CHAPTER SIX

The Effect of Steroid Hormones on Ovarian Follicle Development

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Abstract

Ovarian follicular cell undergoes extensive proliferation and differentiation during the period that the follicle evolved from the primordial state to its ovulatory phase and then corpus luteum status. During this process, different and various levels of steroid hormones in follicular fluid, or obtained from circulation and adjacent cells as granulosa and theca cells, or from environment and diet will all affect the process of follicular growth and development. Differential steroid hormones might have differential effects

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on ovarian folliculogenesis via the effect on granulosa cell growth and follicular fluid formation that involves cell proliferation, apoptosis, and angiogenesis within the follicle. In this chapter we further discuss the role of various steroid hormones such as estrogens, progesterone, and androgens on ovarian follicular growth and development. Various stages of follicle development that might be disturbed by the steroid hormones are also discussed in this chapter.

1. INTRODUCTION

The progression of a primordial follicle to the ovulatory stage follicle requires nearly 1 year in female. During the course of folliculogenesis, growth is achieved by cell proliferation of granulosa cells and formation of follicular fluid, whereas development involves cell proliferation, apoptosis, and angiogenesis within the follicle. In this chapter we discuss the role of various steroid hormones such as estrogens, progesterone, and androgens on follicular growth and development.

2. ESTROGEN EFFECT ON OVARIAN FOLLICLE DEVELOPMENT

2.1 Estrogen Does Not Have a Direct Effect on Granulosa Cell Proliferation

The levels of steroid hormones in follicular fluid obtained from women with normal ovarian function are closely related to the follicular size and health of the aspirated follicles, and also correlated to the phase of the menstrual cycle in which the follicular fluid sample obtained. A significant correlation exists between the granulosa cell DNA contents and concentration of estradiol (E2) in follicular fluid. Such correlation is restricted to nonpreovulatory follicles with a diameter >6 mm. An abundance of evidence supports the notion that E2 acts as a local mitogen in follicles (Westergaard, Christensen, & McNatty, 1986).

Ovarian follicular cell undergoes extensive proliferation and differentiation from the time that the follicle escapes from the primordial state to its ovulatory capacity. A study examined the dynamic modification of the phosphorylation state of the histone H3 N-terminal tail in granulosa cells (GCs) during follicular development. Estradiol of the rat ovarian follicles induces the activity of the mitotic kinase and thus provides evidence for mitotic regulation in follicle development (Aurora et al., 2005).

Estrogen functions via activation of two estrogen receptors (estrogen receptor-alpha [ER-α] and estrogen receptor-beta [ER-β]). ER-β is widely distributed in tissues throughout the body, including tissues previously considered to be estrogen-insensitive. The epithelium of ovarian granulosa cells is ER-α-negative, but ER-β-positive and ER-β appear to be involved in important physiologic processes, such as differentiation, extracellular matrix organization, and stromal–epithelial communication. In tissues in which both ERs are expressed, the two receptors counteract each other. In the uterus, mammary gland, and immune system, ER-α promotes proliferation, whereas ER-β has proapoptotic and prodifferentiating functions (Morani, Warner, & Gustafsson, 2008).

Estrogen plays an important role in the aging of estrogen-responsive tissues. Disruption of the aromatase gene caused estrogen deficiency in mice results in a significant inhibition of telomerase maintenance of telomeres in mouse ovaries in a tissue-specific manner. Such inhibition entails a significant shortening of telomeres and compromised proliferation in the ovarian follicular granulosa cells. Decreased levels of protooncogene c-Myc and telomerase reverse transcriptase were noted in response to estrogen deficiency by gene expression analysis. Estrogen replacement therapy leads to an increase in telomerase reverse transcriptase gene expression, telomerase activity, telomere length, and ovarian tissue growth, thereby restore ovary development to normal (Bayne et al., 2011).

Primordial follicles are generated early in life and remain dormant for prolonged periods in the mammal ovaries. The growth of primordial follicles resumes via primordial follicle activation, and growth continues until the preovulatory stage under regulation of estrogen and other growth factors. The phosphatidylinositol-3 kinase (PI3K)/acute transforming retrovirus thymoma protein kinase (Akt) signaling pathway is activated in GCs through the process of ovarian follicular growth. A p110δ isoform of the PI3K catalytic subunit encoded by the Pik3cd gene knockout strategy was used to identify the role of PI3K in the development of follicle (Li et al., 2013). Pik3cd-null female mice are subfertile, exhibit fewer growing follicles and more atretic antral follicles in the ovary, and respond poorly to exogenous gonadotropins as follicle-stimulating hormone (FSH) stimulation in vitro compared with controls. p110δ isoform of the PI3K catalytic subunit is crucial for the PI3K pathway in both FSH- and E2-stimulated follicle growth in ovarian granulosa cells; however, the p110δ isoform is not required for primordial follicle activation and oocyte development (Li et al., 2013). Therefore, estrogen does not have a direct effect on GC

proliferation, which may due to the lack of ER-α; however, estrogen increases telomerase reverse transcriptase gene expression, telomerase activity, telomere length, mitotic kinase activation, and PI3K signaling, all of which are critical for follicle development.

