

Role of the *N, N'*-dimethylbiguanide metformin in the treatment of female prepuberal BALB/c mice hyperandrogenized with dehydroepiandrosterone

Valeria Sander, Carolina Griselda Luchetti, María Emilia Solano, Evelin Elia, Guillermo Di Girolamo¹, Claudio Gonzalez¹ and Alicia Beatriz Motta

Centro de Estudios Farmacológicos y Botánicos (CEFyBO), Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Buenos Aires, Argentina and ¹Facultad de Medicina, Departamento de Farmacología, Buenos Aires, Argentina

Correspondence should be addressed to A B Motta, Laboratorio de Fisiopatología Ovárica, Centro de Estudios Farmacológicos y Botánicos (CEFyBO), UBA-CONICET, Paraguay 2155, (1121) Buenos Aires, Argentina; Email: aliciabmotta@yahoo.com.ar

Abstract

The present study investigated the role of the *N, N'*-dimethylbiguanide metformin (50 mg/100 g body weight in 0.05 ml water, given orally with a cannula) in the prevention of endocrine and immune disorders provoked by the hyperandrogenization with dehydroepiandrosterone (DHEA) in prepuberal BALB/c mice. The treatment with DHEA (6 mg/100 g body weight in 0.1 ml oil) for 20 consecutive days, recreates a mouse model that resembles some aspects of the human polycystic ovary syndrome (PCOS). The treatment with DHEA did not modify either body mass index (BMI) or blood glucose levels, but did increase fasting insulin levels when compared with controls. Markers of ovarian function – serum estradiol (E), progesterone (P) and ovarian prostaglandin E (PGE) – were evaluated. The treatment with DHEA increased serum E and P levels while ovarian PGE diminished. When metformin was administered together with DHEA, serum insulin, E and P levels, and ovarian PGE values did not differ when compared with controls. Using flow cytometry assays we found that the treatment with DHEA diminished the percentage of the CD4 + T lymphocyte population and increased the percentage of the CD8 + T lymphocyte population from both ovarian tissue and retroperitoneal lymph nodes. However, when metformin was administered together with DHEA, the percentages of CD4 + and CD8 + T lymphocyte populations from both ovarian tissue and retroperitoneal lymph nodes were similar to those observed in controls. Finally, when DHEA was administered alone it increased the serum tumor necrosis factor- α (TNF- α) levels when compared with controls; however, when metformin was administered together with DHEA, serum TNF- α levels were similar to controls. These results indicate that metformin is able, directly or indirectly, to avoid the endocrine and immune alterations produced when mice are hyperandrogenized with DHEA.

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Introduction

Polycystic ovary syndrome (PCOS) – which is characterized by hyperandrogenemia, hirsutism, oligo- or amenorrhea and anovulation – is one of the most common endocrinological diseases encountered in premenopausal women (Franks 1995, Asuncion *et al.* 2000). It has been reported that hyperinsulinemia, frequently associated with PCOS, increases both the risk of cardiovascular diseases and the development of diabetes mellitus (Abbott *et al.* 2002). Although during the last decade several clues have emerged from human and animal studies, little is known about the etiology and pathophysiology of PCOS. The battery of animal models used for the study of polycystic ovaries has allowed researchers to investigate different

aspects of the pathology (Billiar *et al.* 1985, Szkiewicz & Uilenbroek 1998, Weil *et al.* 1999, West *et al.* 2001, Abbott *et al.* 2002). After it was found that dehydroepiandrosterone (DHEA) levels were increased in women with PCOS (Malesh & Greenblatt 1962), Roy *et al.* (1962) produced an animal model using DHEA for induction of PCOS. Subsequent studies established that the DHEA–PCOS murine model exhibits some of the salient features of human PCOS, such as hyperandrogenism, abnormal maturation of ovarian follicles and anovulation (Lee *et al.* 1991, 1998, Anderson *et al.* 1992, Henmi *et al.* 2001). These findings, together with the fact that increasing evidence indicates that DHEA has, in addition, potent immunoregulatory functions (Meikle *et al.* 1992, Okabe *et al.* 1995, Hernandez Pardo *et al.* 1998, Zhang *et al.* 1999) led

us to use the DHEA–mice model to study some aspects related to the endocrine and immune responses involved.

In a previous study, we have demonstrated that treatment of prepuberal BALB/c mice with DHEA alters ovarian morphology, induces ovarian cysts, increases serum 17 beta estradiol (E) and progesterone (P) levels, and modifies ovarian prostaglandin E (PGE) concentration. Therefore, the treatment with DHEA led to a selective ovarian CD4 + and CD8 + T lymphocyte expression characterized by both a diminution of the percentage of CD4 + lymphocytes and an increase in the percentage of CD8 + T lymphocytes (Luchetti *et al.* 2004). In view of our previous studies and those reported by Lu *et al.* (2002), who demonstrated that production of cytokines by B cells is controlled by an enriched CD8 + T lymphocyte population and steroid hormones, experiments were designed to evaluate whether DHEA was able to lead to a selective T lymphocyte population that could, in turn, regulate cytokine production. Considering the fact that tumor necrosis factor- α (TNF- α) regulates androgen production by theca cells (Sayin *et al.* 2003) and that it has been reported to be associated with PCOS (Deshpande *et al.* 2000, Araya *et al.* 2002, Korhonen *et al.* 2002, Peral *et al.* 2002), the aim of this study was to investigate the concentration of serum TNF- α after treatment with DHEA.

