EXPRESSION AND ROLE OF APELIN AND ITS RECEPTOR IN REPRODUCTIVE ORGANS OF FEMALE MICE

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EXPRESSION AND ROLE OF APELIN AND ITS RECEPTOR IN REPRODUCTIVE ORGANS OF FEMALE MICE

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Submitted

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

CERTIFICATE

I certify that the thesis entitled "**Expression and role of apelin and its receptor in reproductive organs of female mice**" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **Anima Borgohain** is a record of research work carried out during the period of 2020-2024 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or institution of higher learning.

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September, 2023

I, **Anima Borgohain**, 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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(ANIMA BORGOHAIN)

CONTENTS

List of figures

List of Tables

General Introduction

Reproduction is regulated by endocrine glands through secreting various autocrine, paracrine hormones and intracrine factors (Unniappan, 2009). In mammals, reproduction is mainly driven and regulated by the hypothalamic-pituitary-gonadal/ovary (HPG/HPO) axis, which comprised of the hypothalamus, pituitary and ovary or testis that coordinates the endocrine/autocrine/paracrine/intracrine actions (Wang et al., 2021). The hypothalamus, a central region of brain secretes gonadotropin releasing hormone (GnRH) from GnRH neuron that regulates the anterior pituitary and stimulates the release of gonadotropin hormones, follicle stimulating hormones (FSH) and luteinizing hormone (LH) from gonadotropes. Further, the gonadotropins regulate the downstream secretion of gonadal hormones, synthesized by the gonads that ultimately regulate the mammalian reproductive function (Oyola and Handa, 2017). FSH directly stimulates the ovarian granulosa cells to regulate estradiol release, while LH helps the follicle development and ovulation. In human, the HPG axis shows three activation periods are during foetal life, after birth or mini-puberty and during puberty (Guimiot et al., 2012).

Reproduction is a high energy demanding process and subsequently influenced by multiple factors secreted by endocrine glands. In addition to the hormones produced from the HPG axis, a number of internal and external substances or factors of the gonads are able to modulate the gonadal development (Su et al., 2009; Clelland and Peng, 2009). Adipose tissue is the largest endocrine organ which regulates glucose homeostasis, steroid production, the immune system, hematopoesis, and reproductive function (Bohler et al., 2010). Adipose tissue is majorly made up of adipocytes and also contains preadipocytes (fat stem cells), macrophages, fibroblasts, blood cells, and endothelial cells (Feve, 2005; Ali et al., 2013). Previously, Adipose tissue was considered as an energy-preserving tissue in the form of fat (triacylglycerols). Furthermore, adipose tissue releases numerous substances that act in a paracrine, autocrine, endocrine, and vasocrine way to maintain metabolic homeostasis (Ronti et al., 2006). It has been commonly known that excess or insufficient white adipose tissue

influences sexual maturation, fertility, and puberty in various species (Mircea et al., 2007). Adipose tissue is now known to be a promising and active endocrine gland rather as an energy reservoir which secretes cytokines influencing the fertility through endocrine function (Kershaw and Flier 2004; Fischer-Posovszky et al., 2004). These substances include immunomodulatory proteins, collectively known as 'adipokines' or 'adipocytokines' (Esler et al., 2006). It has been shown that adipokine plays very important role in mammalian reproduction like pigs, cows, goats, rodents and human (Budak et al., 2006; Rak et al., 2017; Cheng et al., 2016; Dupont et al. 2015).

Adipokine or adipocytokines

Adipocytes, derived adipokines, such as adiponectin, leptin, resistin, visfatin, chemerin and apelin, as well as inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) are basically cytokines, a product of adipocytes and adipose stromal tissue, collectively known as adipocytokines (Al-Mansoori et al., 2022; Yamauchi et al., 2014). Many studies have shown that adipokines are involved in adipocyte differentiation, energy metabolism, insulin resistance, inflammation, immunity, cancer, and angiogenesis (Kelesidis et al., 2006; Badman and Flier 2007; Fantuzzi, 2013; Dupont et al., 2012). The new roles of adipokines have lastly emerged and gaining much attention to developed a better understanding of the immunoregulatory functions of adipokines in the field of fertility and reproduction (Budak et al., 2006). These adipokines such as leptin, adiponectin, apelin, chemerin, resistin, vaspin and visfatin are suggested to be involved in the reproductive function through regulating gonadal functions and the hypothalamic-pituitary axis (Reverchon et al., 2014; Estienne et al., 2019; Tersigni et al., 2011). Meanwhile, apelin is emerging much attention in the field of fertility and reproduction as well therapeutic target for reproductive disorder, metabolic disease, neurological disease and other pathological condition (Wang et al., 2020; Liu et al., 2020; Falcão-Pires et al., 2010; Castan-Laurell et al., 2019; Hu et al., 2016; Li et al., 2022; Yan et al., 2020). Apelin has gained increasing attention due to its numerous distinct biological activities in a variety of

organs. Consistent with its putative role as an adipokine, apelin has been appears as a beneficial adipokine with anti-insulin resistance properties, and thus as a promising therapeutic target in metabolic disorders (Zhu et al., 2011; Bertrand et al., 2018).

Apelin and its distribution

In 1998, Tatemoto and his co-workers isolated and characterized a peptide from bovine stomach extracts as a ligand for a G protein-coupled orphan receptor, now designated as apelin receptor (APJ). In human, apelin protein is encoded by the APLN gene located on chromosome Xq 25-26 (Tatemoto et al., 1998). The APLN gene encodes a 77 amino acid protein known as preproapelin that is subjected to proteolytic processing to produce apelin in its different physiologically active isoforms, including apelin 36, 17, 13 and pyroglutamate apelin-13 (Tatemoto et al., 1998; Castan-Laurell et al., 2012). Apelin, a novel bioactive endogenous peptide and shows its physiological effects by binding to its receptor APJ (Lv et al., 2020). Apelin is mainly produced by adipocytes while some other tissues like heart, lung, stomach, muscle, mammary gland, testis, ovary, uterus, placenta, and brain have shown the apelin expression (mRNA and protein) (Kurowska et al., 2018, Wang et al., 2020). In rat, apelin (mRNA and peptide) was observed within many regions of the central nervous system (CNS) including the spinal cord, midbrain, nucleus accumbens, cortex, striatum, hippocampus and cerebellum (De Mota et al., 2000; Medhurst et al., 2003) and expressed highly in the pituitary, pineal gland, hypothalamus specifically arcuate nucleus, supraoptic nucleus (SON) and paravetricular nucleus (PVN), mammary gland, lung, heart, uterus and ovary (Reaux et al., 2002; De Mota et al., 2004, Habata et al., 1999; O'Carroll et al., 2000; Kawamata et al., 2001; Medhurst et al., 2003; Kacar et al., 2018; Shuang et al., 2016).In mice, apelin expression was distributed within the hypothalamus (PVN and SON) and pituitary of the CNS, adrenal gland, kidney, lung, stomach, intestine, ovary and uterus (Pope et al., 2012; Balci et al., 2023).

In human, apelin was also found within the CNS regions with high expression in the amygdala, substantianigra, spinal cord, corpus callosum, and pituitary gland. Apelin expression has been also reported within the various cells of placenta, lung, kidney, heart, ovary, uterus and the cardiovascular system specifically in vascular and endocardial endothelial cells (Medhurst et al., 2003; De Falco et al., 2002; Roche et al., 2016; Hehir and Morrison, 2012; Kleinz and Davenport, 2004). It has been suggested that circulating apelin level might also be contributed by magnocellular neurons (De Mota et al., 2004), gastrointestinal tract (Susaki et al., 2005) and adipocytes (Boucher et al., 2005).

Apelin receptor (APJ) and signaling pathways

Apelin exert its biological effects by binding to its receptor known as apelin receptor (APJ, APLNR or AR). The APJ was discovered by O'Dowd and his team (1993) based on its sequence homology (40–50% in the transmembrane region) with the angiotensin II receptor (AT1R). APJ was identified as a class A (rhodopsin-like) G-protein coupled receptor (GPCR) and found in many rat brain cells. The receptor was deemed as an orphan GPCR in the absence of an identified cognate ligand after the finding that angiotensin II does not binds to this receptor (O'Dowd et al. 1993). The APJ retained as an orphan GPCR until the discovery of its endogenous ligand, apelin and named later as apelin receptor (Tatemoto et al. 1998). The APJ is encoded by APLNR gene (also known as AGTRL1, APJR, APJ, and FLJ90771) and located on chromosome 11q12 in human, chromosome 2E1 in mice and chromosome 3q24 in rat with 90% similarities (Pitkin et al., 2010; Hosoya et al., 2000).

APJ signaling shows surprising effects even sometimes its contradictory. APJ shows preference for several G-protein (Gα, Gβ, and Gγ) and APJ signaling being heterologous, mediates divergent effects in different cell types and species through activation of many signaling pathways (Tatemoto et al., 1998). Like other GPCR, APJ signaling requires β-arrestin as a signal transducer and shows β-arrestin-dependent

signaling for biological function. It has been shown that apelin mediated APJ signal transduction activate through a combination of Gαi protein subtypes and phosphorylation of APJ at the C-terminal region then recruit the β-arrestins (Chapman et al., 2014; Habata et al., 1999; Chen et al., 2020). APJ can bind to the various isoforms of its known endogenous ligands, apelin and Toddler/ ELABELA and exerts qualitatively different signals (Chapman et al., 2014). Apelinergic (apelin/APJ) system has three important signaling pathways, phosphorylation of phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), reduction of cyclic adenosine monophosphate (cAMP) and activation of phospholipase C-β (PLC-β) (Chapman et al., 2014). At the downstream of APJ, two forms of pertussis toxin-sensitive Gα protein (Gαi/o, Gαq/11) mediate distinct signals transduction (Masri et al., 2002). The Gαi/o activates PI3K/Aktthat mediates both cell survival and nitric oxide (NO) induced vasodilation (Liu et al., 2010). Moreover, Gαi/o inhibits adenylatecyclase, which results in decrease in cAMP as well as in protein kinase A (PKA), which may control glucose homeostasis (Masri et al., 2002). Gαq/11 triggers PLC-β hydrolyze phosphatidylinositol 4, 5-bisphosphate into second messengerdiacylglycerol and inositol trisphosphate, which enhances the release of calcium (Ca^{2+}) from intracellular storage and activates protein kinase C (PKC) (Carpéné et al., 2007).

Previous reports suggested that apelin-13 showed inhibitory effect on forskolin's stimulation of 3′,5′-cAMP by binding APJ to the Gi/o protein (Tatemoto et al., 1998). APJ signaling with apelin isoforms apelin-13 and -36 decreased cAMP productions thereby unable to generate calcium (Ca^{2+}) mobilization in Chinese hamster ovary (CHO) cells (Habata et al., 1999). In contrast, these isoforms showed increased calcium (Ca^{2+}) levels in neurons and the human embryonic kidney cell line (HEK-293) by binding to APJ (Masri et al., 2006). Furthermore, apelin mediated APJ signaling in CHO and HEK-293 cell lines found to be generates through Gαi2 via activation of the extracellular signal-regulated kinase (ERK 1/2) pathway (Foussal et al., 2010). In addition, the apelinergic system initiates PI3K, Akt, 5′AMP-activated kinase (AMPK) phosphorylation and the ribosomal S6 kinase (p70S6K) pathways during cell

proliferation and apoptosis process (Masri et al., 2006; Yang et al., 2016) andapelin/APJ system also mediated by phospholipase C –phosphokinaase C (PLC-PKC) pathway in the positive inotropic effect (Szokodi et al. 2002).

Apelin, hypothalamus and pituitary

Hypothalamic GnRH neurons are the origin of the endocrine function of female reproduction, which primarily receive projections from the arcuate, paraventricular (PVN), supraoptic (SON), and medial preoptic nuclei of the hypothalamus (Jin & Yang, 2014). It was also well illustrated that apelin and APJ peptides are positive regulator of female reproduction through HPO axis and many studies have reported that the HPO axis has primary site for apelin action (Mehri et al., 2023). The critical roles of apelin/APJ system in reproductive control can be revealed by their extensive expression in the same hypothalamic nucleus group. In rat, B78/apj, a homologue to human APJ was expressed in the PVN, SON and the pituitary especially in the anterior lobe and also in the intermediate lobe and posterior lobe (O'Carroll et al., 2000; De Mota et al., 2000). An immunochemistry study revealed that apelin immunoreactive fibre was mainly detected in the median eminence region of hypothalamus and also in the SON, the magnocellular and parvocellular parts of the PVN, periventricular, suprachiasmatic, ventromedial, dorsomedial, nucleic, and retrochiasmatic (Reaux et al., 2002; Newson et al., 2009). A study by Pope and co-author (2012) demonstrated that APJ mRNA has a restricted distribution in the PVN, SON of hypothalamus and the pituitary gland showed high to moderate apelin mRNA expression in the anterior to posterior pituitary, respectively. Apelin and APJ (mRNA and protein) distribution was found in the ovarian cells and played an important role in regulating ovarian function (Kurowska et al. 2018). It was also well illustrated that apelin peptide is a positive regulator of female reproduction. The hypothalamic expression of Apelin/APJ genes indicates the role in the regulation of releasing hormones (Newson et al., 2009). Furthermore, intracerebroventricular and intraperitoneal infusion of apelin-13 suppressed the FSH and LH secretion in anterior pituitary of rats without disruption of the GnRH concentration

(Taheri et al., 2002; Tekin et al., 2017). Due to the structural and functional similarities between apelin and GnRH, it was suspected that apelin could be a competitive inhibitor in the anterior pituitary for GnRH receptor (Cho et al., 2007; Wang et al., 2020). In buffalo ovary, apelin and APJ was found stimulates estrogen and progesterone production (Gupta et al., 2023). The apelin/APJ system showed stimulatory effect on the steroidogenesis after apelin receptor antagonist treatment (Shokrollahi et al., 2022). The apelinergic system might have autocrine or paracrine effects with 3-beta-hydroxysteroid dehydrogenase (3β-HSD) in the luteal phase of the estrous cycle of porcine (Różycka et al., 2018). There is a dearth of literature whether apelinergic system could directly or indirectly regulate female reproduction through modulating HPO axis.

Ovary and Apelin

The female gonads, ovary is central to female reproductive function, the primary female reproductive organs (Baillet et al., 2012). The female fertility is depends on the oocyte reservoir of the neonatal ovary. Ovary links the reproductive and non-reproductive organs during ovarian cycle. The majority of estrogens in the body are produced in the ovarian growing follicle, and after ovulation, the remaining follicle develops into the corpus luteum, a transitory endocrine structure that secretes progesterone, which is essential for the initiation and establishment of pregnancy (Anderson and Hirshfield, 1992). Successful female reproduction is based on a complex cyclical pattern of hormonal communication and feedback that is conducted by the ovary in response to hormones secreted from the anterior pituitary, which are in turn controlled by the hypothalamus (Stefansdottir et al., 2014). Furthermore, ovarian function has been found to be regulated directly or indirectly by the apelinergic system (Shokrollahi et al., 2022).

The apelin/APJ system has been demonstrated in the ovary of many mammalian species including humans, rhesus monkeys, bovines, porcines and rodents (Roche et al., 2016; Kurowska et al., 2018; Schilffarth et al., 2009; Rak et al., 2017; Shuang et al., 2016). Apelin and its receptor, APJ expression was found in the bovine corpus luteum during

estrous cycle and suggested to involved in the maturation of CL and regulation of the luteolytic cascade of corpus luteum (Shirasuna et al., 2008). In bovine ovary, the apelin mRNA expression was found in the thecal cells of estrogen-inactive dominant follicles while APJ express in the granulosa of estrogen-inactive dominant follicles and showed increased APJ level in thecal cells during follicular growth (Shimizu et al., 2009). The expression of apelinergic system in the cultured thecal cells was regulated by LH hormone; in contrast progesterone and FSH hormones stimulate the expression of APJ mRNA in the cultured granulosa cells (Shimizu et al., 2009). Another report have supported that apelin and APJ suggested to have angiogenic effect and regulated the corpus luteum formation and function during estrous cycle. It might have involved in the selection of preovulatory follicle through inducing proliferation of capillaries and supporting the dominant follicular growth. The expression of Apelin and APJ in the bovine ovarian cells during estrous cycle showed higher than during pregnancy and might directed to be regulated by estrogen concentration (Schilffarth et al., 2009). Roche and co-authors (2017) reported that apelin and APJ expressed in the granulosa and oocytes and induces the secretion of progesterone from luteinizing granulosa cells. *In vitro* experiment indicated that apelinergic system inhibits oocyte maturation and progesterone secretion from cumulus cells in the ovary of cow. The apelin/APJ expression was influenced by insulin-like factor1 (IGF1) and FSH (Kurowska et al. 2018). In buffalo ovary, apelin and APJ was found in the ovarian follicles and corpora lutea and stimulates estrogen, progesterone production and granulosa survival (Gupta et al., 2022). The apelin and APJ abundance was co-related with the size of the ovarian follicles and the apelin/APJ system showed stimulatory effect on the steroidogenesis after apelin receptor antagonist treatment (Shokrollahi et al., 2022). In porcine, apelin in the follicular fluid and apelin/APJ increased with follicular growth and found in the granulosa cells, thecal cells and oocytes and APJ was also found in the zona pellucida layer of the ovary (Rak et al., 2017). Like bovine, apelin and APJ expression depends on the growth of the corpus luteum and stimulates luteal progesterone secretion and suggested auto/paracrine effect of apelin with 3β-HSD in the luteal phase of the estrous

cycle of porcine (Różycka et al., 2018). Roche and co-authors (2016) found that apelin and APJ (gene and protein) localization in human ovarian cells and granulosa cell lines. Apelin and APJ immunolocalization were high in the primary, medium and mature follicles. They demonstrated that high expression of APJ was found in the oocytes, granulosa cells (cytoplasn and nuclei), and cumulus than thecal cells of human. Apelin and APJ system was also found in the rat ovarian cells and it has stimulatory effect in the proliferation and inhibitory effect on apoptosis of granulosa cells via PI3/Akt signaling pathway (Shuang et al., 2016). However, expression of apelin and APJ has not shown in the mice and requires further investigation.

Uterus and Apelin

In mammals, the uterus, a major part of female reproductive tract is a vital organ for reproduction. Rather, transport, storage, and capacitation of spermatozoa and delivery of the conceptus at parturition, the uterus produce prostaglandin F2α, the luteolysin required for ovarian cyclicity and develops placenta for conceptus growth and development (Bartol, 1999). Despite the significance of the uterus for the fertility, the hormonal, cellular, and molecular mechanisms that regulating uterus development and functioning through autocrine/paracrine factors is relatively less exposed. Recently, apelin and its receptor were observed to exhibit potential role in the uterus of rat, mouse, ewe and human (Kacar et al., 2018; Balci et al., 2023; Mercati et al., 2019; Hehir et al., 2012).

Firstly, the APJ expression was detected in a rat uterus by RT-PCR technique (Hosoya et al., 2000). Later other reports demonstrated that the endometrial glandular cells were shown to have higher apelin expression during the secretory phase, whereas the stromal cells maintained low levels of apelin (Kawamata et al., 2001; Ozkan et al., 2013). The apelin and APJ were found and exhibits a large differential expression pattern in the different types of ewe uterine cells (Mercati et al., 2019). Therefore, there were intriguing considerations regarding the possible roles of apelin in controlling

reproduction. In ewe, apelin and APJ localization and expression was found in the uterine lining epithelium and the glands of the endometrium (Mercati et al., 2019). Apelin may suppress both spontaneous and oxytocin-induced contraction in human myometrial fibers and play important role in vasodilation role *in vitro* (Hehir& Morrison, 2012). Apelin has been found to regulate spatio-temporal role in spiral arterioles maturation and interstitial edema in endometrium (Wang et al. 2020). In contrast, Kacar and co-author (2018) reported that serum apelin might exert an inotropic effect in myometrial layer via PKC-mediated intracellular Ca^{2+}amplication in rat uterus. The apelin and APJ was found in the same area of the uterus suggesting the autocrine and paracrine effects which serve to control endometrial activity in response to changes during the estrous cycle (Mercati et al., 2018). Due to the angiogenesis and vasodilation properties of apelin could be one of the causes in triggering the symptoms of endometriosis (Wang et al., 2020). Future investigation is needed regarding uterine glands produce apelin into the lumen, which might provide nourishment for the developing foetus.

Furthermore, the apelin and its receptor, APJ were reported to express in the human placenta (Mlyczyńska et al., 2021). One study showed that apelin has potent utero-relaxant effect and it was greater at initial state of pregnancy than late pregnancy (Asalah et al., 2020). Apelin played role in the placental hormone production and secretion (Dawid et al., 2021). One immunohistochemical and molecular biology study demonstrated that the apelin and APJ expression was found in the epithelial cells of the maternal portion in bitch placenta at middle and late gestation (Troisi et al., 2020). Apelin/ APJ system has been reported as a key factor responsible for placental angiogenesis, placental vessels tone and during exchange of oxygen and nutrients through the placenta (Eberle et al., 2019). The apelinergic system is crucial for placental growth and function throughout pregnancy, as well as for the cardiogenesis and vasculogenesis of the embryo. Placenta-associated pregnancy problems, such intrauterine growth restriction and preeclampsia (PE), appeared to be associated to the

apelinergic system (Wang et al., 2017; Pécheux et al., 2023). To best of our knowledge uterine apelin system has not been investigated in the mice.

Apelin and Pathophysiology (polycystic ovary syndrome)

The reproductive tract is tightly coupled with energy balance, and thereby metabolic abnormalities can lead to the development of pathophysiologies. Apelin regulates fertility and reproductive functions in physiological and pathological conditions through its autocrine and/or paracrine effects (Kurowska et al., 2018). Therefore, targeting this pathway is a current demand and a novel outlook that could provide more treatment options for improving reproductive capacity in metabolic disorders like polycystic ovarian syndrome (PCOS).

PCOS is one of the most common (about 5–20%) reproductive disorders in women of reproductive age that cause infertility (Franks et al., 2008). PCOS was first elucidated by Stein and Leventhal in 1935 and noticed symptoms of excessive hair, obesity, and ovaries covered with cysts. PCOS is the major endocrinopathy of fertile women, expressed with hyperandrogenism, chronic oligo or anovulation, polycystic ovaries, irregular menstruation, subfertility, hyperinsulinism, obesity, hirsutism. PCOS also lead to the risk of type2 diabetes, cardiovascular and biliary tract diseases, endometrial cancer and preeclampsia (Azziz et al., 2016; Dravecká et al., 2021).PCOS has heterogenous condition and multifactorial intervention, therefore the etiology of PCOS is still not defined clearly (Caldwell et al., 2014).Previous reports suggested that apelin found to be regulated with the occurrence of obesity and insulin resistance and established a strong link with PCOS (Sun et al., 2015; Karimi et al. 2018). Bongrani and co-author reported that apelin and APJ was increased in women with PCOS condition and positively correlated with follicle count (Bongrani et al., 2019). Circulating serum apelin levels in PCOS condition seems contradictory. Some studies reported elevated serum apelin levels in the obese PCOS (Gören et al., 2012; Liu et al., 2020; Dravecká et al., 2021). On the other hand, serum apelin showed low level in lean PCOS patients

(Choi et al., 2012; Chang et al., 2011). Other reports also suggested that the increased expression of apelin in atretic follicles stimulates steroidogenesis in granulosa cells of obese PCOS patients (Roche et al., 2016). Apelin shown to regulate the metabolism of glucose and lipid, further modulate insulin secretion during PCOS condition. Apelin might be a protective factor that elevated during obese and dyslipidaemia condition to subsequently control the lipid abnormalities of the blood in PCOS patients (Liu et al., 2020). Some reports suggested that elevated apelin level might be positively co-related with homeostatic model assessment for insulin resistance (HOMA-IR) and body mass index (BMI) (Sun et al., 2015; Roche et al., 2016; Bongrani et al., 2019), whereas, another study reported that low serum apelin level might be positively correlated with HOMA-IR and BMI (Altinkaya et al., 2014). Elevated plasma apelin in PCOS may be associated with androgenic obesity, increased adiposity, impaired LH/FSH interaction, impacts of the hypothalamo-hypophyseal axis, and compensatory mechanisms resulting from PCOS metabolic abnormalities (Dravecká et al., 2021). The inconsistent results observed in published literature might be ascribed to many factors such as research design, PCOS stage, sample size, patient genetics, and apelin assessment methods (Wang et al., 2020).

Rationale of the work

Expression of apelin and APJ has been shown in the several tissues including the brain, kidney, pancreas, breast, heart and ovary. However, there is insufficiency of literatures regarding expression and role of apelin and APJ in the reproductive tissue of mice. It has been documented that the intraovarian factors relating to proliferation and apoptosis exhibit striking alterations in the postnatal ovary of rodents (De Felici et al., 1999; Coucouvanis et al., 1993). The process of ovarian cell division and proliferation is tightly controlled. Furthermore, postnatal developmental changes of apelin and APJ in ovary and uterus have not been investigated in any mammalian species. However, it has been shown that expression of apelin and APJ are developmentally regulated in the mice testis and it has stimulatory role in early pubertal stage and inhibitory at adult stage (Das

et al., 2022; 2023). In adult mice testis, apelin has been suggested to regulate the spermiogenesis (Das et al., 2024). The previous study showed that visfatin, other adipokines expression is developmentally regulated in the mice ovary and the levels of ovarian visfatin showed elevated expression in the PCOS (Annie et al., 2019; 2020; 2021). Therefore, it would be logical to analyze the postnatal developmental changes of apelin and APJ in female mice reproductive tissues.

Estrous cycle of rodent is short and there is a modulation of circulating hormones, due to which it gives a good model to study the changes in the various ovarian and uterine factors. Previous studies showed that in the bovine ovary, the expression level of apelin and apelin receptor changes during the estrous cycle is significantly higher compared to the one during pregnancy (Schilffarth et al., 2009; Shirasuna, et al., 2008). Thus, it also logical to hypothesize the ovarian apelin and APJ would also be modulating during estrous cycle in the ovary and uterus as well. Further, the ovarian function is regulated by gonadotropins, the effect of each of the two gonadotropins, FSH and LH on the ovarian apelin and APJ would be very important to study.

The uterus undergoes drastic changes in cell proliferation and differentiation during estrous cycles which are under the endocrine control by ovarian hormones. 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 (Kotlarczyk et al., 2021). This led us to hypothesize that the ovarian steroids, estrogen and progesterone would modulate apelin and APJ expression in ovariectomized mice uterus.

Therefore, this dissertation provided an understandable knowledge about the role of apelin system in female reproductive organ of mice at different developmental stage along with pathogenesis of PCOS in mice model. To the best of our knowledge, no previous study has been conducted on the role of apelin system in the reproductive organ of mice.

CHAPTER 1

Title

Postnatal developmental expression and localization of apelin and apelin receptor protein in the ovary and uterus of mice

***Molecular Reproduction and Development, 90 (2023) 42-52.**

Chapter 1

Introduction

The postnatal developmental processes for ovary and uterus are under regulation of various autocrine, paracrine and endocrine factors. Although morphogenesis of reproductive organs begins in antenatal period, it is keep developing and differentiating until after birth (Hu et al., 2004). Ovary development includes several cellular and molecular events commence at antepartum with a pool of fixed numbered primordial follicles consisting an oocyte until birth (Hirshfield., 1991; Marion and Gier., 1971). Most of the oocytes die in fetal ovary facilitated by the program cell death and it has been shown that apoptosis is the major process of germ cell loss in fetal mouse ovary along with germ cell extrusion and autophagy (Coucouvanis et al., 1993; De Pol et al., 1997; Pepling et al., 2001; Wordinger et al., 1990; Lobascio et al., 2007). In rodent, a few primordial follicles begin to grow during the early neonatal period and growth is extended until the follicle meets one of two fates-ovulation or atresia (Fortune, 1994).

Uterine morphogenesis depends on the gestation length and the interval between birth and puberty of mice (Gray et al., 2001). At birth, the uterus (a specialization of the mesodermally derived paramesonephric or Müllerian ducts) of mice consists of a simple epithelium supported by undifferentiated mesenchyme (Mossman., 1989; Bartol et al., 1993). The postnatal proliferation of uterus has two peaks; one is ovary-independent during neonatal period and second is ovarian steroids-dependent at puberty (Bartol et al., 1999; Wu et al., 1988). The endocrinological, cellular, and molecular mechanisms regulating postnatal uterine development are not clear in all mammals (Hu et al., 2004). Thus, the study of postnatal expression of various factor are required to elucidate the role of internal factor on development.

In recent years, the involvement and role of adipokines are emerging to the forefront in the field of fertility and reproduction. Many studies suggested that adipokines such as leptin (Tersigni et al., 2011; Spicer et al., 2001), visfatin (Reverchon et al., 2013; Annie et al., 2020), resistin (Maillard et al., 2011; Niles et al.,2012; Reverchon et al., 2013), adiponectin (Ledoux et al., 2006; Chabrolle et al., 2007., Lord et al., 2005., Takemura et

al., 2006., Schmidt et al., 2008), chemerin (Goralski et al., 2007; Reverchon et al.,2012; Carlino et al., 2012) and apelin (Wang etal., 2020; Shokrollahi et al., 2022; Kurowska et al., 2018; Roche et al., 2016) are expressed and play important role in ovary, uterus, placenta. Apelin is expressed in various types of tissues and organs such as the central nervous system and peripheral tissues, including the hypothalamus, adipose tissue, skeletal muscle, digestive system, ovary, uterus, placenta and mammary gland (Cobellis et al., 2007: Mercati et al., 2018). Apelin, encodes 36 amino acids, was discovered as an endogenous ligand of the orphan G protein-coupled receptor known as apelin receptor or APJ or APLNR. Apelin was first isolated from bovine stomach (Tatemoto et al., 1998). In human, APJ gene encodes a seven-transmembrane protein closely related to the angiotensin receptor (O'Dowd et al., 1993). The expression of apelin/APJ in granulosa cells, thecal cells and luteal cells of ovary in bovine (Shimizu et al., 2009) and ewes (Mercati et al., 2019) have shown to be elevated with follicular growth. It has been shown that apelin regulates steroidogenesis of granulosa cells and corpus luteum, as an auto/paracrine factor (Wang et al., 2020; Shokrollahi et al., 2022; Rózycka et al., 2018). It has also been shown that apelin regulates steroidogenesis via 3β HSD, AMPKa activation and Akt and MAPK3/1 signaling (Roche et al., 2016; Rak et al., 2017; Roche et al., 2017). APJ has been found in the ampullary secretory cells of oviduct, which might have important role in fertilization and implantation (Mercati et al., 2019). Apelin expression upraised in the secretory phase of glandular cells of uterus, however, remained constantly low in stromal cells (Kawamata et al., 2001; Ozkan et al., 2013; Mercati et al., 2019). Apelin inhibit both spontaneous and oxytocin-induced contraction in human myometrial fibers of uterus (Hehir and Morrison 2012).

These evidences suggest important role of apelin and APJ in the ovary and uterus. However, no study has shown the age dependent or postnatal developmental changes in the ovarian or uterine apelin along with its receptor in the female rodent species. Therefore, our aim was to investigate the changes in the postnatal localization and expression ofapelin and APJ in the ovary and uterus of female Swiss albino mice.

Materials and methods

Animals and ethical statement

Breeding sets of Swiss albino mice were housed in accordance with the protocols approved by the Mizoram University Institutional Animal Ethical Committee (Protocol Number, MZU/IAEC/ 2020/12), Mizoram University, Mizoram, India. These mice were kept under standard laboratory condition of 12 hours light-dark cycle with maintained temperature of 25 ± 2°C and provided with food and water ad libitum.

Sample collection

The female mice of post natal day (PND) 1 (n=10), 7 (n=10), 14 (n=10), 21 (n=5), 42 (n=5) and 65 (n=5) were sacrificed from the reared colony and ovary, uterus and serum samples were collected. One side of the uterus and ovary from five animals per group was fixed in Bouin's solution for immunohistochemistry. For PND1, 7, 14 groups remaining 15 ovaries and uterus were made into 5 samples by pooling 3 ovaries and 3 uteri for western blotting. For PND21, 42, 65 groups, remaining 5 ovaries and uteri from each group were used for western blotting analysis.

Immunohistochemistry

Ovary and uterus were embedded in paraffin as previously described by Tepekoy et al., (2015). Briefly, the uterus and ovary samples were dehydrated in 3 different grades of alcohol (70%, 90% and 100%) for 1hour with two changes each, cleaned with xylene and embedded in paraffin wax. The paraffin-embedded blocks of tissue were then sectioned into a thin ribbon of 7μm thickness with Leica rotary microtome (model RM2125 RTS). The tissue sections were spread properly in warm water (maximum 45°C) and mounted onto previously poly-l-lysine coated slides kept in slide warming table at 37°C.

The slides were processed for immunohistochemistry, after deparaffinization in xylene; tissue sections were rehydrated with alcohol (100%, 90% and 70%) for 10 min each with two changes and hydrated in distilled water followed by 3% H_2O_2 in methanol to block the endogenous peroxidase. For blocking nonspecific binding, the slides were then treated with goat serum 1:100 for 30 min at room temperature and incubated in primary antibody (anti-apelin receptor 1:50, lot# ABD43, Millipore and anti-apelin 1:50, cat#SAB4301741 Sigma Aldrich, USA) diluted with PBS at 4◦C for overnight. Unbound primary antibody were wash off with PBS and incubated with secondary antibody (goat anti-rabbit, Cat# PI-1000, Vector Laboratories, Burlingame, CA, United State) for 3 h at room temperature. Tissue sections were incubated in 0.6 mg/mL solution of 3, 3-diaminobenzidine tetra hydrochloride dihydrate (DAB) in Tris–HCl (pH 7.6) and 0.01 % H2O2at room temperature until brown color developed and the reaction stopped by putting the slides in distilled water. Counterstaining was done with hematoxylin for 10 sec and wash with running water. The stained slides were then dehydrated (70%, 90% and 100%), clean in xylene for twice 10 min each. Finally mount with DPX and examined using a Nikon binocular microscope (ModelE200; Nikon, Tokyo, Japan).

Western blot analysis

Immunoblotting for uterus and ovary was performed following Jeremy et al., (2017). Briefly, 20% (w/v) tissue homogenates were prepared in the lysis buffer (0.01M Tris-HCl (pH 7.6), 1 mMEDTA (pH 8.0), 1M NaCl,1 µg/ml aprotinin,100 µg/ml phenylmethylsulfonyl fluoride and distilled water) and Bradford method (Bradford., 1976) was used to estimate the protein concentrations of each sample. Equal protein (50µg) was resolved for each sample by using 12% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel electrophoresis and the resolved proteins were then transferred onto the polyvinylidene fluoride (PVDF) membrane using wet apparatus for 14 hours. The membranes were blocked with 5% nonfat skimmed-milk in PBST (cat# GRM1254-500G; HiMedia Laboratory private limited, Mumbai) for 30min at the room temperature and incubated with the primary antibody

anti-apelin receptor (1:1000, lot# ABD43, Millipore and anti-apelin 1:50, cat#SAB4301741 Sigma Aldrich, USA) for overnight at 4°C. Unbound antibody was washing out with PBST and then incubated with horseradish peroxidase conjugated secondary antibodies (1:4000, goat anti-rabbit, Cat# PI-1000, Vector Laboratories, Burlingame, CA, USA) for 4 hours. After washing free secondary antibody with PBST the membranes were incubated with electrochemiluminescence and developed onto X-ray film. ImageJ software (imagej.nih.gov/) was used to quantify the band intensities with respect to loading control after stripping and reprobing the membranes with β-Tubulin (1:1500, cat#E7; DSHB, university of Iowa, department of Biology, USA) and secondary antibody (1:4000, Goat anti-mouse, CAT#E-AB1001, Elabscience, Houston, Texas, USA).

Estimation of circulating level of apelin

The serum collected from all the PND groups were estimated for apelin concentration by enzyme linked immunosorbent assay. Commercially available mouse apelin EIA kit (cat # RAB0018; Sigma-Aldrich Co. LLC, USA) was used following instruction manual. Briefly, 100µl of anti-Apelin C-Terminus was added to each well and incubated for overnight at 4°C. The unbound antibody was washed off with wash buffer for4 times (300µl each) then incubated with standard, positive control and serum (100µl each) into the respective wells for overnight at 4°C. After washing, the wells were incubated with 100µl of HRP-Streptavidin solution for 45 min at room temperature with gentle shaking and then discarded the solution proceed with washing and added 100µl of TMB substrate reagent to each well for 30min at room temperature in the dark with gentle shaking manually. Then the reaction was stopped with 50µl of stop solution (0.2M sulfuric acid) and immediately read the absorbance at 450nm by using a Microplate Reader (Erba Lisa Scan EM, Trans Asia Biomedical Ltd, Mumbai, Maharashtra, India).

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 9 and all the data were expressed as mean ± standard error mean. One-way Analysis of variance (ANOVA) followed by Tukey's test was used to analyze the differences between the data. P value less than 0.05 was considered as significant.

Results

Immunolocalization of apelin in the ovary

Immunolocalization of apelin showed a distinct pattern of variation in the immunostaining (Table 1.1). In order to analyze the localization of apelin in the postnatal ovaries, different types and stages of ovarian follicles were analyzed. Apelin protein was present in the primordial follicles all the stages analyzed. Primordial follicles showed mild immunostaining in PND1, PND21, PND42, and PND65 whereas, primordial follicles at PND7 and PND14 showed moderate immunostaining (Fig.1.1 A1-A6). Primary follicle at PND7, PND14 and PND65 showed moderate immunostaining of apelin, with a very mild immunostaining at PND21 and PND42 (Fig.1.1 B1-B5). The secondary follicles at PND7 showed moderate immunostaining, whereas, apelin staining showed mild intensity at PND21 and PND42. The strong immunostaining of apelin was observed in the secondary follicles at PND14 and PND65 (Fig.1.1 C1-C5). The antral follicles at PND21 showed mild staining in the oocyte, granulosa and thecal cells. Oocytes, granulosa and thecal cells of PND42 and PND65 showed intense immunostaining of apelin (Fig. 1 D1-D3). The corpus luteum at PND65 showed intense immunostaining in the luteal cells, whereas, corpus luteum of PND42 showed moderate immunostaining of apelin (Fig.1.1 E1-E2).

Immunolocalization of APJ in the ovary

Immunolocalization of APJ showed moderate staining in the primordial follicle in the ovary of PND14, primordial follicle of other postnatal ovaries exhibited mild

immunostaining of APJ (Fig. 1.2 A1-A6). Primary follicles at PND7, PND21 and PND42 showed mild immunostaining of APJ, whereas, primary follicles at PND14 and PND65 showed moderate staining of APJ (Fig.1.2 B1-B5). At PND21 secondary follicles showed very faint immunostaining of APJ, whereas, PND7, PND14, PND42, PND65 showed moderate to intense staining of APJ in the secondary follicles (Fig. 1.2 C1-C5). Antral follicles showed intense staining at PND65, whereas, moderate staining of APJ was observed at PND21 and PND42 (Fig. 1.2 D1-D3). Corpus luteum of PND65 showed intense staining, whereas, mild staining in the PND42 (Fig. 1.2 E1-E2) (Table 1.2).

Immunostaining of apelin in the uterus

Immunolocalization of apelin in the uterus of postnatal stages showed marked variation in the different compartments (Table 1.3). Luminal epithelium of PND1 and PND7 showed mild and moderate staining respectively. At PND14 intense immunostaining was observed followed by faint immunostaining at PND21 and PND42. However, luminal epithelium showed intense staining at PND65. Stromal cells at PND14 and PND42 showed mild immunostaining, whereas, at other stages faint immunostaining of apelin was observed. Endometrium at PND21 showed faint immunostaing and at other stages showed mild to moderate immunostaining. At PND7 and PND21 Myometrium showed faint immunostaining and other stages showed mild to moderate staining of apelin. PND7, PND21 and PND42 showed faint immunostaining in the perimetrium and PND14 and PND65 showed mild staining of apelin. Uterine gland showed moderate immunostaining at PND14 and PND65, whereas PND21 and PND42 showed mild immunostaining of apelin (Fig. 1.3 A-J).

Immunostaining of apelin receptor in the uterus

Immunolocalization of APJ showed variation in the different compartment and different stages of the uterus (Table 1.4). Luminal epithelium and stromal cells of PND14 and PND65 showed intense immunostaining, PND1, PND7 showed moderate, whereas,

PND21 and PND42 showed mild immunostaining of APJ. At PND14, endometrium showed intense staining of APJ, whereas PND7, PND14 and PND65 showed moderate staining and PND21 showed faint immunostaining of APJ. In the myometrium, PND14 and PND65 showed intense immunostaining. However, other stages showed mild immunostaining of APJ in the myometrium. Faint immunostaining was observed in the perimetrium at PND21 and PND42, moderate staining at PND7 and intense immunostaining of apelin at PND14 and PND65. Immunostaining of APJ was also found in the uterine gland. PND14 and PND65 showed intense immunostaining of APJ in the uterine gland, whereas, PND21 and PND42 showed moderate staining (Fig. 1.4 A-J).

Expression of apelin receptor in the ovary and uterus by western blotting

Expression of APJ was found to be significantly ($p<0.05$) elevated in the ovaries of PND7, PND14 and PND65 compared to PND1, PND21 and PND42, whereas, expression of APJ was significantly ($p<0.05$) lower compared to all other groups (Fig. 1.5).

Uterine expression of APJ increased significantly ($p<0.05$) at PND65 compared to other postnatal stages and also PND14 is significantly ($p<0.05$) elevated compared to PND1, PND7, PND21 and PND42, whereas, PND14 significantly ($p<0.05$) decreased compared to PND65 (Fig. 1.6).

Estimation of apelin in the serum by ELISA

The circulating apelin was detected to be significantly ($p<0.05$) low (469.02±7.51 ng/mL) at PND1 compared to other groups of postnatal stages, PND7 (946.16±53.51 ng/mL), PND14 (949.03±30.45 ng/mL), PND21 (836.58±54.44 ng/mL), PND42 (755.15±55.71 ng/mL) and PND65 (1026.11±118.69 ng/mL). There is no significant ($p<0.05$) difference between other groups (Fig. 1.7).

Chapter 1

Discussion

The present study has investigated the localization and expression of apelin and APJ in the ovary and uterus of mice at different postnatal ages, PND1, 7, 14, 21, 42 and 65. Our results showed the presence of apelin and APJ in the ovary and uterus from PND1 to PND65, with observable changes in their localization and abundance. However, the circulating apelin levels did not show any changes during postnatal ages. Thus, the presence of apelin and APJ suggest the possible role in uterus as well as ovary development in the mice. To best of our knowledge, the expression and localization of apelin and APJ has not been investigated in the uterus and ovary during postnatal changes of any mammalian species. However, the previous studies have shown the expression and localization of apelin and APJ in the porcine and bovine ovarian follicles (Rak et al., 2017; Shirasuna et al., 2008). Uterine presence of apelin and APJ has also been shown in the dog, rat, mice and human (Troisi et al., 2020; Medhurst et al., 2003; Yamaleyeva et al., 2015).

Immunohistochemical localization of apelin and APJ showed its presence in the primordial, primary, secondary and antral follicles along with corpus luteum. The primary and secondary ovarian follicles showed a very distinct pattern of apelin and APJ from PND7 to 65. These follicles showed low abundance of apelin and APJ in the granulosa, thecal and oocytes of PND21, whereas, apelin and APJ showed more abundance at PND7,14, 42and 65. This finding suggests stage dependent role of apelin and APJ in the follicular development. The physiological significance of low apelin and APJ at PND21 needs further investigation. It has been shown that rodent ovarian granulosa cells and follicles under apoptosis and atresia at PND21 due to low FSH levels (Picut et al., 2015). Thus, it might be suggested that low apelin and APJ at PND21 could also contributes to the apoptosis and atresia; however, it is very speculative, based on the only immunohistochemical study. It has also been shown that apelin promotes granulosa cell and proliferation and inhibits apoptosis in the rat ovary (Shuang et al., 2016). The increase expression of apelin and APJ from PND21 to PND65 could be due to increased

gonadotropin levels. Furthermore, corpus luteum of PND42 and PND65 showed mild to intense staining in the luteal cells. These results are in agreement with previous reports, which showed the expression of APJ in the bovine ovarian follicle, corpus luteum as well as in human ovarian follicle (Shirasuna et al., 2015; Shimizu et al., 2009; Schilffarth et al., 2009; Roche et al., 2016). The presence of APJ and apelin in the granulosa, thecal and corpus luteum of mice ovary also suggest its possible role in estrogen and progesterone secretion. It has been shown that apelin stimulate estrogen and progesterone secretion in the porcine and human ovarian cells (Rak et al., 2017; Roche et al., 2016). Based on the physiological role of apelin in steroidogenesis, cell proliferation and apoptosis (Tang et al., 2007), it might be hypothesized that apelin has potential to regulates during these processes of steroidogenesis, cell proliferation and apoptosis in the mice ovary during postnatal stages. Our study has shown only the localization of apelin and APJ in the ovary, and this was limitation of the study to propose the further role of apelin and APJ in the postnatal ovarian development.

Our results also showed the presence of apelin and APJ in the uterine compartments from PND1 to PND65, with maximum expression at PND65. The staining of apelin and APJ showed a very conspicuous observation at PND21, where immunostaining of apelin and APJ was mild in the various parts of uterus. The overall expression and localization study of apelin and APJ showed an increasing trend from PND1 to PND14 followed by a decline in PND21 and elevation in the expression PND21 onwards. These results are same as we noticed the ovary; however, its physiological significance needs further investigation. It has been shown that adipokines such as leptin, ghrelin, including apelin, inhibits human uterine contraction in vitro (Hehir, and Morrison, 2012). Other study on rats, have shown that the levels of apelin increases at the end of pregnancy and increased apelin induces uterine muscle contraction (Kacar et al., 2018). However, based on these studies, it might be suggested that apelin could modulate the uterine contraction in the adults. Since, apelin, is an angiogenic factor, which helps in the formation of blood vessels (Wysocka et al., 2018), thus, it might also be suggested that apelin could be involved in the formation of blood vessels in the mice uterus during postnatal periods. It

is known that apelin also regulates cell proliferation and apoptosis (Tang et al., 2007). As uterus undergoes dramatic changes (proliferation and apoptosis) during postnatal stages as well as during estrous cycle (Da Costa et al., 2007; Wu et al., 2017), thus, it can be suggested that uterine apelin regulates the proliferation and apoptosis. It should also be noted that expression of apelin system has been shown in the different compartment of uterus; therefore, it is logical to propose the differential role of apelin based on its presence. It has been shown that postnatal uterine development comprises of differentiation and development of the endometrial stroma, myometrium from the mesenchyme along with the adenogenesis or endometrial glandular epithelium from the luminal epithelium, which involving overlapping positive and negative changes in uterine gene expression (Gray et al., 2001; Hu et al., 2004). It has also been shown that postnatal proliferation of uterus has two peaks, are ovary-independent during neonatal period and ovarian steroids-dependent at puberty (Bartol et al., 1999; Wu et al., 1988). Thus, possible involvement of apelin in these processes would be important and interesting to elucidate further.

