EXPRESSION AND ROLE OF VISFATIN IN THE TESTIS OF MICE

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EXPRESSION AND ROLE OF VISFATIN IN THE TESTIS OF MICE

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Submitted

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

CERTIFICATE

I certify that the thesis entitled **"Expression and role of visfatin in the testis of mice"** submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **Vanlalrempuia** is a record of research work carried out during the period of 2021- 2024 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

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I **VANLALREMPUIA**, 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 basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of **Doctor of Philosophy in Zoology.**

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(VANLALREMPUIA)

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Abbreviations

µm	micrometer
17βHSD	17β-Hyroxysteroid dehydrogenase
3βHSD	3β-Hyroxysteroid dehydrogenase
ANOVA	One-way analysis of Variance
AR	Androgen receptor
BCL2	B-cell lymphoma 2
BMI	Body mass index
BrdU	Bromodeoxyuridine
CYP11A1	Cytochrome P450
CYP19A1	Aromatase
DAB	Diaminobezidine
DPX	Dibutylphthalate Polystyrene Xylene
EAT	Epididymal adipose tissue
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
DHEA	Dehydroepiandrosterone
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FK866	Visfatin inhibitor
FSH	Follicle-stimulating hormone
g	centrifugal force
g	Gram

GEH	Germinal epithelium height
GCNA	Germ cell nuclear antigen
GnRH	Gonadotropin-releasing hormone
GnRHR	Gonadotropin-releasing hormone receptor
GSI	Gonadosomatic index
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-testicular
HRP	Horse-radish Peroxidase
IGF-1	Insulin-like Growth Factor-1
IgG	Immunoglobulin G
Kg	Kilogram
LβT2	LbetaT2 Mouse Pituitary Gonadotrope Cell Line
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LD	Lumen diameter
MCF-7	Michigan Cancer Foundation-7
mg	Milligram
ml	Milliliter
mM	Millimol
NAMPT	Nicotinamide phosphoribosyltransferase
NF-κB	Nuclear factor kappa B
ng	Nanogram
nM	Nanomol
PBS	Phosphate-buffered saline

PCNA	Proliferating cell nuclear antigen
PCOS	Polycystic ovarian syndrome
pg	Picogram
PMSF	Phenylmethylsulphonyl fluoride
PND	Postnatal development
PVDF	Polyvinylidene fluoride
rpm	Revolutions per minute
SAT	Subcutaneous adipose tissue
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
STD	Seminiferous tubule diameter
STAR	Steroidogenic acute regulatory protein
T2DM	Type 2 diabetes mellitus
VAT	Visceral adipose tissue
VP	Visfatin Peptide

General Introduction

Reproduction and fertility are highly regulated processes that requires the coordination of several hormones that interacting in unison and rhythmic manner. Long before birth, this regulation begins, and it continues throughout an individual life, even when they are no longer fertile **(Boehm et al., 2015; Clarke and Dhillo, 2016).** The regulation of the reproductive system is complex and adipose tissue metabolism have essential role in reproduction as there is a close link between metabolism and reproduction **(Trayhurn et al., 2006; Rosen and Spiegelman, 2006).** The adipose tissue is now considered not only as a storage for lipids but also an endocrine tissue for their secretion of mediators namely adipokines and these adipokines are known for their influence on glucose homeostasis, angiogenesis, energy metabolism and reproduction **(Wallace et al., 2020; Louveau et al., 2016; Pereira et al., 2020).** In fact, earlier studies have demonstrated that the majority of adipokines and their receptors are expressed in the testis, particularly in seminiferous tubes, and more precisely in Leydig and Sertoli cells as well as on spermatozoa **(Dupont et al., 2015)**. And the increased in adipokines secretion from white adipose tissue affects a number of functions linked to obesity, including reproduction **(Palin et al., 2012).**

Adipokines And Reproduction

In recent years, there has been a lot of interest in the complex relationship between human reproductive processes and adipokines **(Singh et al., 2018)**, and these adipokines have a functional role at every level of the reproductive axis, including the gonad and the hypothalamic-pituitary axis. The essential roles play by adipokines like leptin and adiponectin in various physiological processes, including reproduction, have already been covered in numerous reviews **(Messinis and Milingos, 1999; Tena-Sempere and Barreiro, 2002; Barbe et al., 2019; Kawwass el al., 2015)**. Furthermore, Chemerin, visfatin, resistin, and apelin are also adipokines that have also been found and acknowledged as significant regulator of energy metabolism, moreover earlier studies have emphasized their role in reproductive processes, whether in healthy or unhealthy functions **(Tsatsanis et al., 2015; Elfassy**

et al.,2018; Reverchon el al., 2014; campos et al., 2008). It has been demonstrated that these adipokines and their receptors are expressed in peripheral tissues as well as the reproductive tract, which includes the gonads and hypothalamus-pituitary in both male and female humans and other several species, suggesting their regulatory roles which in turn may be helpful for the explanation of certain reproductive problems **(Estienne et al., 2019)**.

Visfatin

In the present studies we focus on a novel adipokines namely visfatin also known as NAMPT (nicotinamide phosphoribosyltransferase), which was discovered by Fukuhara et al**. (2005)** and it presence have been observed in hypothalamic area and pituitary of mouse. Furthermore, visfatin is also found in LβT2 mouse gonadotrophin cells and have plays a vital role in downregulating the LH production by pituitary **(Maillard et al., 2017).** Therefore, it is hypothesized that visfatin could be essential regulator for reproductive function by acting on the hypothalamo-pituitary level.

Additionally, visfatin is also have a crucial in controlling angiogenesis, cellular proliferation, and apoptosis in mammalian cells **(Bowlby et al., 2012; Mohammadi et al., 2015)**. Visfatin downregulation inhibits tumour growth and encourages apoptosis in cancer cells. Consequently, certain visfatin inhibitors may be used as adjuvant treatment approaches **(Tan et al., 2013).** However, other reports showed that exogenous visfatin promotes the growth of prostate cancer cells **(Patel et al., 2010)**. In addition, research has demonstrated that injecting visfatin can boost food intake without having a major impact on blood glucose levels. Thus, visfatin may regulate appetite without directly affecting glucose metabolism, which suggests that it may interact with other hormones involved in hunger signalling **(Butler and Volkoff, 2023).** According to reports visfatin might be useful biomarker for identifying metabolic syndrome and related conditions, especially in adults who are overweight or obese **(Abdalla et al. 2022)**. Meanwhile, previous studies reported that visfatin mRNA and protein expression in pituitary gonadotroph cells along with heart and skeletal muscles, and might be the primary sources of visfatin in mice rather than the adipose tissues which further suggested that, physiological levels of

these hormones can impact mouse fertility by reducing gonadotroph cell LH production **(Maillard et al., 2017).** An in vitro investigation in chicken model has demonstrated that visfatin, either at the basal level or upon IGF-1 stimulation, suppresses the synthesis of progesterone in granulosa cells via STAR and 3βHSD **(Diot et al., 2015).** In contrast to the chicken model, visfatin promotes in vitro steroidogenesis and may have an influence on IGF-1 by raising the expression of STAR and 3βHSD and secreting more estrogen and progesterone **(Reverchon et al., 2016)**. Besides, Dawid et al. **(2024)** reported that visfatin could be a possible marker for the diagnosis of pregnancy problems. In addition, review reported suggested that visfatin functions could be a controlling factor in male and female reproductive function **(Shpakov et al., 2019).**

Visfatin And Female Reproductive Function

Several studies have shown that visfatin have role in controlling the female reproductive function and the presence of visfatin is found in the ovaries of human and animal model **(Estienne et al., 2015; Mlyczynska et al., 2022).** Its expression is also seen in the uterus of mice and during the reproductive cycle the expression varies, in fact, it seems that visfatin expression is regulated by estrogen and Progesterone, which would account for differences in the expression of visfatin during the reproductive cycle **(Annie et al., 2019).** In human, the expression of visfatin is also observed on Granulosa cells, cumulus cells, and oocytes although theca cells express it less frequently and recombinant visfatin enhances the synthesis of estrogen and Progesterone, in the presence of IGF-1 in human luteinized granulosa cells **(Reverchon et al., 2013)**. GnRH production from the hypothalamus and corpus luteum also seems to effect by the plasma level and expression of visfatin in porcine, suggesting that the animal's hormonal milieu, specifically during the oestrous cycle phase and the early stages of pregnancy, affects the visfatin level **(Kaminski et al., 2021; Mlyczynska et al., 2023)**. Furthermore, in immature pig’s ovaries the expression of visfatin was also high and immunolocalization of visfatin showed strong immunostaining in primordial follicles along with primary and secondary follicles, thus this suggests the possible role of visfatin from the embryonic stages in ovarian function **(Mlyczynska et al., 2023)**.

Visfatin is also showed to have an effective role in varies female reproductive pathologies, despite inconsistent evidence, reports from meta-analysis showed individuals with metabolic syndrome, cardiovascular disease, overweight/obesity, and insulin resistance have considerably higher plasma visfatin levels **(Chang et al., 2011).** In woman with PCOS, regardless of BMI the expression of visfatin in the adipose tissue was elevated **(Tan et al., 2006),** however, a positive correlation between plasma LH and circulating visfatin have been reports, which suggested that visfatin might also plays a crucial role in the dysregulation of the hypothalamo-pituitary-ovarian axis seen in PCOS **(Panidis et al., 2008).** Interestingly, Annie et al. **(2021)** showed that in vivo visfatin inhibition in PCOS induced mice have enhance the glucose metabolism while supressing the biosynthesis of androgen indicating that visfatin might have ability to improve PCOS associated pathogenesis. Nevertheless, reports from earlier studies showed that visfatin levels are directly correlated with polycystic ovarian syndrome, gestational diabetes, and fetal development in the uterus (**Sartori et al., 2016)**.

Visfatin and Male Reproduction

Visfatin presence is also seen in the testis of human and rodent species along with the testis of prepubescent and adult chicken, and its more expression have been observed in the Leydig cells and Sertoli cells **(Jeremy et., 2017; Riammer et al., 2016; Ocon-Grove et al., 2010)**, moreover, it was observed that this adipokines have possible effect on fertility of male, since higher levels of visfatin were linked to lower-quality sperm in rats with diabetes and obesity **(Abdel-Fadeil et al., 2019)**. Anagnostopoulou et al. **(2022)** showed sperm count and sperm concentration were significantly correlated negatively with plasma visfatin levels, however the plasma visfatin levels of fertile and infertile males did not reveal significant differences. Furthermore, its existence and regulation in the spermatozoa of human is shown to be a stages dependent **(Riammer et al., 2016).** In addition, the expression of this adipokines was observed to decline in the testis of ageing induces rodent model and significant decreased was also shown in the levels of testosterone and Leydig cells, suggesting that visfatin also have a vital role testicular ageing by modulating the steroidogenesis and spermatogenesis **(Jeremy et al., 2017)**.

Recent research of this adipokines in the diabetic and obese rats showed visfatin level was negatively corelated with quality of semen along with levels of LH and testosterone and positively associated with degenerative changes in testis, indicating that visfatin might have a potential role male infertility associated metabolic syndrome **(Dutta et al., 2021).** However, Sun et al. **(2007)** proposed that regulation of visfatin during pathological conditions in rodents and human might likely to be different. Furthermore, over the past decade a fair amount of research in the relationship between female reproductive function has been done, meanwhile research on various adipokines including visfatin on male side had been deficient **(Bhattacharya et al., 2020).** Therefore, exploring more knowledge on the role of visfatin in male reproduction is indeed necessary, so our research focus on visfatin relation with male reproductive function in male mice model. We have investigated the role of visfatin in the testis during the infantile stages and prepubertal stages along with the adult by inhibiting the function of visfatin, using a specific inhibitor Fk866.

Rationale of work

Visfatin is an adipokine which is predominantly produced and secreted by visceral fat and also known as nicotinamide phosphoribosyl transferase (NAMPT) **(Fukuhara et al., 2005; Diot et al., 2015)**. Recent studies have elucidated the role of visfatin in ovarian and testicular steroidogenesis **(Diot et al., 2015; Reverchon et al., 2016; Hameed et al., 2012)**. The expression of visfatin has been shown in the testes of rat, pig, mice, chicken and human **(Gurusubramanian and Roy 2014; Chen et al., 2007; Revello et al., 2007; Ocon-Grove et al., 2010; Thomas et al., 2013)**. Despite its role in gonadal steroidogenesis, visfatin has been shown to be involved in aging processes **(Lu et al., 2012)**. It has been suggested that visfatin has potential to slow down aging process by augmenting sirtuins activities (**Yang et al., 2006; Ho et al., 2009)** and also facilitates progress of aging **(Lu et al., 2012).** Further, it has been shown that visfatin administration improves quality of oocytes and fertility in aged female mice **(Choi et al., 2012)**. The functional significance of intra- and extracellular visfatin in human reproduction, however, has not been defined yet. It has been suggested that visfatin expression in human spermatozoa and

testes, depends on their maturation stage and visfatin has impact on sperm viability, motility, fertilization capacity and induction of apoptosis. Visfatin expresses in human spermatozoa in a maturation-dependent manner **(Riammer et al., 2016)**.

The pubmed search on **"Visfatin, Testis, and Postnatal"** showed that no item found. This showed the novelty of the present work. Furthermore, a search on "visfatin, testis, postnatal" showed two publications, and out two one was from our laboratory, which showed that dexamethasone down-regulates testicular visfatin in mice model **(Annie et al., 2019)** and other paper by **Adeghate (2008)**, which showed the first evidence of visfatin expression in the testis. Chemerin and chemerin receptors in rat testis showed their expression in developmentally regulated and highly expressed in Leydig cell **(Li et al., 2014)**. Leptin, expression was also shown developmentally regulated, imprinted by the neonatal endocrine milieu and sensitive to regulation by leptin and gonadotropins **(El-Hefnawy et al., 2002, Tena-Sempere et al., 2001)**. Resistin, which an adipokine is also in increased both basal and choriogonadotropin-stimulated testosterone secretion and their expression also changes during the different post-natal period in the testis **(Nogueiras et al., 2004)**. As it has also been shown that visfatin expression is sage dependent in chicken testis and mice ovary **(Ocon-Grove et al., 2010; Annie et al., 2020, 2021)**, thus question arises, whether the expression of visfatin in the mice testis is developmentally regulated. If expression of visfatin is developmentally regulated, then the following questions are pertinent.

1. What is role visfatin in the pre-pubertal testis in relation to proliferation and apoptosis and steroidogenesis?

2. Does it regulate steroidogenesis, proliferation and apoptosis in the testis of adult mice?

This research gap also prompted us to think that whether the expression of visfatin in the testis of mice is steroid hormone dependent, if so, then how does visfatin and androgen do the crosstalk?

Chapter 1

Evidence of the inhibitory role of visfatin in the testicular activity of mice during the infantile stage

Introduction

Visfatin is an adipokine secreted from the visceral adipose tissue and its expression has been observed in various tissues including the testis of chicken, rats, and mice (**Ocon-Grove et al., 2010; Gurusubramanian and Roy 2014; Annie et al., 2019)**. Mammalian testis development is a complex and sophisticated process, which may be required several regulatory factors to ensue normal spermatogenesis in the later stage of life, and various testicular markers are modulated in the postnatal developmental stages **(Kim et al., 2009; Hutson et al., 2013; Gong et al., 2013)**. It has been shown that testicular visfatin in the chicken testis has been linked to sexual maturation (**Ocon-Grove et al., 2010)**. Furthermore, earlier studies have demonstrated that the expression of ovarian visfatin significantly declined with the advance in the age of females, and visfatin co-administration at the time of superovulation upsurges developmental competence of oocytes which ovulated in a dose-dependent manner in the aged mice but not young mice **(Choi et al., 2012)**.

Thus, it is hypothesized that testicular visfatin expression may also be developmentally regulated. Although, it has been suggested that postnatal testicular development even differs between closely related species and is largely associated with testicular organization and progress of spermatogenesis, which establishes species differences **(Montoto et al., 2012)**. Hameed et al. **(2012)** have reported visfatin upsurges in testicular steroidogenesis in purified rat Leydig cells. Regardless of its role in gonadal steroidogenesis, it has been known to be implicated in the process of aging **(Liu et al., 2012)** and other disorders including diabetes, obesity, inflammation, and cancer **(Garten et al., 2011; Moschen et al., 2010; Busso et al., 2008; Bi et al., 2010)**.The expression of visfatin was also seen in the testis of humans and the spermatozoa shows a decrease in the level of visfatin in comparison to the immature spermatozoa, which suggested that visfatin could be a decent marker for impaired male fertility **(Riammer et al., 2016)**.

Despite having scant literature on the testis, visfatin has been shown that visfatin induces apoptosis of endothelial progenitor cells through the regulation of NF-κB and this pro- apoptotic effects can be inhibited by treatment with Fk866, an inhibitor

for visfatin **(Sun et al., 2017)**. Visfatin has also been shown to regulate the proliferation of human breast cancer cells and endometrial cancer cells **(Kim et al.., 2010; Wang et al., 2016)**. In the pancreatic cell line, visfatin has been shown to inhibit apoptosis and increased its proliferation as well **(Cheng et al., 2011)**. It has also been reported that cell proliferation and apoptosis in the postnatal testis are important processes **(França et al., 2000; Ruwanpura et al., 2008)**. To the best of our knowledge, postnatal changes in the testicular visfatin have not been investigated in any mammalian species. However, our laboratory has shown that mice's ovarian visfatin is developmentally regulated and plays a substantial role in ovarian proliferation and apoptosis during the infantile period **(Annie et al., 2021)**. It has been shown that during the first wave of spermatogenesis, germ cell apoptosis 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)**. It has been shown that the early phase of testicular activity requires a balance of cell death and survival **(Wright et al., 2007)**. Furthermore, the deregulation early phase of apoptosis and cell differentiation in the testis might result in impaired spermatogenesis in the later stage of life **(Coultas et al., 2005, Rodriguez et al., 1997, Russell et al., 2002; Wright et al., 2007)**. Moreover, the presence of testicular visfatin along with other adipokines such as leptin, resistin, and adiponectin, suggest a role of these adipokines as endogenous factors in the testis, thus, their possible in the early postnatal period, such as juvenile stage is important to study.

Therefore, the present study aimed to investigate the developmental expression of visfatin in the mice testis, and also to unravel the role of visfatin in relation to hormone secretion, proliferation, and apoptosis in the testis of infantile mice.

Materials and methods

Swiss albino mice were used for this experiment, mice were housed in conventional polypropylene ventilated cages which is maintained under the controlled condition of 12 hours dark and 12 hours light cycle at 25 ± 2°C, food and water were provided *ad libitum*. All the procedures were approved (process number: MZUIAEC 21-22-05) and carried out under the guidelines given by Mizoram University Institutional

Animal Ethical committee (MZUIAEC), Mizoram University, Mizoram, India. Animals were allowed to breed and pregnant mice were observed regularly for new-born pups and the male pups were collected on their postnatal days (n=10 per PND7 and for rest groups, n=5) of 7, 14, 21, 42 and 65 days. Animals were then given a mild anesthesia and sacrificed immediately, the testis and serum were collected for further analysis. The body weight and testis weight of each group were also observed.

in *In vivo* treatment of visfatin inhibitor

To examine the role of visfatin during infantile stage, FK866, a visfatin inhibitor, was used. FK866 was given i.p. at dose of 1.5mg/kg FK866 on PND 14 for 7 days **(Ohanna et al., 2018).** Animals were distributed into two groups (n=5 per group) Fk866 and control group, Fk866 group received intraperitoneal injection of 1.5mg/kg FK866 and in the same manner normal saline was given to the control group as a vehicle control. The weight of the body and testis were measured at the time of sacrificed. The dose and duration of FK866 was based on our previous study **(Annie et al., 2021)**.

Histopathology of testis

For histopathological studies, testis fixed with Bouin's fluid was used. The tissues were transferred to 70% ethanol before 24 hours to avoid brittleness, followed by a series of dehydration ion graded ethanol (90% and 100%) twice for one hour each. The tissues were then cleared in xylene for a minute and embedded in paraffin wax block. The tissues blocks were sectioned at 7 µm thickness with Leica microtome (model RM2125 RTS) and fixed in a sterile glass slide coated with poly-l-lysine, and then undergoes tissues processing and staining with hematoxylin and eosin (**Bancroft and Gamble, 2002)**. The sections were then observed under a microscope and photographed using camera-mounted binocular microscope (Model E200, Nikon, Tokyo, Japan).

Histomorphometry

The same histological sections were used to performed histomorphometrical studies, seminiferous tubule diameter (STD) **(Guneli et al., 2008)**, germinal epithelium height (GEH) **(Franca et al., 2003)** and lumen diameter (LD) was measured using ocular micrometer of 40X magnification. Thirty round tubular section were selected for measurement of LD, STD and GEH**.**

Immunohistochemistry

The Bouin's fixed testis sample was processed for immunohistochemical studies as described previously **(Jeremy et al., 2019)**. Tissue samples were dehydrated in graded ethanol in the order of increasing concentrations (90% and 100%) twice for 1 hour each, fully dehydrated samples were immersed in xylene and then infiltrated with molten paraffin. The tissues were sectioned using Leica rotary microtome (model RM2125 RTS) at 7 µm thick and then embedded in a clean glass slide. After the tissue slides undergo a dehydration process it was kept in PBS for 10 minutes and incubated with blocking buffer [goat serum 1:100 diluted in PBS (Lot# A0515, Santa Cruz Biotechnology, Inc., CA, USA)] for 30 minutes , then the slides were incubated overnight at 4°C in a humidified chamber using primary antibody against Visfatin (NAMPT)(1:100; rabbit polyclonal antibody, Cat# PA5-30940, Invitrogen, Waltham, Massachusetts, United States), Bcl2(1:100; mouse polyclonal antibody, Cat# sc-7382, Santa Cruz Biotechnology, Inc. Dallas, USA), GCNA(1:200; mouse polyclonal antibody, cat# 10D9G11, DSHB, University of Iowa, Dept of Biology, Iowa, United States), 17β HSD(1:400; rabbit polyclonal antibody, cat# STJ110000, St John's Laboratory Ltd, London, United Kingdom), Androgen receptor (1:100; rabbit polyclonal antibody, Cat# **PA5-16363,** Invitrogen, Waltham, Massachusetts, United States). After overnight incubation at 4°C, slides were washed in PBS and probed with Horse-radish Peroxidase (HRP) conjugated secondary IgG antibody at 1:400 dilution (goat anti-mouse, Cat# PI-2000 and goat anti-rabbit, Cat# PI-1000, Vector Laboratories, Burlingame, CA, United State) for 3 hours at room temperature. The unbound antibody was washed off by PBS and again incubated at room temperature in a solution containing 0.6 mg/ ml solution of 3, 3-diaminobenzidine

tetra hydrochloride dihydrate (DAB) in Tris–HCl (pH 7.6) and 0.01% H_2O_2 till brown colour developed. The slides were counter stained with hematoxylin except for GCNA and Androgen receptor and dehydration was done using different graded ethanol in increasing order of concentrations and then cleared in xylene. It was then mounted with DPX and the slides were examined and photographed using camera-mounted binocular microscope (Model E200, Nikon, Tokyo, Japan). GCNA staining area was calculated with ImageJ software (imagej.nih.gov.), DAB stained area for GCNA in testis was obtained by using threshold tool of ImageJ as previously described **(Jensen, 2013)** and the percentage area was represented. A total of five testis sections were used for both control and FK866 treated group which were photographed at 10x magnification for each testis, area mentioned above refers to the total image field covered with tissue under 10x magnifications excluding the non-image area.

Estimation of sex hormone levels (Testosterone and Estrogen)

The blood serum obtained from in vivo treatment of FK866 were assessed for testosterone and estrogen, quantification of total serum concentration was done by using commercial enzyme linked immunosorbent assay kit (Testosterone Cat# DKO002, DiaMetra, Italy; Estradiol Cat # DKO003, DiaMetra, Italy). The absorbance levels were taken at 450 nm using microplate ELISA reader (Erba Lisa Scan EM, Trans Asia Biomedical Ltd, Mumbai, Maharashtra, India).

Estimation of circulating and testicular visfatin levels

Testis homogenate from each developmental stages were centrifuged at 12,000*g* for 20 minutes, the supernatant was collected. The homogenate and serum were used for the determination of visfatin concentrations with Mouse Visfatin ELISA kit (Cat No: K02-0598; Los Angeles). 40 µl of the samples were loaded to the wells along with 10 µl Biotin conjugate, while 50 µl of standards were also loaded. Then, 50 µl of HPR conjugate was loaded to sample and standard wells but not in the blank, it was incubated in 37°C for 1 hour. It was then washed with 1x wash buffer provided with the kit followed by the addition of 50 µl each of substrate A and B including blank well, and then incubated in the dark at 37°C for 10 minutes. After incubation, stop

solution was added and absorbance was taken at 450 nm using ELISA reader. Testicular visfatin was expressed as ng/mg of protein.

Western blot analysis

Testis collected at the end of in vivo treatment were homogenate in a chilled suspension buffer containing 150 mM Sodium chloride (NaCl), 50 mM Tris-Hydrochloric acid (HCl), pH 8.0, 0.1 % SDS,1 g/mL Aprotinin, 1 mM (PMSF) and 1 mM (EDTA). Then, centrifuged at 4 °C at 12,000*g* for 15 minutes and supernatants were collected. Protein estimation was done by Bradford method **(Bradford, 1976)**, the samples were then denatured in SDS-sample buffer (62·5 mM Tris, 2% SDS, 10 % glycerol) by boiling for 10 minutes. 50 µg/well of each month were loaded in 10 % SDS-PAGE along with protein marker and the gel was electrophoresed at 100V for 3 hours. The resolved proteins were then transferred onto a polyvinylidineflouride membrane (Millipore India Pvt. Ltd., Bangalore, India), using wet transfer at 4° C overnight. The membranes were blocked with 5 % non-fat dry milk with PBS (10 mM, 7.5 pH) and 0.1 % Tween 20 and incubated for 30 minutes at room temperature, after which it was then incubated with primary antibodies; BCL2 (1:2000; mouse polyclonal antibody, cat# sc-7382, Santa Cruz Biotechnology, Dallas, Texas, United States), aromatase (1:2000; rabbit polyclonal antibody, cat# E-AB-64300, Elabscience, Houston, Texas, United States), androgen receptor (1:100; rabbit polyclonal antibody, Cat# **PA5-16363,** Invitrogen, Waltham, Massachusetts, United States), estrogen receptor α (1;500; mouse polyclonal antibody, cat# Bz1, DSHB, University of Iowa, Dept. of Biology, Iowa, United States), estrogen receptor β (1:500; mouse polyclonal antibody, cat# CWK-F12, DSHB, University of Iowa, Dept. of Biology, Iowa, United States), β-Tubulin (1:1500; mouse polyclonal antibody, cat# E7, DSHB, University of Iowa, Dept of Biology, Iowa, United States) for overnight at 4°C. The membrane blots were then washed with PBS-Tween 20 for 2 changes and incubated with horse-radish peroxidase conjugated secondary antibody (1:4000) (goat anti-mouse, cat# E-AB-1001, Elabscience, Houston, Texas, United States; Goat anti-rabbit, cat# E-AB-1102, Elabscience, Houston, Texas, United States), for 4 hours at room temperature. After washing with PBS-Tween, the blots were finally detected by chemiluminescence (ECL) (cat no- 1705060, BioRad,

Hercules, CA, USA) and developed with x-ray film. The protein band was then analysed with Image J software (1.38x, NIH, Bethesda, MD, USA). The density of each band was normalized to the density of the β-Tubulin band that was used as an internal control.

Statistical analysis

All the data were expressed as the mean ± standard error of the mean. Analysis of data was done by One-way analysis of variance (ANOVA) followed by Tukey's test and students't-test. The level of significance was considered as $p<0.05$. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego,CA,USA).

Results

Postnatal changes in body weight, testis weight, testicular visfatin, and circulating visfatin levels

Body weight showed a significant increase from PND7 to PND65 and testis weight also showed a significant increase from PND14 to PND65, but no significant changes were observed in PND7 and PND14 **(Figure-1.1A, B)**. The analysis of serum showed no variation in the circulating visfatin from PND7 to PND65. The changes in the body weight with circulating visfatin levels did not show a significant correlation ($r=0.22$, $p>0.05$) **(Figure-1.1B)**. However, the concentration of testicular visfatin showed variation during postnatal stages and showed a significant positive correlation with testis weight ($r=0.48$, $p<0.05$). The testicular visfatin did not show a significant change from PND7 to PND14, however, the testicular visfatin concentration showed a significant ($p<0.05$) increase at PND21 and PND65 compared to the PND7-14 **(Figure-1.1A).**

Postnatal localization of testicular visfatin

Since the quantification of the testicular showed observable variation, thus, we analyzed the presence of testicular visfatin by immunohistochemistry. From PND7 to PND65, testicular visfatin showed its presence in the interstitium and seminiferous

tubule as well. The intense immunostaining of visfatin was observed in the Leydig cells of PND7, 14, 42, and 65 **(Figure-1.2A, B, D, E)**, whereas faint immunostaining of visfatin was observed in the Leydig cell at PND21 **(Figure-1.2C).** The germ cells at different postnatal age also showed an abundance of visfatin, the spermatocytes at PND14, 21, 42 and 65 showed moderate staining, whereas spermatid at PND21, 42 and 65 also showed moderate staining. The spermatogonia at PND7 showed intense immunostaining of visfatin.

Effect of visfatin inhibition by FK866 (PND14-PND21) on testis and body weight

The inhibition of visfatin by FK866 during PND14 to PND21 did not show a significant change in body and testis weight **(Figure-1.3A, B).** However, testis weight was slightly higher in the FK866 treated group.

Effect of visfatin inhibition by FK866 (PND14-PND21) on testis histology and histomorphometric parameters (Seminiferous tubule diameter, Germinal epithelium height, and Lumen diameter)

The histological examination of the control testis showed a normal arrangement of germ cells up to round spermatid in the seminiferous tubules and normal histoarchitecture in the interstitium **(Figure-1.4A).** The treatment of FK866, visfatin inhibitor showed some degenerative changes in the seminiferous tubules **(Figure-1.4B)**. FK866 treated mice testis showed delamination and darkly stained pyknotic cells.

The treatment of FK866 significantly ($p<0.05$) increased the seminiferous tubule diameter compared to the control **(Figure-1.4C)**, however germinal epithelium height and lumen diameter were unaffected after FK866 treatment **(Figure-1.4D, E).**

Effect of visfatin inhibition by FK866 (PND14-PND21) on circulating testosterone and estrogen levels

To unravel the effect of visfatin on testicular steroidogenesis during PND14-PND21, circulating levels of testosterone and estrogen were measured after FK866 treatment.

The inhibition of visfatin by FK866 significantly ($p<0.05$) increased the circulating testosterone levels compared to the control **(Figure-1.5A)**, however, the levels of circulating estrogen did not show significant changes **(Figure-1.5B**).

Effect of visfatin inhibition by FK866 (PND14-PND21) on the localization and expression of BCl2 and cleaved caspase3

The immunostaining of BCl2 was abundantly observed in the primary spermatocytes and Leydig cells of FK866 treated mice **(Figure-1.6B)** than in control testis **(Figure-1.6A).** Western blot analysis showed expression of BCl2 and cleaved caspase3 was significantly ($p<0.05$) increased in the FK866 treated testis compared to the control testis **(Figure-1.6C, D).**

Effect of visfatin inhibition by FK866 (PND14-PND21) on the localization of GCNA

To unravel the role of visfatin on germ cell proliferation, GCNA was immunolocalized. The immunostaining of GCNA showed intense staining as well as more GCNA-positive cells in the testis of FK866-treated mice than control **(Figure-1.7A, B).** The semi-quantification of GCNA staining also showed a significant ($p<0.05$) increase in the percentage area in the testis of FK866-treated mice compared to the control **(Figure-1.7C)**.

Effect of visfatin inhibition by FK866 (PND14-PND21) on the localization of 17βHSD and androgen receptor

The immunostaining of 17βHSD showed moderate staining in the Leydig cells of the control testis than FK866 treated testis **(Figure-1.8A, B).** Immunolocalization of AR showed increased abundance in the testis of FK866-treated mice **(Figure-1.8C, D).**

Effect of visfatin inhibition by FK866 (PND14-PND21) on the expression of aromatase, AR, ERα and ERβ

The expression of aromatase decreased significantly ($p<0.05$) in the testis of FK866 treated mice compared to the control **(Figure-1.9A).** The expression of AR and ERβ increased significantly ($p<0.05$) in the testis of FK866 treated mice compared to the control **(Figure-1.9B, C).** However, increased expression of ERα was also observed in the FK866-treated mice testis, although it was not significant **(Figure-1.9D)**.

