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ARTICLE

Hyperandrogenism alters intraovarian parameters during early folliculogenesis in mice

Denise Belgorosky [a], Valeria Analía Sander [a], María Paula Di Yorio [b], Alicia G Faletti [b], Alicia B Motta [a,*]

[a] *Laboratorio de Fisiopatología Ovárica, Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Departamento de Farmacología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121ABG Buenos Aires, Argentina;*
[b] *Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Medicina, UBA, Paraguay 2155, 1121ABG Buenos Aires, Argentina*
* Corresponding author. *E-mail address:* aliciabmotta@yahoo.com.ar (AB Motta).

Alicia Beatriz Motta received her PhD from the University of Buenos Aires, Argentina. Her early research interests were in studying the mechanisms involved in corpus luteum regression. As a post-doctoral researcher she began to work on the mechanisms involved in the outcome and development of polycystic ovary syndrome. In that context, she developed rodent models to study this pathology during the prepuberal stage and in early pregnancy. She is currently a researcher for the Argentina National Research Council (CONICET) and Director of the Laboratory of Ovarian Physiopathology, Center for Pharmacological and Botanical Studies (CEFYBO), Faculty of Medicine, University of Buenos Aires.

Abstract This study aimed to investigate how hyperandrogenism affects early folliculogenesis. Hyperandrogenism was induced in prepuberal female BALB/c mice by daily s.c. injection of dehydroepiandrosterone (60 mg/kg body weight in 0.1 ml sesame oil) for 10 consecutive days. Although hyperandrogenism increased the growth rate of primary follicles, it also increased ovarian oxidative stress (evaluated by the increase in lipid peroxidation, the decrease in superoxide dismutase activity and the fact that glutathione content was not modified). By using the annexin V/cytometry assay it was found that the excess of androgens decreased viable ovarian cells and increased early apoptotic ones. The increased lipid peroxidation induced enhanced ovarian prostaglandin E production. In addition, hyperandrogenism increased the number of T lymphocytes that infiltrate ovarian tissue and modified their phenotype (decreased CD4+ or helper and increased the suppressor/cytotoxic CD8+). The excess of androgens decreased the ovarian expression of the long isoform of leptin receptor (Ob-Rb, the only isoform expressed in the ovarian tissue) when compared with controls. All these alterations increased serum concentrations of oestradiol, a pro-apoptotic agent. It is concluded that the excess of androgens impairs early follicular development by modulating some endocrine and immune parameters that are either directly or indirectly related to follicular atresia.

KEYWORDS: dehydroepiandrosterone, folliculogenesis, hyperandrogenism, oestradiol, polycystic ovary syndrome, progesterone

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Introduction

Folliculogenesis starts when follicles leave the pool of resting follicles in order to enter the growth phase. Most follicles fail to complete this maturation scheme and die in a process termed atresia (Gougeon, 1996).

Polycystic ovary syndrome (PCOS) is a heterogeneous disease characterized by hyperandrogenaemia, hirsutism, oligo- or amenorrhoea and anovulation and is frequently associated with hyperinsulinaemia, insulin resistance syndrome, increased cardiovascular risk and diabetes mellitus (Franks, 1995). PCOS follicles fail to mature even when they are exposed to normal serum and intrafollicular bio- and immunoactive follicle-stimulating hormone concentrations, suggesting the involvement of local modulators within the ovary (Erickson et al., 1992; Hsueh, 1986; Mason et al., 1994).

Since dehydroepiandrosterone (DHEA) is one of the most abundant androgens produced by ovaries of women with PCOS, a rodent model was developed by injection of a dose of DHEA equivalent to that of women with PCOS (Roy et al., 1962). Subsequent studies established that the DHEA-PCOS murine model exhibits many of the salient features of human PCOS (Anderson et al., 1992; Elia et al., 2006; Henmi et al., 2001; Lee et al., 1991, 1998; Luchetti et al., 2004; Sander et al., 2005, 2006; Solano et al., 2006). Previous studies have reported that hyperandrogenization with DHEA during the peri-ovulatory period prevents ovulation by inducing a pro-oxidant and pro-inflammatory status (Elia et al., 2006; Luchetti et al., 2004; Sander et al., 2005, 2006; Solano et al., 2006). The present study aimed to investigate some of the intraovarian parameters, particularly those involved in atresia by using the DHEA-PCOS murine model.

It is known that morphological changes and tissue remodelling are necessary physiological events within the membranes in all aerobic cells. Reactive oxygen species (ROS), which are toxic oxygen-derived products, include the production of superoxide radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) (Halliwell and Gutteridge, 1984). The accumulation of ROS — as a consequence of the uncontrolled lipid peroxidation of cellular membranes — is referred to as oxidative stress. Protection against ROS in cells is provided by enzymes (superoxide dismutase (SOD), catalase and glutathione peroxidase), metabolites (glutathione; GSH) and/or vitamins (Agarwal et al., 2005). Considering that increased oxidative stress leads to the loss of gonadotrophin receptors and the loss of follicular function (Wang et al., 1991), this study was interested in investigating whether hyperandrogenism induces an ovarian oxidant/antioxidant imbalance and/or apoptotic events that impair early folliculogenesis.

Prostaglandins play an essential role in the rupture of ovarian follicles associated with ovulation (Husein and Kridli, 2003; Medan et al., 2003). Although it has been well established that prostaglandins exert their functions via enhancing the production of ROS (Sawada and Carlson, 1991; Sugino et al., 1996), the relationship between prostaglandins and the response of the antioxidant defences remains controversial (Al-Gubory et al., 2004; Garrel et al., 2007; Sugino et al., 2004). These observations led us to study the effect of hyperandrogenism in the production of prostaglandin E (PGE) during early folliculogenesis.

The interaction between the reproductive functions and the immune response has been largely reported (Fassnacht et al., 2006; Khan et al., 2008; Son and Roby, 2006; Wu et al., 2004; among others). In fact, leukocytes regulate ovulation (Brannstrom and Enskog, 2002) and the hormonal status modulates the phenotype of T lymphocytes (Lu et al., 2002; Luchetti et al., 2004; Solano et al., 2008; Yan et al., 2000). Moreover, the expression of the T phenotype modulates follicular atresia in normal-cycling human ovaries (Suzuki et al., 1998). These findings prompted us to study whether hyperandrogenism is able to alter the T phenotype and thus indirectly to increase early follicular atresia.