Conclusion

In conclusion, although the present study is preliminary examination of apelin and APJ in the ovary and uterus of mice at different postnatal stages, however, this is the first report on the postnatal developmental changes of apelin and APJ in any mammalian species. Our result showed that apelin and APJ expressions in the ovary and uterus are developmentally regulated from PND1 to PND65. The expression of apelin and APJ was increased in the mature ovary and uterus, it can be suggested that apelin and APJ are involved in physiological processes of the ovary and uterus. Different localization of apelin and APJ in somatic and germ cell compartments of ovary as well as in the different compartment of uterus suggests its distinct roles for apelin in various cell types. Interestingly, decreased expression of apelin and APJ at PND21 is matter of further investigation.

Chapter 1

Summary

Postnatal ovarian and uterine development is crucial to accomplished female fertility. Thus, the investigations of factors that present in pre-pubertal stages are important as it might be responsible for the regulation of ovarian and uterine function. Apelin, an adipokine and its receptor (APJ) are present in female reproductive organs. However, no study has reported its postnatal expression in uterus and ovary. Thus, we investigated the postnatal developmental changes in expression and localization of apelin and APJ in the ovary and uterus of mice. Postnatal ovary and uterus were collected from postnatal day (PND) 1, 7, 14, 21, 42, 65 and performed western blotting and immunohistochemistry. Uterine APJ is elevated in PND14 and PND65, whereas, ovarian APJ elevated in PND7, PND14 and PND65. Apelin expression in both ovary and uterus showed intense staining at PND65 and PND14. Our results showed that apelin and APJ abundance was lower at PND21 in uterus and ovary. In conclusion, apelin and APJ are developmentally regulated in the ovary and uterus, and its localization in the different compartments of ovary and uterus suggest its distribution specific physiological role in the uterus and ovary.

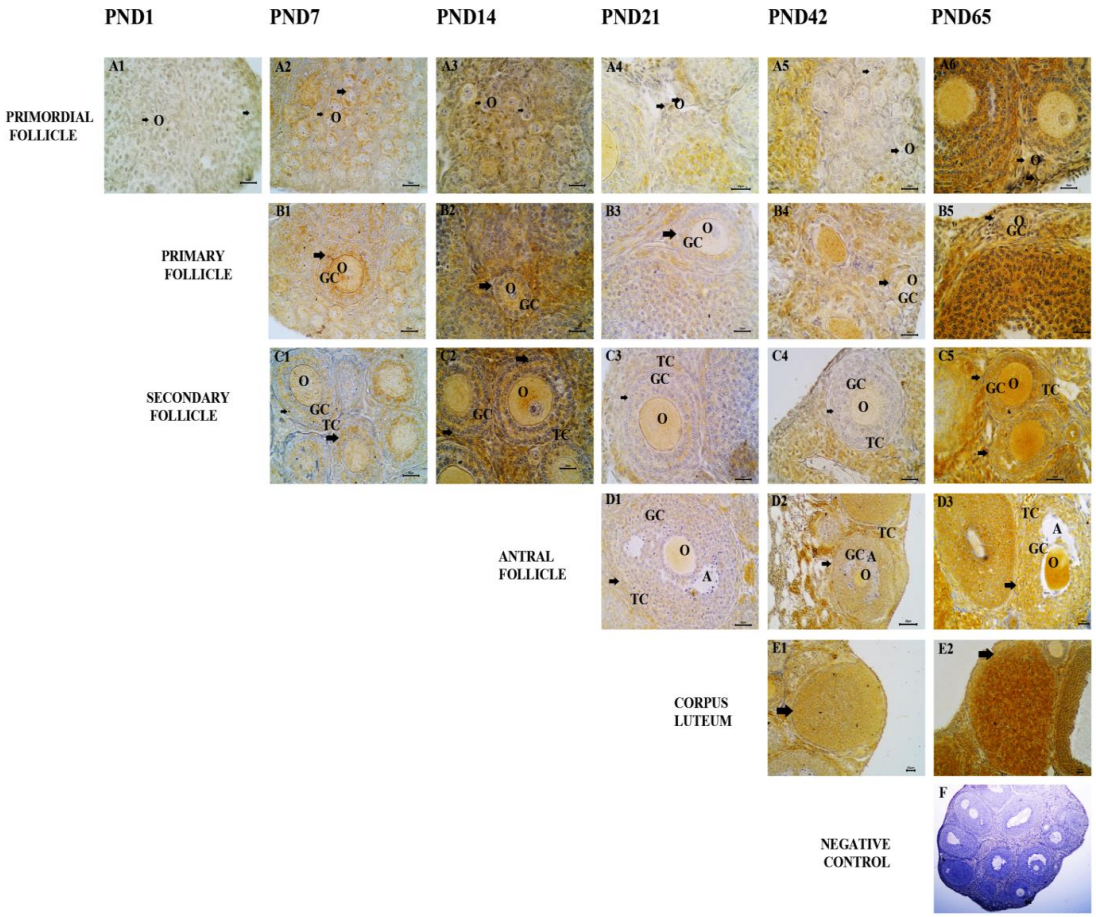

Fig. 1.1: Localization of apelin in ovarian follicles at different postnatal stages.(A1-A6) Primordial follicles from PND1 to PND65(A1–A6) at 60X magnification; (B1–B5)primary follicles from PND7 to PND65at 60X magnification; (C1–C5) secondary follicles from PND7 to PND65 at 60X magnification;(D1–D3)antral follicles from PND21 to PND65 at 40X magnification; (E1–E2)corpus luteum of PND42 and PND65 at 20X magnification; (F) 4X magnificationof negative control of PND65; O, oocyte; GC, granulosa cells; TC, theca cells; A, antrum; PND, postnatal day; black arrow indicate the follicle.

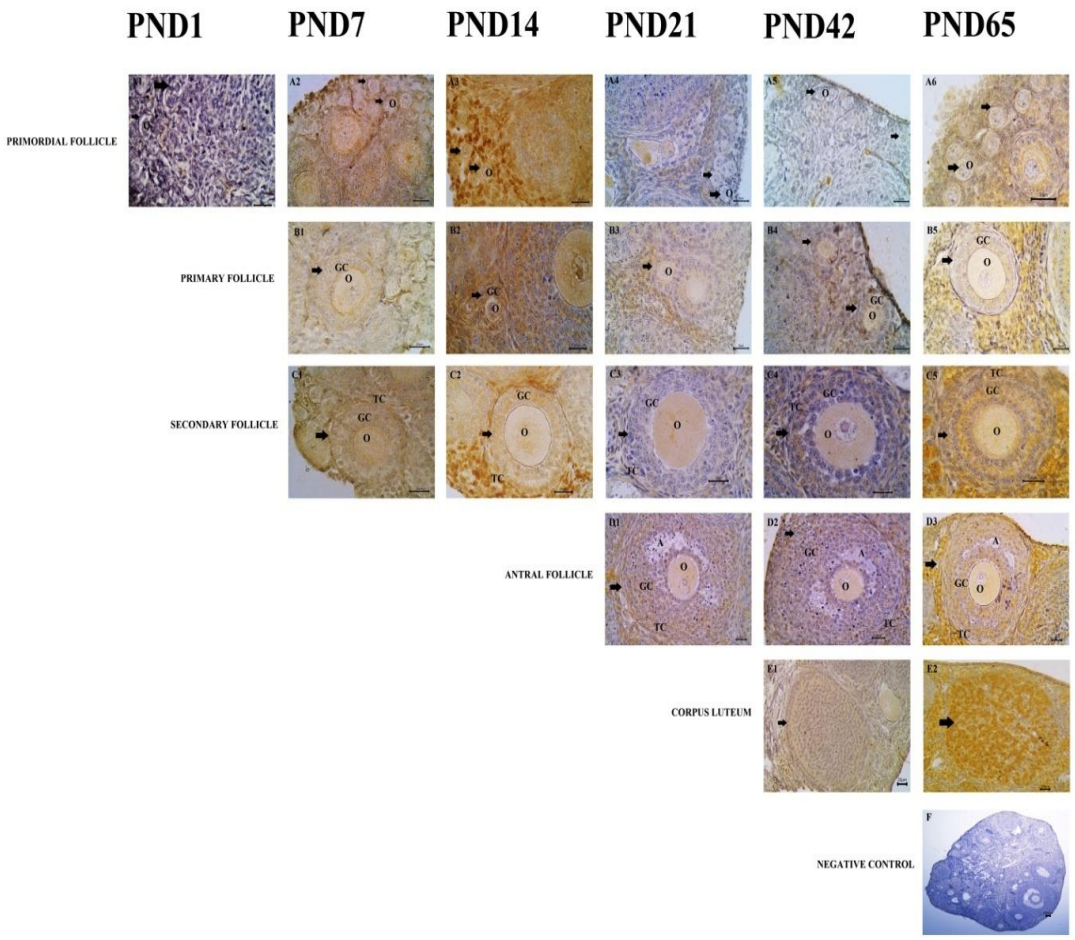

Fig. 1.2: Localization of APJ in ovarian follicles at different postnatal stages. (A1-A6) Primordial follicles from PND1 to PND65 (A1–A6) at 60X magnification; (B1–B5) primary follicles from PND7 to PND65 at 60X magnification; (C1–C5) secondary follicles from PND7 to PND65 at 60X magnification; (D1–D3) antral follicles from PND21 to PND65 at 40X magnification; (E1–E2) corpus luteum of PND42 and PND65 at 20X magnification; (F) 4X magnification of negative control of PND65; O, oocyte; GC, granulosa cells; TC, theca cells; A, antrum; PND, postnatal day, black arrow indicate the follicle.

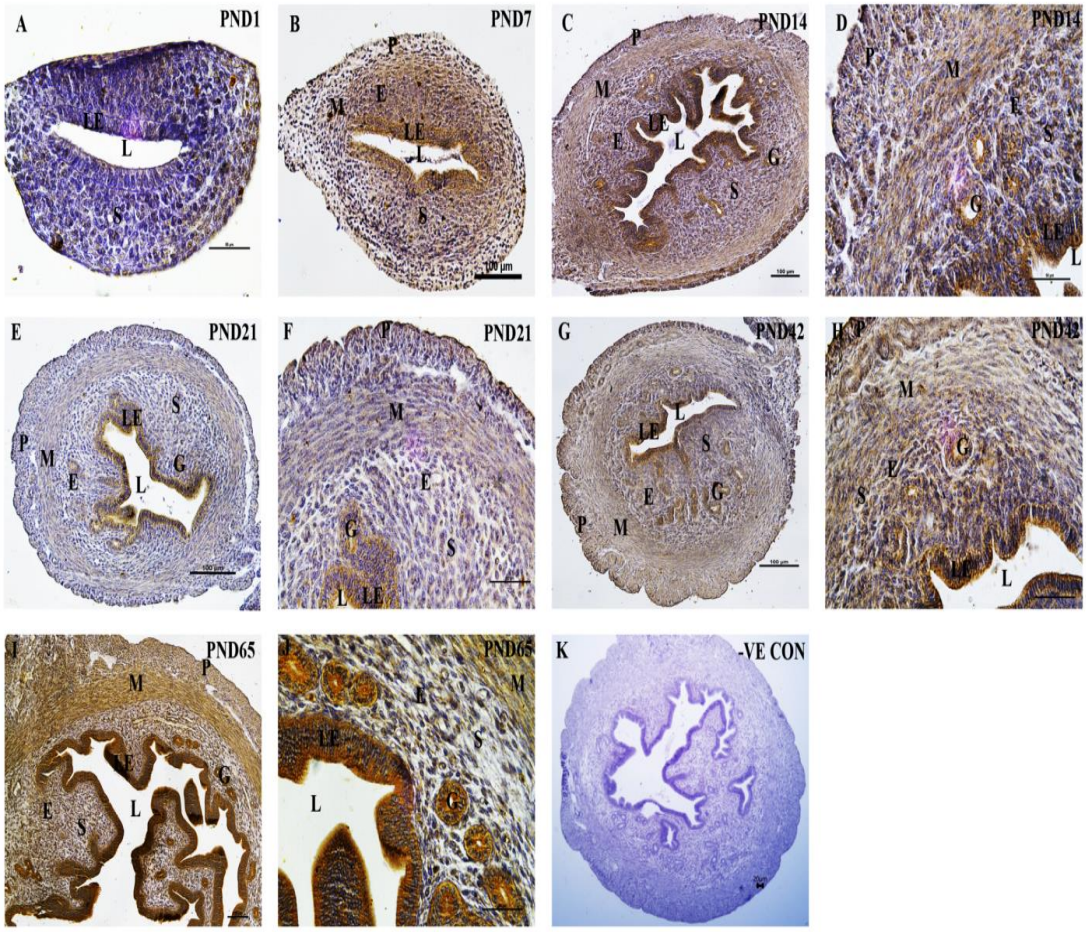

Fig.1.3: Apelin immunostaining in different postnatal stages of mouse uterus. (A) 40X magnification of PND1 uterine section; (B) 10X magnification of PND7 uterine section; (C-D) 10X magnification and (D) 40X magnification of PND14 uterine sections; (E-F) 10X magnification and 40X magnification of PND21uterine sections; (G-H) 10X magnification and 40X magnification of PND42 uterine sections; (I-J) 10X magnification and 40X magnification of PND65 uterine sections; (K) 4X magnification of negative control of PND65 uterus. L, lumen; LE, luminal epithelium; G, gland;S, stromal cell; E, endometrium; M, myometrium; P, perimetrium.

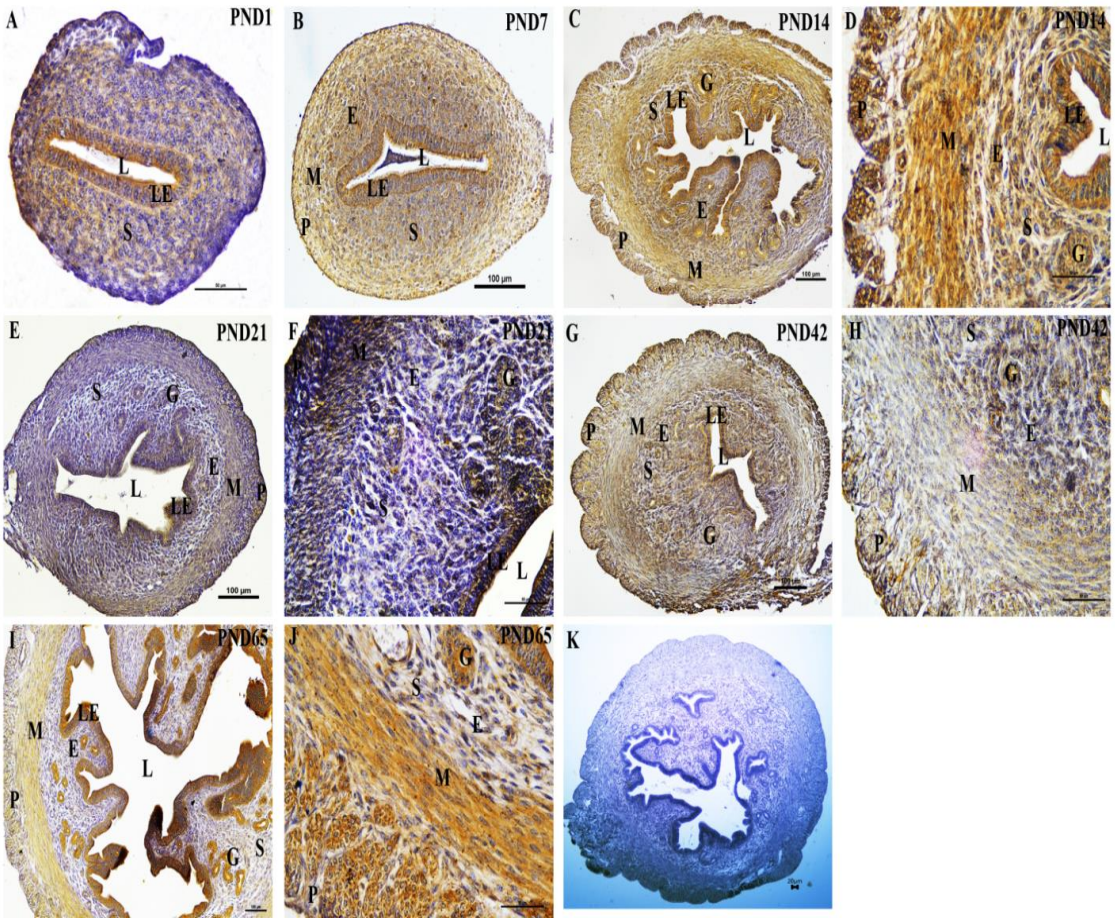

Fig. 1.4: Immunostaining of APJ in different postnatal stages of mouse uterus. (A) 40X magnification of PND1 uterine section; (B) 10X magnification of PND7 uterine section; (C-D) 10X magnification and (D) 40X magnification of PND14 uterine sections; (E-F) 10X magnification and 40X magnification of PND21 uterine sections; (G-H) 10X magnification and 40X magnification of PND42 uterine sections; (I-J) 10X magnification and 40X magnification of PND65 uterine sections; (K) 4X magnification of negative control of PND65 uterus. L, lumen; LE luminal epithelium; G, gland;S, stromal cell; E, endometrium; M, myometrium; P, perimetrium.

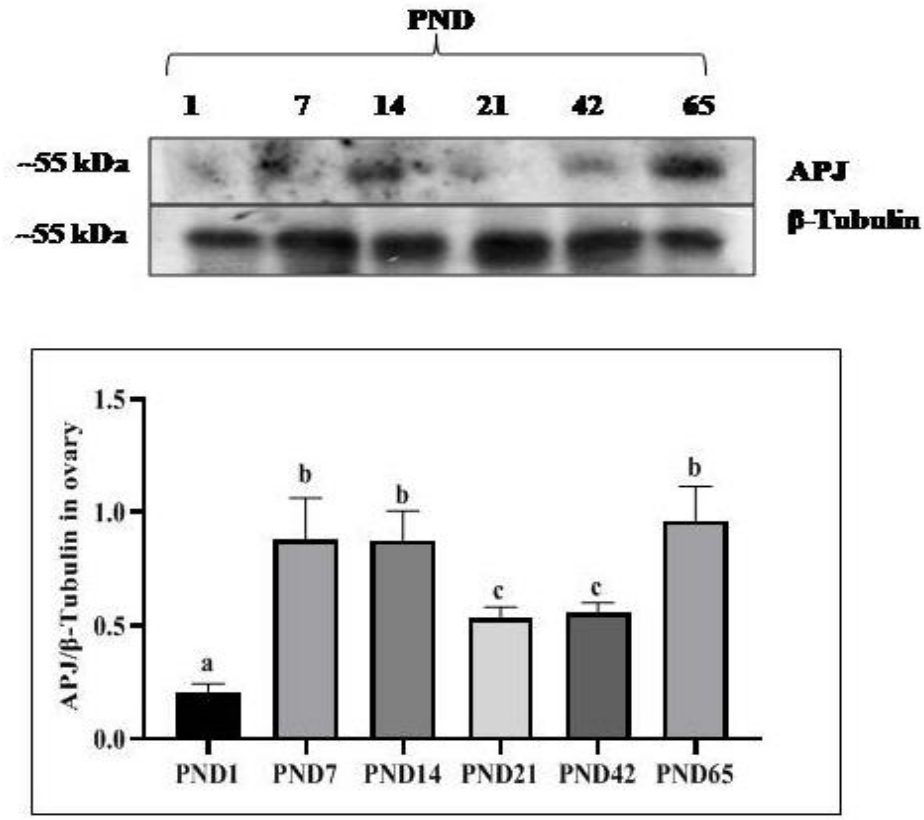

Fig. 1.5: Expression of APJ in ovary at different postnatal stages of mouse. In ovary, PND7, PND14 and PND65 showed a significantly increased ($p<0.05$) expression in APJ compared to other groups. PND21 and PND42 showed significantly decreased ($p<0.05$) expression compared to PND7, PND14 and PND65 and showed significant increased ($p<0.05$) compared to PND1.The data are represented as the mean ± SEM. Different alphabet (a,b,c) showed significant difference ($p<0.05$).

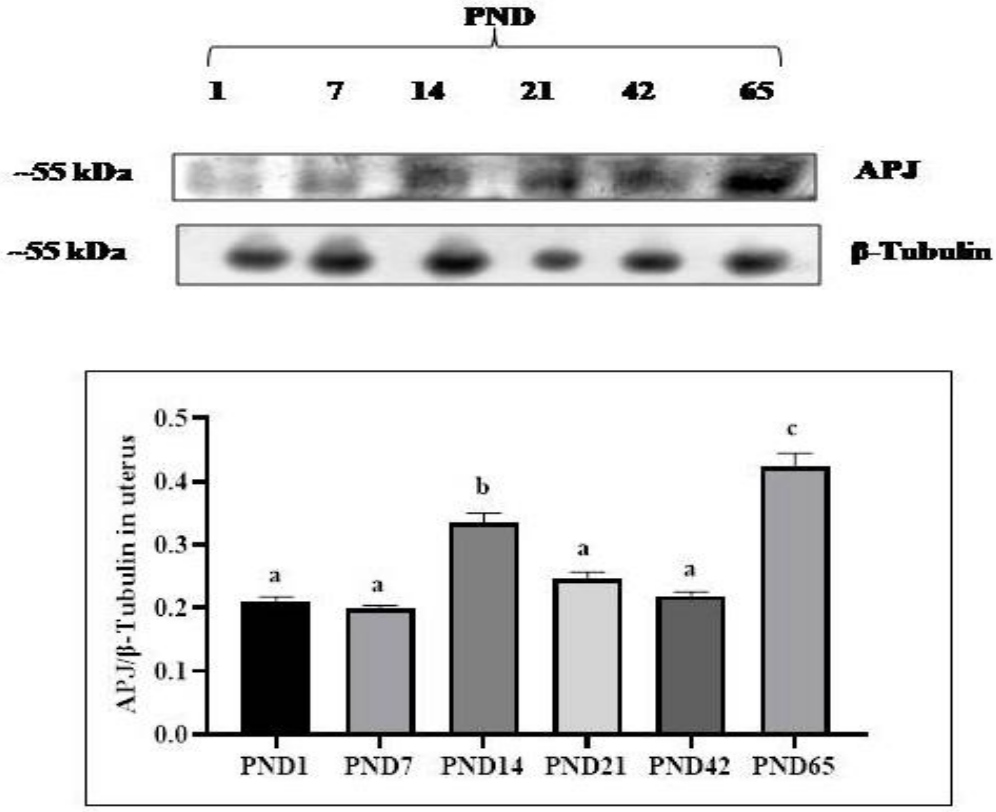

Fig. 1.6: Expression of APJ in uterus at different postnatal stages of mouse. In uterus, PND65 showed a significant increase ($p<0.05$) in APJ compared to other groups as well as PND14 also showed a significant increase ($p<0.05$) compared to other group but showed significantly decreased ($p<0.05$) compared to PND65 group. The data are represented as the mean ± SEM. Different alphabet (a, b, c) showed significant difference ($p<0.05$).

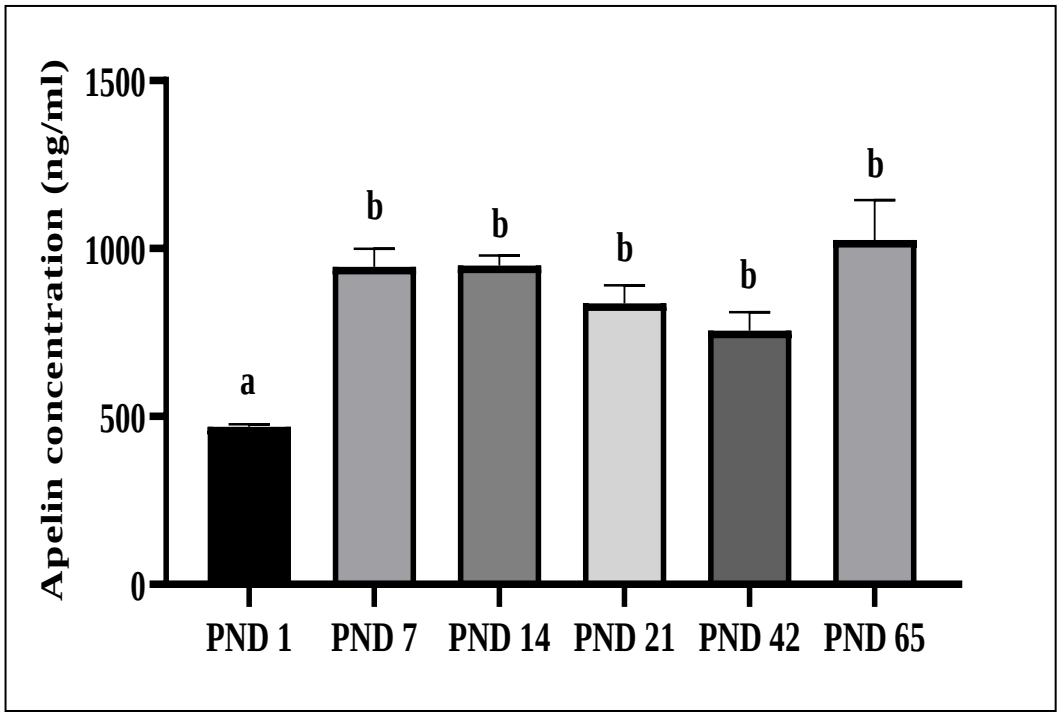

Fig. 1.7: Estimation of circulating level of apelinin postnatal developmental stages of mouse. Serum apelin level showed significantly increased in all the groups compared to PND1.The data are represented as the mean ± SEM. Different alphabet (a,b) showed significant difference ($p<0.05$).

TABLE 1.1: Distribution of immunohistochemical staining of apelin in the ovary at different postnatal stages.

Group/ Follicle	PND1	PND7	PND14	PND21	PND42	PND65
Primordial follicle	+	++	++	+	+	+
Primary follicle		++	++	+	+	++
Secondary follicle		++	+++	+	+	+++
Antral follicle				+	++	+++
Corpus luteum					++	+++

Score for intensity of immunoreactivity are as follows: +, mild; ++, moderate; +++, intense; PND: postnatal day.

TABLE 1.2: Distribution of immunohistochemical staining of apelin receptor in the ovary at different postnatal stages.

Group/Follicle	PND1	PND7	PND14	PND21	PND42	PND65
Primordial follicle	+	+	++	+	+	+
Primary follicle		+	++	+	+	++
Secondary follicle		++	+++	+	++	+++
Antral follicle				++	++	+++
Corpus luteum					+	+++

Score for intensity of immunoreactivity are as follows: +, mild; ++, moderate; +++, intense; PND: postnatal day.

TABLE 1.3: Distribution of immunohistochemical staining of apelin in the uterus at different postnatal stages.

Group/Part of Uterus	PND1	PND7	PND14	PND21	PND42	PND5
Luminal epithelium	+	++	+++	+	+	+++
Stromal cell	+	+	++	+	++	+
Endometrium		++	+++	+	++	++
Myometrium		+	+++	+	++	+++
Perimetrium		+	++	+	+	++
Gland			++	+	+	++

Score for intensity of immunoreactivity are as follows: +, mild; ++, moderate; +++, intense; PND: postnatal day.

TABLE 1.4: Distribution of immunohistochemical staining of apelin receptor in the uterus at different postnatal stages.

Group/Part of Uterus	PND1	PND7	PND14	PND21	PND42	PND5
Luminal epithelium	++	++	+++	+	++	+++
Stromal cell	++	++	+++	+	+	++
Endometrium		++	+++	+	++	++
Myometrium		+	+++	+	+	+++
Perimetrium		++	+++	+	+	+++
Gland			+++	++	++	+++

Score for intensity of immunoreactivity are as follows: +, mild; ++, moderate; +++, intense; PND: postnatal day.

CHAPTER 2

Title

Possible role of apelin on the ovarian steroidogenesis and uterine apoptosis of infantile mice: An in vitro study.

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Chapter 2

Introduction

The female fertility determines by the oocyte reservoir of neonatal ovary. Mammalian ovary contains a fixed number of highly specialized oocytes and the multi-potential stromal cells which further contribute to the follicles (Hirshfield., 1991; Perez et al., 1999). Although, formation of mice ovary begins during the twelfth to eighteenth day of gestation, however, folliculogenesis and steroidogenesis are very likely to change during postnatal stages (Odor and Blandau, 1969a, Mannan and O'Shaughnessy., 1991). The ovary grows in size and undergoes a remarkable development in about 5 weeks after birth that contains large follicles (Graafian follicle) and oocytes (eggs or ova) which eventually ovulate during puberty. The number of oocytes gradually decline from pre-pubertal to adult life (Peters., 1969; Tilly, 2001). Previous reports mentioned that estrogen regulates the initial recruitment of follicle and then the follicle development depends on follicle stimulating hormone (FSH), which is mostly cooperates with intra-ovarian factors (Peters., 1969; Kumar et al., 1997; McGee and Hsueh, 2000; Hsueh et al., 2015).

In mammals, normal postnatal uterine development is also important for the reproduction. At birth, uterus contains only undifferentiated mesenchyme cells with a simple epithelium lined by central lumen. Uterine adenogenesis in the rodent is also a postnatal event, which takes place around postnatal day 7 (PND7) (Vue and Behringer, 2020; Gray et al., 2001). Uterine glands are important for survival and development of the mammalian embryo (Gray et al., 2001). Mechanism undertaking neonatal uterine proliferation, differentiation and adenogenesis is a complex process including involvement of many factors such as growth factors, steroid hormones and also a number of genes (Stewart et al., 2011). Although, enormous progress can be seen in understanding the events and regulations of ovary and uterine function in the adult, the regulation of earlystage development is still seems to be ambiguous. Furthermore, pubertal ovarian and uterine function might be dependent on the factors present in the pre-pubertal stages (Annie et al., 2020). One of the promising factors that regulate the

reproduction is known as adipokine. Earlier study demonstrated that adipose tissue is the main source of adipokines, other organs including female reproductive organs can also synthesized adipokines (Campos et al., 2008). Adipokines involved in the regulation of gonad function and also regulate reproductive axis (Tersigni et al., 2011). In female, adipokines regulates the ovarian dynamics, cell proliferation and folliculogenesis and also involved in the ovulation process (Campos et al., 2008). It has been shown that adipokines such as chemerin, visfatin, resistin and apelin are able to regulate reproductive functions (Estienne et al., 2019; Reverchon et al., 2013; Annie et al., 2020). The adipokines, apelin, was isolated from bovine stomach and which is a ligand for apelin receptor (APJ) (Tatemotoet al., 1998). APJ was considered as an orphan G-protein coupled receptor at the time of its discovery (O'Dowd et al. 1993). Apelin/APJ system is abundantly distributed in female reproductive system including ovary, uterus, oviduct and placenta, and hypothalamo-pituitary-ovary (HPO) axis (Roche et al., 2016; Yang et al., 2019; Wang et al., 2022; Anima 2022;2023). The APJ belongs to a class A (rhodopsin-like) G-protein-coupled receptor with a wide presence in the various organs and it has been shown that APJ mediated signal transduction produced by a combination of Gαi protein subtypes (Chapman et al., 2014; Habata et al., 1999). β-arrestins play a crucial role as signal transducers, and G-protein-coupled receptor mediated biological functions also requires β-arrestin-dependent signaling. The apelin mediated APJ activation occurs thorough Gαi pathway, and phosphorylation of APJ, which occurs at the C-terminal region, is critical for the recruitment of β-arrestins (Chen et al., 2020).Apelin has been reported to regulate steroidogenesis including secretion of gonadotropin releasing hormone, gonadotropin and steroid hormones and also play role in oocyte maturation (Bertrand, Valet &Castan-Laurell, 2015; Yang et al., 2019).One study in rat showed that, apelin promotes the proliferation of granulosa cells and suppress apoptosis (Shuang et al. 2016). Apelin (APLN) and APJ demonstrated to elevate in granulosa cells, thecal cells and luteal cells during follicular growth in bovine (Shimizu et al., 2009; Mercati et al 2019) and porcine (Rak et al., 2017). In bovine, apelin system has been shown to regulate in the corpus luteum (Shirasuna et al., 2008;

Schilffarth et al., 2009). Apelin also shown to inhibits human uterine contractility in an *in vitro* experiment (Hehir et al., 2012). A recent study suggested that apelin/APJ system expressed in uterus during peri-implantation and might be involved in the implantation process and decidualisation (Balci et al., 2023). Although, a few studies on the apelin have been reported in the adult ovary and uterus, and still there is a dearth of literature on the role of apelin during postnatal development of ovary and uterus. Our recent study demonstrated the expression of apelin and APJ in the postnatal ovary and uterus and found to be developmentally regulated at different postnatal stages. Apelin expression was found to be elevated in postnatal day 14 (PND14) and in adult (PND65) (Anima et al., 2022); however, the functions this elevated apelin and APJ at PND14 has not been investigated.Thus, we hypothesized that apelin might regulate steroidogenesis, apoptosis, and proliferation in the ovary and uterus an early developmental stage. Therefore, the present study investigated the possible role of apelin on the ovarian steroidogenesis and uterine apoptosis of infantile mice by *in vitro* approach.

Materials and methods

Animals and ethical statement

Swiss albino inbred mice were used in this experiment and housed in Mizoram University Animal house following the protocols approved (Protocol Number, MZU/IAEC/ 2020/12) by the Mizoram University Institutional Animal Ethical Committee 8, Mizoram University, Mizoram, India and compiled with ARRIVE guidelines. The mice were maintained under controlled laboratory condition of 12h light/dark cycle at 25 ± 2 °C temperature and with water and food ad libitum. Breeding sets were observed regularly for new born pups. 18 female pups on PND14 were collected and sacrificed under mild anesthesia then ovaries and uteri were harvested immediately for *in vitro* study.

Experimental design

To explore the role of apelin in proliferation, apoptosis and steroidogenesis, apelin activity was blocked with apelin inhibitor or apelin receptor antagonist, 4-oxo-6-(pyrimidin-2-ylthio) methyl)-4H-pyran-3-yl 4-nitrobenzoate (ML221) in the PND14. The ovary and uterus from PND14 were cultured in the presence of ML221 (cat# SML0919, Sigma-Aldrich Chemicals Pvt Ltd, United States). The ovaries (N=24) and uterus (N=12) were cleaned carefully from adhering fats and other tissue. The ovaries (n = 6/group) and uterus (equal fragments 1-2 mm, n=6/group) were then cultured for 24 hours following previous method (Islam et al., 2017; Singh et al., 2015). In brief, tissues were cultured in a medium containing the mixture of Dulbecco Modified Eagle'sMedium and Ham's F-12 (cat no-AL155 G, Himedia, Mumbai, India) with 100 U/mL penicillin, 100 µg/mL streptomycin and 0.1 % BSA (Sigma Aldrich St Louis, USA) at 37°C temperature, 5% CO_2 and a humidified atmosphere with 95% air. Both ovary and uterus were cultured in three groups: (1) Control group (Con) ovaries, cultured only in media, (2) 3µM of ML221 treated group (ML3µM) and (3) 50µM of ML221treated group (ML50µM), cultured in the presence of ML221 at a dose of 3µM and 50µM, respectively. The doses of ML221 were selected from the previous reports by (Ishimaru et al., 2017; Anima et al., 2023). The culture was done with all the precautionary measures to prevent contamination of cultured media. The ovaries were cultured as one ovary per 1mL of media and one fragment of uterus per 1mL of media. After 24 hours of culture, ovaries and uteri were harvested and cleaned with PBS. Two ovaries and 2 fragment of uterus per group were fixed in Bouin's fluid for Immunohistochemistry analysis and the remaining per groups were frozen immediately at-20 °C for western blot analysis. Media were then collected for ELISA. The experiment was repeated two times.

Slide preparation and Immunohistochemistry

Fixed ovary and uterus samples were used for the immunohistochemistry evaluation. In order to prevent brittleness, the samples were transferred to 70% ethanol within 24 hours

and proceed to block making following paraffin embedded method (Bancroft & Gamble, 2002). Tissue paraffin blocks were cut into 5-7μM thick ribbons using a Leica rotary microtome (RM2125 RTS). The tissue sections were mounted onto previously poly-l-lysin coated slides, spread out evenly in warm water (maximum temperature 45°C), and then placed on the slide warming table at 37°C.

The slides containing tissue sections were processed for immunohistochemical localization of using previously described method by Gurusubramanian and Roy (2014). Briefly stated, the slides were deparaffinized in xylene, and then subjected to a series of graded alcohol treatments (100%, 90%, 70%), followed by a 10-minute treatment with 3% H2O2 in methanol to inhibit endogenous peroxidase. Sections were blocked with goat serum followed by incubation with primary antibodies (Table.1) for overnight at 4°C. The unbound antibody was washed off with PBS and incubated with the respective secondary antibodies except for 3β-HSD antibody (Table.1) for 4 hours at room temperature. After a second PBS wash, the slides were incubated with the chromagen substrate (0.1% 3 3′ diaminobenzidine tetrahydrochloride (DAB) in 0.5 M Tris 7.6 and 0.01% H2O2) until a brown color formed. Hematoxylin (except for PCNA and GCNA antibodies) was then counterstained the slides for 30 seconds. The slides were then washed in xylene, dehydrated with various degrees of alcohol (70%, 90%, and 100%), and mounted with DPX. The slides were examined and photographed using a microscope (iScope series, Euromex, Netherlands). The DAB-stained area of immunostained slides was quantified using Image J's threshold tool. The data was shown as a percentage of the staining area. The negative control section was replaced with non-immune IgG (mice or rabbit) in place of primary antibody.

Western blot analysis

Following Jeremy et al. (2017), western blotting was done to examine the expression of the estrogen receptors (ERα and ERβ) and B-cell lymphoma 2 (BCL2). Briefly, the froze ovary and uterus samples were homogenate (20%, w/v) with the lysis buffer (0.01M Tris-HCl (pH 7.6), 1mMEDTA (pH 8.0), 1M NaCl, 100μg/ml phenylmethylsulfonyl

fluoride and distilled water) and estimated the protein following Bradford method (Bradford., 1976) by spectrophotometer (BioSpectrometer, Eppendorf, Germany). Equal quantities (50μg) of protein from each sample were resolved into a 12% sodium dodecyl sulfate-polyacrylamide (SDS) gel that had already been prepared, and the resolved protein was then transferred onto a PVDF membrane using a wet transfer apparatus. The membranes were blocked with 5% nonfat skimmed milk in PBST (Cat# GRM1254-500G; HiMedia Laboratory private limited, Mumbai), which then allowed the primary antibody (Table.1) to bind for an overnight period at 4°C. The membranes were washed off with PBST and then incubated with respective horseradish peroxidase-conjugated secondary antibodies (Table.1) for 4 hours. PBST was used to wash any unbound secondary antibody. The membranes underwent a 5-minute electrochemiluminescence incubation procedure before being developed onto X-ray film. The band intensities were quantified by using ImageJ software (imagej.nih.gov/) with respect to loading control after stripping and reprobing the membranes with β-Tubulin (Table.1).

Quantification of steroid hormones (Estrogen, Progesterone, testosterone, androstenedione) by Enzyme-linked Immunosorbent Assay (ELISA)

Commercially available ELISA kits were used to quantify the released steroid hormone level in the media collected after the *in vitro* culture. Estradiol (Cat# DKO003, DiaMetra, Italy), Progesterone(Cat # RH-351, DSI, Saronno, Italy), testosterone (Cat# DKO002, Diametra, Italy) and androstenedione (Cat# DKO008, DiaMetra, Italy) kits were used following the instruction manual provided with the kit.Absorbancewas read at 450 nm of wavelength by a Microplate Reader (Erba Lisa Scan EM, Trans Asia Biomedical Ltd, Mumbai, Maharashtra, India). The intra-assay variability for estrogen, progesterone, testosterone and androstenedione were ≤ 9%, ≤ 4%, ≤ 7.0% and 10.0%, respectively. The inter-assay variability for estrogen, progesterone, testosterone and androstenedione were ≤ 10%, ≤ 9.3%, ≤ 8.3% and ≤ 9.3%, respectively.

Statistical analysis

All the numerical experimental data were expressed as mean ± standard error mean (SEM). The statistical significance was assessed using the GraphPad Prism 9 software by one-way analysis of variance (ANOVA) following Tukey's test. A significant difference was considered if the P value is less than 0.05 ($p < 0.05$).

Result

Effect of APJ antagonist (ML221) on the ovarian steroid hormones (estrogen, progesterone, testosterone and androstenedione) secretion

Effect of APJ inhibition on the ovarian steroid hormones (estrogen, progesterone, testosterone, and androstenedione) secretion by infantile ovary. Hormones were quantified by ELISA in the media after ML221 *in vitro* treatment. Estrogen secretion showed a significant ($p<0.05$) decrease in the 50μM treated ML221 group (11.28±0.08 pg/ml) compared to the control group (48.47±1.23 pg/ml)and 3μM treated ML221 group (57.99±1.77 pg/ml). However, the low concentration of ML221 (3μM) did not show significant ($p>0.05$) change compared to control group (Fig.2.1A).

The ML221 treated groups showed a significant ($p<0.05$) increased level of progesterone as compared to the control group (1.26±0.11 pg/ml). However, there was no significant ($p>0.05$) change between the 3μM(2.32±0.06 pg/ml) and 50μM(2.46±0.16 pg/ml) of ML221 treated groups (Fig.2.1B).

The 50μM treated ML221 group (1.66±0.39 nmol/L) showed a significant ($p<0.05$) decreased level of testosterone as compared to the control group (3.93±0.02 nmol/L) and 3μM of ML221 treated group (3.08±0.25 nmol/L), whereas, there was no significant

($p>0.05$) change found between 3μM treated ML221 group and control group (Fig.2.1C).

The 50μM treated ML221 group (0.19±0.008 ng/ml) showed a significant ($p<0.05$) decreased level of androstenedione as compared to the control group (0.23±0.004 ng/ml) and the 3μM of ML221 treated group (0.24±0.007 ng/ml). However, the low concentration of ML221 (3μM) group did not show significant ($p>0.05$) change compared to control group (Fig.2.1D).

Effect of APJ antagonist (ML221) on the immunostaining of3β-HSD, 17β-HSD and aromatase in the infantile ovary

To unravel the effect of apelin on ovarian steroidogenesis, APJ was inhibited with ML221, and steroidogenicmarkers3β-HSD, 17β-HSD, and aromatase were observed by immunohistochemical analysis. The 3β-HSD staining was observed in the granulosa cells, thecal cells and the oocytes. Immunostaining of 3β-HSD showed intense immunostaining in the 50μM and 3μM of ML221 treated groups (Fig.2.2c, b) and faintly stained in the control group (Fig.2.2a). The ML221 treated groups showed a significant ($p<0.05$) increased stained % area of 3β-HSDas compared to the control group. However, there was no significant ($p>0.05$) change between the 50μM and 3μM of ML221 treated groups (Fig.2.2e).

The 17β-HSD staining was observed in the granulosa cells, thecal cells and oocytes. Immunostaining of 17β-HSD showed moderate immunostaining in the 50μM and 3μM of ML221 treated group (Fig.2.3c, b) and mild staining in the control group (Fig.2.3a). The ML221 treated groups showed a significant ($p<0.05$) increased stained % area of 17β-HSD as compared to the control group (Fig.2.3e). However, there was no significant ($p>0.05$) change between the 50μM and 3μM of ML221 treated groups.

The immunostaining of aromatase was observed in the granulosa cells and oocytes of the infantile ovary. Aromatase showed moderate immunostaining in the 50μM and 3μM of ML221 treated groups (Fig.2.4c, b) and mild staining in the control group (Fig.2.4a).

The ML221 treated groups showed significant ($p<0.05$) increased stained % area of aromatase as compared to the control group (Fig.2.4e). However, there was no significant ($p>0.05$) change between the 50μM and 3μM of ML221 treated groups.

Effect of APJ antagonist (ML221) on the expression of 17β-HSD and aromatase in the infantile ovary

Expression of 17β-HSD showed a significant ($p<0.05$) increased in the 50μM of ML221 treated group as compared to the 3μM of ML221 treated group and control group, whereas, there was no significant ($p>0.05$) change observed between the control group and the 3μM of ML221 treated group(Fig.2.5A).

The expression of aromatase showed a dose-dependent increased in the ovary after apelin inhibition by ML221 treatment. The 50μM of ML221 treated group showed significant ($p<0.05$) increased expression of aromatase as compared to the 3μM of ML221 treated group and control group. Whereas, the 3μM of ML221 treated group also showed significant ($p<0.05$) increased expression of aromatase as compared to the control group (Fig.2.5B).

Effect of APJ antagonist (ML221) on the expression of estrogen receptors (ERα and ERβ) in the infantile ovary

Expression of ERα showed a dose-dependent downregulation in the PND14 mice ovary after apelin inhibition by ML221 treatment. The ERα expressionin the 50μM of ML221 treated group significantly ($p<0.05$) decreased as compared to the 3μM of ML221 treated and control groups. Whereas, the 3μM of ML221 treated group also showed significant ($p<0.05$) decreased in ERα expression as compared to the control group (Fig.2.6A). The ERβ expression showed a significant ($p<0.05$) decreased in both the ML221 treated group as compared to the control group, whereas, no significant ($p>0.05$) change was observed between the 50μM of ML221 treated group and the 3μM of

ML221 treated group. It should be noted that despite non-significant changes between both the ML221 treated groups, low dose of ML221 deceased the expression Erβ than higher dose. This result indicated that low dose of ML221 (ML3μM) group might be physiologically more potent for ERβ regulation than high concentration (ML50μM) (Fig.2.6B).

Effect of APJ antagonist (ML221) on the anti-apoptotic marker, BCL2 in the infantile mice ovary

To explore the role of apelin in the apoptosis of the ovary, APJ was inhibited by ML221 and anti-apoptotic marker BCL2 was checked. The BCL2 expression showed a dose-dependent downregulation in the ML221 treated groups. The 50μM of the ML221 treated group showed significantly ($p<0.05$) decreased BCL2 expression as compared to the 3μM of ML221 treated group and control group. Whereas, the 3μM of ML221 treated group also showed significantly ($p<0.05$) decreased BCL2 expression as compared to the control group (Fig.2.6C).