2.2 Antiapoptosis Effect of Estrogen on Granulosa Cells

Apoptotic cell death is a mechanism underlying ovarian follicle atresia. In a study that using hypophysectomized and diethylstilbestrol (DES) capsules implanted immature rates to study the regulation of follicle cell apoptosis by sex steroids (Billig, Furuta, & Hsueh, 1993). In granulosa cells, an increase in apoptotic DNA fragmentation was seen 12 h after withdrawal of DES implants, followed by a 25-fold increase at 48 h. In situ analysis of DNA fragmentation on histologic sections of ovaries demonstrated that apoptosis induced by DES withdrawal is confined to the granulosa cells in early antral and preantral follicles. No increase in DNA breakdown was detected in thecal cells and interstitial tissues or GCs of primordial and primary follicles. In contrast, replacement with DES or estradiol benzoate completely prevented the observed ovarian weight loss and suppressed the increase in GC apoptosis. Treatment with estradiol benzoate suppressed apoptosis 2 days after removal of DES implants in a dose-dependent fashion. Furthermore, the antiatretogenic effect of estrogen was blocked by treatment with testosterone (0.5 mg twice daily), which increased ovarian apoptotic DNA fragmentation and decreased ovarian weight in DES-treated animals in a time-dependent manner. Therefore, sex steroids play an important role in the regulation of apoptotic cell death of ovary, with estrogens preventing apoptosis and androgens antagonizing the effect of estrogens (Billig et al., 1993).

Several para/autocrine effects of estrogen within the ovary include increased ovarian weight, stimulation of GC growth, augmentation of FSH action, and attenuation of apoptosis through regulation of gonadotropin synthesis and secretion from the hypothalamic–pituitary axis.

ERα is present in all three components of the murine hypothalamic–pituitary–ovarian axis. In contrast, ERβ is easily detectable in ovarian granulosa cells but is low-to-absent in the pituitary of the adult mouse. The different expression pattern for the two ERs suggests the presence of separate roles for each ER in the regulation of ovarian function. The lack of ER-α in the hypothalamic–pituitary axis of ER-α-knockout (α-ERKO) mice results in chronic elevation of serum luteinizing hormone (LH) and is the primary basis for the ovarian phenotype with features of polycystic follicles and

anovulation. Prolonged treatment with a gonadotropin releasing hormone (GnRH) antagonist reduces serum LH levels and prevents the α-ERKO cystic ovarian phenotype. To investigate a direct role for ER-α within the ovary, immature α-ERKO females were stimulated to ovulate with exogenous gonadotropins. The ovulatory capacity in immature α-ERKO females is reduced compared with age-matched wild-type; however, oocytes collected from α-ERKO mice were able to undergo successful in vitro fertilization. A similar discrepancy in oocyte yield was observed after superovulation of peripubertal wild-type and α-ERKO females. In addition, ovaries from immature superovulated α-ERKO females had several ovulatory, but unruptured follicles.

These studies indicated that treatment of α-ERKO females with a GnRH antagonist decreased the serum LH levels to within the wild-type range and also prevented development of the characteristic cystic and hemorrhagic follicle phenotype. A lack of functional ER-α within the ovary had no effect on the regulation of ER-β, LH-receptor, cyclin-D2, P450-side chain cleavage enzyme, prostaglandin synthase (PGS)-2, and progesterone receptor (PGR) which are required for follicular maturation and ovulation. Reduced number of ovulations following the administration of exogenous gonadotropins in the α-ERKO suggests an intraovarian role for ER-α in follicular development and ovulation (Couse, Bunch, Lindzey, Schomberg, & Korach, 1999).

NF-kappaB has also been implicated as an important regulator of genes controlling apoptosis, and estradiol may modulate NF-kappaB activation. After assay the NF-kappaB activity in relation to the levels of E2 and follicle growth on days 4, 6, and 8 of the first follicular wave following ovulation, the concentration of E2 in follicular fluid decreased and the activity of NF-kappaB in GCs lowered in accompany with the follicle growth. NF-kappaB activity in vitro and a minimal incidence of apoptosis (<4%), as measured by the Annexin V and TUNEL assays, are associated with production of E2; however, inhibition of NF-kappaB in vitro leads to suppression of apoptosis. These results indicate that follicular NF-kappaB activation is associated with E2 production (Valdez & Turzillo, 2005). Experiments were also conducted to test whether or not E2 protects GCs from Fas ligand (FasL)-induced apoptosis and whether or not protection involves modulation of the cell cycle of proliferation. Treatment of cultured bovine GCs with E2 decreases susceptibility to FasL-induced apoptosis. The effects of E2 are mediated through the estrogen receptor and are not mediated by stimulation of IGF production. E2 also increases the

percentage of cells progressing from the G1-to-S phase of the cell cycle and increases expression of cyclin D2 protein and the cell proliferation marker, Ki67. Progression from the G1-to-S phase of the cell cycle was necessary for the protective effect of E2. Blocking progression from the G1-to-S phase with the cdk2 inhibitor, roscovitine, or blocking cells in the S phase with hydroxyurea, prevents protection by E2. E2 stimulates transition from the G1-to-S phase and protects against apoptosis in ovarian granulosa cells only when cell cycle progression is unperturbed (Quirk, Cowan, & Harman, 2006).

