ROLE OF VISFATIN IN REPRODUCTIVE ORGANS OF FEMALE MICE

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ROLE OF VISFATIN IN REPRODUCTIVE ORGANS OF FEMALE MICE

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

CERTIFICATE

I certify that the thesis entitled "**Role of visfatin in reproductive organs of female mice**" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **Annie Lalrawngbawli** is a record of research work carried out during the period of 2016-2021 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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January, 2021

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

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

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Preface and Consolidated Abstract

Mammalian reproduction is regulated by a number of factors. Among the various factors, adipokines are the important factors which are linked in the regulation of male and female reproduction. These adipokines are mainly secreted by the adipose tissue and regulates several biological functions. Not only the adipose tissue, adipokines are also known to be expressed by the gonads and regulates gonadal function in an autocrine and paracrine manner. Visfatin is also an adipokine, which is expressed in the ovary of human, hen, turkey and rodents. Furthermore, visfatin, expression has also been shown in pig uterus. The role of visfatin has been shown in the gonadal steroidogenesis in human and chicken. It has also been shown that the expression of visfatin is developmentally regulated in the gonads. Visfatin is also known to regulate proliferation and apoptosis in various tissues and cells. It has also been shown that levels of visfatin are deregulated in the ovarian pathogenesis such as polycystic ovary syndrome in human. Despite its expression in ovary and uterus of some organisms, there is dearth of literature on the role of visfatin in laboratory animals such as rat and mice. The present dissertation has undertaken the work to explore the role of visfatin in laboratory mice. As visfatin has been shown in the ovary, then a question arises, whether visfatin expression changes during postnatal ovarian development? Since mice estrous cycle is well characterized and various factors also changes during the estrous cycle, so a question arises again, how does the expression of ovarian visfatin changes during the estrous cycle in ovary and uterus of mice? Furthermore, uterine factor and its function is largely dependent on gonadal steroids, thus it may be hypothesized that uterine visfatin expression may also be modulated by the ovarian steroids. Whether the deregulated levels of visfatin in pathogenesis could also be targeted for management of PCOS, is still not clearly known. These are the questions and research gaps, which prompted us to work on the visfatin in the gonadal and uterine functions by using a laboratory mice model.

The study incorporated in this thesis is thus broadly divided into five chapters. **Chapter 1** describes postnatal changes in ovarian visfatin and its role on the ovarian steroidogenesis, proliferation and apoptosis. **Chapter 2** describes the detailed role of ovarian visfatin in the regulation of ovarian steroidogenesis, proliferation and

apoptosis by in vitro and in vivo study. **Chapter 3** describes the changes in ovarian visfatin during estrous cycle and its potential role in the proestrus with respect to proliferation and apoptosis. **Chapter 4** describes the estrous cycle mediated changes in the uterine visfatin and its regulation by ovarian steroids such as estrogen and progesterone. **Chapter 5** showed that ovarian and circulating visfatin are elevated in the letrozole-induced hyperandrogenised mice and further this chapter showed that inhibition of elevated visfatin by its inhibitor, FK866 could be important for management of PCOS.

Chapter-1: Visfatin protein may be responsible for suppression of proliferation and apoptosis in the infantile mice ovary

Visfatin is an important adipokine, which is expressed in different tissues including ovary of mammals. The postnatal ovary in rodents undergoes dramatic changes of intra-ovarian factors in relation to proliferation and apoptosis. There are studies which showed that gonadal visfatin changes in postnatal life. However, role of visfatin in the early postnatal period i.e. infantile period has not been studied. Therefore, the present study was aimed to explore the role of visfatin in the early postnatal ovarian functions. Furthermore, to explore the role of visfatin, the endogenous visfatin was inhibited from PND14-PND21 by FK866 with dose of 1.5 mg/kg. Our results showed gain in body weight and ovarian weight after visfatin inhibition. The inhibition of visfatin increased the ovarian proliferation (increase in PCNA, GCNA expression and BrdU incorporation) and apoptosis (increase in BAX and active caspase3 expression). Moreover, visfatin inhibition decreased the expression of anti-apoptotic/survival protein, BCL2 in the ovary. These findings suggest that visfatin in the infantile ovary may suppress the proliferation and apoptosis by up-regulating BCL2 expression. An interesting finding has been observed that circulating estrogen and progesterone remain unaffected, although visfatin inhibition up-regulated ER-β and down-regulated ER-α. It may also be suggested that visfatin could regulates proliferation and apoptosis via modulating estrogen signaling. In conclusion, visfatin inhibits the proliferation and apoptosis without modulating the ovarian steroid biosynthesis and visfatin mediated BCL2

expression could also be mechanism to preserve the good quality follicle in early postnatal period.

Chapter-2: Inhibition of visfatin/NAMPT affects ovarian proliferation, apoptosis, and steroidogenesis in pre-pubertal mice ovary

Pubertal ovarian function might be dependent on the factors present in the pre-pubertal stages. Visfatin regulates ovarian steroidogenesis in adult. To date, no study has investigated the role of visfatin either in pre-pubertal or pubertal mice ovary. Thus, we investigated the role of visfatin in pre-pubertal mice ovary in relation to steroidogenesis and proliferation and apoptosis in vitro by inhibiting the endogenous visfatin by a specific inhibitor, FK866. Inhibition of visfatin increased the estrogen secretion and also up-regulated the expression of CYP11A1, 17β-HSD and CYP19A1 in mice ovary. Furthermore, active caspase3 was up-regulated along with the down-regulation of BAX and BCL2 in the pre-pubertal ovary after visfatin inhibition. The expression of GCNA, PCNA, and BrdU labeling was also decreased by FK866 treatment. These results suggest that visfatin inhibits steroidogenesis, increases proliferation, and suppresses apoptosis in the pre-pubertal mice ovary. So, visfatin is a new regulator of ovary function in pre-pubertal mice.

Chapter-3: Changes in the localization of ovarian visfatin protein and its possible role during estrous cycle of mice

Visfatin is a crucial adipokine, which also regulates ovarian functions in many animals. Mice estrous cycle is characterized by a dynamic complex physiological process in the reproductive system. Expression of various factors changes during the estrous cycle in the ovary. To the best of our knowledge, no previous study has been conducted on the expression of visfatin in mice ovaries during the estrous cycle. Therefore, we investigated the localization and expression of visfatin protein in the ovary of mice during the estrous cycle. Western blot analysis showed the elevated expression of visfatin in proestrus and lowest in diestrus. Immunohistochemical

localization of visfatin showed intense staining in the corpus luteum of proestrus and diestrus ovaries. Thecal cells, granulosa cells, and oocytes also showed the presence of visfatin. Expression of ovarian visfatin was correlated to BCL2 and active caspase3 expression and exhibited a significant positive correlation. Furthermore, *in vivo* inhibition of visfatin by FK866 in the proestrus ovary down-regulated active caspase3 and PCNA expression, and up-regulated the BCL2 expression. These results suggest the role of visfatin in the proliferation and apoptosis of the follicles and specific localization of visfatin in the corpus luteum also indicate its role in corpus luteum function, which may be in progesterone biosynthesis and regression of old corpus luteum. However, further study is required to support these findings. In conclusion, visfatin may also be regulating follicular growth during the estrous cycle by regulating proliferation and apoptosis.

Chapter-4: Estrogen and progesterone dependent expression of visfatin/NAMPT regulates proliferation and apoptosis in mice uterus during estrous cycle

Visfatin is an adipokine which has an endocrine effect on reproductive functions and regulates ovarian steroidogenesis. There is scant information about the expression, regulation, and functions of visfatin in the mammalian uterus. The present study examined expression and localization of visfatin in the mouse uterus at various stages of the natural estrous cycle, effects of estrogen and progesterone on localization and expression of visfatin in the ovariectomised mouse uterus and effect of visfatin inhibition by a specific inhibitor, FK866 on proliferation and apoptosis in the uterus. Western blot analysis of visfatin showed high expression in proestrus and metestrus while it declined in estrus and diestrus. Immulocalization study also showed strong immunostaining in the cells of endometrium, myometrium, luminal and glandular epithelium during proestrus and metestrus that estrus and diestrus. The uterine visfatin expression closely related to the increased estrogen levels in proestrus and suppressed when progesterone rose to a high level in diestrus. The treatment with estrogen to ovariectomised mice up-regulates visfatin, PCNA, and

active caspase3 whereas progesterone up-regulates PCNA and down-regulates visfatin and active caspase3 expression in mouse uterus. The co-treatment with estrogen and progesterone up-regulates visfatin and down-regulates PCNA and active caspase3. In vitro study showed endogenous visfatin inhibition by FK866 increased expression of PCNA and BCL2 increased catalase activity while FK866 treatment decreased expression of Active caspase3 and BAX with decreased SOD and GPx activity. BrdU labeling showed that inhibition of visfatin modulates the uterine proliferation. This study showed that expression of visfatin protein is steroid dependent in mouse uterus which is involved in the regulation of proliferation and apoptosis via modulating antioxidant system in the uterus of mice during the reproductive cycle.

Chapter-5: Inhibition of visfatin by FK866 mitigates pathogenesis of cystic ovary in letrozole-induced hyperandrogenised mice

Polycystic ovary syndrome (PCOS) is one of the common reproductive disorders in the female of reproductive age, which is characterized by hyperandrogenism, insulin resistance, cystic ovary and infertility. The levels of pro-inflammatory adipokines such as visfatin have been shown to be elevated in the PCOS conditions in human and animal as well. Therefore, it is hypothesized that inhibition of PCOS associated hypervisfatinemia, might ameliorate the pathogenesis of PCOS. In the present study, letrozole induced hyperandrogenised PCOS like mice model have been used to unravel the effects of visfatin inhibition. The results of this study showed that visfatin inhibition in PCOS has suppressed the secretion of androgens, androstenedione and testosterone levels. Furthermore, the histological study also showed that visfatin inhibition suppressed the cyst formation and promotes corpus luteum formation. Visfatin inhibition has also suppressed the apoptosis and increases the expression of anti apoptotic protein BCL2 along with increase in the proliferation (GCNA expression elevated). The visfatin inhibition has increased the intraovarian glucose content, which was supported by the increase in the ovarian GLUT8 expression. The *in vitro* study has also supported the *in vivo* findings where visfatin

inhibition by FK866 suppressed the androgen production from PCOS ovary. In conclusion, this is the first report, which showed that inhibition of visfatin by FK866 in hyperandrogenised mice ameliorates the pathogenesis of PCOS. Thus, it may be suggested that visfatin inhibition could have a therapeutic potential in the management of PCOS along with other intervention.

General Introduction

Reproduction is a highly regulated process by the endocrine factors, secreted from the different endocrine glands. Adipose tissue is considered an endocrine gland, and adipocytokines or adipokines are the class of cytokine, secreted by adipose tissue, which regulates the metabolic and reproductive functions in males and females **(Scheja and Heeren, 2019; Ntaios et al., 2013; Trujillo and Scherer, 2006)**. Various reports show that adipokines are involved in the reproductive functions in normal and pathological conditions **(Tsatsanis et al., 2015; Elfassy et al., 2018; Reverchon et al., 2014; Campos et al., 2008)**. It has been shown that various adipokines are expressed in the ovarian cells, and they can modulate ovarian physiology in animals, like pigs, cows, goats, ewes, chickens, turkeys and rodents **(Rak et al., 2017a; Cheng et al., 2016)**. It has also been postulated that adipokines may be beneficial for the regulation of reproductive functions, and there is a need to explore the role of various adipokines in reproduction **(Campos et al., 2008)**.

Types of adipokines

There are various adipokines, such as leptin, adiponectin, resistin, chemerin, tumor-necrosis factor, interleukin, apelin, vaspin, and visfatin. The four known adipokines, adiponectin, visfatin, omentin and vaspin, all increase the tissue sensitivity to insulin, and thus are described as 'beneficial' adipokines **(Campos et al., 2008)**. Adipokines act in a paracrine, autocrine, and endocrine fashion, influencing cytokine and chemokine secretions and hormonal and growth factors, along with lipid and glucose metabolism **(Pereira et al., 2014)**. There is strong support for the role of adiponectin in ovarian and placental functions. There is evidence for direct effects of this adipokine on the late stages of folliculogenesis, and additive interactions of adiponectin with insulin and gonadotropins in inducing periovulatory changes in ovarian follicles. Despite adipose tissue, these adipokines are also known to be expressed in the gonads and suggested to play an essential role in gonadal functions **(Bongrani et al., 2019)**.

Visfatin

Visfatin is one of the new members of adipokines, which was discovered by **Fukuhara et al. (2005)**. It was shown that visceral fat secretes more visfatin than subcutaneous. It has further been suggested that visfatin stimulates glucose uptake in muscle and fat cells and inhibits glucose release from hepatocytes **(Fukuhara et al., 2005; Hug and Lodish, 2005)**. It has also been suggested that visfatin regulates glucose metabolism by binding to the insulin receptor other than insulin site, however this mechanism of visfatin is still not clear due to the retraction of published initial work **(Fukuhara et al., 2007)**. Before being named as visfatin, this adipokine was known as pre-B cell colony-enhancing factor (PBEF), and nicotinamide phosphoribosyltransferase (NAMPT) **(Samal et al., 1994)**; visfatin is a highly conserved, 52 kDa protein expressed in a variety of tissues and cell types, including adipocytes, lymphocytes, bone marrow, liver, muscle, trophoblast, and fetal membranes **(Fukuhara et al., 2005)**.

Furthermore, the crystal structure of visfatin (PBEF/NAMPT) showed the visfatin as an important enzyme in nicotinamide adenine dinucleotide (NAD) biosynthesis **(Wang et al., 2006)**. Visfatin, on the other hand, is also known as a cytokine hormone and acts as an important enzyme which is involved in obesity, diabetes and immune disorders **(Berndt et al., 2005)**. It has been shown that mammalian visfatin exists in two forms, the intra- and extracellular forms, iNAMPT and eNAMPT **(Revollo et al., 2007)**. The brown adipose tissue in mice has been shown to have the highest expression of iNAMPT than liver and kidneys. The low levels of visfatin have been shown to be expressed in the white adipose tissue (WAT), lungs, spleen, testis, and skeletal muscle. It has been shown that intracellular visfatin is involved in the NAD biosynthesis and plays an important role in sirtuin activation in mitochondria, and extracellular visfatin function is not very clear, as this is secreted by the number of normal cell types, such as adipocytes, hepatocytes, myocytes, pancreatic cells, neurons and immune cells **(Revollo et al., 2007; Yoon et al., 2015)**.

Visfatin and Ovary

Despite the wider distribution of visfatin in the different tissues and cells, visfatin still needs to be explored in relation to ovarian physiology. Visfatin has been shown to be expressed in the different tissues of hen in a sex-dependent manner along with ovary, and it has been shown that visfatin suppressed the progesterone synthesis in hen granulosa cells **(Ons et al., 2010; Diot et al., 2015b)**. In the ovary of turkey, visfatin has also been shown to be expressed, and its level declines during the laying cycle **(Diot et al., 2015a)**. In the mammalian species, visfatin has been reported in the ovaries of cow, buffalo, human, rat and mice **(Reverchon et al., 2016; Thakre et al., 2020; Shen et al., 2010; Nejabati et al., 2020; Choi et al., 2012)**. In the cow ovary, visfatin has been shown to stimulate steroidogenesis and its other potentialities of insulin-like growth factor-1 (IGF-1) effects by increasing steroidogenic markers, Steroidogenic acute Regulatory Protein (StAR) and 3β-hydroxysteroid dehydrogenase (3β-HSD) expression, as well as estrogen and progesterone secretion **(Reverchon et al., 2016)**. In human, it has been shown that visfatin is mainly expressed in the granulosa cells, with less expression in cumulus cells, oocytes, and theca cells. It has also been suggested that visfatin in human ovary could have functioned as it was reported in the bovine ovary; where visfatin has a positive effect on steroidogenesis **(Reverchon et al., 2013)**. To the best of our knowledge, very less research has been done on visfatin and the ovary with respect to mice and rat. There were two reports on the effect of visfatin in mice ovary. The first report by **Choi et al. (2012)** showed that administration of visfatin during super-ovulation improves developmental competency of oocytes and fertility potential in aged female mice and second very recently, **Park et al. (2020)** showed that visfatin restores fertility in aged mice. Thus, these two studies have suggested some vital role of visfatin in mice ovary. Even in the rat model, the role of visfatin has been unexplored in the ovary. Therefore, further study is required to unravel the role of visfatin in ovarian physiology.

Adipokine, Visfatin and Uterus

Not only the ovary rather different adipokines and their receptors are present in the female reproductive tract of different species. As the various adipokines are known to regulate the ovarian function such as steroidogenesis, and oocytes maturation, in the same way, these adipokines are also present in the uterus and placenta where they could create a favourable environment for embryonic implantation and play a key role in maternal-fetal communication and gestation **(Reverchon et al., 2014)**. The role and expression of adipokines have also not been explored much, and still, this needs further investigation to warrant the role of adipokines in the uterine physiology. It has been shown that adiponectin receptors (AdipoR1 and AdipoR2) are expressed in the pig endometrium and human glandular epithelium **(Lord et al., 2005; Takemura et al., 2006)**. These adiponectin receptors have also been shown in the uterus of rabbit, pig, human, and rodents **(Schmidt et al., 2008; Takemura et al., 2006; Kim et al., 2011)**. It has been hypothesized that low adiponectin receptor expression could lead to implantation failure in human **(dos Santos et al., 2012)**. These studies suggest that a change in the expression of adiponectin and/or its receptors may be involved in endometrial receptivity **(Kim et al., 2011; dos Santos et al., 2012)**. Leptin and its receptor have also been shown in the canine uterus and placenta during pregnancy and it has been suggested that it might play an important role in various cell types in a paracrine/autocrine manner **(Balogh et al., 2015)**. Resistin expression has been documented in the sheep uterus and further has been suggested to be involved in the functionality of the uterus, which is also affected by the animal's nutritional status **(Dall'Aglio et al., 2019)**. The human uterus has also been shown to produce chemerin, and it is differentially expressed by decidual cells during early pregnancy. Chemerin production in the uterus is up-regulated during decidualization, suggesting an important role in vascular remodelling during early pregnancy **(Carlino et al., 2012)**. Visfatin expression has been shown in the pig uterus **(Palin et al., 2008)**. Only a few data are available on the potential role of visfatin in the uterine physiology. In rat and human myometrium, visfatin has been shown to inhibit the contractility, and it has been suggested that increased output of

visfatin and leptin in obese pregnant women may impair uterine contractility **(Mumtaz et al., 2015)**.

Adipokine, Visfatin and Polycystic ovary

Polycystic ovary syndrome (PCOS) is a pathological condition which prevails in the women of reproductive age. This is a heterogeneous pathological condition, which is characterized by acne, hirsutism, hyperandrogenemia, polycystic ovaries, anovulation, and infertility **(Goodarzi et al., 2011)**. Despite its above features, PCOS has also been shown to be closely associated with metabolic syndromes such as insulin resistance, hyperinsulinemia, dyslipidemia, and obesity. Additionally, it has been shown that altered metabolic profiles in PCOS patients, where increase glycolysis and disturbance in amino acids metabolism has been reported **(Sun et al., 2012; Zhao et al., 2012)**. Therefore, PCOS can lead to an increased risk of developing type 2 diabetes mellitus (T2DM) and cardiovascular disease in patients **(Diamanti-Kandarakis and Dunaif, 2012)**. Since PCOS has been linked to obesity, and it is well known that fat mass massively accumulated to store energy, and on the other hand, fat cell/adipose tissue is not an only energy depot rather it can be considered as an endocrine gland, which secrets several adipokines, such as leptin, adiponectin, resistin, chemerin, tumor necrosis factor, omentin, apelin, retinol-binding protein-4 and visfatin. It has been shown that abnormal levels of adipokines are strongly associated with insulin resistance, type 2 diabetes and PCOS **(Chen et al., 2013)**. The adiposity associated pathogenesis in PCOS is an outcome of adipose tissue dysfunction which leads to an over-production of pro-inflammatory adipokines such as tumor necrosis factorα (TNF-α), and the reduced expression of some 'beneficial adipokines' such as adiponectin. It has been reported that several identified adipose-specific and some of the non-adipose-specific adipokines in female reproduction may act as link between obesity and PCOS; other adipokines, such as TNFα, IL6, and IL1β, are also known for their association with inflammation and PCOS **(Chen et al., 2013; Bohler et al., 2010)**.

The data from the human studies showed that some circulating adipokines are elevated, and some are inhibited. Many studies have shown that leptin levels are

elevated in the PCOS patient that control **(Brannian and Hansen, 2002; Pehlivanov and Mitkov, 2009; Yildizhan et al., 2011)** and some studies found there was no change in the serum leptin between PCOS and control **(Svendsen et al., 2012; Carmin et al., 2009)**. The data of serum adiponectin levels in relation to PCOS and controls are still controversial **(Orio 2003; Spranger et al., 2004; Pinhas-Hamiel et al., 2009, Lecke et al., 2011)**. It has been shown that serum adiponectin concentrations are reduced in PCOS patients compared with controls in some studies **(Ardawi and Rouzi, 2005; Escobar-Morreale et al., 2006; Pinhas-Hamiel et al., 2009; Manneras-Holm et al., 2011)**, whereas some study has shown that, serum adiponectin concentrations did not show the change between PCOS and controls **(Orio, 2003; Lecke et al., 2011)**. Similarly, for resistin also some reports showed that there was no significant change either in ovarian and circulating resistin between PCOS and control **(Panidis et al., 2004; Escobar-Morreale et al., 2006; Seow et al., 2007; Zhang et al., 2011)**, furthermore, **Escobar-Morreale et al. (2006)** showed that serum resistin levels were increased in overweight and obese women compared with lean subjects, irrespective of their PCOS or controls status. The plasma omentin-1 concentrations were found to be decreased in PCOS and insulin-resistant women compared with control subjects **(Tan et al., 2008; Choi et al., 2011)**. Chemerin showed a significant increase in serum levels, as well as in subcutaneous, and omental adipose tissue showed increase mRNA level and protein expression in the PCOS **(Tan et al., 2009)**.

As other adipokines data are conflicting in relation to PCOS, the same way data on visfatin and PCOS are also not conclusive. The circulating visfatin has been shown to be significantly increased in overweight/obesity, type 2 diabetes, metabolic syndrome, and cardiovascular diseases conditions **(Chang et al., 2011)**. On the other hand, in some other studies, plasma visfatin levels in obese subjects were reported to reduce with weight loss **(Haider et al., 2006; Choi et al., 2007)**. In the PCOS women increase gene expression of visfatin along with elevated plasma visfatin has also been reported **(Tan et al., 2006; Carmina et al., 2009; Ozkaya et al., 2010; Seow et al., 2011)**. However, some studies did not find a difference in plasma or

serum visfatin levels between patients with PCOS and control groups **(Gudducu et al., 2012; Lajunen et al., 2012; Olszanecka-Glinianowicz et al., 2012)**.

Visfatin exhibits pro-inflammatory properties and modulates immune functions and further it has been reported that visfatin induces NF-κB signalling in human endothelial cells and activated MMP-2/9, indicating its possible role in the pathogenesis of PCOS with its pro-inflammatory characteristics **(Adya et al., 2008; Chen et al., 2013)**. Recently it has been shown that visfatin concentration decreases in follicular fluid of the PCOS women, and it was suggested that visfatin has a positive effect on female reproductive function **(Bongrani et al., 2019)**. Although very fewer reports are available on visfatin and experimental model of PCOS. The laboratory animals such as rat and mice are frequently used to study the PCOS like pathogenesis. In a very recent study by **Nejabati et al. (2020)** showed PCOS rat had significantly higher visfatin expression in the ovary and adipose tissue. As it has been shown that rodent models of human pathological conditions, such as PCOS, may replicate many or most clinical characteristics of that disorder **(Walters et al., 2012)**, therefore, it may be suggested that visfatin further deserve study on a rodent model of PCOS for conclusive information.

Biological functions of visfatin

Visfatin has been shown to have a plethora of biological functions. It has been shown that visfatin being a pro-inflammatory cytokine, it stimulates cell proliferation through AKT/PI3K and ERK/MAPK pathway in cancer cell lines and it also protects against apoptosis in these cells **(Gholinejad et al., 2017)**. In the endothelial progenitor cells visfatin decreased the expression of the anti-apoptotic gene, Bcl-2, and increased expression of the pro-apoptotic gene, caspase-3 and it has been suggested that visfatin regulates the process of cell death **(Sun et al., 2017)**. The cell proliferation process may be regulated by visfatin, in the mouse pancreatic cell line, visfatin increase β-cell proliferation and inhibits apoptosis **(Cheng et al., 2011)**. Visfatin can also act as a survival factor in different cell types, such as visfatin protects the macrophages from ER stress-induced apoptosis by activating an IL6/STAT3 signalling pathway **(Li et al., 2008)**. Visfatin also contributes to cellular

resistance against genotoxic/oxidative stress and may increase the survival of immune cell **(Rongvaux et al., 2008)**. FK866 is a potent Nampt catalytic inhibitor, which prevented visfatin-mediated cell protection **(Yang et al., 2007)**. The cardioprotective effects of visfatin have also been shown in the murine hearts and isolated murine cardiomyocytes **(Lim et al., 2008)**. It has also been shown that visfatin prolong the life span of smooth muscle cells by activating SIRT1 and restricting the accumulation of p53 **(Van der Veer et al., 2007)**. Visfatin also has a vital role in the normal glucose-dependent insulin secretion, and β-cell function **(Revollo et al., 2007)**. It has also been shown that visfatin is a helpful tool to preserve islet function and regulate insulin secretion **(Tanaka and Nabeshima, 2007)**. Visfatin has multifunctional activities and exerts its pro-survival action by different mechanisms depending on the apoptotic protection. In the gonads of some animals, including human, visfatin has also been shown to regulate steroidogenesis and gametogenesis. The direct role of visfatin on testosterone production by Leydig cells has also been documented **(Hameed et al., 2012)**. In the male chicken, the circulating visfatin significantly increased in the adult compared to the pre-pubertal stage and has also been hypothesized about its role in spermatogenesis and steroidogenesis **(Ocón-Grove et al., 2010)**. However, in the hen ovary circulating visfatin was lower in adult than juvenile. In hen ovary, visfatin decreased the progesterone biosynthesis and had no effect on granulosa cell proliferation **(Diot et al., 2015b)**. The human ovary also expresses visfatin in the granulosa cells, and it has been shown that visfatin increases IGF-1-induced steroidogenesis and cell proliferation through the AMPK/SIRT1 signalling pathway **(Reverchon et al., 2013)**. The data from bovine granulosa cell also showed that recombinant visfatin increases the secretion of progesterone and estradiol, which was associated with an increase in steroidogenic markers **(Reverchon et al., 2016)**. In a recent study, the role of visfatin on ovarian steroidogenesis of water buffalo has also been shown that visfatin stimulates estradiol and progesterone secretion by ovarian cells **(Thakre et al., 2020)**. These studies on the functional aspects of visfatin also suggest its role in reproductive organs of mice.

Rationale of work

It is well known that postnatal rodent ovary shows dramatic changes in intra-ovarian factors in relation to proliferation and apoptosis. The ovarian cell proliferation and death are a finely regulated process **(De Felici et al., 2000; Coucouvanis et al., 1993)**. There is some evidence that adipokine like tumour necrosis factor-related protein 3, adiponectin and resistin regulates ovarian proliferation and apoptosis **(Mao et al., 2018; Singh et al., 2015; Reverchon et al., 2013; Rak-Mardyła et al., 2014; Rak et al., 2017b)**. The previous study showed that visfatin levels are lower in the pre-pubertal stage and increases in adolescence **(Nourbakhsh et al., 2015; Giapros et al., 2012)**. It has been shown that adipokine like leptin accelerates follicular growth by suppressing the apoptosis and proliferation in the pre-pubertal rat **(Almog et al., 2001)**. Another adipokine such as TGF-β1 has been shown to influence ovarian follicular growth and differentiation in postnatal and immature ovarian models **(Rosairo et al., 2008)**. Expression of various factors changes during the estrous cycle in the ovary and uterus as well. The uterine functions are known to be regulated by the ovarian steroids. In addition, the deregulation of visfatin has been shown in the PCOS condition.

Therefore, the present dissertation evaluated the possible role of visfatin in infantile, pre-pubertal and adult ovary along with the uterus. The present dissertation also elucidated the role of visfatin in the pathogenesis of PCOS in letrozole induced hyperandrogenism mice model. To the best of our knowledge, no previous study has been conducted on the role of visfatin in mice reproductive organ.

Visfatin protein may be responsible for suppression of proliferation and apoptosis in the infantile mice ovary

Introduction

The mammalian ovary has been evolved to serve the two functions, namely, oogenessis and steroidogenesis; and ovarian development is a complex process, which includes several cellular and molecular events **(McLaren, 2000)**. In fact, oogenessis begins in embryonic period and at birth oocytes are naked or surrounded by somatic cells, as primordial follicles. These follicles are from a pool of primordial germ cells, which formed during early gestation **(Cohen and Holloway, 2010; Gondos et al., 1986; Motta et al., 1997)**. It has been shown that female born with fixed number of oocytes and after birth most of the oocytes die in fetal ovary, and the proliferation and death of germ cells in the ovary are strictly coordinated and controlled by various factors **(De Felici et al., 2000, De Felici et al., 2005, De Felici et al., 2007)**. It has been shown that germ cell loss is facilitated by the program cell death, and apoptosis is thought to be major process for germ cell loss in fetal mouse ovary **(Coucouvanis et al., 1993; De Pol et al., 1997; Pepling and Spradling, 2001)** along with other process like germ cell extrusion and autophagy **(Wordinger et al., 1990, Lobascio et al., 2007)**. It has been demonstrated that in the new born mice apoptosis is very prominent and coincides with follicle formation **(Ratts et al., 1995, Pepling and Spradling, 2001)**.

It has been shown that number of germ cells/oocytes number between P2 and P20 remains unchanged **(Rodrigues et al., 2009)**. Furthermore, **Faddy et al. (1987)** have suggested that the primordial follicle pool decreases between days 14 and 42 postpartum. These finding suggests decreased proliferation in early postnatal ovary. However, Johnson et al. **(2004)** have shown that oocytes and follicles renewal occurs in the postnatal mice ovary. It has also been suggested that 42 apoptotic related genes are up regulated in adult ovary than infantile ovary, which may be involved in the ovarian functions **(Pan et al., 2014, Hussein, 2005)**. There may be some intra ovarian factors, which could be involved in the ovarian proliferation and apoptosis in the infantile ovary. It has been show that intra-ovarian adipokines from peri-ovarian

adipose tissue regulates folliculogenesis in mice **(Yang et al., 2018)**. Visfatin is also an adipokine, which is also expressed in the mammalian and non-mammalian ovary **(Reverchon et al., 2013; Annie et al., 2020a; Ons et al., 2010; Diot et al., 2015a)**. The expression of visfatin has been shown to be developmentally regulated in the gonads, including mice ovary **(Ocón-Grove et al., 2010; Diot et al., 2015b; Choi et al., 2012; Annie et al., 2020b)**. Other adipokine such as TGF-β1 has been shown to influence ovarian follicular growth and differentiation in postnatal and immature ovarian models **(Rosairo et al., 2008)**. Leptin is also an adipokine, which is expressed in the ovary **(Ryan et al., 2002; Phoophitphong et al., 2017)** furthermore it may regulate postnatal organ development including ovary **(Attig et al., 2011)**. However, the role of visfatin in early postnatal or infantile period has not been investigated in any mammalian species. It has been shown that visfatin regulates plethora of biological functions including proliferation and apoptosis in the reproductive organ **(Gholinejad et al., 2017; Cheng et al., 2011; Annie et al., 2019, Annie et al., 2020)**.

As visfatin is present in the ovary, and postnatal ovary undergo apoptosis and proliferation, thus question arises, whether visfatin in involved in the proliferation and apoptosis in the infantile ovary. Thus, we hypothesize that visfatin could be involved in the regulation of proliferation and apoptosis in the infantile ovary. The rationale to hypothesize the role of visfatin in infantile ovary (PND14-PND21) lies on the fact that recently we have evinced that visfatin showed an increasing trend from postnatal day 14 to 21. Therefore, the present study aimed to investigate the role of ovarian visfatin in proliferation, apoptosis and steroidogenesis in infantile mice.

Materials and methods

Animals and study design

Mice were housed at 25°C in conventional polypropylene ventilated cages on 12L:12D cycles. The mice were provided food and water bottles ad libitum. All animal procedures were approved (process number: MZUIAEC16-17-10) by the

Mizoram University Institutional Animal Ethical committee (MZUIAEC), Mizoram University, Mizoram, India. Pregnant female Swiss albino mice were regularly monitored for their delivery, and pups were collected on their postnatal days of 1 (n=10), 7, 14, 21 and 42 days (n=5). Mice were mildly euthanized and sacrificed immediately, where serum and organs were collected for further analysis.

In vivo treatment of visfatin inhibitor

An investigation on the effect of visfatin inhibitor (FK866, Cat # F8557, Sigma Aldrich, St. Louis, MO, USA) during pre-pubertal development of ovaries was performed by giving 1.5 mg/kg FK866 i.p on PND 14 for 7 days **(Ohanna et al., 2018)**. The FK866 was first dissolved in DMSO (10 mg/ml) and before use; it was diluted in the normal saline (1:9). The solution of DMSO and saline (1:9) was used as vehicle control in the same manner. This selection was after observing an increased trend in the concentration of ovarian visfatin protein from PND14-PND21. Body weights and ovary weights were measured at the time of collection of organs.

BrdU labeling

Another set of experiment was designed after *in vivo* treatment where BrdU labeling was done on the last day, 3 h prior to sacrifice which were separated in sub-groups- Brdu only and Brdu +FK866 groups. The dose for Brdu was given 100 mg/kg body weight (Sisco Research Laboratories, Mumbai, India) as performed in our previous paper **(Annie et al., 2020b)**.

Estimation of sex hormone levels (Estrogen and Progesterone)

The serum procured from *in vivo* treatment of FK866 were estimated for estrogen and progesterone and measured by using commercial enzyme linked immunosorbent assay kit (Estradiol Cat # DKO003, DiaMetra, Italy; Progesterone Cat # RH-351, DSI, Saronno, Italy). The intra assay coefficient of variation and cross reactivity for these kits is 3.5% and 0.004% respectively. Absorbance levels were read at 450nm using a Microplate Reader (Erba Lisa Scan EM, Trans Asia Biomedical Ltd, Mumbai, Maharashtra, India).

Estimation of circulating and ovarian visfatin levels

The ovaries for each developmental stage were homogenized (10 %, w/v) in phosphate buffered saline (pH 7.4) and centrifuged at 10,000*g* for 20 min, and the supernatant was collected for ovarian visfatin estimation. The protein was also estimated in the homogenate by Bradford to represent the ovarian visfatin as ng/mg of protein. Serum and the homogenate were analyzed for estimation of visfatin concentrations by using a commercial Mouse Visfatin ELISA kit (Cat # K02-0598; KinesisDx, Los Angeles, CA, USA). In brief, 40 µl of samples (serum samples and the supernatant of ovarian homogenate) and 50 µl of standard were loaded to each well along with 10 µl of Biotin conjugate. After the addition of samples and standard, 50 µl of HRP conjugate was loaded to both sample and standard, except blank well and incubated in 37 ° C for 1 h. After incubation, the wells were then washed 4 times with 1x wash buffer provided with kit, and added 50 µl each of TMB substrate A (H_2O_2 solution provided in kit) and B (Tetramethylbenzidine) including blank well. It was then incubated for 10 min at 37°C in dark, and after incubation, stop solution was added. The wells were immediately read for absorbance at 450 nm using ELISA reader. The protocol was followed as per manufacturer's instruction.

Immunohistochemistry and immunofluorescence

Ovary samples were fixed in Bouin's fluid for 24 h and processed into paraffinised tissue block. Immunohistochemistry of PCNA was performed with ImmunoCruz Rabbit ABC staining kit (Lot# SC-2018; Santa Cruz Biotechnology) **(Annie et al., 2020b)**. Tissue sections in ribbons were first embedded in cleaned slides, which were later deparaffinised and rehydrated with different grades of alcohol (100%, 90%, and 70%). After complete hydration, it was treated with 3% H_2O_2 in methanol to block the endogenous peroxidase. The tissue slides were incubated with goat-blocking serum for 30 min, followed by primary antibody (PCNA, 1:100, Cat # SC-7907; Santa Cruz Biotechnology, Santa Cruz, CA) incubation at 4°C overnight. After primary wash, slides were incubated with goat anti-rabbit immunoglobulin G secondary antibody, and horseradish avidin- peroxidase conjugate for 30 mins

respectively at the room temperature. DAB (diaminobezidine tetrahydrochloride) was prepared in 0.5 M Tris-HCl, pH 7.6, and 0.01% H_2O_2 and used as chromogenic substrate for staining the tissue sections. After mounting, images were captured by using Nikon binocular microscope (Model E200, Nikon, Tokyo, Japan). The semi-quantification of PCNA and BrdU staining were performed by the ImageJ software (imagej.nih.gov.). The DAB stained area for PCNA in the ovary, and BrdU were obtained by using threshold tool of ImageJ as described previously (**Jensen, 2013**) and represented in percentage area. The five ovarian sections of control as well as FK866 treated group were photographed at 10x magnification for each ovaries (n=5, control; n=5, FK866 treated). The area mentioned refers to the total image field covered with tissue under 10x magnifications without non-image area.

BrdU staining for immunofluorescence

Following similar steps till rehydration, tissue sections were treated with 2 N HCl for 1 h at 37°C and 0.1M borate buffer for 10 min at room temperature. After several washes in PBS, the slides were incubated in blocking goat serum for 30 min and then incubated with anti-BrdU antibody (mouse monoclonal G3G4, Developmental Studies Hybridoma Bank (DSHB), University of Iowa) at 4°C overnight in a wet chamber. Slides were washed in PBS and then incubated in secondary antibody (goat anti-mouse FITC conjugated, 1:200, Cat # E-AB-1015, Elabscience Biotechnology Inc., Wuhan, Hubei, China) for 3 h at room temperature. After secondary antibody incubation, slides were washed in PBS and immersed in it for 5 min. Counterstaining was done with DAPI prepared in 0.1% Mcllvaine's solution for 10 min and immediately mounted for observation with a Nikon fluorescence microscope (Eclipse E200, Nikon, Tokyo, Japan). For quantification, three ovarian sections were photographed at 40x magnification for each ovaries (n=3, control; n=3, FK866 treated) from control BrdU as well as FK866+BrdU treated group. Total image field observed with tissue under 40x magnifications without non image area was considered for area percentage calculation.

Western blot analysis

Ovaries of mice collected after *in vivo* treatment were homogenated 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)**. The protein samples were run in 10% SDS-PAGE, 50 µg per well and then transferred in polyvinylidene fluoride membrane (Millipore India Pvt. Ltd., India) using wet transfer. The membranes were blocked using skimmed milk solution for 30 min at room temperature, and then incubated with primary antibodies: ER-α (1:1,000; Cat # E-AB-31380, Elabscience), ER-β (1:1,000; Cat # CWK-F12, DSHB, Department of Biology, IA), BCL2 (1:500; rabbit polyclonal antibody, Cat # EPP10828, Elabscience, Houston, Texas, USA), BAX (1:1000; rabbit polyclonal antibody, Cat # SC6236; Santa Cruz Biotechnology Inc, Dallas, USA), Active caspase3(1:1000; mouse polyclonal antibody, Cat # STJ97448, St. John's Lab, London, UK), PCNA (1:1000; rabbit polyclonal IgG, Cat # sc7907, Santa Cruz Biotechnology Inc., Dallas, USA), GCNA (1:2000; mouse polyclonal antibody, Cat # 10D9G11, DSHB, Department of Biology, Iowa), at 4°C overnight. After primary incubation, membranes were washed several times with PBST, and incubated with secondary antibodies conjugated with horseradish peroxidase (goat anti-mouse, 1:4000, Merck Specialties Pvt. Ltd, Mumbai, India; goat anti-rabbit conjugated with HRP, 1:4000, Cat # PI-1000, Vector Laboratories, Burlingame, CA, USA). X-ray film was used for developing the membranes to give protein bands. The band intensities were quantified by using the ImageJ software (**imagej.nih.gov/**).

***In vitro* study**

To support the *in vivo* findings, mice ovaries (PND16) were excised, cleaned and cultured in the presence of 10 nM FK866 (Cat # F8557, Sigma Aldrich, St Louis, USA). The excised ovaries (n=4 per group) were cultured for 24 h as the method described earlier **(Annie et al., 2020b)**. Ovaries were cultured in a mixture of Dulbecco Modified Eagle's Medium and Ham's F-12 (Cat # AL155G, Himedia, Mumbai, India) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 0.1% BSA (Sigma Aldrich St Louis, USA). After initial incubation for 2 h at 37°C, culture

medium was discarded, and ovaries (one per tube) were finally cultured in 1 ml of medium in a humidified atmosphere with 95% air and 5% CO_2 for 24 h at 37°C. The dose of FK866 was selected based on our previous study **(Annie et al., 2020b)**. After 24 h, ovaries were harvested and cleaned with PBS and ovaries were freezed at -20° C for western blot analysis and media were also harvested for estrogen and progesterone estimation.

Statistical analysis

Data were expressed as the mean ± standard error of the mean. Analysis of data was done by ANOVA followed by post-hoc Tukey's test and students' t-test. The level of significance was considered as $p<0.05$. Statistical analysis was performed using GraphPad Prism 8.

Results

Postnatal changes in the circulating and ovarian visfatin

The circulating visfatin levels significantly ($p<0.05$) decreased in the PND7 group compared to the PND1, followed by an increase in PND14 and the levels of visfatin did not change in PND21, after that again showed an elevation ($p<0.05$) in PND42 compared to the PND7 and PND21 **(Figure-1.1A)**. However, ovarian visfatin showed a significant ($p<0.05$) lowest concentration in the PND14 compared to the other groups **(Figure-1.1B)**.

Effect of *in vivo* inhibition of visfatin by FK866 on the body weight, ovarian weight and circulating estrogen and progesterone levels and expression of ER-α and ER-β

The inhibition of endogenous visfatin by FK866 from PND14-PND21 significantly ($p<0.05$) increased the body and ovary weight compared to the control **(Figure-1.2A-B)**. Inhibition of visfatin did not change the circulating estrogen and progesterone levels **(Figure-1.2C-D)**.

