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Effect of DHEA and metformin on corpus luteum in mice

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Abstract

We evaluated the effect of hyperandrogenism in ovaries with functional and regressing corpora lutea (CL) and the action of metformin in preventing these possible alterations using a mouse model. To obtain a CL functional for 9 ± 1 days, immature female mice of the BALB/c strain were injected i.p. with 10 IU/mouse of pregnant mare's serum gonadotropin (PMSG). DHEA (60 mg/kg body weight s.c., 24 and 48 h prior to kill) decreased both serum progesterone (P) and estradiol (E_2) levels and increased the activity of superoxide dismutase (SOD) from ovaries with functional CL (on day 5 after PMSG). It increased P and E_2 and the activities of SOD and catalase (CAT) and decreased lipoperoxidation of ovaries with regressing CL (on day 9 after PMSG). Treatment with DHEA did not affect the production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or PGE by ovaries with functional CL, whereas DHEA decreased $PGF_{2\alpha}$ and increased PGE production by ovaries with regressing CL. Metformin (50 mg/kg body weight, orally) given together with DHEA restored E_2 levels from mice with ovaries with functional CL and serum P, $PGF_{2\alpha}$ and PGE levels, and oxidative balance in mice with ovaries with regressing CL. Metformin alone was able to modulate serum P and E_2 levels, lipoperoxidation, SOD and CAT, and the 5,5-dimethyl-1-pyrroline *N*-oxide/$^{\cdot}$OH signal. These findings suggest that hyperandrogenism is able to induce or to rescue CL from luteolysis and metformin treatment is able to prevent these effects.
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Introduction

Luteolysis, or corpus luteum (CL) regression, a normal and necessary event in the mammalian reproductive cycle, is related to a decline in serum progesterone (P) production (functional luteolysis) followed by morphological changes and tissue remodeling events within the luteal tissue (structural luteolysis) (McCracken *et al.* 1999). The generation of reactive oxygen species (ROS), such as the superoxide radical ($O_2^{\cdot -}$), H_2O_2, and hydroxyl radical ($^{\cdot}OH$), increases during functional luteolysis (Motta *et al.* 2001*a*, Minegishi *et al.* 2002). Accumulation of ROS is a consequence of the uncontrolled lipid peroxidation (LPO) of cellular membranes. The increased LPO leads to the loss of gonadotropin receptors, a decreased production of cAMP, and finally to the loss of luteal function (Wang *et al.* 1991). Protection against oxidative stress in cells is provided by enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase), metabolites (glutathione: GSH), or vitamins (Agarwal *et al.* 2005). Enhanced LPO leads to the accumulation of the radical superoxide ($O_2^{\cdot -}$). SOD is responsible for the elimination of cytotoxic active oxygen; $O_2^{\cdot -}$ by catalysing the dismutation of the superoxide radical to oxygen and H_2O_2. This is an important step of the antioxidant cascade since the accumulation of $O_2^{\cdot -}$ leads to the formation of peroxynitrite, one of the most aggressive ROS. The accumulation of H_2O_2 is prevented by CAT. This enzyme turns H_2O_2 into water and oxygen. The accumulation of H_2O_2 leads to the formation of $^{\cdot}OH$, another aggressive radical. GSH is an antioxidant metabolite involved in reactions of oxide reduction, thus neutralizing oxidant species. It has been reported that antioxidant defenses are regulated by hormones (Motta & Gimeno 1997, Motta *et al.* 2001*a*, 2001*b*, Tam *et al.* 2003, Estevez *et al.* 2004, Duarte *et al.* 2005).

Prostaglandins (PGs) also regulate luteal function (Wiltbank & Ottobre 2003). The mechanisms leading to luteolysis are species specific and complex; nevertheless, PGs play an important role in its regulation. The addition of PGE to cultured ovine (Weems *et al.* 1997), bovine (Kotwica *et al.* 2006), human (Endo *et al.* 1988), and rat luteal cells (Hurwitz *et al.* 2002) increases the production of P. In contrast, the release of $PGF_{2\alpha}$ initiates luteal regression in domestic livestock (Knickerbocker *et al.* 1988), pigs (Gadsby *et al.* 1990), bovines (Hansel 1996), mice (Sugimoto *et al.* 1997), and women (Ristimaki *et al.* 1997). It has been well established that $PGF_{2\alpha}$ is responsible for the loss of luteal functions via enhancing the production of ROS (Sawada & Carlson 1991, Sugino *et al.* 1996). However, the relationship

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between PGs and the response of the antioxidant defenses in different systems is controversial (Sugino *et al*. 2004, Al-Gubory *et al*. 2005, Garrel *et al*. 2007).

