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Metformin prevents embryonic resorption induced by hyperandrogenisation with dehydroepiandrosterone in mice

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Abstract. The present study examined the mechanism by which metformin prevents dehydroepiandrosterone (DHEA)-induced embryonic resorption in mice. Treatment with DHEA (6 mg/100 g bodyweight, 24 and 48 h post implantation) induced $88 \pm 1\%$ embryonic resorption and the diminution of both serum oestradiol (E) and progesterone (P) levels. However, when metformin (50 mg/kg bodyweight) was given together with DHEA, embryo resorption ($43 \pm 3\%$ *v.* $35 \pm 5\%$ in controls) and both serum E and P levels were not significantly different from controls. Glucose and insulin levels were increased in the DHEA-treated mice but when metformin was administered together with DHEA these parameters were similar to control values. Treatment with DHEA increased ovarian oxidative stress and diminished uterine nitric oxide synthase (NOS) activity; however, when metformin was administered together with DHEA, both ovarian oxidative stress and uterine NOS activity were not different from controls. Metformin treatment did not modify the percentage of $CD4^+$ and $CD8^+$ T cells from both axillar and retroperitoneal lymph nodes but prevented the increase of serum tumour necrosis factor α produced in DHEA-treated mice. These results show that metformin acts in DHEA-induced embryonic resorption in mice by modulating endocrine parameters, ovarian oxidative stress and uterine NOS activity.

Extra keywords: lymphocyte, miscarriage, nitric oxide.

Introduction

Dehydroepiandrosterone sulfate (DHEAS) and dehydroepiandrosterone (DHEA) are androgen precursors mainly secreted from the adrenal cortex in humans (Nieschlag *et al.* 1973). It has been suggested that DHEAS is a reservoir and a precursor of DHEA, in turn a precursor of sex steroids (Miki *et al.* 2002). During pregnancy, DHEA and DHEAS have immunoregulatory actions on the maternal immune system (Nieschlag *et al.* 1974; Facchinetti *et al.* 1986; Tagawa *et al.* 2004). Not only maternal adrenal glands (Nieschlag *et al.* 1973) but also early placenta and fetal adrenal glands are capable of converting cholesterol to pregnenolone to DHEA (Loganath *et al.* 2002). Therefore, serum DHEA suppresses immune reactions by modifying cytokine levels and thus contributing to the development of gestation (Nieschlag *et al.* 1974; Du *et al.* 2001; Tagawa *et al.* 2004). 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). Recently, DHEA has been shown to bind and activate the oestrogen receptor (Frantz *et al.* 2005; Seely *et al.* 2005).

It has been demonstrated that androgens regulate immune homeostasis and that DHEA favours both the Th2 immune response and cytokine production (Du *et al.* 2001). However, abnormally increased levels of DHEA lead to an imbalance in ovarian function that results in miscarriage (Sir-Petermann *et al.* 2002). Moreover, it has been reported that increased levels of androgens result in recurrent miscarriages by detrimental effects on endometrial function (Okon *et al.* 1998).

Polycystic ovary syndrome (PCOS), which is characterised by hyperandrogenemia, induces oligomenorrhea or amenorrhea, anovulation and recurrent miscarriages. It is estimated that 6–10% of women in their reproductive ages are affected by this pathology (Franks 1995; Asuncion *et al.* 2000). After it was found that DHEA levels were increased in women with PCOS (Malesh and Greenblatt 1962), Roy *et al.* (1962) produced an animal model using DHEA for the 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 follicules and anovulation (Lee *et al.*

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1991; Anderson *et al*. 1992; Lee *et al*. 1998; Henmi *et al*. 2001). In a previous study (Sander *et al*. 2005) we found that hyperandrogenisation with DHEA of early pregnant BALB/c mice induced embryonic resorption. In addition, it is correlated with diminution of both serum progesterone (P) levels and ovarian antioxidant status (evaluated by gluthatione content) (Sander *et al*. 2005).

Multiple concomitant therapies have been applied in PCOS to address the variety of symptoms and achieve better results. Recent studies have investigated the role of a type of insulin-sensitising agent: the biguanides. The use of *N*,*N′*-dimethylbiguanide (metformin) is becoming increasingly accepted and widespread. It has been shown that metformin effectively restores insulin sensitivity in insulin-resistant women with PCOS (Fedorcsak *et al*. 2003; Harborne *et al*. 2003*a*; Lord *et al*. 2003). Furthermore, a wide range of benefits in metabolic, reproductive and clinical measures has been reported (Harborne *et al*. 2003*b*). Considering that there is an increasing number of women with PCOS that become pregnant after metformin treatment (Vandermolen *et al*. 2001; Fedorcsak *et al*. 2003; Harborne *et al*. 2003*b*), it is expected that the use of this biguanide in and around the time of pregnancy will increase (Mc Carthy *et al*. 2004); however, the clinical practice is ahead of the knowledge of the mechanism involved.

In view of data presented, the purpose of the present report was to investigate the actions of metformin in DHEA-induced embryonic resorption in early pregnant mice. We have focused the study on different ovarian, uterine and immune parameters involved in the development of pregnancy. To determine the role of metformin on ovarian functionality, serum P and oestradiol (E) levels were quantified.

Metformin modulates insulin concentration and as, in turn, insulin controls ovarian steroidogenesis (Willis and Franks 1995), it can be said that metformin acts indirectly on steroidogenic activity (La Marca *et al*. 2002). However, recently Mansfield *et al*. (2003) have demonstrated that metformin also exerts a direct effect on ovarian cells. Thus, in the present report we also designed experiments to evaluate the effects of metformin treatment on parameters related to ovarian steroidogenesis, such as fasting glucose and serum insulin levels.

