ORIGINAL ARTICLE

Early ovarian follicular development in prepubertal Wistar rats acutely exposed to androgens

L. Paixão[1], L. M. Velez[2], B. R. Santos[1], C. Tusset[1], S. B. Lecke[1,3], A. B. Motta[2] and P. M. Spritzer[1,4]*

[1]*Gynecological Endocrinology Unit, Division of Endocrinology, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil*
[2]*Laboratorio de Fisio-patología Ovárica, Centro de Estudios Farmacológicos y Botánicos, Universidad de Buenos Aires, Buenos Aires, Argentina*
[3]*Department of Diagnostic Methods, Federal University of Health Sciences of Porto Alegre, Porto Alegre, Brazil*
[4]*Laboratory of Molecular Endocrinology, Department of Physiology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil*

Androgens may directly modulate early ovarian follicular development in preantral stages and androgen excess before puberty may disrupt this physiological process. Therefore, the aim of this study was to investigate the dynamics of follicular morphology and circulating androgen and estradiol levels in prepubertal Wistar rats acutely exposed to androgens. Prepubertal female Wistar rats were distributed into three groups: control, equine chorionic gonadotropin (eCG) intervention and eCG plus dehydroepiandrosterone (DHEA) intervention (eCG + DHEA). Serum DHEA, testosterone and estradiol levels were determined, and ovarian morphology and morphometry were assessed. The eCG + DHEA group presented increased serum estradiol and testosterone levels as compared with the control group ($P < 0.01$), and higher serum DHEA concentration *v.* the eCG-only and control groups ($P < 0.01$). In addition, the eCG + DHEA group had a higher number of, and larger-sized, primary and secondary follicles as compared with the control group ($P < 0.05$). The eCG group presented intermediate values for number and size of primary and secondary follicles, without significant differences as compared with the other two groups. The number of antral follicles was higher in the eCG + DHEA and eCG groups *v.* controls ($P < 0.05$). The number of primordial, atretic and cystic follicles were similar in all groups. In conclusion, the present experimental model using an acute eCG + DHEA intervention was useful to investigate events involved in initial follicular development under hyperandrogenic conditions, and could provide a reliable tool to study defective follicular development with possible deleterious reproductive consequences later in life.

Introduction

Ovarian follicular development is crucial to female reproductive healthiness and this event depends on several factors such as growth factor and hormone signals. At the beginning, the transition of primordial follicles to secondary stages is controlled by some growth factor as bone morphogenic protein 15 (BMP15), kit ligand, BMP4 and hormones as insulin and growth hormone. All of them are important to maintain oocytes and granulosa cells (GCs) survival through initial follicular development.[1] Physiological levels of androgens have also been linked to a role on modulating the expression and activity of genes related to growth and development of ovarian follicles in mammals.[2,3]

In turn, androgen excess may disturb physiological mechanisms of follicular development leading to chronic anovulation, as observed in human clinical diseases. One example is the polycystic ovary syndrome (PCOS), a prevalent condition in which impaired follicular growth and high circulating androgen levels lead to menstrual disturbances and anovulatory infertility.[4]

Evidence indicates that prenatal or prepubertal androgen excess may be involved with the pathophysiology of PCOS.[5–7] In addition, some animal models of PCOS have been developed by using experimental exposure of high androgen levels.[8–12] In this sense, the study of ovarian dysfunction promoted by animal models of androgen interventions may provide interesting insights into the initial pathogenic mechanisms of PCOS. Indeed, the pathological ovarian features of PCOS include arrest of follicular development with accumulation of early and mid-antral stage follicles.[10] Abnormalities in cellular investments of the ovarian follicle, such as theca cell (TC), GC and oocyte layers during early follicular development may be a primary cause of ovarian dysfunction leading to reduced responsiveness to follicle-stimulating hormone (FSH) and luteinizing hormone stimulation.[13] Nevertheless, it is not clear whether reproductive hormone abnormalities are a primary or secondary reflex of follicular disruption, or whether and to what degree androgen over-production may modulate GCs activity.[14]

Because of these many uncertainties, animal models are still crucial for the study of PCOS. In fact, the observation that dehydroepiandrosterone (DHEA) levels are increased in women with PCOS[15] led to the development of a PCOS animal model using postnatal treatment with DHEA, the first androgen to rise in the early female pubertal period.[16]

*Address for correspondence: P. M. Spritzer, Division of Endocrinology, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, 90035 003, Porto Alegre, RS, Brazil.
(Email spritzer@ufrgs.br)

This postnatal DHEA treatment produced different degrees of ovarian morphological changes, such as cystic ovaries with thin GC layer and consistent with anovulation,[17–20] increased ovarian stroma and increased number of atretic follicles. Animals also exhibit altered steroidogenesis with high serum estrogen and androgen levels.[21] However, these animal models are limited by the need for long experimental periods, precluding the observation of direct androgen effect on early follicular growth and development. Thus, animal models using short periods of follicular stimulation would be useful to investigate this aspect.