Leptin was initially known to be a satiety hormone regulating both food intake and energy expenditure, but it is now known that this protein plays an important role in reproduction. Leptin is recognized by the leptin receptor (Ob-R; Tartaglia et al., 1995), which is a product of the diabetes (db) gene (Chen et al., 1996; Chua et al., 1996). A previous report found that leptin is able to differentially modulate the expression of its own receptors in the reproductive axis (Di Yorio et al., 2008). A reduction in the folliculogenesis, possibly as a consequence of the altered expression of leptin and its isoform receptor genes has been recently reported (da Silveira et al., 2009). Taken together, these observations led us to study whether hyperandrogenism is able to alter the expression of ovarian leptin receptor as a modulator of early folliculogenesis.

Materials and methods

Animals and experimental protocol

In order to study the effects of high concentrations of circulating androgens in early folliculogenesis, a dehydroepiandrosterone (DHEA) mouse model that has been previously used in the study laboratory was used (Elia et al., 2006, 2008; Luchetti et al., 2004; Sander et al., 2006). Briefly, female prepuberal (22 days old) mice of the BALB/c strain were injected (s.c.) daily with DHEA (60 mg/kg body weight) dissolved in 0.1 ml sesame oil (DHEA group). The treatment with DHEA (dose and frequency) has been assessed previously (Sander et al., 2005). The control group consisted of mice injected with 0.1 ml of vehicle (sesame oil). Mice (75 per group: control and DHEA treated) were housed under controlled temperature (22°C) and illumination (14-h light, 10-h darkness; lights on at 5 a.m.) and were allowed free access to rat chow (Purina; Nestle) and water. All procedures involving animals were approved by the animal care and use committee of the National Research Council (CONICET). After 10 days of DHEA treatment, mice were killed by decapitation. Trunk blood was collected and serum was separated by centrifugation at 1000*g* for 15 min and stored at −20°C until determination of progesterone and oestradiol concentrations by radioimmunoassay (RIA). Freshly dissected ovaries were divided as follows: five from each group (control and DHEA) were immediately fixed in 4% (w/v) paraformaldehyde for morphological studies, 10 from each group were immediately used to determine viability and cellular apoptosis by annexin V assay, 10 were immediately processed to determine CD4+ and CD8+ T cells by flow cytometry assay and 50 were frozen at −70°C until 10 were

used to quantify PGE production by RIA, 10 for lipid peroxidation index, 10 for SOD activity, 10 for total GSH content and 10 for the Ob-R expression by Western blotting. All the experiments were repeated three times.

Morphological studies of ovarian tissue

To study the effect of hyperandrogenization on early folliculogenesis, five ovaries from the control and five from the DHEA-treated groups, fixed as described above, were consecutively cut (6 μm per section, for each ovary the sections were taken from the middle and each extreme of the sample) and placed on gelatin-coated slides (Biobond, British Biocell International, Cardiff) and air dried for 2 h before being fixed for 5 min in acetone at 4°C. Then, consecutive sections of each ovary were washed in phosphate-buffered saline (PBS; 137 mmol/l NaCl, 2.7 mmol/l KCl, 4.3 mmol/l $Na_2HPO_4.7H_2O$, 1.4 mmol/l KH_2PO_4, pH 7.3) and stained with haematoxylin and eosin (DAKO Corporation, Carpinteria, CA, USA) for histological analysis. This resulted in 40 sections for each ovary. For morphological analysis, the sections were chosen as follows: five from each extreme and five from the middle of each ovary. Five ovaries from the control and five from the DHEA treated group were observed.

Oxidative stress of ovarian tissue

Lipid peroxidation

The amount of malondialdehyde (MDA), which is formed from the breakdown of fatty acids is taken as an index of lipid peroxidation and oxidative stress. The method quantifies the amount of MDA that reacts with trichloroacetic acid/thiobarbituric acid/HCl (15% w/v; 0.375% w/v and 0.25 mol/l respectively) yielding a red compound that absorbs at 535 nm (Motta et al., 2001). Briefly, 350 μl of homogenates obtained from ovaries (one ovary per determination and 10 ovaries per group in 0.5% v/v trichloroacetic acid) were treated with trichloroacetic acid/ thiobarbituric acid/HCl and heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000*g* for 10 min. Absorbance of samples was determined at 535 nm. Content of thiobarbituric acid reactants is expressed as mol MDA formed/mg protein.

Superoxide dismutase activity

SOD enzymes are a group of metalloenzymes that detoxify ROS through the conversion of superoxide radical to peroxide oxygen and molecular oxygen. Total SOD activity was assayed by a spectrophotometric method based on the inhibition of a superoxide-induced epinephrine oxidation (Misra and Fridovich, 1972). Radical superoxide serves as a chain propagation species for the autoxidation of epinephrine to adrenochrome. SOD competes with this reaction, thus decelerating the adrenochrome formation. One unit of SOD is defined as the amount of extract that inhibits the rate of adrenochrome formation by 50%. Briefly, one ovary per determination (10 ovaries per group) was homogenized in 50 mmol/l phosphate buffer (pH 7.2) and centrifuged at 1000*g* for 10 min at 4°C. Supernatants were collected and 0.05 mol/l carbonate buffer (pH 10.2) containing 0.1 mmol/l EDTA and followed by 30 mmol/l epinephrine in 0.05% v/v acetic acid was added. The change in SOD activity was measured at 480 nm for 4 min. The control was made following the same protocol but samples were replaced by carbonate buffer, pH 10.2. To calculate units of SOD in the samples, it has been defined that 1 unit (U) of SOD = V control/V sample − 1, where V is the slope of the change in absorbance in the absence and presence of ovarian homogenates.