The fact that metformin is an aminoguanidine-related compound gives it the additional ability to act as a scavenger of reactive oxygen species (ROS) (Faure *et al*. 1999; Ceriello 2000; Srividhya *et al*. 2002; Bonnefont-Rousselot *et al*. 2003). However, these previous studies have described a systemic action of metformin as a scavenger of ROS, and no evidence of metformin effect on ovarian tissue has been reported. In the present study we investigated whether metformin was able to modulate ovarian functionality by regulating the ovarian oxidant–antioxidant balance. We then determined the oxidant status (measured by the lipid peroxidation index) and the antioxidant response (quantified by glutathione production and catalase activity) of ovarian tissue from DHEA-treated and DHEA + metformin-treated mice.

Nitric oxide (NO) plays important roles during pregnancy in implantation, decidualisation, vasodilatation of decidual, placental and uterine vessels and myometrial relaxation (Sladek *et al*. 1997; Chwalisz *et al*. 1999; Chwalisz and Garfield 2000; Ogando *et al*. 2003). It has also been reported that NO participates in vascular invasion of the trophoblast (Ariel *et al*. 1998) and infection control during pregnancy (Nowicki *et al*. 1997). The vascular function is regulated by steroid hormones (Simoncini and Genazzani 2000, 2003) and, in particular, oestradiol stimulates NO synthesis (Kleinert *et al*. 1998; Simoncini and Genazzani 2000). However, it has been found that some of the effects of DHEA depend on its conversion to oestrogens and androgens, and on the recruitment of their respective receptors (Hayashi *et al*. 2000) and recently it has been demonstrated that DHEA binds to oestrogen receptors (Frantz *et al*. 2005; Seely *et al*. 2005). In addition, Simoncini and Genazzani (2003) have reported that DHEA has direct genomic and non-genomic effects on the vascular wall through the NO system (Simoncini and Genazzani 2003). These findings, coupled with the fact that hyperandrogenisation induces detrimental changes in the endometrial tissue (Okon *et al*. 1998), led us to evaluate the effect of hyperandrogenisation with DHEA on the activity of the enzyme responsible for NO synthesis, nitric oxide synthase (NOS). As it has recently been found that metformin is able to modulate vasodilator agents by improving total NOS activity in the mesentery of diabetic rats (Sartoretto *et al*. 2005) and that the NO system has been proposed as an intermediary of the metformin action (Zou *et al*. 2004), we hypothesised that metformin could modulate uterine NOS activity of early hyperandrogenised pregnant mice.

It is well known that pregnancy is associated with changes in the immune response. It seems likely that these changes are necessary to accommodate the semi-allogeneic blastocyst. The general accepted idea has always been that type 1 cytokines, which are involved in cellular immune responses, are harmful for pregnancy whereas type 2 cytokines, which are involved in humoral immune responses, are protective for the fetus (Veenstra van Nieuwenhoven *et al*. 2002). These findings, together with our previous results that treatment of prepuberal BALB/c mice with DHEA alters the percentages of $CD4^+$ and $CD8^+$ T lymphocytes from retroperitoneal lymphoid nodes (Luchetti *et al*. 2004) and the fact that DHEA immunomodulates the development of gestation (Nieschlag *et al*. 1974; Meikle *et al*. 1992; Okabe *et al*. 1995; Du *et al*. 2001; Tagawa *et al*. 2004), led us to investigate whether treatment with DHEA could alter the percentages of $CD4^+$ and $CD8^+$ T lymphocytes in retroperitoneal lymphoid nodes of early pregnant mice. In view of the fact that metformin modulates lymphocyte differentiation (Stefanovic *et al*. 1999), we were interested in knowing whether metformin could regulate $CD4^+$ and $CD8^+$ T lymphocyte expression in retroperitoneal

lymphoid nodes of early pregnant mice. In addition, considering the action of the type 1 cytokine tumour necrosis factor α (TNFα) on ovarian steroidogenesis (Spaczynski *et al*. 1999; Peral *et al*. 2002; Sayin *et al*. 2003) and its relationship with both PCOS (Deshpande *et al.* 2000; Araya *et al*. 2002; Korhonen *et al*. 2002; Peral *et al*. 2002) and pregnancy (Kinalski *et al*. 2005; Zenclussen *et al*. 2005), we also studied the effect of both DHEA and metformin treatment on serum TNFα levels of early pregnant mice.

In summary, the aim of the present work was to assess the efficacy of metformin in modulating some parameters altered after hyperandrogenisation of early pregnant mice. The investigations were focused on ovarian functionality (sex steroids, the glucose : insulin ratio, ovarian oxidative stress), uterine status (activity of uterine NOS) and the immune response (percentages of $CD4^+$ and $CD8^+$ T lymphocytes from ovaries and lymph nodes, and serum TNFα concentration).

Materials and methods

Animals and experimental protocol

In order to study the effects of high levels of circulating androgens in the development of early pregnancy, DHEA was injected during the post-implantatory period as described previously (Sander *et al*. 2005). Briefly, BALB/c 8–12-week-old virgin female mice were paired with 8–12-week-old BALB/c males. The day of appearance of a coital plug was taken as Day 0 of pregnancy. The implantation developed on Day 5 of pregnancy. Pregnant mice (30 per group: control, DHEA and DHEA + metformin) were housed under controlled temperature (22°C) and illumination (14 : 10 h L : D cycle; lights on at 0500 hours) and were allowed free access to rat chow (Purina, Buenos Aires, Argentina) and water. All procedures involving animals were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*. At Day 6 of pregnancy, mice were divided into the following three groups:

1. *Control*: Animals were injected s.c. with 0.1 mL of sesame oil and given 0.05 mL of water orally by canulla on Days 6 and 7 of pregnancy.
2. *DHEA*: Animals were injected s.c. with DHEA (6 mg/100 g bodyweight dissolved in 0.10 mL sesame oil) on Days 6 and 7 of pregnancy. The treatment with DHEA (doses and frequency) was assessed previously (Sander *et al*. 2005).
3. *DHEA + metformin*: Animals were injected s.c. with DHEA (6 mg/kg bodyweight dissolved in 0.10 mL sesame oil) and given metformin (50 mg/kg bodyweight in 0.05 mL of water given orally with a canulla) on Days 6 and 7 of pregnancy. The doses of metformin administered were equivalent to those used in women with PCOS.

