the detection of apelin in the human and rat Leydig cells **(Habata et al., 1999; Medhurst et al., 2003)**, the

results on APJ expression in human and rodent testicular cells is contradictory. A number of authors indicated either a low level of expression of this receptor in the testes, or its absence in them (**Medhurst et al., 2003; Pope et al., 2012)**, which makes the possibility of a direct effect of apelin on testicular cells doubtful. In this regard, the detection of AJP expression in the testes of the control and diabetic mice is an important argument in favor of the presence of a functionally active apelin signaling system in testicular cells. This assumption is confirmed by the data of other authors in relation to the testes of the rats (**Akkan et al, 2020; Brzoskwinia et al., 2020**). We detected an increase in the APJ expression in T1DM, which may indicate the role of this system in the development of diabetes-associated testicular dysfunctions. Since it is known that T1DM suppresses the functions of Leydig cells and reduces the secretion of testosterone, an increase in the intratesticular level of apelin and a more intense immunostaining of apelin and APJ in diabetic testes as compared to the control testes, observed by us, indicate possible inhibitory role of apelin in the biosynthesis of testosterone.

Currently, there is a little information about the regulatory effect of apelin on the male reproductive system, and this influence is mainly associated with the effects of apelin on the hypothalamic and pituitary components of the HPG axis. Thus, it has been shown that high doses of apelin-13 cause a decrease in testosterone production by the testes, inhibiting the secretion of LH by the anterior pituitary gland **(Sandal et al., 2015)**. It was also found that treatment with apelin-13 lowers the levels of LH, FSH and testosterone and, thus, may have a negative effect on reproductive functions **(Tekin et al., 2017)**. Taking into account the presence of apelin and APJ receptors in the testes, it can be concluded that apelin is able to act not only on the hypothalamic and pituitary components of the gonadal axis, but also on its peripheral links, the gonads, as shown for other adipokines, including leptin, adiponectin and visfatin **(Dupont et al., 2015; Jeremy et al., 2017; Zhang &Gong 2018; Estienne et al., 2019)**.

We have established a direct relationship between high levels of intratesticular apelin and decreased testosterone production in diabetic mice. At the same time, in the *in vitro* experiments with cultured explants isolated from the diabetic testis, it was

shown that ML221, an antagonist of APJ, significantly increased the secretion of testosterone by these explants, and the level of the hormone even exceeded that in the control, while apelin-13 was ineffective. Thus, this is the first report that a decrease in the apelin signaling activity in the testes by the APJ receptor antagonist may have significant therapeutic potential to treat the reproductive dysfunctions, at least in T1DMconditions. Previously, it was also suggested that antagonists of apelin receptor could be used to treat infertility, but in this case only the hypothalamic mechanisms of action of apelin were taken into account **(Sandal et al., 2015)**.

It should be noted that we measured the total concentration of apelin in the testes, including its long, less active forms (for example, apelin-36), as well as more active shortened forms (apelin-13), which is usually used in pharmacological studies **(Tatemoto et al., 1998; Kurowska et al., 2018)**. It cannot be ruled out that the pattern of apelin isoforms in the testes can also change in diabetes, which affects the specific activity of peptides of the apelin family and the spectrum of their physiological effects, and this should be the subject of further research.

Our results showed that the proliferation of germ cells in the testes of diabetic mice is significantly reduced. Two proliferation markers, PCNA and GCNA, were very low expressed in diabetic testes. In the testes of diabetic mice, we also observed an increased expression of the pro-apoptotic enzyme caspase-3 and a decrease in the expression of the anti-apoptotic protein BCL2. These data indicate that infertility associated with diabetes may be due to an imbalance in the apoptosis and proliferation of germ cells. Other authors also showed that in diabetes, the testes have decreased proliferation and increased proapoptotic activity, which is due to increased oxidative stress, inflammation and endoplasmic reticulum stress **(Zhao et al., 2018; Nna et al., 2020)**, and ultimately may lead to **(Sajadi et al., 2019)**.

We assume that a high level of apelin may be one of the factors in the decrease in proliferative activity and increased apoptosis in the testes. Despite the fact that currently there is no data on the relationship between the levels of apelin and the activity of growth and apoptotic factors in the testes, there is information on such relationships in the other tissues. It was shown thatapelin inhibits the proliferation of smooth muscle cells in the rat pulmonary artery during hypoxia **(Zhang et al, 2014)**,

and in diabetes, a high level of apelin promotes apoptosis in podocytes (**Liu et al., 2017**). On the other hand, there is evidence that apelin stimulates the proliferation of granulosa cells and myocardial cells **(Shuang et al., 2016; Yin et al., 2018)**, and inhibits apoptosis in granulosa cells and human osteoblast cells **(Xie et al., 2007; Shuang et al., 2016)**. We believe that in diabetic conditions, a high level of apelin in the blood and testes may promote apoptosis and reduce the proliferation of germ cells in the testes, while the regulatory effects of normal concentrations of apelin on apoptosis and proliferation may change, which requires further research. It should be noted that apelin is a pro-angiogenic factor **(Mughal and O'Rourke 2018)**, and high levels of apelin can cause ischemic testicular damage in diabetes. Apelin receptor antagonists have been shown to prevent ischemic retinopathy in mice **(Ishimaru et al., 2017)**. Based on this, it can be assumed that the antagonist ML221 can weaken or prevent pathological angiogenesis in the testes caused by hypoxia and pro-inflammatory factors. Our study of the potentiating effect of ML221 on steroidogenesis in the *in vitro* conditions suggests that apelin receptor antagonists may have the potential to improve androgenic status in diabetes. To confirm this, the *in vivo* studies of its effect on testicular steroidogenesis and spermatogenesis in normal and pathological are needed.

Thus, the present study opens up opportunities for studying the role of apelin and it’s signaling system in the regulation of testicular steroidogenesis, spermatogenesis, and testicular angiogenesis both in normal conditions and in pathological conditions induced by diabetes. The testicular hyperapelinemia we discovered can make a significant contribution to the pathogenesis of the testes, as a result of which inhibition of the apelin receptor by the ML221 antagonist can improve the functioning of the male reproductive system through the improvement of testicular steroidogenesis.

Summary

Type 1 diabetes mellitus (T1DM) is a metabolic disorder with severe hyperglycemia, one of the complications of which is testicular dysfunctions, androgen deficiency and decreased male fertility. In the diabetic testes, the expression and signaling pathways of leptin and a number of other adipokines are significantly changed. However, there is no information on the localization and expression of adipokine, apelin and its receptor (APJ) in the diabetic testes, although there is information on the involvement of apelin in the regulation of reproductive functions. The aim of this study was to investigate the expression and localization of apelin and APJ in the testes of mice with streptozotocin-induced T1DM and to estimate the effects of agonist (apelin-13) and antagonist (ML221) of APJ on the testosterone production by diabetic testis explants in the *in vitro* conditions. We first detected the expression of apelin and its receptor in the mouse testes, and showed an increased intratesticular expression of apelin and APJ along with the reduced testosterone secretion in T1DM. Using immunohistochemical approach, we showed that apelin and APJ are localized in the Leydig and germ cells, and in diabetes, the amount of these proteins was significantly higher than in the control mice. The diabetic testes had a decrease in germ cell proliferation (the reduced PCNA and GCNA levels) and an increase in apoptosis (the increased active caspase-3 and decreased BCL2 levels). These results suggest an involvement of apelin and APJ in T1DM-induced testicular pathogenesis. Treatment of the cultured testis explants with ML221 significantly increased the testosterone secretion, whereas apelin-13 was ineffective. Thus, hyperapelinemia in the testes can significantly contribute to testicular pathogenesis in T1DM, and pharmacological inhibition of apelin receptors can improve testicular steroidogenesis.

Figure 4.1. The changes in the body weight (A), testes weight (B), blood glucose (C) and circulating testosterone levels (D) in the control and diabetic groups of adult Swiss albino male mice.

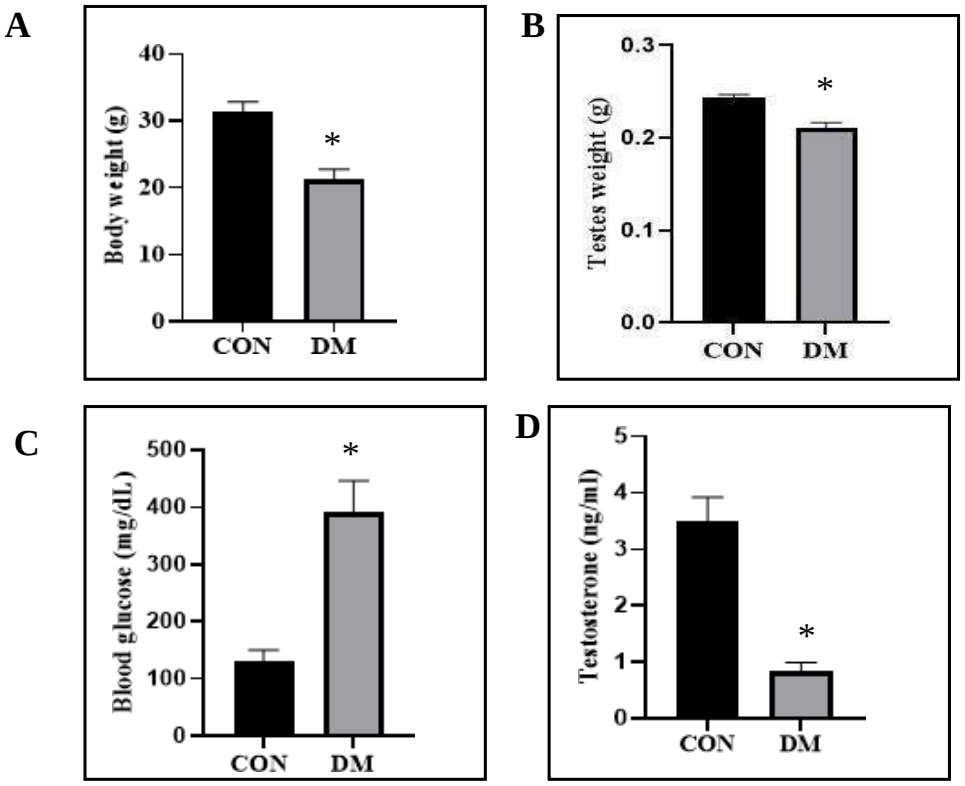

A
Body weight (g)
40
30
20
10
0
*
CON
DM
B
Testes weight (g)
0.3
0.2
0.1
0.0
*
CON
DM
C
Blood glucose (mg/dL)
500
400
300
200
100
0
*
CON
DM
D
Testosterone (ng/ml)
5
4
3
2
1
0
*
CON
DM

Figure 4.2 Localization and levels of apelin in the testes of control and diabetic mice

The immunolocalization of apelin showed intense staining in the Leydig cells (L) of control (A) and diabetic testes (B). In the diabetic testes, pronounce abundance of apelin can also be observed in the other cell types, such as spermatogonia (Spg), primary spermatocytes (Ps), round spermatid (Rs) and sperm (Sp) (B). The semi-quantization (C) and quantification (D) of apelin also showed significantly increased levels of this adipokine in the diabetic testes compared to the control testes.

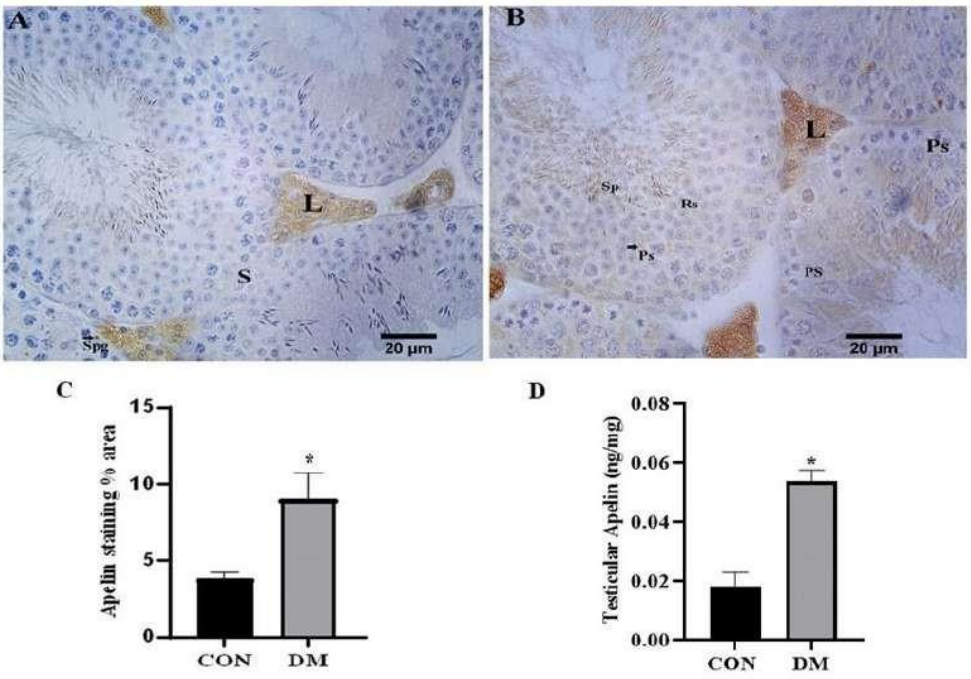

A
L
S
Spg
20 µm
B
L
Ps
Sp
Rs
Ps
PS
20 µm
C
Apelin staining % area
15
10
5
0
*
CON
DM
D
Testicular Apelin (ng/mg)
0.08
0.06
0.04
0.02
0.00
*
CON
DM

Figure 4.3 Localization and expression of apelin receptor (APJ) in the testes of control and diabetic mice

The immunolocalization of APJ was observed in the Leydig cells (L), primary spermatocytes (Ps), round spermatid (Rs) and sperm (Sp) in the control (A-B) and diabetic testes (C-D). The perinuclear staining of APJ can be seen in the control testes (B, red arrow) and diabetic testes (C, Ps). The diabetic testes showed mild increase staining of APJ in the Leydig cells (L), primary spermatocytes and elongated spermatid cells (C). The diabetic testes also showed multinucleate giant cells (M) with moderate increase in APJ immunostaining, and some vacuole can also be seen in the tests of diabetic mice. The negative control (E) showed no immunostaining in the testes (the primary antibody was replaced with 1% rabbit non-immune IgG).

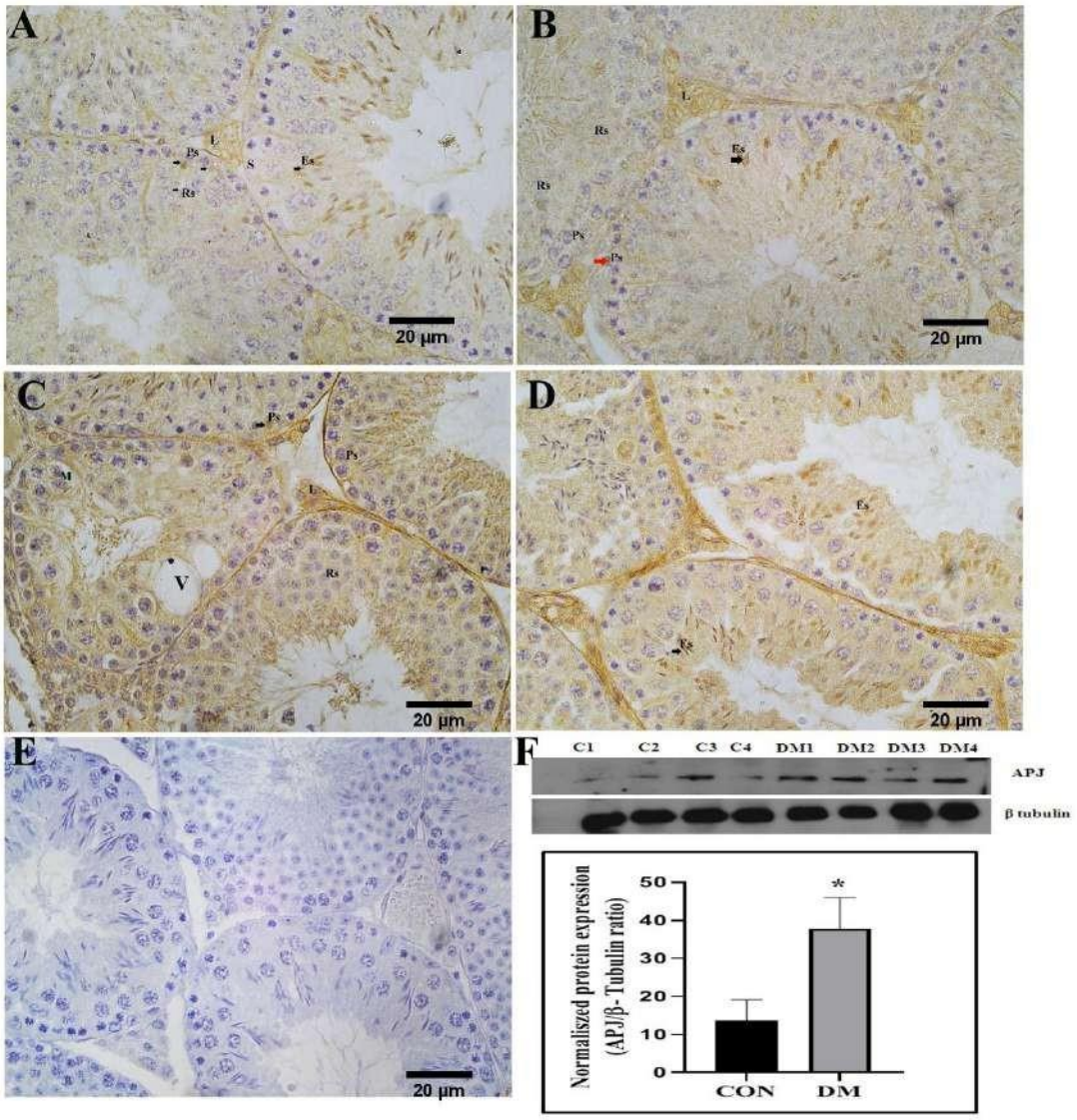

A
B
C
D
E
F
20 µm
C1 C2 C3 C4 DM1 DM2 DM3 DM4
APJ
β tubulin
Normalisized protein expression (APJ/β-Tubulin ratio)
50
40
30
20
10
0
CON
DM
*

Figure 4.4 Immunolocalization of proliferation markers (PCNA and GCNA) in the testes of control and diabetic mice

PCNA and GCNA immunostaining showed many positive cells in the control testes (A, D), whereas the diabetic testes showed very few and weakly stained cells (arrow, B, E). The negative control for PCNA (C) and GCNA (F) showed no immunostaining, and was replaced with 1% non-immune rabbit IgG and mouse IgG, respectively. The semi-quantification study of PCNA (G) and GCNA (H) immunostaining showed a significantly higher percentage of stained area in the control testes than in the diabetic testes.

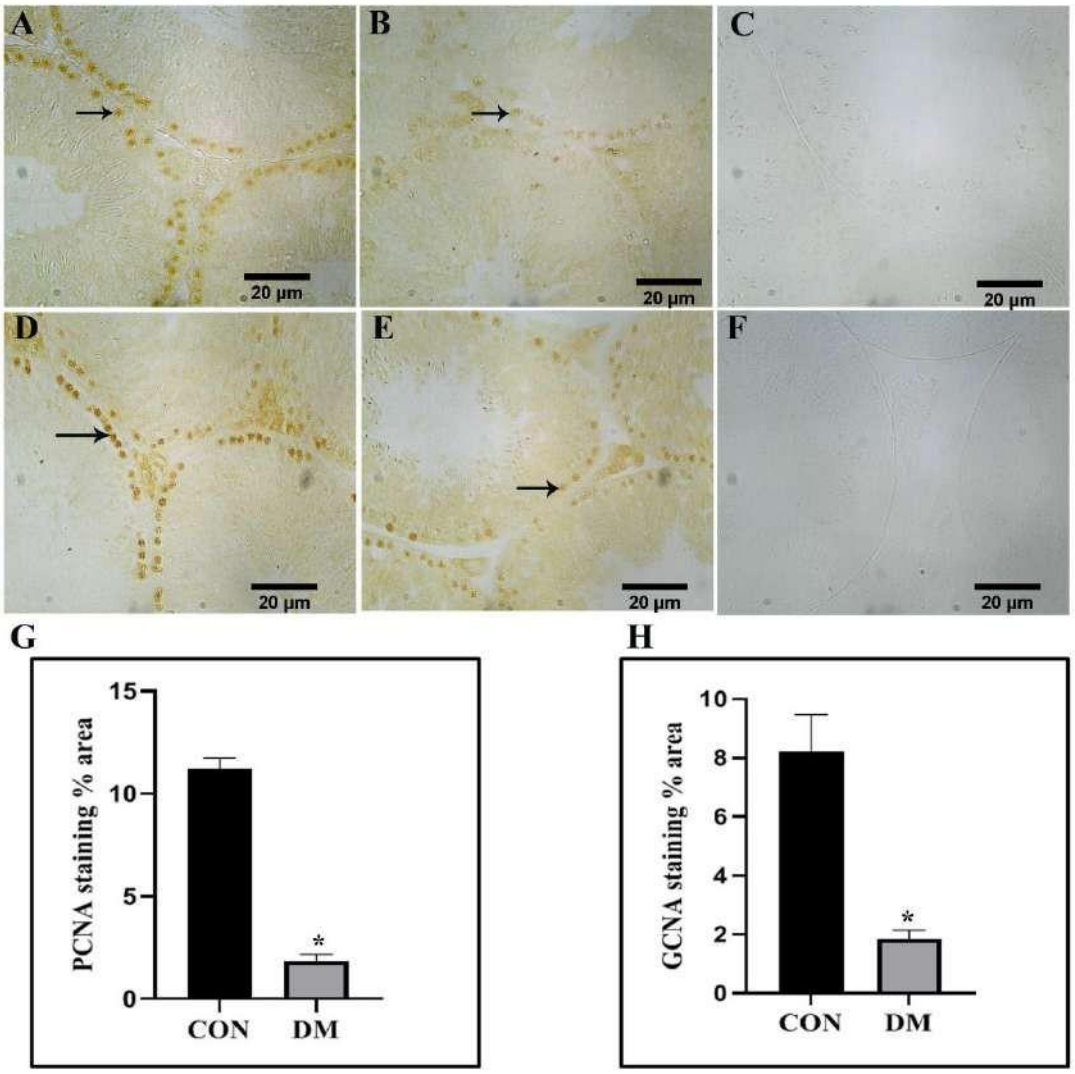

A
B
C
D
E
F
20 µm
G
PCNA staining % area
15
10
5
0
*
CON
DM
H
GCNA staining % area
10
8
6
4
2
0
*
CON
DM

Figure 4.5 Expression of BCL2 and active form of caspase-3 in the testes of control and diabetic mice

The expression of BCL2 was significantly lower and active caspase-3 was significantly higher in the diabetic testes than in the control testes.

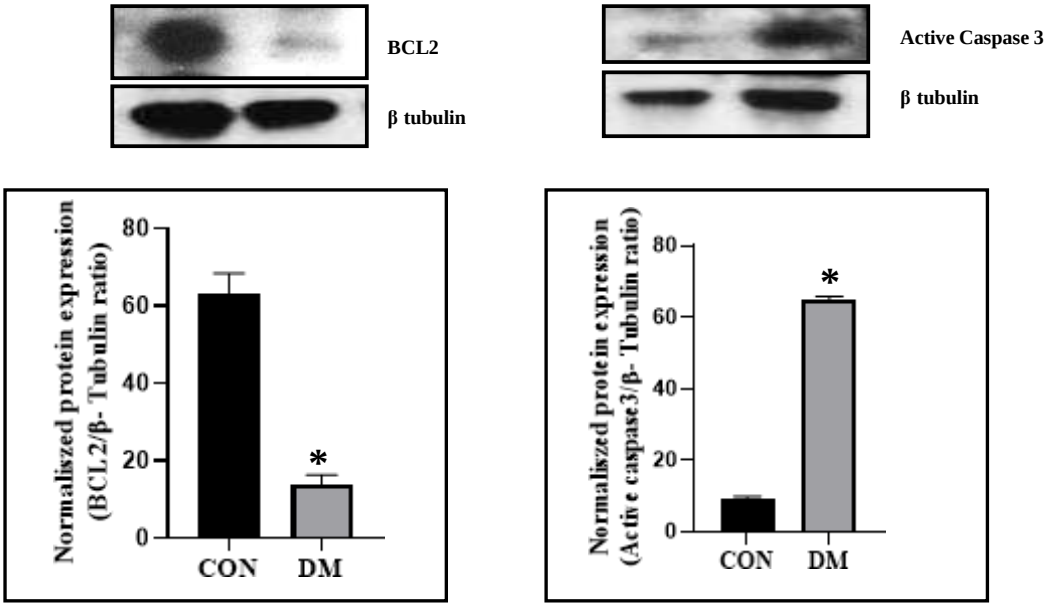
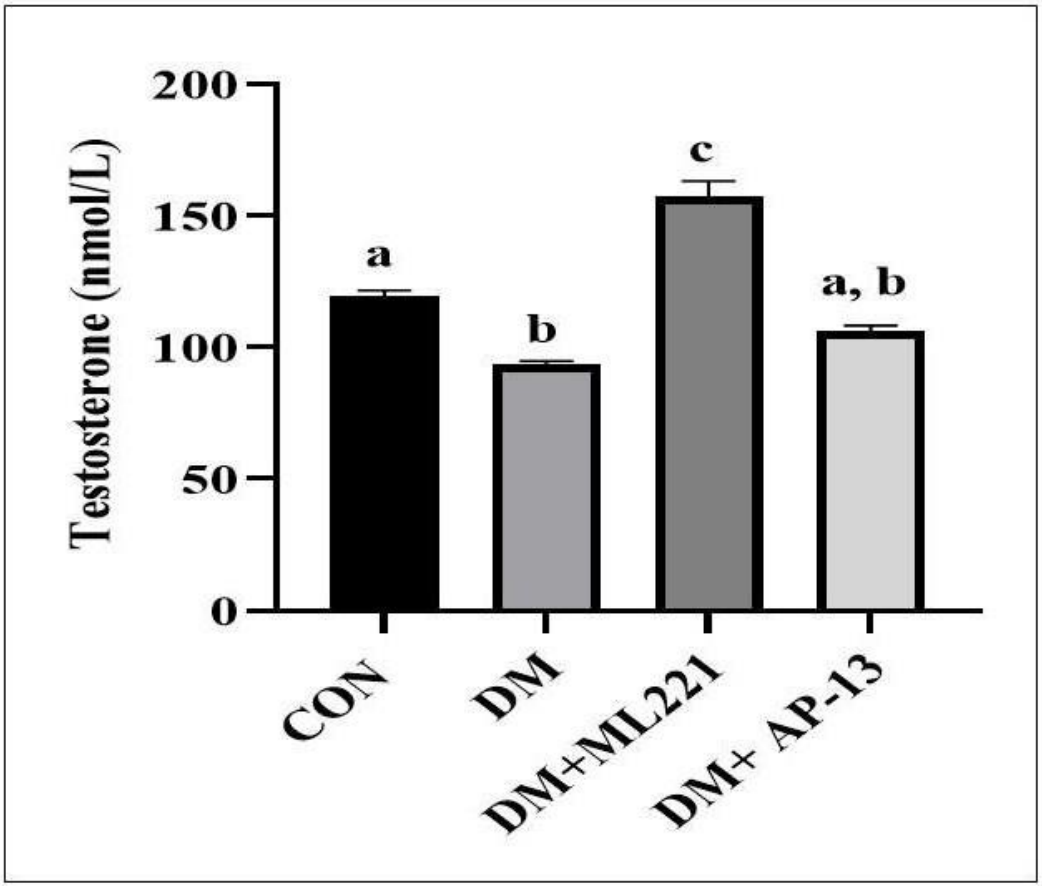

BCL2
β tubulin
Active Caspase 3
β tubulin
Normaliszed protein expression
(BCL2/β- Tubulin ratio)
80
60
40
20
0
*
CON
DM
Normaliszed protein expression
(Active caspase3/β- Tubulin ratio)
80
60
40
20
0
*
CON
DM

Figure 4.6 Effect of APJ-antagonist ML221 and apelin-13 on the testosterone secretion by the cultured testis explants *in vitro*

The testosterone secretion by the untreated diabetic testis explants (DM) was significantly decreased compared to the control testis explants (CON). The treatment of the diabetic testis explants with the APJ-antagonist ML221 (DM+ML221) increased the testosterone levels higher than in the other investigated groups. The treatment of the diabetic testis explantswith the APJ-agonist aplein-13 (DM+AP-13) did not affect testosterone levels compared to the untreated explants.


Testosterone (nmol/L)
200
150
100
50
0
a
b
c
a, b
CON
DM
DM+ML221
DM+ AP-13

Consolidated Summary

1. The presence of apelin and its receptor, APJ, in the testis can indeed suggest that apelin signalling may be involved in regulating the growth and differentiation of testicular cells during early development.

2. Apelin and APJ is developmentally regulated, suggesting a role in the regulation of testicular development during postnatal age.

3. The circulating apelin levels change over time during the postnatal period, with the lowest levels observed at Postnatal Day 1 (PND1) and the highest levels at Postnatal Day 42 (PND42).

4. Among testosterone, estrogen and androstenedione the circulating testosterone increases from PND1 to PND42 due to the development of steroidogenic potential of testis.

5. The relationship between the expression of testicular APJ and the levels of circulating apelin, testosterone, and androstenedione are positively correlated each other.

6. The APJ receptor inhibition using the antagonist ML221 during PND14-PND20 has detrimental effects on seminiferous tubule diameter, germinal epithelium height and lumen diameter, suggesting that apelin signaling is crucial for normal testicular development during this period.

7. Inhibition of the APJ receptor during PND14-PND20 has several effects, including suppressing testicular cell proliferation by supressing the expression of proliferation markers PCNA and GCNA, increasing apoptosis, and changing estrogen secretion.

8. The decrease in p-Akt abundance in response to ML221 treatment implies that apelin, the natural ligand for the APJ, involved in signaling during the developmental period from PND14 to PND20.

9. ML221 treatment had a minor, statistically insignificant impact on the expression of estrogen receptors ERα and ERβ, suggesting a relatively weak link between apelin system and estrogen receptor expression.

10. This study suggests that both *in vivo* and *in vitro* treatment with ML221 resulted in a decrease in the expression of the androgen receptor. This provides insight into a potential interaction between the apelin system and the androgen receptor signaling pathways, indicating that apelin activity might be involved in regulating the expression of the androgen receptor.

11. The treatment of ML221 in adult mice resulted a decrease in body weight, suggesting apelin system plays a role in regulating factors related to metabolism.

12. ML221 treatment led to a significant increase in sperm concentration suggests that endogenous apelin had an inhibitory role on sperm production and testicular activity in the adult mice.

13. Our findings indicate that inhibiting apelin action has significant effects on hormone levels, including an increase in circulating and intra-testicular testosterone and estrogen levels, as well as a decline in circulating estrogen levels. These results suggest that ML221 has a stimulatory role in testicular steroid biosynthesis.