A rodent model of acute gonadotropin hyperstimulation and hyperandrogenism has been recently developed.[22–24] In that experimental model, a two-hormone treatment was administered to immature female animals aged 22–25 days: equine chorionic gonadotropin (eCG), an efficient interspecies inductor of follicular recruitment,[25] and DHEA, in order to promote a hyperandrogenic condition.[24] However, that study did not focus on the prepubertal stage, and therefore missed the opportunity to promote a folliculogenesis spurt after the onset of physiological follicular development in the ovaries. In the present study, we aimed to investigate the dynamics of follicular morphology and ovarian function in prepubertal Wistar rats, aged 25–28 days, acutely treated with the same previously validated concentrations of eCG and DHEA.

Materials and methods

All procedures involving animals were conducted in accordance with local regulations (National Council on Research Animal Care, CONCEA, Brazil). The study was approved by the Ethics Committee at Hospital de Clínicas de Porto Alegre (Brazil).

Experimental animal and procedures

In total, 20 prepubertal (25–28 days old) female Wistar rats were divided into three groups, that received one single dose of the following treatments, between 8 and 10 am: intraperitoneal injection of 25 IU of eCG alone (Novormon, Buenos Aires, Argentina) in 0.1 ml of saline solution (eCG group, $n = 7$);[26] subcutaneous injection of 60 mg/kg of DHEA (Sigma–Aldrich, MO, USA) in 0.1 ml of sunflower oil together with 25 IU of eCG (eCG + DHEA group, $n = 7$);[22] and a group with no hormones and subcutaneous injection of vehicle (sunflower oil) (control group, $n = 6$).

Rats were housed under controlled temperature (21°C) and lighting (12 h light/12 h dark cycle; lights from 7 am until 7 pm), with free access to chow (Nuvilab CR-1, PR, Brazil) and water until the experimental day.

Animals were anesthetized with isoflurane and killed by decapitation 8 h after the treatments. Trunk blood was collected and serum was separated by centrifugation at 2500 *g* for 10 min and stored at −80°C until DHEA, testosterone and estradiol measurements. The left ovarian tissue from each rat was dissected and immediately fixed in 10% buffered formalin for morphological and morphometric studies.

Hormonal assays

Radioimmunoassays (RIA; Immunotech SAS, Marseille, France and Diagnostics Systems Laboratories, TX, USA) were used to measure serum DHEA, testosterone and estradiol, with sensitivity of 0.06 ng/ml, 0.02 ng/ml and 2.20 pg/ml, respectively. Intra- and interassay coefficients of variation were ≤ 10%. RIAs were performed according to manufacturer instructions, using control serum samples.

Morphological and morphometric analysis of follicles and oocytes

Formalin-fixed ovaries were dehydrated in ethanol and embedded in paraffin. Serial 5-μm thick sections were prepared on microscope slides and stained with hematoxylin–eosin (Laborclin, PR, Brazil; Vetec, RJ, Brazil).

The identification of follicles was based on strict criteria.[27] Five-μm step sections were mounted at 50 μm intervals onto microscope slides. This interval was chosen to prevent counting the same structure twice. Two trained investigators, who were blinded to treatment group, carried out the morphological analyses (Olympus BX41, Tokyo, Japan).

Follicles were classified as primordial, primary, secondary, antral, atretic and cystic.[28] Primordial follicles were those with one oocyte surrounded by flattened GCs. Primary follicles presented a single layer of cuboidal GCs, whereas secondary follicles had two or more GC layers. Antral follicles were recognized by the presence of both visible one single vacuole and oocyte nuclei. Atretic follicles were defined as follicles with >5% of the GCs having pyknotic nuclei. Cystic follicles were characterized as those with a large antral cavity, thickened theca interna layer and attenuated GC layer.