Western blot analysis showed that visfatin inhibition by FK866 significantly ($p<0.05$) increased the expression of ER-β, expression of ER-α was found to be significantly ($p<0.05$) decreased **(Figure-1.3A-B)**.

Effect of *in vivo* visfatin inhibition on the expression of BCL2, BAX and Active caspase3 proteins in the ovary

In order to confirm the role of visfatin on apoptosis, anti-apoptotic (BCL2) and pro-apoptotic (BAX and Active caspase3) protein expressions were analyzed by western blot after visfatin inhibition. Western blot analysis showed the expression of BCL2 was significantly ($p<.05$) down-regulated after FK866 treatment compared to the control **(Figure-1.4A)**, inhibition of visfatin significantly ($p<0.05$) up-regulated the expression of BAX and Active caspase3 **(Figure-1.4B-C)**.

Effect of *in vivo* inhibition of visfatin by FK866 on the expression of ovarian PCNA, GCNA, and BrdU incorporation

To confirm the exact role of ovarian visfatin on the proliferation in infantile period, visfatin was inhibited by *in vivo* injection of FK866. Western blot analysis showed that the inhibition of ovarian visfatin significantly ($p<0.05$) increased the PCNA and GCNA expression compared to the control group **(Figure-1.5A-B)**.

Immunolocalization of PCNA also showed staining in the thecal, granulosa and oocytes of control and FK866 treated ovary (Fig.6A-D). The semi-quantification analysis of PCNA stained area also showed a significant ($p<.05$) increased in the FK866 group compared to the control **(Figure-1.6E)**.

Effect of *in vivo* inhibition of visfatin by FK866 on the BrdU incorporation

To confirm whether visfatin was involved in the ovarian cell proliferation, we have performed the BrdU labeling study and results showed that more BrdU positive cells were found in the ovary of FK866 treated mice compared to the control **(Figure-1.7A-B)**. The semi quantification analysis of BrdU stained area also showed a significant ($p<.05$) increased in the FK866 group compared to the control **(Figure-1.7C)**.

Effect of *in vitro* inhibition of visfatin by FK866 on the progesterone, estrogen secretion and on the expression of PCNA and Active caspase3

To support the findings of *in vivo* study, *in vitro* study was performed. The *in vitro* inhibition of visfatin showed no significant change in the progesterone secretion by the ovary **(Figure-1.8A)**. Although estrogen secretion seems to be decreased but no significant change was observed **(Figure-1.8B)**. The expression of PCNA was up regulated ($p<.05$) and Active caspase3 was down regulated ($p<.05$) after visfatin inhibition compared to the control.

Discussion

The present study was aimed to investigate the circulating and ovarian visfatin in the different postnatal ages with particular role in the infantile ovary of mice (PND14-PND21). The infantile period from PND14 to PND21 was selected; because concentration of ovarian visfatin protein showed an increasing trend from PND14-PND21, however, circulating visfatin levels did not show any change from PND14-PND21. Earlier it has been shown that there is no correlation between visfatin plasma and follicular fluid levels **(Shen et al., 2010)**. In this experiment, we have inhibited the endogenous visfatin by FK866 treatment from PND14-PND21 to unravel the role of visfatin in infantile ovary of mice. Our results for the first time showed that ovarian visfatin varies in the early postnatal period. This result is in agreement with the previous study which demonstrated the postnatal changes in the visfatin protein in ovary and testis **(Ocón-Grove et al., 2010; Diot et al., 2015b; Choi et al., 2012)**. However, the role of visfatin in early postnatal has not been investigated. The presence of visfatin in the infantile ovary suggests its possible involvement in the ovarian functions, such as proliferation, apoptosis and steroid biosynthesis.

To unravel the possible role of visfatin in the infantile ovary, we have inhibited the endogenous visfatin from PND14-PND21 by FK866 treatment, and our results demonstrated that body weight and ovary weight showed an increase in the visfatin inhibited group. The increase in the body weight showed that visfatin might be inhibiting body growth in early postnatal period. It has been shown that visfatin

induces anorexia and reduces body weight in mice by enhancing activities of POMC neurons **(Tu et al., 2017)**. Although the role of visfatin in fetal development is not known and it has been shown that visfatin has positive relation with body weight and adipose tissue mass, furthermore, it may have active role for visfatin in fetal growth **(Briana et al., 2007)**.

The increase in the ovary weight has prompted us to hypothesized that visfatin may have some role in the proliferation, and apoptosis as well. Since the ovarian hormone has been shown to regulate the proliferation and apoptosis, thus we have measured the circulating estrogen and progesterone hormone levels after visfatin inhibition, moreover, circulating estrogen and progesterone levels did not show any change. Our *in vitro* study also showed that visfatin inhibition did not affect progesterone secretion by PND16 ovary, whereas estrogen secretion seems to be decreased, however it was not significant. Therefore, these results suggest that, in infantile ovary visfatin may not be regulating the ovarian steroidogenesis and visfatin may have age dependent role in ovarian steroidogenesis, nevertheless, previous study showed that visfatin regulates ovarian steroidogenesis **(Diot et al., 2015b; Reverchon et al., 2016)**. The western blot analysis of two proliferation makers, PCNA and GCNA was up-regulated after visfatin inhibition by FK866, this result was further supported by the immunolocalization of PCNA in the ovary, which also showed increase in the PCNA localization in the thecal and granulosa cells of infantile ovary. The expression of PCNA was also up regulated by FK866 treatment *in vitro*. To further clarify the role of visfatin in the proliferation, BrdU labeling study was done and BrdU also showed more incorporation in the ovary of FK866 treated mice. These results suggest that visfatin might be inhibiting the ovarian cell proliferation in infantile ovary. This result is in agreement with our previous report which showed that visfatin may have anti proliferative role in the uterus **(Annie et al., 2019)**. In contrast, there are various reports, which have shown the proliferative role of visfatin in the many tissues and cell lines **(Wang et al., 2009; Xie et al., 2007; Bułdak et al., 2013; Patel et al., 2010)**. Our recent study moreover showed that visfatin inhibition in proestrus and pre-pubertal ovary decreases the ovarian proliferation **(Annie et al., 2020a; Annie et al., 2020b)**. As the circulating estrogen

and progesterone levels did not show any change, and estrogen regulates ovarian growth and proliferation, therefore, these results further prompted us to investigate the expression of two receptors for estrogen i.e. estrogen receptor-α and β. It has been shown that expression of both ER-α and ER-β are developmentally regulated in mice and ovary, more specifically ER-β in the mice ovary increases with follicular maturation, and granulosa cells population **(Jefferson et al., 2000, Jefferson et al., 2002)**. Our results also showed that visfatin inhibition increases the expression of ER-β and increase ER-β could be responsible for increase ovarian proliferation. It has also been suggested that ER-β may play important role in pre-ovulatory maturation **(Khristi et al., 2018)**. However, visfatin inhibition decreases the expression of ER-α in the infantile ovary, there is very scant information available on the visfatin mediated regulation of estrogen receptor expression. In this context, a recent study showed that visfatin increases the phosphorylation of ER-α in the MCF-7 breast cancer cells without affecting the expression of ER-α **(Zangooei et al., 2018)**. Thus, further study would be required to unravel the exact regulation of estrogen receptor in mice ovary by visfatin.

Despite the anti-proliferative role of visfatin in the infantile ovary, our results also provided an evidence of visfatin in the ovarian apoptosis. It has been shown that apoptosis involved in ovarian function such as atresia, follicle loss and folliculogenesis and also been suggested that imbalances between cell proliferation and apoptosis could lead to pathology **(Hussein 2005; Hsu and Hsueh, 1997)**. The visfatin inhibition by FK866 showed down-regulation of the survival protein, BCL2 and up-regulation of the apoptotic proteins, Active caspase3 and BAX in the ovary. Our *in vitro* study also showed that visfatin inhibition by FK866 up regulates active caspase3 expression in the infantile mice ovary. These results suggest that ovarian visfatin in infantile mice may inhibits the apoptosis and increased BCL2 expression further suggest that visfatin may also be involving in the selection of follicle pools by inhibiting the apoptosis. Previous study also suggested that postpartum ovarian follicle loss by apoptosis leads to the establishment of a fixed follicle reserve which is progressively depleted during the reproductive lifespan **(Reynaud and Driancourt 2000; Tilly 2001; Qu et al., 2007)**. Visfatin has also been shown to

inhibits the apoptosis in placenta and it has been emphasized that visfatin is a beneficial factor preventing apoptosis under inflammatory conditions in the placenta **(Ognjanovic and Bryant-Greenwood, 2002; Romacho et al., 2013)**. It has also been shown that during postnatal period from PND6-PND42, follicle number did not change **(Rodrigues et al., 2009)**. Thus, it may also be suggested that low ovarian proliferation in infantile ovary, visfatin might also be involved. Visfatin has also been shown to up regulate the antiapoptotic/survival protein BCL2 and down regulates apoptotic proteins, BAX and caspase in pancreatic β-cell line, and extend the life span of cell as survival protein **(Cheng et al., 2011; van der Veer et al., 2007)**. The expression of PCNA and GCNA showed a significant positive correlation with the expression of BAX, active caspase3 and ER-β, whereas expression of PCNA and GCNA showed a significant negative correlation with BCL2 and ER-α (Table-1). Recently, it has also been proposed that visfatin may be involved for the good quality production the activation of primordial follicles **(Park et al., 2020)**. Not only visfatin, other adipokine like resistin has also been shown to inhibit apoptosis and acts as survival factor by up regulating BCL2 expression in the porcine ovary **(Rak et al., 2015)**.

In conclusion, the present experiment for the first time showed the role of ovarian visfatin in the infantile ovary in relation to steroidogenesis, proliferation and apoptosis. The results of the current study showed ovarian visfatin in infantile may inhibit the proliferation and apoptosis without modulating the ovarian steroid biosynthesis. Furthermore, inhibition of apoptosis could be due to the increase in the survival protein, BCL2, which may be involved in the survival of follicle of good quality.

Summary

Visfatin is an important adipokine, which is expressed in different tissues including ovary of mammals. The postnatal ovary in rodents undergoes dramatic changes of intra-ovarian factors in relation to proliferation and apoptosis. There are studies which showed that gonadal visfatin changes in postnatal life. However, role of visfatin in the early postnatal period i.e. infantile period has not been studied. Therefore, the present study was aimed to explore the role of visfatin in the early postnatal ovarian functions. Furthermore, to explore the role of visfatin, the endogenous visfatin was inhibited from PND14-PND21 by FK866 with dose of 1.5 mg/kg. Our results showed gain in body weight and ovarian weight after visfatin inhibition. The inhibition of visfatin increased the ovarian proliferation (increase in PCNA, GCNA expression and BrdU incorporation) and apoptosis (increase in BAX and Active caspase3 expression). Moreover, visfatin inhibition decreased the expression of antiapoptotic/survival protein, BCL2 in the ovary. These findings suggest that visfatin in the infantile ovary may suppress the proliferation and apoptosis by up-regulating BCL2 expression. An interesting finding has been observed that circulating estrogen and progesterone remain unaffected, although visfatin inhibition up-regulated ER-β and down-regulated ER-α. It may also be suggested that visfatin could regulates proliferation and apoptosis via modulating estrogen signaling. In conclusion, visfatin inhibits the proliferation and apoptosis without modulating the ovarian steroid biosynthesis and visfatin mediated BCL2 expression could also be one mechanism to preserve the good quality follicle in early postnatal period.

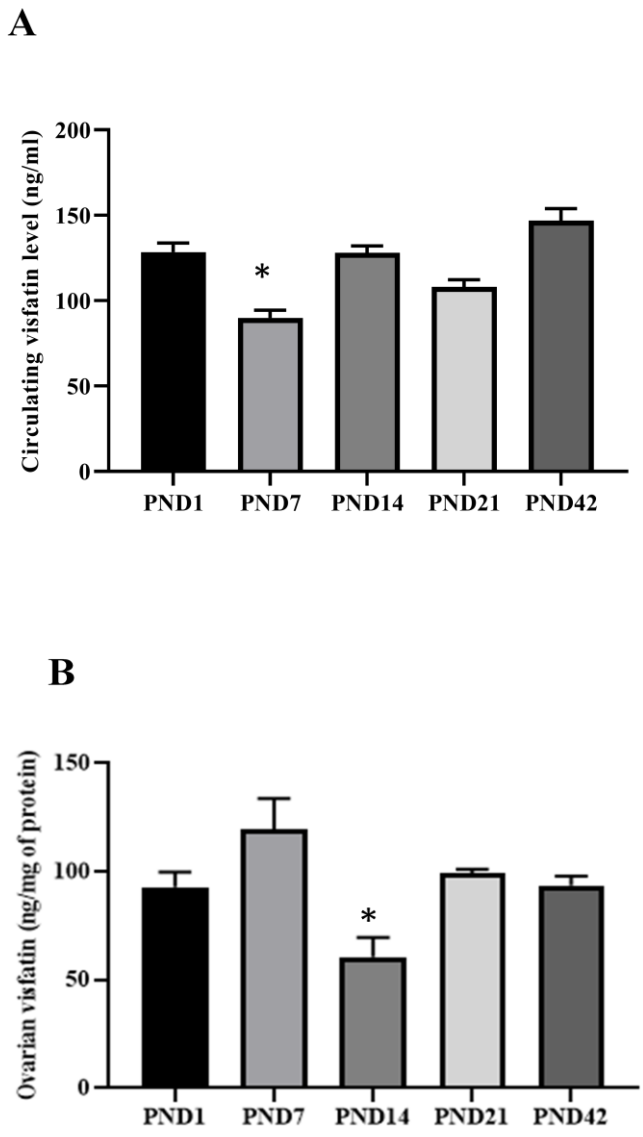


Figure-1.1- Changes in the circulating **(A)** and ovarian visfatin **(B)** in different postnatal age. The circulating visfatin showed a significant (*, $p<0.05$) decreased at PND7 compared to the PND1, 14, 42. The ovarian visfatin exhibited a significant (*, $p<0.05$) decreased at PND14 compared to the other groups. Data are represented as mean ± SEM (n=5).

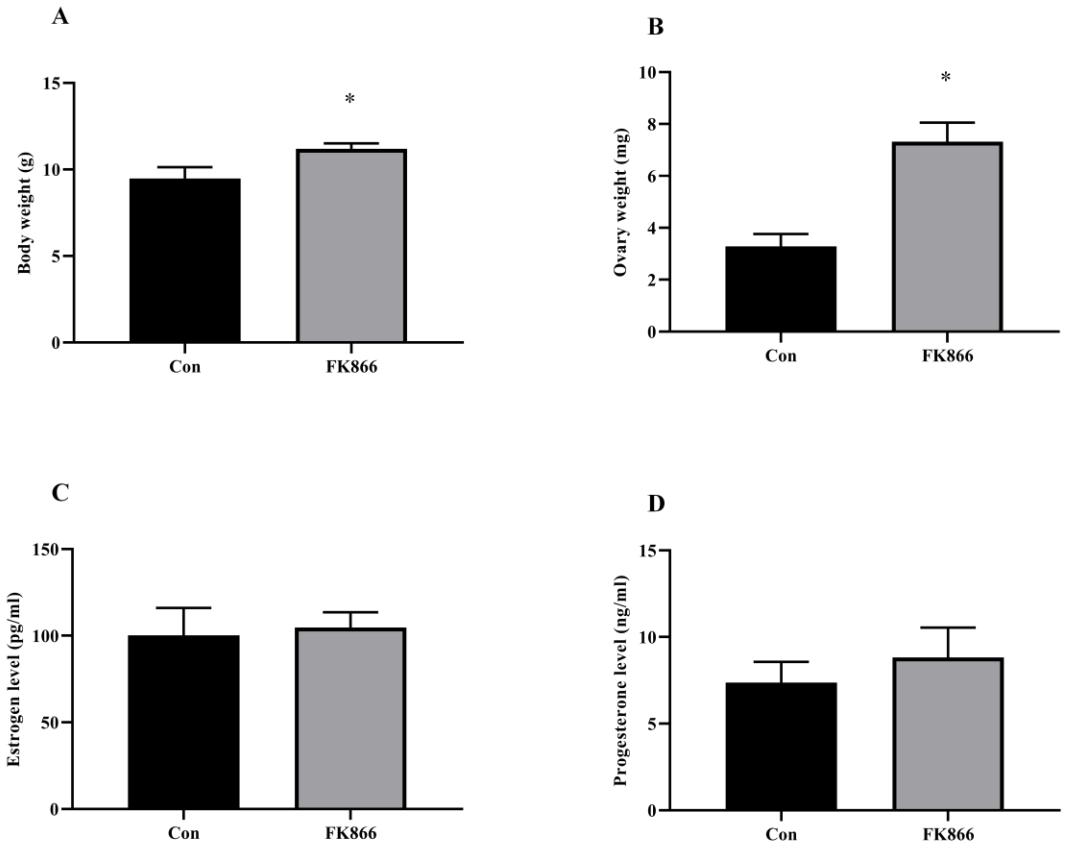


Figure-1.2- Effect of *in vivo* inhibition of visfatin by FK866 (1.5 mg/kg, from PND14-PND21) on the body weight **(A)**, ovarian weight **(B)** and circulating estrogen **(C)** and progesterone levels **(D)**. The inhibition of visfatin significantly (*, $p<0.05$) increased the body weight and ovarian weight compared to the control. However, visfatin inhibition did not change circulating estrogen and progesterone levels. Data are represented as mean ± SEM (n=5).

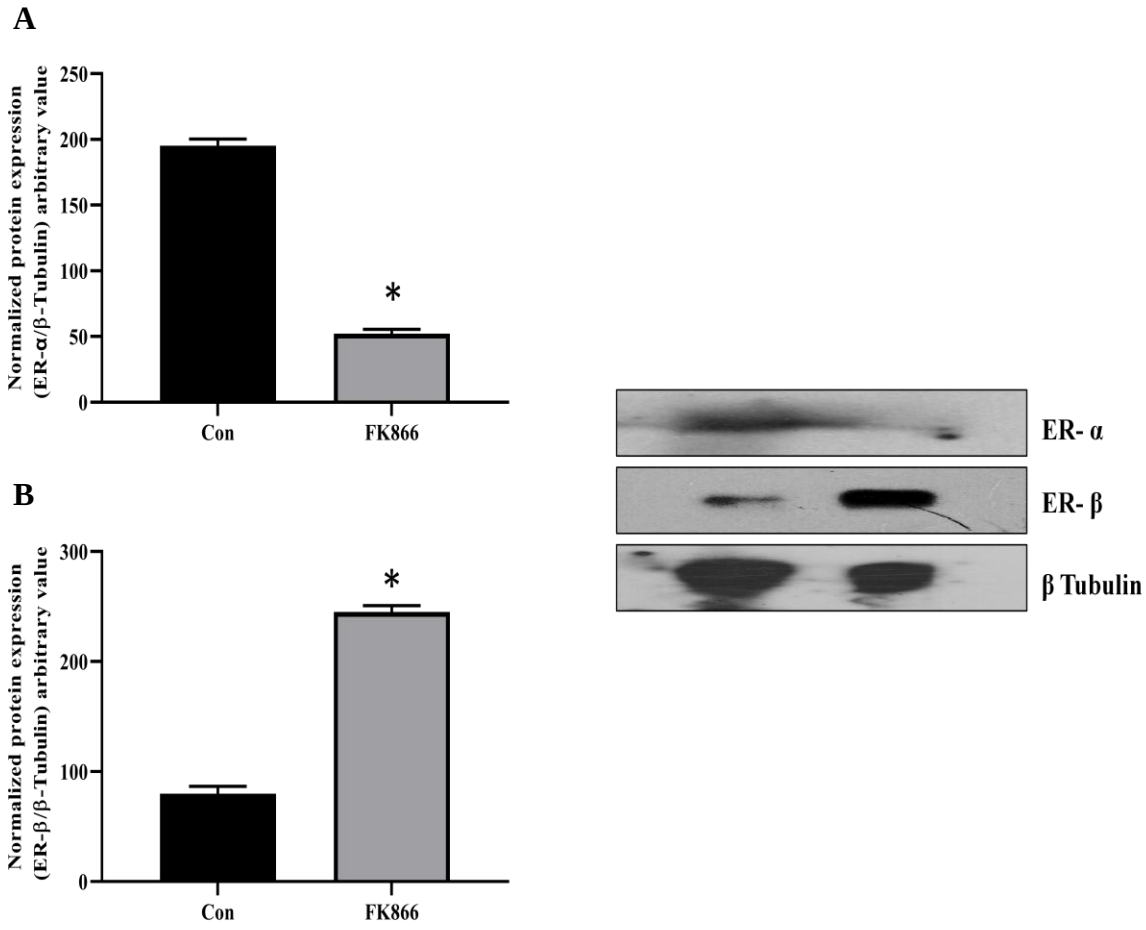


Figure-1.3- Effect of *in vivo* inhibition of visfatin by FK866 (1.5 mg/kg, from PND14-PND21) on the expression of ER-α **(A)** and ER-β **(B)** by western blot analysis. The histogram represents densitometric analysis of visfatin, and data are represented as mean ± SEM (n=5). Expression of ER-α significantly (*, $p<0.05$) down-regulated and ER-β was significantly (*, $p<0.05$) up-regulated after visfatin inhibition.

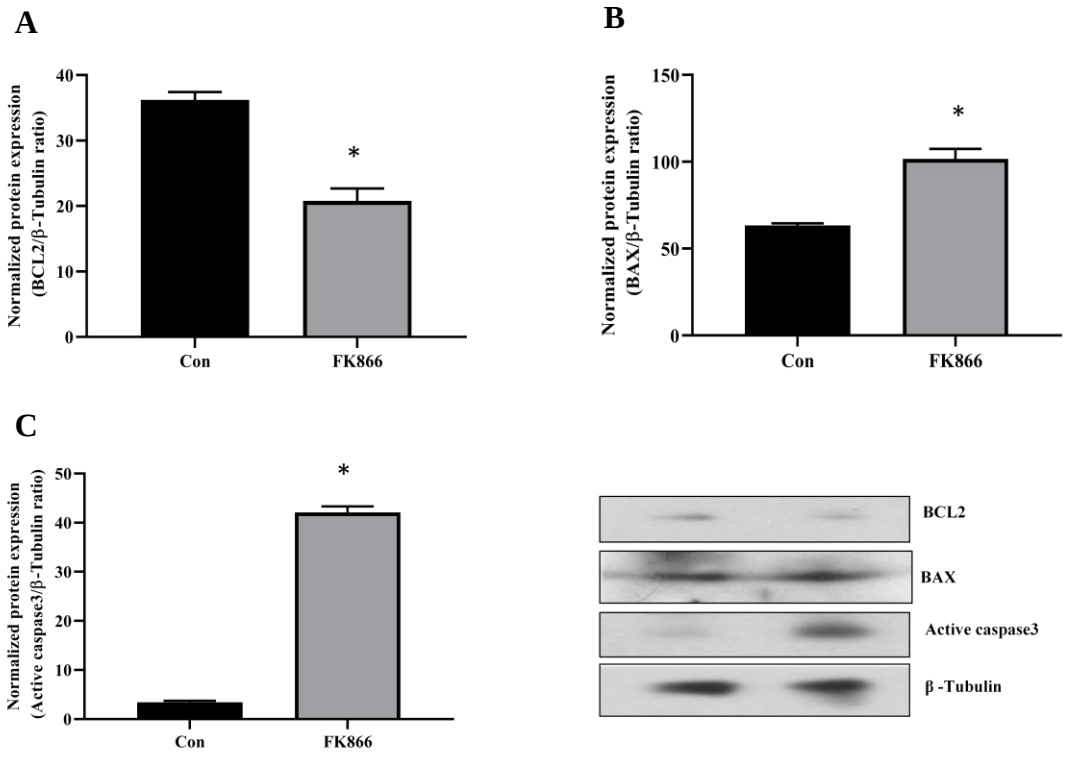


Figure-1.4- Effect of *in vivo* inhibition of visfatin by FK866 (1.5 mg/kg, from PND14-PND21) on the expression of BCL2, BAX and active caspase3 proteins in the ovary. The histogram represents densitometric analysis of BCL2 **(A)**, BAX **(B)** and active caspase3 **(C)** after western blot and data are represented as mean ± SEM (n=5). Visfatin inhibition significantly (*, $p<0.05$) down-regulated the expression of BCL2, and significantly (*, $p<0.05$) up-regulated the expression of BAX and active caspase3 compared to the control.

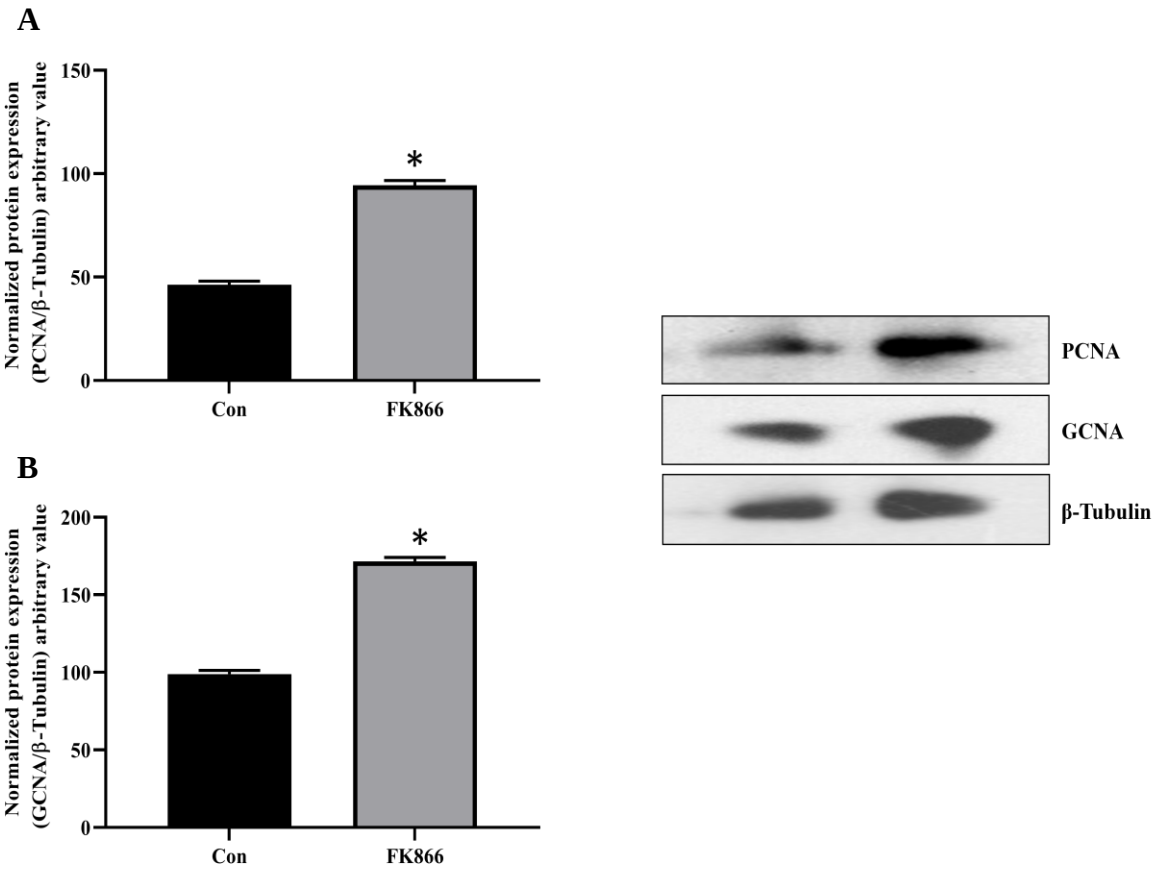


Figure-1.5- Effect of *in vivo* inhibition of visfatin by FK866 (1.5 mg/kg, from PND14-PND21) on the expression of PCNA and GCNA proteins in the ovary. The histogram represents densitometric analysis of PCNA **(A)**, GCNA **(B)** after western blot and data are represented as mean ± SEM (n=5). Visfatin inhibition significantly (*, $p<0.05$) up-regulated the expression of PCNA and GCNA compared to the control.

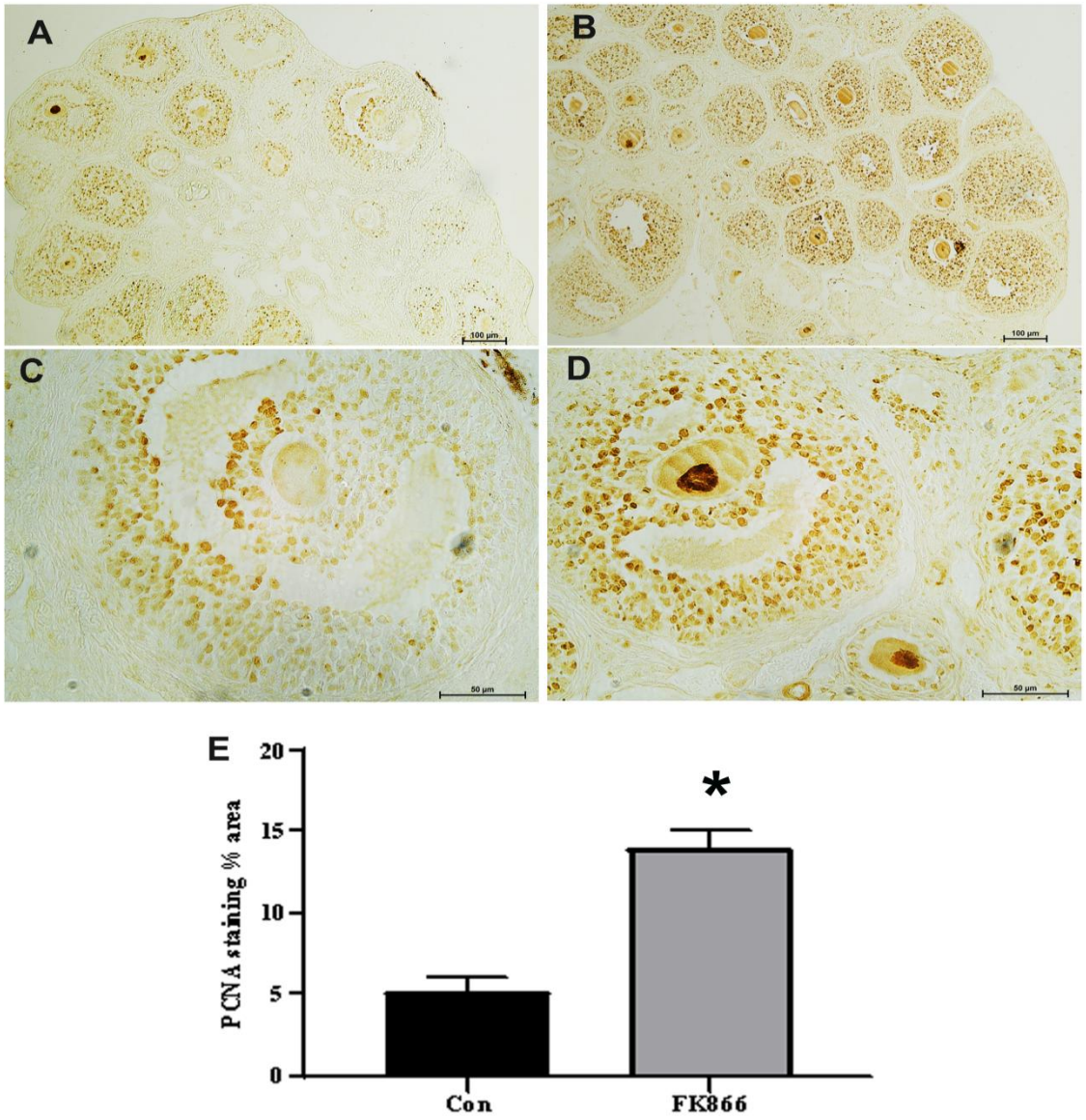


Figure-1.6- Effect of *in vivo* inhibition of visfatin by FK866 (1.5 mg/kg, from PND14-PND21) on the localization of PCNA proteins in the ovary. Visfatin inhibition showed intense immunostaining in the granulosa cells, thecal cells and oocytes (**B**, 10x magnification, **D**, 40x Magnification) than control ovary (**A, B**). The semi-quantification of PCNA stained area by ImageJ showed significant (*, $p<0.05$) increase in the FK866 group compared to control group (**E**).

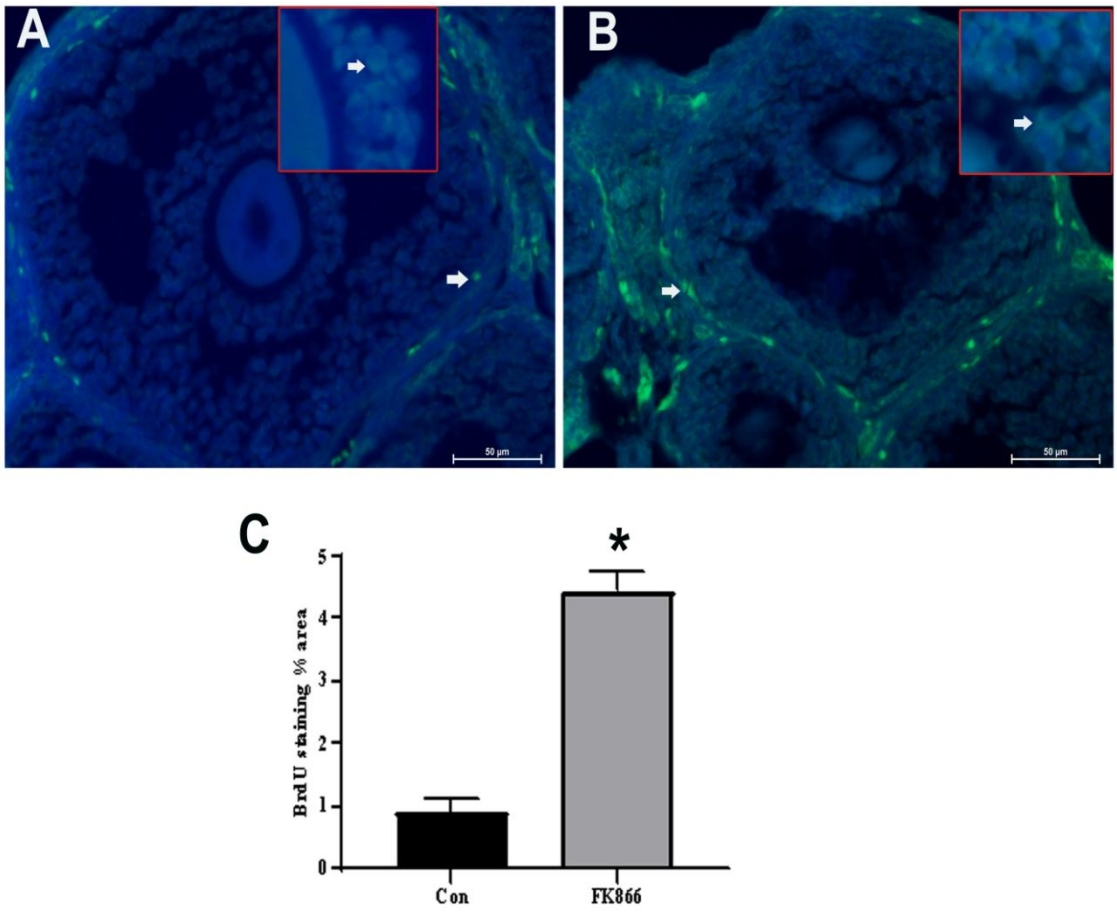


Figure-1.7- Effect of *in vivo* inhibition of visfatin by FK866 (1.5 mg/kg, from PND14-PND21) on the BrdU incorporation in the ovary. Visfatin inhibition showed many BrdU positive cells (arrowhead) in the FK866 treated ovary **(B)** than control ovary **(A)**. The arrow in the inset shows BrdU positive granulosa cells. The quantification of BrdU **(C)** stained area by ImageJ showed significant (*, $p<0.05$) increase in the BrdU staining compared to control group.

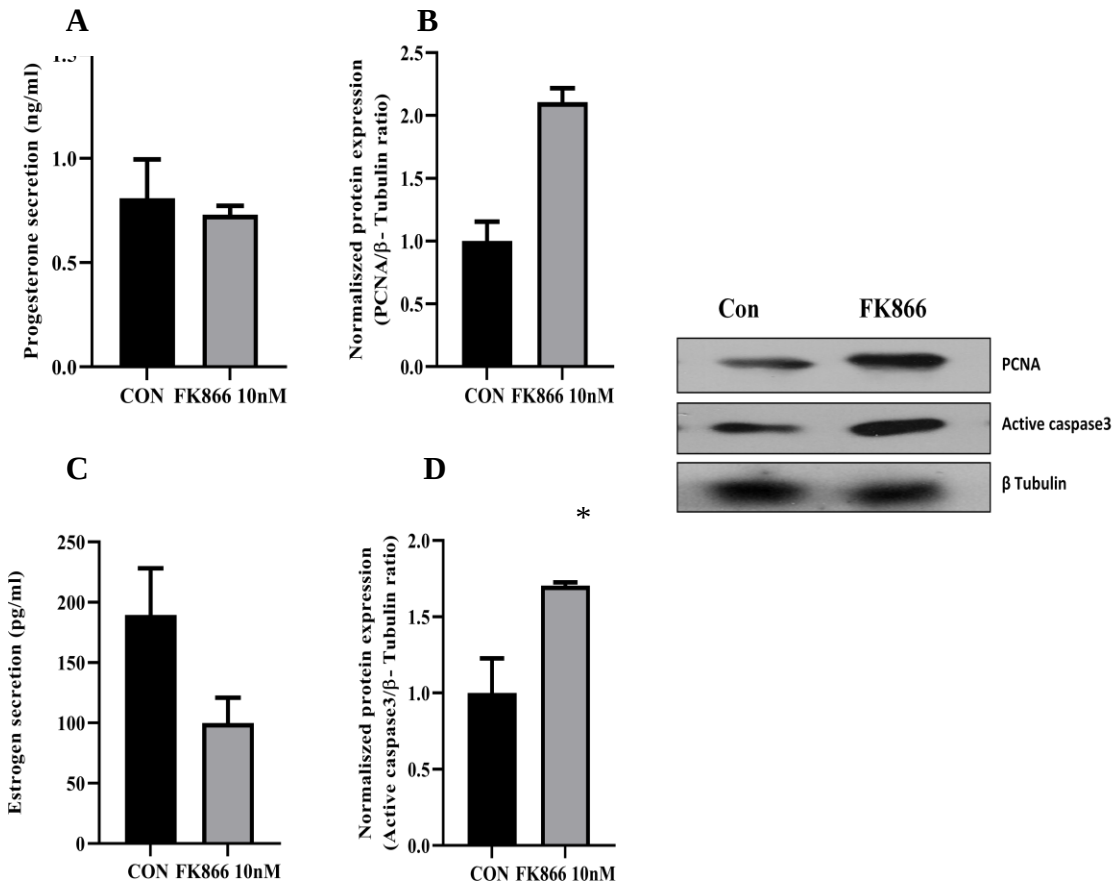


Figure-1.8- Effect of *in vitro* inhibition of visfatin by FK866 (10 nM) on the hormone secretion and on the PCNA and Active caspase3 expression. The *in vitro* inhibition of visfatin by the FK866 did affect the progesterone **(A)** and estrogen **(B)** secretion by the ovary (PND16). Visfatin inhibition significantly (*, $p<0.05$) up-regulated the expression of PCNA **(C)** and Active caspase3 **(D)** compared to the control.

Table 1: Correlation of the proliferative markers with pro-apoptotic and anti-apoptotic factors

	BCL2	**BAX**	**Active caspase3**	**ER-α**	**ER-β**
PCNA	-0.916858 p<0.05	0.95782 p<0.05	0.996399773 p<0.05	-0.988641261 p<0.05	0.99639977 p<0.05
GCNA	-0.899338 p<0.05	0.95665 p<0.05	0.992426131 p<0.05	-0.977842656 p<0.05	0.99242613 p<0.05

Inhibition of visfatin/NAMPT affects ovarian proliferation, apoptosis, and steroidogenesis in pre-pubertal mice ovary

Introduction

The development of mammalian ovaries is unique to female gonads with respect to a fixed number of oocytes that gradually decline from pre-pubertal to adult life **(Tilly, 2001; Broekmans et al., 2009)**. This loss may be crucial for the reproductive life of an organism. In rodents, the oocyte growth is initiated as the primordial follicle population's appearance from the neonatal stage and further develops into the primary and secondary follicles **(Peters, 1969; Mauleon et al., 1969)**. It has been shown that intra-ovarian factors and estrogen regulate the initial recruitment of follicles, and later on, this process is dependent on the cyclic release of FSH **(McGee et al., 2000; Hsueh et al., 2015; Webb et al., 2004; Dierich et al., 1998; Kumar et al., 1997)**. Thus, it was advocated that the study of pre-pubertal steroidogenesis and folliculogenesis are required to unravel the role of intra-ovarian factors **(Ojima et al., 2019)**.