14. ML221 treatment led to an increase in the levels of both FSH and LH. This indicates that the inhibition of apelin action by ML221 influenced the regulation of these pituitary hormones in the adult mice.

15. Treatment with ML221 led to an increase in the abundance of 3βHSD within Leydig cells, it indicates that apelin signaling could be involved in regulating the enzymatic processes necessary for the production of testosterone within the testes.

16. ML221 treatment had an impact on spermatogenesis by increasing the number of seminiferous epithelium cycle stage VII/VIII, at a higher dose, it also led to an increase in sperm concentration.

17. The markers associated with cell proliferation, such as PCNA, GCNA, and BrdU, were up-regulated by ML221 treatment, suggests that apelin might suppress germ cell proliferation in the adult testis.

18. ML221 treatment increased the expression of BCL2 and decreased the expression of BAX, active caspase 3 and NF-κB. This suggests that apelin promote apoptosis in adult testis.

19. Treatment with ML221 increased the testicular antioxidant enzymes, catalase and GPx and oxidative stress. This implies that ML221 has a protective effect against oxidative stress.

20. Our results suggest that apelin-mediated suppression of the androgen receptor in the adult testis may have multiple downstream effects, including increased apoptosis, oxidative stress, and inhibited germ cell proliferation. This study sheds light on the potential inhibitory role of apelin signaling in testicular function and physiology.

21. Based on the findings of chapter I and II, it can be suggested that apelin have differential role in the testicular activity depending the stage. In early developmental stage, apelin has a stimulatory and at adult stage apelin has inhibitory role in the testis.

22. The immunostaining of APJ found in two specific regions within the testes: the interstitium and the seminiferous tubules. It suggests that apelin may play a role in testicular steroidogenesis and regulates spermatogenesis.

23. The immunolocalization of APJ in various cells within the seminiferous tubule such as primary spermatocytes, round spermatids, elongated spermatids and in mature sperm indicates a potentially significant role for the apelin signaling pathway in the regulation of male reproductive processes, from the early development of sperm to their final maturation and function.

24. Our findings suggest that the expression of APJ is not uniform but rather stage- and cell-specific within the seminiferous epithelium. The round spermatids from step 1 to 4 at stages I-IV showed moderate immunostaining of APJ. The

immunostaining of APJ in spermatid steps 5-6 at stage V-VI showed mild staining, however, 7-8 spermatids showed very faint immunostaining of APJ. The immunostaing of APJ again showed a mild staining in the steps 9 spermatids followed a moderate immunostaining of APJ in 10-12 steps of spermatids (stage X-XII). The spermatids steps from 13 to 16 showed either faint or no immunostaining of APJ.

25. APJ immunostaining is less in spermatids at stages VII-VIII suggests that apelin expression may involve in signaling pathways that facilitate the detachment and release of mature spermatids from the seminiferous epithelium.

26. The increase in APJ abundance from spermatid steps 9 to 12, which corresponds to the period when round spermatids begin to elongate and reach maximum elongation at step 12, suggests that apelin may have potential role in chromatin remodeling for round spermatids. Chromatin remodeling is a crucial process in spermatogenesis, involving changes in the structure and packaging of DNA to facilitate the formation of mature sperm.

27. The intra-testicular inhibition of ML221 resulted some seminiferous tubules devoid of round and elongated spermatids at low dose. At higher doses some seminiferous tubules were devoid of round spermatid and showed only sperm suggests role of apelin signaling in spermatid differentiation.

28. Since APJ has shown specific distribution, dependent on the stage of seminiferous tubules, thus, it can be suggested the apelin signalling is required for elongation of spermatid and might have a crucial role in spermiogenesis. Moreover, as indicated that apelin has stage dependent role during early postnatal and adult stage, it can also be suggested that within the seminiferous tubules apelin would be mainly involved in the spermiogenesis (stimulatory role) and in the interstitium apelin may inhibit the testicular steroidogenesis by modulating Leydig cells (a inhibitor role). This is very interesting finding which require further study.

29. Our study showed that streptozotocin-induced T1DM in mice leads to hyperglycemia, testicular structural changes, and a decrease in testosterone secretion, which is consistent with the effects seen in diabetic patients and other experimental

models of diabetes. These findings contribute to our understanding of the systemic effects of diabetes on the male reproductive system.

30. The levels of apelin and the expression of APJ receptor in the testes of diabetic mice were significantly higher than in the control group.

31. A moderate increase in the content of apelin and APJ in testicular cells of diabetic mice was shown compared to control animals. Along with this, it was shown that apelin is localized within the germ cells of the control and diabetic testes.

32. Since it is known that T1DM suppresses the functions of Leydig cells and reduces the secretion of testosterone, an increase in the intratesticular level of apelin and a more intense immunostaining of apelin and APJ in diabetic testes as compared to the control testes, observed, indicate possible inhibitory role of apelin in the biosynthesis of testosterone.

32. Our results showed that the proliferation of germ cells in the testes of diabetic mice is significantly reduced. Two proliferation markers, PCNA and GCNA, were very low expressed in diabetic testes.

33. In the testes of diabetic mice, we also observed an increased expression of the pro-apoptotic enzyme caspase-3 and a decrease in the expression of the anti-apoptotic protein BCL2.

34. The *in vitro* experiments with cultured explants isolated from the diabetic testis, it was shown that ML221, an antagonist of APJ, significantly increased the secretion of testosterone by these explants, and the level of the hormone even exceeded that in the control, while apelin-13 was ineffective. Thus, this is the first report that a decrease in the apelin signaling activity in the testes by the APJ receptor antagonist may have significant therapeutic potential to treat the reproductive dysfunctions.

Conclusion

The present dissertation for the first time showed the localization and expression of apelin and APJ protein in the testis of mice, along with its possible role in the testicular function. The results showed the testicular apelin and APJ expression is developmentally regulated and in the adult its expression is also dependent different stages of seminiferous epithelium cycle. Inhibition of the APJ receptor during PND14-PND20 suppressed testicular cell proliferation by and, increased apoptosis. This study showed that both *in vivo* and *in vitro* treatment with ML221 (APJ antagonist) to early developmental stage decreased in the testicular expression of AR. These results suggest that apelin activity might be involved in AR signaling. Based on data, it is clear that apelin signaling plays a stimulatory role in the testicular activity in early developmental stage (juvenile phase). However, the inhibition of apelin signaling by ML221 resulted in increased sperm concentration, circulating testosterone, LH, and FSH levels, and increase in the abundance of 3βHSD within Leydig cells, suggest that apelin has an inhibitory role in the testicular steroidogenesis. These finding also suggest that apelin has inhibitory role on the testicular steroidogenesis by suppressing pituitary gonadotropin secretion. Furthermore, ML221 treatment also increased the germ cell proliferation marker in the adult testis, which coincides with increased expression of Bcl2, AR and decreased expression of active caspase 3, Bax and NF-κB. Treatment with ML221 also increased the testicular antioxidant enzymes, catalase and GPx and oxidative stress. Our study is the first attempt to unravel the role of the endogenous apelin system on steroidogenesis, proliferation, apoptosis and oxidative stress. Our study showed that apelin might suppress germ cell proliferation and increases apoptosis in adult testis. These results confirmed an inhibitory role of apelin in the mice testis.

In addition, the immulocalization of APJ showed a seminiferous tubule stage dependent localization of APJ in the spermatids. Since the increased APJ abundance coincides with elongation of round spermatid, thus it might be suggested that apelin signaling could be important for this transformation and might play important role in spermiogenesis. To confirm the role of APJ on the spermiogenesis, the intra-testicular injection of ML221 was performed. ML221 treated testis exhibited noticeable changes. Few seminiferous tubules were devoid of round and elongated spermatids at low dose. Moreover, at higher doses some seminiferous

tubules were devoid of round spermatid and showed only sperm. These results suggest that APJ expression in the round spermatid involves in the spermiogenesis. As stated above an inhibitory role of apelin signaling in the testicular proliferation and steroidogenesis in adult mice testis, it seems that apelin signaling is deferential in nature. Within seminiferous tubules it might stimulates spermiogenesis and in the interstitium it inhibits testicular testosterone secretion by acting on the Leydig cells. Thus, testicular apelin system plays important role in developmental stage and cell dependent manner. Furthermore, the testicular apelin system was up-regulated in the diabetic condition, which coincides with decreased testosterone secretion, proliferation and increased apoptosis. These results suggest that testicular apelin system is deregulated in the diabetic testis. Moreover, treatment of the cultured testis explants of diabetic mice with ML221 significantly increased the testosterone secretion, whereas apelin-13 was ineffective. Thus, it can be suggested that pharmacological inhibition of apelin receptors can improve testicular steroidogenesis in the diabetic testis and apelin system could be a target for management of diabetic associated testicular impairment.

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PUBLICATIONS

1. **Das, Milirani,** Chuckles Ch Marak, Malsawmhriatzuala Jeremy, Guruswami Gurusubramanian, and **Vikas Kumar Roy**. **"Heat-induced changes in the expression and localisation of PGC-1α in the mice testis."** Andrologia (2020): 13713.

2. **Das, Milirani,** Lalrawngbawli Annie, Kira V. Derkach, Alexander O. Shpakov, Guruswami Gurusubramanian, and **Vikas Kumar Roy. "Expression and localization of apelin and its receptor in the testes of diabetic mice and its possible role in steroidogenesis."** Cytokine 144 (2021): 155554.

3. **Das, Milirani,** Guruswami Gurusubramanian, and **Vikas Kumar Roy. "Postnatal developmental expression of apelin receptor proteins and its role in juvenile mice testis."** The Journal of Steroid Biochemistry and Molecular Biology (2022): 106178.

4. **Das, Milirani**, Guruswami Gurusubramanian, and **Vikas Kumar Roy**. "**Apelin receptor antagonist (ML221) treatment has a stimulatory effect on the testicular proliferation, antioxidants system and steroidogenesis in adult mice."** Neuropeptides (2023): 102354.

CONFERENCE ATTENDED

1. International conference on “Reproductive biology, comparative endocrinology and development” and 39th meeting of the society for Reproductive Biology and Comparative Endocrinology.

2. International conference on Reproductive Health with Emphasis on “Innovations in Reproductive Science and Technologies: Hope, Risk and Responsibilities”. Organised by Ravenshaw University, Cuttack, Odisha.

Received: 30 April 2020 | Revised: 18 May 2020 | Accepted: 20 May 2020

DOI: 10.1111/and.13713

ORIGINAL ARTICLE

First International Journal of Andrology ANDROLOGIA WILEY

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Abstract

The functions of mammalian testis are temperature-sensitive. There are various testicular factors, which express in response to heat as a mechanism of defence. PGC-1α and HSP70 have poetical role in the protection from oxidative stress in various tissues, including testis. The expression of PGC-1α and HSP70 has been shown in the testis, and it has also been documented that heat modulates the expression of PGC-1α and HSP70. However, heat-dependent changes in the localisation and expression of PGC-1α have not been investigated so far. Thus, we studied the expression and localisation pattern of PGC-1α in the testis of heat-treated mice along with marker of proliferation (PCNA, GCNA), serum testosterone levels, MDA levels and HSP70. The results showed a significant increase in PGC-1α and HSP70 and MDA levels in the testis of heat-treated mice along with a decrease in PCNA, GCNA and serum testosterone levels. The immunolocalisation study showed intense immunostaining of PGC-1α in the Leydig cell and germ cells of the heat-treated testis, with pronounced damaged in the histoarchitecture. The results showed that increase expression of PGC-1α in germ cells and Leydig cells of testis could be a counter mechanism to cope up with oxidative stress in coordination with HSP70.

KEYWORDS

heat stress, HSP70, PGC-1α, testis

1 | INTRODUCTION

The reproductive activity of vertebrate's testis is regulated by the temperature and any rise in the temperature of scrota or testis impairs spermatogenesis, because in the mammalian testis, temperature is <2–8°C from general body temperature (Banks, King, Irvine, & Saunders, 2005; Costa et al., 2018; Hansen, 2009; Ivel, 2007). The temperature increase in external testis has been associated with poor semen quality in human and in mice; it leads to poor embryo quality as well (Momen, Ananian, Fahmy, & Mostafa, 2010; Takahashi, 2012). Similarly, either increase in body temperature or increase in internal temperature of testis due to cryptorchidism exhibit deleterious effect on testis (Setchell, 1998). In our previous study, we have shown that experimentally induced heat stress on testes causes oxidative stress, decreased serum testosterone and decelerate testicular cell proliferation (Roy, Verma, & Krishna, 2017).

Furthermore, the elevated temperature in the testis increases the levels of reactive oxygen species (ROS) and reduces antioxidant enzymes and these imbalances may lead to infertility due to impaired spermatogenesis and it has also been shown that increase temperature promotes apoptosis and DNA damage to the spermatozoa (Shadmehr, Tabatabaei, Hosseinifar, Tabandeh, & Amiri, 2018; Henkel et al., 2005; Ngoula et al., 2020; Pérez-Crespo, Pintado, & Gutiérrez- Adán, 2008; Paul, Teng, & Saunders, 2009; Takahashi, 2012; Henkel, 2011) . It has been suggested that the scrotal hyperthermia alters various gene expression in a complex manner in the testis (Durairajanayagam, Agarwal, & Ong, 2015). In mice, hyperthermia induces the expression of genes associated with stress

response, whereas other genes associated with apoptosis and DNA repair are downregulated (Rockett et al., 2001).

Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α) is an important regulator of mitochondrial biogenesis and also involves in the regulation of genes for energy metabolism (Puigserver, 2005). It also decreases ROS production by upregulating genes for ROS detoxification (Austin & St-Pierre, 2012; St-Pierre et al., 2006). It has also been shown that the elevated expression of PGC-1α protects annulus fibrosus cells from apoptosis and mitophagy (Xu et al., 2019). It was first identified in brown adipose tissue as a direct coactivator of PPARγ gene (Puigserver et al., 1998). Heat shock protein 70 (HSP70) is a molecular chaperone that assists protein folding and transport. Research suggests the heat shock proteins required for spermatogenesis and expression elevated during the stressed conditions like temperature increase, radiation and chemical exposure (Legare, Thabet, & Sullivan, 2004). Both PGC-1α and HSP70 expressions are induced by heat (Liu & Brooks, 2012); however, heat-dependent expression and localisation pattern of PGC-1α have not been studied in any mammalian testis. Moreover, we have shown that testicular functions may require PGC-1α for antioxidant defence system and apoptosis in the mice (Annie, Gurusubramanian, & Roy, 2019; Roy et al., 2017). Thus, it may be hypothesised that, like other factors, expression of PGC-1α may be modulated by scrotal hyperthermia in the mice. Therefore, we investigated the detailed cellular localisation and expression of PGC-1α in the heat-treated mouse testis.

2 | MATERIALS AND METHODS

2.1 | Animal maintenance and experimental design

The animals used in this study were 3-month-old male Swiss albino mice (Body weight 25–30 g). Mice were kept in aerated polypropylene cage with ad libitum access to food and water at 25 ± 5°C temperature under 12/12 hr (h) light/dark daily cycle. All experimental procedures were conducted in compliance with the protocols approved (MZUIAEC16-17–11) by the Mizoram University Institutional Animal Ethical Committee (MZUIAEC).

Mice were randomly divided into two experimental groups. The control group (CN, $n = 5$) and a heat-treated group (HS, $n = 5$). After mild anaesthesia, the lower part of the body with the scrotum was submerged in a temperature-controlling incubator at 43°C for 15 min (HS group). The heat treatment of mice was given as a method described earlier (Roy et al., 2017). Control group animals were given sham treatment for 15 min at 25°C water. The animals were carefully wiped dry after the heat treatment and placed on a well-ventilated mat (25°C) until they had fully recovered from the anaesthesia.

2.2 | Sample collection

Mice were sacrificed under mild anaesthesia after 14 days of heat treatment. The collected blood was centrifuged for serum collection. One testicle from each animal was removed and frozen at −20°C for later use, and testicle of other side was fixed in a Bouin's solution for 24 hr for histology and immunohistochemical study.

2.3 | Estimation of lipid peroxidation

Malondialdehyde (MDA) level was estimated in the testis as method described by Ohkawa, Ohishi, and Yagi (1979) with minor modifications (Sőnmez, Türk, & Yüce, 2005). Briefly, one volume of the testis lysate (10% w/v) was mixed with two volumes of reagents. The reagents were containing 15%, w/v trichloroacetic acid in 0.25 N HCl and 0.375%, w/v thiobarbituric acid in 0.25 N HCl. After mixing, samples were vortexed and boiled for 15 min at 100°C. After cooling, sample was centrifuged and the absorbance of supernatant was measured at 532 nm against the blank. A blank solution was taken without the testis lysate. The concentration of MDA was expressed as nmol/mg protein.

2.4 | Histopathology of testis

Bouin's fixed testis was processed for routine paraffin sectioning and sectioned at 7 μm thick by Leica rotary microtome (RM2125 RTS). The sections were spread on glass slides for haematoxylin and eosin (H&E) staining. After staining, the slides were mounted in DPX and observed under Nikon microscope (Model E200, Nikon) and photographs were taken for analysis (Bancroft & Gamble, 2002).

For histomorphometric analyses, tubular sections that were round or nearly round from H&E slides were selected and the diameter and the germinal epithelial height of 50 seminiferous tubules were measured using an ocular micrometer of 10× magnification (Jeremy, Gurusubramanian, & Roy, 2019a, 2019b).

2.5 | Immunohistochemistry

Tissue sections from control and heat stress groups were processed for immunohistochemical localisation of PGC-1α, PCNA and GCNA as method described previously (Annie et al., 2019; Roy et al., 2017). After rehydration with ethanol of different grades, slides were incubated with blocking goat serum for 30 min at room temperature. After blocking, slides were incubated with rabbit polyclonal PCNA antibody (1:100, Cat#SC-9707, Santa Cruz), mouse monoclonal GCNA (1:100, Cat#10D9G11, DSHB) and rabbit polyclonal PGC-1α (1:500, kind gift from Daniel P. Kelly, Washington University) overnight at 4°C. The slides were washed with PBS and incubated with secondary antibody (for PCNA and PGC-1α, 3 hr at RT with HRP-conjugated goat anti-rabbit IgG, Cat#PI-1000, 1:500, Vector Laboratories) and for GCNA, HRP-conjugated goat anti-mouse IgG (1:500, Cat#SC-2005, Santa Cruz) for 3 hr at RT. After secondary incubation, slides were washed thoroughly with PBS and incubated with 0.05% diaminobenzidine (DAB) solution (3, 3'- diaminobenzidine in 50 mmol/L

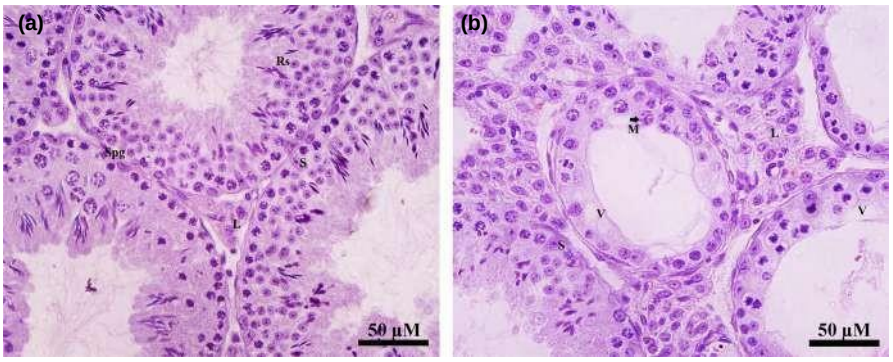

FIGURE 1 Histopathological assessment of testicular tissues of the mice in control (CN) groups and heat-treated group (HS): (a) Normal histoarchitecture of testicular tissue in animals in the control group; (b) severe germ cell lost indicated by thinner germinal epithelium height, multinucleated giant cell (M) and vacuolisation (V) in the testicular tissue of the heat-treated mice; and H&E, (magnification ×40), bar: 50 μm

Tris pH 7.6) for 5 min. After that, slides were counter-stained with haematoxylin for PGC-1*α* and all slides were dehydrated with ethanol and mounted with DPX and photographs were taken with Nikon microscope (E200, Nikon).

2.6 | Western blotting

Immunoblotting of testicular tissue was performed based on the previous report (Jeremy et al., 2019a, 2019b). In brief, decapsulated testicular tissues were homogenised in a weight-adjusted volume of suspension buffer (50 mmol/L Tris – HCl, with pH 8.0, 150 mmol/L of NaCl, 1 mmol/L EDTA 1 g/ml aprotinin, 1 mmol/L phenylmethylsulphonyl fluoride (PMSF) and 0.1% SDS). Equal amounts of tissue homogenate, as determined by the Bradford method (Bradford, 1976), were run on a 10% SDS-PAGE gel, transferred to a PVDF membrane (Semi-Dry Blotting MX-1295–01, Medox) for 30 min. Membranes were then incubated with 5% nonfat skimmed milk in PBST for 30 min, and the membranes were probed primary antibodies HSP70 (at dilution 1:1,000, Cat#SC-32239, Santa Cruz) and PGC-1*α* (at dilution 1:1,000) overnight at 4ºC. After primary incubation, membranes were washed with PBST and further kept in secondary antibody; for PGC-1*α*, goat anti-rabbit conjugated with HRP was used (1:4,000, Cat#PI-1000, 1:500, Vector Laboratories); and for HSP70, goat anti-mouse conjugated with HRP was used. Membranes were washed with PBST and incubated in enhanced chemiluminescence (ECL) solutions (Cat#1705061, Bio-Rad) as per the manufacturer's instructions and then developed onto X-ray film. The band was quantified with ImageJ software. Equal protein amount loading was ascertained by reprobing each membrane with β-tubulin (1:4,000, Cat# E-AB-20033, Elabscience) and was used for normalisation.

2.7 | Testosterone estimation

The levels of testosterone were estimated in the collected serum with commercial kit as per manufacturer's instruction (Cat# DKO002, DiaMetra). Briefly, 25 μl of both calibrator (C_0-C_4) and serum from each group were loaded into each well and incubated at 37°C for 1 hr after the addition of 100 μl of working conjugate solution. The contents were discarded and washed three times with 1X wash buffer. 100 μl of TMB substrate was added and incubated at RT in dark for 15 min. The reaction was stopped by adding 100 μl stop solution, and the absorbance of the solution was read at 450 nm against blank using ELISA microplate reader (Erba Lisa Scan EM, Transasia Biomedical Ltd). The intra-assay and inter-assay precision levels of the kit are ≤7.0% and ≤8.35% respectively.

2.8 | Statistical analysis

Data were represented as mean ± *SEM*. The Kolmogorov–Smirnov test was used to analyse the normal distributions of the data ($p > .05$). The Student *t* test was performed to analyse the data by GraphPad Prism7. The *p*-value less than 0.05 ($p < .05$) was considered significant. The (Pearson correlation) correlation analysis of PGC-1*α* expression with MDA levels, testosterone levels and HSP70 expression was also performed.

3 | RESULTS

3.1 | Effect of heat stress on testicular histology

Single acute heat exposure to mice showed severe changes in the histoarchitecture of the testis. Heat treatment caused several pathological signs in the mice testis; seminiferous tubules of control mice (Figure 1a) showed normal histoarchitecture, while seminiferous tubules of heat-treated testis showed devoid of germs cells, vacuolisation, delamination and detachment of spermatid in the lumen of seminiferous tubules (Figure 1b).

Furthermore, the histomorphometric analysis also showed a significant reduction in the seminiferous tubule diameter (t-value = 3.327, $df = 8$, $p < .05$) (Figure 2a) and germinal epithelial

height (t-value = 6.113, *df* = 8, $p < .05$) (Figure 2b) of the heat-treated group in comparison with the control group testis.

3.2 | Effect of heat treatment on the immunolocalisation of PCNA and GCNA

To confirm the effect of heat on spermatogenesis, two proliferating markers PCNA and GCNA were localised in the testis. Immunolocalisation of PCNA and GCNA showed a strong circular dividing germ cell nucleus along the periphery of seminiferous tubules of control group testis (Figure 3a and c), whereas heat treatment reduced the PCNA and GCNA immunostaining in the seminiferous tubules (Figure 3b and d).

3.3 | Effect of heat treatment on the immunolocalisation of PGC-1*α*

The immunohistochemical study showed that PGC-1*α* immunostaining was shown in the Leydig cells, primary spermatocytes and round spermatids of control testis (Figure 4a). The heat treatment group testis showed a noticeable variation in the immunostaining of PGC-1*α* in the interstitium and seminiferous tubules (Figure 4b–e). The Leydig cells adjacent to the damaged seminiferous tubules showed strong immunostaining of PGC-1*α* (Figure 4b), whereas Leydig cells adjacent to the unaffected seminiferous tubules showed immunostaining of PGC-1*α* similar to control group (Figure 4e). Similarly, within the damaged seminiferous tubules of the heat-treated testis, primary spermatocytes, multinucleated cells and detached spermatids in the lumen showed strong immunostaining of PGC-1*α* (Figure 4b–d) compared with unaffected seminiferous tubules of the heat-treated testis (Figure 4e) and control testis (Figure 4a).

3.4 | Effect of heat treatment on the expression of HSP70 and PGC-1*α*

The expression of HSP70 (t-value = 4.335, *df* = 6, $p < .05$) (Figure 5a) and PGC-1*α* (t-value = 4.201, *df* = 6, $p < .05$) (Figure 5b) showed a significant upregulation in the testis of heat-treated mice compared with the control mice.

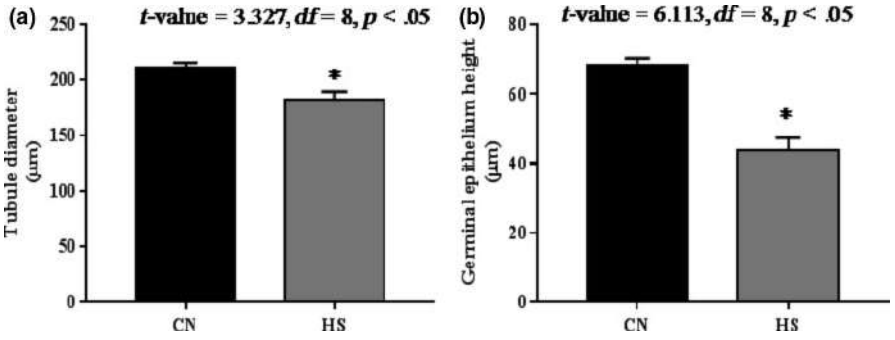

FIGURE 2 Histomorphometric comparisons between the mice in control (CN) group and heat-treated group (HS): The seminiferous tubule diameter (a) and germinal epithelium height (b) significantly decreased in heat-treated animals (HS) compared with the control group (CN).* statistically significant ($p < .05$) between the CN and HS groups

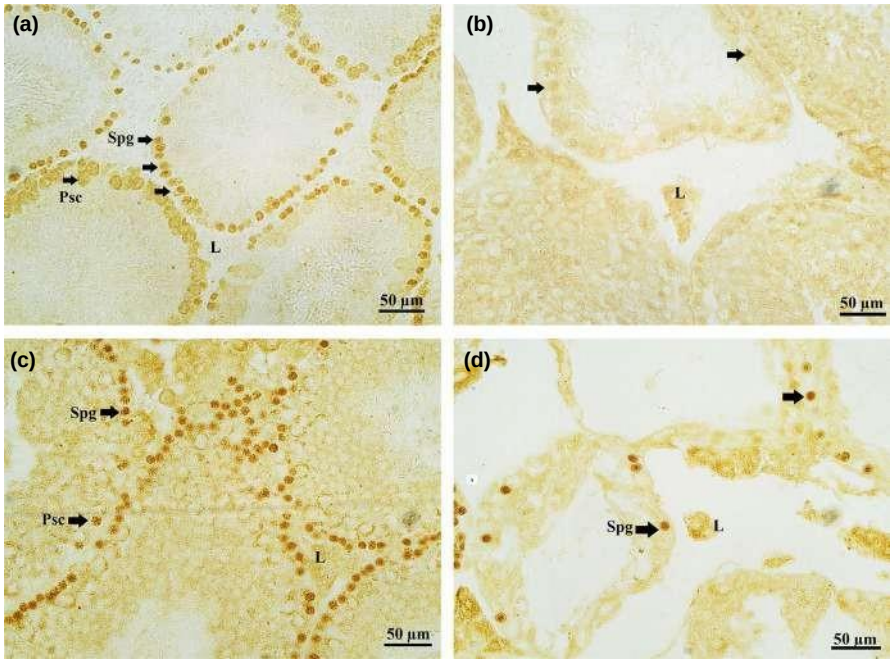

FIGURE 3 Immunohistochemical assessments of PCNA (a & b) and GCNA (c & d) in the testis of heat-treated animals (HS) and control groups (CN): (a) Strong immunoreactions for PCNA in the control group (CN) in the spermatogonia (Spg) and spermatocytes (Spc) (Arrow); (b) less intense immunostaining of PCNA in the heat-treated group; (c) immunostaining of GCNA confined to spermatogonia (Spg) and spermatocytes (Spc) in the control group (CN) (arrow); and (d) a few cells in the seminiferous epithelium showed immunostaining in the heat-treated group. (magnification ×40), bar: 50 μm

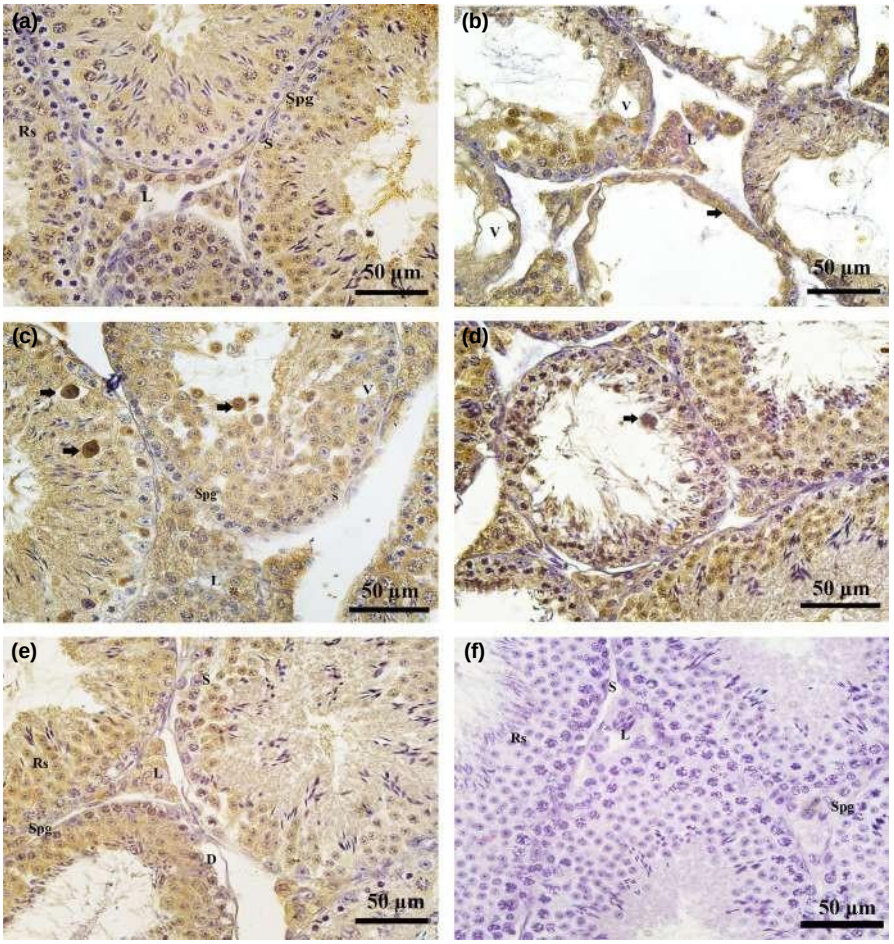

FIGURE 4 Immunohistochemical study of PGC-1α in the testis tissues of the control (a) and heat-treated animals (b-e): (a) control group testis showed moderate immunoreactions in the Leydig cells (L), spermatocytes (Spc) and round spermatids (Rs) (arrow): In the heat-treated group, strong immunoreactions in Leydig cell (L) bordered by damaged tubule (b) (arrowhead); (c-e) intense immunostaining in spermatocytes (Spc), multinucleated cells (M) and spermatids (Sp)(arrow); unaffected seminiferous tubules of heat-treated mice testis showed similar immunoreactions to that of control groups (e); and the negative control (f) showed no immunostaining. (magnification ×40), bar: 50 µm

3.5 | Effect of heat treatment on the circulating testosterone levels and oxidative stress (MDA levels)

The heat-treated mice showed a significant (t-value = 4.233, *df* = 8, $p < .05$) decrease in the circulating testosterone levels (Figure 6a) compared with the control mice. The testicular oxidative stress as a measure of malondialdehyde levels (Figure 6b) showed a significant (t-value = 3.587, *df* = 8, $p < .05$) increase in the heat-treated testis compared with the control testis.

