EFFECTS OF DEVELOPMENTAL EXPOSURE TO ESTROGENIC ENDOCRINE-DISRUPTING CHEMICALS METHOXYCHLOR AND BISPHENOL A DURING FETAL AND NEONATAL PERIODS ON OVARIAN FOLLICULOGENESIS AND REPRODUCTIVE PARAMETERS IN RATS

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ABSTRACT OF THE THESIS

EFFECTS OF DEVELOPMENTAL EXPOSURE TO ESTROGENIC ENDOCRINE-DISRUPTING CHEMICALS METHOXYCHLOR AND BISPHENOL A DURING FETAL AND NEONATAL PERIODS ON OVARIAN FOLLICULOGENESIS AND REPRODUCTIVE PARAMETERS IN RATS

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Endocrine-disrupting chemicals (EDCs) are synthetic or natural compounds that can be found in the environment in various forms, such as pesticides [e.g., methoxychlor (MXC)], plasticizers [e.g., bisphenol A (BPA)], or pharmaceutical agents [e.g., diethylstilbestrol (DES), a potent synthetic estrogen that was prescribed to pregnant women between 1940s and 1970s. Previous studies indicated that the ovary is particularly vulnerable to estrogenic EDC exposures during fetal and neonatal periods. To investigate the effects of estrogenic EDCs in two critical developmental windows, we performed two experiments, using timed-pregnant Fisher CDF rats. In Experiment 1, we exposed the dams to 50 μg/kg/day (Low) BPA or 50 mg/kg/day (High) BPA, or vehicle from embryonic day (E) 18 to 21, and the pups from postnatal day (PND) 0 to 7. In Experiment 2, we exposed animals to 0.1μg/kg/day DES, 75 mg/kg/day MXC, 50 mg/kg/day BPA, or vehicle from E11 to PND7. We then examined reproductive parameters, including pubertal age, regularity of reproductive cycles, and follicular composition. Expression of critical ovarian markers, including estrogen receptor 1 (ESR1), Mullerian inhibiting substance (MIS), cytochrome P450 side chain cleavage (P450scc), luteinizing hormone receptor (LHR), and proliferating cell nuclear antigen (PCNA) were also examined.

In Experiment 1, we found that there were no changes in age at puberty or estrous cyclicity in the both Low and High BPA-treated females. However, there was a decrease ($p < 0.05$) in primary follicles in High BPA-treated rats. In addition, there was an increase in atretic follicle numbers ($p < 0.05$) in the Low and High BPA-treated females suggesting that there was an effect on follicular dynamics. Moreover, a short window of ovarian exposure to BPA did not show any statistically significant effects on expression of ovarian markers except ESR1which was decreased in primary follicles in Low BPA-treated females.

In Experiment 2, our results demonstrated that DES- and MXC-treated females had an accelerated onset of puberty and altered estrous cyclicity. Although there was no effect on the litter size, MXC-treated females showed a strong trend towards reduction in litter size ($p = 0.07$) as compared to control. MXC caused a decrease in steroid hormone levels. DES, MXC, and BPA exposures altered follicular dynamics. There was an increase in atretic follicles in DES and BPA-treated females. In addition, the number of corpora lutea (CL) was reduced ($p < 0.01$) in MXC-treated females while the total follicle numbers were not altered. There were alterations in ovarian molecular markers. Expression of MIS significantly increased in secondary and pre-antral follicles in MXC-treated group. Furthermore, expression of P450scc decreased in pre- and early-antral follicles in MXC-treated females while the expression increased in CL in BPA-treated females.

Overall, the results show that developmental exposure to estrogenic-EDCs affects female reproduction and ovarian follicular dynamics. In addition, DES and MXC can alter the age of puberty, and regulatory of reproductive cycles, which may lead to female fertility problems.

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ABBREVIATIONS

EDC	Endocrine-disrupting chemicals
MXC	Methoxychlor
DES	Diethylstilbestrol
BPA	Bisphenol A
PGC	Primordial Germ Cell
E	Embryonic Day
PND	Postnatal Day
GD	Gestational Day
ESR 1	Estrogen Receptor 1
ESR 2	Estrogen Receptor 2
LHR	Luteinizing Hormone Receptor
P450scc	Cytochrome P450 Side Chain Cleavage
MIS	Müllerian Inhibiting Substance
PCNA	Proliferating Cell Nuclear Antigen
GC	Granulosa Cell
TC	Theca Cell
HPG axis	Hypothalamic-Pituitary-Gonadal Axis
GnRH	Gonadotropin-Releasing Hormone
FSH	Follicle Stimulating Hormone
LH	Luteinizing Hormone
E2	Estradiol
P4	Progesterone
PE	Persistent Estrus
PD	Persistent Diestrus

TTP	Prolonged Time-to-Pregnancy
EPA	US Environmental Protection Agency
LOAEL	Lowest Observed Adverse Effect Level
H & E	Hematoxylin and Eosin
IHC	Immunohistochemistry
RIA	Radioimmunoassay
PBS	Phosphate-Buffered Saline
dpc	Day Post-Coitum
i.p.	Intraperitoneal
s.c.	Subcutaneous
ng/ml	Nanogram per milliliter
pg/ml	Picogram per milliliter
cpm	Count per minutes

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CHAPTER 1: INTRODUCTION

1.1. Overview

The ovary is one of the most important organs for female fertility due to its responsibility for gamete production and hormone biosynthesis. The development and maturation of ovarian follicles is called folliculogenesis [1]. Early embryonic developmental events are vital to folliculogenesis that is directly related to female fertility and reproductive functions because these events control the size of the follicular pool [2], which in turn determines the reproductive lifespan of a female [3]. Early folliculogenesis and follicular pool size are regulated by the ovarian steroid hormones, such as estrogens. The ovarian hormones are essential for follicular development [4]. Endogenous estrogen binds to estrogen receptors (ESR1 and ESR2) to mediate its actions. While ESR1 is primarily expressed in theca cells, ESR2 is expressed in granulosa cells [5]. These two ESRs regulate follicular functions in the ovary [6].

According to the U.S. Environmental Protection Agency (EPA), an endocrine-disrupting chemical (EDC) is defined as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process". Recently, there is a heightened concern, regarding the adverse health effects of EDCs. The potential adverse effects include cancer, infertility, and obesity and metabolic disorders [7, 8]. EDCs are found in various forms in the environment; pesticides (e.g., methoxychlor, MXC) [2, 9], pharmaceuticals (e.g., diethylstilbestrol, DES) [10, 11], and plasticizers (e.g., bisphenol A, BPA) [8]. EDCs, such as MXC, BPA, and DES, can alter ovarian function by acting as agonists or antagonists for steroid hormone receptors, especially for ESR1 and ESR2 [12].

It has been reported that MXC alters ESR1 and causing ovarian toxicity particularly targeting antral follicles. MXC inhibits follicular growth and causes a high number of atretic follicles [13]. EDCs also affect the levels and responses of reproductive hormones [2, 14]. It was shown that high MXC exposure during development causes a reduction in ovulation in response to exogenous gonadotropins, suggesting that they directly affect the ovary [2]. In addition, EDCs may affect the estrogen biosynthetic pathways by changing the expression of enzymes involved in the synthesis of estrogens in the ovary [15].

The aim of this research was to focus on the effects of developmental exposure to estrogenic EDCs on the ovarian function in two critical fetal and neonatal developmental windows. To this end, the effects of three EDCs (DES, MXC, and BPA) were investigated. The next few sections will highlight the literature on ovarian follicular development and the effects of EDCs on critical developmental processes.

1.2. Ovarian Follicular Development

1.2.1. Embryonic Germ Cell Migration

The interactions of primordial germ cells (PGCs), and somatic cells are required for the differentiation of mammalian gonad [16]. The PGCs, the precursor cells for the gametes, originate from the extra-embryonic mesoderm [17, 18]. In mice, starting with E9.5, PGCs migrate, while proliferating, through the hindgut to the genital ridge, an embryonic structure that differentiates into the bipotential gonad [19]. The migration of PGCs ends at around E13.5 [20]. The somatic cells of the gonad undergo sexual differentiation, which depends on the absence or presence of the *Sry* gene [21]. The germ cell mitosis continues in the gonad and proliferating PGCs in females, which are now referred to as oogonia, remains as cluster of cells interconnected with each other with cytoplasmic bridges [22]. Following a series of mitotic divisions, the oogonia enter meiosis and are arrested at the early diplotene phase of the first meiotic prophase and form oocyte nests [23, 24]. The oocyte nests are the source of the primordial follicles that would form later during development.

The early critical follicular developmental processes consist of oocyte nest breakdown and assembly of primordial follicles, and the transition of the primordial to primary follicle stage [25]. These process are regulated by maternal ovarian steroid hormones, more prominently estrogens [26], and are important for determining the ovarian capacity and lifespan of female reproduction [24].

1.2.2. Oocyte Nest Breakdown and Primordial Follicle Assembly

The formation of primordial follicles is mediated by oocyte apoptosis, known as oocyte nest breakdown [27], which starts at E16.5, in mice [22]. During late gestational and early postnatal stages, the oocytes within nests undergoes apoptosis, which reduces the number of oocytes by ~2/3. Remaining oocytes are individually enclosed by somatic cells (pre-granulosa cells) forming primordial follicles [28]. Primordial follicle formation is completed between PND3 and 7, making up the initial follicular pool [29]. Each primordial follicle consists of a single oocyte at the center surrounded by a single layer of pre-granulosa cells (GCs).

The maternal estrogens play a role in oocyte nest breakdown and primordial follicle assembly. While high levels of maternal estradiol maintain oocyte nests in the fetal ovary, the levels of estradiol drop at birth [30]. The low levels of estrogen allow the formation of the primordial follicles in the neonatal ovary. For example, previous studies showed that the high level of estradiol inhibited oocyte nest breakdown and reduced the formation of follicular assembly [26, 30]. In addition, other steroid hormones such as, progesterone [31], and estrogenic EDCs, such as DES [32] and genistein inhibit follicular assembly [30].

1.2.3. Follicular Activation

Once formed, most primordial follicles remain quiescent, but some are activated immediately in neonatal period between PND4-7. Those remaining follicles are activated throughout the reproductive life of a female in cohorts until the follicular pool is exhausted [24]. Those follicles that are activated undergo follicular growth and transition from primordial to primary follicular stage [33]. During this transition, the pre-granulosa cells proliferate and convert to the cuboidal granulosa cells [31, 34]. Once this process is initiated, the follicles continues through the developmental pathway, resulting either ovulation or death by the follicular atresia [35]. Thus, the control of this transition is critical for a healthy female reproductive life. Estrogens are also known to regulate this transition [31]. Thus, this process is likely to be influenced by the exposure to estrogenic EDCs.

Following follicular activation, the oocyte becomes larger, and the second somatic cell type, the theca cells are recruited from the surrounding interstitial tissue [25]. The granulosa and theca cell collaboration is essential for larger quantities of estradiol at later developmental stages of folliculogenesis. In addition, follicular development regulated by hypothalamic-pituitary-gonadal (HPG) axis in which gonadotropin-releasing hormone (GnRH) stimulate gonadotropins, namely follicle stimulating hormone (FSH) and luteinizing hormone (LH), are required for optimum steroid production as well as cellular changes and maturation of follicles [36]. The LH and FSH integrate function of the component of the follicles [37].

1.2.4. Pre-antral and Antral Follicle Development

Pre-antral and antral follicular stages are characterized by the rapid cell proliferation of the granulosa cells, which forms multiple layers [38]. This is followed by the formation of an antrum or fluid-filled space in the follicles [35, 38]. The antrum space and its fluid may help to the ovulation and serve as a vehicle for nutrient [39].

1.2.5. Follicular Selection, Maturation, and Atresia

The selection of the antral follicle that is destined to further growth and ovulation requires complex regulation [40], involving both FSH and LH [41]. In the absence of appropriate gonadotropic stimulation, follicles do not develop beyond the early antral stage and atresia occurs [38]. It appears that at earlier stages FSH receptor density, and at later stages LH receptor (LHR) densities play critical roles in follicle selection [42]. Actions of FSH, which is on the granulosa layer, is responsible for the follicular growth, and action of LH, which is primarily on the thecal layer (at the periovulatory stage it also acts on the granulosa cells) is required for the final maturation and ovulation. The FSH actions on granulosa during at late stages of follicular growth lead to increased aromatase activity. During this stage, the estradiol production from granulosa reaches to its peak, and these cells also gain LH-responsiveness during their final maturation and ovulation [43].

EDCs can adversely affect the final maturation and ovulation. For example, previous study shown high dose MXC (100 mg/ kg/day) significantly reduced LHR expression in large antral follicles [2], which is associated with reduced ovulation and litter size.

1.2.6. Ovulation and Corpus Luteum Formation

The process of the ovulation is a complex phenomenon, and a number of regulatory factors involve in this phenomenon [44]. The high level of the estrogen from dominant follicles initiates the LH surge and, to a lesser extent, FSH surge, that induces numerous cellular and molecular changes in the follicle [45]. Following ovulation, the remnants of the ruptured (ovulated) follicular cells, the remaining granulosa and theca cells undergo a differentiation as known as luteinization [46]. During the luteinization, granulosa cells lose their aromatase activity and hence capacity to produce estradiol. Along with theca cells, these cells become luteal cells and form the corpus luteum (CL). The CL, which has a rich vascularization, is primary source of progesterone and essential for the maintenance of pregnancy [47].

1.2.7. Reproductive Senescence

The reproductive senescence is a natural physiological event and occurs with aging [48]. Reproductive aging is dependent on the quality and quantity of the follicular pool and oocyte numbers. The depletion of this pool leads to reproductive senescence [49]. Normal reproductive senescence begins at 10 to 12 months in rats [49]. The loss of ovarian follicles is controlled by hypothalamic, or pituitary function [50]. Especially, GnRH has a crucial role on this control mechanism [49]. However, there is a growing concern about EDCs in the environment may influence early reproductive aging [51]. For instance, it was shown that MXC exposure alters the follicular pool and leads to early reproductive senescence in rats [2].

1.3. Ovarian Markers

This section will give a brief overview of ovarian markers that play roles in critical ovarian functions, including folliculogenesis (ESR1, MIS), steroidogenesis (P450scc), and ovulation (LHR). Brief information on PCNA, a cell proliferation marker, will also be provided. These markers are selected because they play critical roles in ovarian functions. In addition, previous *in vivo* or *in vitro* studies show that EDCs alter their expression in the ovary.

1.3.1. Estrogen Receptors

Both subtypes of estrogen receptors (ESR1 and ESR2) are directly related to folliculogenesis. These receptors are required for the maintenance of ovarian cells [52] are encoded by separate genes. Their expression profiles are different based on their sites on the follicular cells [53]. ESR1 is localized in the theca cells, and significant ESR1 expression can be detectable at 14 day post-coitum (dpc) and PND12 in rat ovaries [54]. They affect multiple cellular functions in target tissues, such as growth and differentiation of cells [55, 56]. ESR2 plays an important role at many stages of folliculogenesis from follicle activation to FSH-mediated follicle maturation and steroid production [57], and is localized in granulosa cells, starting with primary follicular stage [58].

EDCs, actions on ESRs can be estrogenic or antiestrogenic. MXC and it major metabolite HPTE (2,2-bis-p-hydroxyphenyl)-1,1,1-trichloroethane) acts on ESR1 as an agonist while HPTE acts on ESR2 as an antagonist [59]. Moreover, HPTE can interfere with estrogen signaling in multiple ways, including reduction of ESR2 protein levels and estradiol biosynthesis in the granulosa cells [60].

1.3.2. Cytochrome P450 Side Chain Cleavage

Cytochrome P450 side chain cleavage (P450scc), a mitochondrial enzyme, has a crucial role in the production of steroid hormones [61]. Its function is to convert cholesterol into pregnenolone [62], which serves as the common substrate for the biosynthesis of progesterone and estrogens. P450scc mRNA is primarily expressed in the theca/interstitial cells of the antral follicles and CL [63]. Its activity increases during follicular development in the theca cells. In the preovulatory follicles, the expression of P450ssc is also induced in granulosa cells by gonadotropins [63].

EDCs have effects on the P450scc expression. Developmental MXC exposure reduces the P450scc immunoreactivity in adult rat ovary [2]. In addition, using rat granulosa cell cultures MXC metabolite HPTE was shown to reduce P450scc mRNA levels, which is associated with reduced progesterone and estradiol production [64].

1.3.3. Mullerian Inhibiting Substance

Müllerian inhibiting substance (MIS) also known as an anti-Müllerian hormone (AMH) [65] is a member of the transforming growth factor (TGF)-beta superfamily [29]. MIS is an important marker that plays a crucial regulatory role in primordial follicle recruitment [66, 67], and dominant follicle selection in the ovary in mice [29]. MIS is expressed in granulosa cells in growing follicles between stages of primary and early antral follicles [67, 68]. Its expression gradually disappears in large antral follicles [29]. MIS is inhibitory for the primordial follicle activation, which is supported by gene deletion studies. MIS-null mouse strain depletes their primordial follicular reserves much faster than wild-type mouse [69]. In addition, MIS-treated ovaries in culture contain 40% less growing follicles than control animals [67]. Developmental exposure to estradiol or

MXC stimulates expression of MIS in growing follicles including primary, secondary, pre-antral, and antral follicles in the ovary [66, 70].

1.3.3. Luteinizing Hormone Receptor

Luteinizing hormone (LH) is a gonadotropin produced from the pituitary gland. Responsiveness to LH is mediated by LHR, which is a member seven-transmembrane-spanning receptor family, whose mRNA first appears in the ovary around PND7 in rats [71]. LHR expression in the ovary is essential for the follicular development, particularly follicular maturation beyond the antral follicle stages, and ovulation and formation of CL [72]. During the antral follicle stage, LHR expression increases in theca cells. At periovulatory follicular stage, LHR also expressed granulosa cells, especially those surrounding the antral space wall, known as mural granulosa cells [43].

1.3.4. Proliferating Cell Nuclear Antigen

Proliferating cell nuclear antigen (PCNA), a nuclear protein with an essential role in DNA replication, and cell proliferation. Its immunoreactivity marks early events in the follicular and oocyte growths, and appears in the pre-granulosa cells of the primary follicles that just start to grow [73]. PCNA is used to examine the proliferation of the pre-granulosa cell and oocyte apoptosis.

1.4. BPA, MXC, and DES as Estrogenic EDCs

MXC [1,1,1-trichloro-2, 2-bis (4-methoxyphenyl) ethane] is a chlorinated hydrocarbon pesticide used as a replacement of dichlorodiphenyltrichloroethane. Although MXC's production in the USA has stopped in early 2000s, it is produced and used in countries in Asia, and Africa [74, 75]. Thus, MXC can be transferred through natural and commercial routes. Women with agricultural work histories, which are likely to be exposed to organochlorine pesticides, show reduced fertility rate [76].