Polycystic ovary syndrome (PCOS) in women is characterized by hyperandrogenemia, hirsutism, oligo- or amenorrhea, and anovulation (Franks 1995, Asuncion *et al*. 2000). The ovarian function of women with PCOS is altered. It has been reported that hyperandrogenism leads to abnormal length of estrus cycles and anovulation in rats (Lee *et al*. 1991, 1998, Henmi *et al*. 2001) and high rates of recurrent miscarriages in early pregnant women with PCOS (Glueck *et al*. 2002). In previous studies, we have reported that hyperandrogenization of prepuberal BALB/c mice by treatment with DHEA induces endocrine and immune disturbances in mouse ovarian tissue (Luchetti *et al*. 2004, Sander *et al*. 2006) and embryo resorption of early pregnant mice (Sander *et al*. 2005, Solano *et al*. 2006). The first aim of the present study was to assess the mechanisms by which hyperandrogenization affects the lifespan of CL. Particularly, we were interested in studying the impact of an excess of androgens in the oxidant–antioxidant balance and its relationship with the PG system.

Multiple concomitant therapies have been applied in PCOS women 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. Although the use of metformin (*N*,*N*′-dimethylbiguanide) is becoming accepted and widespread, it has been clinically used without a complete understanding of the mechanisms involved. Metformin reduces insulin resistance (Fedorcsak *et al*. 2003, Harbone *et al*. 2003, Lord *et al*. 2003), affects ovarian steroidogenesis (La Marca *et al*. 2002, Mansfield *et al*. 2003, Elia *et al*. 2006, Sander *et al*. 2006, Tosca *et al*. 2006*a*), and restores ovulation of anovulatory women with PCOS (Palomba *et al*. 2004, 2006). The treatment with metformin is also able to improve uterine vascularization in PCOS patients (Palomba *et al*. 2006). In addition, metformin prevents early embryo resorption in women with PCOS (Jakubowicz *et al*. 2004) by regulating both the PG and NO systems (inducible and constitutive nitric oxide synthase activity), the expression of progesterone-induced blocking factor, and the production of cytokines (Luchetti *et al*. 2008). Thus, our second objective was to investigate the action of metformin in preventing the ovarian dysfunctions produced by hyperandrogenism.

Results

Effect of hyperandrogenism and metformin on P and estradiol levels

In mice with functional CL (day 5), the hyperandrogenization with DHEA decreased both serum P and estradiol (E_2) levels when compared to controls (Fig. 1A and B respectively). Metformin alone decreased serum P and E_2 levels and metformin administered together with DHEA prevented the effect of DHEA on E_2 levels, whereas the levels of P remained lower than controls (Fig. 1A and B).

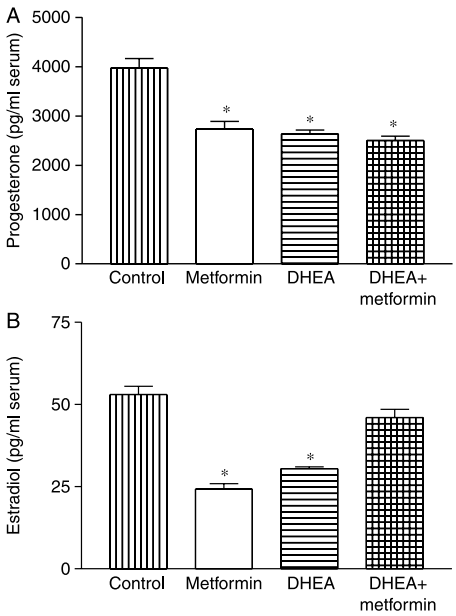

Figure 1 (A) Serum progesterone levels from mice with ovaries with functional corpora lutea treated with vehicle (control), metformin, DHEA, and DHEA+metformin. (B) Serum estradiol levels from mice with ovaries with functional corpora lutea treated with metformin, DHEA, and DHEA+metformin. Each column represents the mean ±S.E.M. of ten measurements from different animals, $^*P<0.001$.

The hyperandrogenization with DHEA increased both serum P and E_2 levels when compared to controls in mice with ovaries with regressing CL (Fig. 2A and B respectively). Metformin alone increased serum P and E_2 levels and metformin administered together with DHEA prevented the effect of DHEA on serum P levels, whereas the levels of E_2 remained higher than controls (Fig. 2A and B).

