

1 **Altered expression of Bcl-2 and Bax in follicles within**
2 **dehydroepiandrosterone-induced polycystic ovaries in rats**

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15 Running title: Bcl-2 apoptotic protein levels in PCOS ovaries
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Abstract

Polycystic ovary syndrome (PCOS) is a heterogeneous disease characterized by hyperandrogenemia, hirsutism, oligo- or amenorrhea, insulin resistance and anovulation. The aim of the present study was to evaluate if the balance between the ovarian expression of Bax (proapoptotic protein) and Bcl-2 (antiapoptotic protein) is altered in a PCOS model developed in rats by DHEA administration. In addition, the ovarian morphology and the circulating progesterone levels were evaluated. Histological studies confirmed the presence of follicular cysts, atretic follicles and the absence of corpora lutea in the ovaries from the PCOS group and a significant decrease in circulating progesterone levels. Immunohistochemical studies showed that the expression of Bcl-2 and Bax were mainly localized in granulosa cells of antral follicles in both groups. Bax expression was greater in preantral and antral follicles from PCOS ovarian sections than in Controls. In contrast, intense Bcl-2 immunostaining was observed in Control antral follicles, while Bcl-2 protein was either absent in preantral follicles or weakly expressed in antral follicles from PCOS rats. These results were partially confirmed by western studies. Data revealed that the ovarian level of Bcl-2 protein was lower in PCOS than in Control, and that there were no differences in Bax ovarian levels between groups. However, Bax/ Bcl-2 ratio was significantly higher in PCOS group than in Control group. In conclusion, an increase in ovarian apoptosis through an imbalance among the Bcl-2 family members may be involved in the transformation of growing follicles in cystic follicles in the ovaries from DHEA-induced PCOS rats.

1 Introduction

2
3 Infertility affects 10% of human couples, a proportion of which are due to ovarian
4 dysfunction. Polycystic ovary syndrome (PCOS) represents a remarkable 75% of cases
5 of anovulatory infertility (Dunaif and Thomas, 2001; Ehrmann, 2005). This is a
6 heterogeneous disease frequently characterized by hyperandrogenemia, hirsutism, oligo-
7 or amenorrhea, insulin resistance and anovulation. However, the lack of a clear etiology
8 associated with these syndromes has led to multiple treatments with few effective
9 therapies for infertility.

10 Apoptosis is a physiological process that plays a critical role in the maintenance of
11 homeostasis in multicellular organisms (Schwartzman and Cidlowski, 1993). Signaling
12 for apoptosis occurs through multiple independent pathways that converge in a common
13 machinery of cell destruction. For example, several genes belonging to the *bcl-2* family
14 may function as intracellular mediators of cell survival. The protein products of the *bcl-2*
15 and *Bax* genes have been described as anti- or proapoptotic factors, respectively
16 (Korsmeyer, 1992; Reed, 1994; Boise et al., 1993). Depending on the balance of these
17 proteins, the activation of the initiator caspase 9 and the sequential activation of several
18 effector caspases such as caspase 3 or 7, which promote apoptosis in various systems,
19 occurs (Budihardjo et al., 1999).

20 Mahesh (Mahesh, 1966) and Greenblatt (Greenblatt and Mahesh, 1974) showed that
21 dehydroepiandrosterone (DHEA) and its sulfate derivative are among the first
22 androgens that increase during puberty in most patients with PCOS. It has been
23 demonstrated that the injection of DHEA to immature rats produces an animal model of
24 PCOS that exhibits the prominent features of human PCOS- i.e., the follicles undergo
25 atresia or exhibit various stages of cystogenesis up to complete pathological
26 transformation into follicular cysts (Roy et al., 1962; Anderson et al., 1992). Thus, this
27 model appears to be appropriate to study the process of follicular atresia in PCOS
28 stages.

29 The ovarian cycle is characterized by extensive tissue remodeling, where 99% of
30 mammalian follicles fail to ovulate and become atretic by a hormonally controlled
31 apoptotic process (Hsueh et al., 1996). Although Bcl-2 family protein members are
32 involved in this process, their significance in PCOS development remains unclear.
33 Accordingly, the aim of the present study was to evaluate if the balance between the
34 ovarian expression of Bax (a proapoptotic protein) and Bcl-2 (an antiapoptotic protein)
35 is altered in a PCOS model developed in rats by DHEA administration. In addition, the
36 ovarian morphology and the circulating progesterone levels were evaluated.