3.6 | Correlation analysis of MDA and HSP70 with PGC-1α

The expression of PGC-1α showed a significant positive correlation with MDA levels ($r = .75$; $p = .0012$) and HSP70 ($r = .77$; $p = .009$), whereas it showed a significant negative correlation with serum testosterone levels ($r = .69$; $p = .024$).

4 | DISCUSSIONS

In this study, we investigated the detailed cellular localisation and expression of PGC-1α in the mice testis after acute scrotal heat exposure. We have also measured the expression of HSP70 and serum testosterone levels along with two proliferation markers GCNA and PCNA, in the mice testis. Our results showed that heat severely affected the histoarchitecture of testis in terms of vacuolisation, delamination, loss of germ cells and formation of multinucleated giant cells. These results coincide with previous studies, which also documented scrotal hyperthermia causes degenerative changes in the rodent testis (Kanter & Aktas, 2009; Paul et al., 2009; Zhang et al., 2012).

The immunohistochemical analysis of PGC-1α showed very conspicuous staining in the different cell types of heat-treated mice testis. The Western blot results also showed an overall increase in the expression for PGC-1α in the mice testis. Heat-treated testis showed intense immunostaining of PGC-1α in the Leydig cell, primary spermatocytes, round spermatid and sloughed germ in the lumen of damaged seminiferous tubules. However, some seminiferous tubules of heat-treated mice showed mild staining in these cell types and resembled the control mice testis. The reason for this immunostaining pattern would be that all the seminiferous tubules are not affected by acute heat stress. Although most of tubules are prone to heat-induced damage, and the expression of PGC-1α could not be elevated in the tubules which might have escaped the heat stress, so, some of the seminiferous tubules of heat-treated mice testis resembles to the control seminiferous tubules. Further study is required

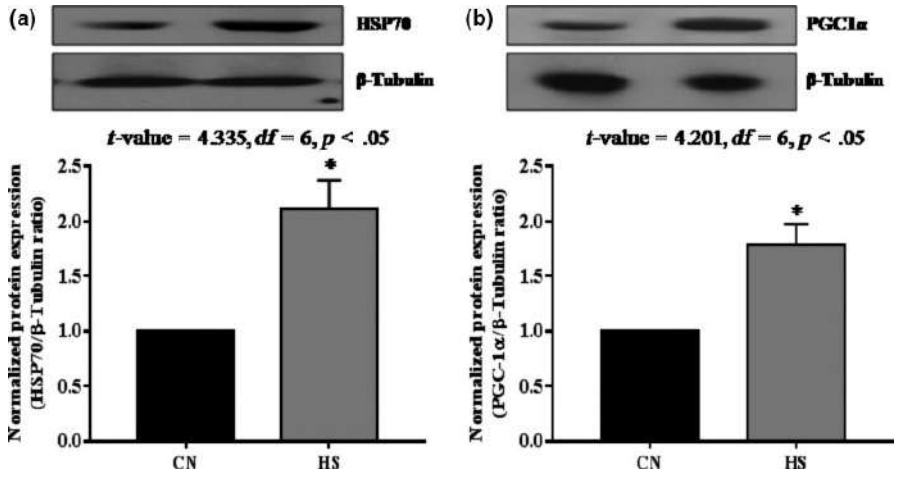

FIGURE 5 Immunoblotting of HSP70 and PGC-1α: The expression of both HSP70 (a) and PGC-1α (b) significantly decreased in the heat-treated group (HS) compared with the control group (CN). *Statistically significant ($p < .05$) between CN and HS groups

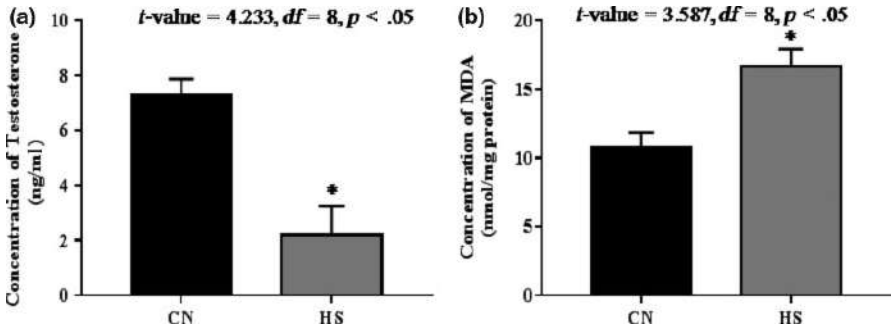

FIGURE 6 Comparisons of circulating testosterone and level of MDA between control groups (CN) and heat-treated group (HS): (a) The level of circulating testosterone level is significantly higher in control groups (CN) compared with the heat-treated group (HS); (b) the MDA level in the heat-treated group (HS) significantly increased compared with that of the control group (CN). *Statistically significant ($p < .05$) between CN and HS groups

to confirm this statement. It has been shown that PGC-1α belongs to a family of transcription coactivators, which has a plethora of biological functions (Liang & Ward, 2006). Its expression is regulated by mainly cold exposure and over-feeding (Cannon, Houstek, & Nedergaard, 1998; Rothwell & Stock, 1979). Our results showed that scrotal hyperthermia upregulated the expression of PGC-1α in the mice testis. The previous study has also shown that mild heat induces PGC-1α expression in the myotubes and its expression may be modulated by temperature fluctuation (Liu & Brooks, 2012).

Our results also showed a decrease in GCNA and PCNA in the testis of heat-treated mice. The circulating testosterone levels also decreased in the mice subjected to scrotal hyperthermia. The heat-induced decrease in PCNA and GCNA of testis showed a decrease in spermatogenesis. This decrease in spermatogenesis could be due to decrease in testosterone levels and increase in oxidative stress (Kumar Roy et al., 2016; Kanter, 2013). Further testicular oxidative stress (MDA levels) and HSP70 showed a significant increase in the mice, subjected to heat treatment. Oxidative stress ($r = .75$; $p = .0012$) and HSP70 expression showed a positive correlation with PGC-1α expression ($r = .77$; $p = .009$). It is well documented that scrotal hyperthermia increases testicular oxidative stress and decreases circulating testosterone levels (Paul et al., 2009; Kumar roy et al., 2016; Rao et al., 2015). Heat shock proteins are molecular chaperones, which expression increases in response to various stimuli (Anckar & Sistonen, 2011). It has been shown that PGC-1α can stimulate the expression of HSP70 after heat stimuli in the mouse fibroblast cells (Xu, Ma, Bagattin, & Mueller, 2016). Despite its function in thermogenesis, PGC-1α has been described as a marker of mitochondrial biogenesis and also increases reactive oxygen species, the scavenging potential in many cell types and which may further increase the cell survival (Chen et al., 2011; Mastropasqua, Girolimetti, & Shoshan, 2018). Based on our results of immunohistochemistry, which showed that heat-induced damaged germ cells and Leydig cell strongly stained with PGC-1α, it can be hypothesised that heat-induced increase in PGC-1α expression may be a counter mechanism to prevent cell death from oxidative stress. The previous study has also suggested that an increased PGC-1α expression may protect neural cells from oxidative stress-mediated cell death (St-Pierre et al., 2006). To confirm the precise role of heat-induced PGC-1α expression in the testis concerning apoptosis requires further study. The increased HSP70, along with PGC-1α expression, also suggests that PGC-1α may be required to modulate the expression of HSP70 to protect the testicular cells from heat stress as it has been reported in the mouse fibroblast cells (Xu et al.,2016).

A recent study has also shown that over-expression of PGC-1α ameliorates oxidative stress in the TM4 cells and may protect

testicular cells from oxidative stress (Liu et al., 2017). The previous study from our laboratory showed that dexamethasone decreases the testicular PGC-1α expression and correlated with increase oxidative stress (Annie et al., 2019). However, in this study, the expression of PGC-1α showed a positive correlation with oxidative stress and negative correlation (r = .69; p = .024) with serum testosterone levels. Another study from our laboratory also showed that carnitine treatment increases the PGC-1α-mediated antioxidant system, serum testosterone levels and anti-apoptotic protein Bcl2 in the mice testis (Roy et al., 2017). Despite an increase of PGC-1α in the Leydig cell of the heat-treated testis, the levels of testosterone did not increase; however, the previous report suggested the role of PGC-1α in the testicular and ovarian steroidogenesis (Roy et al., 2017; Yazawa et al., 2010). It could also be suggested that increase PGC-1α in the Leydig cell may be a preventive mechanism to counter oxidative stress, and the pathway of steroidogenesis may also be compromised over the preventive mechanism, which leads to a decrease in testosterone biosynthesis. Thus, it can also be suggested that the expression of PGC-1α may be differentially regulated in the testis, depending on the type of stimuli received by the testis.

In conclusion, to the best of our knowledge, this is the first report on the heat-induced expression and localisation of PGC-1α in the mice testis. The results of the present study showed that increase expression of PGC-1α in germ cells and Leydig cells of testis could be a counter mechanism to cope up with oxidative stress in coordination with HSP70. Furthermore, it can be suggested that most of the seminiferous tubules and Leydig cells are damaged by heat-induced oxidative stress, which may lead to a decrease in spermatogenesis and steroidogenesis, and increased expression and localisation of PGC-1α in germ cells and Leydig cells may reflect as a counter mechanism to prevent cell death. However, whether the increased PGC-1α in the germ cells and Leydig cells could protect or facilitate the removal of damaged cells in heat stress requires further investigation.

ACKNOWLEDG EMENT

The gift of PGC-1α antibody from Prof. Daniel P. Kelly and Teresa Leone, Washington University, USA, is greatly acknowledged. The infrastructure facility to Department of Zoology, Mizoram University, by DST-FIST programme, New Delhi, is greatly acknowledged.

CONFLIC T OF INTEREST

The authors declared no conflict of interest.

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How to cite this article: Das M, Marak CC, Jeremy M, Gurusubramanian G, Roy VK. Heat-induced changes in the expression and localisation of PGC-1*α* in the mice testis. *Andrologia*. 2020;00:e13713. https://doi.org/10.1111/and.13713

Cytokine 144 (2021) 155554

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Expression and localization of apelin and its receptor in the testes of diabetic mice and its possible role in steroidogenesis

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ARTICLEINFO

Keywords:
Apelin
Apelin receptor
Diabetes
Testis
Testosterone

ABSTRACT

Type 1 diabetes mellitus (T1DM) is a metabolic disorder with severe hyperglycemia, one of the complications of which is testicular dysfunctions, androgen deficiency and decreased male fertility. In the diabetic testes, the expression and signaling pathways of leptin and a number of other adipokines are significantly changed. However, there is no information on the localization and expression of adipokine, apelin and its receptor (APJ) in the diabetic testes, although there is information on the involvement of apelin in the regulation of reproductive functions. The aim of this study was to investigate the expression and localization of apelin and APJ in the testes of mice with streptozotocin-induced T1DM and to estimate the effects of agonist (apelin-13) and antagonist (ML221) of APJ on the testosterone production by diabetic testis explants in the *in vitro* conditions. We first detected the expression of apelin and its receptor in the mouse testes, and showed an increased intratesticular expression of apelin and APJ along with the reduced testosterone secretion in T1DM. Using imunohistochemical approach, we showed that apelin and APJ are localized in the Leydig and germ cells, and in diabetes, the amount of these proteins was significantly higher than in the control mice. The diabetic testes had a decrease in germ cell proliferation (the reduced PCNA and GCNA levels) and an increase in apoptosis (the increased active caspase-3 and decreased BCL2 levels). These results suggest an involvement of apelin and APJ in T1DM-induced testicular pathogenesis. Treatment of the cultured testis explants with ML221 significantly increased the testosterone secretion, whereas apelin-13 was ineffective. Thus, hyperapelinemia in the testes can significantly contribute to testicular pathogenesis in T1DM, and pharmacological inhibition of apelin receptors can improve testicular steroidogenesis.

1. Introduction

Cytokines are small secreted proteins from immune cells that have an important role in cell communications [1]. Like immune cells, the adipose tissue also secretes a variety of cytokines such as leptin, visfatin, adiponectin, resistin, apelin and chemerin, commonly referred to as adipokines or adipocytokines [2]. In humans and animals, these adipokines/cytokines regulate metabolic processes [3–5] and are involved in the control of a wide range of physiological functions, including reproduction [2,6]. Since these adipokines regulate the male and female gametogenesis and steroidogenesis, modulating the activity of the hypothalamic-pituitary–gonadal (HPG) axis in both normal and pathological conditions [2,7–9], they are considered as the important regulators of reproductive functions. There is evidence that adipokines, such as leptin, visfatin, adiponectin, and chemerin, as well as some components of their signaling cascades are expressed in the testes [10–14]. Along with other adipokines, peptides of the apelin family, which are encoded by the proapelin gene *APLN*, were found in the testes of humans and rats [14–18]. However, the data on the expression of the apelin receptor (APJ receptor) encoded by the *APLNR* gene in the testes are contradictory [17–19]. Therefore, the question of whether the testes are a direct target for apelin has not been fully resolved. It is important to note that there are no data on the localization of apelin and its receptor in the testes of mice, despite the fact that mice, including transgenic ones, are one of the most frequently used experimental animals for studying reproductive functions.

Type 1 diabetes mellitus (T1DM) is a metabolic disorder characterized by acute hyperglycemia and insulin deficiency. In human and

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https://doi.org/10.1016/j.cyto.2021.155554
Received 7 April 2021; Received in revised form 22 April 2021; Accepted 23 April 2021
Available online 4 May 2021

experimental animals, T1DM not only causes dysfunctions of the cardiovascular, renal and nervous systems [20,21], but also leads to the reproductive dysfunctions, including impaired testicular steroidogenesis and spermatogenesis [22–27]. In diabetic conditions, the expression of adipokines changes significantly, including in the testes [26–29]. We have previously shown that in the testes of rats and mice with T1DM, the levels of visfatin, leptin and leptin receptor were reduced [26,29], while in the testes of rats with T2DM, the level of leptin was increased and the expression of leptin receptor was decreased, which was due to leptin resistance [27]. The treatment of diabetic rats and mice with metformin led to normalization of leptin system in the testes and improvement of the testicular functions [26,27]. Other authors have shown that leptin treatment of diabetic mice with leptin deficiency improves testicular function [30]. There are studies that show a decrease in the expression of adiponectin and its receptor in the testes in T1DM [31], and the restoration of testicular functions after treatment with adiponectin [32,33]. However, there is no information available on the expression of apelin and its receptor in the diabetic testes, as well as on the possible regulatory effects of apelin receptor ligands on testicular functions. We hypothesized that the expression and levels of apelin and APJ receptor in the testes of mice with T1DM can be altered, and such changes may indicate the involvement of the intratesticular apelin system in the etiology and pathogenesis of male reproductive disorders in diabetic pathology.

Therefore, the aim of the present work was to investigate the localization and expression of apelin and its receptor, APJ, in the testes of mice with streptozotocin (STZ)-induced T1DM and to study the effects of agonist (apelin-13) and antagonist (ML221) of APJ receptor on the testosterone production by the cultured explants isolated from the diabetic testes.

2. Materials method

2.1. Animals

The adult Swiss albino male mice were selected for this study. The animals were maintained in aerated polypropylene cage with *ad libitum* food and water, under standard experimental conditions (12 hrs light–dark cycles, 25 ± 2 °C). The animals were handled according to the protocol (MZUIAEC-8) approved by Mizoram University Institutional Animal Ethical Committee (MZUIAEC), Mizoram University, Mizoram, India.

2.2. Diabetes induction in mice model and sample collection

Healthy adult mice were weighed and randomly divided into two groups: control (CON)) and diabetes (DM). After 24 hr fasting blood glucose was measured before diabetes induction using glucometer (BG-03, Dr. Morpen). The mice of DM group was injected with four consecutive doses of streptozotocin (STZ) (75 mg/Kg) [26,27] which was dissolved in 0.1 M sodium citrate buffer (pH 4.4). Two weeks after STZ injection, the mice with > 15 mM blood glucose were considered as diabetic. The control animals were given vehicle only (sodium citrate buffer). At the end of experiment, the mice were sacrificed (CON, n = 5; DM, n = 5), and the tissues were collected and stored at —20 °C and fixed in Bouin's Fluid. The serum sample extracted from blood by centrifugation and stored at —20 °C for further analysis.

2.3. In vitro study

To study the effects of apelin-13 and APJ inhibitor (ML221) on testosterone secretion, the second set of experiment was performed. The diabetic group included mice that were treated with STZ four times, similar to that described above, and which had blood glucose levels > 15 mM. The mice were sacrificed after mild anesthesia, and the testes from the control (n = 4) and diabetic mice (n = 4) were taken out and cleaned of adhering tissue. The testes were cut in to equal pieces and testes explants cultured were performed from control and diabetic mice. Further, the testes explants were cultured for 24 hrs according to the method described earlier [34]. In brief, the testes were cut in equal fragments and cultured in the presence of apelin-13 (1 μg/ml) or ML221 (1 μM) (cat # SML0919, Sigma Aldrich, St Louis, USA) in a DMEM: Ham'F12 medium (1:1) with penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 for 24 h at 37 °C. The fragments of diabetic testes were cultured in the presence of apelin-13, and the dose of 1 μg/ml was selected from previous study [35], or in the presence of ML221, APJ antagonist, and in this case the dose of 1 μM was also selected from previous studies [36,37]. After successful cultivation, the tissue and media were harvested for further study.

2.4. Immunohistochemistry of PCNA, GCNA, apelin and apelin receptor and quantification by image J

The excised testis was embedded in paraffin for immunohistochemistry as described earlier [38] and sectioned at 7 μM thick by Leica rotary microtome (RM2125 RTS). The testis sections were spread on a glass slide previously coated with poly-*L*-lysine. The slides were processed for immunolocalization of PCNA, GCNA, apelin and apelin receptor (APJ). PCNA, apelin and APJ receptor were developed by ABC staining kit (Lot# sc 2018; Santa Cruz Biotechnology, CA, USA). Briefly, the sections were deparaffinised and rehydrated by following the earlier method, and then the tissue sections were blocked with goat serum (Lot# H2114; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at the room temperature. After blocking, the tissue sections were incubated with the primary antibodies to apelin (1:50, cat # SAB4301741; Sigma Aldrich, St Louis, USA), APJ (1:100, cat # ABD43; EMD Millipore Corporation, USA) and PCNA (1:100, cat # SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA, USA), for overnight at 4 °C in humidified chamber, and then the primary antibody was washed with phosphate-buffered saline (PBS), and the tissue sections were incubated with biotinylated goat anti-rabbit immunoglobulin secondary antibody for 30 min at the room temperature. After rinsing in PBS, the slides were incubated with horseradish avidin-peroxidase conjugate for 30 min at the room temperature, and then the slides were washed. The GCNA immunolocalization was performed by the primary antibody to GCNA (1:200, cat # 10D9G11; DSHB, University of Iowa, Iowa, USA) followed by the goat anti-mouse secondary antibody conjugated with horseradish peroxidase. After incubation the slides were washed and developed by using 0.05% diaminobenzidine (DAB) solution (3,3′-diaminobenzidine tetrahydrochloride, in Tris-HCl buffer, pH 7.6). For immunohistochemical determination of apelin and APJ, the tissue sections were counterstained with hematoxylin, and then the obtained sections were dehydrated and mounted by DPX. The negative control slides was also run with 1% mouse non-immune IgG for GCNA and 1% rabbit non-immune IgG for PCNA, apelin or APJ to check the specificity of the primary antibody. The slides were observed and photographed by Nikon microscope (E200, Nikon, Japan).

The semi-quantification of PCNA and GCNA staining in the testes of the control and diabetic mice was executed by ImageJ software. The stained area by DAB for PCNA and GCNA in the testis was acquired by using threshold tool of ImageJ as described previously [39], and the data was presented as percentage area of PCNA and GCNA staining. The percentage area for immunostaining was described earlier for the other tissues [26]. In brief, the image was opened in the Image J and image was converted to 8-bits followed by selection of "Image–Adjust–Threshold. After selecting the threshold, the sliders in the pop up window was manually moved to get stained area. Then, it was clicked on apply followed by selection of Analyze–Set Measurements. The limit to Threshold, area and area fraction option was selected. The percentage stained area was obtained by selecting analyze and measure from menu of Image J. Similarly, for apelin immunohistochemistry, where section

was counter stained with hematoxylin, the color deconvolution plugin tool was used and H-DAB vector was selected and resulted in to three images, the DAB stained image was selected and percentage area was obtained as it was obtained for PCNA and GCNA. The quantification of apelin was done same way after using a color deconvolution tool [40].

2.5. Western blot analysis

Western blotting of testis tissue was performed by following the standard protocol of **Jeremy et al, 2019** [38]. Briefly, 10% (w/v) tissue homogenate were prepared with lysis buffer which contains 0.01 M Tris-HCl (pH 7.6), 1.0 mM EDTA, (pH 8.0),0.1 M NaCl, 1 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and the protein concentrations were estimated by Bradford method [41], The equal amount (50 μg) of protein from each groups loaded to each well along with molecular weight marker in a 10% SDS-PAGE. The resolved proteins were transferred to PVDF membrane using wet transfer apparatus for 12 hrs, after successful protein transfer the membrane blocked for 30 min at the room temperature with 5% non-fat skimmed milk prepared using PBST (cat # GRM1254-500G; HiMedia Laboratory private limited, Mumbai, India) and then overnight incubated at 4 °C with the primary antibodies to apelin receptor (APJ) (1:2000, cat # ABD43; EMD Millipore Corporation, USA), antiapoptotic marker BCL2 (1:2000,cat # SC7382; Elabscience Wuhan, China) and proapoptotic marker – active form of caspase-3 (1:1000, cat # STJ97448; St. John's Lab London, UK). Then the primary antibody was washed for 30 min with PBST, and the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase at the room temperature for 2 hrs. After incubation the membranes were washed and developed onto X-ray film by using the electrochemiluminescence (ECL) (cat # 1705060; BioRad, Hercules, CA, USA) method. The band intensities were quantified by using the ImageJ software (imagej.nih.gov/). The membranes were stripped and reprobed for β-Tubulin (1:4000, cat # E-AB-20033; Elabscience, Houston, Texas, USA) for loading control. Immunoblotting of the cultured explants isolated from the diabetic testes were performed following above protocol.

2.6. Hormone assay

Testosterone concentration was estimated in the serum from *in vivo* study and in media from *in vitro* study by using a commercial human testosterone kit (cat # EIA K209; Xema-Medica Co. Ltd, Moscow, Russia) according to manufacturer's instruction. The apelin levels in the testes of control and diabetic animals were measured by using mouse apelin EIA kit (cat # RAB0018; Sigma-Aldrich Co. LLC, USA) as per instruction manual.

2.7. Statistical analysis

Using GraphPad Prism7, all statistical analyses were performed and all numerical data were expressed as mean±*SEM*. The normal distributions of the data were analyzed by the Shapiro-Wilk normality test. To compare the data from different groups, One-way Analysis of variance (ANOVA) followed by Tukey's test was used for *in vitro* testosterone assay, and the Student's *t* test was used for *in vivo* study. The data were considered as significant at the $p < 0.05$.

3. Results

3.1. Changes in the body weight, testis weight, blood glucose and circulating testosterone levels

The diabetic mice exhibited a significant decline in the body ($t = 4.821$, $df = 8$, $p = 0.0013$) and testis weight ($t = 4.647$, $df = 8$, $p = 0.0035$) compared to the control mice (Fig. 1**A-B**). The blood glucose levels ($t = 4.413$, $df = 8$, $p = 0.0022$) in diabetic mice showed a significant elevation compared to the control animals, whereas the

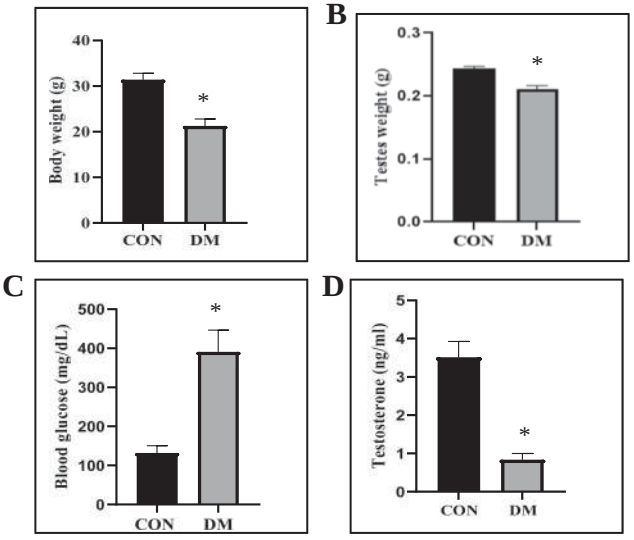

Fig. 1. The changes in the body weight (A), testes weight (B), blood glucose (C) and circulating testosterone levels (D) in the control and diabetic groups of adult Swiss albino male mice. * -the difference between control and diabetic groups is significant at $p < 0.05$. The data are presented as the mean ± SEM, n = 5 per group.

circulating testosterone concentration ($t = 5.907$, $df = 8$, $p < 0.0001$) showed a significant decline in the diabetic mice compared to the control mice (Fig. 1**C-D**).

3.2. Changes in the localization pattern and intratesticular apelin concentration

In order to find out the distribution of apelin in the diabetic testis, immunolocalization of apelin was performed. The immunolocalization of apelin showed moderate staining in the Leydig cells and faint staining in the sperm of control testis, whereas in the diabetic testis, intense immunostaining in the Leydig cells, primary spermatocytes, round spermatid and sperm was detected (Fig. 2**A-B**). The semi quantitative analysis showed an increase in the stained area in the diabetic testis compared to the control testis ($t = 2.982$, $df = 8$, $p = 0.0175$) (Fig. 2**C**). Furthermore, the quantitative analysis also showed elevated concentration of apelin in the diabetic testis compared to the control testis ($t = 5.853$, $df = 8$, $p = 0.0004$) (Fig. 2**D**).

3.3. Changes in the localization pattern and expression of apelin receptor (APJ)

To gain further insight to the testicular apelin system in the diabetes, the expression and localization of APJ was also performed. Immunohistochemical study showed the presence of APJ in the Leydig cells, primary spermatocytes, elongated spermatid and sperm in the control (Fig. 3**A-B**) and diabetic testis (Fig. 3**C-D**). The immunostaining of APJ was found to be more pronounced in the different cells types of diabetic testis than in the control testis. The severely damaged tubules of diabetic testis showed intense immunostaining in the multinucleate cells. In the diabetic testis APJ immunostaining was very strong in the covering of seminiferous tubules.

The expression of APJ was found be significantly higher ($t = 2.437$, $df = 6$, $p = 0.0408$) in the diabetic testis compared to the control testis, as measured by western blot analysis (Fig. 3**F**).

3.4. Changes in the proliferating markers, PCNA and GCNA

The immunolocalization of two proliferation markers, PCNA ($t = 14.73$, $df = 8$, $p < 0.0001$) (Fig. 4**A-B**) and GCNA ($t = 4.986$, $df = 8$, $p <$

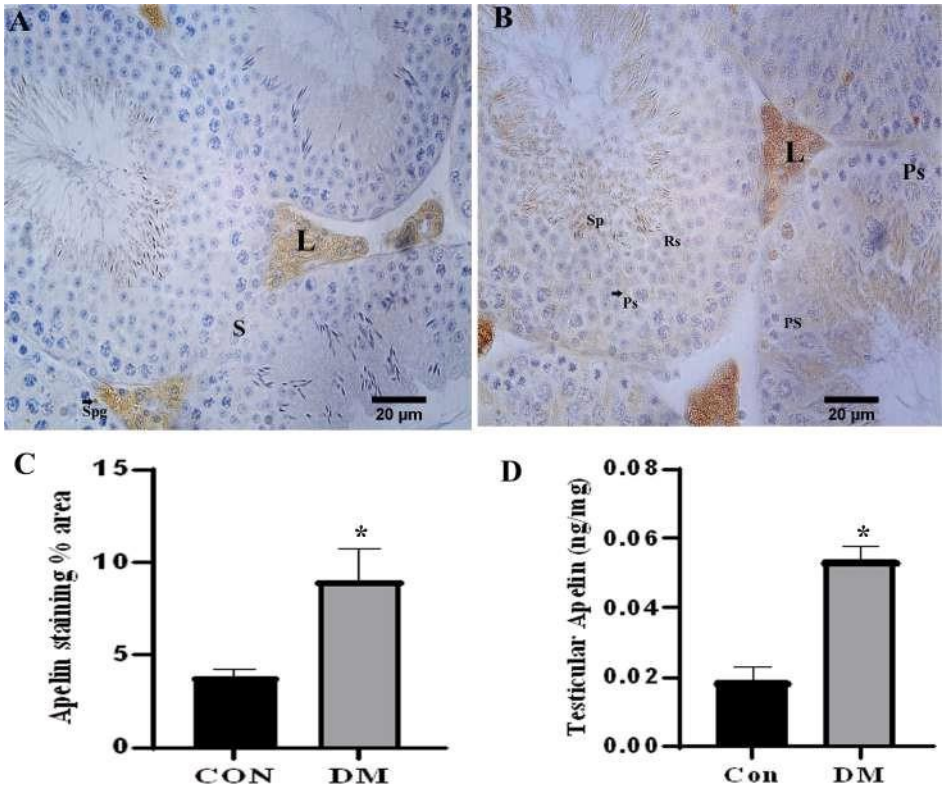

Fig. 2. Localization and levels of apelin in the testes of control and diabetic mice. The immunolocalization of apelin showed intense staining in the Leydig cells (L) of control (A) and diabetic testes (B). In the diabetic testes, pronounce abundance of apelin can also be observed in the other cell types, such as spermatogonia (Spg), primary spermatocytes (Ps), round spermatid (Rs) and sperm (Sp) (B). The semi-quantization (C) and quantification (D) of apelin also showed significantly increased levels of this adipokine in the diabetic testes compared to the control testes. * -the difference between control and diabetic groups is significant at $p < 0.05$. The data are presented as the mean ± SEM, n = 5 per group.