Adipokines are now shown to be involved in the ovarian dynamics, regulating cell proliferation, folliculogenesis and ovulation leading to the formation of functional corpus luteum **(Campos et al., 2008; Berisha et al., 2005)**. Visfatin, also known as Nampt (Nicotinamide Phosphoribosyltransferase), is an adipokine of the visceral adipose tissue, mostly expressed in the liver, bone marrow, 3T3-L1 cells, and amniotic epithelial cells **(Kendal and Bryant-Greenwood, 2007; Dahl et al., 2012; Kralisch et al., 2005)**. Adipokine has been shown to be involved in reproduction **(Campos et al., 2008; Reverchon et al., 2014)**. Expression of visfatin has been shown in the reproductive organs of adult females such as oocyte, granulosa, and thecal cells of the human ovarian follicle as well as placenta and myometrium **(Reverchon et al., 2013; Esplin et al., 2005; Fasshauer et al., 2007)**. The circulating visfatin proteins and testicular visfatin gene increase from pre-pubertal to adult stage in the chicken; on the

other hand, circulating visfatin decreases in aged hens and mice **(Ocón-Grove et al., 2010; Diot et al., 2015b; Choi et al., 2012)**. The ovarian visfatin regulates steroidogenesis in the hen, turkey and human **(Dupont et al., 2015; Diot et al., 2015a; Reverchon et al., 2013)** by regulating the steroidogenic markers, StAR (Steroidogenic acute regulatory protein), and 3βHSD **(Stocco et al., 2001)**. Visfatin stimulates insulin-like growth factor 1-induced estrogen biosynthesis in the human granulosa cells and bovine granulosa cells **(Reverchon et al., 2013; Reverchon et al., 2016)**. It has been emphasized that early exposure of estrogen may be important for reproductive tissue to function optimally in later stages of life and circulating estrogen levels during postnatal stages changes dramatically in the rodents with high levels at birth followed by decline and again a peak at pre-pubertal age **(Schulz et al., 2009; Ojeda et al., 1980; Prevot et al., 2015; Bell, 2018; Döhler and Wuttke, 1975; Walker et al., 2012)**. However, high estrogen levels at birth were suggested from non-ovarian tissue **(Weisz and Gunsalus, 1973; Konkle and McCarthy, 2011)**. In mice, the circulating estrogen was not detected at birth; however, its levels increase at postnatal age day seven, and ovarian steroidogenic enzymes activities increase from postnatal day 7 to 21, which is before puberty **(Dutta et al., 2014; Mannan and O'Shaughnessy, 1991)**. Thus, it is obvious that pre-pubertal mouse ovary at 21 days old has machinery for ovarian estrogen biosynthesis, and it is also evident that visfatin expression has been shown in the pre-pubertal ovary of other species, so it is logical to hypothesize that pre-pubertal ovarian visfatin may also modulate the estrogen synthesis in mice. However, the postnatal developmental or age-dependent changes in the circulating or ovarian visfatin have not been reported in the rodent species.

It has been shown that estrogen is another intra-ovarian factor involved in the regulation of follicular development **(Krege et al., 1998; Lubahn et al., 1993)**. However, the decrease in ovarian estradiol production does not necessarily affect the growth of follicles, but any imbalance caused by hormones can impair oocyte development **(Tingen et al., 2009)**.

Follicular development and its survival depend on various intra-ovarian autocrine and paracrine factors signaling, which further regulates proliferation and apoptosis **(Hussein, 2005; Giebel et al., 1997)**. There are shreds of evidence that intra-ovarian adipokine, such as C1q/tumor necrosis factor-related protein 3, adiponectin, and resistin regulates ovarian proliferation and apoptosis **(Mao et al., 2018; Singh et al., 2015; Rak-Mardyła et al., 2014; Rak et al., 2017b)**. It has also been suggested that visfatin levels are lower in the pre-pubertal stage and increases in adolescence **(Nourbakhsh et al., 2015; Giapros et al., 2012)**. Therefore, we suggested that visfatin may play an essential role in puberty. In addition to that, visfatin induces cell proliferation **(Reverchon et al., 2014)** and inhibits apoptosis **(Cheng et al., 2011; Rongvaux et al., 2002)**. Furthermore, leptin has been shown to accelerate follicular growth by suppressing apoptosis and proliferation in the pre-pubertal rat **(Almog et al., 2001)**. So, a question arises whether visfatin can also regulate or do have some role in ovarian proliferation and apoptosis in pre-pubertal mice.

Thus, we hypothesized that visfatin expression might be changed during pre-pubertal stages and further, it may be a potential regulator of estrogen biosynthesis, apoptosis, and proliferation in the pre-pubertal ovary. Therefore, the present study investigated whether visfatin is involved in pre-pubertal proliferation, apoptosis, and steroidogenesis.

Materials and Methods

Animals

The female mice of age 1 (PND1), 7 (PND7), 14 (PND14) and 21 days old (PND21) were collected from the reared colony, which is maintained under the controlled condition of constant 12L: 12D cycle (Animal House, Mizoram University, India). These mice were handled in accordance with protocols approved (process number: MZUIAEC17-18-08) by the Mizoram University Institutional Animal Ethical Committee (MZUIAEC), Mizoram University, Mizoram, India. Ovaries were harvested (n=4) from postnatal days, PND1, 7, 14 and 21 female mice **(Tepekoy et al., 2014)** for

developmental expression of visfatin. Further, immature female Swiss albino mice (21 days, n=27) were used for *in vitro* experiment.

***In vitro* study**

To explore the role of visfatin in proliferation, apoptosis, and steroidogenesis, ovaries from immature female mice of 21 days old were cultured in the presence of visfatin inhibitor FK866 (Cat # F8557, Sigma Aldrich, St Louis, USA) collected after sacrificing with mild anesthesia. The excised ovaries (n=4 per group) were cleaned of any adhering tissue and cultured for 24 h as the method described earlier **(Singh et al., 2015)**. Ovaries were cultured in a mixture of Dulbecco Modified Eagle's Medium and Ham's F-12 (cat no-AL155G, Himedia, Mumbai, India) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 0.1% BSA (Sigma Aldrich St Louis, USA). After initial incubation for 2 h at 37°C, the culture medium was discarded, and ovaries (one per tube) were finally cultured in 1 ml of medium in a humidified atmosphere with 95% air and 5% CO_2 for 24 h at 37°C. Three groups were cultured:(1) Control group ovaries, cultured only in media, and group (2) and (3) ovaries were cultured in the presence of visfatin inhibitor FK866 at the dose of 1nM and 10 nM respectively. The doses of FK866 were selected based on the previous study **(Okumura et al., 2012; Annie et al., 2019)**. Each treatment and control group was run in 6 sets, and the experiment was repeated two times. After 24 h, ovaries were harvested and cleaned with PBS, and ovaries were frozen at -20° C for western blot analysis and also fixed in 10% NBF (neutral buffered formalin) for immunohistochemistry of visfatin. Media were also harvested for estrogen estimation.

Estrogen estimation in cultured media

The estrogen level was measured from the cultured media by using a commercial enzyme-linked immunosorbent assay kit (Estradiol Cat # DKO003, DiaMetra, Italy). In brief, 25 µl of the media, standard, and control was loaded to the wells and incubated with conjugate (200 µl) for 2 h with mild shaking (500-700 rpm) at room temperature. The wells were then aspirated and washed with wash solution 3 times, followed by the

addition of 100 µl Tetramethylbenzidine (TMB) substrate to each well and incubated for another 30 min room temperature. The reaction was stopped by adding 100 µl of Stop solution (0.2 M sulfuric acid) and the absorbance was measured at 450 nm using a Microplate Reader (Erba Lisa Scan EM, Trans Asia Biomedical Ltd, Mumbai, Maharashtra, India). The intra assay and inter assay coefficient of variation is ≤ 9% and ≤10% respectively.

Immunohistochemistry

Cultured ovaries were fixed in NBF and embedded in paraffin. Paraffin blocks were cut into thin ribbons of 5-7µm, mounted onto positively charged slides, and put on the slide warming table at 37°C. Tissue sections were processed with xylene for deparaffinization and rehydrated with different grades of alcohol for immunohistochemistry of visfatin **(Gurusubramanian and Roy, 2014)**. The ovary sections were blocked with 3% hydrogen peroxide solution and washed with phosphate-buffered saline (PBS). The sections were then blocked for non-specific binding with blocking goat serum 1:100 for 30 min at room temperature and incubated in primary antibody (visfatin 1:200; Cat # V9139, Sigma Aldrich., St Louis, USA) diluted with PBS at 4°C overnight. The slides were washed in PBS and incubated with secondary antibody (goat anti-rabbit IgG, 1:400, Cat # 114380011730, Genei Laboratories Pvt Ltd, Bangalore, India) for 3h at room temperature. Tissue sections were then incubated in 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 for 1 min at room temperature and the reaction stopped by putting the slides in distilled water. Counterstaining was done with hematoxylin for 5 min and subsequently dehydrated in increasing grades of alcohol and cleared in xylene. The slides were then mounted with DPX and the ovary sections were photographed using the microscope (Eclipse E200, Nikon, Tokyo, Japan). The BrdU positive thecal and interstitial cells were counted in the ovaries (n=4 per group) of all the groups. Three different sections from each ovary were counted for every treatment group.

BrdU incorporation and immunofluorescence

To confirm the role of visfatin on pre-pubertal ovarian proliferation, a different set of experiments was performed. Pre-pubertal mice ovaries (n=3 per group) were also cultured in the media containing DMEM:Ham'F12 along with BrdU (Sisco Research Laboratories, Mumbai, India; 1.5 μg/ml) in the presence of visfatin inhibitor, FK866 with dose 1nM and 10nM respectively. The concentration of BrdU was selected from the previous study **(Annie et al., 2019)**. *In vitro* culture was conducted for 24 h at 37°C in 5% CO_2. The tissues were harvested and fixed in NBF for immunofluorescence study. Tissue sections that were made in paraffin were treated in xylene and hydrated with 100% and 95% alcohol and rinsed in distilled water. Denaturation with 2 N HCl was done for 1 h at 37°C and incubated in 0.1 M borate buffer for 10 min. The sections were washed with PBS and incubated with blocking goat serum (1:100) for 30 min and anti-BrdU antibody (mouse monoclonal G3G4, Developmental Studies Hybridoma Bank (DSHB), University of Iowa) at 1:400 for overnight at 4°C. After the slides were washed with PBS, a secondary antibody (goat anti-mouse FITC conjugated, 1:200, Cat # E-AB-1015, Elabscience) was added and incubated for 3h at room temperature. The sections were washed with PBS and counterstained and mounted in antifade mounting medium with DAPI (Cat # ENZ-53003-M010, Enzolifesciences, NY, USA). After mounting, the slides were observed, and photographs were taken under Nikon fluorescence microscope attached with a camera (Eclipse E200, Nikon, Tokyo, Japan). In brief, the five ovarian sections were photographed at 40x magnifications for each ovary from control, 1 nM and 10 nM FK866 groups. The BrdU positive thecal and interstitial cells were counted in each section.

Western Blotting

Ovaries harvested from the culture and different postnatal age (PND1, 7 and 14 and 21) were homogenized for each group in lysis buffer (50 Mm Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1 g/ml Aprotinin, 1 mM PMSF and 1 mM EDTA), centrifuged for 10

min at 10,000rpm in 4°C for western blot analysis as described earlier **(Annie et al., 2019)**. The concentration of proteins from the supernatant in the samples was analyzed by Bradford's method **(Bradforf, 1976)**. The samples were then denatured in SDS-sample buffer (62·5 mM Tris, 2% SDS, 10% glycerol, 1% mercaptoethanol and 0·003% Bromophenol Blue, pH 6·8) by boiling for 5 min. 50 µg/well of each groups were loaded in 10% SDS-PAGE alongwith protein marker and the gel was electrophoresed at 100 V for 2 h. The resolved proteins were then transferred onto a polyvinylidineflouride membrane (Millipore India Pvt. Ltd., Bangalore, India), using Medox-Bio Mini Semi-Dry Blotting MX-1295-01 for 25 min. The blots were then blocked with 5% non-fat dry milk with PBS (10 mM, 7.5 pH) and 0.1% Tween20 and incubated for 30 min at room temperature, after which it was then incubated with primary antibodies diluted in blocking buffer for overnight at 4°C. The details of antibodies have been given in **Table-1**. The blots were washed with PBS-Tween20 for 2 changes and incubated with horse-radish peroxidase-conjugated secondary antibody (1:4000) for 3 h at room temperature. After washing with PBS-Tween20, the blots were finally detected by chemiluminescence (ECL) (Cat # 1705060, BioRad, Hercules, CA, USA) and developed with x-ray film. The protein band was then analyzed with ImageJ 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

Quantitative data were obtained by densitometric analysis of the bands for protein expressions and estrogen levels were expressed as mean ±SEM. Data were analyzed by one-way ANOVA between the groups, followed by post hoc Tukey's test. The level of significance was considered as $p<0.05$. Correlation analysis was also performed. Statistical analysis was performed using GraphPad Prism software.

Results

Postnatal changes in the expression of ovarian visfatin

Western blot analysis of ovarian visfatin showed a significant ($p<0.05$) increased expression at PND21 compared to the PND1, PND7, and PND14. Although visfatin expression at PND14 showed a decreasing trend compared to PND1 and PND7, however, it was not statistically significant **(Figure-2.1)**.

Effects of visfatin inhibition (by FK866) on the expression of BCL2, BAX, and Active caspase3 proteins

To confirm the role of visfatin on apoptosis in pre-pubertal mice ovary, the ovaries were cultured in the presence of visfatin inhibitor (FK866). Visfatin inhibition markedly affects the expression of anti-apoptotic (BCL2) and pro-apoptotic (BAX and Active caspase3) proteins in the ovaries of pre-pubertal mice. The expression of BCL2 showed a significant ($p<0.05$) down-regulation by both the doses (1nM and 10 nM) of FK866 compared to the control group **(Figure-2.2A)**. BAX expression is also significantly ($p<0.05$) down-regulated by FK866 at both doses compared to the control group **(Figure-2.2B).** However, the expression of Active caspase3 was significantly ($p<0.05$) up-regulated by 1nM and 10 nM doses of FK866 compared to the control group **(Figure-2.2C).**

Effects of visfatin inhibition by FK866 on BrdU incorporation and the expression of GCNA and PCNA proteins

To confirm the role of visfatin in ovarian proliferation in pre-pubertal mice, we checked the BrdU incorporation in the pre-pubertal ovary in the presence of a visfatin inhibitor. Visfatin inhibition showed suppressive proliferation in the pre-pubertal ovary (**Figure-2.3F, I**), which was reflected as few BrdU positive cells; however, in control ovaries, many BrdU positive cells were observed (**Figure-2.3C**). Furthermore, the BrdU positive thecal and interstitial cells were counted in each group, and the total number of BrdU

positive cells and thecal cells showed a significant decrease in the FK866 groups (1 nM and 10 nM) compared to the control (**Figure-2.3C**). However, the number of BrdU positive interstitial cells did not show significant change.

To unravel the effects of visfatin on pre-pubertal ovarian proliferation, the action of visfatin was inhibited with FK866 treatment, and markers of proliferation, GCNA, and PCNA were measured by western blot analysis. Visfatin inhibition by FK866 at a dose of 10 nM significantly ($p<0.05$) decreased the expression of GCNA compared to control and 1 nM groups (**Figure-2.3K).** The expression of PCNA showed a significant ($p<0.05$) dose-dependent decrease after FK866 treatment compared to the control group (**Figure-2.3L).**

Effects of visfatin inhibition by FK866 on the expression and localization of visfatin proteins

To investigate whether the inhibition of visfatin by specific inhibitor, FK866, affects visfatin expression and localization in the pre-pubertal mice ovary, we have checked the expression of visfatin in control, 1 nM and 10 nM groups. The treatment with FK866 significantly ($p<0.05$) decreased the expression of visfatin in the pre-pubertal mice ovary in a dose-dependent manner **(Figure-2.4)**.

Immunolocalization of visfatin also showed a marked variation in the pre-pubertal mice ovary after FK866 treatment *in vitro*. The control ovary showed intense immunostaining in the oocyte and mild immunostaining in the thecal cells and granulosa cells (**Figure-2.5A**). The treatment of 1nM dose of FK866 showed faint or weak visfatin immunostaining in the thecal cells and no immunoreactivity in granulosa cells (**Figure-2.5B**). The treatment with a higher dose of FK866 also showed a faint or no immunostaining at all in the ovary (**Figure-2.5C**).

Effects of visfatin inhibition by FK866 on estrogen synthesis and expression of ER-α and ER-β

To investigate whether the visfatin has a role in ovarian steroidogenesis in pre-pubertal mice ovary, we measured the estrogen secretion after FK866 treatment to pre-pubertal mice ovary *in vitro*. Estrogen secretion was significantly ($p<0.05$) increased after visfatin inhibition by both the doses of FK866 (1 nM and 10 nM) compared to the control (**Figure-2.6A**).

The expression of ER-α showed a significant ($p<0.05$) increase by FK866 treatment compared to the control group (**Figure-2.6B**). However, the expression of ER-β significantly ($p<0.05$) decreased in 1 nM group compared to control and 10 nM groups (**Figure-2.6C**).

Effects of visfatin inhibition on the expression of ovarian steroidogenic markers (StAR, CYP11A1, 17β-HSD, and CYP19A1)

To find out the exact role of visfatin on pre-pubertal ovarian steroidogenesis, after *in vitro* treatment with FK866 to the ovary, we measured the expression of steroidogenic makers such as StAR, CYP11A1 (Cytochrome P450 Family 11 Subfamily A member 1), 17βHSD and CYP19A1 (Cytochrome P450 Family 11 Subfamily A member 1) by western blot analysis. Treatment with FK866 at the dose of 1 nM significantly ($p<0.05$) decreased the expression of StAR compared to the control, and a dose of 10 nM significantly ($p<0.05$) increased StAR expression compared to control and 1 nM groups (**Figure-2.7A**). Furthermore, the expression of CYP11A1, 17β-HSD and CYP19A1 significantly ($p<0.05$) increased by FK866 treatment in a dose-dependent manner compared to control (**Figure-2.7B, C, D**).

Correlation Analysis

Correlation analysis of visfatin protein expression after FK866 treatment showed that expression of visfatin showed significant negative correlation with the expression of steroidogenic markers (CYP11A1, $r= -0.69$, $p<0.05$; 17β-HSD, $r= -0.77$, $p<0.05$; CYP19A1, $r= -0.65$, $p<0.05$) and showed no significant correlation with StAR

expression (r=0.45, p>0.05). The expression of visfatin also showed a significant correlation with expression of proliferating markers (GCNA, r=0.59, p<0.05; PCNA, r=0.82, p<0.05), anti-apoptotic factor (BCL2, r=0.98, p<0.05) and pro-apoptotic markers (BAX, r=0.98, p<0.05; Active caspase3, r=-0.90, p<0.05). This correlation has been shown in **Table-3.**

Discussion

For the first time, the present study investigated changes in the ovarian visfatin expression during pre-pubertal stages and investigated the possible role of visfatin in pre-pubertal mice ovary in relation to proliferation, apoptosis, and steroidogenesis by *in vitro* approach. We have measured the expression of proliferating markers (GCNA, PCNA, and BrdU labeling), anti-apoptotic marker (BCL2), pro-apoptotic markers (BAX and Active caspase3), estrogen secretion along with markers of steroidogenesis (StAR, CYP11A1, 17βHSD and CYP19A1) in pre-pubertal ovary after inhibition of endogenous visfatin by specific inhibitor, FK866.

The *in vivo* study showed that the expression of visfatin changes during different pre-pubertal stages, with the highest expression at the postnatal age of 21. To best of our knowledge, there is no report on changes in the ovarian visfatin during pre-pubertal stages in rodents; however, some previous studies showed that gonadal visfatin might be developmentally regulated in the chicken and mice as well **(Ocón-Grove et al., 2010; Diot et al., 2015b; Choi et al., 2012)**. However, there is still a need to analyze the expression of ovarian visfatin from birth to adulthood. Since our *in vivo* study showed its highest expression at postnatal day 21, therefore, we further carried out the *in vitro* study to elucidate the possible role of visfatin in pre-pubertal mice ovary by using visfatin inhibitor, FK866. The results of our study revealed that inhibition of endogenous visfatin by FK866 up-regulated the expression of Active caspase3 and down-regulated the expression of BAX and BCL2 in pre-pubertal mice ovary. The active capase3 is the executor of apoptosis, and BCL2 is an anti-apoptotic protein; thus, it can be suggested

that visfatin would suppress the apoptosis in pre-pubertal mice ovary. In mammalian ovary, proliferation, and cell death by apoptosis **(Perez et al., 1999)** is a continuous process, and it has been shown that follicles are eliminated in pre-pubertal mice ovary with poor oocyte quality **(Bristol-Gould et al., 2006)**. Furthermore, it has also been shown that caspase3 is expressed in secondary follicles, not in primordial follicles of pre-pubertal mice ovary, and suggested that the cell death process is dependent on developmental stages and cell types in pre-pubertal ovary **(Tingen et al., 2009)**. It is believed that ovary at birth contains a finite number of primordial follicles, which is established due to proliferation in the fetal ovary; furthermore, in early postnatal life, this reserve of follicles decreases **(Kerr et al., 2006)**. It has been shown that pre-pubertal mice ovary losses a significant amount of primordial follicles at postnatal day six, and about 5100 primordial follicles are lost by day 19 **(Bristol-Gould et al., 2006)**. The pre-pubertal mice ovary has been shown that postnatal follicle loss is mediated by apoptosis **(Coucouvanis et al., 1993; Pepling et al., 2001; Felici et al., 2005)**; however, non-classical apoptotic pathways are also involved in the small follicle death in pre-pubertal mice ovary with multiple mechanisms **(Tingen et al., 2009; Rodrigues et al., 2009)**. Visfatin has also been shown to have an anti-apoptotic role in the spleen of normal rats **(Xiao et al., 2015)**. Our results also showed that inhibition of visfatin decreases the expression of proliferating markers, GCNA, and PCNA. Immunolocalization of visfatin also showed mild staining in the oocytes, thecal and granulosa cells after treatment of FK866. These results suggest that visfatin might stimulate the proliferation in the pre-pubertal mice ovary. A study has been shown that visfatin stimulates the proliferation of human granulosa cells, and FK866 eliminated the visfatin-induced proliferation **(Reverchon et al., 2013)**. The previous study has also reported visfatin as a survival factor for various cell types and inhibits apoptosis in different cell types **(Park et al., 2020; Rongvaux et al., 2002; Cheng et al., 2011)**. Therefore, it may be suggested that visfatin inhibits apoptosis by increasing BCL2 expression and by suppressing the expression of Active caspase3. Our immunohistochemical study showed the presence of visfatin in the oocytes; therefore, we can suggest that the presence of visfatin in oocytes

might suppress apoptosis for selecting the follicle of good quality oocytes before puberty **(Choi et al., 2012; Park et al., 2020)**.

In a recent study, we have shown that visfatin stimulates apoptosis and decreases proliferation in mice uterus **(Annie et al., 2019)**. Moreover, in the present study visfatin inhibition affected the thecal cells proliferation, not granulosa cells as observed by BrdU labeling. However, previous study also showed that granulosa cells of pre-pubertal mouse ovary at 21 days old do not exhibit pronounced proliferation, as shown by KI67 immunostaining in the granulosa cells **(Da Silva-Buttkus et al., 2008)**. Diot et al. **(2015b)** also showed that NAMPT/visfatin does not alter granulosa cell proliferation in hen, although it is present in both thecal and granulosa cells, on the other hand, visfatin may increase IGF-induced granulosa cell proliferation in human **(Reverchon et al., 2013).** Ovarian thecal cells provide structural support, and their proliferation begins in the early secondary stage of a follicle and plays an important role in folliculogenesis during developmental stages **(Young et al., 2010; Fraser et al., 2009)**. Visfatin also induces cell proliferation and suppresses apoptosis in the cancer cells and studies in the porcine, bovine, and ovine also showed that thecal cell proliferates more in *in vitro* conditions **(Campbell et al., 1995; Campbell et al., 1998; Wrathall et al., 1995; Smith et al., 2005; Gholinejad et al., 2017)**. Our BrdU labeling study was performed in vitro only for 24 h, whether long term in vitro study in the presence of FK866 would affect granulosa cell proliferation or not, requires further investigation. Thus, it may be suggested that visfatin could influence thecal cell proliferation in pre-pubertal mouse ovary.

The changes in the postnatal follicular development have been associated with ovarian steroidogenesis in rodents **(Mannan and O'Shaughnessy, 1991)**. It has been shown that intraovarian factors such as steroids and regulatory peptide, including adipokines (leptin, resistin), regulate follicular development, and it is required for the onset of puberty **(Rak et al., 2017b; Webb et al., 2004; Sarkar et al., 2010)**. The role of ovarian visfatin in steroidogenesis and folliculogenesis has not been explored in the pre-pubertal stage.

However, visfatin has been shown to influence sexual maturity in chicken testis **(Ocón-Grove et al., 2010)**. Thus, we hypothesize that visfatin could be one of the important local regulators of ovarian function during pre-pubertal time. Our results also showed that inhibition of pre-pubertal ovarian visfatin up-regulated the expressions of CYP11A1, 17βHSD, CYP19A1, and increased the estrogen secretion. However, inhibition of visfatin showed a biphasic effect on the expression of StAR. These results clearly showed that visfatin regulates ovarian estrogen synthesis by regulating ovarian CYP11A1, 17βHSD and CYP19A1. Since the final step of estrogen biosynthesis involves the conversion of testosterone to estrogen by CYP19A, so it seems that CYP19A1 could be the one important target for estrogen biosynthesis by visfatin. A study by Diot et al., **(2015b)** also showed that visfatin inhibits progesterone secretion by hen granulosa cells. In another study, it has been shown that visfatin stimulates ovarian estrogen secretion in bovine granulosa cells **(Reverchon et al., 2016)**. Since many of the earlier studies has not been performed during the pre-pubertal time, but on different species, thus it further may be suggested that the role of visfatin in ovarian steroidogenesis would be stage and species-dependent. Our study was focused on the pre-pubertal ovary and we have not investigated the role of visfatin in adult ovary, so it would be interesting to show whether visfatin exhibit the same role in the adult ovary, in mice particular.

Our study further showed that treatment of FK866 decreased the expression and localization of visfatin in the ovary of pre-pubertal mice. However, previous studies showed that *in vitro* FK866 treatment to 3T3-L1 pre-adipocytes and melanoma cells has no effect on the localization and expression of NAMPT **(Svoboda et al., 2019; Ohanna et al., 2018)**. Furthermore, the *in vivo* treatment of FK866 to control rats also showed no effect on visfatin localization, while induced visfatin expression by ischemia-reperfusion was canceled by the visfatin inhibitor, FK866 **(Wu et al., 2017)**. In a very similar study by Esposito et al. **(2012)**, FK866 treatment down-regulates injury-induced visfatin expression in the spinal cord of rats; however, no expression was observed in the control rats. It could be noticed from the above studies that elevated visfatin expression was

down-regulated by FK866; thus, it may also be suggested that FK866 treatment decreases the expression of visfatin protein in pre-pubertal mice ovary since there is a discrepancy on the role FK866 on the visfatin expression, therefore, further study would be required to unravel the mechanism of FK866 mediated downregulation of visfatin. Since estrogen requires two receptors ER-α and -β, to exhibit biological functions, thus we have also checked the expression of these two receptors. Our results showed that visfatin inhibition increased the expression of ER-α, and the expression of ER-β showed a decrease only in 1 nM group. The modulation of ERs expression in the pre-pubertal ovary might be regulated by estrogen. The developmental expression of ER-α and -β have been reported in mice ovary **(Jefferson et al., 2000)** and it has also been proven that ER-α and -β are required for early ovarian folliculogenesis and maintain the germ cell and somatic cells in the postnatal ovary of mice and hamster **(Dupont et al., 2000; Couse et al., 1999; Yang et al., 2004)**.

The results of the present study showed that pre-pubertal ovarian visfatin suppress estrogen biosynthesis, increases proliferation, and decrease apoptosis. Further, the reduced expression of visfatin after FK866 treatment showed a significant negative correlation with the expression of steroidogenic markers, CYP11A1, 17βHSD and CYP19A1, and apoptotic marker, Active caspase3; however, the expression of visfatin showed a significant positive correlation with expression of BCl2, an anti-apoptotic protein, and with the expression of PCNA and GCNA, which are markers of proliferation. Whether visfatin targets proliferation and apoptosis and steroidogenesis independently or the regulation of proliferation and apoptosis are mediated via estrogen signaling is not clear from this study, and further investigation is needed to explore this possibility. Visfatin/NAMPT has also been shown to acts as an enzyme in NAD pathway **(Dahl et al., 2012)** and sirtuins, which belongs to the enzyme family and has NAD-dependent protein deacetylase activity and splitting NAD during each deacetylation cycle **(Imai et al., 2000; Landry et al., 2000; Smith et al., 2000)**. There are seven homologs of mammalian sirtuins which has been reported, and SIRT1 is one of them, which regulation is mostly dependent on the NAD concentration and therefore

depends on the activity of visfatin/NAMPT **(Dahl et al., 2012; Revollo et al., 2004; Zhang et al., 2009)**. Expression of SIRT1, SIRT3, and SIRT6 has been shown in the mice ovary (**Zhang et al., 2016)**. Since the function of visfatin has been shown to be mediated by SIRT1 in the human and bovine ovarian cells **(Reverchon et al., 2013; Reverchon et al., 2016)**, thus, it is logical to hypothesize that in pre-pubertal mice ovary, sirtuins could also be involved in the steroidogenesis, proliferation, and apoptosis, however, in this experiment, sirtuins expression and activity has not been shown, therefore an explorative study is required to confirm this hypothesis. To the best of our knowledge, there are only two reports on *in vivo* treatment of visfatin to mice by Choi et al, **(2012)** and Park et al., **(2020)** both of these studies showed that visfatin improves the oocytes quality and fertility outcome in aged mice. Furthermore, Choi et al. **(2012)** showed that visfatin treatment did not improve the retrieved blastocysts number in young mice of age between 6-11 weeks; however, they suggested the ovarian visfatin expression in young mice may be enough to induce normal follicular development for the production of good-quality oocytes. Although, they have not reported the effect visfatin has on ovarian estrogen biosynthesis; however, the role of visfatin in ovarian steroidogenesis has been described by Reverchon et al., **(Reverchon et al., 2013; Reverchon et al., 2016)** in human and bovine ovarian cells along with its role in proliferation. They showed visfatin treatment increased IGF-1- -stimulated estradiol production in hCGs. Our study showed that FK866 treatment increased estrogen secretion, our study showed visfatin has a stimulatory role in ovarian cell proliferation, in particular thecal cells and an inhibitory role in estrogen secretion. Thus, it may be suggested that that visfatin has an inhibitory role in estrogen secretion in pre-pubertal age; furthermore, it would be interesting to study the age-dependent role of visfatin mice ovary.

In conclusion, the findings of this study suggest that the presence of visfatin in pre-pubertal mice ovary directly modulates ovarian steroidogenesis and folliculogenesis; therefore, visfatin could be one of the regulators of ovarian functions in pre-pubertal

animals. Since this is an *in vitro* study, an *in vivo* study would be required to support the present findings.

Summary

Pubertal ovarian function might be dependent on the factors present in the pre-pubertal stages. Visfatin regulates ovarian steroidogenesis in adult. To date, no study has investigated the role of visfatin either in pre-pubertal or pubertal mice ovary. Thus, we investigated the role of visfatin in pre-pubertal mice ovary in relation to steroidogenesis and proliferation and apoptosis *in vitro* by inhibiting the endogenous visfatin by a specific inhibitor, FK866. Inhibition of visfatin increased the estrogen secretion and also up-regulated the expression of CYP11A1, 17βHSD and CYP19A1 in mice ovary. Furthermore, Active caspase3 was up-regulated along with the down-regulation of BAX and BCL2 in the pre-pubertal ovary after visfatin inhibition. The expression of GCNA, PCNA, and BrdU labeling was also decreased by FK866 treatment. These results suggest that visfatin inhibits steroidogenesis, increases proliferation, and suppresses apoptosis in the pre-pubertal mice ovary. So, visfatin is a new regulator of ovary function in pre-pubertal mice.

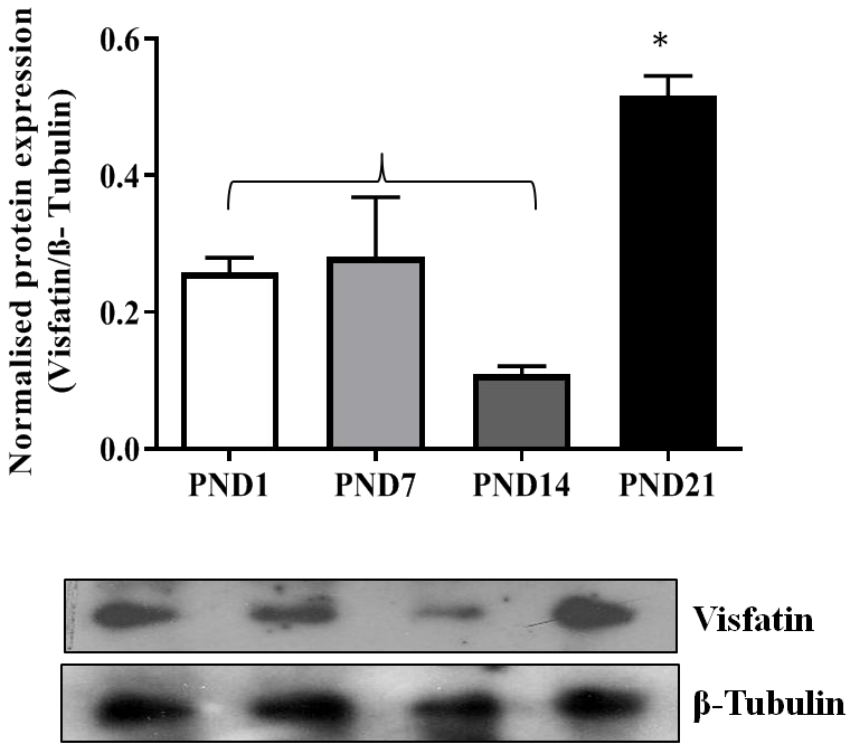


Figure-2.1- Postnatal changes in ovarian visfatin expression. Densitometric analysis showed a significant highest (*, $p<0.05$) expression of visfatin at postnatal day 21 (PND21). The density in each band was normalized to the density of the β-Tubulin band. Data are expressed as mean± SEM.

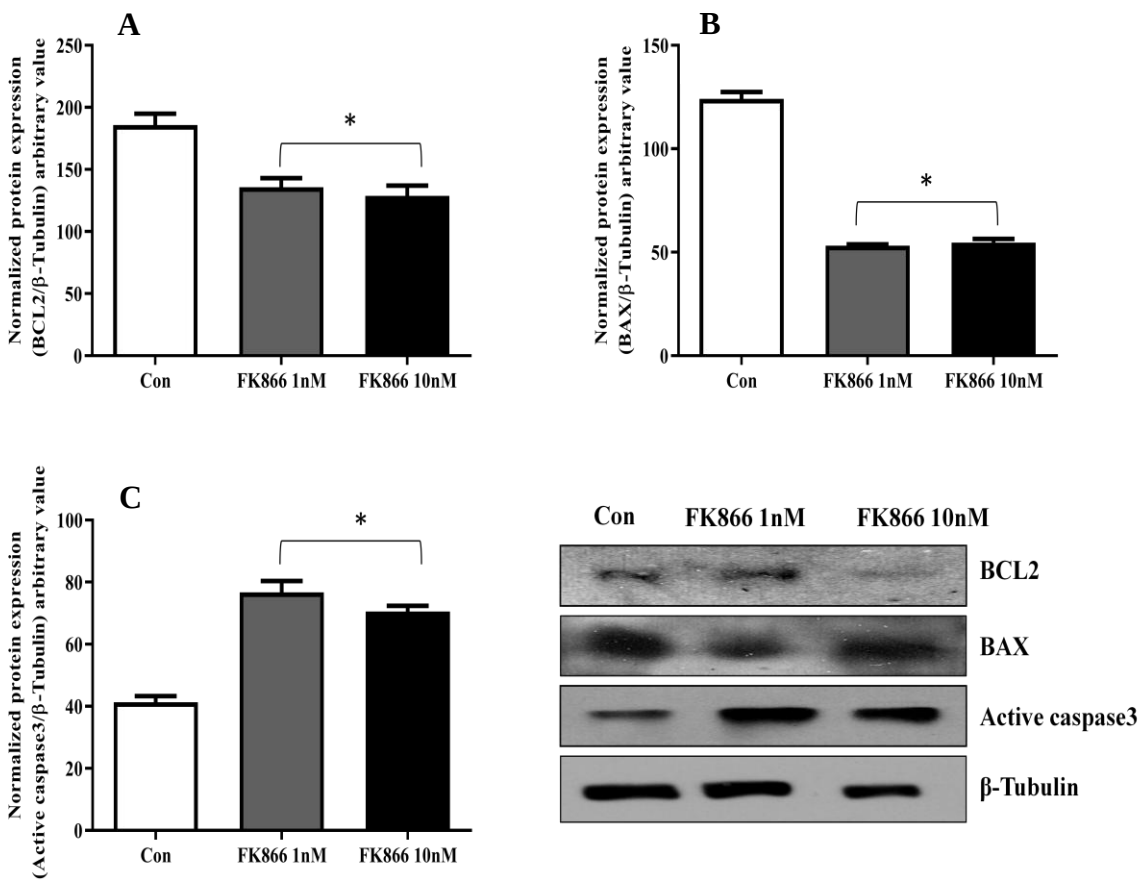


Figure-2.2- Effect of visfatin inhibition by FK866 (1 nM and 10 nM) on the expression of anti- and pro-apoptotic proteins in pre-pubertal mice ovary by western blot analyses. Densitometric analyses of BCL2 expression **(A)**, BAX expression **(B)** and Active caspase3 expression **(C)**. The density in each band was normalized to the density of the β-Tubulin band. Data are expressed as mean± SEM (n=4). * On the bar showed a significant difference as compared to the control (Con) group ($p<0.05$).

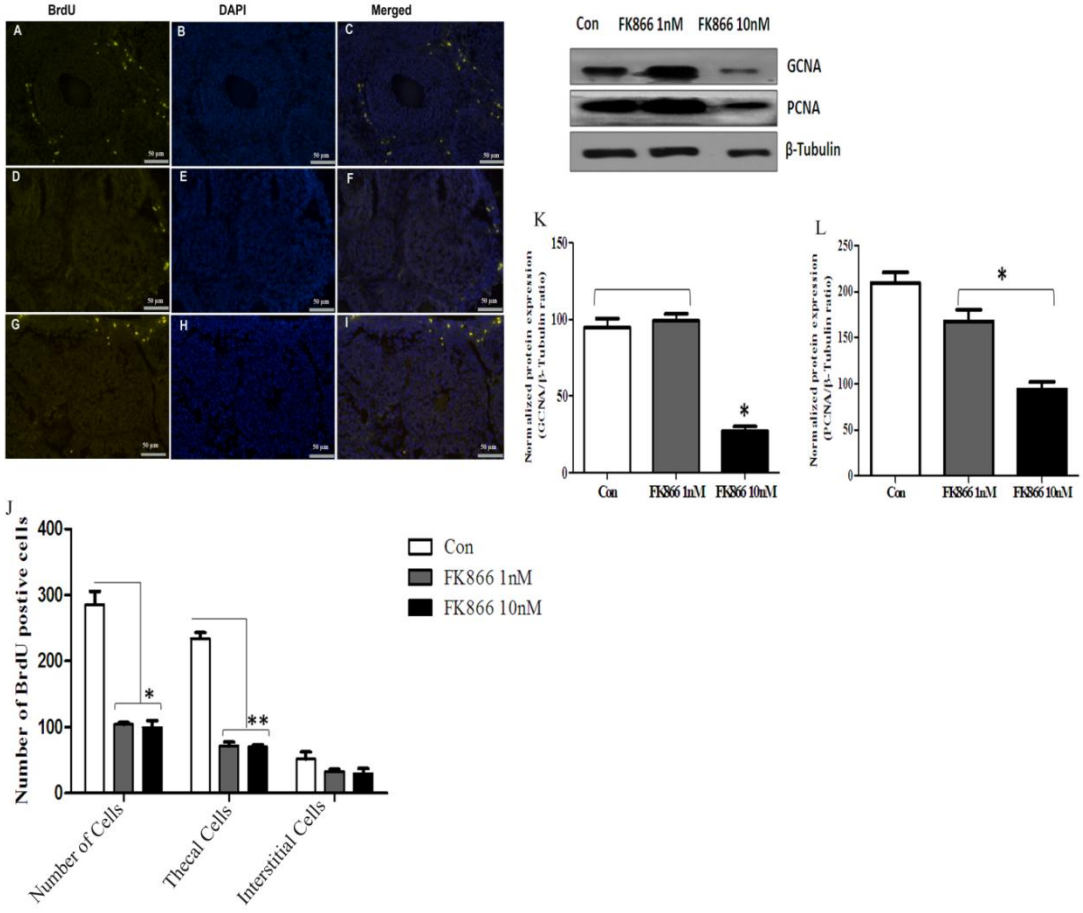


Figure-2.3- Effect of visfatin inhibition on BrdU incorporation in pre-pubertal ovary. The treatment of FK866 showed suppressed proliferation as evidenced by few BrdU positive cells in the ovary of 1 nM **(F)** and 10 nM **(I)** groups. However, control ovary section showed many BrdU positive cells **(C)**. FK866 treatment significantly decreased BrdU positive total cells (*, $p<0.05$) and thecal cells (**, $p<0.05$) compared to the control and, number of BrdU positive interstitial cells did not show the significant change **(J)**. Expression of proliferating markers after visfatin inhibition by FK866 (1 nM and 10 nM). Densitometric analyses of GCNA expression **(K)**, PCNA expression **(L)**. The density in each band was normalized to the density of the β-Tubulin band. Data are expressed as mean± SEM (n=4). * On the bar showed a significant difference as compared to the control group ($p<0.05$).