Glutathione content

The GSH assay was carried out as previously described (Motta et al., 2001). The reduced form of GSH comprises the bulk of cellular protein sulphydryl groups. Thus, the measurement of acid-soluble thiol is used for the estimation of GSH content in tissue extracts. Briefly, supernatants (50 μl/point, 10 ovaries/group) were incubated with 800 μl of 1.5 mol/l Tris buffer (pH 7.4) containing 50 μl of 5×10^{-3} mol/l NADPH and 6 IU of GSH reductase. The reaction involves the enzymatic reduction of the oxidized form to GSH. When Ellman's reagent (a sulphydryl reagent 5,5-dithiobis-2 nitrobenzoic acid; Sigma, USA) is added to the incubation medium, the chromophoric product resulting from this reaction develops a molar absorption at 412 nm that is linear for the first 6 min, after which the reaction remains constant. Results are expressed as mmoles GSH/mg protein.

Determination of viability, apoptosis and necrosis of ovarian cells

In order to determine cellular viability, early apoptosis and late apoptosis or necrosis, suspensions of ovarian cells were treated with an annexin V kit (Calbiochem, Gibbstown, NJ, USA) immediately after collection. The kit contains both fluorescein isothiocyanate (FITC) conjugated to annexin V and propidium iodide (PI). Viable ovarian cells do not bind FITC—annexin V and do not stain nuclear formation with PI, while late apoptotic or necrotic cells bind FITC—annexinV but also stain their nuclei with PI (because of the permeabilization of cellular membranes during necrosis). Early apoptotic cells bind FITC—annexin V but exclude PI. Quantification is carried out by flow cytometry, as the FITC signal can be detected by the FITC detector at 518 nm, while PI can be detected at 620 nm and can be detected by the phycoerythrin fluorescence detector. Viable cells are shown in the lower left-hand quadrant of the dot-plot graph, early apoptotic cells in the lower right-hand quadrant and necrotic or apoptotic cells in the upper right-hand quadrant. All procedures were carried out following the manufacturer's instructions. Briefly, 1×10^6 ovarian cells in 1 ml of Roswell Park Memorial Institute 1640 from each treatment was incubated in polypropylene tubes with 1.5 ml annexin V for 15 min at room temperature (18—24°C) in the dark. Cells were then centrifuged at 100*g* for 5 min at room temperature, the media removed and cells resuspended in 500 ml PBS (pH 7.4). Finally, each sample was added to 10 ml PI and then placed on ice away from light. Flow cytometry assays were carried out immediately. Fluorescence analysis was evaluated with FACScan and Winmdi 2·8 software (http://facs.scripps.edu/software.html) and results are expressed as the percentage of cells at different stages.

Determination of ovarian prostaglandin E by radioimmunoassay

The measurement of PGE was carried out in incubation media, since it has been previously determined that both homogenates and incubated tissues reflected ovarian PGE concentrations (Luchetti et al., 2004). Briefly, the tissue (each ovary) was weighed and incubated in Krebs–Ringer bicarbonate (KRB) with glucose (11.0 mmol/l) as external substrate (pH 7.0) for 1 h in a Dubnoff metabolic shaker under an atmosphere of 5% CO_2 in 95% O_2 at 37°C. At the end of the incubation period, the tissue was removed and the solution acidified to pH 3.0 with 1 mol/l HCl and extracted for prostaglandin determination three times with 1 volume of ethyl acetate. Pooled ethyl acetate extracts were dried under an atmosphere of N_2 and stored at −20°C until RIA was performed. Prostaglandin E was quantified using a rabbit antiserum from Sigma. Sensitivity was 10 pg/tube and cross-reactivity was 100% PGE and <0.1% with other prostaglandins. Results are expressed as pg PGE/mg protein.

Determination of ovarian CD4+ and CD8+ T lymphocytes by flow cytometry

To carry out the flow cytometry assay, ovarian cells must be dispersed. Briefly, ovaries (from 10 control and 10 DHEA-treated animals) were enzymatically dissociated in culture medium (medium 199, 25 mmol/l $NaHCO_3$, 26 mmol/l HEPES and 50 IU/ml penicillin) with trypsin-free collagenase (740 IU/100 mg tissue) and DNase (14 IU/100 mg tissue). After 90 min, ovarian cells were washed twice with culture medium, twice with Dulbecco's PBS free of Ca and Mg ions and twice with culture medium containing EDTA (1 mmol/l). To remove blood cells, suspensions were applied to a Ficoll Hypaque gradient 1.077 (Sigma), centrifuged at 400*g* for 45 min and washed with PBS/0.1% bovine serum albumin. Cells were counted in a haemocytometer, >80% viability was assessed by the trypan blue exclusion method and then the cells were processed by direct immunofluorescence. Then, 100 μl of each cell suspension (control or DHEA-treated), at a concentration of 10^6 cells/ml, was incubated for 30 min at 4°C with: (i) 30 μl of phycoerythrin (PE) rat immunoglobulin G2a (IgG2a) isotype control plus 30 μl of FITC rat IgG2a isotype control (eBioscience, USA) corresponding to the isotype control sample; or (ii) 4 μl (8 μg protein) PE anti-mouse-CD4 plus 4 μl (8 μg protein) FITC anti-mouse-CD8 T-cell monoclonal antibody (eBioscience); corresponding to either the control or the DHEA assay. Antibodies were used at saturating concentration as established after titration by flow cytometry. Then, samples were washed with PBS and PBS-EDTA, fixed with 4% paraformaldehyde and stored at 4°C in the dark until analysis within 6 days of labelling. Fluorescence analysis was evaluated with FACScan and the Winmdi 2.8 software. Lymphocytes were analysed using different physical characteristics such as size and complexity by gating using forward (cell size) and side scatter (cell complexity) parameters. Flow cytometric analysis was performed using standard fluorescence 1 (FITC anti-mouse-CD8+ T lymphocytes) and fluorescence 2 (PE anti-mouse-CD4+ T lymphocytes). The analysis was based on the measurement of 50,000 nucleated cells/assay where the specific region of the T lymphocyte was characterized by means of its size and complexity. The percentage of positively labelled cells was calculated by subtracting signals from non-specifically labelled cells.