Effect of hyperandrogenism and metformin on ovarian oxidative stress

In ovarian tissue with functional CL, none of the treatments were able to modify ovarian LPO (Fig. 3A). Hyperandrogenization with DHEA increased SOD activity when compared to the control group (Fig. 3B). Metformin alone or administered together with DHEA increased SOD activity when compared to the control group (Fig. 3B) and none of the treatments were able to modify CAT activity (Fig. 3C).

In ovarian tissue with regressing CL, both metformin alone and DHEA decreased ovarian LPO when compared to controls (Fig. 4A). However, when metformin was administered together with DHEA, the ovarian LPO levels did not differ from that of controls (Fig. 4A). DHEA increased both SOD and CAT activities

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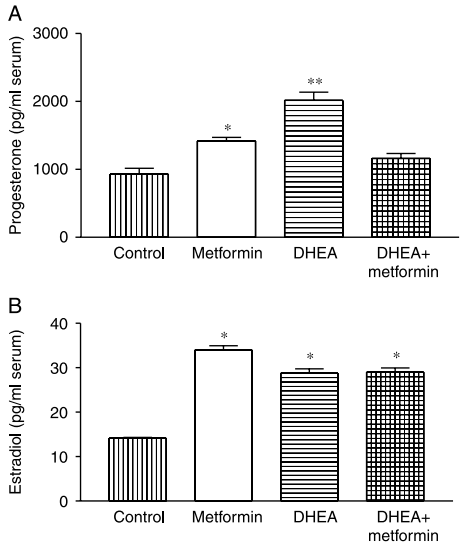

Figure 2 (A) Serum progesterone levels from mice with ovaries with regressing corpora lutea treated with vehicle (control), metformin, DHEA, and DHEA+metformin. (B) Serum estradiol levels from mice with ovaries with regressing corpora lutea treated with metformin, DHEA, and DHEA+metformin. Each column represents the mean ±S.E.M. of ten measurements from different animals, *,**$P<0.001$.

when compared to the control group (Fig. 4B and C), and metformin alone increased CAT activity but not SOD activity (Fig. 4C). When metformin was administered together with DHEA, SOD activity remained higher than that of controls, whereas CAT activity was similar to that of control values (Fig. 4B and C).

Effect of hyperandrogenism and metformin in ovarian ˙OH scavenging

5,5-dimethyl-1-pyrroline *N*-oxide (DMPO)/˙OH adduct was increased by all the treatments in ovarian tissue with functional CL (Fig. 5A and E–H). These data revealed that DHEA decreased the capacity to scavenge ˙OH and that metformin was not able to prevent the effect of DHEA in ovaries with functional CL.

In contrast with ovarian tissue with functional CL, in ovarian tissue with regressing CL, the electron spin resonance (ESR) signal intensity of the DMPO/˙OH adduct corresponding to both the metformin alone and DHEA group was decreased when compared to the control group (Fig. 5B and I–K). These data revealed that DHEA increased the capacity to scavenge ˙OH in ovaries with regressing CL. Metformin given together with DHEA prevented the action of DHEA on ESR signal (Fig. 5B, I, and L).

In order to address whether DHEA and metformin act direct or indirectly as ovarian ˙OH scavenger, we also assayed the effect of increasing concentrations of DHEA and metformin in medium free of ovarian homogenates. We found that neither DHEA nor metformin generated any change in the signal of the adduct DMPO/˙OH when compared to the control reaction (Fenton+DMPO) (data not shown).

Ovarian $PGF_{2\alpha}$ and PGE concentration

We investigated whether the luteolytic prostanoid $PGF_{2\alpha}$ and the luteotrophic PGE were regulated by DHEA and metformin. None of the treatments were able to modify the $PGF_{2\alpha}$ or PGE produced by ovaries with functional CL (Fig. 6A and B respectively). However, DHEA decreased $PGF_{2\alpha}$ levels and increased the levels of ovarian PGE produced by ovaries with regressing CL (Fig. 6C and D respectively). When metformin was administered together with DHEA, $PGF_{2\alpha}$ and PGE levels were similar to controls (Fig. 6C and D respectively).