At Day 8 of pregnancy, animals from the three groups were anaesthetised with ether and killed by cervical dislocation. Blood was collected and glucose levels were determined immediately. Serum was then isolated and stored at −70°C until assayed for P, E, insulin and serum TNFα determinations. Lymphoid tissues (axillar and retroperitoneal nodes) isolated from twenty animals from each group were immediately collected and fixed in 4% (w/v) paraformaldehyde to further determine $CD4^+$ and $CD8^+$ T lymphocyte expression by flow cytometry. The ovarian tissues were immediately treated for determination of oxidant–antioxidant parameters: lipid peroxidation index, glutathione production and catalase activity. Ten uterine tissues from each group were collected and stored at −70°C until NOS determination and ten uterine tissues were immediately fixed in 4% (w/v) paraformaldehyde to stain with haematoxylin and eosin for histological analysis of embryo resorption. All experiments were repeated three times.

In order to investigate whether both of the controls and the metformin treatment produced any abnormal effects, two additional control groups of ten animals each were analysed:

1. *Additional control I*: Animals were injected s.c. with 0.1 mL of sesame oil and given 0.05 mL of water orally by canulla on Days 6 and 7 of pregnancy.
2. *Additional control II*: Animals were injected s.c. with DHEA (6 mg/kg bodyweight dissolved in 0.10 mL sesame oil) and given metformin (50 mg/kg bodyweight in 0.05 mL of water given orally with a canulla) on Days 6 and 7 of pregnancy.

Pregnant mice from these two groups (additional controls I and II) were allowed to proceed to parturition. We found that these animals exhibited normal labour and size and number of pups. These results allowed us to ensure that neither the vehicle controls nor metformin exerted abnormal effects during the development of pregnancy.

Uterine morphology and embryonic resorption

To study the effect of DHEA treatment on the implantation process, uterine tissues from the three groups of animals were separated and fixed as described above. Tissues were cut consecutively (6 μm per section) and placed on gelatin-coated slides (Biobond; British Biocell International, Cardiff, UK), air-dried for 2 h and later treated with acetone at 4°C for 5 min. In order to evaluate the resorption rate, only sections that passed through the centre of the implantation sites were selected to be stained with haematoxylin and eosin (DAKO Corporation, Carpinteria, CA, USA).

Oestradiol and progesterone determination

To evaluate the effect of both hyperandrogenisation with DHEA and metformin treatment on ovarian function, serum E and P levels were evaluated as described previously (Luchetti *et al*. 2004). Briefly, the blood was allowed to clot and the serum removed and frozen until P and E concentrations were determined by radioimmunoassay. Both antisera were provided by Dr G. D. Niswender (Colorado State University, Fort Collins, CO, USA). Progesterone antiserum was highly specific for P with low cross reactivity: <2.0% for α-dihydro-progesterone and deoxycorticosterone, and 1.0% for other steroids normally present in serum. As sensitivity was 5–10 pg $tube^{-1}$, 2–5 μL of serum was assayed routinely. Oestradiol antiserum showed low cross reactivity: <1% for progesterone and testosterone, <5% for 17α-oestradiol and oestriol and <10% for oestrone. Oestradiol was expressed as pg mL^{-1} serum and progesterone as ng mL^{-1} serum.

Assays for glucose and insulin quantification

Fasting blood glucose was determined in the control, DHEA and DHEA + metformin groups using Haemo–Glukotest (Roche, Mannheim, Germany) test strips for visual determination in the range of 20–800 mg/100 mL (1–44 mM). The test principle uses the glucose oxidase–peroxidase reaction. Results are expressed in mM.

Serum insulin levels were assayed using the Coat-A-Count insulin method (Diagnostic Products Corporation, Los Angeles, CA, USA) following the manufacturer's instructions. Briefly, the determination is 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 radiolabelled insulin. The samples were counted in a gamma counter. Analytical sensitivity was 1.2 μIU insulin mL^{-1} serum. Results were expressed as pg mL^{-1} serum.

Oxidative stress-related parameters

Lipid peroxidation

Homogenates of ovarian tissue (ten animals from each group; two ovaries per point) were treated with trichloroacetic acid : thiobarbituric acid : HCl (15% w/v : 0.375% w/v : 0.25 M) and heated for 20 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000*g* for 10 min. The amount of malondialdehyde (MDA) formed from the breakdown of polyunsatured fatty acids was taken as an index of peroxidation reaction. The method used (Motta *et al.* 2001*a*) is based on the quantification of the red compound formed when MDA reacts with trichloroacetic acid : thiobarbituric acid : HCl, which absorbs at 535 nm. The results were expressed as μmol MDA formed g^{-1} ovarian tissue.

Antioxidant parameters

Glutathione content and catalase activity. To evaluate ovarian antioxidant status samples were processed as described previously (Luchetti *et al.* 2004). Briefly, ten ovaries from each group of treatments were homogenised on ice in three volumes of the homogenisation buffer (150 mM ethylenediamine tetraacetic acid (EDTA), 1 mM β-mercaptoethanol, 20 mM Tris Buffer, 500 mM sucrose). After centrifugation at 1000*g*, 4°C for 10 min, the supernatants were separated and frozen at −70°C until glutathione (GSH), catalase (CAT) and protein determinations.

Ovarian glutathione production. The GSH assay was carried out as described previously (Luchetti *et al.* 2004). Briefly, 100 μL of supernatant obtained as described above was incubated with buffer 1.75 M Tris (pH 7.4) containing NADPH and glutathione reductase. The reaction involves the enzymatic reduction of the oxidized form (GSSG) to GSH. When Ellman's reagent (a sulphydryl reagent 5,5-dithiobis-2 nitrobenzoic acid; Sigma, St Louis, MO, USA) is added to the incubation medium, the chromophoric product resulting from this reaction develops a molar absorption at 412 nm that is linear to the first 6 min; after this, the reaction remains constant. Results were expressed as nmol GSH mg^{-1} protein.