Complementary DNA array was used to study the pattern of ovarian mRNA expression from day-2 and -4 Swiss mice to study the genes involved in ovarian primordial-to-primary follicle transition. The expression profiles of 1176 genes in neonatal mouse ovaries on days 2 and 4, which contain primordial and primary follicles, respectively, were compared. The results revealed that 26% of the genes were differentially expressed between day-2 and -4 ovaries, with 19% upregulated and 7% downregulated on day 4. Analysis of differentially expressed genes revealed that the primordial-to-primary follicle stage transition is associated with induction in the expression of mainly growth factors, immune-related factors, hormone and hormone receptors, and signal transducers. The transition is also associated with proliferation of granulosa cells and absence of apoptosis. The primary follicles express ER-β and are responsive to estrogen actions in vitro with respect to the increase in the number of primary follicles and GC proliferation (Dharma, Modi, & Nandedkar, 2009).

The antifolliculogenic effect of oral dienogest was evaluated by using female cynomolgus monkeys started since day 7 of the menstrual cycle. In this study, the plasma E2 level declined within 24 h after dinogest treatment, while dienogest did not decrease the FSH level prior to the E2 decline. After the decline in the E2 level, the low level of E2 was sustained for >11 days. It is thought that a single oral dose of dienogest induces atresia of the dominant follicle. On histologic examination, three animals that showed a decline in the E2 level also revealed apoptotic changes in GCs with scattered aromatase expression in the ovarian dominant follicles. Such finding suggested that dienogest inhibits the plasma E2 level by inducing the atresia of the ovarian dominant follicle directly (Sasagawa et al., 2008).

During mouse embryonic development, oocytes develop in germline cysts, formed by several rounds of cell division and incomplete cytokinesis. Shortly after birth, cysts break down and individual oocytes are enclosed by

GCs to form primordial follicles. At the same time, two-thirds of the oocytes die by apoptosis, with only one-third surviving. The steroid hormones, E2 and P4, as well as the phytoestrogen, genistein, inhibit cyst breakdown and primordial follicle assembly. E2 could exert its effect through the effects of membrane-bound estrogen receptors to inhibit the cyst breakdown and therefore involved in estrogen signaling in neonatal oocyte development (Chen, Breen, & Pepling, 2009).

The cytotoxic drug, cis-platinum, could induce premature ovarian failure by reducing the viability of human granulosa cells. Steroid hormones as E2 and P4 decreased in human granulosa-luteal cells which incubated with cis-platinum. The E2 production was more pronounced than P4 with cis-platinum treatment. Such findings demonstrated that cis-platinum induces apoptosis of human granulosa-luteal cells in culture with impaired steroidogenesis, which may be one mechanism by which a cis-platinum-containing regimen induces premature ovarian failure (Chatterjee, Helal, Mobberley, Ryder, & Bajoria, 2014).

CCAAT enhancer binding proteins (CEBP)-β has been identified in the ovary and is critical for follicular growth, ovulation, and luteinization in mice. In the CEBP-β gene knocked down porcine GCs, knockdown of CEBP-β significantly increased the expression of p-ERK1/2 and arrested the GCs at the S phase of the cell cycle, but had no effects on cell apoptosis. More importantly, knockdown of CEBP-β markedly downregulated the expression of E2 and P4 in the culture medium. Messenger RNA expression of bcl-2 (antiapoptosis), StAR, and Runx2 (steroid hormone synthesis) was upregulated, while genes related to apoptosis (caspase-3 and p53), hormonal synthesis (CYP11A1), and cell cycle (cyclinA1, cyclinB1, and cyclinD1) were downregulated, suggesting that knockdown of CEBP-β may inhibit apoptosis, and regulate the cell cycle and hormone secretions at the transcriptional level in porcine GCs (Zhen et al., 2014).

2.3 Effect of Estrogen on Follicular Angiogenesis

The rapid, controlled, and cyclic nature of angiogenesis in the ovarian follicle suggests the potential for sex steroids to influence neovascularization. Angiogenesis is regulated by a local balance between the levels of endogenous stimulators and inhibitors. There is limited data regarding the direct effect of estrogen on angiogenesis of ovarian GCs; however, existing evidence suggests that estrogens stimulate angiogenesis via effects on endothelial cells. Estradiol-17beta [E(2)β] and its metabolites, which are sequentially

synthesized by cytochrome P450s and catechol-*O*-methyltransferase (COMT) to form 2- and 4-hydroxyestradiol [OHE(2)] and 2- and 4-methoxestradiol [ME(2)], are elevated during pregnancy. A study investigated whether or not cytochrome P450s and COMT are expressed in uterine artery endothelial cells, and whether or not E(2)β and its metabolites modulate cell proliferation via ER-α and/or ER-β and play roles in physiologic uterine angiogenesis during pregnancy by using the 5-bromodeoxyuridine proliferation assay (Jobe et al., 2010). Using confocal microscopy and Western blot analyses to determine enzyme locations and levels, we observed CYP1A1, CYP1A2, CYP1B1, CYP3A4, and COMT expression in uterine artery endothelial cells; however, all of these expressions were similar between nonpregnant and pregnant uterine artery endothelial cells. E(2)β, 2-OHE(2), 4-OHE(2), and 4-ME(2) treatments stimulated proliferation in pregnant, but not nonpregnant uterine artery endothelial cells in a concentration-dependent fashion; 2-ME(2) did not stimulate proliferation in either cell type. Proliferative responses of pregnant uterine artery endothelial cells to E(2)β were solely mediated by ER-β, whereas responses to E(2)β metabolites were neither ER-α- nor ER-β-mediated. This study demonstrated an important vascular role for E(2)β, its cytochrome P450- and COMT-derived metabolites, and ER-β in uterine angiogenesis regulation during pregnancy that may be dysfunctional in preeclampsia and other cardiovascular disorders (Jobe et al., 2010).