BPA (Bisphenol-A) is a plasticizer [77] used as a component of polycarbonate plastic and epoxy resins [78, 79] in the manufactures of polycarbonate baby bottles, food and beverage containers, thermal print papers, and composites [80]. It is one of the highest-volume chemicals produced worldwide, by about over 6 million pounds per year [81]. Even though BPA has been used commercially since 1957, the importance of its effect on human health has been recently realized. BPA has been detected in human serum, urine, breast milk, and ovarian follicular fluids [82]. There are studies suggesting BPA serum or urinary concentration is adversely associated with gamete quality and steroidogenic capacity in women undergoing IVF procedure, however, the evidence is inconclusive [83].

DES is a non-steroidal synthetic estrogen, which was used between 1940s to 1970s, prescribed at doses of 5–150 mg⁄day to prevent miscarriages in human [84]. Their babies were exposed to DES *in utero* causing the formation of plethora of pathologies later in life resulting in the ovarian dysfunctions. These pathologies were replicated in model experimental animals, especially in mice reported by numerous studies [85]. In addition, neonatal exposure to a single dose of DES, which shows estrogenic activity *in vivo*, induces adverse effects on female rats in a dose-dependent manner [86].

1.4.1. *In Vivo* Studies Investigating Effects of DES, MXC, and BPA on Reproductive Parameters

In vivo studies with animals shown that transient, fetal, and neonatal EDC exposures to MXC [2], DES [87], and BPA [88] affect female reproductive parameters, such as ovarian weight, age of puberty, regularity of estrous cycle, pregnancy rate, and litter size. In adult rats, MXC exposure (2500 or 5000ppm) interferes with their normal estrous cycle and reduces the mating rate and litter size [89]. In another study in which female rats were exposed during fetal and neonatal exposure to a dose of MXC (E19-PND7) that has shown accelerated vaginal opening, irregular estrous cycles, reduced litter size, and early reproductive aging [2]. The exposure also leads to an alteration in folliculogenesis and increased pre- and early antral follicles and reduced number of corpora lutea (CL). In other studies of MXC showed persistent vaginal estrus [90] pregnancy loss [91] and ovarian atrophy due to inhibition of folliculogenesis causing to atretic follicles and decrease ovulation [90].

Previous studies have also shown that early postnatal BPA exposure affects ovarian folliculogenesis (e.g., increased large antral-like follicles and reduced the number of CL) without affecting GnRH neurons [92], suggesting a direct effect in the ovary. In another study where exposure to low dose BPA (0.5 μg/kg and 50 μg/kg) *in utero* between E10.5 and 17.5 altered the ovarian morphology and reduced the number of primordial follicles at birth in the mouse [93]. A chronic exposure model (100 day) starting with E7 that is also with low (10 mg/kg/day) and high (100 mg/kg/day) BPA exposure led to increased atresia level and decreased number of follicles in rat ovary [94].

There is a study in mice showing that various dose of DES was exposed neonatally (PND 1-5) causing hemorrhagic cysts and lack of CL [87]. There was also reduction of some litters and number of oocytes ovulated, which were dose-dependent [95].

1.5. Objectives

This study has two aims. The first aim is to determine the effects of DES, MXC, and BPA exposures during the critical window of ovarian development on female reproductive parameters. To this end, we measured pubertal age, estrous cyclicity, litter size, and pregnancy rate. The second aim is to understand the effects of DES, MXC, and BPA exposures in the ovary. To this end, we determined changes in follicular composition and expression of various ovarian markers, such as ESR1, MIS, P450scc, and LHR. To achieve these aims, we conducted two experiments as described in next chapter, Chapter 2.

CHAPTER 2: EFFECTS OF DEVELOPMENTAL EXPOSURE TO ESTROGENIC ENDOCRINE-DISRUPTING CHEMICALS METHOXYCHLOR AND BISPHENOL A DURING FETAL AND NEONATAL PERIODS ON OVARIAN FOLLICULOGENESIS AND REPRODUCTIVE PARAMETERS IN RATS

2.1. Introduction

Epidemiological studies show that exposure to environmental EDCs are associated with the problems of the development and function of the female reproductive system [96, 97]. For example, there is a 2- to 3-fold increase in prolonged time-to-pregnancy (TTP) and spontaneous abortion among female greenhouse workers [97]. Maternal exposures to environmental chemicals during pregnancy are associated with preterm labor, decreased birth weight, and intrauterine growth retardation [98]. It has also been reported that there is an increase in infertility among the women who worked in the agricultural industry in their past, involving exposure to organochlorine pesticides [76]. In addition, during developmental exposure EDCs can have long lasting effects on female reproduction [99, 100]. The mechanism for the delayed effect is not exactly known, but it may occur in any organ in female reproductive systems, including in the ovary.

Studies in laboratory animals show that developmental exposures to EDCs can have long-lasting effects in the ovary. Previous work from our lab has shown that the late fetal and neonatal exposure (E19-PND7) to 100 mg/kg/day MXC increased the number peri-antral follicles and reduced number of CLs, which were associated with changes in several reproductive parameters including accelerated puberty, irregular estrous cyclicity, reduced litter size, and early reproductive aging [2]. Neonatal exposure to BPA (50 µg or 50 mg/kg/day) between PND0 and 3, induces hemorrhagic, enlarged and degenerated follicles in rat ovary [101]. *In utero* exposure to relatively low dose of BPA (1 µg/kg/day) between E9 and 16 induced cystic follicles in mice [102]. Single injection of 10 µg/kg DES during E15 lead to various ovarian histopathological conditions, including lack of CL at 7 months of age [103].

Depending on the exposure window, EDCs during development can affect various processes in the ovary. Fetal ovary differentiation from the bipotential gonad starts around mid-gestation, embryonic day (E) 11 in rats [104]. Around this time, germ cells migrate to the gonad and then rapidly proliferate forming the oocyte nest. The oocytes enter meiosis and arrest at the diplotene stage of prophase I [23]. Starting around E16.5, ~2/3 of oocytes in a nest are eliminated by apoptosis, which is known as oocyte nest breakdown [28]. The remaining oocytes form primordial follicles, which is completed between PND4 and 7 [105]. A cohort of primordial follicles is activated and recruited into growing follicles continuously throughout the reproductive life of a female [49]. The activated follicles undergo a number of series and rapid cellular differentiation, proliferation, and growth. These events occur through various stages of folliculogenesis, incorporating two main functions of the ovary: steroidogenesis and gametogenesis, including the ovulation [29].

Some of the critical markers of the ovarian functions include ESR1, MIS, P450scc, LHR, and PCNA. ESR1 regulates steroid production and folliculogenesis: evidence shows that it regulates androgen production to prevent hyperandrogenic environment in the ovary and to reduce follicular atresia [106]. MIS also regulates folliculogenesis and is assumed to slow down the follicular recruitment and maturation [107] It was shown that *in vitro* MIS-treated ovaries contained 40% less growing follicles than control ovaries [67]. P450scc is a rate-limiting enzyme in steroid biosynthesis pathway [108], and is known to be affected by EDCs [2]. LHR is critical for growth and differentiation at the final stages of folliculogenesis and for ovulation [43]. Rapid and significant cell proliferation especially in granulosa cells, takes place during folliculogenesis. Therefore, PCNA can be used as a marker of cell proliferation [73].

Sex steroids, primarily estradiol, regulates all stages of folliculogenesis. From the developmental perspective, the role of estradiol is the most pronounced in the oocyte nest breakdown and primordial follicle formation [26]. Estrogenic EDCs are also known to affect these critical early folliculogenesis events [29]. For instance, DES exposure during the neonatal period causes abnormal multiple oocyte follicles in adult mice [109]. Similar effects of genistein and BPA were shown. Neonatal genistein treatment altered early folliculogenesis in mice by inhibiting oocyte nest breakdown and increased oocyte survival [110]. BPA exposure also inhibits germ cell nest breakdown by reducing apoptosis in cultured neonatal mouse ovaries *in vitro* [111]. Similarly*, in utero* BPA exposure disrupts germ cell nest breakdown and reduces fertility with age [112].

In the current study, the effects of estrogenic EDCs MXC and BPA on the ovarian development and function and female reproductive parameters were investigated by conducting two experiments.

In Experiment 1, we examined the effects of exposure to two doses of BPA (Low BPA: 50 µg/kg/day and High BPA: 50 mg/kg/day) between E19 and PND7 on pubertal age, regularity of estrous cycle, and ovarian folliculogenesis, including critical ovarian markers.

In Experiment 2, we studied the effects of exposure to MXC (75 mg/kg/day), BPA (50 mg/kg/day), and DES (0.1µg/kg/day) between E11 and PND 7 on reproductive parameters, folliculogenesis and ovarian markers. To this end, the age of puberty, estrous cyclicity, litter size, and follicular dynamics in the ovary were studied. In addition, the protein expression of ESR-1, P450scc, MIS, LHR and PCNA were determined to have a better understanding of the potential effects of estrogenic EDCs on ovarian folliculogenesis and female reproduction.

2.2. Material and Methods

2.2.1. Chemicals

All chemicals (BPA, MXC, DES, dimethyl sulfoxide (DMSO), sesame oil, and corn oil) were purchased from Sigma-Aldrich.

2.2.2. Animals

Eight- to twelve-week-old Fischer CDF female rats (Charles River, Wilmington, MA) were maintained on a 14-hour light/10-hour dark cycle and fed, *ad libitum*. A reduced isoflavone diet was provided in order to minimize possible effects of phytoestrogens (Purina, 5V01, Brentwood, MO). The estrous cycles of the rats were followed daily, and individual females were mated with untreated males starting on the day of proestrus. A sperm-positive vaginal smear day was designated as embryonic day 0 (E0). All animal care and treatment protocols were carried out in accordance with institutional guidelines and were approved by Rutgers University Institutional Animal Care and Use Committee. Rats that were similarly prepared were used in two experiments: *Experiment 1* and *Experiment 2.*

2.2.3. Experiment 1:

Experimental Design and Treatments and Selection of Doses

In *Experiment 1*, as shown in Figure 1A, timed-pregnant inbred Fisher CDF females received one of two different treatment dosages of BPA: 50 µg/kg/day (Low BPA; n = 3 litters) and 50 mg/kg/day (High BPA; n = 4 litters) in 1 ml vehicle (an emulsion of 10% ethanol (EtOH) and 90% corn oil) per kg body weight. Control animals (n = 5 litters) received vehicle alone. Some litters contained more than one female pups, which were used in the study. Number of total female pups as well as litter used in each treatment group was given in Table 1. Daily treatments were administered (intraperitoneal; i.p.) to the pregnant dams between E18 and 21; and to the neonates (subcutaneous; s.c.) within 8 hours of birth (considered PND0) and continued until PND7. These two doses of BPA were selected, because the 50 mg/kg/day BPA dose is established by EPA as the lowest observed adverse effect level (LOAEL), and the 50 μg/kg/day BPA is the reference dose and considered to be safe for human exposure [101, 113]. E18-PND7 was chosen because early ovarian developmental events, such as oocyte nest breakdown, follicular assembly, and initial follicular activation take place during this window.

Determination of Onset of Puberty and Regularity of Estrous Cycles

Starting PND28, all female pups were followed daily for their vaginal opening as an indication of pubertal age to determine if developmental exposures to BPA alters the age of puberty, because estrogenic EDCs are known to alter pubertal age [2, 114].

After vaginal opening, to determine the % normal cycles, the stages of estrous cycles were followed by vaginal cytology as described previously [2]. The vaginal cytology samples were classified into proestrus - characterized by the presence of mostly round, nucleated vaginal epithelial cells; estrus - characterized by the presence of large numbers of irregularly shaped keratinized epithelial cells that lost their nucleus; and diestrus - characterized by the presence leucocytes, and reduced number of epithelial cells. In a normal estrous cycle (Normal), females show an estrus every 4-5 days.

Abnormal cycles include persistent estrus (PE) and persistent diestrus (PD), which are characterized by 5 or more consecutive days of estrous- or diestrous-pattern of vaginal cytology, respectively; and prolonged cycle (Prolonged) - characterized by interestrus intervals greater than 5 days, and a persistent estrus or persistent diestrus was not observed. Regularity of estrous cycles was expressed as % normal cycle (i.e., # normal cycles / # of total cycles x 100), prolonged cycle (i.e., # prolonged cycles / # of total cycles x 100), persistent estrus (i.e., # persistent estrous cycles / # of normal cycles x 100), and persistent diestrus (i.e., # persistent diestrous cycles / # of normal cycles x 100) for each treatment group.

Tissue Collection and Histological Processing

On a proestrous day between PND 50 and 60 after their third estrous cycle, the rats were weighed and killed and one of the ovaries were collected for histology. The ovaries were cleaned out of the surrounding tissues and weighed. Bouine's-fixed, paraffin-embedded ovaries were serially sectioned (5 µm thick) and were mounted onto Superfrost microscope slides. Representative histological sections were used either for follicle counting or for immunohistochemistry (IHC).

Follicle Counting and Classification

For each animal, three histological sections, ~100µm apart from each other and located at the largest cross-sectional area of the ovary were stained with hematoxylin and eosin (H & E) and examined by light microscopy for follicle counting and classification for each treatment group as previously described with some modifications [2]. Briefly, follicles were classified into as primordial - an oocyte surrounded by squamous pre-granulosa cells, only one of which is an enlarged (cuboidal); primary - a relatively larger oocyte surrounded by at least two or more cuboidal granulosa cells that form no more than single layer (at the early primary follicle stage, some of the granulosa cells may still be squamous); secondary - an oocyte surrounded by more than one but no more than two layers of cuboidal granulosa cells; pre-antral - an oocyte surrounded by more than two layers of granulosa cells with no apparent antrum; early antral - an oocyte surrounded by multiple layers of granulosa cells with an antrum that occupies no more than 1/3 of the follicle; mid-to-late antral follicle: a follicle with oocyte and an antrum that occupies more than 1/3 of the follicular space. Atretic follicles, which are defined as either a follicle with apparently healthy oocyte but more than 10% of pyknotic/apoptotic granulosa cells and shrinking granulosa layer or an antral follicle with disintegrating or fragmented oocyte, separated from cumulus cell layer and severe shrinkage of granulosa cell layer, were also counted. These two categories of atretic follicles were combined. The number of follicles in each stage of folliculogenesis was expressed as the percentage of total follicle number. Corpora lutea were also counted and expressed as percentage of total follicles.

Immunocytochemistry

To better understand the ovarian phenotype, the expression level of certain ovarian proteins, including ESR1, P450scc, MIS, LHR (only in Experiment 2), and PCNA immunohistochemistry was performed. Sections were de-paraffinized in Citrisolv (D-limonene, Fisher) and rehydrated in PBS for 10 minutes. Antigen retrieval was performed by microwaving slides in 0.01 M sodium citrate buffer (pH 6.0) for 6 and ½ minutes at high and for 10 minutes with medium power. The sections were blocked with 1.5% non-immune serum (goat serum for ESR1, P450scc, LHR, and PCNA; donkey serum for MIS) for 30 minutes and incubated with the primary antibodies at room temperature overnight in a humidified chamber (see Table 2, for detailed information on primary antibodies, including dilution and source). In negative control sections, primary antibody was replaced with PBS. Biotinylated anti-rabbit or anti-donkey secondary antibodies were added at 1:100 dilutions and incubated for 60 minutes at room temperature. Detection was done with Strep-Alexa-488 (green). All sections were counterstained with ethidium homodimer-2 (EthD-2, Invitrogen) and imaged using a Nikon Eclipse E800 microscope with epifluorescent attachments and suitable filters for Strep-Alexa-488 (green) and EthD-2 detection (red). Images were acquired with a Nikon DXM1200F camera with ACT1 software (Version 2) at equal exposure levels.

Quantification of Immunohistochemical Staining Intensity

Mean staining intensity measurements were made using ImageJ (NIH) on Alexa-488-stained images. ESR1 staining intensity was measured in theca layers of primary, secondary, pre-antral and early antral follicles and CL. LHR and P450scc staining intensity were measured for the theca layer of pre-antral and early antral follicles, and CL. MIS staining intensity was measured in granulosa layer of primary, secondary, pre-antral and early antral follicles. All healthy follicles that are positively identifiable in Alexa-488-stained sections were included in the measurements. The polygonal selection tool was used to select the respective structures. When the granulosa layer was selected, oocyte and antral space were excluded. As for the theca layer, the entire layer for each follicle was selected. The rectangular selection tool was used for measurements in CL. All images were in RGB mode and results were calculated using brightness values.

For proliferation index, PCNA immunostaining was used for both granulosa and theca layers of primary, secondary, pre-antral and early antral follicles. Briefly, the percent ratio of PCNA-positive cells to total (i.e., EthD-2-positive) cell numbers were determined for a given follicle stages. Not all sections had follicles in mid-to-large antral stage. Therefore, this stage was not included in any of the IHC analyses.

2.2.4. Experiment 2:

Experiment 2 was performed similar to *Experiment 1,* with the following main differences: (1) two additional EDCs (MXC and DES) were used and (2) the daily treatment period was longer, between E11 and PND7. In addition to endpoints examined in *Experiments 1,* serum hormone levels were measured and additional reproductive parameters were assessed.

Experimental Design and Treatments and Selection of Doses

In *Experiment #2*, as shown in Figure 1B, the timed-pregnant females received one of the following three EDCs or two control vehicles. The EDCs and doses were as follows: BPA (50 mg/ kg/day; n = 8 litters) and DES (0.1 µg/kg/day; n = 6 litters) in 1 ml vehicle/kg BW of EtOH: corn oil (1:9) and MXC (75 mg/kg/day; n = 8 litters) in a vehicle of DMSO: sesame oil (1:2). There were two subgroups of control animals receiving either the EtOH: corn oil vehicle (n = 5 litters) or the DMSO: sesame oil vehicle (n = 5 litters). Similar to *Experiment 1,* some litters contained more than one female pups, which were used in the study (Table 1). For data analysis and presentation, data from two control groups were combined (see *Statistical Analysis*). Daily treatments were administered (i.p.) to the pregnant dams between E11 and E21 and to the neonates (s.c.) within 8 h of birth (considered PND0) and continued until PND7. The dose of DES used (0.1 µg) is lower than what was used in women for therapeutic purposes [98], but is known to be effective in rodents as an estrogenic EDC [115]. The dose of MXC (75 mg) was selected, as similar doses have been shown to have reproductive effects when used in other developmental exposure windows [2, 66]. As compared to *Experiment 1,* the treatment window was expanded to include the gonadal differentiation period, as it is likely to be vulnerable to EDC exposures.

In *Experiment 2, Determination of Onset of Puberty and Regularity of Estrous Cycles* and *Tissue Collection and Histological Processing* were performed similar to *Experiment 1,* except that tissue (and serum) collection was performed on a proestrous day between PND70 and 90. One or more littermates killed for the collection of ovaries and serum. The age, on which the animal was killed, was determined by estrous cyclicity. Due to perturbations in normal estrous cycles by EDCs (e.g., MXC) treatments, the collection period was spread to a wider window than the *Experiment 1*. In addition, *Follicle Counting and Classification,* and *Immunocytochemistry and Quantification of Immunohistochemical Staining Intensity* were also performed similar to the *Experiment 1.*

Radioimmunoassay.