For morphometric evaluation, 15 structures of each follicle stage were chosen in each group for analysis. Follicular diameters were determined as the average of two measurements at a right angle across the midpoints. In both primordial and primary follicles, the diameter was measured from the outer layer of GCs. For secondary and antral stages, the diameter was measured from the outer wall of the thecal layer. All diameters were measured using the Image-Pro Plus 4.5 Software (Media Cybernetics, MD, USA), after calibration with a stage micrometer (Media Cybernetics, MD, USA).

Statistical analysis

Statistical analyses were carried out using GraphPAD PRISM 5 Software (San Diego, CA, USA). Data were tested for normal distribution using the Shapiro–Wilk test and described as median and interquartile range (25–75%). Non-parametric analysis was carried out with Kruskal–Wallis test for comparing the control, eCG and eCG + DHEA groups. Dunn's *post-hoc* test was used as the adjustment procedure for multiple comparisons. Data were

considered statistically significant at $P < 0.05$. The P for trend was estimated for the comparisons among the groups.

Results

Effect of treatments on DHEA, testosterone and estradiol levels

Animals treated with eCG + DHEA presented higher serum estradiol [61 (38–596) pg/ml] and testosterone [4.2 (2.3–17.0) ng/ml] levels in comparison with the control group [8.0 (5.6–10.0) pg/ml and 0.09 (0.09–0.11) ng/ml]. Animals under eCG stimulation showed intermediate levels of these steroid hormones [10.0 (8.5–15.0) pg/ml and 0.23 (0.18–0.25) ng/ml]

(Fig. 1a and 1b). Serum DHEA levels were elevated in eCG + DHEA [23.00 (21.00–23.00) ng/ml] in relation to the other two groups [0.69 (0.40–2.40) and 0.60 (0.56–0.72) ng/ml, respectively, for control and eCG groups) (Fig. 1c).

Morphological analysis of ovarian follicles

Table 1 shows the number of follicles according to follicular stage and treatment. The total number of primary and secondary follicles was higher in the eCG + DHEA group than in the control group (Table 1; Fig. 2). The eCG group did not differ from either the control or the eCG + DHEA group regarding the number of primary and secondary follicles.
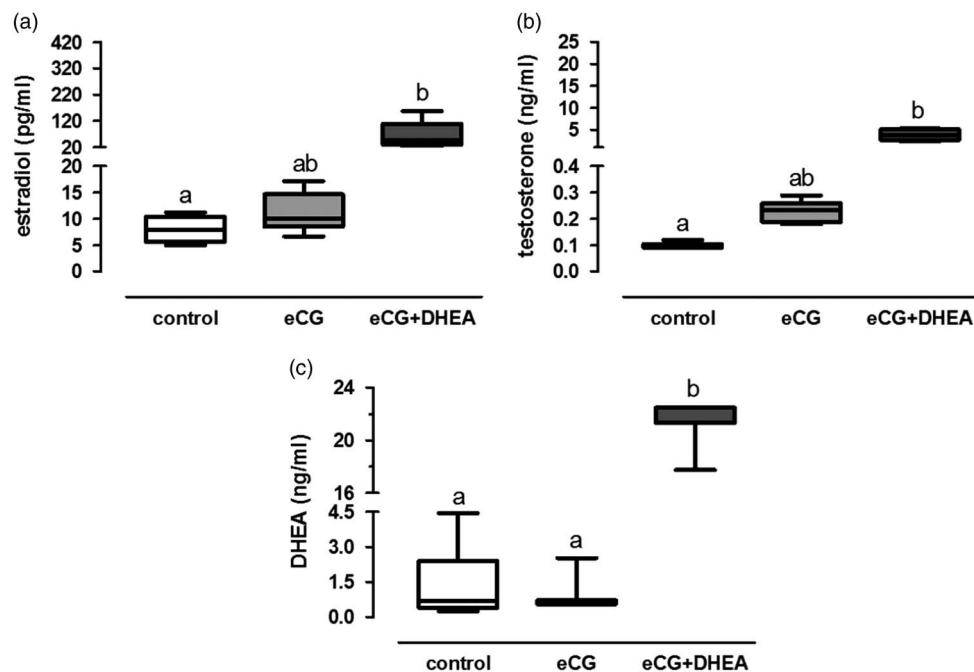
Fig. 1. Serum estradiol (*a*), testosterone (*b*) and dehydroepiandrosterone (DHEA) (*c*) levels from control (*n* = 6), equine chorionic gonadotropin (eCG) (*n* = 7) and eCG + DHEA (*n* = 7) groups. Values are expressed as median and 25–75% interquartile range (lower and upper limit of the box); maximum and minimum values are shown by the limits of vertical lines. $P < 0.01$ by Kruskal–Wallis test. Different letters indicate significant differences ($P < 0.05$).