Multiple concomitant therapies have been applied in PCOS to address the variety of symptoms and to achieve better results. Recent studies have investigated the role of a family of insulin-sensitizing agents: the biguanides. Thus, the use of metformin (*N,N'*-dimethylbiguanide) is becoming increasingly accepted and widespread. However, this drug is being clinically used without a complete understanding of the mechanisms involved. Metformin has been shown to be useful in the reduction of insulin resistance by restoring insulin sensitivity (Fedorcsak *et al.* 2003, Harbone *et al.* 2003, Lord *et al.* 2003). In addition, it has been demonstrated that metformin is able, either directly or indirectly, to regulate ovarian steroidogenesis (La Marca *et al.* 2002, Mansfield *et al.* 2003).

Controversial results have been reported with regards to metformin and its relationship with the immune system. In patients with type-2 diabetes, the biguanide enhances the tyrosine kinase activity of the insulin receptor by modulating the plasma cell differentiation antigen (PC-1) (Stefanovic *et al.* 1999). Nevertheless, Ruat *et al.* (2003) have failed to demonstrate any relationship between metformin and proliferation assays of T cells of lymph nodes.

The aim of the present work was to assess the efficacy of metformin on some immune and endocrine parameters altered after hyperandrogenization with DHEA. We therefore evaluated body mass index (BMI), the glucose:insulin ratio (in order to determine the homeostasis model assessment (HOMA) index), and serum E and P levels (as indicators of ovarian function). We also quantified ovarian PGE production, because PGE is not only involved in inflammatory processes and displays immunomodulatory

properties, but is also altered in PCOS (Navarra *et al.* 1996, Wojtowicz-Praga 1997). With regards to the immune system we also designed experiments to investigate the role of metformin in modulating both the CD4 + and CD8 + T cell population of ovarian tissue and secondary immune tissues (axillar and retroperitoneal lymph nodes). In addition, the production of the pro-inflammatory cytokine TNF- α was evaluated.

Materials and Methods

Animals and experimental protocol

The hyperandrogenized environment of PCOS was reproduced in mice by injection of DHEA (Luchetti *et al.* 2004). Briefly, female prepuberal (25 days old) mice of the BALB/c strain were injected daily with DHEA (6 mg/100 g body weight, dissolved in 0.10 ml sesame oil) for 20 consecutive days (DHEA group). The animals of the DHEA + metformin group were injected with DHEA and given metformin orally (50 mg/100 g body weight in 0.05 ml water, given orally with a cannula) for 20 days. The dose of metformin administered was equivalent to that used in the treatment of women with PCOS. The controls consisted of three groups: (a) animals injected with oil (0.1 ml) and given water orally (0.05 ml) for 20 consecutive days (control vehicle); (b) the metformin-alone group which consisted of mice treated orally with 50 mg metformin/kg body weight in 0.05 ml water for 20 days; (c) the untreated group formed by animals that did not receive treatment. Mice were housed under controlled temperature (22 °C) and illumination (14 h light:10 h darkness; lights on at 0500 h) and were allowed free access to Purina rat chow and water. All procedures involving animals were conducted in accordance with the Animal Care and Use Committee of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) 1996.

Throughout the whole treatment, the animals were weighed daily and, in addition, the stage of the sexual cycle was determined. Vaginal smears were taken daily up to the day of autopsy. As previously found (Luchetti *et al.* 2004), mice treated with DHEA remained in constant estrus. In contrast, mice administered with DHEA together with metformin showed irregular sexual cycles. None of the animals in the experimental groups displayed a complete sexual cycle at the time of killing. These findings led us to use, for the control and metformin-alone groups, only those animals that at the time of killing were in the estrus stage of the sexual cycle. In other words, in order to compare the different groups, we have treated sufficient animals from groups other than the DHEA-alone group to ensure that there were ten animals per group at estrus stage on the day of killing.

After 20 days of treatment, ten mice (45 days old) per group were anesthetized with ether and killed by decapitation. Blood was collected and fasting glucose levels were immediately determined. Then, serum was isolated

and stored at -70°C until assayed for E, P, insulin and TNF- α . Twenty freshly dissected ovaries per group were divided as follows: ten ovaries of each group were prepared to evaluate CD4+ and CD8+ T lymphocyte expression by flow cytometry assay; ten ovarian tissues were immediately frozen at -70°C until PGE radioimmunoassay. Lymphoid tissues (axillar and retroperitoneal nodes) from the ten animals of each group were immediately collected and processed to determine the CD4+ and CD8+ T cell expression by flow cytometry assay. All experiments were repeated three times.

Assays for glucose and insulin quantification

Fasting blood glucose was determined by using the Haemoglukotest (Roche) test strips for visual determination in the range of 20–800 mg/100 ml (1–44 mmol/l). The test principle uses the glucose-oxidase/peroxidase reaction. Results are expressed in millimoles of glucose per liter.

Serum insulin levels were assayed by the Coat-A-Count insulin method (Diagnostic Products Corporation, LA, USA) following the manufacturer's instructions. Briefly, the determination consists of a solid-phase ^{125}I radioimmunoassay designed for the quantitative measurement of insulin in serum. The antibody is immobilized to the wall of a polypropylene tube and simply decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabeled insulin. The samples were counted in a gamma counter. Analytical sensitivity was 1.2 μIU insulin/serum ml.

E and P determination

Serum E levels were evaluated as described previously (Luchetti *et al.* 2004). Briefly, the blood was allowed to clot and the serum removed and frozen until E and P concentrations were determined by radioimmunoassay. Antisera were provided by Dr G D Niswender (Colorado State University, Fort Collins, CO, USA). The E antiserum showed low cross-reactivity: <1% for P and testosterone, <5% for estriol and <10% for estrone. The P antiserum was highly specific and showed low cross-reactivity: <2% for 20- α -dihydro- progesterone and deoxycorticosterone, and 1% for other steroids normally present in serum. Results are expressed in nanograms per serum milliliter.