37 Materials and Methods

38 Animal Treatments and Tissue Collection

39
40 General care and housing of rats was carried out at the Instituto de Biología y Medicina
41 Experimental (IByME), Buenos Aires, Argentina. Prepubertal rats were from our
42 colony. Immature female Sprague-Dawley rats (21-23 days old) were allowed food and
43 water ad libitum, kept at room temperature (21-23 °C) on a 12L:12D cycle. To induce
44 the hyperandrogenic PCOS condition the protocol developed by (Henmi et al., 2001)
45 was used. Rats were injected s.c. with DHEA (PCOS group, 6 mg/100 g body
46 weight/0.2 ml sesame oil, n=4-6) every evening for 15 days. Control animals were
47 injected with 0.2 ml of sesame oil every evening for an equivalent length of time
48 (Control group, n=4-6). There were no signs of inflammation at the injection sites
49 throughout the experiment. The animals were either euthanized by CO₂ asphyxiation or
50

1 decapitated to obtain blood samples for steroid extraction. The plasma was stored at -20
2 °C for subsequent RIA analysis. Ovaries were removed and cleaned of adhering tissue;
3 one ovary per rat was used for Western immunoblot assays and the other for
4 immunohistochemical assays.

6 Immunohistochemistry

7 Ovaries from both the control and the PCOS group were obtained and immediately
8 fixed in 4% neutral buffered formalin for 12 h and then embedded in paraffin. Five-
9 micron step sections were mounted at 50-µm intervals onto microscope slides to prevent
10 counting the same follicle twice, according to Woodruff *et al.* (Woodruff *et al.*, 1990).
11 Tissue sections were treated as previously described (Abramovich *et al.*, 2010). Sections
12 were incubated with goat polyclonal anti-Bcl-2 (1:100) or rabbit polyclonal anti-Bax
13 (1:100) primary antibodies. Specificity for Bcl-2 and Bax was supported by the loss of
14 staining in follicular tissue preabsorbed with their respective peptides from Santa Cruz
15 Biotechnology, Inc. (Santa Cruz, CA).

16 Morphological characteristics of preantral follicles (PF) include their size (PF: 120-200
17 µm) and an oocyte surrounded by 2-4 layers of granulosa cells. Antral follicles (AF) are
18 defined as follicles of 200-500 µm in diameter with a well-defined and thick granulosa
19 layer and an internal and external thecal layer (Hughes Jr. and Gorospe, 1991;
20 Hirshfield and Midgley, Jr., 1978). Cystic follicles were characterized by a thickened
21 theca interna layer and a diminished granulosa cell layer.

22 Atresia was defined as the presence of >10 pyknotic granulosa cells; in the smallest
23 follicles, the criterion for atresia was a degenerated oocyte and/or precocious antrum
24 formation (Andreu *et al.*, 1998).

26 Western blot

27 Five ovaries per group were resuspended in 350µl of lysis buffer (20 mM Tris-HCl [pH
28 8], 137 mM NaCl, 1% NP-40, and 10% glycerol) supplemented with protease inhibitors
29 (0.5 mM PMSF, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM
30 N-p-tosyl-lysine chloromethyl ketone, and 0.025 mM ML-1-tosylamide-2-phenyl-
31 ethylchloromethyl ketone) (all the products were obtained from Sigma) and
32 homogenized with an Ultra-Turrax (IKA Werk, Breisgau, Germany) homogenizer.
33 Samples were centrifuged at 4°C for 10 min at 10 000 x g, and the resulting pellets
34 discarded. Protein concentration in the supernatant was measured by the Bradford assay.
35 Western blot technique was performed as previously described (Abramovich *et al.*,
36 2010). Blots were incubated with anti-Bax (1/200); anti Bcl-2 (1/500) and anti-actin B
37 (1/1.000) primary antibodies. Blots were then incubated with anti-rabbit or anti-mouse
38 antibodies conjugated with horseradish peroxidase (1:1000) and finally detected by
39 chemiluminescence and autoradiography with an x-ray film.

41 Quantification for the Western Immunoblot

42 In each experiment, equal amounts of protein were loaded for all samples, and protein
43 extracts from the PCOS and Control groups were loaded on the same gel. For
44 quantification, a screening was performed on blots with an x-ray film using different
45 times of exposure to optimize the signal. The density of each band was normalized to
46 the density of the actin B band that was used as an internal control. The levels of protein
47 were compared and analyzed with densitometric studies by Scion Image for Windows
48 (Scion Corporation, NIH, Washington, D.C.). Optical density data are expressed as
49 arbitrary units ± SEM.