Vascular endothelial growth factor (VEGF), another factor that plays a pivotal role in the regulation of microvascular permeability and angiogenesis, processes essential for normal endometrial growth and implantation. Estrogen could stimulate VEGF expression in the uterus at the transcriptional level; however, the VEGF gene promoter lacks a consensus estrogen response element. Chromatin immunoprecipitation showed that both Sp1 and Sp3 were associated with a proximal, GC-rich region of the promoter of VEGF gene before E2 treatment. Both the α- and β-subunits of the transcription factor, hypoxia-inducible factor 1 (HIF-1), which regulates VEGF expression in response to hypoxia and several hormones and growth factors, were present in the uterus. E2 can induce recruitment of the α- and β-subunits of HIF-1 to the VEGF promoter, which contains the hypoxia response element to which HIF-1 binds. This binding was transient, matching the pattern of E2-induced VEGF expression. These results indicate that HIF-1 is an important mediator of E2-induced VEGF expression in the uterus (Kazi, Jones, & Koos, 2005).

2.4 Antiangiogenesis Effect of Estrogen Metabolites

2-Methoxyestradiol (2-ME) is an estradiol metabolite with antiangiogenic and antitumor activity. 2-ME is formed by GC COMT activity and is present in the normal follicle at high concentrations. In this unique microenvironment, 2-ME may regulate selected cell types via autocrine and/or paracrine action. By in vitro culture studies using primary cultures of hormone- and/or growth factor-stimulated porcine GCs, as well as two types of endothelial cells, primary cultures of porcine endothelial cells, and a spontaneously transformed rabbit endothelial vascular cell line, 2-ME or E2 exert differential mitotic and/or apoptotic effects on endothelial cells and GC (Shang, Konidari, & Schomberg, 2001). The 2-ME, but not E2, suppressed epidermal growth factor-stimulated rabbit endothelial vascular cells and epidermal growth factor/insulin-stimulated porcine endothelial cell proliferation. In contrast, 2-ME did not attenuate FSH/INS-stimulated porcine GC proliferation. E2 was not antimitotic to porcine GCs and was nonapoptotic in either cell type. 2-ME significantly increased apoptosis of rabbit endothelial vascular cells and attenuated the cell movement. These results show that 2-ME may have autocrine and/or paracrine action at its site of production and as a candidate ovarian angiogenesis inhibitor (Shang et al., 2001).

Cultured GCs are able to produce 2-ME under hypoxic conditions. 2-ME could be a local effector in determining the fine tuning responsible for follicle angiogenesis (Basini et al., 2011). A study was conducted to quantify 2-ME in human corpus lutea (CL) of different ages, determine the expression of cytochrome-P450-1A1 (CYP1A1) and COMT in CL, and clarify the action of 2-ME on VEGF secretion and luteal angiogenesis. CL were collected from 15 women via minilaparotomy for tubal sterilization. Granulosa lutein cells were harvested 36 h after hCG administration in patients undergoing IVF. The results demonstrated that plasma levels of E2 decreased in the late luteal phase in association with an increase in luteal tissue 2-ME concentrations. There was a significant reduction of angiogenic activity in late CL. There was no significant variation in CYP1A1 and COMT expression in all CL. At physiologic doses, 2-ME inhibited basal VEGF by granulosa lutein cells and diminished the angiogenic activity in conditioned media, but did not prevent VEGF production stimulated by hCG. The study demonstrated the role of 2-ME in physiologic luteolysis by reducing angiogenesis through inhibition of new vessel growth (Basini et al., 2008; Kohen et al., 2013).

3. ANDROGEN EFFECT ON OVARIAN FOLLICLE DEVELOPMENT

3.1 Apoptotic Phenomenon in Follicles and Corpora Lutea

Follicles and CL were evaluated for the presence of DNA fragmentation and characteristics of apoptosis in a clinical study that enrolled 116 human ovarian follicles (primordial to dominant) and 5 CL from a total of 27 premenopausal women (Yuan & Giudice, 1997). Follicle functional status was evaluated by determining follicle sizes and follicular fluid androgen-to-estrogen (A:E) ratios. No apoptosis was observed in 67 primordial, primary, or secondary follicles. Positive staining for DNA fragmentation was found in a limited number of GCs in 0.1–2 mm follicles, whereas abundant staining in GCs was detected in 2.1–9.9 mm follicles. In contrast, no DNA fragmentation was detected in dominant follicles (10–16 mm). The frequency of apoptosis in follicles was calculated to be 37% in 0.1–2 mm follicles, 50% in 2.1–5 mm follicles, and 27% in 5.1–9.9 mm follicles. Abundant low-molecular weight DNA laddering was only found in androgen-dominant follicles and not in estrogen-dominant follicles. Positive staining for DNA fragmentation and low-molecular weight DNA laddering was observed in degenerating, but not healthy appearing CL. In the former, DNA fragmentation was found primarily in large luteal cells. These data suggest that follicular atresia in human ovaries results from normal programmed cell death and primarily occurs in the GC layers of the early antral and antral follicles <10 mm in size. Furthermore, because apoptosis occurs as early as the 200-mm stage, follicle selection may begin as early as the initial formation of the antrum. The results also suggest that degeneration of the CL occurs by apoptotic mechanisms (Yuan & Giudice, 1997).