Western blot analysis for leptin receptor

Soluble tissue extracts were prepared as described previously (Di Yorio et al., 2008). Briefly, ovaries were homogenized in 20 mmol/l ice-cold Tris–HCl buffer (pH 7.4), containing 0.25 mmol/l sucrose, 1 mmol/l EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 mg/ml phenylmethylsulphonyl fluoride and 10 μg/ml trypsin inhibitors. The homogenates were sonicated and centrifuged at 7800*g* at 4°C for 15 min and protein concentration in the supernatant was determined by the Bradford method (1976) with bovine serum albumin as the standard. Homogenates were boiled for 5 min in buffer containing 0.3% (w/v) bromophenol blue and 1% (v/v) β-mercaptoethanol. Equal amounts of protein (100 μg) were loaded onto 4% (w/v) 0.125 mol/l Tris–HCl (pH 6.8) stacking polyacrylamide gel, followed by a 7.5% (w/v) 0.375 mol/l Tris–HCl (pH 8.8) separating polyacrylamide gel. Following electropshoresis, proteins were transferred to polyvinylidene fluoride membrane (Bio-Rad Laboratories, USA) for 60 min in a cold chamber using a Bio-Rad transblot apparatus. Membranes were first blocked at 4°C overnight in Tris-HCl/saline (50:150 mmol/l; pH 7.5) containing 5% (w/v) of milk powder and then incubated at 4°C overnight with an antibody raised in rabbit against Ob-R (H-300) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The final dilution of the antibody was 1:200. The membranes were washed four times for 15 min each in Tris-HCl/saline containing 0.1% (v/v) Tween 20 (pH 7.5; TTBS). Negative controls were carried out by omitting the incubation with the primary antibody. No bands were detected. Then, the sections were incubated for 1 h at room temperature with goat anti-rabbit IgG (1:2500) as the secondary antibody (Santa Cruz Biotechnology). The antibody was then washed off in TTBS and the immunoreactive bands were visualized using chemiluminescence detection reagents (Sigma) and exposed to Kodak X-OMAT film. Before re-use, membranes were stripped, blocked and manipulated according to the manufacturer's instructions. Membranes were incubated with anti-actin antibody (A2066; Sigma). Molecular weight standards (Kaleidoscope St; Bio-Rad Laboratories) were run under the same conditions to identify the protein bands. Blots were scanned using a scanning UMAX Astra 12205 and densitometry was analysed using a Dekmate III Sigma Gel software package (Jandel Scientific Soft-ware; Sa11 Rafael, CA, USA). The data were normalized to -actin protein concentrations in each sample to prevent procedural variability.

Serum progesterone and oestradiol concentrations by radioimmunoassay

In order to evaluate the steroidogenic activity, both serum progesterone and oestradiol concentrations were measured by specific RIA as described before (Motta et al., 1999) with highly specific antisera. The cross-reactivity of the progesterone antiserum was <2.0% for 20 α-dihydroprogesterone and deoxycorticosterone and 1.0% for other steroids normally

present in serum. The cross-reactivity of the oestradiol antiserum was <1% for progesterone and testosterone, <5% for 17-α-oestradiol and oestriol and <10% for oestrone. Since both sensitivities were 5—10 pg/tube, 2—5 l of serum was routinely assayed. Results are expressed as pg/ml serum.

Ovarian protein concentration

Ovarian protein concentration was determined as described by Bradford (1976).

Statistical analysis

Statistical analyses were carried out by using the Instat program (GraphPAD software, San Diego, CA, USA). Student's *t*-test and one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test were performed in order to assess the statistical significance of differences between means. $P < 0.05$ was considered significant.

Results

Morphology

Figure 1**a** shows a representative section of ovarian tissue from the control group. The general appearance of the normal tissue histology: a central medulla consisting of a fibromuscular stroma and a large number of blood vessels. The peripheral cortex contained large numbers of follicles in various stages of development. Histological examination of ovaries from hyperandrogenized mice revealed an increase in the number of small primary follicles located in the cortex (Figure 1**b**).

Lipid peroxidation index

As a measure of the ovarian damage induced by the excess of androgens the oxidant/antioxidant balance was evaluated. It was found that hyperandrogenism increased lipid peroxidation, decreased SOD activity and did not modify GSH content when compared with controls (Figure 2**a**—**c**).

Determination of viability, apoptosis and necrosis

The FITC—annexin V assay was used to determine whether hyperandrogenism induces early apoptosis, late apoptosis or necrosis during early folliculogenesis. Figure 3**a** illustrates a representative analysis of samples from both control and hyperandrogenized mice. Viable cells are shown in the lower left-hand quadrant of the dot-plot graph, early apoptotic cells in the lower right-hand quadrant and necrotic or apoptotic cells in the upper right-hand quadrant. Hyperandrogenism decreased ovarian cell viability and increased early apoptosis when compared with controls (Figure 3**b**). Therefore, hyperandrogenism did not modify late apoptosis or necrosis when compared with controls.

Ovarian prostaglandin E production

Hyperandrogenism increased ovarian PGE production when compared with controls (Figure 4).

Ovarian lymphocyte T infiltration and immunophenotype

Considering that the immune system and its network of secretor products play an active role in early folliculogenesis, this study was interested in possible changes in the phenotype of T lymphocytes as a consequence of hyperandrogenization with DHEA. Flow cytometry was used to study the number and phenotype of T lymphocytes. In control samples (over 50,000 events analysed), 42 ± 3% were lymphocytes, and in treated samples (for the same region over 50,000 total events), 64 ± 2% were lymphocytes, thus indicating a significant ($P < 0.001$) increase in T-lymphocyte infiltration. Figure 5 illustrates a representative analysis of samples from both controls and hyperandrogenized mice using forward scatter (cell sizes) and side scatter (granularity) parameters. The dot-plot analysis shows that CD4+ T and CD8+ T lymphocytes represented 51 ± 2% and 49 ± 3%, respectively, of the total T cells in control animals (Figure 5**a**). Hyperandrogenization with DHEA decreased the population of the CD4+ T-cell subset (17 ± 1%) and increased the CD8+ T-cell subset (83 ± 4%) (Figure 5**a** and **b**).