0.0001) (Fig. 4**D-E**) showed a significant decrease in their expression in the diabetic testis compared to the control testis.

3.5. Changes in the anti-apoptotic (BCL2) and pro-apoptotic (active caspase3) protein

The expression of BCL2 was down regulated ($t = 8.464$, $df = 6$, $p < 0.0001$) (Fig. 5**A**) and active form of caspase-3 ($t = 40.92$, $df = 6$, $p < 0.0001$) (Fig. 5**B**) was up regulated in the diabetic testis, as compared to the control.

3.6. Effect of apelin-13 and ML221 on the testosterone secretion- an in vitro study

The untreated diabetic testis explants showed a significant ($p < 0.05$) decline in testosterone secretion compared to the control testis explants. The treatment of the diabetic testis explants with apelin-13 (1 μg/ml) did not show changes in testosterone secretion compared to the untreated diabetic and control testis explants. The treatment of the diabetic testis explants with 1 μM of APJ antagonist (ML221**,** 5-[(4-nitrobenzoyl) oxy]-2-[(2-pyrimidinylthio)methyl]-4H-pyran-4-one**)** significantly stimulated the testosterone secretion compared to control testis explants and to untreated and apelin-13-treated explants isolated from diabetic mice ($p < 0.05$) (Fig. 6).

4. Discussion

In the present study, we first demonstrated the comparative immunolocalization of apelin and its receptor (APJ) in the testes of control and diabetic mice and assessed the intratesticular levels of apelin and APJ, and then compared these levels with testosterone secretion and the proliferative activity of spermatogenic cells. In order to study the role of the apelin system in the functions of Leydig cells, the effect of apelin-13, the most active form of peptides of the apelin family, and ML221, a low molecular weight antagonist of APJ receptor, on testosterone production in the testis explants obtained from the diabetic mice was studied in the *in vitro* conditions.

We have shown that streptozotocin-induced T1DM leads to hyperglycemia, disorganization of the testicular architecture, and a decrease in testosterone secretion in mice, which is fully consistent with the results of previous studies on diabetic patients and animals with experimental models of diabetes [22,24–27]. At the same time, the levels of apelin and the expression of APJ receptor in the testes of diabetic mice were significantly higher than in the control group, and this is the first report of an increase in the expression of apelin signaling system components in the testes in the conditions of metabolic disorders. Earlier, an increase in the level of apelin and its receptor was shown in the adipose tissue of rats with streptozotocin-induced diabetes [42].

Using immunohistochemical methods, a moderate increase in the content of apelin and APJ in testicular cells of diabetic mice was shown compared to control animals. Along with this, it was shown that apelin is localized within the germ cells of the control and diabetic testes. Earlier, the expression of APJ was found in the brain, ovaries, kidney, pancreas, heart and breast, and is stimulated by insulin, estrogens, glucocorticoids, cAMP and some transcriptional factors [43]. Recently, there have been reports on the distribution of apelin and APJ in the testes of rats [14,18], but such information was not available for mice prior to our study. In addition, despite the detection of apelin in the human and rat Leydig cells [16,17], the results on APJ expression in human and rodent testicular cells is contradictory. A number of authors indicated either a low level of expression of this receptor in the testes, or its absence in them [17,19], which makes the possibility of a direct effect of apelin on testicular cells doubtful. In this regard, the detection of AJP expression in the testes of the control and diabetic mice is an important argument in favor of the presence of a functionally active apelin signaling system in testicular cells. This assumption is confirmed by the data of other authors in relation to the testes of the rats [14,18].We detected an increase in the APJ expression in T1DM, which may indicate the role of this system in the development of diabetes-associated testicular dysfunctions. Since it is known that T1DM suppresses the functions of Leydig

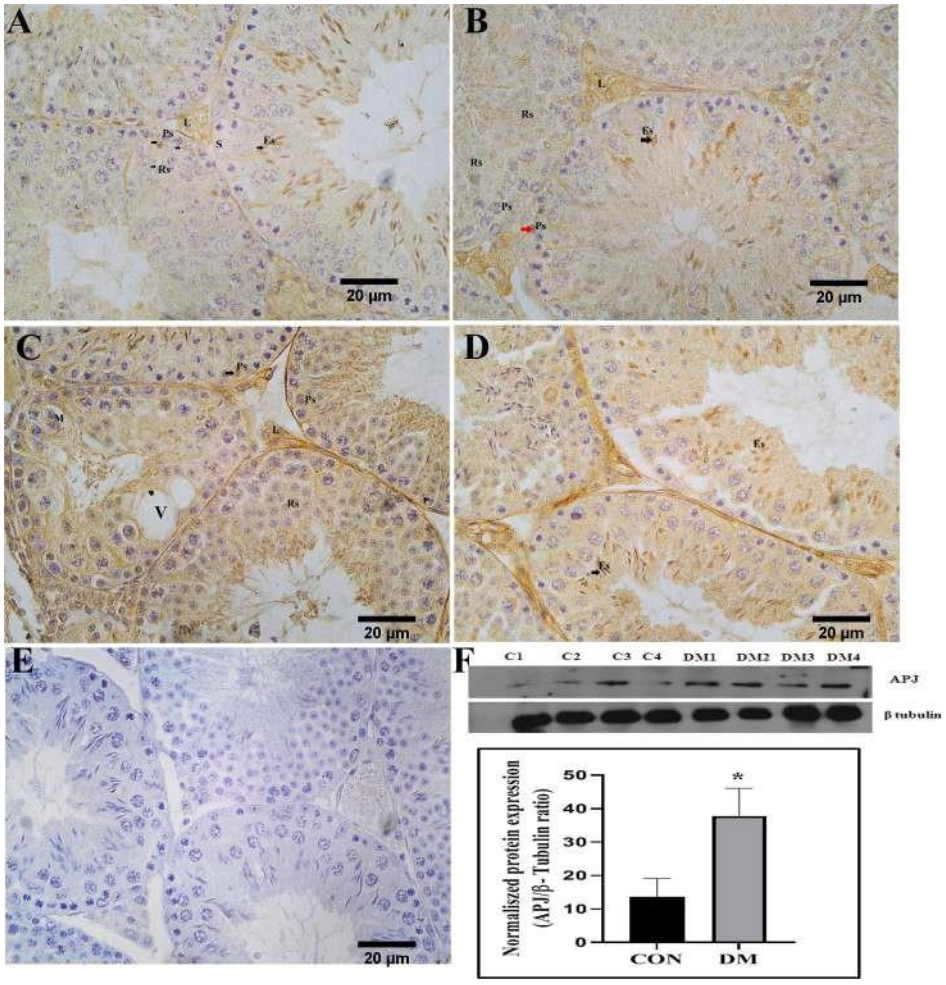

Fig. 3. Localization and expression of apelin receptor (APJ) in the testes of control and diabetic mice. The immunolocalization of APJ was observed in the Leydig cells (L), primary spermatocytes (Ps), round spermatid (Rs) and sperm (Sp) in the control (A-B) and diabetic testes (C-D). The perinuclear staining of APJ can be seen in the control testes (B, red arrow) and diabetic testes (C, Ps). The diabetic testes showed mild increase staining of APJ in the Leydig cells (L), primary spermatocytes and elongated spermatid cells (C). The diabetic testes also showed multinucleate giant cells (M) with moderate increase in APJ immunostaining, and some vacuole can also be seen in the tests of diabetic mice. The negative control (E) showed no immunostaining in the testes (the primary antibody was replaced with 1% rabbit non-immune IgG). Western blot analysis of APJ was significantly higher in the diabetic testes compared to the control testes. * -the difference between control and diabetic groups is significant at $p < 0.05$. Data are presented as the mean ± SEM, n = 4 per group.

cells and reduces the secretion of testosterone, an increase in the intratesticular level of apelin and a more intense immunostaining of apelin and APJ in diabetic testes as compared to the control testes, observed by us, indicate possible inhibitory role of apelin in the biosynthesis of testosterone.

Currently, there is a little information about the regulatory effect of apelin on the male reproductive system, and this influence is mainly associated with the effects of apelin on the hypothalamic and pituitary components of the HPG axis. Thus, it has been shown that high doses of apelin-13 cause a decrease in testosterone production by the testes, inhibiting the secretion of LH by the anterior pituitary gland [44]. It was also found that treatment with apelin-13 lowers the levels of LH, FSH and testosterone and, thus, may have a negative effect on reproductive functions [45]. Taking into account the presence of apelin and APJ receptors in the testes, it can be concluded that apelin is able to act not only on the hypothalamic and pituitary components of the gonadal axis, but also on its peripheral links, the gonads, as shown for other adipokines, including leptin, adiponectin and visfatin [2,6,11,46].

We have established a direct relationship between high levels of intratesticular apelin and decreased testosterone production in diabetic mice. At the same time, in the *in vitro* experiments with cultured explants isolated from the diabetic testis, it was shown that ML221, an antagonist of APJ, significantly increased the secretion of testosterone by these explants, and the level of the hormone even exceeded that in the control, while apelin-13 was ineffective. Thus, this is the first report that a decrease in the apelin signaling activity in the testes by the APJ receptor antagonist may have significant therapeutic potential to treat the reproductive dysfunctions, at least in T1DMconditions. Previously, it was also suggested that antagonists of apelin receptor could be used to treat infertility, but in this case only the hypothalamic mechanisms of action of apelin were taken into account [44].

It should be noted that we measured the total concentration of apelin in the testes, including its long, less active forms (for example, apelin-36), as well as more active shortened forms (apelin-13), which is usually used in pharmacological studies [15,47]. It cannot be ruled out that the pattern of apelin isoforms in the testes can also change in diabetes, which affects the specific activity of peptides of the apelin family and the spectrum of their physiological effects, and this should be the subject of further research.

Our results showed that the proliferation of germ cells in the testes of diabetic mice is significantly reduced. Two proliferation markers, PCNA and GCNA, were very low expressed in diabetic testes. In the testes of diabetic mice, we also observed an increased expression of the pro-apoptotic enzyme caspase-3 and a decrease in the expression of the anti-apoptotic protein BCL2. These data indicate that infertility associated with diabetes may be due to an imbalance in the apoptosis and proliferation of germ cells. Other authors also showed that in diabetes, the testes have decreased proliferation and increased proapoptotic activity, which is due to increased oxidative stress, inflammation and endoplasmic reticulum stress [48,49], and ultimately may lead to infertility [50].

We assume that a high level of apelin may be one of the factors in the decrease in proliferative activity and increased apoptosis in the testes. Despite the fact that currently there is no data on the relationship between the levels of apelin and the activity of growth and apoptotic factors in the testes, there is information on such relationships in the

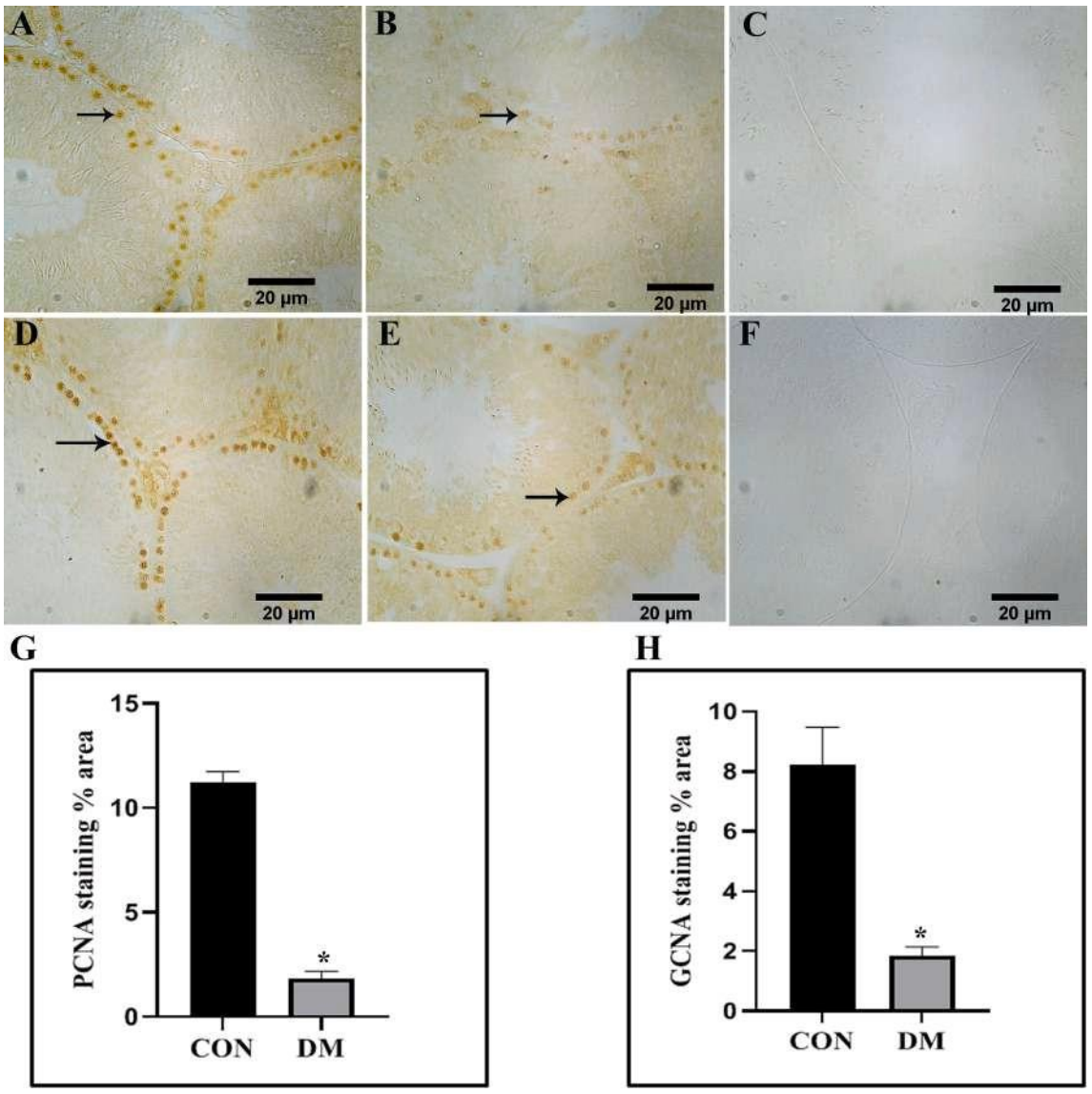

Fig. 4. Immunolocalization of proliferation markers (PCNA and GCNA) in the testes of control and diabetic mice. PCNA and GCNA immunostaining showed many positive cells in the control testes (A, D), whereas the diabetic testes showed very few and weakly stained cells (arrow, B,E). The negative control for PCNA (C) and GCNA (F) showed no immunostaining, and was replaced with 1% non-immune rabbit IgG and mouse IgG, respectively. The semi-quantification study of PCNA (G) and GCNA (H) immunostaining showed a significantly higher percentage of stained area in the control testes than in the diabetic testes. * -the difference between control and diabetic groups is significant at $p < 0.05$. The data are presented as the mean ± SEM, n = 5 per group.

other tissues. It was shown thatapelin inhibits the proliferation of smooth muscle cells in the rat pulmonary artery during hypoxia [51], and in diabetes, a high level of apelin promotes apoptosis in podocytes [52]. On the other hand, there is evidence that apelin stimulates the proliferation of granulosa cells and myocardial cells [53,54], and inhibits apoptosis in granulosa cells and human osteoblast cells [53,55]. We believe that in diabetic conditions, a high level of apelin in the blood and testes may promote apoptosis and reduce the proliferation of germ cells in the testes, while the regulatory effects of normal concentrations of apelin on apoptosis and proliferation may change, which requires further research. It should be noted that apelin is a pro-angiogenic factor [56], and high levels of apelin can cause ischemic testicular damage in diabetes. Apelin receptor antagonists have been shown to prevent ischemic retinopathy in mice [36]. Based on this, it can be assumed that the antagonist ML221 can weaken or prevent pathological angiogenesis in the testes caused by hypoxia and pro-inflammatory factors. Our study of the potentiating effect of ML221 on steroidogenesis in the *in vitro* conditions suggests that apelin receptor antagonists may have the potential to improve androgenic status in diabetes. To confirm this, the *in vivo* studies of its effect on testicular steroidogenesis and spermatogenesis in normal and pathological are needed.

Thus, the present study opens up opportunities for studying the role of apelin and its signaling system in the regulation of testicular steroidogenesis, spermatogenesis, and testicular angiogenesis both in normal conditions and in pathological conditions induced by diabetes. The testicular hyperapelinemia we discovered can make a significant contribution to the pathogenesis of the testes, as a result of which inhibition of the apelin receptor by the ML221 antagonist can improve the functioning of the male reproductive system through the improvement of testicular steroidogenesis.

Credit author statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work for the content, including concept, design, analysis, writing, and revision of

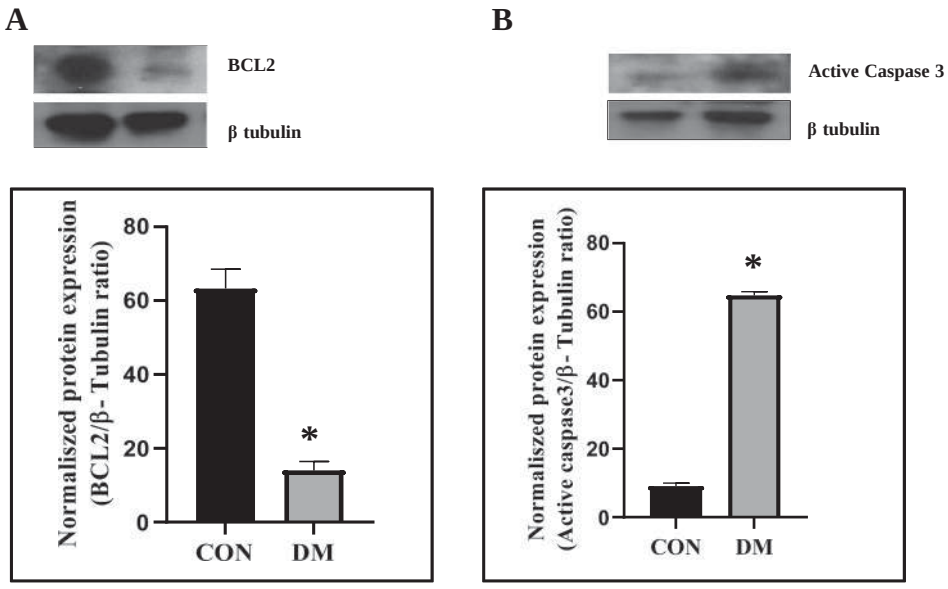

Fig. 5. Expression of BCL2 and active form of caspase-3 in the testes of control and diabetic mice. The expression of BCL2 was significantly lower and active caspase-3 was significantly higher in the diabetic testes than in the control testes. * - the difference between control and diabetic groups is significant at $p < 0.05$. The data are presented as the mean ± SEM, n = 4 per group.

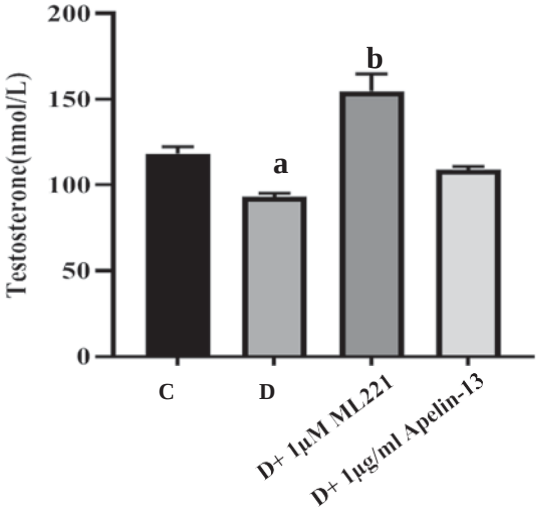

Fig. 6. Effect of APJ-antagonist ML221 and apelin-13 on the testosterone secretion by the cultured testis explants *in vitro*. The testosterone secretion by the untreated diabetic testis explants (D) was significantly decreased compared to the control testis explants (C). The treatment of the diabetic testis explants with the APJ-antagonist ML221 (1 μM) increased the testosterone levels higher than in the other investigated groups. The treatment of the diabetic testis explants with the APJ-agonist aplein-13 (1 μg/ml) did not affect testosterone levels compared to the untreated explants. [a] – the difference between groups C and D is significant at $p < 0.05$; [b] - the difference between groups D + 1 μM ML221 and the other groups is significant at $p < 0.05$. The data are presented as mean ± SEM, n = 4 per group.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors greatly acknowledged the funding for the present study by DST, New Delhi under Indo-Russian International Research Project. DST, Grant/Award Number: INT/RUS/RFBR/P-310; Russian Foundation for Basic Research, Grant/Award Number: 18-515-45004 IND-a. We also acknowledge the infrastructure facility provided to Department of Zoology, Mizoram University under DST-FIST program by DST, New Delhi, India.

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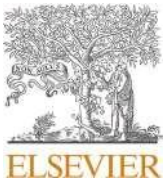

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Postnatal developmental expression of apelin receptor proteins and its role in juvenile mice testis

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ARTICLEINFO

Keywords:
Apelin
Proliferation
Postnatal
Testis
Testosterone
ML221

ABSTRACT

The expression of apelin system has been shown in the adult testis of rat and mice. It has also been emphasized that regulation of testicular activity in early stages is important to sustain normal testicular activity in adulthood. Since the expression of apelin receptor (APJ) has been shown in the adult testis, moreover, developmental expression of APJ and its role has not been explored yet. Thus, we have examined the testicular expression of APJ during postnatal stages with special reference to proliferation, apoptosis and hormone secretion in early postnatal stage. Postnatal analysis showed that circulating apelin was lowest at PND1 and maximum at PND42. Among testosterone, estrogen and androstenedione, only circulating testosterone showed a gradual increase from PND1 to PND42. Testicular expression of APJ was also developmenatly regulated from PND1 to PND42, revealing a positive correlation with circulating apelin, testosterone, and androstenedione. Immunohistochemical study showed that APJ was mainly confined to Leydig cells of early postnatal stages, whereas, seminiferous tubules at PND42 showed immunostaining in the round spermatids. APJ inhibition from PND14-PND20 by ML221 suppressed the testicular proliferation, increased apoptosis and increased estrogen secretion. However, expression of AR was down-regulated by ML221 treatment. Furthermore, ML221 decreased the abundance of p-Akt. *In vitro* study also showed that APJ antagonist, ML221 decreased AR expression. These results suggests that apelin signaling during early developmental stages might be required to stimulate the germ cell proliferation, and inhibition of apoptosis. Both in vivo and in vitro study have shown that expression of AR was regulated by apelin signaling. Since the first wave spermatogenesis involves proliferation and apoptosis, therefore, further study would be required to unravel the exact mechanism of apelin mediated regulation of testicular activity during early postnatal stages. In conclusion, the present results are an indicative of apelin mediated signaling during early postnatal stage for regulation of germ cell proliferation, apoptosis and AR expression.

1. Introduction

Spermatogenesis in testis known be initiated at neonatal stages, where quiescent germ start differentiation, which results in appearance of first wave of spermatogenesis at postnatal (PND) age of 6 weeks [22,23]. It has been reported that at juvenile stage (PND20), round spermatids appear whereas sperm appears at PND35 in the mice testis [1]. It has been emphasized that the first wave of spermatogenesis at early stage is a well-regulated and synchronized processes, where at different stage of postnatal life, germ cells proliferate and differentiate, and this is an important event for maturation of germ cells [20,42]. The establishment of successful first wave of spermatogenesis is critical for continuous spermatogenesis throughout adulthood [19,43,7]. Furthermore, this successful wave of spermatogenesis requires appropriate germ, Sertoli cell number, and the number of these cells is established by apoptosis. Apoptosis is important and constant feature of testis during early developmental stages and adulthood as well [35]. Germ cell apoptosis during first wave of spermatogenesis is required to eliminate

Abbreviations: Akt, Protein Kinase B, PKB; ANOVA, Analysis of Variance; APJ, apelin receptor; AR, androgen receptor; BCL2, B-cell lymphoma-2; Caspase-3, cysteine dependent aspartate directed protease-3; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DMEM, Dulbecco's Modified Eagle Medium; ECL, electrochemiluminescence; EDTA, Ethylenediamine tetraacetic acid; ERα and β, estrogen receptor α and β; GCNA, Germ Cell Nuclear Antigen; ML221, apelin receptor antagonist; NaCl, sodium chloride; p-Akt, Phosphorylated Akt; PCNA, Proliferating Cell Nuclear Antigen; PND, Postnatal development; PVDF, Polyvinylidene fluoride; RPM, Revolutions Per Minute; SDS-PAGE, Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis.

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https://doi.org/10.1016/j.jsbmb.2022.106178
Received 18 August 2022; Received in revised form 8 September 2022; Accepted 9 September 2022
Available online 12 September 2022
0960-0760/

the abnormal germ cells from the testis [15,28,49,50]. Therefore, regulation of testicular activity by various endogenous factors in early postnatal period, such as juvenile stage is important to study.

It is believed that endogenous factors, such as hormones, mainly testosterone, estrogen, and follicle-stimulating hormone along with other endocrine and paracrine/autocrine players also regulate testicular activity in relation to germ cell proliferation and apoptosis [5,45]. It has been evidenced that differentiation of Leydig cell occurs after initial differentiation of Sertoli cells [40]. Despite germ cell proliferation, and apoptosis, pre-pubertal testis also synthesizes testosterone by Leydig cells. Testis of both rat [13] and mice [11] has been shown to synthesize testosterone from 15.5dpc and 14.5dpc respectively. It has also been shown that treatment of testosterone suppresses the apoptosis during first wave of spermatogenesis [15]. Furthermore, not only testosterone rather estrogen also regulates proliferation and maturation of cells in the testis [16].

Apelin receptor, (APJ) is a classical heterotrimeric guanine nucleotide binding protein (G-protein) coupled receptor (GPCR), and the physiological function and signal transduction produced by the combination of Gαi protein subtypes [9]. It has been shown that APJ signaling is mediated by activation of Gαi2 or Gαi3 through molecular rearrangements, however, APJ signaling activation might also be mediated by Gαo and Gαq through classical dissociation model [3]. The expression of apelin and apelin receptor (APJ) has been shown in the testis of rat and mice [30,34,46]. Localization study has shown the presence of Apelin and AP in the Leydig cell as well as in the seminiferous tubules, which suggests a possible role of apelin and APJ in testicular steroidogenesis and germ cell proliferation in the adulthood [30,46]. To best of our knowledge, there is paucity of literatures on role of apelin on the testis. A previous study by Sandal et al. (2015) [48] has shown that apelin 13, infusion in male rats suppresses testosterone synthesis by decreasing LH secretion. These studies have provided evidence of apelin and APJ in the testis with potential role in adult stage, however, the ontogeny of apelin and APJ in postnatal stage and its function remains unclear in the mouse during early developmental stages. Apelin has been shown to inhibit apoptosis and promotes proliferation in rat granulosa cells as well as in mouse osteoblastic cell line via Akt signaling [44,53]. Since the first wave spermatogenesis involves, Leydig cell steroidogenesis, germ cell proliferation and germ cell loss by apoptosis, thus question arises, whether apelin can influences these functions during first wave of spermatogenesis or not. Moreover, it is also important to analyze the developmental expression of apelin and its receptor.

Therefore, the aims of present study were to investigate the developmental expression of apelin and APJ in the mice testis, also to unravel the role of apelin and APJ during first wave of spermatogenesis in relation to hormone secretion, proliferation and apoptosis.

2. Materials method

2.1. Animals

Breeding cages were set with healthy adult Swiss albino mice and were maintained in aerated polypropylene cage with ad libitum food and water, under standard experimental conditions (12 hrs. light-dark cycles, 25 ± 2°C). All the animal experiment was approved by Mizoram University Institutional Animal Ethical Committee (MZUIAEC-9), Mizoram University, Mizoram, India. Male pups of different postnatal day 1 (PND1), 7 (PND7), 14 (PND14), 21 (PND21) and 42 (PND42) were used for the postnatal study.

2.2. Sample collection

Mice at PND1 (n = 10), PND7 (n = 10), PND 14 (n = 5), PND 21 (n = 5), PND 42 (n = 5), were sacrificed. One side of testis was fixed in Bouin's fluid for immunohistochemistry and contralateral testis was freeze for western blot analysis. The serum sample was collected from blood by centrifugation at 2500 RPM and stored at −20°C for hormone assays.

2.3. In vivo study

To unravel the role of apelin receptor (APJ) in the testis during juvenile stage, the antagonist of APJ (ML221) was given from PND14 to 20. Male mice PND14 were randomly divided into two 1. Control group (CON, n= 5) and 2. Treated group (ML221, n= 5). ML221 (cat # SML0919, Sigma Aldrich, St Louis, USA) dissolved in DMSO and then diluted with double distilled water. ML221 was given at dose of 150 μg/kg based on previous study [6]. The duration was for 7 days, from PND14 to 20, because during PND14 to 20, expression of APJ was up regulated, therefore, this duration was selected for ML221 treatment. Equal amount of vector (DMSO diluted with distilled water) was injected to the control animals. On 21th day the body weight of the animals was taken then sacrificed. Testes were collected, weighed and stored in Bouin's fixative solution for histology and immunohistochemistry and at −20°C for western blotting. Serum samples were collected after centrifugation of the blood for different hormone assays. Gonadosomatic Index was calculated using the formula.

GSI (%) = (Testes Weight/Body Weight)*100·

2.4. In vitro study

In vitro culture of mice testes was performed to study the direct effect of ML221 on testicular activity. The testes were taken from PND 18 mice and were cleaned of adhering tissue. Testes excised, were cultured in two groups, first were untreated (CON) and second were apelin receptor antagonist (ML221) treated group. Tissues were cut in to equal pieces, and the testes explants were cultured for 24 h according to the method described earlier [32]. briefly, the testes were cut in equal fragments and cultured as control with no treatment and ML221 (50 μM) [14,27] (cat # SML0919, Sigma Aldrich, St Louis, USA) in a DMEM: Ham'F12 medium (1:1) with penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 at 37 °C for 24 h. The tissue and media were harvested stored at −20 °C for western blot analysis and hormone assay.