Discussion

The present study was undertaken to improve our understanding on the effect of the excess of androgens and that of metformin treatment in two well-defined

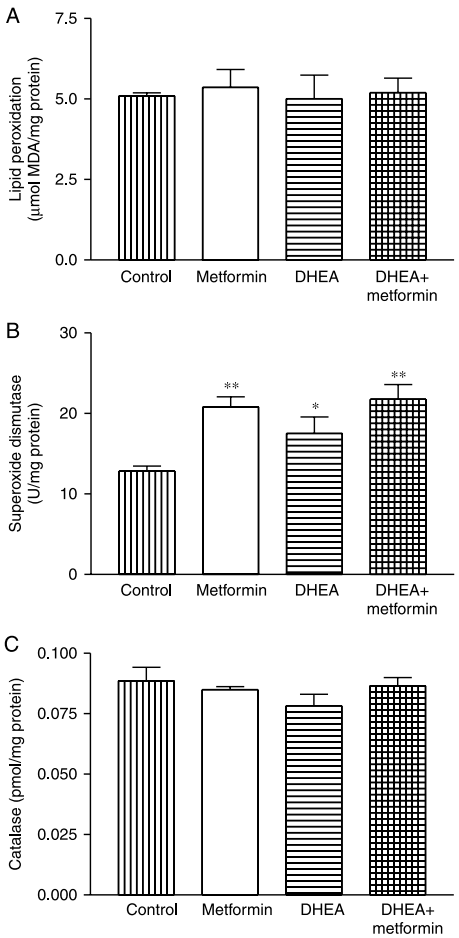

Figure 3 (A) Lipid peroxidation of ovarian tissue with functional corpora lutea from mice treated with vehicle (control), metformin, DHEA, and DHEA+metformin. (B) Ovarian superoxide dismutase activity of ovarian tissue with functional corpora lutea from mice treated with vehicle (control), metformin, DHEA, and DHEA+metformin. *$P<0.05$; **$P<0.001$. (C) Ovarian catalase activity of ovarian tissue with functional corpora lutea from mice treated with metformin, DHEA, and DHEA+metformin. Each column represents the mean ±S.E.M. of ten measurements from different animals.

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metabolic and hormonal states of CL lifespan. We found a dual effect of hyperandrogenism in modulating P and E_2 synthesis. During the functional stage of CL development, DHEA decreased both serum P and E_2 levels (Fig. 1). In agreement with these findings, it has been reported that women with PCOS show lower levels of P in the early luteal phase when compared to healthy controls (Joseph-Horne *et al.* 2002, Lunn *et al.* 2002). Moreover, the inability of women with PCOS to sustain early pregnancy is correlated to the decrease in ovarian steroidogenesis during the luteal phase (Franks 1995, Meenakumari *et al.* 2004).

Contrary to the functional stage, during the regressing stage of CL development, DHEA enhanced serum P and E_2 levels (Fig. 2). According to this finding, it has been reported that an excess of androgens leads to more prolonged sexual cycles in PCOS patients by rescuing the CL from luteolysis (Manikkam *et al.* 2006). The ability of DHEA to stimulate E_2 secretion has also been previously reported in human granulose cells (Bonser *et al.* 2000), and the mechanism proposed is the enhanced expression of P450 aromatase by DHEA (Elbeltagy *et al.* 2007). Moreover, Genazzani *et al.* (2006) have recently reported

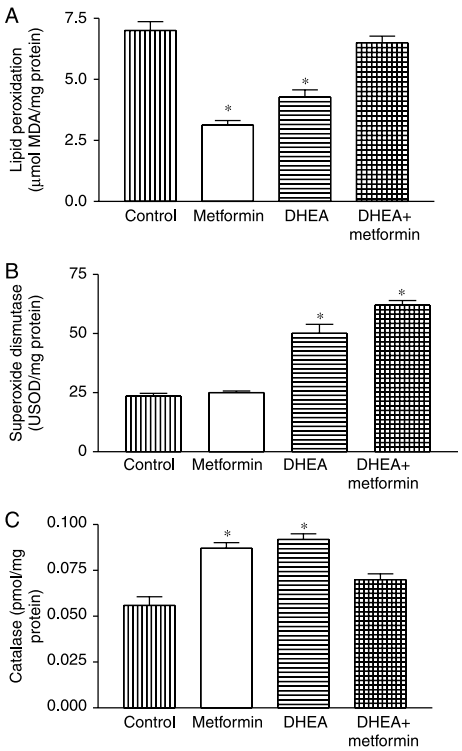

Figure 4 (A) Lipid peroxidation of ovarian tissue with regressing corpora lutea from mice treated with vehicle (control), metformin, DHEA, and DHEA + metformin, *$P<0.001$. (B) Ovarian superoxide dismutase activity of ovarian tissue with regressing corpora lutea from mice treated with vehicle (control), metformin, DHEA, and DHEA + metformin, *$P<0.001$ (C) Ovarian catalase activity of ovarian tissue with regressing corpora lutea from mice treated with vehicle (control), metformin, DHEA, and DHEA + metformin *$P<0.001$. Each column represents the mean ± S.E.M. of ten measurements from different animals.