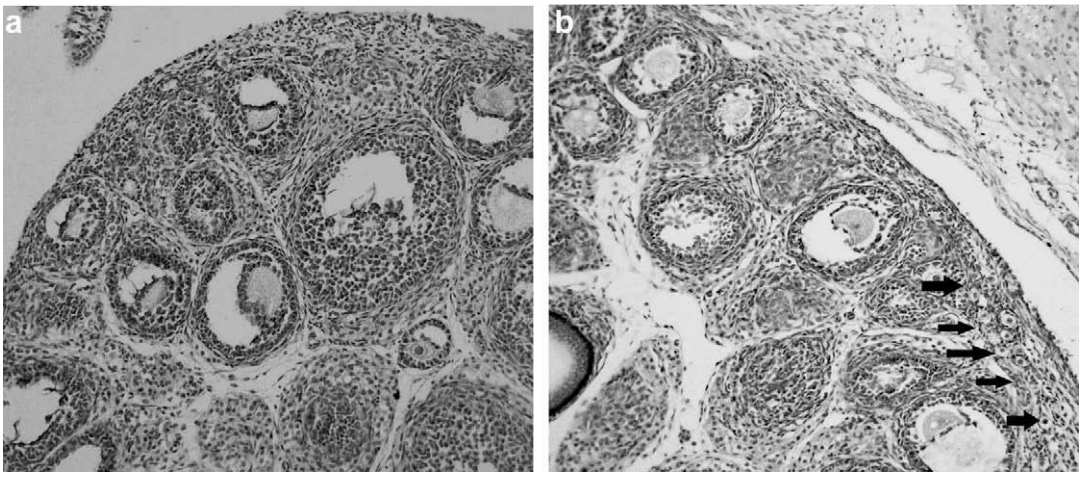

Figure 1 (**a**) A representative ovarian tissue section of a mouse injected with sesame oil for 10 consecutive days (×100) and (**b**) an ovarian section from a mouse treated with dehydroepiandrosterone for 10 consecutive days (×100); Arrows = primary follicles.

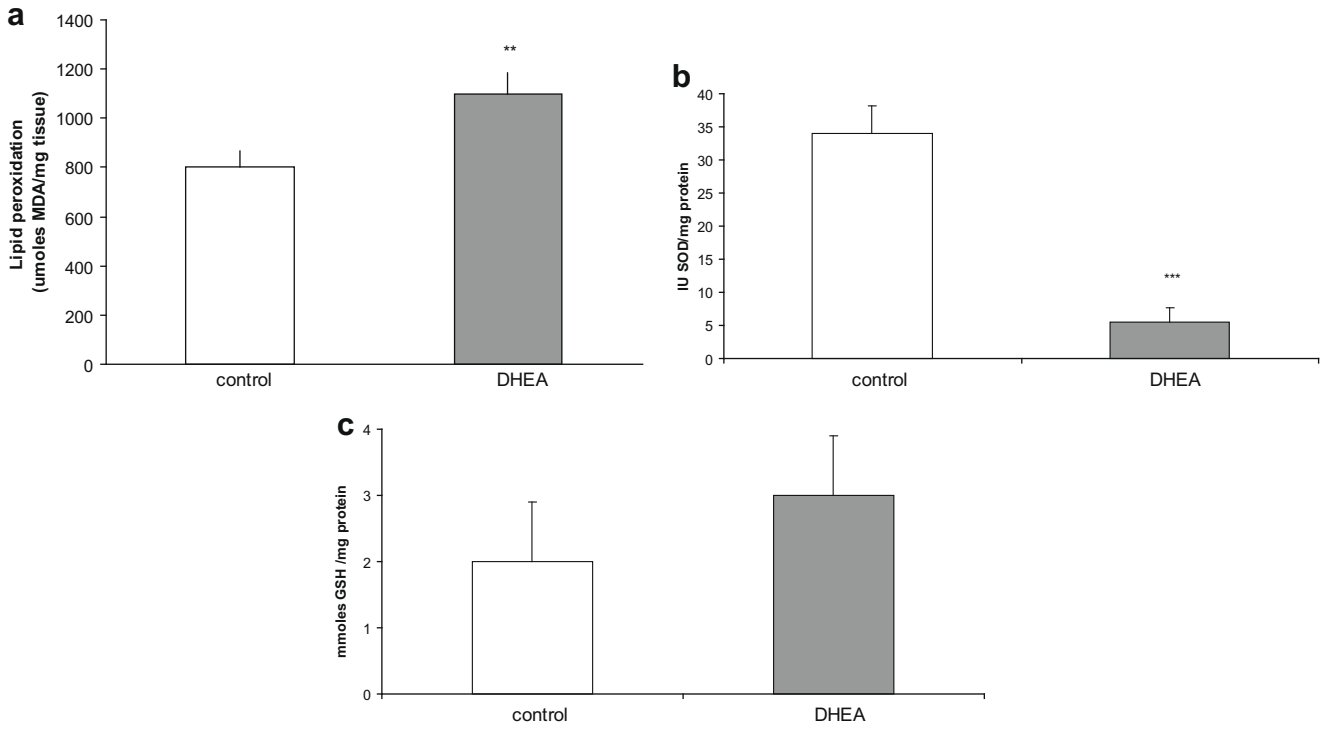

Figure 2 Oxidative stress from control and dehydroepiandrosterone (DHEA)-treated mice. (**a**) Lipid peroxidation index, (**b**) superoxide dismutase activity and (**c**) total glutathione content. Each column represents mean ± SEM of 10 measurements from different animals. MDA = malondialdehyde; $^{**}P < 0.001$; $^{***}P < 0.0001$.