Catalase activity. Catalase is a ubiquitous antioxidant enzyme that is present in most aerobic cells. CAT is involved in the detoxification of hydrogen peroxide, which is a toxic product of both normal aerobic metabolism and pathogenic ROS production. This enzyme catalyses the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water (catalytic activity). The method is based on the reaction of the enzyme (contained in 100 μL of the homogenates prepared as described above) with 100 μL of 3 M H_2O_2. The product resulting from this reaction develops a molar absorption at 240 nm.

Nitric oxide synthase activity

Nitric oxide synthase activity in uterine pooled homogenate was determined by monitoring the formation of L-[^{14}C]citrulline from L-[^{14}C]arginine as described previously (Motta *et al.* 2001*b*). Briefly, the frozen tissue was homogenised at 0°C in three volumes of 50 mM HEPES, 1 mM dithiothreitol, 1 M NADPH (pH 7.5) and L-valina (50 mM). Samples were incubated at 37°C for 15 min with 10 μM L-[^{14}C]arginine (11.1×10^{-3} Bq). The samples were then applied to 1 mL DOWEX AG50W-X8 resin (Na^+ form; BioRad, Hercules, CA, USA). The radioactivity was measured by liquid scintillation counting. Results were expressed as pmol g^{-1} min^{-1}.

Flow cytometry

To carry out the flow cytometry assays, lymph nodes (axillar and retroperitoneal) must be dispersed (Luchetti *et al.* 2004). Briefly, tissues were dissociated enzymatically in culture medium (Medium 199/25 mM HEPES/26 mM $NaHCO_3$ and 50 IU mL^{-1} penicillin) with trypsin-free collagenase (740 IU per 100 mg tissue) and DNAse (14 IU per 100 mg tissue). After 90 min cells were washed twice with culture medium, twice with Delbucco's modified Eagle medium with phosphate-buffered saline (PBS) free of Ca^{2+} and Mg^{2+} and twice with culture medium containing EDTA (1 mM). To remove blood cells, suspensions were applied to a Ficoll-hystopaque 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 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 from each treated group was incubated at 10^6 cells mL^{-1} for 30 min at 4°C with 4 μL phycoerythrin (PE)-conjugated anti-mouse CD4 + 4 μL fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 (eBioscience, San Diego, CA, USA), corresponding to control, DHEA and DHEA + metformin assays according to the cellular suspension. The isotype control was formed by 30 μL of PE-conjugated rat IgG2a, k isotype control + 30 μL of FITC-conjugated rat IgG2a, k isotype control (eBioscience). Antibodies were used at saturating concentration as was 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 labelling. Fluorescence analysis was evaluated with FACScan and the Winmdi 2.8 software (J. Trotter, Cytometry Laboratories, Purdue University, West Lafayette, IN, USA). Lymph suspensions were analysed using different physical characteristics (i.e., size and complexity) using forward (FSC: cell size) and side scatter (SSC: cell complexity) parameters. The settings were thus correlated with cellular size and granularity of mouse T lymphocytes. The resulting gate and quadrant were maintained during the analysis. Flow cytometric determination was performed using standard fluorescence 1 (FL1: FITC-conjugated anti-mouse T lymphocyte $CD8^+$) and fluorescence 2 (FL2: PE-conjugated anti-mouse T lymphocyte $CD4^+$). The analysis was based on quantification of 10 000 cells per point.

Serum tumour necrosis factor α determination

An enzyme immunometric assay (EIA; Assay Design's mouse, Ann Arbor, MI, USA) for TNFα was used to quantify the serum cytokine from samples of the three experimental groups. Results were expressed as pg mL^{-1} serum.

Protein quantification

Protein levels were determined in ovarian homogenates using the method of Bradford (1976).

Statistical analyses

Statistical analyses were carried out using the Instat program (GraphPAD software, San Diego, CA, USA). The Newman–Keuls test was used to compare all pairs of columns and $P < 0.05$ was considered significant. All results are presented as the mean ± s.e.m.

Results

Metformin treatment on embryonic resorption

Dehydroepiandrosterone injected 24 and 48 h after implantation induced 88 ± 1% embryonic resorption *v.* 35 ± 5% in controls. However, when metformin was administered together with DHEA, mice showed 43 ± 3% embryonic resorption (not significantly different from control values).

Role of metformin in ovarian function: serum oestradiol and progesterone levels

As a measure of ovarian function after androgenisation with DHEA, E and P were measured in serum samples by