3.2 Nuclear Progesterone Receptor Mediates the Susceptibility to Apoptosis in Luteinizing GCs

A study investigated if PGR-mediated effects are involved in regulating the susceptibility to apoptosis in LH receptor-stimulated human luteinizing GCs (Svensson, Markström, Shao, Andersson, & Billig, 2001). Luteinizing GCs were isolated from follicular aspirates after oocyte removal. The cells were treated with or without PGR antagonist, progesterone, androgens, or steroid to evaluate their effects.

Addition of the PR antagonists (RU 486 or Org 31710) in vitro to human luteinizing GCs caused an increase in caspase-3 activity and an increase in

internucleosomal DNA fragmentation. No effect on DNA fragmentation was noted after addition of dexamethasone, dihydrotestosterone, or picrotoxin. Such findings revealed that nuclear PGR-mediated effects are involved in regulating the susceptibility to apoptosis in LH receptor-stimulated human luteinizing GCs (Svensson et al., 2001).

3.3 Granulosa Cells Significantly Decreased With Age

The incidence of apoptotic GCs associated with the women's age on ovarian fecundity. In a study that enrolled 21 normoovulatory women underwent ovulation induction for standard IVF and were divided into four groups according to their ages. Granulosa cells were isolated by follicle aspiration after the ovarian hyperstimulation with gonadotrophins and tested for the incidence of apoptosis by using fluorescence microscopy (Sadraie, Saito, Kaneko, Saito, & Hiroi, 2000). In this study, GCs in the older women revealed a significant increase in the number of apoptotic cells. Though the endometrial thickness and follicular E2, P4, and free testosterone levels were not significantly different among the four different age groups, the number of total oocytes and the number of mature oocytes obtained significantly decreased with age (Sadraie et al., 2000).

3.4 Androgens Stimulate Early Primate Follicle Development

Excess androgens are associated with a characteristic morphology of polyfollicular ovaries. However, if such feature is due to direct androgen action on follicular development or due to interference with gonadotropin release at the level of the pituitary or hypothalamus is not clear. To elucidate potential androgen effects on the ovary, using in situ hybridization, the cellular localization of androgen receptor (AR) mRNA was studied in rhesus monkey. Researchers compared the relative abundance of AR transcripts in monkeys during follicular and luteal phases of the menstrual cycle and in monkeys treated with testosterone to investigate the regulation of ovarian AR gene expression (Weil et al., 1998). AR mRNA expression was most abundant in GCs of healthy preantral and antral follicles in the primate ovary. Theca interna and stromal cells also expressed AR mRNA, but to a lesser degree than GCs. No significant cycle stage effects were noted in AR mRNA levels. The AR mRNA level was significantly increased in GCs and decreased in theca interna and stromal cells of testosterone-treated monkeys. GC AR mRNA abundance was positively correlated with expression of the proliferation-specific antigen Ki-67 and negatively correlated

with GC apoptosis. These data show that primate ovary AR gene expression is most abundant in GCs of healthy growing follicles, where its expression is upregulated by testosterone. The positive correlation between granulosa AR gene expression and cell proliferation and the negative correlation with programmed cell death suggest that androgens stimulate early primate follicle development (Weil et al., 1998).

In sheep, excess prenatal testosterone leads to increased ovarian follicular recruitment and persistence, and finally leads to reproductive dysfunction. In a study which injected twice weekly with testosterone propionate or dihydrotestosterone (DHT) propionate from days 30 to 90 of gestation in pregnant sheep to investigate the follicular disruption and the developmental ontogeny of ovarian proliferation and apoptotic factors (Salvetti, Ortega, Veiga-Lopez, & Padmanabhan, 2012). Prenatal testosterone treatment induced changes in expression of proliferative and apoptotic markers by assessing the changes in ovarian expression of proliferating cell nuclear antigen (PCNA), BCL2, BAX, activated CASP3, and FAS/FASLG at prenatal, postnatal, prepubertal, and after puberty stages. Prenatal testosterone, but not DHT, increased PCNA and decreased BCL2 in granulosa/theca cells of antral follicles postnatally at 10- and 21-month age, but decreased CASP3 in granulosa/theca cells of antral follicles at prepubertal and after puberty. Both treatments decreased BAX immunostaining in GCs of fetal primordial/primary follicles. No differential effect of FAS expression at any developmental time point in any follicular compartment. The effects on BAX appear to be programmed by androgenic actions and PCNA, BCL2, and CASP3 by estrogenic actions of testosterone. Overall, the findings demonstrate that fetal exposure to excess testosterone disrupts the ovarian proliferation and apoptosis balance, thus providing a basis for the follicular disruptions (Salvetti et al., 2012).