Discussion

Adipokines have emerged as regulators of gonadal function in many mammalian and non-mammalian species. In the present study, we have investigated the developmental expression of testicular visfatin along with its possible role in testicular activity in infantile testis. The expression of testicular visfatin by western blot and immunohistochemistry showed an appearance at PND7. Localization of visfatin in the seminiferous tubules as well as in the interstitium suggests the possible involvement of visfatin in steroidogenesis and spermatogenesis. We have observed variation in the localization and expression of visfatin in the testis. Immunohistochemical analysis was more intense in the postnatal stages of PND7 and 42, whereas the moderate intensity of visfatin was also observed in the testis of PND65. To unravel this discrepancy in the testicular visfatin by western blot and immunohistochemistry, the testicular tissue visfatin was measured by ELISA. The results of testicular visfatin showed an increase from PND7 to PND14 and onwards, however, the testicular visfatin did not show a significant change in PND42 and PND65. The circulating visfatin did not show age-dependent variation as well as a significant correlation with body weight. Furthermore, the changes in the postnatal testicular visfatin showed a significant positive correlation with testis weight ($r=0.48$, $p<0.05$). These findings suggest that mouse testicular visfatin expression and content is dependent on the postnatal age, and more likely it increases with age. In a recent study we have shown the expression of ovarian visfatin also changes with age **(Annie et al., 2021)**. The developmental expression of visfatin mice testis has not

been explored earlier. A previous study in chicken has shown that adult testis has increased expression of the visfatin gene and circulating visfatin levels than pre-pubertal testis **(Ocón-Grove et al., 2010)**. However, in their study, testicular visfatin did not show a change in visfatin protein expression by Western blot analysis. It has been suggested that visfatin might have an influence on sexual maturation in chicken testis by regulating testicular spermatogenesis and steroidogenesis **(Ocón-Grove et al., 2010)**. The role of visfatin in the testis of rat and mice have also been suggested by our groups **(Jeremy et al., 2017; Annie et al., 2019).**

Since the expression of testicular visfatin was upregulated with the progress of developmental stages, therefore, to unravel the role of visfatin, an inhibitor of visfatin, FK866 has been used to inhibit the visfatin function. Inhibition of visfatin from PND14-PND21 did not affect the testis and body weight, however, testis weight showed a slightly increasing trend after visfatin inhibition. These results are in partial agreement with our recent study in females, where inhibition of visfatin increased body and ovarian weight **(Annie et al., 2021)**. The slight change in testis weight has promoted us to examine the histological changes. The morphometrical study of the testis exhibited a slight increase in seminiferous tubule diameter without affection germinal epithelium height and lumen diameter. The gross examination of the testis showed some degenerative changes like delamination and a darkly stained nucleus, which could be pyknotic. Testicular functions are regulated by steroid hormones, testosterone, and estrogen **(Cooke and Walker, 2021)**. It has been shown that both of these hormones are required for the regulation of spermatogenesis **(Carreau and Hess 2010)**. Thus, we have examined the circulating testosterone and estrogen after visfatin inhibition. Inhibition of visfatin increased circulating testosterone and estrogen, however, testosterone increase was significant. These results suggest the inhibitory role of visfatin in testicular steroidogenesis. Visfatin has been shown to regulate gonadal steroidogenesis in males and females as well. In contrast to our result, in isolated rat Leydig cells, visfatin has been shown to stimulate testosterone secretion **(Hameed et al., 2012)**. The direct influence of visfatin on testicular steroidogenesis has not been reported in mice of any developmental stages. Our previous study on female mice has shown no effect of

visfatin on ovarian estrogen and progesterone secretion during the infantile period **(Annie et al., 2021)**. Visfatin has been shown to inhibit basal and IGF-induced progesterone secretion by hen granulosa cells and estrogen secretion in pre-pubertal mice **(Diot et al., 2015; Annie et al., 2020)**. Thus, it seems that visfatin might have a stage and gender-dependent role in gonadal steroidogenesis.

To unravel the reason behind the increased testosterone after visfatin inhibition by FK866, we have analyzed the expression and localization of 17βHSD and aromatase (CYP19A1). The inhibition of visfatin decreased the expression of 17βHSD and aromatase. The deceased aromatase could be the possible reason for the increased testosterone after visfatin inhibition; however, 17βHSD converts androstenedione to testosterone, therefore decreased 17βHSD with increased testosterone levels remains to be investigated. Furthermore, these results showed that visfatin may suppress testicular testosterone secretion by up-regulating aromatase expression in infantile mice. A previous study has shown that visfatin inhibition increased aromatase expression in pre-pubertal mice ovary **(Annie et al., 2020)**. On the other hand, visfatin has been shown to increase the aromatase expression in granulosa cells of buffalo ovary and suggested to have a stimulatory role of visfatin in estradiol and progesterone synthesis **(Thakre et al., 2021)**. It has been sown that the rise of testosterone synthesis started at PND30 and reaches a high concentration at PND40 in mice **(Bell et al., 2018)**. This rise of circulating testosterone in mice has been suggested due to the appearance of active adult Leydig cells at PND25, which become the major androgen source **(Wen et al., 2016)**. Therefore, it may be suggested that during the infantile stage, visfatin could be one of the regulators of testosterone suppression. Furthermore, the role of visfatin in fetal and adult Leydig cell-mediated testosterone biosynthesis would be an important question for further investigation.

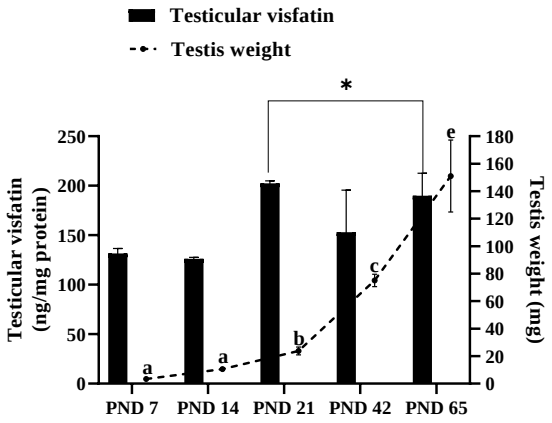
It is well known that testosterone and estrogen mediate signaling by AR and ERα and ERβ. These receptors have already been shown to express in the testis **(Zhou et al., 1996; Mahmoud et al., 2015)**. Thus, we have also investigated the expression of these receptors after visfatin inhibition. Visfatin inhibition had up-regulated the expression of AR and ERβ in the testis. These findings further suggest the inhibitory

role of visfatin in androgen and estrogen signaling in infantile testis, which might be important for the regulation of spermatogenesis. It has been reported that AR is present in the different cell types of postnatal mice testis **(Zhou et al., 1996)**. Our immunohistochemical study also showed the abundance of AR presence in the FK866-treated mice testis. This result further supports the hypothesis of decreased androgen signaling by visfatin in the infantile testis.

Our data also showed visfatin during the infantile stage suppressed germ cell proliferation, as visfatin inhibitors increased the expression of GCNA. Visfatin has also been shown to regulate cell proliferation either by stimulating or inhibiting cell proliferation. Our previous studies in mice ovary, and uterus has shown that visfatin suppresses proliferation **(Annie et al., 2019; 2021)**. In other non-reproductive cells and tissues, visfatin has been shown to stimulate cell proliferation **(Patel et al., 2010; Buldak et al., 2013; Xie et al., 2007; Wang et al., 2009).** Apart from cell proliferation, visfatin also regulates apoptosis **(Annie et al., 2021)**. Inhibition of visfatin increased the expression of cleaved caspase3 in the infantile mice testis; this result is in agreement with our previous report on mice, where visfatin inhibition increases the apoptosis in infantile mice ovary **(Annie et al., 2021)**. However, the expression of BCl2 was also up regulated by visfatin inhibition, which is in contrast with our previous study **(Annie et al., 2021)**. It is generally expected that expression BCl2 should decrease to facilitate apoptosis by increasing caspsas3. The physiological significance of these results remains to be investigated. As we have detected the expression of cleaved caspase3 after visfatin inhibition, therefore, anti-apoptotic role visfatin in mice testis might be hypothesized.

In conclusion, this study first time showed the role of testicular visfatin in infantile mice with respect to testosterone secretion, proliferation, and apoptosis. Overall, the suppression of apoptosis and germ cell proliferation in the infantile mice testis could be due to the visfatin-mediated down-regulation of expression of AR and ERβ.

A



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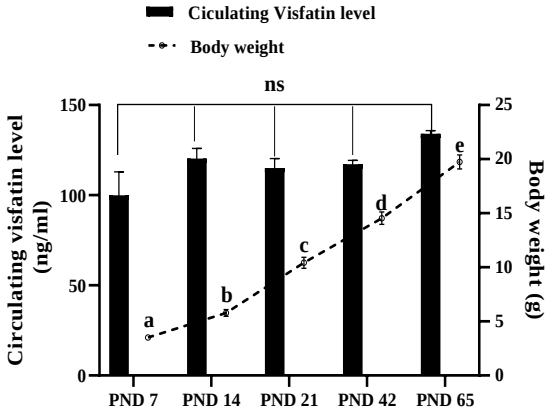

Figure-1.1- Changes in the body weight, testis weight, circulating and testicular visfatin levels. (A). The testicular visfatin concentration showed significant ($p<0.05$) highest concentration at PND21 and PND65 (*, $p<0.05$vs PND7, PND14 and PND42). Testis weight showed a significant increase from PND14 to PND65 (B). Circulating visfatin level did not show significant variation, while the body weight showed significant changes from PND7 to PND65. The data are represented as mean±SEM (n=5 per group). Different letters in the graph showed significant differences ($p<0.05$).

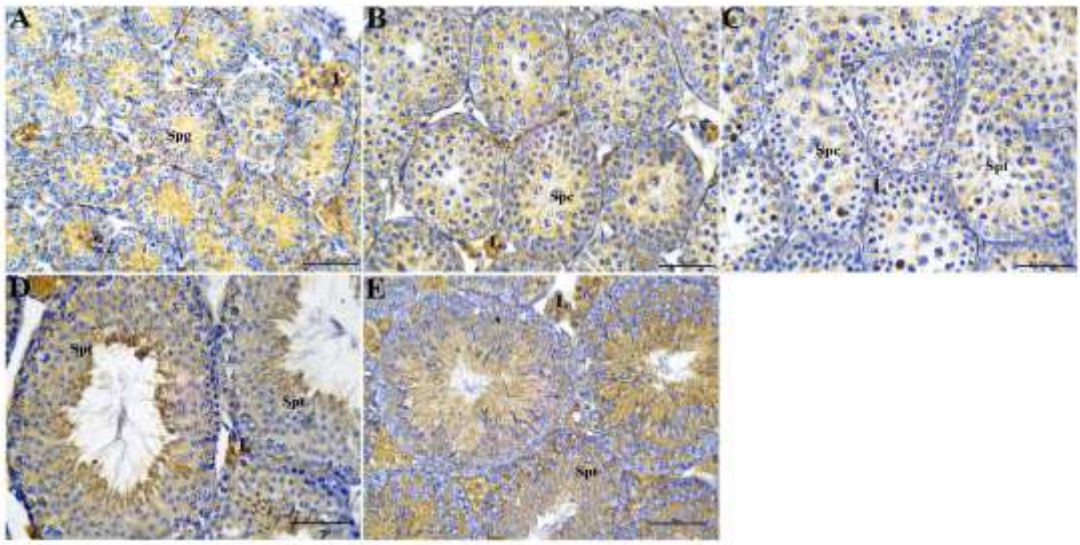

Figure-1.2- Immunolocalization of visfatin in postnatal testis. The Leydig cells (L) showed moderate immunostaining at PND7 (A), PND14 (B), PND42 (D), and PND65 (E). Mild staining was found in the Leydig cell (L) at PND21 (C). Spermatogonia (Spg) at PND7 showed strong immunostaining (A). Spermatocytes at PND14 (B), PND21 (C), PND42 (D), and PND65 (E) showed moderate immunostaining. Immunostaining of visfatin showed mild staining in the spermatid (spt) at PND21 and PND42 where as strong immunostaining of visfatin was observed in PND65.

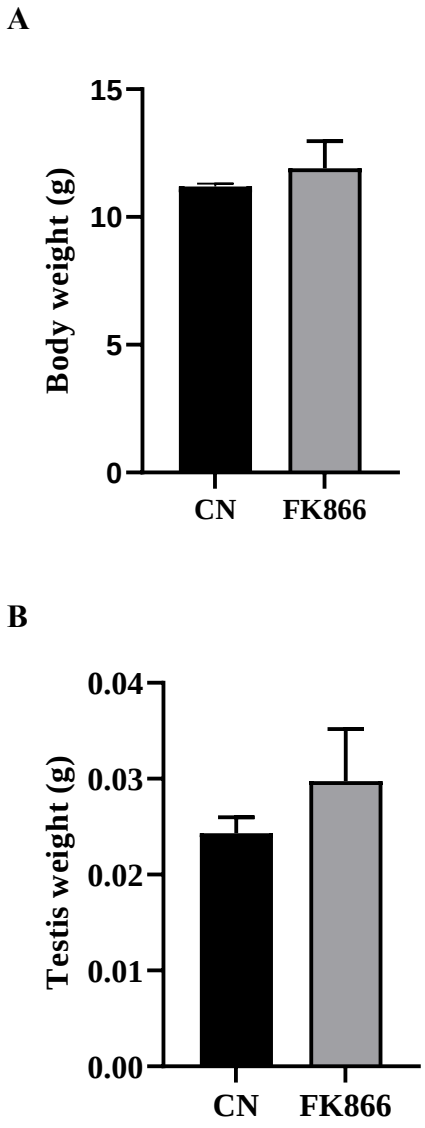

Figure-1.3- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on the body weight and testis weight. (A). Body weight, (B) testis weight did not show significant changes in FK866 treated mice compare to the control group. Data are represented as mean±SEM (n=5 per group).

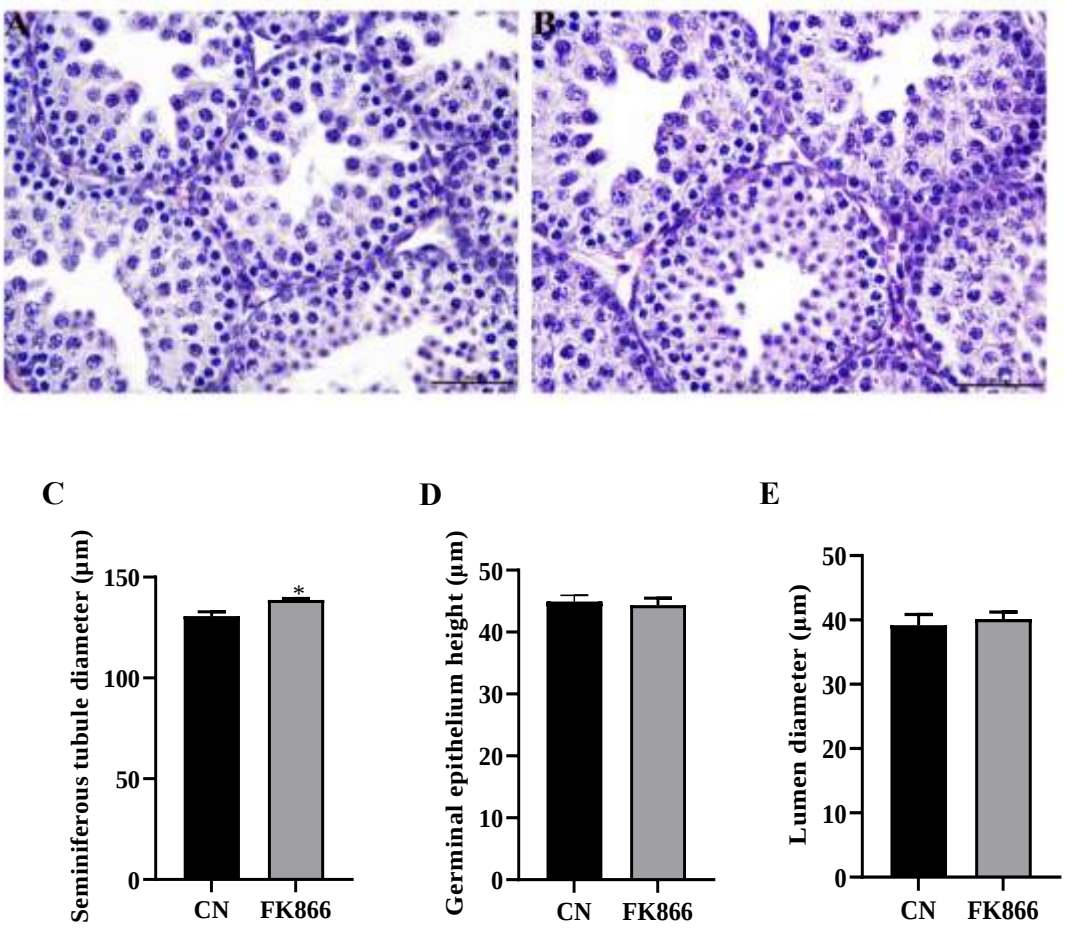

Figure-1.4- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on the testicular histology and morphometric analysis. (A) Control testis showed a normal arrangement of germ cells, whereas (B) FK866 treated mice testis showed degenerative changes in the seminiferous tubule (delamination, Dl; arrow showed pyknotic nucleus). (C) Morphometric analysis of the testis showed a significant (*, $p<0.05$) increase in seminiferous tubule diameter in FK866 compared to Control. (D) Germinal epithelium height and (E) tubule diameter did not show significant ($p>0.05$). Data are represented as mean±SEM (n=5 per group).

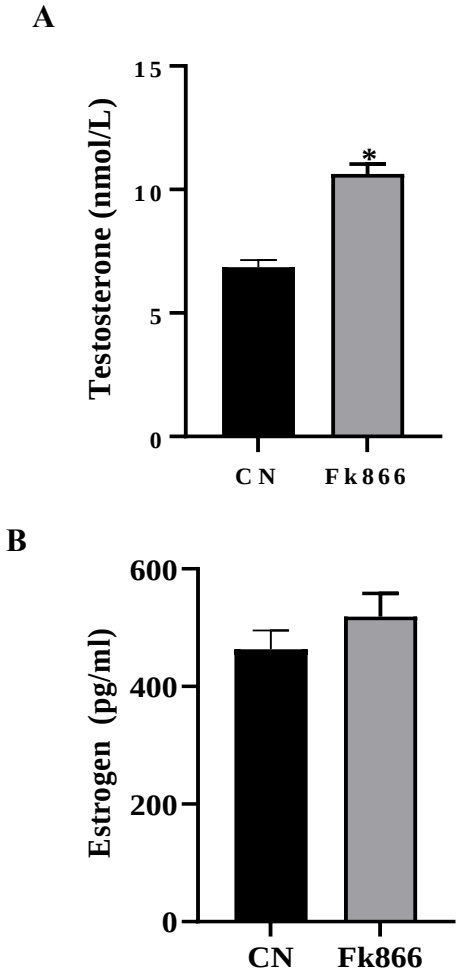

Figure-1.5- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on circulating testosterone and estrogen levels. (A) The circulating testosterone levels of visfatin showed a significant (*$p<0.05$) increase in the FK866 group compared to the control. Data are represented as mean±SEM (n=5 per group).

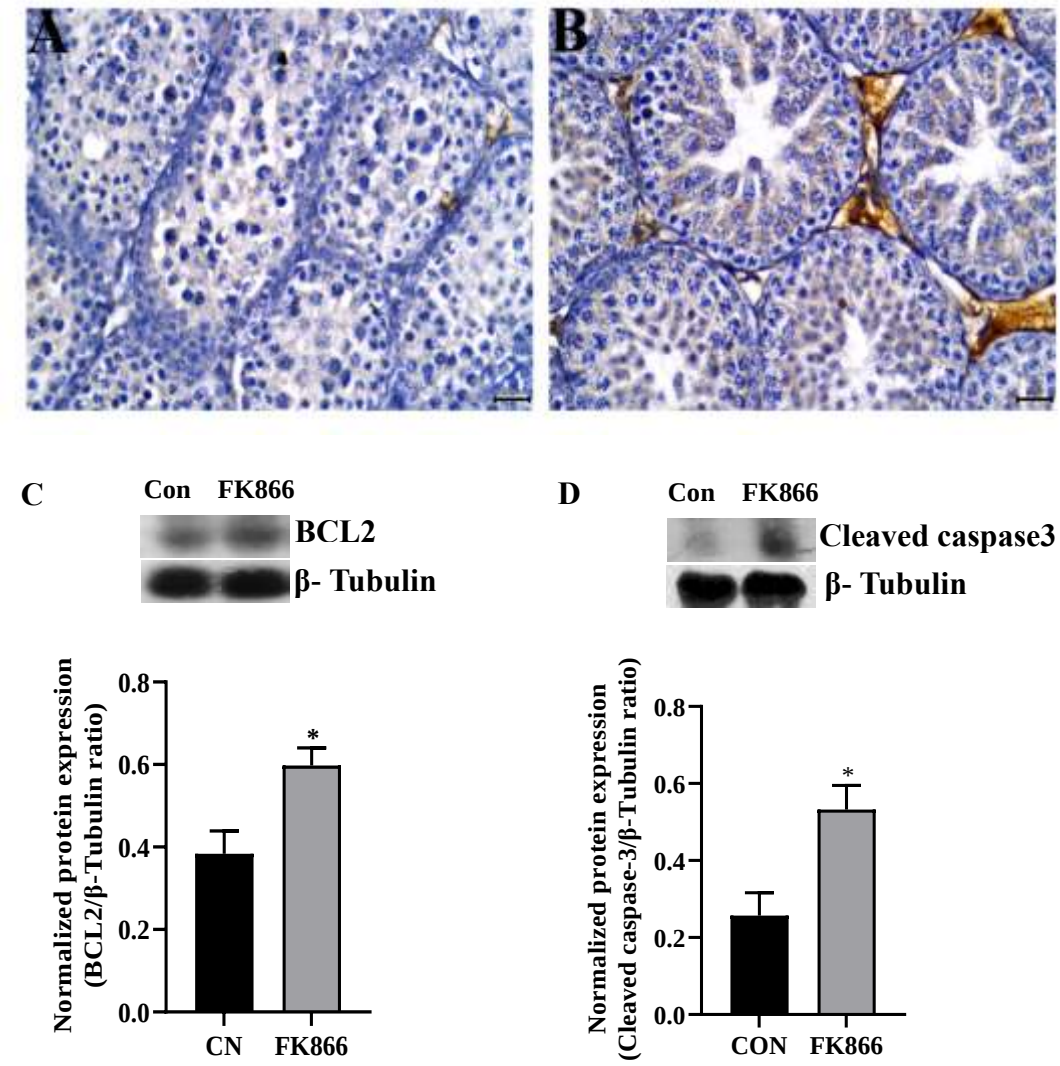

Figure-1.6- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on the localization and expression of BCl2 and cleaved caspase3. (A) Immunostaining of BCL2 showed intense staining of Leydig cells and spermatocytes in the FK866 group than the control group (B). (C) The expression of BCl2 was significantly (*$p<0.05$) higher in the FK866 group compare to the control group and (D) the expression of cleaved caspase3 was significantly (*$p<0.05$) higher in the FK866 group compare to the control group. Data are represented as mean±SEM (n=5 per group).

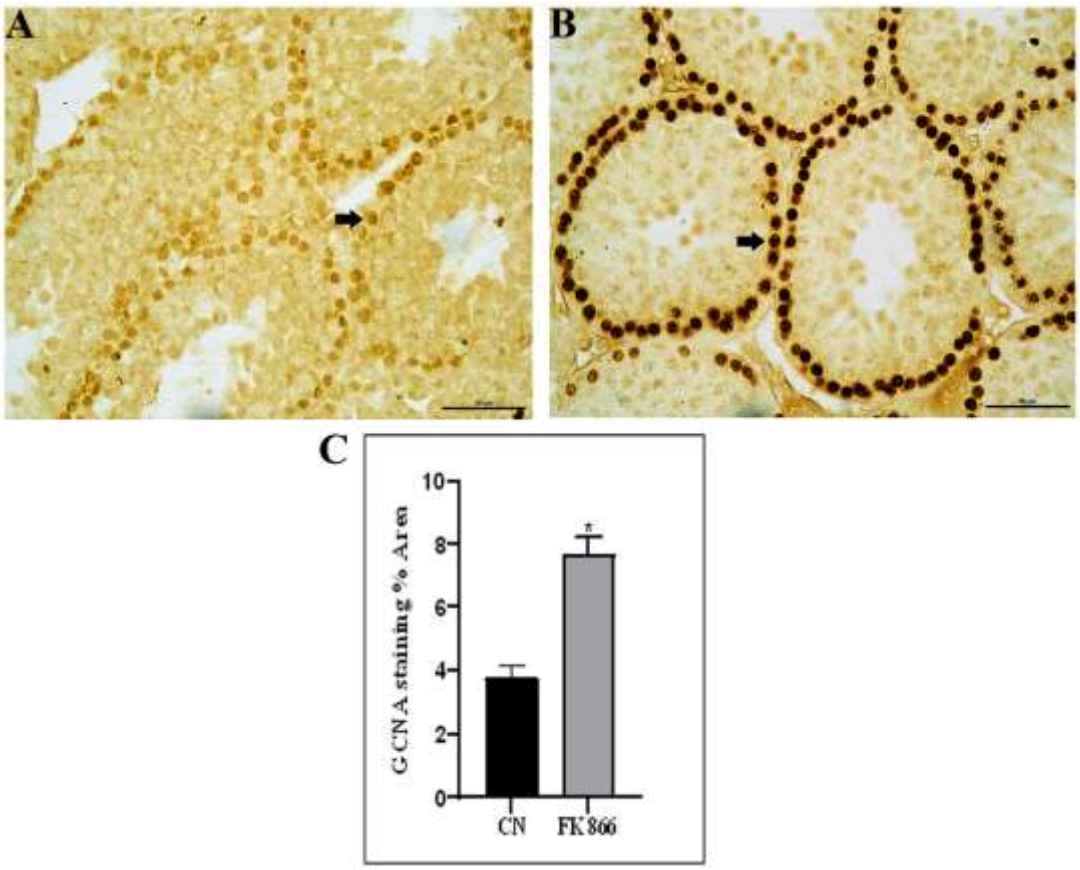

Figure-1.7- Effect of visfatin inhibition FK866 (1.5 mg/kg) on the localization of GCNA. GCNA immunolocalization showed intense staining and more GCNA-positive cells in the FK866 group (B) than in the control group (A). (C) semi-quantification of GCNA staining showed a significant (*$p<0.05$) increase in FK866 group compared to control. Data represented as mean±SEM (n=5 per group).

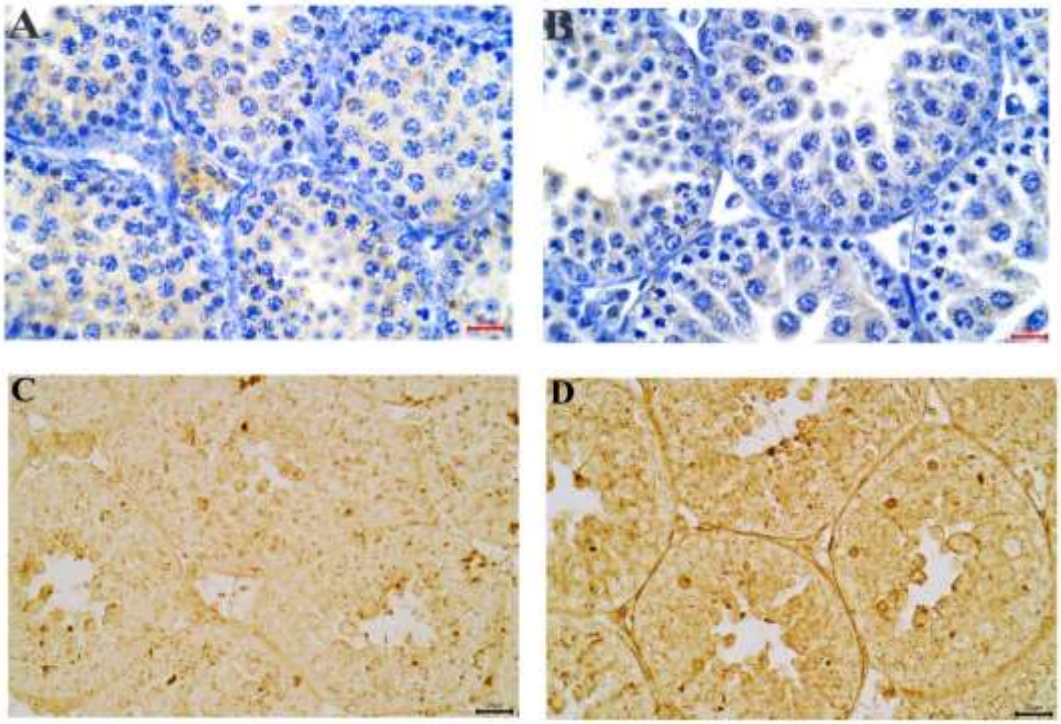

Figure-1.8- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on the localization of 17βHSD and AR. Immunolocalization of 17βHSD showed more staining in the Leydig cells of control (A) than FK866 (B) group. The seminiferous tubule of FK866 treated group (C) showed more staining of AR compared to the control group (D).

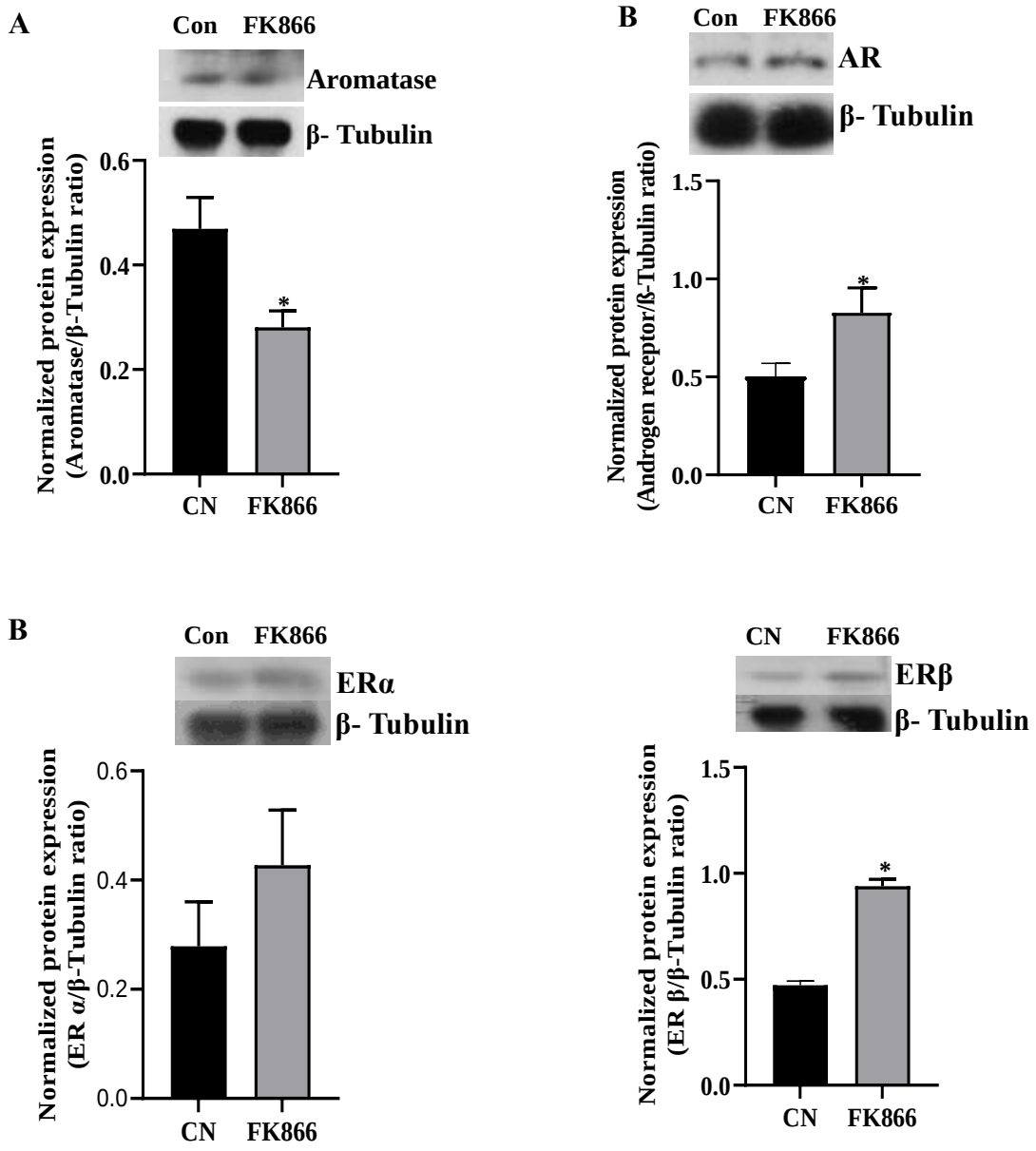

Figure-1.9- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on the expression of aromatase, AR, ERα, and ERβ. (A) Expression of aromatase significantly (*$p<0.05$) declined in the FK866-treated mice compared to the control**.** (B) The expression of AR was significantly (*$p<0.05$) higher in the testis of the FK866 group compared to the control group. (C) Expression of ERβ significantly (*$p<0.05$) increased in the FK866 group compared to the control. (D) Expression of ERα was also higher in the FK866 group compared to the control, but there was no significant ($p>0.05$) change. All the data were represented as mean±SEM (n=5 per group).

Chapter 2

Differential effect of visfatin inhibition on the testicular androgen and estrogen receptors expression in early pubertal mice

Introduction

It is well-known that adipokines, secreted by the adipose tissues have a vital role in energy and metabolic processes. Studies have shown the involvement of these hormones in regulating reproductive function and their implication in explaining some reproductive disorders **(Estienne et al., 2019).** Visfatin is an adipokine that is believed to have a stimulating role in spermatogenesis via testosterone production **(Hameed et al., 2012)**. Visfatin also affects insulin-like growth factor-1-induced steroidogenesis in bovine granulosa cells **(Reverchon et al., 2013)**. Dutta *et al*. **(2021)** reviewed shows that during metabolic disorders visfatin may play a significant role in the regulation of male fertility as its expression was noted in the testis and in hypothalamic areas is related to reproductive and energy homeostasis.

It is also shown that visfatin treatment has improved the quality of oocytes and ovarian function in 18-month-old female mice, clearly indicating that visfatin enhanced the fertility potential in aged mice **(Park et al., 2020).** Visfatin has a protective effect on stress-induced gonadal dysfunction **(Safya et al., 2018)**. Ocon-Grove *et al.* **(2010)** also provided the evidence that increased testicular visfatin and plasma visfatin levels were associated with sexual maturation in boiler breeder chicken; they also found that the quantity of testicular visfatin mRNA in adults was fourfold higher in comparison with the pre-pubertal chickens. Adipokines, particularly leptin and adiponectin, appear to cause early puberty in obese girls and boys by influencing the hypothalamic-pituitary-gonadal (HPG) axis **(Nieuwenhuis et al., 2020)**. Leptin has been shown to act as a permissive factor in pubertal maturation and reproduction **(Elias et al., 2013)**.It has been suggested that might have local effects on the function of testis that can also modulate testosterone synthesis and spermatogenesis and could be critical for puberty initiation **(Zhang et al., 2018)**.The expression of the adiponectin gene in the testes appears to be regulated by developmental signals and hormonal influences. Before puberty, moderate mRNA levels of adiponectin were observed in the rat testis **(Caminos et al., 2008)**. Whether visfatin may have a local role in the mice testis at pubertal age has not been investigated.