Trunk blood was collected at the time of tissue collection and kept at 4°C for 2 h. The blood was centrifuged at approximately 400xg for 15 minutes for serum separation at 4°C and the serum was stored at −80°C until used for measuring progesterone and estradiol concentrations. Progesterone was measured in 100 µl and estradiol was measured in 200 µl serum samples using commercially available radioimmunoassay (RIA) kits, according to the manufacturer’s instructions. Serum concentrations of progesterone and estradiol were measured using Coat-A-Count RIA kits (Siemens Medical Solutions). Radioactivity was measured in counts per minute (cpm) and the standard curve was used to generate serum concentration values nanograms per milliliters (ng/ml) for progesterone (sensitivity for the progesterone RIA assay was 0.02 ng/ml) and picograms per milliliters (pg/ml) for estradiol (sensitivity for the estradiol RIA assay was 8 pg/ml).

Determination of Litter Size and Pregnancy Rate

Additional littermates from the same litters, from which other littermate(s) were killed for tissue and serum collection, were used to assess these reproductive parameters. Following vaginal opening, the estrous cycles were followed similarly as described in *Experiment 1*. On a proestrous day, following at least 3 regular cycles, each female was mated with a non-treated male that is proven to be fertile. A sperm-positive vaginal sample next day was considered to be the gestational day (GD) 0. Females showing 7 days of consecutive diestrus are considered pregnant. Pregnancy was confirmed on GD14 with an observation of distended abdomen and flanks of rats. In our colony, the birth usually takes place on the afternoon of the GD21. The next morning (PND0), the cages were checked for the delivery of the litter. If delivered, the pups were sexed and counted, including any dead ones, which gives the litter size for that particular dam. The females, who did not show sperm-positive vaginal cytology on GD0, and/or continued to display regular cycles, instead of 7 days of consecutive diestrus following the mating, or did not show any sign of pregnancy on GD14, were considered not pregnant. Non-pregnant females' estrous cycles were followed and mated in a similar manner as described above for two additional times, before considered infertile. *Pregnancy Rate* for each treatment group was determined, by dividing number of successful delivery of litter to the number of sperm-positive mating.

2.2.5. Statistical Analysis for Experiment 1 and 2

Data for pubertal age, body and organ weights, litter size, total follicle number, percentage of CL, intensity of IHC staining, proliferation index for PCNA, and RIA were expressed as mean ± SEM for each group and analyzed with GraphPad Prism Software (GraphPad Software, San Diego, CA), using a one-way ANOVA followed by Dunnett's multiple comparison test. Percentage of follicle number was analyzed using two-way ANOVA followed by Tukey's multiple comparison test. Early stage follicles (primordial, primary, and secondary) and late stage (pre-antral, early-antral, and mid to late-antral) follicles and atretic follicles were analyzed as a group with two-way ANOVA followed by Tukey's multiple comparison test. Atretic follicles included in the late stage follicles because most atretic follicles content in most studies originate from late stage follicles. The percent normal cycles and pregnancy rate was analyzed using Chi-Square analysis. In studies in *Experiment 1,* except IHC studies, 8-12 females from 3-5 separate litters were used for each group. The *Experiment 2* started with 13-26 females from 5-8 litters for each group, which are used for assessment of pubertal age and regularity of estrous cycles. For subsequent experiments, the littermates within each litter were randomly assigned either for tissue and serum collection or for mating with an untreated male to assess additional reproductive parameters (see Table 1 for number of pups for each treatment for each study). Combined data from littermates within each litter were used as a replicate. In addition, since the two vehicle control groups in *Experiment 2* did not show statistically significant differences between each other, they were combined and used as a single "control" group. IHC, including quantifications were repeated 2-3 times, using ovarian sections from 3-11 pups coming from 3-5 litters for *Experiment 1* and using ovarian sections from 4-10 pups belonging to 3-6 litters for *Experiment 2*. A statistically significant difference was confirmed at $p < 0.05$.

2.3. Results

2.3.1. Experiment 1

In *Experiment 1*, we examined the effects of two doses of BPA (50 µg or 50 mg per kg body weight) exposures between E18 and PND7 on pubertal age and regularity of estrous cycles and ovarian folliculogenesis and protein expression.

Pubertal Age and Regularity of Estrous Cycle

Neither of the BPA doses significantly affected the pubertal age (mean ± SEM) as designated the day of vaginal opening (Figure 2A). Control animals reached puberty at 36.8 ± 0.4, while the low- and high-BPA exposed animal reached puberty at 36.9 ± 0.4 and 36.00 ± 0.7, respectively, which were not significantly different ($p < 0.05$) from the control animals (Figure 2A). In addition, the regularity of cycles, measured as the percentage of normal cycles over all cycles observed, were not adversely affected by BPA treatments (Figure 2B). The % cycles for the control animals were 84.4 ± 5.4 and 50 µg/kg/day or 50 mg/kg/day BPA-treated females were 89.6 ± 5.1 or 90.3 ± 5.0, respectively.

The Effects of BPA on Body Weights and Ovarian Weights

At the time of the tissue collection, the body weight and ovarian weights were measured. The BPA treatments did not affect body weight or ovarian weight ($p > 0.05$) (Figure 3A). The control animal had body weight (g, mean ± SEM) 131.9 ± 2.7 and low and high BPA had 133.9 ± 4.04 and 131.4 ± 4.3, respectively. The ovarian weights (mg;

mean ± SEM) for control, low BPA and high BPA treated females were 61.3 ± 3.6; 62.3 ± 2.4; and 57.1 ± 3.8, respectively (not shown). The normalized ovarian weights (organ weight / body weight) were not significantly affected by BPA treatments either (Figure 3B).

Effects of BPA on Ovarian Follicular Numbers and Composition

In order to determine the effects of developmental exposure to BPA on ovarian follicular number and composition, the follicles in control and 50 µg or 50 mg BPA-treated ovaries were counted and classified in the ovaries collected between PND50 and 60, as described in Materials and Methods. All follicle counts were reported as a percentage of the total follicle numbers (Figure 4 AB). The percent ratio of CL to the total follicle numbers per section (Figure 4C). and percent of atretic follicles per section were also reported (Figure 4B).

There was a reduction in the percentage of primary follicles in the high BPA-treated females (41.16 ± 1.4) as compared to control (44.94 ± 0.9; $p < 0.5$; Figure 4A). In addition, atretic follicle numbers showed an increase ($p < 0.01$) in 50 µg/kg/day BPA (55.7 ± 4.2) and 50 mg/kg/day BPA (53.0 ± 2.8) as compare to control (41.5 ± 2.5) (Figure 4B). However, there were no effects on either total follicle number per section or percent ratio of CL to total follicle numbers ($p > 0.05$; Figure 4C-D).

Immunohistochemistry (IHC) and Quantification of IHC Staining Intensities

To better understand the effects BPA in the ovary, we examined the effects on the expression of levels of ovarian proteins, including, ESR1, P450scc, and MIS, and staining intensities for these proteins were measured. In addition, using IHC for cell proliferation marker PCNA, the proliferation indices were determined in the ovaries.

Estrogen Receptor Alpha (ESR1)

The ESR1immunoreactivity was observed in thecal cells and CL (Figure 5). The ESR1 staining intensities were measured in the theca cells of primary, secondary, pre-antral, and early antral follicles and CL. Only significant effect of BPA was observed in the primary follicles. Low dose BPA reduced the staining intensity from 45.19 ± 2.6 to 27.49 ± 2.8 ($p < 0.05$; Figure 5). The BPA treatments did not alter the ESR1 staining in any other follicular stages or in CL.

Cytochrome P450 Side Chain Cleavage Enzyme (P450scc)

Immunolocalization of P450scc was observed in theca/interstitial cells, surrounding pre-antral and early antral follicles and CL in the ovaries. The P450scc is also observed in large (mid-to-late) antral follicles. However, there were not adequate number of mid-to-late antral follicles to conduct statistical analysis in all groups. Therefore, this follicular stage was excluded from the study.

The BPA treatments appeared not to affect the immunolocalization of the P450scc in any stages of follicles or CL as compared to control ovaries (Figure 6). In addition, even though there were some trends towards reduction of intensity in high BPA group, neither of BPA treatments significantly altered staining intensities for P450scc in any follicular stages or in CL (Figure 6; $p > 0.05$).

Mullerian Inhibiting Substances (MIS)

Mullerian inhibiting substance is primarily expressed in the granulosa cells of growing follicles, including primary through early antral follicles, which was confirmed in our studies (Figure 7). However, the staining intensities in any stages of folliculogenesis were not altered by BPA treatments ($p > 0.05$).

Proliferating Cell Nuclear Antigen (PCNA) IHC and Proliferation Index

PCNA immunohistochemistry was used to assess the proliferation and proliferation indices were calculated in granulosa and theca cells of primary, secondary, pre-antral, and early antral follicles (Figure 8A, presentative figures were shown in early antral follicles). The proliferation indices varied between 38% and 78% among three groups (0, 50 µg, and 50 mg BPA) and various cell and follicular groups (Figure 8B). However, no effects of BPA treatments on proliferation indices were observed ($p > 0.05$), suggesting that developmental BPA exposures did not alter cell proliferation the granulosa and theca cells associated with these follicular stages.

2.3.2. Experiment 2

In *Experiment 2*, we examined the effects of daily exposures to DES (0.1 µg/kg/day), MXC (75 mg/kg/day) and BPA (50 mg/kg/day) between E11 and PND7 on various reproductive parameters, including pubertal age, regularity of estrous cycles, litter size, and pregnancy rate; ovarian folliculogenesis and protein expression, and serum progesterone and estradiol levels.

Pubertal Age and Regularity of Estrous Cycle

In *Experiment 2*, the pubertal age (mean ± SEM; days) of females was significantly advanced when treated with 0.1µg/kg/day DES and 75 mg/kg/day MXC (DES, 35.0 ± 0.4, $p < 0.05$ and MXC, 33.6 ± 0.4, $p < 0.001$) as compare to control (36.8 ± 0.2, Figure 9A). Furthermore, exposure to DES and MXC resulted significant alterations in estrous cycle patterns (Figure 9B). While control females showed 95% Normal and 5% Prolonged cycles, DES-treated females showed 83.8% Normal ($p < 0.05$), 11.2% PE ($p < 0.05$), 2.3% PD, and 2.7% Prolonged; and MXC-treated females showed 74.1% Normal ($p < 0.001$), 14.5% PE ($p < 0.01$), 2.8% PD, and 8.6% Prolonged cycles. In contrast, cycle pattern of BPA-exposed females was not different from control ($p > 0.05$) and showed 89.6% Normal and 10.4% Prolonged cycles.

The Effects of EDCs on Body Weight and Ovarian Weights

The body weights and ovarian weight at the time of tissue collection was determined (Figure 10). The body weights (gr, mean ± SEM) were 163.7 ± 1.7, 175.9 ± 4.8, 182.3 ± 7.2 and 163.5 ± 1.4 for control, DES-, MXC- and BPA-exposed females, respectively (Figure 10A). The body weight of MXC-exposed group was significantly higher ($p < 0.05$). The ovarian weights (mg, mean ± SEM)) were 76.5 ± 4.0, 67.3 ± 6.4, 42.5 ± 3.2, and 61.0 ± 3.1 for control, DES-, MXC- and BPA-exposed females, respectively (not shown). The ovarian weights of MXC-exposed ovaries were significantly ($p < 0.005$) lower as compared to control. When the ovarian weights were normalized by the body weights, the values for DES- and BPA-exposed groups ($p < 0.05$) as well as MXC-exposed group ($p < 0.001$) were significantly smaller than the control (Figure 10B). One caveat with these data is the following: As described in Materials and Methods, to reduce or eliminate the effects of the stage of estrous cycle on the ovary or serum hormone levels, the tissues were aimed to be collected on proestrus day. However, as reported above, in DES- and MXC-exposed females, the regularity of estrous cycles were disturbed. Thus, the tissue collection period was spread into a wider window (PND70-90) as compared to *Experiment 1*, which introduced additional variations to the body weights, and possibly, to the organ weights. Therefore, the data should be interpreted with caution.

The Effects on Follicular Composition and Dynamics in the Ovary

In *Experiment 2*, the follicular composition of MXC and BPA-treated females demonstrated alterations in early follicular development. Specifically, percentage of primordial follicles in 75 mg/kg/day MXC-treated ovaries (32.3 ± 4.0; $p < 0.05$) and 50 mg/kg/day BPA-treated ovaries (30.5 ± 1.4; $p < 0.01$) were significantly lower than percentage of primordial follicles in control ovaries (39.4 ± 1.7; Figure 11A). In addition, the percentage of primary follicles were higher in MXC exposed ovaries (38.9 ± 1.2) as compare to control ovaries (29.8 ± 1.2; $p < 0.01$. Although the percentages of follicles in any other stages were not altered due to EDC treatments, exposure to 0.1 µg/kg/day DES and 50 mg/kg day BPA exposure increased atretic follicle numbers as compare to control (26.6 ±5.2 for DES or 22.8 ± 2.1 for BPA *versus* 15.2 ± 2.0 for control; Figure 11B, $p < 0.01$). Furthermore, exposure to MXC resulted in a significant ($p < 0.01$) decrease in the percentage of CL (6.1 ± 1.7; Figure 11C) as a compared to control (32.8 ± 3.1), which is consistent with the noticeable trend towards reduced litter size in MXC-treated females. However, the total number of follicles did not change significantly due to exposures to DES, MXC, or BPA ($p > 0.05$; Figure 11D).

The Effects on Litter Size and Pregnancy Rate

The litter size (mean ± SEM, number of pups per litter) for Control, DES-, MXC-, and BPA-exposed females were 9.6 ± 0.6; 9.9 ± 0.8; 7.0 ± 1.5; and 9.00 ± 0.7, respectively (Figure 12A). MXC-exposed animals showed a trend ($p < 0.07$; *t*-test) for reduction in litter size, but the difference was not significant when tested with one-way ANOVA ($p > 0.05$). There was a reduction in pregnancy rate of MXC-treated females (90.7%), however, the reduction was not significant as compared to control (100%, Figure 12B). DES and BPA exposures did not reduce the litter size or pregnancy rate.

Immunohistochemistry (IHC) and Quantification of IHC Staining Intensities

To determine the effects of DES, MXC and BPA on localization and levels of ovarian proteins that are critical for ovarian functions, we performed IHC for ESR1, P450scc, MIS, and LHR and quantified the staining intensities with ImageJ. In addition to examine the effects on granulosa and theca cell proliferation, we performed IHC for PCNA and calculated proliferation indices for each treatment group.

Estrogen Receptor Alpha (ESR1)

We localized ESR1 protein in the theca cell for primary, secondary, pre-antral and early antral follicles and CL with IHC (Figure 13), for which the staining intensities were evaluated (Figure 13). The BPA treatment stimulated ESR1 protein in theca cells of secondary follicles as compared to control ($p < 0.05$). The staining intensity for BPA-exposed group was 51.08 ± 4.3 while control group was 34.9 ± 2.5. The staining intensity ESR1 in secondary follicles DES (35.1 ± 6.6) was similar to control. While MXC-treated group (49.7 ± 1.9) was relatively higher, the difference was not statistically significant. The ESR1 expression levels in the theca cells of early antral follicles were also altered by EDC treatment. As compare to the control (26.30 ± 1.9), while DES-exposure inhibited ESR1 protein expression (14.84 ± 1.4), BPA treatment stimulated it (35.43 ± 2.5). The ESR1 staining intensity for MXC-treated ovaries were very similar (26.9 ± 3.1) to the control ovaries. Furthermore, BPA exposure significantly increased CL's ESR1 expression (37.36 ± 4.04) as compare to the control (26.74 ± 2.45). In contrast, DES (22.1 ± 3.6) and MXC (22.5 ± 1.7) did not significantly alter ESR1 levels in CL.

Cytochrome P450 Side Chain Cleavage (P450scc)

In *Experiment 2*, the P450scc levels were significantly affected ($p < 0.05$) in theca/interstitial cells of pre-antral and early antral follicles as well as CL by EDC exposures (Figure 14). MXC reduced P450scc expression in pre-antral and early follicles (17.0 ± 3.8 and 17.2 ± 2.6) as compared to control (33.6 ± 4.5 and 28.6 ± 1,4). P450scc levels in pre-antral and early antral follicles of DES- (27.9 ± 2.8 and 27.4 ± 2.9) and BPA-exposed ovaries (34.3 ± 3.8 and 30.6 ± 3.9) were not significantly altered. In contrast, in CL, BPA significantly stimulated P450scc expression ($p < 0.05$). P450scc levels in BPA-exposed ovaries were 45.2 ± 2.5 while the level was 34.6 ± 2.0 in control ovaries. The P450scc level in CL of DES- and MXC-exposed animals were 24.6 ± 3.2 and 35.7 ± 6.5, respectively, which were not significantly different than those of control ovaries (Figure 14).

Mullerian Inhibiting Substances (MIS)

As expected, Mullerian-inhibiting substance was observed in the granulosa cells of the primary, secondary, pre-antral and early antral follicles (Figure 15). It appeared that EDC exposure altered the MIS levels. Quantification of the MIS levels has shown that, MXC significantly stimulated MIS protein levels in secondary follicles (64.2 ± 6.3) and pre-antral follicles (41.2 ± 5.2) as compare to control values (40.6 ± 3.6 for secondary and 27.0 ± 2.3 for pre-antral follicles). In addition, BPA-treated ovaries had significantly higher MIS in secondary follicles (63.6 ± 12.8; $p < 0.05$). MIS levels were not significantly altered in primary and early-antral follicles.

Luteinizing Hormone Receptor (LHR)

Luteinizing hormone receptor is expressed the theca/interstitial cells of follicles, and CL was not significantly affected by any EDCs treatment (Figure 16; $p > 0.05$).

Proliferating Cell Nuclear Antigen (PCNA) IHC and Proliferation Index

To assess the role of cell proliferation on the effect of EDCs on follicular composition of atresia, the effects of developmental exposure to EDCs on the granulosa and theca cell proliferation were assessed using PCNA immunohistochemistry (Figure 17A, representative pictures from early-antral follicles). In addition, the proliferation indices were calculated (Figures 17B). The percent of PCNA-positivity, as an indication of actively proliferating cell numbers, ranged from ~50% to ~85%. However, there were no statistically significant effects of any of EDCs exposure on cell proliferation ($p > 0.05$), suggesting that the effects of developmental EDCs on follicular compositions or atresia are unlikely to be mediated with alterations in cell proliferation in granulosa or theca cells.

Effects on Serum Progesterone and Estradiol

To determine whether steroidogenic function of the ovary is affected by developmental EDC exposures, as suggested by reduced P450scc levels in EDC-exposed ovaries, we measured serum progesterone and estradiol levels by RIA. Although there was no effect of EDCs on either progesterone or estradiol in serum were not statistically significant ($p > 0.05$; one-way ANOVA), there was strong trend for reduction in levels of these steroids in MXC-exposed animals (with t test; $p = 0.012$ for progesterone and $p = 0.06$ for estradiol; Figure 18A-B). The progesterone (ng/ml) for control, DES-, MXC-, and BPA-exposed females were 10.2 ± 2.1, 8.4 ± 2.5, 4.1 ± 0.8, and 10.8 ± 4.3, respectively. The estradiol (pg/ml) for control, DES, MXC, and BPA- exposed females were 13.9 ± 1.3, 13.7 ± 2.5, 9.6 ± 1.6, and 18.5 ± 3.0, respectively.