3.5 Androgen Factors Involved in Follicular Arrest

The effects of testosterone, DHT, and 17β-estradiol on human ovarian stromal tissue survival in culture and their effects on cell death were investigated by tissue cultures (Otala et al., 2004). The study revealed that ARs were detected in the ovarian stroma and GCs of the primordial follicles, although they were more clearly seen in primary follicles and more advanced stage follicles. Testosterone only marginally suppressed ovarian tissue apoptosis in vitro. DHT was more effective than testosterone, whereas 17β-estradiol had no notable effect on the viability of the tissue. The effects of androgens

on the ovarian tissue may be mediated through ARs because blocking the receptors with an AR antagonist reversed the suppressive effect of DHT (Otala et al., 2004; Salvetti et al., 2012).

Our previous study investigated the effects of androgens on metabolism, as well as factors involved in follicular arrest and the reduced number of estrus cycles (Chen et al., 2015). The DHT-treated rats had fewer estrus cycles, a higher number of large arrested follicles, and an increase in body weight gain compared with dehydroepiandrostenedione (DHEA)- and placebo-treated rats. In cultured rat GCs, DHT suppressed FSH-induced GC proliferation and increased the accumulation of cells in the G2/M phase. DHT decreased phosphorylated Akt (p-Akt) and cyclin D1 levels by increasing PTEN. DHT-promoted PTEN expression was regulated by peroxisome proliferator-activated receptor-gamma (PPAR-γ) in GCs. In the large follicles of the DHT-treated rats, the expression of PPAR-γ and PTEN was higher, but the expression of p-Akt and PCNA was lower. Thus, DHT and DHEA produced differential effects on metabolism in prepubertal female rats, such as the clinical manifestations of women with PCOS. DHT treatment may affect ovarian follicular maturation by altering GC proliferation via the regulation of enhancing PPAR-γ-dependent PTEN/p-Akt expression in the GCs (Chen et al., 2015). Basic studies have implied that DHT may be useful for enhancing human ovarian tissue survival in vitro.

4. PROGESTERONE EFFECT ON OVULATION

4.1 Clinical Evidence of Progesterone Effect on Ovulation

The relationships and interindividual variations in urinary and serum reproductive hormone levels relative to ultrasound-observed ovulation in menstrual cycles of normally menstruating women were studied previously (Roos et al., 2015). This study enrolled 40 normally menstruating and aged between 18- and 49-year-old women without known subfertility. The enrolled subjected collected daily urine and bloody samples for hormone profiles and transvaginal ultrasound for a complete menstrual cycle. Serum LH, P4, E2, urinary LH, pregnanediol-3-glucuronide, and estrone-3-glucuronide were measured. As shown in this close observational study (Roos et al., 2015), menstrual cycle length varied from 22 to 37 days (median, 27 days) and ovulation that was evidenced by ultrasound ranged from day 8 to 26 (median, day 15). Serum and urinary hormone profiles showed excellent agreement. Estrogen and LH peaks in urine and serum showed a range of signal characteristics across the study group before

and after ovulation. The rise in estrogen and LH always occurred before ovulation; the rise in P4 from baseline always occurred after ovulation. The study demonstrated that the beginning of the surge in serum and urinary LH was an excellent predictor of ovulation. The rise in P4 and pregnanediol-3-glucuronide above baseline was a consistent marker of luteinization, thus confirming ovulation. Both LH and P4 surges delivered clear, sharp signals in all volunteers, allowing reliable detection and confirmation of ovulation (Roos et al., 2015).

In a clinical study that enrolled 302 women aged between 20 and 40 years old and classified into two groups according to ovulatory status (Sanchez et al., 2016). This study was conducted to assess the correlation between low levels of P4 and ovulation by ultrasound monitoring in infertile patients with regular menstrual cycles. All related factors as age, body mass index, duration of infertility, FSH, thyroid-stimulating hormone, LH, and E2 were comparable between two groups. The study results revealed that there was a significant association between the percentage of ovulation by ultrasound monitoring and the percentages of patients who presented with low levels of P4. The study suggested that low serum levels of P4 are associated with a low percentage of ovulation in infertile women with regular menstrual cycles and women with unexplained infertility (Sanchez et al., 2016).

Another clinical study was conducted to identify an accurate P4 threshold to confirm ovulation in the assessment of a woman's fertility (Leiva, Bouchard, Boehringer, Abulla, & Ecochard, 2015). The study included 107 women over 326 menstrual cycles and tracked daily first morning urine, changes in observed cervical mucus discharge, serum P4, and ultrasonography to identify the day of ovulation. The study revealed that a random serum P4 level >5 ng/mL confirmed ovulation (Leiva et al., 2015).

4.2 Molecular Mechanism of Progesterone Effect on Ovulation

Ovulation is a precisely timed process by which a mature oocyte is released from an ovarian follicle. This process is initiated by the pituitary surge of LH, temporally associated with transcriptional regulation of numerous genes, and is presumed to involve the synthesis and/or activation of specific proteases that degrade the follicle wall. The PGR, a nuclear receptor transcription factor, is induced in GCs of preovulatory follicles in response to the LH surge and has been shown to be essential for ovulation. P4 is activated through the PGR. The study conducted on nonluteinized GCs collected from monkeys undergoing controlled ovarian stimulation protocols demonstrated that the

nuclear PGR appears to mediate P4 action in the dominant follicle promoting ovulation in primates. The in vitro and in vivo effects of PGR knockdown in luteinized granulosa cells also support the hypothesis that P4 enhances its own synthesis in the primate CL by promoting luteinization (Bishop, Hennebold, Kahl, & Stouffer, 2016).