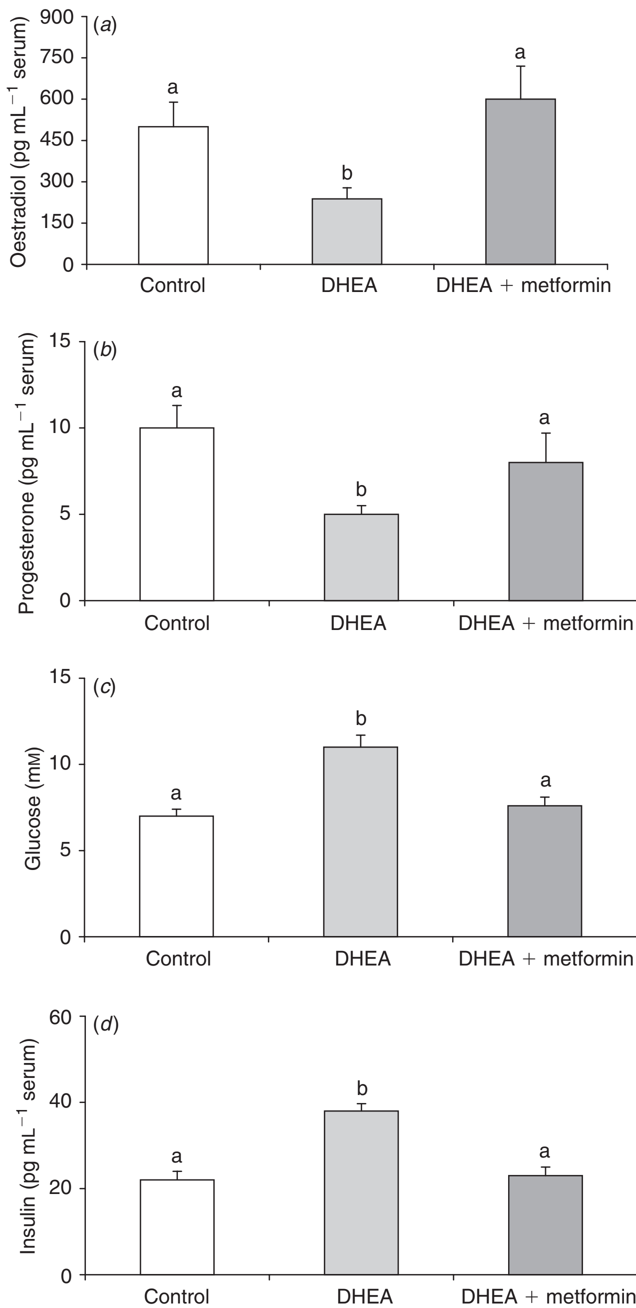

Fig. 1. Serum (*a*) oestradiol and (*b*) progesterone levels. (*c*) Fasting glucose and (*d*) serum insulin from control, dehydroepiandrosterone (DHEA) and DHEA + metformin-treated pregnant mice. Each column represents the mean + s.e.m. of 10 measurements from different animals, a *v.* b is significantly different ($P < 0.001$). Assays were carried out twice.

radioimmunoassay. Treatment with DHEA significantly ($P < 0.001$) diminished both serum E and P levels of early pregnant mice compared to controls (Fig. 1*a*,*b*). However, when mice were treated with DHEA + metformin, serum E (Fig. 1*a*) and P levels (Fig. 1*b*) showed no significant differences compared with control values.

Fasting glucose and serum insulin levels in response to metformin treatment

Fasting glucose was increased ($P < 0.001$) in those pregnant mice treated with DHEA. However, when metformin was administered orally together with DHEA, glucose levels showed the same values as those from animals from the control group (Fig. 1*c*). Similarly, the serum insulin concentration was higher in sera of animals from the DHEA group than in sera of animals from the control group. When metformin was administered together with DHEA, no significant difference was found compared with the control group (Fig. 1*d*).

Role of metformin on oxidative stress parameters

To evaluate whether androgenisation was able to disrupt the ovarian oxidant–antioxidant balance and the role of metformin in restoring these parameters, lipid peroxidation, GSH production and CAT activity were evaluated in mice from the three groups. Figure 2*a* represents ovarian MDA formation as a representative index of lipid peroxidation. It can be seen that androgenisation with DHEA significantly ($P < 0.001$) increased lipid peroxidation of ovarian tissue, whereas metformin given together with DHEA showed oxidative stress-like control values (Fig. 2*a*). Concomitantly, DHEA injection significantly ($P < 0.001$) diminished ovarian GSH production, whereas metformin partially reverted the diminution of the ovarian antioxidant metabolite levels (Fig. 2*b*). Moreover, CAT was also significantly ($P < 0.001$) diminished after androgenisation, whereas the DHEA + metformin group showed no significant differences when compared to the control group (Fig. 2*c*).

Effect of metformin on uterine relaxation: role of nitric oxide

In order to evaluate the effect of androgenisation on uterine relaxation and the possible role of metformin treatment, the activity of NOS (the enzyme that produces the relaxing muscular product NO) was evaluated in animals from the three groups. Whereas DHEA significantly ($P < 0.001$) diminished uterine NOS activity (DHEA 0.21 ± 0.05 pmol g^{-1} min^{-1} *v.* control 0.36 ± 0.06 pmol g^{-1} min^{-1}; $P < 0.05$), DHEA + metformin did not produce significant changes in enzymatic activity (0.39 ± 0.02 pmol g^{-1} min^{-1}) compared to the controls.

Regulation of metformin in T lymphocyte expression from lymph nodes

In view of the fundamental role of the immune system in the development of pregnancy, we evaluated the percentages of CD4^{+} and CD8^{+} T lymphocytes from both axillar (Fig. 3*a*) and retroperitoneal (Fig. 3*b*) lymph nodes.

The flow cytometry analysis of axillar lymph nodes (Fig. 3*a*) showed higher levels ($P < 0.001$) of CD4^{+} than CD8^{+} T lymphocytes in the three treatments. The control