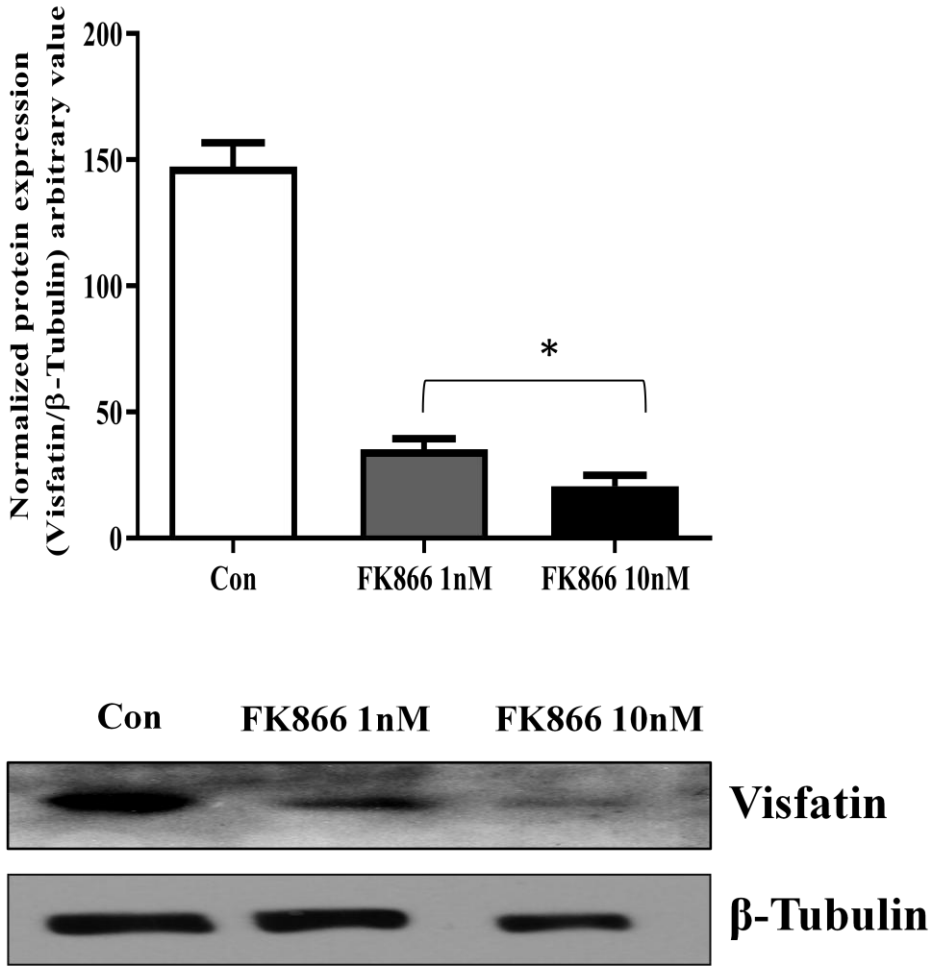


Figure-2.4- Expression of visfatin after FK866 (1 nM and 10 nM) treatment. Densitometric analyses of visfatin expression showed a significant decrease in the ovarian expression (*$p<0.05$, compared to the control).The density in each band was normalized to the density of the β-Tubulin band. Data are expressed as mean± SEM (n=4).

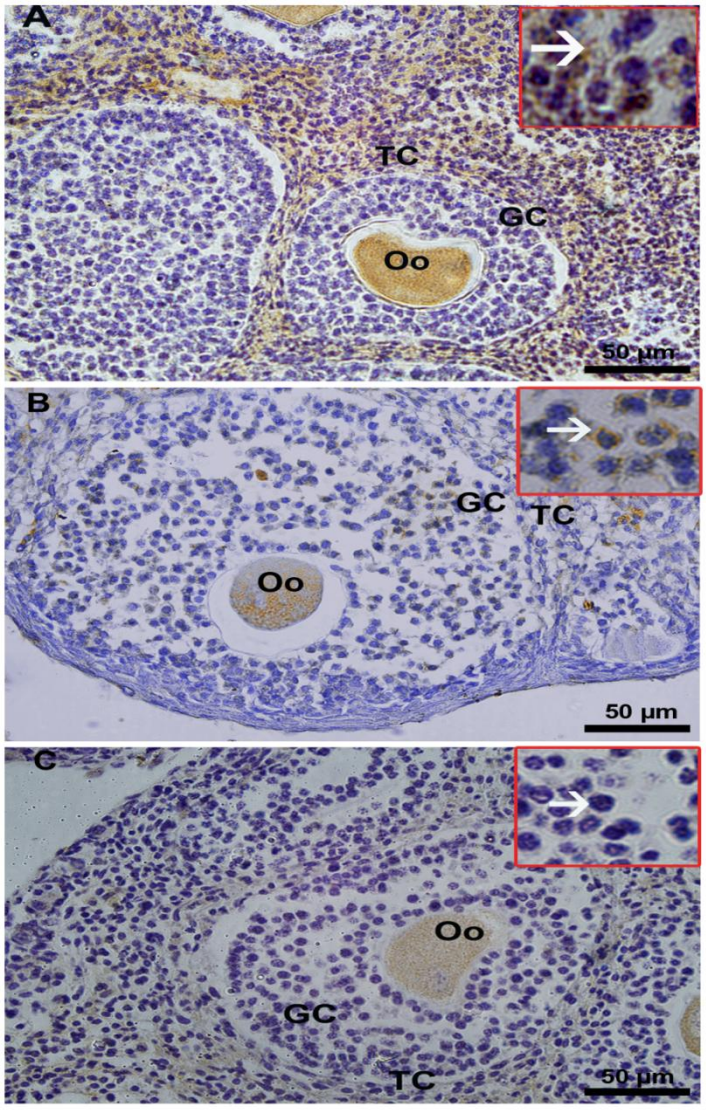


Figure-2.5- Localization of visfatin in the pre-pubertal ovary after FK866 treatment. Immunolocalization of visfatin showed intense staining in the thecal cells and oocytes and mild in granulosa cells of the control ovary **(A)**. After treatment with FK866 (visfatin inhibitor), visfatin showed mild to faint staining in the 1 nM and 10 nM groups, respectively **(B, C)**. The white arrow in the inset shows the visfatin in granulosa cells (A, B). Oo- oocyte; GC-Granulosa cells; TC- Theca cells.

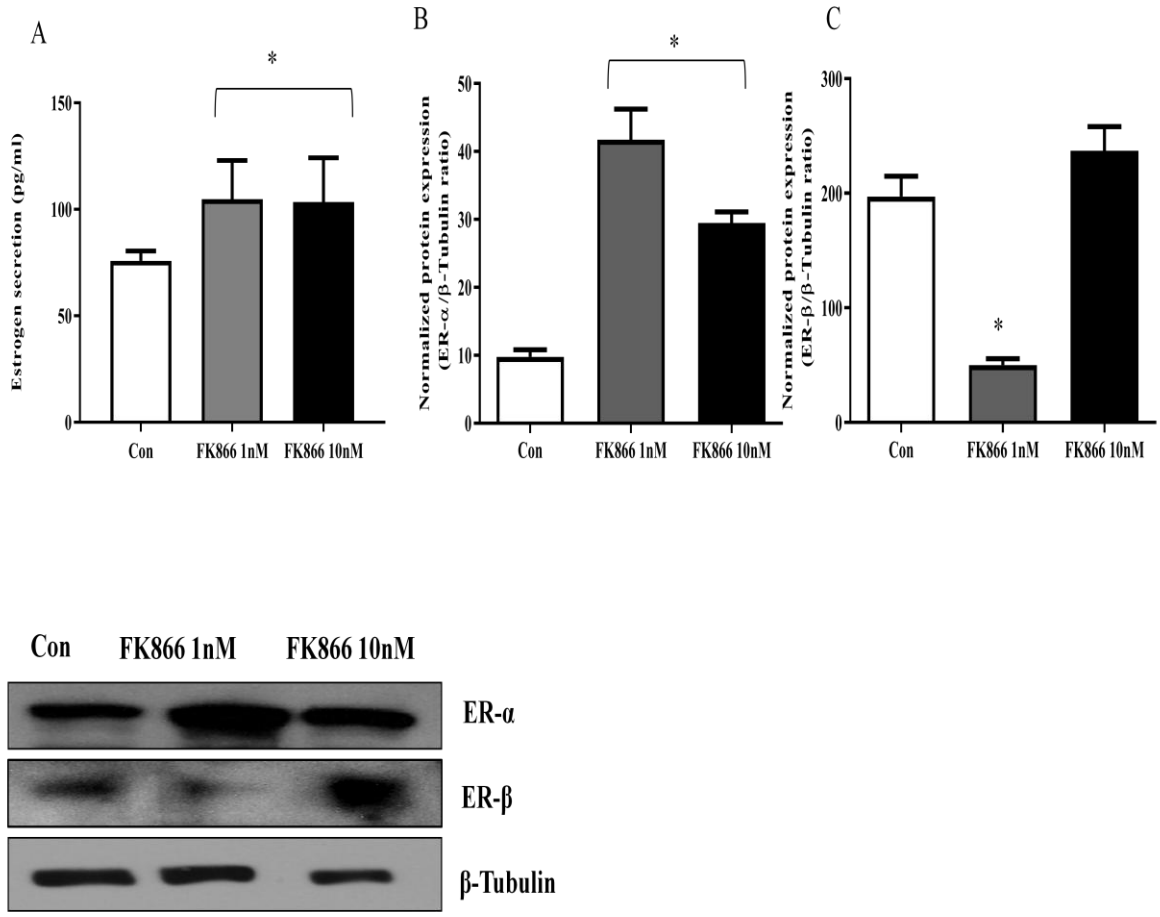


Figure-2.6- Estrogen secretion and expression of ER-α and ER-β by FK866 (1 nM and 10 nM). **A.** Estrogen secretion showed a significant (*$p<0.05$, n=6) increase after treatment of FK866 *in vitro*. **B** and **C** showed the densitometric analyses of ER-α and ER-β expression, respectively. The density in each band was normalized to the density of the β-Tubulin band. Data are expressed as mean± SEM (n=4). * On the bar showed a significant difference as compared to the control group ($p<0.05$).

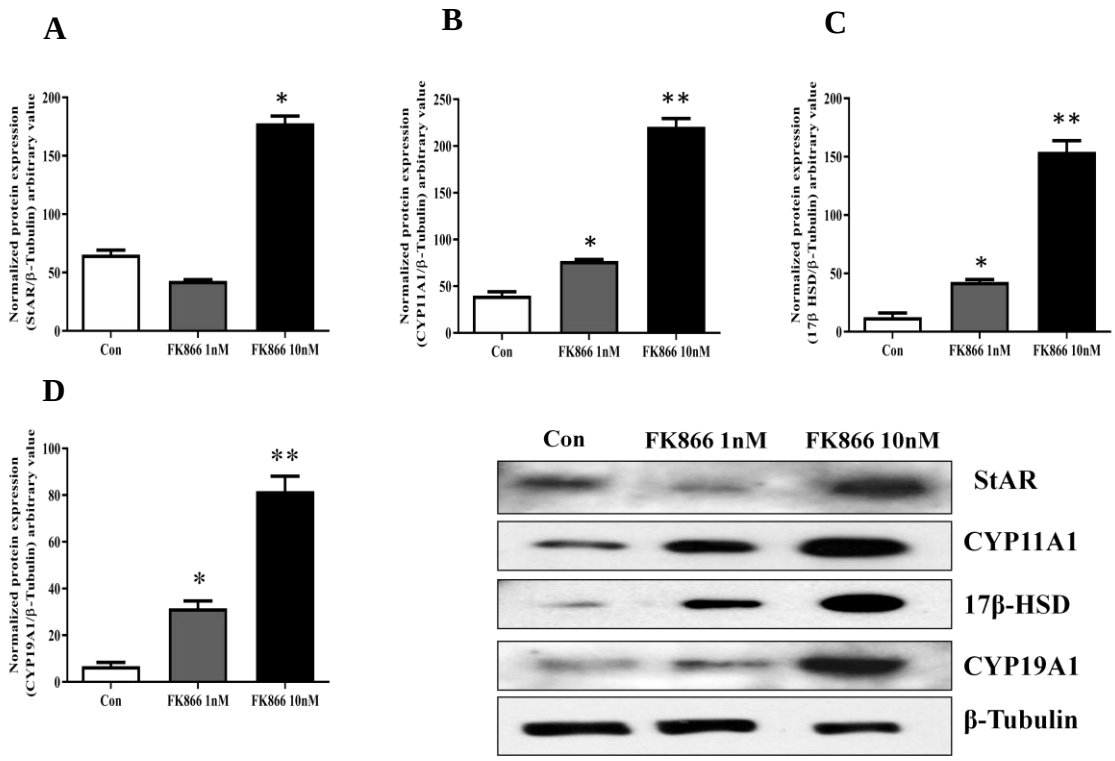


Figure-2.7- Effect of visfatin inhibition by FK866 (1 nM and 10 nM) on the expression of steroidogenic proteins in pre-pubertal mice ovary by western blot analyses. Densitometric analyses of StAR expression **(A)**, CYP11A1 expression (**B**), and 17β-HSD **(C)** and CYP19A1 (**D**). The density in each band was normalized to the density of the β-Tubulin band. Data are expressed as mean± SEM (n=4). * On the bar showed a significant difference as compared to the control and 10 nM groups ($p<0.05$) and ** showed significant difference as compared to control and 1 nM groups.

Table 2: Details of antibodies used for Western Blot analysis

Antibody	Host	Catalog No.	Supplier	Address	Dilution
Primary antibodies					
BCL2	Mouse	SC-7382	Elabscience	Wuhan, China	1:1000
BAX	Rabbit	SC-6236	Santa Cruz Biotechnology Inc.	Dallas, USA	1:1000
Active Caspase3	Mouse	STJ97448	St. John's Lab	London, UK	1:1000
GCNA	Mouse	10D9G11	DSHB	Dept of Biology, Iowa	1:2000
PCNA	Rabbit	SC-7907	Santa Cruz Biotechnology Inc.	Dallas, USA	1:1000
Visfatin	Rabbit	V9139	Sigma-Aldrich	St. Louis, MO, USA	1:1000
ER-α	Rabbit	E-AB-33615	Elabscience	Wuhan, China	1:2000
ER-β	Mouse	CWK-F12	DSHB	Dept of Biology, Iowa	1:2000
StaR	Rabbit	STJ191473	St. John's Lab	London, UK	1:5000
CYP11A1	Rabbit	BS3608R	Bios Antibodies Inc	Woburn, MA, USA	1:1000
17β-HSD	Rabbit	STJ110000	St. John's Lab	London, UK	1:5000
CYP19A1	Rabbit	E-AB-31086	Elabscience	Wuhan, China	1:1000
β-Tubulin	Mouse	E-AB-27380	Elabscience	Wuhan, China	1:1000
Secondary antibodies					
Rabbit IgG-hrp	goat	114380011730	Genei Lab Pvt Ltd	Bangalore, India	1:4000
Mouse IgG-hrp	goat	114068001A	Genei Lab Pvt Ltd	Bangalore, India	1:4000

Table-3: Correlation between the expression of visfatin with steroidogenic markers, anti-pro-apoptotic markers and proliferating markers in the pre-pubertal mice ovary after visfatin inhibition by FK866 *in vitro*.

Expression	Steroidogenic markers			
	StAR	CYP11A1	17β-HSD	CYP19A1
Visfatin	r= -0.45, p>0.05	r= -0.69, p<0.05	r= -0.77, p<0.05	r= -0.65, p<0.05

Expression	Anti- and Pro-apoptotic markers			Proliferating markers	
	BCL2	BAX	Active caspase3	GCNA	PCNA
Visfatin	r= 0.98, p<0.05	r= 0.90, p<0.05	r= -0.90, p<0.05	r= 0.59, p<0.05	r= 0.82, p<0.05

Changes in the localization of ovarian visfatin protein and its possible role during estrous cycle of mice

Introduction

Adipokines are the major secretory or circulating products of adipose tissues, which play an essential role in metabolism and reproductive functions **(Bohler et al., 2010; Campos et al., 2008; Budak et al., 2006)**. It has also been reported that the locally produced adipokines regulate reproductive function in autocrine and paracrine manners in the testis and ovary **(Chen et al., 2013; Seroussi et al., 2016; Caminos et al., 2008)**. Visfatin is an important adipokine, also known as NAMPT (nicotinamidephosphoribosyltransferase). Other than adipose tissue, visfatin is expressed in the various reproductive tissues, uterus, ovary, and testis **(Annie et al., 2019; Reverchon et al., 2014; Diot et al., 2015a; Jeremy et al., 2017)**. Now, it is evident that visfatin is expressed in the gonads and also involved in the regulation of ovarian steroidogenesis in hen, buffalo, and human granulosa cells **(Diot et al., 2015b; Thakre, 2018; Reverchon et al., 2013)**. It has been shown that hCG can enhance visfatin expression in the granulosa and luteal cells **(Shen et al., 2010)**. Not only in the ovary, has visfatin also been suggested to play an important role in testicular steroidogenesis in the chicken **(Oco´n-Grove et al., 2010)**. In humans, visfatin levels were found to be significantly higher in seminal plasma than in serum **(Thomas et al., 2013)**, however, visfatin levels during the menstrual cycle do not show a significant change. Moreover, an increase in the level of visfatin was found during the luteal phase **(Sramkova et al., 2015)**.

Visfatin has also been shown to regulate cell proliferation and apoptosis in mammalian cells **(Lee et al., 2011)**. It has also been demonstrated that visfatin might have a dual role in the regulation of apoptosis. In some tissue, visfatin has been shown to induce apoptosis, and in other tissue, it may inhibit apoptosis **(Sun et al., 2017; Cheng et al., 2011)**. Visfatin expression was found to be higher in various cancerous cells, which

indicates its role in the cell proliferation **(Zhang et al., 2014; Lu et al., 2014)**. On, the other hand, visfatin was found to inhibit the growth of hepatoma cells **(Lin et al., 2015)**. The mammalian ovary is a dynamic structure, which undergoes proliferation and apoptosis during the oogenesis, and there are various factors, including adipokines, which are involved in the regulation of proliferation and apoptosis in the ovary **(Edson et al., 2009; Kurowska et al., 2018)**. The estrous cycle of rodents has been shown to last for 4-5 days and the ovary undergo dramatic changes during this short period of time **(Bertolinand Murphy, 2014)**. Furthermore, estrous cycle associated apoptosis has been linked to the changes in hormone secretions **(Martimbeau and Tilly, 1997)**.

There are many ovarian factors that changes during the estrous cycle **(Ramirez and Sawyer, 1965; Gruenberg et al., 1983; Hohos et al., 2019; Carlock et al., 2014)**. Since the ovarian factors change during the cycle and visfatin is also expressed in the ovarian cells of other animals; thus, it is logical to hypothesize that the expression of visfatin may also be changing in the ovary during the estrous cycle of mice. Despite the presence of visfatin in the ovary of hen, humans, and mice, no study has conducted the cyclic changes in the ovary of mice during the estrous cycle. To the best of our knowledge, this will be the first report on the localization of visfatin protein in the rodent ovary. However, recently we have shown that expression of visfatin changes in the uterus of mice during the estrous cycle and also involved in the regulation of proliferation and apoptosis of uterine cells during the estrous cycle **(Annie et al., 2019)**.

Therefore, this study aimed to investigate the localization and expression of visfatin protein in the mice ovary during the estrous cycle and to find out whether ovary visfatin is involved in ovarian proliferation and apoptosis.

Materials and methods

Animals and sample collections

3-months old female Swiss albino mice were used for this study and handled in compliance with protocols approved by the Mizoram University Institutional Animal

Ethical Committee (MZUIAEC17-18-08), Mizoram University, Mizoram. All mice were kept under standard laboratory conditions of 12 h light: 12 h dark cycle and 25±2°C. For observation of the estrous cycle, the vaginal smear was taken and classified into groups according to their stages as per **Goldman et al. (2007)**, proestrus stage (P), estrus stage (E), metestrus stage (M), and diestrus stage (D). For each stage, six mice (n=5) were taken for the study in each group. Mice were sacrificed immediately following each specific observation.

***In vivo* treatment**

To find out the role of ovarian visfatin, a specific inhibitor of visfatin, FK866 was given to the mice. A total of 10 mice were divided into two groups (n=5 per group): control and FK866 group. The female mice for this experiment were all in the proestrus stage during an estrous cycle, which is in consideration for their uniformity in sampling, and the highest expression of visfatin was also found in the proestrus stage. Control groups were given vehicles. FK866 group were given an intraperitoneal injection of FK866 at a dose of 1.5 mg/kg body weight, and mice were sacrificed 6 h after the treatment **(Ohanna et al., 2018)**. Since the proestrus period in mice has been reported for less than 24 h and it changes dramatically **(Cora et al., 2015)** and furthermore, the half-life of visfatin inhibitor, FK866 has been reported from 7.9–76.5 h in the blood **(Holen et al., 2008, Chen et al., 2017)**, therefore, animals were sacrificed after 6 h of FK866 treatment.

At the end of the experiment, the animals were anesthetized with a mixture of 90 mg/kg Ketamine and 4.5 mg/kg Xylazine in intraperitoneal injection **(Clouthier and Wicha, 2012)**. Ovaries were collected and immediately fixed in Bouin's fixative for immunocytochemistry and frozen at -20°C for western blot analysis.

BrdU labeling

To further confirm the role of visfatin in the proliferation, BrdU labeling study was done in the presence of visfatin inhibitor, FK866. A total of 10 mice were taken (n=5) and

divided into two groups, first BrdU control group, which was injected with BrdU at 100 mg/Kg body weight (Sisco Research Laboratories, Mumbai, India) and second group BrdU+FK866 group, which were injected BrdU (100 mg/kg) and FK866 (1.5 mg/kg). BrdU was given 3 h before sacrifice, and FK866 was given in the second group, 6 hours before sacrifice and control group was given only saline. After treatment, mice were sacrificed, and ovaries were fixed in Bouin's solution for immunofluorescence. Ovaries were embedded in the paraffin and sectioned at 6 µm. For immunofluorescence, after rehydration, slides were denatured with 2 N HCl for 1 h at 37°C, after which it was incubated in 0.1M borate buffer for 10 min at room temperature. Sections were washed in PBS and incubated in blocking serum for 30 min at room temperature. After blocking, slides were incubated with anti-BrdU antibody (mouse monoclonal G3G4, Developmental Studies Hybridoma Bank (DSHB), University of Iowa) at 4°C overnight. After washing in PBS, slides were incubated in secondary antibody (goat anti-mouse FITC conjugated, 1:200, Cat # E-AB-1015, Elabscience Biotechnology Inc. Wuhan, Hubei, China) for 3 h at room temperature. After incubation, slides were washed in PBS and then dipped in McIlvaine's solution for 5 min. Tissue sections were counterstained with DAPI for 10 min and mounted in an antifade mounting medium for observation with a Nikon fluorescence microscope (Eclipse E200, Nikon, Tokyo, Japan). The quantification of BrdU staining in the ovarian sections of different groups was performed by ImageJ software (imagej.nih.gov.). The FITC stained area for BrdU in the ovary was obtained by using the threshold tool of ImageJ as described previously **(Jensen, 2013; Glastras et al., 2017)**.

Immunohistochemistry

Fixed tissues in Bouin'ssolutionwereparaffinized and cut in ribbon sections of6µm thickness with Leica microtome (model RM2125 RTS). As per methods described by previous reports, the sections were dewaxed and rehydrated in descending grades of alcohol **(Gurusubramanian and Roy, 2014)**. To reduce non-specific binding, slides were incubated in blocking serum for 30min followed by incubation with primary

antibody at 4^{o}C overnight in the wet chamber; PCNA (rabbit polyclonal IgG; SC-7907, Santa Cruz Biotechnology Inc., Dallas, Texas, USA), visfatin (rabbit polyclonal IgG, Cat # V9139, Sigma-Aldrich, MO, USA), 1:100 dilution with phosphate-buffered saline (PBS). The tissue sections were then washed and incubated at room temperature with horse-radish peroxidase-conjugated goat-anti-rabbit secondary antibody (Cat # PI-1000, 1:500, Vector Laboratories, Burlingame, CA, USA). 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_2within 10 min at room temperature. Hematoxylin counterstain was done for visfatin and dipped in 0.3% acetate solution and 0.3% ammonia solution respectively and then allowed for bluing in running water for 5 min. The negative control section was also run to confirm the specific binding, where 1 % non-immune rabbit serum was used in place of primary antibody **(Gurusubramanian and Roy, 2014)**. The slides were dehydrated in alcohol, cleared in xylene, and mounted with DPX. The semi-quantification of PCNA staining in the ovary of control and FK866 treated groups was performed by ImageJ software. The DAB stained area for PCNA in the testis was obtained by using threshold tool of ImageJ as described previously **(Jensen, 2013)** and the data were presented as percentage area of PCNA staining, and the percentage area for immunostaining has also been described for other tissue by ImageJ **(Glastras et al.,2017)**.

Western blot analysis

The ovary samples collected at the end of the experiments were homogenized in suspension buffer containing 50 Mm Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1 g/ml Aprotinin, 1 mM PMSF and 1 mM EDTA. Homogenized samples were centrifuged at 10,000 rpm for 10 min. Protein concentration was estimated following Bradford's method **(Bradford, 1976)**. Samples were denatured in gel loading buffer (62·5 mM Tris, 2% SDS, 10% glycerol, 1% - mercaptoethanol and 0·003% Bromophenol Blue, pH 6·8), then loaded 50 µg/well and run in 10% SDS-PAGE with protein marker at 100V. Electrophoresed gels were then transferred on PVDF (polyvinylidineflouride) membrane

(Millipore India Pvt. Ltd., India) using Semi-Dry apparatus for 30 min. Transferred membranes were then blocked for non-specific binding with skim milk solution (5% non-fat dry milk with PBS and 0.1% Tween20) for 30 min and primary antibody incubation with visfatin BCL2 (1:500; rabbit polyclonal antibody, Cat # EPP10828, Elabscience), active caspase3 (1:1000; mouse polyclonal antibody, Cat # STJ97448, St. John's Lab, London, UK), PCNA (1:1000)were done at 4^{o}C overnight. The membranes were washed with PBS-Tween20 and then incubated with horse-radish peroxidase-conjugated secondary antibody (goat anti-mouse, 1:4000, Merck Specialties Pvt. Ltd, Mumbai, India; goat anti-rabbit conjugated with HRP, 1:4000, Cat # PI-1000, 1:500, Vector Laboratories) for 3 h at room temperature. After incubation, membranes were washed with PBS-Tween20 and developed with ECL detection method. The X-ray film used for visualizing the protein band was scanned and analyzed using ImageJ software (imagej.nih.gov/). The western blot experiment was replicated three times for each protein.

Statistical analysis

GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses, and quantitative data were expressed as mean±SEM. To compare the data from different groups, One-way Analysis of variance (ANOVA) followed by post-hoc Tukey's test and student's t-test was used. A statistically significant difference was considered if $p<0.05$.

Results

Localization and expression of visfatin in the ovary during estrous

The localization of visfatin in mice ovaries during the estrous cycle demonstrated the presence of different cell types during the estrous cycle **(Figure-3.1a-h)**. The immunolocalization study showed the strongest (++++) staining in the corpus luteum (CL) of proestrus **(Figure-3.1a-b)** and intense (+++) in the diestrus ovaries (CL) **(Figure-3.1g-h)**. The thecal (Tc), granulosa cell (Gc), and oocytes (Oo) also showed

moderate (++) in the proestrus (a-b) and estrus ovary (c-d), however, faint immunostaining was observed in the metestrus (e-f) and diestrus (g-h) ovary.

Western blot analysis of ovarian visfatin proteins exhibited a marked variation throughout the estrous cycle (One-way ANOVA, $F_{3,16}$=201.5, p<0.0001). One-way ANOVA followed by post hoc analysis (Tukey's test) of visfatin expression showed a peak with significant abundance in the proestrus compared to estrus (Q=23.72, p<0.0001), metestrus (Q=32.90, p<0.0001), and diestrus (Q=24.98, p<0.0001). The expression of visfatin was significantly lowest in the metestrus compared to proestrus (p<0.0001), estrus (Q=9.18, p<0.0001) and diestrus (Q=7.92, p=0.0002) **(Figure-3.2)**. The expression of visfatin protein in estrus and diestrus did not show a significant change from each other.

Changes in the expression of BCl2 and active caspase3 proteins in the ovary during estrous

Expressions of BCL2 **(Figure-3.3a)** and active caspase3 **(Figure-3.3b)** proteins also showed significant changes throughout the estrous cycle (for, BCL2, One-way ANOVA**,** $F_{3,16}$=119.1, p<0.0001, for active caspase3, $F_{3,16}$=471.3, p<0.0001). One-way ANOVA followed post hoc Tukey's test of BCL2 proteins showed significant highest expression in the proestrus compared to estrus (Q=7.94, p<0.0001), metestrus (Q=12.12, p<0.0001), and diestrus (Q=26.06, p<0.0001) and lowest in the diestrus compared to proestrus (p<0.0001), estrus (Q=18.11, p<0.0001), and metestrus (p<0.0001). However, expression in estrus and metestrus did vary significantly from each other.

The expression of active caspase3 proteins also showed a decreasing trend from proestrus to diestrus with significant highest expression in the proestrus compared to estrus (Q= 11.27, p=), metestrus (Q=38.96, p<0.0001), and diestrus (Q=45.26, p<0.0001) and lowest in the diestrus compared to proestrus (p<0.0001), estrus (Q=33.99, p<0.0001), and metestrus (Q=6.30, p<0.0001).The correlation study of visfatin showed a

significant positive correlation (r=0.6194, p<0.05) with BCl2 **(Figure-3.3c)** and active casapse3 **(Figure-3.3d)** (r=0.7733, p<0.05) in the ovary during estrous cycle.

Effect of *in vivo* inhibition of visfatin by FK866 on the expression of ovarian BCL2, PCNA, and active caspase3 proteins

To confirm the exact role of ovarian visfatin on proliferation and apoptosis, ovarian visfatin was inhibited by *in vivo* injection of FK866. The inhibition of ovarian visfatin significantly increased (t=11.05, df=8, p<0.0001), the BCL2 **(Figure-3.4a)** expression compared to the control group. However, visfatin inhibition by FK866 significantly decreased PCNA (t=10.89, df=8, p<0.0001) **(Figure-3.4c)**, and active caspase3 (t=6.45df=8, p<0.0001) **(Figure-3.4b)** expression compared to the control group.

Effect of *in vivo* inhibition of visfatin by FK866 on ovarian BrdU incorporation and PCNA immunolocalization

Further, to confirm the role of visfatin on the ovarian proliferation, BrdU labeling study was done in the presence of FK866 along with PCNA immunolocalization. The inhibition of visfatin showed a few of BrdU incorporation in the ovary (**Figure-3.5b)**; however, control, ovaries showed many BrdU positive cells **(Figure-3.5a)**. The inhibition of visfatin also showed a decrease in PCNA positive cells in the ovary **(Figure-3.5h)**. The quantification of BrdU and PCNA stained area in the control and FK866 treated proestrus ovary showed that FK866 treatment significantly decreased the BrdU staining (t=2.745 df=, p<0.05) and PCNA staining (t=3.681 df=8, p<0.05) compared to the control group (**Figure-3.5j-k)**.

Discussion

This is the first study that examined the expression and localization of visfatin protein in the ovary of mice during the estrous cycle and an attempt was made to unravel the role of visfatin in the ovarian proliferation and apoptosis. The immunohistochemical study showed the presence of visfatin in different cell types of the ovary during the estrous

cycle with a distinct pattern of localization. The ovaries of proestrus mice showed intense immunostaining in the corpus luteum and moderate in the thecal cells, granulosa cells, and oocytes. Thus, the localization of visfatin suggests a role in the corpus luteum and in follicle functions. The previous study has shown that visfatin in the granulosa and thecal cells of hen and humans regulate ovarian steroid biosynthesis **(Diot et al., 2015b; Reverchon et al., 2013)**.

Furthermore, western blot analysis showed a peak in visfatin expression in the ovary of proestrus and with intense staining in corpus luteum. Since it has been demonstrated that corpus luteum in mice ovary survive two to four generations, and many generations of corpus luteum may be present in the ovary at any time of the cycle **(Deanesly, 1930; Mircea et al., 2009; Bertolin and Murphy, 2014)**, thus the corpus luteum of proestrus represent an old generation, and increased expression of visfatin may suggest its role in luteolysis. However, there is no report, whether visfatin promotes luteolysis in mice, although another adipokine, like apelin, has been shown to regulate luteolysis in bovine corpus luteum **(Shirasuna et al., 2008)**. In the corpus luteum of buffalo, it was also observed that visfatin was localized in the late luteal stage **(Thakre, 2018)**. These findings suggest that visfatin might regulate corpus luteum functions. The thecal and granulosa cells of growing follicles in the proestrus showed the presence of visfatin; thus, it might also be involved in the proliferation, apoptosis, and steroidogenesis. The expression of visfatin decreases from proestrus to estrus, as showed by western blot analysis, and immunohistochemical staining of visfatin showed an increase in thecal and granulosa cells of proestrus and estrus. In metestrus, expression of visfatin was lowest; however, in the corpus luteum, immunostaining of visfatin showed an increased expression and started to increase more in diestrus with strong immunostaining in the corpus luteum. Thus, it gives evidence of visfatin in corpus luteum function, which may be involved in the progesterone biosynthesis in the initial stage, and in a later stage, it may inhibit progesterone biosynthesis and may promote luteolysis and degeneration of corpus luteum as well. However, this statement requires further study to confirm. It has been shown that apoptosis is involved in the ovarian function, including oogenesis,

folliculogenesis follicular atresia, and luteolysis **(Hussein and Mahmoud, 2005; Tilly et al., 1997; Tilly and Hirshfield, 1996)**.

The expression of visfatin was correlated with BCL2 and active caspase3 expression during the estrous cycle to establish a link between visfatin expression and apoptosis. Our results showed that visfatin showed a significant positive correlation with BCl2 ($r= 0.6194$, $p<0.05$), and active caspase3 ($r= 0.7733$, $p<0.05$). Expression of BCL2 and active caspase3 was high in proestrus and estrus and lowest in diestrus, this result is in partial agreement with a previous report, which showed the lowest expression of BCL2 and highest expression of caspase3 in the diestrus ovary of rat **(Slot et al., 2006)**. In the study by Peluffo et al. **(2006)**, it has been shown that the old corpus luteum of rat ovary in estrus attains a peak of caspase3, which was associated with a decline in progesterone and functional regression of corpus luteum. It has also been shown that progesterone suppresses active caspase3 activity, apoptosis, and luteal degeneration **(Svensson et al., 2001; Okuda et al., 2004; Robker et al., 2000; Young and Stouffer, 2004)**. Since corpus luteum in the proestrus ovary may not be involved in the active progesterone biosynthesis, increased visfatin, may inhibit progesterone biosynthesis in proestrus and estrus **(Annie et al., 2019)** or accelerate luteal degeneration (increased active caspase3). Furthermore, it has been shown that visfatin also regulates apoptosis and cell proliferation in other tissues, including the uterus **(Annie et al., 2019; Lim et al., 2008; Rongvaux et al., 2008; Cheng et al., 2011)**. Based on the finding of the present study, it remains unclear whether visfatin is directly involved in the regression of corpus luteum, or it may facilitate the degeneration of corpus luteum by suppressing progesterone biosynthesis. This remains the limitation of our study; however, this is the first report to show changes in ovarian visfatin during the estrous cycle of rodents with possible functions.

The localization of visfatin in the thecal and granulosa cells also suggests its role in proliferation, apoptosis, and steroidogenesis **(Reverchon et al., 2013; Reverchon et al., 2016; Cheng et al., 2011)**. To confirm the role of visfatin in the ovary of mice, we have

injected a specific visfatin inhibitor, FK866, in the proestrus stage of mice. The proestrus stage was selected based on elevated expression of visfatin. After inhibition of visfatin, our results showed that the expression of PCNA decreases, and expression of active caspase3 and BCL2 increases. These findings suggest that visfatin increases cell proliferation in the ovary of proestrus, which may be promoting follicular growth, and on the other hand, visfatin in proestrus may also be promoting apoptosis of corpus luteum and follicles as well, by down-regulating the survival factor, BCL2. Previous study also showed that increase in PCNA expression in proestrus and estrus suggests increased proliferation than diestrus ovaries **(Asensio et al., 2018)**. Despite being known for proliferation, PCNA is also involved in DNA replication and repair **(Gary et al., 1997; Umar et al., 1996)**. It has been shown that PCNA is an important protein that is critical for managing the replication fork and other sites of DNA synthesis **(Boehm et al., 2016)**. Thus, PCNA as a marker of proliferation has limitations; however, some studies considered the PCNA, a marker of proliferation **(Kurki et al., 1986; Bravo et al., 1987)**. Therefore, decreased PCNA in the ovary after FK866 treatment might also suggest impaired DNA repair along with decreased proliferation. Our BrdU labeling and its quantification further suggest that inhibition of visfatin decreases the proliferation in the proestrus ovary. The previous study has even shown that visfatin increased proliferation of human granulosa cells, and inhibition of visfatin by FK866 also showed suppression of proliferation **(Reverchon et al., 2013)**. Visfatin may act as a survival factor and inhibits apoptosis in different cell types **(Lim et al., 2008; Rongvaux et al., 2008; Cheng et al., 2011)**. In the correlation study between the expression of visfatin to BCL2 and active caspase3, it seems that visfatin could up-regulate BCL2 and active caspase3 expression and might be suggested that visfatin acts as a survival factor and also promotes apoptosis. However, our *in vivo* results, where visfatin was inhibited by high dose of FK866 in the proestrus ovary, only active caspase3 expression was decreased, which suggests that visfatin up-regulates active caspase3 and may facilitate apoptosis, although, expression of BCL2 was increased. In the logical comparison of BCL2 expression up-regulated by visfatin during estrous and its increased expression of

BCL2 after FK866 treatment in proestrus, a discrepancy has been reflected, this could be due to a sudden response of ovarian cell to escape from death by up-regulating BCL2 expression after the FK866 treatment and during estrous, visfatin could be regulating BCL2 by other mechanisms and probably other factors could have also been involved. However, further study would be required to confirm this explanation. Recently, our *in vitro* study on the uterus showed that inhibition of visfatin decreases the active caspase3 expression and increases the BCL2 and PCNA expression **(Annie et al., 2019)**. It is evident that visfatin regulates apoptosis in ovary and uterus, and the discrepancy in PCNA expression may be due to the different tissues studied (uterus vs. ovary), and different experimental approaches have been utilized (in vivo vs. in vitro inhibition). Our results of visfatin mediated apoptosis are in agreement with another study, which has also shown that visfatin induces apoptosis in endothelial progenitor cells by up-regulating caspase3 and down-regulating BCL2 expression **(Sun et al., 2017)**.

In conclusion, we have, for the first time, showing the changes in the localization and expression of visfatin proteins in the mice ovary during the estrous cycle. The increased expression of visfatin in the corpus luteum suggests its role in the regulation of progesterone biosynthesis and in the regression of corpus luteum. Furthermore, visfatin may also be regulating follicular growth during the estrous cycle by regulating proliferation and apoptosis.

Summary

Visfatin is a crucial adipokine, which also regulates ovarian functions in many animals. Mice estrous cycle is characterized by a dynamic complex physiological process in the reproductive system. Expression of various factors changes during the estrous cycle in the ovary. To the best of our knowledge, no previous study has been conducted on the expression of visfatin in mice ovaries during the estrous cycle. Therefore, we investigated the localization and expression of visfatin protein in the ovary of mice during the estrous cycle. Western blot analysis showed the elevated expression of visfatin in proestrus and lowest in diestrus. Immunohistochemical localization of visfatin showed intense staining in the corpus luteum of proestrus and diestrus ovaries. Thecal cells, granulosa cells, and oocytes also showed the presence of visfatin. Expression of ovarian visfatin was correlated to BCL2 and active caspase3 expression and exhibited a significant positive correlation. Furthermore, *in vivo* inhibition of visfatin by FK866 in the proestrus ovary down-regulated Active caspase3 and PCNA expression, and up-regulated the BCL2 expression. These results suggest the role of visfatin in the proliferation and apoptosis of the follicles and specific localization of visfatin in the corpus luteum also indicates its role in corpus luteum function, which may be in progesterone biosynthesis and regression of old corpus luteum. However, further study is required to support these findings. In conclusion, visfatin may also be regulating follicular growth during the estrous cycle by regulating proliferation and apoptosis.

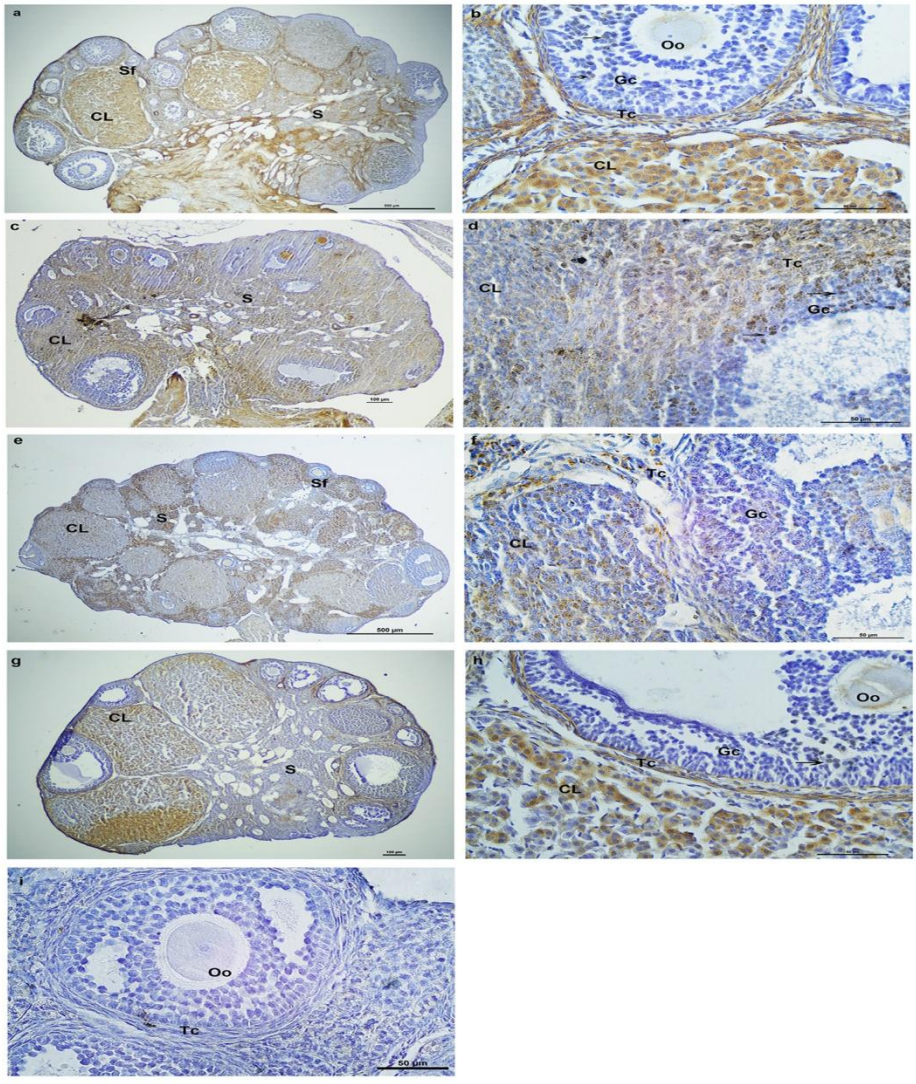


Figure-3.1- Immunolocalization of visfatin in the ovary of mice during the estrous cycle. Corpus luteum (CL) of proestrus ovary showed intense immunostaining in the luteal cells **(a-b)**, and moderate staining in the stroma (S), thecal cell (Tc) and granulosa cell (Gc). Estrus stage showed mild to faint staining in Oocyte (Oo), and moderate in the thecal cells (Tc), and granulosa cells (Gc) **(c-d)**. Metestrus ovary showed moderate immunostaining in corpus luteum and mild in the stroma **(e-f)**. Diestrus stage showed intense staining at corpus luteum, stroma, and faint in thecal cells **(g-h)**. The negative control ovary section (primary was omitted with 1% non-immune rabbit serum) showed no immunostaining **(i)**.