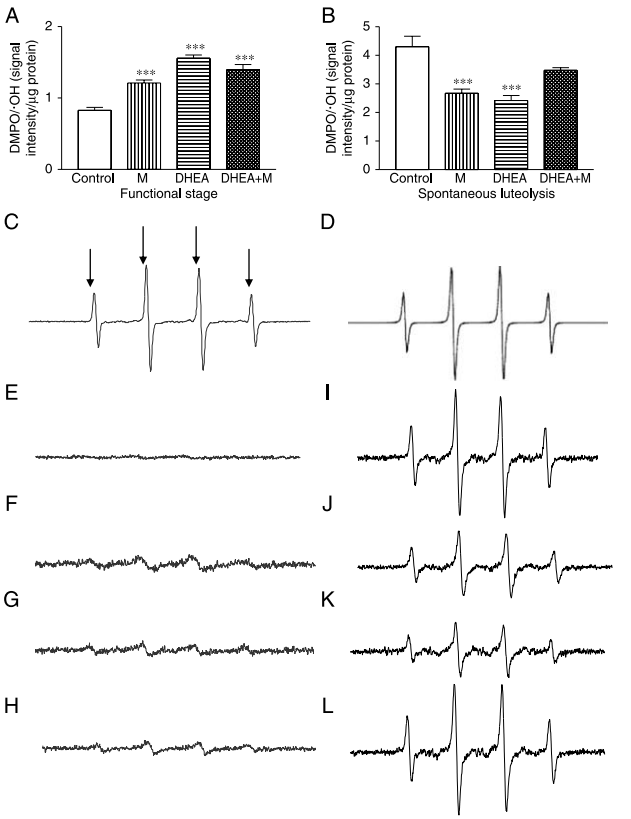

Figure 5 Detection of DMPO/˙OH adducts by Fenton reaction of ovarian tissue with functional corpora luteum. (A) Mean signal intensities of DMPO/˙OH; (C) ESR spectrum of DMPO/˙OH adduct generated by the Fenton reaction alone; (D) simulated ESR spectrum of 3C. Experimental electron spin resonance spectra obtained from ovarian tissue at functional stage of CL development from the control group (E), the Metformin group (F), the DHEA group (G), and the DHEA + metformin group (H). ***$P<0.001$ versus control group. The arrows in spectrum 3B indicate the lines corresponding to the hyperfine splitting of DMPO/˙OH adduct. Detection of DMPO/˙OH adducts by Fenton reaction of ovarian tissue with regressing corpora lutea. (B) Mean signal intensities of DMPO/˙OH; experimental electron spin resonance spectra obtained from ovarian tissue at luteal regression from the control group (I), the metformin group (J), the DHEA group (K), and the DHEA + metformin group (L). ***$P<0.001$ versus control group. The hyperfine interaction constant values obtained from simulation and fits were $a_N = 15.0$ Gauss and $a_{H\beta} = 14$.

that administration of DHEA in postmenopausal women is capable of increasing circulating levels of androgens and progestins.

The dual effect of DHEA in regulating ovarian function has been reported in our previous studies, where hyperandrogenization with DHEA was able both to decrease serum P and E_2 levels from early pregnant mice – state of enhanced steroidogenesis – (Solano *et al.* 2006) and to increase both steroids from prepuberal female mice – state of poor steroidogenesis – (Sander *et al.* 2006). Comparing these data with the present results, it appears that DHEA inhibits ovarian steroidogenesis when it is high in young fully functional CL, but stimulates it when it is low in CL nearing the end of their lifespan.