1 Serum progesterone levels

2 Following ether extraction, serum steroid levels were measured by RIA in rats from the
3 Control and PCOS groups, as described previously (Saragueta *et al.*, 1989; Irusta *et al.*,
4 2003). Progesterone was measured using a specific antibody supplied by Dr. G. D.
5 Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State
6 University, Fort Collins, CO). Under these conditions, the intra-assay and inter-assay
7 variations were 8.0 and 14.2% respectively.

9 Data Analysis

10 Data are expressed as the mean \pm SEM. Three different experiments with 4-6 ovaries
11 each were carried out for Western blot analysis and representative gels are shown in the
12 figures. Statistical analysis was performed with a paired t-Test using the GraphPad
13 Prism program version 4.00. Values of $p < 0.05$ were considered significantly different.

15 Results

17 Morphological Studies and Serum Progesterone Levels

18 Ovarian histology confirmed the presence of follicular cysts and many atretic follicles
19 and the absence of corpora lutea in the rat ovaries from the PCOS group (Fig. 1B). In
20 contrast, Control ovaries showed the presence of numerous growing follicles and some
21 corpora lutea (Fig. 1A).

22 Progesterone concentration in serum was measured after 15 days of DHEA treatment
23 (PCOS group) and in control animals (Control group). A significant decrease in
24 circulating progesterone levels was observed in the PCOS group (Control: 11.7 ± 2.3
25 ng/ml; PCOS: 4.8 ± 1.3 ng/ml, $n=8$, $p < 0.05$).

27 Analysis of Bcl-2 and Bax in the ovaries from the PCOS and Control groups

28 Figure 2 shows the immunohistochemical staining for Bcl-2 in ovarian sections from
29 PCOS and Control rats during the different follicular stages. In the Control group, we
30 found that Bcl-2 was expressed in granulosa cells (Gc) at all follicular stages. In
31 contrast, no immunostaining for Bcl-2 was observed in thecal cells (Tc) (Fig. 2). In
32 preantral follicles we detected minimal expression of this protein (Fig. 2A). However,
33 intense immunostaining for Bcl-2 was observed in antral follicles (Fig. 2C). In PCOS
34 rats, Bcl-2 protein was either absent in preantral follicles (Fig. 2B) or weakly expressed
35 in Gc in antral follicles (Fig. 2D). In contrast, atretic follicles showed and intense
36 staining for Bcl-2 in ovarian sections from both groups (Fig. 2E-F).

37 Figure 3 shows the immunohistochemical staining for Bax in ovarian sections from
38 Control and PCOS rats. In Gc from the Control group, Bax protein was either absent in
39 preantral follicles (Fig. 3A) or moderately expressed in antral follicles (Fig. 3C). In
40 contrast, Bax expression was greater in Gc from preantral and antral PCOS follicles
41 (Fig. 3B & 3D). Antral ovarian follicles from Control and PCOS rats showed a weak
42 Bax expression in Tc, whereas preantral follicles showed total absence of expression
43 (Fig. 3A-D). In addition, atretic follicles showed an intense staining for Bax in ovarian
44 sections from both groups (Fig. 3E-F).

45 In addition, follicular cysts from the rat ovaries of the PCOS group showed a weak
46 expression of Bcl-2 in Gc (Fig. 2H) and an intense expression of Bax (Fig. 3H).

1 Figure 4 shows the expression of ovarian Bcl-2 and Bax protein determined by Western
 2 immunoblot. No differences in Bax ovarian levels were detected between groups (Fig.
 3 4A). However, expression of Bcl-2 protein was lower ($p < 0.05$) in ovaries from PCOS
 4 rats than in those from Control rats (Fig. 4B). In addition, the Bax/ Bcl-2 ratio was
 5 significantly increased in the PCOS group in comparison to the Control group (Fig. 4C).

6 7 Discussion

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10 The present study investigated the protein expression levels and cellular localization of
 11 Bcl-2 and Bax in ovaries from DHEA-induced PCOS rats. In order to correlate these
 12 results with morphological and functional ovarian changes, ovarian structures were also
 13 examined and serum progesterone levels measured.