Expression of visfatin is now well known in the gonads of mice, rats, bovine, porcine, and chicken **(Annie et al., 2020; Jeremy et al., 2017; Thakre et al., 2021; Ons et al., 2010; Mlyczyńska et al., 2023)**. Recently, we have shown that visfatin inhibits steroidogenesis, promotes proliferation, and inhibits apoptosis in the ovary of pre-pubertal mice. Visfatin is therefore a novel regulator of ovarian function in pre-pubertal mice **(Annie et al., 2020)**. Thus, it can be hypothesized that like ovarian function, visfatin might also regulate testicular function in mice at early pubertal age. In a recent study, we have shown that visfatin has an inhibitory role in testicular function at infantile age **(Rempuia et al., 2023)**. However, in infantile mice, Annie *et al*. **(2021)** show that ovarian visfatin inhibits apoptosis and proliferation without regulating ovarian steroid biosynthesis. Testis harbors the process of apoptosis and proliferation in different age groups of mice (**Blanco-Rodriguez et al., 1998; Jeyaraj et al., 2003)**. An early apoptotic wave of germ cells after birth (about 3-4 weeks) may be needed for determining the later proper pattern of spermatogenesis **(Russell et al., 2002)**. Thus, it would be important to unravel the role of visfatin on the testicular proliferation, apoptosis, and steroidogenesis in early pubertal age. Therefore, the present study used pre-pubertal (25 days old) male mice to unravel the role of visfatin on the testicular activity.

Materials and Methods

Animal maintenance

All the procedures were carried out under the guidelines given by Mizoram University Institutional Animal Ethical Committee (Protocol Approval number-MZUIAEC 21-22-05), Mizoram University, Mizoram, India. 25-day-old male Swiss albino mice were used for this experiment, mice were collected from a reared colony maintained under controlled conditions with a 12 h light: 12 h dark cycle and 25±2°C, food and water were provided *ad libitum*.

In vivo treatment

To find out the role of visfatin in early pubertal age, the pre-pubertal mice aged, post natal days 25 (PND25) were selected to be treated with FK866, a specific inhibitor of visfatin. Sixteen mice were divided into two groups: Control group (n=8) was given normal saline as a vehicle and FK866 (n=8) group was administered visfatin

inhibitor (FK866) at the dose of 1.5mg/kg by intraperitoneal injection for 10 days from PND25 to PND35. The dose of FK866 was selected based on our recent study **(Rempuia et al., 2023)**. After 24 hours of the last dose, mice were euthanized. Body weight and testis weight were also measured. One side of the testis and adipose tissues were fixed in Bouins fluid for histological and immunohistochemical analysis and other side of the testis were freeze for western blot analysis. Blood was also collected and centrifuged at 3000 g for serum collection, which was used for hormonal assay.

Histology

A histopathological study was performed using testis fixed with the Bouin's fluid. Tissues were transferred to 70% ethanol after fixing it for 24 hours to avoid brittleness. The tissues were then dehydrated in a series of graded ethanol (90% and 100%) twice for one hour each, which were then cleared in xylene for a minute and embedded in a paraffin wax block. Tissue blocks were sectioned at 7 µm thickness with Leica microtome (model RM2125 RTS) and then fixed at a sterile glass slides coated with poly-l-lysine. The tissues fixed in glass slides then undergo tissues processing and staining with hematoxylin and eosin **(Bancroft et al., 2002)**. Sections were observed under a microscope and photographed using camera-mounted binocular microscope (Model E200, Nikon, Tokyo, Japan).

Histomorphometry

The same histological sections were used for histomorphometrical analysis, seminiferous tubules diameter (STD) **(Guneli et al., 2008)**, germinal epithelium height (GEH) **(França et al., 2003)** and lumen diameter (LD) was measured with ocular micrometer of 40X magnification. Thirty round tubular sections were selected, and measurements were taken for STD, GEH and LD.

Immunohistochemistry

For immunohistochemical studies, Bouin's fixed testis and adipose tissues (subcutaneous, visceral and epididymal) samples were used as described previously

(Jeremy et al., 2019). The tissues sample was dehydrated in a series of ethanol (90% and 100%) twice for 1 hour each. The dehydrated tissue samples were then immersed in xylene and infiltrated with molten paraffin. The samples were then sectioned using Leica rotary microtome (model RM2125 RTS) at 7 µm thick and embedded in a sterile glass slide. The tissue slides then undergo dehydration process after which it was kept in PBS (1X) for 10 minutes followed by incubation in blocking buffer [goat serum 1:100 diluted in PBS (Lot# A0515, Santa Cruz Biotechnology, Inc., CA, USA)] for 30 minutes, the slides were incubated overnight at 4°C in a humidified chamber using primary antibody against Bcl2 (1:100; mouse polyclonal antibody, Cat# sc-7382, Santa Cruz Biotechnology, Inc. Dallas, USA), cleaved caspase3 (1: 100; rabbit polyclonal antibody, cat# E-AB-30004, Elabscience, Houston, Texas, United States), GCNA (1:200; mouse polyclonal antibody, cat# 10D9G11, DSHB, University of Iowa, Dept of Biology, Iowa, United States), PCNA (1:100, cat# SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA), 17βHSD (1:400; rabbit polyclonal antibody, cat# STJ110000, St John's Laboratory Ltd, London, United Kingdom), 3βHSD (1 : 200, Santa Cruz Biotech. Cat# Sc-515120 HRP lot #J1117), androgen receptor (1:100; rabbit polyclonal antibody, Cat# **PA5-16363,** Invitrogen, Waltham, Massachusetts, United States), visfatin (NAMPT) (1:100; rabbit polyclonal antibody, Cat# PA5-30940, Invitrogen, Waltham, Massachusetts, United States). After overnight incubation at 4°C, slides were washed in PBS and probed with Horse-radish Peroxidase (HRP) conjugated secondary IgG antibody at 1:400 dilution (goat anti-mouse, Cat# PI-2000 and goat anti-rabbit, Cat# PI-1000, Vector Laboratories, Burlingame, CA, United State) for 3 hours at room temperature, the unbound antibody was washed off by PBS followed by incubation in a solution containing 0.6 mg/ ml solution of 3, 3-diaminobenzidine tetra hydrochloride dihydrate (DAB) in Tris–HCl (pH 7.6) and 0.01% H_2O_2 at room temperature till brown color developed, the reaction was stopped with distilled water and counterstained with hematoxylin except for GCNA and PCNA, slides were then dehydrated using graded ethanol in increasing order of concentrations and then cleared in xylene. It was then mounted with DPX and the slides were examined and photographed using a camera-mounted binocular microscope (Model E200, Nikon, Tokyo, Japan). Semi-quantification of PCNA and GCNA in the testes was executed

by ImageJ software. The stained area by DAB in the testis was calculated by using threshold tool of ImageJ as described previously **(Jensen et al., 2013)**.

BrdU labelling and staining

BrdU labelling was also done on the last day of in vivo treatment; 12 hours prior to sacrifice mice were separated in sub-groups- BrdU only (n=3) and BrdU + FK866 groups (n=3). The dose were given 100 mg/Kg body weight (Sisco Research Laboratories, Mumbai, India) as performed previously by Annie et al**. (2020)**.

The tissues section from BrdU experiment undergo rehydration in graded series of alcohol, followed by incubation for 60 minutes in 2N HCl at 37℃ and neutralized in 0.1M in Borate buffer for 10 minutes. Slides were then washed in phosphate buffer solution (PBS) and incubated with blocking buffer [goat serum 1:100 diluted in PBS (Lot# A0515, Santa Cruz Biotechnology, Inc., CA, USA) for 30 minutes in humidified chamber. Blocked tissue section was incubated overnight with BrdU anti-body (1:30 cat# G3G4, DSHB, University of Iowa, Dept. of Biology, Iowa, United States) at 4℃. After overnight incubation, slides were washed in PBS and probed with Horse-radish Peroxidase (HRP) conjugated secondary IgG antibody at 1:400 dilution (goat anti-mouse, cat# E-AB-1001, Elabscience, Houston, Texas, United States) for 3 hours and washed is PBS for 10 minutes. Slides were again incubated in a solution containing 0.6 mg/ ml solution of 3, 3-diaminobenzidine tetra hydrochloride dihydrate (DAB) in Tris–HCl (pH 7.6) and 0.01% H_2O_2 at room temperature till brown colour developed and the reaction was stopped with distilled water. Slides were then dehydrated in graded series of alcohol, cleared in xylene and mount with DPX. The stained area by DAB in the testis was calculated using threshold tool of ImageJ as described by **(Jensen et al., 2013)**.

Estimation of circulating sex hormone (Testosterone, Estrogen and Androstenedione) and visfatin levels

Blood serum from *in vivo* treatment of FK866 was used for measurement of testosterone, estrogen and androstenedione levels. Quantification of total serum concentration was done using commercial enzyme linked immunosorbent assay kit

(Testosterone Cat # EIA K209; Xema-Medica Co.Ltd, Moscow, Russia); (Estradiol Cat # DKO003, DiaMetra, Italy); (Androstenedione Cat # DKO008, DiaMetra, Italy). Circulating visfatin was also measured with a Mouse Visfatin ELISA kit (Cat No: K02-0598; Los Angeles). Absorbance levels were measure at 450 nm using microplate ELISA reader (Erba Lisa Scan EM, Trans Asia Biomedical Ltd, Mumbai, Maharashtra, India).

Western blot analysis

The testes samples collected at the end of in vivo treatment were homogenate with lysis buffer (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 PMSF) and protein estimation was done by Bradford method **(Bradford, 1976)**. Gel loading buffer (1M Tris-Cl pH 6.80, 2 Mercaptoethanol, 10% SDS, glycerol and dH2O) was added to the sample homogenate in equal ratio after protein estimation and boiled for 10 minutes. 50 µg/well of samples were loaded in 10% SDS-PAGE along with protein marker and thenelectrophoresed at 100V for 3 hours and resolved proteins were transferred in a polyvinylidineflouride membrane (Millipore India Pvt. Ltd., Bangalore, India), by using wet transfer at 4° C for overnight. The membranes blots were then blocked in 5 % non-fat dry milk with PBS (10 mM, 7.5 pH) and 0.1 % Tween 20 solution for 30 minutes at room temperature, followed by incubation with primary antibodies; BCL2 (1:2000; mouse polyclonal antibody, cat# sc-7382, Santa Cruz Biotechnology, Dallas, Texas, United States), cleaved caspase3 (1: 1000; rabbit polyclonal antibody, cat# E-AB-30004, Elabscience, Houston, Texas, United States), aromatase (1:2000; rabbit polyclonal antibody, cat# E-AB-64300, Elabscience, Houston, Texas, United States), androgen receptor (1:100; rabbit polyclonal antibody, Cat# **PA5-16363,** Invitrogen, Waltham, Massachusetts, United States), estrogen receptor α (1;500; mouse polyclonal antibody, cat# Bz1, DSHB, University of Iowa, Dept. of Biology, Iowa, United States), estrogen receptor β (1:500; mouse polyclonal antibody, cat# CWK-F12, DSHB, University of Iowa, Dept. of Biology, Iowa, United States), visfatin (NAMPT) (1:100; rabbit polyclonal antibody, Cat# PA5-30940, Invitrogen, Waltham, Massachusetts, United States), β-Tubulin (1:1500; mouse polyclonal antibody, cat# E7, DSHB, University of Iowa, Dept of Biology, Iowa, United States)

for overnight at 4°C.The blots were then washed with PBS-Tween 20 for 2 changes, and incubated with horse-radish peroxidase conjugated secondary antibody (1:4000) (goat anti-mouse, cat# E-AB-1001, Elabscience, Houston, Texas, United States; Goat anti-rabbit, cat# E-AB-1102, Elabscience, Houston, Texas, United States), for 4 hours at room temperature. After washing, the blots were finally detected by chemiluminescence (ECL) (cat no- 1705060, BioRad, Hercules, CA, USA) and developed in x-ray film. The protein band was analysed with Image J software (1.38x, NIH, Bethesda, MD, USA). The density of each band was normalized to the density of the β-Tubulin band that was used as an internal control.

Statistical analysis

All the data were expressed as the mean ± standard error of the mean. Analysis of data was done by students't-test. The level of significance was considered as $p<0.05$. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego,CA,USA).

Results

Effect of visfatin inhibition by FK866 (PND25-PND35) on the testis and body weight

To unravel the effect of visfatin inhibition, on the testis and body weight; both body weight and testis were analyzed. The inhibition of visfatin by FK866 during PND25 to PND35 showed changes in the body and testis weight. The body weight of FK866 treated mice was significantly ($p<0.05$) increased compared to the control mice **(Figure-2.1A)**. The testis weight was also higher in the FK866 treated mice but the data showed no statistically significant difference with control mice **(Figure-2.1B).**

Effect of visfatin inhibition by FK866 (PND25-PND35) on testis histology and histomorphometric parameters (Seminiferous tubule diameter, Germinal epithelium height, and Lumen diameter)

Whether the testicular histology and histomorphometric parameters were affected by visfatin inhibition, the testis histology and histomorphometric parameters were measured. The histological examination of control testis and testis of FK866 treated

mice showed regular arrangement of cells **(Figure-2.2A).** However, some of the seminiferous tubules of FK866 treated mice testis showed darkly stained germ cells near the lumen **(Figure-2.2B)**. The treatment of FK866 significantly ($p<0.05$) increased the seminiferous tubule diameter, germinal epithelium height and lumen diameter compared to the control group **(Figure-2.2C, D, E)**.

Effect of visfatin inhibition by FK866 (PND 25-PND 35) on the circulating testosterone, estrogen, and androstenedione levels

Sexual hormones play a vital role in the maintenance and regulation of spermatogenesis. In order to unravel the effect of visfatin on testicular steroidogenesis during PND25-PND35, the circulating levels of testosterone, estrogen, and androstenedione levels were examined after the FK866 treatment. The levels of circulating testosterone and androstenedione were slightly higher in control group but did not show any significant changes **(Figure-2.3A, C)**, however, inhibition of visfatin by FK866 significantly ($p<0.05$) increased the circulating estrogen levels compared to the control group **(Figure-2.3B)**.

Effect of visfatin inhibition by FK866 (PND25-PND35) on the localization and expression of BCl2 and cleaved caspase3

To unravel the effect of visfatin inhibition on the testicular apoptosis, expression of BCl2 and cleaved capsase3 were analysed. The immunostaining of BCl2 and cleaved caspase3 (~20kDa) was abundantly observed in the Leydig cells and spermatocytes of FK866 treated mice compared to control testis **(Figure-2.4A, B, C, D)**. Western blot analysis also showed significantly ($p<0.05$) higher expression of BCl2 and cleaved caspase3 in FK866 treated group compared to the control group **(Figure-2.4E, F)**

Effects of visfatin inhibition by FK866 (PND25-PND35) on BrdU incorporation and the localization of GCNA and PCNA proteins

To study the role of visfatin in testicular proliferation in pre-pubertal mice, BrdU incorporation was checked in the pre-pubertal testis in the presence of a visfatin inhibitor. Increased BrdU positive cells were observed in the testis of FK866 treated

mice compared to control mice **(Figure-2.5A, B)**. BrdU stained areas increased significantly ($p<0.05$) in FK866 group compared to the control **(Figure-2.5C)**. The immunostaining of PCNA and GCNA also showed a significant change in FK866 group. Immunostaining of GCNA and PCNA showed intense staining in FK866-treated mice compared to the control group **(Figure-2.5D, E, G, H)**. The semi-quantification of GCNA and PCNA also significantly ($p<0.05$) increased in the FK866 treated group than control group **(Figure-2.5F, I)**.

Effect of visfatin inhibition by FK866 (PND 25-PND 35) on the localization of 17βHSD and 3βHSD

To access the changes in the steroidogenic markers, 17βHSD and 3βHSD immunostaining was performed. The immunostaining of 17βHSD showed intense staining in the Leydig cells of FK866-treated mice than control **(Figure-2.6A, B)**. The immunostaining of 3βHSD also showed a similar trend, moderate staining was observed in Leydig cells of control testis while intense staining was seen in the Leydig cells of FK866 treated mice testis **(Figure-2.6C, D)**.

Effect of visfatin inhibition by FK866 (PND25-PND35) on the expression of aromatase, AR, ERα and ERβ

The expression of ERα and ERβ significantly ($p<0.05$) increased in the testis of FK866-treated mice compared to the control mice **(Figure-2.7C, D)**. The expression of aromatase also increased significantly ($p<0.05$) in the Fk866-treated mice compared to the control **(Figure-2.7A)**. However, the expression of AR significantly ($p<0.05$) decreased in the FK866-treated mice **(Figure-2.7B)**.

Effect of visfatin inhibition by FK866 (PND 25-PND 35) on the localization of AR

Immunolocalization of AR showed increased abundance in the testis of control mice whereas moderate staining was observed in FK866-treated mice **(Figure-2.8A, B)**. The negative control showed no immunostaining of AR **(Figure-2.8C)**.

Effect of visfatin inhibition by FK866 (PND25-PND35) on the localization of visfatin in the Subcutaneous adipose tissue (SAT), Visceral adipose tissue (VAT) and Epididymal adipose tissue (EAT)

Immunostaining of visfatin showed mild staining in the adipose tissues (SAT, VAT, EAT) of control mice, whereas no immunostaining was observed in the adipose tissues (SAT, VAT, EAT) of FK866-treated mice **(Figure-2.9A-F).**

Effect of visfatin inhibition by FK866 (PND25-PND35) on the localization, expression of testicular visfatin and circulating visfatin levels

Immunolocalization of visfatin showed strong immunostaining in the primary spermatocytes, round spermatid and Leydig cells of control mice testis **(Figure-2.10A),** whereas mild immunostaining of visfatin was observed in these cells of FK866-treated mice testis **(Figure-2.10B)**. The expression of testicular visfatin was significantly ($p<0.05$) decreased in FK866-treated mice compared to the control mice **(Figure-2.10C)**. The circulating visfatin levels were also slightly lower in FK866-treated group but did not show any significant changes with control group **(Figure-2.10D)**.

Discussion

The present study has investigated the possible involvement of visfatin on the testis during early pubertal age by using visfatin inhibitor (FK866) from PND25 to PND35 to male mice. Expression of visfatin in the testis of various mammals has been well documented **(Gurusubramanian et al., 2014; Jeremy et al., 2017; Estienne et al., 2019)**. In a recent study, we have shown that visfatin has an inhibitory role on testicular activity in the infantile stage **(Rempuia et al., 2023)**. In non-mammalian testis (chicken) visfatin is expressed in the nucleus of Sertoli cells and Leydig cells in pre-pubertal and adult stages and is suggested to have a role in sexual maturation **(Ocon-Grove *et al.*, 2010)**. Our recent studies have shown that the expression of visfatin in the testis and ovary of mice has been shown to be developmentally regulated **(Annie et al., 2020; Rempuia et al., 2023)**. Visfatin inhibits steroidogenesis, promotes proliferation, and decreases apoptosis in the pre-pubertal

mouse ovary. Thus, visfatin was suggested as a novel regulator of ovarian function in pre-pubertal mice **(Annie et al., 2020)**. However, the possible of role of visfatin in the early pubertal mice testis has not been investigated.

FK866 treatment from PND25-35 increased the body and testis weight, thus, mice could gain body weight due to increase food consumption. Visfatin is known to decrease food intake and body weight in mice via stimulating POMC neurons in conjunction with microglial activation **(Tu et al., 2017)**. However, intraperitoneal injections of visfatin increased food consumption in goldfish **(Butler et al., 2023)**. Our recent study, showed visfatin inhibition from PND14-PND21 (infantile stage) had no effect on testis or body weight; however, testis weight increased to some extent following visfatin inhibition **(Rempuia et al., 2023)**. These findings are consistent with a previous study on female infantile mice in which visfatin inhibition increased body and ovarian weight **(Annie et al., 2021)**. These findings also suggest that visfatin may play a role in the control of feeding and energy homeostasis. The increase in the testis weight of FK866 treated mice, although it was non-significant; also suggests that visfatin affects the testis. The histological section of FK866 treated testis did not show much gross observable changes in the histoarchitecture, except that some of the tubules showed darkly stained germ cells near lumen. However, the histomorphometric parameters were increased in the FK866-treated mice testis. Thus, FK866 treatment might have a stimulatory role in the testicular proliferation. These findings are supported by increased BrdU labeling and increased PCNA and GCNA in the testis of FK866-treated mice. Thus, it seems that visfatin also have an inhibitory role in testicular proliferation as in juvenile mice testis **(Rempuia et al., 2023)**. However, visfatin stimulates cell proliferation in various cancer cells **(Gholinejad et al., 2017; Wang et al., 2013; Zhao et al., 2020)**. Moreover, visfatin suppresses the cell proliferation in reproductive tissues like ovary, uterus and testis **(Annie et al., 2019; Annie et al., 2021; Rempuia et al., 2023)**. The expression of Bcl2 and cleaved caspase3 also increased in the testis of FK866-treated mice. The increased expression of caspase 3 suggests increased apoptosis in the testis of FK866-treated mice. It has been shown that visfatin inhibition by FK866 increased apoptosis in the ovary and testis of infantile mice **(Annie et al., 2021; Rempuia et**

al., 2023). Further study would be required to find an explanation of increased apoptosis despite increased expression of BCl2 by FK866. However, our recent study has shown that FK866 treatment increased the expression of BCl2 and caspase3 in the infantile mice testis **(Rempuia et al., 2023)**. The increased proliferation could also be a reason for increased apoptosis in the FK866-treated mice testis.

Furthermore, the circulating testosterone and androstenedione was unaffected by the FK866 treatment, however, the levels of circulating estrogen was significantly elevated by the FK866 treatment. It is well known that testosterone is aromatized to estrogen by an enzyme aromatase (CYP19A1). Our results showed that FK866 treatment also increased the expression of CYP19A1, suggesting that estrogen level increased due to increased CYP19A1 expression. The markers of steroidogenesis, 3βHSD and 17βHSD also showed increased abundance in the Leydig cells of FK866-treated mice testis. Thus, visfatin might have an inhibitory role in testicular steroidogenesis during early pubertal age. Our recent study has shown that FK866 treatment increased the testosterone levels along with decreased expression of aromatase **(Rempuia et al., 2023)**. To best of our knowledge, only our laboratory has investigated the role of visfatin in the gonadal steroidogenesis of mice at the infantile stage and in early pubertal stage **(Annie et al., 2021; Rempuia et al., 2023)**. It has been shown that visfatin stimulates testicular steroidogenesis in the adult mice **(Hameed et al., 2012)**. Visfatin has also been shown to effect ovarian steroidogenesis in human and bovine granulosa cells **(Reverchon et al., 2013; 2016)**. Thus, it can be suggested that visfatin has potential to influence the gonadal steroidogenesis.

Despite no change in the circulating testosterone levels, the expression of testicular AR was significantly decreased in the FK866-treated mice. Thus, it is evident that visfatin up-regulates the testicular AR expression in early pubertal mice. On the other hand, the increased circulating levels of estrogen in the FK866 treated mice coincide with up-regulation of ERα and ERβ. Since the visfatin inhibition increases the estrogen levels along with up-regulation of ERs, thus, it is also evident that visfatin might have an inhibitory role in estrogen signaling while stimulatory on the AR signaling during early pubertalage. It has been suggested that androgen-dependent

changes are important in the mammalian testis around pubertal onset, in human and mice **(Edelsztein et al., 2019)**. It has been shown that AR expressed in the spermatogenic cell and peaked at the age of puberty in the rats **(Abd EL-Meseeh et al., 2016)**. Similar to animal models, androgen activity is essential for early pubertal maturation of Sertoli cells and the start of the spermatogenic process in humans as well **(Lapoirie et al., 2021)**. Thus, based on the findings and other reports on androgen, it might be suggested that visfatin mediated signaling of AR could be important for spermatogenic process in mice at early pubertal stage. Visfatin is widely expressed in ovarian follicles of pre-pubertal and mature pigs and suggested that visfatin might have an important role in early stages **(Mlyczyńska et al., 2023)**. It should be noted that visfatin mediated regulation of AR and ERs in the testis and ovary of mice have only been reported by our group **(Annie et al., 2020; 2021; Rempuia et al., 2023)**. Previous study has shown that inhibition of visfatin-FK866 had no effect on ERα phosphorylation; however, it was suggested that visfatin might increase ER signaling in the presence of estrogen in MCF-7 cell line **(Zangooei et al., 2018)**. These results suggest that visfatin modulate the ER signaling. Cavaco et al. **(2019)** shown that AR and ERs are expressed in somatic and germinal testicular cells, respectively. Estrogen has also been considered an important factor for testicular functions **(Akingbemi et al., 2005)**. It has also been shown that endogenous estrogens inhibit steroidogenesis through the ER by acting directly on the testis throughout foetal and neonatal development **(Delbès et al., 2005)**. Thus, it can be suggested from our results that visfatin-mediated inhibition of estrogen signaling could also be important for testicular functions at an early stage of puberty in mice. It should also be noted that most of the parameters studied in the present work showed an inhibitory role of visfatin, despite stimulated androgen signaling. Thus, the role of visfatin in early pubertal mice testis seems to be complex with respect to androgen and estrogen signaling. Since testicular visfatin action was the main concern of study, therefore, we have evaluated the localization and expression of testicular visfatin. FK866 treatment down-regulated the expression of testicular visfatin. Moreover, we have not analysed the gene expression in the testis after visfatin inhibition; therefore, this decrease was really due to down-regulation of testicular visfatin or from other sources, could not be confirmed. We cannot exclude

possibility of testicular visfatin through circulation, thus, we have also analysed the immunolocalization of visfatin in the subcutaneous, visceral and epididymal adipose tissue along with circulating visfatin levels. The abundance of visfatin in these adipose tissues was down-regulated after FK866 treatment, moreover, circulating visfatin was also slightly decreased but it was not significant. Therefore, we may conclude that FK866 decreases the visfatin expression in adipose tissues and decrease visfatin expression by FK866 in the testis could be due to decrease visfatin synthesis by adipose tissue and testis as well. Our previous study has also showed that FK866 treatment decreased the visfatin abundance in mice ovary **(Annie et al., 2020).**

In conclusion, visfatin has a differential role on the expression of testicular AR and ERs at early pubertal stage of mice.FK866-mediated decreased AR expression and increased ERs expression also suggests that visfatin stimulate androgen signaling than estrogen signaling in the early pubertal stage of mice. The other parameters like apoptosis, proliferation, and estrogen synthesis seems to be inhibited by visfatin, despite increased expression of AR, which needs further investigation.

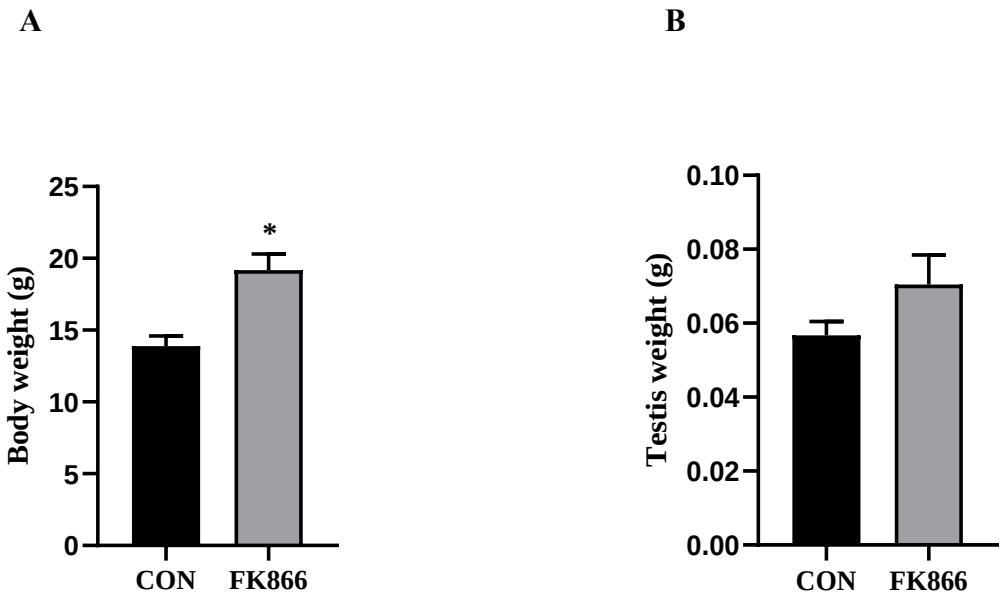

Figure-2.1- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on the body weight and testis weight. (A) Body weight showed significant ($p<0.05$) increased in FK866-treated mice compared to control group. (B) Testis weight did not show significant changes in FK866-treated mice compared to control group. Data are represented as mean±SEM (n=5 per group).

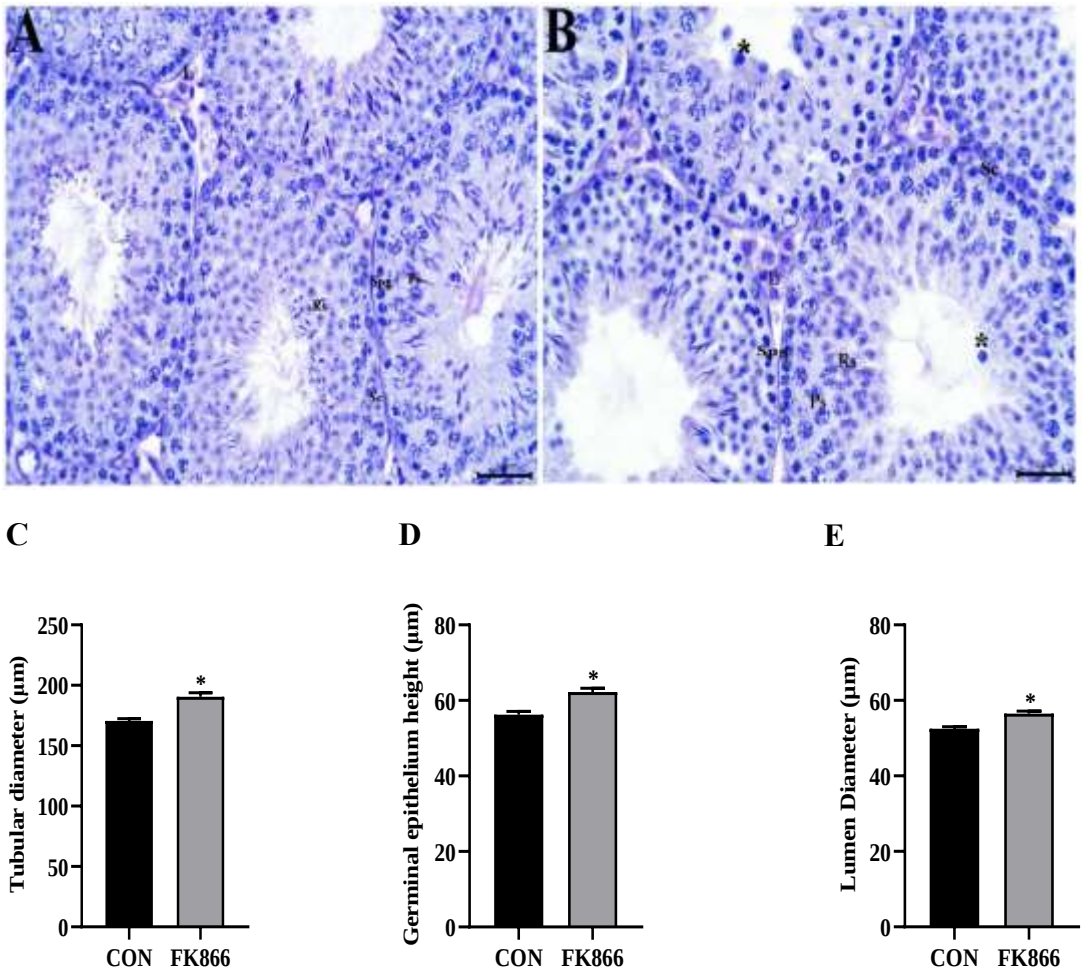

Figure-2.2-Effect of visfatin inhibition by FK866 (1.5 mg/kg) on the testicular histology and morphometric analysis. (A) Control testis showed a normal arrangement of germ cells, whereas darkly stained germ cells (*) were observed near the lumen of (B) FK866 treated group. Morphometric analysis of the testis showed significant ($p<0.05$) increased in (C) seminiferous tubule diameter, (D) germinal epithelium height and (E) lumen diameter in FK866 compared to Control. Data are represented as mean±SEM (n=5 per group). (Spg- Spermatogonia, Ps- Primary spermatocytes, Rs-Round spermatid, L- Leydig cells, Sc- Sertoli cells).

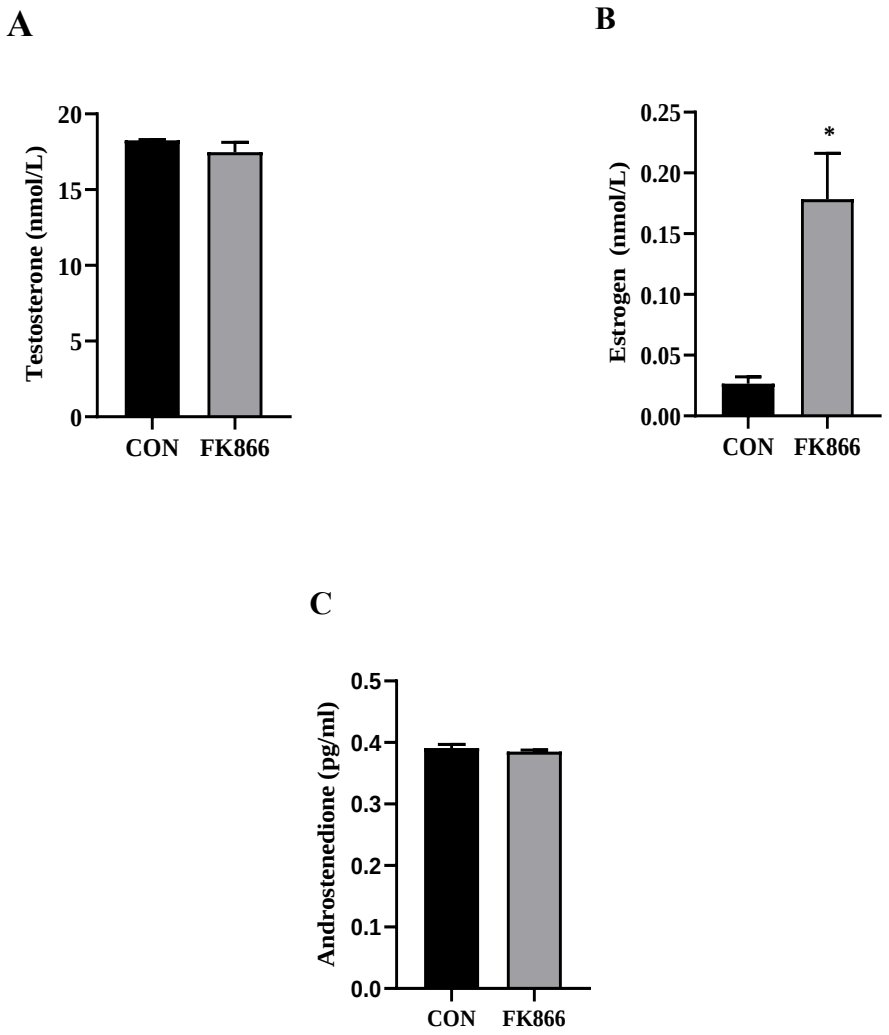

Figure-2.3- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on circulating testosterone, estrogen and Androstenedione levels. The circulating (A) testosterone levels did not showed significant changes between control group and the FK866 treated group, however, significant ($p<0.05$) increased was found in the levels of circulating (B) estrogen of FK866 treated group compared to control group.(C) Androstenedione levels did not show significant changes in the FK866 treated group compared to control group. Data are represented as mean±SEM (n=5 per group).