**Table 1.** *Number of follicles according to follicular stage and treatment*

Follicle	Control (*n* = 6)	%	eCG (*n* = 7)	%	eCG + DHEA (*n* = 7)	%	*P*
Primordial	47 (21–105)	57.2	175 (84–181)	59.7	117 (83–242)	65.9	0.07
Primary	9.0 (6.0–24)[a]	13.0	25 (16–34)[ab]	10.4	33 (25–39)[b]	14.0	0.04
Secondary	6.0 (3.5–8.5)[a]	5.8	15 (13–18)[ab]	6.5	19 (10–22)[b]	6.1	0.01
Antral	28 (15–31)[a]	23.0	44 (30–64)[b]	23.1	36 (32–42)[b]	13.6	0.04
Atretic	0.00 (0–1)	0.4	0.00 (0–0)	0	0.00 (0–0.25)	0.1	0.5
Cystic	0.00 (0–1.5)	0.6	0.00 (0–1.5)	0.3	0.00 (0–1)	0.4	0.9

eCG, equine chorionic gonadotropin; DHEA, dehydroepiandrosterone.
Values are expressed as median and interquartile range (25–75%) and percentage of total number of follicles for each follicle stage and per group (Kruskal–Wallis test).
[a,b]Different superscript letters indicate significant differences ($P < 0.05$).
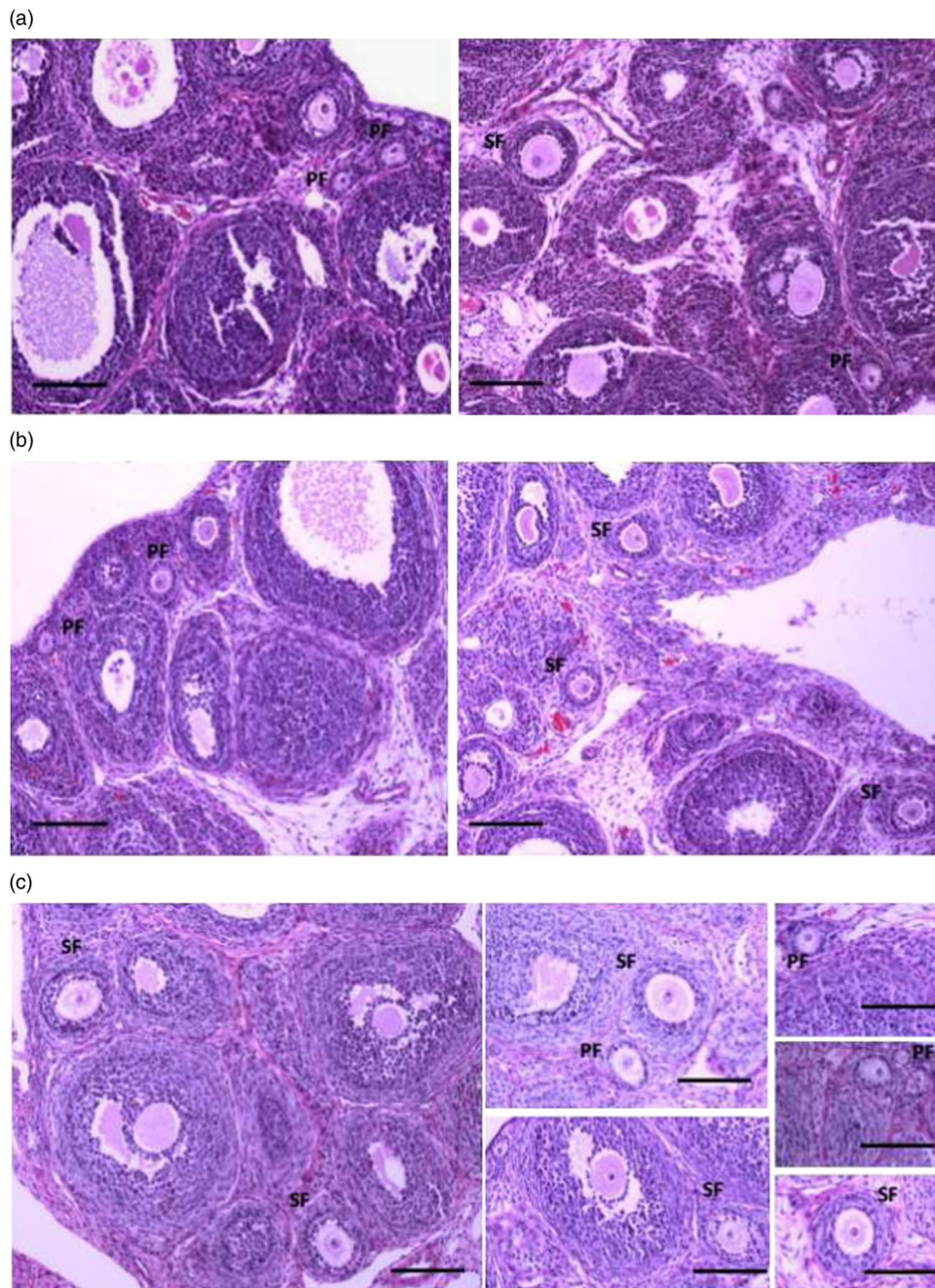
Fig. 2. Representative ovarian micrographs from control (*a*), equine chorionic gonadotropin (eCG) (*b*) and eCG + dehydroepiandrosterone (DHEA) (*c*) groups. PF, primary follicle; SF, secondary follicle. Scale bar is 100 μm.