Prostaglandin radioimmunoassay

The measurement of PGE was carried out in the incubation media of ovarian tissues as previously reported (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 M 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 prostaglandin radioimmunoassay was performed. PGE was quantified using a rabbit antiserum from Sigma. Sensitivity was 10 pg/tube and cross-reactivity was 100% with PGE and <0.1% with other prostaglandins. Results are expressed in picograms PGE per milligram of protein. Ovarian protein content was determined using the Bradford method (1976).

Flow cytometry

To carry out the flow cytometry assays, ovarian tissue and lymph nodes (axillar and retroperitoneal) must be dispersed (Luchetti *et al.* 2004). Briefly, tissues were enzymatically dissociated in culture medium (medium 199, 25 mM Hepes, 26 mM NaHCO_3 and 50 IU/ml penicillin) with collagenase (trypsin-free, 740 IU/100 mg tissue) and DNase (14 IU/100 mg tissue). After 90 min, cells were washed twice with culture medium, twice with Dulbecco–phosphate-buffered saline free of Ca^{2+} and Mg^{2+} (PBS) and twice with culture medium containing EDTA (1 mM). To remove blood cells, suspensions were applied to Ficoll-hystopaque gradient 1.077 (Sigma), centrifuged at 400 *g* for 45 min and washed with PBS/ 0.1% BSA. Cells were counted in a hemocytometer (viability was >80% as assessed by the trypan blue exclusion method) and then processed for direct immunofluorescence. Thus, 100 μl of each cellular suspension, at a concentration of 10^6 cells/ml, were incubated for 30 min at 4°C with: (a) 30 μl phycoerythrin (PE) Rat IgG2a K Isotype Control plus 30 μl fluorescein isothiocyanate (FITC) Rat IgG2a K Isotype Control (eBioscience, San Diego, CA, USA) corresponding to the isotype control sample; or (b) 4 μl PE anti-mouse CD4 plus 4 μl FITC anti-mouse CD8 (eBioscience, USA), corresponding to control, DHEA and DHEA + metformin assay according to the cellular suspension. Antibodies were used at saturating concentrations, as established after titration by flow cytometry. Samples were then washed with PBS and PBS-EDTA, fixed with 4% paraformaldehyde and stored at 4°C in darkness until the analysis was performed within 6 days of labeling. Fluorescence analysis was evaluated with FACScan and WinmDI 2.8 software (Scripps Research Institute). Both ovarian and lymph suspensions were analyzed using different physical characteristics (i.e. size and complexity), using both forward (FSC: cell size) and side scatter (SSC: cell complexity) parameters. Thus, the settings correlated to cellular size and granularity of mouse T lymphocytes. The resulting gate and quadrant were maintained throughout the analysis. Flow cytometric determination was performed using standard fluorescence 1 (FL1; FITC anti-mouse CD8 + T lymphocyte) and fluorescence 2 (FL2; PE-anti-mouse CD4 + T lymphocyte). The analysis was based on quantification of 50 000 cells for each assay for ovarian tissue and 10 000 cells for lymph nodes.

Serum TNF- α determination

A TNF- α enzyme immunoassay (EIA) kit (Assay Design's mouse, MI, USA) was employed following the manufacturer's instructions to quantify serum TNF- α from samples of the three experimental groups. Results are expressed in picograms per serum milliliter.

Statistical analysis

Statistical analyses were carried out using the InStat program (GraphPAD software, San Diego, CA, USA). ANOVA was performed using Newman-Keuls test to compare all pairs of columns and $P < 0.05$ was considered significant. All results are presented as the means \pm S.E.M.

Results

Effect of metformin on BMI, homeostasis model assessment, fasting glucose and insulin levels

These experiments were performed to determine whether the treatment with metformin was able to modify some parameters of the homeostasis and to study the role, if any, of metformin when administered together with DHEA. The weight of the animals was not modified by any of the treatments (Table 1), and thus the BMI (defined as weight (kg)/height (m^2)) was similar in all the groups analyzed (Table 1). Glucose levels did not vary in any of the groups studied (Fig. 1A), while fasting insulin levels increased with DHEA treatment when compared with controls (untreated, control vehicle and metformin-alone groups). HOMA index (defined as: $HOMA = \text{insulin } (\mu\text{UI/ml}) \times \text{glucose (mmol/l)} : \text{BMI (kg/m}^2)$) increased after the treatment with DHEA when compared with controls. When metformin was administered together with DHEA, the effects of DHEA on both insulin levels (Fig. 1B) and the HOMA index were attenuated (Fig. 1C).

Effect of metformin on ovarian function: serum E and P levels and ovarian PGE production

To evaluate both ovarian function after treatment with DHEA and the possible role of metformin as a treatment for the ovarian anomalies produced by DHEA, we designed experiments to quantify serum E and P levels, and ovarian PGE production. Treatment with DHEA for 20 consecutive days increased both serum E and P levels (Fig. 2A and B) when compared with controls (untreated, control vehicle and metformin alone). When metformin

was administered together with DHEA, serum E and P levels showed similar patterns to those of control values (Fig. 2A; DHEA + metformin).

On the other hand, the production of PGE by ovarian tissue was diminished after treatment with DHEA (Fig. 2C), but did not significantly differ from the control values when metformin was administered together with DHEA (Fig. 2C).