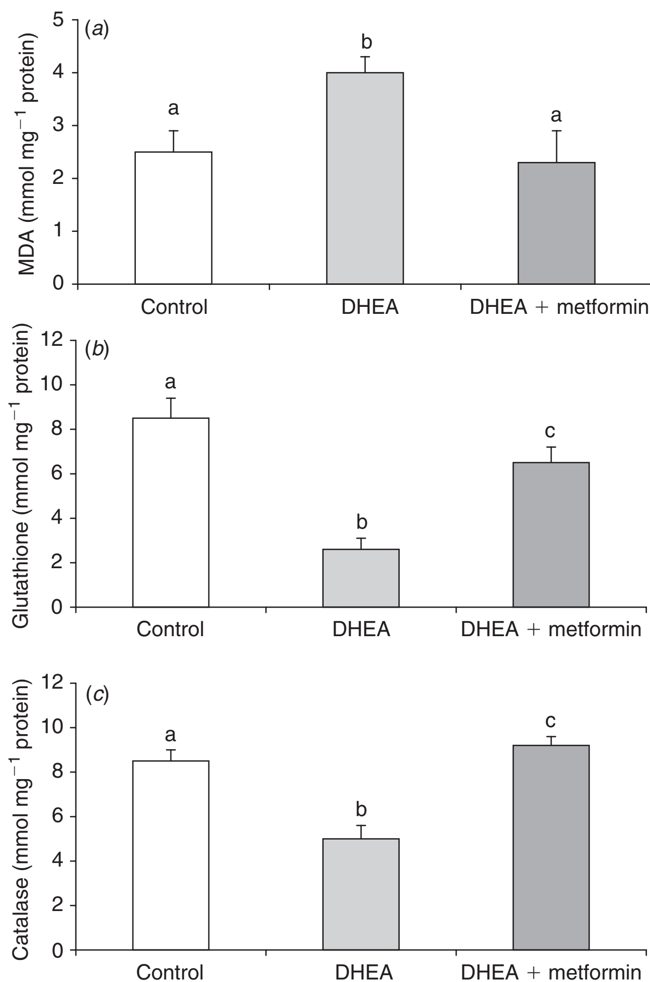

Fig. 2. Parameters of oxidative stress. (*a*) Lipid peroxidation index of ovarian tissue from control, dehydroepiandrosterone (DHEA) and DHEA + metformin-treated pregnant mice. (*b*) Glutathione content of ovarian tissue from control, DHEA and DHEA + metformin-treated pregnant mice. (*c*) Catalase activity of ovarian tissue from control, DHEA and DHEA + metformin-treated pregnant mice. Each column represents the mean + s.e.m. of 10 measurements from different animals, a *v.* b, b *v.* c and c *v.* a are significantly different ($P < 0.001$). Assays were carried out twice. MDA, malondialdehyde.

group displayed 60 ± 6% CD4$^+$ T lymphocytes, whereas CD8$^+$ T lymphocyte expression was 40 ± 6%. This profile was the same as those observed in animals from both the DHEA-treated group (CD4$^+$ T lymphocytes = 62 ± 2% and CD8$^+$ T lymphocytes = 38 ± 2%) and the DHEA + metformin group (CD4$^+$ T lymphocyte expression = 66 ± 6% and CD8$^+$ T lymphocyte expression = 34 ± 6%).

The dot plot analysis of the expression of retroperitoneal lymph nodes (Fig. 3*b*) demonstrated that in mice from the control group, the percentage of CD4$^+$ T lymphocytes was higher than the percentage of CD8$^+$ T lymphocytes (Fig. 3*b*, CD4$^+$ T lymphocytes = 59 ± 3% and CD8$^+$ T lymphocytes = 41 ± 4%, $P < 0.001$). Neither the DHEA (CD4$^+$ T lymphocytes = 64 ± 2% and CD8$^+$ T lymphocytes = 36 ± 2%) nor the DHEA + metformin treatment (CD4$^+$ T lymphocytes = 66 ± 7% and CD8$^+$ T lymphocytes = 33 ± 7%) were able to modify the T lymphocyte populations.

Role of metformin on serum tumour necrosis factor α concentration

Mice injected with DHEA showed increased ($P < 0.001$) serum TNFα levels (118 ± 14 pg mL^{-1} serum) when compared to controls (80 ± 15 pg mL^{-1} serum), whereas metformin administered together with DHEA returned the serum cytokine level (74 ± 15 pg mL^{-1} serum) compared to control values.

Discussion

It is generally agreed that one of the most critical periods of embryonic development in mammals occurs during the first weeks of gestation. Although DHEA is an androgen with modulatory functions during pregnancy (Nieschlag *et al.* 1974; Du *et al.* 2001; Tagawa *et al.* 2004), increased levels of DHEA have been associated with early pregnancy loss (Sir-Petermann *et al.* 2002) and detrimental effects on uterine function (Okon *et al.* 1998).

The biguanide metformin has been used not only as a classic antihyperglucemic drug in the treatment of diabetes, but also to induce ovulation and even during pregnancy in PCOS (Vandermolen *et al.* 2001; Fedorcsak *et al.* 2003; Harborne *et al.* 2003*a*, 2003*b*; Lord *et al.* 2003; Mc Carthy *et al.* 2004). However, its clinical use is being conducted without a complete understanding of the mechanism involved. This investigation examined the mechanisms by which metformin prevents DHEA-induced embryonic resorption in early pregnant mice.

We found that DHEA-induced embryonic resorption in mice involved impaired ovarian function characterised by a diminution of serum P and E levels and an imbalance in the oxidative stress. The corpus luteum, an endocrine gland formed after ovulation, is responsible for synthesising P, which is fundamental for the maintenance of early pregnancy in mammals. Luteal regression or luteolysis, described as the decline in serum P levels, is a mechanism widely studied for its implication in early pregnancy loss. However, it has been observed that E induces and maintains the expression of P receptors (Graham and Clarke 1997), stimulates endometrial tissue, initiates microvascular permeability and angiogenesis (Ferrara 1999), and is essential during the implantation process (Rockwell *et al.* 2002). We can therefore propose that our results suggest an indirect effect of metformin both in the implantation process (by restoring serum E levels) and in corpus luteum development (by protecting the diminution of P levels). Moreover, previous reports have demonstrated the efficacy of metformin in regulating the hormonal pattern in women displayed during the hyperandrogenic process