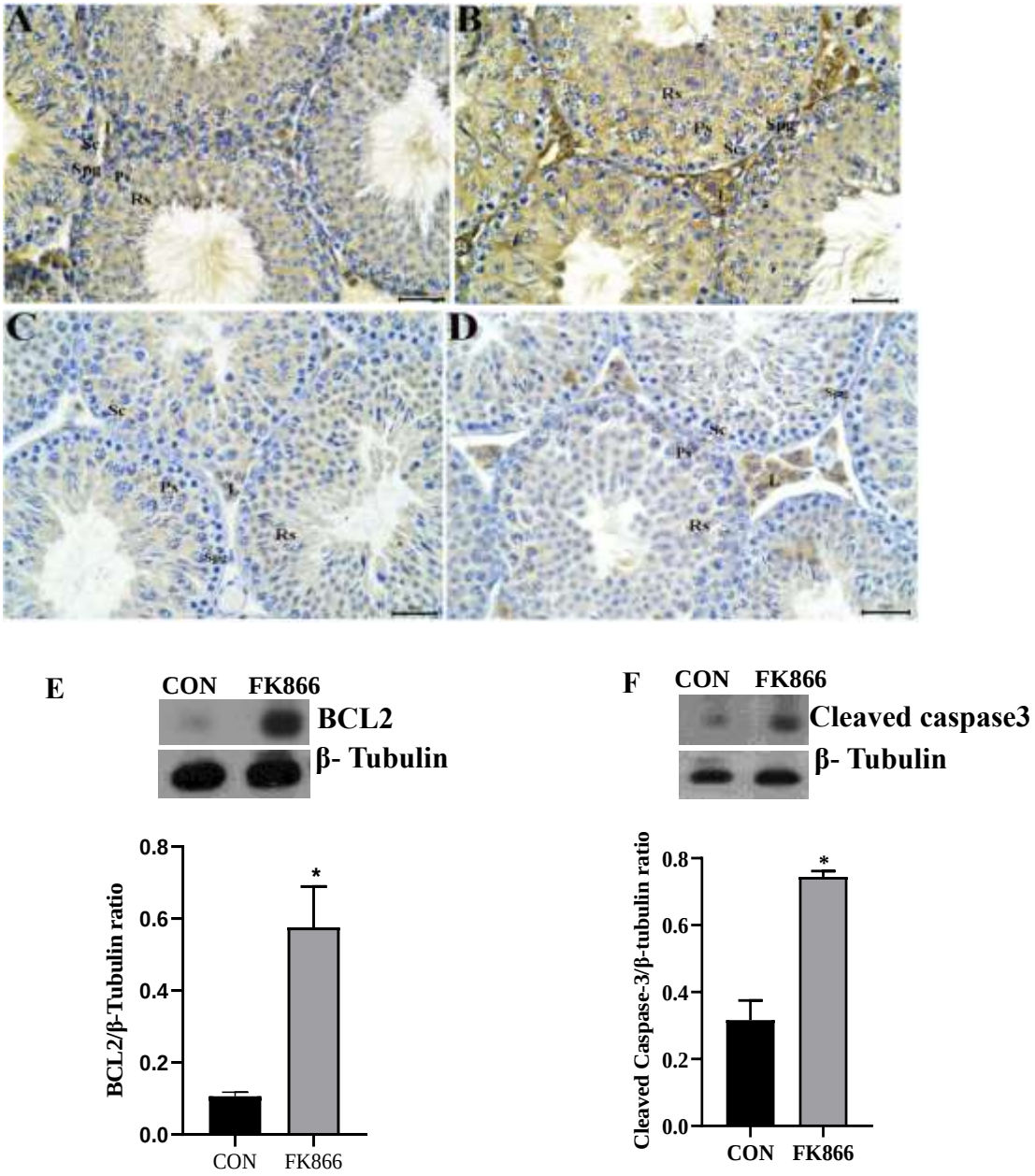

Figure-2.4- Changes in the immunolocalization and expression of BCl2 and cleaved caspase3 after visfatin inhibition by FK866 (1.5 mg/kg). FK866 treatment showed intense immunostaining of (A-B) BCl2 and (C-D) cleaved caspase3 in the Leydig cells and spermatocytes compared to control. Expression of (E) BCl2 and (F) cleaved caspase3 was also increased in the FK866 treated group compared to the control group. Data are represented as mean±SEM (n=5 per group). (Spg- Spermatogonia, Ps- Primary spermatocytes, Rs-Round spermatid, L- Leydig cells, Sc- Sertoli cells).

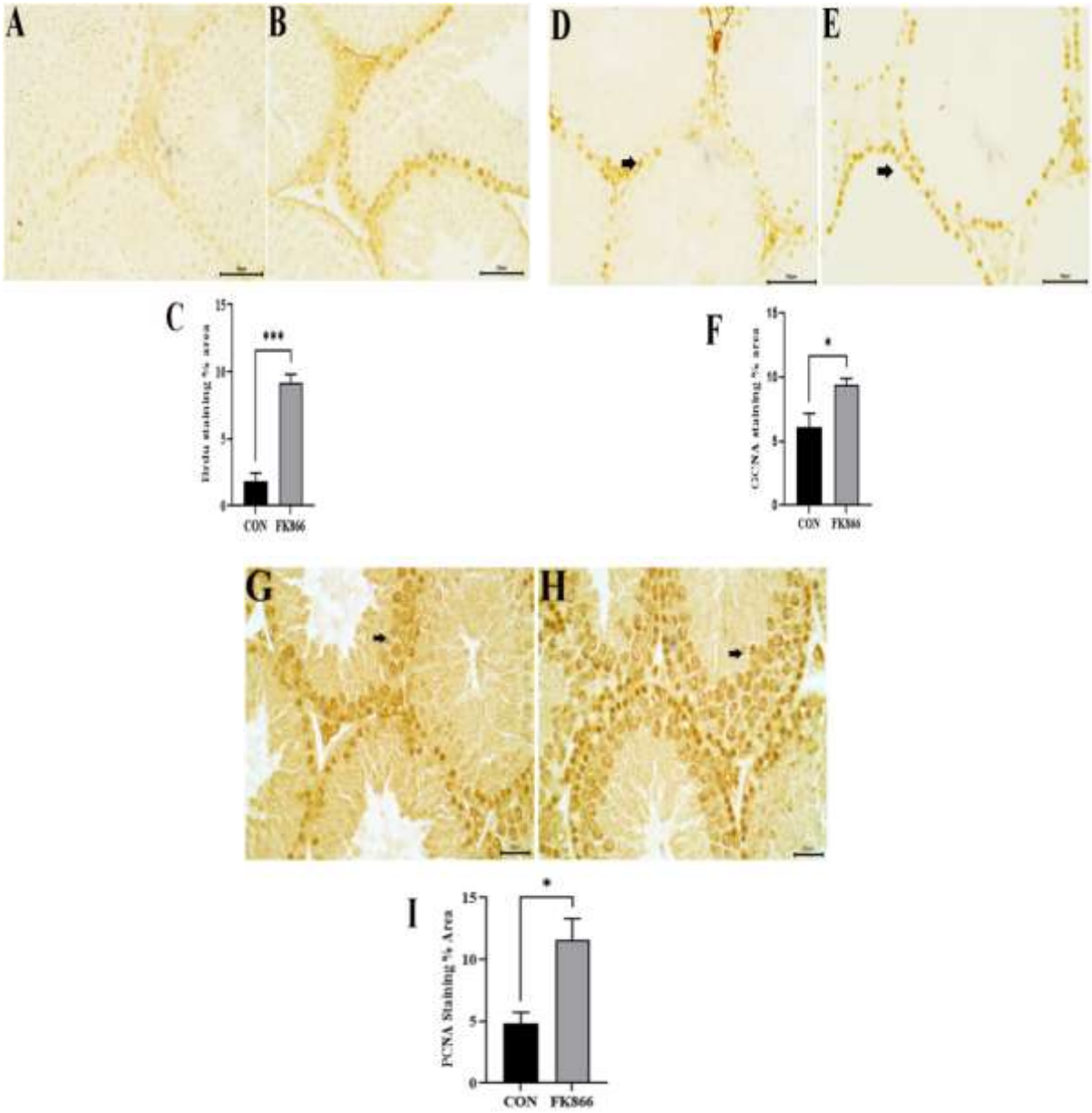

Figure-2.5- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on BrdU incorporation and localization of GCNA and PCNA. The immunostaining of (A-B) BrdU, (D-E) GCNA and (G-H) PCNA showed more positive cells in the testis of FK866 treated group compared to control group. The (C) BrdU, (F) GCNA and (I) PCNA staining areas were also significantly ($p<0.05$) higher in FK866 treated group compared to the control.

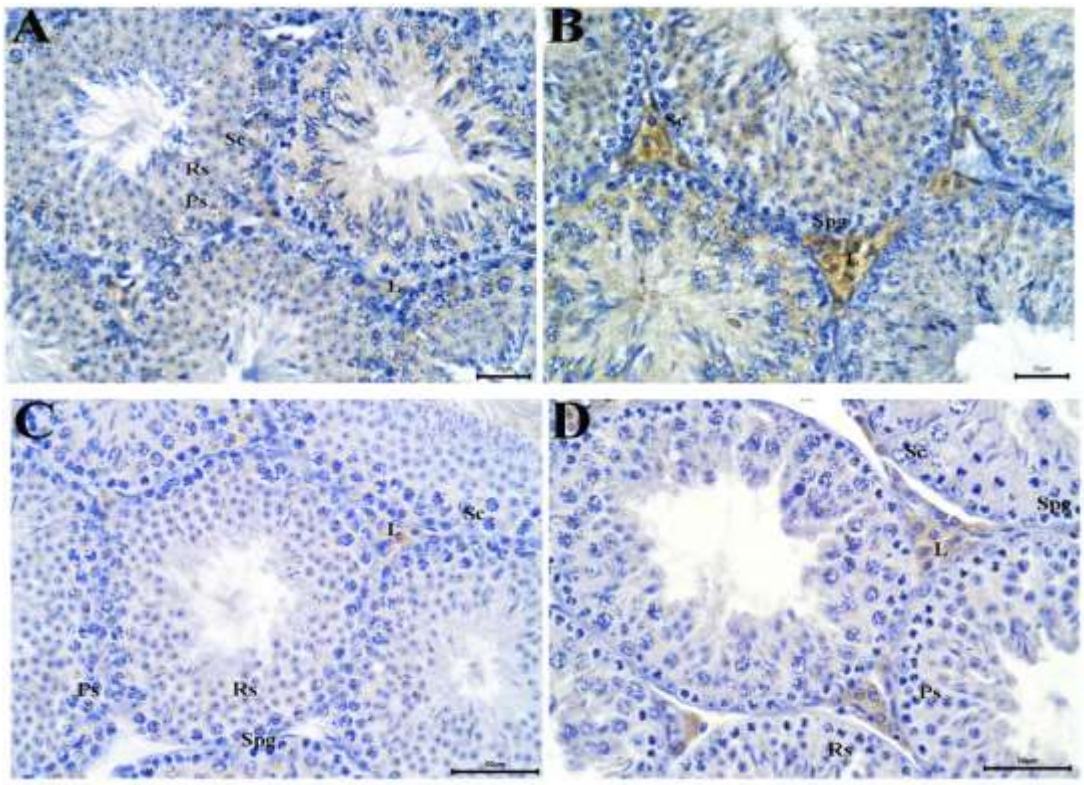

Figure-2.6- Changes in the immunolocalization of 17βHSD and 3βHSD after visfatin inhibition by FK866 (1.5 mg/kg). The immunostaining of 17βHSD was observed more in the Leydig cells of (A) FK866 treated group than (B) control group, the treatment of (C) FK866 also showed an increased abundance of 3βHSD in the Leydig cells compared to Leydig cells of (D) control testis. Data are represented as mean±SEM (n=5 per group). (Spg- Spermatogonia, Ps- Primary spermatocytes, Rs-Round spermatid, L- Leydig cells, Sc- Sertoli cells).

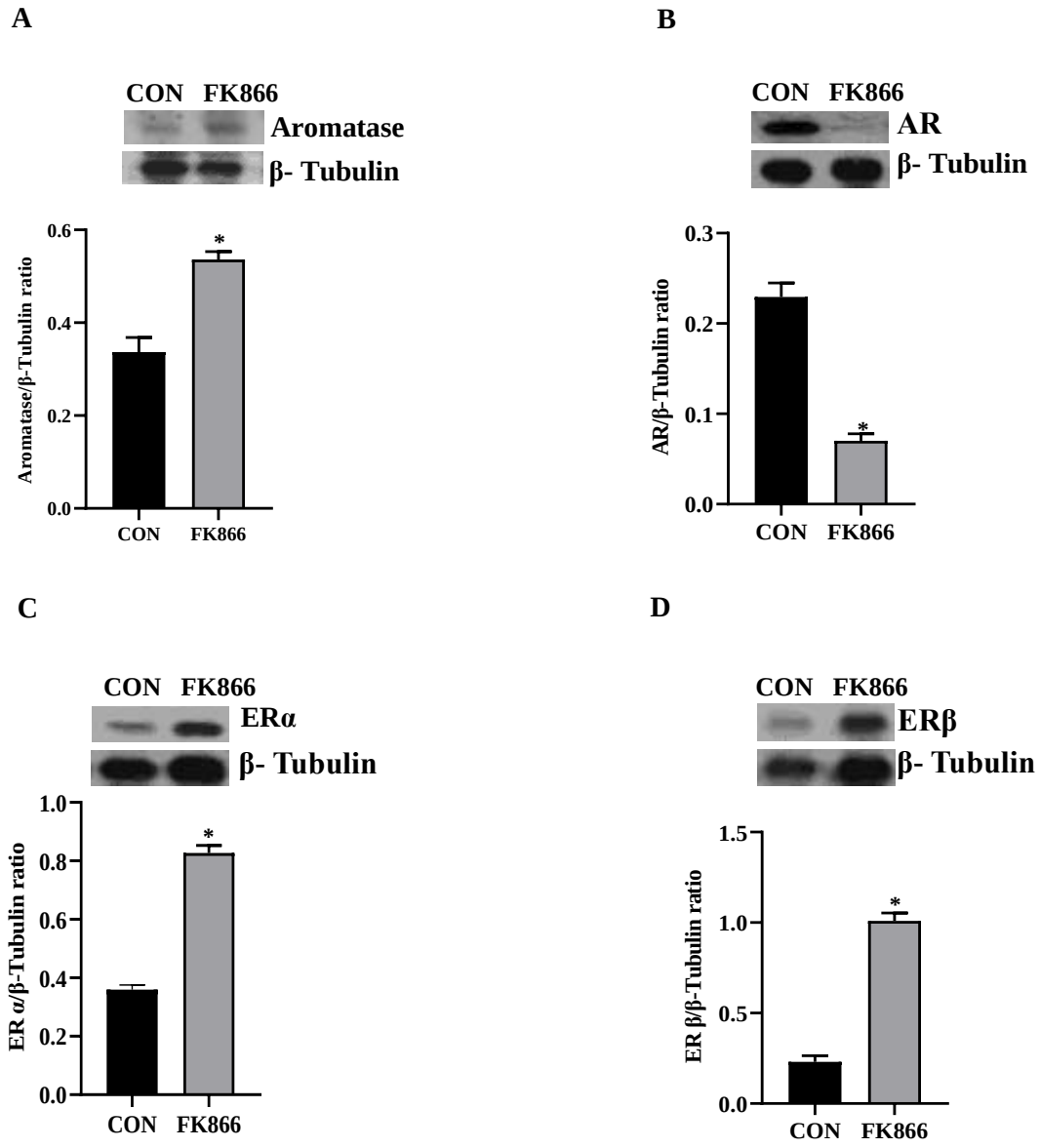

Figure-2.7- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on the expression of aromatase, AR, ERα and ERβ. The expression of (A) aromatase significantly ($p<0.05$) increased in the FK866 treated mice compared to the control**.** The expression of (B) AR was significantly declined in the testis of FK866 group compared to the control group. Expression of (C) ERα and (D) ER β were significantly ($p<0.05$) increased in FK866 group compared to control. Data are represented as mean±SEM (n=5 per group).

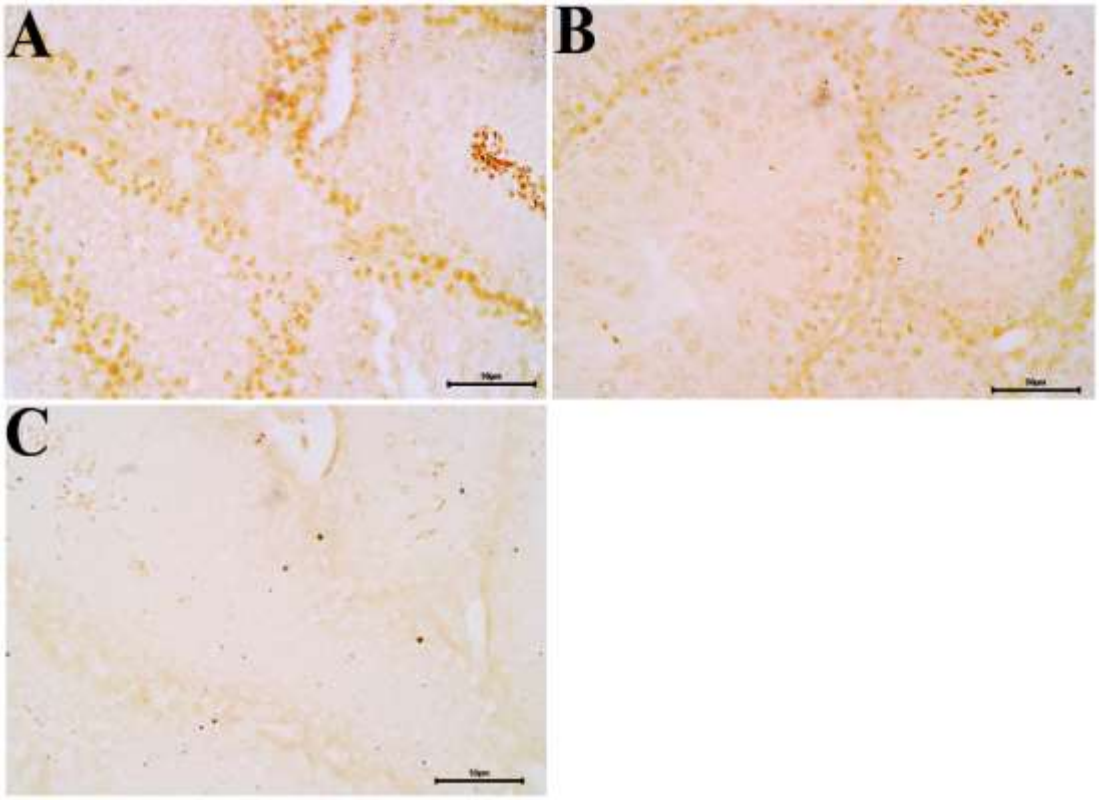

Figure-2.8- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on the localization AR. The seminiferous tubule of control group (A) showed more staining of AR compared to FK866 treated group (B). No staining was found in (C) negative control. Data are represented as mean±SEM (n=5 per group).

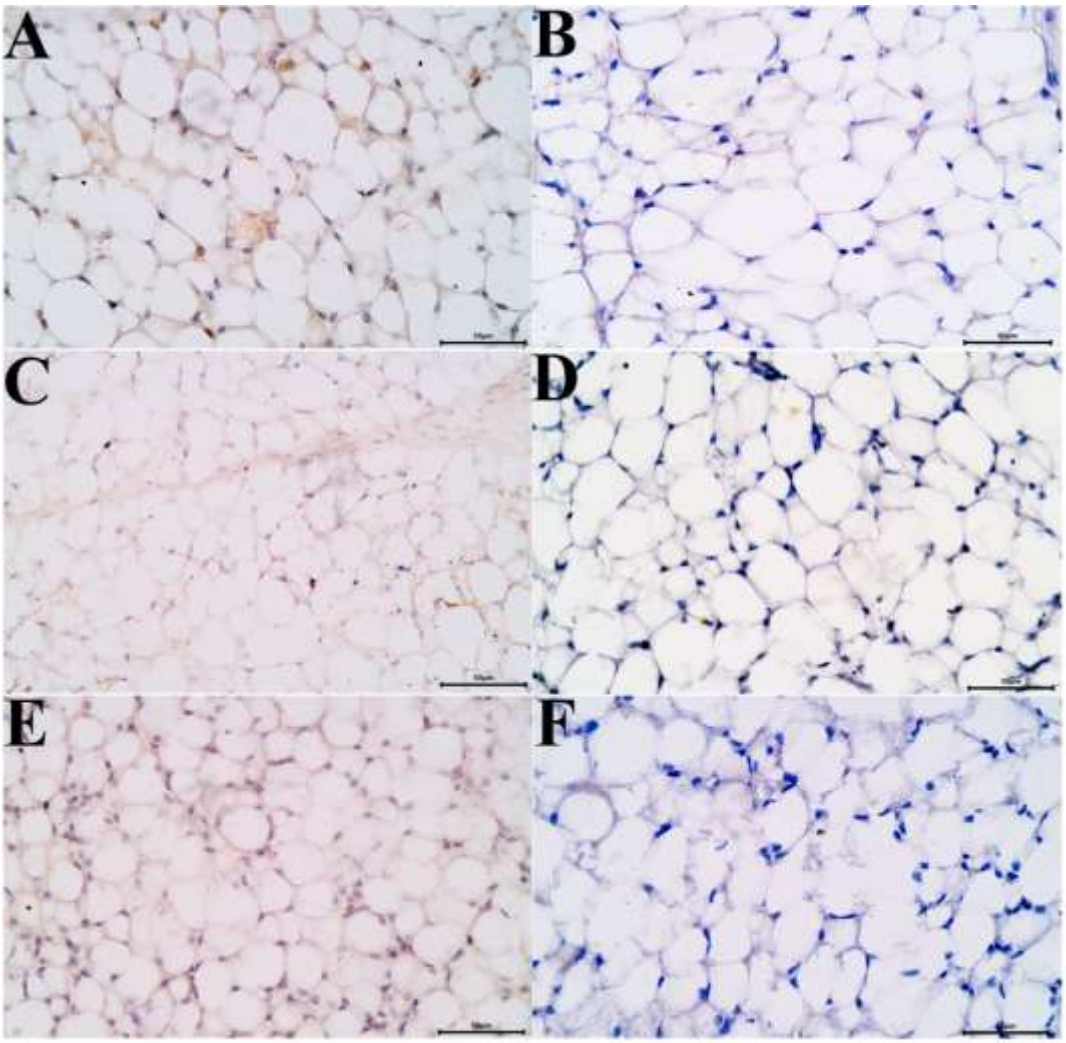

Figure-2.9- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on visfatin localization in the Subcutaneous adipose tissue (SAT), Visceral adipose tissue (VAT) and Epididymal adipose tissue (EAT). Immunolocalization of visfatin showed mild staining in SAT, (A), VAT (C) and EAT (E) of control group. No immunostaing of visfatin was noticed in the SAT (B), VAT (D) and EAT (F) of FK866 treated mice.

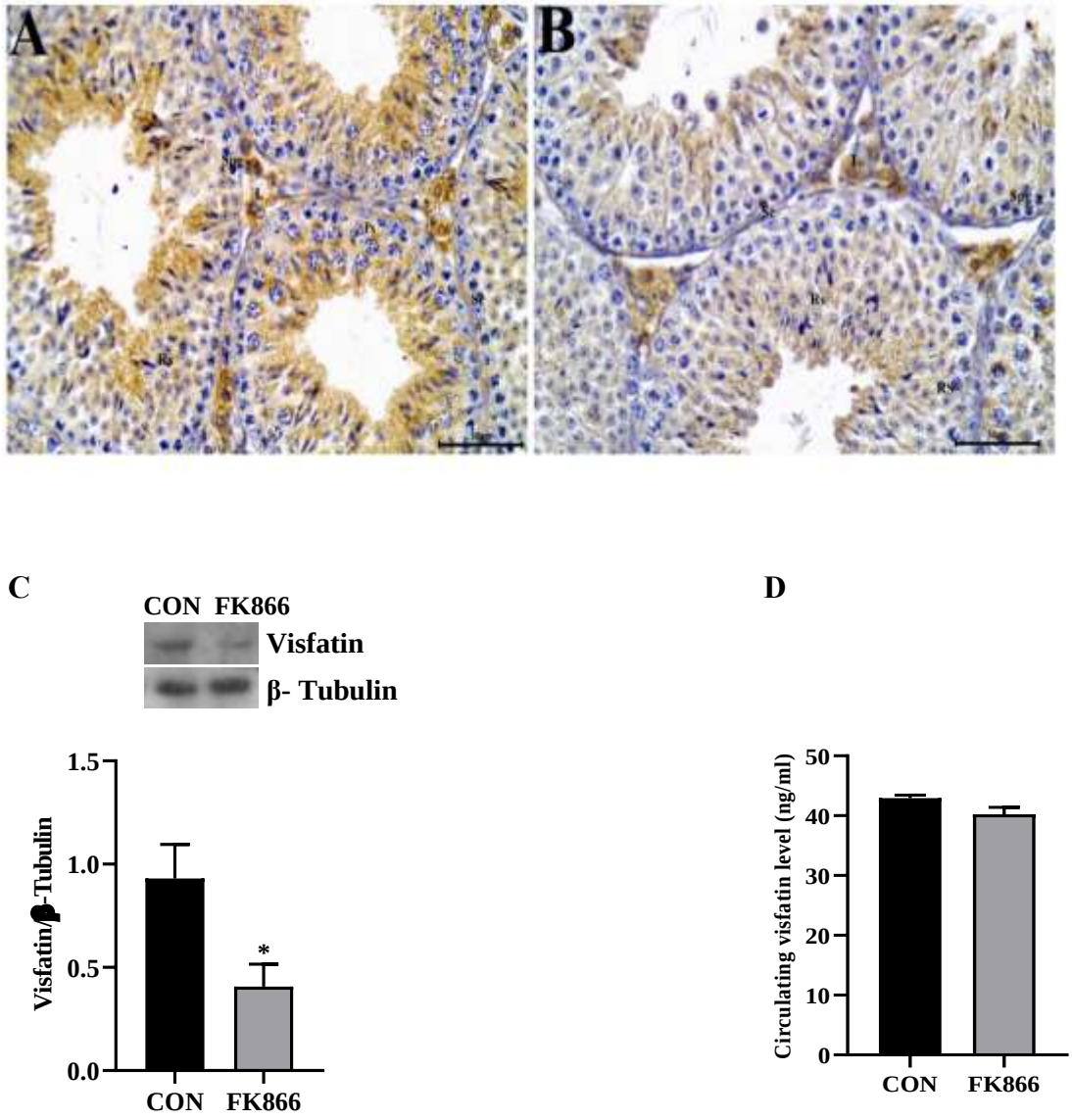

Figure-2.10- Effect of visfatin inhibition by FK866 (1.5 mg/kg) on the immunolocalization, and expression of testicular visfatin and circulating visfatin levels. Immunostaining of visfatin showed intense staining in the, spermatocytes, round spermatid and Leydig cell of control mice testis (A). Mild immunostaining was noticed in the spermatocytes, round spermatid and Leydig of FK866 treated mice testis (B). Expression of testicular visfatin significantly down-regulated in the FK866 treated mice testis compared to the control (C). The circulating visfatin levels slightly decreased in the FK866 treated mice (D). Data are represented as mean±SEM (n=5 per group). (Spg- Spermatogonia, Ps- Primary spermatocytes, Rs- Round spermatid, L- Leydig cells, Sc- Sertoli cells).

Chapter 3

Intra-testicular visfatin inhibition disrupts androgen and estrogen signalling in the mouse testis

Introduction

Adipokines secreted mainly from the white adipose tissues are an endocrine factor that plays a vital role in energy metabolism and serves as an important link between male reproduction and energy metabolism **(Bongrani et al., 2019).** Visfatin was one of the adipokines expressed in the gonads in the like pigs, bovine, human that play a vital role in energy metabolism as well as in reproductive functions such as folliculogenesis, spermatogenesis and steroidogenesis in the ovary and testis of turkeys and chickens **(Diot et al., 2015; Ocon-Grove et al., 2010).** In chicken, the testicular and plasma visfatin levels were also found to significantly elevate as the chicken attained sexual maturity (**Ocon-Grove et al., 2010)**. Studies have also demonstrated that the sperm concentration and sperm count in men from infertile couples have a negative correlation with seminal plasma visfatin levels **(Anagnostopoulou et al., 2022).** It is suggested that human testicular cells also produce visfatin since its level was significantly higher in the seminal plasma compared to the serum level **(Thomas et al., 2013).** In male rodent, the expression of visfatin was found in the sperm, spermatocytes and Leydig cells of rat testis; it is also shown that visfatin increases testosterone synthesis via direct stimulation of Leydig cell of rats **(Gurusubramanian and Roy, 2014; Hameed et al., 2012; Jeremy et al., 2017; Safya and Radwan, 2018).**

Visfatin expression has also been shown in different cell types of mice testis **(Annie et al., 2019)**. Maillard et al **(2017)** have shown that fertility in female mice could be affected by visfatin concentration via decreasing the production of LH from gonadotroph cells. Our laboratory has shown that visfatin differentially regulates ovarian functions in mice at different developmental stage **(Annie et al., 2020a; 2020b; 2021).** Visfatin treatment aged-female mice ameliorate folliculogenesis, oocytes quality, and ovarian function along with fertility potential (**Reverchon et al., 2016; Choi et al., 2012, Park et al., 2020).** To best of our knowledge, there is dearth of data on the role of visfatin in adult mice testis and thus it requires further investigation. Recently we have shown that testicular visfatin expression are developmentally regulated in the mice, where visfatin has inhibitory role in the

testicular functions in the infantile mice and at early pubertal stage, visfatin also regulates testicular functions by modulating androgen and estrogen receptor **(Rempuia et al., 2023;2024).**

Safya and Radwa, **(2018)** have also shown the protective role of visfatin against stress-induced gonadal impairment in the male rats. In male mice, it has been shown that dexamethasone mediated decreased testosterone levels was associated with decreased testicular visfatin and suggested that visfatin regulates testosterone secretion **(Annie et al., 2019).** The heat-stressed induced testosterone synthesis was also associated with decreased testicular visfatin expression in the mice **(Jerang et al., 2024)**. Based on the above-mentioned literatures, it is evident that visfatin regulates gonadal functions in different species; however, the direct role of visfatin has not been investigated in the testicular functions of adult mice testis. It has been shown that the intra-testicular injection of any agent is effective mode to study the direct role in the testis without systemic metabolism **(Russell et al., 1987).** Thus, it is hypothesised that disruption of intra-testicular visfatin by FK866 would modulate the testicular functions such as hormone secretion, proliferation and apoptosis. Therefore, the aim of the present study was to investigate the direct role of visfatin in mice testicular functions by intra-testicular injection of visfatin inhibitor, FK866.

Materials and Methods

Animal maintenance

All the procedures were carried out according to the guidelines provided by the Mizoram University Institutional Animal Ethical Committee (Protocol Approval number- MZUIAEC 21-22-05), Mizoram University, Mizoram, India and were performed in accordance with relevant guidelines and regulations, and the arrive guideline 2.0. Animals were kept in the animal house facility where the room temperature was maintained at 25±2°C, with the availability of food and water provided ad libitum. The room was well-ventilated and maintained a 12-hour light-dark cycle. 15 males, healthy Swiss albino mice, at least 3 months old were included in the study.

Experimental design

To find out the direct role of visfatin in mice's testis, an intra-testicular injection of FK866, a visfatin inhibitor, was used. The adult 3 months old male Swiss albino mice with body weight 22-25 g were selected. Mice were anesthetized by ketamine and xylazine injection for intra-testicular injections; the needle was inserted into one pole of the testis and pushed in the opposite direction for the uniform distribution of FK866 throughout the testicular tissue. Mice were randomly divided into three groups (n=5 per group):-

1. Control (CON) group received PBS as a vehicle.
2. 24H FK866 group-Received intra-testicular FK866 at doses 1.5 mg/kg in both testis and post 24 hours, animals were sacrificed.
3. 1W FK866 group-Received intra-testicular FK866 at doses 1.5 mg/kg in both testis and post 1 week FK866 group animals were sacrificed. The dose of FK866 was selected from a previous study, where it was given intra-peritoneal **(Rempuia et al., 2023).**

Animals were sacrificed immediately by decapitation under mild anaesthesia (ketamine and xylazine), blood was collected, and the serum was separated out from blood sample within 1 hour after centrifugation at 300 rpm and stored at –20° C until it was used for hormonal analysis. Testis was dissected out from the scrotal sacs, testis weight were recorded and kept in freeze or fix (Bouin’s fluid for 24 hours). This was later used for further analysis i.e. histopathological examination, immunohistochemistry, immunoblots and enzyme assay.