14 Histological studies confirmed the presence of follicular cysts and many atretic follicles
 15 and the absence of corpora lutea in the ovaries from the PCOS group and, as a
 16 consequence, a significant decrease in the circulating progesterone levels. Our data
 17 constitute the first evidence that these alterations are associated with changes in the
 18 expression of ovarian apoptotic proteins of the Bcl-2 family. We demonstrated by
 19 immunohistochemical studies that the expression of Bcl-2 and Bax was mainly
 20 localized in granulosa cells (Gc) of antral follicles both in control and PCOS rats,
 21 pointing out the importance of Gc during the process of follicular atresia and
 22 cystogenesis observed in PCOS ovaries. However, Bax expression was greater in Gc of
 23 preantral and antral follicles from PCOS ovarian sections than in those from the Control
 24 group. In contrast, intense Bcl-2 immunostaining was observed in Control antral
 25 follicles, while Bcl-2 protein was either absent in preantral follicles or weakly
 26 expressed in antral follicles from PCOS rats. These results were partially confirmed by
 27 western immunoblot studies. Our data revealed that the ovarian level of Bcl-2 protein
 28 was lower in ovaries from PCOS rats than in those from Control rats, and that no
 29 changes in Bax ovarian levels were detected between groups. The different expression
 30 of Bax observed using immunohistochemical assays could be due to the fact that for
 31 Western blots the whole ovary was used, while immunohistochemical studies detect the
 32 staining in each follicular stage. A possible dilution could take place when the total
 33 ovarian level of Bax was measured. Nevertheless, the Bax/ Bcl-2 ratio measured by
 34 Western blots was significantly higher in the PCOS group than in the Control group.

35
36 Tilly et al have demonstrated that follicular atresia is associated with an imbalance
 37 among the Bcl-2 family members. Gonadotropin treatment inhibits granulosa cell
 38 apoptosis and follicular atresia mediated by the reduction of granulosa Bax expression
 39 while maintaining the levels of Bcl-2 and Bcl-xL (Tilly, 1996; Tilly et al., 1995). Thus,
 40 it was postulated that the intrinsic pathway of apoptosis would be involved in follicular
 41 atresia (Tilly et al., 1995; Tilly, 1996). In addition, in a previous study, we have shown
 42 that the local inhibition of Vascular Endothelial Growth Factor A (VEGFA) activity
 43 appears to cause an increase in ovarian apoptosis through an imbalance among the Bcl-
 44 2 family members, thus leading a larger number of follicles to atresia (Abramovich et
 45 al., 2006). Moreover, in vivo GnRH agonist treatment caused an increase in follicular
 46 atresia and apoptosis process in follicles from eCG- treated rats, which is correlated
 47 with a decrease in the ratio of anti-apoptotic: proapoptotic proteins, as observed for the
 48 Bcl-xL/Bcl-xS pair (Parborell et al., 2002). A similar mechanism could take part in the
 49 development of the PCOS state induced by high levels of androgens (Tilly and Tilly,
 50 1995; Parborell et al., 2002). Accordingly, the present data indicate that the intrinsic

1 pathway participates in the transformation of growing follicles in cystic follicles in the
 2 ovaries from DHEA-induced PCOS rats. In an earlier study, Honnma et al (Honnma et al.
 3 al., 2006) demonstrated that the extrinsic apoptotic pathway is also involved in the
 4 mechanisms of follicular atresia in rat ovaries affected by PCOS induced by DHEA. In
 5 the same animal PCOS model used in this study, these authors found an increase in the
 6 number of apoptotic follicle cells and in the ovarian levels of the proapoptotic protein
 7 FasL and the processed caspase-8 protein. All these data lead us to suggest that both
 8 pathways may be involved in the development of the PCOS stage. In addition, our
 9 results are in agreement with those obtained by Salvetti et al. in a different PCOS
 10 model. In rats exposed to continuous light they described changes in the ovarian
 11 expression of Bcl-2 family members using immunodetection assays (Salvetti et al.,
 12 2009).

13 Apoptosis is related to the oxidant-antioxidant equilibrium. Thus, damage to biological
 14 systems caused by the excess of reactive oxygen species (ROS) produce cellular
 15 oxidative stress. It has been reported that hyperandrogenization of BALB/c mice with
 16 DHEA prevents ovulation by increasing ovarian oxidative stress and altering the
 17 endocrine and immune systems (Elia et al., 2006; Luchetti et al., 2004; Sander et al.,
 18 2005; Solano et al., 2006; Belgorosky et al., 2010). These alterations could be
 19 associated with the changes in the expression of the apoptotic proteins described in this
 20 study.