2.4. Discussion

In this study, we examined the developmental effects of estrogenic EDCs in the ovary and on female reproduction in rats. We conducted two experiments: *Experiment 1* and *Experiment 2*. In *Experiment 1*, rats were exposed to two doses of BPA (50 µg/kg/day, Low BPA or 50 mg/kg/day, High BPA) between E18 and PND7 and we examined the pubertal age, regularity of estrous cycles, body and ovarian weights, folliculogenesis, protein levels of markers of ovarian (ESR1, P450scc, and MIS) and of a cell proliferation (PCNA) using IHC. Neither Low BPA nor High BPA significantly affected female reproductive parameters and body and ovarian weights. BPA exposures had limited effects on follicular composition: a significant reduction in the percentage of primary follicles by Low BPA, without affecting the other stages of folliculogenesis, and an increase in atretic follicles by both doses of BPA. In addition, we observed a significant reduction in ESR1 protein expression in the theca cells of primary follicles of Low BPA-treated ovaries. However, considering lack of any effects on examined reproductive parameters and body and organ weights, the significance of the limited effects on folliculogenesis and ESR1 expression is unclear. Thus, we will focus the rest of the discussion onto the *Experiment 2*, and draw comparisons between the two experiments as needed.

In *Experiment 2,* following exposures to three different EDCs, 0.1 µg/kg/day DES, 75 mg/kg/day MXC, and 50 mg/kg/day BPA between E11 and PND7, we examined several reproductive parameters: pubertal age, regularity of estrous cycles, litter size, and pregnancy rate. In addition, on a proestrous day between PND70 and 90, we collected the ovary and serum and determined follicular composition and measure protein levels of several ovarian factors, including ESR1, P450scc, MIS, and LHR and a cell proliferation marker PCNA in the ovary using IHC and serum concentration of progesterone and estradiol using RIA. During the tissue collection, we also measured body and ovarian weights. We observed effects on reproductive parameters and body and organ weights as well as effects on folliculogenesis and protein levels in the ovary. The effects were EDC-dependent.

DES and MXC treatment advanced of pubertal age and resulted in irregular estrous cyclicity. A previous report from our lab has shown that 100 mg/kg/day MXC between E19 and PND7 significantly advances pubertal age and increases irregular estrous cyclicity [2]. Others have reported that female rats exposed to similar doses of MXC (50-150 mg/kg/day) during perinatal and juvenile periods [116] or peripubertal periods [117] showed early vaginal opening. Studies by Chapin and co-workers also reported that 80-85% of the animals showed irregular cyclicity [116]. The higher incidence of irregular cycles in the study by Chapin and co-workers can be attributed to longer treatment period. In addition, in our previous study, rats that were exposed to 1 mg/kg/day estradiol benzoate, a long-lasting estradiol also showed early puberty [2]. In that study since the dose of estradiol was relatively high, none of the exposed animal showed regular estrous cyclicity. In contrast, in the current study, although DES treatment caused advancement of pubertal age, about 84 % of the cycles of these females were normal, suggesting that the dose of DES was relatively low. In addition, both DES and BPA caused an increased in persistent estrus. In contrast, BPA-treated animals did not show any advancement in their pubertal age or irregularity in their estrous cycles. The lack of abnormalities in gross reproductive parameters is in agreement with findings of the *Experiment 1*. However, the studies examining effects of BPA on pubertal age (and regularity of cycles) from other labs show mixed results. Some reported that developmental exposure to BPA (400 mg/kg/day) had no effect on pubertal age [117] while others suggest that developmental exposure to BPA (50 µg/kg/day) during neonatal period causes significant advances in vaginal opening [101]. The reasons for apparent discrepancy for the effect of BPA on pubertal age can be subtle differences between experimental conditions such as strain of mice or diet used, which are factors known to affect the susceptibility to estrogens or estrogenic EDCs [118].

Some of the abnormalities in reproductive parameters, such as accelerated puberty or increased irregular estrous cycles, suggest effects on hypothalamus and/or pituitary, as the developmental exposure to estrogens or estrogenic EDCs can affect the hypothalamus and/or pituitary [119, 120]. A recent collaborative study from our lab showed that MXC treatment altered gene expression involving energy homeostasis and reproduction in arcuate nucleus [121]. Thus, the potential effects on the hypothalamus and/or pituitary should be kept in mind in the interpretation of the results.

Serum progesterone and estradiol levels were assessed at the time of tissue collection. MXC inhibited progesterone and showed a trend towards reduction of estradiol. DES and BPA did not have an effect on serum hormone levels. The effects on steroid levels shows some parallelism between P450scc results.

The follicular composition was affected by all three EDCs. MXC reduced primordial follicles, and increased primary follicles, while reducing number CL in the ovary. This suggests that although MXC is likely to stimulate the activation of primordial follicles, these follicles fail to mature and ovulate. A strong trend towards the reduction of total follicle numbers supports this notion. However, lack of effect of MXC on atretic follicles requires further investigation. In our previous study, we have shown that MXC alter follicular composition, increasing the number of periantral follicles, without affecting the early stage follicles [2]. The difference in follicular dynamics observed in these two studies may be related to differences in exposure windows and doses of MXC. However, in our previous study [2], MXC-treatment similarly reduced the number of CLs. The reduction in CL suggests a reduction in ovulation, which is supported by observation of a strong trend towards the reduction of litter size and a trend in reduction in pregnancy rate in MXC-treated group in the current study.

Similar to MXC, BPA also reduced the number of primordial follicles, and increased atretic follicles. In contrast, BPA did not significantly affect number of follicles in any other follicular stages or CL. This suggests that BPA stimulated activation, but activated follicles underwent atresia rather than ovulate. Previous studies are consistent with these results. For example, 20 mg/kg/day BPA between PND1 and 7 reduced the number of primordial follicles [122], which is consistent with our observations in the current experiment. Another study showed that exposure to various doses of BPA (10-160 mg/kg day) for 7 days during prepubertal period caused dose-dependent reduction for all stages follicles and CL, and increase in atretic follicles [123]. In contrast, DES also stimulated the number of atretic follicles, without affecting the percentage of follicles in any stages of folliculogenesis or of CL. Previous studies have reported that DES treatment during perinatal period cause significant effects on ovarian folliculogenesis. For example, Rodriguez and co-workers [122], reported that 0.2 µg/kg/day DES caused a reduction in primordial follicles and increase in growing follicles. Lack of the effect of

DES on folliculogenesis in the current study is likely to be related with the relatively lower doses of DES.

We have examined several ovarian markers, including ESR1, P450scc, MIS, and LHR to understand the molecular basis of alterations of folliculogenesis, steroidogenesis and ovulation by EDCs. MXC had no effects in ESR1 at any stage of follicular development, which is in agreement with our previous study [2]. However, BPA has stimulated ESR1 in theca cells of secondary and early antral follicles as well as in CL. Previous report by Rodriguez and co-workers has shown that neonatal BPA exposure increases ESR1 protein levels in rat ovary [122]. In contrast the effect of DES on ESR1 expression was inhibitory in early antral follicles. The opposite effect of BPA and DES might be their differential actions of these two EDCs on estrogen receptors. While DES is pure agonist for ESR1 and ESR2, BPA's actions on estrogen receptors are subtype-specific, acting as mixed agonist-antagonist [101, 124].

Next we examined the effect of EDCs on P450scc, one of the key enzymes in steroid production in the ovary. MXC reduced the protein levels of P450scc in pre-antral and early antral follicles. This result is consistent with previous reports from our lab with *in vivo* studies using MXC [2] as well as *in vitro* studies with rat granulosa cells using the major metabolite of MXC, HPTE [10, 64]. In addition, it is also consistent with our observation in the current study for the reduction of serum progesterone. In contrast, BPA stimulated the P450scc levels in luteal cells. Previous reports on the effect of BPA using *in vitro* rat luteal cell and mice antral follicle culture are controversial. While BPA inhibited P450scc mRNA in mice antral follicles [61], it has no effects on P450scc activity in rat luteal cells [125]. Considering that BPA did not have any effect on progesterone production in the current study, this issue to be further investigated. Finally, contrary to our expectations, DES did not have any effects on the P450scc, because previous studies neonatal exposure to DES inhibits P450 expression in the ovary [126].

MIS is a paracrine factor, belonging to TGFbeta superfamily. MIS is expressed in granulosa cells of growing follicles. It is proposed that MIS inhibits primordial follicle activation and FSH-stimulated growth of periantral follicles [127]. In the current study, MXC stimulated MIS expression in secondary and early antral follicles. This is consistent with findings from previous studies with MXC from our lab [2, 66] as well as studies with estrogenic chemicals from other laboratories [70]. However, it is an apparent contradiction in the current study that while MXC appears to stimulate primordial follicle activation, it also stimulates the production of MIS, which is inhibitory for the activation of primordial follicles. This discrepancy is needed to be further investigated. Similar to MXC, BPA also stimulated MIS protein expression in secondary follicles. Previous studies showed that prepubertal BPA exposure in rats stimulates MIS mRNA and protein levels in 35-day-old ovaries [123], supports the current finding. In contrast, DES-treatment did not affect the MIS levels in the ovary. It is known that exposures to estrogens or estrogenic compounds during development stimulate AMH production later in life in the ovary [126]. Therefore, this result was unexpected. Once again, the potential reason for this result can be relatively low dose of DES exposure in the current study.

Developmental MXC exposure did not alter LHR. This is different from results we observed in our previous work that 100 mg/kg/day MXC between E18-PND7 down-regulated LHR protein expression [2], which might be due the difference in duration of the exposure or in dosage of MXC. Similar to MXC, BPA or DES did not affect the protein LHR. To the best of our knowledge there are no reports from a comparable study for the effects of BPA or DES on LHR level in the rat ovary. Similarly, none of the EDCs tested had a significant effect on cell proliferation as measured by PCNA immunostaining. The previous reports suggest that while MXC inhibits cell proliferation and PCNA levels in cultured mice antral follicles, [128], it stimulates cell proliferation in surface epithelium in adult mice ovary [129]. However, there are several differences between the cited studies and our current study, such as *in vitro* versus *in vivo* treatment and adult versus developmental exposure, can account for the differential effects. Limited or no information is available on the developmental effects of BPA or DES on cell proliferation of PCNA expression in the ovary. However, it is reported that while daily DES exposure stimulates granulosa cell proliferation within 24-48 hours of exposure, continuous exposure beyond 48 hour inhibits cell proliferation [130]. Considering that BPA and DES increased atretic follicle number and lack of effects on PCNA, it is possible that increased follicular atresia may be a result of increased cell death, which needs to be further investigated.

Overall, exposure to estrogenic EDCs exposure during fetal neonatal period affect reproductive parameters and folliculogenesis in the ovary, which are EDC-dependent and exposure window-dependent. EDC-dependent effect may be related to EDCs agonistic and/or antagonistic actions against estrogen receptors. In addition, there appears to be some discrepancies in this study, such as increased primordial follicle activation in the MXC-treated ovaries, in the face of increased MIS stimulation, which requires further investigation.

Table 1: Number of litters and pups used for each experiment, treatment, and endpoint.

	Age of Puberty, Estrous Cycle		**Litter Size, Pregnancy Rate**		**Follicle Count, RIA**	
	# of litters[1]	**# of pups**	**# of litters[1]**	**# of pups**	**# of litters[1]**	**# of pups**
Experiment 1						
Control	5	12			5	12
Low BPA (50 µg/kg)	3	9			3	9
High BPA (50 mg/kg)	4	8			4	8
Experiment 2						
Control (DMSO)[2]	5	13	5	9	4	4
Control (EtOH)[2]	5	15	5	10	3	5
DES (0.1 µg/kg)[3]	6	20	6	11	6	10
MXC (75 mg/kg)[3]	8	16	8	10	4	7
BPA (50 mg/kg)	8	26	8	17	6	9

[1] Data from littermates combined and used as one replicate.

[2] Since data from two control groups (i.e., DMSO, and EtOH) were not statistically different. Two control groups were combined for data analyses.

[3] The total number of pups used in “Litter Size and Pregnancy Rate” and “Follicle Count and RIA” studies exceed the original number of pups used in “Age of Puberty and Estrous Cycle” studies, because when litter size and pregnancy rates were determined, one female from DES- and one female from MXC-treated groups failed to become pregnant after three mating attempts, which were also used in tissue and serum collection.

Table 2 Antibodies Used for Immunohistochemistry

Target protein	Supplier	Catalog number	Animal source	Concentration
ESR1	Santa Cruz Biotechnology	sc-542	Rabbit	1:50
P450scc	Millipore	ABD236	Rabbit	1:100
MIS	Santa Cruz Biotechnology	sc-6886	Donkey	1:100
PCNA	Santa Cruz Biotechnology	sc-7907	Rabbit	1:100
LHR	Santa Cruz Biotechnology	sc-25828	Rabbit	1:50

Figure 1: Timeline for treatments and experimental design for Experiment 1 and 2

In Experiment 1**(A),** Timed-pregnant females were treated daily with 50 µg or 50 mg/kg BPA or a vehicle (Oil: DMSO; 2:1;1ml/kg) between embryonic day (E) 18 and postnatal day (PND) 7 as described in **Material and Methods**. Starting from PND28, pubertal age was measured by monitoring vaginal opening day, and estrous cyclicity was followed. The treated females and control animals were dissected for collection of ovary between PND50 and 60, on a proestrus day. Control; n = 5 litters, 12 pups; Low BPA; n = 3 litters, 9 pups; and High BPA; n = 4 litters, 8 pups (see Table 1 for details on litter and pup numbers). In Experiment 2 **(B)**, Timed-pregnant females were treated daily with 0.1µg/kg DES, 75 mg/kg MXC, and 50 mg/kg BPA, or two vehicles (DMSO: oil; 1:2 for MXC or EtOH: oil; 1:9 for DES and BPA) between E11 and PND7 as described. Starting from PND28, pubertal age was measured by monitoring vaginal opening day, and estrous cyclicity was followed. Some animals were mated for assessment of pregnancy rate and litter size, and others were dissected for collection of ovary and serum between PND70 and 90, on a proestrus day. DES; n = 6 litters, 20 pups; MXC; n = 8 litters, 16 pups; BPA; n = 8 litters, 26 pups; and EtOH; n = 5 litters, 15 pups; or DMSO; n = 5 litters, 13 pups (see Table 1 for details on litter and pup numbers). Two vehicle controls were combined for data analysis.

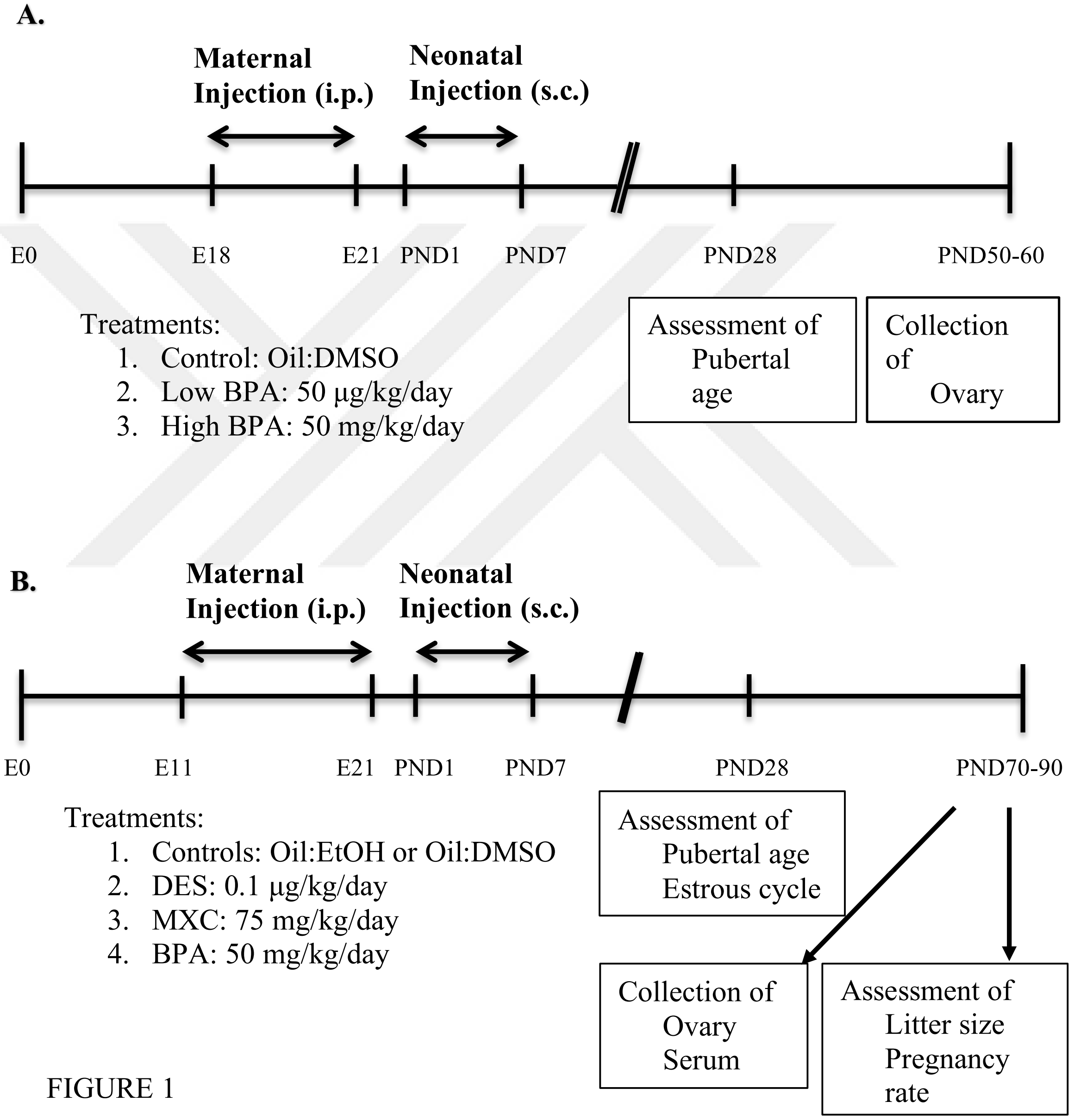
A.
Maternal Injection (i.p.)
Neonatal Injection (s.c.)
E0
E18
E21
PND1
PND7
PND28
PND50-60
Treatments:
1. Control: Oil:DMSO
2. Low BPA: 50 µg/kg/day
3. High BPA: 50 mg/kg/day
Assessment of Pubertal age
Collection of Ovary
B.
Maternal Injection (i.p.)
Neonatal Injection (s.c.)
E0
E11
E21
PND1
PND7
PND28
PND70-90
Treatments:
1. Controls: Oil:EtOH or Oil:DMSO
2. DES: 0.1 µg/kg/day
3. MXC: 75 mg/kg/day
4. BPA: 50 mg/kg/day
Assessment of Pubertal age Estrous cycle
Collection of Ovary Serum
Assessment of Litter size Pregnancy rate

FIGURE 1

Experiment 1

Figure 2: The effects of fetal and neonatal exposure to BPA on the onset of puberty and the regularity of estrous cycles

Timed-pregnant females were treated daily with 50 µg or 50 mg/kg BPA or a vehicle between embryonic day (E) 18 and postnatal day (PND) 7 as described in **Material and Methods.** Vaginal opening was monitored as an indication of pubertal age **(A)**. Regularity of estrous cycles was monitored with vaginal cytology **(B)**. Percent of cycle types, including normal (Normal), prolonged (Prolonged), and persistent diestrus (PD) were calculated as also described. Treatments had no significant effects on pubertal age or regularity of estrous cycles.