The total number of antral follicles was also higher in the eCG and eCG + DHEA groups compared with controls. Primordial, atretic and cystic follicles were similar in all groups (Table 1).

Morphometric analysis of ovarian follicles

Primordial follicle diameter was larger in the eCG and eCG + DHEA groups compared with controls (Table 2). The size of primary and secondary follicles was greater in eCG + DHEA animals compared with controls, whereas the diameter of primary follicles was similar in the eCG group in comparison with both controls and eCG + DHEA animals (Table 2; Fig. 2). Antral follicular diameter was similar in all groups.

Discussion

In the present study, acute administration of eCG plus androgen affected initial follicular recruitment, causing an increase in

Table 2. *Follicular diameter in* μ*m according to follicular stage and treatment*

Follicle	Control (n = 6)	eCG (n = 7)	eCG + DHEA (n = 7)	P
Primordial	15 (12–16)[a]	17 (16–19)[b]	17 (16–18)[b]	0.01
Primary	35 (30–44)[a]	41 (36–50)[ab]	46 (37–52)[b]	0.04
Secondary	88 (75–103)[a]	107 (104–125)[b]	108 (95–122)[b]	0.001
Antral	192 (175–219)	200 (168–284)	274 (218–339)	0.05

eCG, equine chorionic gonadotropin; DHEA, dehydroepiandrosterone.

Values are expressed as median and interquartile range (25–75%) of follicular diameter for each follicle stage and per group (Kruskal–Wallis test).

[a,b]Different superscript letters indicate significant differences ($P < 0.05$).

the number of primary, secondary and antral follicles in ovaries of prepubertal rats. A similar animal model has been previously described in immature Sprague Dawley rats (22–25 days of age), showing early apoptosis of antral follicles.[22] The present study is the first to employ both morphological and morphometric analysis in order to measure follicular growth in different stages of follicular development after acute stimulation with eCG plus DHEA in Wistar rats. The decision to use this rat strain is warranted by the fact that this is the experimental strain for which the largest amount of reproductive physiology data are available.

Moreover, our study was carried out specifically with prepubertal animals (25–28 days of age). In prepubertal 25–30-day-old animals, the hypothalamus–pituitary–ovary axis is undergoing maturation; follicular development has initiated, and both antral and atretic follicles are already present. Therefore, eCG stimulation may be very useful to promote follicular development in terms of speed and quantity.[23] Actually, the study of Gal *et al.*,[23] using eCG stimulation followed by human chorionic gonadotropin administration, resulted in a growth spurt of peri-ovulatory follicles. Interestingly, in the present study, stimulation with eCG only produced a slightly and non-significant increase in the proportion of small follicles. This probably occurred because of the short-term duration of the experimental model, with no sufficient time to allow growing follicles could secrete androgens. Nevertheless, eCG stimulation served as priming for the stimulation with DHEA,[29,30] generating an androgen excess state and promoting greater ovarian morphological changes. In turn, although we did not tested the effect of isolated DHEA treatment, it is important to point out that prepubertal animals present only an early slight follicular growth. Based on evidence from previous studies using the same treatments,[22–24] we hypothesize that a priming effect of eCG would be needed to detect a DHEA effect on ovarian changes. Further studies are needed to confirm this hypothesis.