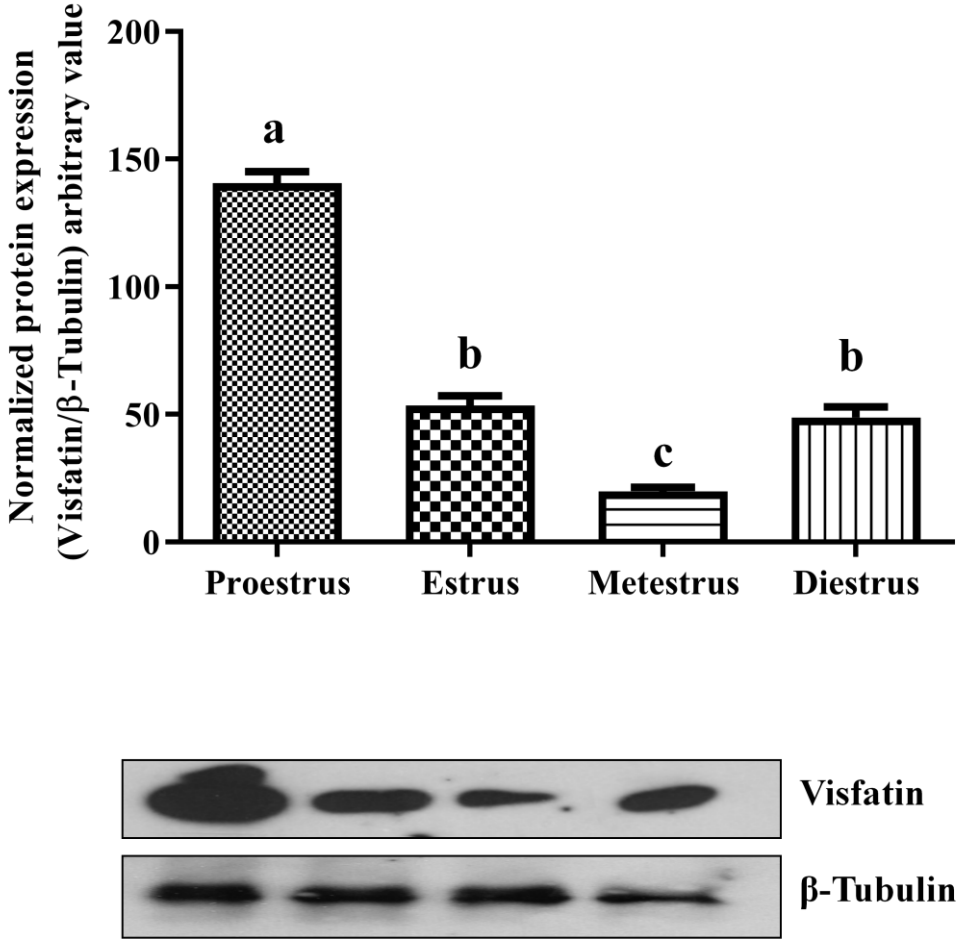


Figure-3.2- Western blot analysis of visfatin protein in the ovary during the estrous cycle. The histogram represents densitometric analysis of visfatin, and data are represented as mean ± SEM (n=5). Proestrus ovary showed significantly highest expression ($p<0.05$, a vs. b,c,d).

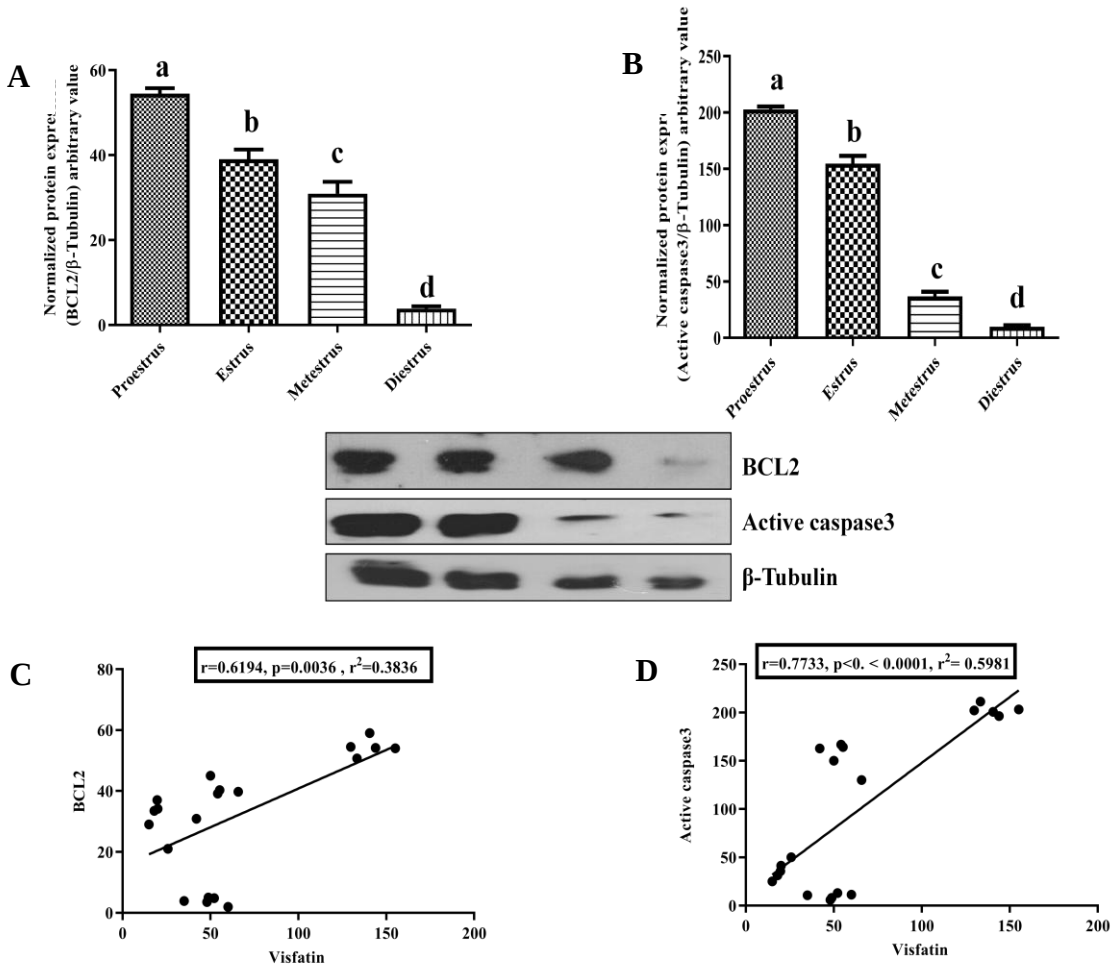


Figure-3.3- Western blot analysis of BCL2 and active caspase3 in the ovary during the estrous cycle. The histogram represents densitometric analysis of visfatin, and data are represented as mean ± SEM (n=5). BCL2 (A) and Active caspase3 (B) showed significantly highest expression in proestrus ovary compared to other stages ($p<0.05$, a vs. b,c,d). Expression of visfatin showed significant positive correlation with BCL2 (C) ($r=0.6194$, $p=0.0036$) and Active caspase3 (D) $r=0.7733$, $p<0.0001$).

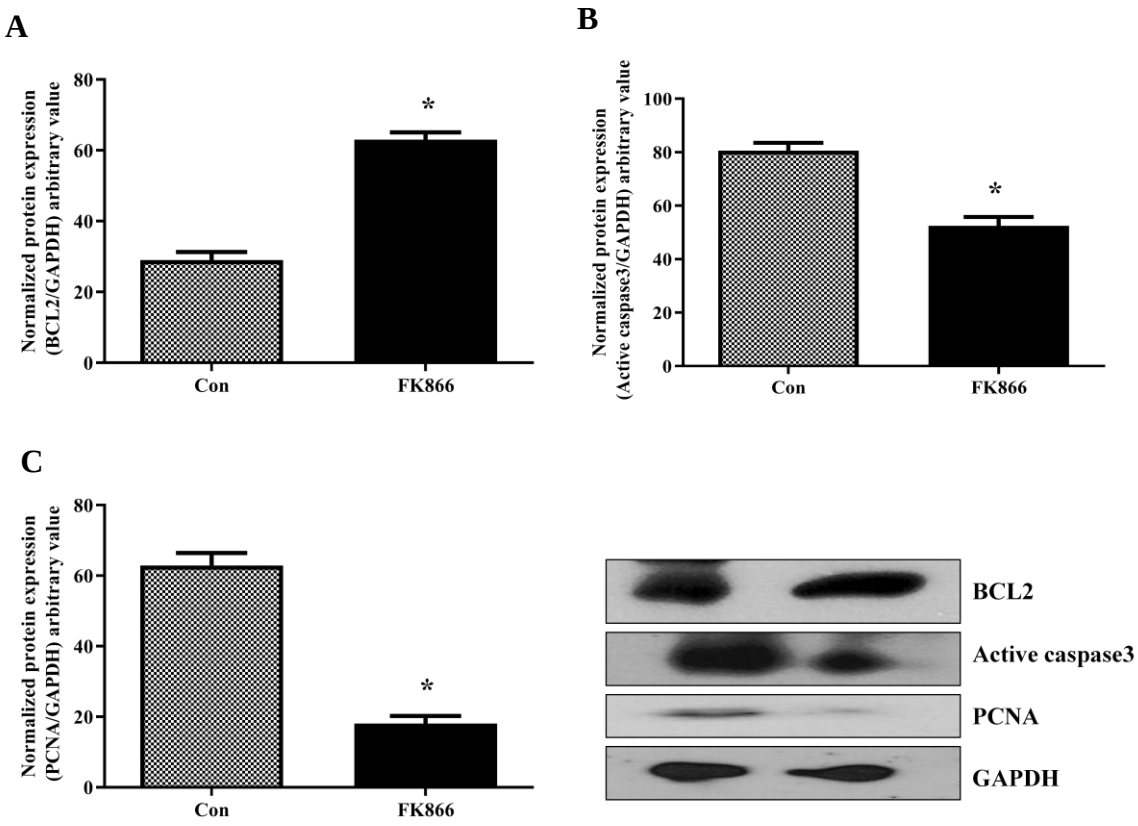


Figure-3.4- Effect of visfatin inhibition by FK866 on the expression of BCL2, Active caspase3, and PCNA. The histogram represents densitometric analysis of visfatin, and data are represented as mean ± SEM (n=5). FK866 significantly increased the expression of BCL2 **(A)** (*, $p<0.05$ vs. control), whereas FK866 significantly decreased the expression of Active caspase3 **(B)** and PCNA **(C)** (*, $p<0.05$ vs. control).

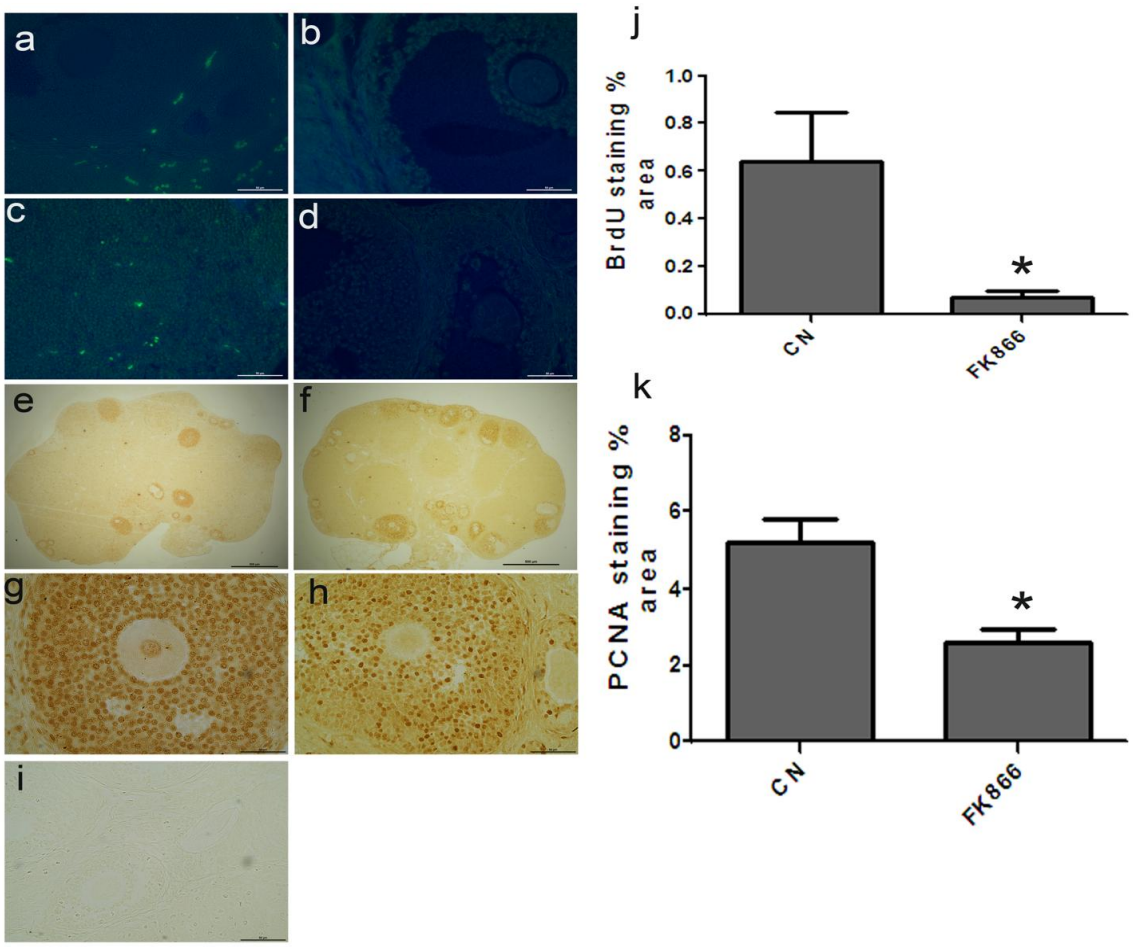


Figure-3.5- *In vivo* BrdU incorporation showed many BrdU positive cells (arrowhead) in the control ovary **(a)**, whereas in vivo inhibition of visfatin by FK866 showed no BrdU positive cells in the ovary **(b)**. The positive control tissue of lymph node showed BrdU positive cells **(c)**. The negative control section of ovary (BrdU antibody replaced with 1% mice serum) showed no BrdU positive cells **(d)**. Immunolocalization of PCNA showed strong immunostaining in the ovary **(e,g)** (arrowhead), whereas inhibition of visfatin (by KF866) showed faint immunostaining in the ovarian section **(f,h)**. The negative control section of ovary (PCNA antibody replaced with 1 % rabbit serum) showed no PCNA staining **(i)**. The quantification of BrdU **(j)** and PCNA **(k)** stained area by ImageJ showed significant (*, $p<0.05$) decrease in the BrdU and PCNA staining compared to control group.

Estrogen and progesterone dependent expression of visfatin/NAMPT regulates proliferation and apoptosis in mice uterus during estrous cycle

Introduction

Adipose tissue acts as an endocrine organ that secretes biologically active molecules, adipokines **(Ouchi et al., 2011)**. Various adipokines such as leptin, adiponectin, resistin have been detected in placenta, skeletal muscle, brain, endothelium as well as in gonads and exert specific functions **(Galic et al., 2010; Ouchi et al., 2011; Coelho et al., 2013)**. Adipokines also contributes in energy metabolism **(Kershaw et al., 2004)**, steroidogenesis **(Reverchon et al., 2014)** as well as regulation of gonadotropin secretion during the estrous cycle **(Kiezun et al., 2014)**.

Visfatin is an adipokine, also called as nicotinamidephosphoribosyltransferase (NAMPT) secreted by the visceral fat, which was first identified in mice and humans **(Fukuhara et al., 2005)**. Visfatin was also known formerly as pre-B cell colony enhancing factor which fascilitates nicotinamide adenine nucleotide biosynthetic activities in mammalian cells and immune cells **(Samal et al., 1994; Rongvaux et al., 2002)**. Like other adipokines, visfatin has been shown to be expressed in gonads of rodents **(Jeremy et al., 2017; Yang et al., 2015)**. In chickens, visfatin gene expressions have been found to be sex or tissue- dependent with a higher level in females than males **(Diot et al., 2015)**. Visfatin has been shown to be widely expressed in the ovarian cortex and granulosa especially during the growth of follicles. Visfatin has been localized in thecal and granulosa cells of ovary, where it has been shown to regulate steroidogenesis and folliculogenesis **(Ons et al., 2010; Ocon-Grove et al., 2010)**. This indicates the role of visfatin in reproduction **(Song et al., 2012)**. As expression of visfatin has been shown in ovary and a scant report is available on the expression of visfatin in the uterus. Thus, the expression of visfatin in the uterus needs to be highlighted and its function needs to be explored with changes in female reproductive tract during estrous cycle. Although,

Palin et al. **(2008)** observed the expression of visfatin in pig uterus; its function is still unknown.

The circulating levels of visfatin have been correlated with intrauterine growth during fetal development **(Malamitsi et al., 2007)**. Yang et al. **(2015)** found that the distribution of visfatin in the muscle layers of rat myometrium and uterine glands suggests visfatin role in muscle formation and participates in uterine gland secretion. However, its expression in mouse uterus is yet to be investigated.

The uterus undergoes drastic changes in cell proliferation and differentiation during estrous cycles under the endocrine control by ovarian hormones **(Marcus, 1973)**. Increased visfatin expression has been shown to promote cell proliferation in endometrial cancer by activating their signalling pathway **(Wang et al., 2016)**. Visfatin also regulates cell apoptosis in vascular smooth muscle cells **(Wang et al., 2009)** but its role in proliferation and apoptosis in uterus has not been studied. Since, the reproductive organ is well regulated by gonadal hormones, such as estrogen and progesterone during the estrous cycle, and there is evidence of the expression of visfatin in uterus. Thus, it would be interesting to unravel whether expression of visfatin is hormone-dependent. To the best of our knowledge, visfatin expression and the effect of visfatin on uterine function have never been studied in relation to estrous cycle. We hypothesized that uterine visfatin expression is steroid dependent and might be involved in uterine proliferation and apoptosis. Therefore, the aims of the present study were to investigate the expression and localization of uterine visfatin during estrous cycle of mice, the effect of steroid on visfatin expression, and role of visfatin in proliferation and apoptosis.

Materials and methods

Ethic statement

All animals included in the present study were handled and received humane care in compliance with protocols approved by the Mizoram University Institutional Animal Ethical committee (MZUIAEC), Mizoram University, Mizoram, India.

Animal maintenance and estrous cycle determination

Female swiss albino mice were inbred and kept in laboratory conditions of temperature and light change intervals of 25±2°C and 12 h light: 12 h dark cycles respectively, with food and water *ad libitum*. Matured and virgin female mice were checked for 3-4 regular estrous cycles, by obtaining vaginal smears daily checked under microscope which showed four distinct stages such as proestrus, estrus, metestrus and diestrus as described by **Goldman et al. (2007)**. Mice which showed regular estrous cycle were selected for the study.

Sample collection

Uterine tissues collected from mice showing distinct phases of the estrous cycle stages (n=4 per group) were thoroughly cleansed and freezed at -20°C while the other half was fixed in 10% NBF (neutral buffered formalin) for 24 h and stored in 70% ethanol. Blood samples were collected followed by centrifugation for 10 min at 10,000 rpm, and serum was pipetted out for storage till further analysis.

Ovariectomy and steroid hormone treatment

Healthy mice weighing between 25-28 g of 3 months old, (n=16) irrespective of their estrous stages were anaesthetized with xylaxine: ketamine concoct and bilateral ovariectomy was performed. The mice were checked the next day for complete recovery and rested for two weeks to obviate steroid hormones in circulation. At the end of two weeks, vaginal smear were checked and noted. The mice were then randomly categorized into four groups: (1) control group, were injected vehicle alone i.e., sesame oil. (2) Estradiol group (E_2), were injected subcutaneously 30 ng/g body weight estradiol dissolved in vehicle. (3) P_4 group, Progesterone was given in 150 µg/g body weight. (4) E_2+ P_4 group, both estrogen and progesterone were administered for 3 consecutive days **(Huang et al., 1999)**. The mice were sacrificed 24 h after the last injection. The uteri were also collected from different groups of the ovariectomised mice for Western blot

and enzyme assay. Progesterone (Cat # P0478) and β-estradiol (Cat # E0025) were obtained from Tokyo Chemical Industry Co., Ltd, Tokyo, Japan.

***In vitro* study**

To establish the role of visfatin in proliferation, apoptosis and antioxidant system, uterus tissue (n=3 per group) was cultured in the presence of visfatin inhibitor, FK866, as per Nakamura et al. **(2008)**. In brief, the uterine horns were removed and cleaned in PBS, and the uteri were cut into 2-3 mm and cultured for 24 h in DMEM: Ham'F12 medium (1:1) containing penicillin (100 U/ml) and streptomycin (100µg/ml). Visfatin inhibitor (FK866) was added in two doses at 1 nM and 10 nM in triplicate culture tubes **(Okumora et al., 2012)**. The uteri were fixed in 10% NBF and freeze for enzyme assay and Western blot.

Immunohistochemistry

Uteri of estrous cycle and ovariectomy experiment were fixed in 10% NBF after 24 h were stored in 70% alcohol. The tissues were further dehydrated in different grades of alcohol i.e. 70%, 90%, 100% respectively for 1 h, two changes each. Dehydrated tissues were immediately immersed in xylene and then embedded in paraffin wax and sectioned into a thin ribbon of 6µm thickness with Leica rotary microtome (model RM2125 RTS) as method described earlier **(Gurusubramanian and Roy, 2014)**. The sections were introduced to sequential deparaffinization with xylene and rehydrated in different grades of alcohol of 100%, 90%, 70% of two changes each for 10 min and placed in methanol + 3% hydrogen peroxide. Slides were then incubated in blocking goat serum 1:100 (Cat # A0515, Santa Cruz Biotechnology, Inc., CA, USA) with phosphate-buffered saline (PBS) for 30 min at room temperature in a humid chamber. Incubation with primary antibody was done at the dilution of 1:200 for visfatin (rabbit polyclonal IgG, Cat # V9139, Sigma-Aldrich, MO, USA), overnight at 4°C. Primary antibody wash with PBS is done for 15 min at 2 changes. Secondary-biotinylated antibody (Goat anti-rabbit IgG, Cat # H0113, Santa Cruz Biotechnology) of 1:50 dilution with both blocking and PBS

was applied for 90 min. The tissue sections were then washed and incubated with avidin-biotinylated-hrp-conjugated (Cat # E3013, Santa Cruz Biotechnology) at 1:50 dilution which was prepared atleast 30 min before use, and incubated with 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 for 10 min at room temperature. The slides were subsequently counterstained with Hematoxylin for 1 min and different grades of alcohol for dehydration and two changes of xylene, each 10 min; after which it is then mounted with DPX and observed under the microscope (Model E200, Nikon, Tokyo, Japan).

Bromodeoxyuridine (BrdU) labeling in *in vitro*

Uterus cultures were prepared with DMEM: Ham'F12 medium supplemented with 5-Bromo-2-Deoxyuridine (BrdU; Sisco Research Laboratories, Mumbai, India; 1.5 μg/ml) and incubated for 24 h at 37°C in 5% CO_2 **(Arai et al., 2015)**. The tissues are harvested and fixed in NBF for 24 h and processed for immunostaining. The sections were deparrafinisedand hydrated with 100% and 95%, rinsed in distilled water. DNA was denatured by incubating the sections in 2 N HCl for 1 h at 37°C. Acid neutralization was done by immersing in 0.1 M borate buffer for 10 min. After washing with PBS, slides were then incubated with blocking goat serum at 1:100 dilution for 30 min and incubated with anti-BrdU at 1:400 dilution with PBS (mouse monoclonal G3G4, Developmental Studies Hybridoma Bank (DSHB), University of Iowa) for overnight at 4°C. The sections were then incubated with secondary antibody (goat anti-mouse FITC conjugated, 1:200, Cat # E-AB-1015, Elabscience Biotechnology Inc., Wuhan, Hubei, China) for 3h at room temperature. Counterstaining was done with DAPI prepared in 0.1% Mcllvaine's solution for 10 min and immediately mounted for observation with a Nikon fluorescence microscope (Eclipse E200, Nikon, Tokyo, Japan).

Western blot analysis

The uterus samples collected during estrous cycle, after ovariectomy and *in vitro* treatments were homogenized with suspension buffer (50 Mm Tris–HCl, pH 8.0, 150

mM NaCl, 0.1% SDS, 1 g/ml Aprotinin, 1 mM PMSF and 1 mM EDTA) and centrifuged at 10,000 rpm for 10 min. Protein concentrations of the uterus sample was determined by Bradford's method **(Bradford 1976)** and subjected for Western Blot analysis **(Jeremy et al., 2017)**. The samples were denatured in SDS-sample buffer (62·5 mM Tris, 2% SDS, 10% glycerol, 1% - mercaptoethanol and 0·003% Bromophenol Blue, pH 6.8) in a final concentration of 500 µg/ml and loaded (50 µg/well) in 10% SDS-PAGE with protein marker at 100 V. The resolved sample was then transferred onto a polyvinylidineflouride membrane (Millipore India Pvt. Ltd., Bangalore, India), using Medox-Bio Mini Semi Dry Blotting MX-1295-01 for 30 min. The membranes were then blocked with 5% non-fat dry milk with PBS (10 mM, 7.5 pH) and 0.1% Tween 20 for 30 min at room temperature, and incubated with visfatin (1:1000 dilution in blocking buffer; Cat # V9139, Sigma-Aldrich), BCL2 (1:500; rabbit polyclonal antibody, Cat # EPP10828, Elabscience), BAX (1:1000; rabbit polyclonal IgG, Cat # SC-6236, Elabscience), Active caspase3 (1:500; polyclonal antibody rabbit, Cat # ENC006, Elabscience), PCNA (1:1000; rabbit polyclonal IgG, Cat # SC-7907, Santa Cruz Biotechnology), tubulin (1:2000; mouse monoclonal antibody, Cat # AF0344, Elabscience) overnight at 4°C. The membranes were washed two times with PBS-Tween20, and incubated with horse-radish peroxidase conjugated goat-anti-rabbit secondary antibody (1:4000; Merck Specialties Pvt. Ltd, Mumbai, India) for 4 h at room temperature. After three washes with PBS-Tween20, the membranes were incubated and developed with enhanced chemiluminescence (ECL) detection system (Cat # 1705060, BioRad, Hercules, CA, USA) with X-ray film to visualize the protein band. The protein band was then scanned and analyzed with the software ImageJ (1.38x, NIH, Bethesda, MD, USA). β-Tubulin was used as the loading control for all the samples in Western blot.

ELISA

The serum hormone estrogen and progesterone were measured by using enzyme linked immunosorbent assay kit (Estradiol Cat # DKO003, DiaMetra, Italy; Progesterone Cat #

RH-351, DSI, Saronno, Italy) as per manufacturer's instructions. The absorption level was determined with a MicroplateReader at 450 nm. Both E_2 and P_4 have their intra assay coefficient of variation being 3.5% and cross reactivity of 0.004%.

Enzyme Assay

The activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase in the uterus samples of ovariectomy and *in vitro* experiments were performed by following Weydert and Cullen method of non-denaturing polyacrylamide gel electrophoresis. For SOD activity, uterus homogenate were loaded 50 µg protein per well of 12% non-denaturing PAGE. After electrophoresis, subsequent staining of the gels to substrate specific staining of SOD bands was performed as described before **(Beauchamp and Fridovich, 1971)**. The gels were completely immersed in the staining mixture, which consists of 2.5 mM nitrobluetetrazolium (NBT), 28 mM riboflavin and 28 mM tetramethylethylenediamine (TEMED) and incubated for 20 min in the dark. After incubation, the gels were then exposed to an illuminated light so as to develop an achromatic band in contrast to the dark blue background which may corresponds to SOD protein in the gel. For catalase activity, uterus tissue extracts of each experimental groups containing 50 µg proteins were loaded and electrophoresed in an 8% non-denaturing PAGE. The gels were first soaked in 0.003% H_2O_2 for 10 min, and then incubated in a catalase specific staining mixture solution with 2% potassium ferricyanide and 2% ferric chloride **(Weydert and Cullen, 2010)**. Achromatic catalase bands developed against a blue-green background of the gel and the band intensity was quantified by ImageJ software. Determination of GPx level was performed as per the method described by **Sun et al. (1988)**. Uterine tissue extracts containing 50 µg protein was electrophoresed in a 10% non-denaturing PAGE. After electrophoresis, the gels were incubated in the staining mixture composed of 50 mM Tris–Cl buffer (pH 7.9), 3 mM GSH, 0.004% H_2O_2, 1.2 mM NBT and 1.6 mM PMS. Achromatic bands corresponded to GPx specificity was observed on the violet-

blue background of the gel. The intensity of the band suggests the GPx level, quantified using ImageJ software.

Statistical analysis

All data were expressed as mean ±SEM and were analyzed by one way ANOVA followed by post hoc Tukey's test to compare the data from different groups. The probability $p<0.05$ was set as the level of significance.

Results

Immunolocalization and expression of visfatin in uterus throughout the estrous cycle

To reveal the cellular localization pattern of visfatin in the mouse uterus during estrous cycle, an immulocalization study was performed **(Figure-4.1)**. Immunolocalization study detected the variation in immunostaining of visfatin throughout the estrous cycle from intense to low immunostaining based on the stage and compartments of uterus. The immunostaining of visfatin was observed in different compartments of uterus: endometrium, myometrium, glandular epithelium and luminal epithelium of uterus. Endometrium and myometrium of proestrus uterus showed intense immunostaining of visfatin **(Figure-4.1a-c)** whereas uterus of estrus showed faint immunostaining of visfatin **(Figure-4.1d-f)** in glandular epithelium and luminal epithelium of estrus than in proestrus. Further, as estrous cycle progresses to metestrus stage, visfatin showed intense immunostaining in endometrium and myometrium while strong immunostaining was observed in the glandular and luminal epithelium **(Figure-4.1g-i)** than in proestrus, estrus and diestrus. At diestrus, visfatin showed faint immunostaining in the endometrium, myometrium, glandular and luminal epithelium **(Figure-4.1j-l)**. Western blot analysis of visfatin expression showed significant ($p<0.05$) increased in proestrus and metestrus uterus than in estrus and diestrus. The expression of visfatin was lowest in diestrus uterus than in the other stages of estrous cycle **(Figure-4.2)**.

Levels of circulating estrogen and progesterone during estrous cycle

Circulating estrogen levels showed a remarkably higher concentration in the proestrus stage and metestrus during the estrous cycle **(Figure-4.3a)**. Serum progesterone levels showed a peak in diestrus stage **(Figure-4.3b)**.

Effect of ovarian steroids, estrogen and progesterone on visfatin expression in mouse uterus

The level of ovarian steroids varies throughout the estrous cycle and the biological action of estrogen and progesterone are inevitable for regulation of the uterine physiology. This led us to examine the effect of ovarian steroids, estrogen and progesterone on visfatin expression in ovariectomised mice. In control uterus, immunolocalization of visfatin showed moderate staining in endometrium, myometrium and glandular epithelium, whereas faint staining of visfatin was observed in stroma and luminal epithelium **(Figure-4.4a-c)**. The treatment of estrogen at a dose of 30 ng/g body weight showed intense immunostaining of visfatin in all the compartments of uterus, glandular epithelium and luminal epithelium in particular **(Figure-4.4d-f)**. The treatment of progesterone alone of 150 µg/g body weight showed mild immunostaining of visfatin in myometrium and stroma, and no staining of visfatin in glandular epithelium and luminal epithelium **(Figure-4.4g-i)**. The combined treatment with estrogen and progesterone showed resumption of visfatin staining in glandular epithelium and luminal epithelium of uterus **(Figure-4.4j-l)**.

Western blot analysis showed estrogen alone treatment to the ovariectomised mice significantly ($p<0.05$) increased visfatin expression in the uterus as compared with control mice. While progesterone alone treatment significantly ($p<0.05$) decreased visfatin expression in the uterus of ovariectomised mice as compared with control. However, treatment with both estrogen and progesterone significantly ($p<0.05$) increased the visfatin expression as compared to control, estrogen alone and progesterone alone treatment **(Figure-4.5).**

Expression of PCNA, BCL2, BAX and Active caspase3 in uterus throughout the estrous cycle

The expression of PCNA (proliferating marker), BCL2 (anti-apoptotic marker), BAX and Active caspase3 (apoptotic markers) showed marked variation throughout the estrous cycle. Expression of PCNA showed a significant ($p<0.05$) increase in proestrus and diestrus as compared with estrus and metestrus uterus **(Figure-4.6a)**. Expression of BCL2 protein showed increased levels in metestrus than the other stages of estrous **(Figure-4.6b)**. The expression of BAX protein increased in proestrus and metestrus, and showed lowest levels in the estrus stage. Further, at diestrus, expression of BAX showed significant ($p<0.05$) increase with respect to estrus **(Figure-4.6c)**. Expression of Active caspase3 increased significantly ($p<0.05$) in estrus than in the other stages of estrous cycle. At metestrus and diestrus, Active caspase3 expression was significantly ($p<0.05$) lower than the other stages **(Figure-4.6d)**.

Effect of ovarian steroids, estrogen and progesterone on PCNA, BCL2, BAX and Active caspase3 expression in mouse uterus

Expression of PCNA, BCL2, BAX and Active caspase3 showed marked variation in uterus of ovariectomised mice by ovarian steroids, estrogen and progesterone. Treatment with estrogen or progesterone alone significantly ($p<0.05$) increased the PCNA expression than the control uterus, however, combined treatment with estrogen and progesterone significantly ($p<0.05$) decreased the PCNA expression in uterus when compared to estrogen or progesterone alone treated uterus **(Figure-4.7a)**. BCL2 protein level is increased by estrogen alone treatment and progesterone alone decreased the expression of BCL2. The combined treatment of estrogen and progesterone increased BCL2 protein expression than the progesterone alone treatment **(Figure-4.7b)**. BAX protein showed increased expression by estrogen alone and combined treatment with estrogen and progesterone; while progesterone alone decreased BAX expression than both estrogen alone and combined treatment with estrogen and progesterone **(Figure-4.7c)**. Expression of Active caspase3 showed significant ($p<0.05$) increase by estrogen

alone treatment and significant ($p<0.05$) decrease by progesterone alone treatment, whereas treatment with both estrogen and progesterone together decreased the expression of Active caspase3 than the other treatments **(Figure-4.7d)**.

Effect of ovarian steroids, estrogen and progesterone on antioxidant enzymes (SOD, GPx and catalase) in the uterus of ovariectomised mice

The treatment with estrogen, progesterone and estrogen with progesterone to ovariectomised mice showed significant ($p<0.05$) increase in SOD activity as compared to control **(Figure-4.8a)**. Estrogen alone and progesterone alone treatment significantly ($p<0.05$) decreased as compared with control, whereas treatment of both estrogen and progesterone significantly ($p<0.05$) decreased GPx activity as compared to all the other groups **(Figure-4.8b)**. In contrast to GPx, catalase activity was increased by estrogen as well as progesterone, whereas combined treatment with progesterone and estrogen significantly ($p<0.05$) decreased catalase activity as compared to control and estrogen alone group **(Figure-4.8c)**.

Effect of visfatin inhibitor (FK866) on PCNA, BCL2, BAX and Active caspase3 expression in mouse uterus

To access the role of visfatin on uterine proliferation and apoptosis, the uterine explants culture was performed in the presence of visfatin inhibitor, where proliferation marker (PCNA), apoptotic (BAX and Active caspase3) and anti-apoptotic (BCL2) markers were measured by Western blot analysis. The treatment of visfatin inhibitor (1 nM and 10 nM) showed a significant ($p<0.05$) dose dependent increase in PCNA and BCL2 expression in the uterus than in control **(Figure-4.9a, b)**. However, two doses of visfatin inhibitors, 1nM and 10 nM decreased the expression of BAX and Active caspase3 in a dose dependent manner **(Figure-4.9c, d)**.

Effect of visfatin inhibitor (FK866) on antioxidant enzymes (SOD, GPx and catalase)

The changes in the levels of antioxidative enzymes (SOD, GPx and catalase) in the control and FK866 (1 nM and 10 nM)-treated mice uterus were studied by in-gel assay followed by densitometric analysis. The results showed a dose-dependent significant ($p<0.05$) decrease in the levels of SOD and GPx in the uterus of FK866-treated mice uterus as compared to control **(Figure-4.10a, b)**. The catalase level increased significantly ($p<0.05$) only with high dose of FK866, but not in low dose, as compared with the control **(Figure-4.10c)**.

Effect of visfatin inhibitor (FK866) on BrdU incorporation

Effect of visfatin inhibitor, FK866 on BrdU incorporation in uterus showed marked variation **(Figure-4.11a-d**). Treatment with 1 nM dose of FK866 stimulated proliferation, which was clearly shown by BrdU positive cells **(Figure-4.11c**). Whereas, treatment with 10 nM dose of FK866 suppressed proliferation, evidenced by few BrdU positive cells in uterus **(Figure-4.11d**).

Discussion

The present study investigated the expression and localisation pattern of visfatin in mice uterus during estrous cycle, effects of ovarian steroids on the expression and localisation pattern of visfatin in ovariectomised mice uterus along with expression of PCNA, BAX, BCL2 and Active caspase3 in natural estrous cycle as well as on ovariectomised mice uterus. Further, the present study also showed the effects of visfatin inhibitor, FK866 on uterine antioxidant enzymes (SOD, GPx and Catalase) activity and the expression of PCNA, BCL2, BAX and Active caspase3 in *in vitro* condition.

The present study is the first study to demonstrate the expression and localisation of visfatin in mouse uterus and its responsiveness to exogenous estrogen and progesterone treatments. Immunolocalization study showed localisation pattern of visfatin varies and

depends on the stage of the estrous cycle. The intense immunostaining of visfatin has been observed in proestrus and metestrus uterus than in estrus and diestrus. These results suggest that change in visfatin expression during estrus cycle might be involved in uterine physiology to prepare and facilitate gamete transport and implantation. Visfatin is an adipokine and several studies have shown that adiponectin is one adipokine which is expressed in the uterus of different species such as pig **(Lord et al., 2005)**, rabbit **(Schmidt et al., 2008)**, rodents **(Kim et al., 2011)** and human **(Takemura et al., 2006)**. Similarly, another adipokine, chemerin expression has also been shown in human uterus **(Carlino et al., 2012)**. The previous study suggested that the expression of adipokines could be involved in implantation of the embryo **(Santos et al., 2012)**. To the best of our knowledge, there is a limited report on the expression of uterine visfatin; however, visfatin expression has been shown in pig uterus **(Palin et al., 2008)** and suggested that like other adipokine, visfatin may have potential role in the reproductive tract **(Reverchon et al., 2014; Campos et al., 2008)**.

The result of Western blot analysis of visfatin coincides with immunolocalisation study, which showed decreased expression of visfatin in the uterus of estrus and diestrus stage, and expression of visfatin showed a peak during proestrus and metestrus. During proestrus, the peak of visfatin coincided with the increased expression of PCNA and elevated levels of serum estrogen and decreased expression of visfatin might be linked to increased PCNA expression in diestrus.

Increased visfatin expression during proestrus and decrease in estrus also coincides with increased expression of Active caspase3, which suggests that visfatin might play apoptotic role in visfatin mediated cell proliferation and apoptosis. It has been shown that cell proliferation increase from diestrus toward proestrus, whereas apoptotic death decrease at proestrus and increase at estrus with a decrease in PCNA expression **(Sandow et al., 1979; Sato et al., 1997; Lai et al., 2000)**. PCNA plays an important role in DNA replication, cell division and in DNA repair mechanisms **(Boehm et al., 2016)**. It has been shown that tissue remodeling during the estrous cycle is

accompanied by cell proliferation, apoptosis, and DNA replication **(Yip et al., 2013)**. Whether the elevated PCNA expression during proestrus and diestrus is involved in proliferation or DNA repair needs further study.

The present study showed a decline in the expression of Active caspase3 protein, from metestrus to diestrus, which coincides with decreased expression of visfatin and increased progesterone levels during a normal estrous cycle from metestrus to diestrus **(Wood et al., 2007)**. It has also been shown that progesterone suppresses apoptosis and uterine glandular cells showed the highest apoptotic index at estrus and low apoptotic index at metestrus and diestrus **(Rotello et al., 1992; Sato et al., 1997; Dharma et al., 2001)**. This finding suggests that from metestrus to diestrus stage visfatin might also be involved in apoptosis of uterus. Further, it might be suggested that visfatin could be involved in estrogen and progesterone regulated cyclic proliferation and death of uterine epithelial cells during estrous cycle. It has been reported that exogenous administration of visfatin increases inflammatory cytokines and prevented apoptosis induced by chronic distension, labor, or infection in the placenta **(Ognjanovic and Bryant-Greenwood, 2002)**. Increased caspase3 activation has been shown in rat mucosa and suggested that visfatin regulates apoptosis and cell proliferation **(Zhou et al., 2017)**. Recently visfatin has been shown to induce proliferation in breast cancer cells and demonstrated that visfatin treatment increase cell proliferation via PI3K and MEK1/2 pathway **(Gholinejad et al., 2017)**.