Mice lacking PGR fail to ovulate and are infertile. In a study that using mice model to elucidate PGR-regulated genes in the ovulation process, matrix metalloproteinases (MMP-2 and MMP-9) were found not being targets of PGR during ovulation, but the other two proteases (ADAMTS-1 [a disintegrin and metalloproteinase with thrombospondin-like motifs] and cathepsin L [a lysosomal cysteine protease]) were noted to be involved in the PGR action as transcriptional targets (Robker et al., 2000). ADAMTS-1 is induced after LH stimulation in GCs of preovulatory FSH, but the highest levels of cathepsin L mRNA occur in preovulatory follicles in response to LH in a PGR-dependent manner. The identification of two regulated proteases in the ovary, together with abnormal expression in anovulatory PGR knockout mice, suggests that each plays a critical role in follicular rupture and represents a major advance in our understanding of the proteolytic events that control ovulation (Robker et al., 2000).

Progesterone acting through its nuclear receptor plays an essential role in ovulation by mediating the expression of genes involved in ovulation and/or luteal formation (Mishra, Park, Wilson, & Jo, 2015). Microarray analysis using rat GCs treated with hCG with and without PGR antagonist (RU486) showed that the transcript of Xlr5c-like mRNA was downregulated by RU486 (Mishra et al., 2015). The Xlr5c-like mRNA level is transiently upregulated in GCs of periovulatory follicles after hCG stimulation in PMSG-primed rat ovaries. The transient induction of Xlr5c-like mRNA was mimicked by hCG treatment in cultured GCs from preovulatory ovaries. LH-activated PKA, MEK, PI3K, and p38 signaling is involved in the increase in Xlr5c-like mRNA, while the increase in Xlr5c-like mRNA was abolished by RU486. The inhibitory effect of RU486 was reversed by synthetic progestin (medroxyprogesterone), but not by dexamethasone. Furthermore, mutation of SP1/SP3 and PGR response element sites in the promoter region of Xlr5c-like decreased Xlr5c-like reporter activity. RU486 also inhibited Xlr5c-like reporter activity. A ChIP assay verified binding of PGR and SP3 to the Xlr5c-like promoter in periovulatory GCs. Xlr5c-like knockdown in GC cultures resulted in reduced levels of mRNA for Snap25, Cxcr4, and Adamts1. Progesterone/PGR mediates the LH-induced increase in Xlr5c-like mRNA.

In turn, Xlr5c-like is involved in regulating the expression of specific ovulatory genes, such as Snap25, Cxcr4, and Adamts1, possibly acting in the nucleus of periovulatory GCs (Mishra et al., 2015).

There are three types of local factors induced by the mid-cycle LH surge and play a critical role in ovulation and/or luteinization of the primate follicle (Stouffer, Xu, & Duffy, 2007). The ovulatory gonadotropin surge increases prostaglandin (PG) levels in follicles prior to rupture. LH stimulation lead to the synthesis of the "inducible" form of PG G/H synthase (PGS2), other aspects of PG synthesis (notably a phospholipase A2 [cPLA2], and a PGE synthase [PGES]) and metabolism (15-hydroxy PG dehydrogenase [HPGD]) also appear to be LH-regulated and may control the timing of the PG rise in the ovulatory follicle. Intrafollicular ablation and replacement of PGs suggest that PGE2 is essential for release of the oocyte, but not necessary for follicle rupture or luteinization. Novel PGE-regulated genes have been identified in macaque GCs, including adipose differentiation-related protein. Similar studies indicate that the rise in P4 synthesis, as well as the induction of the genomic PGR in GCs, is essential for both ovulation and luteinization of the primate follicle. Progesterone could control cell cycle activity (via cyclin B1 and cyclin-dependent kinase inhibitor p27), lipid metabolism, proteases, and protease inhibitors (matrix metalloproteinase or MMP1; tissue inhibitor of MMP or TIMP1) and cell health in the GC layer. Two classes of angiogenic factors, originally proposed as important for embryonic and pathologic (tumorigenic) vasculogenesis, appear induced in the granulosa layer of the preovulatory follicle, i.e., VEGF and angiopoietin (ANGPT). Local injection of antagonists to VEGF (soluble VEGF receptor) and ANGPT (the natural antagonist ANGPT2) into the preovulatory follicle suppressed ovulation and luteinization in monkeys, possibly by disrupting the structure–function of existing vessels or preventing angiogenesis in the avascular granulosa layer (Stouffer et al., 2007).

4.3 Progesterone Increased Immune Protection When Fertilization and Implantation

To evaluate the leukocyte count on the day of ovulation, blood samples were obtained from healthy and regularly cycling women at the following three times: during menstruation, ovulation, and in the mid-luteal phase to investigate the levels of white blood cells (WBCs), neutrophils, lymphocytes, mixed cells, P4, and E2. The results demonstrated that compared to menstruation, WBCs and neutrophils increased around ovulation and remained stable in the mid-luteal phase, whereas lymphocyte and mixed cell counts did not change throughout the menstrual cycle. As for the association

between sex hormone variation with leukocyte changes and menstruation and ovulation, it was found a positive correlation existed between E2 and WBC, and negative correlations existed between P4 and WBCs and between P4 and the neutrophil count, but not between ovulation and the mid-luteal phase. The results showed that peripheral leukocyte changes taking place in the second half of the cycle are already observable on the day of ovulation and are associated with sex hormone variation. For such reason, it could be speculated that these changes accompanied with elevated progesterone levels may lead to increased immune protection against pathogens at a time when fertilization and implantation occur (Nowak, Borkowska, & Pawlowski, 2016).