Role of metformin in ovarian CD4 + and CD8 + T lymphocyte expression

Flow cytometry analysis was employed to determine both the effect of DHEA treatment on the expression of ovarian CD4 + (or helpers) and CD8 + (or cytotoxic/suppressors) T lymphocytes and the role of metformin when administered together with DHEA. For the 50 000 cells analyzed, we found that the control groups showed equivalent percentages of ovarian CD4 + and CD8 + T lymphocytes (Fig. 3). However, treatment with DHEA diminished the percentage of CD4 + T cells ($17 \pm 5\%$) and increased the percentage of CD8 + T cells ($83 \pm 4\%$) when compared with controls (Fig. 3). In contrast, when metformin was administered together with DHEA, we observed the same percentages of CD4 + and CD8 + T cells as in the controls (Fig. 3).

Role of metformin in T lymphocyte expression in lymph nodes

In order to establish whether the effects of DHEA treatment and the administration of metformin together with DHEA also involved secondary lymphoid tissues, the percentages of CD4 + and CD8 + T cells were quantified in both axillar and retroperitoneal lymph nodes. Figure 4A illustrates the flow cytometry analysis of axillar nodes. It can be seen that all the control groups yielded equivalent percentages of CD4 + and CD8 + T lymphocytes, and that neither the treatment with DHEA nor treatment with DHEA and metformin together were able to modify the percentages of CD4 + and CD8 + T lymphocytes from axillar nodes.

The flow cytometry analysis of retroperitoneal lymph nodes (Fig. 4B) showed equivalent percentages of CD4 + and CD8 + T lymphocytes in all the control groups. However, treatment with DHEA diminished the percentage of CD4 + T cells, and increased the CD8 + T cells from retroperitoneal lymph nodes (Fig. 4B; CD4 +, $24 \pm 6\%$; CD8 +, $64 \pm 4\%$). In contrast, when metformin was administered together with DHEA, the percentages of

Table 1 Effect of DHEA on BMI.

	Untreated	Control vehicle	Metformin-alone	DHEA	DHEA + metformin
Age (days)	45	45	45	45	45
Weight (g)	14.0 \pm 1.2	15.0 \pm 3.0	14.0 \pm 2.5	14.8 \pm 1.8	15.2 \pm 2.4
BMI (kg/m ²)	5.6 \pm 0.2	5.7 \pm 0.5	5.7 \pm 0.4	5.4 \pm 0.6	5.3 \pm 0.8

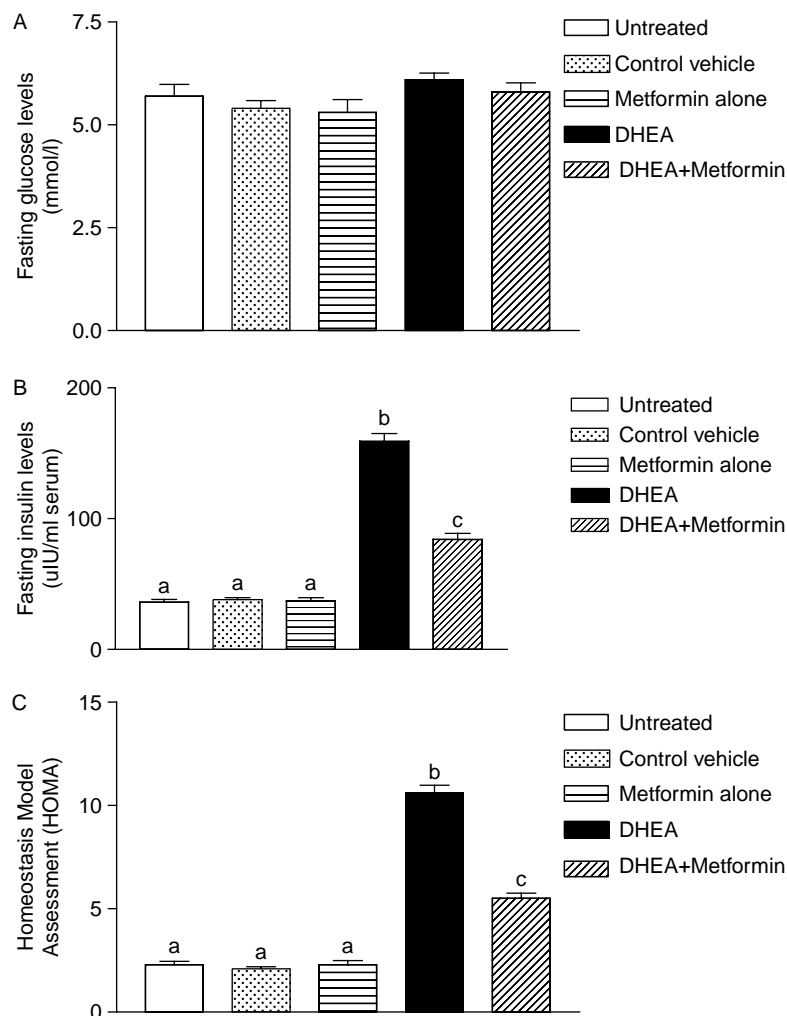


Figure 1 Role of DHEA in (A) fasting glucose levels, (B) fasting insulin levels and (C) HOMA index and effect of metformin treatment. $P < 0.001$, b vs a; $P < 0.05$, c vs a. Each group represents the mean \pm S.E.M. of ten different animals.

CD4 + and CD8 + T cells from retroperitoneal lymph nodes were similar to those of controls (Fig. 4B).

Role of metformin on serum TNF- α concentration

As shown in Fig. 5, mice injected with DHEA for 20 days showed increased serum TNF- α concentration when compared with controls, while those injected with metformin together with DHEA showed a concentration similar to that of controls.