Effect of APJ antagonist (ML221) on the apoptotic marker active caspase3 in the infantile mice ovary

Immunolocalization of active caspase3 showed variation of expression in the control and ML221 treated groups. Immunostaining of active caspase3 was observed in the granulosa cells, thecal cells, oocytes, and interstitial cells of infantile mice ovary. The ML221 treated groups showed intense staining of active caspase3and the control group showed moderate immunostaining of active caspase3 respectively (Fig.2.7a-f). The ovaries ofML221 treated groups showed significant ($p<0.05$) increased stained % area of active caspase3 as compared to the control group. However, there was no significant ($p>0.05$) change between the 50μM and 3μM of ML221 treated groups (Fig.2.7h).

Effect of APJ antagonist (ML221) on the proliferation of the infantile mice ovary

To unravel the effect of apelin on ovarian proliferation after APJ inhibition with ML221, proliferation marker PCNA and GCNA were observed by immunohistochemical analysis. Immunostaining of PCNA showed intense immunostaining in the ML221-treated groups and moderate staining in the control group (Fig.2.8A). The ovaries of ML221 treated groups showed significant ($p<0.05$) increased stained % area of PCNA as compared to the control group. However, there was no significant ($p>0.05$) change between the 50μM and 3μM of ML221 treated groups (Fig.2.8B). It should be noted that despite non-significant changes between both the ML221 treated groups, low dose of ML221 increased PCNA stained area than higher dose. This result indicated that low dose of ML221 (ML3μM) group might be physiologically more potent for ERβ regulation than high concentration (ML50μM).

Immunostaining of GCNA showed intense immunostaining in the 50μM of ML221 treated group and moderate staining in the control group and the 3μM of ML221 treated group (Fig.2.9A). The ovaries of ML221 treated groups showed significant ($p<0.05$) increased stained % area of GCNA as compared to the control group. However, there was no significant ($p>0.05$) change between the 50μM and 3μM of ML221 treated groups (Fig.2.9B).

Effect of APJ antagonist (ML221) on the expression of estrogen receptors (ERα and ERβ) in the infantile mice uterus

Expression of ERs showed downregulation in the PND14uterus after *in vitro* APJ inhibition by ML221 treatment. The ML221 treated group showed significant ($p<0.05$) decreased expression of ERs as compared to the control group. Whereas, there was no significant ($p>0.05$) change observed between the 3μM of ML221 and the 50μM of ML221treated groups (Fig.2.10A, B).

Effect of APJ antagonist (ML221) on the anti-apoptotic marker BCL2 in the infantile mice uterus

To explore the role of apelin in uterine apoptosis, the anti-apoptotic marker BCL2 was checked after APJ inhibition. The 50μM of ML221 treated group showed significantly ($p<0.05$) decreased BCL2 expression as compared to the 3μM of ML221 treated group and control group. Whereas, there was no significant ($p>0.05$) change observed between the control and the 3μM of ML221 treated group (Fig.2.10C).

Effect of APJ antagonist (ML221) on the apoptotic markers active caspase3 in the infantile mice uterus

Immunolocalization of active caspase3 showed variation of expression in the control and ML221 treated groups. Immunostaining of active caspase3 was observed in the endometrium, myometrium, perimetrium, uterine gland and also in the luminal epithelium. The ML221 treated group showed moderate staining of active caspase3 and very mild staining was observed in the control group (Fig.2.11a-f).The uterus of ML221 treated groups showed a significant ($p<0.05$) increased stained % area of active caspase3 as compared to the control group. However, there was no significant ($p>0.05$) change of stained % area between the 50μM and 3μM of ML221 treated groups (Fig.2.11h).

Effect of APJ antagonist (ML221) on the proliferation marker PCNA in the infantile mice uterus

To explore the role of apelin in uterine proliferation, PCNA was checked after APJ inhibition. The ML221 treated groups showed significantly ($p<0.05$) increased PCNA expression as compared to the control group. Whereas, there was no significant ($p>0.05$) change observed between the 3μM and the 50μM of ML221 treated groups (Fig.2.12).

Discussion

The postnatal ovarian and uterine development depends on the various factors, which might act as an autocrine and paracrine manner. Adipokines have emerged as regulator

of postnatal development and many experimental animal models of developmental programming have been used to study changes in adipokine profiles (Reynolds and Vickers 2019). Recently we have shown that apelin and APJ are developmentally regulated in the ovary and uterus of mice (Anima et al., 2022). We have shown the two peaks of APJ system in the ovary and uterus of mice; one at early postnatal stage PND14 and other at adult (Anima et al., 2022). Since functional significance of APJ in ovary and uterus in early postnatal stage has not been investigated, therefore, the present study has attempted to unravel the physiological significance of apelin system in the ovary and uterus of mice at early postnatal stage by in vitro approaches. APJ, antagonist, ML221 has been used to unravel the possible role of apelin system in the uterus and ovary of mice at early postnatal stage on the steroidogenesis, proliferation and apoptosis. The treatment of ML221 decreased the secretion of estrogen, testosterone and androstenedione while secretion of progesterone was elevated. It has been shown that the circulating estrogen, progesterone and testosterone levels during different postnatal age of female mice exhibits different pattern (Bell 2018). It has been shown that mice ovary from PND7-PND21 acquires the enzymes activities for steroidogenesis (Mannan et al., 1991). The levels of estrogen in first week of mice were found to be low (Dutta et al., 2014) and estrogen secretion from PND26-PND29 (Ahima et al., 1997). The circulating testosterone in different strain was also found to be low from birth to PND10 and showed increased at PND21 (Corbier et al., 1992; Poling and Kauffman 2012; Pang and Tang 1984; Jean-Faucher et al., 1978). The levels of progesterone were found be elevated at birth followed by decline at PND7; however, gonadotropin mediated progesterone secretion has been reported to initiates at PND10 (Dutta et al., 2014) and elevated subsequently (Mannan and O'Shaughnessy 1988). These results suggest that apelin modulates ovarian steroid biosynthesis in the early postnatal stage (PND14), probably may be involved in the promoting estrogen secretion and suppressing progesterone. Since progesterone is synthesized from pregnenolone by 3βHSD, thus we have also analysed the localization of 3βHSD in the ovary after ML221 treatment. Our results showed that ML221 treatment increased the 3βHSD abundance which also

supports the findings on the elevated progesterone secretion by ML221 treatment. We have also analysed the 17βHSD and aromatase expression by immunohistochemistry and western blot and the abundance and expression of 17βHSD and aromatase was increased after ML221 treatment. However, increased aromatase and 17βHSD followed by decreased estrogen androstenedione secretion remains unclear from our findings. This is very important limitation of our study, which could not explain this discrepancy.

Estrogen has been shown play important role in the postnatal ovary and both receptors (ERα and β) are necessary for the maintenance of germ and somatic cells (Couse et al., 1999). APJ antagonist treatment also down-regulated the expression of ERα and β in the ovary of PND14, which coincides with decreased estrogen secretion and these results suggest the decreased estrogen signaling by APJ antagonist. Since inhibition seems to decrease the estrogen signaling, thus, endogenous apelin signaling could be involved in the stimulated estrogen signaling in the ovary of PND14 mice. It has also been shown that estrogen signaling and apoptosis can be linked to Bcl2 protein (Chen et al., 2009). Our results also showed that APJ, antagonist decreased the expression of Bcl2 and increased the abundance of active caspse3 in the PND14 ovary. These findings suggest that apelin signaling could be involved in the maintenance and survival of ovarian follicles by suppressing apoptosis. The localization of two proliferation markers PCNA and GCNA were also down-regulated by APJ antagonist, which suggest that apelin mediated suppression of ovarian cell proliferation. Our result is consistent with previous study which demonstrated apelin can inhibit apoptosis in granulosa cells of rat ovary via PI3/Akt signaling pathway (Shuang et al., 2016). Apelin also found to inhibit apoptosis in osteoblastic cell of humans and mice (Tang et al., 2007; Xie et al., 2007).

In addition to ovary, we have also investigated the effect of APJ inhibition on the uterine ERs expression along with active caspase3 and Bcl2. During the postnatal period and the estrous cycle, the uterus experiences significant changes, including proliferation and apoptosis (Roberto da Costa et al., 2007; Wu et al., 2017). Recently we have also shown the expression of apelin system in the uterus at different postnatal stages with elevation

at PND14 and we also suggested that uterine apelin regulates the apoptosis (Anima et al., 2022). To best of our knowledge, no work has been done on the adipokine in relation to postnatal uterine functions; however, various adipokines are known to express in the uterus of adult and play important role in uterine contractility and placenta (Estienne et al., 2019). It has been shown that postnatal uterine growth may dependent the various factors including hormones (Franco et al., 2011; Vue and Behringer, 2020; Stewart et al., 2011). Our results showed that inhibition of APJ, down-regulated the expression of ERs, and Bcl2 and increase the abundance of active caspse3 in the uterus of mice at PND14. These findings suggest that endogenous apelin and estrogen signaling could suppress the apoptosis in the uterus by up-regulation of Bcl2 expression. Whether apelin directly or via estrogen signaling regulates the uterine apoptosis in the PND14 mice remains unclear and further study would be required to unravel the exact mechanism. Our result showed that the expression of proliferation marker PCNA was up-regulated by APJ antagonist, which suggest that apelin mediated suppression of uterine cell proliferation. The uterine development is a dynamic event which continues reproductively viable period of a female's life and this process occurs over an extended time rather than only during a defined postnatal period (Stewart et al., 2011). The postnatal uterine gland attains a normal growth in the ovariectomized and/or adrenalectomized rat at PND6; however, decreased uterine growth was also observed (Branham et al., 1995). It has been suggested that postnatal estrogen and progesterone is required for uterine growth to occur (Stewart et al., 2011). On the other hand, it has also been reported that postnatal (neonatal) uterine development is estrogen-independent (Bigsby et al., 1985). It would have been interesting and necessary to investigate whether the effect of apelin could be reversed by apelin supplementation. This experiment would have further strengthened the role of apelin in the ovarian and uterine physiology of infantile mice. This is also an important limitation of present study which requires further investigation. It should also be noted that present study has shown the role of apelin on the ovarian and uterine apoptosis by investigating Bcl2 and active caspase3. However, it has also been reported that the progression of apoptosis and the

time of this histone release from DNA are highly correlated and DNA fragmentation may be initiated during apoptosis (Wu et al., 2002; Rogakou et al., 2000). Thus, histone fragmentation study would have further strengthened the apelin mediated apoptosis in ovary and uterus of infantile mice.

Conclusion

In conclusion, our results showed that apelin signaling would be important for ovarian estrogen synthesis in the juvenile mice (PND14). Furthermore, apelin signaling suppresses the ovarian and uterine apoptosis via up-regulating Bcl2 expression in PND14 mice. It should also be noted that inhibition of apelin signaling by ML221 also decreased the estrogen signaling in the ovary and uterus, which was indicated by down-regulation of estrogen receptor. Finding also suggested that apelin might suppress cell proliferation in infantile ovary and uterus.

Summary

The expression of adipokines is well-known in the ovary and uterus. Recently we have shown that apelin and its receptor, APJ are developmentally regulated in the ovary and uterus of mice with elevation at postnatal day 14 (PND14). However, its role in the ovary and uterus of PND14 has not been investigated. Thus, we aimed to unravel the role of the apelin system (by APJ antagonist, ML221) on ovarian steroid secretion, proliferation, and apoptosis along with its role in uterine apoptosis in PND14 mice by *in vitro* approaches. The treatment of ML221 decreased estrogen, testosterone, and androstenedione secretion while increasing the progesterone secretion from the infantile ovary. These results suggest that apelin signaling would be important for ovarian estrogen synthesis in infantile mice (PND14). The abundance of 3β-HSD, 17β-HSD, aromatase, and active caspase3 increased in the infantile ovary after ML221 treatment. The expression of ERs and BCL2 were also down-regulated by ML221 treatment. The decreased BCL2 and increased active caspase3 by ML221 suggest the suppressive role of apelin on ovarian apoptosis. The APJ antagonist treatment also down-regulated the

ERs expression in the uterus along with increased active caspase3 and decreased BCL2 expression. The APJ antagonist treatment also up-regulated the PCNA expression in the uterus. In conclusion, apelin signaling inhabits the ovarian and uterine apoptosis via estrogen signaling in the ovary and uterus.

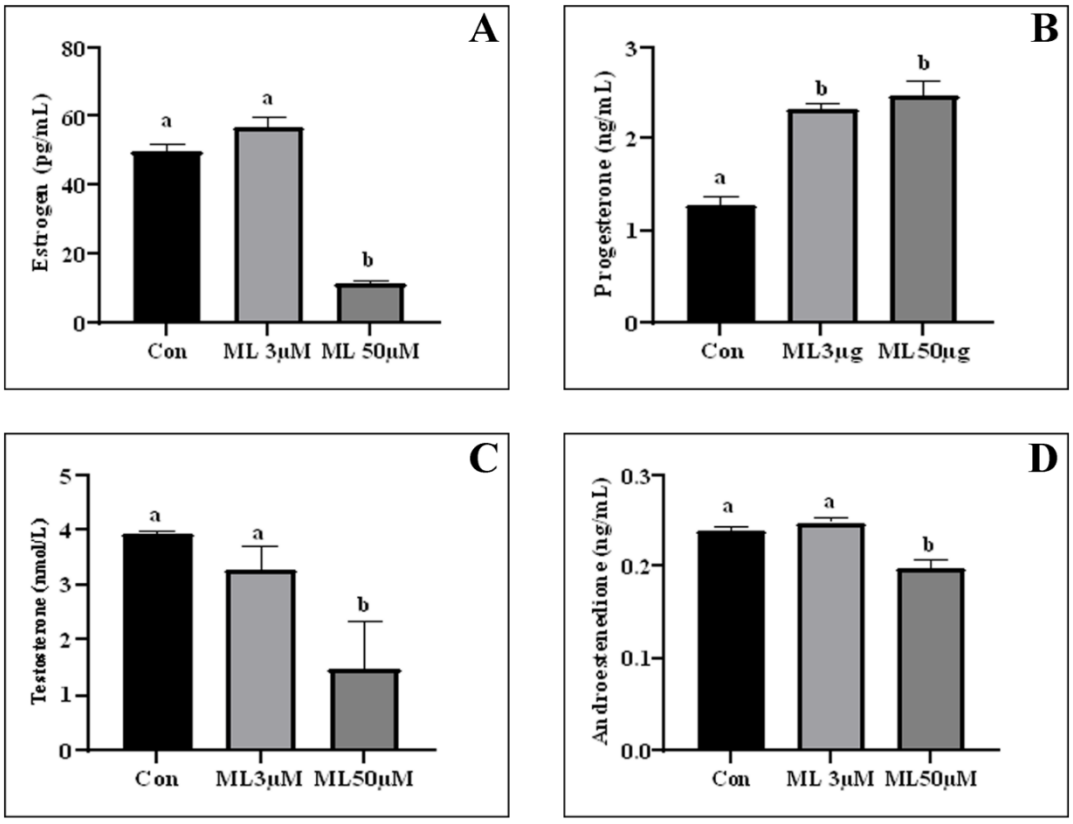

Fig.2.1. Effect of APJ antagonist on infantile ovarian steroid secretion *in vitro*. A, Estrogen secretion showed a significant ($p < 0.05$, $n = 8$) decrease after treatment of 50μM of ML221. B, Progesterone secretion showed a significant ($p < 0.05$, $n = 8$) increase after treatment of ML221. C, Testosterone secretion showed a significant ($p < 0.05$, $n = 8$) decrease after treatment of 50μM of ML221. D, Androstenedione secretion showed a significant ($p < 0.05$, $n = 8$) decrease after treatment of 50μM of ML221. Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

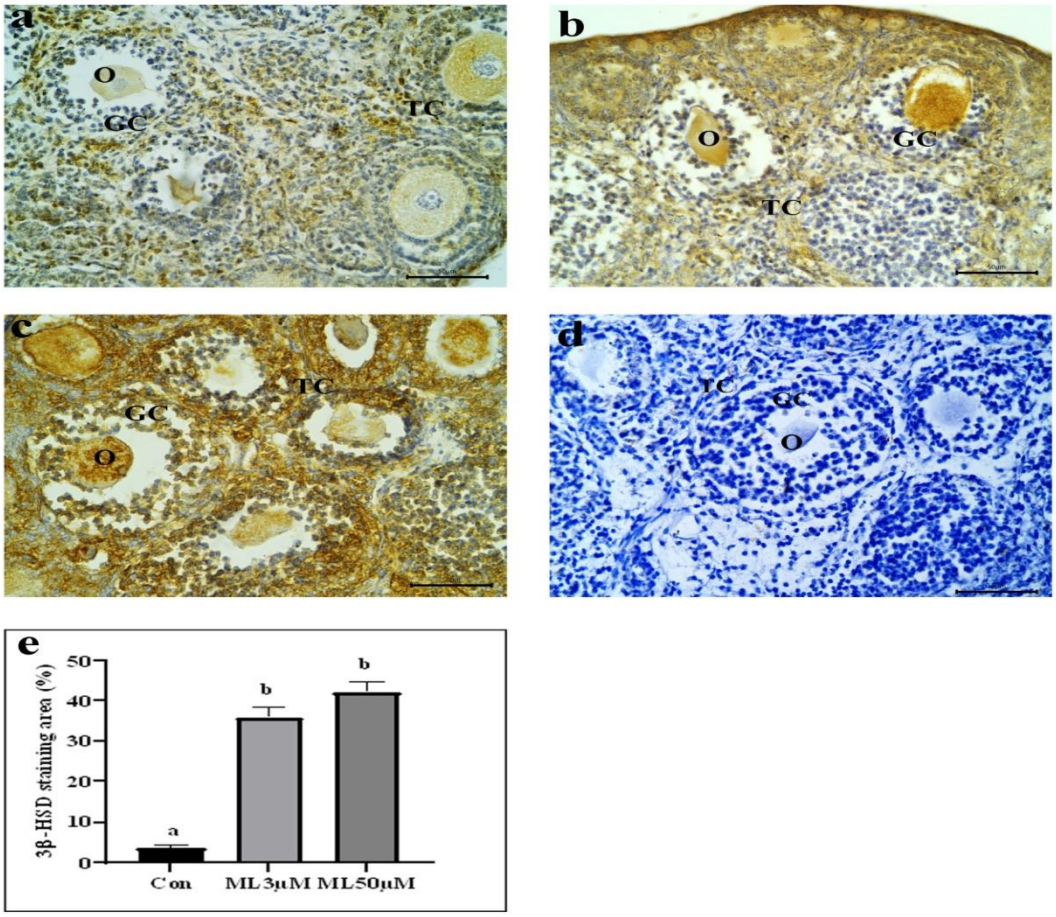

Fig.2.2. Localization of 3β-HSD on the infantile ovary after APJ antagonist (ML221) treatment (a-e). a, b, c and d showed ovaries of the control group, 3μM of ML221 group, 50μM of ML221 group and negative control, respectively at a magnification of 40X. O-oocyte; GC-Granulosa cells; TC-Theca cells. d showed an estimation of the stained percentage area of 3β-HSD. Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

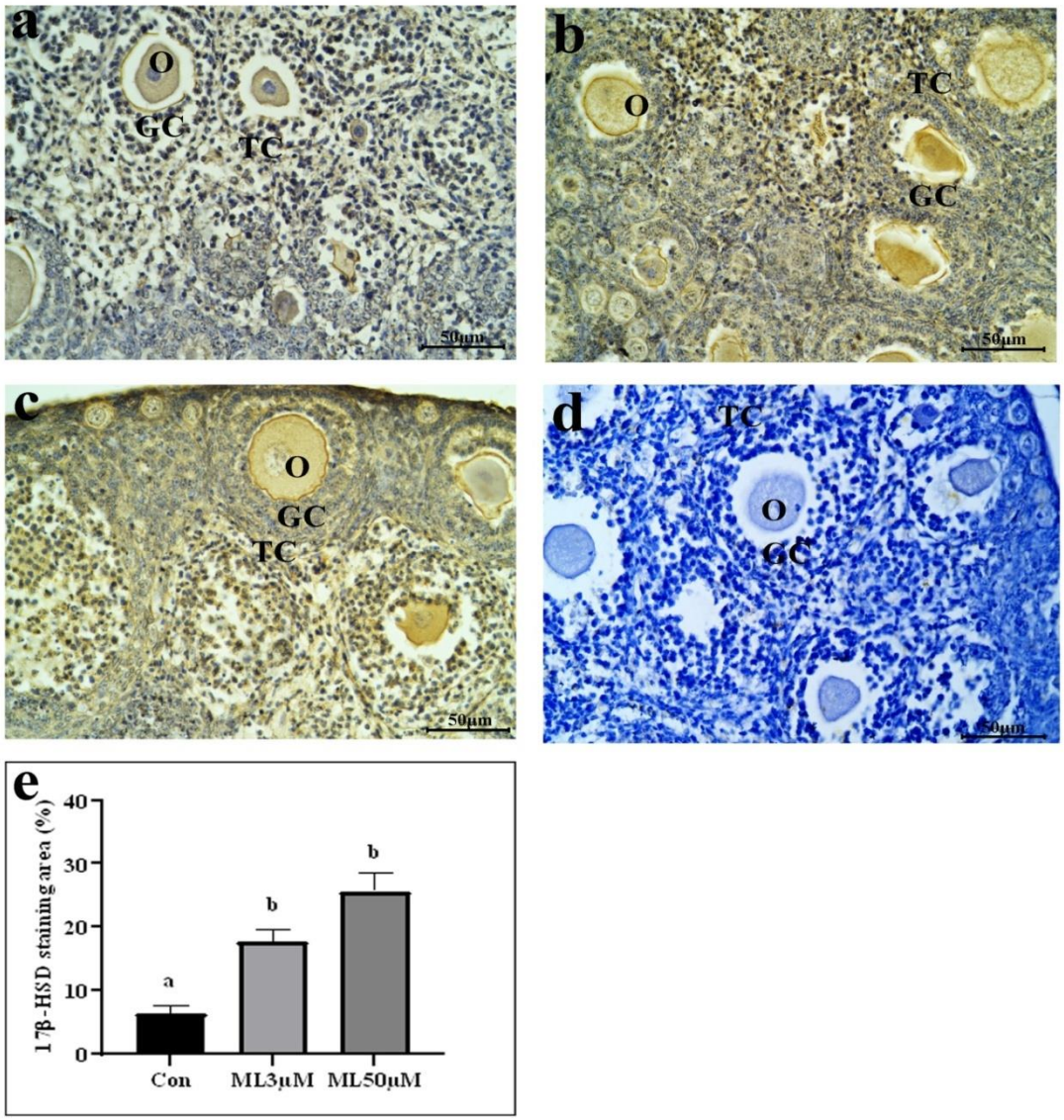

Fig.2.3. Localization of 17β-HSD on the infantile ovary after APJ antagonist (ML221) treatment (a-e). a, b, c and d showed ovaries of the control group, 3µM of ML221 group, 50µM of ML221 group and negative control, respectively at a magnification of 40X. O-oocyte; GC-Granulosa cells; TC-Theca cells. e showed an estimation of the stained percentage area of 17β-HSD. Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

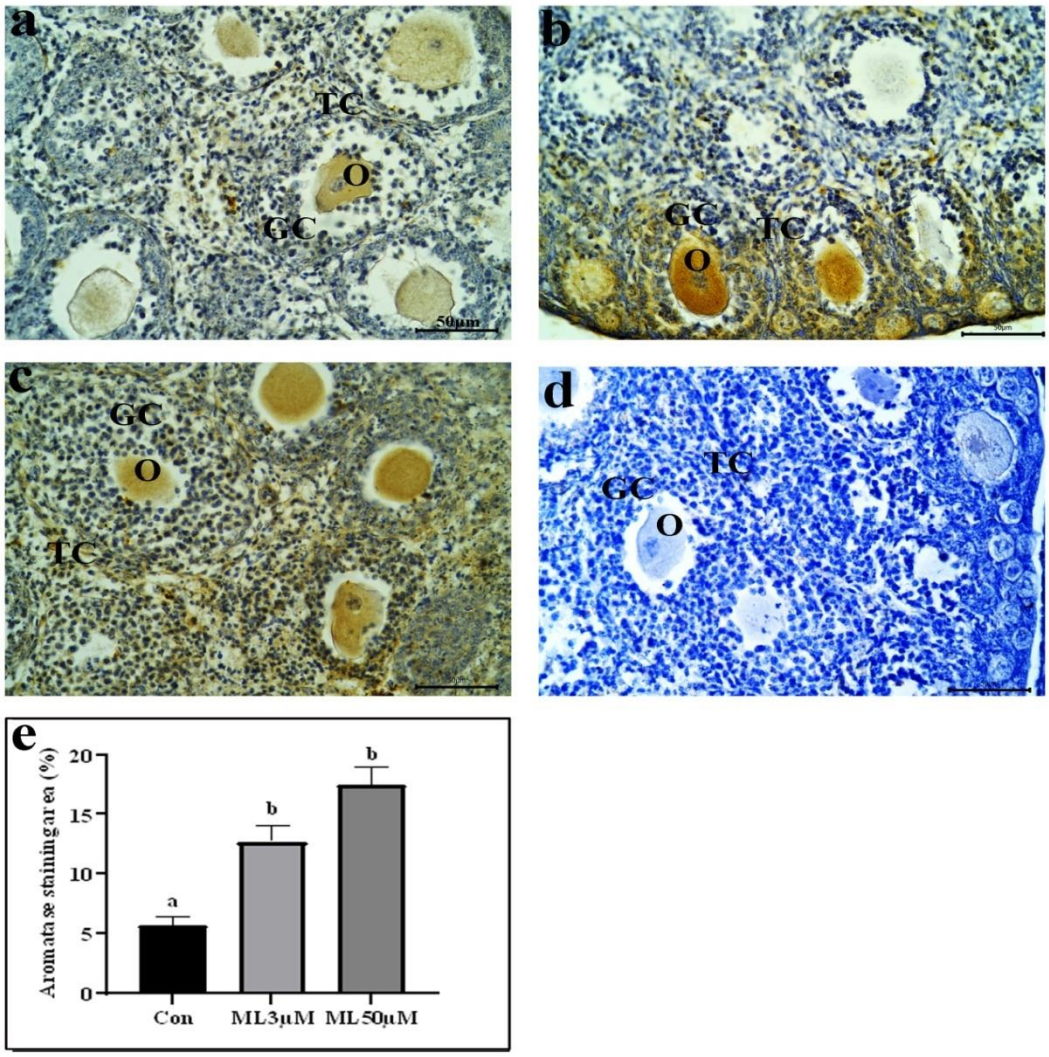

Fig.2.4. Localization of aromatase on infantile ovary after APJ antagonist (ML221) *in vitro* treatment (a-e). a, b, c and d showed ovaries of the control group, 3µM of ML221 group, 50µM of ML221 group and negative control, respectively at a magnification of 40X. O-oocyte; GC-Granulosa cells; TC-Theca cells. e showed an estimation of the stained percentage area of aromatase. Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

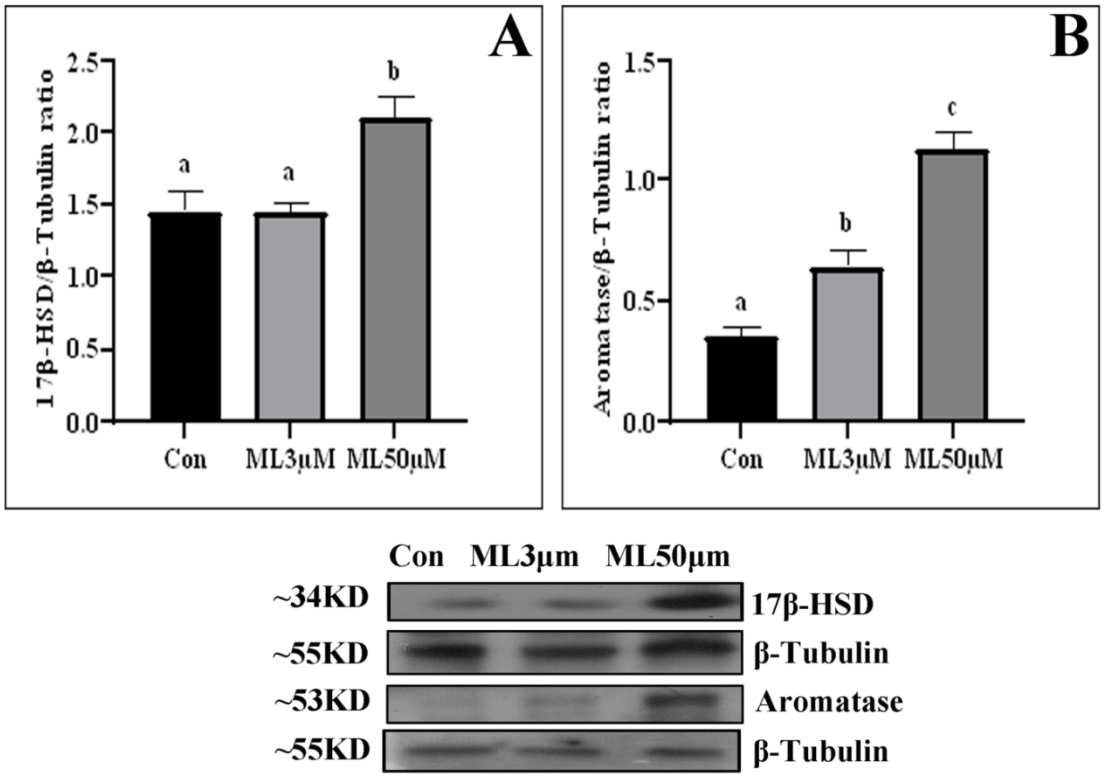

Fig.2.5. Expression of 17β-HSD (A) and aromatase (B) in the infantile ovary after APJ antagonist (ML221) in vitro treatment. The 17β-HSD showed significant ($p<0.05$) increased expression in the 50μM of ML221 treated group. The expression of aromatase showed dose dependent significant ($p<0.05$) increased in the ML221 treated groups. The band density was normalized with the β-tubulin band density. Data are expressed as mean ± SEM. Different alphabets (a,b,c) showed a significant difference ($p < 0.05$).

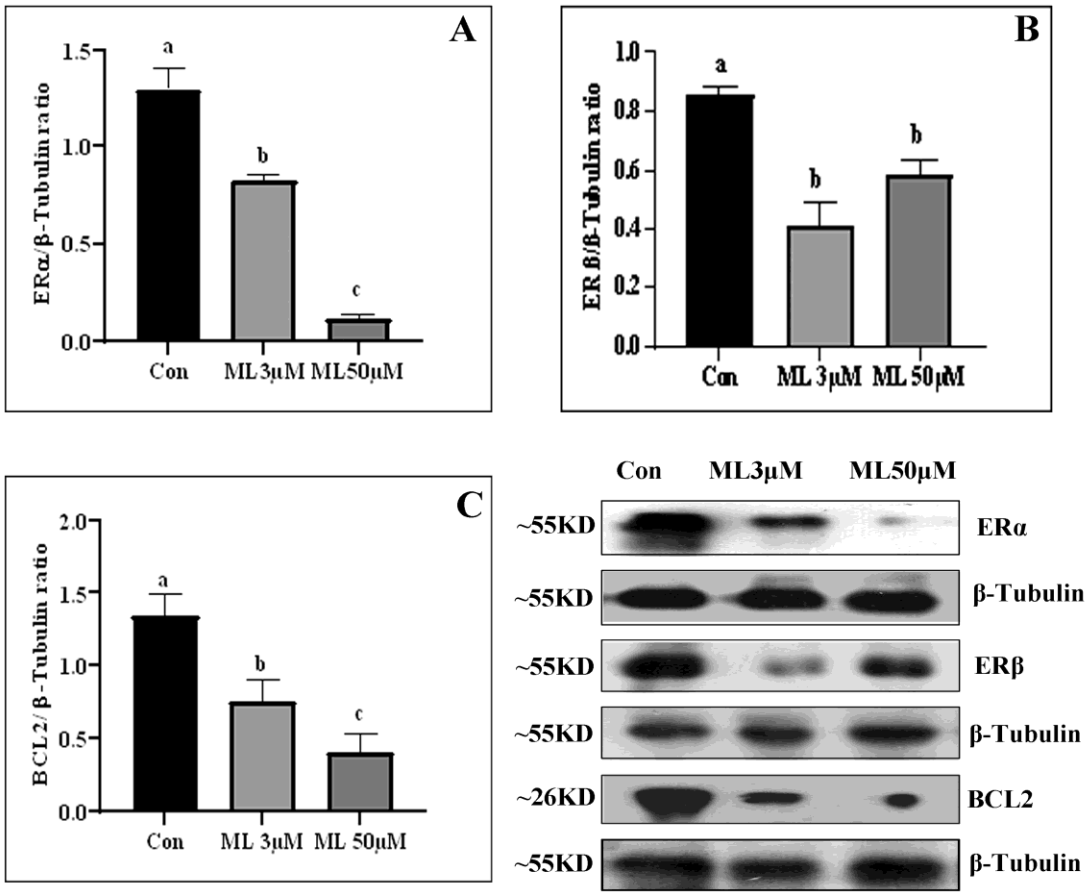

Fig.2.6. Expression of estrogen receptors ERα (A), ERβ (B) and BCL2 (C) in the infantile ovary after APJ antagonist (ML221) *in vitro* treatment. ERα showed significant ($p<0.05$) downregulation dose-dependently after ML221 treatment. The ML221 treated groups showed significant ($p<0.05$) decreased expression of ERβ in the infantile ovary, whereas, no significant ($p>0.05$) change observed between the ML221 treated group. BCL2 showed significant ($P<0.05$) downregulation dose-dependently after ML221 treatment. The band density was normalized with the β-tubulin band density. Data are expressed as mean ± SEM. Different alphabets (a,b,c) showed a significant difference ($p < 0.05$).

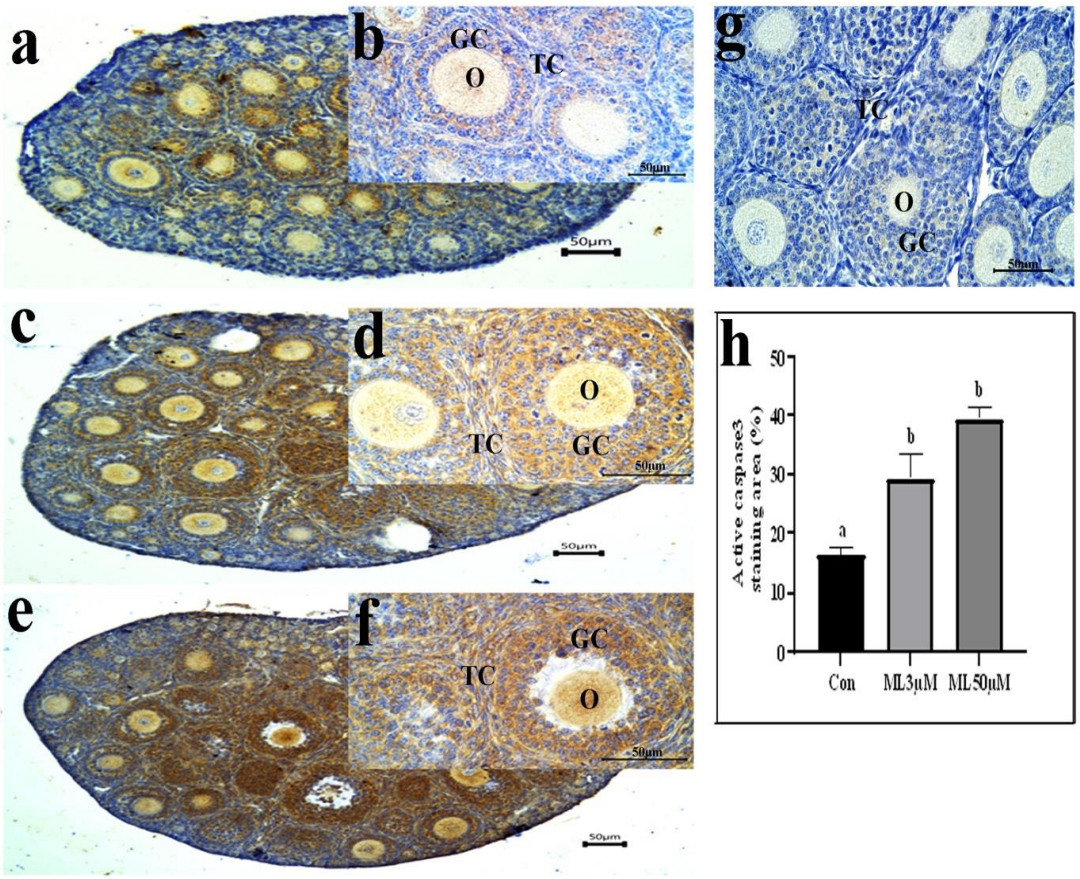

Fig.2.7. Localization of active caspase3 on infantile ovary after APJ antagonist (ML221) *in vitro* treatment (a, h). a-g showed immunolocalization of active caspase3 in the ovaries of the control group (a at 10X and b at 60X magnification), 3µM of ML221 group (c at 10X and d at 60X magnification), 50µM of ML221 group (e at 10X and f at 60X magnification) and negative control (g at 40X magnification). O- oocyte; GC-Granulosa cells; TC-Theca cells. h showed an estimation of the stained percentage area of active caspase3 in the ovary. Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

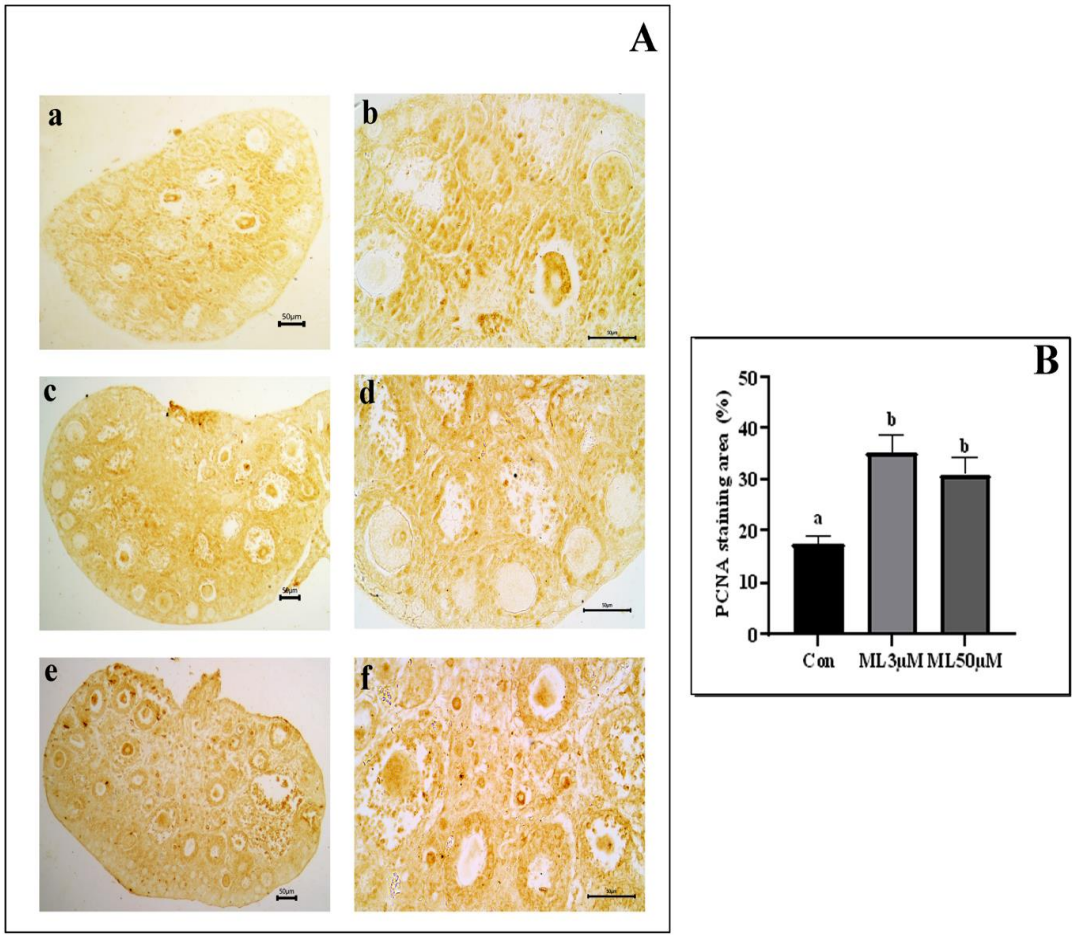

Fig.2.8. Localization of PCNA on the infantile ovary after APJ antagonist (ML221) *in vitro* treatment (A, B). A showed immunolocalization of PCNA in the ovaries of the control group (a at 10X and b at 40X magnification), 3μM of ML221 group (c at 10X and d at 40X magnification) and 50μM of ML221 group (e at 10X and f at 40X magnification). B showed an estimation of the stained percentage area of PCNA in the ovary. Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

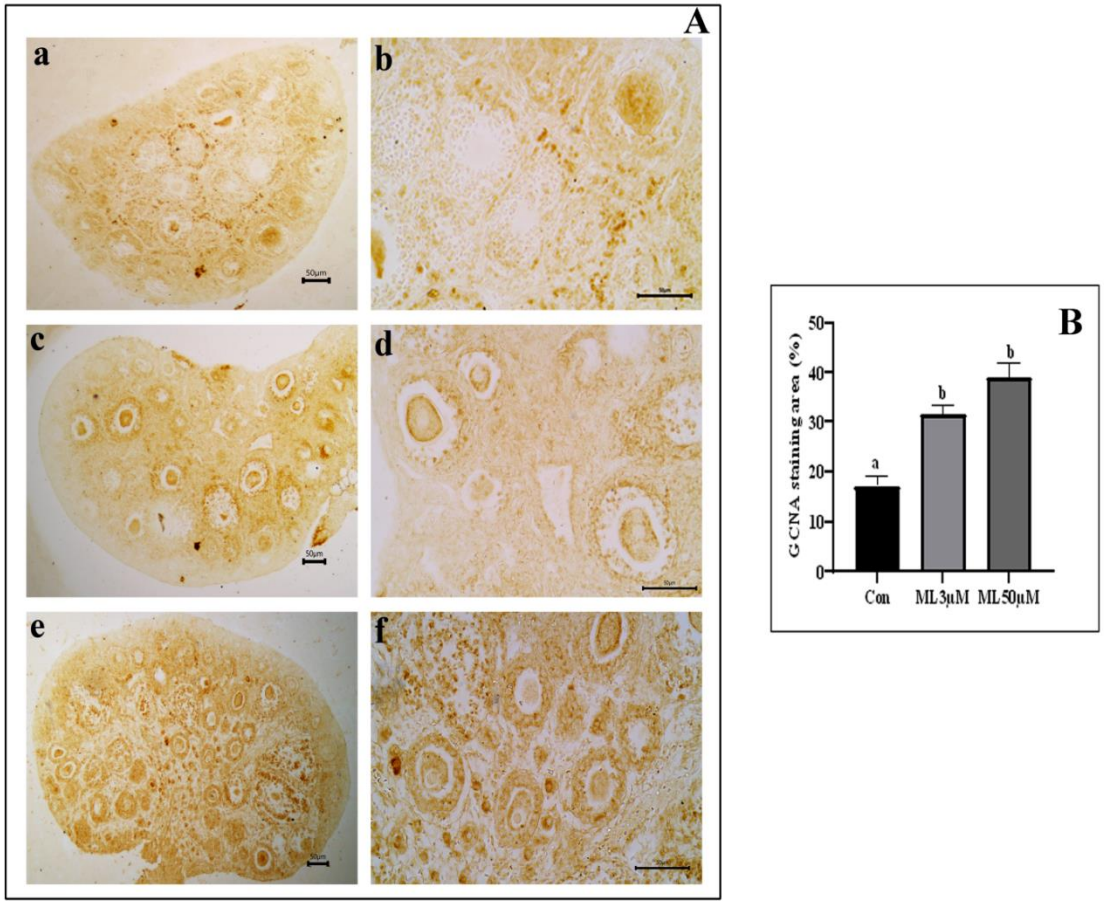

Fig.2.9. Localization of GCNA on the infantile ovary after APJ antagonist (ML221) *in vitro* treatment (A, B). A showed immunolocalization of GCNA in the ovaries of the control group (a at 10X and b at 40X magnification), 3μM of ML221 group (c at 10X and d at 40X magnification) and 50μM of ML221 group (e at 10X and f at 40X magnification). B showed an estimation of the stained percentage area of GCNA in the ovary. Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

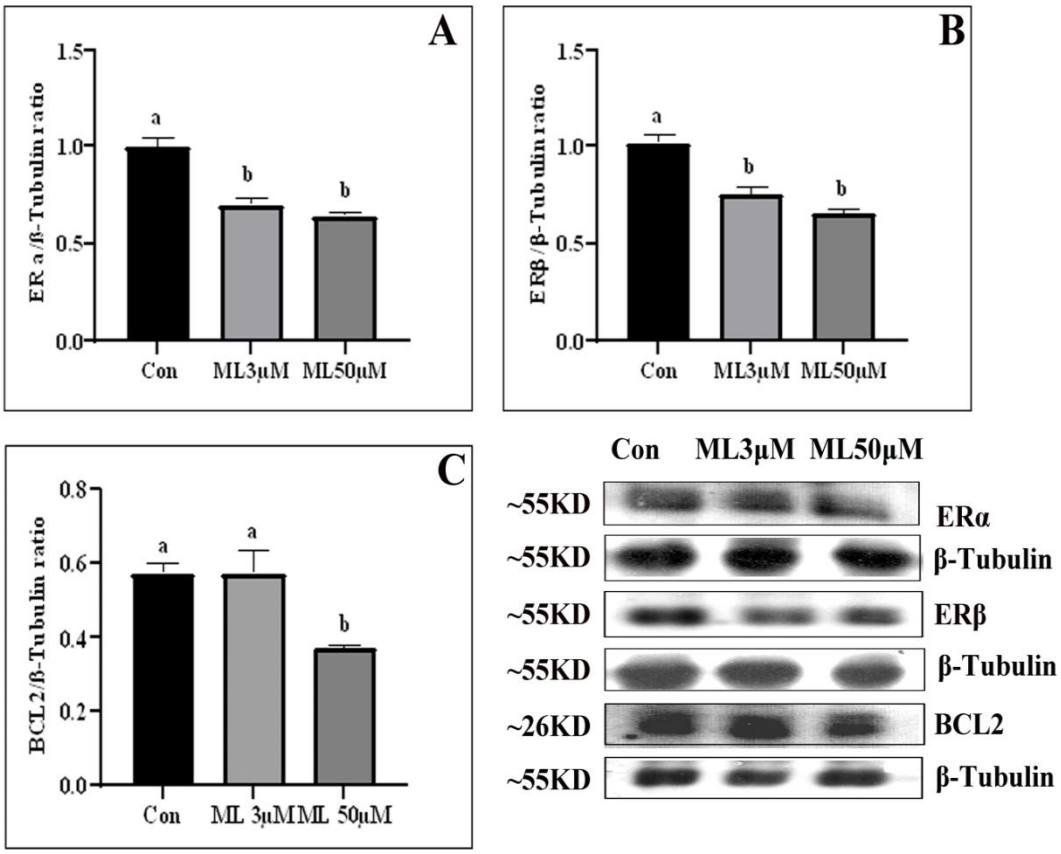

Fig.2.10. Expression of estrogen receptors ERα (A), ERβ (B) and BCL2 (C) in the infantile uterus after APJ antagonist (ML221) *in vitro* treatment. ERα showed significant ($p<0.05$) decreased expression after ML221 treatment. The ML221 treated groups showed significant ($p<0.05$) decreased expression of ERβ in the infantile uterus. The 50µM of ML221 treated group showed significant ($p<0.05$) decreased BCL2 expression in the infantile uterus.The band density was normalized with the β-tubulin band density. Data are expressed as mean ± SEM. Different alphabets (a,b,c) showed a significant difference ($p < 0.05$).