2.5. Histology and histomorphometric study

The fixed testes were properly dehydrated by gradually ethanol grade, embedded in paraffin for histomorphometry and immunohistochemistry as earlier described [31]. Then the paraffin block was sectioned at 7 μM thick by Leica rotary microtome (RM2125 RTS). The sections were spread on a glass slide coated with poly-*L*-lysine and incubated overnight at 37 °C. Hematoxylin and Eosin staining was performed by following deparaffinization, rehydration, staining, dehydration, clearing; mount with DPX [21]. The mounted slides were observed and seminiferous tubule diameter, germinal epithelial height and lumen diameter were measured by using an ocular micrometer. Fifty round or nearly round seminiferous tubules were randomly chosen from each group and seminiferous tubule diameter, germinal epithelial height and lumen diameter measured.

2.6. Immunolocalization of apelin, apelin receptor (APJ), PCNA and GCNA

Apelin and apelin receptor (APJ) were localized in postnatal developmental groups (PND1, 7, 14, 21 and 42) and the proliferating markers, PCNA and GCNA were performed in in vivo treatment groups (CON and ml 221) as described earlier [26]. Briefly, the spread sections were deparaffinized in xylene, rehydrated in ethanol and distilled water

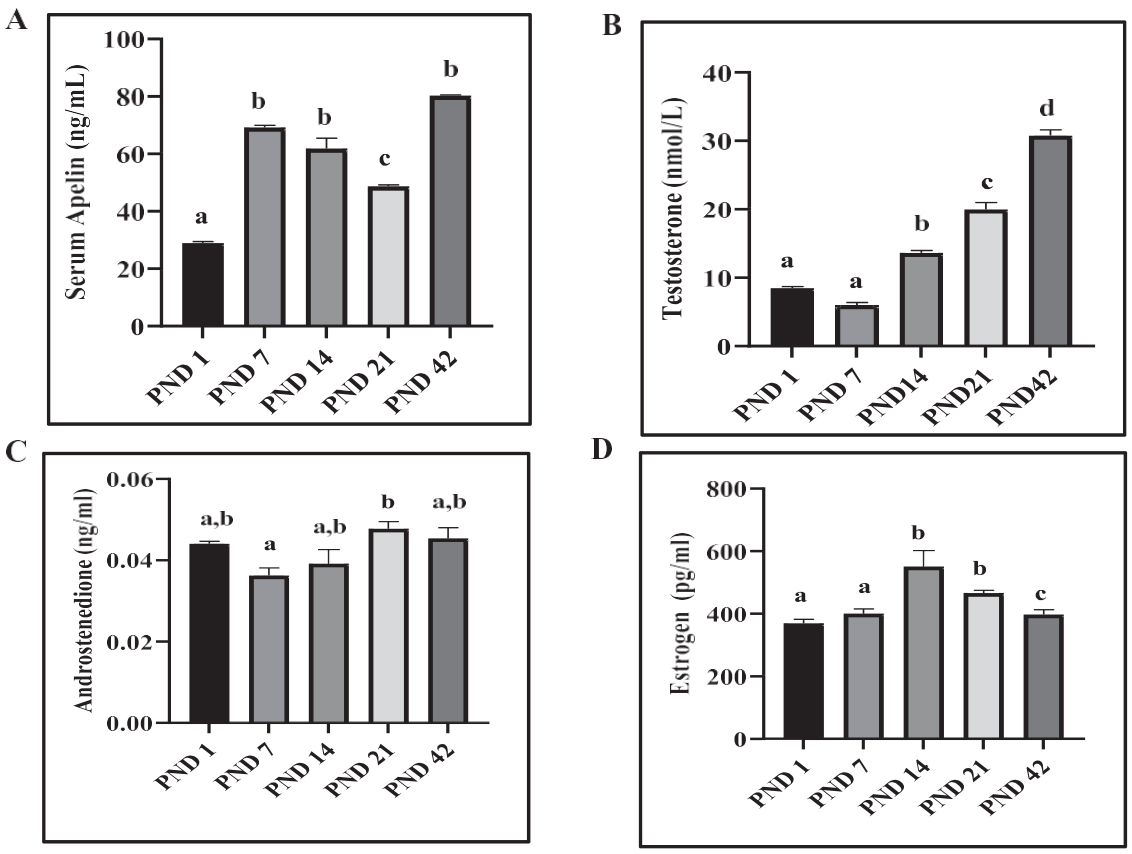

Fig. 1. Postnatal day dependent changes in the circulating apelin, testosterone, androstenedione and estrogen levels. The circulating apelin levels showed a significant ($p < 0.05$) decrease at PND1 compared to the PND7–42 (A). The circulating testosterone levels did not show significant change between PND1 and PND7, however, testosterone levels showed stage dependent significant ($p < 0.05$) increase from PND14 to PND42 compared to the PND1–7 (B). The circulating androstenedione levels did not show stage dependent marked variation (C). The circulating estrogen levels showed significant ($p < 0.05$) increase at PND14–21 compared to the other stages (D). Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups.

and blocked by goat serum (1:100, Lot# H2114; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at the room temperature. Then, the tissue sections were incubated with the primary antibodies to apelin (1:50, cat # SAB4301741; Sigma Aldrich, St Louis, USA), APJ (1:100, cat # ABD43; EMD Millipore Corporation, USA) and PCNA (1:100, cat # SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA, USA)for overnight at 4°C.The primary antibody wash was done with phosphate-buffered saline (PBS), and the tissue sections were incubated with Horseradish peroxidase (HRP) tagged goat anti-rabbit immunoglobulin secondary antibody for 3 h at the room temperature. After secondary incubation the sections was washed in PBS. The GCNA immunolocalization was done by the primary antibody GCNA (1:200, cat # 10D9G11; DSHB, University of Iowa, Iowa, USA) followed by the goat anti-mouse secondary antibody conjugated with horseradish peroxidase. Then the slides were washed in PBS and developed by using 0.05% diaminobenzidine (DAB) solution (3,3′-diaminobenzidine tetrahydrochloride, in Tris-HCl buffer, pH 7.6 and hydrogen peroxide). The apelin and APJ sections were counterstained with hematoxylin, and then the stained sections were dehydrated and mounted by DPX. The negative control slides were run with 1% rabbit non-immune IgG for apelin, APJ to check the specificity of the primary antibody. The slides were observed and photographed by Nikon microscope (E200, Nikon, Japan). The PCNA and GCNA staining in control and ML221 mice testes were calculated by ImageJ software. The stained area by DAB for PCNA and GCNA in the testis was acquired by using threshold tool of ImageJ as described previously [10], and the data was presented as percentage area of PCNA and GCNA staining. The percentage area for immunostaining was described earlier [25].

2.7. Western blot analysis

Western blotting was performed in testis sample by following the standard protocol of Jeremy et al., 2019 [32]. Briefly, 10% (w/v) tissue homogenate were prepared with lysis buffer, containing 0.01 M Tris-HCl (pH 7.6), 1.0 mM EDTA, (pH 8.0), 0.1 M NaCl, 100 μg/ml phenylmethylsulfonyl fluoride, and the protein concentrations were estimated by Bradford method (Bradford 1976), The equal amount (50 μg) of protein from each groups loaded to each well along with molecular weight marker in a 10% SDS-PAGE. The resolved proteins were transferred to PVDF membrane using wet transfer apparatus for 12 hrs., after successful protein transfer the membrane blocked for 30 min at the room temperature with 5% non-fat skimmed milk prepared using PBST(cat # GRM1254–500 G; HiMedia Laboratory private limited, Mumbai, India) and then overnight incubated at 4°C with the primary antibodies to apelin receptor (APJ) (1:2000, cat # ABD43; EMD Millipore Corporation, USA), androgen receptor (1:250, Ref # PA5–16363; Thermo Fisher Scientific, USA), antiapoptotic marker BCL2 (1:2000,cat # SC7382; Elabscience Wuhan, China) and proapoptotic marker – active caspase-3 (1:1000, cat # STJ97448; St. John's Lab London, UK), estrogen receptor α (1:500, cat # P03372, DSHB, University of Iowa, Iowa,USA) and estrogen receptor β (1:500, cat # CWK-F12, DSHB, University of Iowa, Iowa,USA). For phosphorylated Akt protein the membranes were blocked in 1X TBS 1% casein blocker (cat # 161–0782, Bio-Rad Laboratories, USA) for 30 mins and then incubated with p-Akt Ser473(1:500, cat # 4060 T, Cell Signaling Technology, USA). Then the primary antibody was washed for 30 min with PBST, and the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase at the room temperature for 2 hrs. After incubation the

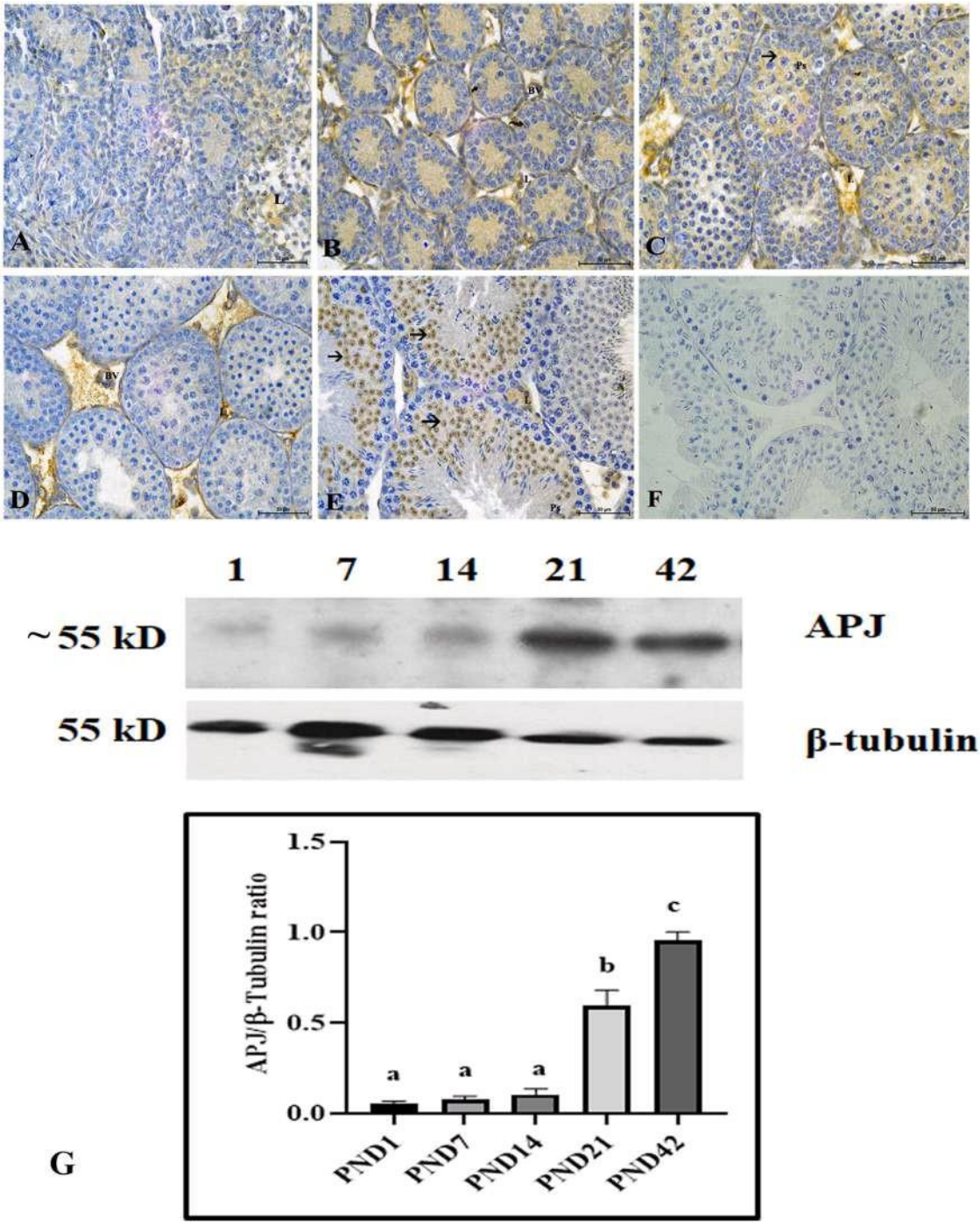

Fig. 2. Postnatal day dependent changes in the expression and localization of testicular apelin receptor. Expression of APJ showed a stage dependent increase, however, from PND1 to PND14, it was not significant. The expression of APJ showed a significant increase in PND21 and PND42 compared to the other groups. At PND42, expression of APJ was significantly highest. Data are expressed as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups (G). Localization of APJ was observed in the Leydig cells (L) from PND1-PND42 with faint immunostaining at PND42 (A-E). Germ cells, mainly spermatocytes also showed mild immunostaining (arrow) at PND14 (C), whereas, at PND21, no immunostaining was seen in seminiferous tubules (D). PND42 testis showed moderate immunostaining in the round spermatids (arrow) (E). The negative control (F). L. Leydig cell, Ps. Primary spermatocytes, BV. Blood Vessels.

membranes were washed and developed onto X-ray film by using the electrochemiluminescence (ECL) (cat # 1705060; Bio-Rad, Hercules, CA, USA) method. The band intensities were quantified by using the ImageJ software (imagej.nih.gov/). Total Akt protein performed in the membrane after p-Akt Ser473 and probed with Akt (1:1000, cat # 9272 S, Cell Signaling Technology, USA). The membranes were stripped and reprobed for β-Tubulin (1:4000, cat # E-AB-20033; Elabscience, Houston, Texas, USA) for loading control. Immunoblotting of AR, ER α and β were performed in testis explants by following the same method.

2.8. Hormone assay

Hormone concentration such as testosterone and estrogen was estimated in the serum from PND groups, APJ inhibitor in vivo study and in media from in vitro study by using a commercial human testosterone kit (cat # EIA K209; Xema-Medica Co.Ltd, Moscow, Russia) and human

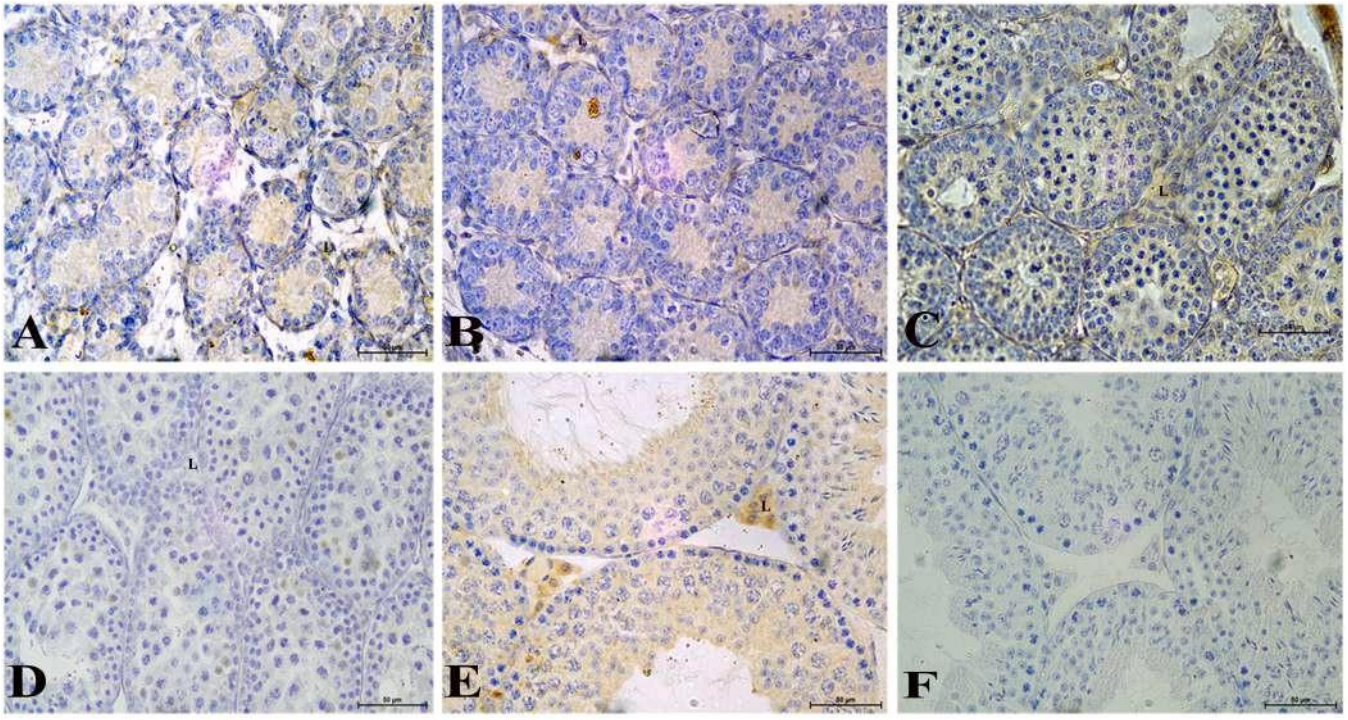

Fig. 3. Postnatal day dependent changes in the localization of testicular apelin. Immunolocalization of apelin also showed mild immunostaining in the Leydig cells (L) at PND1, 7, and 14 (A-C). No immunostaining was observed at PND21 (D), whereas, Leydig cells at PND42 showed moderate immunostaining (E). The negative control (F).

estrogen kit (cat # DKO 003; DiaMetra Immunodiagnostic Systems (IDS) Ltd Boldon, UK) according to manufacturer's instruction. Sensitivity of the assay was assessed as 0.15nmol/l. The circulatory apelin levels were measured in PND 1 to PND 42 animals by using mouse apelin EIA kit (cat # RAB0018; Sigma-Aldrich Co. LLC, USA) as per instruction manual. Minimum detectable concentration is 5.84 pg/ml. This kit shows no cross reactivity with any of the cytokines.

2.9. Statistical analysis

Using GraphPad Prism9, all statistical analyses were performed and all numerical data were expressed as mean±*SEM*. To compare the data from different groups, One-way Analysis of variance (ANOVA) followed by Tukey's test was used for in vitro testosterone assay, and the Student's *t* test was used for in vivo study. The normal distributions of the data were analyzed by the Shapiro-Wilk normality test. The data were considered as significant at the $p < 0.05$.

3. Results

3.1. Changes in the circulating apelin, and steroids (Testosterone, estrogen and androstenedione) hormone levels

The circulating apelin levels were minimum at PND1 and maximum at PND42. The levels of apelin at PND7 and PND14 did not show a significant changes, whereas, a decline in apelin levels was noticed at PND21 compared to the PND7, 14 and 42 (Fig. 1A). The circulating testosterone levels showed highest at PND42 compared to the other PND groups (Fig. 1B). However, the circulating androstenedione didn't show marked variation. On the other hand estrogen levels showed significant ($p < 0.05$) increase at PND14 and 21 compare to other postnatal stages (Fig. 1C-D).

3.2. Expression and localization of apelin receptor (APJ) during postnatal stages

Western blot analysis showed presence of APJ in the testis from PND1 to PND42. The expression of APJ was significantly ($p < 0.05$) elevated at PND21 and PND42, compared to the other groups. The expression of testicular APJ from PND1 to PND14 showed a mild elevation, however, it was not significant (Fig. 2 R1G).

Immunohistochemical localization of testicular APJ also showed the presence from PND1 to PND42. The localization of testicular APJ from PND1 to PND7 (Fig. 2 R1A-B) was mainly confined to the interstitium, with moderate staining in the Leydig cells. At PND14, spermatocytes and Leydig cells showed mild staining (Fig. 2 R1C). However, at PND21, APJ immunostaining was confined to Leydig cells only (Fig. 2 R1D). At PND42, a faint immunolocalization of APJ was observed in the Leydig cells. However moderate staining of APJ was observed in round spermatid showed moderate immunostaining (Fig. 2 R1E).

In order to analyze testicular apelin, immunohistochemistry of apelin was performed. Immunolocalization of apelin showed faint immunostaining in the interstitium from PND1 to PND14 (Fig. 3A-C), however, at PND21 no immunostaining was observed (Fig. 3D). Apelin at PND42 showed a moderate immunostaining in the Leydig cells (Fig. 3E).

To gain further insight about the involvement of apelin system in testicular steroidogenesis during postnatal stages, a correlation study was performed between the expression of testicular APJ expression and circulating steroid levels. The result of correlation study showed a significant positive correlation of postnatal testicular APJ expression with circulating apelin ($r = 0.4782$, $P = 0.0156$), testosterone ($r = 0.9388$, $P < 0.0001$) and androstenedione levels ($r = 0.4438$, $P = 0.0263$) (Supplementary file). However, expression of testicular APJ did not show significant correlation with estrogen levels ($r = -0.1038$, $P = 0.6216$) (Supplementary file).

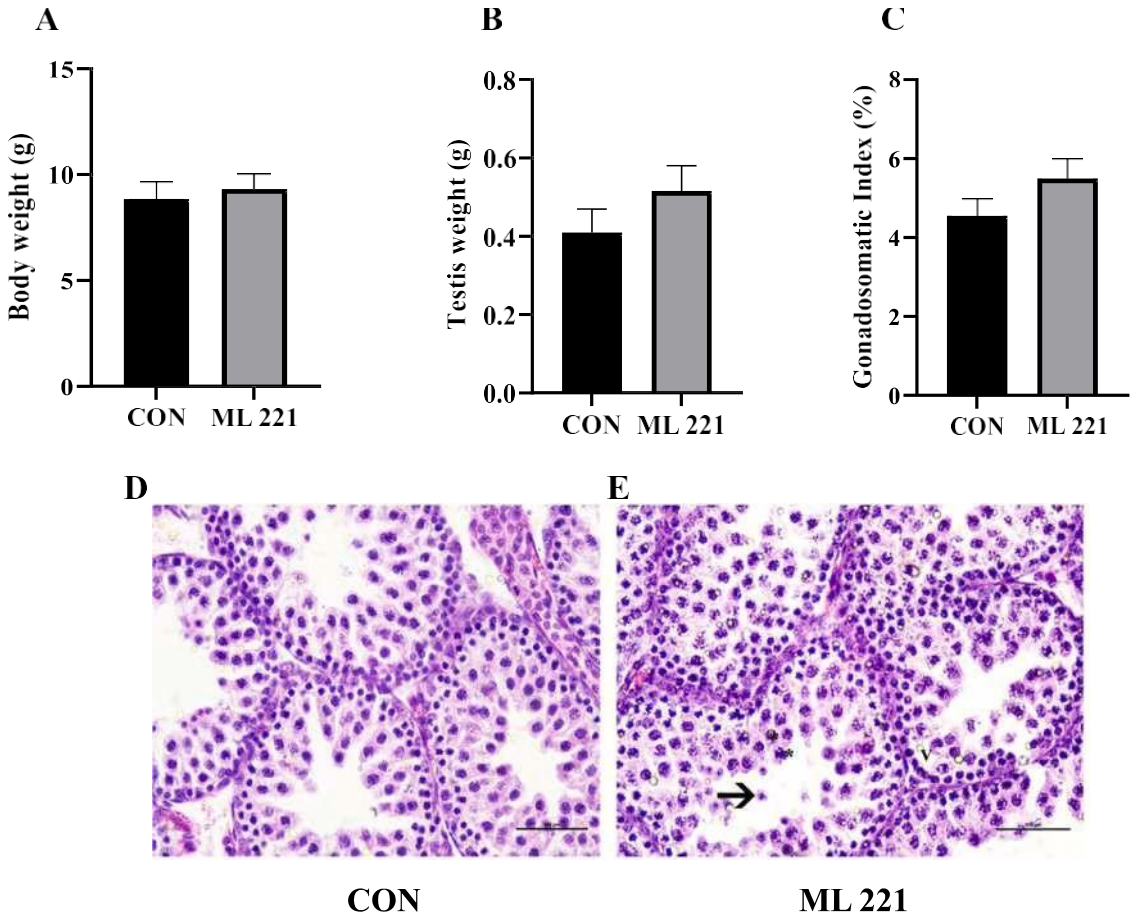

Fig. 4. Changes in body weight, testis weight, gonad somatic index and testicular histology after apelin receptor antagonist treatment. The treatment of ML221 did not show significant ($p > 0.05$) change on body weight (A), testis weight (B) and GSI (C). Data are expressed as mean±SEM. The control mice testis showed normal histoarchitecture with presence of primary spermatocytes, round spermatid, Sertoli cells Leydig cells (D). The testicular section of ML221 treated mice showed some degenerative changes, vacuole (V), sloughing of germ cell in the lumen (arrow) and darkly stained cells (*) (E).

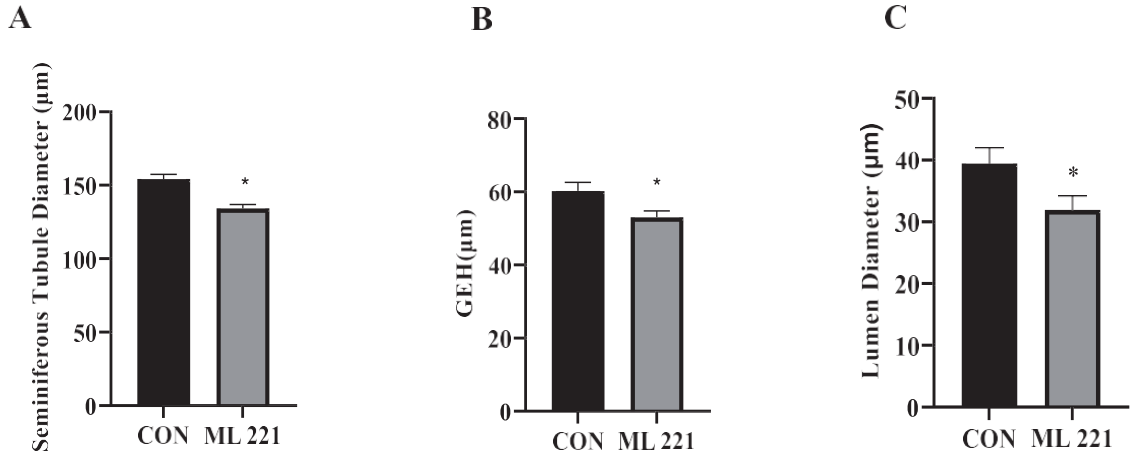

Fig. 5. Change in testicular histomorphometric parameters after apelin receptor antagonist treatment. APJ antagonist, ML221 significantly decreased seminiferous tubule diameter (Fig. 5A), germinal epithelium height (Fig. 5B) and luminal diameter (Fig. 5C).Data are expressed as mean±SEM. (*, $p < 0.05$ vs CON).

3.3. Effects of APJ inhibition by ML221 on body weight, testis weight and gonad somatic index (GSI)

In order to unravel the role of apelin on testis during early postnatal stages, APJ antagonist, (ML221) was given from PND14-PND20. The treatment of ML221 did not show any significant change in body weight, testis weight and GSI (Fig. 4 R2A-C).

3.4. Effects of APJ inhibition by ML221 on testis histology, seminiferous tubule diameter, germinal epithelium height and lumen diameter

The treatment of APJ antagonist, (ML221) during PND14-PND20 showed observable changes in the testis. The histological section of testis exhibited vacuole (V), sloughing of germ cell in the lumen (arrow) and darkly stained germ cells (*), which could be pyknotic cells (Fig. 4

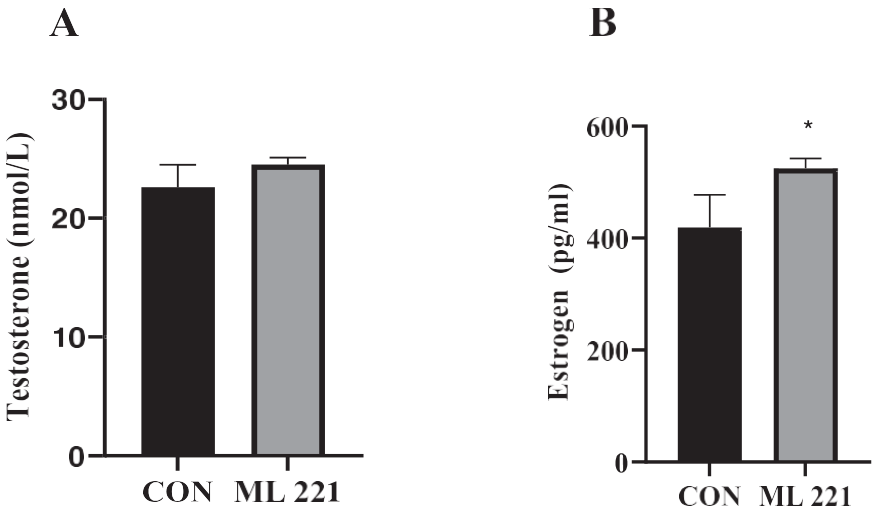

Fig. 6. Change in testosterone and estrogen secretion after apelin receptor antagonist treatment. APJ antagonist, ML221 significantly increased estrogen secretion; however, testosterone secretion did not change. Data are expressed as mean±SEM. (*, $p < 0.05$ vs CON).

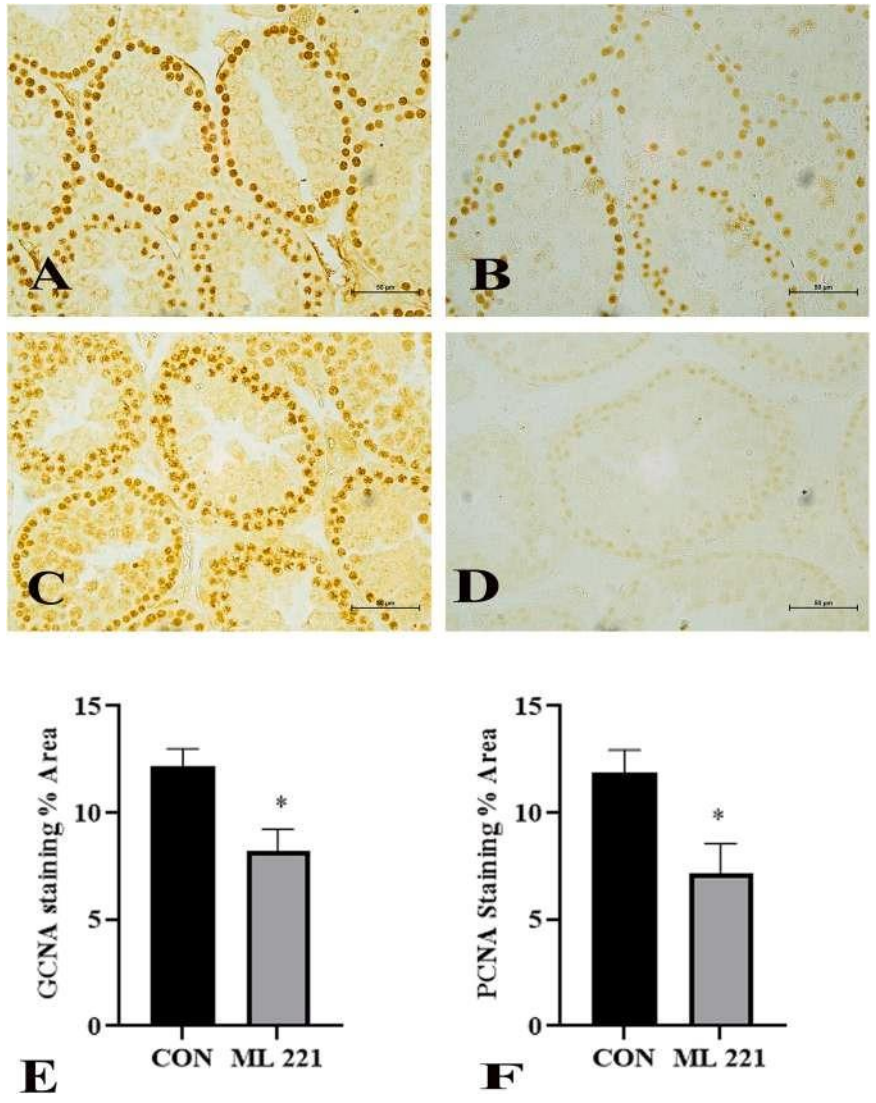

Fig. 7. Change in GCNA and PCNA expression after apelin receptor antagonist treatment. APJ antagonist, ML221 treatment showed mild immunostaining of PCNA in the testis (D), whereas GCNA showed faint immunostaining (B). The semi quantitative measurement of GCNA (E) and PCNA (F) stained area showed a significant decrease in ML221 treated group compared to the control. Data are expressed as mean±SEM. (*, $p < 0.05$ vs CON).