Discussion

In this work, we studied some aspects related to both the endocrine and the immune responses in a polycystic ovarian condition induced by hyperandrogenization with DHEA in BALB/c mice. The aim of the present study was also to investigate the ability of metformin to modulate these aspects.

The mechanism by which administration of DHEA brings about the cascade of hormonal events that produces ovarian failure remains unknown. However, it is

known that the experimental model used in this work reproduces the main aspects of human PCOS (Lee *et al.* 1991, 1998, Anderson *et al.* 1992, Henmi *et al.* 2001). Our data show that the hyperandrogenic environment recreated by the treatment with DHEA did not modify the weight of the animals or, consequently, the BMI. We could then infer that neither hyperlipidemia nor obesity, which can sometimes be associated with PCOS (Franks *et al.* 1997, Abbott *et al.* 2002), were induced in this animal model.

Both hyperinsulinemia and hyperandrogenism play a pathogenic role in PCOS since they contribute to anovulation (Shoupe *et al.* 1983, Dunaif *et al.* 1989, Franks *et al.* 2000), impair folliculogenesis and affect follicular development (Dunaif *et al.* 1989, Gougeon 1996, Musso *et al.* 2005). For these reasons, insulin-sensitizing drugs such as metformin can improve the rate of spontaneous ovulation (De Leo *et al.* 1999, Glueck *et al.* 1999, Vandermolen *et al.* 2001). Although we did not directly assess insulin sensitivity, we measured surrogate markers of insulin sensitivity such as fasting serum insulin, fasting blood glucose and the HOMA index (which reflects the glucose–insulin

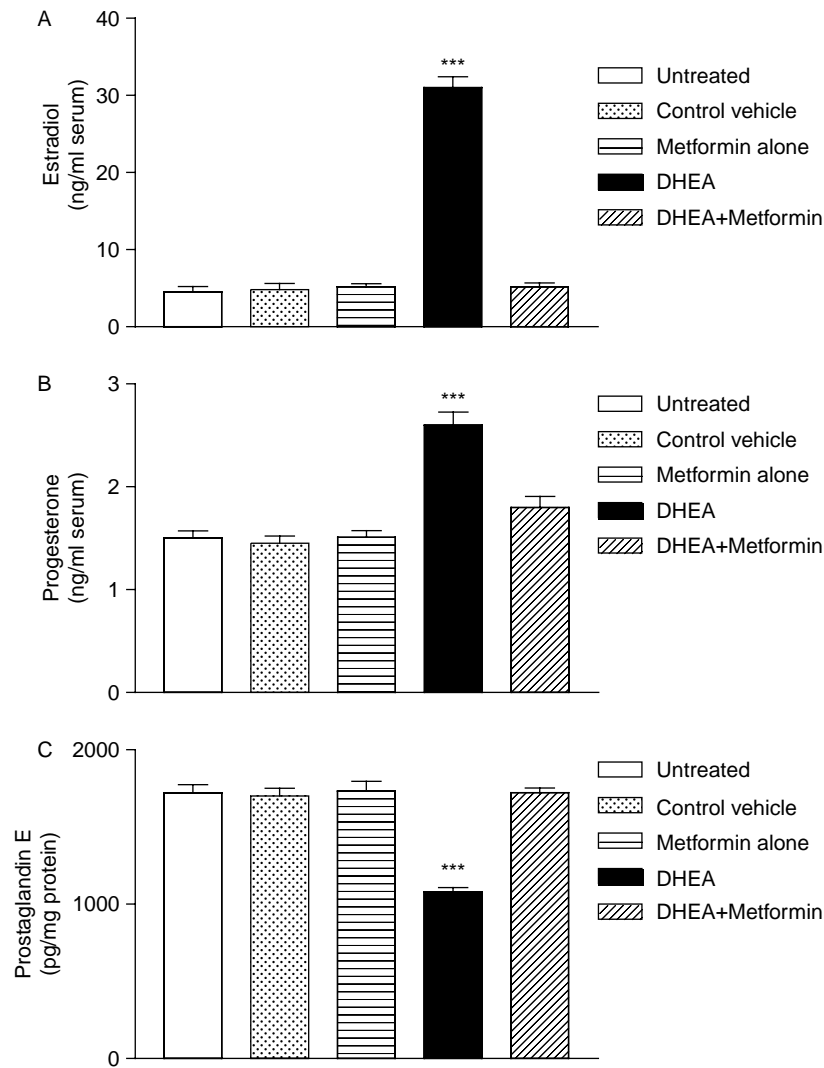


Figure 2 (A) Serum 17 beta estradiol levels from mice from control groups, and the DHEA and DHEA + metformin groups. (B) Serum progesterone levels from mice from control groups, and the DHEA and DHEA + metformin groups. (C) Ovarian PGE concentration from mice from control groups, and the DHEA and DHEA + metformin groups. Each column represents the mean \pm S.E.M. of ten measurements from different animals. *** $P < 0.001$.

relationship). Our results showed that treatment with DHEA increased the serum insulin levels without affecting fasting glucose, while treatment with metformin and DHEA together led to a HOMA index similar to that of controls. Although metformin is usually used for the treatment of chronic obese, insulin-resistant type-2 diabetic, and PCOS patients (Fedorcak *et al.* 2003, Harbone *et al.* 2003, Lord *et al.* 2003), the role of this drug during conditions of normal glucose concentration remains unknown. Our findings are in agreement with previous studies that have demonstrated that metformin increases peripheral insulin sensitivity in non-diabetic women with PCOS (Diamanti-Kandarakis *et al.* 1998, Moghetti *et al.* 2000, Vandermolen *et al.* 2001).