A study was conducted to characterize the immune cell types within the primate corpus luteum (CL, 36). Luteal tissue was collected from rhesus females at discrete intervals during the luteal phase of the natural menstrual cycle and incubated with fluorescently labeled antibodies specific for immune cell surface proteins (CD11b [neutrophils and monocytes/macrophages], CD14 [monocytes/macrophages], CD16 [natural killer {NK} cells], CD20 [B-lymphocytes], and CD3epsilon [T-lymphocytes]) for analysis by flow cytometry. The results revealed that the number of CD11b(+) and CD14(+) cells increased significantly 3–4 days after serum P4 concentrations declined to <0.3 ng/mL. CD16(+) cells were the most abundant immune cell type in the CL during the mid- and mid–late luteal phases and were threefold increased 3–4 days after serum P4 decreased to baseline levels. CD3epsilon(+) cells tended to increase 3–4 days after P4 decline. The number of CD11b(+) and CD14(+) cells of CL increased after GnRH antagonist treatment, whereas progestin, but not estrogen, replacement suppressed the number of CD11b(+), CD14(+), and CD16(+) cells. The number of CD3epsilon(+) cells of CL was not changed with either GnRH antagonist or steroid treatment. These data suggest that an increased number of innate immune cells in primate CL after P4 synthesis declines play a role in onset of structural regression of the primate CL (Bishop, Xu, Molskness, Stouffer, & Hennebold, 2015).

4.4 Effects of Estradiol, Progesterone, and Corpus Luteum on the Dominant Follicle

To determine the effects of E2, P4, presence of a CL, and size of a dominant follicle on the characteristics and patterns of GnRH-induced LH release and subsequent ovulation, there was a study using lactating dairy cows with a GnRH-induced LH release and hourly collected blood samples for hormone assay were analyzed (Stevenson & Pulley, 2016). Injections of GnRH

induced LH release during 6 h after each injection in all study subjects. The concentrations of LH induced by GnRH were inhibited at greater concentrations of P4 and in the presence of a CL. In contrast, GnRH-induced LH concentrations were increased when E2 was ≥4.0 pg/mL, but relatively unaffected by the size of the dominant follicle. Furthermore, the resulting incidences of ovulation were decreased at greater P4 concentrations and the presence of a CL, and increased at greater E2 concentrations and presence of a dominant follicle. In cows with or without a CL, the presence a dominant follicle did not increase the mean LH concentration or incidence of ovulation. While when premature P4 concentration was elevated at the time of GnRH treatment, subsequent LH concentrations and ovulation were suppressed. At a lower concentration of P4 or at higher concentration of E2, the pregnancy outcomes after timed artificial insemination were improved with similar incidence of ovulation. Such findings suggest a greater P4 or lesser at the time of artificial insemination may suppress pregnancy chance by other mechanisms.

Ovarian steroid hormones are major regulators of the physiology of the oviduct and reproductive events occurring within the oviduct. A study was conducted to establish an entire steroid profiling of the bovine oviductal fluid during the estrous cycle (Lamy et al., 2016). Contralateral and ipsilateral (to the CL or preovulatory follicle) oviducts of cows were classified into four stages of the estrous cycle: postovulatory, mid-luteal, late luteal, and preovulatory on the basis of the ovarian morphology and intrafollicular steroid concentrations. The concentrations of P4 in ipsilateral oviductal fluid increased from postovulatory to mid-luteal, then decreased from late-luteal to preovulatory, and were 4–16 times higher than the contralateral oviductal fluid. Most P4 metabolites followed similar patterns of variation. The concentrations of preovulatory estradiol-17β were significantly higher compared with all other stages, with no difference regarding the side of ovulation. Concentrations of androstenedione displayed a pattern similar to that of E2, whereas other androgens, estrone, and corticoids did not vary between stages or sides. The results demonstrated a highly concentrated and fluctuating hormonal environment in the bovine oviductal fluid (Lamy et al., 2016).

5. CONCLUSION

Degeneration of the CL occurs by apoptotic mechanisms. The nuclear PGR-mediated effects are involved in regulating the susceptibility to apoptosis in LH receptor-stimulated human luteinizing GCs. Apoptotic GCs increased while the number of total oocytes and the number of mature

oocytes significantly decreased with age. However, E2, P4, and free testosterone levels were not significantly changed with age. The positive correlation between granulosa AR gene expression and cell proliferation and negative correlation with apoptosis suggest that androgens stimulate early primate follicle development; however, fetal exposure to excess testosterone disrupts the ovarian proliferation/apoptosis balance. DHT may be useful for enhancing human ovarian tissue survival in vitro.

The surge of serum P4 is a signal for confirmation of ovulation. The level of P4 or E2 is critical for the time of artificial insemination. The expression of the PGR is critically involved in proteolytic events that control ovulation. Progesterone/PGR mediate the LH-induced increase in the expression of specific ovulatory genes. The effect of P4 mediates immune protection against pathogens at a time when fertilization and implantation typically occur. Understanding the cascade of P4 leading to ovulation and luteinization of the primate follicle may help to consider novel ovary-selective approaches to human reproduction.

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