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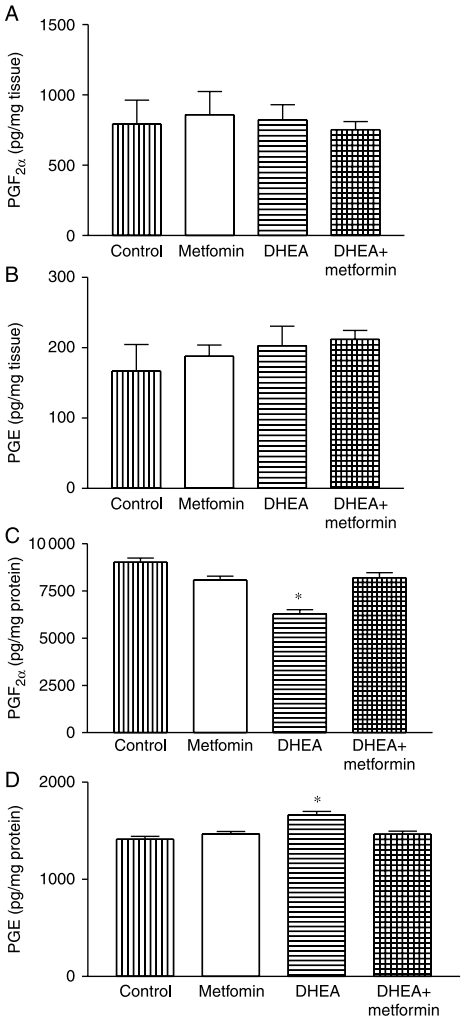

Figure 6 Effect of DHEA and metformin on (A) prostaglandin $F_{2\alpha}$ and (B) prostaglandin E from ovaries with functional corpora luteum (C) prostaglandin $F_{2\alpha}$ and (D) prostaglandin E from ovaries with regressing corpora lutea. Each column represents the mean ± S.E.M. of three measurements from five pooled ovaries from different animals, $^*P<0.001$.

With respect to metformin treatment, we found that during the functional stage of CL development, metformin given together with DHEA prevented the decrease of E_2 while it did not prevent DHEA-induced decrease on serum P levels (Fig. 1). Controversial results have been reported on the action of metformin in modulating ovarian steroidogenesis during the luteal phase. While Meenakumari *et al.* (2004) demonstrated that metformin restores P levels during the functional stage of CL development in PCOS patients, Vrbíková *et al.* (2001) reported no significant changes in basal steroid production. Our results are in agreement with those reported by Tosca *et al.* (2006*b*), who found that metformin increases E_2 secretion of no-stimulated granulose cells. Conversely to that observed in the functional phase, we found that DHEA increased both serum P and E_2 levels during luteolysis (Fig. 2). The mechanisms by which metformin regulates ovarian steroidogenesis remain unknown. It has been demonstrated that metformin regulates P synthesis by modulating the phosphorylation of AMP-activated protein kinase (AMPK; Elia *et al.* 2006, Tosca *et al.* 2006*a*, 2006*b*). However, AMPK activation does not seem to be essential for regulating E_2 production (Tosca *et al.* 2006*b*). It has been reported that metformin regulates E_2 levels by decreasing the amount of CYP19A1 protein by an AMPK-independent mechanism (La Marca *et al.* 2002, Tosca *et al.* 2006*a*, 2006*b*). Unexpectedly, we found that metformin alone modified ovarian steroid synthesis (Figs 1 and 2). In fact, it has been reported that metformin alone modulates ovarian steroid synthesis in no-stimulated human leukocytes (Bonnefont-Rousselot *et al.* 2003), no-stimulated granulosa cells (Tosca *et al.* 2006*b*), and no-stimulated endometriotic stromal cells (Takemura *et al.* 2007). However, the mechanisms involved remain unknown and studies are being designed to clarify this point.

Considering that ROS modulate the lifespan of CL (Riley & Berhman 1991, Sawada & Carlson 1991, Minegishi *et al.* 2002), we also quantified the ovarian oxidative status. As we expected, and like in the case of ovarian steroidogenesis, we found that both DHEA and metformin regulate the balance of ROS according to the endocrine status. During the functional stage of CL development, the hyperandrogenization with DHEA and metformin treatment increased SOD activity (Fig. 3b). Since this enzyme detoxifies the cells from $O_2^{\bullet-}$ accumulation, it represents the first pathway to be regulated. The fact that during the functional stage of CL development all the treatments increased SOD activity verifies this point (Fig. 3B). On the other hand, hyperandrogenism and metformin decreased the capacity of ovarian tissue with functional CL to scavenge $^{\bullet}OH$ (Fig. 5G and F); however, LPO was not increased (Fig. 3A), perhaps because the capacity to scavenge $O_2^{\bullet-}$ – one of the most aggressive ROS – is higher in ovaries with functional CL than in those ovaries with regressing CL (Fig. 3B).