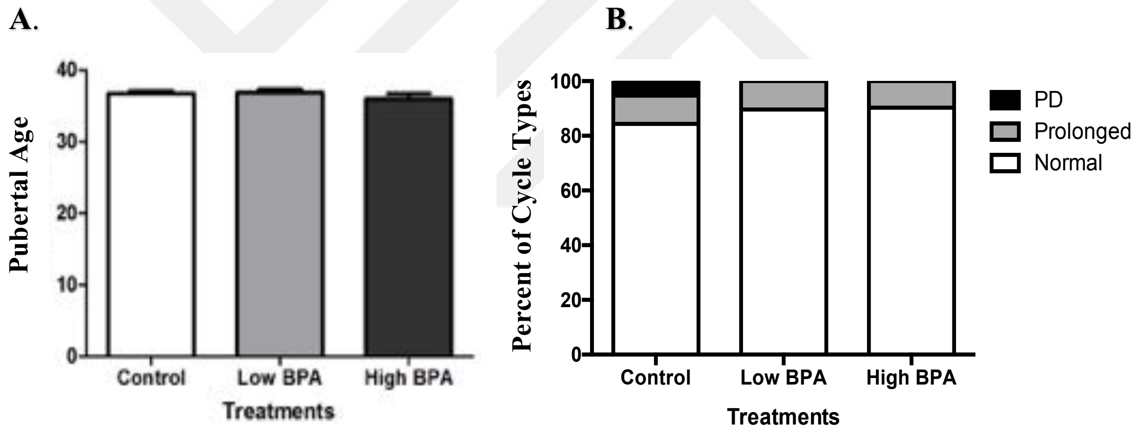
A.
Pubertal Age
40
30
20
10
0
Control
Low BPA
High BPA
Treatments
B.
Percent of Cycle Types
100
80
60
40
20
0
Control
Low BPA
High BPA
Treatments
PD
Prolonged
Normal

FIGURE 2

Figure 3: The Effects of BPA on Body Weights and Ovarian Weights

Timed-pregnant females were treated daily with 50 µg or 50 mg/kg BPA or a vehicle between embryonic day (E) 18 and postnatal day (PND) 7 as described in **Material and Methods.** At the time of tissue collection between PND50-60, body weight (BW) was measured **(A).** In addition, ovarian weight (OW; mg) also measured and normalized OW/ body weights (g) **(B)**. Treated animals did not have a significant effect on BW or normalized ovarian weight.

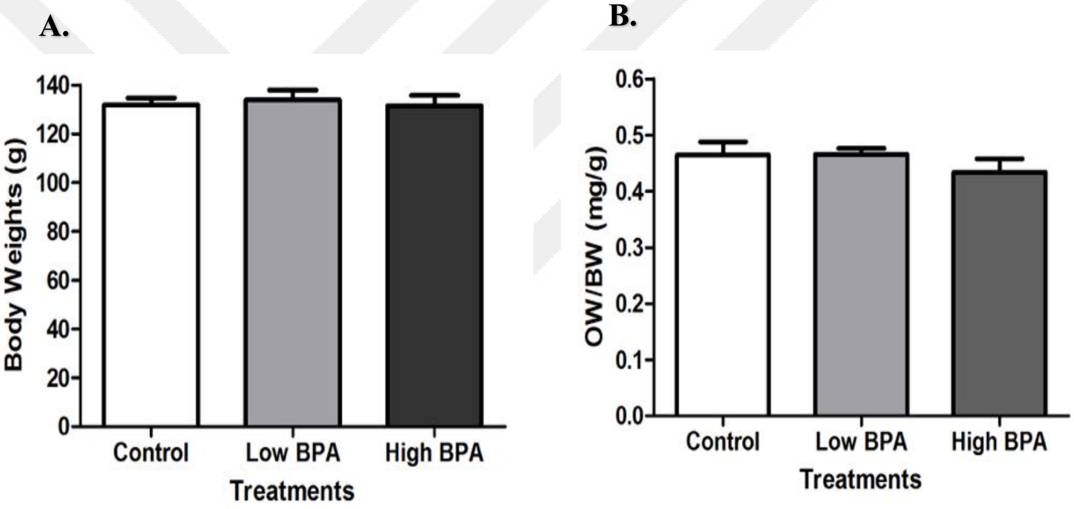
A.
Body Weights (g)
140
120
100
80
60
40
20
0
Control
Low BPA
High BPA
Treatments
B.
OW/BW (mg/g)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
Low BPA
High BPA
Treatments

FIGURE 3

Figure 4: Effects of BPA on Ovarian Follicular Composition

Timed-pregnant females were treated daily with 50 µg or 50 mg/kg BPA or a vehicle between embryonic day (E) 18 and postnatal day (PND) 7 as described in **Material and Methods**. The treated females and control animals were dissected for ovary collection between PND50 and 60, on a proestrus day. Hematoxylin and eosin-stained sections were used to follicle counting and classification, which evaluated for percentage of follicles in all stages of folliculogenesis. Follicles of early stages of development **(A**; primordial, primary, secondary), and antral stage follicles (pre-antral, early-antral, and mid to late antral follicles) and atretic follicles **(B)** were analyzed together, as the most atretic follicles, and produced from antral stage follicles. Percentage of corpora lutea (CL) **(C)** as well as total follicles number **(D)** were determined. Statistical analysis of follicular composition was performed using GraphPad Prism using two-way ANOVA, Tukey's multiple comparison test. *= $p < 0.05$, **< 0.01.

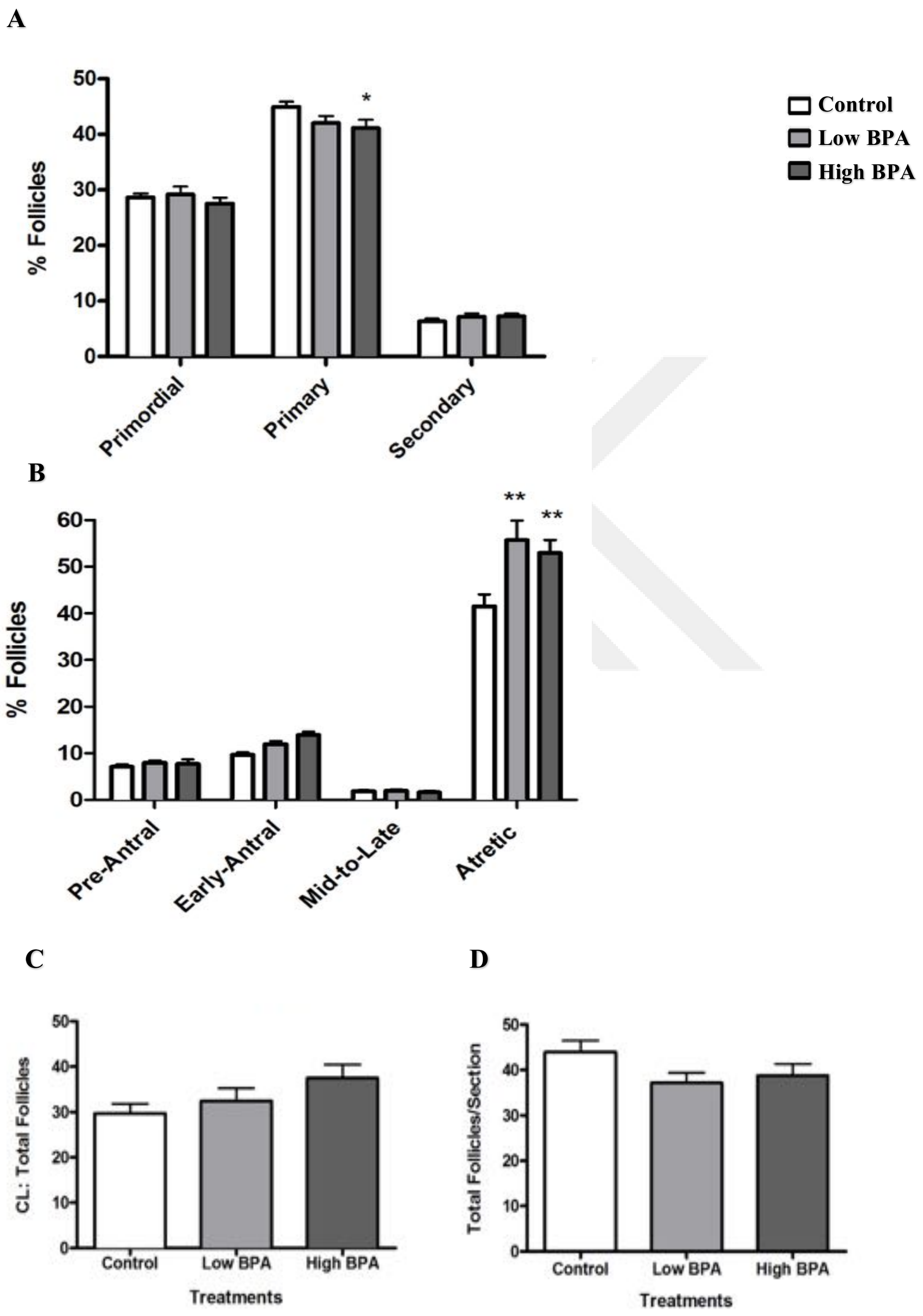
A
% Follicles
50
40
30
20
10
0
*
Primordial
Primary
Secondary
Control
Low BPA
High BPA
B
% Follicles
60
50
40
30
20
10
0
**
**
Pre-Antral
Early-Antral
Mid-to-Late
Atretic
C
CL: Total Follicles
50
40
30
20
10
0
Control
Low BPA
High BPA
Treatments
D
Total Follicles/Section
50
40
30
20
10
0
Control
Low BPA
High BPA
Treatments

FIGURE 4

Figure 5: Effects of BPA on ESR1 Protein Expression in the Ovary

Effects of daily BPA exposure between embryonic day (E) 18 and postnatal day (PND) 7 on ovarian estrogen receptor 1 protein expression were assessed using quantitative immunohistochemistry (IHC) as described in **Material and Methods**. Representative pictures were shown. ESR1 normally expressed in theca cells in primary, secondary, pre-antral, and early-antral follicles, and in the corpora lutea (CL) (arrows). Quantification of the IHC pictures with ImageJ showed that Low BPA reduced ESR1 protein levels in theca cells of primary follicles. *= $p < 0.05$.

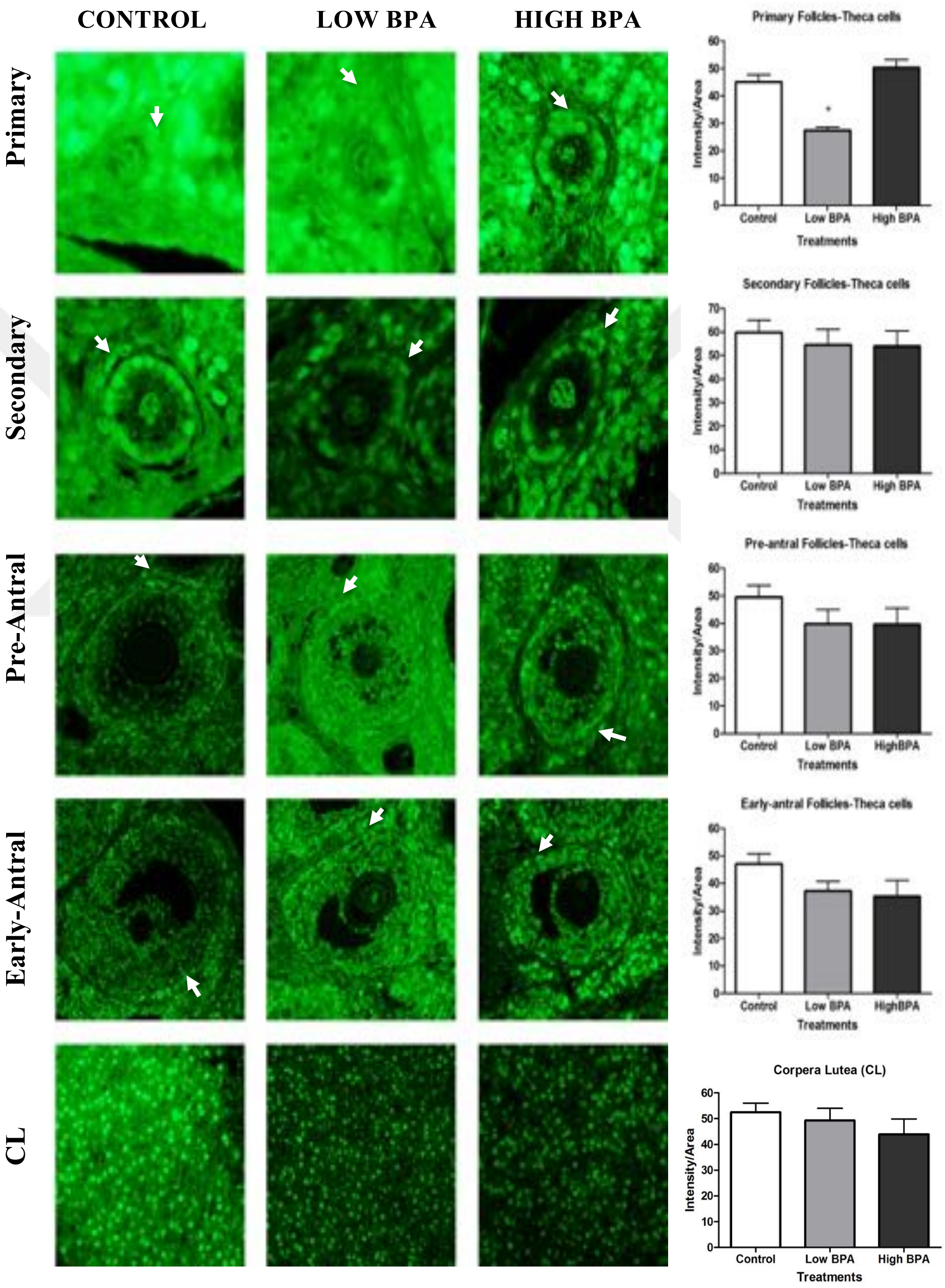
CONTROL
LOW BPA
HIGH BPA
Primary
Secondary
Pre-Antral
Early-Antral
CL
Primary Follicles-Theca cells
Intensity/Area
0
10
20
30
40
50
60
*
Control
Low BPA
High BPA
Treatments
Secondary Follicles-Theca cells
Intensity/Area
0
10
20
30
40
50
60
70
Control
Low BPA
High BPA
Treatments
Pre-antral Follicles-Theca cells
Intensity/Area
0
10
20
30
40
50
60
Control
Low BPA
HighBPA
Treatments
Early-antral Follicles-Theca cells
Intensity/Area
0
10
20
30
40
50
60
Control
Low BPA
HighBPA
Treatments
Corpera Lutea (CL)
Intensity/Area
0
10
20
30
40
50
60
Control
Low BPA
High BPA
Treatments

FIGURE 5

Figure 6: Effects of BPA on P450scc Protein Expression in the Ovary

Effects of daily BPA exposure between embryonic day (E) 18 and postnatal day (PND) 7 on ovarian cytochrome P450 side chain cleavage protein expression were assessed using quantitative IHC as described in **Material and Methods**. Representative pictures were shown. Immunolocalization of P450scc was observed in theca/interstitial cells of developing follicles, including pre-antral and early-antral follicles and in the corpora lutea (CL) (arrows). Intensity of staining were measured with ImageJ which showed no significant effects of BPA-treatments.

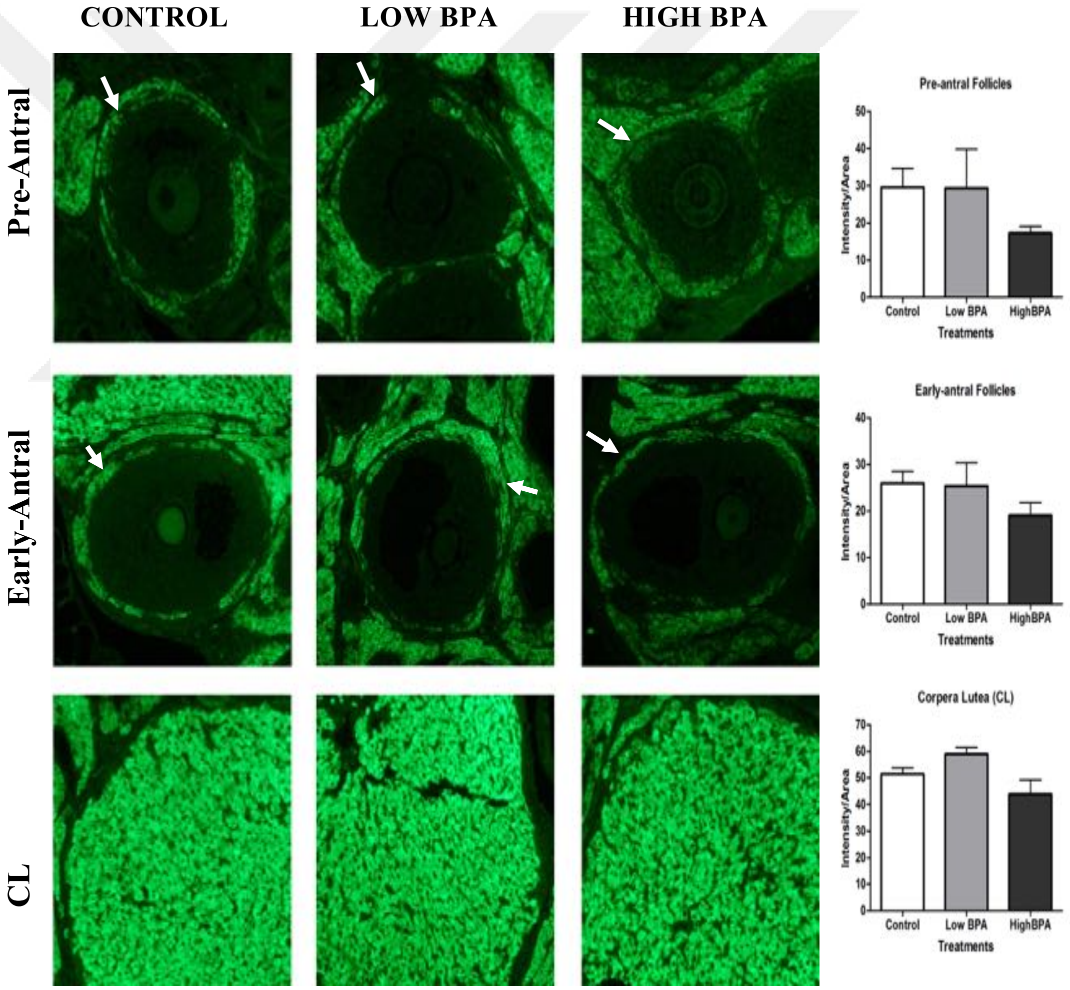
CONTROL
LOW BPA
HIGH BPA
Pre-Antral
Early-Antral
CL
Pre-antral Follicles
Early-antral Follicles
Corpera Lutea (CL)
Intensity/Area
0
10
20
30
40
50
60
70
Control
Low BPA
HighBPA
Treatments

FIGURE 6

Figure 7: Effects of BPA on MIS Protein Expression in the Ovary

Effects of daily BPA exposure between embryonic day (E) 18 and postnatal day (PND) 7 on ovarian Müllerian inhibiting substance protein expression were assessed using quantitative IHC as described in **Material and Methods**. Representative pictures were shown. Immunolocalization of MIS was observed in granulosa cells of early stage of follicles, including primary, secondary follicles and developing follicles, including pre-antral and early-antral follicles (arrows). Using ImageJ software staining intensity were measured, which showed no statistically significant differences in MIS expression between BPA-treated or control ovaries.