Since metformin modulates insulin concentration and, in turn, insulin controls ovarian steroidogenesis, it can be said that metformin acts indirectly on the steroidogenic activity of theca and granulosa cells (La Marca *et al.* 2002). However, recently, Mansfield *et al.* (2003) have

demonstrated that metformin exerts a direct effect on cultured ovarian cells. Therefore, we could speculate that metformin would regulate ovarian steroidogenesis both by modulating insulin levels and by acting directly on ovarian cells. These two pathways would contribute to ensure the accuracy of ovarian function. However, we are designing further experiments to clarify both this point and the molecular mechanisms involved in the action of metformin.

The data presented here show that mice from the DHEA group exhibited increased levels of both serum E and P and were in constant estrus. In view of the fact that only those follicles that show significant amounts of aromatizable androgens and low production of E are classified as selectable follicles (i.e. appropriate to ovulate) (Gougeon 1996), we can assume that in addition to the hyperandrogenized environment created by the daily injection of DHEA, the enhanced concentration of serum E would result in unfavorable conditions for producing follicles

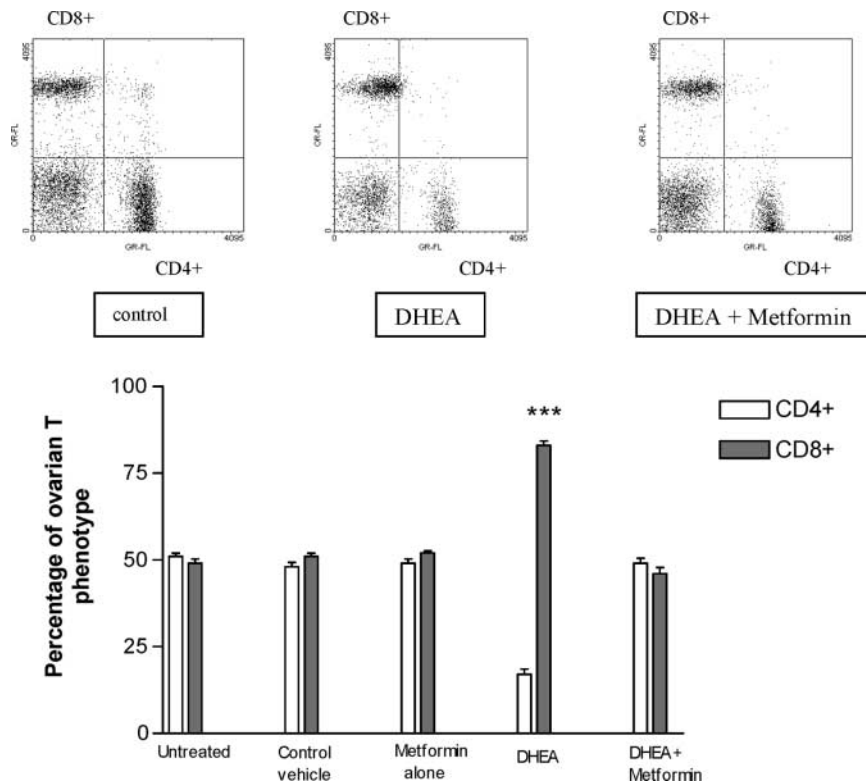


Figure 3 Percentage of ovarian T cell phenotypes from mice from control groups (untreated, control vehicle and metformin alone), and from the DHEA and DHEA + metformin groups, determined by flow cytometry assay. Each column represents the mean \pm S.E.M. of ten measurements from different animals. *** $P < 0.001$. Upper panels show a representative flow cytometry analysis using forward (FSC; cell size) and side scatter (SSC; cell complexity) parameters, and dotplot analysis using both standard fluorescence 1 (FL1; FITC anti-mouse CD8+ T lymphocyte) and 2 (FL2; PE anti-mouse CD4+ T lymphocyte).

destined for ovulation. Lee *et al.* (1992), who worked with the same animal model, have reported a similar hormonal profile. This profile suggests an increased steroidogenic activity, which is widely described in PCOS (Franks *et al.* 2000, Abbott *et al.* 2002). Evidence shows that follicles from anovulatory women with PCOS hypersecrete E when compared with size-matched follicles from normal ovaries or polycystic ovaries from ovulatory women (Franks *et al.* 2000, Mendonca *et al.* 2004, Doi *et al.* 2005). These findings also support those of the current study.

In the present work, also in accordance with previous findings (Vandermolen *et al.* 2001, Harbone *et al.* 2003, Kazerooni & Dehghan-Kooshkghazi 2003, Weerakiet *et al.* 2004), we demonstrate that the administration of metformin together with DHEA prevented the effect of hyperandrogenization, i.e. the increase of serum E and P levels. As discussed above, these data would represent the result of both indirect and direct actions of metformin in modulating ovarian steroidogenesis.

Since animals from the DHEA group remained at constant estrus throughout the treatment, we suggest that the hyperandrogenization induced with DHEA resulted in anovulation. Conversely, neither the animals from the control groups nor those from the DHEA + metformin group showed a complete sexual cycle. However, they did show irregular cycles. Therefore, we suggest that the administration of metformin together with DHEA creates an endocrine condition that allows the animals to start to cycle, and that the irregularity of sexual cycles could be attributed to the immature condition of the animals

(45 days old at the time of killing) rather than to inefficiency of the treatment with metformin.