Histopathological analysis

Histopathological studies were performed using testes fixed with the Bouin’s fluid from each group (n=5 per group). Tissues were transferred to 70% ethanol after 24 hours to avoid brittleness, which undergo further dehydration in a series of graded ethanol (90% and 100%) twice for 1 hour each. The tissues were then cleared in xylene for a minute and embedded in a paraffin wax block. Tissue blocks were sectioned at 7 µm thickness with Leica microtome (model RM2125 RTS) and then

fixed at sterile glass slides coated with poly-l-lysine. The tissues fixed in glass slides then undergo tissue processing and staining with hematoxylin and eosin **(Bancroft and Gamble, 2002) .** Sections were then observed under a microscope and photographed using a camera-mounted binocular microscope (Model E200, Nikon, Tokyo, Japan).

Histomorphometry

The same histological sections (n=5 per group) were used for histomorphometric studies; seminiferous tubules diameter (STD) **(Guneli et al., 2008)**, germinal epithelium height (GEH) **(Franca et al., 2003)** and lumen diameter (LD) were measured with an ocular micrometer at 40X magnification. Thirty round seminiferous tubules were selected, and measurements were taken for LD, STD and GEH.

Immunohistochemistry

Fixed testis (n=5 per group) were also used for immunohistochemical studies, processed for paraffinized tissue block and sectioned with microtome as described previously **(Jeremy et al., 2019).** The tissue sections were embedded in sterile glass slides coated with poly-L-lysine and incubated at a slide warming table overnight at 37°C. The slides were then deparaffinized with xylene and rehydrated with 100%, 90% and 70% alcohol for 2 changes at 10 min each. After which it was kept in PBS (1X) for 10 minutes and incubated in goat-blocking serum [goat serum 1:100 diluted in PBS (Lot# A0515, Santa Cruz Biotechnology, Inc., CA, USA)] for 30 minutes at room temperature, followed by overnight incubation at 4°C in a humidified chamber using primary antibodies. The details of antibodies used for immunohistochemistry have been shown in **Table 1.A (**All the antibodies used were commercially procured and have been used in mice testis). Slides were then undergo primary antibody washed in PBS and probed with HRP-conjugated secondary antibodies (**Table 1.A**, goat anti-rabbit IgG secondary antibody and goat anti-mouse IgG anti-mouse secondary antibody for respective primary antibodies) for 3 hours at room temperature. Unbound antibody was washed off by PBS, followed by incubation in a solution containing 0.6 mg/ ml solution of 3, 3-diaminobenzidine tetra hydrochloride

dihydrate (DAB) in Tris–HCl (pH 7.6) and 0.01% H_2O_2 at room temperature till brown colour developed. The reaction was stopped using distilled water and counterstained with hematoxylin except for GCNA, PCNA and AR. Slides were then dehydrated in a series of graded alcohols (70%, 90% and 100%), cleared in xylene and mounted with DPX for observation under a Nikon binocular microscope (Model E200, Nikon, Tokyo, Japan).

Hormone and visfatin Assays

Serum testosterone, estrogen, androstenedione and progesterone (n=5 per group) levels were also measured by commercial ELISA kits. Quantification of serum testosterone, androstenedione and estrogen were estimated by using commercial ELISA kit as per manufacturer's instruction kit (Testosterone Cat# DKO002, DiaMetra, Italy; androstenedione Cat# DKO008, DiaMetra, Italy, Estradiol Cat# DKO003, DiaMetra, Italy). Analytical sensitivity of testosterone ELISA kit i,e. lowest concentration that could be distinguished from calibrator 0 is 0.10 ng/mL at 95% confidence limit, and the assay measuring range is 0.10 ng/mL to 8.63 ng/mL. Intra assay and inter assay coefficient of variation were ≤7.0% and ≤8.3% respectively for testosterone. Analytical sensitivity of androstenedione ELISA kit i,e. lowest concentration that could be distinguished from calibrator 0 is 0.10 ng/mL at 95% confidence limit, the assay measuring range is 0.60 ng/mL to 2.7 ng/mL. Intra assay and inter assay coefficient of variation were ≤10.0% and ≤9.5% respectively for androstenedione. Analytical sensitivity of estradiol ELISA kit i,e. lowest concentration that could be distinguished from calibrator 0 is 8.68 pg/mL at the 95% confidence limit. The assay measuring range is 29.6 pg/mL to 2000 pg/mL. Intra assay and inter assay coefficient of variation were ≤9% % and ≤10% respectively for estrogen.

Serum progesterone (Cat# 100-02, Alkor Bio, Russia) was also estimated by using commercial ELISA kit as per manufacturer's instruction. Analytical sensitivity of SteroidEIA-Progesterone kit i,e. lowest concentration that could be distinguished from calibrator 0 is 0.5 nmol/L. The assay measuring range is 0.5 nmol/L to 100 nmol/L. Intra assay and inter assay coefficient of variation were ≤8.8% % and ≤11.5% respectively for progesterone. Absorbance levels were read at 450nm using a

Microplate ELISA Reader (Erba Lisa Scan EM, Trans Asia Biomedical Ltd, Mumbai, Maharashtra, India).

Circulating visfatin levels was also measured with a Mouse Visfatin ELISA kit (Cat No: K02-0598; Los Angeles). 40 µl of samples and 10 µl of conjugate were loaded in each well, 50 µl HPR conjugate was added to sample well and was incubated for 1 hour at 37°C. The wells were then washed with washing buffer, 50 µl of substrate A and B were added to each well and was incubating in the dark for 10 min at 37°C, after that stop solution was added. Absorbance was read at 450 nm using ELISA reader. Sensitivity of mouse visfatin ELISA kit is 7.413 pg/mL. The assay measuring range is 75 pg/mL to 1200pg/mL.

Western blot analysis

Testis tissue collected after visfatin inhibition were processed for western blot, samples were homogenate with lysis buffer (0.01 M Tris-HCl, pH 7.6, 1.0 mM EDTA, pH 8.0, 0.1 M NaCl, 100 µg/ml PMSF) and protein concentrations were estimated by Bradford method **(Bradford, 1976)**. To the sample homogenate, gel loading buffer (1M Tris-Cl pH 6.80, 2 Mercaptoethanol, 10% SDS, glycerol and dH2O) was added in equal ratio after protein estimation and boiled for 10 minutes. Samples (50 µg/well) were then loaded along with the protein marker and resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoresed at 100V for 3 hours. The resolved proteins were transferred in a polyvinylidine flouride membrane (Millipore India Pvt. Ltd., Bangalore, India) by using wet transfer for overnight at 4° C. Membrane blocking was done with 5 % non-fat dry milk with PBS 1X (10 mM, 7.5 pH) and 0.1 % Tween 20 solution for 30 min at room temperature, followed by overnight incubation at 4° C with primary antibodies. The details of antibodies used for western blotting have been shown in **Table 1.B** (All the antibodies used were commercially procured and have been used in mice testis). The membrane blots were then washed in PBS-Tween 20 for 2 changes and incubated with horse-radish peroxidase conjugated secondary antibody **(Table 1.B)**, at room temperature for 4 hours. After secondary washing was done, the blots were then finally detected by chemiluminescence (ECL) (cat no- 1705060,

BioRad, Hercules, CA, USA) and developed in x-ray film. ImageJ software (1.38x, NIH, Bethesda, MD, USA) was used for quantification of protein band. The density of each band was normalized to the density of the β-Tubulin band that was used as an internal control.

Steroidogenic enzyme assay

Testicular steroidogenic enzymes (n=5 per group) (3βHSD and 17βHSD) activities were also determined. Testicular tissue was homogenized using lysis buffer (0.01 M Tris-HCl, pH 7.6, 1.0 mM EDTA, pH 8.0, 0.1 M NaCl, 100 µg/ml PMSF) and the homogenate was centrifuged at 12, 000 *g* for 15 minutes at 4° C. The supernatants were then collected, and protein concentration was measured by the Bradford method **(Bradford, 1976)**. The activity of 3βHSD was measured by following the previous method given by Talalay et al, **(1962)**; samples containing 200 µg of protein was mixed with 250 µl of 0.01M Phosphate-Buffered Saline (pH 8.9) and 30 µg Dehydroepiandrosterone (DHEA) (TCI, America, United States, CAT #:D0044) dissolved in 10 µl of ethanol was also added to the mixture. The enzyme activity was then determined after addition of 50 µl of NAD (0.5 µM) at 340 nm in spectrophotometer against the blank. The enzyme activity was expressed as µmol/ min/mg protein.

The activity of 17βHSD was also determined using method described by Jarabak et al, **(1962)**. The supernatant containing 200 µg protein was mixed with 250 µl of 0.01M Phosphate-Buffered Saline (pH 10.2) and 10 µl of ethanol that contained 0.3 µM testosterone (HiMedia, Maharashtra, India, SKU. RM1848-5G). Enzyme activity was then determined after addition of 50 µl of NAD (0.5 µM) at 340 nm in spectrophotometer against the blank and was expressed as µmol/min/mg protein.

Statistical analysis

All data were expressed as the mean ± standard error of the mean, and analysis of data was done by One-way analysis of variance (ANOVA) followed by Post hoc

Tukey's test. Distribution of normality was checked and all the data were passed by Shapiro-Wilk test. The level of significance was considered as $p<0.05$. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

Results

Effect of intra-testicular visfatin inhibition by FK866 on the body and testis weight

Intra-testicular inhibition of visfatin did not show significant changes in the body weight after 24 hours compared to control mice, a significant decrease in the body weight was observed after 1 week of visfatin inhibition compared to post 24 hours and control groups **(Figure-3.1A)**. The testis weight was significantly ($p<0.05$) decreased in FK866 treated mice in both 2H FK866 and 1W FK866 groups compared to control group (**Figure-3.1B).** However, GSI to body weight was significantly decreased in 24H FK866 compared to control and 1W FK866 group **(Figure-3.1C)**

Effects of intra-testicular visfatin inhibition by FK866 the testis histology and histomorphometric parameters

To unravel whether the intra-testicular visfatin affects the testicular histology and histomorphometric parameters, both histopathological and histomorphometric analysis of the testis was done after FK866 administration. The histological examination of the control testis showed a regular and normal histoarchitecture of the testis **(Figure-3.2A)**. The seminiferous tubules, after 24 hours of intra-testicular treatment with FK866, showed noticeable changes in the testicular anatomy; some tubules showed lessening round spermatids and sperm **(Figure-3.2B)**. However, no significant structural deformation was seen in the testis after 1 week of intra-testicular treatment with FK866 **(Figure-3.2C)**.

Histomorphometric examination also showed significant changes in the seminiferous tubule diameter (STD), germinal epithelium height (GEH) and lumen diameter (LD) after intra-testicular administration of FK866. The STD and LD of the 24H FK866

group showed a significant ($p<0.05$) increase compared to 1W FK866 and the control group. However, the STD and LD of the 1W FK866 group were significantly ($p<0.05$) decreased compared to the control group **(Figure-3.2D, F)**. The GEH was significantly ($p<0.05$) declined in the 24H FK866 group compared to the control and 1W FK866 group; the GEH of the 1W FK866 group also showed significant ($p<0.05$) decreased compared to the control group **(Figure-3.2E)**.

Effect of intra-testicular visfatin inhibition by FK866 on the circulating testosterone, estrogen, androstenedione, progesterone and visfatin levels

In order to unravel the effect of visfatin on testicular steroidogenesis after intra-testicular administration of FK866, the circulating levels of testosterone, estrogen, and androstenedione levels were examined. The levels of male hormone, testosterone significantly ($p<0.05$) declined after 24 hours of intra-testicular treatment of FK866 compared to the control group, however elevation in the level of testosterone was observed after 1 week, but no statistically significant difference was seen compared to the control group **(Figure-3.3A)**. The circulating estrogen levels were found to be significantly ($p<0.05$) lower after 24 hours and 1 week of intra-testicular administration of FK866 compared to the control group **(Figure-3.3B)**. The levels of circulating androstenedione did not show any significant changes with the control group after visfatin inhibition **(Figure-3.3C)**. However, inhibition of visfatin by FK866 significantly ($p<0.05$) increased the circulating progesterone levels after 24 hours and 1 week compared to the control group **(Figure-3.3D)**. The circulating visfatin levels showed a slight decline post 1 week of FK866 treatment, however, it was not significant **(Figure-3.3E)**.

Effect of intra-testicular visfatin inhibition by FK866 on the localization, expression and activity of steroidogenic enzymes (3βHSD and 17βHSD)

To evaluate the Leydig cell activity in relation to steroidogenesis, immunostaining of 3βHSD was performed**.** The immunostaining of 3βHSD exhibited its abundance in the Leydig cells of the control group compared to 24H FK866 and 1W FK866 group **(Figure-3.4A, B, C)**. The enzyme activity of 3βHSD and 17βHSD was also significantly ($p<0.05$) decreased in 24H FK866 and 1W FK866 group compared to

the control group **(Figure-3.4D, E)**. The expression of 17βHSD was also significantly ($p<0.05$) decreased in 1W FK866 group compared to the control and 24H FK866 groups **(Figure-3.4F)**.

Effect of intra-testicular visfatin inhibition by FK866 on the localization and expression of aromatase, cleaved caspase3, Bcl2 and visfatin

The immunostaining of aromatase showed intense staining in the Leydig cells of control **(Figure-3.5A)** and 24H FK866 **(Figure-3.5B)** group, a mild immunostaining was observed in the Leydig cells of 1W FK866 **(Figure-3.5C)**. The expression of aromatase and visfatin was also decreased significantly ($p<0.05$) in 1W FK866 group compared to control and 24H FK866 group **(Figure-3.5D, E)**. The expression of cleaved caspase3 was significantly ($p<0.05$) declined in the 1W FK866 group compared to other groups, a slight increase was also observed in the 24H FK866 group from the control group; however, the data did not show any significant changes **(Figure-3.5F)**. The expression of Bcl2 was significantly ($p<0.05$) decreased in 24H FK866 group compared to the control group, a further significant ($p<0.05$) decreased in the expression of Bcl2 from control and 24H FK866 group was observed in the 1W FK866 group **(Figure-3.5G)**.

Effect of intra-testicular visfatin inhibition by FK866 on the localization and expression of AR

Immunolocalization of AR showed increased abundance in the testis of control and 24H FK866 group, whereas mild staining was observed in 1W FK866 group **(Figure-3.6A, B, C)**. The expression of AR also significantly ($p<0.05$) declined in the 1W FK866 group compared to the control and 1W FK866 group **(Figure-3.6D)**.

Effect of intra-testicular visfatin inhibition by FK866 on the expression of ERs

To unravel the effect of intra-testicular administration of FK866 on estrogen receptors, the expression of ERα and β were analyzed by western blot methods. The expression of ERα and ERβ significantly ($p<0.05$) decreased in the testis of 1W FK866 group compared to the control and 24H FK866 group **(Figure-3.7A, B)**. No

significant changes were observed in the expression of ERs between the control and 24H FK866 groups.

Effect of intra-testicular visfatin inhibition by FK866 on the localization of GCNA and PCNA proteins

To evaluate the testicular germ cell proliferation in the testis after intra-testicular administration of FK866, the immunolocalization of GCNA and PCNA was performed. The localization of GCNA showed mild immunostaining in the 24H FK866 group, an increase in the immunostaining was observed in the testis of Control **(Figure-3.8A)** and 1W FK866 **(Figure-3.8C)** group compared to 24H FK866 group **(Figure-3.8B)**. The proliferating marker, PCNA also showed decreased immunostaining in 24H FK866 group **(Figure-3.8E)** compared to control **(Figure-3.8D)** and 1W FK866 group **(Figure-3.8F)**.

Discussion

The present study has investigated visfatin's direct role in mice's testis by intra-testicular injection of visfatin, inhibitor, FK866. The expression of testicular visfatin has been shown in various species including mice **(Jeremy et al., 2017; Riammer et al., 2016; Ocon-Grove et al., 2010; Rempuia et al., 2023).** *In vitro* study has shown that testicular visfatin regulates steroidogenesis in the Leydig cells of rat **(Hameed et al., 2012).** However, the direct role of visfatin on the testis functions at the adult stage has not been shown in any species. The intra-testicular injection of FK866 led to pathological changes in the testis histoarchitecture along with morphometric parameters after 24 hours. However, the testis did not show structural damage after 1 week. It should be noted that sperm and round spermatids showed depletion mainly after visfatin inhibition. Thus, visfatin have an essential role in the testicular functions by regulating spermatid and sperm maturation. Previous studies have also demonstrated that visfatin is mainly localized in these cells (spermatid and sperm) and pathological condition like diabetes and aging has pronounced negative impact on the testicular functions **(Gurusubramanian et al., 2014; Jeremy et al., 2017).**

The testis is a proliferating organ, and histoarchitecture changes after visfatin inhibition have prompted us to examine the proliferation. The localization of two proliferating markers, GCNA and PCNA, was decreased only in the post-24H FK866 treatment. These results demonstrated that visfatin is important for germ cell proliferation in adult mice testis. Visfatin has also been shown to stimulate human granulosa cells **(Reverchon et al., 2013)** and cancer cells (**Zhao et al., 2020)**. However, recently we demonstrated that visfatin inhibition by FK866 stimulated germ cell proliferation in mice's infantile and early pubertal testis, which suggest its anti-proliferative role **(Rempuia et al., 2023; 2024).** Moreover, previous study has also shown that visfatin stimulates germ cell proliferation in the ovary of early pubertal mice **(Annie et al., 2019).** Thus, it may be suggested that visfatin has a differential role in the germ cell proliferation in the testis depending on the developmental stage. Germ cell proliferation and cell death by apoptosis is an integral process of spermatogenesis **(Martinčič et al., 2001; Shaha et al., 2010)**. It has also been demonstrated that despite cell proliferation, visfatin also regulates apoptosis **(Kang et al., 2024)**. The expression of cleaved caspase3 was increased, and Bcl2 expression was decreased in the testis of post 24H FK866 treatment. These results show visfatin inhibits apoptosis of germ cells in the testis. This finding coincides with our previous results, where visfatin suppresses apoptosis in the testis of infantile and pubertal mice **(Rempuia et al., 2023; 2024).**

Furthermore, after 1 week of FK866 treatment, testicular expressions of cleaved caspase and Bcl2 were down-regulated compared to the control. Despite normal testicular proliferation, the suppressed apoptosis in the testis after 1 week of FK866 treatment needs further investigation. However, it may also be suggested that even after 1 week of FK866 treatment, testicular functions have not been resumed nearly to control. Since we have not observed the effects of FK866 after 1 week, thus suggesting further resumption of testicular functions would be only speculative. These points are the important limitations of the present work.

The levels of progesterone increased in the FK866-treated groups. Progesterone is synthesized from pregnenolone by the 3βHSD enzyme **(Payne and Hales, 2004).** Thus, we have measured the enzyme activity. The enzyme activity of 3βHSD and

abundance in the Leydig cells decreased after 24 hours and 1 week of FK866 treatment. Increased progesterone with decreased 3βHSD enzyme activity remains unclear from our findings.

The androstenedione levels also remained unchanged in both groups, including the control group. The testosterone and estrogen levels declined in the post-24 hours of the FK866-treated group, followed by elevated testosterone levels in a post-a-week group. However, estrogen levels remained declined. Since testosterone is formed from androstenedione by the 17βHSD enzyme, testosterone is aromatized to estrogen by aromatase **(Payne and Hales 2004)**. Our results showed that the enzyme activity of 17βHSD and expression decreased in the post-24 hours of FK866 treatment, which coincides with low testosterone levels. These results suggest that visfatin has a stimulatory role in testosterone biosynthesis by elevating 17βHSD enzymes. The role of visfatin in testicular testosterone synthesis is scant. However, previous studies have shown the stimulatory role of visfatin in the Leydig cells **(Hameed et al., 2012).**

Despite no change in the aromatase expression, the estrogen levels in the post-24 hours of the FK866 group could be due to low testosterone levels. The 17βHSD enzyme activity was lowest in the post-1 week of FK866 treatment. However, the elevated testosterone levels in this group could be due to decreased aromatase expression. The reason for reduced aromatase, 17βHSD, and 3βHSD enzymes in the post 1-week of FK866 treated group remain unknown. However, it may be suggested that the effects of visfatin inhibition could not be resumed to a normal level, even after 1 week of FK866 treatment. We have shown that visfatin inhibition decreased aromatase expression in infantile mice testis **(Rempuia et al., 2023)**; however, in the early pubertal testis, visfatin inhibition elevates aromatase expression **(Rempuia et al., 2024).** Our results also showed that FK866 treatment decreased testicular visfatin expression in post-1-week group; however, the circulating visfatin was not changed. The testicular expression of AR decreased after 1 week of FK866 treatment, along with decreased expression of ERs. These findings suggest that visfatin regulates the testicular AR and ER expression. Our recent study also showed that visfatin regulates the AR and ER expression differentially in the pubertal mice testis **(Rempuia et al.,**

2023). Most of our parameters were found to deviate from the control in the post-24 hours and 1 week of FK866 treatment, and these parameters could be attained up to control. Thus, the effect of visfatin inhibition could be prolonged, and further time-dependent study would be required to observe the lasting effects. It should also be noted that the expression of various protein showed changes in the FK866 treated testis, and it has also been suggested that ERβ expression at protein levels is not proper due to insufficient antibody validation of ERβ **(Andersson et al., 2017),** thus, gene expression of corresponding protein would have been a worthy to study. Although, this is an important limitation of our study.

To the best of our knowledge, data on the visfatin-mediated direct regulation of testicular functions are scant on mammals; however, to date, only Hameed et al. **(2012)** have shown the direct role of visfatin on testicular steroidogenesis. In conclusion, our results showed that visfatin regulates testicular steroidogenesis, proliferation and apoptosis in the adult testis and might have a stimulatory role in the testis. Furthermore, role of visfatin in the testicular functions in relation to pathological condition such diabetes and aging, would be important for better understanding of pathology of testis.

Table 1.A: Details of antibodies used for Immunohistochemical analysis.

Primary antibodies: Catalogue: Source	**Dilution of primary antibody**	**Secondary antibody: Catalogue: Source Dilution 1:400**	**Reference**
3βHSD-HRP (cat# sc-515120 HRP), Santa Cruz Biotechnology, Dallas, Texas, United States	1:50	_	Nicy et al., **2022**
Aromatase (cat# E-AB-64300), Elabscience, Houston, Texas, United States	1:200	Goat anti-rabbit (cat# E-AB-1102), Elabscience, Houston, Texas, United States	Marak et al., **2023**
Androgen receptor (PA5-16363), Invitrogen	1:50	Goat anti-rabbit (cat# E-AB-1102), Elabscience, Houston, Texas, United States	Rempuia et al., **2023**
GCNA (cat# 10D9G11), DSHB, University of Iowa, Dept. of Biology, Iowa, United States	1:200	Goat anti-mouse (cat# E-AB-1001), Elabscience, Houston, Texas, United States	Nicy et al., **2022**
PCNA (cat# sc-7907), Santa Cruz Biotechnology, Dallas, Texas, United States	1:100	Goat anti-rabbit (cat# E-AB-1102), Elabscience, Houston, Texas, United States	Nicy et al., **2022**

Table 1.B: **Details of antibodies used for western blot analysis.**

Primary antibodies: Catalogue: Source	**Dilution of primary antibody**	**Secondary antibody: Catalogue: Source Dilution 1:4000**	**Reference**
Aromatase (cat# E-AB-64300), Elabscience, Houston, Texas, United States	1:2000	Goat anti-rabbit (cat# E-AB-1102), Elabscience, Houston, Texas, United States	Rempuia et al., **2023**
Androgen receptor (PA5-16363), Invitrogen	1:250	Goat anti-rabbit (cat# E-AB-1102), Elabscience, Houston, Texas, United States	Rempuia et al., **2023**
ERα (cat# Bz1), DSHB, University of Iowa, Dept of Biology, Iowa, United States)	1:500	Goat anti-mouse (cat# E-AB-1001), Elabscience, Houston, Texas, United States	Rempuia et al., **2023**
ERβ (cat# CWK-F12), DSHB, University of Iowa, Dept of Biology, Iowa, United States)	1:500	Goat anti-mouse (cat# E-AB-1001), Elabscience, Houston, Texas, United States	Rempuia et al., **2023**
BCL2 (cat# sc-7382), (Santa Cruz Biotechnology, Dallas, Texas, United States)	1:1000	Goat anti-mouse (cat# E-AB-1001), Elabscience, Houston, Texas, United States	Rempuia et al., **2023**
Cleaved caspase3 (cat# E-AB-30004), Elabscience, Houston, Texas, United States)	1: 1000	Goat anti-rabbit (cat# E-AB-1102), Elabscience, Houston, Texas, United States	Rempuia et al., **2023**
Visfatin (NAMPT) (Cat# PA5-30940, Invitrogen, Wal- 264 tham, Massachusetts, United States)	1: 1000	Goat anti-rabbit (cat# E-AB-1102), Elabscience, Houston, Texas, United States	Rempuia et al., **2024**
17β-HSD (cat# STJ110000, St John's Laboratory Ltd, London, United Kingdom)	1: 1000	Goat anti-rabbit (cat# E-AB-1102), Elabscience, Houston, Texas, United States	Rempuia et al., **2024**
β-Tubulin (cat# E7, DSHB, University of Iowa, Dept of Biology, Iowa, United States)	1:1500	Goat anti-mouse (cat# E-AB-1001), Elabscience, Houston, Texas, United States	Rempuia et al., **2023**

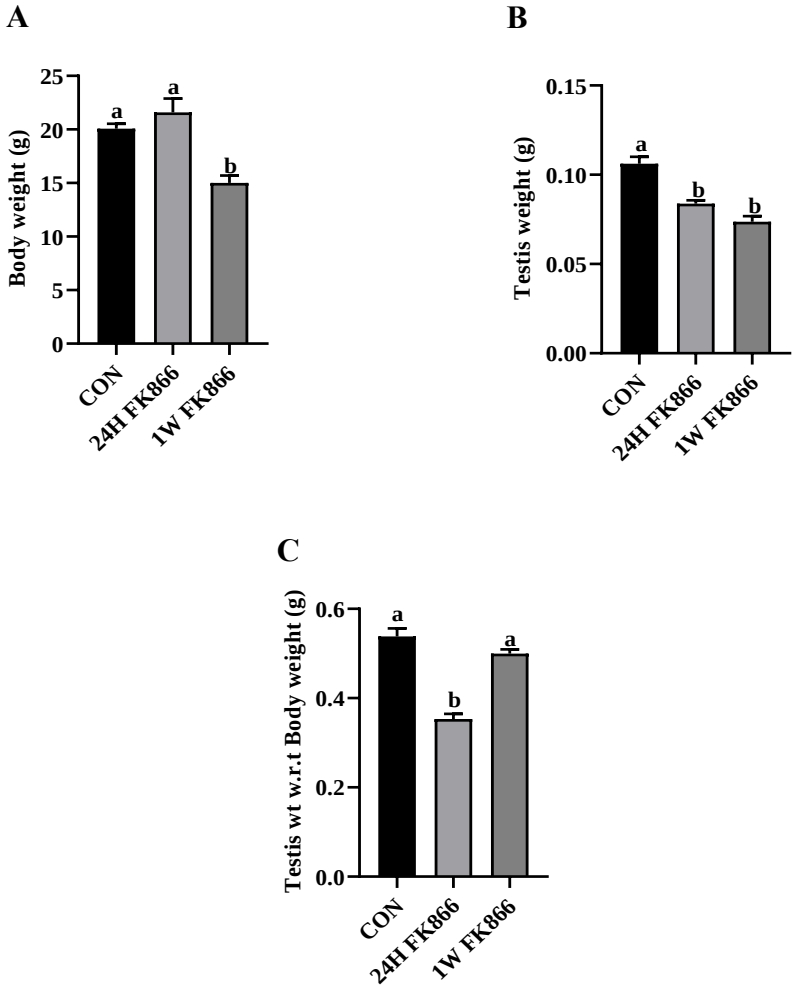

Figure-3.1- Changes in the body and testis weight after intra-testicular visfatin inhibition by FK866 (1.5 mg/kg). (A) The body weight of 1W FK866 group was significantly ($p<0.05$) decreased compared to control and 24H FK866 group. No significant change was shown between the control and 24H FK866 groups. (B) Testis weight also showed a significant ($p<0.05$) decrease in 24H FK866 and 1W FK866 group compared to the control group. (C) Testis weight with reference to body weight was significantly ($p<0.05$) decreased in 24H FK866 compared to the other group. Data are represented as mean±SEM (n=5 per group), and analysis of data was done by One-way analysis of variance (ANOVA) followed by Tukey's test.

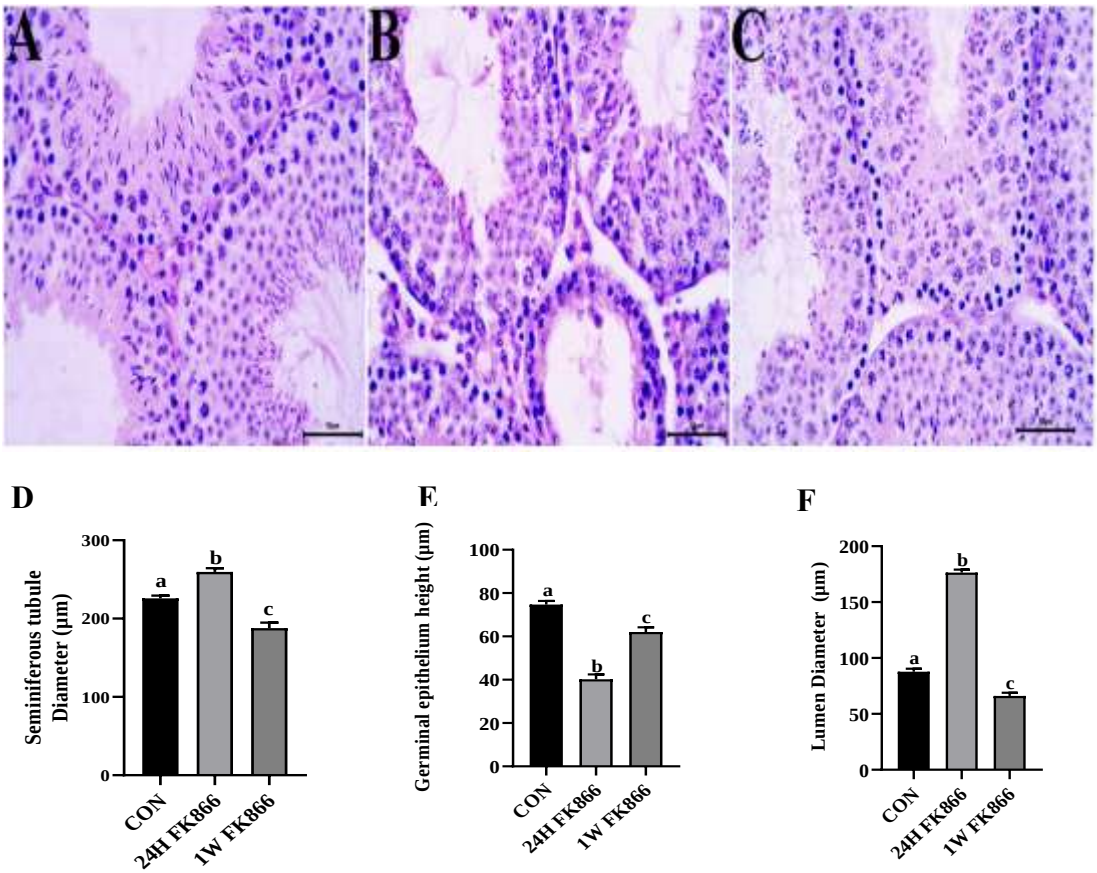

Figure-3.2- Effect of intra-testicular visfatin inhibition by FK866 (1.5 mg/kg) on the testicular histology and morphometric analysis. (A) The gross anatomy of the testis from the control group showed normal histoarchitecture of the testis. (B) The testicular anatomy after 24 hours of intra-testicular treatment with FK866 showed some depletion of round spermatids and sperm cells. (C) The testis after 1 week of intra-testicular treatment with FK866 did not showed any noticeable degenerative changes. (D) The seminiferous tubule diameter showed significant ($p<0.05$) elevation in 24H FK866 group followed by a significant ($p<0.05$) declined in 1W FK866 group compared to control group. (E) Germinal epithelium height was significantly ($p<0.05$) decreased in both treated groups compared to control group. (F) The lumen diameter was significantly ($p<0.05$) increased in 24H FK866 group compared to control group and lumen diameter of 1W FK866 was significantly ($p<0.05$) decreased in 1W FK866 compared to control group. Data are represented as mean±SEM (n=5 per group), and analysis of data was done by One-way analysis of variance (ANOVA) followed by Tukey's test.