Expression of ovarian Ob receptor

The expression of leptin receptors was evaluated in ovaries from the control and hyperandrogenized mice by Western blot analysis using Ob-R antibody reactive against a recombinant protein corresponding to amino acids 541—840, mapping a region within an internal domain of Ob-R. Both control and hyperandrogenized ovaries showed protein bands at 150 kDa consistent with the predicted size of Ob-Rb, which is considered to be the long isoform of the leptin receptor (Di Yorio et al., 2008). On the other hand, hyperandrogenization decreased the ovarian expression of the Ob-Rb receptor when compared with controls (Figure 6**a** and **b**). No evidence of other OB-R isoforms was found in either the control or in hyperandrogenized ovaries.

Serum progesterone and oestradiol concentrations

Serum progesterone and oestradiol concentrations were quantified in order to assess how the altered endocrine and immune parameters influence early folliculogenesis. Hyperandrogenization with DHEA did not modify serum progesterone concentrations but increased serum oestradiol concentrations when compared with controls (Figure 7**a** and **b**).

Discussion

Although it is well known in polycystic ovarian pathology that follicles fail to mature and enter atresia, the mechanisms triggering atresia of resting follicles remain unknown. In the present study, histological examinations showed enhanced growth of the primary follicles located in the ovarian cortex. In parallel, studies have shown increased oxidative stress, represented by increased lipid peroxidation, and a diminished activity of SOD (the enzyme that detoxifies the cells to $O_2^{\cdot}$, a strong oxidant radical that in combination with nitric oxide synthesizes peroxynitrite radical) (Beckman et al., 1990). For these reasons, the decrease in SOD activity demonstrates the accumulation of $O_2^{\cdot}$ and a high degree of oxidative damage. The fact that hyperandrogenism decreased ovarian cell viability and increased early apoptosis confirmed this damage. On the other hand, hyperandrogenism did not have any effect on late apoptosis or necrosis. In agreement with these findings, Walters et al. (2008) reported that androgens enhance follicular atresia by activating endonuclease activity, and Tilly and Tilly (1995) demonstrated that enhanced oxidative stress favours the expression of death-inducer genes, which control the initiation of apoptosis. These data demonstrated that hyperandrogenism during early folliculogenesis increases oxidative stress and enhances early apoptosis.

As has been previously found during the peri-ovulatory period (Elia et al., 2006; Luchetti et al., 2004; Sander et al., 2006), hyperandrogenization increased ovarian PGE concentrations during early folliculogenesis. This may be analysed as a consequence of the enhanced lipid peroxidation of ovarian cells that in turn release arachidonic acid, the substrate of PGE. The increase in ovarian PGE production results in both an enhanced recruitment of T lymphocytes infiltrating the ovarian tissue — as described in other systems (Gutzmer et al., 2004; Vancheri et al., 2004) — and in the regulation of the

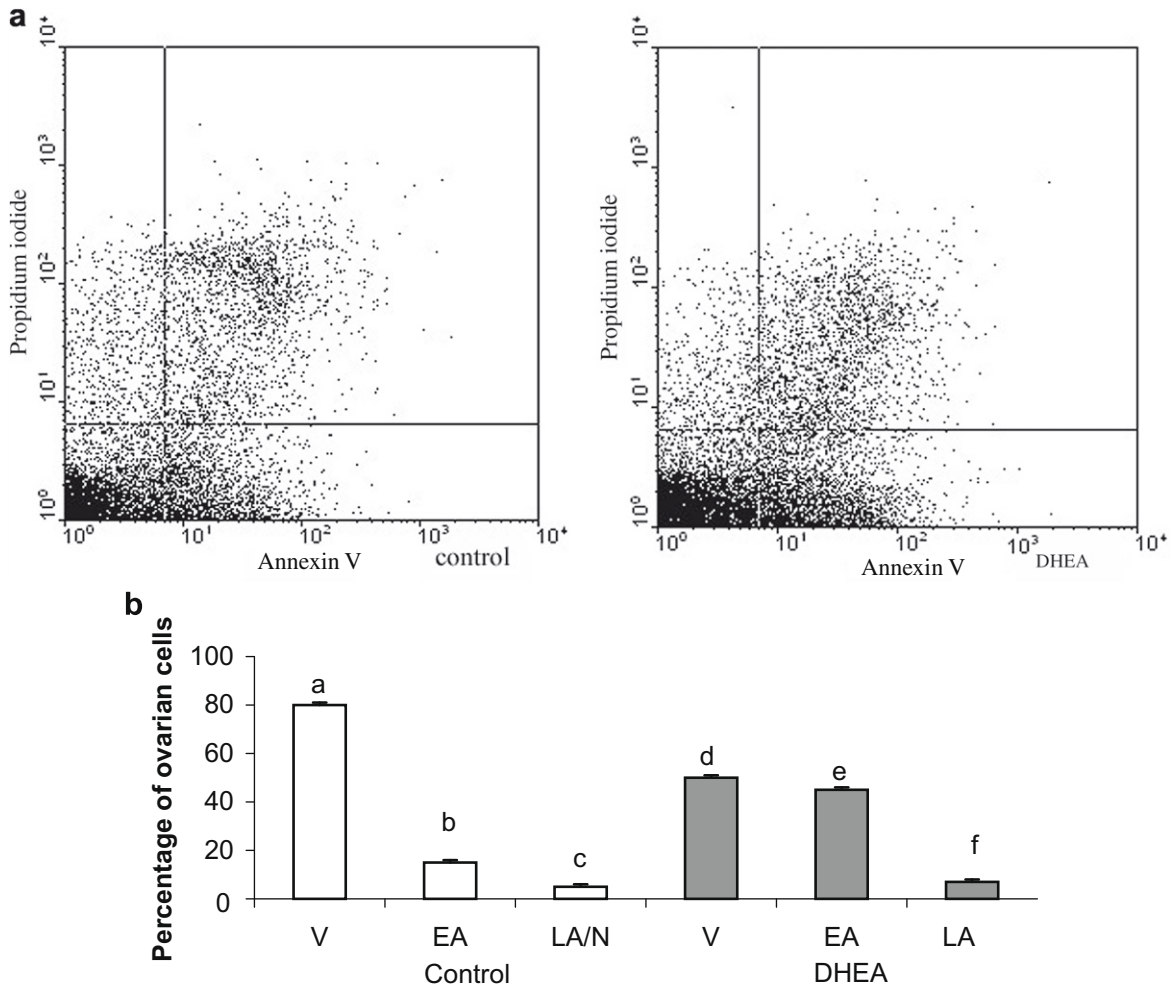

Figure 3 Modulation of ovarian cellular viability, early apoptosis and late apoptosis/necrosis by dehydroepiandrosterone using the FITC—annexin V assay. (**a**) A dot-plot analysis and (**b**) percentages of ovarian cells, a versus d $P < 0.0001$, b versus e $P < 0.0001$. DHEA = dehydroepiandrosterone; EA = early apoptosis; LA/N = late apoptosis/necrosis; V = cellular viability.