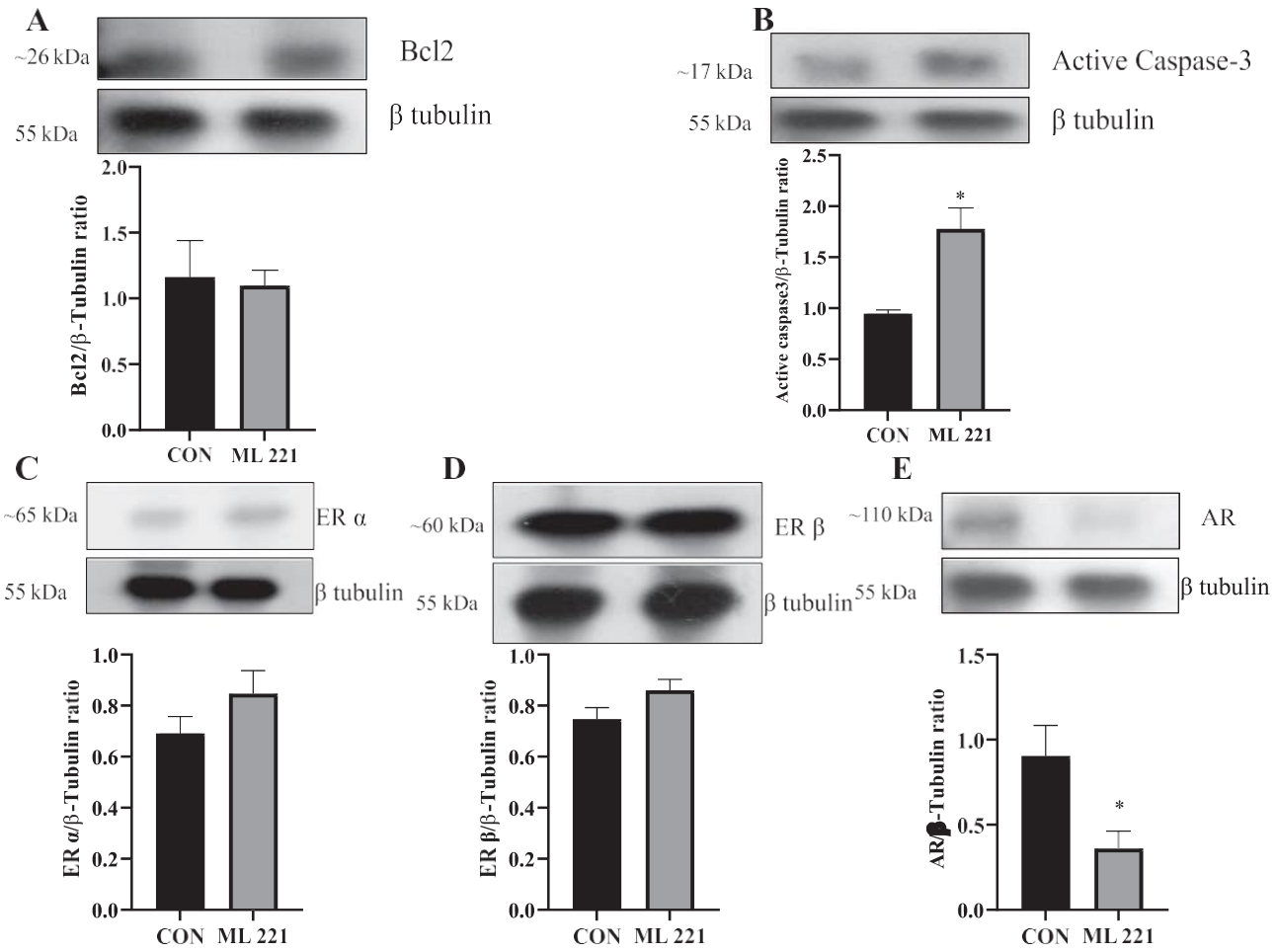

Fig. 8. Change in the expression of BCl2, active caspase3, ERα, ERβ and AR after apelin receptor antagonist treatment. Histogram represents densitometric analysis of blots. Expression of Bcl2, ERα, and ERβ did not show significant change. Expression of active caspase3 was significantly up-regulated (B) and expression of AR was significantly down-regulated (E) by APJ antagonist. Data are expressed as mean±SEM. (*, $p < 0.05$ vs CON).

R2D-E). The histomorphometrical measurements of seminiferous tubule diameter, germinal epithelium height and lumen diameter was significantly ($p < 0.05$) decreased in the ML221 treated group compared to the control (Fig. 5A-C).

3.5. *Effects of APJ inhibition by ML221 on testosterone and estrogen secretion*

Furthermore, to unravel the role of apelin on hormone secretion during early postnatal period, testosterone and estrogen were measured after ML221 treatment. The treatment of ML221 significantly ($p < 0.0$) increased the estrogen secretion without significant change in testosterone secretion (Fig. 6A-B).

3.6. *Effects of APJ inhibition by ML221 on testicular proliferation*

Since testis is diving organ, therefore, expression markers of two germ cell proliferations, GCNA and PCNA were performed by immunohistochemistry. Immunolocalization of GCNA exhibited abundance in the control testis, whereas in the testis of ML221 mice GCNA immunostaining was very faint. Similarly the abundance of PCNA was also observed in the control testis compared to the ML221 treated testis (Fig. 7A-D).

Semi-quantitative analysis of GCNA and PCNA by Image J also showed a significant ($p < 0.05$) decrease in the GCNA and PCNA percentage area in ML221 treated group compared to the control testis (Fig. 7E-F).

3.7. *Effects of APJ inhibition by ML221 on the expression of BCL2, active caspase3, ERα, ERβ and AR*

It is evident that testis undergoes apoptosis during early postnatal period, thus, marker of antiapoptosis, BCL2 and apoptosis, active caspase 3 was performed by Western blot analysis after ML221 treatment. The expression of BCl2 was unaffected after ML221 treatment, whereas, expression of active caspase3 was significantly ($p < 0.05$) up-regulated in ML221 group compared to the control group (Fig. 8A-B).

It is also well known that testosterone and estrogen regulates testicular activity, thus receptor for androgen, AR and receptors for estrogen ERα, ERβ were also analyzed by western blot analysis. ML221 treatment slightly elevated the expression of ERα, ERβ; moreover, this increase was not statistically significant (Fig. 8C-D). On the other hand, expression of AR was significantly decreased in ML221 group compared to the control (Fig. 8E).

3.8. *Effects of APJ inhibition by ML221 on the Akt phosphorylation Ser473 (p-Akt S473)*

It has been shown that apelin exhibit biological effect via Akt phosphorylation; therefore, Akt phosphorylation Ser473 was measured by Western blot analysis after ML221 treatment. The APJ, antagonist, ML221 significantly ($p < 0.05$) decreases Akt phosphorylation compared to the control (Fig. 9).

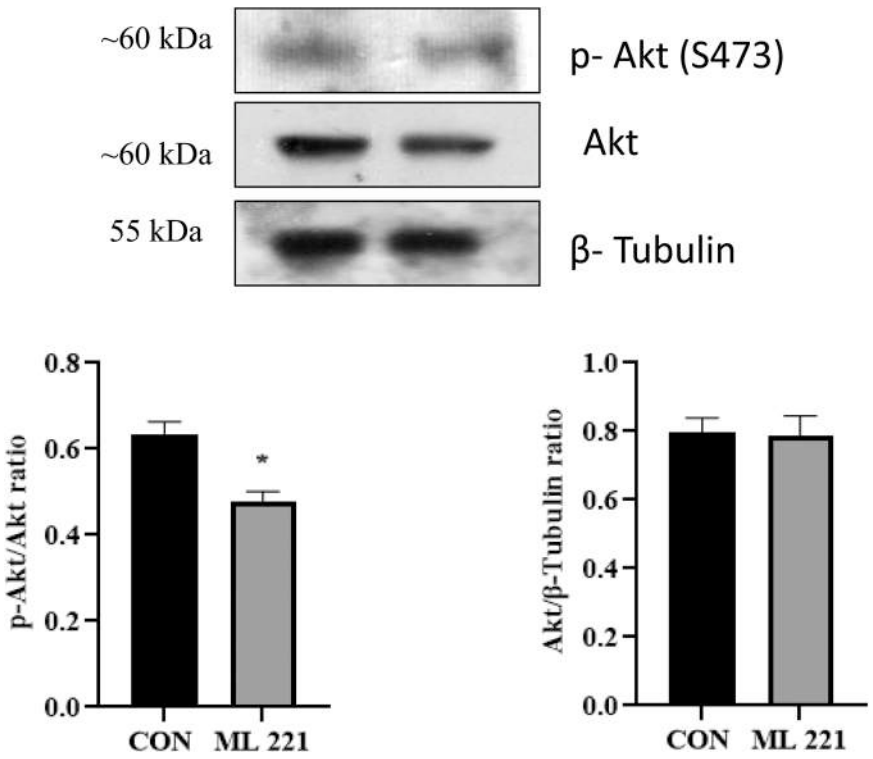

Fig. 9. Change in the expression of Akt phosphorylation Ser473 (p-Akt S473) after apelin receptor antagonist treatment. ML221 treatment significantly decrease the abundance of p-Akt S473 compared to the control. Data are expressed as mean±SEM. (*, $p < 0.05$ vs CON).

3.9. Effects of APJ inhibition by ML221 on the testosterone, estrogen secretion and on the AR, ERα and ERβ: An in vitro study

In order to strengthen the findings of in vivo study, in vitro study was conducted on PND 18 testis. The treatment of ML221 to the testis fragment, showed no significant change in testosterone and estrogen secretion. Expression of ERs showed a mild elevation in ML221 treated groups compared to the control, however, it was not significant. Expression of AR was significantly ($p < 0.05$) down-regulated after ML221 treatment compared to the control (Fig. 10A-E).

4. Discussion

The present study has undertaken to analyze the expression and distribution of apelin and its receptor in the testis of mice during postnatal developmental periods. Apelin is one of the important cytokine, which is expressed in the various organs such as brain, ovaries, kidney, pancreas, heart and breast [36]. The expression of apelin and its receptor has also been shown in the testis of mice and rats [30,34,46]. Despite its expression in the different organs, including testis, and human Leydig cells, the expression and localization of apelin and apelin receptor has not been investigated in the testis during postnatal periods. To best of our knowledge, this is a first attempt to unravel the expression and distribution of apelin in the testis of mice during postnatal developmental stages. Our results showed that the circulating apelin was minimum at PND1 and levels increased again at PND42. As the our aim was also to analyze the expression of APJ in the testis, and western blot analysis showed that expression of APJ was significantly increased in the testis of PND21, and PND42 compared to the PND1–14. This finding suggests that apelin signaling would be important in the testis from PND14-PND42, most likely during first wave of spermatogenesis. Immunohistochemical study showed presence of APJ in the testis from PND1 to PND42, in the Leydig cells, as well as in germ cells at PND14 and PND42. The immunostaining of apelin was observed only in the Leydig cells of PND42, however, the circulating apelin levels was increased in other age groups compared to the PND1. The correlation study of testicular APJ and circulating apelin showed a positive correlation. Based on these results, it may be suggested that apelin receptor signaling would also be important for testicular growth as well as steroid biosynthesis. Whether, the circulating apelin or locally synthesized apelin could mediate APJ activity in the Leydig cells of postnatal testis, this is not clear from the present study. Similarly, the ligand for APJ on germ cells at PND 42, whether comes from circulation or locally synthesized source, needs to be investigated further. Since we do not have data on whether apelin can cross blood testis barrier or not, thus, emphasizing role of circulatory or local apelin would be very speculative.

In order to find out a possible relationship between apelin system, and the circulating the steroids hormones, the levels of testosterone, estrogen and androstenedione were also measured. Furthermore, the circulating the circulating testosterone, and androstenedione levels showed a significant positive correlation with expression of APJ. These observations provide an evidence for stimulatory role of apelin signaling in steroid biosynthesis during postnatal developmental stages. It has previously been reported that apelin may modulate the testosterone biosynthesis in male rats [48]. The short term (7 and 14 days) treatment of apelin to adult male rats, decreases the circulating testosterone levels [2,48]. It has been suggested that apelin might a negative impact on reproductive functions [47,48]. Our recent study has shown that apelin treatment increases testosterone biosynthesis from diabetic mice testis [34]. Since there is no report on developmental expression of apelin system in any mammalian species, and moreover, our study showed a progressive increase in the APJ expression from PND1 to PND42 in mice testis, therefore, more study is needed to explore the exact role of apelin system in the testicular physiology. It may be suggested that there may be developmental stage dependent role of apelin in the testis.

In order to unravel the role of apelin in early developmental stages, apelin receptor antagonist (ML221) treatment was given from PND14 to PND20. Since, we have observed a sharp increase in the expression of APJ in the testis from PND14-PND21, therefore, the treatment of ML221

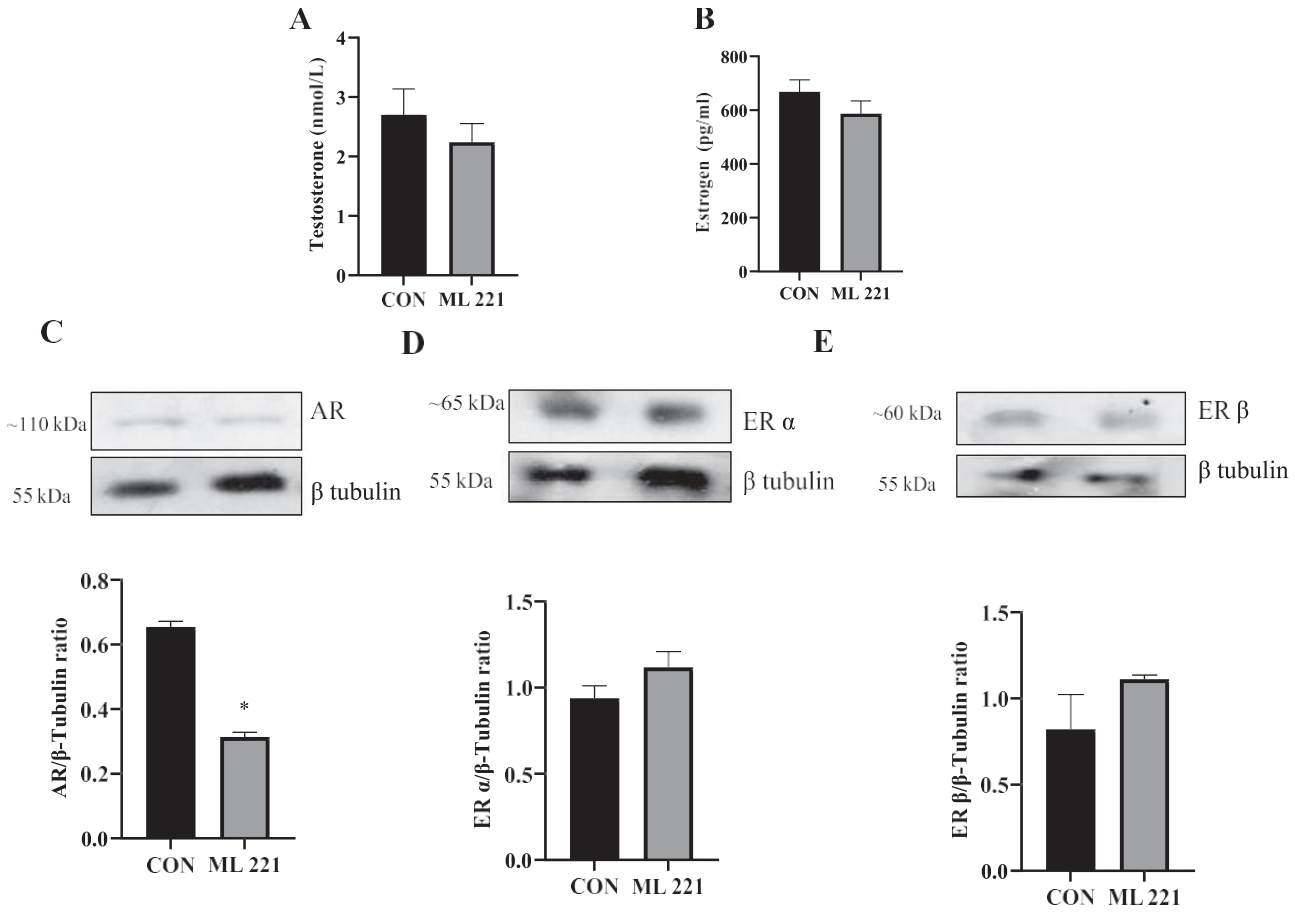

Fig. 10. Changes in the testosterone, estrogen secretion and AR, ERα and ERβ expression after apelin receptor antagonist treatment. The in vitro treatment of ML221 did not affect testosterone and estrogen secretion. Expression of AR was significantly down-regulated by ML221 and expression of ERs did not significant change. Data are expressed as mean±SEM. (*, $p < 0.05$ vs CON).

was given during this period. Our findings showed that the treatment of APJ antagonist, ML221 had no effect on the testis and body weight. Interestingly, seminiferous tubule diameter, germinal epithelium height and lumen diameter was in decreased by ML221 treatment. These observations suggest a possible role of apelin signaling in the testis of early developmental stages. To best of our knowledge, this is the first report on role of apelin in testis during early developmental stages. It has been shown that the first wave of spermatogenesis starts in murine testis from a few days later from birth [2]. It has also been shown that pachytene spermatocytes appear around at PND14, round spermatids appear at PND20 ND sperm appears at PND35 [23]. Since the first wave of spermatogenesis includes germ cell proliferation, and apoptosis in the rodent testis [15,24]. We have accessed the proliferation in the testis ML221 treatment by immunohistochemistry of two proliferating markers GCNA and PCNA. Our finding revealed that inhibition of apelin signaling by ML221, decreased germ cell proliferation. In other words, apelin seems to play important role in the germ cell proliferation during first wave of spermatogenesis. The direct involvement of apelin in germ cell proliferation has not been investigated. However, it has been shown that apelin is an angiogenic factor, which induces endothelial cell proliferation [37]. The apelin agonist, apelin-13 peptide has been shown to stimulate proliferation in breast cancer line [52]. Hypoxia induced apelin expression has also been shown to regulate endothelial cell proliferation [33]. Thus, exact mechanism is not very clear from the present study, how apelin regulates germ cell proliferation. Furthermore, ML221 treatment decrease the abundance of p-Akt, which suggests that apelin could be involvement in signaling from PND14-PND20. Previous study has also shown that apelin stimulates muscle cell proliferation via Akt signaling [8] and also inhibits apoptosis via Akt pathway [18].

Since the proliferation and apoptosis are simultaneous processes, therefore, expression of antiapoptotic protein, Bcl2 and pro-apoptotic protein, active caspase3 was analyzed after ML221 treatment. The expression of Bcl2 was not changed; however, expression of active caspas3 was up-regulated in the ML221 treated testis. These results suggest that apelin receptor antagonist increase apoptosis and decrease germ cell proliferation in the testis of early developmental stages. In other words, apelin increases the proliferation and decreases apoptosis in the testis of early developmental stages. Furthermore, it may be hypothesized that apelin mediated stimulated proliferation and decreased apoptosis might have a regulatory role for quality control of spermatogenesis. It has been previously suggested that early loss of germ cells in the rodent testis is requisite for establishment of functional spermatogenesis [2,15]. It has also been shown that testis early developmental stages secrets androgen by fetal Leydig cells up to PND25 [18,38]. Thus, question arises whether apelin can regulates steroidogenesis in testis of early developmental stages, therefore, the levels of circulating of testosterone and estrogen was measured after ML221 treatment. Our results showed that treatment of apelin receptor antagonist increased the circulating estrogen levels without change in circulating testosterone levels. In order to gain further role apelin on testicular steroidogenesis, the explants of testis was cultured with ML221. The in vitro treatment of ML221 did not show effect on testosterone and estrogen secretion[39].

It is well known that testosterone and estrogen acts via androgen and estrogen receptors. The expression of AR has been detected in the mice testis first at age of PND14 in spermatogonia, Sertoli cells, and myoid

cells, whereas as Leydig cells express AR at PND21 [51]. In human testis also, ontogeny of AR is similar to rodents and plays important role in Sertoli cells maturation along with initiation of spermatogenesis at adult [17,29,41]. Similarly, it has been shown that expression of ER in the mice testis is developmentally regulated [4,12]. Therefore, we have analyzed the expression of AR, ERα and ERβ in mice testis after in vivo treatment of apelin receptor antagonist. Our results showed that ML221 treatment down-regulated the AR expression in the testis, whereas, expression of ERs was unchanged. To gain further insight on expression of these receptors after ML221 treatment, in vitro was performed. The in vitro study also showed that treatment of apelin receptor antagonist, ML221 down-regulated expression of AR and expression of ERs were unchanged. These results of in vivo and in vitro study showed that apelin directly regulates the testicular AR expression. However, exact mechanism of apelin mediated regulation of AR expression needs further study. It may also be suggested that apelin mediated AR might be important to regulates the proliferation and apoptosis in early stages of testis. The significance of these events with respect adult spermatogenesis required further study.

In conclusion, this is first study on the developmental expression of apelin and apelin receptor in the testis. Furthermore, the present showed first time role of apelin on early stages of testicular developments. Our study unravel that apelin might promotes germ cell proliferation and suppresses apoptosis during first wave of spermatogenesis. The apelin mediated regulation of proliferation and apoptosis might be largely dependent on androgen signaling by AR.

CRediT authorship contribution statement

Vikas Kumar Roy, Milirani Das, Guruswami Gurusubramanian: Conceptualization and design of the study. **Milirani Das, Vikas Kumar Roy**: Execution of experimental procedures, Data collection. **Milirani Das, Vikas Kumar Roy, Guruswami Gurusubramanian**: Analysis of data, Discussion and manuscript preparation.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgments

The research facility to Department of Zoology, MZU from DST-FIST, New Delhi is greatly acknowledged.

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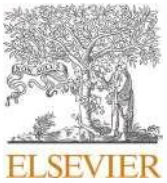

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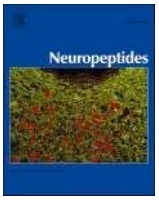

Apelin receptor antagonist (ML221) treatment has a stimulatory effect on the testicular proliferation, antioxidants system and steroidogenesis in adult mice

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ARTICLEINFO

Keywords:
Apelin
Proliferation
Apoptosis
Testis
Testosterone
ML221

ABSTRACT

The expression of apelin and its receptor (APJ) has been shown in the hypothalamus-pituitary-testicular axis. It has also been suggested apelin and APJ are neuropeptide factors. The presence of apelin and APJ in the seminiferous tubules and interstitium might be predicted to act as a local regulator of testicular activity, although the function is not well known in the mice testis. In the present study, we have investigated the effects of APJ, antagonist, ML221 on the gonadotropin levels, testicular steroidogenesis, proliferation, apoptosis and antioxidant system. Our results showed that inhibition of APJ by ML221 increased the sperm concentration, circulating testosterone, FSH, LH levels and intra-testicular testosterone concentration. Furthermore, ML221 treatment stimulates the germ cell proliferation and antioxidant system in the testis. The expression of BCL2, AR was up-regulated whereas, the expression of BAX and active caspase3 was down-regulated after ML221 treatment. Immunohistocehmical analysis of AR also showed increase abundance in the spermatogonia, primary spermatocytes and Leydig cells of 150 μg/kg dose group. These findings suggest that in adult testis, the apelin system might have an inhibitory role in germ cell proliferation and a stimulatory role in apoptosis. It might also be suggested that the apelin system could be involved in the disposal mechanism for damaged germ cells during spermatogenesis via the down-regulation of AR.

1. Introduction

The various regulatory factors for testicular activity in males have been reported, like gonadotropin releasing hormone (GnRH) from the hypothalamus, gonadotropin from the pituitary and steroid hormones, testosterone and estrogen by the testis itself. Despite this, regulatory control of testis by GnRH and gonadotropin from hypothalamus, and pituitary respectively, and testis also express a variety of cytokines such as leptin, adiponectin, resistin, visfatin and nesfatin, which regulates testicular functions in an autocrine and paracrine manner. Apelin is an endogenous ligand for a G-protein coupled receptor (APJ), which was first reported in bovine stomach extracts (Tatemoto et al., 1998). Apelin, which is made up of sections and fragments with varying numbers of amino acids (such as apelin-13, apelin-17, and apelin-36), is derived from a preproapelin with 77 amino acids (Chen et al., 2023; Zhou et al., 2021). Apelin mRNA and proteins have also been shown in the white adipose tissue and are called adipocytokines (Czarzasta et al., 2019).

It has been shown that apelin can be synthesized in the hypothalamus, pituitary, ovary and testis in several species (Kurowska et al., 2018). The presence of apelin in the hypothalamic area like the supraoptic and the paraventricular nuclei suggested it as a neuropeptide factor (De Mota et al., 2004). Not only apelin, but rather mRNA expression of APJ has also been shown in the hypothalamic area of rat brains (Czarzasta and Cudnoch-Jedrzejewska, 2014; De Mota et al., 2000; O'Carroll et al., 2013; Mohseni et al., 2021). It has been suggested that the presence of apelin and APJ in the hypothalamus, and anterior pituitary might have a regulatory role in the FSH and LH secretion from the anterior pituitary (De Mota et al., 2000; Sandal et al., 2015). The direct evidence of apelin-mediated LH and FSH release from the pituitary has been shown by Sandal et al. (2015), where an intracerebroventricular infusion of apelin-13 peptide was given and it was shown that apelin 13 decreased LH secretion. It has also been shown that intraperitoneal injection of apelin 13 decreased testosterone and gonadotropin and suggested that the apelin system might have an inhibitory role in the testicular activity (Tekin et al., 2017). It has also been shown that the apelin system gets elevated in diabetic-mediated

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https://doi.org/10.1016/j.npep.2023.102354
Received 4 April 2023; Received in revised form 4 June 2023; Accepted 18 June 2023
Available online 20 June 2023

testicular impairment in rodents and humans (Das et al., 2021; Song et al., 2022). The role of apelin in apoptosis, insulin secretion, oxidative stress and angiogenesis has been involved in the pathogenesis of diabetic complications (Li et al., 2022).

Recently, from our laboratory as well as from other laboratories, the presence of apelin and APJ have been shown in the testis of rat, mice, and dogs (Das et al., 2021; Das et al., 2022; Brzoskwinia et al., 2020; Troisi et al., 2022a, 2022b). Our recent study has shown that expression of testicular apelin and APJ is developmentally regulated in the mice testis and regulates the testicular steroidogenesis, proliferation and apoptosis in juvenile mice (Das et al., 2022). It should be noted that the presence of apelin and APJ in the testis in different compartments suggested some regulatory role of the apelin system in steroidogenesis and spermatogenesis. However, to the best of our knowledge, no study has been conducted to decipher the role of the endogenous apelin system in testicular activity. The previous studies (Sandal et al., 2015; Tekin et al., 2017) showed the effect of exogenous apelin-13 peptides on testicular activity, although the role of the endogenous apelin system in the testicular activity is yet to be investigated in the adult.

Therefore, the present study investigated the role of the endogenous apelin system on the testicular activity in adult mice by using the APJ antagonist, ML221.

2. Materials and methods

2.1. Animals

Adult male Swiss albino mice were used in this study. Mice were handled according to the protocol (MZUIAEC-9), approved by Mizoram University Institutional Animal Ethical Committee (MZUIAEC), Mizoram University, Mizoram, India and all animal experiments was complied with the ARRIVE guidelines. The mice were maintained in an aerated polypropylene cage under standard experimental conditions (12 h light-dark cycles, temperature 25 ± 2 °C) with ad libitum food and water.

2.2. Experimental design

This study investigated the role of apelin in spermatogenesis and steroidogenesis by blocking the apelin receptor (APJ) with the antagonist (ML221) according to the previous study (Hall et al., 2017). Mice were randomly divided into 3 groups, Group-1: Vehicle control (Con, n = 8), Group-2: ML221 15 μg/kg (ML-15, n = 8) and Group 3: ML221 150 μg/kg (ML-150, n=8). The APJ antagonist ML221 (cat # SML0919, Sigma Aldrich, St Louis, USA) was prepared by dissolving in DMSO and then diluted with double distilled water. The treatment was given for one spermatogenic cycle (35 days) intraperitoneally. The treated mice were received ML221 in 15 μg/kg body weight and 150 μg/kg body weight in 100 μl volume and equal volume of vehicle (DMSO diluted with distilled water) was injected intraperitoneally to the control mice for 35 days.

2.3. BrdU incorporation

To investigate the effect of ML221 on germ cell proliferation BrdU labelling was done. Before 12 h of the sacrifice, three mice from each group were intraperitoneally injected with BrdU (200 mg/kg) (Balu et al., 2009) dissolved in phosphate buffer saline (PBS).

2.4. Sample collection

After 24 h of the last treatment mice's body weight was measured and sacrificed, and the testes were weighed. The sperm parameters were performed immediately. The testes were collected and fixed in Bouin's solution for immunohistochemical studies and stored at −20 °C for western blot analysis. Serum was collected for the hormone assays.

2.5. Sperm parameters

The caudal epididymis was collected and minced in PBS with a fine scissor for the sperm analysis. In brief, the caudal portion of epididymis was dissected out and minced in 250 μl PBS (10 mM PBS, pH 7.4) maintained at 37 °C and a drop of it was immediately placed in clean slide covering with the cover slip and observed the sperm motility within 5min. The stock solution was further diluted in 200 μl PBS and a drop of it was observed in Neubauer chamber for analysis of sperm concentration as per the standard protocol of WHO laboratory manual (1999). Sperm motility and concentration was calculated by using the following formula.

$$\text{Motility (\%)} = (\text{Number of motile spermatozoa} / \text{Total number of spermatozoa}) \times 100$$

$$\text{Concentration of sperm} = \text{Average number of spermatozoa counted (N)} \times \text{multiplication factor (10000)} \times \text{dilution factor (20)}$$

2.6. Hormone assays

Circulating and intra-testicular testosterone, and estrogen was estimated by using the human testosterone (cat # EIA K209; Xema-Medica Co. Ltd., Moscow, Russia) and estrogen (cat # EIA K206; Xema-Medica Co. Ltd., Moscow, Russia) kit as per manufacturer instruction. Circulating LH and FSH were estimated using Mouse LH (cat # *E*-EL-M3053, Elabscience,) and Mouse FSH (cat #E-EL-M0511, Elabscience) ELISA kit.

