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# Sex steroid receptors and apoptosis-related proteins are differentially expressed in polycystic ovaries of adult dogs

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ABSTRACT

In Polycystic Ovaries (PCOs), the dynamics of sex hormone receptors and follicle-related apoptotic signaling remain unknown. In this study, we investigated the expression of androgen receptors (AR), estrogen receptors (ERα and ERβ), and apoptosis-related molecules (BAX, active caspase-3, Bcl-2 and Survivin) on different follicular stages of PCOs in adult dogs. Clinical evidences of high estradiol and testosterone levels, persistent estrus and vaginal discharge were observed. Inhibin B immunolabeling was increased in primary and 2 to 5-mm follicles, and a marked epithelial hyperplasia was common in the ovarian surface. Ovarian epithelia and primary follicles showed low expression of AR, ERα, and ERβ, whereas a moderate immunoexpression of AR was found in theca cells of secondary follicles and cysts. In PCOs, growing follicles displayed ERα expression, and secondary follicles exhibited higher ERβ expression. In addition, while few ERα-positive cells were found in the cysts, ERβ was moderately expressed in growing follicles and cysts. BAX was upregulated in the ovarian epithelium, primary follicles, and in the wall of follicular cysts. Active caspase-3 was significantly downregulated in the epithelium, primary follicles, and follicular cysts, whereas growing follicles had a strong immunoexpression in the granulosa cells. Bcl-2 and survivin were increased in the epithelium and primary follicles, and only survivin was upregulated in secondary and growing follicles. While Bcl-2 had a diffuse immunexpression in the follicular cysts, survivin was overexpressed by these cells. We concluded that sex steroid receptors and apoptotic proteins are differentially expressed in the follicles of adult dogs with PCOs.



## 1. Introduction

Polycystic ovary syndrome is a common pathophysiological condition affecting women at reproductive age, characterized by chronic anovulation, polycystic ovaries, hyperandrogenism, and insulin resistance (Magoffin and Jakimiuk, 1998). Although it is a heterogeneous disorder of unclear etiology, there is evidence of genetic component as well as environmental factors (e.g. alterations in the expression and activity of sex steroid receptors (Xita et al., 2002; Palioura and Diamanti-Kandarakis, 2013)).

Laboratory examination has detected an increase in androgen levels, which are linked to inhibition of follicle development, anovulation, menstrual changes, and microcysts in the ovaries (Lombardi et al., 2012), but no description of their ovarian receptors is provided. Importantly, experimental evidence indicated that intrauterine exposure to androgens is associated with development of PCOs (Xita and Tsatsoulis, 2006).

In canines, the diagnosis of bitches with follicular cysts often occurs around 8 years of age. Concurrent diseases observed in dogs bearing ovarian cysts include cystic endometrial hyperplasia, pyometra, mammary neoplasia, ovarian and uterine neoplasias, and skin alterations, characteristic of hyperestrogenism, lichenification, and hyperkeratosis (Johnston et al., 2001). In animal models and humans, it is well-established the role of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and their receptor gene polymorphisms on follicular development and PCOs (Bogovich, 2007; Singhasena et al., 2014; Wu et al., 2014). Unfortunately, there is no information concerning sex hormone receptors related to PCOs in dogs. Because sex steroid receptors orchestrate a variety of ovarian events such as folliculogenesis, luteogenesis, and even ovulation, a better knowledge of how these receptors work in canine PCOs might be useful for exploring new strategies of treatment.

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Apoptosis is a natural event responsible for ovarian functions. There are many hormonal and intracellular factors coordinating life and cell death during the development and maintenance of ovarian activity (Tilly, 1996). In experimental PCOs, the involvement of Bcl-2, an anti-apoptotic member, seems to be important during the transformation of growing follicles into cystic follicles (Bas et al., 2011). Bcl-2 activity is associated with another recently described protein termed BAX, which probably acts independently of Bcl-2 heterodimerization to induce apoptosis (Tilly, 1996). Survivin is a member of the inhibitor of apoptosis protein (IAP) family involved in mitosis regulation and inhibition of apoptosis (Jiang et al., 2014). Conditional deletion of survivin in granulosa cells is associated with compromised follicle development and ovulation leading to defective ovarian function and subfertility (Jiang et al., 2014). To date, no study has been conducted to trace the relationship between apoptotic and anti-apoptotic signals in different follicles of PCOs.

We therefore aimed to investigate the expression and distribution of sex steroid receptors (AR, ERα and β) and apoptosis-related molecules (BAX, Active caspase-3, Bcl-2, and Survivin) in adult dogs with PCOs. The dog is considered an excellent model for comparative studies of reproductive biology (Kirchhoff, 2002).

## 2. Materials and methods

### 2.1. Animal and tissues

Seven 7-year-old female dogs were presented with history of persistent estrus and vaginal discharge. Samples of the PCOs and control ovaries ($n = 07$/group) were obtained from adult Rottweiler dogs (*Canis familiaris*) during castration surgery performed at the Clinical Hospital of the Veterinary Medical School of UNESP at Botucatu. Large fragments of ovaries were collected, and rapidly immersed in 10% (v/v) buffered formalin as fixative solution.