Our study showed elevated levels of progesterone in diestrus and this finding coincides with previous reports where progesterone levels showed a peak in diestrus phase of cycling mice **(Fata et al., 2001; Wood et al., 2007; Schwartz et al., 2004)**. However, other studies also imply that progesterone levels attain peak during proestrus **(Kosaka et al., 1988)** and estrus **(Zenclussen et al., 2014)** in cycling mice. The discrepancy in circulating progesterone levels in mice by various studies needs further investigation.

Expression of uterine visfatin showed a close association with circulating steroid hormones, such as estrogen and progesterone levels. This prompted us to investigate the

effect of estrogen and progesterone on uterine visfatin expression. Estrogen and progesterone have been shown to modulate visfatin expression in 3T3-L1 cells and suggested that estriol, estradiol and progesterone exert a synergistic effect on visfatin gene expression **(Zhou and Seidel, 2010)**, and it has been shown that estrogen expression in resistin is through the estrogen receptor **(Chen et al., 2006)**. Our results showed for the first time that estrogen up-regulates and progesterone down-regulates visfatin expression in uterus of ovariectomised mice, and no similar study has been conducted. In contrast, it has also been shown that estradiol does not affect visfatin gene expression **(Jung et al., 2013)**. These results are partly inconsistent with our finding that visfatin expression was directly influenced by estradiol and progesterone. In the present study, the expression of visfatin has been examined at protein levels and not at transcript levels, whether the change in expression of visfatin could be due to change in visfatin gene expression or alterations in protein turnover, this requires further study.

The findings of the estrous cycle experiment can be further correlated to the results of ovariectomy experiments, and showed concurrence of estrogen-stimulated expression of visfatin with an increase in expression of proliferating marker (PCNA), anti-apoptotic marker (BCL2) and apoptotic markers (BAX and Active caspase3) and antioxidant enzymes (SOD and catalase) activities. Several studies have shown that visfatin stimulates cell proliferation in various cell types such as osteoblasts **(Xie et al., 2007)**, vascular smooth muscle cells **(Wang et al., 2009)**, Me45 cell **(Buldak et al., 2013)** and prostate cancer cells (PC-3 line) **(Patel et al., 2010)**. Xiao et al. **(2015)** demonstrated that visfatin has pro-apoptotic role in lymphocytes under normal condition by activating caspase3 and play an anti-apoptotic role in LPS-treated lymphocytes by inhibiting Active caspase3. Thus, it may be suggested that estrogen stimulated visfatin expression could be involved in the process of proliferation (increase PCNA) and maintain a balance between cell survival (increase BCL2) and apoptosis (increase BAX and Active caspase3). On the other hand, progesterone mediated decrease in visfatin might suppress apoptosis (decrease BAX and Active caspase3), increase proliferation (increase PCNA) and cell survival (decrease BCL2) in the mice uterus. Estrogen along with progesterone

decreased Active caspase3 and PCNA expression, suggesting that visfatin regulates cell proliferation and apoptosis in mice uterus. The cell proliferation, differentiation and death of uterine epithelium are regulated by estrogen and progesterone **(Wood et al., 2007)**. Visfatin has also been shown to exhibit an anti-apoptotic role in MIN6 cells via a kinase-dependent signaling pathway **(Cheng et al., 2011)**. The role of visfatin in uterine proliferation and apoptosis has not been investigated so far.

Recently, visfatin has been linked to modulating antioxidant enzymes and suggested that it could be a marker of oxidative stress **(Marseglia et al.,2016)**. Buldak et al. **(2013)** showed that visfatin at various concentration exhibit a redox adaptation response, which leads to an up-regulation of the antioxidant enzymes. These studied led us to examine the activity of antioxidant enzymes (SOD, GPx and catalase). Progesterone mediated decrease in visfatin expression coincides with a decrease in expression of BCL2, BAX, Active caspase3 as well as decreased activities of catalase and SOD (in comparison to estrogen only). In dog uterus, it has been suggested that steroid-dependent SOD, GPx and catalase could involve in different pathways regulating the endometrial cycle **(Santos et al., 2016; Al-Gubory et al., 2017)**. However, combined estrogen and progesterone treatment mediated increase in visfatin expression is related to decreased catalase and GPx activities. These results suggested that visfatin might be involved as an essential regulator in steroid mediated apoptosis, proliferation and antioxidant homeostasis.

To establish whether visfatin was involved in the modulation of the antioxidant system, proliferation and apoptosis, *in vitro* study was conducted to unravel the mechanism. The *in vitro* study clearly showed that inhibition of visfatin by a specific inhibitor (FK866) increased the expression of anti-apoptotic marker (BCL2) and proliferation marker (PCNA), and decreased apoptotic markers BAX and Active caspase3. To confirm the role of visfatin on proliferation, BrdU labeling study was performed, which showed that mild inhibition of visfatin by low dose of FK866 stimulated the cell proliferation and inhibition of visfatin by high dose of FK866 suppressed the proliferation, as noticed by

BrdU positive cells. These results showed that visfatin is involved in the regulation of cell proliferation and apoptosis; however, treatment with visfatin inhibitor FK866 increases PCNA expression in dose dependent manner and suggests the anti-proliferative role of visfatin. As our result of BrdU labeling confirmed that abolition of visfatin action could suppress proliferation, an increased in PCNA could be involved in DNA repair since it has an important role in DNA repair mechanism **(Boehm et al., 2016)**. Another study has also shown that visfatin inhibitor FK866 inhibits the proliferation of lung adenocarcinoma cells **(Okumura et al., 2012)**.

It has been shown that visfatin inhibitors increase the activities of caspase3 and decreased SOD **(Feng et al., 2016)**. *In vitro* study results also showed that visfatin inhibitor decreased SOD and GPx activity and increased catalase activity. This suggests the overall decrease in antioxidant capacity. Despite the decrease in antioxidant capacity, visfatin inhibitor decreased apoptosis and increased proliferation; this finding is yet to be investigated. However, our results are in agreement with the previous report that treatment with visfatin decreased levels of the anti-apoptotic gene, BCL2, and increased expression levels of the pro-apoptotic gene, caspase3, as well as an increased BAX and treatment with visfatin inhibitor, suppresses expression of cleaved caspase3 and BAX and increased BCL2 in endothelial progenitor cells **(Sun et al., 2017)**. Esposito et al. **(2012)** demonstrated that FK866 treatment to mice suppress apoptosis by down-regulating pro-apoptotic marker, BAX and up-regulating anti-apoptotic marker, BCL2, in spinal cord injury model. Recently, FK866 mediated cell proliferation has been documented in the tubular cell **(Benito-Martin et al., 2014)**. Thus, it may be suggested that endogenous visfatin modulates cell death and cell proliferation during estrous cycle of mice. To the best of our knowledge, this is the first study to show the effect of visfatin inhibitor on pro-and anti-apoptotic markers, proliferation marker and antioxidative enzyme in the uterus of any mammalian species.

In conclusion, the present study showed that the expression of uterine visfatin is regulated by estrogen and progesterone. This study provides the first evidence that

steroid-dependent expression of visfatin might be involved in the regulation of proliferation and apoptosis in the uterus of mice during reproductive cycle and would be important for uterine changes takes place during cycle for successful reproduction.

Summary

Visfatin is an adipokine which has an endocrine effect on reproductive functions and regulates ovarian steroidogenesis. There is scant information about the expression, regulation, and functions of visfatin in the mammalian uterus. The present study examined expression and localisation of visfatin in the mouse uterus at various stages of the natural estrous cycle, effects of estrogen and progesterone on localisation and expression of visfatin in the ovariectomised mouse uterus and effect of visfatin inhibition by a specific inhibitor, FK866 on proliferation and apoptosis in the uterus. Western blot analysis of visfatin showed high expression in proestrus and metestrus while it declined in estrus and diestrus. Immulocalisation study also showed strong immunostaining in the cells of the endometrium, myometrium, luminal and glandular epithelium during proestrus and metestrus that estrus and diestrus. The uterine visfatin expression closely related to the increased estrogen levels in proestrus and suppressed when progesterone rose to a high level in diestrus. The treatment with estrogen to ovariectomised mice up-regulates visfatin, PCNA, and Active caspase3 whereas progesterone up-regulates PCNA and down-regulates visfatin and Active caspase3 expression in mouse uterus. The co-treatment with estrogen and progesterone up-regulates visfatin and down-regulates PCNA and Active caspase3. *In vitro* study showed endogenous visfatin inhibition by FK866 increased expression of PCNA and BCL2 increased catalase activity while FK866 treatment decreased expression of Active caspase3 and BAX with decreased SOD and GPx activity. BrdU labeling showed that inhibition of visfatin modulates the uterine proliferation. This study showed that expression of visfatin protein is steroid dependent in mouse uterus, which is involved in the regulation of proliferation and apoptosis via modulating antioxidant system in the uterus of mice during the reproductive cycle.

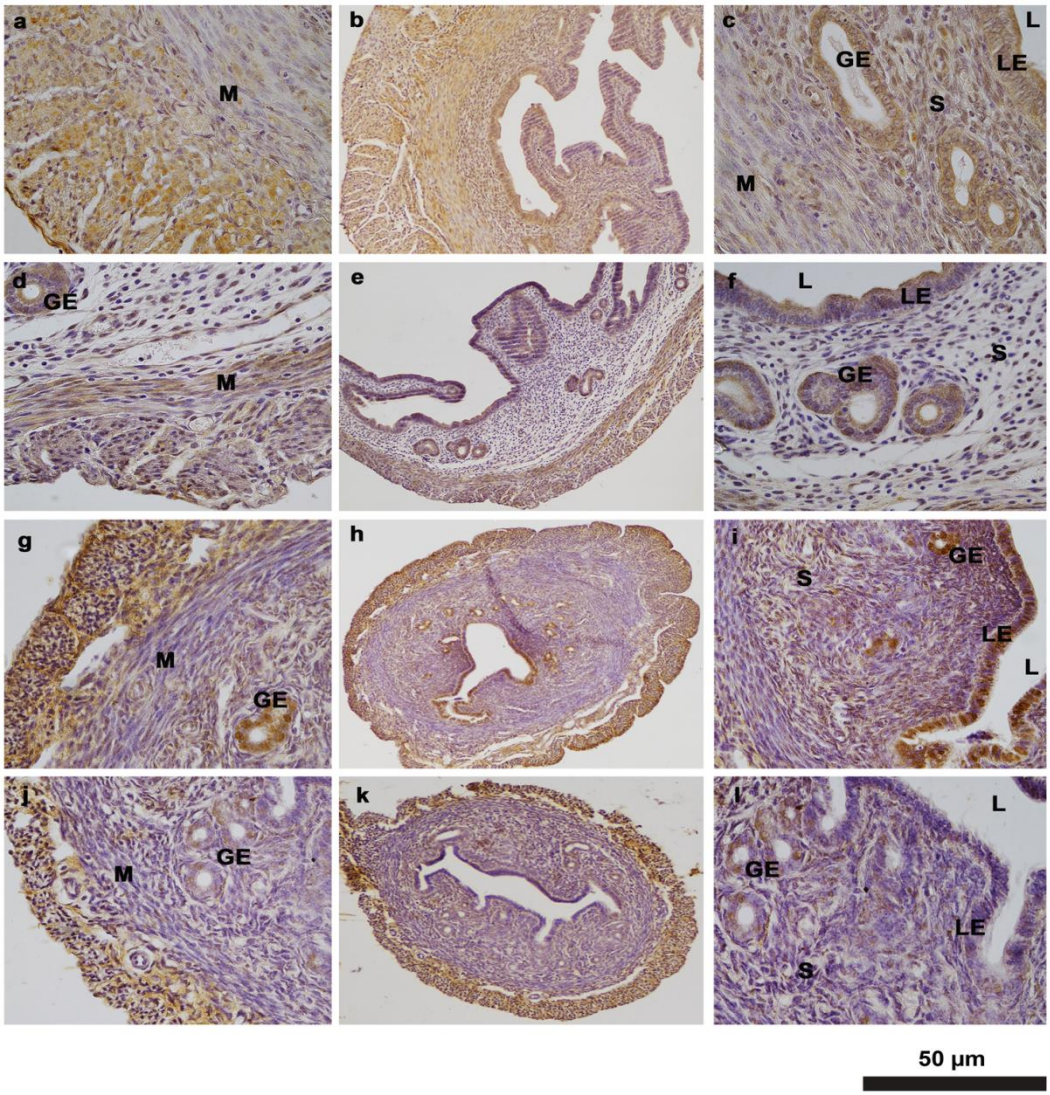


Figure-4.1- Localization of visfatin in the mouse uterus at different stages of the estrous cycle. Uterine sections showed intense immunostaining during proestrus (**a,c** at 40x and **b** at 10x magnification), faint during estrus (**d,f** at 40x and **e** at 10x magnification), intense during metestrus (**g,i** at 40x and **h** at 10x magnification), and low during diestrus stages (**j,l** at 40x and **b** at 10x magnification) in different compartments. M, myometrium; GE, glandular epithelium; LE, luminal epithelium; S, stroma; L, Lumen.

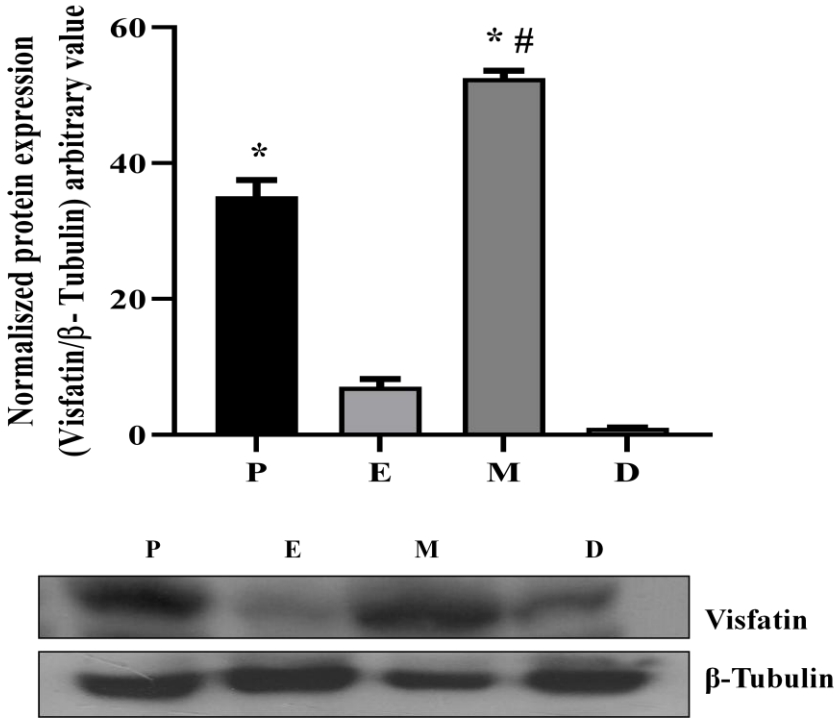


Figure-4.2- Differential expression of visfatin in the mouse uterus during the estrous cycle. Histogram represents densitometric analysis of visfatin expression. The data are represented as the means ± SEM. The band intensity values showed peak during proestrus and metestrus. *$p<0.05$ (proestrus as compared with the estrus and diestrus); *# $p<0.05$ (metestrus as compared with proestrus, estrus and diestrus).P, proestrus; E, estrus; M, metestrus; D, diestrus.

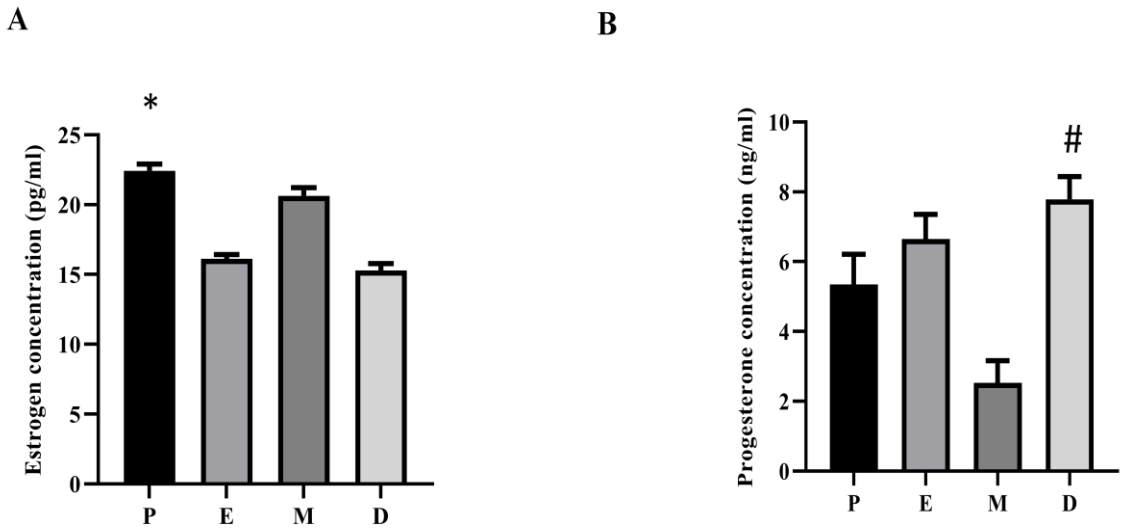


Figure-4.3-Estrous cycle dependent changes in serum estrogen and progesterone levels. Serum concentration of circulating estrogen showed significant (*$p<0.05$) elevated levels in proestrus (A) and progesterone showed significant (# $p<0.05$) highest levels in diestrus as compared with other stages.

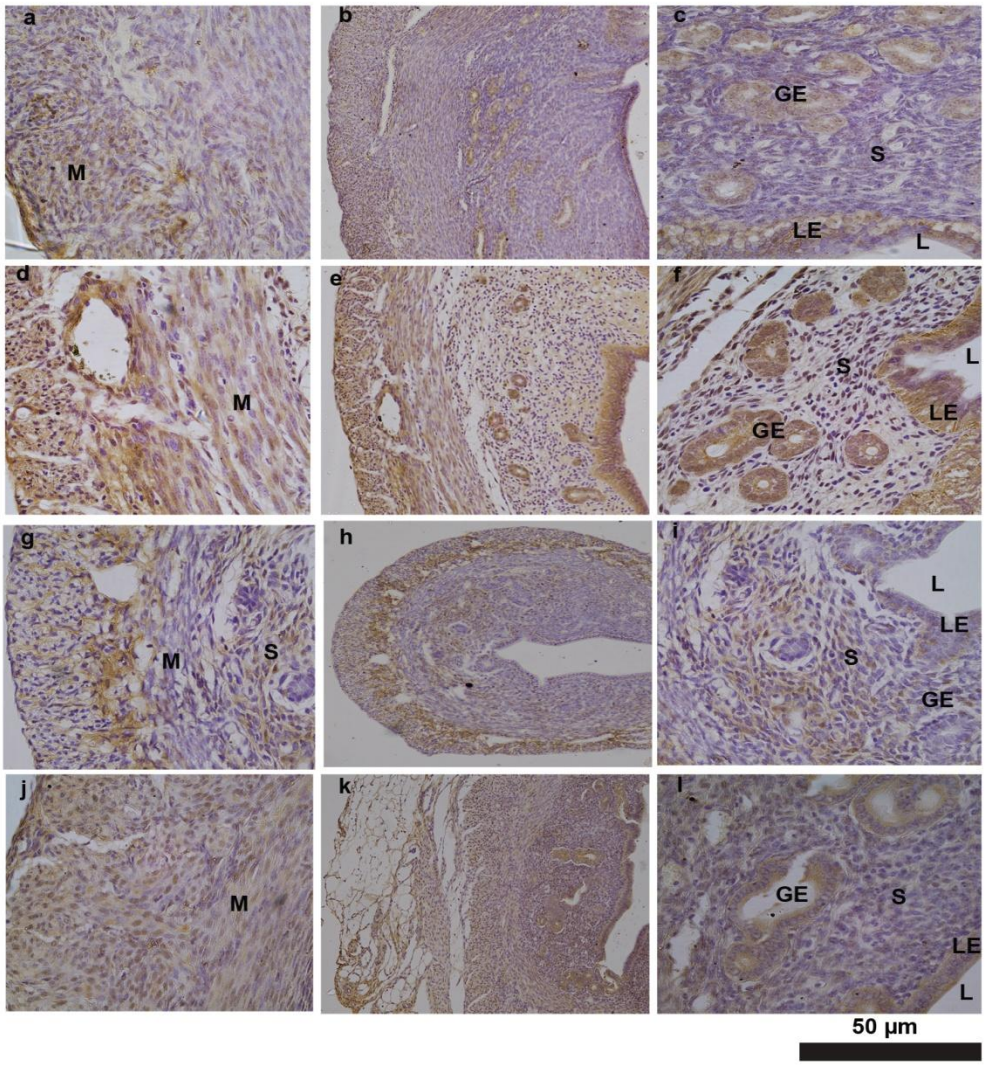


Figure-4.4-Exogenous estrogen and progesterone changes the localization pattern of visfatin in the uterus of ovariectomised mice. Uterine sections of ovariectomised mice without treatment showed mild immunostaining (**a,c** at 40x and **b** at 10x magnification), intense immunostaining after estrogen treatment (**d,f** at 40x and **e** at 10x magnification), faint immunostaining after progesterone treatment (**g,i** at 40x and **h** at 10x magnification), and mild immunostaining after combined treatment with estrogen and progesterone (**j,l** at 40x and **b** at 10x magnification) in different compartments. M, myometrium; GE, glandular epithelium; LE, luminal epithelium; S, stroma; L, Lumen.

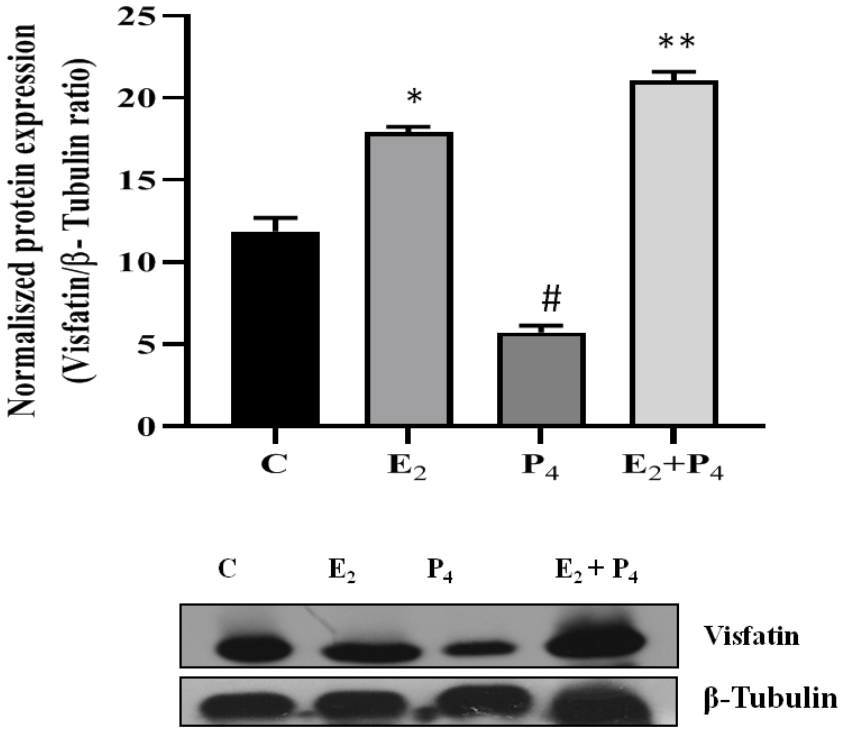


Figure-4.5-Regulation of visfatin expression in the ovariectomised mice uterus by exogenous estrogen and progesterone. Histogram represents densitometric analysis of visfatin expression. The data are represented as the means ± SEM. *p<0.05, estrogen (E_2) alone significantly increased visfatin expression in the uterus as compared with control (C) and progesterone (P_4) treated mice. # p<0.05, P_4 alone treatment showed significant decrease visfatin expression as compared with C, E_2 and E_2+P_4. ** p<0.05, E_2+P_4 showed significant increased as compared with C, E_2 and P_4. C, control group; E_2, Estrogen alone group; P_4, Progesterone alone group; E_2+ P_4, estrogen and progesterone combined group.

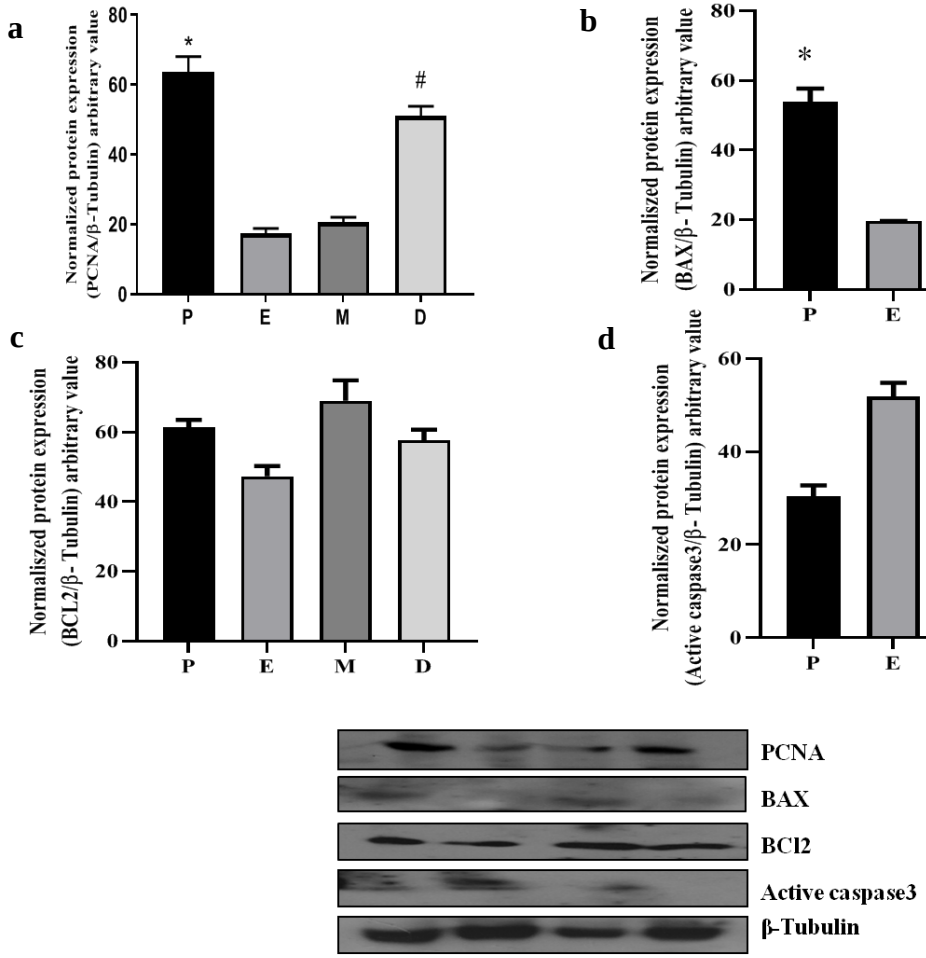


Figure-4.6-Changes in the expression of PCNA, BCL2, BAX and active caspase3 in uterus throughout the estrous cycle. Histogram represents densitometric analysis of PCNA, BCL2, BAX and Active caspase3 expression. The data are represented as the means ± SEM. **(a)** PCNA showed a significant increase in P (*p<0.05) as compared with E, M and D and in D (#p<0.05) as compared with E and M. **(b)** The expression of BCL2 showed insignificant change during estrous cycle. **(c)** BAX showed significant increase in P and M (*p<0.05) compared with E and D. **(d)** Active caspase3 showed significant decrease in M and D (*p<0.05) as compared with P and E. P, proestrus; E, estrus; M, metestrus; D, diestrus.

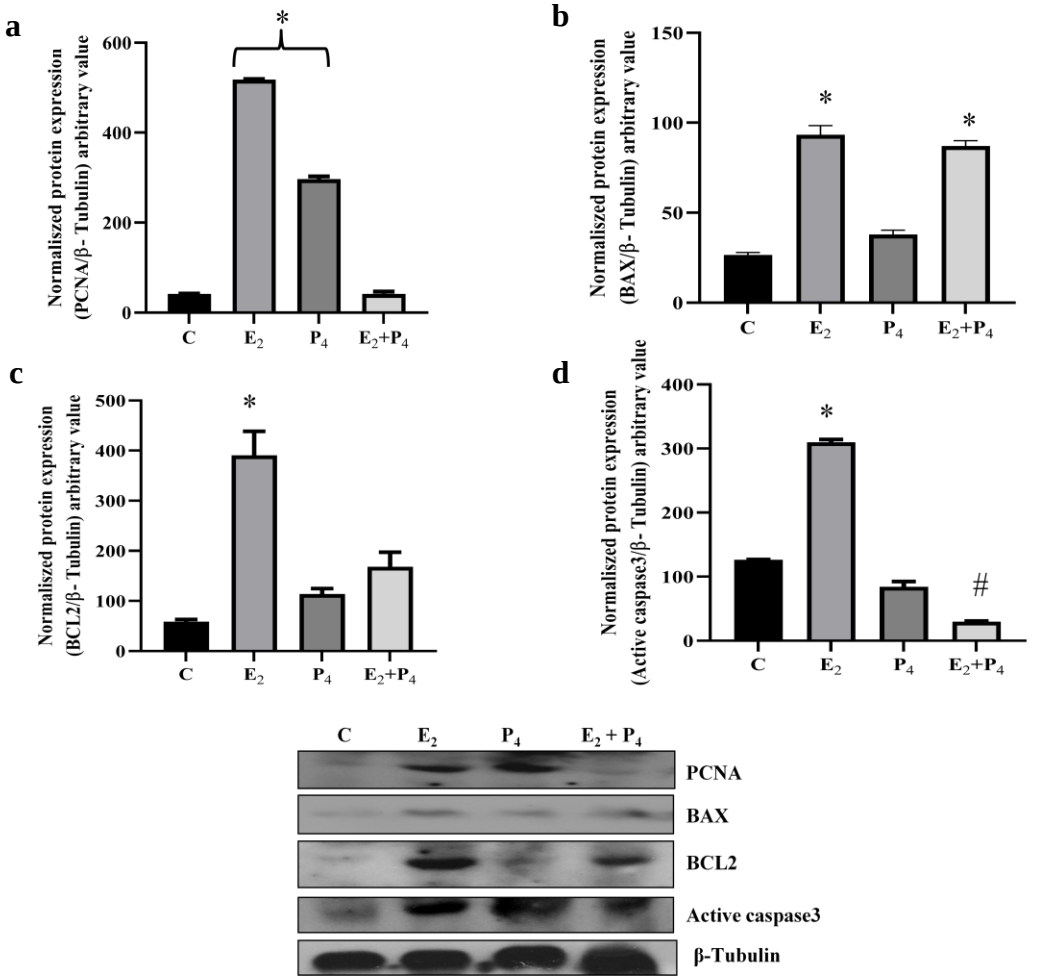


Figure-4.7-Regulation of PCNA, BCL2, BAX and Active caspase3 expression in the ovariectomised mice uterus by estrogen and progesterone. Histogram represents densitometric analysis of PCNA, BCL2, BAX and Active caspase3 expression. The data are represented as the means ± SEM. (**a**) PCNA showed significant increase in expression (*p<0.05) with E_2 and P_4 treatment as compared with C and E_2+P_4. **(b)** BCL2 expression significantly increased in (*p<0.05) E_2 as compared with C, P_4 and E_2+P_4. **(c)** BAX expression showed increased in E_2 (*p<0.05) as compared with C and P_4 and highest in E_2+P_4 (#p<0.05) as compared with C, E_2 and P_4. **(d)** Active caspase3 showed significant increase in E_2 (*P<0.05) as compared with C, P_4 and E_2 + P_4. C, control group; E_2, Estrogen alone group; P_4, Progesterone alone group; E_2+ P_4, estrogen and progesterone combined group.

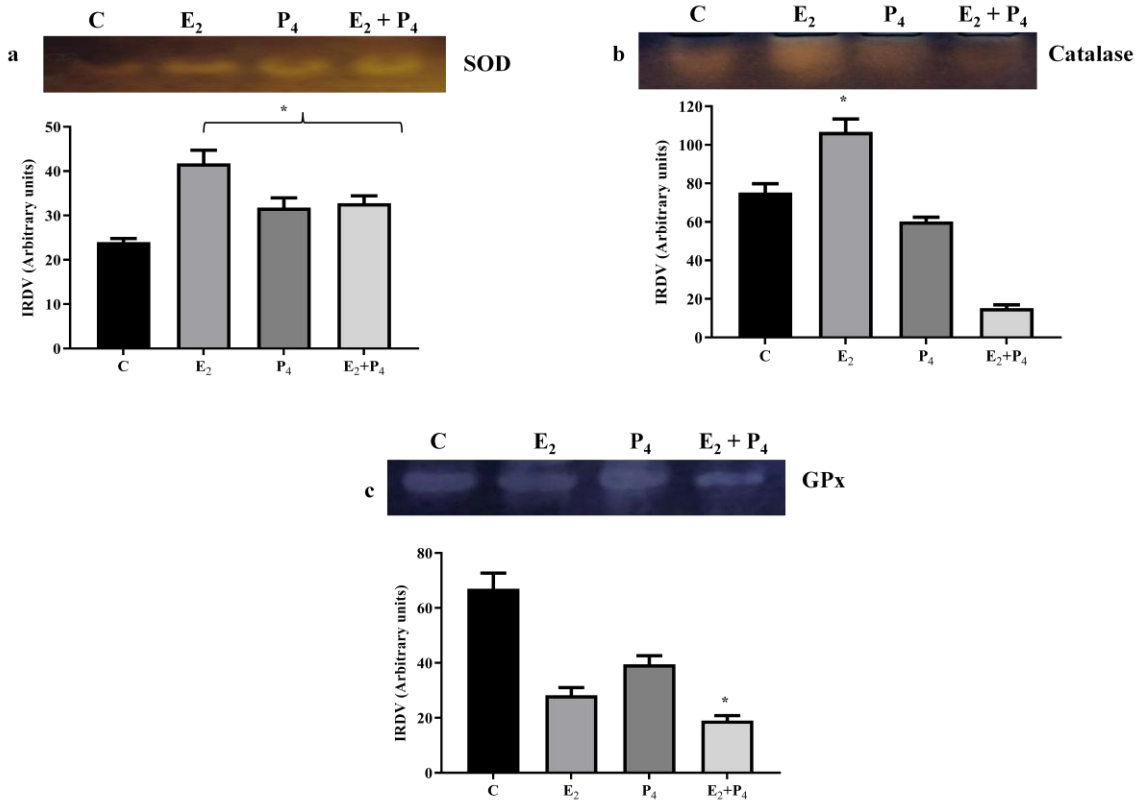


Figure-4.8-Estrogen and progesterone regulates antioxidant enzymes in the uterus of ovariectomised mice. Representative images of gels are shown, in addition to histograms representing densitometric analysis of the bands. The data are represented as the means ± SEM. **(a)** Treatment with E_2, P_4 and E_2+P_4 showed significant increase in SOD (*p<0.05) as compared with C. **(b)** E_2+P_4 showed significant decreased in SOD (*p<0.05) as compared with C, E_2 and P_4. **(c)**E_2 showed significant increase in catalase (*p<0.05) as compared with C, P_4 and E_2+P_4. C, control group; E_2, Estrogen alone group; P_4, Progesterone alone group; E_2+ P_4, estrogen and progesterone combined group.

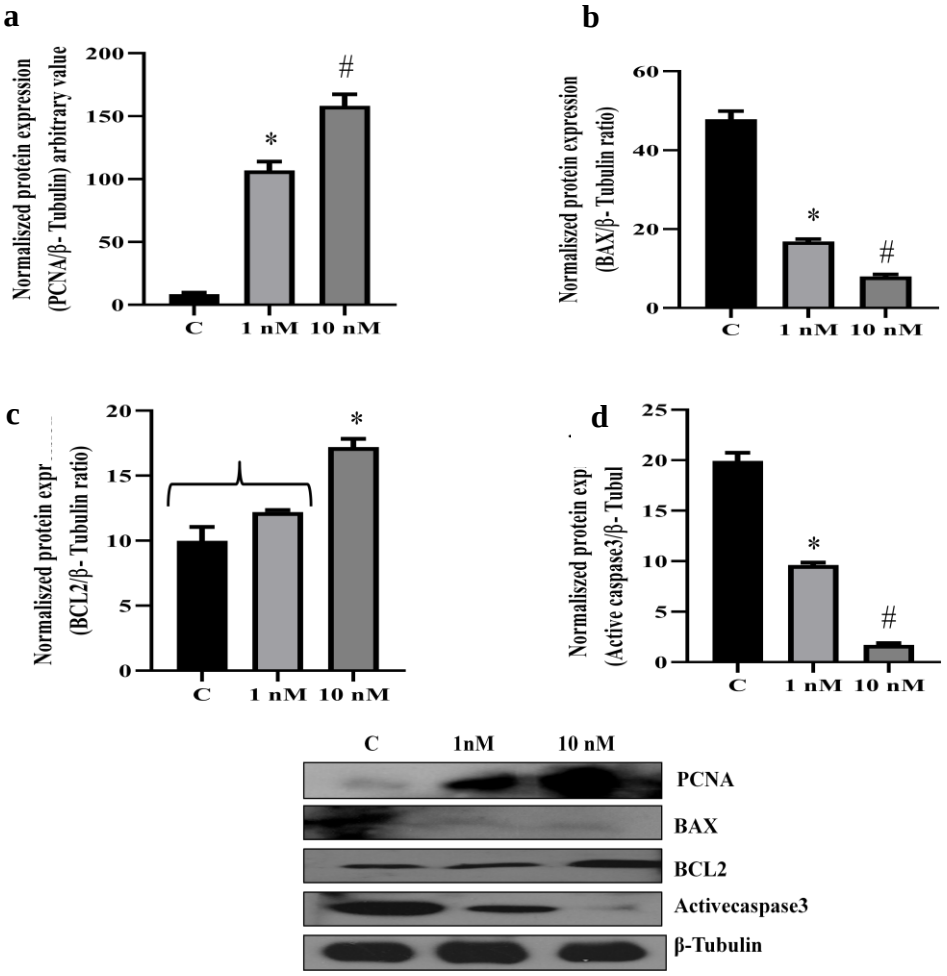


Figure-4.9- Visfatin inhibitor causes the increase in PCNA and BCL2 and decrease in BAX and Active caspase3 expression in the mice uterus. Histogram represents densitometric analysis of PCNA, BCL2, BAX and Active caspase3 expression. The data are represented as the means ± SEM. **(a)** PCNA expression showed a significant dose dependent increase (* $p<0.05$ compared with control), (#$p<0.05$ compared with 10 nM). **(b)** BCL2 showed increased expression (*$p<0.05$) in 10 nM dose as compared with control and 1 nM dose. **(c)** BAX showed a significant decrease in expression (*$p<0.05$ compared with control), (#$p<0.05$ compared with 1 nM). **(d) A**ctive caspase3 showed a significant decrease in expression (*$p<0.05$ compared with control), (#$p<0.05$ compared with 1 nM). C, control; 1 nM, FK866 1 nM; 10 nM, FK866 10 nM.

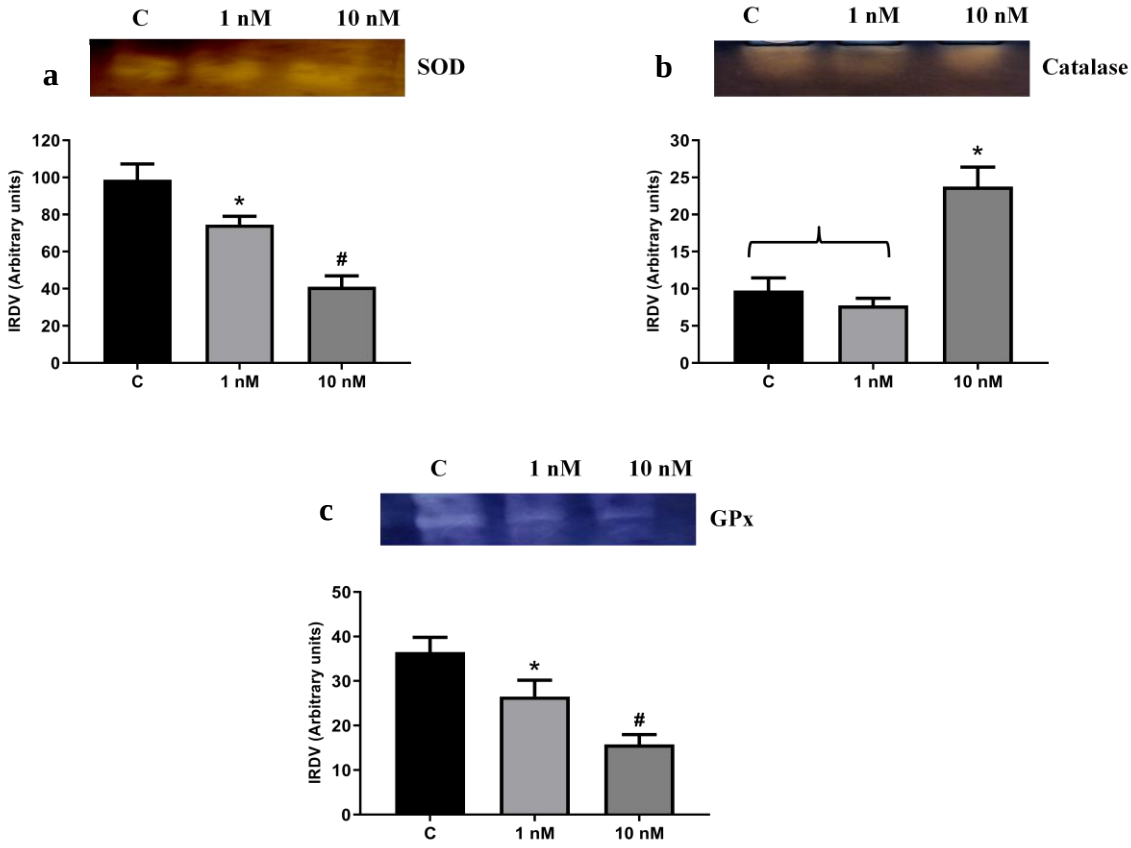


Figure-4.10- Visfatin inhibitor modulates antioxidant enzymes in the mice uterus. Representative images of gels are shown, in addition to histograms representing densitometric analysis of the bands. The data are represented as the means ± SEM. **(a)** SOD showed a significant decrease activity (*p<0.05 compared with control), (#p<0.05 compared with 1 nM). **(b)** Catalase showed significant increase (*p<0.05) in 10 nM dose as compared with control and 1 nM. **(c)** GPx showed a significant decrease activity (*p<0.05 compared with control), (#p<0.05 compared with 1 nM). C, control; 1 nM, FK866 1 nM; 10 nM, FK866 10 nM.