Considering that prostaglandins are involved in the paracrine regulation of the rupture of ovarian follicles associated with ovulation (Priddy & Killick 1993) and that PGE has been reported to have immunomodulatory properties by modulating cytokine production (Kuroda & Yamashita 2003, Lakier Smith 2003, Yang *et al.* 2003), we also evaluated whether the ovarian concentration of PGE was modified by DHEA-induced hyperandrogenization. The fact that the treatment with DHEA reduced ovarian PGE production was an expected result since DHEA-hyperandrogenized animals not only did not start to cycle but also showed increased levels of TNF- α when compared with controls. Although we have previously reported that the treatment with DHEA increased ovarian PGE (Luchetti *et al.* 2004), this apparently controversial result could be explained by the fact that, in that report, the dose of DHEA administered to prepuberal mice was lower than that used here. The dose of DHEA used in the present report correlates better with the concentration of DHEA described in women with PCOS (Malesh & Greenblatt 1962, Roy *et al.* 1962, Lee *et al.* 1991, 1998, Anderson *et al.* 1992, Henmi *et al.* 2001). We also found an inverse relationship between the concentration of DHEA and both the ovarian PGE production and the expression of cyclooxygenase (COX) (the enzyme that synthesizes PGE) (data not shown). The last observation is due to the fact that it has been demonstrated that prostaglandins down-regulate their own

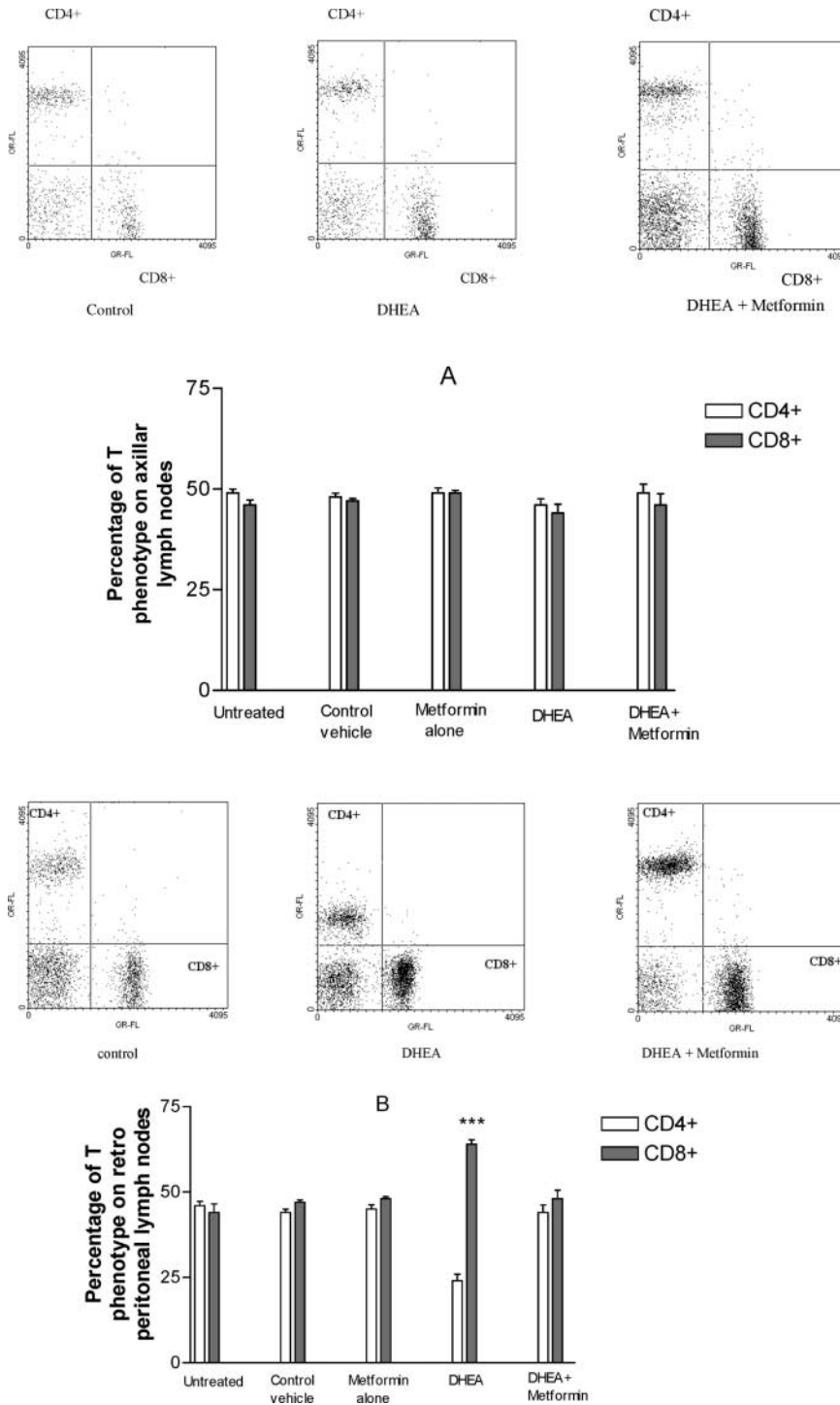


Figure 4 Percentage of (A) T cell phenotypes from axillary lymph nodes and (B) T cell phenotypes from retroperitoneal lymph nodes determined by flow cytometry assay. Each column represents the mean \pm S.E.M. of ten measurements from different animals. *** $P < 0.001$. Upper panels show a representative flow cytometry analysis using forward (FSC; cell size) and side scatter (SSC; cell complexity) parameters, and dot-plot analysis using both standard fluorescence 1 (FL1; FITC anti-mouse CD8+T lymphocyte) and 2 (FL2; PE anti-mouse CD4+T lymphocyte).

synthesis (Ivanov & Romanovsky 2004). We therefore assume that the higher dose of DHEA injected in the present report led to an accumulation of ovarian PGE which in turn could inhibit the expression of COX and consequently the production of ovarian PGE, as observed here.