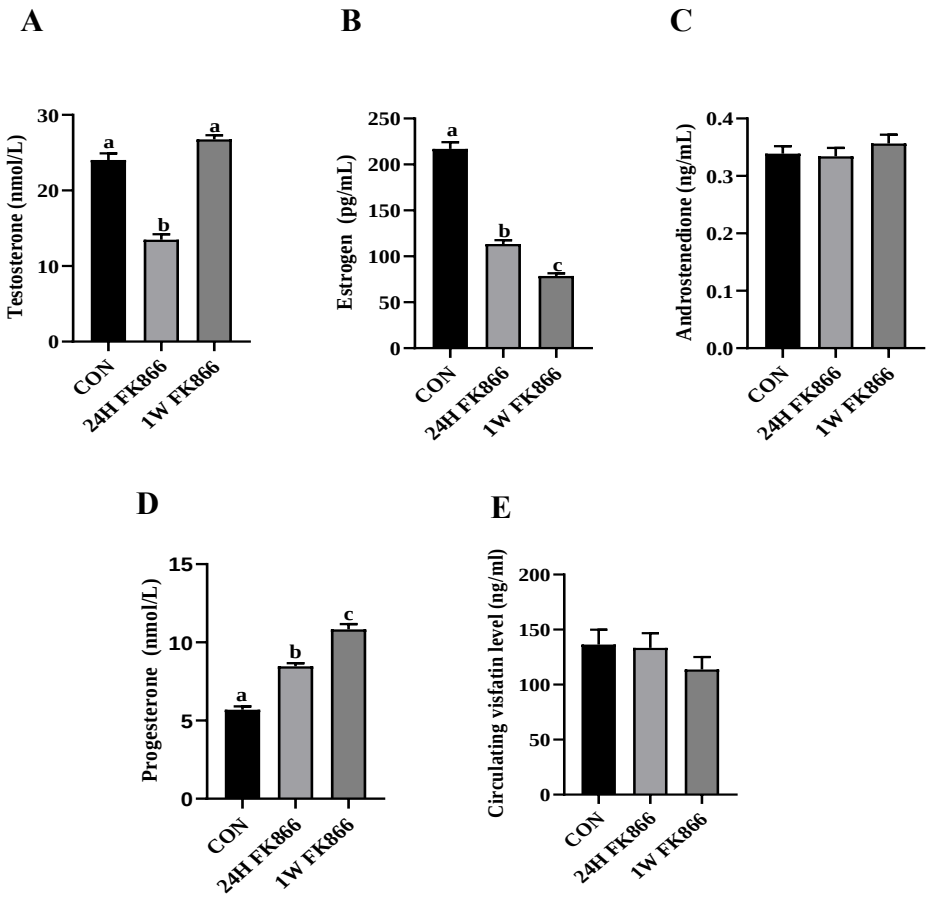

Figure-3.3- Changes in the circulating testosterone, estrogen, androstenedione, progesterone and visfatin levels after intra-testicular visfatin inhibition by FK866 (1.5 mg/kg). (A) The circulating testosterone level was significantly ($p<0.05$) decreased in 24H FK866 group compared to other group, no significant changes was showed between control and 1W FK866 group. (B) Estrogen level was significantly ($p<0.05$) decreased in both FK866 treated group compared to control. (C) Circulating androstenedione levels did not show any significant changes. (D) The progesterone level was significantly ($p<0.05$) increased in 24H FK866 and 1W FK866 group compared control group. (E) The circulating visfatin also showed declined after FK866 treatment but no significant change was observed among the groups. Data are represented as mean±SEM (n=5 per group), and analysis of data was done by One-way analysis of variance (ANOVA) followed by Tukey's test.

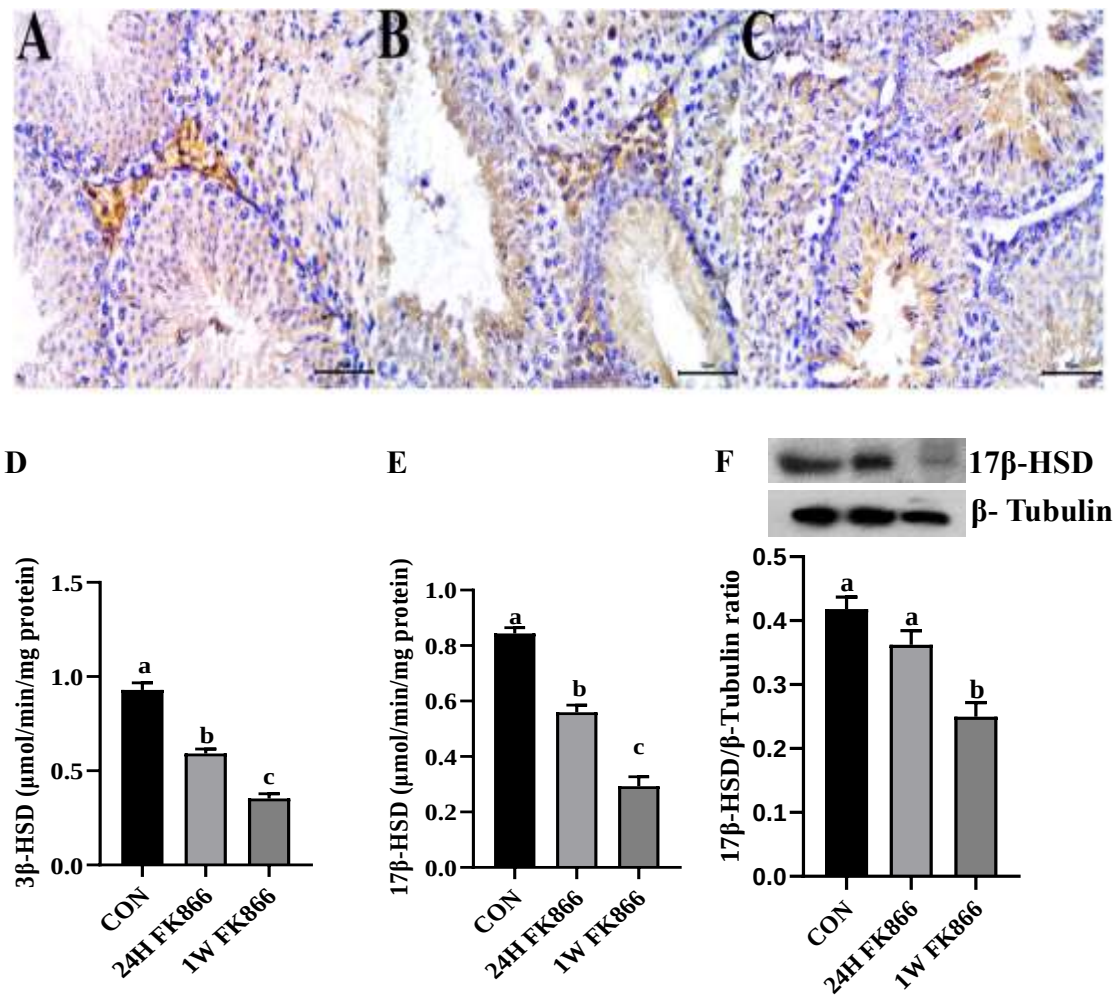

Figure-3.4- Effect of intra-testicular visfatin inhibition by FK866 (1.5 mg/kg) on the localization, expression and activity of steroidogenic enzymes. Immunolocalization of 3βHSD showed intense staining in the Leydig cells of control (A), moderate staining was observed in the Leydig cells of 24H FK866 (B), mild or no staining was showed in the Leydig cells of 1W FK866 group (C). (D) The activity of 3βHSD was significantly ($p<0.05$) decreased in both the FK866 treated group compared to control group. (E) The enzyme activity of 17βHSD also showed a significant ($p<0.05$) decreased in the 24H FK866 and 1W FK866 group compared to the control group. (F) The expression of 17βHSD showed significant ($p<0.05$) decreased in 1W FK866 group compared to control and 24H FK866 group. Data are represented as mean±SEM (n=5 per group).

Data analysis was done using a one-way analysis of variance (ANOVA), followed by Tukey's test.

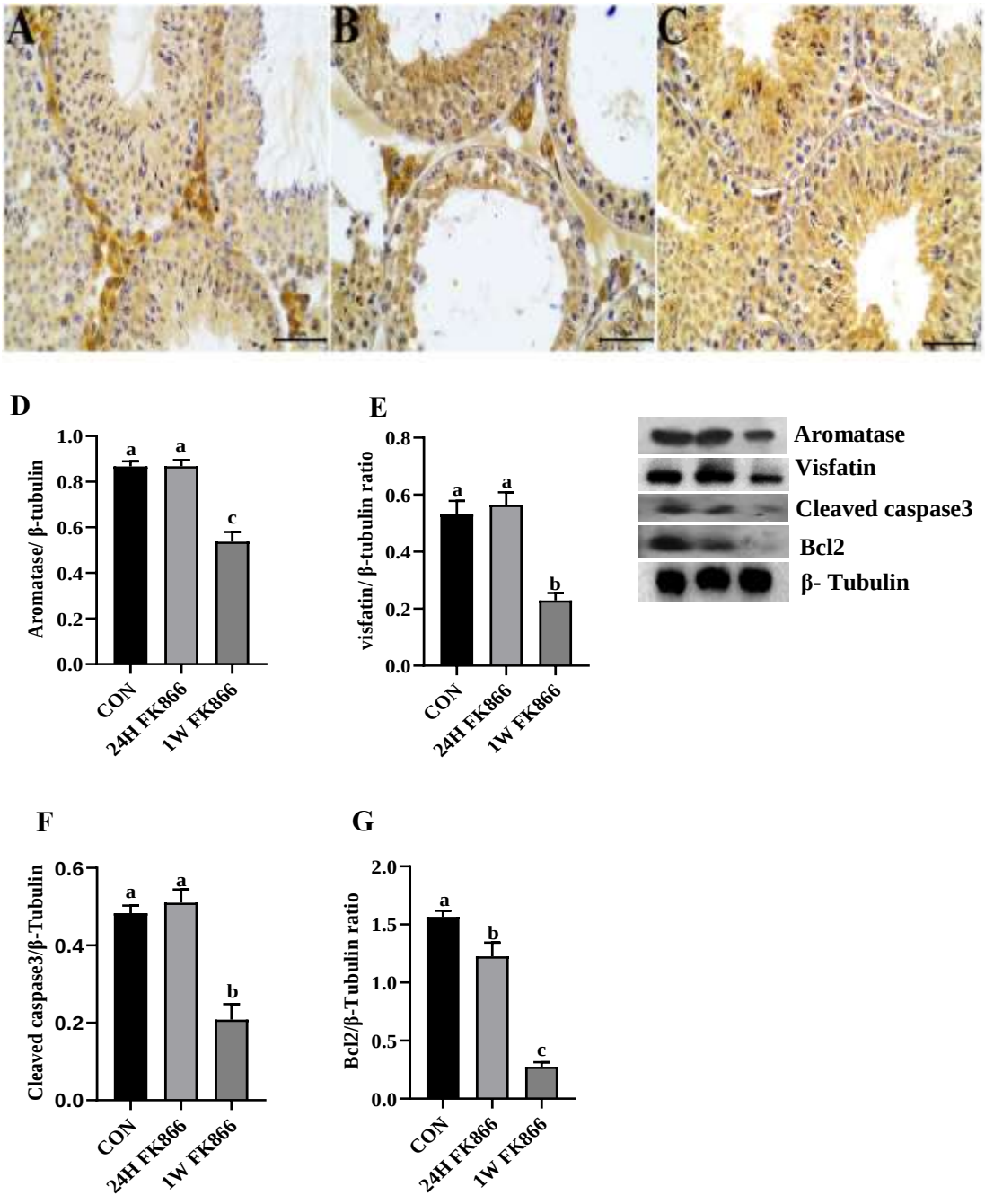

Figure-3.5- Changes in the on the localization and expression of aromatase, cleaved caspase3, Bcl2 and visfatin after intra-testicular visfatin inhibition by FK866 (1.5 mg/kg). Immunostaining of aromatase showed more staining in the

leydig cells of (A) Control and (B) 24H FK866 group compared to (C) 1W FK866 group. (D) The expression of aromatase was significantly ($p<0.05$) decreased in 1W FK866 group compared to control and 24H FK866 group. (E) The expression of visfatin also showed significantly ($p<0.05$) declined in 1W FK866 group compared to control and 24H FK866 group. (F) The expression of cleaved caspase3 was significantly ($p<0.05$) decreased in the 1W FK866 group compared to the other group. (G) Bcl2 expression was significantly ($p<0.05$) decreased in both the treatment groups compared to control group. Data are represented as mean±SEM (n=5 per group), and analysis of data was done by One-way analysis of variance (ANOVA) followed by Tukey's test.

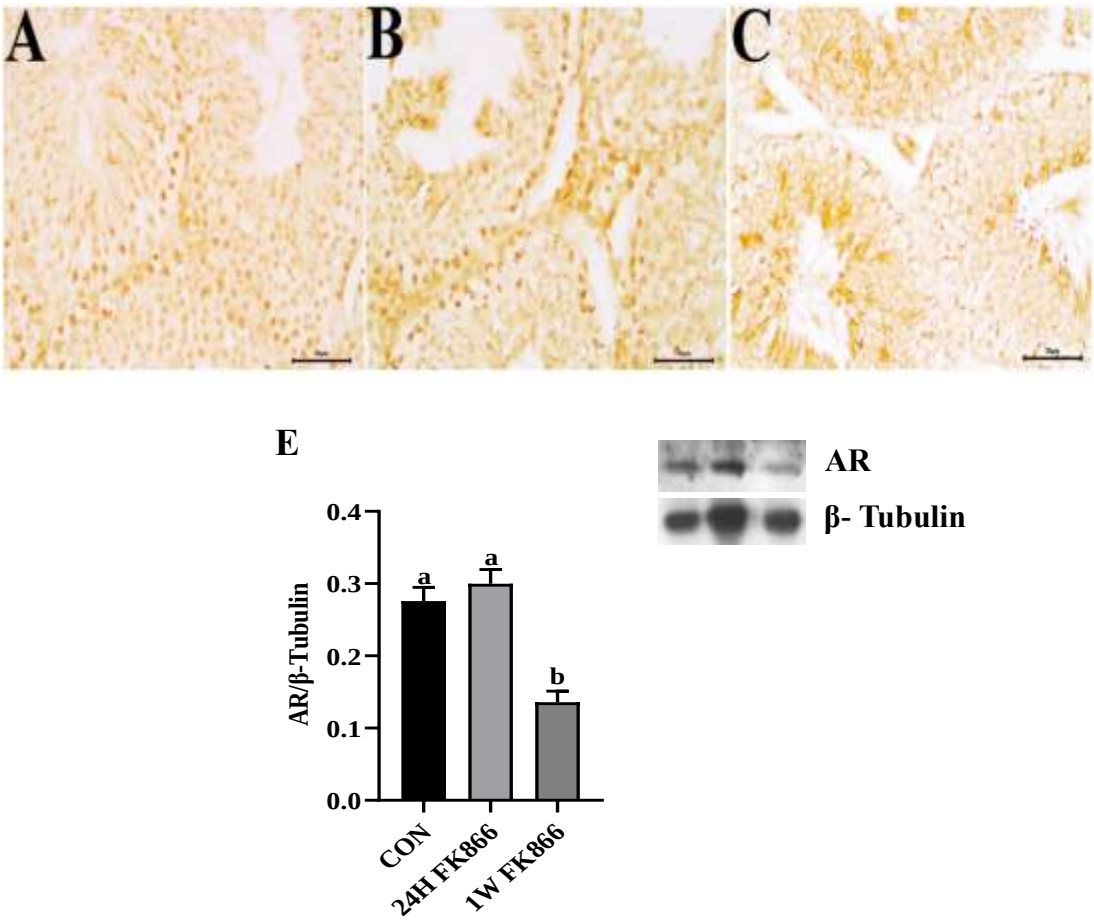

Figure-3.6- Changes in the localization and expression of Androgen receptor (AR) after intra-testicular visfatin inhibition by FK866 (1.5 mg/kg). (A) Control and (B) 24H FK866 group showed increased immunostaining of AR compared to (C) 1W FK866. (D) The expression of AR also showed a significant ($p<0.05$) decrease in the 1W FK866 group compared to the control and 24H FK866 group; no significant change was shown between the control and 24H FK866 group. Data are represented as mean±SEM (n=5 per group), and analysis of data was done by One-way analysis of variance (ANOVA) followed by Tukey's test.

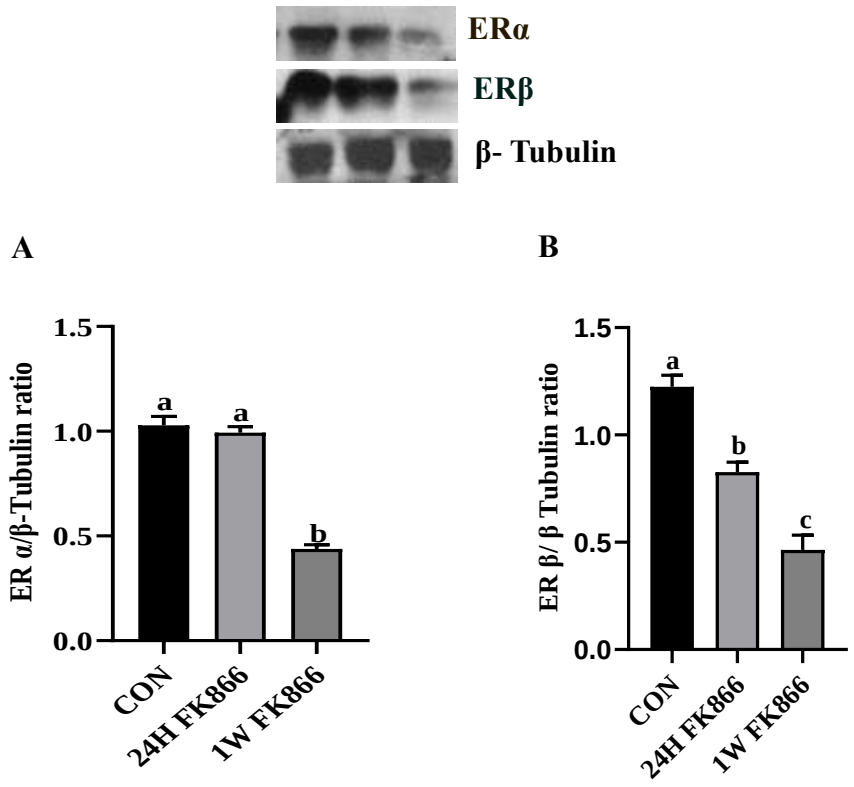

Figure-3.7- Effect of intra-testicular visfatin inhibition by FK866 (1.5 mg/kg) on the expression of Estrogen receptors (ERs). (A) Expression of ERα was significantly ($p<0.05$) decreased in 1W FK866 group compared to control group, no significant change was showed in 24H FK866 group compared to control group. (B) The expression of ERβ also showed a significant ($p<0.05$) decrease in both FK866 treated groups compared to the control group. Data are represented as mean±SEM (n=5 per group), and analysis of data was done by One-way analysis of variance (ANOVA) followed by Tukey's test.

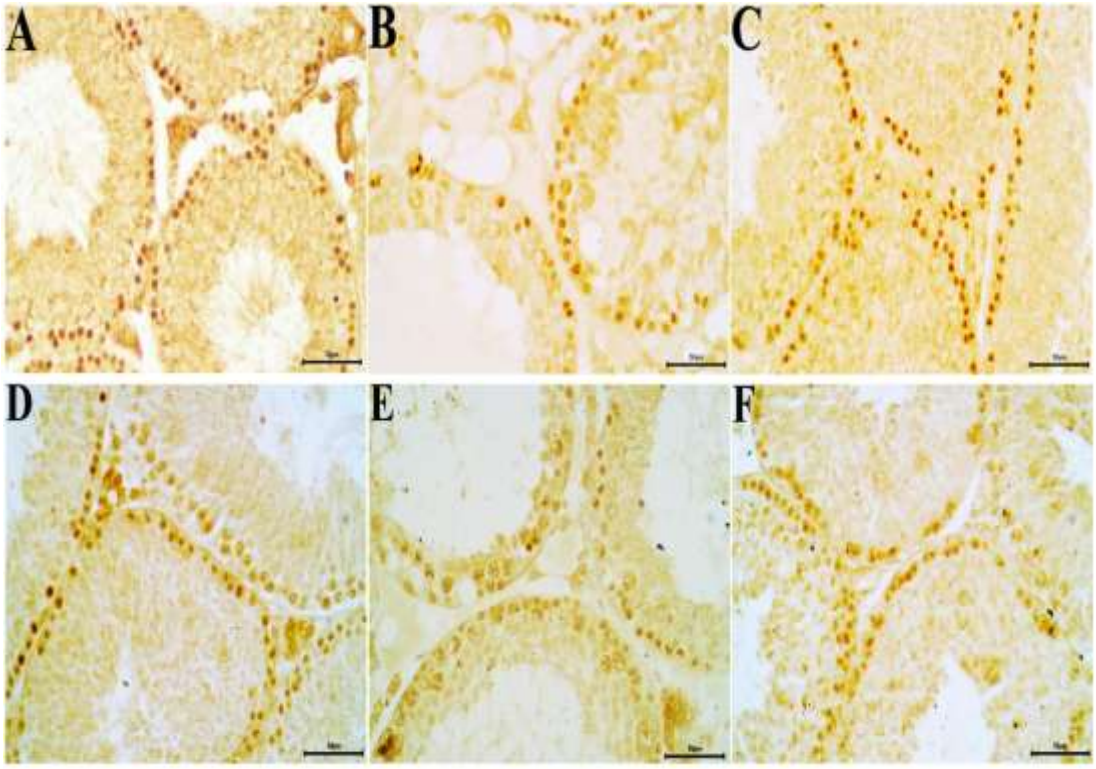

Figure-3.8- Changes in the localization of GCNA and PCNA after intra-testicular visfatin inhibition by FK866 (1.5 mg/kg). (A-C) The immunostaining of GCNA was decreased in 24H FK866 group compared to control and 1W FK866 group. Immunolocalization of PCNA was also decreased in (E) 24H FK866 group compared to (D) control and (F) 1W FK866 group. Data are represented as mean±SEM (n=5 per group).

Chapter 4

Exogenous visfatin modulates hypothalamic-hypophyseal-testicular hormones in mice

Introduction

Adipose tissue derived adipokines like leptin, adiponectin, visfatin and resistin have important role in the HPG axis, especially for testosterone production **(Shpakov et al., 2018).** The expression of these adipokines like leptin, and adiponectin, have also shown in the hypothalamus and pituitary where it regulates GnRH and gonadotropin secretion, LH (luteinizing hormone) and FSH (follicle-stimulating hormone) **(Manfredi-Lozano et al., 2016; Jin et al., 2000; Wen et al.,2008; Lu et al., 2008)**. Although, data on the presence and role of resistin and visfatin on the hypothalamic GnRH and pituitary gonadotropin secretions are less, and requires further investigation. The data on the role of visfatin in male reproductive axis are less compared to the female. Previous studies from our group as well as from other groups have shown that visfatin regulates ovarian proliferation, and steroidogenesis in mice, hen and human ovarian cells **(Annie et al., 2019; Diot et al., 2015; Shen et al., 2010)**. Similarly, visfatin has also been shown regulates testicular steroidogenesis and proliferation in the rodents **(Rempuia et al., 2024; Hameed et al., 2012)**. However, as other adipokines like leptin and adiponectin affects hypothalamic GnRH and pituitary gonadotropin secretion in females, the effects of visfatin have not been evaluated in males in relation to these secretions.

It has been shown that visfatin gene and protein are expressed in the porcine hypothalamus and regulates GnRH secretion depending upon estrous stage and pregnancy **(Kaminski et al., 2021)**. Furthermore, it has also been documented that visfatin in the pig hypothalamus may acts via an autocrine or paracrine effect on GnRH synthesis and supports the possibility that it is a neuromodulator of reproductive processes **(Kaminski et al., 2021)**. The expression of visfatin has also been reported in the mice and chicken hypothalamus **(Maillard et al., 2017; De Sousa Abreu et al., 2009)**. It has also shown that cells of anterior pituitary in porcine also expresses visfatin **(Szymanska et al., 2023)**. Visfatin expression has also been shown in the pituitary of female mice and suggested to affects FSH and LH production **(Maillard et al., 2017)**. However, it is still unknown how does visfatin affects pituitary gonadotropin secretion. It has been shown that visfatin may also be involved in the regulation of GnRH generation, as evidenced by its expression in the

hypothalamic structures that produce GnRH. Moreover, the direct role of visfatin has been shown in the GnRH-I gene expression in chicken after visfatin injections, which was given intracerebroventricularly **(Li et al., 2018)**. Thus, question may arise; does visfatin affects the hypothalamic GnRH and pituitary gonadotropin secretion in male mice. This research gap still needs to be filled. Based on the above description, it is evident that visfatin affects testicular testosterone secretion, hypothalamic GnRH secretion and pituitary gonadotropin secretions in different species and these findings are fragmentary in relation to HPG axis.

The presence of visfatin in the testis, hypothalamus and pituitary also suggests its role as autocrine and paracrine factor in controlling of central axis of reproduction. It has also been suggested that visfatin study on the central axis of reproduction from other species and in relation to other adipokines would be important avenue for research **(Maillard et al., 2017)**. Recently we have shown that expression of testicular visfatin in the mice is developmentally regulated and inhibition of testicular visfatin supresses testosterone secretion **(Rempuia et al., 2023; 2024)**. However, effects of visfatin on HPG axis of male have not been investigated.

Therefore, the present study has investigated the effects of exogenous visfatin on HPG axis of male mice in relation to their secretions.

Materials and methods

Animal maintenance

All the procedures were carried out accordingly to the guidelines provided by Mizoram University Institutional Animal Ethical committee (Protocol Approval number- MZUIAEC 21-22-05), Mizoram University, Mizoram, India. Animals were kept in animal house facility with standard conditions of 12 h light: 12 h dark cycle and 25±2°C, with availability of food and water were provided ad libitum. Male Swiss albino mice, at least 3 months old were used in the study.

Experimental design

To find out the effect of exogenous visfatin on adult male mice, intraperitoneal injection of visfatin (BioVision) at 50 ng per mice was performed for 35 days. The

dose of visfatin was selected from earlier studies **(Choi et al., 2012)**. A total 10 male mice were divided into two groups (n=5 per group): Control (CON) and Visfatin Peptide (VP) treatment group. CON group were given vehicles and VP group received intraperitoneal injection of visfatin peptide for 35 days. At the end of the treatment, animals were anesthetized with a mixture of 90 mg/kg Ketamine and 4.5 mg/kg Xylazine in intraperitoneal injection **(Clouthier et al., 2012)**, body weight were measured and sacrificed immediately by decapitation. Blood was collected and serum sample was separated out from the blood within 1hour after centrifugation at 300 rpm and stored at -20° C for further analysis. Pituitary was collected and fixed in bouin;s fluid. Testis was dissected out from the scrotal sacs; weight was recorded and kept in freeze at -20° C or fix in Bouin's fluid for 24 hours. This was later used for histopathological examination, immunohistochemistry, immunoblots and enzyme assay.

Sperm parameters

The motility percentage of spermatozoa and cauda sperm concentration were analyzed as per method described earlier **(Verma et al., 2014)**. Briefly, cauda epididymis was collected and minced in 250µl 1X PBS maintained at 37℃. A drop of it was placed in a sterile glass slide and covered with a cover slip. The motility of the sperm was then observed in 10 different fields under 40x magnifications within 5 minutes. 10µl of sperm solution was further diluted in 200µl 1X PBS and sperm concentration was observed as per WHO laboratory manual **(1999)**. A drop of sperm suspension was placed in Neubauer chamber and sperm concentration was observed.

Hormone assays

Circulating levels of testosterone, estrogen, androstenedione and progesterone were measured. Quantification of serum testosterone, estrogen, androstenedione and progesterone were estimated by using commercial ELISA kit as per manufacturer's instruction kit (Testosterone Cat# DKO002, DiaMetra, Italy; Estradiol Cat# DKO003, DiaMetra, Italy; Androstenedione Cat# DKO008, DiaMetra, Italy; Progesterone Cat# DKO006, DiaMetra, Italy).Circulating Luteinizing hormone (LH), Follicle-stimulating hormone (FSH) and Gonadotropin-releasing hormone (GnRH)

were also estimated using Mouse LH (cat # E-EL-M3053, Elabscience,), Mouse FSH (cat #E-EL-M0511, Elabscience) and GnRH (cat #E-EL-0071, Elabscience) ELISA kit.

Histopathological examination

Histopathological analysis was performed using testis fixed in Bouin's fluid. Tissues were transferred to 70% ethanol after 24 hours to avoid brittleness, and then undergo further dehydration in series of graded ethanol. The tissues were cleared in xylene and embedded in paraffin wax block, tissue blocks were cut in ribbon sections of 7µm thickness with Leica microtome (model RM2125 RTS) and fixed at sterile glass slides coated with poly -l-lysine. Tissues fixed in glass slides then undergo tissue processing and staining with hematoxylin and eosin as per method described earlier **(Bancroft et al., 2002)**. Tissue sections were then observed under a microscope and photographed using camera-mounted binocular microscope (Model E200, Nikon, Tokyo, Japan).

Immunohistochemistry

Fixed testes and pituitary were used for Immunohistochemical analysis. Tissues were paraffinized and cut in ribbon sections of 7µm thickness with Leica microtome (model RM2125 RTS) and fixed in sterile glass slides, the sections were dewaxed and rehydrated in descending grades of alcohol as per methods described by previous reports **(Jeremy et al., 2019)**. The tissue sections were then incubated for 30 minutes in goat-blocking serum [goat serum 1:100 diluted in PBS (Lot# A0515, Santa Cruz Biotechnology, Inc., CA, USA)] to reduce non- specific binding. The slides then undergo incubation at $4^{o}C$ overnight with primary antibody in the wet chamber; GCNA (1:200; mouse polyclonal antibody, cat# 10D9G11, DSHB, University of Iowa, Dept of Biology, Iowa, United States), PCNA (1:100; rabbit polyclonal antibody, cat# SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA), LH (1:100; rabbit polyclonal antibody, cat# PA5-102674, Invitrogen, Waltham, Massachusetts, United States), FSH (1:100; rabbit polyclonal antibody, cat# PAA830Mu01; Cloud-Clone Corp, Houston, Texas, United States) , AR (1:50; rabbit polyclonal antibody, cat# PAB252Mu01, Cloud-Clone Corp, Houston, Texas, United States), GnRH (1:

100; rabbit polyclonal antibody, cat# E-AB-15701, Elabscience, Houston, Texas, United States).Tissue sections then undergo washed in PBS and incubated with Horse-radish Peroxidase (HRP) conjugated secondary IgG antibody at 1:400 dilution (goat anti-mouse, Cat# E-AB-1001 and goat anti-rabbit, Cat# E-AB-1003, Elabscience, Houston, Texas, United States). Secondary antibody wash was done followed by antigen detection with 3, 3-diaminobenzidine tetrahydrochloride Dihydrate (DAB) in Tris–HCl (pH 7.6) and 0.01% H_2O_2 till brown colour developed at room temperature. The reaction was stopped using distilled water and Hematoxylin counterstain was done except for GCNA, PCNA and AR. The slides were dehydrated in a series of graded ethanol, cleared in xylene, and mounted with DPX. Slides were then observed under a microscope and photographed using camera-mounted binocular microscope (Model E200, Nikon, Tokyo, Japan).

Western blot analysis

The testis tissues collected at the end of experiments were homogenized 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 PMSF and protein concentrations were estimated by Bradford method [22]. The samples were denatured in gel loading buffer (62·5 mMTris, 2% SDS, 10% glycerol, 1% - mercaptoethanol and 0·003% Bromophenol Blue, pH 6·8), Samples were then loaded 50 µg/well and run in 10% SDS-PAGE with protein marker at 100V for 3 hrs, resolved protein were then transferred on PVDF (polyvinylidineflouride) membrane (Millipore India Pvt. Ltd., India) using wet transfer apparatus for overnight at 4 oC. The transferred membranes were blocked for non –specific binding with Skim milk solution (5% non-fat dry milk with PBS and 0.1% Tween 20) for 30 mins at room temperature, followed by overnight incubation with primary antibody; 17β-Hydroxysteroid dehydrogenase 3 (17β-HSD3) (1:1000; rabbit polyclonal antibody, cat# STJ110000, St John's Laboratory Ltd, London, United Kingdom), Cytochrome P450 11A1 (CYP11A1) (1:1000; rabbit polyclonal antibody, cat# PAD545Mu01, Cloud-Clone Corp, Houston, Texas, United States), Luteinizing hormone receptor (LHR) (1:1000; rabbit polyclonal antibody, cat# SC3588, Santa Cruz Biotechnology, Santa Cruz, CA), B-cell lymphoma 2 (Bcl2) (1:1000; mouse polyclonal antibody, Cat# sc-7382, Santa Cruz

Biotechnology, Inc. Dallas, USA), cleaved caspase3 (1: 1000; rabbit polyclonal antibody , cat# E-AB-30004, Elabscience, Houston, Texas, United States), AR (1:250; rabbit polyclonal antibody, cat# PAB252Mu01, Cloud-Clone Corp, Houston, Texas, United States), aromatase (1:2000; rabbit polyclonal antibody, cat# E-AB-64300, Elabscience, Houston, Texas, United States), Estrogen receptor α (ERα) (1;500; mouse polyclonal antibody, cat# Bz1, DSHB, University of Iowa, Dept. of Biology, Iowa, United States), Estrogen receptor β (ERβ) (1:500; mouse polyclonal antibody, cat# CWK-F12, DSHB, University of Iowa, Dept. of Biology, Iowa, United States), Cytochrome P450 17α Hydroxylase/17,20 lyase (CYP17) (1:1000; rabbit polyclonal antibody, cat# AF5210#1937, Affinity Bioscience, Cincinnati, OH, United States),β-Tubulin (1:1500; mouse polyclonal antibody, cat# E7, DSHB, University of Iowa, Dept of Biology, Iowa, United States) for overnight at 4oC. The membranes blots were washed in PBS-Tween20 and incubated with horse-radish peroxidase-conjugated secondary antibody (goat anti-mouse, Cat# E-AB-1001 and goat anti-rabbit, Cat# E-AB-1003, Elabscience, Houston, Texas, United States) for 4 hrs at room temperature. After incubation, secondary antibody washed was done with PBS-Tween20 and developed with ECL detection method. X-ray film used for visualizing the protein band was scanned and ImageJ software (imagej.nih.gov/) was used for quantification of the protein band. The density of each band was normalized to the density of β-Tubulin band which was used as loading control.

Steroidogenic enzyme assay

Testicular Steroidogenic enzymes (3βHSD and 17βHSD) activities were also analysed using method described earlier **(Shivanandappa et al., 1997)**. In brief, 10% testis homogenate was prepared with 0.1 M Tris-HCL buffer. The homogenate was centrifuged at 12,000 *g* for 15 minutes at 4^{o}C, the supernatant was collected and the concentration of protein was measured using Bradford method **(Bradford et al., 1976).** The activity of 3βHSD and 17βHSD was also determined by adding 300 µl of 1mM Dehydroepiandrosterone (DHEA) and 300 µl of 1mM testosterone respectively to sample containing 50 µg of protein, followed by addition of 300 µl of 5mM NAD to the mixture. 500 µl of colour reagent (40mg INT and 0.5 ml Tween20 disssolved in 50 µl distilled water) was also added to it. After incubated for 2 hours at 37 ^{o}C, the

reaction was stopped by addition of 2 ml phosphate buffer and optical density was measured at 490 nm in spectrophotometer against the blank. The enzyme activity was expressed as nmoles of NAD reduced/mg/min.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Using One-step TUNEL In Situ Apoiptosis Kit (Green, FITC) (Catalogue No: E-CK-A320. Elabscience, Houston, Texas, United States), fixed testes tissues were processed for TUNEL assay according to manufacturer's instructions. In brief, after deparaffinization in xylene the tissues sections were then hydrated with a sequential of graded ethanol. Tthen slides were washed with PBS and then incubated with proteinase K (1:100 in 10 mM Tris pH 8) for 20 mins at 37oC. The slides were then washed again with PBS and incubated with DNase buffer (1X) for 10 mins followed by addition of Terminal Deoxynucleotidyl Transferase (TdT) and incubated overnight at 37oC. The slides were the undergo washing with PBS, followed by incubation with DAPI in RT for 10 mins in shading light , the slides were then washed again with PBS and mounted with mounting medium containing n-propyl gallate. The slides were examined and photographed using a Nikon binocular microscope (Model E200, Nikon, Tokyo, Japan). The TUNEL-positive apoptotic cells were observed as green FITC.