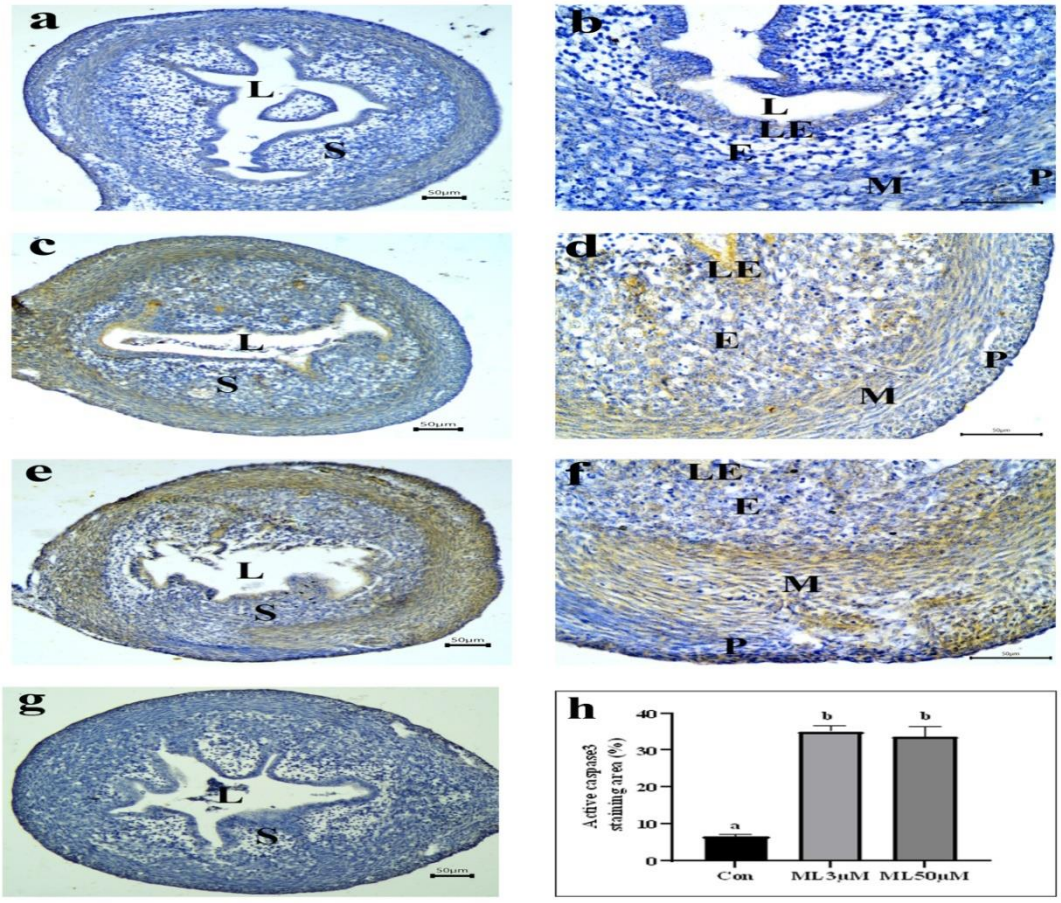

Fig.2.11. Localization of active caspase3 on infantile uterus after APJ antagonist (ML221) *in vitro* treatment (a-h). a-g showed immunolocalization of active caspase3 in the uterus of the control group (a at 10X and b at 40X magnification), 3μM of ML221 group (c at 10X and d at 40X magnification), 50μM of ML221 group (e at 10X and f at 40X magnification) and negative control (g at 10X magnification). E, endometrium; M, myometrium; P, perimetrium; G, uterine gland; LE, luminal epithelium; L, lumen. h showed an estimation of the stained percentage area of active caspase3 in the uterus. Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

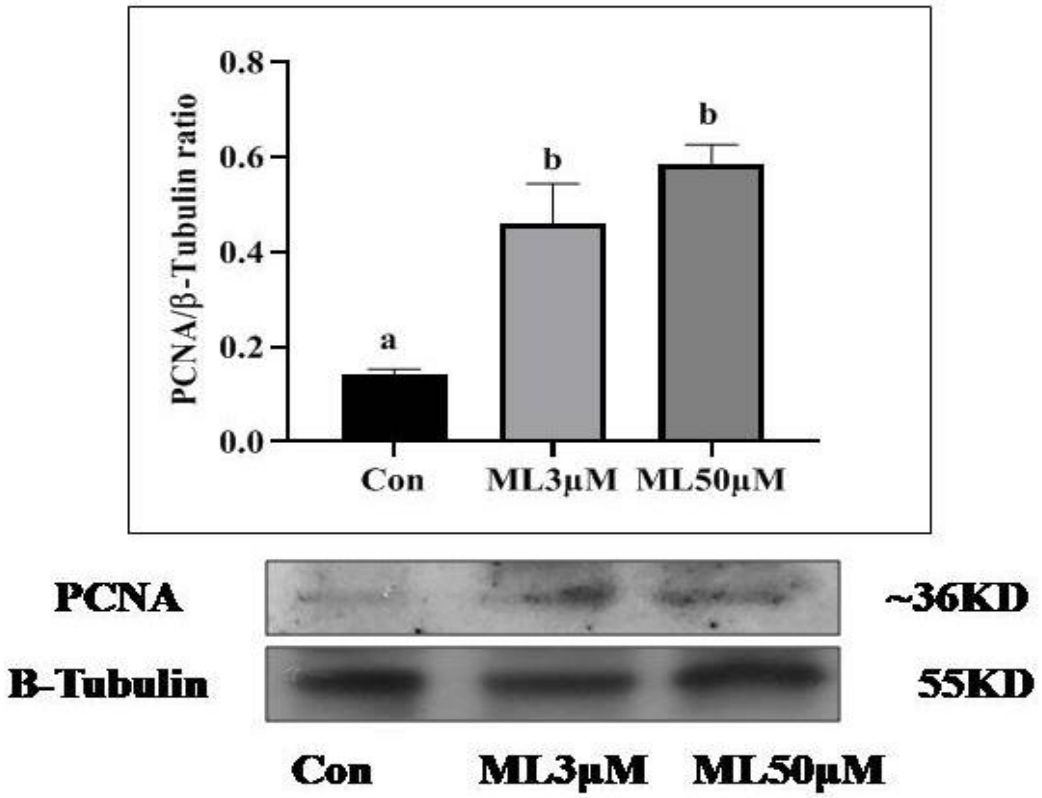

Fig.2.12. Expression of PCNA in the infantile uterus after APJ antagonist (ML221) *in vitro* treatment. PCNA showed significant ($p<0.05$) increased expression after ML221 treatment in the infantile uterus. The band density was normalized with the β-tubulin band density. Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

Table 2.1: Details of antibody used for immunohistochemistry and western blotting analysis.

Primary antibodies, Host, Catalogue, Source	**Host**	**Dilution of primary antibody**	**Secondary antibodies, catalogue, source**	**Dilution of secondary antibody**
For Immunohistochemistry Analysis				
3-beta-hydroxysteroid dehydrogenase (3β-HSD) (HRP conjugated), Cat# sc515120, Santa Cruz, Biotechnology, Dallas, Texas, United States	Mouse Monoclonal IgG	1:200	-	-
17β-Hydroxysteroid dehydrogenases (17β-HSD) Cat# STJ110000, St John's Laboratory Ltd, London, United Kingdom	Rabbit Polyclonal IgG	1:100	Goat anti-rabbit, Cat# E-AB-1102, Elabscience, Houston, Texas, United States	1:200
Aromatase (CYP19A1) Cat #E-AB-31086, ElabScience, Houston,	Rabbit Polyclonal IgG	1:50	Goat anti-rabbit, Cat# E-AB-1102,	1:200

Texas, USA			Elabscience, Houston, Texas, United States	
Active caspase3, Cat #E-AB-22115, ElabScience, Houston, Texas, USA	Mouse Polyclonal IgG	1:100	Goat anti-mouse (cat# E-AB-1001) Elabscience, Houston, Texas, United States	1:400
Proliferating cell nuclear antigen (PCNA), Cat# sc-7907, Santa Cruz Biotechnology, Dallas, Texas, United States	Rabbit Polyclonal IgG	1:100	Goat anti-rabbit, Cat# E-AB-1102, Elabscience, Houston, Texas, United States	1:200
Germ Cell Nuclear Acidic Peptidase (GCNA) Cat# 10D9G11, DSHB, University of Iowa, Dept of Biology, Iowa, United States	Mouse Polyclonal IgG	1:200	Goat anti-mouse (cat# E-AB-1001) Elabscience, Houston, Texas, United States	1:400
For Western Blotting Analysis				
Estrogen receptor α (ERα), Cat# Bz1, DSHB, University of Iowa, Dept of Biology,	Mouse Polyclonal IgG	1:500	Goat anti-mouse (cat# E-AB-1001) Elabscience,	1:4000

Iowa, United States			Houston, Texas, United States	
Estrogen receptor β (ERβ), Cat# CWK-F12, DSHB, University of Iowa, Dept of Biology, Iowa, United States)	Mouse Polyclonal IgG	1:500	Goat anti-mouse (cat# E-AB-1001) Elabscience,	1:4000
B-cell lymphoma 2 (BCL2) Cat# sc-7382, Santa Cruz Biotechnology, Dallas, Texas, United States	Mouse Polyclonal IgG	1:2000	Goat anti-mouse (cat# E-AB-1001) Elabscience,	1:4000
β-Tubulin Cat#E7, DSHB, University of Iowa, Department of Biology, United States)	Mouse Polyclonal IgG	1:1500	Goat anti-mouse (cat# E-AB-1001) Elabscience,	1:4000

CHAPTER 3

Title

Expression of apelin and apelin receptor protein in the hypothalamo–pituitary–ovarian axis during the estrous cycle of mice

*Neuroendocrinology (2023)

Chapter 3

Introduction

Reproductive function of mammals is driven by a neuro-hormonal system consisting hypothalamus, pituitary, and the gonads and this axis is known as the hypothalamic-pituitary-gonadal/ovarian axis (HPG or HPO) (Maggi et al., 2016). The physiological function of the HPO axis is to ensure the cyclic production of gonadotropin releasing hormone (GnRH), gonadotropins and steroid hormones from the respective place for the reproductive activities (Taşkiran et al., 2015). The hypothalamus, a central brain region secretes GnRH, a decapeptide that which controls the anterior pituitary to synthesize and secrets gonadotropin hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH) that ultimately regulates the downstream production and secretion of ovarian steroids and oogenesis (Oyola and Handa, 2017; Parhar et al., 2016; Spaziani et al., 2021; Styne, 2003). However, ovarian steroids do not give direct feedback to GnRH neuron,whereas itrelies on kisspeptin system during ovarian cycle. Kisspeptin neurons of the hypothalamus secrete kisspeptin, a decapeptide which regulates the reproductive axis by stimulating its receptor on the GnRH neurons (Oakley et al., 2009).The feedback signal from ovarian steroids for the GnRH surge and pulse are conveyed through the two population of kisspeptin that exists inanteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC), respectively (Prashar et al., 2023). In mouse, the population of kisspeptin located in the AVPV transduces the positive feedback to kisspeptin neurons that stimulate occurrence of GnRH surge necessary for LH surge just before ovulation and the other population located in the arcuate nucleus receivenegative feedback and is more involved in tonic GnRH secretion (Khan and Kauffman, 2012).

The estrous cycle of mice has four repetitive sequential phases namely proestrus, estrus, metestrus and diestrus and which last for 4–5 days (Bertolin and Murphy, 2014). During estrous cycle the functions of hypothalamus, pituitary and ovary are modulated in relation to their secretions of GnRH, gonadotropin and ovarian steroids respectively (Taşkiran et al., 2015). Although the GnRH-FSH/LH-E2/P4 pathways involved in HPG axis regulation is largely elaborated in mammals, the molecular and cellular events that

interfere in this pathway are still unclear (Ak et al., 2023). Previous study suggested that several neuropeptides are involved in the modulation of this pathway through direct affect in synaptic transmission and/or act in a paracrine manner (Pałasz et al., 2018, 13. Gołyszny et al., 2022). Apelin (APLN), an endogenous peptide isolated from bovine stomach tissue extracts, which has been identified in 1998 as the endogenous ligand of the human orphan APJ receptor (Tatemoto et al., 1998). Apelin receptor (APJ, also known as APLNR, APJR, AGTRL1 and HG11) is a class of G-protein coupled receptor consists of 380 amino acids, which was discovered in 1993 (O'Dowd et al., 1993). Both apelin and its receptor APJ are expressed highly in brain, heart, kidney, liver, placenta, gonads and other reproductive organs in many species (Kawamata et al., 2001; Zeng et al., 2007; Kasai et al., 2008; Lv et al., 2017; Wang et al., 2020). The immunoreactivity of apelin has been identified in certain hypothalamic nuclei and pituitary and it was suggested that this peptide may be a signaling molecule along the hypothalamic-hypophysial axis (Reaux et al., 2002; Reaux-Le Goazigo et al., 2007).

Previous study demonstrated the presence of apelin neurons in the supraoptic (SON) and paraventricular nuclei (PVN) of the rat brain, which are involved in the central control of pituitary hormone release (Reaux et al. 2001; Czarzasta and Cudnoch-Jedrzejewska, 2014; De Mota et al., 2000; O'Carroll et al., 2013). The presence of apelin in the hypothalamic area like the supraoptic and the paraventricular nuclei suggested it as a neuropeptide factor as well (De Mota et al., 2004). The direct evidence of apelin-mediated LH and FSH release from the pituitary has been shown by Sandel et al(2015) in male rats.

The apelin and APJ are also expressed in the granulose cells and oocyte, and controls the progesterone secretion from luteinizing granulosa cells (Roche et al., 2017). Apelin has been shown to stimulate secretion of progesterone and estradiol in bovine ovary (Rak et al., 2017). Thus, the above-mentioned literatures established the presence of apelin and APJ in the HPO axis. There are evidences which suggest that various factors of the HPO axis changes during the estrous cycle(Gusmao et al., 2022; Di Yorio et al., 2008). The

expression of ovarian apelin system has been shown in the bovine estrous cycle (Schilffarth et al., 2009). To best of our knowledge, no study has shown in the expression of apelin system in HPO axis during estrous of mice and other mammals. Thus, the HPG axis in female mice was examined in terms of apelin and APJ expression and localization during estrous cycle phases.

Materials and methods

Animal maintenance and experimental design

In the present study, 32 virgin female Swiss Albino mice of age 3–4 months (weight 25±5 g) were used from the reared colony from laboratory animal house of Mizoram University, India. These mice were handled in according to the protocol approved by the Mizoram University Institutional Animal Ethical Committee (Protocol Number, MZU/IAEC/ 2020/12), Mizoram University, Mizoram, India and all animal experiments was complied with the ARRIVE guidelines. All the mice were maintained under the standard laboratory conditions of 12 hours light and dark, at a temperature of 25±2°C and provided water and food ad libitum.

Chemicals

The Apelin-13 (apelin) peptide (Gln-Arg-Pro-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe) used in this study was purchased from GMR Foundation, Tiruchirappalli, Tamil Nadu, India. The purity of the Apelin-13 (apelin) peptide was ≥95%.

The 4-oxo-6-((pyrimidin-2-ylthio)methyl)-4H-pyran-3-yl 4-nitrobenzoate (ML221), a potent apelin inhibitor or functional antagonist of APJ was purchased from Sigma-Aldrich Chemicals Pvt Ltd, St. Louis, Missouri, United States (cat# SML0919).

The Leuprolide acetate, a GnRH agonist used in this study was purchased from Sun pharmaceutical ind. Ltd, Gujarat, India.

Identification of estrous cycle

The estrous cycle of the mice was observed according to the procedure described by Goldman et al (Goldman et al., 2007). In brief, the vaginal smear of all the mice were taken with dropper at around 8.00-9.30 AM or 5.00-6.00 PM and observed under the microscope. The estrous stages were identified and classified in groups on the basis of their vaginal cytology. The vaginal smear showed oval flat nuclear epithelial cells in the proestrus phase, cornified cells in the estrus phase, cornified and white blood cells in the metestrus and mostly white blood cells in the diestrus.

***In vivo* study**

The mice (n= 8/group) were divided into four groups as proestrus (Pro), estrus (Est), metestrus (Met) and diestrus (Di) according to their vaginal smear observation. All the mice were sacrificed at respective phase of estrous cycle in the evening at around 5.00-6.00 PM and the hypothalamus, pituitary and ovary samples were harvested immediately.The hypothalamus was excised following previous report by Daikoku and Shimizu (1970). The half of the pituitary samples (n=4/group) were fixed in Bouin's fluid for immunohistochemical study. Furthermore, other pituitary (n=4/group) at proestrus and estrus are used for *in vitro* study. The half of the ovaries and hypothalamus (n=4/group) were fixed in Bouin's fluid for immunohistochemical study and remaining were stored at -20°C for western blotting analysis.

***In vitro* study**

To explore the role of apelin in the gonadotropin secretion, the pituitary (n = 4/group) at proestrus and estrus stages were cultured for 24 hours. Briefly, pituitary was cultured in a medium of Dulbecco Modified Eagle's and Ham's F-12 (cat no-AL155 G, HiMedia, Mumbai, India) mixed with 100U/mL penicillin, 100µg/mL streptomycin and 0.1 % BSA (Sigma Aldrich St Louis, USA). Pituitary (1 pituitary per tube) was cultured in 500µL of medium in a humidified atmosphere with 95% air and 5% CO2 for 24 h at 37ºC. The pituitary were divided into four groups: (i) Control group (Con), cultured only in media, (ii) GnRH group, cultured in the presence of GnRH agonist at a dose of

100ng/mL (Slawrys et al., 2017) (iii) GnRH+AP group, cultured in the presence of GnRH agonist and apelin-13 at a dose of 1μg/ml (Li et al., 2018) and (iv) GnRH+ML group, cultured in the presence of GnRH agonist and apelin inhibitor ML221 at a dose of 50μM (Das et al., 2022). After 24 hours, media was collected for ELISA.

Immunohistochemistry

Hypothalamus, ovaries and pituitary were fixed in Bouin's fluid for 24 hour and stored in 70% alcohol. Histological slides were prepared by paraffin embedded method described earlier by Tepekoy et al (Tepekoy et al., 2015).Briefly, samples were dehydrated in alcohol grades (70%, 90% and 100%), cleansed with xylene and paraffin-embedded blocks was prepared. The blocks were sectioned (7μm) with Leica rotary microtome (model RM2125 RTS). The hypothalamic sections were selected at level of median eminence. The tissue sections were then spread in warm water then placed in poly-L-lysine coated slide and kept in slide warming table at 37°C for overnight.

For immunohistochemistry, the slides were deparaffinized in xylene, followed by rehydrated with different alcohol grades (100%, 90% and 70%) and then hydrated in distilled water. The endogenous peroxidase was blocked with 3% H_2O_2 and methanol solution and for blocking the nonspecific binding the slides were then treated with goat serum (1:100 with PBS) at room temperature and incubated in primary antibody (anti-apelin receptor 1:50, cat# ABD43, Millipore and anti-apelin 1:50, cat#SAB4301741, Sigma Aldrich, USA) diluted with PBS at 4◦C for overnight. PBS was used to wash off the unbound primary antibody and incubated with secondary antibody (1:400, goat anti-rabbit, cat# PI-1000, Vector Laboratories, Burlingame, CA, United State). The antibodies for apelin and apelin receptor have been used for the localization of these proteins in the mice (Anima et al., 2023). The slides were then proceeded in DAB solution containing 0.6 mg/mL solution of 3, 3-diaminobenzidine tetra hydrochloride Dihydrate (DAB) in Tris–HCl (pH 7.6) and 0.01 % H_2O_2 until brown color developed and the reaction was stopped by distilled water. Counterstaining was done with hematoxylin. The stained slides were then dehydrated (70%, 90% and 100%), clean in

xylene. Finally mount with DPX and examined using a microscope (iScope series, Euromex, Netherland). The immunostaining was quantified by image J using ImageJ's threshold tool and e DAB-stained area was obtained, and the data was shown as a percentage of the staining area. Absorption control was also performed to confirm the specificity of the apelin antibody. In brief, apelin antibody was pre-incubated with the apelin peptide (1:10 ratio) for 24hr at 4°C. This process allows the antibody to bind with the target antigen and becomes inactive. Then the pre-absorbed antibody was incubated with the tissue instead of primary antibody following above mentioned method.

Western blot analysis

Immunoblotting was performed in the hypothalamus and ovary, as method described earlier(Jeremy et al., 2017). The hypothalamus was excised at depth to optic chiasm and optic tract along the approximate margins from anterior to posterior (optic chiasm to infundibulum) and lateral to the optic tract (Quennell et al., 2011; Ou et al., 2022)**.** Protein concentration was estimated by Bradford method (Bradford, 1976). Equal protein amount (50µg) was loaded and resolved by 12% sodium dodecyl sulfatepolyacrylamide (SDS) gel electrophoresis and the resolved proteins were then transferred onto the polyvinylidene fluoride (PVDF) membrane using wet apparatus. The membranes blocking was done with 5% nonfat skimmed-milk (cat# GRM1254-500G; HiMedia Laboratory private limited, Mumbai) in PBST and incubated with the primary antibody, anti-apelin receptor (1:1000, lot# ABD43, Millipore) and anti-apelin (1:1000, cat#SAB4301741 Sigma Aldrich, USA). PBST solution was used for washing unbound antibody and then incubated with horseradish peroxidase conjugated secondary antibodies (1:4000, goat anti-rabbit, cat# PI-1000, Vector Laboratories, Burlingame, CA, USA). Again, PBST was used to wash free secondary antibody, the membranes were then incubated with electrochemiluminescence and developed onto X-ray film. To quantify the band intensities with respect to loading control, ImageJ software (imagej.nih.gov/) was used. For loading control, the membrane was stripped and reprobed with β-Tubulin (1:1500, cat#E7; DSHB, university of Iowa, Department of

Biology, United States) and secondary antibody (1:4000, Goat anti-mouse, cat#E-AB1001, Elabscience, Houston, Texas, USA).

Estimation of gonadotropin hormone level in cultured media

The media collected from the *in vitro* experiment were estimated for luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentration by enzyme linked immunosorbent assay. Commercially available mouse ELISA kit (Luteinizing Hormone, cat#E-EL-M3053; Follicle Stimulating Hormone, cat# E-EL-M0511, Elabscience, USA) was used following instruction manual. The sensitivity of the luteinizing hormone and follicle stimulating hormone are 0.94 ng/ml and 0.19 ng/ml, respectively. The coefficient of variation for both the hormone kit is <10%.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 9 (GraphPad Software, San Diego,CA,USA). The differences between the data were analyze by One-way Analysis of variance (ANOVA) followed by Tukey's test and the data were expressed as mean ± standard error mean (SEM). $P<0.05$ was considered as significant.

Results

Expression of apelin and apelin receptor (APJ) in the hypothalamus during estrous cycle

The expression of apelin in the hypothalamus during the estrous cycle was found to be significantly ($p<0.05$) increased in the metestrus group as compared to other groups. There was no significant change in apelin expression in the proestrus, estrus and metestrus groups (Fig.3.1A).

The expression of APJ in the hypothalamus during the estrous cycle showed a significant ($P<0.05$) decrease in the metestrus groups as compared to other groups, whereas, in the diestrus group significantly ($p<0.05$) increased as compared to other

groups. There was no significant change in APJ expression between the proestrus and estrus groups (Fig. 3.1B).

Immunolocalization of APJ in the hypothalamus during estrous cycle

Immunostaining of APJ in the hypothalamus was observed in the median eminence during the estrous cycle (Fig.3.2A (a-h)). The APJ immunostaining in the median eminence showed strong staining in the diestrus (g,h), whereas, metestrus showed mild APJ immunostaining (e,f). The APJ showed moderate immunostaining in the proestrus (a,b), and estrus phases (c.d).The percentage (%) area of APJ staining in the hypothalamus was also measured (Fig.3.2B). The Metestrus group showed significant ($p<0.05$) decreased stained % area as compared to other groups. Whereas, there were no significant ($p>0.05$) changes between the proestrus, estrus and diestrus groups.

Immunolocalization of apelin and APJ in the pituitary gland during the estrous cycle

Immunostaining of apelin in the pituitary gland during the estrous cycle was observed in various cell types of the anterior pituitary gland (Fig. 3.3A (a-h)). Apelin distribution was also observed in the intermediate lobe without any visible changes during the estrous cycle. The apelin immunostaining in the anterior lobe of the estrus (c,d) and diestrus (g,h) showed intense immunostaining with abundant distribution of apelin-stained cells, whereas the metestrus (a,b) and proestrus (e,f) group showed faint and considerably less distribution of apelin stained cells. The percentage (%) area of apelin staining in the pituitary was measured (Fig.3.3B). Estrus and diestrus groups showed significant ($p<0.05$) increased stained % area as compared to proestrus and metestrus. Whereas, no significant ($p>0.05$) changes were observed between the proestrus and metestrus groups. Furthermore, there was no significant ($p>0.05$) change between the estrus and diestrus groups.

Immunohistochemistry of APJ in the pituitary gland during the estrous cycle showed variation in the distribution and staining in various cell types of the anterior pituitary gland (Fig. 3.4A (a-h)). Immunostaining of APJ showed intense staining in the anterior lobe of the estrus (c,d) and diestrus (g,h) groups with abundant distribution of APJ stained cells, whereas metestrus (e,f) and proestrus (a,b) group showed mild immunostaining and considerably less distribution of apelin receptor stained cells. The percentage (%) area of apelin receptor staining in the pituitary was measured (Fig.3. 4B). Estrus and diestrus groups showed significant ($p<0.05$) increased stained % area as compared to proestrus and metestrus. However, the proestrus group showed a significant ($p>0.05$) increased % area as compared to the metestrus. There was no significant ($p>0.05$) change observed between the estrus and diestrus groups.

Effect of apelin 13 and ML221 treatment on the GnRH agonist stimulated gonadotropin, secretion by the pituitary at the proestrus phase

The luteinizing hormone (LH) concentration in the cultured media was detected as significantly ($p<0.05$) higher in the only GnRH agonist-treated group (11.64±0.14 ng/mL) and GnRH agonist plus apelin inhibitor (ML221) treated groups (11.26±0.65 ng/mL) compared to the control (1.07±0.23 ng/mL) and GnRH plus apelin treated groups (7.38±0.78 ng/mL) whereas, GnRH plus apelin treated group showed significant ($p<0.0001$) increased than control group. However, there is no significant ($p=0.95$) change between the only GnRH and GnRH plus ML221 treated groups (Fig.3.5A).

The follicle-stimulating hormone (FSH) concentration in the cultured media was detected as significantly ($p<0.05$) higher in the only GnRH treated (29.88±0.34 ng/mL) and GnRH plus ML221 treated groups (36.64±0.71 ng/mL) than the other groups, control (22.03±2.11 ng/mL), and GnRH plus apelin treated group (23.25±2.01 ng/mL). Whereas, the GnRH plus Ml221 treated group showed a significant ($p<0.05$) increase in FSH secretion than the only GnRH group. However, there are no significant ($p=0.94$) changes observed between the control and GnRH plus apelin-treated groups (Fig.3.5B).

Effect of apelin 13 and ML221 treatment on the GnRH agonist stimulated gonadotropin, secretion by pituitary at estrus phase

The luteinizing hormone (LH) concentration in the cultured media was detected as significantly ($p<0.05$) higher in the only GnRH group (15.8±1.66 ng/mL) compared to the other groups, control group (12.27±0.45 ng/mL), GnRH plus apelin treated group (13.45±3.35 ng/mL) and GnRH plusML221 treated group (15.01±3.62 ng/mL). The GnRH plus apelin treated group showed significantly ($p<0.05$) decreased LH levels than other groups. However, GnRH plus ML221 treated groups showed no significant ($p>0.05$) change between the control and GnRH plus apelin-treated groups (Fig.3.6A).

The follicle-stimulating hormone (FSH) concentration in the cultured media was detected as significantly ($p<0.001$) higher in the GnRH plus ML221 treated groups (29.37±0.18 ng/mL) and GnRH plus apelin treated group (26.82±0.5) than the other groups, control (22.02±0.54 ng/mL) and only GnRH (22.39±0.27 ng/mL). Whereas, the GnRH plus apelin treated group showed a significant ($p=0.002$) decrease in FSH secretion than the GnRH plus ML221 treated group. However, there are no significant ($p=0.91$) changes observed between the control and only GnRH groups (Fig.3.6B).

Immunolocalization of apelin and APJ in the ovary during estrous cycle

Immunostaining of apelin in the ovary during the estrous cycle was observed in all the follicles along with the oocytes, granulosa cells and theca cells, and also in the corpus luteum (Fig.3.7A (a-t)). Immunohistochemistry of apelin showed intense staining in the corpus luteum and antral follicle of proestrus (b,c) and diestrus groups (q,r), whereas moderate and mild staining in the corpus luteum and antral follicle of metestrus (l,m) and estrus (g,h) groups, respectively. Granulosa cells, thecal cells and oocytes of proestrus (d,e), metestrus (n,o) and diestrus (s,t) showed moderate immunostaining whereas estrus (i,j) showed very faint staining of apelin. The percentage (%) area of apelin staining in the ovary was measured (Fig.3.7B). Proestrus and diestrus groups showed significant ($p<0.05$) increased stained % area as compared to estrus and

metestrus. However, there was no significant ($p>0.05$) change observed between the estrus and metestrus groups.

Immunohistochemistry of APJ in the ovary during the estrous cycle showed staining in follicles along with the oocytes, granulosa cells and theca cells, and also in the corpus luteum (Fig.3.8A (a-t)). Proestrus (b,c) and diestrus (q,r) showed intense immunostaining of APJ in the corpus luteum and antral follicle, whereas moderate and faint staining in the estrus group (g,h) and metestrus (l,m) group, respectively. Proestrus and diestrus showed moderate immunostaining in the oocytes, granulosa cells, and thecal cells whereas estrus showed very faint staining of apelin in these cells. The percentage (%) area of apelin staining in the ovary was measured (Fig.3.8B). Proestrus and diestrus groups showed significant ($p<0.05$) increased stained % area as compared to estrus and metestrus. However, there was no significant ($p>0.05$) change observed between the estrus and metestrus groups.

Expression of APJ in the ovary during estrous cycle

The expression of APJ in the ovary during the estrous cycle showed a significant ($P<0.05$) decrease in the estrus and metestrus groups as compared to other groups whereas, the diestrus group was found to be significantly ($p<0.05$) increased as compared to other groups. Metestrus and estrus groups showed no significant ($p>0.05$) change in apelin receptor expression in the ovary (Fig.3.9).

Discussion

The present study has investigated the expression and distribution of apelin and its receptor, APJ in the hypothalamus, pituitary and ovary of mice during different phases of estrous cycle. It is well known that female reproductive process is controlled along the HPO (hypothalamo-pituitary-ovarian) axis by GnRH from hypothalamus, FSH and LH from pituitary and ovarian hormones (Taşkiran et al., 2015). Apelin is an endogenous peptide, considered as neuropeptide (Reaux et al., 2001) and adipokines (Dogra et al., 2021) as well. It has been shown that apelin elicits biological action via

binding to its receptor, APJ (Kidoya and Takakura, 2012). Our results showed that expression of apelin and APJ in the hypothalamus did not show change from proestrus to estrus, however, expression of APJ was lower in metestrus along with elevated apelin. Expression of APJ in the hypothalamus was elevated in the diestrus phase along with decreased apelin expression. Our immunohistochemical study also showed mild staining of APJ in the metestrus, strong in the diestrus and moderate in the proestrus and estrus at the level of median eminence. The neuroendocrine system which includes the median eminence serves as a vital link between the hypothalamus and pituitary gland (Clayton, et al., 2022). Previous study has also showed that apelin system are present in the median eminence (De Mota et al., 2004). Western blot analysis and quantification of immunohistochemistry of APJ did not show same trend with respect to quantification in the hypothalamus. Since for western blot entire hypothalamus was selected and immunohistochemistry represent only one area of hypothalamus. This could be the reason of this discrepancy, because apelin systems are also expressed in other hypothalamic areas (De Mota et al., 2004). Taken together, expression of apelin and APJ during metestrus to diestrus could be considered as stimulated apelin signaling in these phases (post ovulatory) of estrous cycle in the hypothalamus. However, the presence of apelin and APJ during estrous also suggest its possible role in the hypothalamus. The exact role of hypothalamic APJ system during the estrous cycle requires further investigation. Previous studies have shown that hypothalamic areas express APJ system in the paraventricular, supraoptic and arcuate (ARC) nuclei (De Mota et al., 2000; Pope et al., 2012). It has also been shown that apelin fibers are present in the close proximity of GnRH neuron, and also suggested that apelin could stimulate bursting and GnRH release in male rats (Abot et al., 2022). Since, we have not measured the physiological role of hypothalamic APJ system in the female mice during estrous cycle on GnRH secretion, thus, it would only be suggestive role of APJ on the GnRH secretion in female mice. Whether apelin stimulates or suppresses the GnRH in female mice remains unclear. This is also an important limitation of the study, which needs subsequent analysis. Moreover, the post-ovulatory elevated apelin system in the

hypothalamus (metestrus and diestrus) might be slowing GnRH secretion. It has also been suggested that GnRH pulse frequency slows down during postovulatory estrous/luteal phase (Herbison, 2020).

Despite, hypothalamus, the present study has also investigated the localization of apelin and APJ in the pituitary. Our results showed that apelin and APJ immunolocalization exhibited abundance in the anterior pituitary during estrus and diestrus phases of estrous. The intermediate lobe (Taheri et al., 2002) also showed immunostaining of apelin and APJ during estrous cycle, without any noticeable changes in the localization. Previous study has also shown the presence of APJ in the anterior pituitary of rats (O'Carroll et al., 2000). The presence of apelin and APJ was also noticed in the posterior pituitary; however, the apelin exhibited low immunostaining in the posterior pituitary. It has been shown that apelinergic nerve endings was found be highest in the inner layer of the median eminence and in the posterior pituitary. Moreover, arginine vasopressin (AVP) neurons also project toward the posterior pituitary (Brailoiu et al., 2002; Reaux et al., 2002). It has also been shown that intracerebroventricularly apelin infusion in the rodents inhibits AVP neuron activity leading low AVP and increased urination (De Mota et al., 2004). It has also been shown that water deprivation increases the apelin immunoreactivity in the hypothalamic nuclei (Goazigo et al., 2004) and inhibits its release. Our results showed a low immunoreactivity of apelin in the posterior pituitary. The exact reason of this low apelin immunoreactivity remains to be investigated in the posterior pituitary. Moreover, it could be suggested that water was given ad libitum in our experiment, thus, apelin expression could have been lower in the posterior pituitary due to decreased apelin synthesis at hypothalamic level. A recent study has suggested that under physiological conditions, apelin and AVP are released in balanced proportions from the magnocellular AVP neurons for water balance (Girault-Sotias et al., 2021); this could be reason of low apelin immunoreactivity in the posterior pituitary during estrous cycle. It would have been interesting and worthy to measure the apelin release from posterior pituitary during estrous cycle to ascertain the reason of low apelin immunoreactivity in the posterior pituitary.

The presence of apelin and APJ in the anterior pituitary during estrus phase might be suggested to have some influence on the gonadotropin secretion. Since we have not performed the co localization of apelin systems in the gonadotrophs along with LH and FSH, thus, only a suggestive role of apelin system in LH and FSH secretion might be proposed. The presence of apelin system has been shown in corticotrophs and the possibility of apelin system in the gonadotrophs has not been ruled out (Reaux-Le Goazigo et al., 2007). It is well known LH surge facilitates the ovulation, thus, whether apelin could influence the LH and FSH secretion from pituitary during estrus, we have performed in vitro study on the pituitary explants. The pituitary explants study was performed in two phases of estrous cycle, proestrus and estrus. Our results showed that, the treatment of apelin 13 has suppressed that GnRH agonist stimulated FSH and LH secretion in proestrus, however, treatment of APJ, antagonist, ML221 increased GnRH stimulated FSH secretion without affecting LH secretion. In the estrus phase, GnRH showed stimulatory effect on LH and no effect on FSH has been observed. It has also been shown that at the end of the follicular phase (proestrus in rodents), pituitary showed more responsiveness to GnRH (Silveira et al., 2017) and LH surge is required for ovulation. Moreover, the treatment with apelin 13 again inhibited GnRH-stimulated LH secretion; surprisingly, ML221 also inhibited GnRH stimulated LH secretion. The ML221 mediated LH inhibition needs further investigation. The secretion of FSH showed a mild increase after apelin 13 and ML221 treatment. To best of our knowledge, our result is first report on the direct role of apelin on LH and FSH secretion from mice pituitary. Our immunolocalization study also showed increase abundance of apelin and APJ in the diestrus, thus, it may be suggested that apelin system could be involved in suppression gonadotropin secretion during diestrus, as next round of folliculogenesis yet to start, however, increase abundance of apelin and APJ during estrus remain unclear with respect to gonadotropin secretion. However, our *in vitro* results suggest that apelin might suppress the gonadotropin section from pituitary. Previous studies have also showed that apelin inhibits LH secretion in male rats (Sandal et al., 2015; Taheri et al., 2003). The expression of apelin system in the gonadotrophs remain unclear from the

present study as well as from earlier study by Reaux-Le Goazigo et al (2007), however, the modulation of pituitary LH and FSH secretion by the apelin system during estrous cycle of mice, prompted us to hypothesize that apelin system might be regulating gonadotropin secretion by modulating corticotrophs functions, although we do not have the supplementary results for this hypothesis. Furthermore, Reaux-Le Goazigo et al (2007) showed that apelin can stimulate basal ACTH secretion. Previous study also proposed that ACTH and cortisol might modulate the GnRH stimulated LH secretion from pig pituitary in vitro, however, only cortisol, not ACTH showed inhibitory effect on LH secretion (Li, 1987). Since the ACTH is derived from POMC, therefore, POMC related or derived peptides (endogenous opiates) might be involved in apelin mediated LH suppression. It has been shown that endogenous opiates exert an inhibitory action on LH secretion during the menstrual cycle (Quigley and Yen, 1980). It should be noted that there was a minor statistical change in GnRH-elicited LH secretion in presence of apelin receptor antagonist. Since this finding was based on our in vitro study and this could be due to a change in osmolarity, thus further study should be required to elicit its role in vivo conditions.

We have also examined the localization of apelin and APJ in the ovary during estrous cycle. The intense immunostaining of apelin and APJ was observed in the corpus luteum as well as in the antral follicles of proestrus phase. Previous studies have also shown that the corpus luteum at proestrus represent the regressing structure (Bagheripour et al., 2017; Gaytan et al., 2017) and the presence of apelin and APJ suggests its role in the regression of corpus luteum. The staining of apelin showed faint immunostaining in the corpus luteum and antral follicles of estrus phase, however, moderate immunostaining of APJ was observed in the corpus luteum and antral follicles of estrus phase. The presence of apelin system in the corpus luteum and follicle suggests that apelin might have modulatory effect of the progesterone and estrogen secretion. Previous studies have also shown that apelin increases the progesterone and estrogen secretion from human granulosa cells, buffalo ovarian follicles and corpus luteum and bovine luteinizing granulosa cells (Roche et al., 2016; Roche et al., 2017; Gupta et al., 2003). It should also

be noted that abundance of apelin and APJ in the corpus luteum from metestrus to diestrus showed an increasing trend in the abundance of apelin system. Based on these findings, it can be suggested that apelin system might be involved in the luteinizing of granulosa cells and maturation of corpus luteum. Our result is in agreement with previous study on the cow ovary where involvement of the apelin-APJ system shown in the maturation of corpus luteum (Shirasuna et al., 2008).The ovarian follicles also showed increase abundance of APJ from metestrus to estrus, which suggest that apelin system could also be involved in the folliculogenesis. Previous study has also suggested apelin system might regulate folliculogenesis and other aspect of ovarian function such as steroid hormone secretion, proliferation, or apoptosis (Kurowska et al., 2018). It has also shown that apelin system has a stimulatory role in bovine ovarian function in vivo and inhibitory role in vitro study (Roche et al., 2017). It should be noted that present study has investigated only the expression of apelin and APJ at protein levels, however, it would have been worthy to analyse the expression of apelin and APJ at gene levels to strengthens the findings. This is limitation of present study, which requires further study.

Conclusion

In conclusion, this is first report on the cyclic changes of apelin system along HPO axis during estrous of mice. Our results showed that expression of apelin system changes in HPO axis during estrous cycle of mice. The pattern of changes of apelin system during estrous cycle showed tissue dependent variation along HPO axis. Based on our findings and support from previous studies, it might be suggested that apelin might have an inhibitory role on hypothalamus and pituitary and stimulatory role on the ovarian functions.

Summary

Apelin is an endogenous peptide, whose expression has been shown in the hypothalamus, pituitary, and ovary; furthermore, it is also called a neuropeptide, binding to apelin receptor (APJ) for various functions. It has been suggested that the

hypothalamus, pituitary and ovarian (HPO) axis is tightly regulated and factors and functions of the HPO axis can be modulated during the estrous cycle to influence reproductive status. To the best of our knowledge, the status of apelin and its receptor, APJ has not been investigated in the HPO axis during the estrous cycle. To explore the expression of apelin and APJ in the HPO axis of mice during the estrous cycle, mice were divided into four groups proestrus (Pro), estrus (Est), metestrus (Met), and diestrus (Di), and apelin and apelin receptor (APJ) were checked. Further, to explore the role of apelin in gonadotropin secretion, an in vitro study of the pituitary was performed at the proestrus and estrus stages. The expression apelin and APJ in the hypothalamus showed elevation during the estrous cycle of post-ovulatory phases, metestrus, and diestrus. The immunolocalization of apelin and APJ in the anterior pituitary showed more abundance in the estrus and diestrus. Our in vitro results showed that GnRH agonist stimulated LH secretion was suppressed by the apelin 13 peptide from the pituitary of proestrus and estrus phases. This suggests an inhibitory role of apelin on gonadotropin secretion. The ovary also showed conspicuous changes in the presence of apelin and APJ during the estrous cycle. The expression of apelin and APJ coincides with folliculogenesis and corpus luteum formation and the expression of the apelin system in the different cell types of the ovary suggests its cell-specific role. Previous studies also showed that apelin has a stimulatory role in ovarian steroid secretion, proliferation, and corpus luteum. Overall our results showed that the apelin system changes along the HPO axis during the estrous cycle and might have an inhibitory at level of hypothalamus and pituitary and a stimulatory role at ovarian level.

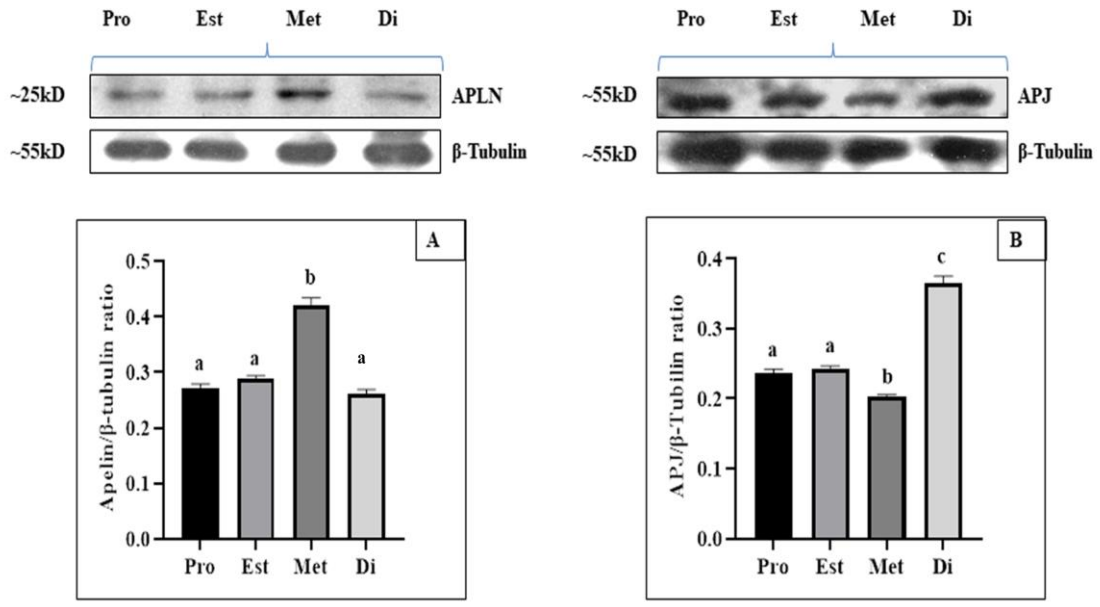

Fig.3.1: Immunoblotting for apelin (A) and APJ (B) in the hypothalamus during estrous cycle. The data are represented as the mean ± SEM. Different alphabet (a,b) showed significant differences ($p<0.05$). Pro, Proestrus group; Est, Estrus group; Met, Metestrus group; Di, Diestrus group. The differences between the data were analyzed by One-way Analysis of variance (ANOVA) followed by Tukey's test.