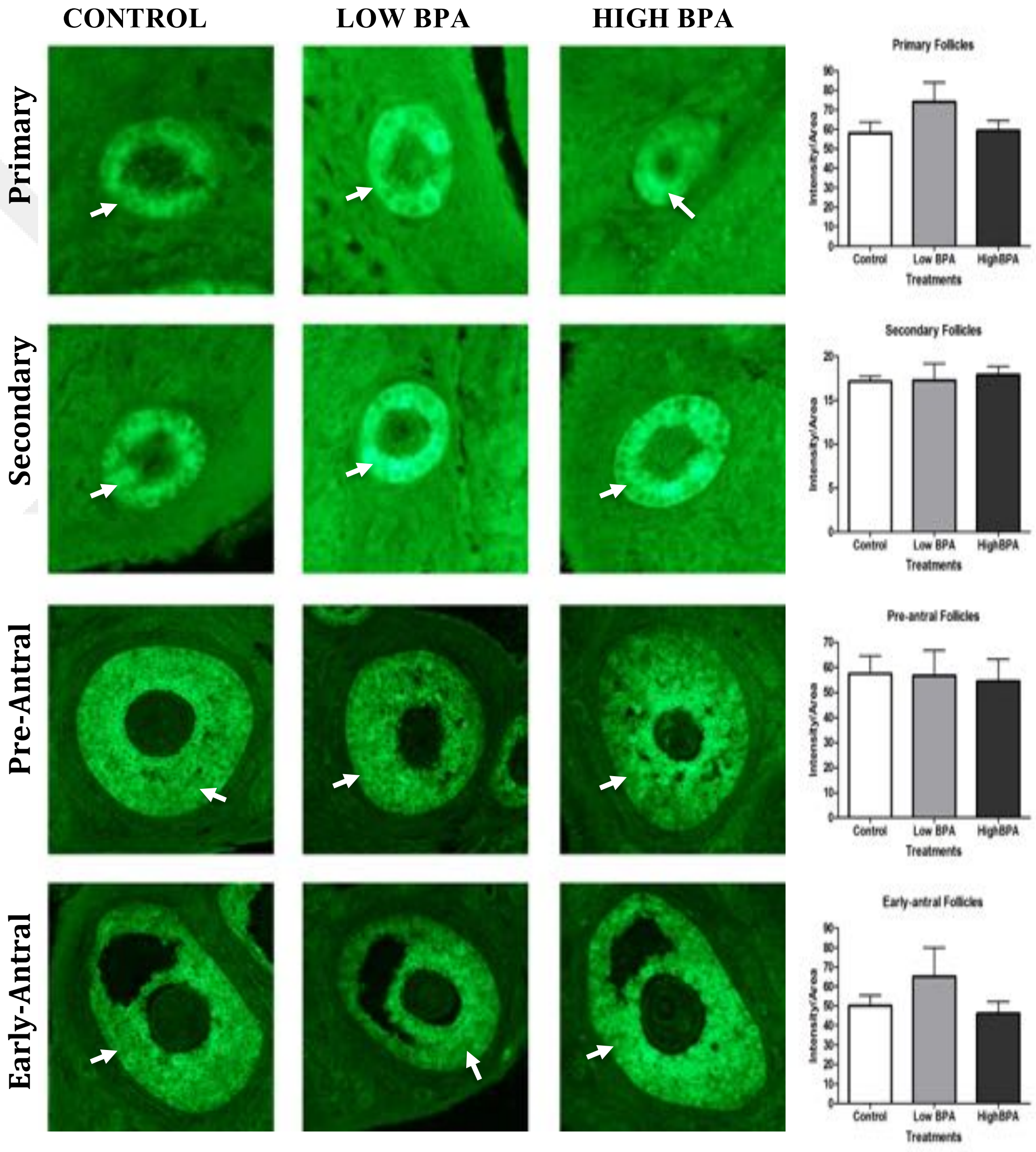
CONTROL
LOW BPA
HIGH BPA
Primary
Secondary
Pre-Antral
Early-Antral
Primary Follicles
Secondary Follicles
Pre-antral Follicles
Early-antral Follicles
Intensity/Area
Control
Low BPA
HighBPA
Treatments
0
10
20
30
40
50
60
70
80
90
5
15

FIGURE 7

Figure 8: Effects of BPA on PCNA Protein Expression in the Ovary

Effects of daily BPA exposure between embryonic day (E) 18 and postnatal day (PND) 7 on ovarian proliferating cell antigen (PCNA) staining were measured using quantitative IHC as described in **Material and Methods**. Representative pictures (**A**; from early-antral follicles) and proliferation indices **(B)** were shown. Proliferating (Alexa-488+) cells were counted and expressed a fraction of all nuclei (ethidium homodimer-2 staining) to generate a proliferation index for the cell type associated the follicular stage. There were no statistically significant differences in the ovaries of treated animals and controls.

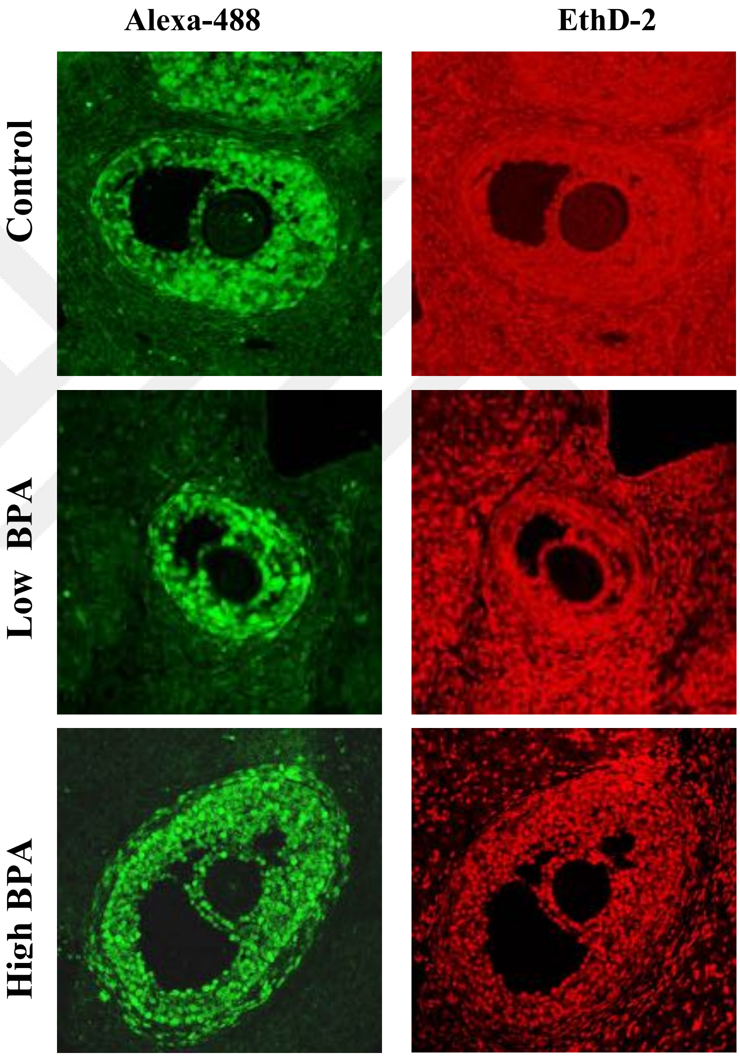

FIGURE 8A

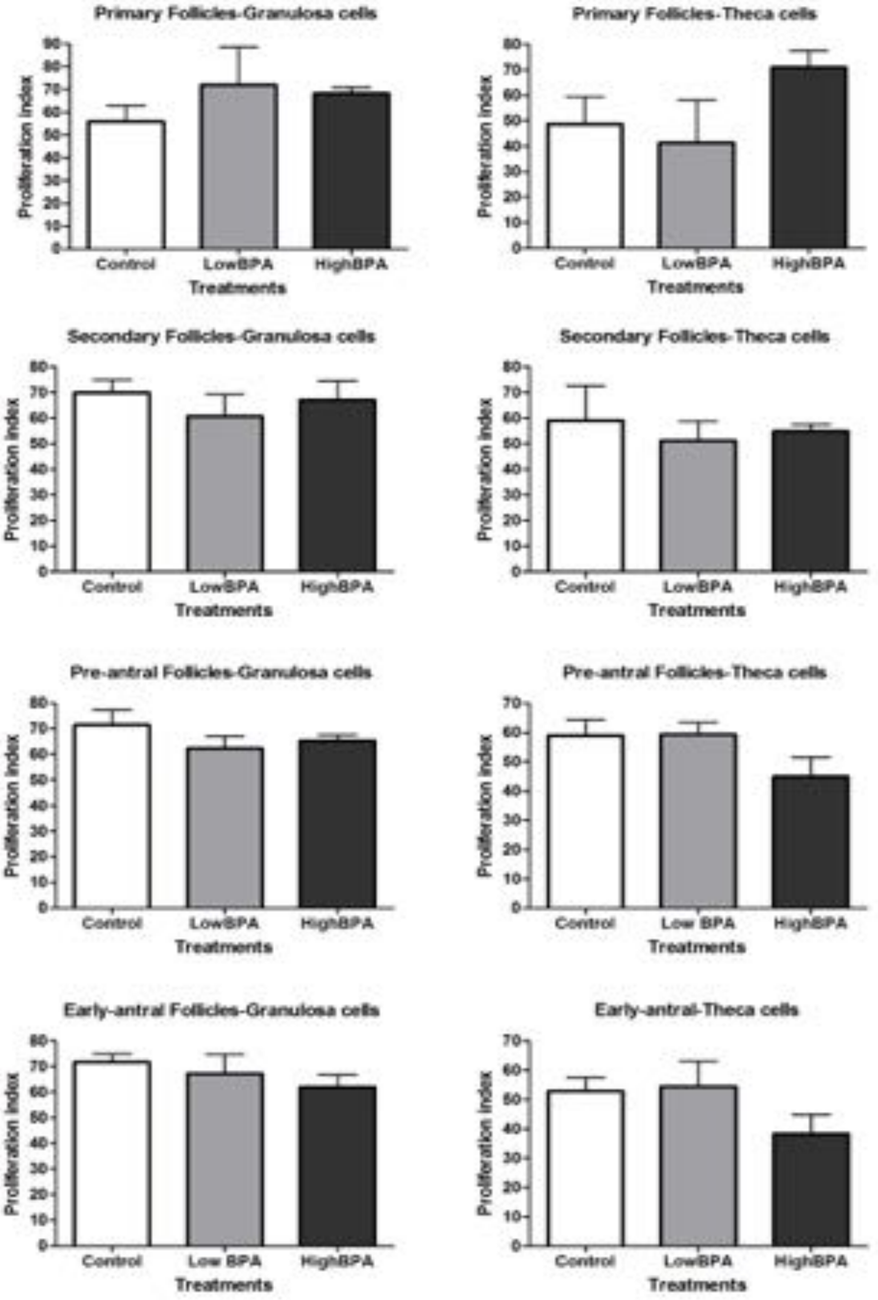
Primary Follicles-Granulosa cells
Primary Follicles-Theca cells
Secondary Follicles-Granulosa cells
Secondary Follicles-Theca cells
Pre-antral Follicles-Granulosa cells
Pre-antral Follicles-Theca cells
Early-antral Follicles-Granulosa cells
Early-antral-Theca cells
Proliferation index
Treatments
Control
LowBPA
Low BPA
HighBPA
0
10
20
30
40
50
60
70
80
90

FIGURE 8B

Experiment 2

Figure 9: The effects of fetal and neonatal exposure to MXC, BPA, and DES on the onset of puberty and the regularity of estrous cycle

Time-pregnant females were treated daily with 0.1µ DES, 75 mg MXC, and 50 mg BPA, or two vehicles (1ml; control) per kg body weight between embryonic day (E) 11 to postnatal day (PND) 7 as described in **Material and Methods**. Vaginal opening was monitored as an indication of pubertal age **(A).** Regularity of estrous cycles was monitored with vaginal cytology. Percent of cycle types, **(B)** including normal (Normal), prolonged, (Prolonged), persistent estrus (PE), and persistent diestrus (PD) were calculated as described. The DES and MXC-treated females accelerated the age of puberty. The DES and MXC-treated females demonstrated statistically significant irregular estrous cyclicity. *= $p < 0.05$ and ****= $p < 0.001$.

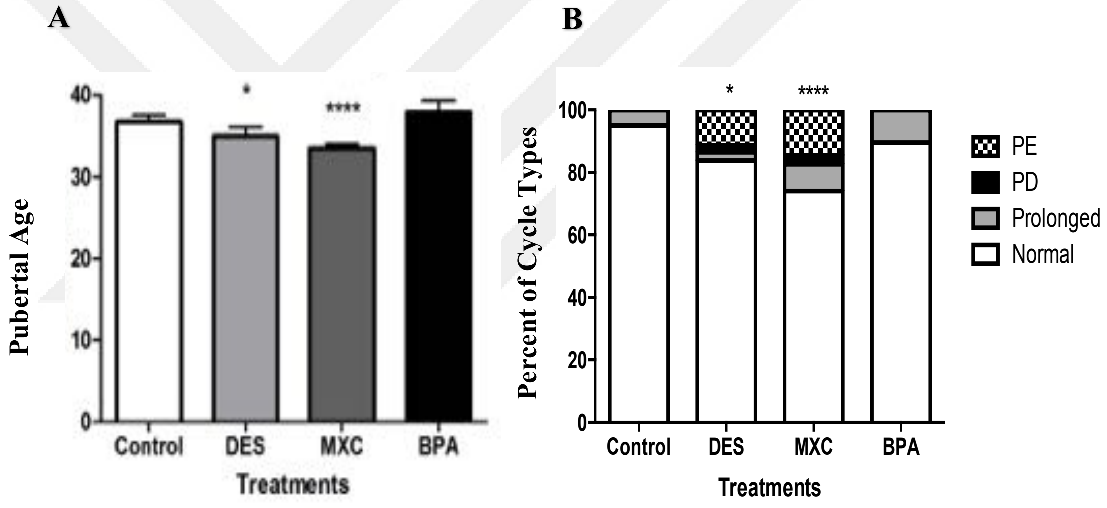
A
Pubertal Age
0
10
20
30
40
*
****
Control
DES
MXC
BPA
Treatments
B
Percent of Cycle Types
0
20
40
60
80
100
*
****
Control
DES
MXC
BPA
Treatments
PE
PD
Prolonged
Normal

FIGURE 9

Figure 10: The effects of fetal and neonatal exposure to DES, MXC, and BPA on body weights and ovarian weights

Timed-pregnant females were treated daily with DES, MXC, and BPA, or the vehicles (1ml; control) as described in **Material and Methods.** At the time of tissue collection, body weight (BW) was measured **(A).** In addition, ovarian weight (OW) was also measured and normalized with BW **(B)**. There was significant increase BW in MXC-treated while DES and BPA-treated did not changed. In addition, normalized ovarian weights were significantly decrease in DES, MXC, and BPA-treated females. *= $p < 0.05$, ***< 0.001

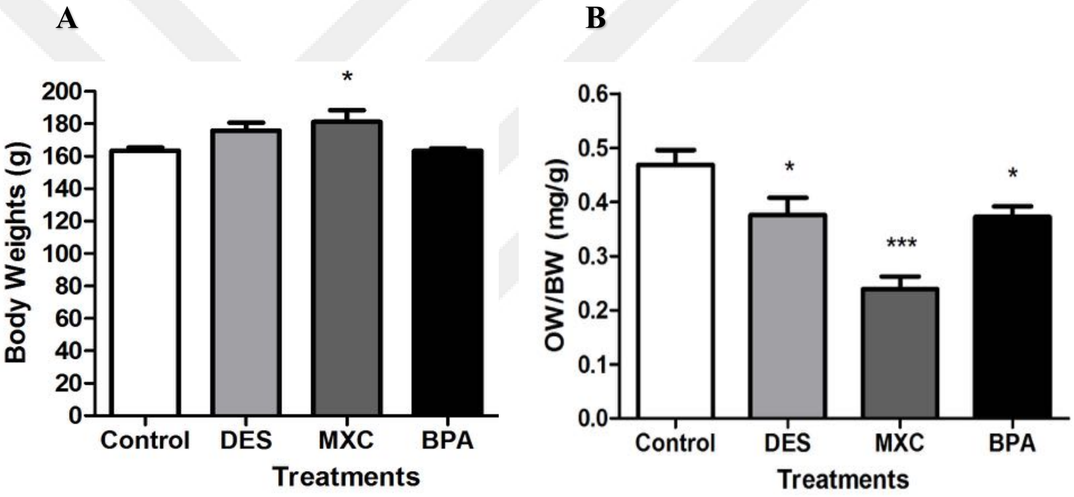
A
Body Weights (g)
200
180
160
140
120
100
80
60
40
20
0
*
Control
DES
MXC
BPA
Treatments
B
OW/BW (mg/g)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
***
*
Control
DES
MXC
BPA
Treatments

FIGURE 10

Figure 11: The effects of fetal and neonatal exposure to DES, MXC, and BPA on the follicular composition

Timed-pregnant females were treated daily with DES, MXC, and BPA, or the vehicles between embryonic day (E) 11 and postnatal day (PND) 7 as described in **Material and Methods**. Hematoxylin and eosin-stained sectioned were used for follicle counting and classification, which evaluated for percentage of developing follicles **(A)** (primordial, primary, and secondary follicles), antral follicles (pre-antral, early-antral, and mid to late antral follicles) and atretic follicles **(B),** percentage of corpora lutea (CL) **(C),** and total number of follicles **(D)** as described. *= $p < 0.05$, **= < 0.01, **** = p <0.001.