Statistical analysis

Data were expressed as the mean ± standard error of the mean. Analysis of data was done by students't-test. The level of significance was considered as $p<0.05$. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego,CA,USA).

Results

Effect of exogenous visfatin administration on body weight, testis weight, sperm motility and concentration

To unravel the effect of exogenous visfatin, on the testis weight, body weight and sperm parameters; both body and testis weight along with sperm motility and concentration were analyzed. The body weight did not shows any significant changes

after visfatin treatment (**Figure-4.1A)**, however, the testis weight of visfatin treated mice was significantly ($p<0.05$) increased compared to control mice (**Figure-4.1B).** The study of sperm parameters showed a significant ($p<0.05$) increased in sperm motility of visfatin treated mice, whereas no significant changes in the sperm concentration was observed between control and visfatin treated mice **(Figure-4.1C, D).**

Effect of exogenous visfatin administration on histology of testis

To uravel the effect of visfatin on the testicular histology, histopathological examination of testis after visfatin administration was performed. The histological examination did not showed significant observable changes after visfatin treatment compared to control group **(Figure-4.2A, B).**

Effect of exogenous visfatin administration on circulating reproductive hormones levels

Reproductive hormones play a vital role in the maintenance and regulation of testicular function. To unravel the effect of exogenous visfatin on testicular function, the circulating levels of testosterone, estrogen, androstenedione, progesterone, LH, FSH and GnRH levels were examined after visfatin treatment. The circulating testosterone level was significantly ($p<0.05$) elevated after visfatin treatment **(Figure-4.3A)**, while the progesterone level showed significant ($p<0.05$) decreased in visfatin treated mice compared to control mice **(Figure-4.3D)**. However, the circulating levels of estrogen and androstenedione levels did not showed significant changes after visfatin treatment **(Figure-4.3B, C)**. The circulating levels of LH and FSH were also significantly ($p<0.05$) declined after visfatin treatment **(Figure-4.3E, F),** while the level of GnRH was significantly ($p<0.05$) increased in visfatin treated mice compared to control mice **(Figure-4.3G)**.

Effect of exogenous visfatin administration on the activity and expression of steroidogenic enzymes and LHR.

To unravel the effect of visfatin on testicular steroidogenesis, the expression and activity of 3βHSD, 17βHSD, CYP17, CYP11A1 and LHR were analyzed. The

enzyme activity of 3βHSD did not show significant changes and 17βHSD was significantly ($p<0.05$) decreased in the visfatin treated mice compared to control mice **(Figure-4.4A, B)**, the expression of 17βHSD and CYP11A1 also showed significant ($p<0.05$) declined after visfatin treatment **(Figure-4.4C, E)**. The expression of CYP17 was significantly ($p<0.05$) up-regulated in the visfatin treated mice testis compared to the control **(Figure-4.4D)**. Moreover, the expression of LHR was also significantly ($p<0.05$) decreased in the visfatin treated mice compared to control mice **(Figure-4.4F)**.

Effect of exogenous visfatin administration on the immunolocalization of GCNA and PCNA

To evaluate the testicular germ cell proliferation in the testis after visfatin administration, immunolocalization of GCNA and PCNA was done. The immunolocalizaton of both GCNA and PCNA exhibit a slight decreased after visfatin treatment, however, no significant observable changes was found between visfatin treated mice and control mice **(Figure-4.5A, B, C, D)**.

Effect of exogenous visfatin administration on the testicular apoptosis

To find out the potential role of visfatin on apoptosis in testis, the expression of apoptotic marker BCL2 and cleaved caspase3 protein were analyzed. The expression of anti-apoptotic marker BCl2 was significantly ($p<0.05$) increased in visfatin treated mice compared to control mice **(Figure-4.6A)**. Moreover, the pro-apoptotic marker cleaved caspase3 expression also showed significant ($p<0.05$) increased after visfatin administration **(Figure-4.6B)**. The TUNEL assay also showed more frequent distribution of apoptotic germ cells like spermatogonia, primary spermatocytes, round spermatid and sperm in visfatin treated testis**(Figure-4.6C)** than control **(Figure-4.6D).**

Effect of exogenous visfatin administration on the immunolocalization of LH and FSH in pituitary

To unravel the effect of visfatin on the pituitary gland in relation to reproductive function, immunolocalization of LH and FSH was performed. The

immunolocalization of LH in the pituitary showed its abundance in the control mice compared to visfatin treated mice **(Figure-4.7A, B)**, however, no significant observable changes was found on the immunolocalization of FSH in the pituitary between control and visfatin treated mice **(Figure-4.7C, D)**.

Effect of exogenous visfatin administration on the immunolocalization AR and GnRHR in pituitary

An immunolocalization study of AR and GnRHR was performed in visfatin treated mice pituitary and compared with the control. The immunostaining of AR in pituitary was less in visfatin treated mice compared to control mice **(Figure-4.8A, B)**.While the immunostaining of GnRHR in the pituitary showed increased abundance after visfatin treatment compared to control mice **(Figure-4.8C, D)**.

Effect of exogenous visfatin administration on the expression of AR, aromatase and ERs

To find out the exact role of visfatin on testicular steroidogenesis, expression of AR, aromatase, ERα and ERβ were analyzed. The expression of AR was significantly ($p<0.05$) declined after visfatin administration **(Figure-4.9A)**. The expression of aromatase was slightly increased after visfatin treatment; however the data did not significant differences compared to control **(Figure-4.9B)**. Moreover, the expression of ERα and ERβ did not showed significant differences between Control and visfatin treated group **(Figure-4.9C, D)**.

Discussion

Visfatin is known adipokines to regulate testicular and ovarian activity in different species **(Shen et al., 2010; Hameed et al., 2012; Ocón-Grove et al., 2010; Riammer et al., 2016; Reverchon et al., 2016)**. Role of visfatin has already been well described in female mice **(Annie et al., 2020; 2021)**. However, the role of visfatin we have also shown in the male mice at pre-pubertal stage earlier **(Rempuia et al., 2024).** Despite some studies from our lab and other as well, still role of visfatin deserve to be investigated in the testicular functions. The present study

investigated the effects of exogenous recombinant visfatin treatment on the hypothalamo-hypophyseal-testicular hormone secretion along with other testicular parameters. The exogenous visfatin stimulated the testicular weight and sperm motility; however, the sperm concentration was slightly elevated, but it did not show significant differences. The increased testis weight, prompted us to analyse the circulating testicular steroid hormones. The circulating testosterone levels showed exalted levels in the visfatin treated mice, while progesterone levels found to be decreased. The levels of androstenedione and estrogen did not show significant change after visfatin treatment. Previous studies have also shown that exogenous adipokines like, leptin, adiponectin and asprosin modulates testicular steroidogenesis **(Wang et al., 2018; Choubey et al., 2019; Maurya et al., 2022)**. Visfatin treatment also decreased LHR, CYP11A1, and 17βHSD expression in the testis. Furthermore, increased testosterone promoted us to analyse the expression of CYP19A1 (aromatase); despite no change in aromatase, the increased testosterone was surprising. Since CYP17 directly regulates testosterone biosynthesis by acting various steps of steroidogenesis **(Liu et al., 2005),** thus, we have also analysed the expression of CYP17. The elevated testosterone coincides with the up-regulated expression of CYP17 in the visfatin treated mice testis. Therefore, it can be suggested that visfatin stimulates the testosterone secretion by elevating CYP17 expression in the mice testis. Moreover, adrenal cortex also secrets small amount of testosterone, thus, whether, visfatin treatment has also affected the adrenal steroidogenesis and contributed to elevated circulating testosterone, remains to be investigated.

The testosterone biosynthesis and testicular functions are regulated by the pituitary gonadotropins, LH and FSH. Visfatin treatment has supressed the LH and FSH secretions from pituitary. The decreased LH secretion and LHR expression in the testis coincide with decreased progesterone levels. Thus, whether high visfatin has stimulatory role in the testicular steroidogenesis remains unclear. Moreover, we have taken only single dose of visfatin, thus, different doses of visfatin would have been important to unravel the exact role of visfatin on the testicular steroidogenesis. However, it has been shown that visfatin is found to increase testosterone levels

(Hameed et al., 2012; Elfassy et al., 2018). Recently we have also shown that intra-testicular inhibition of visfatin by FK866, supresses the testosterone secretion **(Rempuia et al., 2024)**. The functions of testis are well regulated by androgen and estrogen through its receptors **(Wang et al., 2009; Chimento et al., 2014).** Thus, we have analysed the expression of AR and ERs in the testis. The expression of AR was down-regulated by the visfatin treatment and ERs expression was unaffected. Thus, it may be suggested that elevated testosterone has suppressed the testicular AR expression. It has been shown that suppression of androgen by LHRH antagonist decline AR expression in the testis **(Zhu et al., 2000).** However, it has also been shown that moderate to high concentration of testosterone down-regulates AR expression in the peripheral tissue **(Lin et al., 1993)**. Since we have given exogenous visfatin, thus, it seems that elevated visfatin might alter the testicular functions. The markers of proliferation PCNA and GCNA were also unaffected by the visfatin treatment, however, the expression of cleaved caspase3 and Bcl2 were up-regulated. Since we detect cleaved caspase3 real executor of apoptosis, thus, excess visfatin might have stimulated apoptosis by supressing testosterone signalling. The TUNEL assay in our experiments also showed stimulated apoptosis in the visfatin treated mice testis. Furthermore, TUNEL positive cells showed the stimulated apoptosis in the different cell types of the testis It has also been shown that testosterone supresses apoptosis in the testis **(Erkkilä et al., 1997)**. The stimulated expression of Bcl2 might have been a counter mechanism to prevent apoptosis.

The elevated testosterone levels negatively correlated with LH and FSH, it has also been shown that exogenous testosterone supresses LH and FSH secretion **(Pitteloud et al., 2008)**. Moreover, it has also been shown that visfatin supresses the LH secretion in the LβT2 gonadotroph cell line **(Maillard et al., 2017).** Whether, declined gonadotropin secretion was due to elevated testosterone acting on pituitary or visfatin has direct inhibitory role in the pituitary gonadotropin secretion remains to be investigated. Furthermore, gonadotropins secretion is regulated by the hypothalamic GnRH, which acts on pituitary via GnRHR **(Voliotis et al., 2018).** Thus, we have also measured GnRH levels and visfatin treatment elevated the GnRH levels. Despite increased GnRH levels, the suppressed gonadotropin levels were

again surprising. The circulating testosterone and GnRH showed positive correlation, however, it has been suggested that elevated testosterone has inhibitory role in GnRH secretion **(Marques et al., 2022)**. To best of our knowledge, effects or role of visfatin in hypothalamo-pituitary-gonadal axis has not been investigated in males. However, role of visfatin on the hypothalamic-pituitary-gonadal axis, in the females has been suggested and also showed that visfatin gene and protein are expressed in the porcine hypothalamus and may have role in the GnRH secretion **(Kaminski et al., 2021)**. It has also been shown that visfatin injections intra-cerebroventricularly stimulated GnRH-I gene expression in chicks **(Li et al., 2018).** Based on the above description, it may also be suggested that visfatin might have stimulated the hypothalamic GnRH secretion, despite elevated testosterone may be expected to supress its secretion. Since the gonadotropin levels were low, thus, it may also be suggested that high or exogenous visfatin leads to hypogonadotropic like condition in males. Our immunolocalization of GnRHR in the pituitary showed increased abundance. Despite increased GnRH levels and GnRHR in pituitary, the supressed gonadotropin by exogenous visfatin remains unclear. However, it may also be suggested that pituitary exhibits a condition like GnRH resistance **(Vagenakis et al., 2005)**, because of supressed gonadotropin levels. The abundance of FSH in the anterior pituitary did not show change, however, LH abundance was decreased in the pituitary. These findings suggest that secretion and synthesis of LH is compromised after visfatin treatment. As testosterone has shown to suppress the LH secretion, thus we have also analysed the AR abundance in the pituitary. The abundance of AR was less in the visfatin treated pituitary. Therefore, testosterone associated suppression of gonadotropin seems to be superficial and visfatin might have directly supressed the gonadotropin secretion by acting on the pituitary.

In conclusion, this is the first report on the effects of exogenous visfatin (hypervisfatinemia like conditions) on the hypothalamic-pituitary-testicular hormonal secretions in male mice. Although, visfatin has been shown to affects hypothalamus, pituitary and testicular functions and data are fragmentary, as, no study has analysed its effects in HPT axis. Our results showed visfatin has a differential role in the HPT axis, visfatin has stimulatory effects on the hypothalamic

GnRH secretion, and testicular testosterone synthesis, and however, it has an inhibitory role on the pituitary gonadotropin secretions due to GnRH resistance of pituitary. Despite, down-regulation of steroidogenic markers (LHR, 17β-HSD3, CYP11A1), the elevated testosterone levels could be due to elevated CYP17 expression after exogenous visfatin treatment. In addition, the down-regulation of AR expression in the testis also suggests supressed signalling of androgen due to high testosterone. The apoptotic marker cleaved caspase3 also showed stimulated apoptosis, which significance remains unclear. The present results also have limitation in speculating whether hypervisfatinemia would have positive or negative impact on the testicular functions. However, hypervisfatinemia like conditions probably, deregulates gonadotrophins and testosterone secretion along pituitary-testis axis.

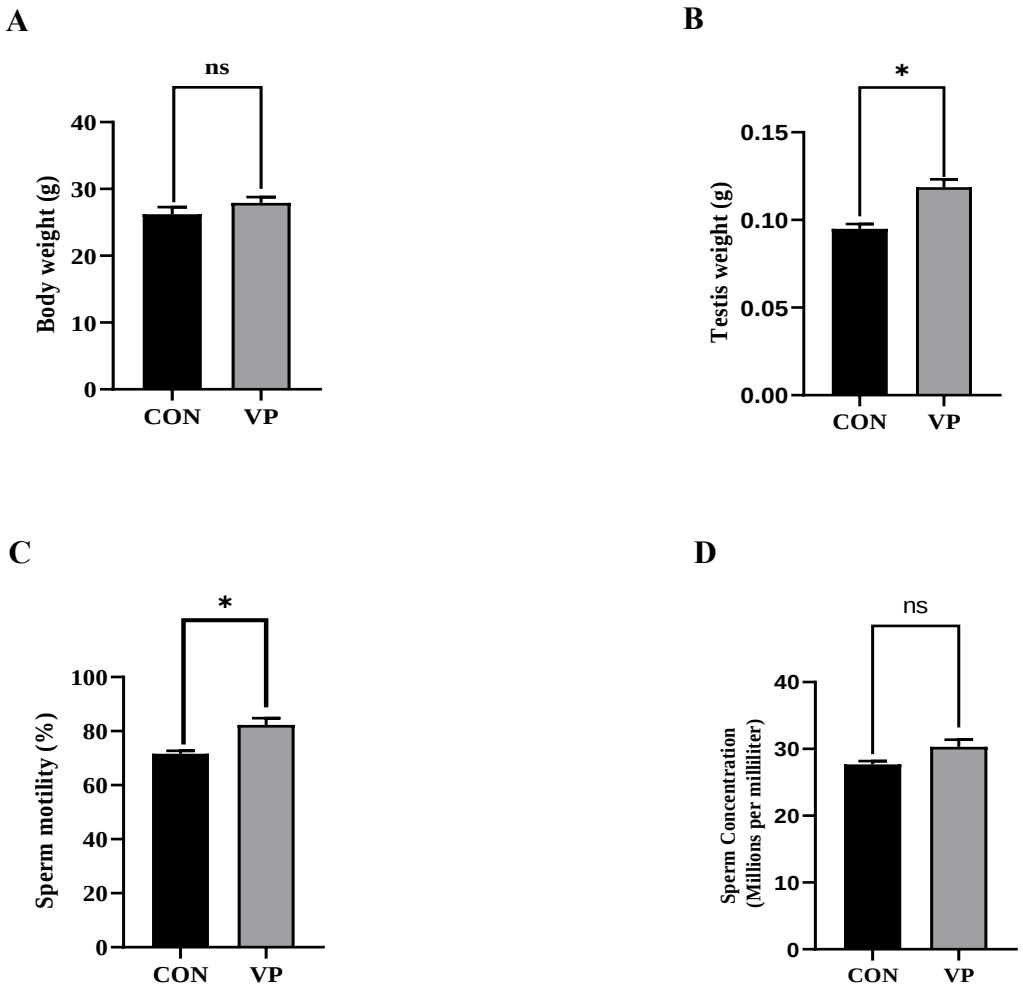

Figure-4.1- Changes in the body weight, testis weight, sperm motility and concentration after exogenous visfatin administration. (A) The body weight did not showed significant changes after visfatin treatment compared to control group. (B) Testis weight was significantly ($p<0.05$) higher in visfatin treated group compared to control group. (C) Sperm motility was significantly ($p<0.05$) increased in treatment group compared to control group. (D) The sperm concentration did not showed significant changes after visfatin treatment compared to control.Data are represented as mean±SEM (n=5 per group), and analysis of data was done by students't-test.

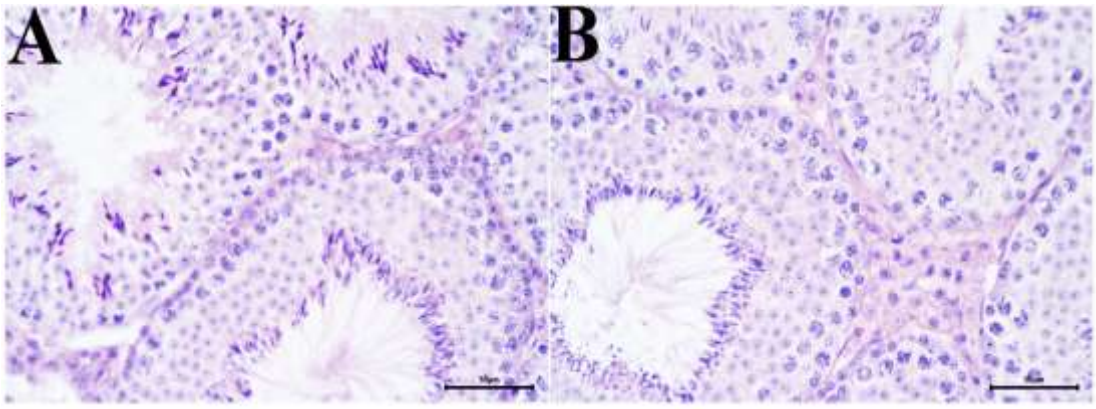

Figure-4.2- Effect of exogenous visfatin administration on testicular histology. (A) The histophathological examination of testis from the control group showed normal histoarchitecture of the testis. (B) The testicular anatomy after visfatin treatment did not showed any noticeable degenerative changes.

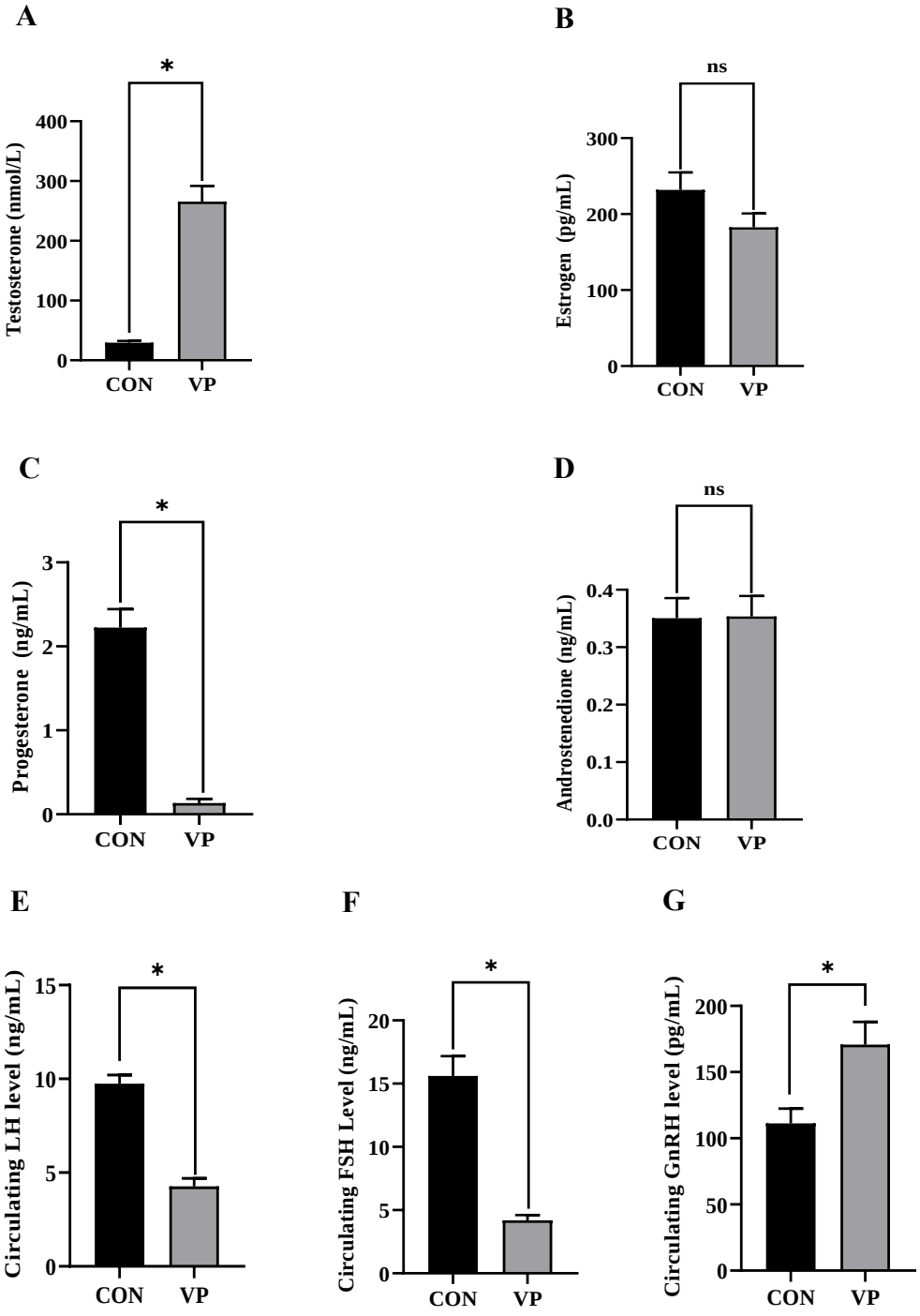

Figure-4.3- Changes in the circulating testosterone, estrogen, androstenedione, progesterone, LH, FSH and GnRH levels after exogenous visfatin administration. (A) The circulating testosterone level was significantly ($p<0.05$) higher in visfatin treated group compared to control group (B) Estrogen level did not

showed significant changes after visfatin administration (C) Circulating androstenedione levels did not show any significant changes. (D) The progesterone level was significantly ($p<0.05$) decreased visfatin treated group compared to control group. (E) The circulating LH level also showed significant ($p<0.05$) declined after visfatin treatment. (F) FSH level was also significantly ($p<0.05$) decreased in treatment group compared to control group. (G) The GnRH level was significantly ($p<0.05$) higher in treatment group compared to control group. Data are represented as mean±SEM (n=5 per group), and analysis of data was done by students't-test.

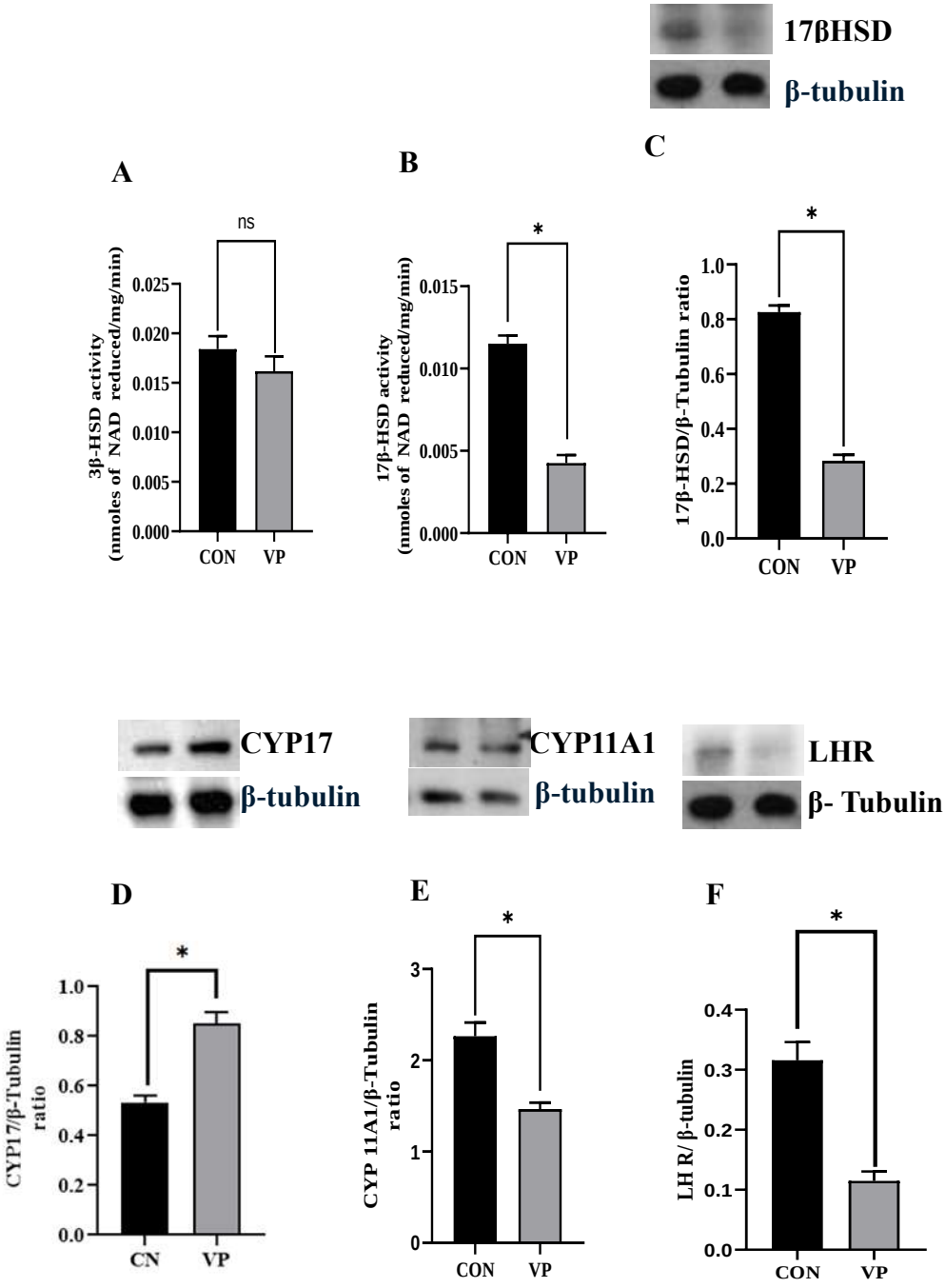

Figure-4.4- Effect of exogenous visfatin administration on the expression and activity of 3βHSD, 17βHSD, CYP17, CYP11A1 and LHR. (A) The enzyme activity of 3βHSD significantly ($p<0.05$) decreased in the visfatin treated group compared to control group. (B) The activity of 17βHSD was also significantly ($p<0.05$) declined after visfatin treatment, the expression of 17βHSD also significant ($p<0.05$) decreased after visfatin administration (C). (D)The expression of CYP17

showed significant ($p<0.05$) increased in visfatin treated group compared to control. (E) The expression of CYP11A1 also showed significant ($p<0.05$) declined in visfatin treated group compared to control group. (F) Expression of LHR was also significantly ($p<0.05$) decreased in visfatin treated group compared to control group. Data are represented as mean±SEM (n=5 per group). Data analysis was done by students't-test.

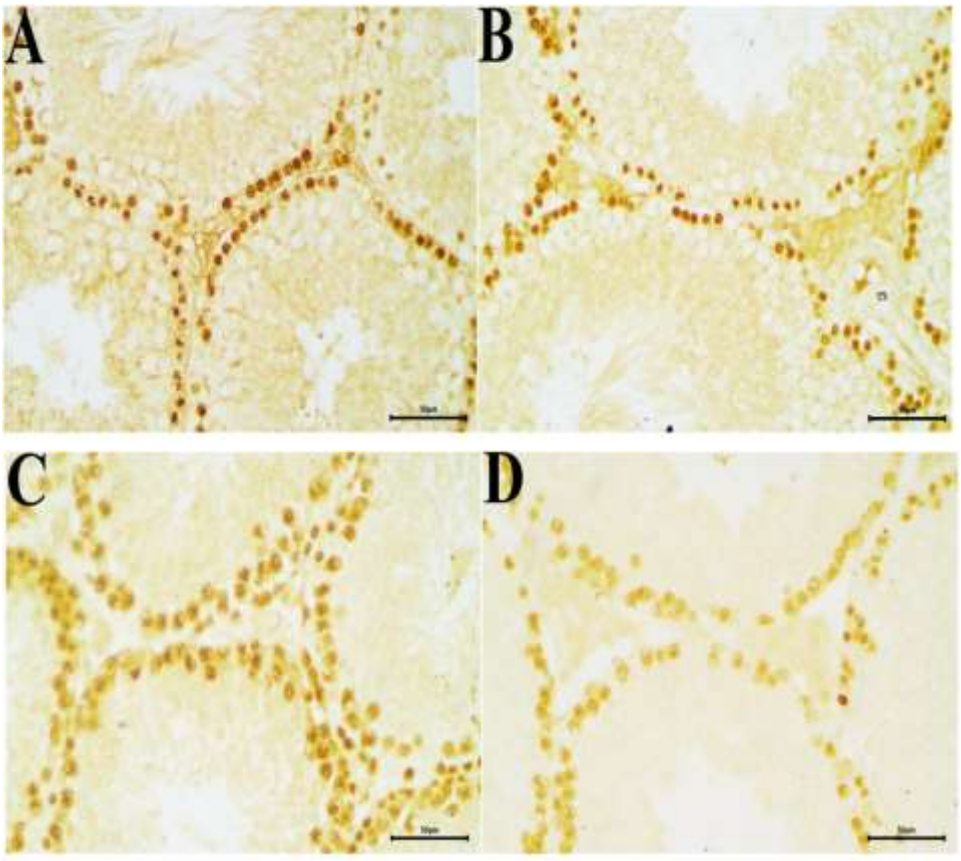

Figure-4.5- Changes in the localization of GCNA and PCNA after exogeneous visfatin administration. The immunolocalization of GCNA showed no significant observable change in (A) Control and (B) visfatin treated group. (C) Control showed increased immunostaining of PCNA compared to (D) Visfatin treated group but no significant change was shown between the groups.

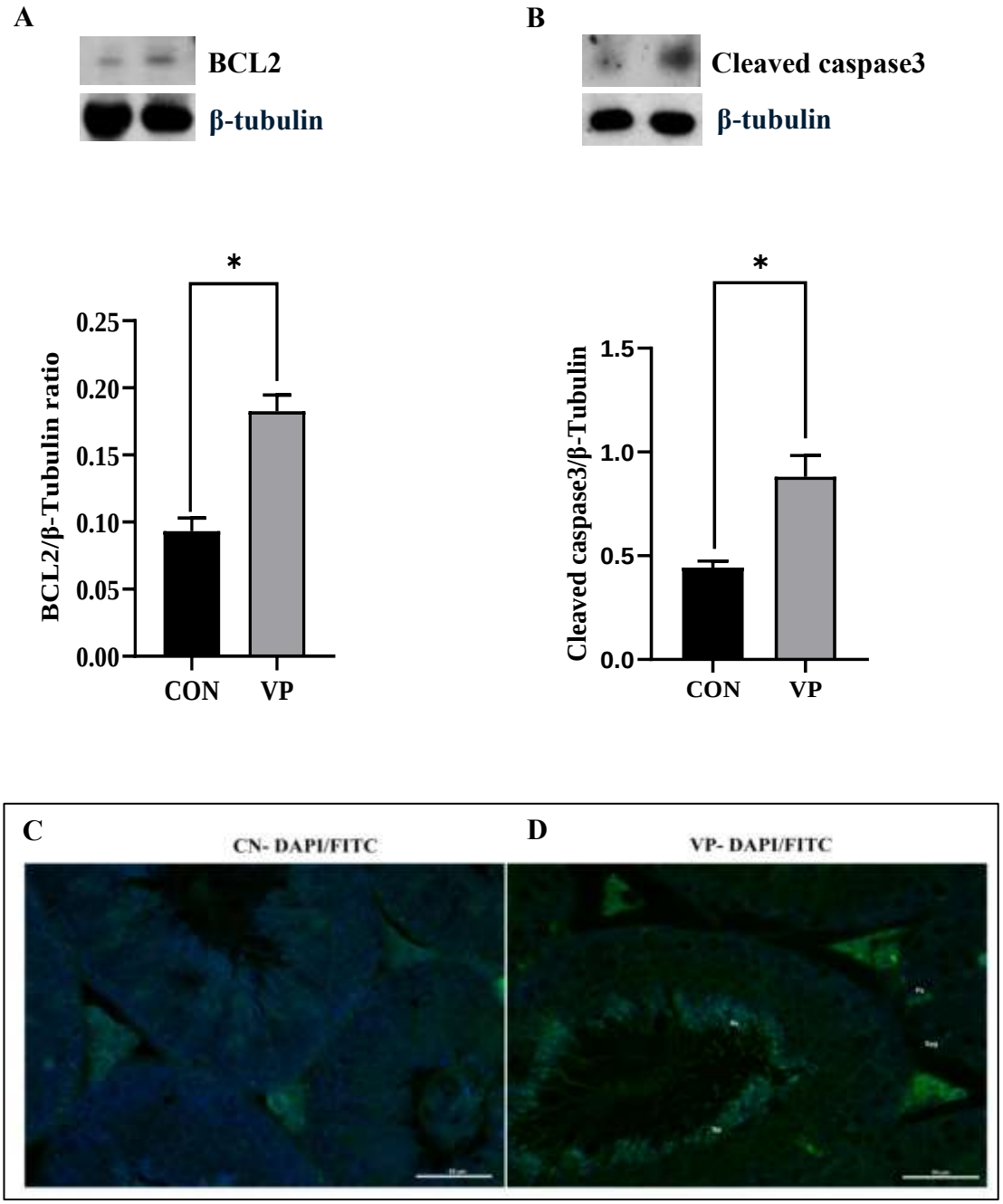

Figure-4.6- Changes on the expression of BCL2, cleaved caspase3 and TUNEL positive cells after exogenous visfatin administration. (A)The expression of BCL2 showed significant ($p<0.05$) declined after visfatin treatment. (B) Expression of

cleaved caspase3 was also significantly ($p<0.05$) decreased in visfatin treated group compared to control. TUNEL assay also showed more apoptotic germ cells in visfatin treated testis (D) than control (C). *- The difference between control and Visfatin treated group is significant at $p< 0.05$. Data are represented as mean±SEM (n=5 per group). Data analysis was done by students't-test. CN- Control group, VP- Visfatin treated group, Spg- Spermatogonia, Sp- Spermatozoa, Rs- Round spermatids, Ps- Primary spermatocytes.