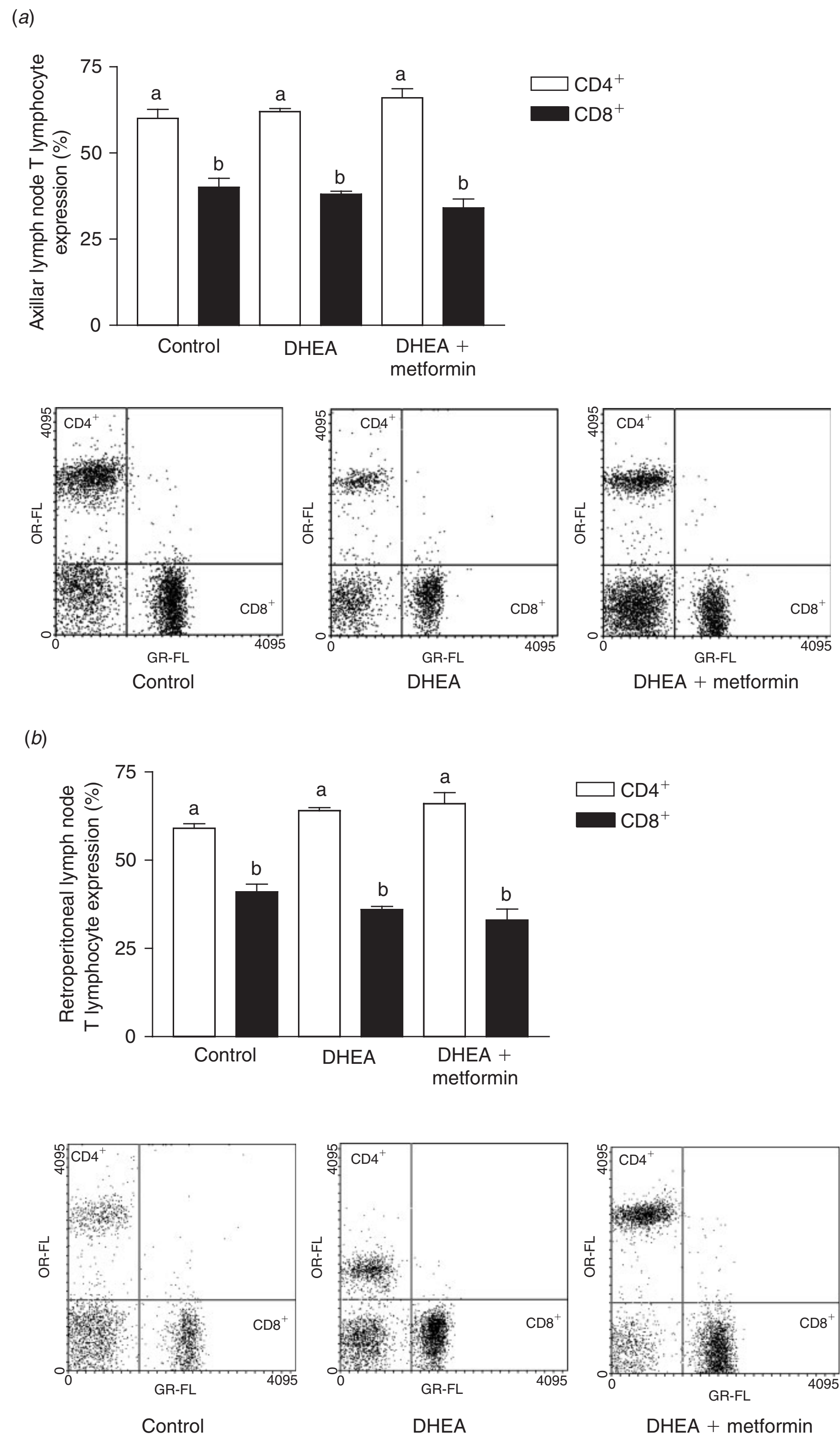

Fig. 3. Flow cytometric analysis using forward (FSC: cell size) and side scatter (SSC: cell complexity) parameters and dot plot analysis using two standard fluorescence: FL1, fluorescein isothiocyanate-conjugated anti-mouse-$CD8^+$ T lymphocytes; and FL2, phycoerythrin-conjugated anti-mouse-$CD4^+$ T lymphocytes. (*a*) Analysis of axillar lymph nodes and (*b*) analysis of retroperitoneal lymph nodes. The suspensions correspond to nodes from control, dehydroepiandrosterone (DHEA) and DHEA + metformin pregnant mice; a *v.* b is significantly different ($P < 0.001$). Assays were carried out twice.

as PCOS (Vandermolen *et al*. 2001; Harborne *et al*. 2003*b*; Kazerooni and Dehghan-Kooshkghazi 2003; Weerakiet *et al.* 2004; Kumari *et al*. 2005).

However, even when the hormonal environment is regulated and women with PCOS become pregnant after the induction of ovulation, they can develop defects in the luteal phase resulting in miscarriage (Homburg *et al*. 1988), probably due to premature luteolysis. Corpus luteum involution has been related to an increased generation of ROS (e.g. O_2^- and H_2O_2) by the intact ovary (Berhman and Preston 1989). One consequence of the production of free radicals in ovarian tissue is lipid peroxidation (Kappus 1985), which occurs within the plasma membrane of luteal cells and may be associated with the observed loss of gonadotrophin receptors, diminished cyclic AMP formation and, therefore, with a decreased steroidogenic ability of the corpus luteum during involution (Auletta and Flint 1988; Wang *et al*. 1991). Protection against ROS in cells is provided by enzymes (superoxide dismutase, CAT and GSH peroxidase), metabolites (GSH) or antioxidant vitamins (Aten *et al*. 1992) and it has been suggested that it is endocrine-regulated (Laloraya *et al*. 1988; Aten *et al*. 1992; Sugino *et al*. 1999). Our results show that hyperandrogenisation with DHEA not only affected P and E production but also increased ovarian lipid peroxidation and diminished protective defenses such as GSH content and CAT activity. Following this, we suggest that hyperandrogenisation during the post-implantation window provokes an imbalance of the ovarian oxidant–antioxidant parameters leading to premature luteolysis and, therefore, to embryo resorption. Metformin has been described as a systemic or hepatic scavenger of ROS (Faure *et al*. 1999; Ceriello 2000; Srividhya *et al*. 2002; Bonnefont Rousselot *et al*. 2003); however, our data represent the first evidence of metformin action on ovarian oxidative stress.