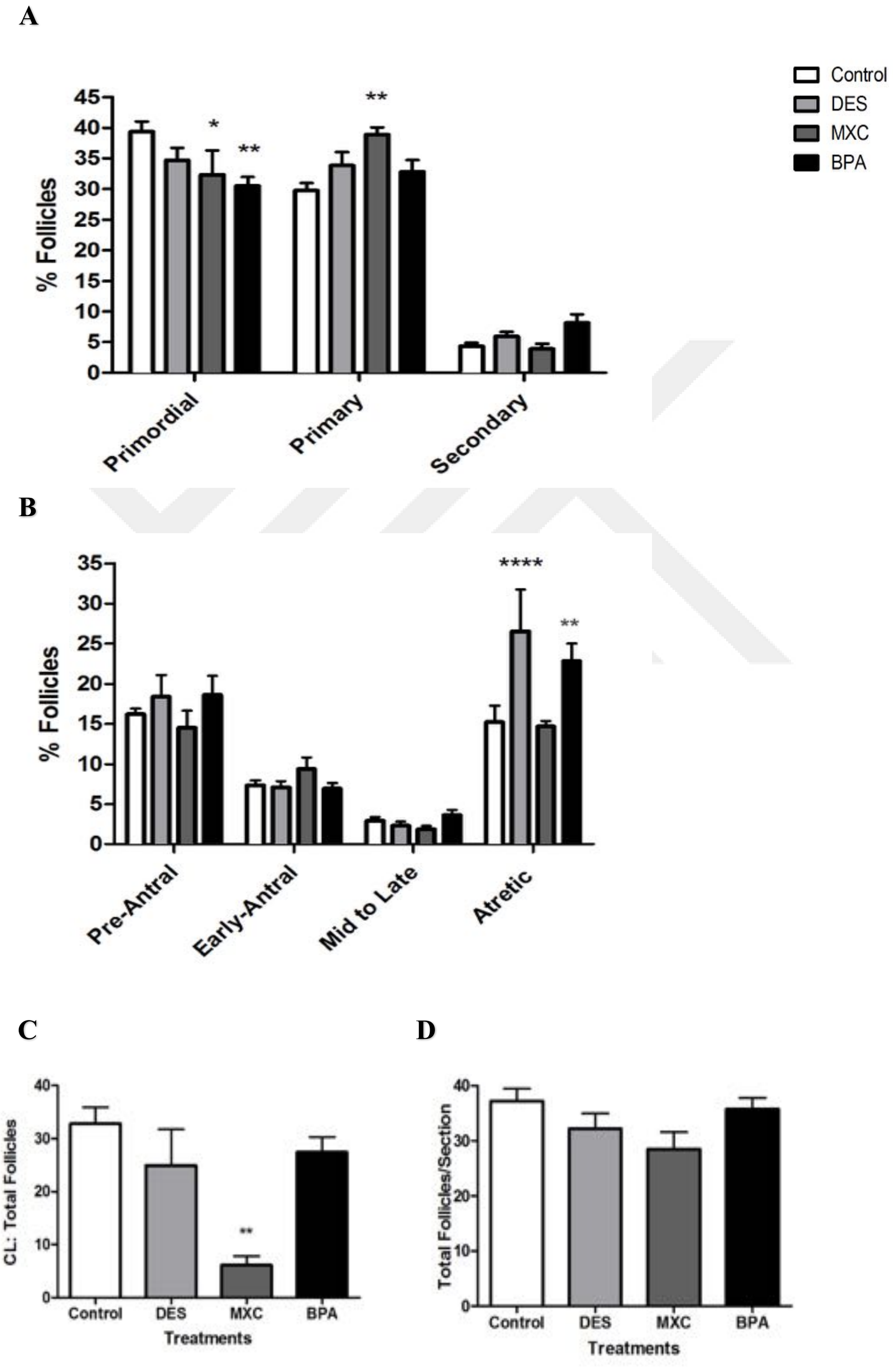
A
Control
DES
MXC
BPA
% Follicles
45
40
35
30
25
20
15
10
5
0
*
**
**
Primordial
Primary
Secondary
B
% Follicles
35
30
25
20
15
10
5
0
****
**
Pre-Antral
Early-Antral
Mid to Late
Atretic
C
CL: Total Follicles
40
30
20
10
0
**
Control
DES
MXC
BPA
Treatments
D
Total Follicles/Section
40
30
20
10
0
Control
DES
MXC
BPA
Treatments

FIGURE 11

Figure 12: The effects of fetal and neonatal exposure to DES, MXC, and BPA on the litter size and pregnancy rate

Timed-pregnant females were treated daily with DES, MXC, and BPA, or the vehicles as described in **Material and Methods**. Litter size (i.e., number of offspring/dam) and pregnancy rate were monitored as described. MXC, DES, and BPA did not significantly change the litter size or pregnancy rate ($p > 0.05$). However, there was a trend for reduction in MXC-treated group who used *t*-test (#, $p < 0.07$).

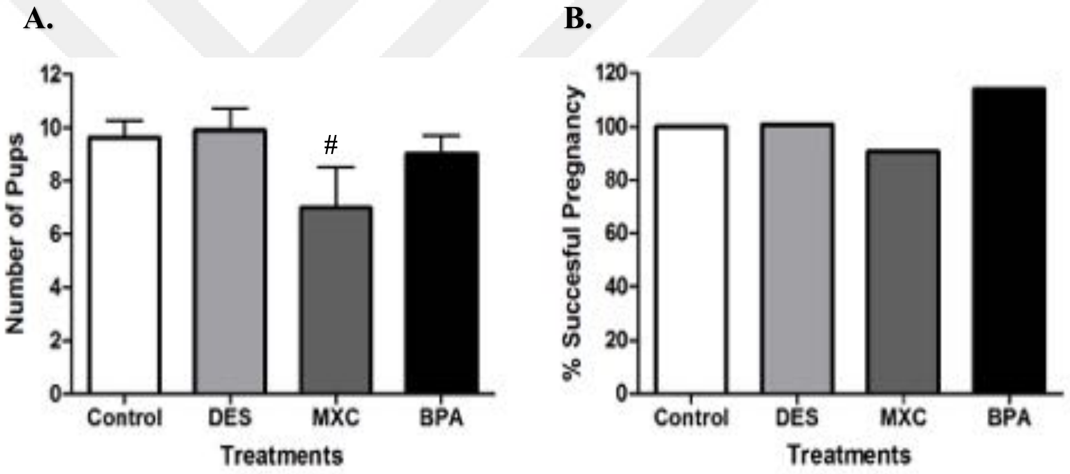
A.
Number of Pups
12
10
8
6
4
2
0
#
Control
DES
MXC
BPA
Treatments
B.
% Succesful Pregnancy
120
100
80
60
40
20
0
Control
DES
MXC
BPA
Treatments

FIGURE 12

Figure 13: Effects of DES, MXC, and BPA on ESR1 Protein Expression in the Ovary

Effects of daily DES, MXC, and BPA exposure between embryonic day (E) 11 and postnatal day (PND) 7 on ovarian estrogen receptor 1 (ESR1) protein expression were assessed using quantitative IHC as described in **Material and Methods**. Representative pictures were shown. ESR1 normally expressed in theca/interstitial cells in primary, secondary, pre-antral, early antral follicles, and corpora lutea (CL) (arrows). The staining was quantified with ImageJ which showed that BPA stimulates secondary, early-antral, and CL. However, DES inhibits ESR1 in early-antral. *= $p < 0.05$.

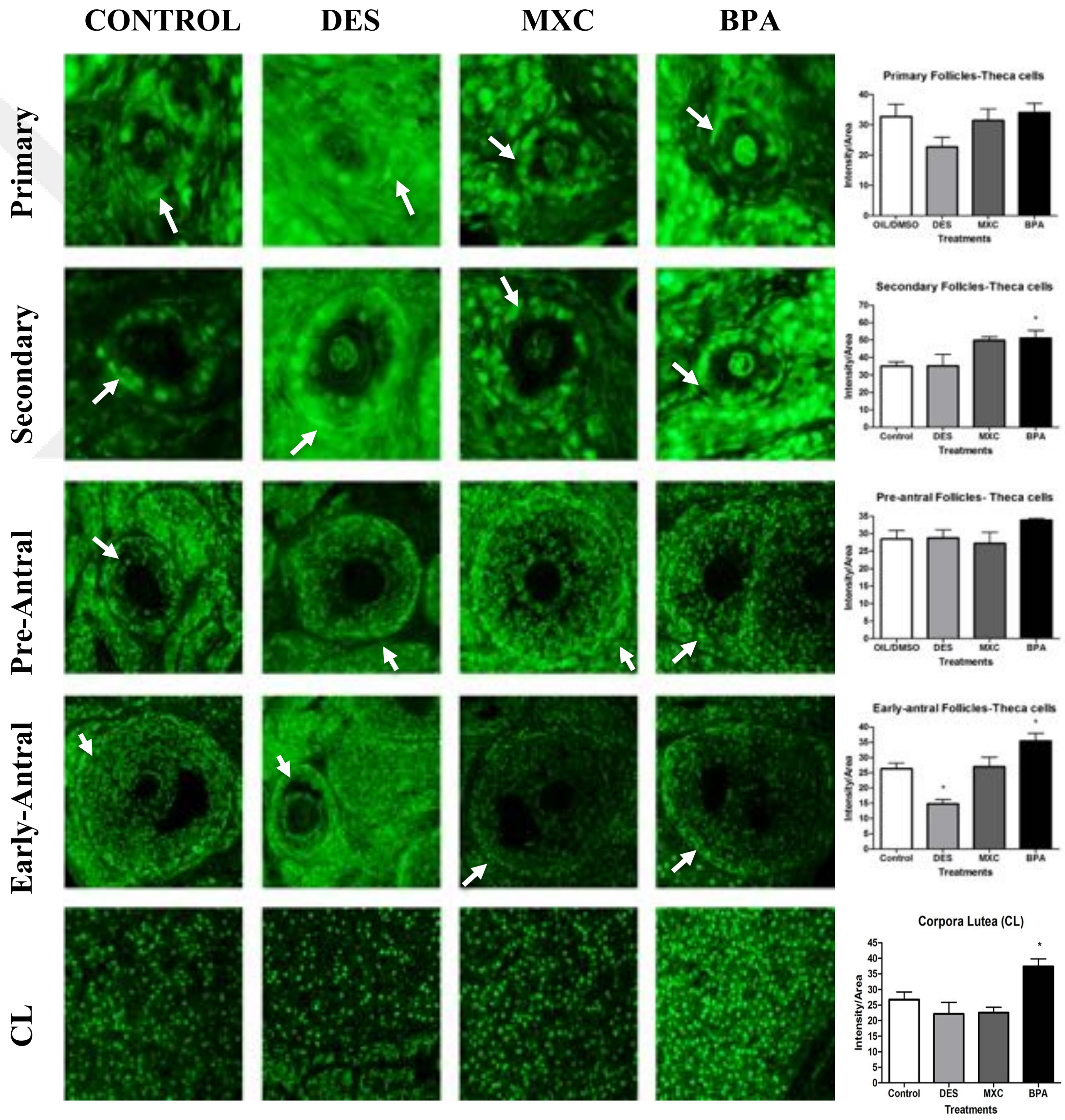
CONTROL
DES
MXC
BPA
Primary
Secondary
Pre-Antral
Early-Antral
CL
Primary Follicles-Theca cells
Intensity/Area
0
10
20
30
40
OIL/DMSO
DES
MXC
BPA
Treatments
Secondary Follicles-Theca cells
Intensity/Area
0
10
20
30
40
50
60
70
*
Control
DES
MXC
BPA
Treatments
Pre-antral Follicles- Theca cells
Intensity/Area
0
5
10
15
20
25
30
35
OIL/DMSO
DES
MXC
BPA
Treatments
Early-antral Follicles-Theca cells
Intensity/Area
0
5
10
15
20
25
30
35
40
*
*
Control
DES
MXC
BPA
Treatments
Corpora Lutea (CL)
Intensity/Area
0
5
10
15
20
25
30
35
40
45
*
Control
DES
MXC
BPA
Treatments

Figure 14: Effects of DES, MXC, and BPA on P450scc Protein Expression in the Ovary

Effects of daily DES, MXC, and BPA exposure between embryonic day (E) 11 and postnatal day (PND) 7 on ovarian cytochrome P450 side chain cleavage (P450scc) protein expression were assessed using quantitative IHC as described in **Material and Methods**. Representative pictures were shown. Immunolocalization of P450scc was observed on theca/interstitial cells of developing follicles, including pre-antral, early-antral follicles and in the corpora lutea (CL) (arrows). Intensity was done ImageJ, which showed a reduction in pre-antral cells in MXC-treated ovaries. In addition, BPA-treated CL showed an increase in P450scc staining intensity. *= $p < 0.05$.

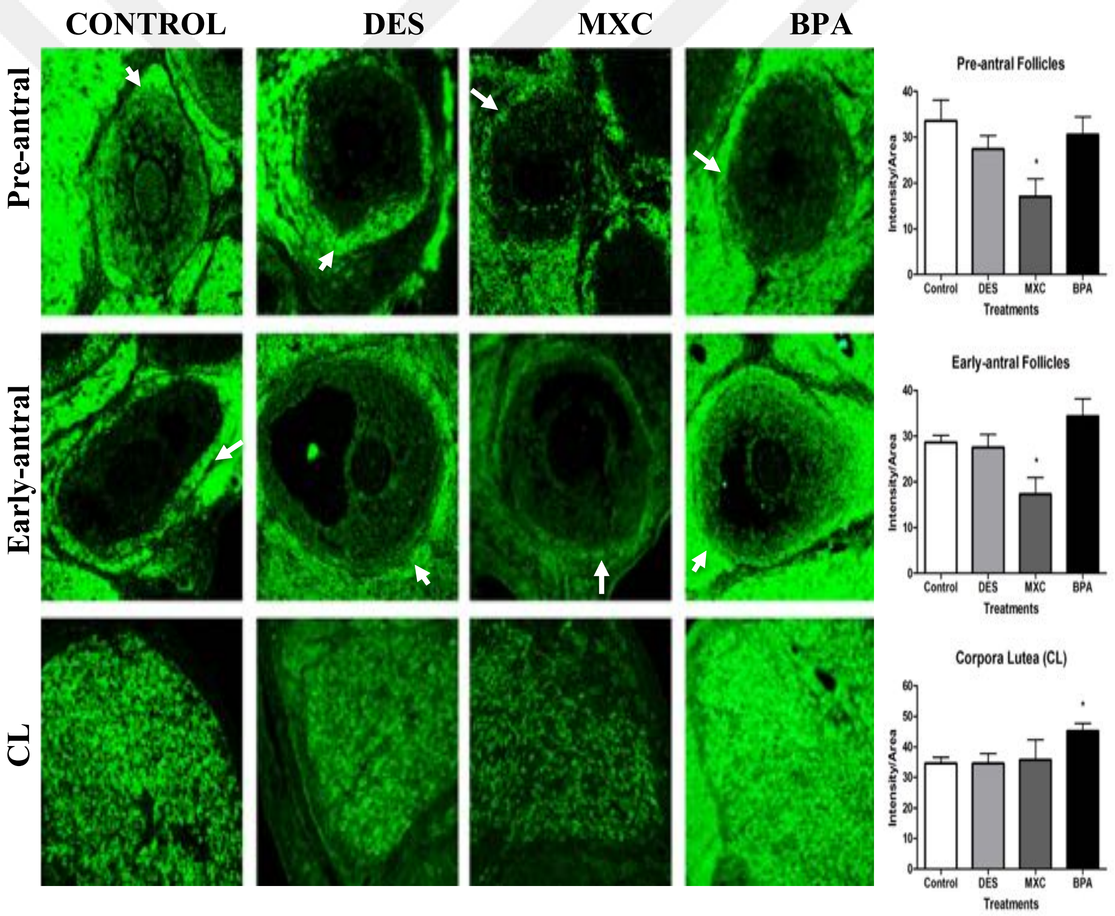
CONTROL
DES
MXC
BPA
Pre-antral
Early-antral
CL
Pre-antral Follicles
Intensity/Area
0
10
20
30
40
*
Control
DES
MXC
BPA
Treatments
Early-antral Follicles
Intensity/Area
0
10
20
30
40
*
Control
DES
MXC
BPA
Treatments
Corpora Lutea (CL)
Intensity/Area
0
10
20
30
40
50
60
*
Control
DES
MXC
BPA
Treatments

FIGURE 14

Figure 15: Effects of DES, MXC, and BPA on MIS Protein Expression in the Ovary

Effects of daily DES, MXC, and BPA exposure between embryonic day (E) 11 and postnatal day (PND) 7 on ovarian Müllerian inhibiting substance (MIS) protein expression were assessed using quantitative IHC as described in **Material and Methods**. Representative pictures were shown. Immunolocalization of MIS was observed in granulosa cells of early stage of follicles, including primary and secondary follicles and in granulosa cells of developing follicles, including pre-antral and early-antral follicles (arrows). *= $p < 0.05$.

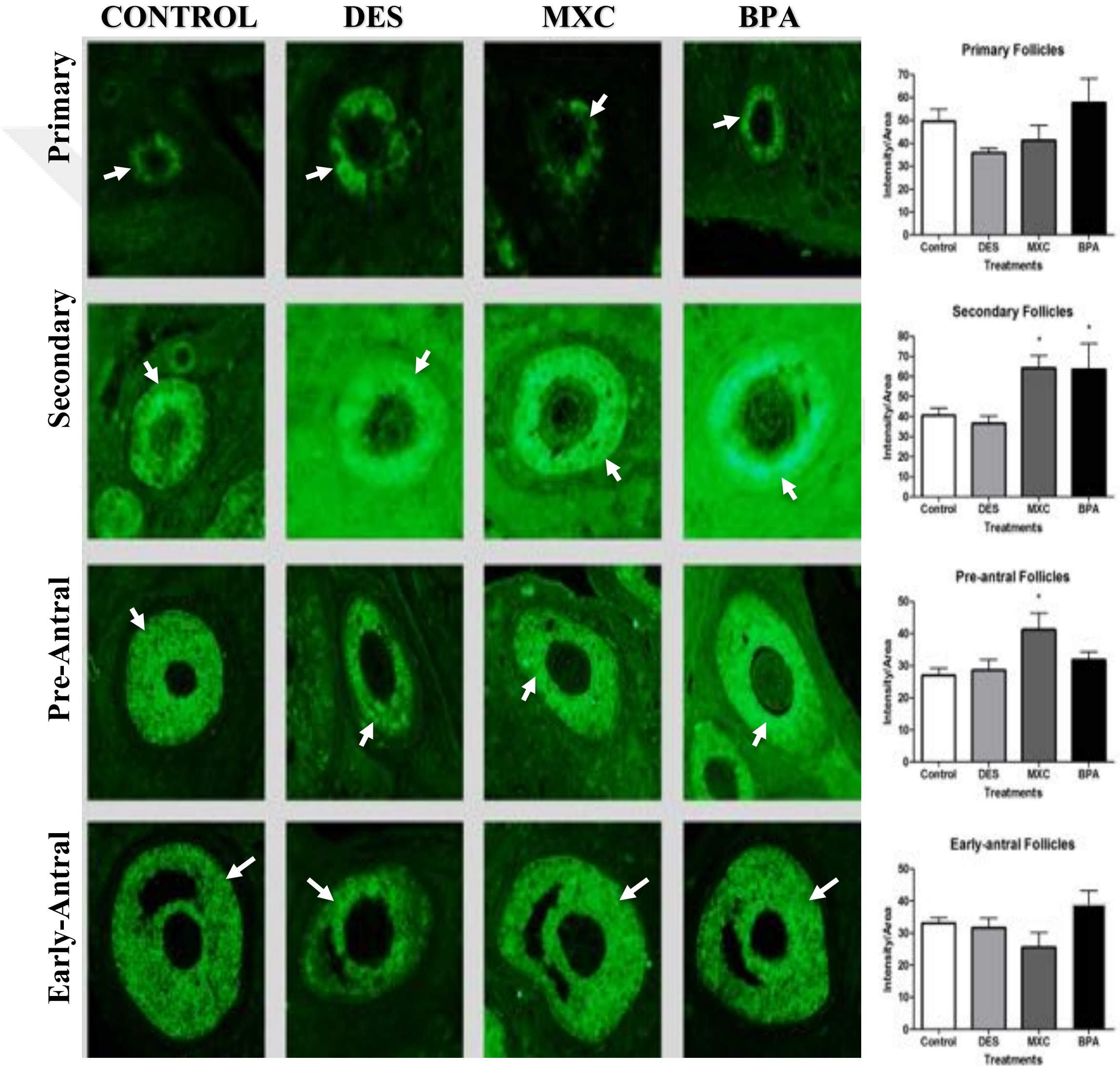
CONTROL
DES
MXC
BPA
Primary
Secondary
Pre-Antral
Early-Antral
Primary Follicles
Secondary Follicles
Pre-antral Follicles
Early-antral Follicles
Intensity/Area
Control
DES
MXC
BPA
Treatments
*

FIGURE 15

Figure 16: Effects of DES, MXC, and BPA on LHR Protein Expression in the Ovary

Effects of daily DES, MXC, and BPA exposure between embryonic day (E) 11 and postnatal day (PND) 7 on ovarian luteinizing hormone receptor protein expression were assessed using quantitative IHC as described in **Material and Methods**. Representative pictures are shown. Immunolocalization of LHR was observed on theca/interstitial cells of developing follicles, including pre-antral, and early-antral follicles (arrows).