Respect to the role of hyperandrogenism prolonging the lifespan of CL, we found that DHEA decreased ovarian LPO (Fig. 4A) and increased both the CAT activity (Fig. 4C) and the capacity of ovarian tissue to scavenge $^{\bullet}OH$ (Fig. 5K). Metformin treatment was able to prevent the decrease in LPO by acting on CAT activity (Fig. 4C) and the capacity to scavenge $^{\bullet}OH$ (Fig. 5L). We also found that metformin alone was able to decrease LPO (Fig. 4A) and increase both the CAT activity (Fig. 4C) and the capacity to scavenge $^{\bullet}OH$ (Fig. 5J). The structure of biguanides gives the molecules the property of being highly reactive to interact with ROS (Bonnefont-Rousselot *et al.* 2003). For this reason, we suggest that metformin alone is able to modulate oxidative stress and to modify ovarian steroidogenesis.

Concurrently with the rescue of CL from luteolysis by DHEA, we found a decrease in $PGF_{2\alpha}$ production and an increase in PGE (Fig. 6C and D). These data are

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consistent with previous findings about the role of $PGF_{2\alpha}$ in the generation of ROS and the luteotrophic role of PGE (Riley & Behrman 1991, Sawada & Carlson 1991, Motta *et al.* 2001*a*, Minegishi *et al.* 2002). Metformin treatment prevented the effects of DHEA since neither LPO (Fig. 4A) nor both PGs (Fig. 6) were different from controls allowing CL to regress.

We conclude that hyperandrogenism acts as a pro-oxidant agent during the functional stage of CL development but as anti-oxidant during spontaneous luteolysis, and our results are in agreement with those reported in other systems (Bonser *et al.* 2000). The dual effect of hyperandrogenism in the balance of ROS coincides with the dual effect of DHEA in regulating ovarian steroidogenesis. Regarding the effect of metformin, our findings suggest that metformin prevents the effects of DHEA mostly through the modulation of ROS leading to the restoration of PG balance that in turn normalizes ovarian steroid synthesis and CL lifespan.

Materials and Methods

Experimental design

Animals were kept and handled according to the NIH publication number 86–23 (revised in 1985 and 1991) and the UK requirements for ethics of animal experimentation (Animal Scientific Procedures, Act 1986). The studies were approved by the 'Independent Committee on Ethics in Research' of the School of Medicine of the University of Buenos Aires. The animal model used has been previously described (Sander *et al.* 2008). Briefly, immature (25–30 days of age) female BALB/c mice were i.p. given 10 IU/mouse of pregnant mare's serum gonadotropin (Sigma Chemical Co). This treatment leads to follicular development and ovulation 48 h later (day 0) and induces mostly the formation of CL that remains functional for 9 ± 1 days. Effects of treatment with DHEA were assessed on either ovaries with functional CL or regressing CL by two s.c. injections of 60 mg DHEA/kg body weight (DHEA group), 24 h apart on days 3 and 4 after ovulation, followed by decapitation on day 5 (functional CL) or on day 7 and 8, followed by decapitation on day 9 (regressing CL).

To investigate the action of metformin, two groups of mice at both stages of CL development received either metformin alone (metformin group; 50 mg/kg body weight in 0.05 ml of water, orally) or in combination with the injection of DHEA (DHEA + metformin group) on day 3 and 4 for mice with ovaries with functional CL and day 7 and 8 for mice with ovaries with regressing CL. The dose of DHEA injected was that previously used by Lee *et al.* (1991, 1998), Henmi *et al.* (2001), Luchetti *et al.* (2004), Sander *et al.* (2005, 2006), Elia *et al.* (2006) and Solano *et al.* (2006) and ensure a hyperandrogenized status. The dose of metformin administered was ensured to that used in the treatment of women with PCOS.

Control groups were two: in one group, mice were given vehicle either orally or s.c. A second control group consisted in mice without treatment. Since no differences were found between the two control groups, the results of both control groups were pooled in only one control group.