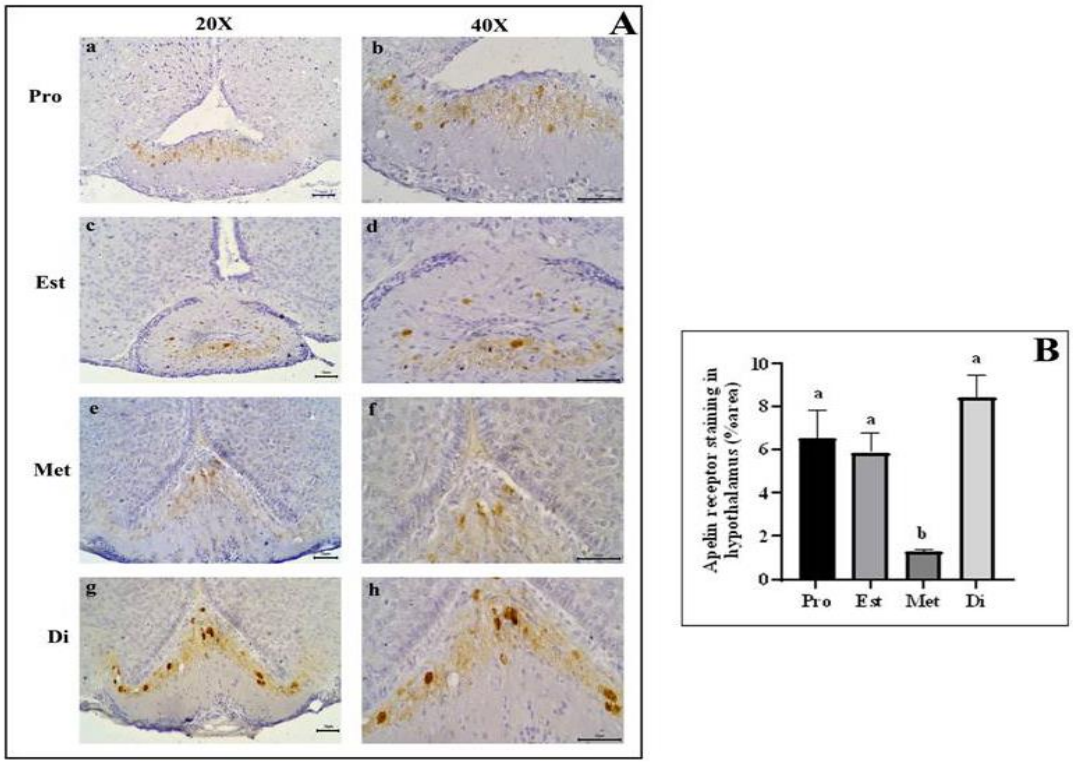

Fig.3.2: Immunostaining of APJ in the brain through median eminence and third ventricle of the hypothalamus during estrous cycle (A). (a,b) Proestrus group at 20X and 40X magnification; (c,d) estrus group at 20X and 40X magnification; (e,f) metestrus group at 20X and 40X magnification; (g,h) diestrus group at 20X and 40X magnification. Pro, Proestrus group; Est, Estrus group; Met, Metestrus group; Di, Diestrus group; ME, Median eminence; 3V, Third ventricle. B shows APJ stained percentage area. The data are represented as the mean ± SEM. Different alphabet (a,b) showed significant differences ($p<0.05$). The differences between the data were analyzed by One-way Analysis of variance (ANOVA) followed by Tukey's test.

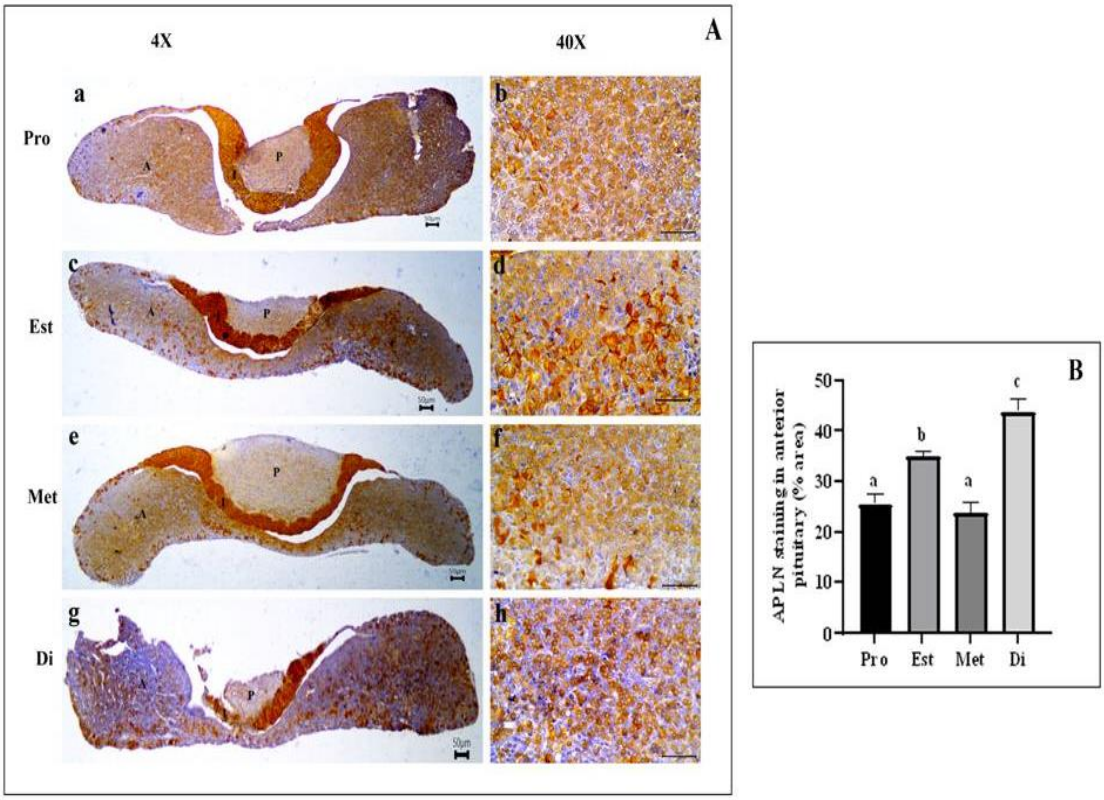

Fig.3.3: Immunostaining of apelin in pituitary gland during estrous cycle (A). (a,b) proestrus group at 4X and 40X magnification; (c,d) estrus group at 4X and 40X magnification; (e,f) metestrus group at 4X and 40X magnification; (G, H) diestrus group at 4X and 40X magnification. Pro, Proestrus group; Est, Estrus group; Met, Metestrus group; Di, Diestrus group, P, Posterior pituitary; I, Intermediate lobe; A, Anterior lobe. B shows apelin stained percentage area. The data are represented as the mean ± SEM. Different alphabet (a,b) showed significant differences ($p<0.05$). The differences between the data were analyzed by One-way Analysis of variance (ANOVA) followed by Tukey's test.

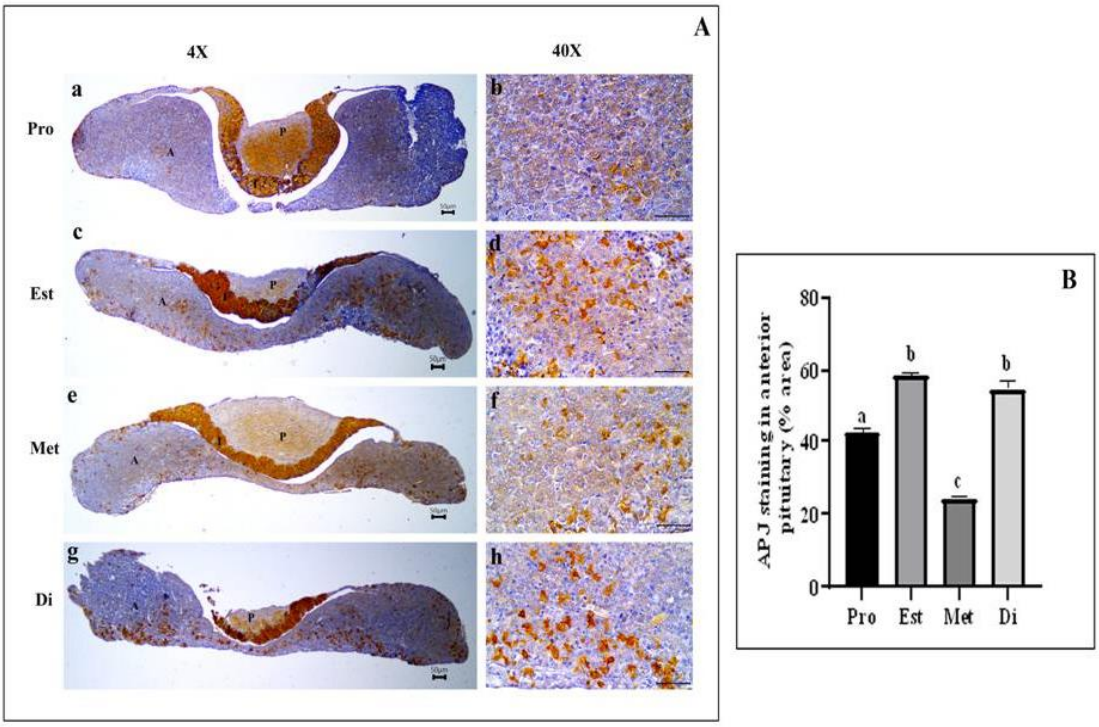

Fig.3.4: Immunostaining of APJ in pituitary gland during estrous cycle (A). (a,b) Proestrus group at 4X and 40X magnification; (c,d) estrus group at 4X and 40X magnification; (e,f) metestrus group at 4X and 40X magnification; (g,h) diestrus group at 4X and 40X magnification. Pro, Proestrus; Est, Estrus; Met, Metestrus; Di, Diestrus, P, Posterior pituitary; I, Intermediate lobe; A, Anterior lobe. B shows apelin receptor stained percentage area. The data are represented as the mean ± SEM. Different alphabet (a,b) showed significant differences ($p<0.05$). The differences between the data were analyzed by One-way Analysis of variance (ANOVA) followed by Tukey's test.

Pro

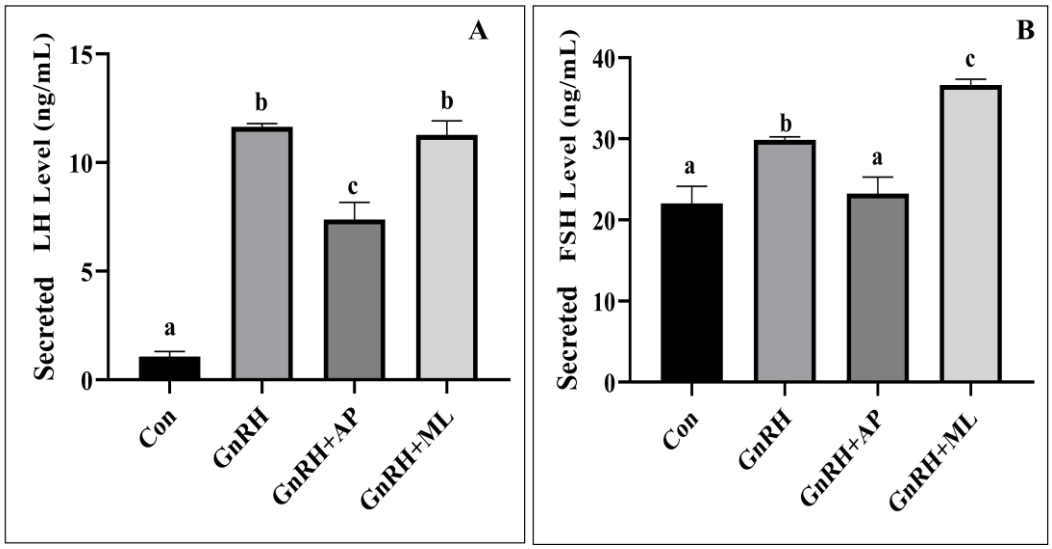

Fig.3.5: Effect of apelin 13 and ML221 treatment on the GnRH agonist stimulated gonadotropin, secretion by pituitary at proestrus phase. (A) Luteinizing hormone; (B) Follicle-stimulating hormone. The data are represented as the mean ± SEM. Different alphabet (a,b, c) showed significant differences ($p<0.05$). CON, Control group; GnRH, Gonadotropin-releasing hormone group; GnRH+AP, apelin-treated gonadotropin-releasing hormone group; GnRH+ML, apelin inhibitor-treated gonadotropin-releasing hormone group. The differences between the data were analyzed by One-way Analysis of variance (ANOVA) followed by Tukey's test.

Est

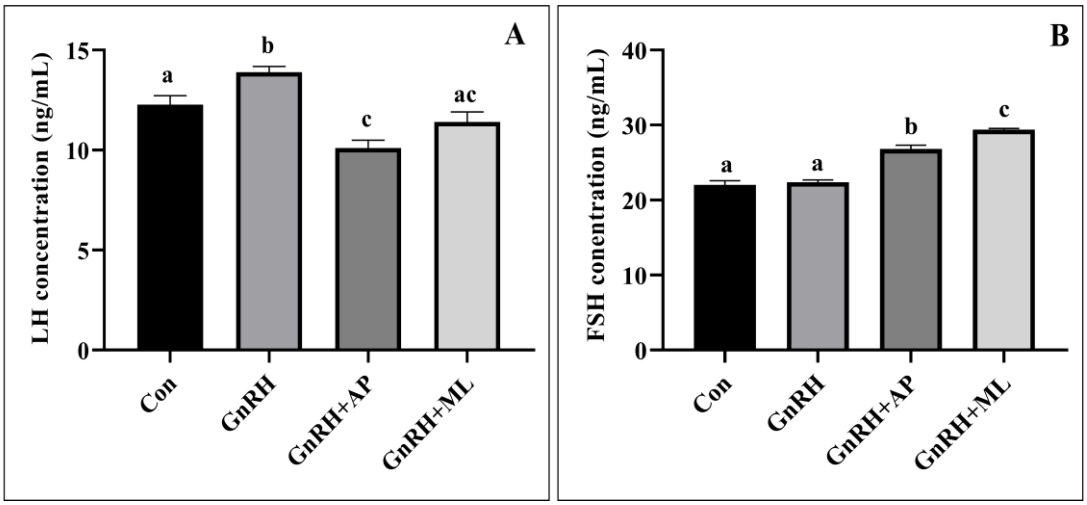

Fig.3.6: Effect of apelin 13 and ML221 treatment on the GnRH agonist stimulated gonadotropin, secretion by pituitary at estrus phase. (A) Luteinizing hormone; (B) Follicle-stimulating hormone. The data are represented as the mean ± SEM. Different alphabet (a, b, c) showed significant differences ($p<0.05$). Con, Control group; GnRH, Gonadotropin-releasing hormone group; GnRH+AP, apelin-treated gonadotropin-releasing hormone group; GnRH+ML, apelin inhibitor-treated gonadotropin-releasing hormone group. The differences between the data were analyzed by One-way Analysis of variance (ANOVA) followed by Tukey's test.

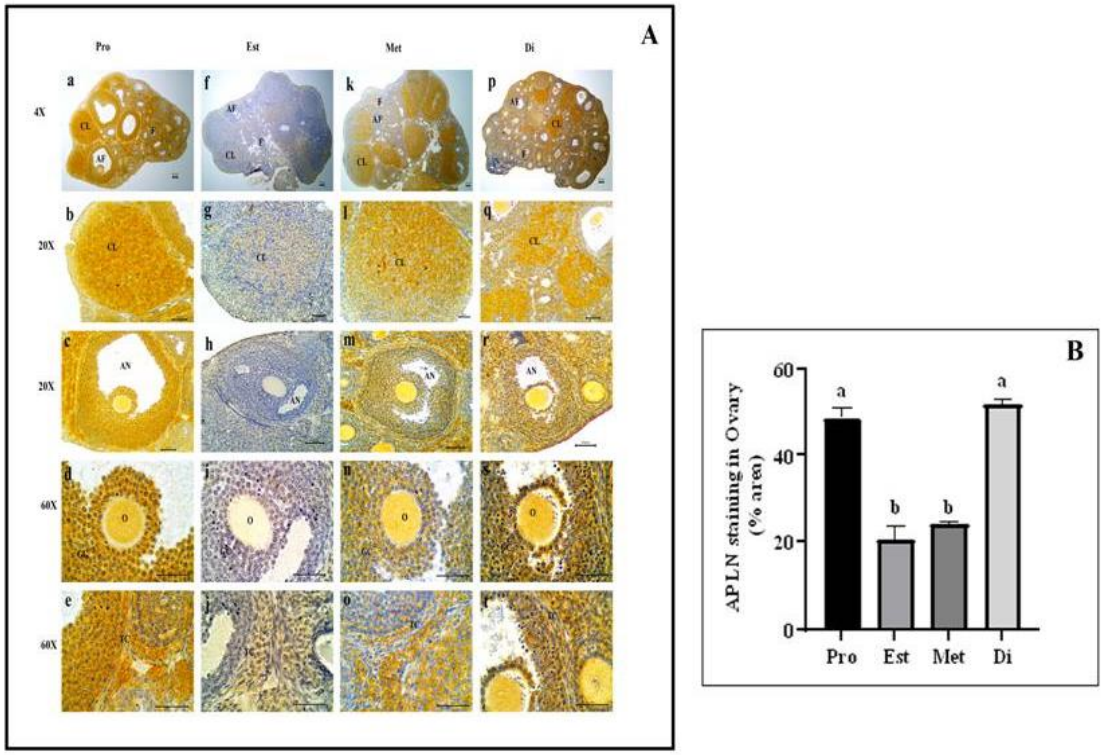

Fig.3.7: Immunostaining of apelin in the ovary during estrous cycle (A). (a-e) Proestrus group, (f-j) estrous group; (k-o) metestrous; (p-t) diestrous. (a, f, k, p) ovary at 4X magnification; (b, g, l, q) corpus luteum at 20X magnification; (c, h, m, r) antral follicle at 20X magnification; (d, i, n, s) oocytes and granulosa cells at 60X magnification; (e, j, o, t) thecal cells at 60X magnification. Pro, Proestrus group; Est, Estrus group; Met, Metestrus group; Di, Diestrus group, AF, Antral follicle; F, Follicle; CL, Corpus luteum; AN, Antrum; O, Oocyte; GC, Granulosa cells; TC, Thecal cells. B shows apelin stained percentage area. The data are represented as the mean ± SEM. Different alphabet (a,b) showed significant differences ($p<0.05$). The differences between the data were analyzed by One-way Analysis of variance (ANOVA) followed by Tukey's test.

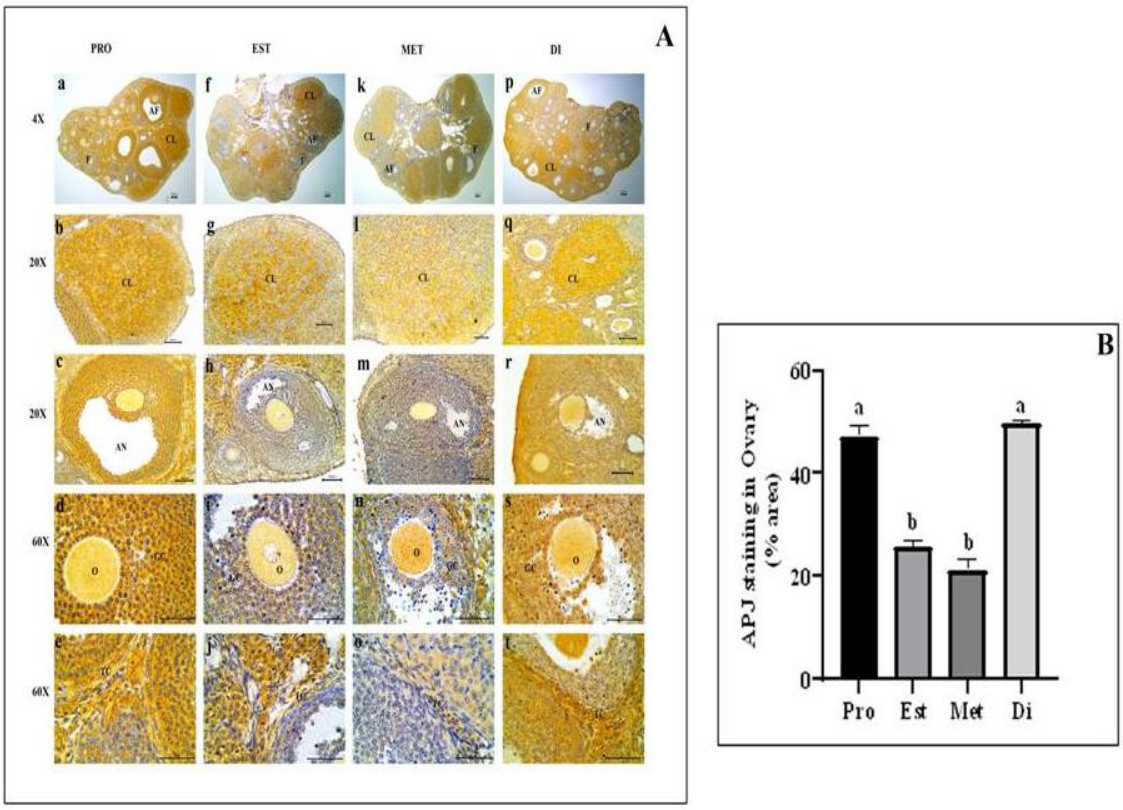

Fig.3.8: Immunostaining of APJ in the ovary during estrous (A). (a-e) Proestrus group, (f-j) estrous group; (k-o) metestrous; (p-t) diestrous. (a, f, k, p) ovary at 4X magnification; (b, g, l, q) corpus luteum at 20X magnification; (c, h, m, r) antral follicle at 20X magnification; (d, i, n, s) oocytes and granulosa cells at 60X magnification; (e, j, o, t) thecal cells at 60X magnification. Pro, Proestrus group; Est, Estrus group; Met, Metestrus group; Di, Diestrus group, AF, Antral follicle; F, Follicle; CL, Corpus luteum; AN, Antrum; O, Oocyte; GC, Granulosa cells; TC, Thecal cells. B shows APJ stained percentage area. The data are represented as the mean ± SEM. Different alphabet (a,b) showed significant differences ($p<0.05$). The differences between the data were analyzed by One-way Analysis of variance (ANOVA) followed by Tukey's test.

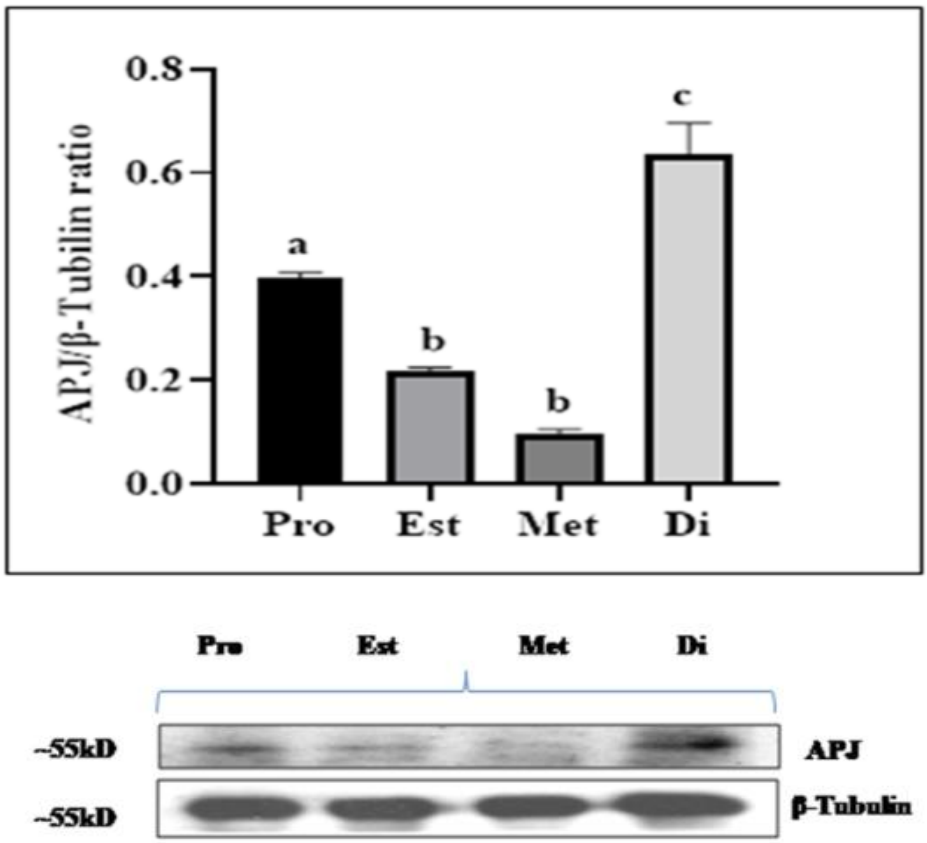

Fig.3.9: Immunoblotting for APJ in the ovary during the estrous cycle. The data are represented as the mean ± SEM. Different alphabet (a,b,c) showed significant differences ($p<0.05$). Pro, Proestrus group; Est, Estrus group; Met, Metestrus group; Di, Diestrus group. The differences between the data were analyzed by One-way Analysis of variance (ANOVA) followed by Tukey's test.

CHAPTER 4

Title

Hormonal dependent expression of apelin and apelin receptor in the ovary and uterus of mice

***Reproductive Biology, (accepted on 17.06.2024)**

Chapter 4

Introduction

Adipose tissue is now considered as an endocrine organ, which secretes hormones/factors known as adipokines and which are involved in a number of physiological processes, including metabolism and reproduction (Kershaw and Flier, 2004; Scheja and Heeren, 2019; Kurowska et al., 2018). A variety of adipokines are secreted mainly by adipocytes, such as leptin, adiponectin, apelin, chemerin, resistin, vaspin and visfatin, which are involved in the reproductive function (Reverchon et al., 2014; Estienne et al., 2019). Out of many adipokines, apelin was isolated first from the bovine stomach and identified as an endogenous ligand for a G protein-coupled receptor called apelin receptor (APJ or APLNR) (Tatemoto et al., 1998). Previously, APJ was considered an orphan receptor and was named after the discovery of apelin (O'Dowd et al., 1993).

Many studies have reported the expression of the cholinergic (apelin/APJ) system in several tissues and organs, including the hypothalamic-pituitary-gonadal axis, reproductive organs such as the ovary, uterus, testes, placenta and mammary gland of several species (Anima et al., 2022, 2023;Brailoiu et al., 2002;Wang et al., 2020; Moretti et al., 2023; Das et al., 2022). Apelin has also been shown to be expressed in the granulosa cells and oocytes to further regulate steroidogenesis in the ovary (Roche et al., 2017; Rak et al., 2017). While, there is very few literature on related or association of apelin with the uterus. Previous reports showed the apelin distribution in rat uterus (Kawamata et al., 2001). Apelin exerts a potent inhibitory effect on human myometrial contractility during early pregnancy (Hehir et al., 2012). Further, it has also been shown that apelin level was increased and induced uterine contraction during the end of pregnancy in rats, which might be affected by the protein kinase C pathway (Kacar et al., 2018). Recently, we have reported the expression of apelin and APJ in the ovary and uterus of mice changes depending on postnatal age (Anima et al., 2022). It has also been shown that the expression of apelin and APJ are expressed in the HPO axis during estrous cycle of mice and apelin suppresses the gonadotropin secretion from the pituitary

(Anima et al., 2023). In the same study, it was hypothesized that apelin might have an inhibitory role in hypothalamic-pituitary secretion and a stimulatory role in ovarian functions, especially in folliculogenesis and corpus luteum formation (Anima et al., 2023). Apelin has been shown to promote ovarian granulosa cell proliferation and inhibit apoptosis via the PI3/Akt signalling pathway (Shuang et al., 2016). Gametogenesis, steroidogenesis, and ovarian development are all regulated by gonadotropins. Follicle-stimulating hormone (FSH) stimulates the growth of ovarian follicles, whereas luteinizing hormone (LH) controls ovulation, preovulatory oocyte maturation, and corpus luteum production (Lee et al., 2021). Since gonadotropin promotes folliculogenesis, various intra-ovarian factors are involved in folliculogenesis (Orisaka et al., 2021). Thus, the question arises whether ovarian apelin and APJ expression are gonadotropin dependent.

It has also been shown that the apelin/APJ system might be involved during embryo development, specifically in angiogenesis and proliferation, implantation, decidualization and the formation of the placenta and tumor angiogenesis (Balci et al., 2023; Kidoya et al., 2008; Kalin et al., 2007; Cox et al., 2006). Uterine proliferation has two peaks: firstly, ovary-independent proliferation at the early period and ovarian steroid-dependent proliferation at puberty as endocrine exposure of neonates compromises reproductive cycles and pregnancy in the adult (Bartol et al., 1999; Wu et al., 1988). The uterus undergoes physiological changes during each reproductive cycle in mammals, and the endometrium of the uterus undergoes repeating synchrony of cellular proliferation, apoptosis, and differentiation, along with extracellular matrix turnover, angiogenesis, and leukocyte infiltration (Dharma et al., 2001; Smith et al., 2009; Evans et al. 1990). The systemic changes during the estrous cycle depend on ovarian steroids, 17β-estradiol (E2) and progesterone (P4) (Wood et al., 2007). However, the expression of apelin and APJ has not been investigated in the uterus during estrous cycle. Furthermore, one study showed that P4 regulates apelin expression not by E2 and APJ was regulated neither by E2 nor P4 (Balci et al., 2023). Thus, it

would be relevant to examine the role of ovarian steroids on uterine expression of apelin and APJ.

It would be logical to investigate the effect of gonadotropin on ovarian apelin and APJ expression and ovarian steroids on the uterine apelin system. Thus, we hypothesized that the apelin and APJ expression might be changed in the uterus during estrous cycle and apelin expression might be dependent on hormones in the uterus and ovary. Therefore, this study aimed to investigate the expression and localization of uterine apelin during the estrous cycle of mice and explore the effect of ovarian steroids on uterine apelin expression and the role of gonadotropin on premature ovarian apelin and APJ expression.

Materials and Methods

Ethical statement and Animal maintenance

Female Swiss albino mice were handled and received humane care in compliance with protocols approved by the Mizoram University Institutional Animal Ethical Committee (Protocol Number, MZU/IAEC/ 2020/12), Mizoram University, Mizoram, India and all animal experiments complied with the ARRIVE guidelines. Mice were 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.

Reagent

β-Estradiol was purchased from Tokyo Chemical Industry Pvt Ltd, Portland, USA (Cat# E0025).

Progesterone was purchased from Tokyo Chemical Industry Pvt Ltd, Portland, USA (Cat# P0478).

Pregnant mare serum gonadotropin was purchased from Prospec-Tany Technogene Ltd, USA (Cat# hor-272-a).

Human chorionic gonadotropin (HCG) hormone was purchased from Lupin Ltd, Mumbai, India.

Experimental design and sample collection

Experiment 1: Estrous cycle experiment

Matured (2-3months) and virgin female mice (N=16) weighing 25 ± 3 g were checked for 3–4 regular estrous cycles by obtaining vaginal smears daily, which showed four distinct stages such as proestrus, estrus, metestrus and diestrus by following the previous method (Goldman et al. 2007). In brief, morning, around 8:00-9:30 am, the vaginal smear of all the mice was taken with a dropper and observed under the microscope. The estrous phases were identified and classified based on their vaginal cytology. The vaginal smear shows oval flat nuclear epithelial cells considered in the proestrus phase, only cornified cells in the estrus phase, cornified and white blood cells together in the metestrus phase, and mostly white blood cells in the diestrus phase. Accordingly, mice (n=4) were grouped as (i) Proestrus (Pro) group, (ii) Estrus (Est) group, (iii) Metestrus (Met) group and (iv) Diestrus (Di) group. All the mice were sacrificed under mild anaesthesia at respective phases of the estrous cycle, and the uterus and blood samples were dissected. One horn of the uterus from each uterus was fixed in Bouin's solution for immunohistochemistry analysis, and the other horn was stored at 20°C for western blotting analysis. Blood samples were centrifuged for 15 minutes at 12000 g, and then the serum was collected carefully and stored at -20 °C for further hormone assay.

Experiment 2: Ovariectomy and Steroid hormone treatment

Healthy, Matured (2-3 months) and virgin female mice (N=16) weighing 25 ± 3 g were used for this experiment irrespective of their estrous stages. The mice were anaesthetized prior to bilateral ovariectomy. The mice were checked daily and rested for two weeks for complete recovery and to avoid steroid hormones in circulation. Vaginal smears were checked and noted. Then, the mice (n=4) were randomly divided into four groups: (i) control group, received vehicle alone, i.e., sesame oil. (ii) Estradiol group (E2), received

30 ng/g body weight of estradiol subcutaneously. (iii) Progesterone group (P4), received subcutaneously in 150 μg/g body weight of progesterone, subcutaneously and (iv) Estrogen plus progesterone group (E2 + P4), received both E2 and P4. The treatment was given for three consecutive days (Annie et al., 2019). After 24 hours of last treatment, all the mice were sacrificed under anaesthesia, and uterus samples were collected. One horn of the uterus from each uterus was fixed in Bouin's solution for immunohistochemistry analysis, and the other horn was stored at 20°C for western blotting analysis.

Experiment 3: Gonadotropin treatment

To further establish the specific effect of gonadotropin (FSH and LH) on the ovarian and uterine apelin and APJ protein, sexually immature (23 days old) mice (N=12) were selected (Wandji et al., 1998). Mice (n=4) were randomly categorised into three groups as (i) Control (Con) group, which received vehicle (distilled water) alone through IP injection, (ii) PMSG treated (PMSG) group, which received 2.5 IU of pregnant mare serum gonadotropin (PMSG) through IP injection, and (iii) PMSG plus HCG treated (PMSG) group, received 2.5 IU of pregnant mare serum gonadotropin (PMSG) followed by 2.5 IU of human chorionic gonadotropin through IP injection after 48 hrs. The treated mice were sacrificed after 24 hours of PMSG or both PMSG and HCG treatment, and the ovaries, uterus and blood samples were collected. Half of the ovary and one horn of each uterus were fixed in Bouin's solution for immunohistochemical analysis, and the remaining half was stored at -20°C for western blotting analysis. Blood samples were centrifuged for 15 minutes at 12000 g; then, the serum was collected carefully and stored at -20 °C for further hormone assay.

Immunohistochemistry

Immunohistochemistry analysis was performed using a method described earlier (Anima et al., 2023). The fixed ovaries and uterus were transferred to 70% alcohol within 24 hours to avoid brittleness and dehydration through 90%, and 100%ethanol and paraffin

blocks were made. The blocks were then sections of 7µm thick and mounted onto cleaned gelatin (1%) coated slides and kept at 37°C for 24 hours. These slides were then used for immunohistochemical analysis of apelin (anti-apelin, 1:50, rabbit polyclonal, cat#SAB4301741, Sigma Aldrich, USA) and APJ (anti-apelin receptor, 1:50, rabbit polyclonal, cat# ABD43, Millipore) primary antibody. The secondary IgG antibody (1:200, goat anti-rabbit, lot# HPO32023, Genei Laboratories Private Limited, India) were used to make the antibody complex and made visible by reaction with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemicals Co., St Louis, USA) in 0.05 M Tris pH 7.6 and 0.1% H2O2 at room temperature. Hematoxylin was used to counterstain the tissue section. The slides were examined and photographed by using a microscope (iScope series, Euromex, Netherlands). The immunostaining of antibodies was quantified using Image J's threshold tool and shown as a percentage of the staining area. The negative control section was replaced with non-immune IgG (rabbit) in place of the primary antibody. Additionally, an absorption (antibody preabsorbed with antigen) control was run to verify the apelin antibody's specificity. In brief, the apelin peptide (1:10 ratio) was pre-incubated for 24 hours at 4°C with the apelin antibody. By binding with the target antigen, this mechanism makes the antibody inactive. The preabsorbed antibody was then treated with the tissue using the previously described procedure instead of the primary antibody.

Western blot analysis

Western blotting was performed for the ovary and uterus of each experiment following a method described earlier (Anima et al., 2023). Tissue homogenate (10%) was prepared with lysis buffer, and protein was estimated using the Bradford method (Bradford, 1976). An equal amount of protein (50 µg) was resolved on 12% SDS-PAGE for electrophoresis and transferred electrophoretically to a polyvinylidene difluoride membrane (PVDF, Millipore, Bangalore, India) using a wet transfer apparatus. Blotted membranes were blocked and incubated with the primary antibody of the anti-apelin receptor(1:1000, rabbit polyclonal, cat# ABD43, Millipore) at 4°C overnight.

Membranes were then washed with PBS-Tween20 buffer and incubated with a secondary antibody (1:2000, goat anti-rabbit, lot# HPO32023, Genei Laboratories Private Limited, India) for 4 hours at room temperature. Immunodetection will be performed with an enhanced chemiluminescence detection system (BioRad, Hercules, USA) and developed onto X-ray film. Experiments were repeated three times, and X-rays were later scanned and then quantified by ImageJ software (imagej.nih.gov/). All immunoblots will be normalized to β-Tubulin antibody (1:1500, cat#E7; DSHB, University of Iowa, Department of Biology, United States) and secondary antibody (1:4000, goat anti-mouse, lot# HPO52023, Genei Laboratories Private Limited, India).

Enzyme-linked immunosorbent assay (ELISA)

ELISA assay was performed to estimate the circulating levels of E2 and P4 hormones for the estrous cycle and gonadotropin experiment. Serum samples were used to estimate the levels of the hormones by using commercial ELISA kit. ELISA assays were performed as per the manufacturer's instructions. The circulating E2 and P4 levels were quantified by using a human Estradiol kit (Cat# DKO003, DiaMetra, Italy) and a Progesterone EIA kit (Cat # DKO006, DiaMetra, Italy), respectively. Microplate Reader (Erba Lisa Scan EM, Trans Asia Biomedical Ltd, Mumbai, Maharashtra, India) was used immediately to read the absorbance, and the absorbance was read at 450 nm wavelength. The intra-assay and inter-assay variability for E2 were ≤ 9% and ≤ 10%, respectively. The intra-assay and inter-assay variability for P4 were ≤4% and ≤ 9.3%, respectively.

Statistical analysis

GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) was used for all the statistical analysis. The data were analyzed using a one-way analysis of variance (ANOVA), followed by multiple comparisons with Tukey's post-hoc test. The distribution of normality was checked, and all the data was passed by the Shapiro-Wilk test. Mean ± standard error mean (SEM) was used to express all the data, and a P value

less than 0.05 was considered a significant difference. The correlation was determined by the Pearson correlation test.

Result

Immunolocalization of apelin and APJ in the uterus during estrous cycle

To reveal the changes in the cellular localization of apelin and APJ in the uterus during estrous cycle, immunohistochemistry analysis was performed. Immunolocalization of apelin showed marked variation in the different compartments and different phases of the uterus during the estrous cycle (Fig.1AH). The luminal epithelium of the metestrus phage showed intense staining, and the proestrus and estrous phases showed moderate staining and mild staining at the diestrus phase. Apelin immunostaining in the stromal cells and endometrium layer of the uterus showed intense staining in the proestrus, estrus and metestrus phases, whereas moderate staining in the diestrus phase. Myometrium and perimetrium layers of the uterus showed intense staining of apelin in the metestrus phase and moderate staining in the proestrus and estrus phases. Diestrus showed mild and moderate staining of apelin in the myometrium and perimetrium layers, respectively. The glandular epithelium of the uterus showed intense staining in the estrus phase and metestrus phases and moderate and mild staining in the proestrus and diestrus phases, respectively.

Immunolocalization of APJ also showed marked variation in different compartments and different phases of the uterus during the estrous cycle (Fig.2AH). The luminal epithelium of the proestrus, oestrous, and diestrus phases showed intense APJ staining and moderate staining in the metestrus phase. APJ immunostaining in the stromal cells and endometrium layer of the uterus showed intense staining in the proestrus and diestrus phases, followed by moderate and mild staining in the estrus and metestrus phases, respectively. The myometrium layer showed moderate immunostaining in the proestrus, estrus and diestrus phases and mild staining in the metestrus phase. The perimetrium layer showed intense immunostaining of APJ in the proestrus, estrus and

diestrus phases and moderate staining in the metestrus phase. The glandular epithelium of the uterus showed intense staining of APJ in the proestrus and moderate staining in the estrus, metestrus and diestrus phases.

Expression of APJ in the uterus during estrous cycle

The APJ protein showed a significant ($p<0.05$) increased expression at the proestrus, estrus and diestrus phases as compared to the metestrus phase. There were no significant ($p>0.05$) changes between the proestrus, estrus and diestrus phases, whereas the diestrus phase showed decreased expression of APJ protein as compared to the proestrus and estrus phases, although it was no significant ($p>0.05$) change (Fig.3).

Correlation test of E2 and P4 with apelin and APJ in the uterus during estrous cycle

To investigate the correlation between E2 and P4 with apelin and APJ in uterus during estrous cycle, Pearson correlation test was conducted. The result showed a significant positive correlation of circulating E2 levels with apelin expression ($r = 0.7573$, $p = 0.0001$) and a negative correlation with APJ expression ($r = -0.589$, $p = 0.0063$).

Circulating P4 levels showed a significant negative correlation with apelin ($r = -0.7344$, $p = 0.0002$) and a negative correlation with APJ expression ($r = -0.01363$, $p = 0.9545$) (Table 1). (For the correlation test, the serum estrogen and progesterone data were taken from our previous paper, Annie et al., 2019, Adopted with License Number-5795360036247).

Effect of ovarian steroids (E2 and P4) on apelin and APJ localization in the uterus of the ovariectomized mice

To reveal the effect of ovarian steroids on apelin and APJ localization in the uterus, ovariectomized mice uterus was used for immunolocalization analysis. Immunolocalization of apelin showed variation in the different compartments of the uterus after ovarian steroid treatment (Fig.4AH). The luminal epithelium, glandular

epithelium, stromal cells, endometrium, myometrium and perimetrium of E2 treated uterus showed intense immunostaining of apelin (Fig.4CD), moderate in the P4 treated uterus (Fig.4EF) and mild staining in the control (Fig.4AB) and E2 plus P4 treated uterus (Fig.4GH).

The luminal epithelium displayed intense staining in the E2 treated group, moderate staining in the P4 alone and E2 plus P4 treated uterus along with faint staining in the control group (Fig.5AH). The stromal cells, myometrium and perimetrium layers showed moderate staining in the E2 treated, faint staining in the control, mild staining in the P4 and E2 plus P4 treated uterus. The endometrium layer of the E2-treated uterus showed moderate staining of APJ, mild staining in the control group, P4 treated and E2 plus P4 treated uterus.

Effect of ovarian steroids (E2 and P4) on APJ expression in the uterus of the ovariectomized mice

To explore the effect of ovarian steroids (E2 and P4) on APJ expression in the mouse uterus, E2 and P4 treatment was given after bilateral ovariectomy. The APJ expression was significantly ($p<0.05$) decreased in the P4 treated group and E2 plus P4 treated group compared to the control group. The E2-treated group showed a significant ($p<0.05$) increased expression of APJ compared to the control group. There was no significant ($p>0.05$) change between the P4 treated group and the group treated with E2 plus P4 (Fig.6).

Effect of exogenous gonadotropin (PMSG and hCG) in the localization of apelin and APJ in the ovary of immature mice

Since the ovarian function is regulated by gonadotropin. Therefore, elucidate of the specific effect of each of the two gonadotropins, FSH and LH on the immature mice ovary, exogenous gonadotropin, PMSG and hCG was given to immature mice.

Immunolocalization of apelin showed staining in granulosa cells, thecal cells, oocyte and corpus luteum. Granulosa cells showed mild staining in the control group and moderate staining in the PMSG plus hCG and the PMSG-treated group. Thecal cells and oocyte showed mild staining of apelin in the control group and moderate staining in the group treated with PMSG plus hCG and the PMSG alone treated group. The corpus luteum showed moderate staining of apelin in the PMSG plus hCG treated group (Fig.7).

Immunolocalization of APJ showed staining in granulosa cells, thecal cells, oocytes and in the corpus luteum. The granulosa cells showed mild staining of APJ in the control group, intense staining in the group treated with PMSG plus hCG and the PMSG alone treated group. Thecal cells showed no staining of APJ in the control group, moderate staining in the PMSG-treated group and mild staining in the group treated with PMSG plus hCG. The oocytes showed no staining in the control group and mild staining in the group treated with PMSG plus hCG and the PMSG-treated group. The corpus luteum showed intense staining of apelin in the PMSG plus hCG-treated group (Fig.8).

Effect of exogenous gonadotropin (PMSG and hCG) in the expression of APJ in the ovary of immature mice

The expression of APJ protein after gonadotropin treatment showed a significant ($p<0.05$) increase expression in the PMSG-treated group and the group treated with PMSG plus hCG as compared to the control group, whereas the PMSG plus hCG-treated group showed a significant ($p<0.05$) decreased as compared to the PMSG treated group (Fig.9).

Effect of exogenous gonadotropin (PMSG and hCG) on the circulating E2 and P4 levels of immature mice

The E2 level showed a significant ($p<0.05$) increase in the group treated with PMSG plus hCG (126.00±0.68pg/ml) and PMSG-treated group (157.09±7.16pg/ml) as compared to the control group (89.49±10.4 pg/ml). However, the group treated with

PMSG plus hCG showed a significant ($p<0.05$) decreased E2 level as compared to the PMSG-treated group (Fig.10A).

The P4 level showed a significant ($p<0.05$) increase in the group treated with PMSG plus hCG (29.00 ± 0.94 pg/ml) and the PMSG-treated group (12.30 ± 0.98 pg/ml)as compared to the control group (5.14 ± 0.49 pg/ml). The group treated with PMSG plus hCG showed significant ($p<0.05$) increased P4 level as compared to the PMSG group (Fig.10B).