21 In summary, our data demonstrate that the Bax/ Bcl-2 ratio was significantly higher in
 22 the ovaries from the PCOS group than in those from the Control group. Thus, changes
 23 in the intrinsic pathway of apoptosis are associated to the ovarian alterations observed
 24 in the ovaries from DHEA-induced PCOS rats.

26 Conclusions

28 Our data suggest that an increase in ovarian apoptosis through an imbalance among the
 29 Bcl-2 family members may be involved in the transformation of growing follicles in
 30 cystic follicles in the ovaries from DHEA-induced PCOS rats.

32 Acknowledgements

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8 Figure legends

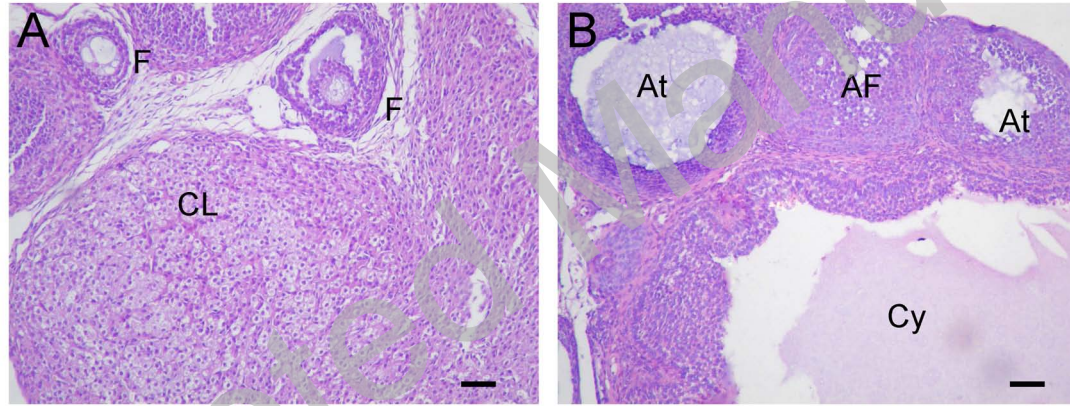
10 **Figure 1.** Representative photomicrographs of ovarian sections stained with
11 hematoxylin-eosin. A: normal cycling control rat treated with vehicle. B: PCOS rat
12 treated with 6 mg DHEA/100 g body weight for 15 days. Note the presence of
13 numerous growing follicles (F) and some corpora lutea (CL) in Fig. 1A and numerous
14 cystic follicles (Cy), atretic follicles (At) and the absence of corpora lutea in Fig. 1B.