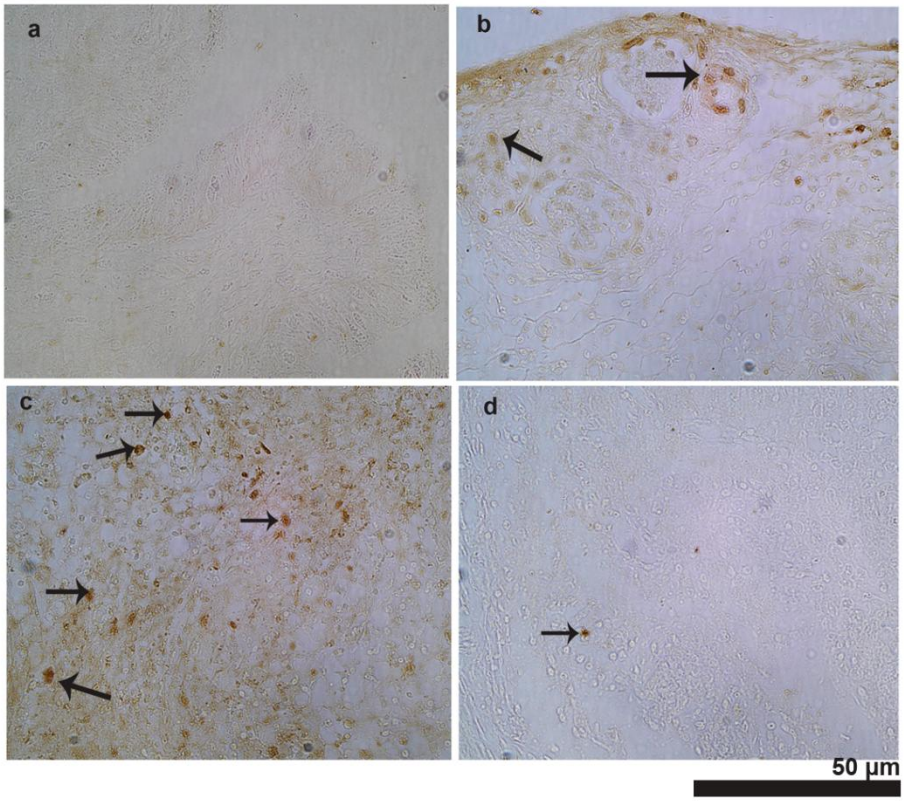


Figure-4.11- Inhibition of visfatin regulates the proliferation in the mice uterus as detected by BrdU incorporation. **(a)** Control section of uterus without BrdU incubation showed no BrdU positive cells. **(b)** Uterus incubated with BrdU showed moderate BrdU positive cells (arrow). **(c)** Uterus incubated with BrdU in presence of 1nM dose of FK866 showed many BrdU positive cells (arrow). **(d)** Uterus incubated with BrdU in presence of 10nM FK866 showed few BrdU positive cells.

Inhibition of visfatin by FK866 mitigates pathogenesis of cystic ovary in letrozole-induced hyperandrogenised mice

Introduction

Polycystic ovary syndrome (PCOS) is one of the common reproductive pathological conditions in female of reproductive age which affects about 5 -20% of woman **(Franks et al., 1996; Azziz et al., 2016)**. The PCOS has been characterized by hyperandrogenism, the presence of cystic follicles in the ovaries and anovulation **(Xu et al., 2011; Escobar-Morreale et al., 2005)**. Despite reproductive impairment, PCOS condition has further been linked to metabolic disorders, such as hyperinsulinaemia, obesity, insulin resistance, dyslipidaemia, and cardiovascular disease **(Chen et al., 2013)**. It has been suggested that insulin resistance is one of the causes of hyperandrogenism, and the elevated high insulin stimulates the androgen production from the ovary leading to the development of PCOS and metabolic disorders **(Bogovich et al., 1999; Nestler et al., 1998; Solorzano et al., 2010)**.

There are various studies, which showed that the rodent model such rat and mice can be used to mimic the PCOS like condition by elevating androgen levels **(Caldwell et al., 2014; Wang et al., 2018; Ward et al., 1978)**. Thus, the hyperandrogenised rodent model has been shown to exhibits many characteristics of PCOS such as hyperandrogenism, insulin resistance, cysts in the ovaries and metabolic disorder **(Torres et al., 2019; Moore et al., 2013)**. Letrozole is an aromatase inhibitor, which blocks the aromatization of estrogen from testosterone. The inhibition of aromatase by letrozole has been used to hyperandrogenised the rodent to exhibits PCOS like conditions **(Baravalle et al., 2006)**. Adiposity and secreted adipokines are also known to be mediators of PCOS-associated pathogenesis **(Spritzer et al., 2015)**. The secreted adipokines such as leptin, adiponectin, resistin, chemerin and visfatin have been shown to regulate the female reproduction in human and animals as well **(Pérez-Pérezet al., 2015; Chabrolle et al., 2009; Reverchon et al., 2012; Reverchon et al., 2013a; Annie**

et al., 2020a). Since PCOS is associated with metabolic syndrome and obesity, so the levels of several adipokines are deregulated in PCOS conditions **(Chen et al., 2013)**. Not only secreted rather, ovarian adipokine has also been shown to fluctuate in the PCOS **(Bongrani et al., 2019)**.

Visfatin is an adipokine, which is mainly secreted by the adipose tissue **(Fukuhara et al., 2005)** andit also expressed in the ovaries of mice, rat, turkey and human **Annie et al., 2020a; Nejabati et al., 2020; Diot et al., 2015; Reverchon et al., 2013a)**. Previously, it has been reported that circulating visfatin and its gene was found to be higher in the PCOS conditions **Tan et al., 2006; Kowalska et al., 2007; Ozkaya et al., 2012)**, however, some studies have shown that circulating visfatin did not vary in PCOS and control **(Guduchu et al., 2012; Lajunen et al., 2012)**. It has been suggested that visfatin is a pro-inflammatory adipokines, which may have possible role in the pathogenesis of PCOS **(Romacho et al., 2009; Adya et al., 2008)**.In a very recent study, it has been shown that ovarian visfatin gene expression was found to be higher in the rat model of PCOS (**Nejabati et al., 2020)**. To the best of our knowledge, there is dearth of evidences on the expression of ovarian visfatin in PCOS model. As the previous study showed elevated visfatin in the PCOS, thus, it is hypothesized that inhibition of visfatin by a specific inhibitor, FK866 might augment the pathogenesis of PCOS in mice models. Therefore, we investigated the effects of visfatin inhibitor, FK866 on the letrozole induced hyperandrogenised PCOS like mice model.

Materials and methods

Animal maintenance

All procedures were carried out according to the guidelines provided bythe Mizoram University Institutional Animal Ethical Committee (Protocol Approval number-MZUIAEC17-18-08), Mizoram University, Mizoram. 3 month old female Swiss albino mice were maintained with a 12 h light: 12 h dark cycle and 25±2°C. Food and water were provided *ad libitum*.

Induction of hyperandrogenism and cystic ovary

Ten mice were divided into two groups: Control group (n=5) was given vehicle only and PCOS group was administered letrozole at the concentration of 1mg/kg i.p **(Reddy et al., 2016)** dissolved in vehicle daily for 21 days. Body weight and ovary weight for each experimental group were recorded before sacrifice. Ovaries were dissected, and cleaned for any adhering fat tissues. The ovaries were fixed in Bouin's solution and also freezed at -20°C for immunoblots and immunohistochemistry. Serum was separated from blood and stored at -20°C for hormonal assays and estimation for metabolic parameters.

***In-vivo* inhibition of FK866 on PCOS mice**

Visfatin inhibition on hyperandrogenised mice was done by administering 1.5 mg/kg FK866 i.p for 2 weeks (n=5) **(Ohanna et al., 2018)**. Control received normal saline for the same period. Body weights and ovary weights were measured at the time of collection of organs.

***In-vitro* culture and treatment with visfatin inhibitor (FK866)**

Ovaries of PCOS mice were cultured 24 h in DMEM: Ham'F12 medium (1:1) containing penicillin (100 U/ml) and streptomycin (100 µg/ml) in an atmosphere of 5% CO_2 at 37°C. Visfatin inhibitor (FK866) and visfatin were in two doses at 1 nM (n=5) and 10 nM (n=5) and 1 ng/ml and 100 ng/ml respectively in triplicate culture tubes **(Okumura et al., 2012).**

Blood glucose, triglycerides and cholesterol estimation

Serum separated from blood was measured for metabolic parameters such as blood glucose, triglycerides and cholesterol levels from commercial kits as per manufacturer instruction (Glucose, Mediclone Biotech Pvt Ltd, Chennai, India, Triglycerides and Cholesterol, Coral Clinical Systems, Uttarakhand, India). Glucose level was measured after mixing samples with Glucose Reagent and incubated at room temperature for 20

min. Absorbance was read at 500 nm. For Triglycerides and cholesterol levels, working solution were first prepared mixing Enzyme reagent 2 into enzyme reagent 1. One ml of this working solution is mixed with 10 µl of samples, and incubated at 37°C for 5 min and read absorbance at 505 nm within 60 min using spectrophotometer.

Visfatin assay- circulating and ovarian visfatin levels

Both the serum and ovarian homogenate of Control and PCOS groups were measured using Mouse Visfatin ELISA kit (Cat # K02-0598; Los Angeles) for visfatin levels (ng/ml for serum, and ng/mg of protein for ovarian homogenate). 40 µl of samples and 10µl of conjugate were loaded in each well. HRP conjugate was added to the well except for blank well and incubated for 1h at 37°C. The wells were washed with wash buffer and 50 µl of substrate A and B were added to each well. After incubating in the dark for 10 min at 37°C, stop solution was added. Absorbance was read at 450 nm using ELISA reader.

Hormone Assays

Serum Testosterone (Cat # DKO002, DiaMetra, Italy) and Androstenedione (Cat # DKO008, DiaMetra, Italy) were estimated by using commercial ELISA kit as per manufacturer's instruction. Serum Progesterone and estrogen were measured by ELISA kit (Estradiol Cat # DKO003, DiaMetra, Italy; Progesterone Cat # RH-351, DSI, Saronno, Italy) following manufacturer's instructions.Absorbance levels were read at 450 nm using a Microplate Reader (Erba Lisa Scan EM, Trans Asia Biomedical Ltd, Mumbai, Maharashtra, India).

Immunohistochemistry

Fixed ovaries were processed for paraffinised tissue block and sectioned with microtome as described previously **(Annie et al., 2020a)**. Tissue sections were embedded in coated poly-L-lysine slides and incubated at slide warming table for 1-2 h at 37°C. Slides were

then allowed for deparaffinisation with xylene and rehydration with 100%, 90% and 70% alcohol for 2 changes at 10 min each. It was then treated with 3% H_2O_2 in methanol to block endogenous peroxidase, and incubated in goat-blocking serum for 30 min at room temperature, followed by visfatin antibody at 1:100 dilution with PBS (rabbit polyclonal IgG, Cat # V9139, Sigma-Aldrich, MO, USA) for 4°C overnight. Primary wash was performed for 2 changes and then incubated with goat anti-rabbit IgG antibody conjugated with hrp for 3h at room temperature. A brown immunostain developed using 3,3- diaminobezidinetetrahydrochloride and hydrogen peroxidase, and counterstained with hematoxylin. It was then dehydrated, cleared in xylene and mounted for observation under Nikon binocular microscope (Model E200, Nikon, Tokyo, Japan).

Histology

Ovaries collected from *in vivo* experiment were fixed in Bouin's fluid for 24h at room temperature. Fixed tissues were dehydrated and processed for embedding in paraffin block. Tissue sections were cut at 7µm in thin ribbon sections with microtome, and spreaded in slides. Paraffin tissue sections were deparaffinised with xylene and sequentially rehydrated with alcohol. It was then stained with Hematoxylin and Eosin for 5 min and undergoes sequential dehydration, cleared in xylene and mounted with DPX for observation under microscope.

Western blot analysis

Ovaries collected from PCOS induction and after FK866 inhibition were processed for western blot, where protein concentrations were determined with Bradford method **(Bradford et al., 1976)**. Samples were then loaded and resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred to polyvinylidene fluoride membrane (Millipore India Pvt. Ltd., India). Membrane blocking was done with skimmed milk solution for 30 min at room temperature. Primary antibodies against androgen receptor (1:1000, Cat # E-AB-33615, Elabscience, Houston, Texas, USA), PCNA(1:1000, rabbit polyclonal IgG, Cat # SC-

7907, Santa Cruz Biotechnology Inc., Dallas, USA), GCNA(1:2000, mouse polyclonal antibody, Cat # 10D9G11, DSHB, Department of Biology, Iowa), GLUT-8 (1:1000, Cat # E-AB-19914 Elabscience, Houston, Texas, USA), BCL2 (1:500; rabbit polyclonal antibody, Cat # EPP10828, Elabscience, Houston, Texas, USA), BAX (1:1000; rabbit polyclonal antibody, Cat # SC-6236; Santa Cruz Biotechnology Inc, Dallas, USA), Active caspase3 (1:1000, mouse polyclonal antibody, Cat # STJ97448, St. John's Lab, London, UK) were done for overnight at 4°C and hrp-conjugated secondary antibodies were used at 1:4000 dilution (goat anti-mouse, Merck Specialties Pvt. Ltd, Mumbai, India; goat anti-rabbit conjugated with HRP, Cat # PI-1000, Vector Laboratories, Burlingame, CA, USA. Bands were visualized with chemiluminescence HRP substrate and normalized with β-Tubulin for loading variations. ImageJ software (**imagej.nih.gov/**) was used for quantification.

Statistical analysis

All analyses were done using student's t-test. Results were represented as the mean ± standard error of the mean and the level of significance was considered as $p<0.05$. Statistical analysis was performed using GraphPad Prism 7 software

Results

Experiment-1. Induction of hyperandrogenism and cystic ovary

Changes in the body weight, ovary weight and metabolic parameters in PCOS

The treatment of letrozole to the female mice significantly increased ($p<0.05$) the serum glucose, triglycerides and cholesterol levels, compared to the control mice. The ovarian tissue glucose concentration showed a significant ($p<0.05$) decreased in letrozole treated mice compared to the control. Furthermore, letrozole treatment did not show marked increase in the body weight, however, the ovarian weight in letrozole treated mice increased ($p<0.05$) compared to the control mice ovary.

Changes in the circulating steroid hormones progesterone, androstenedione, testosterone and estrogen (P_4, A_4, T and E_2) and visfatin in hyperandrogenised mice

The circulating steroid hormones progesterone was found to be significantly ($p<0.05$) low in the letrozole treated mice compared to the control. On the other hand, the levels of androstenedione and testosterone were significantly higher ($p<0.05$) in letrozole treated mice compared to the control, however estrogen levels did not show change in both the groups.

The circulating visfatin was significantly ($p<0.05$) elevated in the hyperandrogenised mice compared to the control.

Expression, concentration, and localization of ovarian visfatin in hyperandrogenised mice ovary

The expression of ovarian visfatin by western blot analysis showed a significant ($p<0.05$) increase in the letrozole treated mice compared to the control. The quantification of intraovarian visfatin also showed significant ($p<0.05$) increase concentration in the hyperandrogenised ovary compared to the control.

Immunohistochemical study also showed an intense staining of visfatin in the ovaries of letrozole treated mice. The intense immunostaining was found in the thecal cells, stromal cells and granulosa cells of letrozole treated ovary. The intense staining of visfatin was around the cystic follicles in the letrozole treated mice. The control ovary showed moderate staining in the different cell types

Changes in the expression of androgen receptor (AR), GLUT8 and proliferating markers, PCNA and GCNA

The expression of AR showed a significant increase in the letrozole ovary compared to the control, however, the expression of PCNA did not show any change in both the groups. The expression was significantly declined ($p<0.05$) in the letrozole treated ovary

compared to the control. Expression of GLUT8 also declined significantly in the ovary of letrozole treated mice compared to the control.

Changes in the expression of anti- and pro- apoptotic proteins (BCL2, BAX and Active caspase3)

The expression of anti-apoptotic protein, BCl2 was significantly ($p<0.05$) down-regulated and pro-apoptotic protein Active caspase3 was significantly ($p<0.05$) up-regulated in the ovary of letrozole treated mice compared to the control. However, expression of BAX showed an increase in the ovary of letrozole treated mice, moreover it was not statistically significant.

Experiment-2. Effect of visfatin inhibitions on hyperandrogenised mice

Effect of visfatin inhibition on the changes in the body weight, ovary weight and metabolic parameters

The treatment of visfatin inhibitor, FK866 to the letrozole induced PCOS mice, showed a significant ($p<0.05$) decline in the blood glucose, triglycerides and cholesterol levels compared to the PCOS control mice. The ovarian tissue concentration also showed a significant ($p<0.05$) increase in the PCOS ovary of FK866 treated mice compared to the PCOS control. Moreover, the body did not significant change in the PCOS mice treated with FK866 and PCOS control. The ovarian weight showed a decline in the PCOS mice treated with FK866 compared to the control.

Effect of visfatin inhibition on the changes in the circulating steroid hormones (P_4, A_4, T and E_2)

The circulating steroid hormones, progesterone, androstenedione and testosterone were significantly ($p<0.05$) decreased in the PCOS mice treated with FK866 compared to the PCOS control. However, the circulating estrogen levels did not change in both the groups.

Effect of visfatin inhibition on the changes in ovarian histology

The treatment of visfatin inhibitor (FK866) to the PCOS mice showed the improvement in the ovarian histology, which was reflected by a few or no cystic follicle in the ovary of PCSO mice treated with FK866, whereas in the PCOS control mice cystic follicle was observed. Furthermore, the treatment of FK866 to the PCOS mice showed the presence of corpus luteum, which was not observed in the ovary of PCOS control mice.

Effect of visfatin inhibition on the changes in the expression of androgen receptor (AR), GLUT8 and proliferating markers, PCNA and GCNA

The expression of AR did not show the significant change in the ovary of PCOS control mice and PCOS mice treated with FK866. The expression of GLUT8 and PCNA was significantly up-regulated in the ovary of PCOS mice treated with FK8866 compared to the PCOS control mice. Whereas expression of GCNA showed a significant decline in the ovary of PCOS mice treated with FK866 compared to the PCOS control mice.

Effect of visfatin inhibition on the changes in the expression of anti and pro-apoptotic proteins (BCL2, BAX and Active caspase3)

The expression of anti-apoptotic protein, BCL2 was significantly up-regulated in the ovary of PCOS mice treated with FK866 compared to the PCOS control mice. Whereas expression of BAX and Active caspase3 showed a significant decline in the ovary of PCOS mice treated with FK866 compared to the PCOS control mice.

Experiment-3. Effect of visfatin inhibitor on steroid hormone secretion by cystic ovary *in vitro*

To confirm the role of visfatin on the ovarian steroid biosynthesis, the cystic ovaries were cultured in presence of visfatin inhibitor. The *in vitro* inhibition of visfatin by two doses (1 nM and 10 nM) of FK866 significantly suppressed the progesterone and testosterone secretion; however, there was not dose dependent effect. The secretion of estrogen and androstenedione did not show the significant change among the groups.

Discussion

The aim of the present study was to explore the potential of visfatin inhibitor, FK866 in the management of ovarian pathogenesis in letrozole induced hyperandrogenised PCOS like mice model. The findings of present study are outcome of three different experiments. In the first experiment, a PCOS mice model was made with letrozole treatment and different parameters were investigated. Our results showed that letrozole treatment induced the cystic ovary with increased levels of androstenedione and testosterone. It is well known that hyperandrogenism and cystic ovary are characteristics of PCOS in laboratory animal and human as well **(Azziz et al., 2016; Cao et al., 2017)**. Letrozole is an aromatase inhibitor, which has been shown to induce the polycystic ovary in the rats and mice **(Manneras et al., 2007; Baravalle et al., 2006; Torres et al., 2019)**. The treatment of letrozole also showed an elevation in the ovary weight and metabolic parameters such as blood glucose, triglycerides and cholesterol levels. It has also been shown that PCOS is a manifestation of metabolic syndrome with hyperlipidemia and hyperglycemia **(Moran et al., 2003)**, our results are in agreement with these studies and showed the successful induction of PCOS like condition in mice. Results of first experiment further showed that PCOS mice had low levels of circulating progesterone without change in the circulating estrogens. Previous study also showed that letrozole induced PCOS rat had low progesterone levels without change in the circulating estrogen **(Nejabati et al., 2020)**. It has also been documented that adipokine levels are deregulated during PCOS condition in human and rodents **(Chen et al., 2013)**. Our protein expression and quantification analysis showed an elevation in the ovarian visfatin in hyperandrogenised mice. Immunohistochemical study also supported the increase ovarian visfatin in hyperandrogenised mice and visfatin showed intense staining in the stromal cells. The circulating visfatin also showed an elevation in the hyperandrogenised mice. The expression of visfatin has been shown in the ovarian cells of mice, bovine, chicken and human **(Song et al., 2014; Reverchon et al., 2016; Reverchon et al., 2013b)**. There are contradictory reports on the levels and expression of visfatin in the PCOS subjects. One study has shown that follicular visfatin was

elevated in the PCOS ovary **(Souma et al., 2014)**, however on the other hand, it has also been shown that visfatin levels was found to be lower in PCOS than control **(Bongrani et al., 2019)**. Visfatin being an adipokine has been suggested to play important role in reproduction alongwith other adipokines **(Dupont et al., 2015)**. Recently, it has been shown that rat PCOS model has increased expression of visfatin gene compared to the control **(Nejabati et al., 2020)**. Our result is in agreement with above report that PCOS ovary has high levels of ovarian visfatin. The elevated intra-ovarian visfatin is an outcome of PCOS associated hyperandrogenism or elevated intra-ovarian visfatin has manifested the PCOS condition like hyperandrogenism, is still a matter of investigation. It has been shown that visfatin could stimulate androgen production in the testis **(Hameed et al., 2012; Gurusubramanian and Roy, 2014)**; however, role of visfatin androgen production in female has not been investigated. Moreover, it may be suggested that visfatin may also promotes androgen biosynthesis in mice model and PCOS associated hyperandrogenism could also lead to hypervisfatinemia. Further study would be interesting to unravel the cross between androgen and visfatin in ovary.

Our results showed that expression of GCNA and PCNA decreases in the PCOS ovary along with increase in the expression of Active caspase3 and androgen receptor. The PCOS associated hypervisfatinemia may further be linked to the increase apoptosis and decrease proliferation. Previous study has also showed that ovarian proliferation decreases and apoptosis increases in the cystic ovary **(Singh et al., 2018)**.The increase of visfatin showed a linked to the pathophysiology of PCOS, this prompted us to explore the effect of visfatin inhibition the PCOS ovary. The results of second experiments showed that the inhibition of visfatin by FK866 decreases the blood glucose, triglycerides and cholesterol along with increase in the ovarian glucose contents and reduction in ovary weight. Furthermore, the histological analysis showed the presence of corpus luteum and reduction of cystic follicle. The circulating androgens, testosterone and androstenedione were decreased by the visfatin inhibition. However, progesterone showed a decline, whereas, estrogen did not change after visfatin inhibition. As the improvement in the ovarian histology was shown by the formation of corpus luteum, the

progesterone level could have been expected more in this group. Previous studies have shown that progesterone level decline in PCOS **(Bas et al., 2011; Zheng et al., 2017)** and any treatment which improve the PCOS condition, progesterone level is increased **(Nejabati et al., 2020)**. The study by Jin et al. **(2019)** showed that without affecting the progesterone secretion, PCOS condition was ameliorated in the mice. The decrease progesterone in the first experiment could be due to increase in ovarian visfatin and previous study has also showed that visfatin inhibit progesterone secretion **(Reverchon et al., 2016; Estienne et al., 1989)**. However, inhibition of visfatin by FK866 further decreased the progesterone secretion, this requires further investigation.

Visfatin has also been shown to regulates the proliferation and apoptosis **(Gholinejad et al., 2017)** and our results showed that visfatin inhibition led to increase in the BCL2 and decrease in the apoptotic markers, BAX and Active casapse3. Furthermore, visfatin inhibition has suppressed the expression of GCNA and increased in the expression of PCNA in the PCOS. It may be suggested that PCOS associated visfatin suppress the proliferation and promotes apoptosis, and inhibition of visfatin by FK866 in the cystic ovary suppress apoptosis and promotes proliferation, and this led to amelioration in the PCOS. Our recent study has also shown that ovary visfatin increase proliferation and suppresses apoptosis in the pre-pubertal mice **(Annie et al., 2020b)**, whereas, in the proestrus ovary, visfatin increases both proliferation and apoptosis **(Annie et al., 2020a)**. These observations further suggest the tissue and stage dependent role visfatin in relation to proliferation and apoptosis.

It has been shown that adipokines are the potential link between reproduction and energy metabolism and including pathophysiology, such as polycystic ovary syndrome, which has been characterized as insulin resistance and hyperandrogenism along with elevated levels of visfatin in PCOS **(Bongrani et al., 2019; Diamanti-Kandarakis et al., 2012; Mannerås-Holm et al., 2011; Kowalska et al., 2007)**. It has further been suggested that elevated visfatin may be involved in the pathogenesis of PCOS **(Kowalska et al., 2007)**. Our results showed that visfatin inhibition in PCOS condition has increased the ovarian

GLUT8 expression and ovarian glucose content. These results suggest that inhibition of visfatin might have improved the insulin resistance and visfatin plays a crucial role in glucose homeostasis in PCOS. Since the PCOS condition is closely linked to imbalances in the circulating hormones levels, therefore, we further investigated the effect of visfatin inhibition on steroid hormone secretion *in vitro* in the third experiment. Our results showed that inhibition of visfatin in PCOS ovary suppresses the secretion of testosterone and progesterone without affecting estrogen and androstenedione. Thus, it seems that inhibition of visfatin could have suppressed the activity of 3β-HSD and 17βHSD in the ovarian steroidogenic pathway. However, previous study showed that in hen ovaries, visfatin inhibits progesterone production in granulosa cells through 3β-HSD and FK866 treatment increases the expression of 3β-HSD **(Dupont, 2015)**.Thus, this explains the decrease progesterone in the first experiment, however, in PCOS condition this mechanism seems to be opposite. Since we have not measured the expression of these markers, so, further study would be required to support this observation.

In conclusion, our study showed the PCOS associated hypervisfatinemia could be an additional factor in the PCOS pathogenesis. Furthermore, our study for the first time showed that *in vivo* inhibition of visfatin in PCOS condition suppresses the androgen biosynthesis, improves glucose metabolism, which might lead to improvement in the PCOS associated pathogenesis. Thus, it further may be suggested that targeting the visfatin could be an also additional therapeutic adjunct in the management of PCOS along with other factors.

Summary

Polycystic ovary syndrome (PCOS) is one of the common reproductive disorders in the female of reproductive age, which is characterized by hyperandrogenism, insulin resistance, cystic ovary and infertility. The levels of pro-inflammatoryadipokines such as visfatin have been shown to be elevated in the PCOS conditions in human and animal as well. Therefore, it is hypothesized that inhibition of PCOS associated hypervisfatinemia, might ameliorates the pathogenesis of PCOS. In the present study, letrozole induced hyperandrogenised PCOS like mice model have been used to unravel the effects of visfatin inhibition. The results of this study showed that visfatin inhibition in PCOS has suppressed the secretion of androgens, androstenedione and testosterone levels. Furthermore, the histological study also showed that visfatin inhibition suppressed the cyst formation and promotes corpus luteum formation. The visfatin inhibition has also suppressed the apoptosis and increases the expression of anti-apoptotic protein BCL2 along with increase in the proliferation (GCNA expression elevated). The visfatin inhibition has increased the intraovarian glucose content, which was supported by the increase in the ovarian GLUT8 expression. The *in vitro* study has also supported the *in vivo* findings where visfatin inhibition by FK866 suppressed the androgen production from PCOS ovary. In conclusion, this is the first report, which showed that inhibition of visfatin by FK866 in hyperandrogenised mice ameliorates the pathogenesis of PCOS. Thus, it may be suggested that visfatin inhibition could have a therapeutic potential in the management of PCOS along with other intervention.

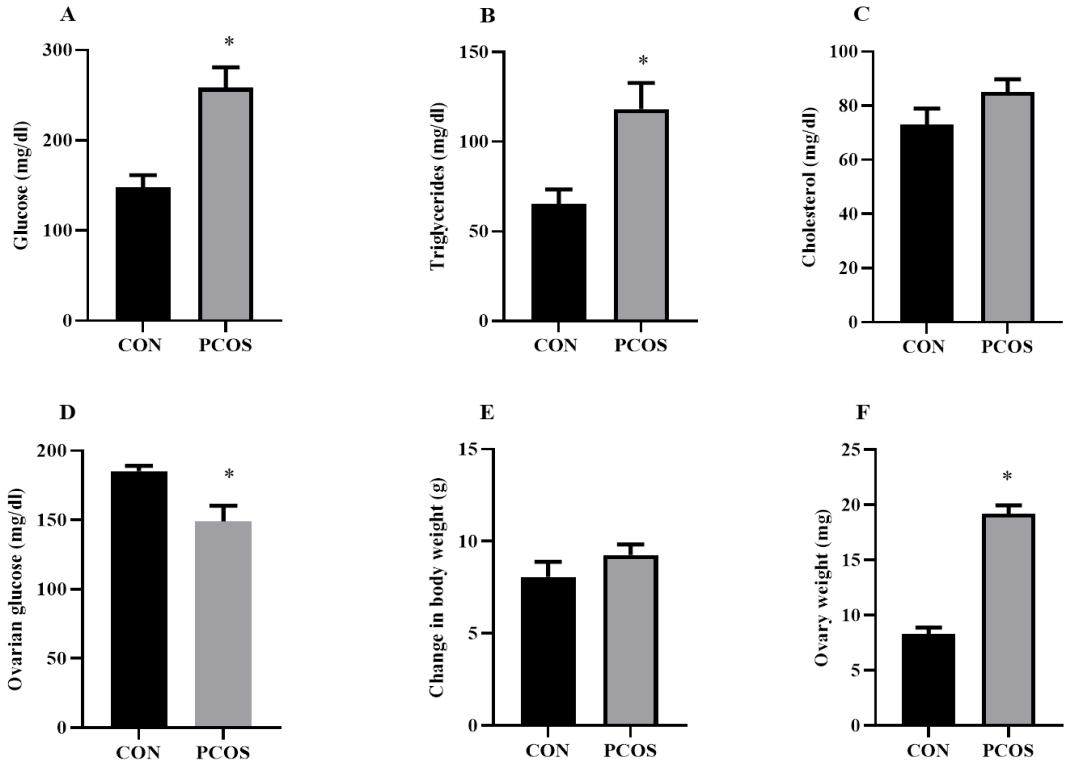


Figure-5.1- Effect of letrozole on the metabolic parameters, body weight and ovary weight. Letrozole treatment significantly (*, $p<0.05$) increase the blood glucose **(A)**, triglycerides **(B)** and cholesterol**(C),** ovary weight **(F)** and compared to the control. The ovarian glucose **(D)** significantly (*, $p<0.05$)decrease in the letrozole treated mice compared to the control. Body weight **(E)** did not show significant change.

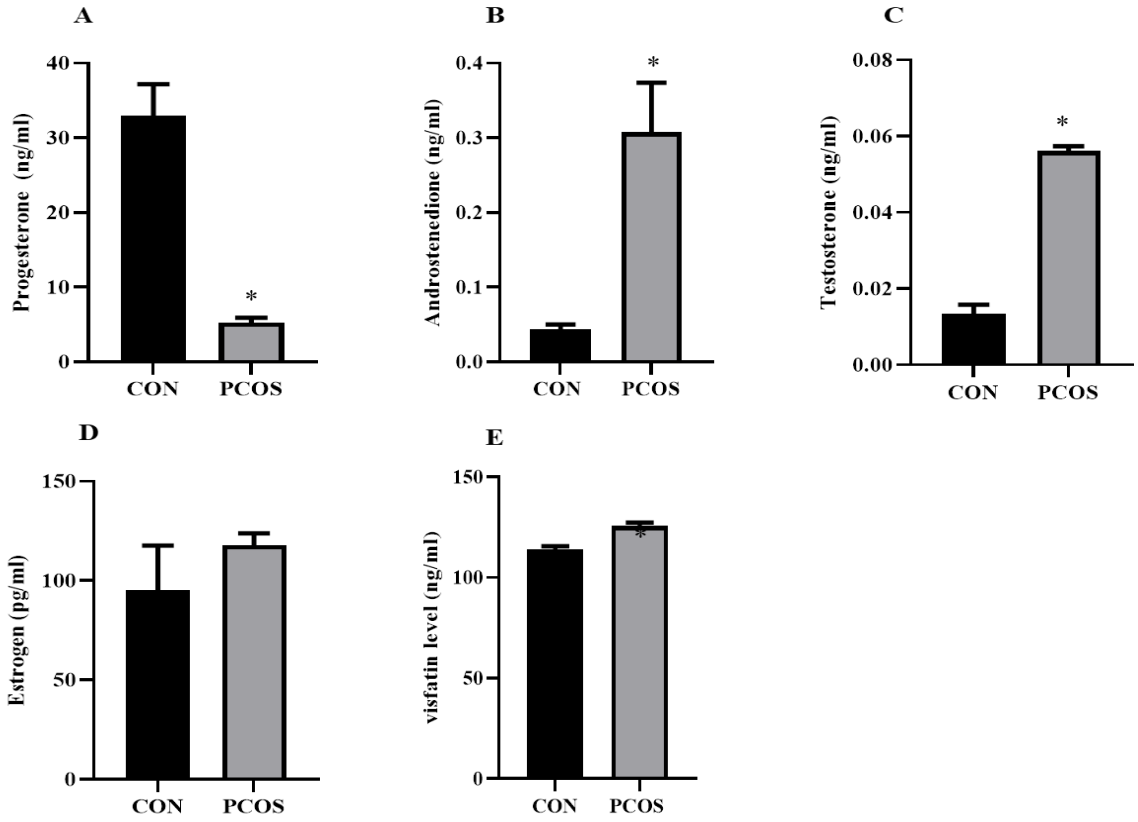


Figure-5.2- Effect of letrozole on the circulating steroid hormone and visfatin levels. Progesterone levels **(A)** significantly (*, $p<0.05$) decreased and androstenedione **(B)**, testosterone **(C)** significantly (*, $p<0.05$) increased in the letrozole treated mice. The circulating estrogen levels **(D)** did not vary. The circulating visfatin levels **(E)** showed a significant (*, $p<0.05$) increase in the letrozole induced PCOS mice.

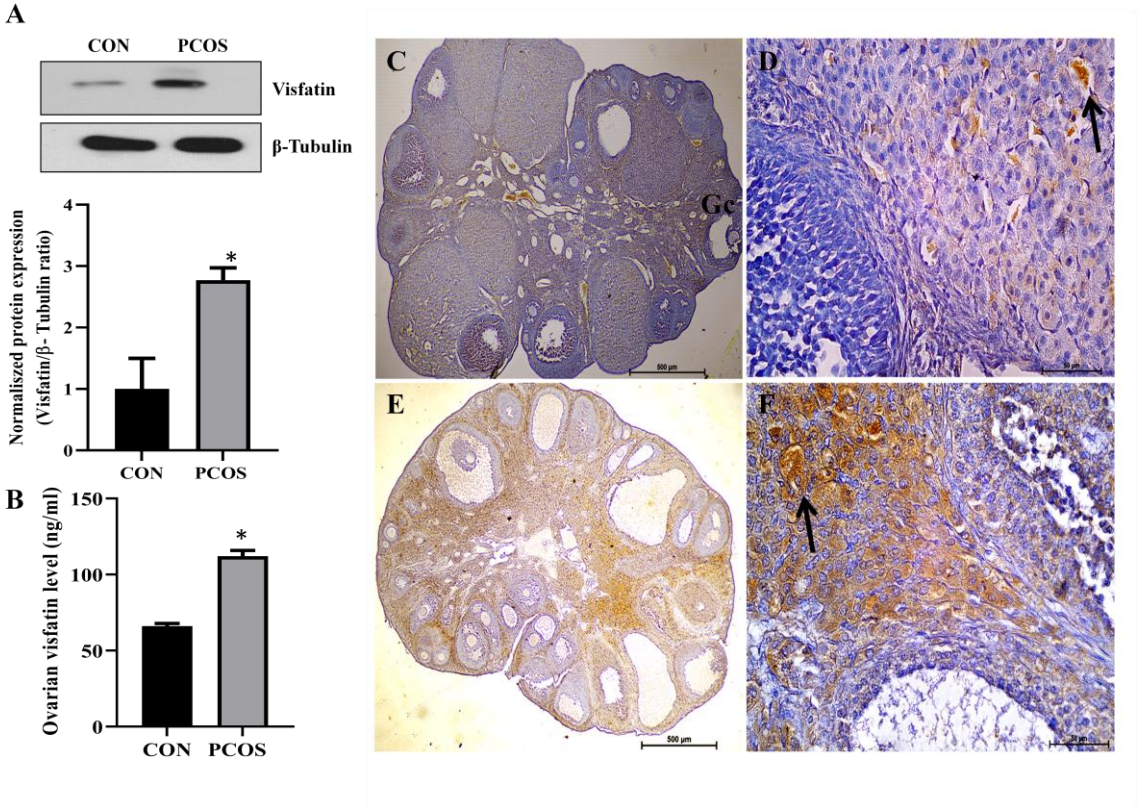


Figure-5.3- Expression, concentration and localization of ovarian visfatin. Expression of ovarian visfatin**(A)** by western blot analysis and concentration of ovarian visfatin**(B)** significantly (*, $p<0.05$) elevated in the letrozole induced hyperandrogenised mice compared to control. Immunohistochemical localization of visfatin showed intense immunostaining in the thecal and stromal cell of hyperandrogenised ovary **(E, F)**. The control ovary exhibited a moderate immunostaining**(C,D)**.

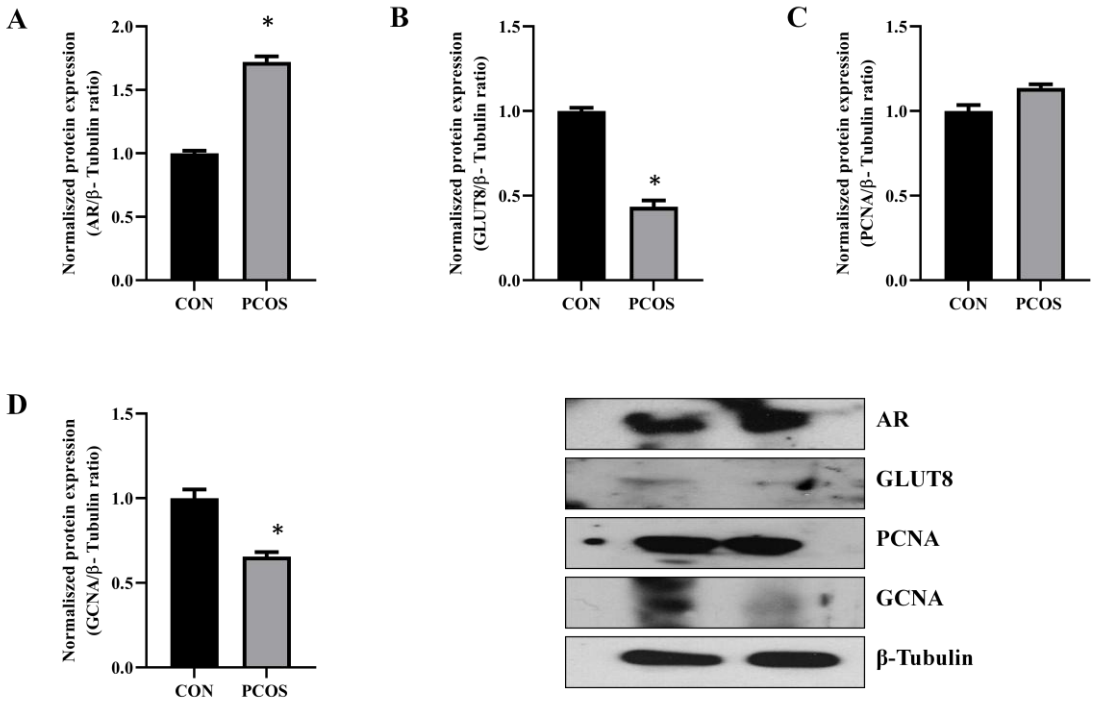


Fig-5.4- Expression of androgen receptor (AR), GLUT8 and proliferating markers, PCNA and GCNA. The expression of AR **(A)** was significantly (*, $p<0.05$) up regulated and expression of GLUT8 **(B)** and GCNA **(D)** were significantly (*, $p<0.05$) down regulated in the hyperandrogenised ovary. Expression of PCNA **(C)** did not show significant change.