On the other hand, we found that animals from the DHEA + metformin group showed a pattern of ovarian PGE synthesis similar to that of the control groups.

Although the action of this biguanide on improving lipid metabolism has been widely reported (Caballero 2004, Dominguez & Sowers 2005, Rautio *et al.* 2005), our data represent the first evidence that metformin modulates the production of ovarian prostaglandins.

TNF has been found to be increased in patients with PCOS (Sayin *et al.* 2003). In addition, a mutation of the TNF receptor has been associated with hyperandrogenism

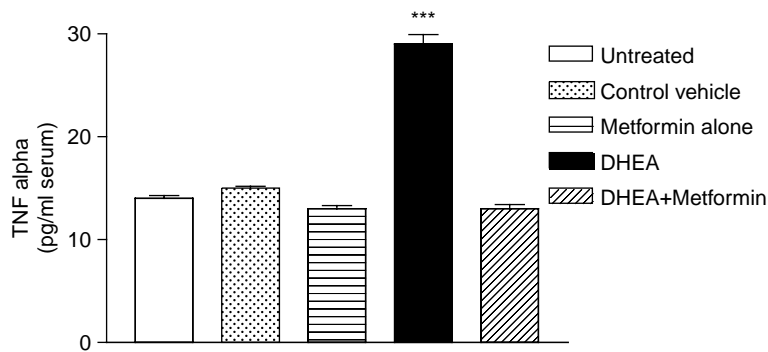


Figure 5 Serum TNF from mice from control groups (untreated, control vehicle and metformin alone), the DHEA group and the DHEA + metformin group. Each column represents the mean+S.E.M. of ten measurements from different animals. *** $P < 0.001$.

(Peral *et al.* 2002). In agreement with these findings, we found that animals treated with DHEA had higher serum TNF- α levels when compared with controls, a fact that correlates both with increased levels of PGE and with anovulatory cycles. It has been reported that TNF- α modulates steroidogenesis of both granulosa and theca-interstitial cells by a mechanism independent of those induced by insulin and insulin-like growth factor-I (IGF-I) (Spaczynski *et al.* 1999). For this reason we could infer that the increase of serum TNF- α observed in animals from the DHEA group would be an additional mechanism to those involved in hyperinsulinemia, which impair ovarian steroidogenesis. In the present work, we demonstrated that the administration of metformin together with DHEA led to serum TNF- α levels similar to control values. In the literature, conflicting results have been reported with regards to the relationship between metformin and the regulation of TNF- α (Cacicedo *et al.* 2004, Bruun *et al.* 2005, Di Gregorio *et al.* 2005, Kiortsis *et al.* 2005).

Finally, the data presented here show that DHEA-induced hyperandrogenization increased the percentage of the CD8 + T population and diminished the percentage of CD4 + T lymphocytes when compared with controls. Although the receptor for DHEA has not been identified yet, a specific DHEA binding activity has been detected in T cells (Meikle *et al.* 1992, Okabe *et al.* 1995) and for this reason we hypothesized that endocrine disturbances could be directly related to T lymphocyte differentiation. It has been documented, for example, that expression of the most mature thymocytes is regulated by P and E levels, and is also related to the high propensity of autoimmune diseases in females (Leposavic *et al.* 2001, Obradovic *et al.* 2001). Moreover, Yan *et al.* (2000) have reported that both autoimmune premature ovarian failure (POF) and insulin-dependent diabetes mellitus (IDDM) patients present increased numbers of CD8 + T cells. In addition, it has been demonstrated that production of cytokines by B cells is controlled by an enriched CD8 + T population (Lu *et al.* 2002).

Data presented here show that metformin treatment resulted in similar percentages of CD4 + and CD8 + T

lymphocytes to those seen in the controls. As metformin modulates P and E levels and since, in turn, P and E control T lymphocyte differentiation (Yan *et al.* 2000, Leposavic *et al.* 2001, Obradovic *et al.* 2001, Lu *et al.* 2002), we can suggest that metformin would act indirectly in modulating the percentages of ovarian CD4 + and CD8 + T lymphocytes. However, other pathways (such as regulation of reactive oxygen species and the induction of AMP-activated protein kinase (AMPK) of T lymphocytes) described in other tissues (Bonfont-Rousselot *et al.* 2003, Cacicedo *et al.* 2004, Huypens *et al.* 2005, McCarthy 2005) must not be disregarded and are currently being studied at our laboratory. Both the fact that the T cell population from axillar nodes was not affected during DHEA-induced hyperandrogenization and that the T cell population from retroperitoneal lymph nodes was affected – and also the fact that hyperandrogenization induced a similar T cell population when samples from retroperitoneal lymph tissue were compared with those obtained from ovarian tissue – lead us to suggest that the local inflammatory status would be contributing to a selective differentiation of T cells. According to this hypothesis, lymph nodes and sex steroids are related to different systems, thus suggesting a coordinated organ-specific and steroid hormone relationship (Chantakru *et al.* 2003). Moreover, the E receptor expressed by follicular dendritic cells in lymph nodes has recently been proposed as a novel pathological marker (Sapino *et al.* 2003) and the deficiency of E caused by ovariectomy or menopause is involved in the T lymphocyte status (Safadi *et al.* 2000). In summary, the present study describes the role of metformin in the regulation of some aspects of the intricate network that relates the endocrine and the immune pathway in a hyperandrogenized environment. As the development of immune cell surface markers is beginning to be used in diagnosis prior to the development of complete ovarian failure, we believe that understanding the role of the immune processes involved in PCOS could also be important in the manipulation of this pathology.

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