2.7. Histology and histomorphological studies

Testis tissue embedded in paraffin and sectioned at 7 μM by following the protocol described earlier (Bancroft and Gamble, 2008). The sections were stained with Hematoxylin and Eosin stain by following the steps of deparaffinization, rehydration, staining, dehydration, clearing and finally DPX mount. Seminiferous tubule diameter and germinal epithelial height were measured and the seminiferous epithelial cycle stage was counted (Das et al., 2022) by using a microscope (E200, Nikon, Japan).

2.8. Immunohistochemical analysis

Immunolocalization of proliferation markers such as BrdU, GCNA, PCNA and steroidogenic marker 3β HSD and androgen receptor (AR) was performed following the protocol described earlier (Jeremy et al., 2019). Briefly, the sections were deparaffinized, rehydrated and blocked with goat serum (1:100, Lot# H2114; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 mins. Sections were incubated with primary antibodies GCNA (1:200, cat # 10D9G11; DSHB, University of Iowa, Iowa, USA), PCNA (1:100, cat # SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA, USA), 3β HSD conjugated with Horseradish Peroxidase (HRP) and AR (1:25, Ref # PA5–16363; Thermo Fisher Scientific, USA), and for BrdU the sections were treated with 2 N HCl at 37 °C and 0.1 M borate buffer after rehydration with grade ethanol and distilled water. Primary antibody of BrdU (1:50, cat # DSHB, University of Iowa, Iowa, USA) incubated at 4 °C overnight. The primary wash was done with PBS and for PCNA sections were incubated with goat anti-rabbit and for BrdU and GCNA goat anti-mouse HRP- Conjugated secondary antibody for 3 h. at room temperature. Then the slides were washed in PBS and developed by using 0.05% diaminobenzidine (DAB) solution (3,3′-diaminobenzidine tetrahydrochloride, in Tris-HCl buffer, pH 7.6 and hydrogen peroxide). After dehydration, the slides were mounted with DPX, observed and photographed using a Euromax microscope. 3β-HSD, BrdU, PCNA, GCNA and AR staining were measured, the stained area by DAB in the testis was acquired by using the threshold tool of ImageJ as described previously (Annie et al., 2019), and the data was presented as

a percentage area of staining.

2.9. Western blot analysis

Western blotting was performed in the testis sample by following the standard protocol of Jeremy et al., 2019. Briefly, 10% (*w*/*v*) tissue homogenate was prepared with lysis buffer, containing 0.01 M Tris-HCl (pH 7.6), 1.0 mM EDTA, (pH 8.0),0.1 M NaCl,100 μg/ml phenylmethylsulfonyl fluoride, and the protein concentrations were estimated by Bradford method (Bradford, 1976), The equal amount (50 μg) of protein from each group loaded to each well along with molecular weight marker in a 12% SDS-PAGE. The resolved proteins were transferred to the PVDF membrane using wet transfer apparatus for 12 h, after successful protein transfer the membrane was blocked for 30 min at room temperature with 5% non-fat skimmed milk prepared using PBST and then overnight incubated at 4 °C with the primary antibodies to the androgen receptor (1:250, Ref # PA5–16363; Thermo Fisher Scientific, USA), antiapoptotic marker BCL2 (1:2000, cat # SC7382; Santa Cruz Biotechnology Inc. Dallas, USA) and proapoptotic marker – active caspase-3 (1:1000, cat #; ElabSciences), BAX (1:100, cat #), NFκB (1:1000, cat #), estrogen receptor α (1:500, cat # P03372, DSHB, University of Iowa, Iowa, USA) and estrogen receptor β (1:500, cat # CWK-F12, DSHB, University of Iowa, Iowa, USA). Then the primary antibody was washed with PBST, and the membranes were incubated with the secondary antibody conjugated with horsera at room temperature for 3 h. After incubation, the membranes were washed and developed onto X-ray film by using the electrochemiluminescence (ECL) (cat # 1705060; BioRad, Hercules, CA, USA) method. The band intensities were quantified by using the ImageJ software (imagej.nih.gov/). The membranes were stripped and reprobed for β-Tubulin (1:4000, cat # *E*-AB-20033; Elabscience, Houston, Texas, USA) for loading control.

2.10. Oxidative stress and enzyme assays

2.10.1. Lipid peroxidation

The oxidative stress marker was measured by estimating malondialdehyde (MDA) levels in the testis by following the previously described method (Ohkawa et al., 1979; Aboul-Soud et al., 2011). The testis tissue was 10% homogenized in PBS. 15% trichloroacetic acid (TCA) and 0.375% thiobarbituric acid (TBA) were prepared in 0.25 N HCl for this experiment. The TCA and TBA solutions were added in a ratio of 1:1:1 with the tissue homogenate and the mixture solution was incubated at 37 °C for 15 mins, cooled and centrifuged at 5000 RPM for 10 min. Then the supernatant absorbance was read at the wavelength 532 nm by using a spectrophotometer (Eppendorf, BioSpectrophotometer). The MDA levels were expressed as nmol/mg of protein.

2.10.2. Catalase activity

The catalase enzyme assay was performed according to the previously described method (Hadwan, 2018). According to the method, 10% tissue homogenate was prepared and incubated with H_2O_2 for 2 min at 37 °C. After this, the reagent working solution (Cobalt (II) solution, sodium hexametaphosphate solution and sodium bicarbonate solution) was added and incubated at room temperature for 10 min in the dark chamber. The absorbance of colour intensity was read at 440 nm. The reagent mixture was taken as blank. Catalase activity was calculated by the following equation.

$$\text{Catalase activity of sample kU} = 2.303/\text{Time} \times \log(\text{standard absorbance}/\text{sample absorbance})$$

Where k = rate constant of a first-order reaction. The final concentration of catalase was expressed according to the protein concentration as kU/ mg protein.

2.10.3. Glutathione peroxidase (GPx)

GPx assay was done according to the previously described method (Nicy et al., 2022). The mixture containing 0.8 mM of EDTA, 10 mM sodium azide, 4 mM reduced GSH and 2.5 mM H_2O_2 of 20 μl each was mixed with 10μl of 10% tissue homogenate and water along with 40 μl of PBS. The solution was incubated at 37 °C for 10min and 50 μl 10% Trichloro acetic acid was added and centrifuged. From the supernatant, 50 μl was pipette out and to it, 300 μl of disodium hydrogen phosphate and 100 μg DNTB were added. The chromophore absorbance intensity was read at 412 nm. The mixture devoid of tissue homogenate was used as the standard. The concentration of GPx was expressed as μmol GSH oxidized/ min/mg protein.

GPx activity=(changes in Absorbance/time× GSH standard× Total reaction volume)/ (Standard absorbance×molecular weight of GSH (307.3235 g/mol) ×vol. of sample ×sample protein concentration in mg). GSH standard was prepared by dissolving 20 mg of GSH in 100 ml of distilled water (0.2 mg/ml).

2.11. Statistical analysis

Using GraphPad Prism9, all statistical analyses were performed and all numerical data were expressed as mean±*SEM*. To compare the data from different groups, a One-way Analysis of variance (ANOVA) followed by Tukey's test was used. The normal distributions of the data were analyzed by the Shapiro-Wilk normality test. The data were considered significant at $p < 0.05$.

3. Results

3.1. Changes in the body weight, testis weight, gonado-somatic index (GSI) and sperm count

Treatment of ML221 (APJ antagonist) with two doses (15 and 150 μg/kg) significantly decreased the percentage body weight increase ($p < 0.05$) in a dose-dependent manner (Fig. 1A). The testis weight did not show a significant change between control and ML221 treated groups (Fig. 1B). ML221 treatment at 150 μg/kg dose significantly ($p < 0.05$) increased GSI compared to the control and 15 μg/kg dose groups (Fig. 1C).

Sperm concentration also showed a significant increase in ML221 treated groups compared to control and exhibited a dose-dependent increase in sperm concentration by ML221 treatment (Fig. 1D).

3.2. Testis histology and morphometric analysis

To analyze the effect of APJ inhibition on the testis; histology and morphometric analysis were also performed. The histological sections of the control and ML221-treated mice testis did not show any gross observable changes (Fig. 2 AC). The morphometrical parameters, seminiferous tubule diameter and germinal epithelium height did not show any change (Fig. 2D, E). However, the number of VII/VIII stages showed a significant increase ($p < 0.05$) in 150 μg/kg ML221 group compared to the other groups (Fig. 2F).

3.3. Changes in the circulating testosterone, estrogen, LH, FSH levels and intra-testicular testosterone and the estrogen concentration

The circulating testosterone levels were found to be significantly ($p < 0.05$) elevated in the ML221 treated groups (15 and 150 μg/kg) compared to the control (Fig. 3A). However, circulating estrogen was found to be significantly ($p < 0.05$) decreased at 150 μg/kg dose compared to the control and 15 μg/kg groups (Fig. 3B). To find out the effect of APJ signaling on the pituitary function, we have measured the gonadotropin, LH and FSH levels. Both doses of ML221 (15 and 150 μg/kg) significantly ($P < 0.05$) increased the levels of LH compared to the control and also exhibited a dose-dependent increase ($P < 0.05$) in

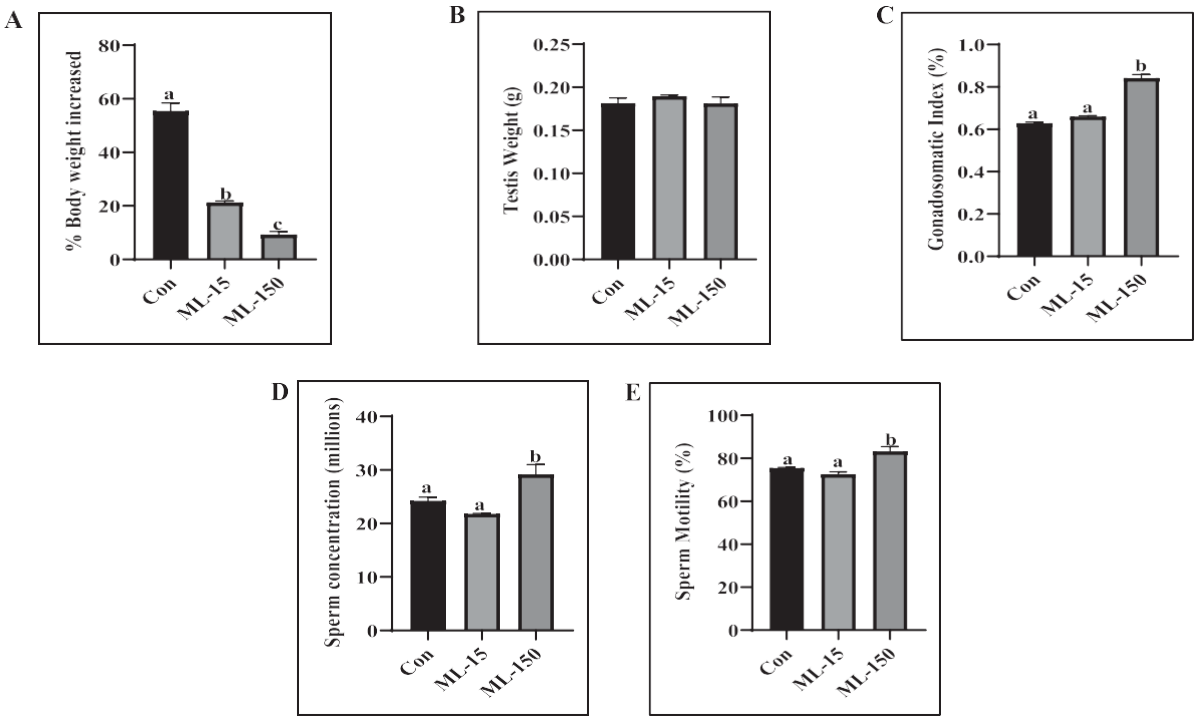

Fig. 1. Effect of ML221 on the body weight (A), testis weight (B), gonado-somatic index (GSI) (C) and sperm count (D).
Treatment of ML221 (APJ antagonist) with two doses (15 and 150 μg/kg), the percentage of body weight increased significantly decreased ($p < 0.05$) compared to the control and two doses of ML221 (A). The testis weight did not show a significant change between the control and ML221-treated groups (B). ML221 treatment at 150 μg/kg dose significantly ($p < 0.05$) increased GSI compared to the control and 15 μg/kg dose groups (C). Sperm concentration also showed a significant increase in ML221 treated groups compared to control (D). Data are expressed as mean ± SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 μg/kg (ML221)-ML-15, 150 μg/kg (ML221) -ML-150.

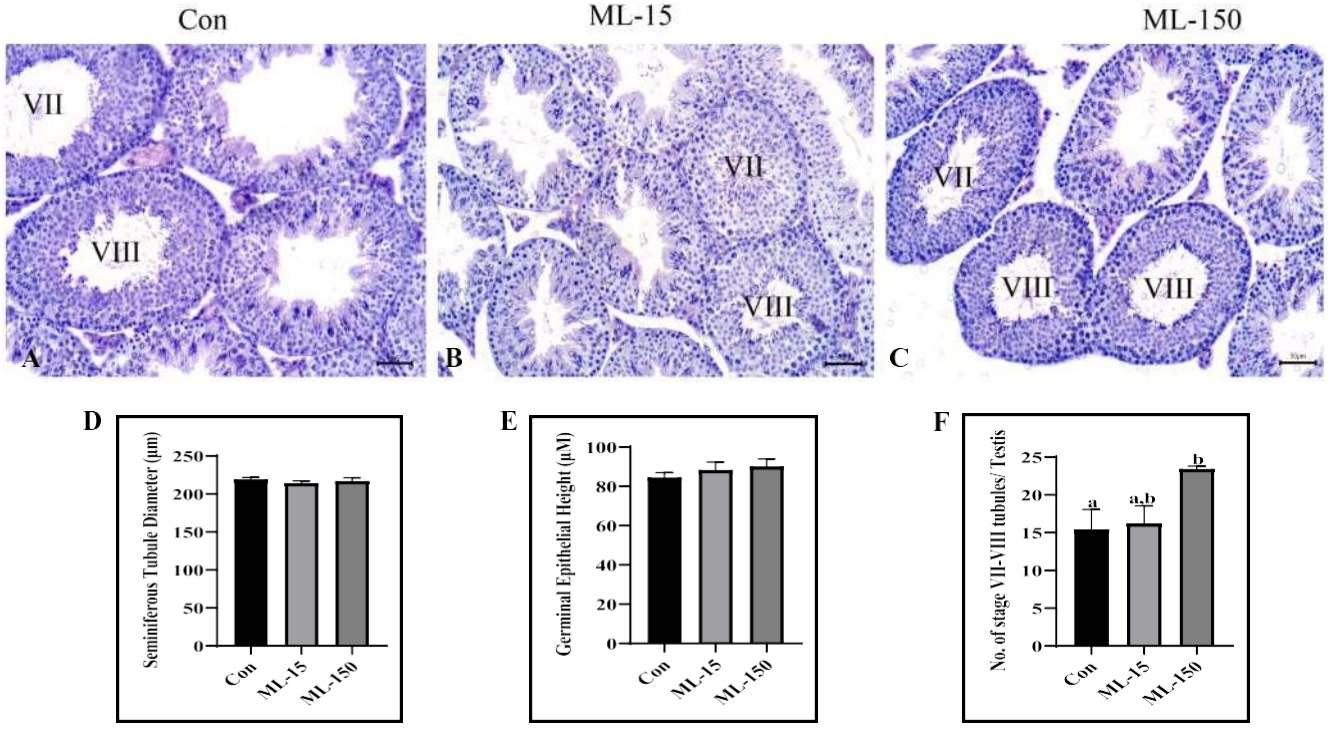

Fig. 2. Change in testicular histoarchitecture (A-C), histomorphometry and seminiferous epithelial cycle stage count.
Treatment of ML221 did not show any gross observable changes in histology (A-C). The seminiferous tubule diameter (D) and germinal epithelium (E) height did not show any change. ML221 treatment at 150 μg/kg dose significantly ($p < 0.05$) increased the no. of VII-VIII seminiferous epithelial cycle stage compared to control and 15 μg/kg dose groups (F). Data are expressed as mean ± SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 μg/kg (ML221)-ML-15, 150 μg/kg (ML221) -ML-150.

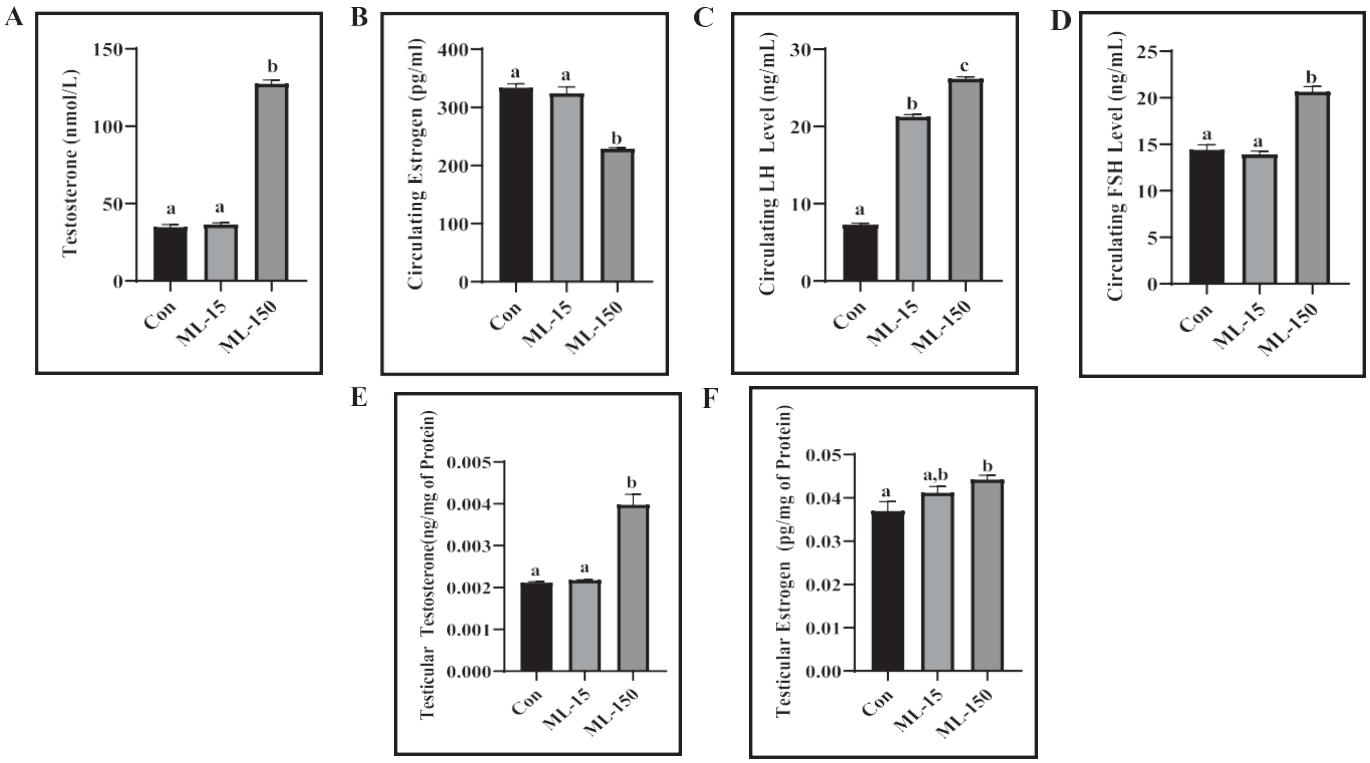

Fig. 3. Treatment of ML221 on circulating testosterone (A), estrogen (B), LH (C), FSH (D) levels and intra-testicular testosterone (C) and estrogen concentration (D). The circulating testosterone levels significantly ($p < 0.05$) elevated in the ML221 treated groups 15 and 150 μg/kg compared to the control (A). However, circulating estrogen was found to be significantly ($p < 0.05$) decreased at 150 μg/kg dose compared to the control and 15 μg/kg groups (B). Both doses of ML221 15 and 150 μg/kg significantly ($P < 0.05$) increased the levels of LH compared to the control groups(C). The levels of FSH were significantly ($p < 0.05$) elevated at the 150 μg/kg dose group compared to the control and 15 μg/kg groups (D). The intra-testicular testosterone concentration showed a significant increase in ML221 treated groups to the control (E). The intra-testicular estrogen concentration was significantly ($p < 0.05$) elevated at 150 μg/kg dose compared to the control and 15 μg/kg groups (F). Data are expressed as mean ± SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 μg/kg (ML221)-ML-15, 150 μg/kg (ML221) -ML-150.

ML221 groups (Fig. 3C). The levels of FSH were significantly elevated at 150 μg/kg dose group compared to the control and 15 μg/kg groups (Fig. 3D). The intra-testicular testosterone concentration showed a significant increase in ML221 treated groups to the control (Fig. 3E). The intra-testicular estrogen concentration was significantly ($p < 0.05$) elevated at 150 μg/kg dose compared to the control and 15 μg/kg groups (Fig. 3F).

3.4. Changes in the oxidative stress (MDA levels) and antioxidant enzymes, GPx and catalase

To unravel the relationship between the apelin system and oxidative stress in the adult testis, we have measured the marker of oxidative stress (malondialdehyde levels) and enzyme activity of two, antioxidant enzymes, GPx and catalase. The treatment of ML221 at both doses, (5 and 150 μg/kg), significantly decreased the MDA levels compared to the control (Fig. 4A). However, the enzymes activity of GPx and catalase

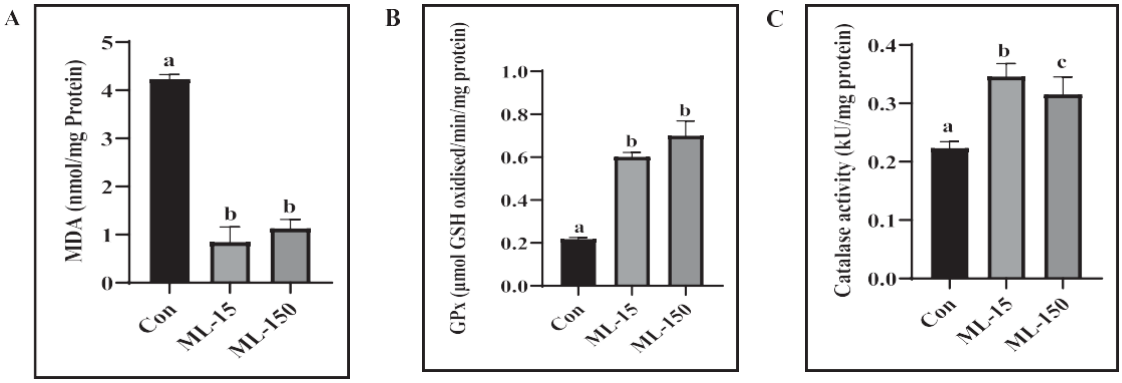

Fig. 4. Effect of ML221 on oxidative stress marker MDA levels (A) and antioxidant enzymes, GPx (B) and catalase (C).
The treatment of ML221 at both doses 15 and 150 μg/kg, significantly decreased the MDA levels compared to the control (A). However, the enzyme activity of GPx and catalase were significantly ($p < 0.05$) elevated at both the doses, 15 and 150 μg/kg of ML221 compared to the control (B, C). Data are expressed as mean ± SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 μg/kg (ML221)-ML-15, 150 μg/kg (ML221) -ML-150.

were significantly ($p < 0.05$) elevated at both the doses, (5 and 150 μg/kg) of ML221 compared to the control (Fig. 4B, C).

3.5. Immunolocalization of 3βHSD

To find out the direct role of the APJ system on the testicular steroid markers, we have accessed the abundance of 3βHSD by immunohistochemistry. The treatment of ML221 showed an increased abundance of 3βHSD proteins in the Leydig cells, whereas moderate staining of 3βHSD was also observed in the Leydig cells of the control testis (Fig. 5A-C). The staining areas were significantly ($p < 0.05$) higher in 150 μg/kg group than control and 15 μg/kg groups (Fig. 5D).

3.6. Changes in the BrdU labelling and PCNA and GCNA localization

Since the testis is proliferating organ and the apelin system has been shown to regulate cell proliferation, therefore, we have measured the markers of cell proliferation, BrdU, PCNA and GCNA. ML221 treatment showed more BrdU (Fig. 6A-C), PCNA (Fig. 6D-F) and GCNA (Fig. 6G-I) positive cells in the testis than control testis. The BrdU, PCNA and GCNA staining areas were significantly ($p < 0.05$) higher in 150 μg/kg dose compared to the control and 15 μg/kg groups (Fig. 6J-L).

3.7. Immunolocalization of AR after ML221 treatment

Immunohistochemistry of AR was performed in control and ML221 treated groups. The intense immunostaining was of AR found in the spermatogonia, primary spermatocytes and Leydig cells of 150 μg/kg dose group (Fig. 7A-C). The staining areas were significantly ($p < 0.05$) higher in 150 μg/kg dose group than control and ML-15 (Fig. 7D).

3.8. Changes in the expression of BCL2, BAX, active caspase3 and NF-κB

To unravel the role of APJ in testicular apoptosis, we have measured the expression of BCL2, BAX and active caspase 3. The expression of BCL2 was significantly ($p < 0.05$) up-regulated by ML221 at a dose of 150 μg/kg compared to the control and 15 μg/kg groups (Fig. 8A). The expression of active caspase 3, BAX and NF-κB were significantly ($p < 0.05$) down-regulated in 150 μg/kg ML treated groups compared to the control and 15 μg/kg groups (Fig. 8B-D).

3.9. Changes in the expression of AR, ERα and ERβ

To unravel the effect of APJ signaling on the steroid receptors' expression, we have measured the expression of AR, ERα and ERβ. Expression of AR was significantly ($p < 0.05$) up-regulated at both doses

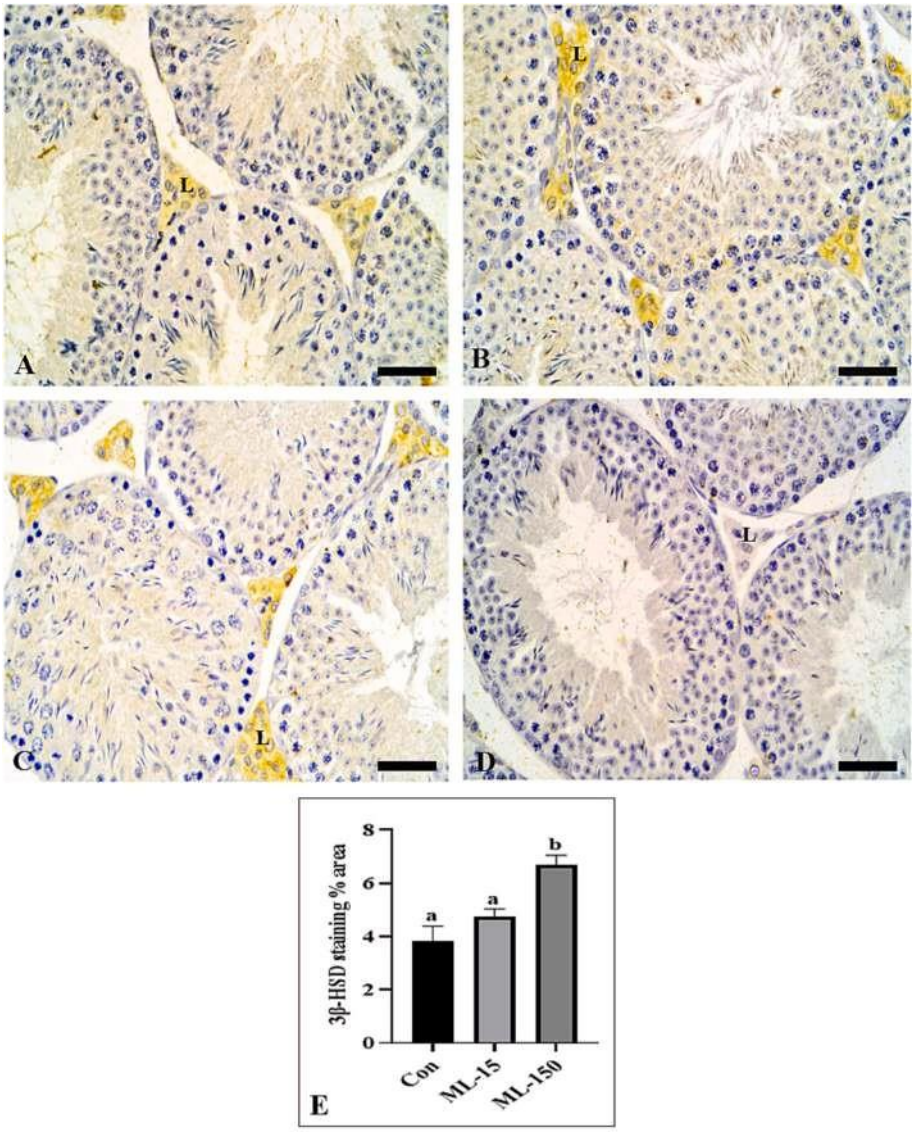

Fig. 5. Change in immunolocalization of 3βHSD after ML221 treatment.
The treatment of ML221 showed an increased abundance of 3βHSD proteins in the Leydig cells, whereas moderate staining of 3βHSD was also observed in the Leydig cells (L) of control testis (A-C). The staining areas were significantly ($p < 0.05$) higher in 150 μg/kg dose compared to the control and 15 μg/kg groups. Data are expressed as mean ± SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 μg/kg (ML221)-ML-15, 150 μg/kg (ML221) -ML-150.