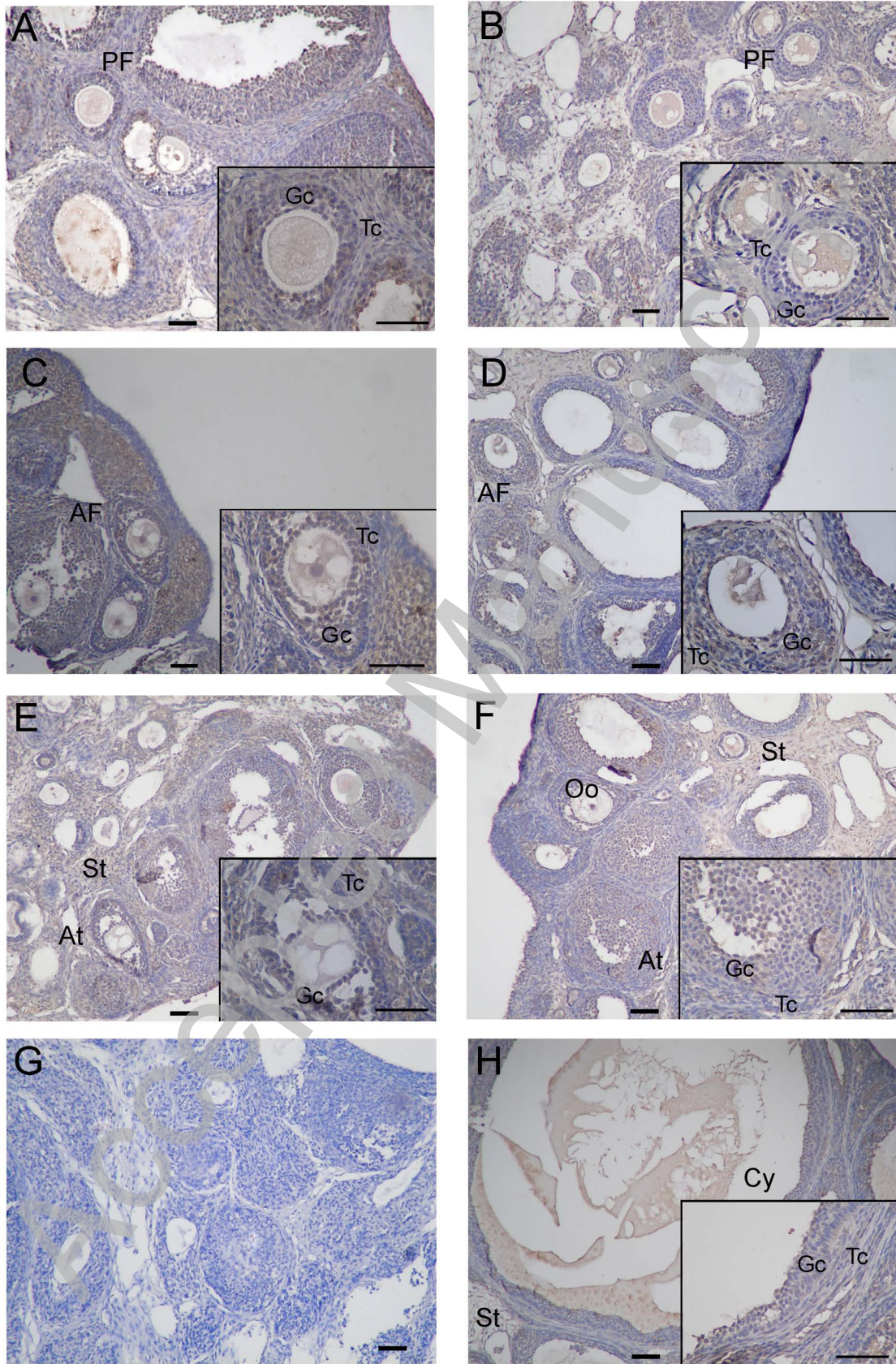
15 **Figure 2.** Immunohistochemical staining for Bcl-2 in representative ovarian sections
16 from Control and PCOS rats showing different follicular stages. Panels A, C and E
17 show representative fields of ovarian sections from Control rats. Panels B, D, F and H
18 show representative fields of ovarian sections from PCOS rats. Panel G: negative
19 control. Specificity for Bcl-2 is supported by the loss of staining in follicular tissue
20 preabsorbed with Bcl-2 peptide (not shown). PF, preantral follicle; AF, antral follicle;
21 At: atretic follicle; Cy: cystic follicles Gc, granulosa cells; Tc, theca cells; Oo, oocyte;
22 St, stroma. *Scale bars represent 50 μm.*

23 **Figure 3.** Immunohistochemical staining for Bax in representative ovarian sections
24 from PCOS and Control rats showing the different follicular stages. Panels A, C and E
25 show representative fields of ovarian sections from Control rats. Panels B, D, F and H
26 show representative fields of ovarian sections from PCOS rats. Panel G: negative
27 control. Specificity for Bax is supported by the loss of staining in follicular tissue in the
28 absence of the primary antibody (not shown). PF, preantral follicle; AF, antral follicle;
29 At: atretic follicle; Cy: cystic follicles; Gc, granulosa cells; Tc, theca cells; Oo, oocyte;
30 St, stroma. *Scale bars represent 50 μm.*