Discussion

The present study has investigated the cyclic expression of apelin and APJ in the mice uterus during the estrous cycle; furthermore, steroid-dependent regulation of apelin and APJ in the uterus and gonadotropin-dependent expression of apelin and APJ has also been investigated. The expression of apelin and APJ in the uterus and ovary of mammalian species has already been shown by various studies (Roche et al., 2017; Schilffarth et al., 2009; Hehir and Morrison, 2012; Shirasuna et al., 2008). Recently, we have shown that the expression of apelin and APJ modulate along the HPO axis during the estrous cycle of mice (Anima et al., 2023). The expression of apelin and APJ has been shown to be developmentally regulated in the uterus of mice (Anima et al., 2022), and it has also been hypothesized that apelin and APJ could be involved in the implantation and decidualization (Balci et al., 2023)**.** However, expression of apelin and APJ has not been investigated in the uterus of mice during estrous cycle. Therefore, it may be hypothesized that the expression and localization of apelin and APJ changes in the uterus during the estrous cycle. The APJ protein expression showed the lowest levels in the metestrus. Immunolocalization of APJ showed mild immunostaining in the stoma, endometrium, and perimetrium during the metestrus phase. The localization of apelin showed intense staining in the metestrus phage, moderate in the proestrus and estrus phages and mild staining in the diestrus phages in the uterus. Apelin showed intense staining in the uterine gland and luminal epithelium of the estrus and metestrus phases. Since observable changes of apelin and APJ were noticed in the uterine gland and

luminal epithelium, apelin/APJ might be regulating the secretary functions of the gland and luminal epithelium during the estrous cycle. Apelin has been shown to regulate blood flow and also control the exocrine secretion from the pancreas (Kapica et al., 2012).

In addition, uterine expression of various factors has been regulated by ovarian steroids (Care et al., 2014)**.** During the estrous cycle, the uterine expression of apelin showed a positive correlation with the circulating E2 level and a negative correlation with the circulating P4 level. Furthermore, the expression of APJ also showed a negative correlation with the circulating E2 level and circulating P4 level in the uterus during the estrous cycle. These findings suggest possible regulation of apelin and APJ by ovarian steroids. Thus, it was also hypothesized that uterine expression of apelin and APJ could be steroid-dependent. Therefore, we have also investigated the effect of E2 and P4 on the uterine apelin/APJ expression. Treatment of E2 to ovariectomized mice significantly increased APJ expression in the uterus compared to control. The treatment of P4 alone or in combination with E2 decreased the expression of uterine APJ compared to the control and E2 alone groups. Our immunohistochemical analysis showed an increased abundance of apelin and APJ in the uterine gland, lumen epithelium, and myometrium of E2-treated mice. The P4 alone decreased the abundance of apelin and APJ in different compartments of the uterus compared to the control and E2 alone groups. However, an abundance of apelin and APJ slightly increased in the luminal epithelium and uterine glands of the E2 and P4-treated uterus compared to the P4-alone group. These findings showed that uterine apelin and APJ expression are regulated by ovarian steroids, where E2 up-regulates apelin and APJ and P4 down-regulates the uterine apelin system. Our previous study has also shown that other adipokines, like visfatin expression, are regulated by the E2 and P4 in the mice uterus (Annie et al., 2019). Recently, it was hypothesized that P4 may control only apelin expression but not E2; moreover, P4 and E2 may not control uterine APJ expression (Balci et al., 2023). The discrepancy between our findings and an earlier study by Balci et al. 2023 could be due to different stages of mice uterus and different experimental approaches studied by us (oestrous cycle-

dependent expression and effect of exogenous steroid on the apelin system), whereas in the earlier study, expression of apelin system was analyzed during pregnancy. These findings also suggest complex regulation of apelin and APJ expression in the uterus.

Likewise, uterus and ovarian functions are regulated by gonadotropin from the pituitary (Messinis et al., 2006). Our previous study has also shown that ovarian apelin and APJ in the mice ovary increase from postnatal day 21 (PND21) to the adult stage. Thus, it would be logical to hypothesize the gonadotropin-dependent augmented expression of apelin and APJ in the ovary of mice. The expression of ovarian apelin and APJ has already been reported in different mammalian ovaries (Roche et al., 2017; Schilffarth et al., 2009). The presence of apelin and APJ in mature ovarian follicles and corpus luteum has been suggested to play a role in hormone secretion, steroidogenesis, and proliferation in the buffalo and pig ovary (Gupta et al., 2023; Rak et al., 2017; Różycka et al., 2018). The treatment of PMSG moderately increased the abundance of apelin in the thecal, granulosa cells and oocytes in the ovary with elevated E2 secretion. Recently, we have also shown that apelin promotes E2 secretion in infantile female mice (Anima et al., 2024). PMSG treatment, followed by HCG treatment, also showed an abundance of apelin in the ovary. The expression of ovarian APJ was found to be intensely regulated by gonadotropin. The expression and localization of APJ showed increased abundance in the thecal, granulosa cells, and oocytes in the PMSG-treated mice; however, the PMSG treatment followed by HCG treatment showed the formation of corpus luteum with increased abundance of APJ and P4 secretion. These findings clearly showed the role of apelin signalling in the corpus luteum formation and its functions. The circulating elevated P4 also coincides with the corpus luteum formation in the immature mice ovary. It has been shown that vascular formation, maturation, and maintenance in the corpus luteum during the estrous cycle may be facilitated by the apelin/APJ system (Shirasuna et al., 2008; Roche et al., 2017). It has also been shown that different isoforms of apelin peptides increased the secretion of E2 and P4 in the buffalo ovary (Shokrollahi et al., 2022). Thus, it may be suggested that gonadotropin-dependent ovarian steroidogenesis and folliculogenesis could be regulated via

apelin/APJ in the mice ovary. To the best of our knowledge, gonadotropin-dependent expression of apelin and its receptor in the ovary and steroid-dependent expression in the uterus has not been investigated.

Conclusion

In conclusion, our study showed that gonadotropin up-regulates the ovarian apelin/APJ system in the mice ovary, which coincides with elevated ovarian steroidogenesis. More particularly, the formation of the corpus luteum involves the apelin and its receptor expression; it might regulate the luteinization of follicles. The uterine expression of apelin and APJ was up-regulated by the E2 and down-regulated by P4 in the ovariectomized mice; these findings showed steroid-dependent regulation of the uterine apelin system in the mice uterus. The function of the uterine apelin system still requires further investigation. Thus, in the normal reproductive cycle, apelin seems to play an important role in ovarian and uterine functions via pituitary gonadotropin and ovarian steroid hormone, respectively.

Chapter 4

Summary

Apelin and APJ have been shown to regulate the female reproductive functions. However, it's uterine expression during estrous cycle and its regulation by ovarian steroid along with gonadotropin dependent its regulation in the ovary has not been investigated. Thus, this study aimed to analyze steroid dependent uterine expression of apelin/APJ in uterus along with estrous cycle. Furthermore, it also aimed to investigate gonadotropin dependent ovarian expression of apelin and APJ. To investigate the uterine expression of apelin and APJ during estrous cycle in mice, uterus at different estrous stage were collected. To explore the ovarian steroids dependent expression of apelin system in the uterus, ovariectomized mice were treated with estrogen at dose of 30 ng/g, progesterone at dose of 150μg/g and combined dose. To study the effect of gonadotropin on ovarian expression of apelin system, immature mice were injected with 2.5 IU of pregnant mare serum gonadotropin (PMSG) alone and both PMSG plus 2.5 IU of chorionic gonadotropin (hCG). Apelin and APJ protein expression are modulated by estrous phases in the uterus. The uterine apelin and APJ expression are up-regulated by estrogen and down-regulated by progesterone. The expression and localization of APJ showed increased abundance in the follicles of PMSG treated mice, however, the PMSG plus hCG treatment showed formation of corpus luteum with increased abundance of APJ and progesterone secretion. The expression of apelin and APJ are regulated by pituitary gonadotropin in the ovary and uterine apelin system by ovarian steroid hormone.

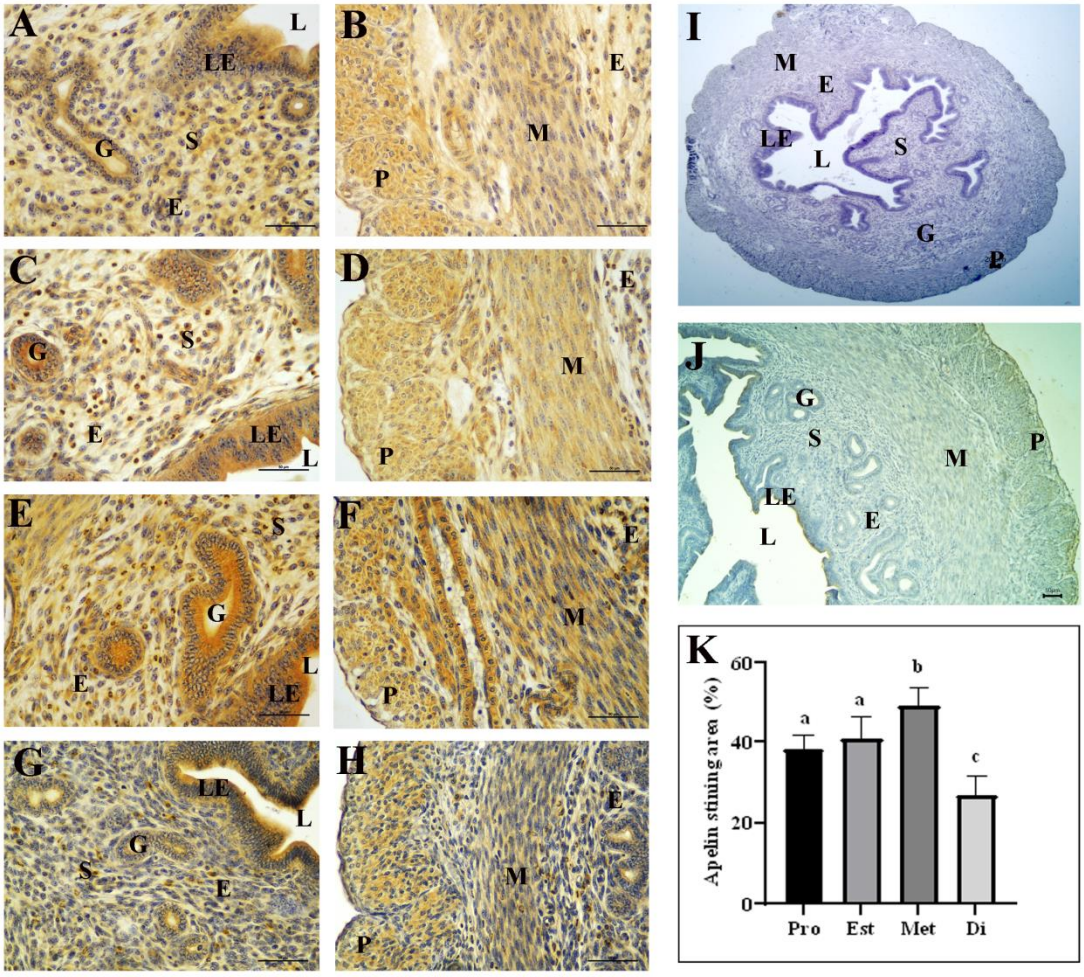

Fig.4.1. Localization of apelin in the uterus during estrous cycle (A-H at 40X magnification). A and B represent the uterus at the proestrus phage, C and D represent the uterus at the estrus phage, E and F represent the uterus at the metestrus phage and G and H represent the uterus at the diestrus phage, I represent negative control (4X), and J represent absorption control (40X). K shows the staining percentage area of apelin. L, lumen; LE luminal epithelium; G, gland; S, stromal cell; E, endometrium, M, myometrium, P, perimetrium; S, stromal cells. Scale bar shows 50μm length. The data are represented as the mean ± SEM. Different alphabet (a, b, c) showed significant difference ($p<0.05$). Pro, Proestrus phase; Est, Estrus phase; Met, Metestrus; Di, Diestrus.

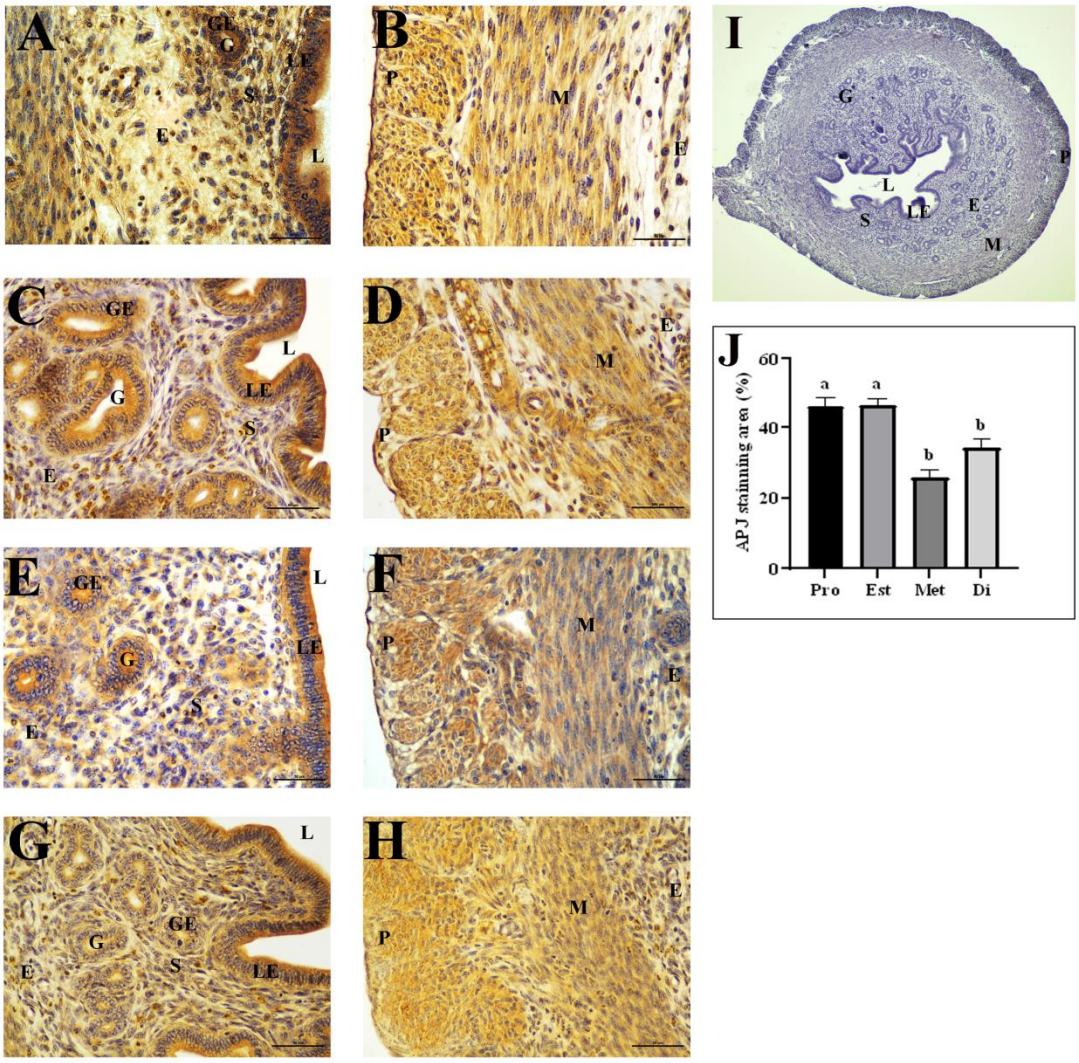

Fig.4.2. Localization of APJ in the uterus during estrous cycle (A-H at 40X magnification). A and B represent the uterus at the proestrus phage, C and D represent the uterus at the estrus phage, E and F represent the uterus at the metestrus phage, and G and H represent the uterus at the diestrus phage. I represent negative control (4X) and J shows the staining percentage area of APJ. L, lumen; LE luminal epithelium; G, gland; S, stromal cell; E, endometrium, M, myometrium, P, perimetrium; S, stromal cells. The scale bar shows 50μm length. The data are represented as the mean ± SEM. Different alphabet (a,b) showed significant difference ($p<0.05$). Pro, Proestrus phase; Est, Estrus phase; Met, Metestrus; Di, Diestrus.

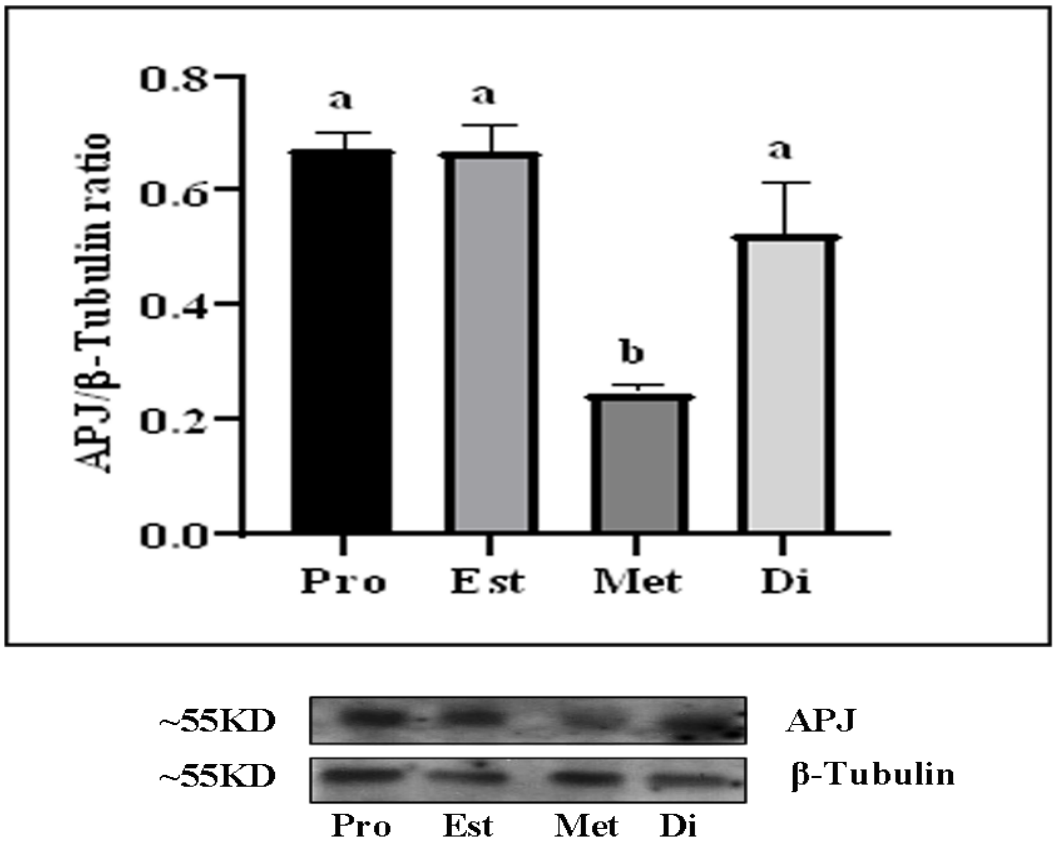

Fig.4.3. Expression of APJ in the uterus during estrous cycle. The data are represented as the mean ± SEM. Different alphabet (a, b) showed significant difference ($p<0.05$). Pro, Proestrus phase; Est, Estrus phase; Met, Metestrus; Di, Diestrus.

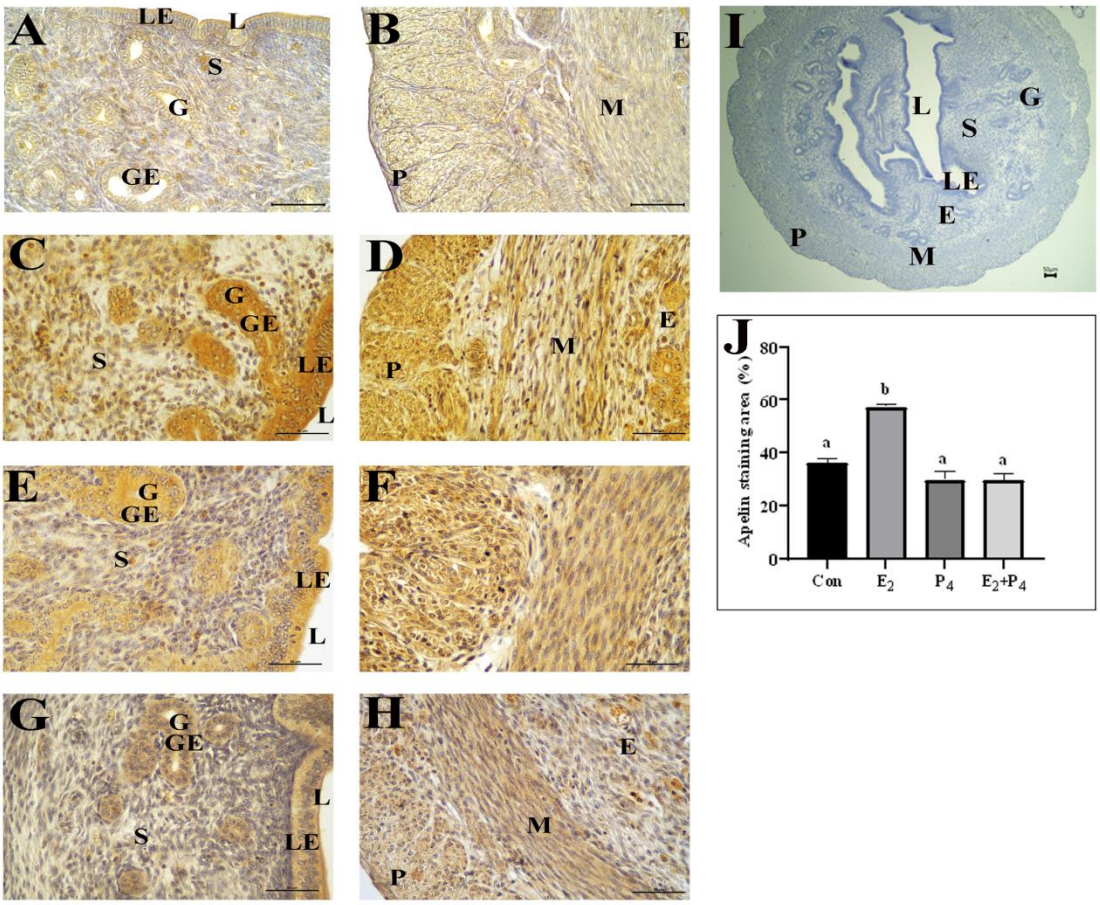

Fig.4.4. Effect of ovarian steroids (E2 and P4) on apelin localization in the uterus of the ovariectomized mice (A-H at 40X magnification). A and B represent the control group uterus, C and D represent the E2 treated group uterus, E and F represent the P4 treated group and G and H represent the E2 plus P4 treated group uterus. I represent absorption control (4X) and J shows the staining percentage area of apelin. L, lumen; LE luminal epithelium; G, gland; S, stromal cell; E, endometrium, M, myometrium, P, perimetrium; S, stromal cells. Scale bar shows 50μm length. The data are represented as the mean ± SEM. Different alphabet (a, b) showed significant difference ($p<0.05$). Con, control group; E2, estrogen-treated group; P4, progesterone-treated group; E2+P4, estrogen plus progesterone-treated group.

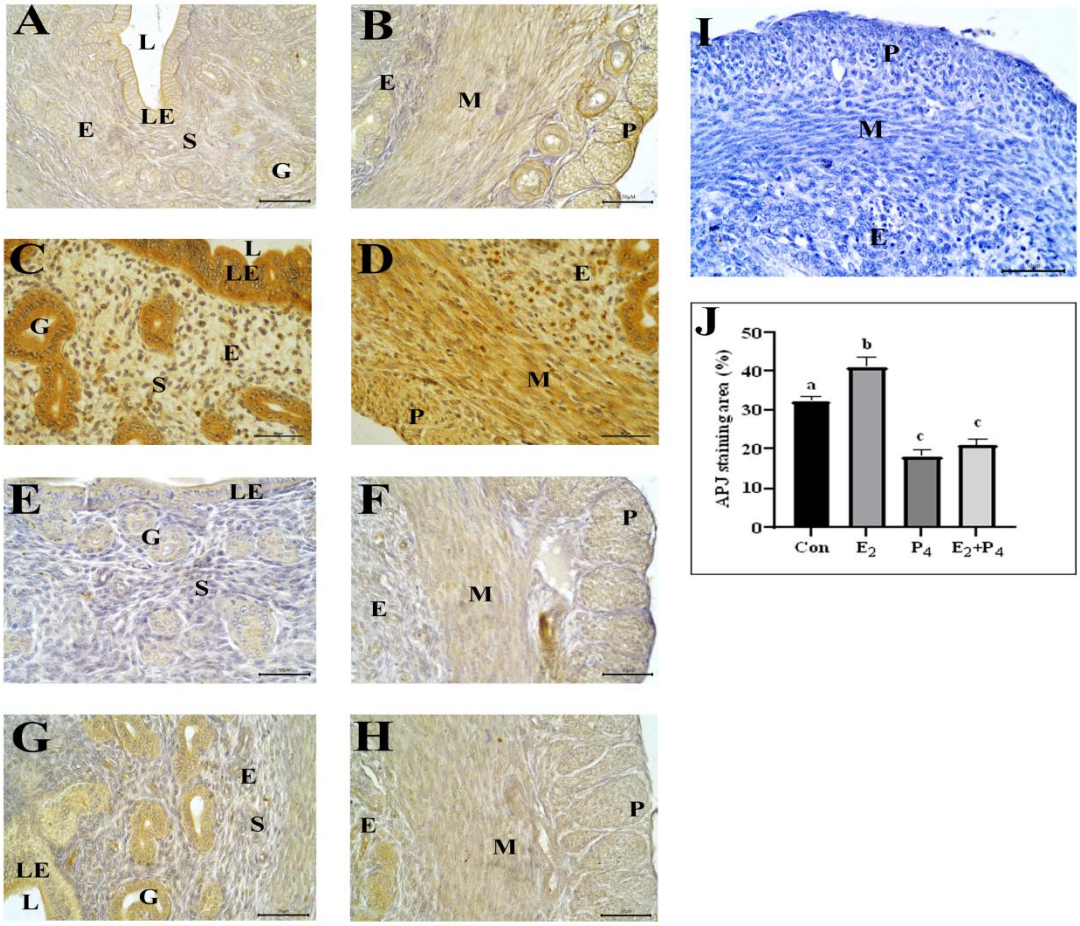

Fig.4.5. Effect of ovarian steroids (E2 and P4) on APJ localization in the uterus of the ovariectomized mice (A-H at 40X magnification). A and B represent the control group uterus, C and D represent the E2 treated group uterus, E and F represent the P4 treated group and G and H represent the E2 plus P4 treated group uterus. I represent absorption control (40X) and J shows the staining percentage area of APJ. L, lumen; LE luminal epithelium; G, gland; S, stromal cell; E, endometrium, M, myometrium, P, perimetrium; S, stromal cells. Scale bar shows 50µm length. The data are represented as the mean ± SEM. Different alphabet (a, b, c) showed significant difference ($p<0.05$). Con, control group; E2, estrogen-treated group; P4, progesterone-treated group; E2+P4, estrogen plus progesterone-treated group.

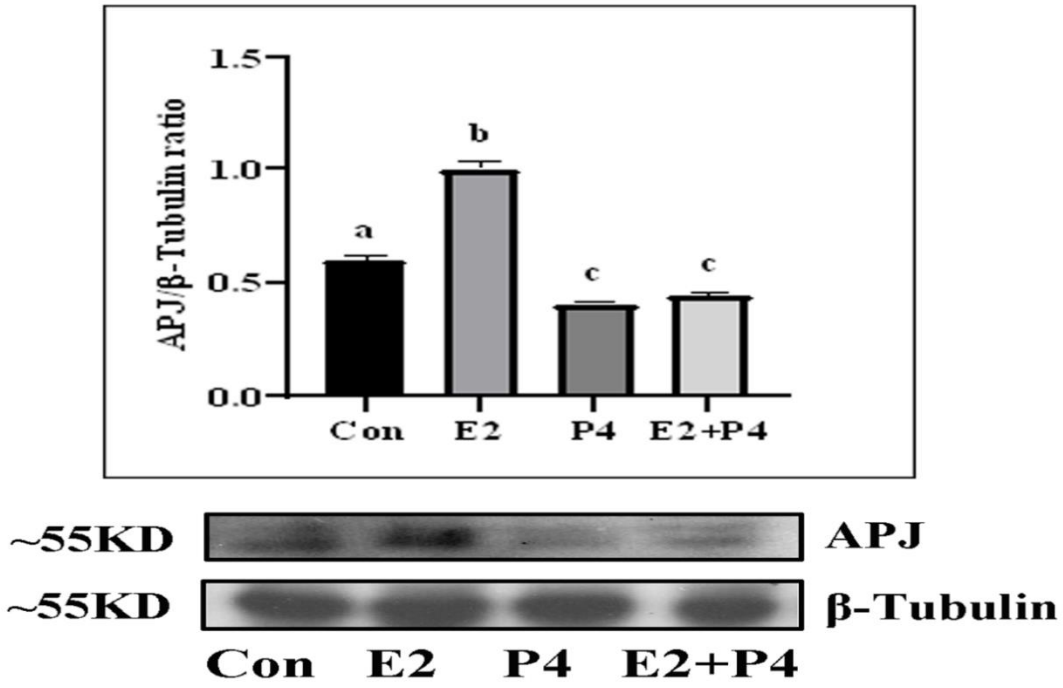

Fig.4.6. Effect of ovarian steroids (estrogen and progesterone) on APJ expression in the uterus of the ovariectomized mice. The data are represented as the mean ± SEM. Different alphabet (a-c) showed significant difference ($p<0.05$). Con, Control group; E2, Estrogen treated group; P4, progesterone treated group; E2+P4, Estrogen plus progesterone treated group.

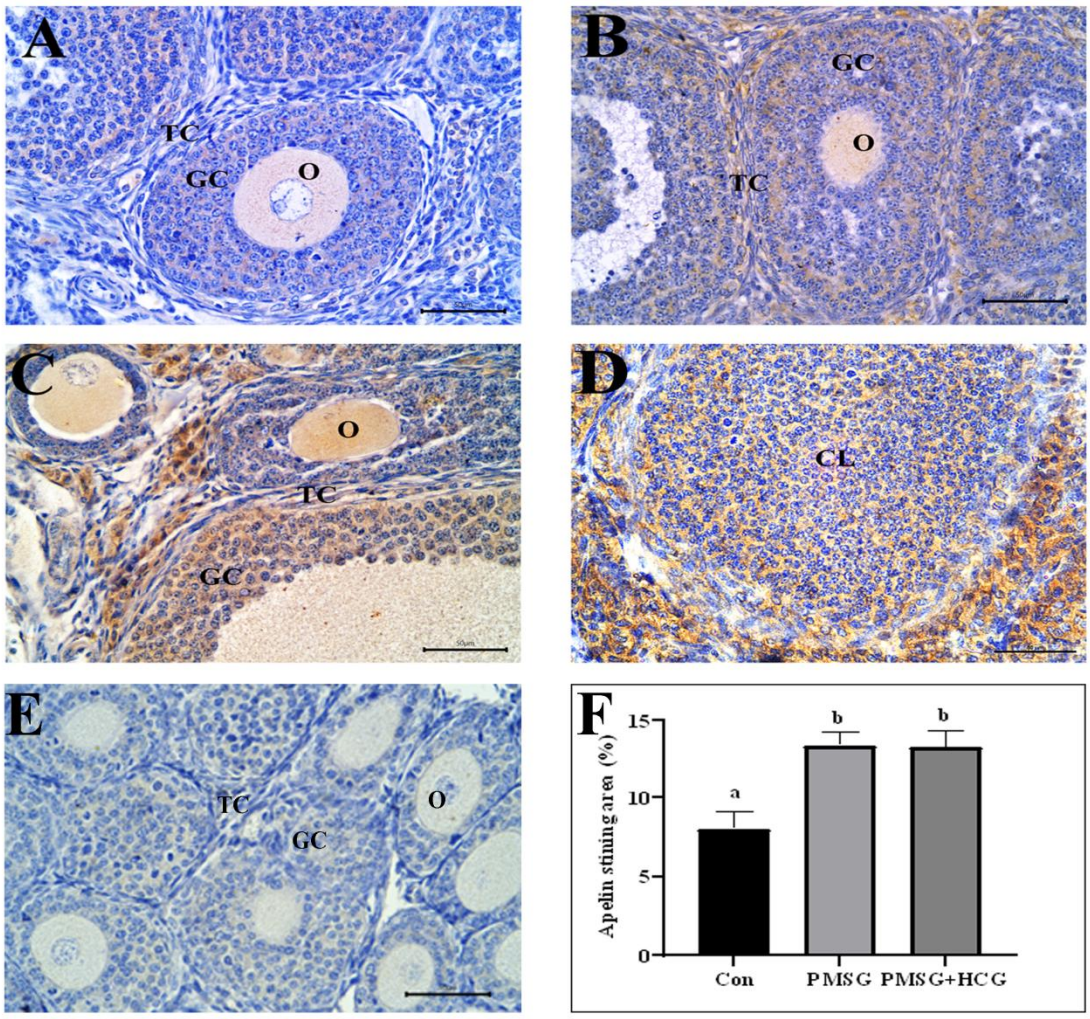

Fig.4.7. Effect of exogenous gonadotropin (PMSG and hCG) in the localization of apelin in the ovary of immature mice (A-D at 40X magnification) (n=4). A shows control group; B, PMSG treated group; C and D, PMSG plus hCG treated group; E, absorption control; F, staining percentage area of apelin. Scale bar shows 50µm length. GC, Granulosa cell; TC, Thecal cells; O, Oocytes; CL, Corpus luteum. The data are represented as the mean ± SEM. Different alphabet (a, b, c) showed significant difference ($p<0.05$).

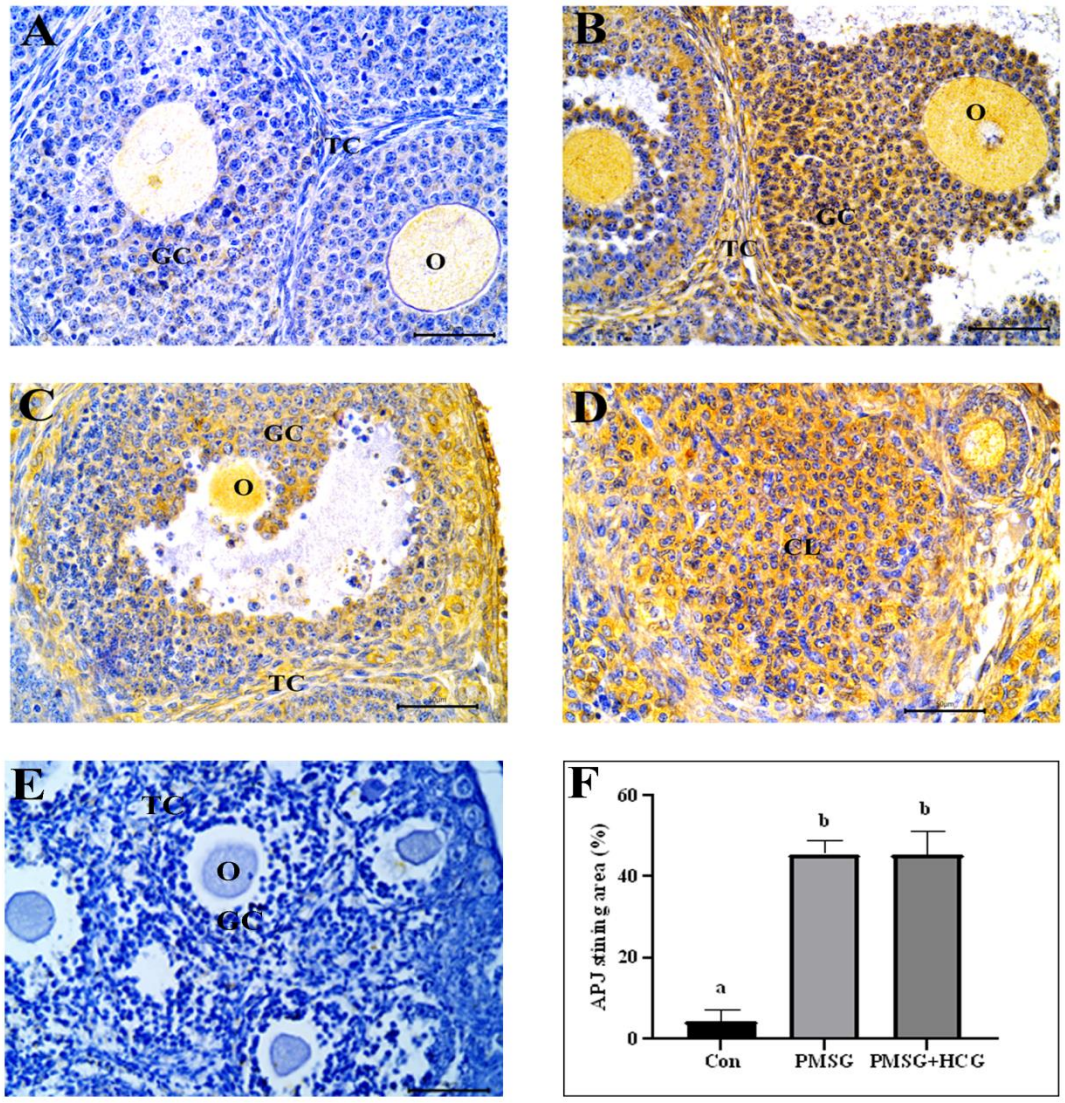

Fig.4.8. Effect of exogenous gonadotropin (PMSG and hCG) in the localization of APJ in the ovary of immature mice (a-d at 40X magnification) (n=4). A, control group; B, PMSG treated group; C and D, follicles and corpus luteum of PMSG plus hCG treated group, respectively. E, absorption control and F, staining percentage area of apelin. The scale bar shows 50µm length. GC, Granulosa cell; TC, Thecal cells; O, Oocytes; CL, Corpus luteum. The data are represented as the mean ± SEM. Different alphabet (a, b, c) showed significant difference ($p<0.05$).

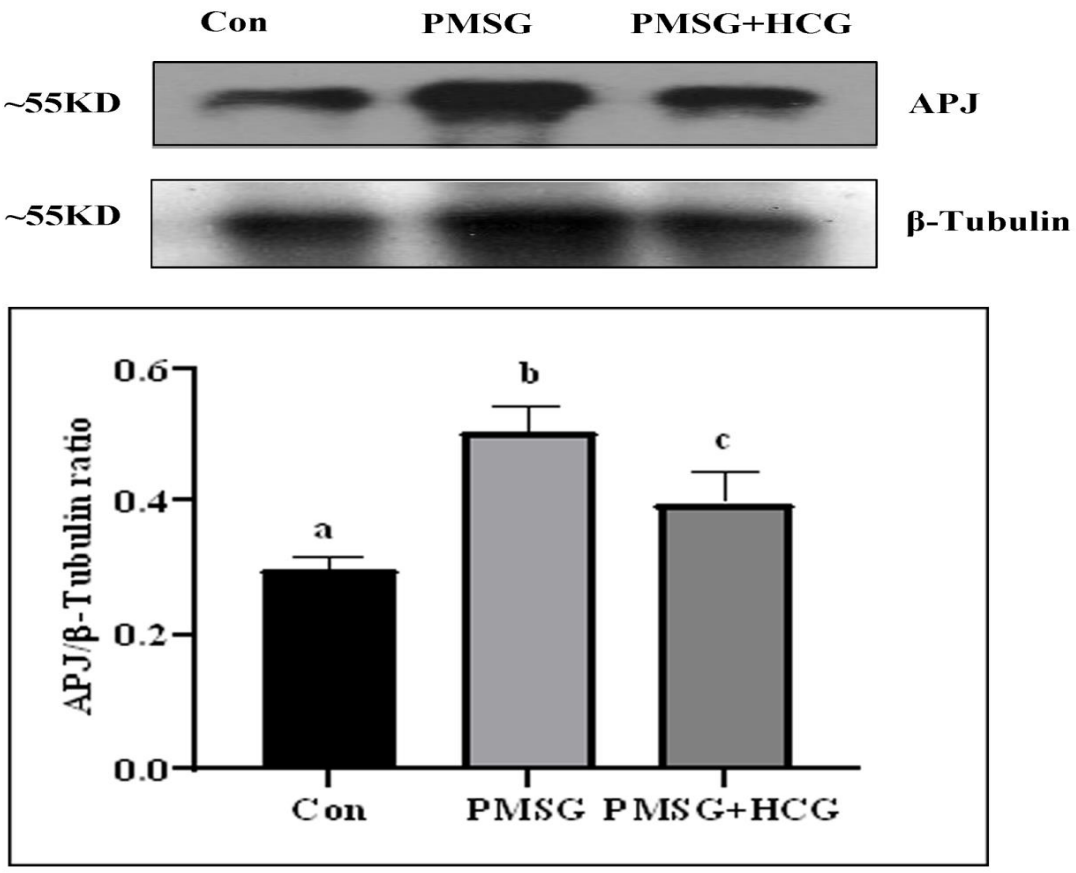

Fig.4.9. Effect of exogenous gonadotropin (PMSG and hCG) on the expression of APJ in the ovary of immature mice. The data are represented as the mean ± SEM. Different alphabet (a, b) showed significant difference ($p<0.05$).Con, Control group; PMSG, PMSG treated group; PMSG+HCG, PMSG plus hCG treated group.

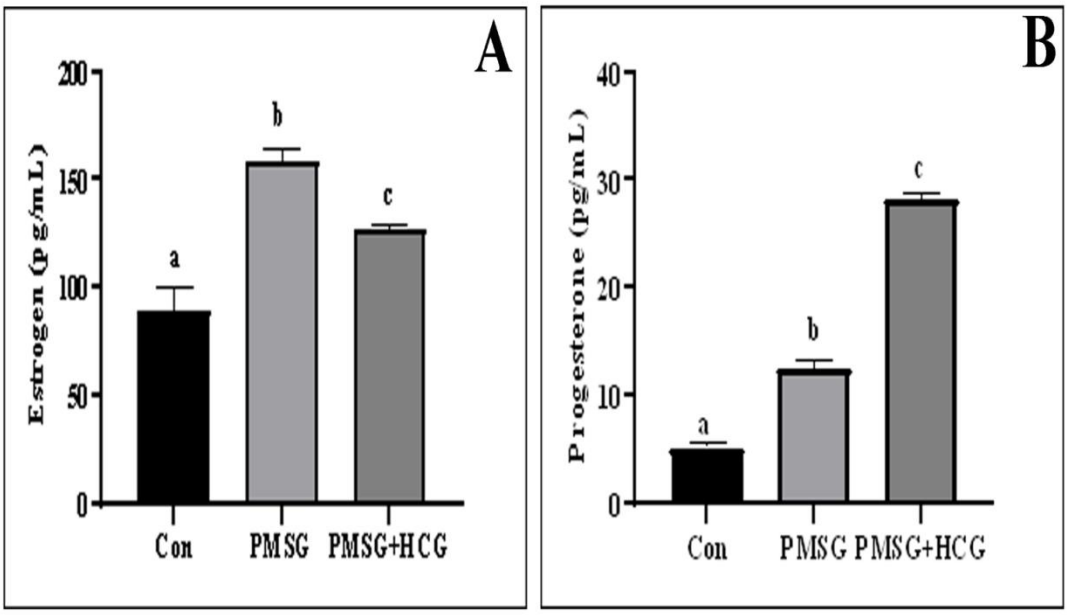

Fig.4.10. Effect of exogenous gonadotropin (PMSG and hCG) on the circulating estrogen (A) and progesterone levels (B) of immature mice (n=4). The data are represented as the mean ± SEM. Different alphabet (a, b, c) showed significant difference ($p<0.05$). Con, Control group; PMSG, PMSG treated group; PMSG+HCG, PMSG plus hCG treated group.

Table 4.1: A correlation study (expressed in terms of Pearson correlation coefficient, r value) of E2 and P4 with apelin and APJ.

Correlation	Apelin(IHC)	APJ (IHC)
E2	r = 0.7573, p = 0.0001*	r = -0.589, p = 0.0063*
P4	r = -0.7344, p = 0.0002*	r = -0.0136, p = 0.9545

P value less than 0.05 is considered as significant (* represent significant change).

Abbreviation: APJ, apelin receptor; E2, estrogen; P4, progesterone; IHC, immunohistochemistry; r, Pearson correlation coefficient.

CHAPTER 5

Title

Apelin receptor modulation mitigates letrozole-induced polycystic ovarian pathogenesis in mice

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Chapter 5

Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common (about 5–20%) and complex endocrinopathy in women of reproductive age. PCOS is the most common cause of female infertility, which includes anovulation, infertility and menstrual cycle abnormalities (Franks et al., 2008) and also associated with metabolic disorder, cystic ovary and hyperandrogenemia (Pasquali, 2018; Escobar-Morreale, 2018; Abbott et al., 2002; Goodarzi et al., 2011). Besides, the etiology of PCOS is still unclear because of the heterogeneous condition and multifactorial intervention (Caldwell et al., 2014). Due to the ethical and logistic limitations on human experimentation, it is challenging to identify the origins and pathogenic mechanisms of PCOS to unravel the optimum or curative therapies. Till date, there is no universal drug for PCOS therapy. Therefore, relevant animal models that can mimic the PCOS condition are being used to study the pathogenesis. Previous studies have suggested that non-human primates and rodent models such as female rat and mice could able to mimic PCOS like condition like cystic ovary with elevating androgen level (Abbott et al., 2013, Franks, 2012). Moreover, hyperandrogenised mouse models have been shown to manifest many characteristics of PCOS like hyperandrogenism, insulin resistance, cysts in the ovaries and metabolic disorder (Annie et al., 2021; Peng et al., 2023). Letrozole is a non-steroidal aromatase inhibitor that prevents the conversion of testosterone and androstenedione to estradiol and estrone respectively (Fisher et al., 2002). Letrozole causes polycystic ovary alterations that are similar to those seen in PCOS condition, including anovulation, thicker theca interna cell layer, and increased ovarian weight (Baravalle et al., 2006; Manneras et al., 2007; Kafali et al., 2004).

Adipose tissue dysfunction has been implicated as a key feature in the PCOS patients (Chazenbalk et al., 2012; Mannerås-Holm et al., 2014). It has been shown that altered adipokine secretion in the PCOS (Olszanecka-Glinianowicz et al., 2013, Hasan and Abd El Hammed, 2018). Previous reports suggested that apelin, an adipocytes-derived hormone found to be regulated with the occurrence of obesity and insulin resistance (Sun et al., 2015; Dravecká et al., 2021). Thereby, it is more likely to have an important

role in PCOS condition. Actually, apelin is an adipokines derived for the first time from bovine stomach extract and identified as an endogenous ligand for an orphan G-protein couple receptor (Tatemoto et al., 1998). This receptor was discovered based on its sequence similarity to angiotensin receptor and named as apelin receptor (APJ) after apelin (O'Dowd et al., 1993).