T phenotype (with increased CD8+ and decreased CD4+ subsets). These data suggest that PGE was able to regulate the ovarian infiltration of T cells as has been reported in other tissues (Kuroda and Yamashita, 2003; Lakier, 2003; Yang et al., 2003). In addition, the increased follicular atresia and early apoptosis could be due to this immune regulation as has been reported in cycling women (Suzuki et al., 1998). Moreover, Lu et al. (2002) found that oestrogen increases the percentage of ovarian CD8+ T-cell population and Chernyshov et al. (2001) described selective changes in the lymphocyte subtype in premature ovarian failure. All these findings, together with the fact that immune mechanisms regulate folliculogenesis (Brannstrom and Enskog, 2002), led us to suggest that hyperandrogenization could alter the early follicular development by regulating the ovarian T-lymphocyte expression.

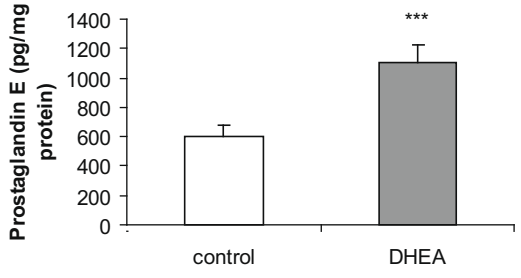

Figure 4 Ovarian prostaglandin E production from control and dehydroepiandrosterone (DHEA)-treated mice. Each column represents mean ± SEM. ***$P < 0.0001$.

It is known that, in granulosa cell cultures from rats (Zachow and Magoffin, 1997) cattle (Spicer and Francisco, 1997) and humans (Agarwal et al., 1999), leptin suppresses the sensitizing effect of insulin and/or insulin growth factor 1 (IGF-1) on FSH-dependent oestradiol production. On the other hand, leptin receptors are sensitive to endocrine changes (Licinio et al., 1998; Messinis et al., 1998; Shimizu et al., 1997). The expression of the long isoform decreases dramatically during ovariectomy (Meli et al., 2004) and the positive or negative effects that leptin exerts during the ovulatory process depends on the regulation of its own receptors (Di Yorio et al., 2008). The present study demonstrated that, similar to adult ovaries (Di Yorio et al., 2008), prepuberal ovaries express the Ob-Rb isoform during early folliculogenesis. The current study also found that hyperandrogenism decreased the Ob-Rb isoform, thus decreasing