In view of their roles in steroidogenesis (Willis and Franks 1995; Spaczynski *et al*. 1999; Peral *et al*. 2002), we also studied the action of DHEA on glucose and insulin levels and on serum TNFα production. DHEA treatment during post-implantation time increased both fasting glucose and serum insulin levels. These results are in agreement with previous reports, which found that hyperandrogenism is frequently associated with hyperinsulinemia, diabetes and insulin resistance (Abbott *et al.* 2002). Moreover, hyperinsulinemia and insulin resistance have been found to be responsible for low progesterone levels during the luteal phase (Meenakumari *et al*. 2004). In addition, we found that metformin treatment administered together with DHEA restored glucose and insulin levels to control values. These findings are in accordance with those of other authors (Harborne *et al*. 2003*a*; Lord *et al*. 2003) who have documented the action of this antidiabetic drug in restoring insulin sensitivity (Fedorcsak *et al*. 2003). Considering the role of insulin in regulating ovarian function, metformin has been considered to act indirectly on steroidogenic activity of theca and granulosa cells (La Marca *et al*. 2002). However, recently Mansfield *et al*. (2003) have proposed that metformin is able to exert a direct effect on ovarian steroidogenesis. We could assume that metformin acting directly on ovarian steroidogenesis and also indirectly by modulating glucose and insulin levels would contribute to ensure the accuracy of ovarian function and to improve the pregnancy rate in hyperandrogenised pregnant mice.

Increased levels of TNFα (Abbott *et al.* 2002; Sayin *et al*. 2003) and also a mutation of TNFα receptor (Peral *et al*. 2002) have been associated with hyperandrogenism. These findings, together with the role of the cytokine as a potent modulator of ovarian functionality, led us to investigate both the effect of hyperandrogenisation and the possible action of metformin treatment on serum TNFα levels. We found that sera from DHEA-treated mice exhibited an increase in TNFα, whereas in the DHEA + metformin group we observed a pattern similar to that of the control group. These results suggest a metformin action to regulate ovarian function, in addition to that of modulation of the glucose-to-insulin ratio and to the direct action on ovarian steroidogenesis. In the literature, controversial results have been documented with regard to the relationship between metformin and the regulation of TNFα (Cacicedo *et al*. 2004; Bruun *et al*. 2005; Di Gregorio *et al*. 2005; Kiortsis *et al*. 2005).

In the present investigation, we found that treatment of early pregnant mice with DHEA resulted in $88 \pm 1\%$ embryonic resorption. We further found that the uterine tissue showed lower NOS activity than healthy controls. However, metformin treatment was able to improve pregnancy rates and to restore NOS activity to control values. Nitric oxide exerts important actions during pregnancy by regulating uterine functions. Thus, it has been described that NO plays fundamental roles during critical steps of pregnancy development such as implantation, decidualisation, vasodilatation of decidual, placental and uterine vessels and myometrial relaxation (Sladek *et al*. 1997; Chwalisz and Garfield 2000). It has been also reported that NO participates in vascular invasion of the trophoblast (Ariel *et al*. 1998) and controls infection processes during pregnancy (Nowicki *et al*. 1997). Although it has been reported previously that the relationship between metformin and the NO system restores vascular function (Caballero 2004) and that metformin activates the phosphorylation of AMP-activated protein kinase via the NO pathway (Fryer *et al*. 2002; Mc Carty 2004; Zou *et al*. 2004), our results represent the first evidence regarding the action of metformin in regulating NOS activity during pregnancy.

As mentioned, DHEA has potent properties as an immunomodulatory molecule (Nieschlag *et al*. 1974; Du *et al*. 2001; Tagawa *et al*. 2004). In fact, we have reported previously (Luchetti *et al*. 2004) that treatment of prepuberal BALB/c mice with DHEA for 20 consecutive days modifies T lymphocyte expression of ovarian tissue, and that these results correlate with those observed on retroperitoneal

lymph nodes but without effect on axillar lymph nodes. These findings led us to hypothesise that increased levels of androgens could lead to the modulation of T lymphocyte expression from lymph nodes. However, in the present study, neither hyperandrogenism nor metformin treatment were able to alter T lymphocyte expression from lymph nodes. Analysing the differences between data from the two hyperandrogenised models, the prepuberal and the early pregnant mice, we can make some conclusions. First, in the previous report the $CD4^+ : CD8^+$ T lymphocyte ratio from retroperitoneal nodes of the control group of prepuberal mice was 1 : 1 (Luchetti *et al*. 2004), whereas the $CD4^+ : CD8^+$ T lymphocyte ratio from retroperitoneal nodes of the control group presented here was 1 : 1.5. These differences were predictable because the immune and endocrine conditions of the two states (prepuberal and pregnant) are essentially different and the prevalence of $CD4^+$ or $CD8^+$ T lymphocyte expression is regulated by hormones as was assessed throughout the sexual cycle (Lawler *et al*. 1999). Moreover, the number of $CD8^+$ T lymphocytes is significantly higher than that of $CD4^+$ T lymphocytes before luteolysis (Lawler *et al*. 1999), although the prevalence of $CD4^+$ T lymphocytes has been described during pregnancy (Du *et al*. 2001). Second, although we previously found that hyperandrogenisation with DHEA increased the percentage of $CD8^+$ T lymphocytes (Luchetti *et al*. 2004), in this investigation we showed that treatment with DHEA did not modify their T lymphocyte patterns. In this respect, we have to consider that although prepuberal mice were administered with DHEA for a longer time (20 days) than pregnant mice (only 2 days), we believe that the pregnant condition (with a slower immune response than the prepuberal condition) is the strongest contribution to these results. Our proposal is in accordance with that reported by Lawler *et al*. (1999) with respect to the immune response being modulated during maternal recognition of pregnancy.

To summarise, the present study suggests that embryonic resorption in hyperandrogenised mice may be attributed to a hostile maternal environment and inappropriate uterine development. We demonstrated that metformin was able to restore, either directly or indirectly, ovarian functionality and uterine reception.

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