Apelinergic system has been found in adipose tissue, male and female reproductive system, placenta, and also in the hypothalamo-pituitary-gonadal (HPO) axis (Sun et al., 2015; Roche et al., 2016; Yang et al., 2019; Anima et al., 2023a; 2023b). Roche et al, (Roche et al., 2016) observed that in the follicular fluid, apelin concentration was elevated along with mRNA expression of apelin and APJ in the PCOS human ovary. Studies suggested that apelin have positive effects on steroidogenesis and proliferation but suppressive to apoptosis (Dravecká et al., 2021). Some studies have found that serum apelin level increases in PCOS patients and apelin could be a potential target for PCOS management (Dravecká et al., 2021; Goren et al., 2012; Liu et al., 2020). In contrast, apelin level also found to be low in lean PCOS patients (Choi et al., 2012; Chang et al., 2011). However, in the rodent PCOS model, apelin system has not been investigated yet. Our previous study has also shown adipokine like visfatin inhibition could ameliorate the PCOS pathogenesis (Annie et al., 2021). Previous studies have also shown that elevated APJ in the diabetes could be managed by its antagonist (Das et al., 2021; Song et al., 2022). Thus, it can be hypothesised that apelin system, more importantly, APJ modulation would be important for the management of hyperandrogenemia associated polycystic ovary. Therefore, objective of the present study was to investigate the effects of APJ agonist, apelin13 and its antagonist ML221, on the letrozole induced hyperandrogenised PCOS like mice model.

Chapter 5

Material and Methods

Drug and Reagents

Letrozole tablets from Unimarck Healthcare Ltd., India was used for inducing hyperandrogenism and cystic ovary to mimic PCOS condition.

The Apelin-13 (apelin) peptide (Gln-Arg-Pro-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe),purity ≥95% was purchased from GMR Foundation, Tiruchirappalli, Tamil Nadu, India.

The ML221, an apelin inhibitor or antagonist of APJ used in this study was purchased from Sigma-Aldrich Chemicals Pvt Ltd, St. Louis, Missouri, United States (cat# SML0919).

Animal maintenance

Swiss albino mice were taken from Mizoram University Animal house following the protocols approved (Protocol Number, MZU/IAEC/ 2020/12) by the Mizoram University Institutional Animal Ethical Committee 8, Mizoram University, Mizoram, India and all animal experiments was conducted following the ARRIVE guidelines. 26 adult virgin female Swiss albino mice of 2–3-month age and average weigh 25g, were used for this study and were kept in a laboratory condition (12 h light: 12 h dark cycle, at 25 ± 2 °C). Food and water were provided ad libitum.

Experimental design

Mice were randomly grouped into four; (i) Control (Con) group (n = 5) was given 100μL of saline (vehicle) orally, (ii) Letrozole-treated group (PCOS) (n=5) was received letrozole orally at a concentration of 1 mg/kg daily for 21 days (Kafali et al., 2004), (iii) Letrozole + apelin treated (PCOS+AP) (n=5) group, was received letrozole orally at a concentration of 1 mg/kg daily for 21 days plus apelin intraperitoneal injection at a dose of 100 μg/kg for 14days (Turtay et al., 2015) and (iv) Letrozole + apelin inhibitor

(PCOS+ML) (n=5) group was received letrozole orally at a concentration of 1 mg/kg daily for 21 days plus intraperitoneal injection of ML221 at a dose of 150 μg/kg for 14days by following Hall et al, (2017). Body weight was taken every day before treatment during the experiment.

Sample collection

On day 36th of experiment, all the mice were sacrificed under mild anaesthesia and body weight was recorded before sacrificed. Ovaries were dissected out without any adhering fat tissue immediately after sacrificed and ovary weight was recorded. Oneof the ovaries from each group was fixed in Bouin's solution for histology and immunohistochemistry analysis. Other ovary was kept in -20°C for western blotting. Serum sample was collected and stored at -20°C for hormonal assays and metabolic parameters quantification.

Co-efficient of ovary weight to body weight

The coefficient of ovary to body weight (BW) was measured after weighing the body and calculated as

Co-efficient of ovary weight to body weight = Wet ovary weight (mg) /BW (g) × 100%

Hormone analysis Enzyme-linked immunosorbent assay (ELISA)

Hormone analysis was done by using commercial ELISA kit as per manufacturer's instruction. Serum samples were used to quantify the circulating hormones levels. The circulating testosterone, androstenedione, estrogen and progesterone levels were quantified by using human Testosterone EIA kit (Cat# DKO002, DiaMetra, Italy), Androstenedione EIA kit (Cat # DKO008, DiaMetra, Italy), Estradiol EIA kit (cat # EIA K208; Xema-Medica Co. Ltd., Moscow, Russia, Cat# DKO003, DiaMetra, Italy), Progesterone EIA kit (Cat # DKO006, DiaMetra, Italy) respectively.

Circulating LH and FSH levels were estimated by using mouse LH (cat# E-EL-M3053, Elabscience,) and Mouse FSH (cat #E-EL-M0511, Elabscience) ELISA kit, respectively. Absorbance was taken at 450 nm using a Microplate Reader (Erba Lisa Scan EM, Trans Asia Biomedical Ltd., Mumbai, Maharashtra, India).

Histology and follicle count

Fixed ovaries were utilized for histological analysis and proceed with paraffin-embedded tissue block making following (Annie et al., 2021). The paraffin-embedded tissue blocks were sectioned at 5-7µm using Leica microtome (model RM2125 RTS) and transferred to previously gelatine (1%) coated slides and incubated on slide warming table for 24 at 37°C. Ovary slides were then preceded for histological analysis and treated with xylene for deparaffinization, rehydrated in different alcohol grades (100%, 90%, and 70%) and hydration followed by hematoxylin and eosin staining. The slides were dehydrated (70%, 90%, and 100%), cleaned in xylene and then mounted with DPX. Dry slides were then used for follicle counting and photographed by using a microscope (iScope series, Euromex, Netherland).

Follicle counting was performed manually. A single section per individual was taken for the follicle count from near the central-medullar zone of the ovaries following previously described method by Trujillo-Vázquezet al, (2023).

Immunohistochemistry

Immunohistochemistry analysis was performed following Anima et al, (2023). Slides were deparaffinised in xylene and rehydrated in different alcohol grades (100%, 90% and 70%) followed by hydration and PBS. The endogenous peroxidase was blocked with 3% H_2O_2 and methanol solution and then incubated in goat serum (1:100 diluted in PBS, lot# A0515, Santa Cruz Biotechnology, Inc. CA, USA) to blocked nonspecific binding at room temperature. After blocking for 30 minutes, the slides were incubated for overnight at 4°C with primary antibody of anti-apelin receptor (1:50, rabbit monoclonal antibody, cat# ABD43, Millipore) and anti- B-cell lymphoma2 (BCL2) (1:100; mouse polyclonal

antibody, cat# sc-7382, Santa Cruz Biotechnology, Inc. Dallas, USA)diluted with PBS. Unbound antibodies were wash off with PBS and then incubated for 4h at room temperature with HRP conjugated secondary IgG antibody (1:200, goat anti-rabbit, lot# HPO32023, Genei Laboratories Private Limited, India and 1:400, goat anti-mouse, lot# HPO52023, Genei Laboratories Private Limited, India) and incubated in 3, 3-diaminobenzidine tetrahydrochloride Dihydrate (DAB) in Tris–HCl (pH 7.6) solution with H_2O_2 till brown color develops. Then the reaction was stopped with distilled water and counterstained with hematoxylin. The slides were then dehydrated, cleared in xylene and mounted with DPX. Immunostaining of the slides were examined and photographed by using a microscope (iScope series, Euromex, Netherland).

Western blot analysis

Immunoblotting for ovaries from both the *in vivo* and *in vitro* experiment was performed following previous method (Anima et al., 2023). Lysis buffer was used for making tissue homogenates (20%) and protein estimation was done by Bradford method (Bradford. 1976). 50µg protein was loaded for each group into 12 and 15 percent sodium dodecyl sulfatepolyacrylamide (SDS) gel electrophoresis and transferred the resolved protein onto the polyvinylidene fluoride (PVDF) membrane by using wet transfer apparatus for 14h at 4°C. Nonfat skimmed-milk (5% diluted with PBST, cat # GRM1254-500G; HiMedia Laboratory private limited, Mumbai) was used for blocking the non-specific binding of the membrane and incubated with primary antibody of anti-apelin receptor (1:1000, rabbit monoclonal antibody, cat# ABD43, Millipore), anti-active caspase3 (1:1000; mouse polyclonal antibody, cat# E-AB-22115, ElabScience, Houston, Texas, USA), anti-androgen receptor (1:200, rabbit monoclonal antibody, cat# PR002, PathnSitu Biotechnologies, India), anti-estrogen receptors (ERα, 1:500, mouse monoclonal antibody, cat# Bz1, DSHB, University of Iowa, Dept of Biology, Iowa, United States and ERβ, 1:500, mouse monoclonal antibody, cat# CWK-F12, DSHB, University of Iowa, Dept of Biology, Iowa, United States) and anti-Bax antibody (1:1000, rabbit monoclonal antibody, cat# PAB343Mu01, Cloud Clone Corp, USA).

After washing unbound antibody with PBST, the membrane was incubated in HRP conjugated secondary antibodies (1:2000, goat anti-rabbit, lot# HPO32023, GeneiLaboratories Private Limited, India and 1:4000, goat anti-mouse, lot# HPO52023, Genei Laboratories Private Limited, India). The membrane was treated with electrochemiluminescence (ECL) for 5minutes and developed onto X-ray film. The band intensities were quantified by ImageJ software (imagej.nih.gov/) with respect to loading control β-Tubulin (1:1500, mouse polyclonal antibody, cat# E7; DSHB, university of Iowa, department of Biology, United States).

Statistical analysis

Statistical analyses were performed using GraphPad prism9 software. The data were carried out using one way analysis of variance (ANOVA) following multigroup comparisons of the differences of mean values among the data and the results were expressed as the mean ± SEM. Statistical significance for all tests was considered at $P<0.05$.

Result

Effect of apelin13 and ML221 on the body weight, ovary weight and ovary co-efficient of letrozole-induced hyperandrogenized mice

The only letrozole-induced hyperandrogenized group showed significant ($p<0.05$) increased in the BW, ovary weight and ovary co-efficient to BW as compared to control group (Fig.5.1). The treatment groups of apelin13 and ML221 significantly ($p<0.05$) decreased the BW of the letrozole-induced hyperandrogenized mice and there was no significant ($p>0.05$) change as compared to control mice (Fig.5.1A). In the ovary weight and ovary co-efficient to BW also showed significant ($p<0.05$) decreased after apelin13 and ML221 treatment as compared to letrozole-induced hyperandrogenized group, whereas, apelin13 treated group showed significant ($p<0.05$) increased in the ovary weight and ovary co-efficient to BW as compared to control group (Fig5.1B, C).

Effect of apelin13 and ML221 on the histomorphology of letrozole-induced hyperandrogenized mice ovary

The histomorphology of ovaries was observed by hematoxylin and eosin staining and the result showed observable changes in the histoarchitecture of the letrozole-induced ovaries. The control group exhibit normal morphological structure with all levels of the follicle (primordial, primary, secondary, mature follicles) and corpus luteum having clear distinguishable shape of the cells. The letrozole-induced hyperandrogenized group exhibited cyst, very few or no corpus luteum, and multiple large antral follicles where the granulosa cells of these large follicles were sparse and condensed around the follicles to form atretic follicles with a large cavity. Furthermore, the treatment groups of apelin13 and ML221 showed improvement in the ovarian histology, which was altered by apparently a very few or no cyst and less atretic antral follicle. The aggregated and thickened intrafollicular granulosa cell layer of large follicles was also displayed improvement. The corpus luteum of the ovary also appeared after the apelin13 and ML221 treatment (Fig.5.2).

The number of all the level of follicles and corpus luteum were found to be different and counted in the ovary of all the groups. Quantification of follicles (primordial (Fig.5.3A), primary (Fig.5.3B), secondary (Fig.5.3C) and mature follicle (Fig.5.3D) showed no significant ($p>0.05$) changes in the letrozole groups after apelin13 and ML221 treatment as compared to control. The large atretic antral follicles count result showed significant ($p<0.05$) increased in the letrozole-induced hyperandrogenized group and the apelin treated group as compared to control, whereas, the ML221 treated group significantly ($p<0.05$) decreased the large atretic antral follicles count as compared to the letrozole-induced hyperandrogenized group and showed no significant ($p>0.05$) change with control group and the apelin treated group (Fig.5.3E). Corpus luteum count result as significant ($p<0.05$) decreased in all other groups as compared to control group, whereas, the ML221 treated group showed significant ($p<0.05$) increased corpus luteum count as compared to the only letrozole-induced group. There were no significant

($p>0.05$) changes obtained between the apelin13 treated group and ML221 treated groups (Fig.5.3E). Cyst was also counted and result showed that the treatment of apelin13 and ML221 significantly ($p<0.05$) reduced the number of cysts in the ovary, whereas, there was no significant ($p<0.05$) change between the apelin13 and ML221 treated groups (Fig5.3F).

Effect of apelin13 and ML221 on circulating steroid hormones of letrozole-induced hyperandrogenized mice

The circulating level of steroid hormones, progesterone, estrogen, androstenedione and testosterone in the female mice were estimated by ELISA assay. The result showed that serum progesterone level was significantly ($p<0.05$) down regulated in the letrozole-induced mice group (3.26 ± 0.68 ng/mL) and the treatment group of apelin13 (3.52 ± 0.3 ng/mL) as compared to control group (14.67 ± 0.43 ng/mL), whereas, the ML221 treatment group (6.06 ± 0.25 ng/mL) significantly ($p<0.05$) upregulated the progesterone level as compared to the letrozole-induced group and the treatment group of apelin13. Furthermore, there was no significant ($p>0.05$) difference between the control and ML221 treated groups (Fig.5.4A).

The circulating level of estrogen result showed that the treatment of apelin13 (0.1 ± 0.02 nmol/L) and ML221 (0.03 ± 0.002 nmol/L) significantly ($p<0.05$) downregulated as compared to the letrozole-induced group (0.26 ± 0.07 nmol/L). Further, the treatment of ML221 showed significant ($p<0.05$) decreased level of estrogen as compared to the treatment group of apelin13. There were no significant ($p>0.05$) changes observed in the treatment group of apelin13 andML221 as compared to control group (0.07 ± 0.02 nmol/L) (Fig.5.4B).

The serum level of androstenedione showed significant ($p<0.05$) elevation in the letrozole-treated group (0.46 ± 0.009 ng/mL) and the apelin13 treated group (0.44 ± 0.03 ng/mL) as compared control group (0.32 ± 0.004 ng/mL), whereas there was no significant ($p<0.05$) change observed between the letrozole treated group and the apelin

treatment group. The treatment of ML221 group (0.40 ± 0.02 ng/mL) showed down regulation of androstenedione level but no significant changes ($p>0.05$) observed as compared to the other groups (Fig.5.4C).

The circulating testosterone level was also quantified. The result showed that the letrozole-treated group (24.07 ± 0.6 pg/mL) significantly ($p<0.05$) elevated testosterone level as compared to the other groups, whereas, the treatment groups of apelin13 (8.6 ± 0.27 pg/mL) and ML221 (12.85 ± 0.86 pg/mL) showed no significant ($p>0.05$) changes as compared to control group (9.7 ± 0.6 pg/mL). The treatment of ML221 group showed a slight significant ($p<0.05$) elevated level of testosterone as compared to the treatment of apelin13 group (Fig.5.4D).

Effect of apelin13 and ML221 on oxidative stress and antioxidant status of letrozole-induced hyperandrogenized mice ovary

To explore the effect of apelin13 and ML221 on oxidative stress and antioxidant status of letrozole-induced hyperandrogenized mice, malondialdehyde (MDA) level, hydroperoxides (H_2O_2) level and antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) levels were measured (Fig.5.5). MDA is commonly known as a marker of oxidative stress and the antioxidant status. The result showed MDA level was significantly ($p<0.05$) elevated in the letrozole-induced group. The treatment groups of aplein13 and ML221 significantly ($p<00.5$) lower the MDA level than the only letrozole-induced group, whereas, the treatment of apelin13 still significantly ($p<0.05$) increased as compared to control group. The treatment of ML221 group showed no significant ($p>0.05$) changes as compared to the control and the apelin13 treated groups (Fig.5.5A).

The level of H_2O_2 showed also significant ($p<0.05$) elevation in the letrozole-induced groups. The treatment of apelin significantly ($p<0.05$) reduced the H_2O_2 level as compared to the only letrozole induced group, whereas, there was no significant ($p<0.05$) changes observed with the control group. The treatment of ML221 group

showed no significant ($p<0.05$) change as compared to the letrozole-induced group (Fig.5.5B).

The antioxidant enzymes SOD (Fig.5.5C)and CAT levels (Fig.5.5D) were significantly ($p<0.05$) downregulated in the letrozole-induced group. The SOD and CAT levels was significantly ($p<0.05$) elevated after the treatment of apelin13 and there were no significant ($p>0.05$) changes between the apelin13 treatment group and control group. The treatment of ML221 group showed no significant ($p>0.05$) change with the letrozole-induced group.

Effect of apelin13 and ML221 on the circulating gonadotropin level of letrozole-induced hyperandrogenized mice

To understand the effect of apelin13 and ML221 on the circulating gonadotropin level of letrozole-induced hyperandrogenized mice, luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels were quantified after apelin13 and ML221 treatment (Fig.5.6). The result showed that LH level was significantly ($p<0.05$) elevated in the letrozole-induced group (3.4 ± 0.73 ng/mL). The treatment of ML221 group (1.7 ± 0.7 ng/mL) significantly ($p<0.05$) lower the LH level and showed no significant ($p>0.05$) change with control group (0.9 ± 0.1 ng/mL). The treatment group of aplein13 (4.2 ± 0.1 ng/mL) showed no significant ($p>0.05$) effect on the LH level as compared to the letrozole-induced group (Fig.5.6A).

The FSH level showed a significant ($p<0.05$) downregulation in the letrozole-induced group (1.1 ± 0.5 ng/mL) as compared to control group. The treatment of apelin13 group (2.2 ± 0.7 ng/mL) significantly ($p<0.05$) upregulated the FSH level than the letrozole-induced group and showed no significant ($p>0.05$) change as compared to control group (3.7 ± 0.12 ng/mL). The treatment of ML221 group (1.5 ± 0.16 ng/mL) no significant ($p>0.05$) change with the letrozole-induced group (Fig.5.6B).

The LH/ FSH ratio is very important for ovulation in the female. Therefore, the LH/ FSH ratio was calculated after apelin13 and ML221 treatment. The result showed that

the LH/FSH ratio was raised (2.28 ± 0.12) in the letrozole-induced group as compared to control group (0.33 ± 0.02). The treatment groups of apelin13 (1.9 ± 0.04) and ML221 (1.16 ± 0.06) significantly ($p<0.05$) lower the LH/ FSH ratio as compared to the only letrozole-induced group. Although the result also showed significant ($p<0.05$) changes in the treatment groups of apelin13 and ML221 as compared to control group but both the group were in the normal range (1-2) (Fig.5.6C).

Effect of apelin13 and ML221 on the apoptosis markers of letrozole-induced hyperandrogenized mice ovary

To explore the effect of apelin13 and ML221 on the apoptosis markers of letrozole-induced hyperandrogenized mice apoptotic markers active caspase3, Bax and anti-apoptotic marker BCL2 were examined. The apoptotic markers active caspase3 (Fig.5.7A) and Bax(Fig.5.7B) showed a significant ($p<0.05$) elevation in the letrozole-induced group as compared to control group. The treatment of apelin13 significant ($p<0.05$) downregulated both the apoptotic markers as compared to the letrozole-induced group, whereas, no significant ($p>0.05$) change was observed with the control group. The ML221 treatment group showed no significant ($p>0.05$) change with the letrozole treated group.

The anti-apoptotic marker BCL2 showed variation of the immunostaining in the ovary and staining was observed in the granulosa cell, thecal cell and luteolytic cell of different follicles. The immunostaining of BCL2 showed intense immunostaining in the control and the apelin13 treated groups and faint staining in the letrozole-induced group and the ML221 treated group (Fig.5.8).

Effect of apelin13 and ML221 on the expression of estrogen receptors and androgen receptor of letrozole-induced hyperandrogenized mice ovary

The expression of estrogen receptors such as ERα (Fig.5.9A)and ERβ (Fig.5.9B) showed significant ($p<0.05$) increased in the letrozole-induced group as compared to control group and found no significant change of ERα with the treatment groups. Furthermore,

the ML221 treatment group showed a significant ($p<0.05$) increased of ERβ expression than the other groups.

The expression of androgen receptor (AR) showed a significant ($p<0.05$) increased in the only letrozole-induced group as compared to control group, whereas the treatment of apelin13 group significantly ($p<0.05$) lower the AR expression and found no significant ($p>0.05$) change with the control group. There was no significant ($p>0.05$) change observed between the treatment of ML221 and the letrozole-induced groups (Fig.5.9C).

Effect of apelin13 and ML221 on the expression of apelin receptor of letrozole-induced hyperandrogenized mice ovary

The expression of apelin receptor (APJ) showed a significant ($p<0.05$) increased in the only letrozole-induced group as compared to control group, whereas the treatment of apelin13 group significantly ($p<0.05$) lower the APJ expression and found no significant ($p>0.05$) change with the control group. There was no significant ($p>0.05$) change observed between the treatment of 221 and the letrozole-induced groups (Fig.5.10).

Discussion

Polycystic ovary syndrome has been associated with the deregulation of various adipokines. Apelin and the apelin receptor may be crucial in the pathophysiology of polycystic ovarian syndrome (Dravecka et al., 2021). However, the apelin level fluctuations or PCOS are not always consistent. Apelin levels in PCOS women were shown to be lower in some studies, but higher in others. Similarly, the apelin levels were shown to be lower in normal-weight PCOS women than in the control group (Choi et al. 2012; Chang et al. 2011). It has also been shown that apelin and APJ expression is higher PCOS subject and correlated with the follicle count (Bongrani et al., 2019). However, there is dearth information on the PCOS animal model and expression of apelin system. In our preliminary findings of hyperandrogenised mice with letrozole, the expression of APJ was higher in the ovary, thus we have analysed the effect of APJ, agonist, (apelin13 peptide) and APJ antagonist, (ML221) on the letrozole induced

hyperandrogenised mice on the ovarian activity. The letrozole-induced hyperandrogenemia has been well established for manifestation of PCOS like pathological conditions in the rodent model and also shown be exhibits reproductive and metabolic disorder of PCOS (Kafali et al., 2004, Baravalle et al., 2006, Peng et al., 2023). Our recent study has also shown that levels of ovarian visfatin, an adipokines, were elevated in the letrozole-induced hyperandrogenised mice and its inhibitor shown to be effective in the amelioration of cystic ovarian syndrome (Annie et al., 2021). The results of present study showed that the treatment with apelin13 and ML221 reduced the ovarian weight, body weight and ovary coefficient compared to the hyperandrogenised mice. Although, these results were surprising that both agonist and antagonist manifest the same findings. Furthermore, we have also analysed the ovarian histology to confirm the effects of agonist and antagonist on the cystic ovary. The letrozole treated ovary showed many cysts and quantification of cysts was higher in the ovary of hyperandrogenised mice. The treatment of apelin13 peptide and ML221 decreased the cysts in the ovary; the quantification of cysts did not show any significant change between apelin 13 and ML221 treated groups. Since the formation of corpus luteum is the evidence of successful ovulation (Lee-Thacker et al., 2020), thus we have also measured the number of corpus luteum. Letrozole treated mice ovary showed a few corpus luteum and treatment of apelin 13 and ML221 increased the corpus luteum number in the ovary, andML221 treated ovary showed increased corpus luteum than apelin 13 treated ovaries. It has also been shown that hyperandrogenism and PCOS increases the number atretic follicles in the ovaries (van Houten et al., 2012 [39]; Yeh and Kim, 1996). In our experiment also letrozole treated mice ovary showed significantly higher atretic follicles and treatment of both aplein13 and ML221 decreased the atretic follicle in the ovary, however, ML221 treated mice ovary exhibited reduced atretic follicles.

The apoptosis is a basic mechanism of ovarian cycle regulation and it has also been shown that rodent model of PCOS, apoptosis occurs in the atretic follicle and cysts (Annie et al., 2021; Singh et al., 2018; Palumbo and Yeh 1995). The expression of

apoptotic markers, active capsase3 and Bax was up-regulated along with decreased Bcl2 immunostaining in the cystic ovary. The treatment of apelin13 decreased the expression of Bax and active caspase3 along with increased Bcl2 immunostaing in the ovary. However, the treatment of ML221 further increased the expression of Bax and active caspase3 in the ovary. These results clearly showed the differential effect of aplein13 and ML221 in the cystic ovary. Based on the findings, it can be suggested that apelin13 suppresses the apoptosis in the hyperandrogenised ovary, however, inhibition of apelin signalling by ML221 increase the apoptosis. It has also been shown that the treatment with apelin13 reduced neuronal death by decreasing caspase expression and increased the Bcl-2/Bax ratio (Shao et al., 2021). In the rat and human ovary, apelin has also been shown to suppress the apoptosis (Shuang et al., 2016; Roche et al., 2016). The treatment with APJ antagonist, ML221 has been shown to stimulate the apoptosis in the juvenile mice testis (Das et al., 2022). It should be noted that our histological study and follicle parameters showed amelioration of cystic ovary by both apelin13 and ML221 in the hyperandrogenised ovary. Despite the same observable effect of apelin and ML221, the discrepancy on the apoptosis remains unclear in the present study. Thus, based on the histological section, it may be hypothesised that ML221 could be involved in the disposal of unhealthy follicle in the hyperandrogenised ovary. The oxidative stress and a decline in the antioxidant system have also proven to be crucial PCOS aetiology (Zeber-Lubecka et al., 2023; Liu et al., 2023).In our findings, apelin13 treatment decreased the letrozole-augmented oxidative stress in the ovary by increasing the antioxidant enzymes, catalase and superoxide dismutase. However, treatment of ML221 still showed elevated oxidative stress, as it was evidenced by the decreased antioxidant enzymes and increased hydrogen peroxide, moreover, MDA was slightly decreased. The stimulated apoptosis coincides with elevated oxidative stress in the ML221 treatment. It has been shown that the increase in oxidative stress is partly caused by the aberrant regulation of caspase, and elevated oxidative stress and lowered levels of certain apoptotic markers may contribute to the development of PCOS (Uyanikoglu et al., 2017; Zeber-Lubecka et al., 2023).The apelin fragments like 13 and 17 have shown to improve the antioxidant status in the

buffalo ovary (Shokrollahi et al., 2023). Moreover, it has also been shown that apelin13 treatment increased the catalase and superoxide dismutase in the cyclophosphamide-inducedcardiorenal toxicity (Kutlay et al., 2022). Here in our study, apelin13 and ML221 exhibited opposite effect on the oxidative stress, despite similar morphological observation of ovarian histology.

The hyperandrogenism and PCOS are often associated hormonal imbalances due to deregulation of HPG axis (Emanuel et al., 2022). Our results also showed elevated levels of testosterone and androstenedione in the letrozole-treated mice; however, the levels of progesterone were lower along slight elevation in the estrogen levels. These imbalances of hormone in PCOS are in agreement with the previous study (Annie et al., 2021; Emanuel et al., 2022).The treatment of ML221 increased the progesterone level; however, apelin13 peptide treatment did not elevate the progesterone section in the letrozole-induced hyperandrogenised mice. Moreover, apelin has been shown to stimulate ovarian progesterone secretion (Roche et al., 2017), thus, it can also be speculated that apelin may have different role in the normal ovary and hyperandrogenised ovary. Furthermore, the estrogen and testosterone secretion were suppressed by the apelin13 and ML221 treatment compared to the letrozole treated groups. The expression of ERs also showed elevated in the apelin13 and ML221 treated groups. It has also been shown that ERs expression is abnormal in ovary of PCOS (Xu et al., 2021). Since the main reason of PCOS is hyperandrogenemia and it was found that the expression of AR elevated in the PCOS conditions (Liu et al., 2015; Luo et al., 2021). Our results showed that apelin13 decreased the expression of AR in the ovary compared to the ovary of letrozole treated mice. ML221 treatment also decreased the AR expression, however, it was not significant compared to the letrozole treated groups and was also higher than apelin13 groups. The amelioration of cystic ovary by apelin13 treatment could be explained by down-regulation of AR, while ML221 mediated amelioration of cystic ovary, remains unclear in the present study.

The abnormal levels of gonadotropin by the pituitary often associated with the PCOS (Hall et al., 1998; Taylor, 2006; Barbieri and Ehrmann, 2018). The rodents' model of PCOS and hyperandrogenism also has elevated LH levels and higher LH/FSH ratio (Shah et al., 2022; Osuka et al., 2019). Our results also showed that ML221 treatment suppresses LH and FSH levels, thereby showing decreased LH/FSH ratio. Moreover, the treatment of apelin13 also showed lower LH/FSH ratio than letrozole treated mice, although LH was elevated and decreased ratio could be attributed to the increased FSH secretion. The modulation of gonadotropin levels occurs via direct action of aplein13 and ML221 at pituitary levels or through ovarian steroid remains to be investigated in the PCOS condition. It has been suggested that apelin has inhibitory role in the gonadotropin secretion (Kurowska et al., 2018). Recently we have shown the direct action of apelin13 on the gonadotropin secretion in the isolated pituitary of female mice and it was inhibitory(Anima et al., 2023). It should also be noted that end points result of cystic ovary amelioration was shown by both apelin13 and ML221 treatment. The reason of this discrepancy remains unclear. It has been shown that apelin/APJ signalling is complex due to different isoforms of endogenous apelin peptide fragments (Chapman et al., 2014; Chen et al., 2020). To study further, we have also analysed the ovarian APJ expression. Our results showed that apelin13 treatment down-regulates the ovarian APJ expression in the letrozole treated mice, however, ML221 did not affect the ovarian APJ expression. These findings suggest that excess of apelin13 could down-regulates the APJ expression; it has also been shown that ligand could down-regulates its own receptor (Krupp et al., 1981). To best of our knowledge apelin13 mediated down-regulation of APJ has not been reported. Thus, our results on the cystic ovarian mitigation by apelin13 and ML221 treatment could be attributed to suppression of APJ signalling either by apelin13 (down-regulation of APJ) and ML221, as APJ antagonist.

Chapter 5

Conclusion

In conclusion, our results showed that PCOS and hyperandrogenemia associated with up-regulation of ovarian APJ, which could also be important factor for PCOS associated pathogenesis. Furthermore, modulation of APJ action either by apelin13 or its receptor antagonist, ML221 could leads to mitigation of cystic ovarian pathogenesis. Despite, more similar observation of ovarian histology by apelin13 and ML221 treatment, both apelin13 and ML221 mediated mitigation have different mechanism, which needs further investigation.

Summary

Polycystic ovarian syndrome (PCOS) is one of the most common (about 5–20%) reproductive disorders in women of reproductive age; it is characterized by polycystic ovaries, hyperandrogenism and oligo/ anovulation. The levels and expression of ovarian adipokines are deregulated in the PCOS. Apelin is an adipokines which acts through its receptor (APJ) and known to express in the various tissues including ovary. It has also been suggested that apelin and APJ could be targeted as therapeutic adjunct for the management of PCOS. However, no study has been conducted on the management of PCOS by targeting apelin system. Thus, we aimed to evaluate its impact on combating the PCOS associated ovarian pathogenesis. The current work employed letrozole-induced-hyperandrogenised PCOS like mice model to investigate the effects of apelin13 and APJ, antagonist ML221. The PCOS model was induced by oral administration of letrozole (1 mg/kg) for 21 days. The total of four experimental groups were made, control, PCOS control, PCOS+aplein13 and PCOS+ML221. The treatment of apelin13 and ML221 was given from day 22 for two weeks. The letrozole-induced PCOS like features such as hyperandrogenism, cystic follicle, decreased corpus luteum, elevated levels of LH/FSH ratio, and up-regulation of ovarian AR expression were ameliorated by apelin13 and ML221 treatment. However, the PCOS-augmented oxidative stress and apoptosis was suppressed by apelin 13 treatments only. ML221 treatment still showed elevated oxidative stress and stimulated apoptosis as reflected by decreased antioxidant

enzymes and increased active caspase 3 and Bax expression. The expression of ERs was elevated in the all groups except control. Furthermore, PCOS model showed elevated expression of APJ and apelin13 treatment down-regulated its own receptor. Overall, by observing the ovarian histology, corpus luteum formation, and decreased androgen levels by both apelin13 and ML221 showed ameliorative effect on the cystic ovary. Despite similar morphological observation of ovarian histology, apelin13 and ML221 exhibited opposite effect on the oxidative stress and apoptosis. Therefore, apelin13 (which down-regulates APJ) and ML221 (an APJ antagonist) may have suppressed APJ signaling, which would account for our findings on the mitigation of poly cystic ovarian syndrome. In conclusion, both apelin13 and ML221 mediated mitigation have different mechanism, which needs further investigation.

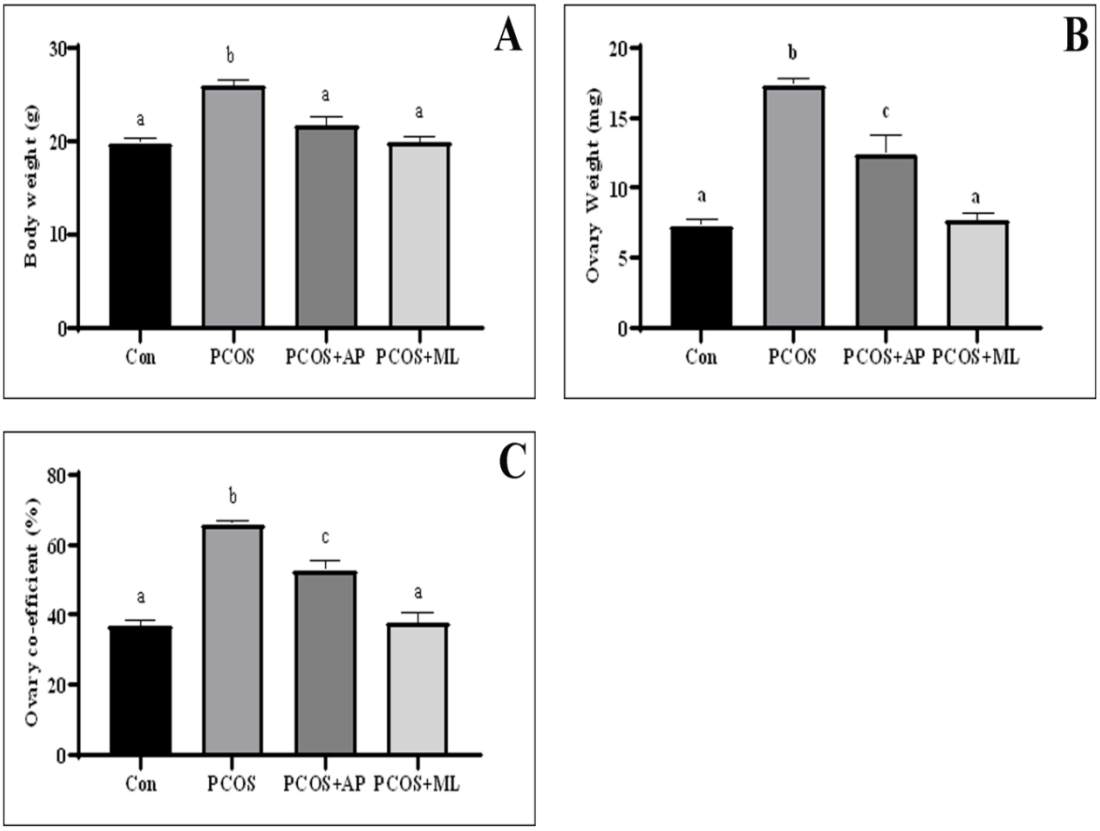


Fig.5.1: Changes in the body weight (A), ovary weight (B) and ovary co-efficient to body weight (C) of letrozole-induced mice after treatment of apelin13 and ML221. Data are expressed as mean ± SEM. Different alphabets (a,b,c) showed a significant difference ($p < 0.05$).

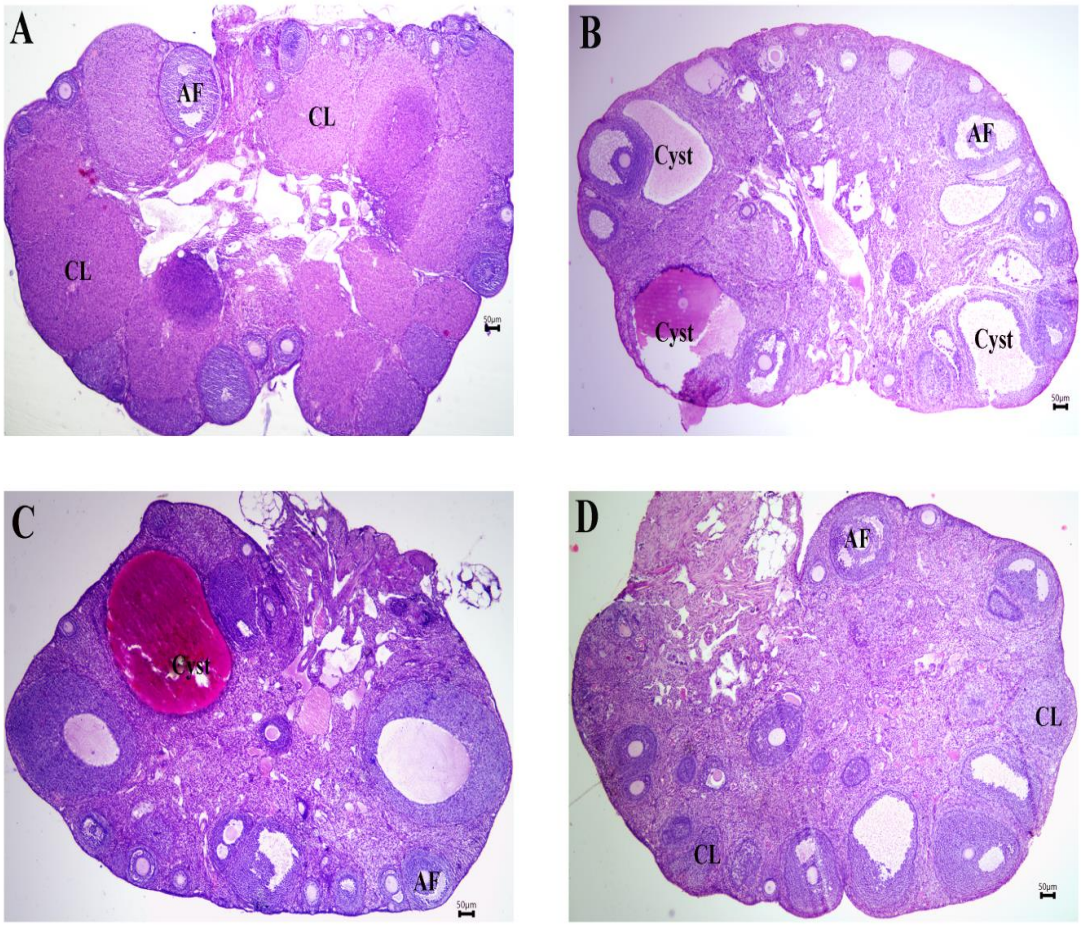


Fig.5.2: Effect of apelin13 and ML221 treatment on histology of letrozole-induced ovary. (A) Con group, (B) PCOS group, (C) PCOS+AP group, and (D) PCOS+ML group. AF, Antral follicle; CL, corpus luteum; Cyst, cystic follicle.

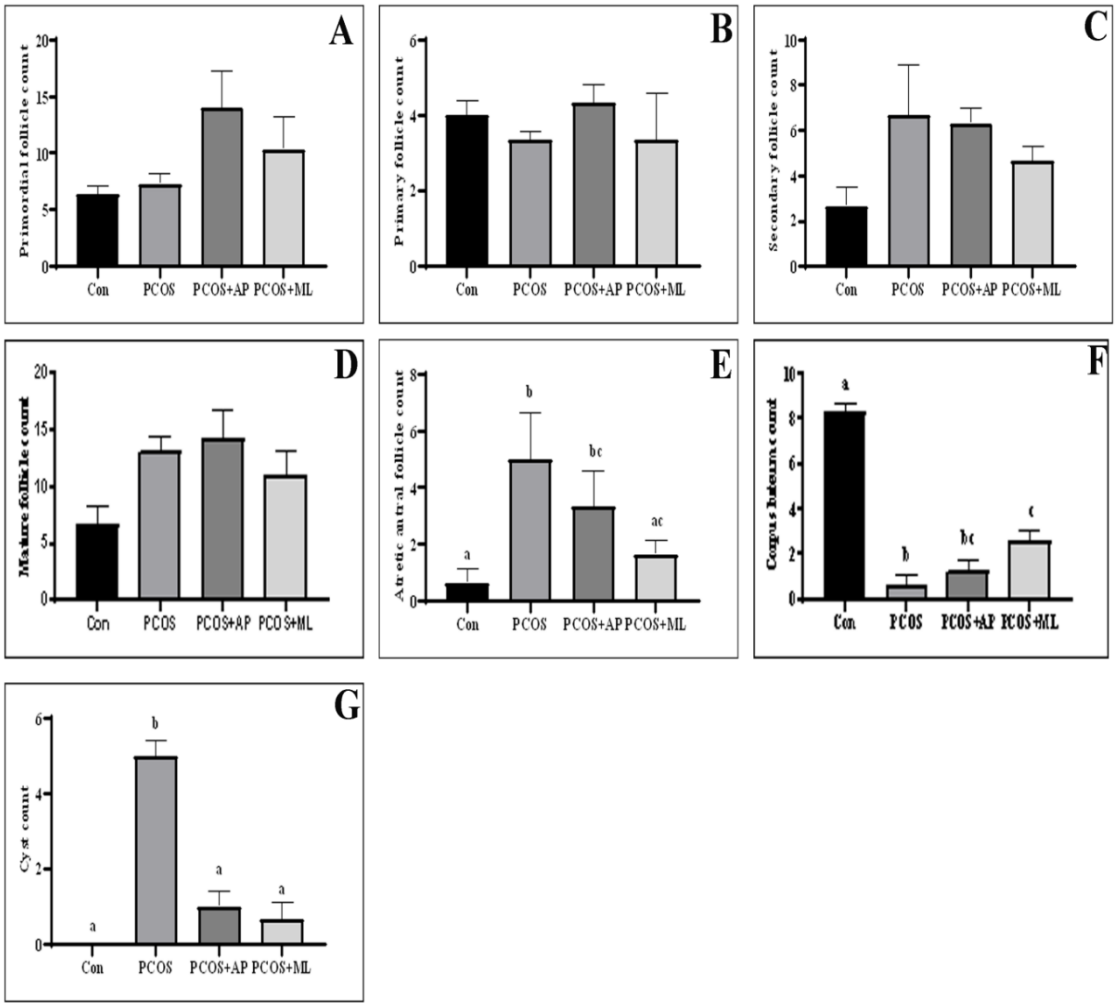


Fig.5.3: Changes in the follicles count, corpus luteum count and cystic follicle count of letrozole-induced ovary after apelin13 and ML221 treatment. (A) Primordial follicle count (B) primordial follicle count, (C) secondary follicle count, (D) mature follicle count, (E) atretic antral follicle count, (F) corpus luteum count and (G) cystic follicle count. Data are expressed as mean ± SEM. Different alphabets (a,b,c) showed a significant difference ($p < 0.05$).

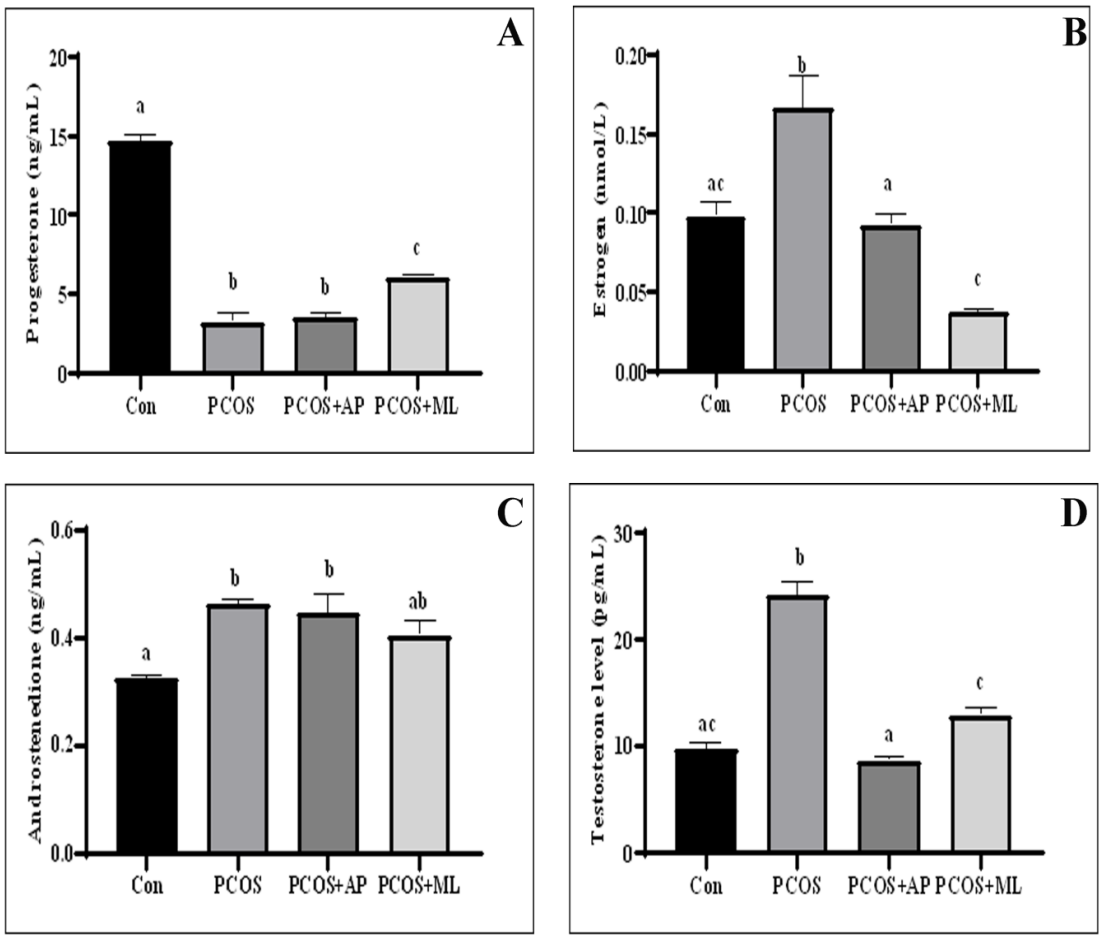


Fig.5.4: Serumsteroid hormones levels of letrozole-induced mice after apelin13 and ML221 treatment. (A) Progesterone level, (B) estrogen level, (C) androstenedione level and (D) testosterone level.Data are expressed as mean ± SEM. Different alphabets (a,b,c) showed a significant difference ($p < 0.05$).