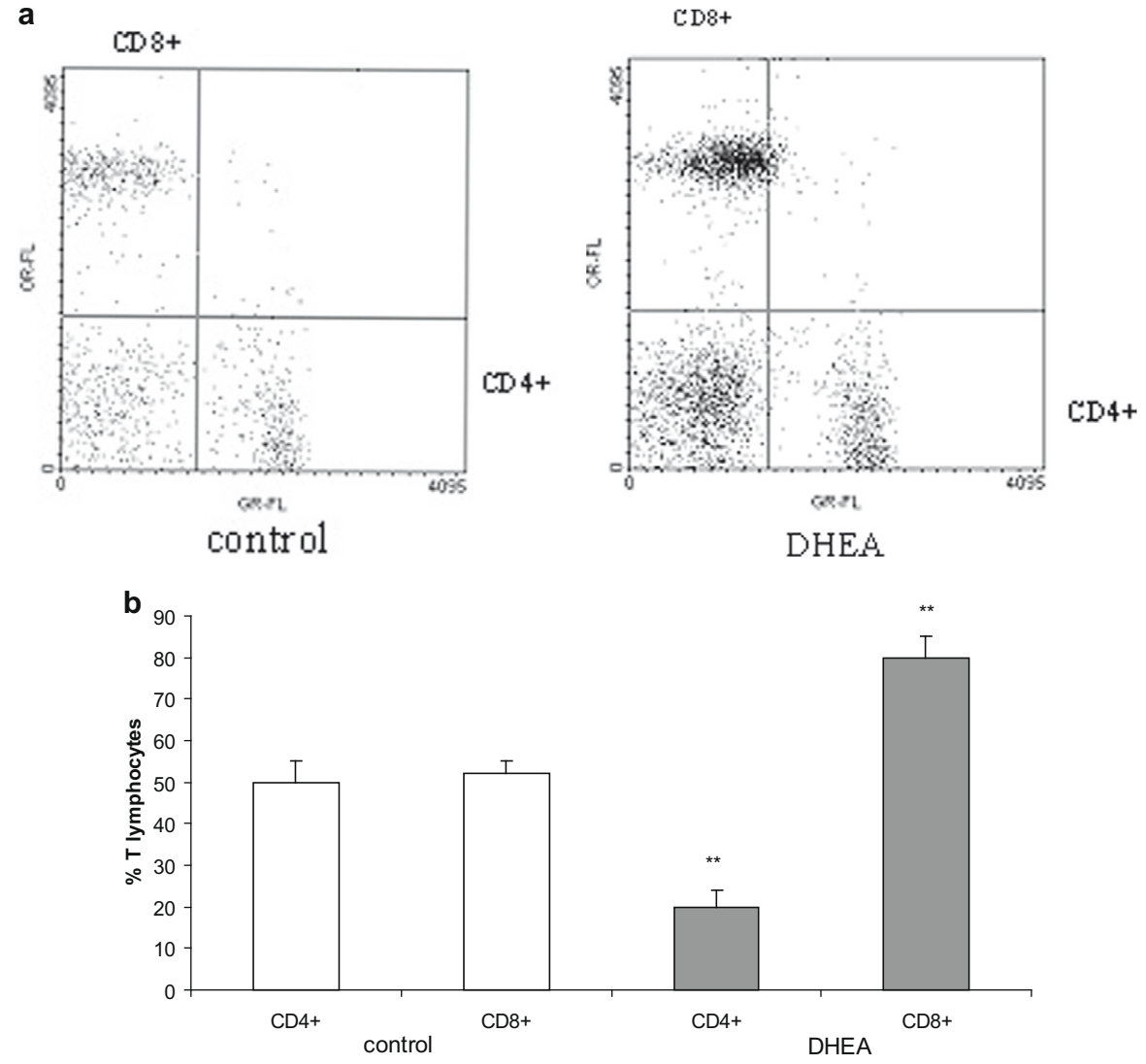

Figure 5 Modulation of ovarian T-lymphocyte infiltration by dehydroepiandrosterone (DHEA). (**a**) Flow cytometry analysis of control and DHEA treatment groups using forward (cell size) and side scatter (cell complexity) parameters and dot-plot analysis using both standard fluorescence with FITC anti-mouse-CD8+ T lymphocyte and phycoerythrin anti-mouse-CD4+ T lymphocyte and (**b**) percentages of T lymphocytes. ***$P < 0.0001$.

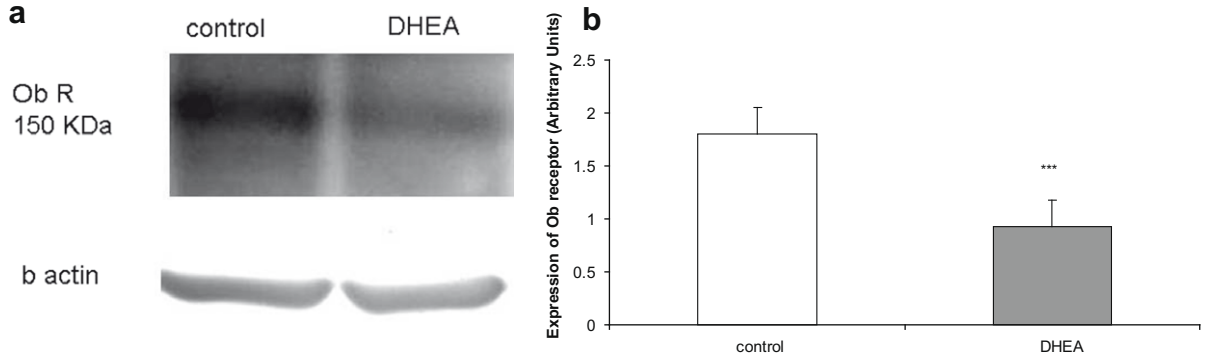

Figure 6 Western blotting for expression of the ovarian leptin receptor (Ob-R) in ovarian tissue from control (oil injected) and dehydroepiandrosterone (DHEA)-treated mice. Bands correspond to the long form; Ob-Rb 150 kDa. (**a**) A representative Western blot and (**b**) integrated optical density of the bands. Each column represents mean ± SEM. ***$P < 0.0001$.

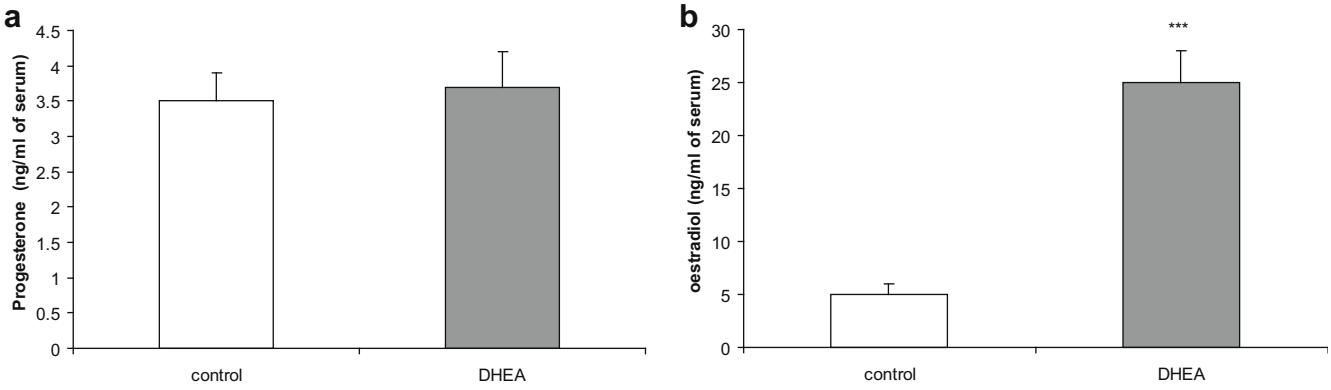

Figure 7 Serum concentrations of (**a**) progesterone and (**b**) oestradiol, from control and dehydroepiandrosterone (DHEA)-treated mice. Each column represents mean ± SEM of 10 measurements from different animals. (*) value is significantly different from control value by analysis of variance; ***$P < 0.0001$.

ovarian sensitivity to leptin. In addition, this study can assume that, as in other systems (Alonso et al., 2007; Meli et al., 2004), sex steroids are able to stimulate different responses towards the same leptin concentration by modulating the Ob-R expression. Finally, the impact of these abnormalities on ovarian steroidogenesis was evaluated and it was found that hyperandrogenism increases serum oestradiol concentrations and that this is related to the increased CD8+ T phenotype that infiltrates the ovarian tissue, as has been previously reported in rhesus macaque ovaries (Lu et al., 2002). This increase in serum oestradiol concentrations regulates the leptin receptor, as has been previously described (Alonso et al., 2007). In summary, the present work describes the complex relationship between some endocrine and immune parameters within the ovarian tissue that directly or indirectly regulate ovarian folliculogenesis.

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