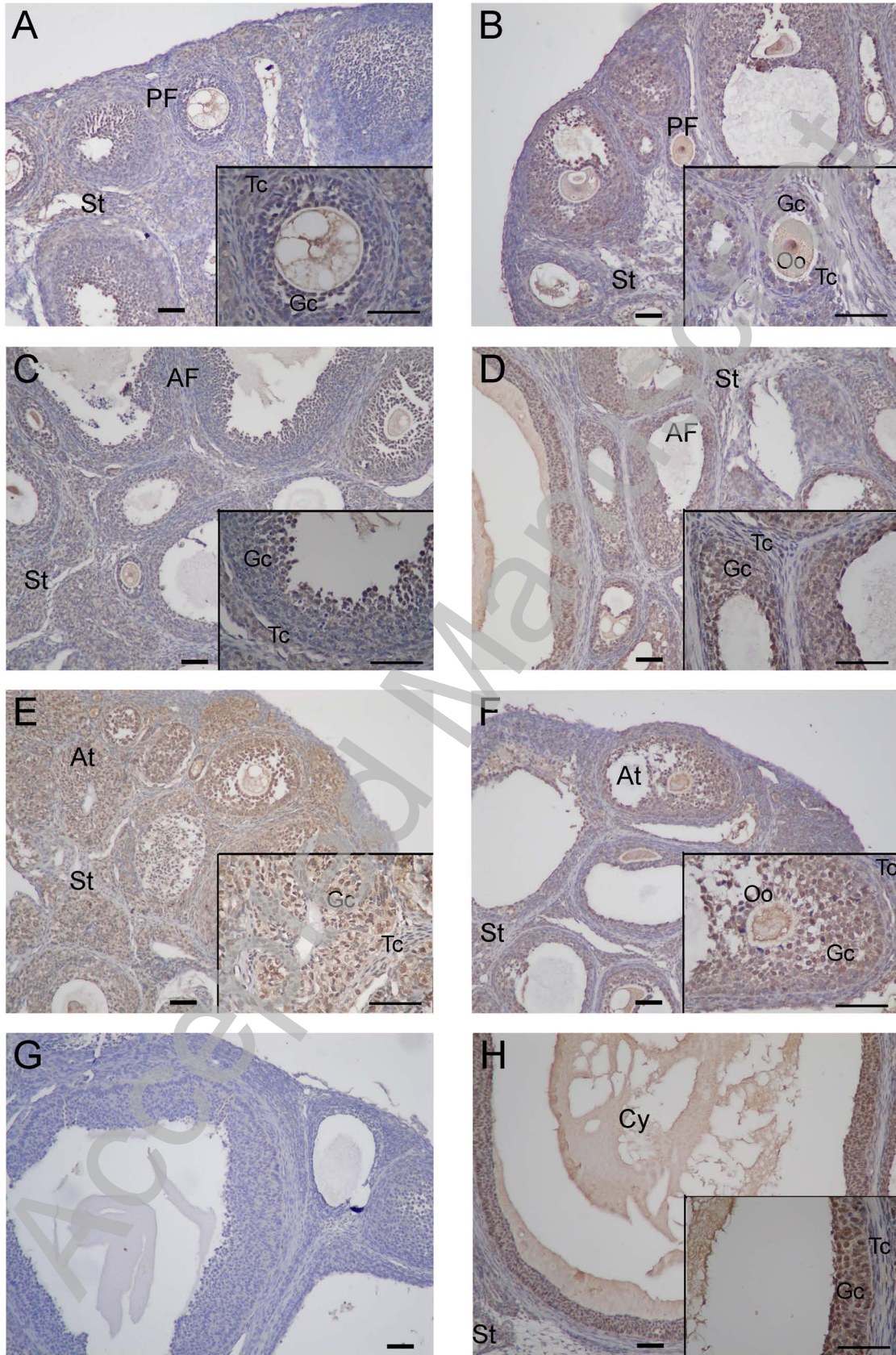
31 **Figure 4.** Effect of DHEA treatment on proapoptotic and antiapoptotic ovarian protein
32 content. After homogenization, proteins were extracted, subjected to 12% SDS-PAGE,
33 and transferred onto nitrocellulose membranes. A) Upper panel: Densitometric
34 quantification of Bax content. Bars represent the mean \pm SEM normalized to actin B.
35 Lower panel: Representative immunoblot of Bax ovarian protein content from control
36 and DHEA-treated rats (PCOS group). Bax protein was visualized with an anti-Bax
37 antibody. No differences in Bax ovarian levels were detected between groups. B) Upper
38 panel: Densitometric quantification of Bcl-2 content. Bars represent the mean \pm SEM
39 normalized to actin B. Lower panel: Representative immunoblot of Bcl-2 protein
40 content in antral follicles from control and DHEA-treated rats (PCOS group). Bcl-2
41 protein was visualized with an anti-Bcl-2 antibody. Levels of Bcl-2 protein were
42 significantly decreased after DHEA treatment (n = 5; p < 0.05). C) Ratio Bax/Bcl-2 in
43 the PCOS and Control groups. The ratio was significantly increased after DHEA
44 treatment (n = 5; p < 0.05).

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