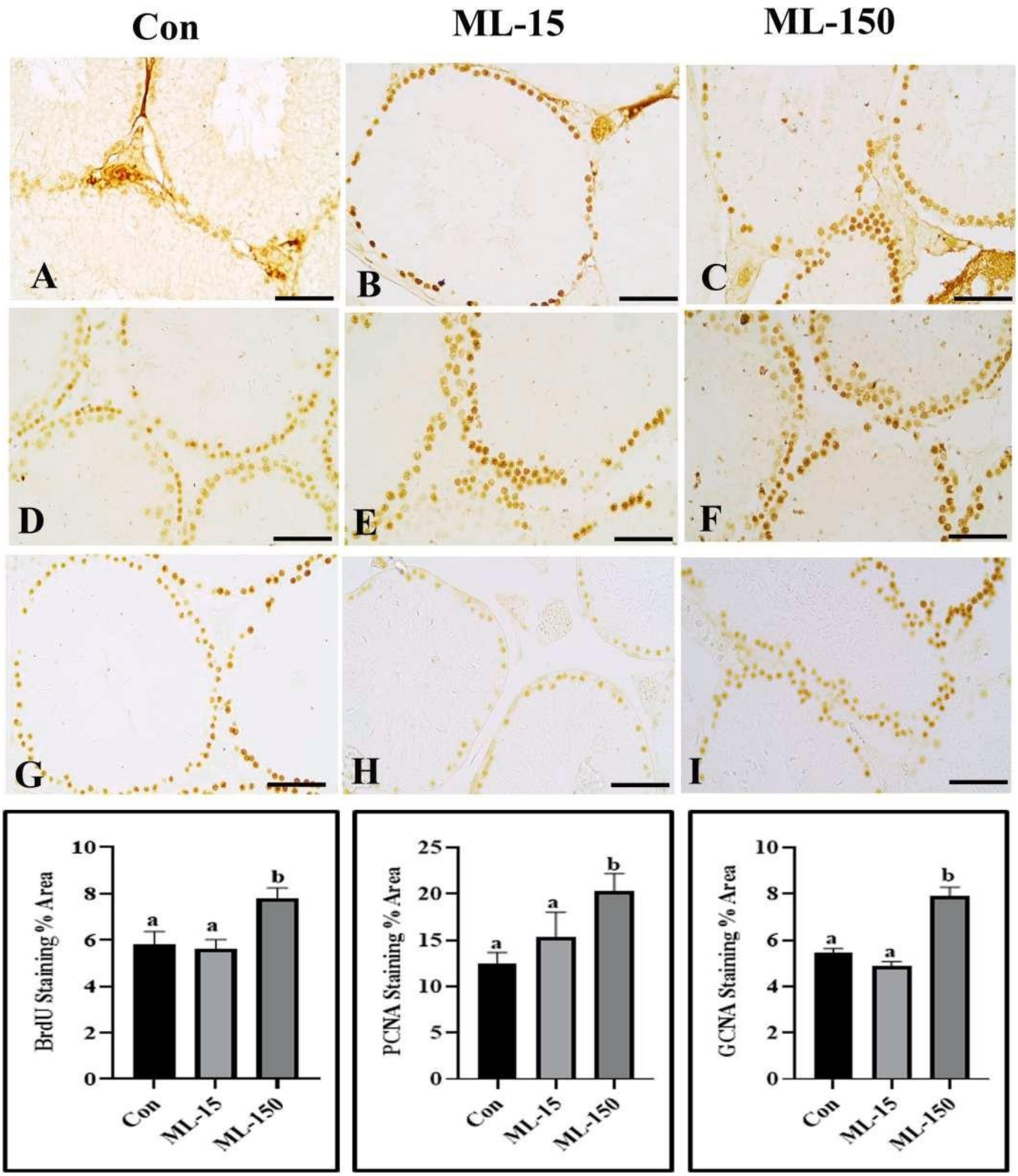

Fig. 6. Changes in the BrdU labelling and PCNA and GCNA by immunohistochemistry.
ML221 treatment showed more BrdU (A-C), PCNA (D–F) and GCNA (G-I) positive cells in the testis than control testis. The BrdU, PCNA and GCNA staining areas were significantly ($p < 0.05$) higher in 150 μg/kg dose compared to the control and 15 μg/kg groups (J-L). Data are expressed as mean ± SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 μg/kg (ML221)-ML-15, 150 μg/kg (ML221) -ML-150.

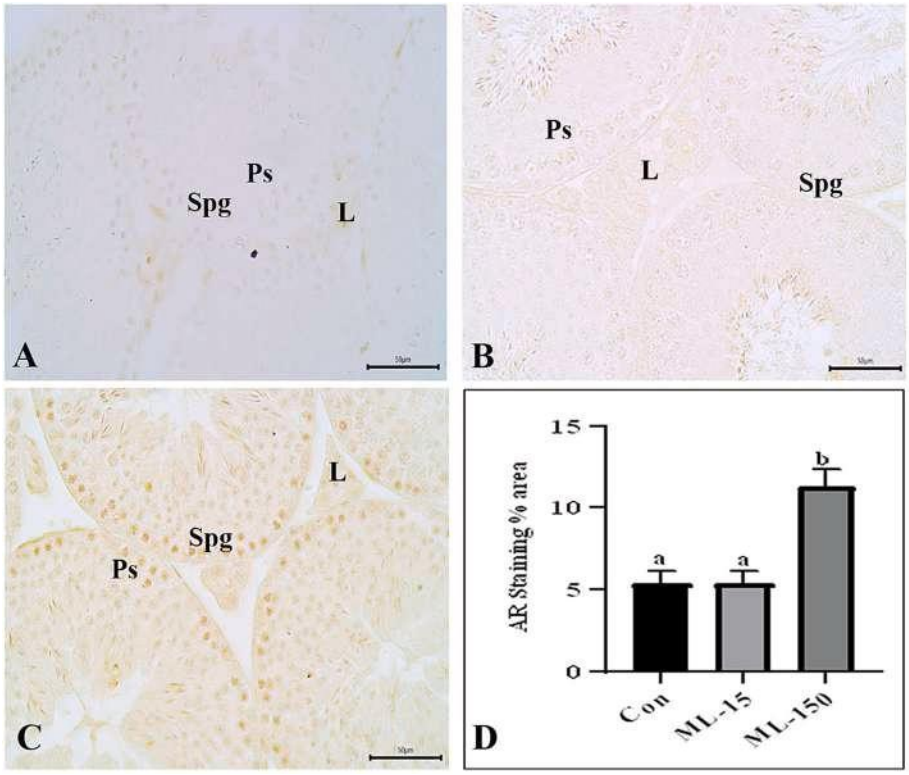

Fig. 7. Changes in the immunolocalization of AR after ML221 treatment.
Immunostaining of AR showed increase abundance in 150 μg/kg dose group than the other two groups (A-C). The staining areas were significantly ($p < 0.05$) higher in 150 μg/kg dose than control and 15 μg/kg groups (D). Data are expressed as mean ± SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 μg/kg (ML221)-ML-15, 150 μg/kg (ML221) -ML-150.
L: Leydig cell; Spg: Spermatogonia; Ps: Primary spermatocytes.

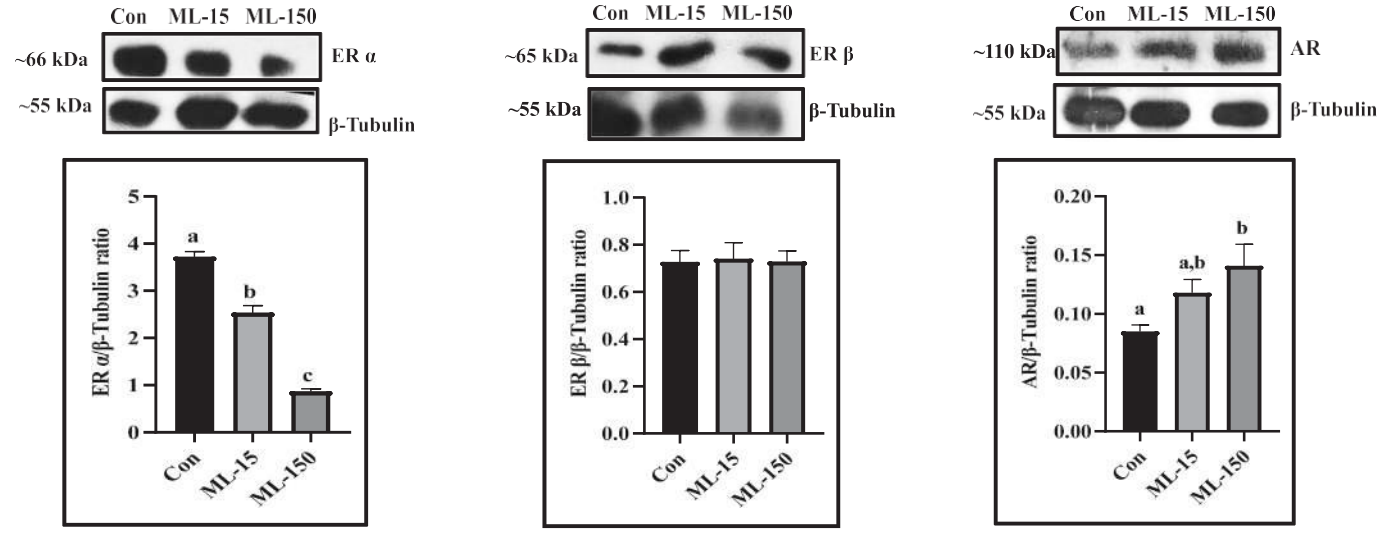

Fig. 8. Changes in the expression of BCL2, BAX, Active Caspase3 and NF-κB after ML221 treatment.
The expression of BCL2 was significantly ($p < 0.05$) up-regulated by ML221 at the dose of 150 μg/kg compared to the control and 15 μg/kg groups (A). The expression of active caspase 3, BAX and NF-κB were significantly ($p < 0.05$) down-regulated in 150 μg/kg ML221 treated groups compared to the control and 15 μg/kg groups (B–D). Data are expressed as mean ± SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 μg/kg (ML221)-ML-15, 150 μg/kg (ML221) -ML-150.

of ML221 (15 and 150 μg/kg) compared to the control (Fig. 9A). However, expression of ERα showed a significant ($p < 0.05$) decline in a dose-dependent manner (Fig. 9B). The expression of ERβ was unchanged in all the groups (Fig. 9C).

4. Discussion

The present study has investigated the effects of APJ antagonist (ML221) treatment on the various testicular. It has been well documented that ML221, a potent APJ antagonist, has widely been used to unravel the biological role of apelin (Hall et al., 2017). The present study is the first attempt to unravel the role of the endogenous apelin system on testicular activity by ML221 treatment on the testicular proliferation, apoptosis, antioxidant system and testicular steroidogenesis as well. It has been documented that testis expresses apelin and its receptor, APJ in the various cell types (Das et al., 2021, 2022), thus suggesting its possible role in the regulation of testicular functions. The treatment of ML221 decreased body weight in a dose-dependent

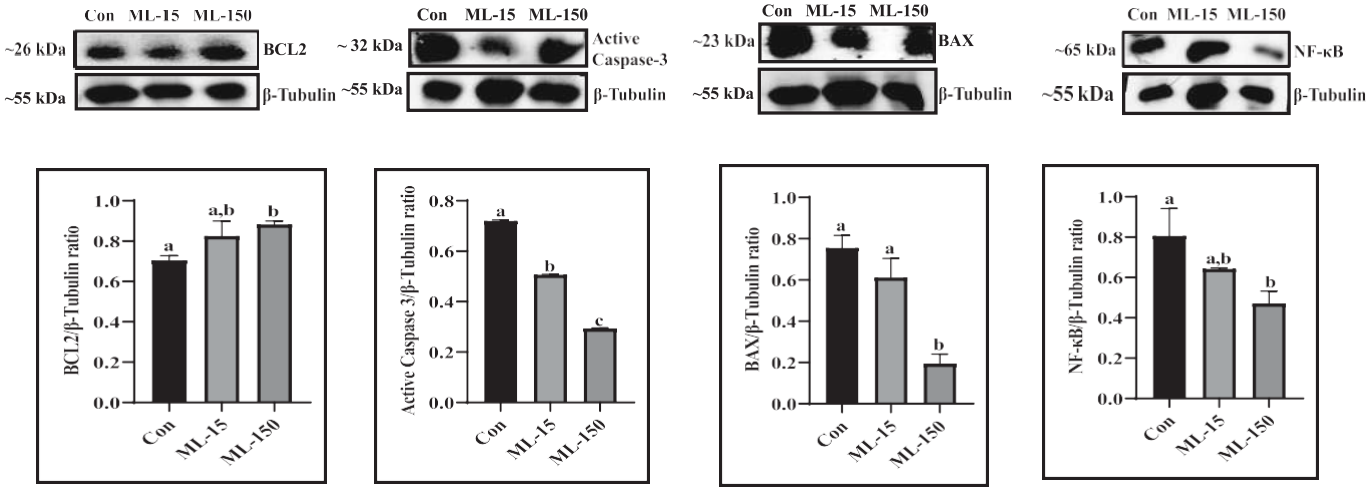

Fig. 9. Changes in the expression of AR, ERα and Erβ after ML221 treatment.
Expression of AR was significantly ($p < 0.05$) up-regulated at both doses of ML221 15 and 150 μg/kg compared to the control (A). However, expression of ERα showed a significant ($p < 0.05$) decline in a dose-dependent manner (B). The expression of ERβ was unchanged in all the groups (C). Data are expressed as mean ± SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups. Groups: Control-Con, 15 μg/kg (ML221)-ML-15, 150 μg/kg (ML221) -ML-150.

manner. This result suggests that inhibition of APJ might have decreased food consumption. Our results are in agreement with the previous report, which showed that apelin 13 peptide treatment increases food consumption and body weight in a rat model (Tekin et al., 2017). The presence of apelin and APJ in the hypothalamic nuclei have been suggested to regulate energy utilization and reproductive behaviour (Pope et al., 2012; Malagon and Vaudry, 2013).

Since we aimed to unravel the role of endogenous apelin on the testicular activity, therefore, we measured various parameters and it was found that testis weight was unaffected by the ML221 treatment, whereas GSI and sperm concentration showed a significant increase in the ML221 treated groups. This observation indicated that endogenous apelin might have an inhibitory role in testicular activity. Earlier studies by Sandal et al. (2015) and Tekin et al. (2017) have also shown that apelin 13 peptide treatments in the rat inhibit reproductive functions. It should be noted that these studies have accessed the role of apelin by infusing exogenous apelin 13 peptides; however, our study has shown the probable role of endogenous apelin on testicular activities. The inhibition of apelin action by ML221 increased the levels of circulating and intra-testicular testosterone and estrogen levels. However, circulating estrogen levels showed a decline. Thus, the inhibition of APJ by ML221 has a stimulatory role in testicular steroid biosynthesis. The testicular steroidogenesis and spermatogenesis are regulated by the pituitary gonadotropin, FSH and LH, and the levels of both FSH and LH increased by the ML221 treatment. These results suggest that apelin acts along the hypothalamic-pituitary-testicular axis and regulate testicular functions. Since we have not estimated the GnRH levels, thus this is the speculative point in the present study. Moreover, it was suggested that reduced testosterone levels by apelin 13 in rats were due to the inhibitory effects of apelin-13 on gonadotropin (LH and FSH) secretion from the anterior pituitary gland (Tekin et al., 2017). It has also been suggested that the hypothalamic-pituitary-gonad (HPG) axis expresses apelin/APJ and might act in an autocrine and/or paracrine manner in the brain, pituitary, ovaries, and testes (Kurowska et al., 2018). To the best of our knowledge, there are two published data which showed the effect of apelin on male reproduction (Tekin et al., 2017; Sandal et al., 2015). Moreover, the expression of apelin and APJ is well-known in the testis of mice, rats and dogs (Das et al., 2022; Brzoskwinia et al., 2020; Troisi et al., 2022a, 2022b). It has also been reported that apelin-mediated signals in the testis of rodents and humans might be deregulated in the pathological condition such as diabetes and inhibition of apelin signaling by ML221 improves the testicular activity (Das et al., 2021: Song et al., 2022). Our results also showed that the abundance of 3βHSD was elevated in the Leydig cells of ML221-treated testis. This finding further strengthens the idea of the local role of apelin signaling in testosterone biosynthesis. Previous studies have also shown that Leydig cells express APJ (Das et al., 2022; Brzoskwinia et al., 2020; Troisi et al., 2022a, 2022b).

The above-mentioned facts on the role of apelin in the testis hinted that apelin has a regulatory rather inhibitory role in hormone secretion. Thus, the question arises, if apelin has an inhibitory role on the testis, then it might also be regulating the germ cell proliferation in the testis. Our histological analysis did not show the observable changes in the testis; however, number of VII/VIII stages were more in the ML221 treated group along with increased sperm concentration at higher dose. These results suggest that ML221 might have some role in germ cell proliferation, thereby increases the sperm concentration. It has been shown that apelin/APJ is a regulator of cell proliferation, apoptosis, pro-inflammatory activity, and revascularization in the liver along with cell proliferation, and apoptosis in the gonads (Lv et al., 2017; Kurowska et al., 2018). Thus, we have accessed the germ cell proliferation by PCNA GCNA and BrdU labelling. Our results showed these markers of cell proliferation were up-regulated after ML221 treatment. These results suggest that apelin might be suppressing germ cell proliferation in the adult testis. To the best of our knowledge, there is no report on the direct role of apelin in testicular germ cell proliferation. Our recent study has shown that apelin stimulates germ cell proliferation and decreases apoptosis in juvenile mice testis (Das et al., 2022). It was also noted in the present study that ML221 treatment increased the expression of BCL2 and decreased the expression of BAX and active caspase 3, suggesting apelin-promoting apoptosis in the adult mice testis. The results of our recent (Das et al., 2022) and present study showed that apelin has an age-dependent role in testicular proliferation and apoptosis. Apelin has been shown to inhibit the proliferation in rat pulmonary arterial smooth muscle cells (Zhang et al., 2014). It has also been shown that the apelin/APJ system may inhibit or stimulate apoptosis under various conditions (Liu et al., 2017). Since the testis undergoes continuous cell proliferation and apoptosis, thus, it might be suggested that endogenous apelin signaling under normal conditions in the adult male testis could be responsible for the disposal of damaged germ cells. However, this statement is only indicative and requires further study to support this statement.

The balance of oxidative stress and the antioxidant system in the testis is critical for spermatogenesis and testicular functions (Aitken and

Roman, 2008a, 2008b; Asadi et al., 2017). It has been shown that oxidative stress and apelin/APJ are closely related (Zhou et al., 2016). Apelin can reduce oxidative stress in cardiomyocytes (Than et al., 2014) and also reduces the synthesis and release of ROS in adipocytes (Foussal et al., 2010). However, it has also been shown that apelin-13 can stimulate the production of ROS in vascular smooth muscle cells (Li et al., 2011). Our results also showed that treatment with ML221 increased the testicular antioxidant enzymes, catalase and GPx and decreases the oxidative stress. These findings suggest that apelin-mediated signaling might be stimulating testicular oxidative stress and apoptosis. We have also measured the expression of NF-κB after ML221 treatment and results showed that APJ inhibition by ML221 decreases the expression of NF-κB. The decreased expression of NF-κB coincides with decreased apoptosis and increased antioxidant and germ cell proliferation in the adult testis. It has been shown that NF-κB regulates germ cell apoptosis in the testis and is suggested to have a pro-apoptotic role (Pentikainen et al., 2002; Rasoulpour and Boekelheide, 2007). It has also been shown that expression of the apelin-13/APJ/NF-κB pathway increased in various tissues under the induction of oxidative stress and also suggested that increased APJ signaling and NF-κB expression could be responsible for increased oxidative stress (El Bakary et al., 2021).

The present study has also investigated the expression of AR, ERα and β after ML221 treatment. Inhibition of APJ increased the expression of AR and decreased the expression of ERα, however, ERβ was unaffected. Since both AR and ERs are important regulators of spermatogenesis, however, in the present study, it seems that apelin-mediated suppression of AR might be associated with increased apoptosis, oxidative stress, and suppressed germ cell proliferation in the adult testis. Our immunolocalization study showed that ML221 treatment increase the abundance of AR in the spermatogonia, primary spermatocytes and Leydig cells of 150 μg/kg dose group. These findings could suggest cell specific role of apelin in the testis. Our recent report showed that apelin signaling up-regulates the expression of AR in the juvenile mice testis. These findings provide evidence of the stage-dependent role of endogenous apelin signaling in testicular functions.

In conclusion, this is the first study on the role of endogenous apelin signaling in adult mice testis. Our study is the first attempt to unravel the role of the endogenous apelin system on steroidogenesis, proliferation, apoptosis and oxidative stress. Our study showed that apelin might suppress germ cell proliferation and increases apoptosis in adult testis. The apelin-mediated apoptosis could be a disposal mechanism for damaged germ cells and the overall role of apelin signaling in adult testis seems to be inhibitory via down-regulation of AR expression.

Credit authorship contribution statement

V.K.R., M.D., G.G. conceptualization and design of the study; M.D., V.K.R. data curation, M.D., V.K.R. formal analysis, M.D., V.K.R execution of experimental procedures, data collection; M.D., V.K.R., G.G. analysis of data, discussion and manuscript preparation.

Funding

No funding was obtained for this study.

Declaration of animal use

All the animal experiment was approved by Mizoram University Institutional Animal Ethical Committee (MZUIAEC-9), Mizoram University, Mizoram, India and all animal experiments was complied with the ARRIVE guidelines.

Declaration of Competing Interest

The authors verify that this work has not been published previously, is not under consideration for publication elsewhere, that it is approved by all authors, and that if accepted for publication, it will not be published elsewhere in the same form without written consent of the copyright-holder.

Data availability

Data will be made available on request.

Acknowledgements

The research facility to Department of Zoology, MZU from DST-FIST, New Delhi is greatly acknowledged.

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Analyzed document	Thesis 30.08.2023.pdf (D173243797)
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DEGREE **:** DOCTOR OF PHILOSOPHY

DEPARTMENT **:** ZOOLOGY

TITLE OF THE THESIS **:** Role of Apelin in spermatogenesis and steroidogenesis in the mice.

DATE OF ADMISSION : 01.08.2019

APPROVAL OF RESEARCH PROPOSAL:

DRC : 04.05.2020

B.O.S : 08.05.2020

SCHOOL BOARD : 12.05.2020

MZU REGISTRATION NO. : 1904353

REGISTRATION No. & DATE : MZU/Ph.D./1410 of 01.08.2019

EXTENSION : Nil

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Preface and Consolidated Abstract

The male reproductive capacity depends on the well-functioning of testis. The testis has been evolved to perform the steroidogenesis and spermatogenesis. There are various factors, from hypothalamus to testis, which are responsible for the well-functioning of testis. Despite the classical regulation of testicular functions along HPT axis, testis expresses various other factors which regulate the testicular function in auto and paracrine manner. In the line of various regulatory factors, adipokines such as leptin, visfatin, resistin, and adiponectin have been shown to regulate testicular functions. These adipokines control a number of biological processes and are mostly secreted by adipose tissue. Not only the adipose tissue, adipokines are also known to be expressed by the gonads and regulates gonadal function in autocrine and paracrine manner. Apelin is an also adipokines, which is expressed in the testis of human, hen, turkey and rodents. In 1998, the apelin and the *APLN* gene encoding preproapelin, a precursor of apelin, were discovered. Due to the site-specific proteolysis, the preproapelin is cleaved to the bioactive forms, such as apelin-36 (the 42–77 region of preproapelin), apelin-17 (61–77), apelin-13 (65–77) and pyroglutaminyl-apelin-13. The regulatory effects of apelin are realized due to its binding to the APJ receptor encoded by the *APLNR* gene. The expression of the APJ is found in the brain, ovaries, testes, kidney, pancreas, heart and breast, and is stimulated by insulin, estrogens, glucocorticoids, cAMP and some transcriptional factors. The pubmed search on expression of apelin and APJ showed that the developmental expression of apelin and APJ has not been investigated yet in the testis any mammalian species. It has also been shown that APJ mRNA expression appears to be higher in infant compared to adult peripheral tissues, thus question arises, what is role apelin in the pre-pubertal testis? Does it regulate steroidogenesis, and proliferation and apoptosis in the testis of pre-pubertal mice? Recently it has been shown that *apelin* is synthesized locally in the hypothalamus, pituitary, ovaries, and *testis* of many species and has autocrine and/or paracrine effects. As in previous study, direct role of apelin has not been investigated so far. Thus, question arises whether apelin impart any direct role in testicular activity and this is the research gap exists despite of previous one publication. Therefore, it is hypothesized that apelin may modulate testicular activity directly. Furthermore, leptin

and adiponectin are shown to be expressed in seminiferous epithelium in a stage dependent manner. Thus, it may also be hypothesized that localization of APJ would be stage dependent. Studies show that visfatin, leptin, and leptin receptor levels are reduced in testes of rats and mice with type 1 diabetes (T1DM), while leptin levels increase in T2DM rats. T1DM is linked to reduced adiponectin expression and restored testicular functioning. There is, however, little information on the expression of apelin and its receptor in diabetic testes, as well as the potential regulatory effects of apelin receptor ligands on testicular activities. We hypothesized that the expression and levels of apelin and APJ receptor may be deregulated in the testes of T1DM mice.

The study incorporated in this thesis is thus broadly divided into four chapters. **Chapter 1** describes postnatal changes in testicular apelin and apelin receptor and its role on the testicular steroidogenesis, proliferation and apoptosis. **Chapter 2** described detail role of testicular apelin signaling in the regulation of testicular steroidogenesis, proliferation and apoptosis by using APJ, antagonist ML221 in adult mice. **Chapter** 3 described the detailed expression pattern of APJ in the adult mice testis in stage dependent manner. **Chapter 4** describes the expression, and localization of apelin and APJ in the diabetic testis along with its role in diabetic-mediated testicular impairment.

Chapter-1: Postnatal developmental expression of apelin receptor proteins and its role in juvenile mice testis

The expression of apelin system has been shown in the adult testis of rat and mice. It has also been emphasized that regulation of testicular activity in early stages is important to sustain normal testicular activity in adulthood. Since the expression of apelin receptor (APJ) has been shown in the adult testis, moreover, developmental expression of APJ and its role has not been explored yet. Thus, we have examined the testicular expression of APJ during postnatal stages with special reference to proliferation, apoptosis and hormone secretion in early postnatal stage. Postnatal analysis showed that circulating apelin was lowest at PND1 and maximum at PND42. Among testosterone, estrogen and androstenedione, only circulating testosterone showed a gradual increase from PND1 to PND42. Testicular expression of APJ was also developmenatly regulated from PND1 to PND42, revealing a positive correlation

with circulating apelin, testosterone, and androstenedione. Immunohistochemical study showed that APJ was mainly confined to Leydig cells of early postnatal stages, whereas, seminiferous tubules at PND42 showed immunostaining in the round spermatids. APJ inhibition from PND14-PND20 by ML221 suppressed the testicular proliferation, increased apoptosis and increased estrogen secretion. However, expression of AR was down-regulated by ML221 treatment. Furthermore, ML221 decreased the abundance of p-Akt. In vitro study also showed that APJ antagonist, ML221 decreased AR expression. These results suggests that apelin signaling during early developmental stages might be required to stimulate the germ cell proliferation, and inhibition of apoptosis. Both in vivo and in vitro study have shown that expression of AR was regulated by apelin signaling. Since the first wave spermatogenesis involves proliferation and apoptosis, therefore, further study would be required to unravel the exact mechanism of apelin mediated regulation of testicular activity during early postnatal stages. In conclusion, the present results are an indicative of apelin mediated signaling during early postnatal stage for regulation of germ cell proliferation, apoptosis and AR expression.

Chapter-2: Apelin receptor antagonist (ML221) treatment has a stimulatory effect on the testicular proliferation, antioxidants system and steroidogenesis in adult mice

The expression of apelin and its receptor (APJ) has been shown in the testis and the apelin system also expresses in the hypothalamus-pituitary-testicular axis. It has also been suggested apelin and APJ as neuropeptide factors. The presence of apelin and APJ in the seminiferous tubules and interstitium might be predicted to act as a local regulator of testicular activity, although the function is not well known in the mice testis. In the present study, we have investigated the effects of APJ, antagonist, ML221 on the gonadotropin levels, testicular steroidogenesis, proliferation, apoptosis and antioxidant system. Our results showed that inhibition of APJ by ML221 increased the sperm concentration, circulating testosterone, FSH, LH levels and intra-testicular testosterone concentration. Furthermore, ML221 treatment stimulates the germ cell

proliferation and antioxidant system in the testis. The expression of Bcl2, AR was up-regulated whereas, the expression of Bax and active caspase3 was down-regulated after ML221 treatment. These findings suggests that in adult testis, the apelin system might have an inhibitory role in germ cell proliferation and a stimulatory role in apoptosis. It might also be suggested that the apelin system could be involved in the disposal mechanism for damaged germ cells during spermatogenesis via the down-regulation of AR.

Chapter-3: Immunolocalization of apelin receptor (APJ) in mouse seminiferous epithelium

The apelin receptor (APJ) belongs to the member of the G protein-coupled receptor family, and expression of APJ has been reported in the different cell type of testis. The seminiferous tubules in the testis can be identified as different stages (I-XII). It has been also suggested that different factors could be expressed in stage and cell specific manner in the seminiferous tubules. Recently, we also shown that expression of APJ is developmenatly regulated in the testis from PND1 to PND42. Therefore, we analysed the expression of APJ in the testis of adult mice by immunohistochemistry. Immunohistochemistry showed that the APJ was highly specific for the round and elongated spermatids with stage dependent changes. The seminiferous tubules at stages I-VII showed APJ immunostaining in the spermatid steps 1 to 8, not steps of 13-16. The seminiferous tubules at stages IX to XII showed APJ immunostaining in the spermatid steps 9 to 12. These results suggested the possible role of APJ in the spermiogenesis process. The intra-testicular administration of APJ antagonist, ML221 showed the a few round spermatids in the seminiferous tubules and some of tubules with complete absence of round spermatid. Overall, we present evidence that APJ expression in spermatid is dependent on the stages of seminiferous epithelium cycle and APJ could be involved in the differentiation of round spermatid to elongated spermatid.

Chapter-4: Expression and localization of apelin and its receptor in the testes of diabetic mice and its possible role in steroidogenesis

Type 1 diabetes mellitus (T1DM) is a metabolic disorder with severe hyperglycemia, one of the complications of which is testicular dysfunctions, androgen deficiency and decreased male fertility. In the diabetic testes, the expression and signaling pathways of leptin and a number of other adipokines are significantly changed. However, there is no information on the localization and expression of adipokine, apelin and its receptor (APJ) in the diabetic testes, although there is information on the involvement of apelin in the regulation of reproductive functions. The aim of this study was to investigate the expression and localization of apelin and APJ in the testes of mice with streptozotocin-induced T1DM and to estimate the effects of agonist (apelin-13) and antagonist (ML221) of APJ on the testosterone production by diabetic testis explants in the in vitro conditions. We first detected the expression of apelin and its receptor in the mouse testes, and showed an increased intratesticular expression of apelin and APJ along with the reduced testosterone secretion in T1DM. Using imunohistochemical approach, we showed that apelin and APJ are localized in the Leydig and germ cells, and in diabetes, the amount of these proteins was significantly higher than in the control mice. The diabetic testes had a decrease in germ cell proliferation (the reduced PCNA and GCNA levels) and an increase in apoptosis (the increased active caspase-3 and decreased BCL2 levels). These results suggest an involvement of apelin and APJ in T1DM-induced testicular pathogenesis. Treatment of the cultured testis explants with ML221 significantly increased the testosterone secretion, whereas apelin-13 was ineffective. Thus, hyperapelinemia in the testes can significantly contribute to testicular pathogenesis in T1DM, and pharmacological inhibition of apelin receptors can improve testicular steroidogenesis.