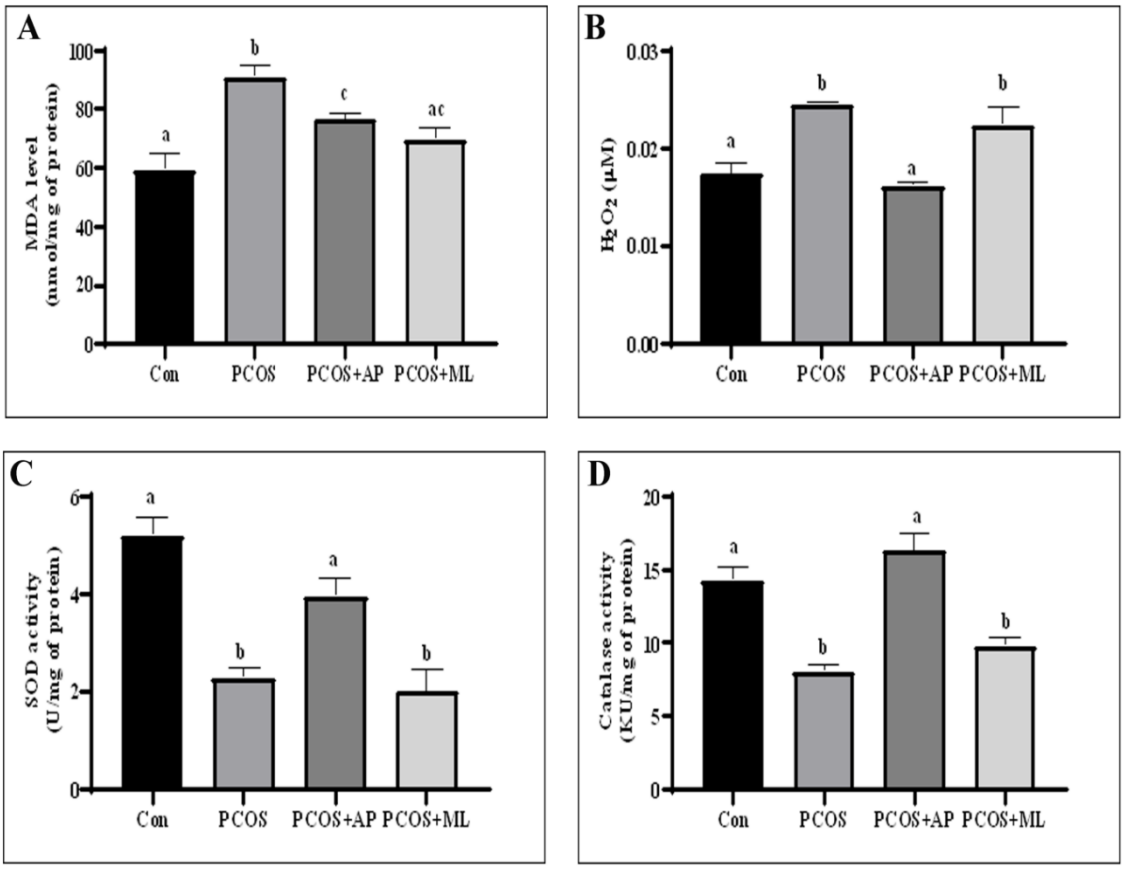


Fig.5.5: Changes in the MDA level (A), H_2O_2 level (B), SOD level (C) and CAT (D) level in letrozole-induced mice ovary after treatment of apelin13 and ML221. Data are expressed as mean ± SEM. Different alphabets (a,b,c) showed a significant difference ($p < 0.05$).

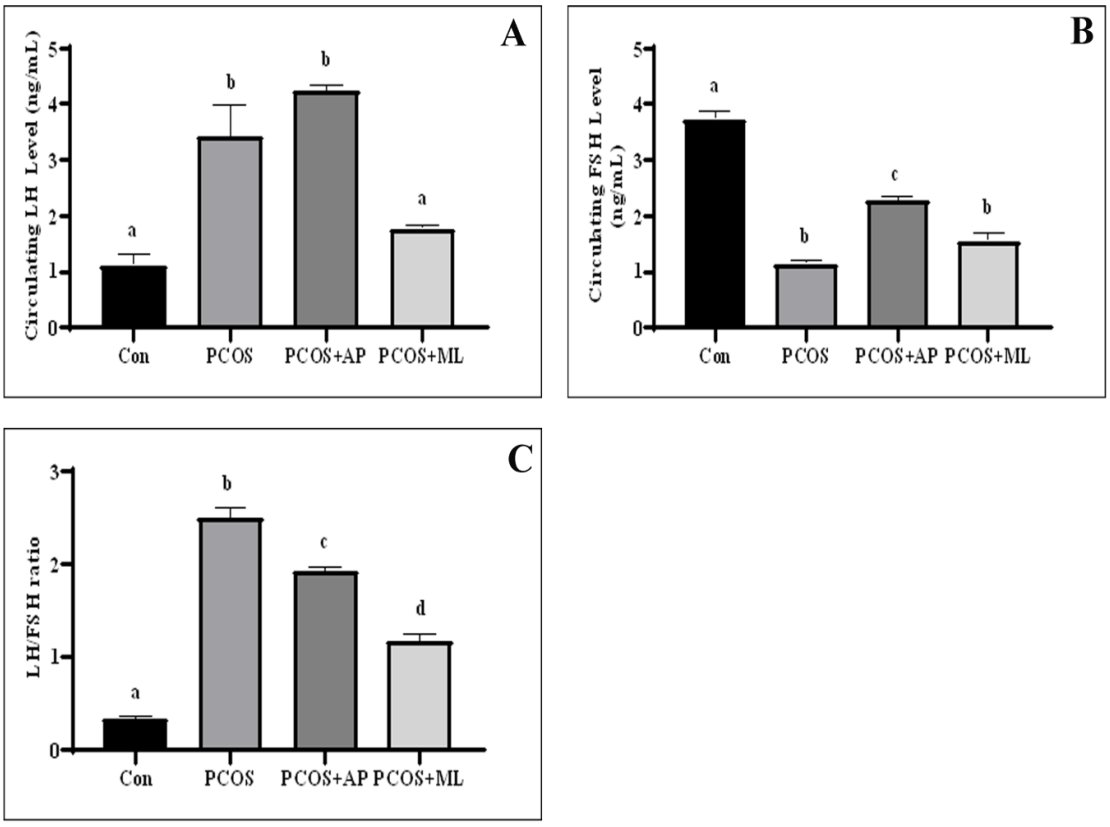


Fig.5.6: Circulating gonadotropin level in the letrozole-induced ovary after treatment of apelin13 and ML221. (A) Circulating LH, (B) circulating FSH and (C) LH/FSH ratio.Data are expressed as mean ± SEM. Different alphabets (a,b,c) showed a significant difference ($p < 0.05$).

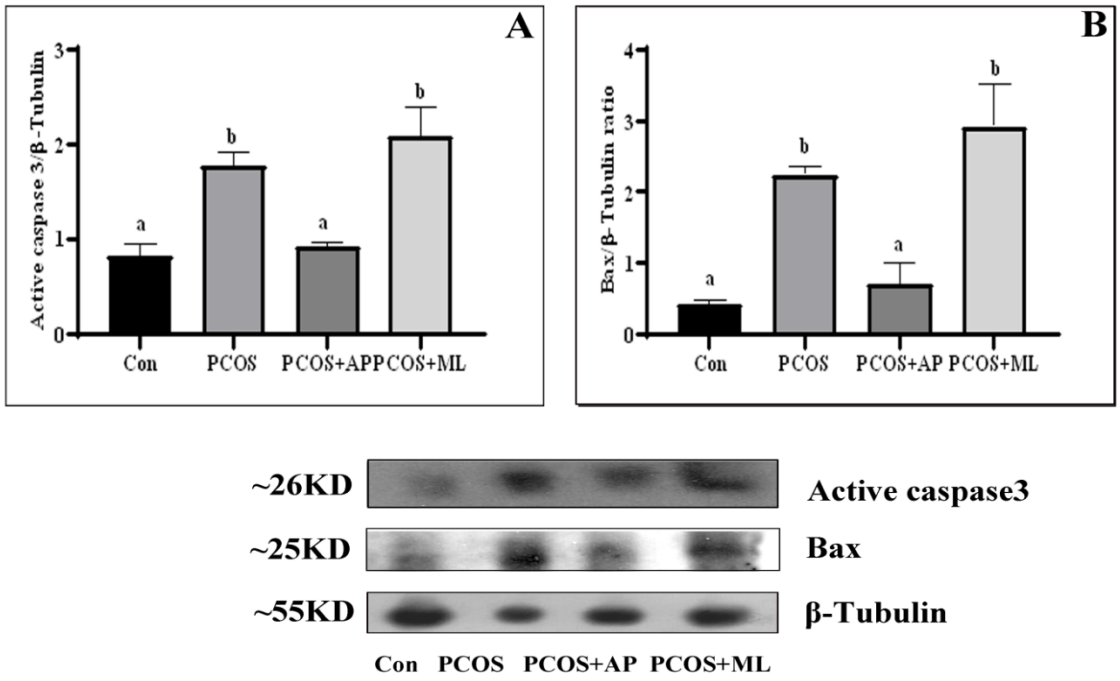


Fig.5.7: Expression of apoptotic markers in the letrozole-induced ovary after apelin13 and ML221 treatment. (A) Active caspase3, (B) Bax.Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

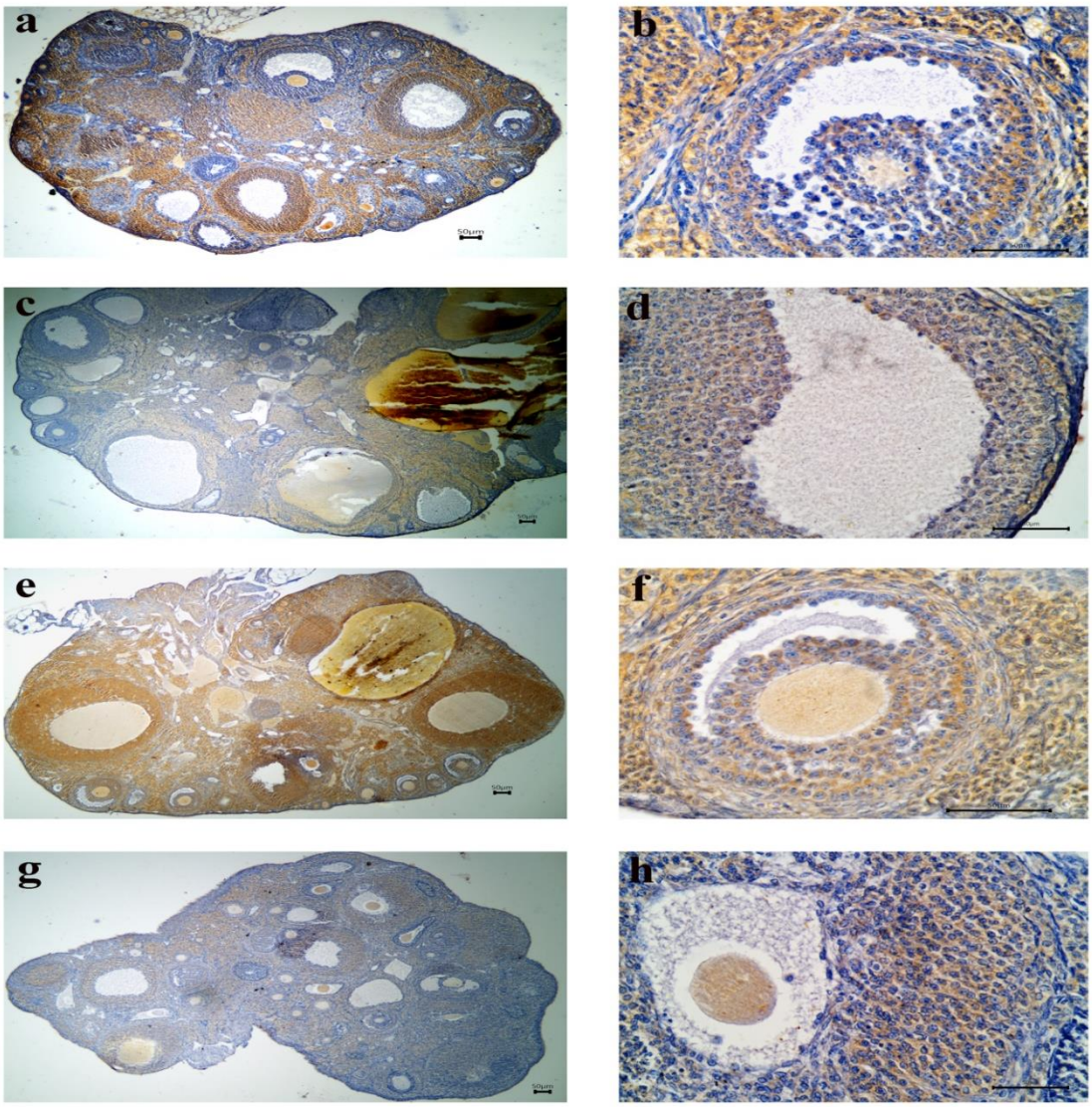


Fig.5.8: Immunolocalization of anti-apoptotic marker BCL2 in the letrozole-induced ovary after apelin13 and ML221 treatment. (A) Con group, (B) PCOS group, (C) PCOS+AP group, and (D) PCOS+ML group.

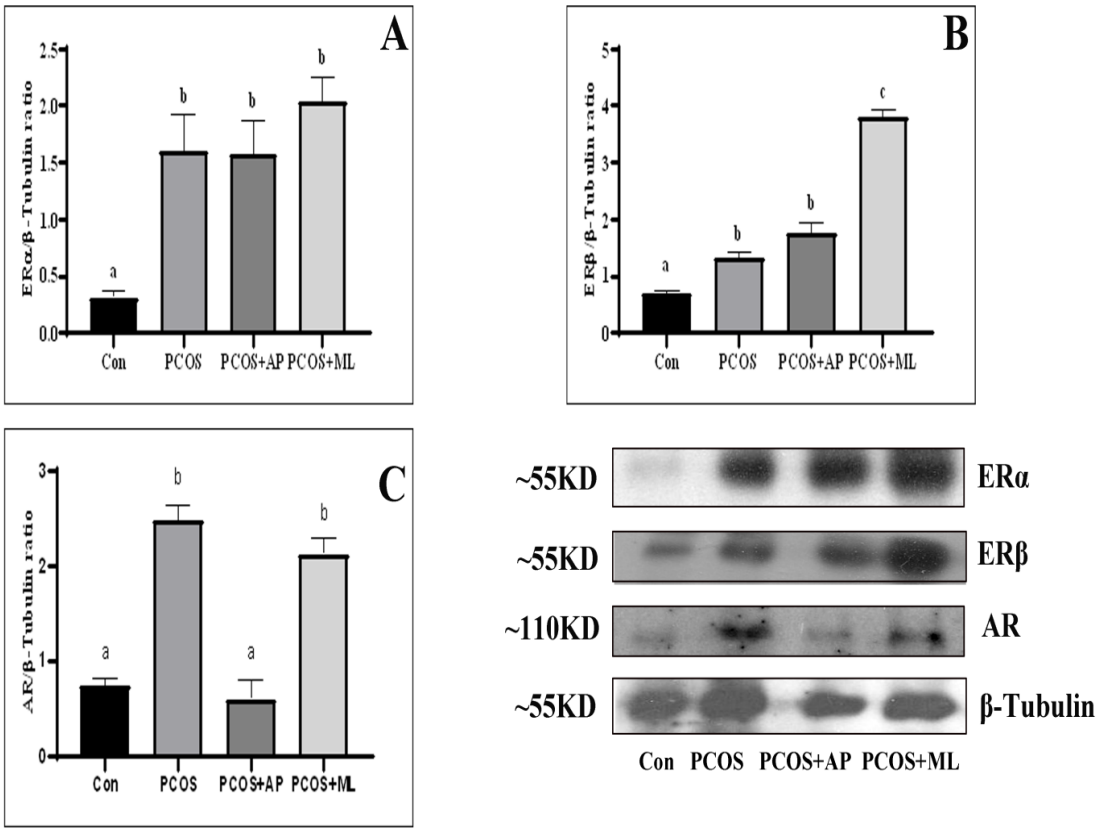


Fig.5.9: Expression of estrogen receptors (ERα and ERβ) and androgen receptor (AR) in the letrozole-induced ovary after apelin13 and ML221 treatment. (A) ERα, (B) ERβ, and (C) AR. Data are expressed as mean ± SEM. Different alphabets (a,b,c) showed a significant difference ($p < 0.05$).

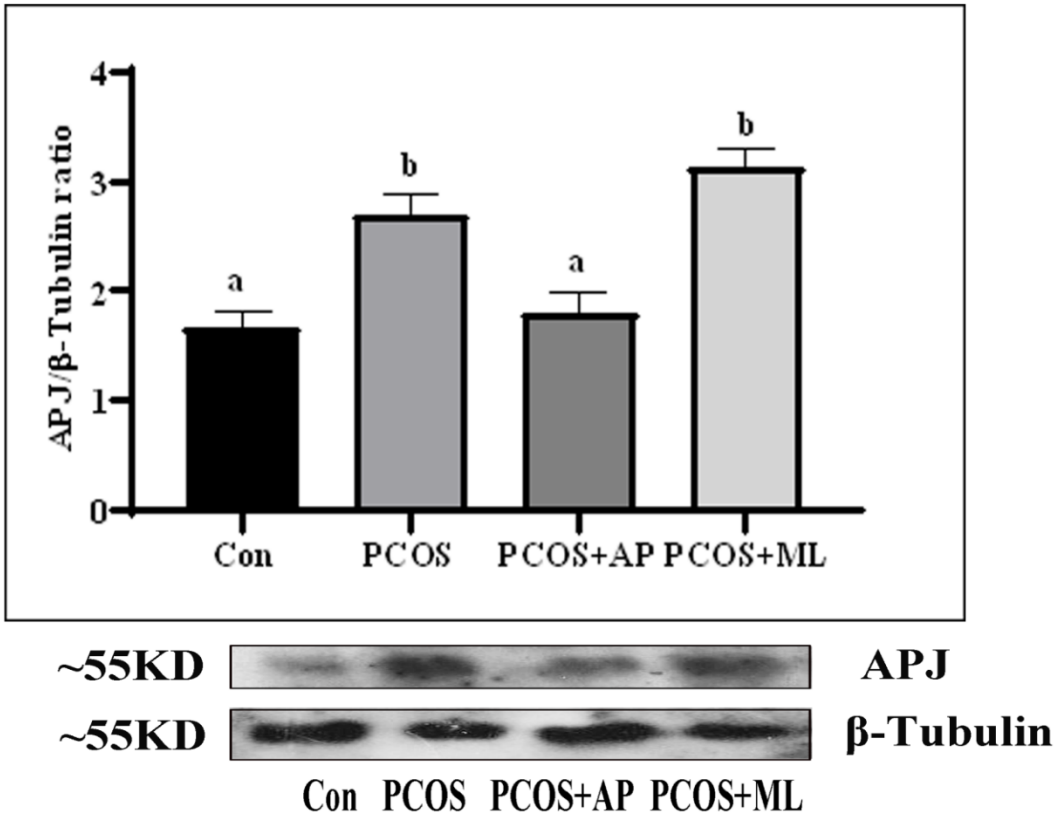


Fig.5.10: Expression of apelin receptor (APJ) in the letrozole-induced ovary after apelin13 and ML221 treatment. Data are expressed as mean ± SEM. Different alphabets (a,b) showed a significant difference ($p < 0.05$).

Consolidated summary

1. The circulating apelin levels did not show any changes during postnatal ages after PND1.
2. Immunolocalization of apelin and APJ in the postnatal ovary showed its presence in the follicles specially, in the granulosa, thecal and oocytes and corpus luteum.
3. Immunolocalization of apelin and APJ showed its presence in the different uterine compartments, with maximum expression at adult.
4. The overall expression and localization of apelin and APJ showed developmental regulation with an increasing trend from PND1 to PND14 followed by a decline in PND21 and elevation in the expression PND21 onwards.
5. The presence of apelin and APJ suggest the possible role in uterus as well as ovary development in the mice.
6. There were two peaks of the apelin system in the ovary and uterus of mice; one at early postnatal stage PND14 and the other at adult PND65.
7. The treatment of ML221 decreased the secretion of estrogen, testosterone, and androstenedione while the secretion of progesterone was elevated. These results suggest that apelin modulates ovarian steroid biosynthesis in the early postnatal stage (PND14), and probably involved in promoting estrogen secretion and suppressing progesterone by reducing 3βHSD.
8. The abundance and expression of 17βHSD and aromatase was increased after ML221 treatment.
9. The ML221 treatment decreased estrogen signaling in the ovary and uterus, which was indicated by the downregulation of estrogen receptors.
10. The localization of two proliferation markers PCNA and GCNA were also down-regulated by APJ antagonist, which suggests that apelin mediated regulation of ovarian and uterine cell proliferation.
11. The apelin signaling suppresses ovarian and uterine apoptosis via up-regulating Bcl2 expression in PND14 mice and down-regulating active caspase3.

12. In adult, the expression of the apelin and APJ showed tissue-dependent changes in the HPO axis during the estrous cycle of mice.
13. The presence of apelin and APJ during estrous also suggests its possible role in the hypothalamus.
14. The expression of apelin and APJ during metestrus to diestrus could be considered as stimulated apelin signalling in these phases (post-ovulatory) of the estrous cycle in the hypothalamus.
15. Apelin and APJ immunolocalization exhibited abundance in the anterior pituitary including the intermediate lobe.
16. Furthermore, the abundance of apelin and APJ in the anterior pituitary during the pre-ovulatory phase (Proestrus and Estrus) suggested having some influence on gonadotropin (LH and FSH) secretion.
17. The treatment of apelin-13 has suppressed the GnRH agonist stimulated FSH andLH secretion in proestrus stage.
18. The treatment of APJ antagonist, ML221 increased GnRH stimulated FSH secretion without affecting LH secretion.
19. In the estrus phase, GnRH agonist showed a stimulatory effect on LH and no effect on FSH has been observed.
20. Thus, apelin might suppress the gonadotropin secretion from the pituitary.
21. The presence of the apelin system in the ovarian corpus luteum and follicle suggests that apelin might have a modulatory effect on progesterone and estrogen secretion.
22. The abundance of apelin and APJ in the corpus luteum from metestrus to diestrus showed an increasing trend in the abundance of the apelin system suggesting that the apelin system involvement in the luteinizing of granulosa cells and the maturation of the corpus luteum.
23. The ovarian follicles showed an increased abundance of APJ from metestrus to estrus, which suggests that the apelin system could also be involved in folliculogenesis.

24. The observable changes of apelin and APJ were noticed in the uterine gland and luminal epithelium suggesting that apelin/APJ might be regulating the secretary functions of gland and luminal epithelium during estrous cycle.
25. Immunolocalization study showed localization of apelin and APJ in the stromal cells, perimetrium, endometrium, myometrium, and epithelial cells of lumen and gland of uterus during estrous cycle.
26. The apelin in uterus have differential expression during the estrous cycle with strong expression in the proestrus and estrus and reduce in the metestrus and diestrus stages.
27. Treatment of estrogen to ovariectomized mice significantly increased APJ expression in the uterus.
28. The treatment of progesterone alone or in combination of estrogen, decreased the expression of uterine APJ.
29. The abundance of apelin and APJ was slightly increased in luminal epithelium and uterine glands of estrogen and progesterone treated uterus than progesterone alone group.
30. The uterine apelin and APJ expression are regulated by the ovarian steroids, where estrogen up-regulates apelin and APJ and progesterone down-regulates uterine apelin system.
31. The treatment of PMSG moderately increased the abundance of apelin in the thecal, granulosa cells and oocytes in the ovary with elevated estrogen secretion.
32. Immunolocalization of apelin upregulated in the ovary of PMSG group while down-regulated after hCG treatment.
33. The APJ expression and localization was upregulated in the ovary of both PMSG and PMSG plus hCG treated group.
34. The PMSG plus hCG treatment showed formation of corpus luteum with increased abundance of APJ and progesterone secretion.
35. The gonadotropin up-regulates the ovarian apelin/APJ system in the mice ovary, which coincides with elevated ovarian steroidogenesis.

36. In normal reproductive cycle apelin seems to play important role in the ovarian and uterine functions via pituitary gonadotropin and ovarian steroid hormone, respectively.
37. The ovarian histology, corpus luteum formation, and decreased androgen levels by both apelin13 and ML221 showed ameliorative effect on the cystic ovary.
38. Furthermore, modulation of APJ action either by apelin13 or its receptor antagonist, ML221 could leads to mitigation of cystic ovarian pathogenesis.
39. The PCOS-augmented oxidative stress and apoptosis was suppressed by apelin13 treatments only.
40. ML221 treatment showed elevated oxidative stress and stimulated apoptosis as reflected by decreased antioxidant enzymes and increased active-caspase3 and Bax expression.
41. The letrozole-induced PCOS like features such as hyperandrogenism, cystic follicle, decreased corpus luteum, elevated levels of LH/FSH ratio, and up-regulation of ovarian AR expression were ameliorated by apelin13 and ML221 treatment.
42. PCOS has been found to be associated with up-regulation of ovarian APJ, which could also be important factor for PCOS associated pathogenesis.
43. The apelin13 and ML221 may have suppressed APJ signalling, which would account for our findings on the mitigation of poly cystic ovarian syndrome.

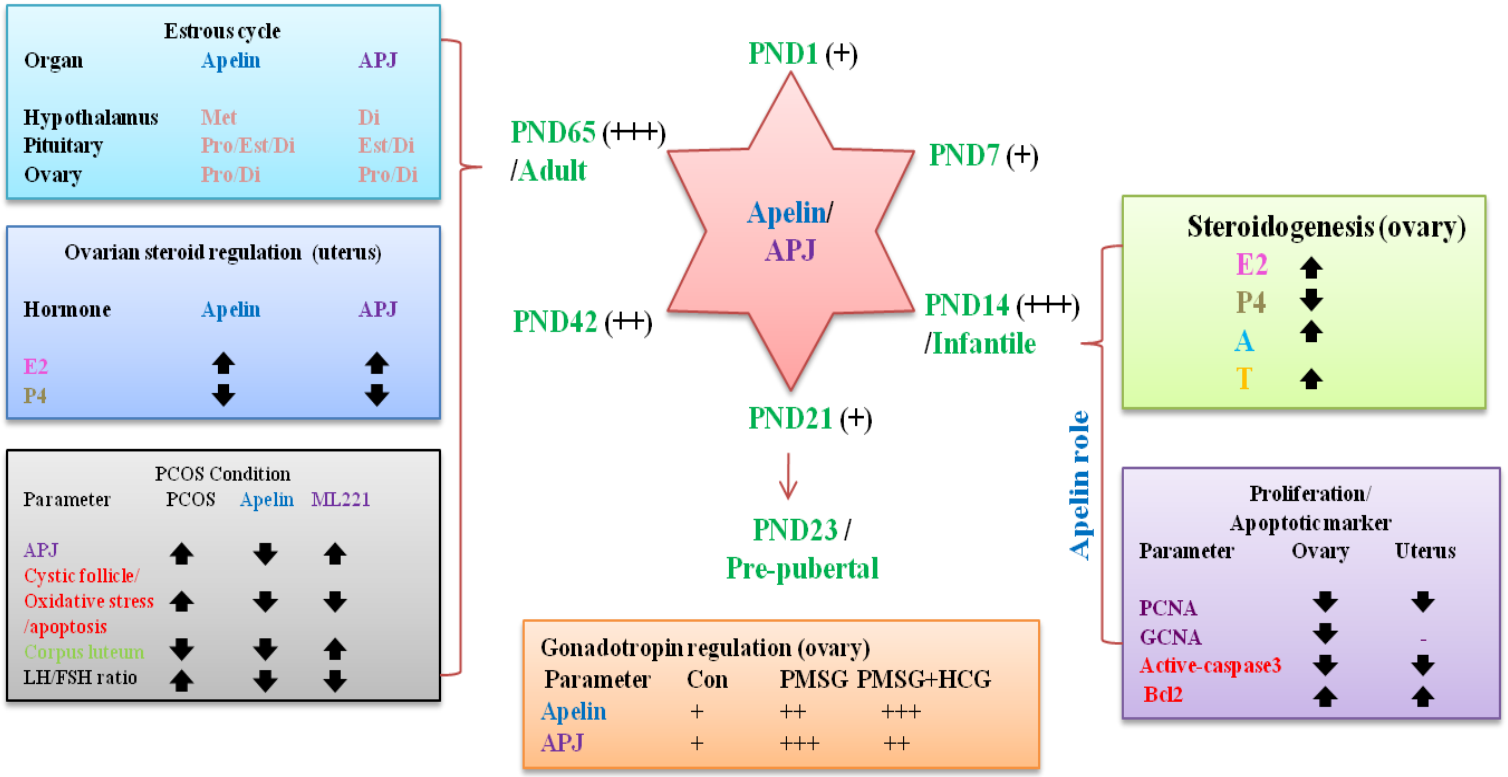
Conclusion

The present study the first time unravelled the localization and expression of the apelin and its receptor, APJ proteins in the mouse ovary, pituitary, hypothalamus and uterus, as well as its potential significance in ovarian, uterine and pituitary function.

The findings demonstrated that ovarian and uterineapelin and APJ expression is developmentally controlled and that their expression was dependent on various stages of the estrous cycle in adults. In the early postnatal stages, the role of apelin in the ovarian and uterine functions was unravelled by using APJ, antagonist, ML221. According to these findings, apelin influences ovarian steroid biosynthesis at the early postnatal period (PND14) and is likely involved in decreasing 3βHSD to increase estrogen release and inhibit progesterone. The findings suggest the apelin mediated regulation of ovarian and uterine cell proliferation in the early postnatal stage.During the mouse estrous cycle, tissue-dependent expression in the HPO axis was observed in the expression of apelin and APJ in adult mice. During estrous cycle the role of apelin on the hypothalamus and pituitary seems to be inhibitory and stimulatory at ovarian levels and it could be involved in the folliculogenesis. The cyclic expression of apelin and APJ in the uterus during estrous cycle may be regulated by ovarian steroids. Furthermore, the ovarian expression of apelin and APJ could also be dependent on the pituitary gonadotropin.

Additionally, ovarian apelin and APJ are up-regulated in pathophysiological conditions like PCOS, which could be one of the causes of the pathogenesis associated with PCOS. The findings demonstrated that inhibiting apelin signalling by apelin 13 peptide or its receptor antagonist, ML221,ameliorated the PCOS associated pathogenesis. It was noted that both apelin13 and Ml221, showed similar observation of ovarian histology,which suggests that mitigation by apelin 13 and ML221 might have different mechanism, which needs further investigation. In conclusion, apelin is a crucial adipokine that, like other adipokines, may regulate reproductive processes in a tissue- and stage-dependent way in normal and pathological conditions.

Schematic representation of apelin system in female mice reproduction



Score for intensity of immunoreactivity are as follows: +, mild; ++, moderate; +++, intense.
PND: postnatal day; E2: Estrogen; P4: Progesterone; A: Androgen; T: Testosterone; ⬇: down-regulation; ⬆: Up-regulation.

Reference

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M.Sc	2020	Mizoram University	Zoology	78.75 %	First

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Published paper

1. Rempuia, V., Anima, B., Jeremy, M., Gurusubramanian, G., Pankaj, P. P., Kharwar, R. K., & Roy, V. K. (2022). Effects of metformin on the uterus of d-galactose-induced aging mice: Histomorphometric, immunohistochemical localization (B-cell lymphoma 2, Bcl2-associated X protein, and active capase3), and oxidative stress study. Journal of Experimental Zoology Part A: Ecological and Integrative Physiology, 337(6), 600-611.
2. Anima, B., Mondal, P., Gurusubramanian, G., & Roy, V. K. (2023). Mechanistic study of copper nanoparticle (CuNP) toxicity on the mouse uterus via apelin signaling. Environmental Science and Pollution Research, 30(38), 88824-88841.
3. Anima, B., Gurusubramanian, G., & Roy, V. K. (2023). Postnatal developmental expression and localization of apelin and apelin receptor protein in the ovary and uterus of mice. Molecular Reproduction and Development, 90(1), 42-52.
4. Anima, B., Gurusubramanian, G., & Roy, V. K. (2023). Expression of apelin and apelin receptor protein in the hypothalamo-pituitary-ovarian axis during estrous cycle of mice. Neuroendocrinology.
5. Anima, B., Gurusubramanian, G., & Roy, V. K. (2024). Possible role of apelin on the ovarian steroidogenesis and uterine apoptosis of infantile mice: An in vitro study. The Journal of Steroid Biochemistry and Molecular Biology, 106463.

Conference /Seminar/Workshop attended

International

1. "International conferences on biodiversity, food security, sustainability & climate changes" on April 25-28, 2023 at AAU, Jorhat.
2. "International Seminar on Recent Advances in Science and Technology (ISRAST) on 16th -18th November 2020 at MZU with North East(India) Academy of Science and Technology(NEAST) (Virtual).

National

3. "National seminar on conservation of biodiversity (NSCB 2022)" on September 2-3, 2022 at NEHU, Tura campus, Meghalaya.

State

4. "4th Mizoram science congress, 2022" on 24-25 November, 2022 at Aijal Club, Aizawl.

Workshop

5. One-week training program on "instruments in biotechnology: theories and practices" organized by department of biotechnology, school of life sciences, Mizoram University.

6. Nanopore sequencing and data analysis: opportunities for rapid biodiversity and biosurveillance programs and local capacity building, organized by Mizoram University, Aizawl

7. Computational genomics with R: a hands on NGS data analysis organized by Mizoram University, Aizawl.

8. The value and interconnections of human, animal, and metataxonomics and metagenomics approach organized by Mizoram University, Aizawl.

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DEPARTMENT **:** ZOOLOGY

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DATE OF ADMISSION : 03.11.2020

APPROVAL OF RESEARCH PROPOSAL:

DRC : 16.04.2021

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Preface and Consolidated Abstract

Adipose tissue is a potential and active endocrine gland rather than an energy reservoir that secretes various adipokines which affect fertility through endocrine, autocrine and paracrine function. Numerous adipokines such as adiponectin, leptin, visfatin and resistin are known to control the activity of the male and female reproduction through hypothalamic-pituitary-gonadal axis. Adipokines are known to be expressed by the gonads in addition to the adipose tissue, and they have an autocrine and paracrine effect on gonadal function. Apelin, an adipokine having pleiotropic effects on several physiological processes and exerts its effect by binding to the deemed orphan G-protein-coupled receptor, now known as apelin receptor (APJ). It plays a significant part in the regulation of reproduction, in females of many species.

Apelin has drawn emerging interest lately due to a wide range of unique biological functions in several organs. Apelin expression (mRNA and protein) has been found in the stomach, brain, pituitary, heart, lung, pancreas, ovary, oviduct, uterus and placenta. More recently, the apelinergic (apelin and apelin receptor) system has been found in the hypothalamus-pituitary-gonadal axis and characterized as a beneficial component regulating both men and female reproduction. Furthermore, apelin and its receptor distribution has been found in the mammalian ovary such as bovine, rhesus monkey, porcine and human specially in the granulosa cells, thecal cells and corpus luteum which is incorporated with ovarian function including steroidogenesis regulation. The intra-ovarian apelin system may have an autocrine function, as shown by the expression of apelin and apelin receptor in corpora lutea and theca cells. Furthermore, the identification of apelin and apelin receptor gene expression in the same subset of luteal cells supports a paracrine effect of apelin.As the role of apelin has been witnessed in the contractility of myocardium and its presence in the uterus of rat and mice suggest some role in uterine remodeling, however, expression of apelin and apelin receptor has not been shown during postnatal period and during estrous cycle of mice and rat. Therefore, present study analyzed for the first time the expression and localization pattern of apelin

and APJ during developmental period including its possible physiological significance and also during estrous cycle as well. It is also logical to examine the role of ovarian steroid on uterine expression of apelin and APJ. Similarly, the ovarian functions are well regulated by gonadotropins, thus it would be logical and important as well to analyze the role of gonadotropin on ovarian apelin and apelin receptor expression. Furthermore, ovarian development is well regulated by plethora of factors, including the adipokine, however, the developmental expression of ovarian apelin and APJ has not been investigated. Thus, the present study would be the first to unravel these aspects of apelin and apelin receptor in mice o.In addition, since the reproductive system and energy balance are closely linked, metabolic disorders may contribute to the development of certain pathophysiologies, including polycystic ovarian syndrome (PCOS).Therefore, apelin intervention could be applied in the future regarding the treatment of diseases of the reproductive system. Apelin found as a beneficial peptide that have potential as a therapeutically target for metabolic diseases due to its anti-insulin resistance capabilities. Thus, we speculate that apelin may play a vital role in pathogenesis of PCOS condition. These are the issues and knowledge gaps that led us to use a model of laboratory mice to study the role of apelin in gonadal and uterine function.

The study incorporated in this thesis is thus broadly divided into five chapters**. Chapter 1**deals with postnatal changes apelin and apelin receptor in the female reproductive organs during developmental period. **Chapter 2** deals with the role of apelin on ovarian steroidogenesis, proliferation and apoptosis as well as uterine proliferation and apoptosis. **Chapter 3** deals withthe expressional changes of the apelin along the hypothalamus-pituitary-ovarian axis during reproductive cycle of mice. **Chapter 4** deals with the changes of apelin and its receptor expression in the uterus during reproductive cycle of mice and gonadotropin hormone dependent ovarian expression of apelin system as well as ovarian hormone dependent apelin expression in the uterus. **Chapter 5**deals with the apelin signaling mitigation of PCOSand to evaluate its impact on combating the PCOS associated ovarian pathogenesis**.**

Abstract

Chapter 1: Postnatal developmental expression and localization of apelin and apelin receptor protein in the ovary and uterus of mice.

Postnatal ovarian and uterine development is crucial to accomplished female fertility. Thus, the investigations of factors that present in pre-pubertal stages are important as it might be responsible for the regulation of ovarian and uterine function. Apelin, an adipokine and its receptor (APJ) are present in female reproductive organs. However, no study has reported its postnatal expression in uterus and ovary. Thus, we investigated the postnatal developmental changes in expression and localization of apelin and APJ in the ovary and uterus of mice.Postnatal ovary and uterus were collected from postnatal day (PND) 1, 7, 14, 21, 42, 65 and performed western blotting and immunohistochemistry. Uterine APJ is elevated in PND14 and PND65, whereas, ovarian APJ elevated in PND7, PND14 and PND65. Apelin expression in both ovary and uterus showed intense staining at PND65 and PND14. Our results showed that apelin and APJ abundance was lower at PND21 in uterus and ovary. In conclusion, apelin and APJ are developmentally regulated in the ovary and uterus, and its localization in the different compartments of ovary and uterus suggest its distribution specific physiological role in the uterus and ovary.

Chapter 2: Possible role of apelin on the ovarian steroidogenesis and uterine apoptosis of infantile mice: An in vitro study.

The expression of adipokines is well-known in the ovary and uterus. Recently we have shown that apelin and its receptor, APJ are developmentally regulated in the ovary and uterus of mice with elevation at postnatal day 14 (PND14). However, its role in the ovary and uterus of PND14 has not been investigated. Thus, we aimed to unravel the role of the apelin system (by APJ antagonist, ML221) on ovarian steroid secretion, proliferation, and apoptosis along with its role in uterine apoptosis in PND14 mice by *in vitro* approaches. The treatment of ML221 decreased estrogen, testosterone, and androstenedione secretion while increasing the progesterone secretion from the infantile ovary. These results suggest that apelin signaling would be important for ovarian

estrogen synthesis in infantile mice (PND14). The abundance of 3β-HSD, 17β-HSD, aromatase, and active caspase3 increased in the infantileovary after ML221 treatment. The expression of ERs and BCL2 were also down-regulated by ML221 treatment. The decreased BCL2 and increased active caspase3 by ML221 suggest the suppressive role of apelin on ovarian apoptosis. The APJ antagonist treatment also down-regulated the ERs expression in the uterus along with increased active caspase3 and decreased BCL2 expression. In conclusion, apelin signaling inhabits the ovarian and uterine apoptosis via estrogen signaling in the ovary and uterus.

Chapter 3: Expression of apelin and apelin receptor protein in the hypothalamo–pituitary–ovarian axis during the estrous cycle of mice.

Apelin is an endogenous peptide, whose expression has been shown in the hypothalamus, pituitary, and ovary; furthermore, it is also called a neuropeptide, binding to apelin receptor (APJ) for various functions. It has been suggested that the hypothalamus, pituitary and ovarian (HPO) axis is tightly regulated and factors and functions of the HPO axis can be modulated during the estrous cycle to influence reproductive status. To the best of our knowledge, the status of apelin and its receptor, APJ has not been investigated in the HPO axis during the estrous cycle. To explore the expression of apelin and APJ in the HPO axis of mice during the estrous cycle, mice were divided into four groupsproestrus (Pro), estrus (Est), metestrus (Met), and diestrus (Di), and apelin and apelin receptor (APJ) were checked. Further, to explore the role of apelin in gonadotropin secretion, an *in vitro* study of the pituitary was performed at the proestrus and estrus stages. The expression apelin and APJ in the hypothalamus showed elevation during the estrous cycle of post-ovulatory phases, metestrus, and diestrus. The immunolocalization of apelin and APJ in the anterior pituitary showed more abundance in the estrus and diestrus. Our in vitro results showed that GnRH agonist stimulated LH secretion was suppressed by the apelin 13 peptide from the pituitary of proestrus and estrus phases. This suggests an inhibitory role of apelin on gonadotropin secretion. The ovary also showed conspicuous changes in the presence of apelin and APJ during the

estrous cycle. The expression of apelin and APJ coincides with folliculogenesis and corpus luteum formation and the expression of the apelin system in the different cell types of the ovary suggests its cell-specific role. Previous studies also showed that apelin has a stimulatory role in ovarian steroid secretion, proliferation, and corpus luteum. Overall our results showed that the apelin system changes along the HPO axis during the estrous cycle and might have an inhibitory at level of hypothalamus and pituitary and a stimulatory role at ovarian level.

Chapter 4:Hormonal dependent expression of apelin and apelin receptor in the ovary and uterus of mice.

Apelin and APJ have been shown to regulate the female reproductive functions. However, it's uterine expression during estrous cycle and its regulation by ovarian steroid along with gonadotropin dependent its regulation in the ovary has not been investigated. Thus study aimed to analyse steroid dependent uterine expression of apelin/APJ in uterus along with estrous cycle. Furthermore, it also aimed to investigate gonadotropin dependent ovarian expression of apelin and APJ. To investigate the uterine expression of apelin and APJ during estrous cycle in mice, uterus at different estrous stage were collected. To explore the ovarian steroids dependent expression of apelin system in the uterus, ovariectomized mice were treated with only estrogen at dose of 30 ng/g, only progesterone at dose of 150 μg/g and combined doses. To study the effect of gonadotropin on ovarian expression of apelin system, immature mice were injected with 2.5 IU of pregnant mare serum gonadotropin (PMSG) alone and both PMSG plus 2.5 IU of chorionic gonadotropin (hCG).Apelin and APJ protein expression are modulated by estrous phases in the uterus. The uterine apelin and APJ expression are up-regulated by estrogen and down-regulated by progesterone. The expression and localization of APJ showed increased abundance in the follicles of PMSG treated mice, however, the PMSG plus HCG treatment showed formation of corpus luteum with increased abundance of APJ and progesterone secretion.The expression of apelin and

APJ are regulated by pituitary gonadotropin in the ovary and uterine apelin system by ovarian steroid hormone.

Chapter 5: Apelin receptor modulation mitigates letrozole-induced polycystic ovarian pathogenesis in mice.

Polycystic ovarian syndrome (PCOS) is one of the most common (about 5–20%) reproductive disorders in women of reproductive age; it is characterized by polycystic ovaries, hyperandrogenism and oligo/ anovulation. The levels and expression of ovarian adipokines are deregulated in the PCOS. Apelin is an adipokines which acts through its receptor (APJ) and known to express in the various tissues including ovary. It has also been suggested that apelin and APJ could be targeted as therapeutic adjunct for the management of PCOS. However, no study has been conducted on the management of PCOS by targeting apelin system. Thus, we aimed to evaluate its impact on combating the PCOS associated ovarian pathogenesis.The current work employed letrozole-induced-hyperandrogenised PCOS like mice model to investigate the effects of apelin13 and APJ, antagonist ML221. The PCOS model was induced by oral administration of letrozole (1 mg/kg) for 21 days. The total of four experimental groups were made, control, PCOS control, PCOS+aplein13 and PCOS+ML221. The treatment of apelin13 and ML221 was given from day 22 for two weeks. The letrozole-induced PCOS like features such as hyperandrogenism, cystic follicle, decreased corpus luteum, elevated levels of LH/FSH ratio, and up-regulation of ovarian AR expression were ameliorated by apelin13 and ML221 treatment. However, the PCOS-augmented oxidative stress and apoptosis was suppresed by apelin 13 treatments only. ML221 treatment still showed elevated oxidative stress and stimulated apoptosis as reflected by decreased antioxidant enzymes and increased active caspase 3 and Bax expression. The expression of ERs was elevated in the all groups except control. Furthermore, PCOS model showed elevated expression of APJ and apelin13 treatment down-regulated its own receptor. Overall, by observing the ovarian histology, corpus luteum formation, and decreased androgen levels by both apelin13 and ML221 showed ameliorative effect on the cystic ovary. Despite similar morphological observation of ovarian histology, apelin13 and ML221

exhibited opposite effect on the oxidative stress and apoptosis. Therefore, apelin13 (which down-regulates APJ) and ML221 (an APJ antagonist) may have suppressed APJ signalling, which would account for our findings on the mitigation of poly cystic ovarian syndrome. In conclusion, both apelin13 and ML221 mediated mitigation have different mechanism, which needs further investigation.