Previous *in vitro* studies suggest that high doses of androgens could impair early follicular development.[31–33] Indeed,

experimental animal models using prenatal exposure to androgens have resulted in reproductive disorders with increased preantral follicles and elevated estradiol and testosterone levels in adult age, resembling PCOS.[33,34] Taken together, these studies and our present results support the hypothesis that prenatal exposure to androgens serves to 'program' both ovaries and adrenals of prepubertal animals to secrete high levels of androgens that interfere with ovarian function.[8,12]

We found significant increases in estradiol, testosterone and DHEA levels in animals treated with eCG plus DHEA in comparison with controls, indicating the consistency of our experimental model. These results are in agreement with those of a previous study[24] with a different female rat strain. Moreover, these findings suggest that follicles remain healthy, with functional cells, following acute exposure to eCG. This was confirmed by our morphological findings and visualization of healthy follicles. Indeed, it is known that biosynthesis of estradiol-like hormones is a basic function of GCs,[1] initiated early in primary follicles. In turn, androgens could be derived from TC secretion from small follicles and adrenal gland. In fact, a recent study[35] has demonstrated significantly increased expression of aromatase in rat ovaries and increased 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-HSD and aromatase messenger RNA (mRNA) levels in adrenal gland. Therefore, DHEA could also be stimulating adrenal cells to synthetize androgens to act as substrate to aromatase enzymes in follicular GCs.

Interestingly, we found an increased proportion of primary, secondary and antral follicles following acute hormonal stimulus with eCG and DHEA. Our results differ slightly from those of a previous study,[24] which reported greater proportion of small follicles. Although Velez *et al.*[24] studied Sprague Dawley rats in the late postnatal age (22–25 days old), in our study Wistar rats were studied at the late prepubertal age (25–28 days old), and presented both small and antral follicles. The presence of higher numbers of primary, secondary and antral follicles in our animals underscores the role of DHEA added to eCG in initial follicular recruitment, despite the acute nature of the intervention. DHEA possibly interacts with androgen receptors expressed in GCs of primary and early antral follicles. Similarly, a previous study[36] observed higher expression of androgen receptors in small follicles. Indeed, acute DHEA treatment seems to enhance intracellular factors to ensure primary to secondary follicular transition.[37] In addition, several studies have shown that androgens mediate local amplification of both insulin-like growth factor-1 (IGF-1) and FSH actions, inducing an increase in IGF-1, IGF-1 receptor and FSH receptor mRNA in the ovaries of experimental animals.[38–40] These findings show that androgens play an important role in modulating growth factors responsible for early folliculogenesis.

An increased diameter of primordial, primary and secondary follicles was also found in our study. We hypothesize that exogenous DHEA amplifies the responsiveness of IGF-1 and perhaps other growth factors by acting on androgen receptors,[41]

and that it stimulates the activity and proliferation of GCs in these small follicles. Amplified proliferation of GCs would be maintained through follicular transitions from primary to secondary follicles.

Limitations of the present study are the absence of molecular analyses such as gene and protein expression in ovarian tissue, which could have provided further mechanistic insight. However, to the best of our knowledge, this is the first study to assess morphological aspects of early follicular development in late prepubertal female Wistar rats acutely stimulated with eCG plus DHEA. The histological analyses were able to demonstrate an initial change that may potentially impair normal follicular development during the ulterior reproductive cycles. Therefore, further studies with this experimental model and longer periods of observation are needed in order to detect whether the observed increased growth of small and early antral follicles is associated with later arrest of preantral follicles throughout the reproductive life course.

Conclusion

In conclusion, the present experimental model using an acute intervention with eCG plus DHEA triggered an increase in the growth and number of small and early antral follicles and could be a reliable tool to study the mechanisms of detrimental follicular development with possible reproductive consequences later in life.

Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (Wistar rats) and have been approved by the institutional committee [Ethics Committee of the Hospital de Clínicas de Porto Alegre, Brazil (GPPG 14-0125)].

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