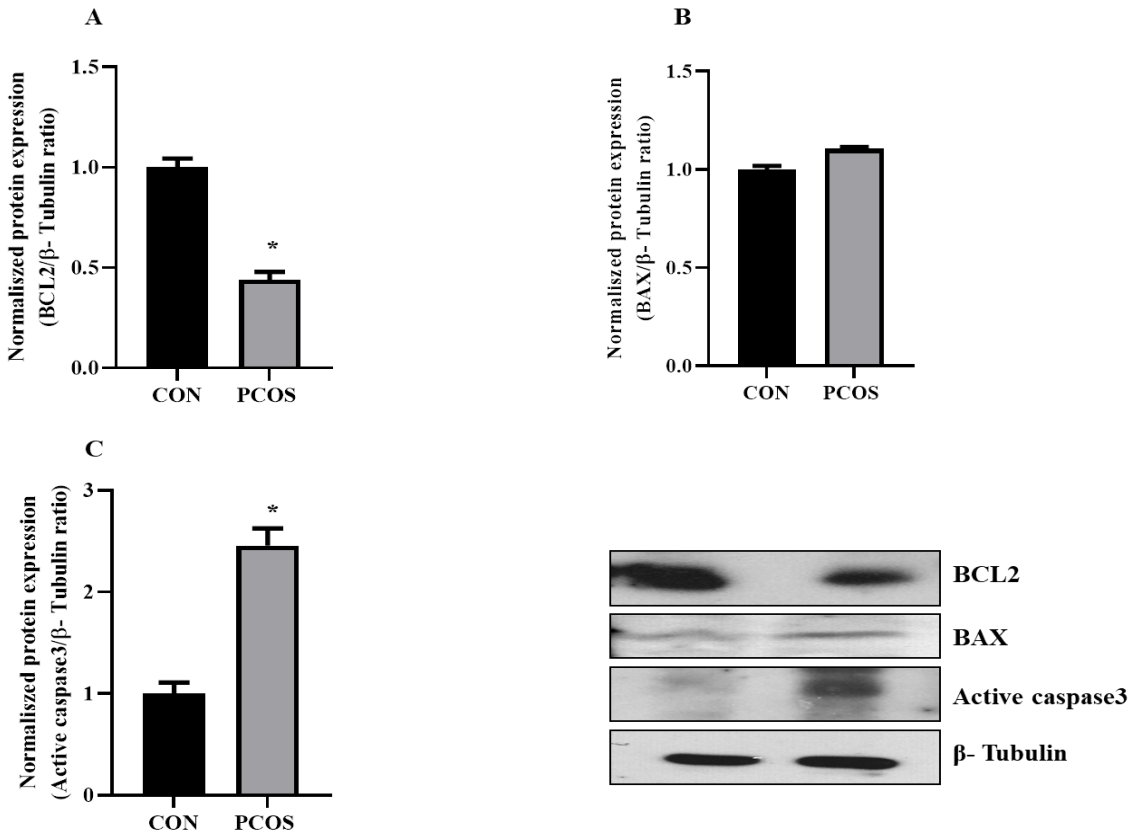


Figure-5.5- Expression of anti (BCL2) and pro apoptotic proteins (BAX and Active caspase3). The expression of BCL2 **(A)** was significantly (*, $p<0.05$) down-regulated and expression of Active caspase3 **(C)** was significantly (*, $p<0.05$) up-regulated in the hyperandrogenised ovary. BAX expression **(B)** did not change significantly.

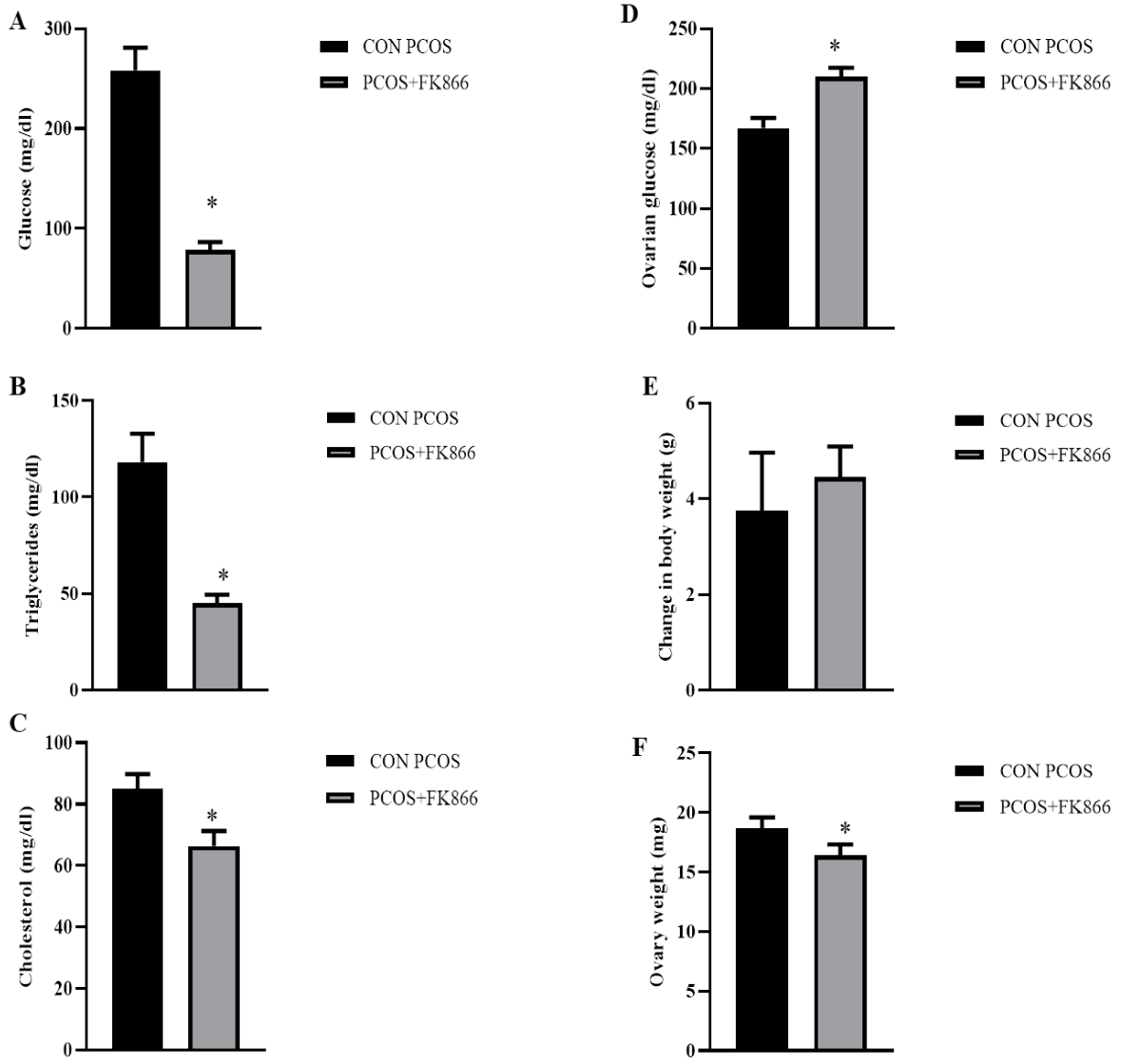


Figure-5.6- Effect of visfatin inhibition (by FK866) on the changes in the body weight, ovary weight and metabolic parameters in the hyperandrogenised mice. The FK866 treatment significantly (*, $p<0.05$) decreased **(A)** blood glucose, triglycerides **(B)**, cholesterol **(C)** and ovary weight **(F)** compared to control. The ovarian glucose **(D)** concentration was significantly (*, $p<0.05$) increased after visfatin inhibition in hyperandrogenised mice ovary. The body did not show significant change **(E)**.

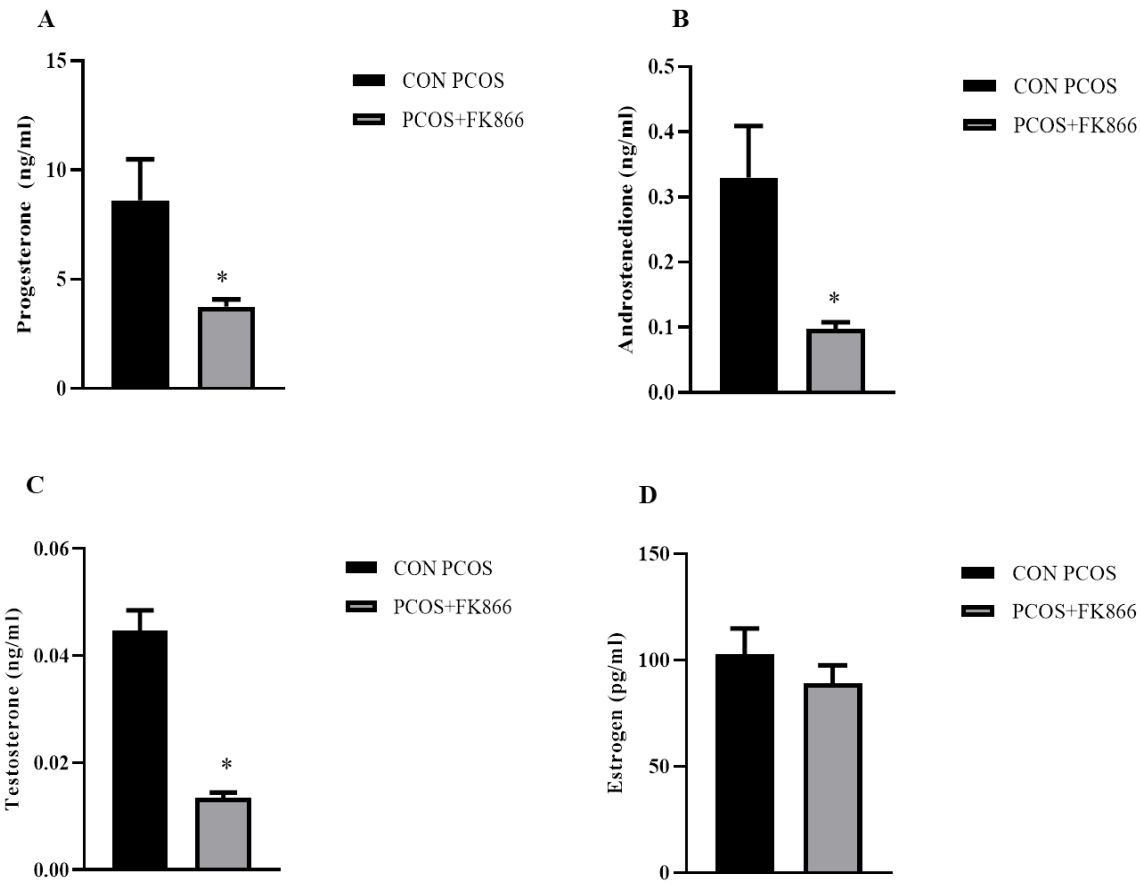


Figure-5.7- Effect of visfatin inhibition (by FK866) on the changes in circulating steroid hormones. The visfatin inhibition by FK866 to hyperandrogenised mice showed a significant (*, $p<0.05$) decrease in the progesterone **(A)**, androstenedione **(B)** and testosterone **(C)**. The estrogen levels **(D)** did not change.

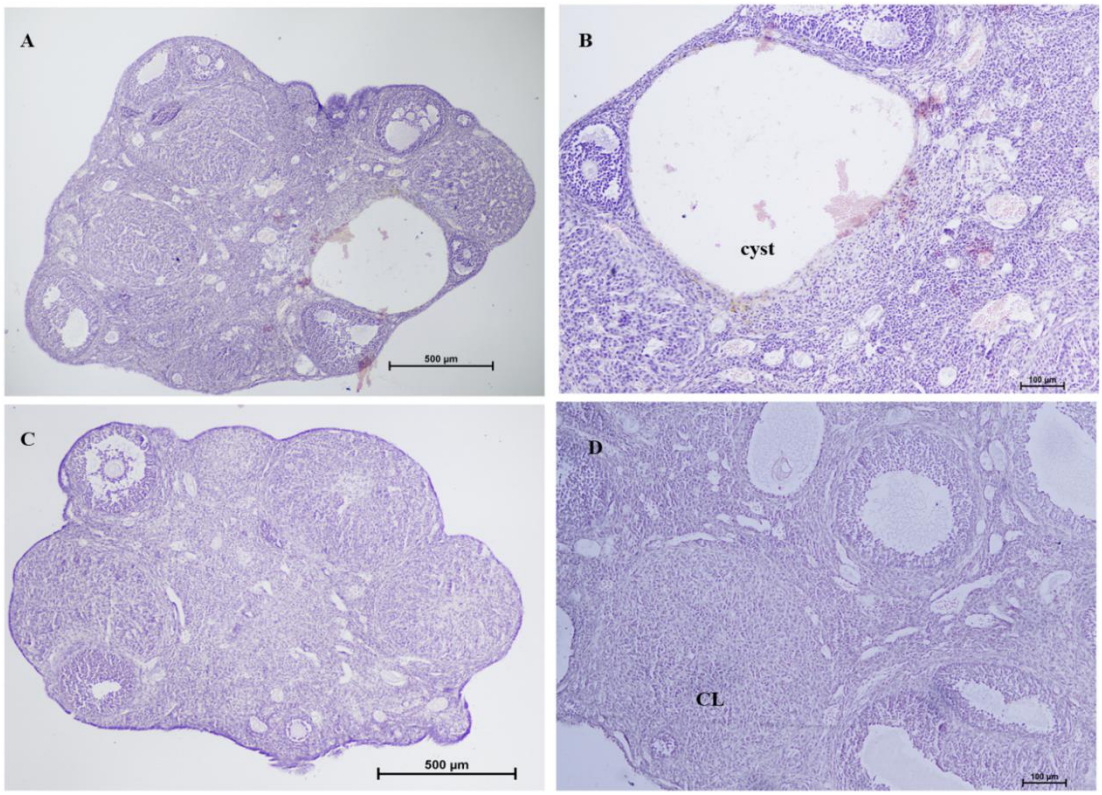


Figure-5.8- Effect of visfatin inhibition (by FK866) on the ovarian histology. The hyperandrogenised mice treated with visfatin inhibitor showed amelioration in cyst formation and formation of corpus luteum**(C, D)**. The hyperandrogenised mice (PCOS control) showed the cyst and no corpus luteum was observed **(A, B)**.

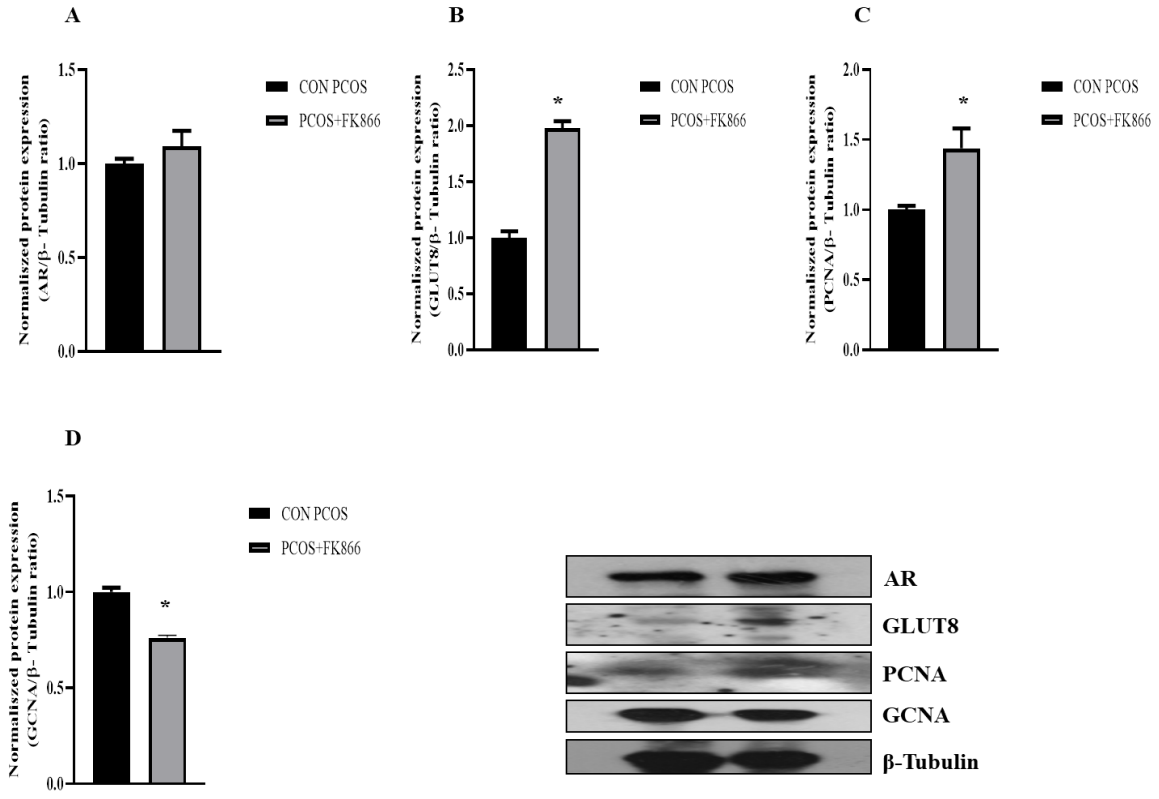


Figure-5.9- Effect of visfatin inhibition (by FK866) on the expression of androgen receptor (AR), GLUT8 and proliferating markers, PCNA and GCNA**.** The expression of AR **(A)** showed no change, however, the expression of GLUT8 **(B)** and PCNA **(C)** were (*, $p<0.05$) significantly up-regulated in hyperandrogenised mice treated with FK866 compared to the control mice. The expression GCNA **(D)** was (*, $p<0.05$) down regulated.

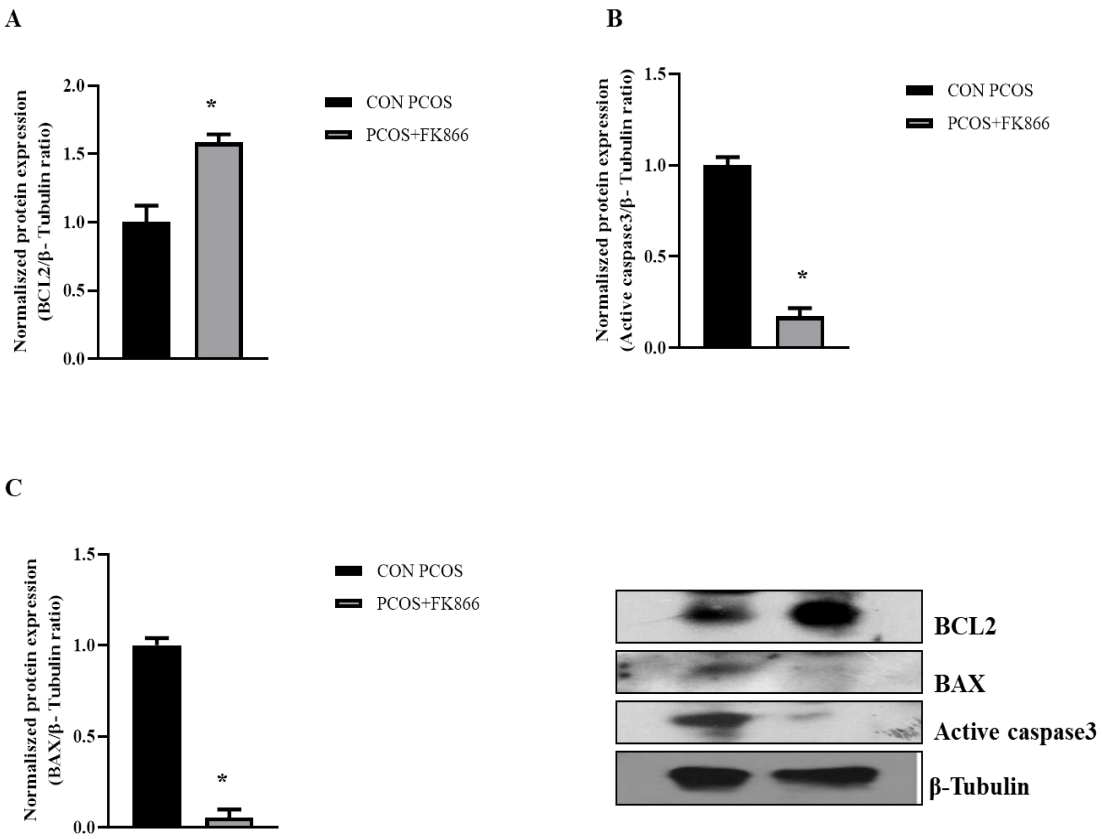


Figure-5.10- Effect of visfatin inhibition (by FK866) on the expression of BCL2, BAX and Active caspase3**.** The expression of BCL2 **(A)** was up regulated (*, $p<0.05$) and expression of BAX **(B)** and Active caspas3 **(C)** were significantly (*, $p<0.05$) down regulated in hyperandrogenised mice treated with FK866 compared to the control mice.

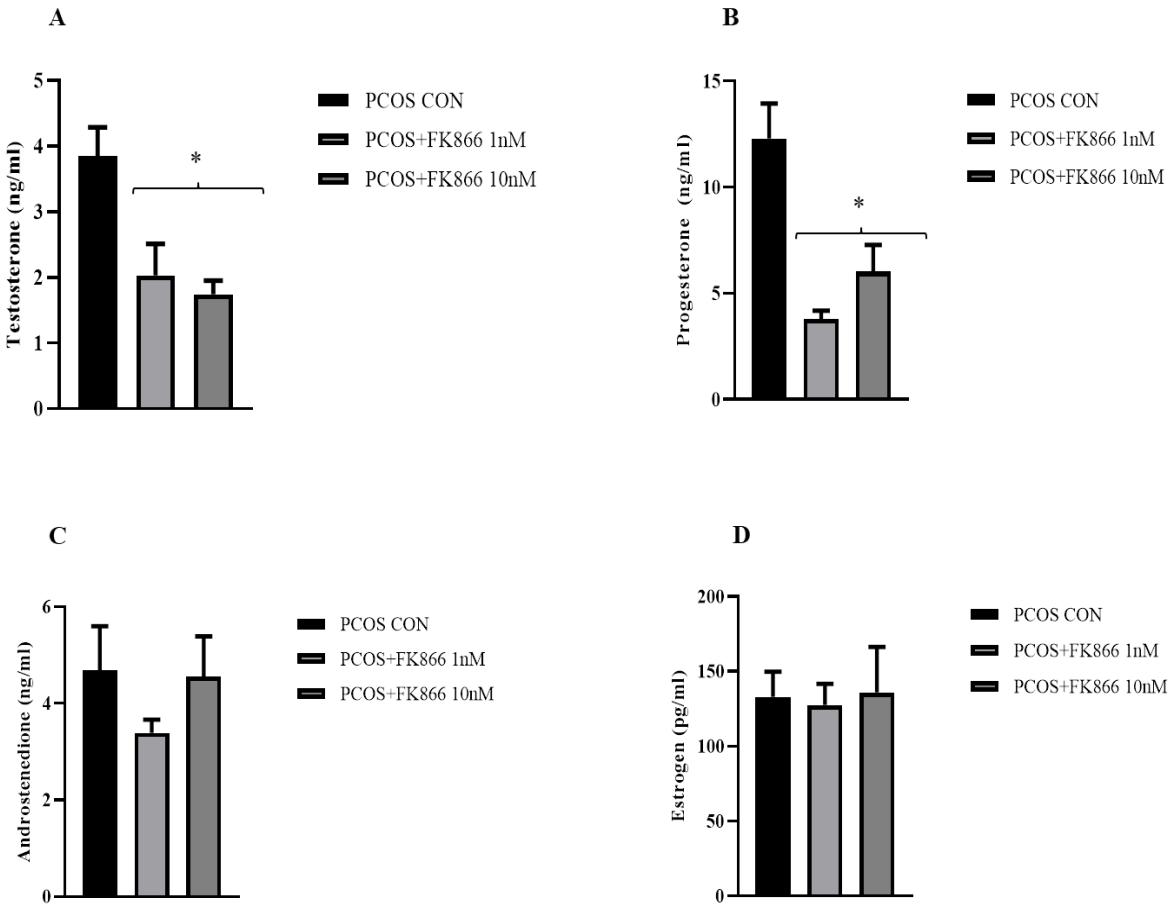


Figure-5.11- The effect of visfatin inhibition by FK866 on the steroid hormone secretion by hyperandrogenised ovary *in vitro*. The visfatin inhibition by two doses (1nM and 10 nM) (*, $p<0.05$) suppressed the testosterone **(A)** and progesterone secretion by hyperandrogenised ovary. The inhibition of visfatin showed no effect on the androstenedione **(C)** and estrogen **(D)** secretion

Consolidated Summary

1. The infantile period from PND14 to PND21, showed a change in concentration of ovarian visfatin protein, with an increasing trend from PND14-PND21; however, circulating visfatin levels did not show any change from PND14-PND21.
2. To unravel the possible role of visfatin in the infantile ovary, we have inhibited the endogenous visfatin from PND14-PND21 by FK866 treatment.
3. Our results demonstrated that body weight and ovary showed an increase in the visfatin inhibited group.
4. The western blot analysis of two proliferation markers, PCNA and GCNA, was up-regulated after visfatin inhibition by FK866. This result was further supported by the immunolocalisation of PCNA in the ovary, which also showed an increase in the PCNA localisation in the thecal and granulosa cells of the infantile ovary.
5. To further clarify the role of visfatin in the proliferation, BrdU labeling study was done and BrdU also showed more incorporation in the ovaries of FK866 treated mice. These results suggest that visfatin might be inhibiting the ovarian cell proliferation in the infantile ovary.
6. The visfatin inhibition by FK866 showed down-regulation of the survival protein, BCL2 and up-regulation of the apoptotic proteins, Active caspase3 and BAX in the ovary.
7. These results suggest that ovarian visfatin in infantile mice may inhibit the apoptosis and increased BCL2 expression further suggest that visfatin may also be involved in the selection of follicle pools by inhibiting the apoptosis.
8. The circulating estrogen and progesterone levels did not show any change after visfatin inhibition.
9. Our results also showed that visfatin inhibition increases the expression of ER-β and increase ER-β could be responsible for increase ovarian proliferation.

10. Estrogen secretion was significantly ($p<0.05$) increased after visfatin inhibition by pre-pubertal mice.
11. The expression of CYP11A1, 17β-HSD and CYP19A1 significantly ($p<0.05$) increased by FK866 treatment in a dose-dependent manner compared to the control.
12. Visfatin inhibition also decreased the expression of PCNA and GCNA in the pre-pubertal mice ovary. These results further supported by BrdU labelling where, the number of BrdU positive cells, especially thecal cell proliferation decreased after visfatin inhibition.
13. Our results showed that inhibition of visfatin increases the proliferation alongwith ER-β in the infantile ovary whereas visfatin inhibition downregulated proliferation in the prepubertal ovary. It has been shown that expression of ER-β in the ovary increases with age and important in preovulatory maturation. The differential expression of PCNA and GCNA by visfatin inhibition in the infantile and pre-pubertal mice maybe due to the differential regulation of ER-β mediated expression by visfatin which could be due to different physiological conditions in these two developmental stages of the ovary.
14. The inhibition of visfatin also down-regulated the BCL2 expression and increased the Active caspase3 expression in pre-pubertal mice ovary.
15. These findings suggest that the presence of visfatin in pre-pubertal mice ovary directly modulates ovarian steroidogenesis and folliculogenesis; therefore, visfatin could be one of the regulators of ovarian functions in pre-pubertal animals.
16. In the adult mice ovary, visfatin expression and localisation changes during the estrous cycle.
17. Visfatin expression showed a peak with significant abundance in the proestrus compared to other stages of estrous.
18. The immunolocalisation study showed the strongest staining in the corpus luteum of proestrus and intense in the diestrus ovaries.

19. However, faint immunostaining was observed in the metestrus and diestrus ovary.
20. The correlation study of visfatin showed a significant positive correlation ($r= 0.6194$, $p<0.05$) with BCl2 and active casapse3 ($r= 0.7733$, $p<0.05$) in the ovary during estrous cycle.
21. Since the proestrus ovary showed the increase expression of visfatin, therefore, to confirm the exact role of ovarian visfatin on the proliferation and apoptosis, ovarian visfatin was inhibited by *in vivo* injection of FK866 in the proestrus stage for 6 h.
22. *In vivo* inhibition of visfatin by FK866 in the proestrus ovary down-regulated Active caspase3 and PCNA expression, and up-regulated the BCL2 expression.
23. The quantification of BrdU and PCNA stained area in control and FK866 treated proestrus ovary showed that FK866 treatment significantly decreased the BrdU staining and PCNA staining compared to the control group.
24. These results suggest the role of visfatin in the proliferation and apoptosis of the follicles in the proestrus and specific localisation of visfatin in the corpus luteum also indicate its role in corpus luteum function, which may be in progesterone biosynthesis and regression of old corpus luteum.
25. However, further study is required to support these findings. In conclusion, visfatin may also be regulating follicular growth during the estrous cycle by regulating proliferation and apoptosis.
26. Like ovary visfatin expression also changes in the uterus during estrous cycle.
27. The immunostaining of visfatin was observed in different compartments of the uterus: endometrium, myometrium, glandular epithelium and luminal epithelium of uterus.
28. Endometrium and myometrium of proestrus uterus showed intense immunostaining of visfatin whereas uterus of estrus showed faint immunostaining of visfatin in glandular epithelium and luminal epithelium of estrus than in proestrus.

29. At diestrus, visfatin showed faint immunostaining in the endometrium, myometrium, glandular and luminal epithelium.
30. Circulating estrogen levels showed a remarkably higher concentration in the proestrus stage and metestrus during the estrous cycle. Serum progesterone levels showed a peak in the diestrus stage.
31. As the circulating hormones change, it seems that uterine visfatin expression may also be regulated by ovarian steroids.
32. This led us to examine the effect of ovarian steroids, estrogen and progesterone on visfatin expression in ovariectomised mice.
33. Western blot analysis showed estrogen-alone treatment to the ovariectomised mice significantly ($p<0.05$) increased visfatin expression in the uterus compared with control mice.
34. Progesterone alone treatment significantly ($p<0.05$) decreased visfatin expression in the uterus of ovariectomised mice compared with control.
35. However, treatment with both estrogen and progesterone significantly ($p<0.05$) increased visfatin expression as compared to control, estrogen-alone and progesterone-alone treatment. These results are supported by immunohistochemical study.
36. To access the role of visfatin on uterine proliferation and apoptosis, the uterine explants culture was performed in the presence of visfatin inhibitor, where proliferation marker (PCNA), apoptotic (BAX and Active caspase3) and anti-apoptotic (BCL2) markers were measured by Western blot analysis.
37. The treatment of visfatin inhibitor showed a significant ($p<0.05$) dose-dependent increase in PCNA and BCL2 expression in the uterus than in control.
38. The two doses of visfatin inhibitors, 1nM and 10 nM decreased the expression of BAX and Active caspase3 in a dose-dependent manner.
39. Effect of visfatin inhibitor, FK866 on BrdU incorporation in uterus showed marked variation. Treatment with 1 nM dose of FK866 stimulated proliferation, which was clearly shown by BrdU positive cells. Whereas treatment with 10 nM

dose of FK866 suppressed proliferation, as evidenced by few BrdU positive cells in the uterus.

40. In conclusion, the present study showed that the expression of uterine visfatin is regulated by estrogen and progesterone. This study provides the first evidence that steroid-dependent expression of visfatin might be involved in the regulation of proliferation and apoptosis in the uterus of mice during reproductive cycle and would be important for uterine changes takes place during cycle for successful reproduction.
41. The levels of pro-inflammatory adipokines such as visfatin have been shown to be elevated in the PCOS conditions in human and animal as well.
42. Therefore, it was hypothesised that inhibition of PCOS associated hypervisfatinemia, might ameliorate the pathogenesis of PCOS.
43. In the letrozole induced PCOS mice, visfatin expression and localisation showed a significant increase compared to the control.
44. Our study showed that the PCOS associated hypervisfatinemia could be an additional factor in the PCOS pathogenesis.
45. Furthermore, our study for the first time showed that *in vivo* inhibition of visfatin in PCOS condition suppresses the androgen biosynthesis, improves glucose metabolism, which might lead to an improvement in the PCOS associated pathogenesis.
46. Thus, it may be suggested that targeting the visfatin could be an additional therapeutic adjunct in the management of PCOS and other factors.
47. The overall role of visfatin can be concluded that visfatin is an important adipokine, which may regulate the ovarian function in tissue and stage dependant manner with respect to proliferation, apoptosis and steroidogenesis.

Conclusion

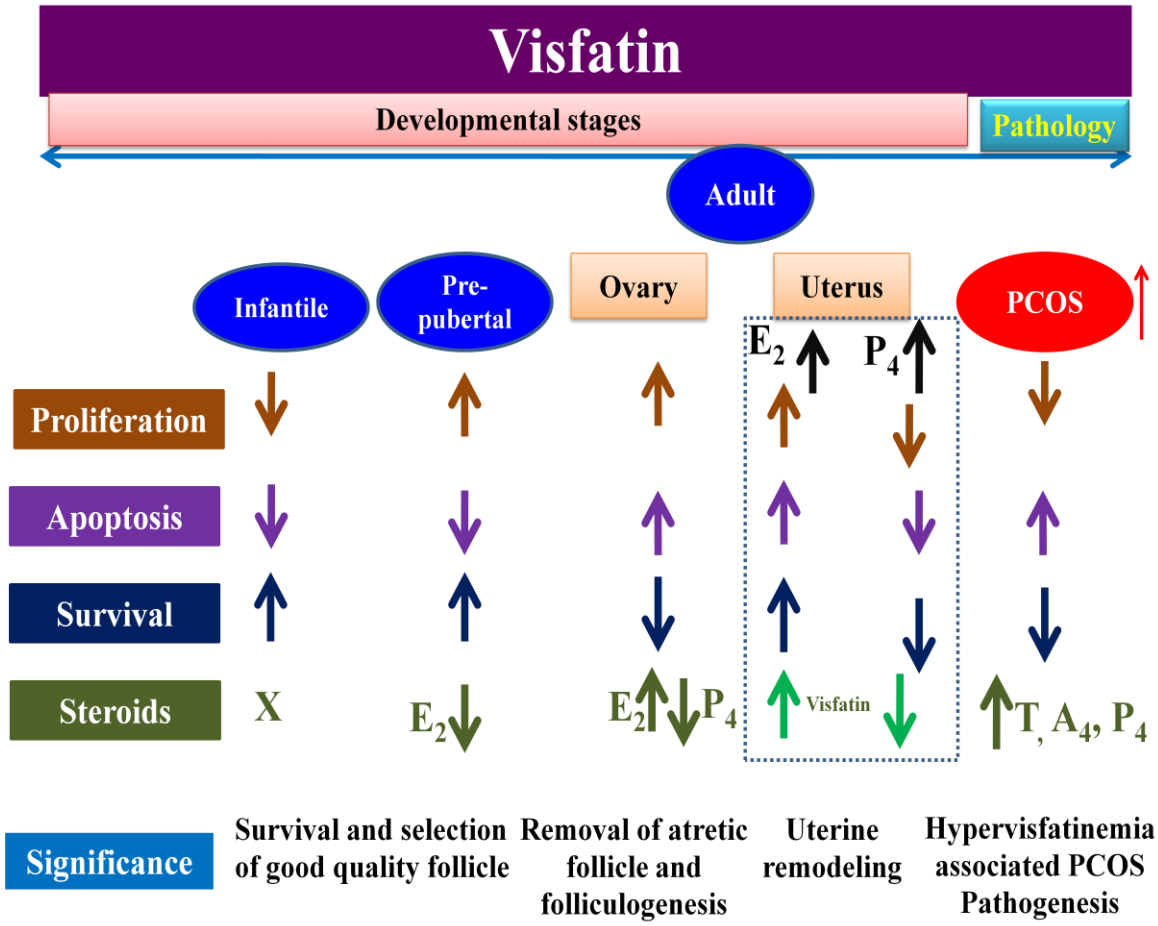
The present dissertation for the first time showed the localization and expression of visfatin protein in ovary and uterus of mice, along with its possible role in the ovarian and uterine function. The ovarian role has been described in relation to proliferation, survival, apoptosis, and steroidogenesis in different stages. However, the uterine role visfatin has been shown in relation to proliferation and apoptosis, and its steroid dependent expression in the uterus.

The results showed the ovarian visfatin expression is developmentally regulated and in the adult its expression is also dependent on different stages of the estrous cycle. In infantile period, visfatin may not have pronounced effect on the ovarian steroidogenesis, moreover, visfatin suppress the ovarian proliferation and apoptosis, and increased the survival factor, BCL2, and it may be suggested that visfatin mediated BCL2 expression could also be a mechanism to preserve the good quality follicle in early postnatal period. On the other hand, in the pre-pubertal mice ovary, visfatin suppress estrogen biosynthesis, increase the proliferation, survival and decrease the apoptosis. It may be suggested that in pre-pubertal mice ovary also, visfatin could be involved in maintaining the good quality follicle. The role of visfatin again changes during the estrous cycle, in particular, during proestrus, visfatin increases proliferation and apoptosis, decreased the survival factor, BCL2. Based on these findings, it may be suggested that visfatin may also be regulating follicular growth during the estrous cycle by regulating proliferation and apoptosis, increase apoptosis, may suggests its role in removal of atretic follicle.

In the uterus, visfatin expression also depends on the stages of estrous cycle, and uterine visfatin expression is regulated by estrogen and progesterone. Estrogen up-regulates, whereas progesterone down-regulates the visfatin expression in the uterus. Thus, it may be suggested that visfatin protein is steroid-dependent in mouse uterus, which is also involved in the regulation of proliferation and apoptosis in the uterus during the reproductive cycle.

Furthermore, in the pathophysiological condition such as PCOS, ovarian and circulating visfatin are up-regulated, which may be one of the reasons for PCOS associated pathogenesis. The results showed that inhibition of visfatin in PCOS mice mitigates the PCOS associated pathogenesis. Thus, it may be suggested that visfatin inhibition could have a therapeutic potential in the management of PCOS along with other intervention.

In conclusion, like other adipokine, visfatin is also an important adipokine which may regulate reproductive functions in a tissue- and stage-dependent manner. The overall role of visfatin has been summarized below.



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Abbreviations

17β-HSD	17β-Hyroxysteroid dehydrogenase
3β-HSD	3β-Hyroxysteroid dehydrogenase
ANOVA	One-way analysis of Variance
AR	Androgen receptor
BAX	Bcl-2-associated X protein
BCL2	B-cell lymphoma 2
DAB	Diaminobezidine
DMEM	Dulbecco Modified Eagle's Medium
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
g	Gram
GCNA	Germ cell nuclear antigen
h	Hour
i.p	Intraperitoneal
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimol

ng	Nanogram
nM	Nanomol
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene fluoride
rpm	revolutions per minute
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
StAR	Steroidogenic acute regulatory protein
TBS	Tris-buffered saline
U	Unit

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HSSLC	2010	MBSE	Science	66.6	First
B.Sc	2013	MZU	Zoology	76.13	First
M.Sc	2015	NEHU	Zoology	69.68	First
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PARTICULARS OF THE CANDIDATE

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DEPARTMENT : ZOOLOGY

TITLE OF THE THESIS : Role of visfatin in reproductive organs of female mice.

DATE OF ADMISSION : 22.08.2016

APPROVAL OF RESEARCH PROPOSAL:

DRC : 04. 04.2017

B.O.S : 19.05.2017

SCHOOL BOARD : 26.05.2017

MZU REGISTRATION NO. : 1607300

Ph. D. REGISTRATION NO. & DATE : MZU/Ph.D/1010 of 26.05.2017

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PUBLICATIONS

1. **L. Annie, G. Gurusubramanian, V.K. Roy,** Estrogen and progesterone dependent expression of visfatin/NAMPT regulates proliferation and apoptosis in mice uterus during estrous cycle, J. Steroid Biochem. Mol. Biol. 185 (2019) 225–236. IF-4.292

2. **L. Annie, G. Gurusubramanian, V.K. Roy,** Changes in the localization of ovarian visfatin protein and its possible role during estrous cycle of mice, Acta Histochem. 122(8) (2020) 151630. IF-2.479

3. **L. Annie, G. Gurusubramanian, V.K. Roy,** Inhibition of visfatin/NAMPT affects ovarian proliferation, apoptosis, and steroidogenesis in pre-pubertal mice ovary, J. Steroid Biochem. Mol. Biol. 204 (2020) 105763. IF-4.292

4. **L. Annie, G. Gurusubramanian, V.K. Roy,** Visfatin protein may be responsible for suppression of proliferation and apoptosis in the infantile mice ovary. Cytokine 140 (2021) 155422. IF-3.861

5. **L. Annie, G. Gurusubramanian, V.K. Roy,** Inhibition of visfatin by FK866 mitigates pathogenesis of cystic ovary in letrozole-induced hyperandrogenised mice. Life Sci. (2021). 276, p. 119409. IF-5.037

CONFERENCES/ SEMINAR/WORKSHOP ATTENDED

International

1. Global Conference on Reproductive health with Focus on Occupational, Environmental and lifestyle Factors - 28th Annual meeting of the Indian Society for the Study of Reproduction and Fertility (ISSRF)
2. Global Conference on Reproductive health with Focus on Occupational, Environmental and lifestyle Factors - 29th Annual meeting of the Indian Society for the Study of Reproduction and Fertility (ISSRF)
3. International Conference on Biodiversity, Environment and Human Health : Innovations and Emerging Trends (BEHIET)

National:

1. Workshop on Science communication by Welcome Trust (DBT Alliance, New Delhi)
2. National Conference on Recent Trends in Zoological Research in North-east India (Dept. of Zoology , NEHU)
3. National Symposium on Avian Biology & Comparative Physiology (Dept. of Zoology, MZU)
4. National seminar on animal handling, maintenance and care (Advanced level state Biotech-Hub Facility, MZU)

State:

1. Outreached Program on Human Health and Biological Timing (Dept. of Zoology, MZU)
2. Workshop on Research methodology and monitoring of Research Projects (Project community, PUC)
3. Mizoram Science Congress (MAS, MISTIC, STAM & BIOCONE)

Mizoram University

Mizoram University Animal Ethical Committee

Prof. G. Gurusubramanian
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Prof. G. Gurusubramanian
Prof. G.S. Solanki
Dr. H.T. Lalremsanga
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Dr. L. Ralte
Dr. V. K. Roy
Dr. Sangnghina
Mr. Peter Vanlalhriata
Dr. A K. Trivedi

Dated 13.06.2017

To Dr. Vikas Kumar Roy
Department of Zoology
Mizoram University
Aizawl - 796004

Sub: Proposal No.**08** entitled: **Expression, regulation and role of Visfatin in the ovary of female mice** on small animal experimentation.

Dear Sir,

It is ti inform you that the Institutional Animal Ethics Committee of Mizoram University has approved your proposal No. **MZUIAEC17-18-08** entitled **"Expression, regulation and role of Visfatin in the ovary of female mice"** in its meeting held on 13.06.2017." in its meeting held on 13.06.2017. You are permitted to undertake experiments on small anima species indicated in your proposal.

Prof. Gurusubramanian
(Chairman)

Chairman
IAEC
Mizoram University

Dr. Amit K. Trivedi
(Member Secretary)

Member Secretary
IAEC
Mizoram University