### 2.2. Morphometry

The PCOs were dissected under a surgical microscope D.F. Vasconcelos, and representative areas were dehydrated using 80%, 90%, 95%, and 100% alcohol for 4 h, followed by diaphanization and paraplast embedding (Oxford Labware, St. Louis, USA). The blocks were sectioned at 5-μm-thickness using a LEICA 2145 microtome, and then stained with Hematoxilin-Eosin (HE). For this procedure, tissue sections were hydrated and stained with hematoxilin for 1 min (nuclear staining) followed by eosin for 1 min (cytoplasm staining). The slides were analyzed and captured by digital photomicroscope Axiophot II Zeiss. For histological analysis and follicle counting, every five slides per ovary in three different areas per section/animal were analyzed using 20× magnification (interval between the sections was 50 μm; Chuffa et al., 2009, 2013a,b). A general classification of the follicles in PCOs consisted of the following categories: (1) primary follicle were comprised of one layer of granulosa cells surrounding the oocyte; (2) secondary follicle were comprised of two to six layers of granulosa cells; (3) growing follicle were comprised of several granulosa cell layers with initial antrum (preantral follicles); (4) atretic follicle showed granulosa cells undergoing apoptosis and oocyte degeneration; (5) ovarian cysts with 2–9 mm in diameter and comprised of fluid collection and surrounded by a very thin follicular wall.

### 2.3. Immunohistochemistry

Sections of PCOs and control ovaries (20 randomly selected sections/ovary/dog) were deparaffinized in xylene based on the areas previously identified during the morphological analysis. Tissues were microwaved (800 W) while immersed in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. After blocking endogenous peroxidase activity, the tissues were incubated with 3% BSA for 1 h to avoid non-specific binding. PCOs sections were then incubated in a humid chamber overnight at 4 °C with primary antibodies (Abcam Inc, MA, USA): mouse monoclonal anti-Pan-cytokeratin (1:100, ab7753), anti-AR (1:100, ab9474), anti-ERα (1:100, ab37438), anti-ERβ (1:100, ab3576), anti-BAX (1:50, ab7977), anti-active caspase-3 (1:100, ab2302), anti-Bcl-2 (1:100, ab7973), or anti-survivin (1:200, ab24479). After immunoreactions, the slides were washed in TBS-T and incubated with secondary biotinylated antibody (1:70 dilution), goat anti-rabbit IgG (Santa Cruz Biotechnology, Calif., USA). The slides were reacted with chromogen diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) for 5 min, and sections were counterstained with hematoxylin. Negative controls were obtained by omission of the primary antibody. IHC results were analyzed under a Zeiss Axiophot II microscope (Carl Zeiss, Oberkochen, Germany) based on the levels of staining intensity as absent, weak, moderate, and strong reactions, and percentage (%) of positive cells for every 1000 or 100 counted cells by specific follicle size were obtained using Axionvision software v.4.8 (Carl Zeiss, Oberkochen, Germany).

### 2.4. Immunofluorescence

PCOs samples ($n = 7$) were washed with phosphate-buffered saline, fixed in 4% paraformaldehyde for 10 min, and permeabilized with PBS at room temperature. Nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) for 60 min. Samples were incubated with anti-inhibin B primary polyclonal antibody (dilution 1:100, overnight at 4 °C) followed by secondary polyclonal anti-rabbit IgG conjugated to FITC (1:200, sc-2012, Santa Cruz Biotechnology Inc., CA, USA) for 1 h at room temperature (RT). Nuclei were stained with 6-diamidino-2-phenylindole (DAPI, 5 min) at RT. For negative immunolabeling, no primary antibody was added. Immunopositive cells were analyzed under a fluorescence microscope (Zeiss II, Oberkochen, Germany) at 40X magnification (excitation 590 nm; emission filter 650 nm) and for DAPI (excitation 365 nm; emission filter 480 nm). The quantification of fluorescence images was performed in different follicle sizes (percentage of positive cells/total cells).

### 2.5. ELISA

Blood samples were collected from cephalic vein and directly placed into conical sterile centrifuge tubes. Afterwards, serum was obtained by centrifugation at 1.200 × *g* for 15 min at 4 °C and stored at −20 °C until they were assayed. Serum levels of Testosterone (Catalog# KA2297, Abnova, Walnut, CA, USA) and 17β-estradiol (Catalog# CA0961 Biotang) were measured by specific enzyme-linked immunoassay (ELISA) and the reading was carried out in an Epoch spectrophotometer (Biotek, Winooski, VT, USA) according to the manufacturer's instructions.

### 2.6. Statistical analysis

The values are presented as the mean ± SD, using total samples for analyses ($n = 07$/group). Student's *t*-test was performed for parametric data and Mann–Whitney *U*-test for non-parametric distribution. Significant results were set at $p < 0.05$. *Sigma Plot Version 12.5* graphing software was used.

## 3. Results

### 3.1. General characteristics of PCOs in adult dog

The clinical characteristics of PCOs are shown in Table 1. While PCOs had a 90.2% increase in ovarian area, there was a severe