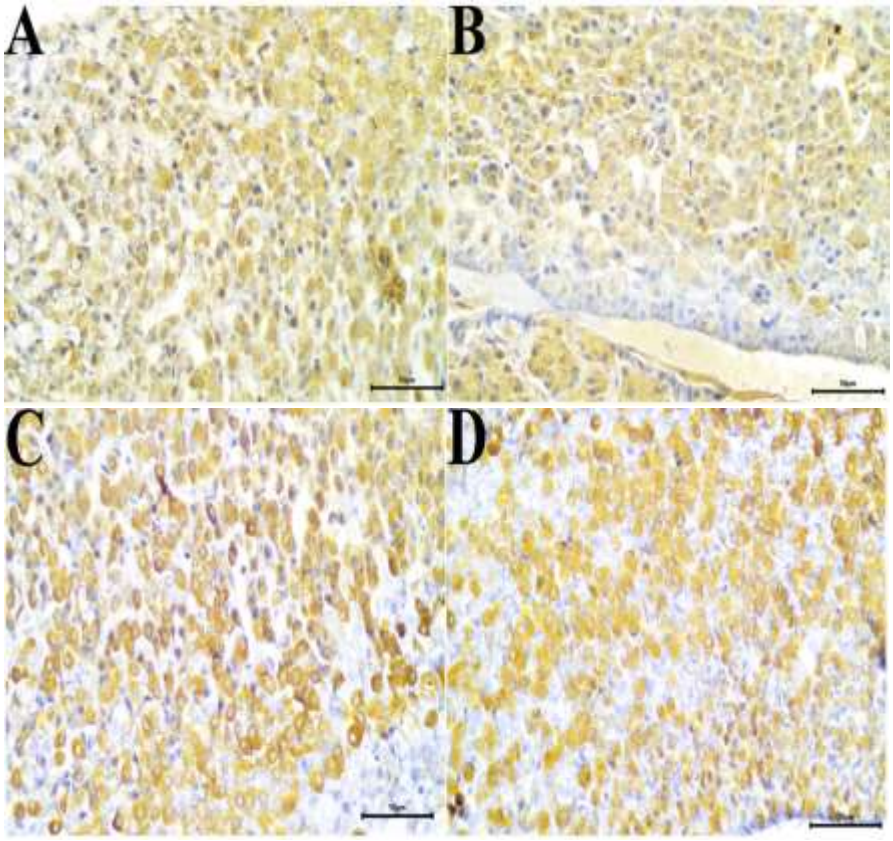

Figure-4.7- Changes on the immunolocalization of LH and FSH in the pituitary after exogenous visfatin administration. The immunostaining of LH in pituitary was more in (A) control group compared to (B) visfatin treated group. (C) Control and (D) visfatin treated group did not significant observable change on the immunostaining of FSH in the pituitary.

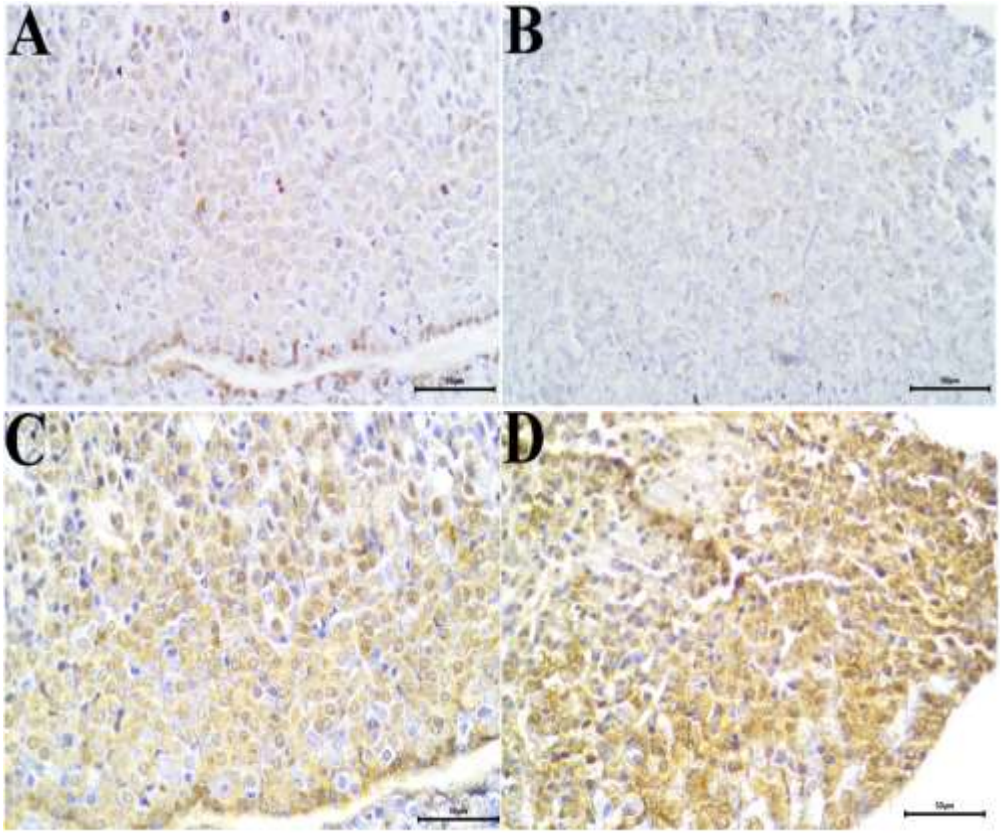

Figure-4.8- Changes on the immunolocalization of AR and GnRHR in the pituitary after exogenous visfatin administration. The immunostaining of AR in pituitary showed its abundance in the (A) control compared to (B) visfatin treated group. Immunolocalization of GnRHR was less in (C) control compared to (D) visfatin treated group.

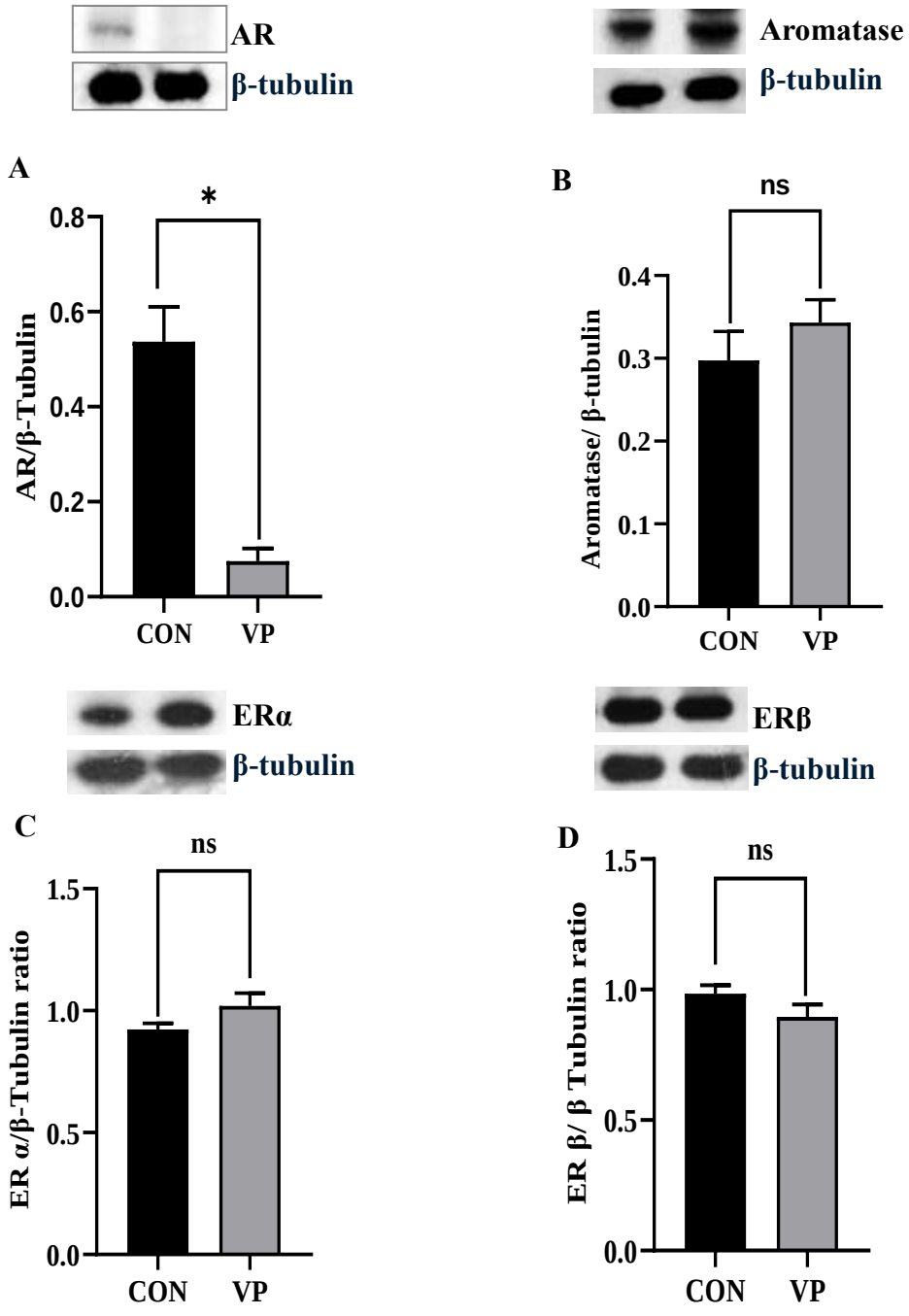

Figure-4.9- Changes on the expression of AR, aromatase and ERs after exogenous visfatin administration. (A) The expression of AR showed significant ($p<0.05$) decreased after visfatin treatment. (B) Expression of aromatase showed slight increased without significant changes after visfatin treatment. (C) The expression of ERα did not showed significant changes in visfatin treated group

compared to control group. (D) The expression of ERβ did not showed significant changes after visfatin administration. Data are represented as mean±SEM (n=5 per group). Data analysis was done by students't-test.

Consolidated summary

1. Immunolocalization of visfatin in the testis from PND7 to PND65 shows its presence in the interstitium and seminiferous tubules, with intense immunostaining of visfatin in the Leydig cells at PND7, PND14, PND42 and PND 65 and the Leydig cells at PND21 showed faint immunostaining of visfatin.
2. The circulating visfatin levels did not show significant variation from PND7 to PND65. However, a peak level was observed at PND65 and lowest level at PND7.
3. Testicular visfatin was increased from PND7 to PND 65, however significant changes were observed only in PND21 and PND65.
4. Furthermore, the changes in the postnatal testicular visfatin showed a significant positive correlation with testis weight ($r=0.48$, $p<0.05$). These findings suggest that mouse testicular visfatin expression and content is dependent on the postnatal age, and more likely it increases with age.
5. To unravel the possible role of visfatin in the testis during the infantile stages, we have inhibited the function of visfatin from PND14 to PND65 by FK866 treatment.
6. The inhibition of visfatin from PND14 to PND21 shows increases in the body weight and testis weight, but our result did not show significant changes in both parameters.
7. Our histological examination of testis shows some degenerative changes along with the increased in Seminiferous tubules diameter after visfatin inhibition during infantile period.
8. The circulating levels of testosterone also significantly ($p<0.05$) increased after FK866 treatment in the infantile mice.
9. Visfatin inhibition by FK866 showed increased expression of pro-apoptotic marker cleaved caspase3, suggesting the anti-apoptotic role of visfatin during infantile stages.

10. The immunohistochemistry analysis of two proliferating markers, GCNA and PCNA, was upregulated in the testis after visfatin inhibition at infantile stages.

11. The expression of aromatase was significantly ($p<0.05$) increased after visfatin inhibition. This result suggested that visfatin suppressed testosterone production by upregulating the aromatase expression at infantile stages.

12. The increased in the immunostaining of 17βHSD in the testis with increased circulating testosterone level need further investigation.

13. The western blot analysis of AR and ERβ also showed significant ($p<0.05$) increased after visfatin inhibition. These findings were supported by increased immunolocalization of AR in the testis and slight increase in the ERα expression in the testis after FK866 treatment.

14. These findings suggested that the supressed proliferation and apoptosis could be due to visfatin mediated downregulation of androgen and estrogen signalling in the infantile mice testis.

15. To access the role of visfatin during early pubertal age, *in vivo* visfatin inhibition was performed during PND25 to PND35 and the circulating steroid hormones levels, proliferating marker (GCNA, PCNA), apoptotic marker (Caspase3) along with anti-apoptotic marker (BCL2) were analysed by ELISA, immunohistochemistry and western blot analysis.

16. Estrogen level was significantly ($p<0.05$) increased after visfatin inhibition during PND25 to PND35, but the circulating levels of testosterone and androstenedione were not affected by visfatin inhibition.

17. The apoptosis marker caspase3 was significantly ($p<0.05$) increased after visfatin inhibition during early pubertal ages, however, the anti-apoptotic marker BCL2 also show to increased. There further studies would be required for the clarification of this results

18. Our data also shows that visfatin inhibition increased GCNA in the testis, suggesting the increased germ cell proliferation after FK866 treatment. This data was supported by increased in BrdU incorporation and PCNA in the testis after inhibiting visfatin from PND25 to PND35.

19. The western blot analysis of steroidogenic marker 17βHSD and 3βHSD increased significantly ($p<0.05$) after visfatin inhibition from PND25 to PND35. This result suggested that visfatin might have an inhibitory role in testicular steroidogenesis during early pubertal stages.

20. Despite no changes in the testosterone levels, the expression of AR was significantly ($p<0.05$) decreased which suggested that visfatin upregulates the testicular androgen signalling at early pubertal stages mice.

21. The upregulation of ERα and Erβ coincide with increasing estrogen levels after visfatin inhibition.

22. The decreased visfatin expression by FK866 in the testis could be due to declined in the visfatin synthesis by adipose tissues and testis.

23. Our results suggested that visfatin have differential role in the androgen and estrogen signalling at early pubertal stages. Visfatin stimulates androgen signalling more than estrogen signalling and the other parameters like apoptosis and proliferation seems to inhibit by visfatin.

24. To unravel the direct role of visfatin in adult mice we performed intra-testicular injection of visfatin inhibition. The intra-testicular injection of FK866 led to pathological changes in the testis histoarchitecture along with morphometric parameters after 24 hours, however the testis did not show further damages after 1 week.

25. Furthermore, the gross examination of testis shows that visfatin might have an essential role in testicular function by regulating the spermatids and sperm maturation, as our histological examination showed the depletion of sperm and spermatids after visfatin inhibition.

26. The localization of two proliferating markers, GCNA and PCNA, was decreased only after 24 hours of visfatin inhibition suggesting that visfatin is important for germ cell proliferation in adult mice testis.

27. The western blot analysis shows the increased expression of cleaved caspase3, and significant ($p<0.05$) decreased in Bcl2 expression after 24 hours of FK866 treatment. These results show visfatin inhibits apoptosis of germ cells in the testis.

28. However, BCL2 and caspase3 remains to declined after 1 week of visfatin inhibition, further studies are needed for the explanation of these findings. Moreover, it may be assumed that the testicular function could not be resume to normal state even after 1 week of visfatin inhibition.

29. The testosterone and estrogen levels declined after 24 hours of the FK866 treatment, followed by elevated testosterone levels in a post-a-week group. However, estrogen levels remained declined.

30. The levels of progesterone increased in the FK866-treated groups while the androstenedione levels was not affected by visfatin inhibition.

31. Our findings showed that the enzyme activity of 17βHSD and expression decreased in the after 24 hours of FK866 treatment, which coincides with low testosterone levels.

32. Despite no change in the aromatase expression, the estrogen levels in the post-24 hours of the FK866 group could be due to low testosterone levels.

33. The 17βHSD enzyme activity was lowest in the post-1 week of FK866 treatment. However, the elevated testosterone levels in this group could be due to decreased aromatase expression.

34. The testicular expression of AR decreased after 1 week of FK866 treatment, along with decreased expression of ERs.

35. Our results showed that visfatin regulates testicular steroidogenesis, proliferation and apoptosis in the adult testis and might have a stimulatory role in the testis.

36. We have shown that expression of testicular visfatin in the mice is developmentally regulated and inhibition of testicular visfatin supresses testosterone secretion. However, effects of visfatin on HPG axis of male have not been investigated. Therefore, we investigated the effects of exogenous visfatin on HPG axis of male mice in relation to their secretions.

37. Visfatin administration have significantly ($p<0.05$) increased the testis weight and sperm motility, however, the body weight and sperm concentration were not affected by exogeneous visfatin administration.

38. Despite significant ($p<0.05$) increased in the testis weight the gross examination of testis did not show much observable changes in the

seminiferous tubules of visfatin treated mice compared to control mice. However, a greater number of Leydig cells were observed in the interstitium of treatment mice testis compared to control.

39. The circulating testosterone levels showed exalted levels in the visfatin treated mice, while progesterone levels was found to be decreased.

40. The levels of androstenedione and estrogen did not show significant changes after visfatin treatment.

41. Visfatin administration also decreased LHR, CYP11A1, and 17βHSD expression in the testis.

42. Since CYP17 directly regulates testosterone biosynthesis by acting various steps of steroidogenesis, thus, we have also analysed the expression of CYP17. The elevated testosterone coincides with the up-regulated expression of CYP17 in the visfatin treated mice testis.

43. Therefore, it can be suggested that visfatin stimulates the testosterone secretion by elevating CYP17 expression in the mice testis.

44. Moreover, adrenal cortex also secrets small amount of testosterone, thus, whether, visfatin treatment has also affected the adrenal steroidogenesis and contributed to elevated circulating testosterone, remains to be investigated.

45. Visfatin treatment has supressed the LH and FSH secretions from pituitary. Moreover, the decreased LH secretion and LHR expression in the testis coincide with decreased progesterone levels.

46. The expression of AR was down-regulated by the visfatin treatment and ERs expression was unaffected. Thus, it may be suggested that elevated testosterone has suppressed the testicular AR expression.

47. The markers of proliferation PCNA and GCNA were also unaffected by the visfatin treatment, however, the expression of cleaved caspase3 and Bcl2 were up-regulated.

48. Since, we detect the increased cleaved caspase3, real executor of apoptosis, thus, excess visfatin might have stimulated apoptosis by supressing testosterone signalling. The increased expression of Bcl2 with increase apoptosis might have been a counter mechanism to prevent apoptosis.

49. The TUNEL assay in our experiments also showed stimulated apoptosis in the visfatin treated mice testis. Furthermore, TUNEL positive cells showed the stimulated apoptosis in the different cell types of the testis.

50. Despite increased GnRH levels, the suppressed gonadotropin levels were surprising. However, the circulating testosterone and GnRH showed positive correlation, and it might be suggested that elevated testosterone has inhibitory role in GnRH secretion.

51. It may also be suggested that visfatin might have stimulated the hypothalamic GnRH secretion, despite elevated testosterone may be expected to supress its secretion. Since the gonadotropin levels were low, thus, it may also be suggested that high or exogenous visfatin leads to hypogonadotropic like condition in males.

52. Despite increased GnRH levels and GnRHR in pituitary, the supressed gonadotropin by exogenous visfatin remains unclear. However, it may also be suggested that pituitary exhibits a condition like GnRH resistance due to supressed gonadotropin levels.

53. The immunolocalization of FSH in the anterior pituitary did not show observable changes after visfatin administration.

54. However, LH abundance was decreased in the pituitary after visfatin treatment. These findings suggest that secretion and synthesis of LH is compromised after visfatin administration.

55. The immunolocalization of AR was less in the visfatin treated pituitary. Therefore, testosterone associated suppression of gonadotropin seems to be superficial and visfatin might have directly supressed the gonadotropin secretion by acting on the pituitary.

56. According to our findings and evidence from earlier studies, we conclude that visfatin might have an inhibitory effect on the pituitary and stimulatory effects on the hypothalamus and testis.

57. Overall, visfatin role on male mice can be concluded that visfatin is an important adipokines that might regulate the testicular function in a stage dependent manner with respect to proliferation, apoptosis and steroidogenesis.

Conclusion

The present dissertation for the first time showed the role of testicular visfatin in infantile, early pubertal and adult mice with respect to proliferation and apoptosis, along with the possible effects of exogenous visfatin (hypervisfatinemia like conditions) on the hypothalamic-pituitary-testicular hormonal secretions in male mice.

The findings demonstrated that expression of testicular visfatin is upregulated with the progress of developmental stages. The role of visfatin in the testicular functions was unravelled by using FK866, a specific visfatin inhibitor. In the infantile mice, visfatin influences testicular steroid biosynthesis and is likely to have an inhibitory role in testicular steroidogenesis. Furthermore, our findings also suggested that visfatin may suppress testicular testosterone secretion by up-regulating aromatase expression in the testis during infantile mice. The suppression of apoptosis and germ cell proliferation in the infantile mice testis could be due to the visfatin-mediated down-regulation of androgen and estrogen signalling. However, during the early pubertal stages visfatin has a differential role in testicular androgen and estrogen, our data suggested that visfatin stimulate androgen signalling than estrogen signalling in the testis and the other parameters like apoptosis and proliferation is also seems to be inhibited by visfatin. In adult, our data showed that visfatin regulate the testicular function by regulating the spermatid along with sperm maturation. Our findings further suggested that in the adult testis visfatin also regulates steroidogenesis, proliferation and apoptosis and might have a stimulatory role in the testis.

In addition, a hypervisfatinemia like conditions has modulated the secretion of hypothalamus, pituitary and testis. Our findings also showed that visfatin inhibits gonadotropin secretion from pituitary, elevated GnRH and testosterone secretion. According to our findings, visfatin may have an inhibitory effect on pituitary and stimulatory effects on the hypothalamus and testis.

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M.Sc	2019	MZU	Zoology	63.75	First
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LIST OF PUBLICATIONS

1. **Rempuia, V., Anima, B., Jeremy, M., Gurusubramanian, G., Pankaj, P. P., Kharwar, R. K., & Roy, V. K.** (2022). Effects of metformin on the uterus of d-galactose-induced aging mice: Histomorphometric, immunohistochemical localization (B-cell lymphoma 2, Bcl2-associated X protein, and active capase3), and oxidative stress study. Journal of experimental zoology. Part A, Ecological and integrative physiology, 337(6), 600–611.
2. **Rempuia, V., Gurusubramanian, G., & Roy, V. K.** (2023). Evidence of the inhibitory role of visfatin in the testicular activity of mice during the infantile stage. The Journal of steroid biochemistry and molecular biology, 231, 106306.
3. **Rempuia, V., Gurusubramanian, G., & Roy, V. K.** (2024). Differential effect of visfatin inhibition on the testicular androgen and estrogen receptors expression in early pubertal mice. Endocrine, 84(3), 1216–1228.
4. **Rempuia, V., Gurusubramanian, G., & Roy, V. K.** (2024). Intra-testicular visfatin inhibition disrupts androgen and estrogen signalling in the mouse testis. Reproductive biology, 24(4), 100956.
5. **Rempuia, V., Gurusubramanian, G., & Roy, V. K**. (2025). Exogenous visfatin suppresses pituitary gonadotrophins and stimulates testosterone secretion in a male mouse. Journal of Neuroendocrinology, p.e70044.

CONFERENCE/SEMINAR/WORKSHOP ATTENDED

Online skill development program on '**In-silico drug designing and molecular dynamics simulations**', 01 –07 DECEMBER, 2021. Cytogene Research & Development Lucknow.

One-Week Training Program on "**Instruments in Biotechnology: Theories and Practices**". Organized by Department of Biotechnology, School of life sciences Mizoram University.

International Conference on "**Reproductive Biology, Comparative Endocrinology Development**". Organized by CSIR-Centre for Cellular and Molecular Biology, University of Hyderabad, 14th – 16th September 2022, CSIR-CCMB, Hyderabad.

International Conference on **Current Trends In Biological Sciences**. 18-20 March, 2024. Organized by School of Life Sciences, Mizoram University.

International Conference on "**Endocrinology, metabolism and reproduction: exploring new frontiers**". organized by Department of Zoology, University of Jammu, October 22th -25th, 2024. Jammu UT J&K, India.

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ABSTARCT

EXPRESSION AND ROLE OF VISFATIN IN THE TESTIS OF MICE

AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF DOCTOR OF PHILOSOPHY

VANLALREMPUIA

MZU REGISTRATION NO.: 1396 of 2014

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DEPARTMENT OF ZOOLOGY

SCHOOL OF LIFE SCIENCES

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EXPRESSION AND ROLE OF VISFATIN IN THE TESTIS OF MICE

By

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Department of Zoology

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Submitted

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

Preface and Consolidated Abstract

A number of factors have control and regulate reproduction in mammals, including hormones, lifestyle factors, and social factors. The molecules secreted mainly by white adipose tissue named adipokines, are also locally synthesized in the reproductive tract and have the ability to affect reproductive processes in male and female. Visfatin is also an adipokines and its expression has been shown in the testis of pigs, chicken, mice and rats. Furthermore, the expression of Visfatin is also seen in human granulosa cells and in cumulus cells along with the oocytes and little in theca cells. Moreover, visfatin concentration is also found to be positively correlated with body weight, testis weight and serum testosterone while negative correlation with the blood glucose concentration. In female, visfatin is shown to modulate the endocrine secretion of hypothalamus, pituitary and ovary, therefore it is considered as an important regulator of reproduction in female. Previous studies in female mice have shown that visfatin suppressed proliferation, apoptosis while increasing the survival of follicle whereas during the pre-pubertal stages it increases proliferation and survival of follicle and suppresses apoptosis and the estrogen secretion. Additionally, visfatin might also have involvement in Type 2 diabetes mellitus (T2DM) -associated testicular function deficit. The level of visfatin is also depends on maturation of pigs and stages of developing ovarian follicle, so a question arises whether visfatin expression is also changes during the postnatal testicular development? It is well known that testicular function is largely dependent on gonadal steroids, therefore it is hypothesized that testicular visfatin might also be modulated by the testicular steroid hormones and may further regulate the testicular steroidogenesis and spermatogenesis. Moreover, the role of visfatin in the testis of male mice is inconsistent. Consequently, the present research investigated the function of visfatin in the testis during infantile, pre pubertal stages and adult by using a laboratory male mice model.

The studies included in this thesis is divided into four chapters. **Chapter 1** describes age dependant changes of testicular visfatin expression and its possible role during

the infantile stages in relation to hormonal secretion, germ cell proliferation and apoptosis. **Chapter 2** illustrates the role of visfatin at early pre-pubertal stages in the regulation of testicular steroidogenesis, proliferation and apoptosis. **Chapter 3** investigated the direct role of visfatin on the testis of adult mice in relation to steroidogenesis and spermatogenesis by intratesticular visfatin inhibition. **Chapter 4** showed the effects of exogenous visfatin on the hypothalamic-pituitary-gonadal (HPG) of male mice in relation to their hormonal secretions and other testicular parameters.

Chapter-1: Expression and localization of visfatin in postnatal developmental testis and its possible role in infantile stages.

Adipokines have emerged as regulators of gonadal function in many mammalian and non-mammalian species. In the present study, we have investigated the developmental expression of testicular and ovarian visfatin along with its possible role in the testicular activity infantile stages. Previously, our group has the extensive role of ovarian visfatin in relation to steroidogenesis, proliferation, and apoptosis in female mice. To the best of our knowledge, no study has shown the role of visfatin in mice testis. Our results from the previous study and present study showed that visfatin in the testis and ovaries are developmentally regulated. To unravel the role of visfatin, we have used FK866, as visfatin inhibitor. FK866 was used as a visfatin inhibitor, to decipher the role of visfatin in the testis of mice. Our results showed that visfatin expression in the testis was developmentally regulated in the testis. Leydig cells as well as germ cell have shown the presence of visfatin in mice testis, which suggest its role in testicular steroidogenesis and spermatogenesis. Furthermore, visfatin inhibition by FK866 significantly increased the testosterone secretion, and expression of AR, Bcl2, and ERα. The expression of GCNA was upregulated by FK866 treatment. These results suggest that visfatin has an inhibitory role in testicular steroidogenesis and germ cell proliferation in the infantile stage. Further research is required to define the precise role of visfatin in infantile mice testis.

Chapter-2: Differential effect of visfatin inhibition on the testicular androgen and estrogen receptors expression in early pubertal mice

It is now well known that visfatin is expressed in the testis and ovary of various animals. Visfatin is known to regulate gonadal functions such as steroidogenesis, proliferation, and apoptosis in the ovary and testis of mice. Recently, we have shown that visfatin has an inhibitory role in the infantile mice testis. It has also been shown that visfatin stimulates testicular steroidogenesis in adult rats. However, the role of visfatin during puberty has not been investigated in relation to the above-mentioned process. The objective of the present study was to examine the effect of visfatin inhibition by FK866 from PND25 to PND35 (pre-pubertal to early pubertal) in male Swiss albino mice on steroidogenesis, proliferation, and apoptosis. Sixteen mice (25 days old) were divided into two groups, one group was given normal saline and the other group was administered with inhibitor of visfatin (FK866) at the dose of 1.5 mg/kg by intraperitoneal injection for 10 days. Histopathological and immunohistochemical analysis, western blot analysis and hormonal assay were done. Visfatin inhibition resulted in increased estrogen secretion, body weight, seminiferous tubule diameter, germinal epithelium height, proliferation along with increased expression of BCl2, casapse3, ERs and aromatase expression in the mice testis. Visfatin inhibition down-regulated the testicular visfatin expression and decreased abundance in the adipose tissues. In conclusion, decreased AR expression and increased ERs expression by FK866, suggest that visfatin might have a stimulatory effect on AR signaling than ERs in the early pubertal stage of mice.

Chapter-3: Intra-testicular visfatin inhibition disrupts androgen and estrogen signalling in the mouse testis

Visfatin is an important adipokines, which has been shown to be expressed in the testis of chicken, human and rodents. Visfatin expresses in the different cell types of testis and known to regulate several biological functions. Nevertheless, the direct role of visfatin in the adult testis has not been studied. In the present, we utilised mouse model to investigate testicular response such as, apoptosis, proliferation, and steroidogenesis in the testis after inhibiting visfatin by intra-testicular injection of FK866. The effects of visfatin inhibition were accessed at 24 hours and one post FK866 treatments. The testicular histoarchitecture showed degenerative changes after 24 hours of FK866 treatment and showed resumption in the architecture after

one week. Similarly, circulating testosterone levels, and proliferating markers suppressed after 24 hours and elevated after one week. The expression of BCl2 was down-regulated along with slightly elevation of caspase3 after 24 hours; however, both proteins still showed suppressed expression after one week. The expression of AR and ERα were down-regulated after one week of FK866 treatment. Furthermore, ERβ expression, 3βHSD, 17βHSD was down-regulated in the both groups compared to the control. Despite, down-regulation of some factors, the testicular proliferation and histoarchitecture showed resumption in the testis. This could be due to increased testosterone secretion by supressing aromatase expression. In conclusion, our result is first report on the direct role of visfatin in the adult testis. Visfatin has stimulatory role in the testosterone synthesis, proliferation in the testis. Moreover, some deregulated factors in the testis after one week of FK866 treatment, despite normal histoarchitecture treatment could be a compensatory mechanism after visfatin inhibitions.

Chapter-4: Exogenous visfatin modulates hypothalamic-hypophyseal-testicular hormones in mice

Visfatin expression has been shown in the testis, hypothalamus and pituitary. However, role of visfatin in HPG axis is fragmentary and no study has shown the effects of visfatin in the HPG axis of male in relation to their hormonal secretions. The present study has investigated the effects of exogenous visfatin, (most likely a state of hypervisfatinemia) on the hormonal secretion of each links of HPG axis in a male mouse. The effects of exogenous visfatin were given for 35 days, which covers one spermatogenic cycle. The circulating testosterone was elevated after visfatin treatment, and there was a down-regulation of AR along with steroidogenic markers in the testis, which coincides with elevated apoptosis. The levels of circulating LH and FSH were also supressed after visfatin treatment. The pituitary showed decreased abundance of GnRHR along with elevated circulating GnRH levels. These conditions suggest GnRH resistance at pituitary levels as low gonadotropin were observed. Furthermore, AR abundance in pituitary of visfatin treated mice also showed decreased abundance, thus it can be suggested that pituitary gonadotropin secretin suppressed by direct action of visfatin rather than via elevated testosterone.

In conclusion, our results showed that exogenous visfatin modulated secretion along HPG axis in a differential manner. Visfatin has stimulatory role in hypothalamic GnRH secretion, inhibitory in pituitary gonadotropin and stimulatory in testosterone secretion from the testis. Thus, conditions similar to hypervisfatinemia likely impair the release of hormones along the HPG axis. The physiological significance of elevated visfatin requires further investigation along HPG axis.