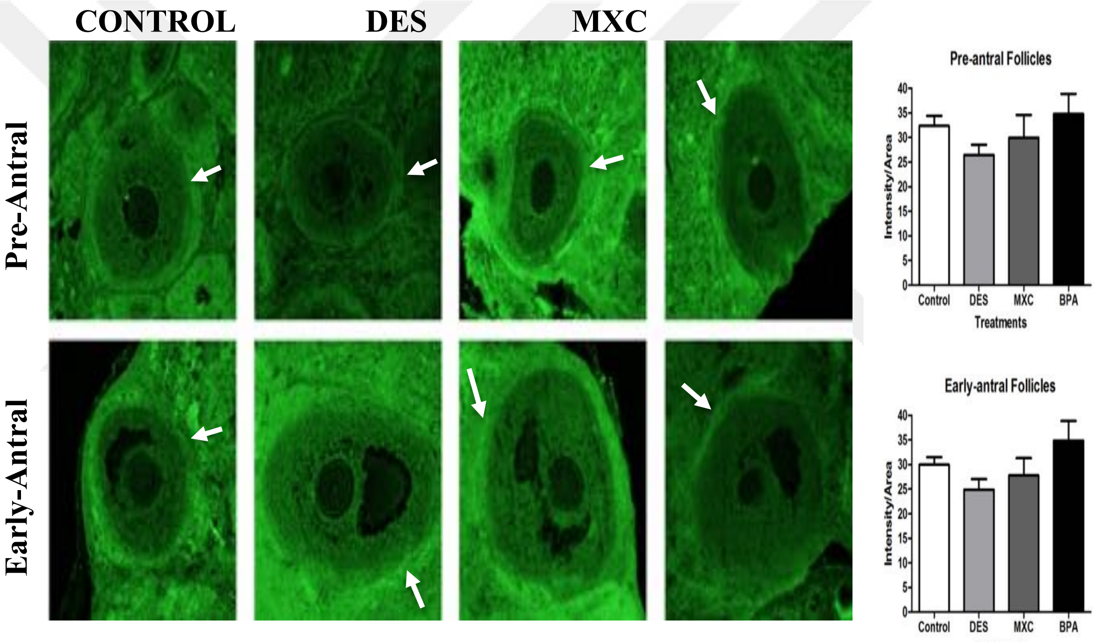
CONTROL
DES
MXC
Pre-Antral
Early-Antral
Pre-antral Follicles
Intensity/Area
0
5
10
15
20
25
30
35
40
Control
DES
MXC
BPA
Treatments
Early-antral Follicles
Intensity/Area
0
5
10
15
20
25
30
35
40
Control
DES
MXC
BPA
Treatments

FIGURE 16

Figure 17: Effects of DES, MXC, and BPA on PCNA Protein Expression in the Ovary

Effects of daily DES, MXC, and BPA exposure between embryonic day (E) 11 and postnatal day (PND) 7 on ovarian proliferating cell antigen (PCNA) staining were measured using quantitative IHC as described in **Material and Methods**. Representative pictures (**A**; from early-antral follicles) and proliferation indices **(B)** were shown. Proliferating (Alexa-488+) cells were counted and expressed a fraction of all nuclei (ethidium homodimer-2 staining) to generate a proliferation index for the cell type associated the follicular stage. There were no statistically significant differences between treated ovaries and control.

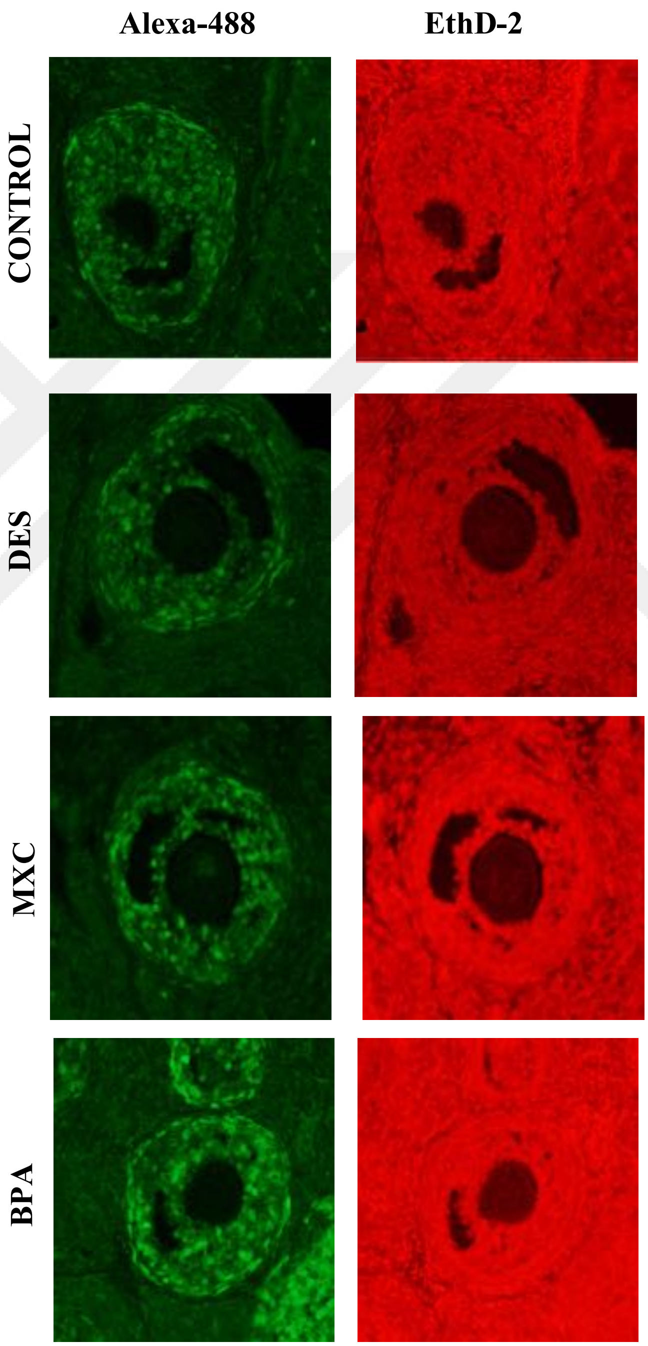

FIGURE 17A

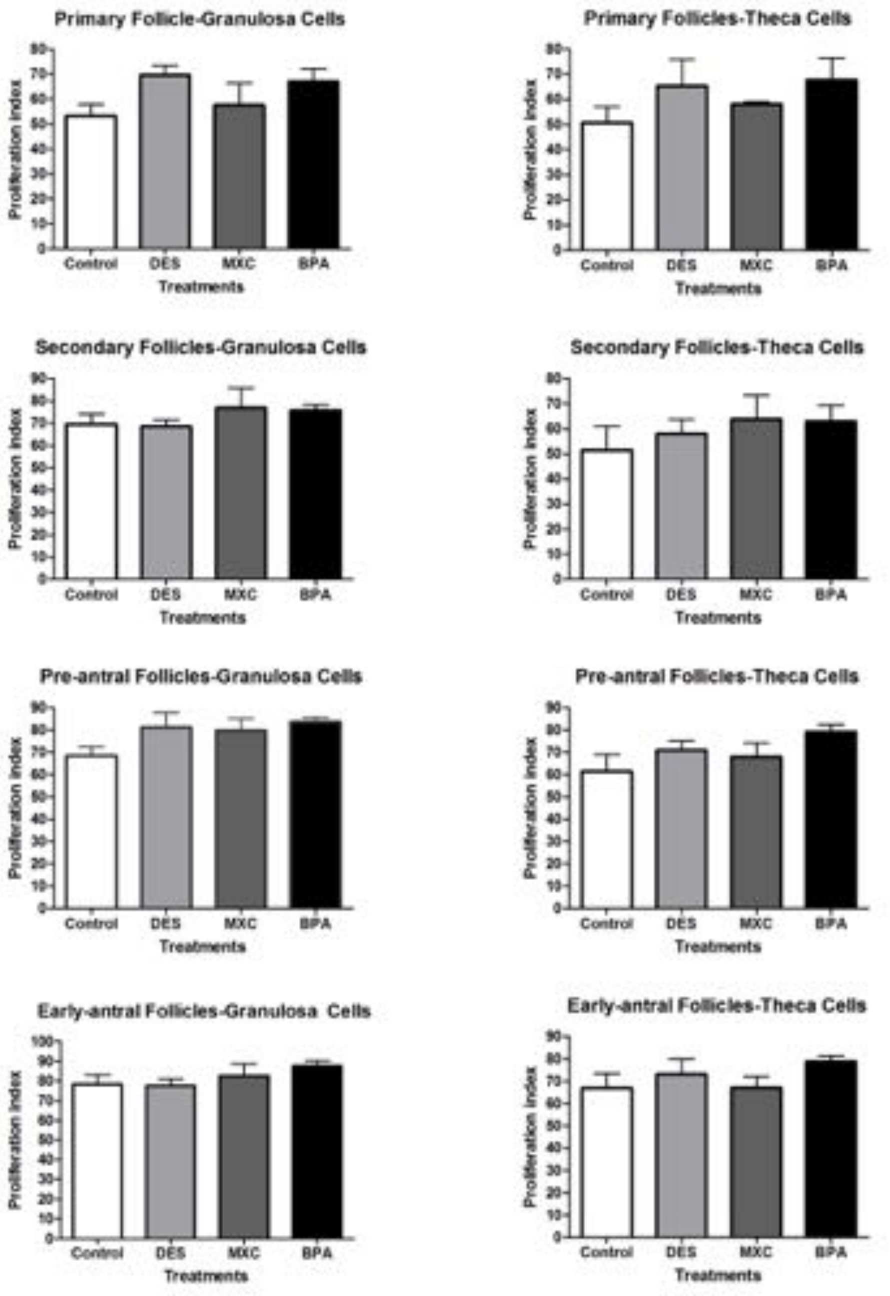
Primary Follicle-Granulosa Cells
Primary Follicles-Theca Cells
Secondary Follicles-Granulosa Cells
Secondary Follicles-Theca Cells
Pre-antral Follicles-Granulosa Cells
Pre-antral Follicles-Theca Cells
Early-antral Follicles-Granulosa Cells
Early-antral Follicles-Theca Cells
Proliferation index
0
10
20
30
40
50
60
70
80
90
100
Control
DES
MXC
BPA
Treatments

FIGURE 17B

Figure 18: The effects of fetal and neonatal exposure to DES, MXC, and BPA on serum hormones

Timed-pregnant females were treated daily with DES, MXC, and BPA, or the vehicles between embryonic day (E) 11 to postnatal day (PND) 7 as described in **Material and Methods**. Serum was collected from DES, MXC, and BPA-treated animals between PND70-90. Concentration of progesterone **(A)** and estradiol **(B)** hormones were measured by RIA. MXC reduces level of progesterone hormone while DES and BPA did not ($p < 0.05$). In addition, level of estradiol did not significantly change. However, there is a trend in MXC-treated demonstrated reduction of estradiol hormone (*= $p < 0.05$, #= $p = 0.06$; *t*-test).

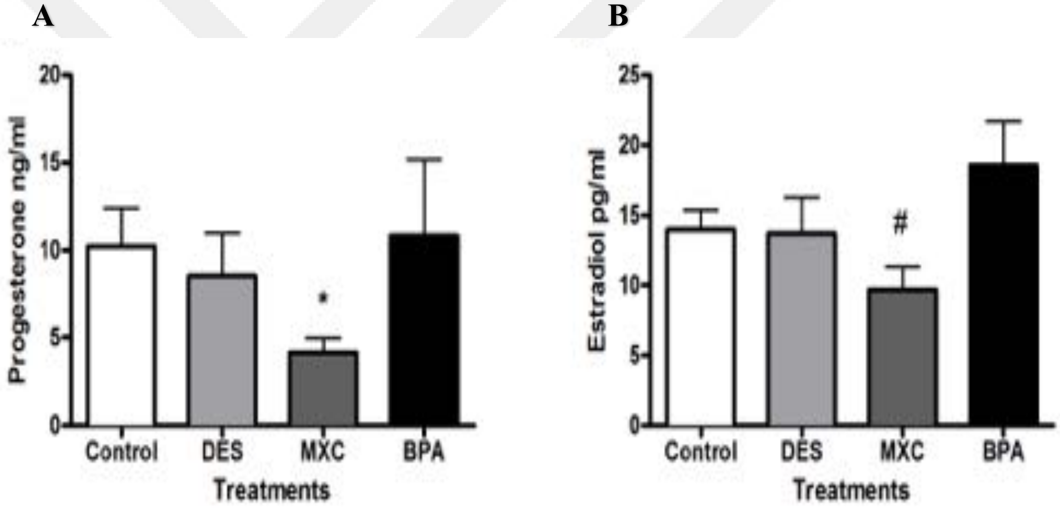
A
B
Progesterone ng/ml
20
15
10
5
0
*
Control
DES
MXC
BPA
Treatments
Estradiol pg/ml
25
20
15
10
5
0
#
Control
DES
MXC
BPA
Treatments

FIGURE 18

CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

The current study shows that exposures to estrogenic EDCs during critical fetal and neonatal periods have effects on ovarian function and fertility in adults. There were some effects on reproductive parameters such as accelerated onset of puberty and irregular estrous cyclicity in response to MXC and DES. The current study focused on the ovary as one of the target organs for estrogenic EDCs and a potential source for the alterations in female reproductive parameters. However, estrogenic EDCs are known to affect other reproductive organs, including the hypothalamus and pituitary, which control the onset of the puberty and reproductive cycles. Through expression of neuropeptides, such as kisspeptin (Kiss1), kisspeptin neurons directly innervate and stimulate GnRH in the preoptic area of the hypothalamus [131]. Furthermore, kisspeptin neurons express the estrogen receptors which can be altered by EDCs. Thus, the effects of MXC and DES on the hypothalamus can be in part responsible for the accelerated puberty. A recent collaborative study with our lab, examining the effects of BPA, DES, and MXC on arcuate nucleus gene expression related to reproduction and energy homeostasis found MXC reduce a gene (Adipor1) expression in the arcuate nucleus, potentially reducing the inhibition of HPG axis and Kiss1, causing advance puberty [121]. This suggests that the effects of EDCs on HPG axis as related to female reproduction should be taken into consideration when explaining our current results.

In the present study, EDCs had some alteration on folliculogenesis and ovulation, which included an acceleration of follicular activation, an increasing number of atretic follicles, and reduced number of CL. In addition, we observed that MIS levels were elevated in EDC-treated ovaries. The accelerated follicular activation and increased MIS levels appear to be contradictory, which needs to be further investigated. In addition, there were increase in the number of atretic follicles. The critical role of FSH in the antral follicle survival is well known [132]. Therefore, future studies should examine the expression levels of FSH receptor in the ovary or serum FSH levels. Similarly, we have found a reduction in the number of CL, suggesting a deficiency in the ovulation, in which LHR plays a vital role. However, LHR expression did not alter. It is possible that effects on additional molecules which are upstream (e.g., serum LH levels) or downstream to LHR (e.g., molecules in the intracellular signaling pathway for LHR) should be investigated in future.

We found that a reduction of expression of P450scc in EDC-treated ovaries. While significant reduction in progesterone and strong trend in estradiol levels support this observation, other enzymes in steroidogenic pathway, such as HSD3B and P450aromatase, might be further investigated to verify the effects of EDCs on the steroidogenesis. These enzymes involve in estradiol production. In addition, the ovary expresses both estrogen receptor subtypes (ESR1 and ESR2). While both receptors have roles in ovarian functions, the role of ESR2 considered to be more ovary specific [133]. We examined the ESR1 protein levels and observed some changes in DES- and BPA-treated ovaries. We also attempted to determine the effects on ESR2, using IHC, however the antibody showed excessive non-specific binding and the results were inconclusive. To better understand the effects in the ovary, ESR2 expression should be reinvestigated with new ESR2 antibodies.

There is accumulating evidence in the field showing exposure to EDCs during development have consequences on reproductive health later in life. This concept falls within the realm of the Developmental Origins of Health and Disease or The Barker

Hypothesis. This hypothesis suggests that developmental environment, whether nutritional, chemical or psychological, have long lasting consequences on health and disease states. Epigenetic mechanisms were proposed to explain the long-lasting effects of developmental conditions, including EDC-exposures. Epigenetics is “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence” [134]. There are three common epigenetic mechanisms which are DNA methylation, post-translational modifications of histone proteins (histone modifications), and non-coding RNA (ncRNA). The most studied mechanism is DNA methylation which alters binding of the transcription factor to DNA, resulting in alteration in gene regulation. Previous work from our lab [29, 135] and others [136] have shown evidence that EDC-induced physiological and gene expression changes are associated with DNA methylation alterations in the ovary and other endocrine organs. Therefore, in order to fully understand the effects of EDCs, epigenetic changes will be studied in the future.

Along with current findings the future studies will enhance our understanding for mechanism of action of the estrogenic EDCs in the ovary and female reproductive function.

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