Housing of animals and sample collection

Thirty animals/group of each stage of CL development were housed under controlled temperature (22 °C) and light (14 h light:10 h darkness cycles; lights on at 0500 h) and allowed free access to Purina Rat Chow and water. Animals from the four groups (control, metformin alone, DHEA alone, and DHEA + metformin) of each stage were killed by decapitation and trunk blood was collected. Serum was isolated and stored at −70 °C until assayed for P and E_2. Freshly dissected ovaries (taken from different animals in all cases) were divided as follows: ten ovaries per group were immediately collected and processed to evaluate LPO, ten for SOD, ten for CAT, and ten to quantify total antioxidant capacity to scavenge $^{\cdot}OH$. Finally, 20 ovaries per group were immediately frozen at −70 °C until assayed for $PGF_{2\alpha}$ and PGE by RIA.

Progesterone and E_2 RIA

We evaluated serum concentration of P and E_2 by specific RIA as described previously (Luchetti *et al.* 2004). Both antisera, which were highly specific, were kindly provided by Dr G D Niswender (Colorado State University, Fort Collins, CO, USA). The cross-reactivity of P antiserum was <2.0% for 20 α-dihydroprogesterone and deoxycorticosterone. The cross-reactivity of E_2 antiserum was <1% for P and testosterone, <5% for 17-α estradiol and estriol, and <10% for estrone. Since both sensitivities were 5–10 pg/tube, 2–5 µl serum were routinely assayed. Results are expressed as pg/ml serum.

Oxidative stress-related parameters

Lipid peroxidation

The amount of malondialdehyde (MDA), which is formed from the breakdown of fatty acids, may be taken as an index of LPO. 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 M respectively), yielding a red compound that absorbs at 535 nm (Motta *et al.* 2001*a*). Single ovaries were homogenized in 850 µl 0.5% v/v trichloroacetic acid; 350 µl homogenates 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 µmol MDA formed/mg protein.

SOD activity

SODs are a group of metalloenzymes that detoxify ROS through the conversion of $O_2^{\cdot-}$ to H_2O_2 and molecular oxygen. Total SOD activity was assayed by a spectrophotometric method based on the inhibition of a superoxide-induced epinephrine oxidation (Misra & Fridovich 1972). $O_2^{\cdot-}$ serves as 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 (ten ovaries per group) was homogenized

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in 50 mM phosphate buffer (pH 7.2) and centrifuged at 800 ***g*** for 10 min at 4 °C. Supernatants were collected and 0.05 M carbonate buffer (pH 10.2) containing 0.1 mM EDTA and followed by 30 mM 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

$$1 \text{ unit (U) of SOD} = V_{control}/V_{sample} - 1$$

V is the slope of the change in absorbance in the absence and in the presence of ovarian homogenates.

CAT activity

The technique used is based on the ability of CAT to catalyse the conversion of H_2O_2 to molecular oxygen and water (Solano *et al.* 2006). Briefly, one ovary per determination (ten ovaries per group) was homogenized as previously described for the SOD assay. Homogenates (100 µl/point) were incubated with 3 ml of 50 mM phosphate buffer (pH 7.2) and 100 µl of 3 M H_2O_2. Since H_2O_2 absorbs at 240 nm, its consumption was monitored by a spectrophotometer for 1 min at 10 s intervals. Results are expressed as pmol/mg protein.

Electron spin resonance assay

Ten ovaries per group were assayed to determine the antioxidant capacity of the ovarian tissue to scavenge $^{\bullet}OH$. Each ovary per determination (ten ovaries per group) was homogenized in a solution of DMPO 200 mM. Immediately, $FeSO_4$ (final concentration = 3 mM) and H_2O_2 (final concentration = 150 mM) were added in order to generate $^{\bullet}OH$ by a Fenton reaction (equation 1). The electron spin resonance spectra of the spin adduct DMPO/($^{\bullet}OH$) (equation 2) were recorded 2 min after the addition of H_2O_2. Measurements were obtained at 20 °C using an X-band Electron Spin Resonance Spectrometer Bruker ECS 106. The spectrometer settings were: center field 3483 G, sweep width 80 G, microwave power 10 mW, microwave frequency 9.76 GHz, conversion time 2.56 ms, time constant 2.56 ms, modulation frequency 50 KHz, modulation amplitude 0.103 G, gain 2.00×10^4, and resolution 1024 points. All spectra were the accumulation of ten scans. DMPO/$^{\bullet}OH$ signal intensity was measured as the total height of the low-field peak (h_{+1}) in the first derivative spectrum of the ^{14}N triplet. Results were interpreted in terms of total $^{\bullet}OH$ scavenger capacity in the sample, being a higher DMPO/$^{\bullet}OH$ signal the consequence of a decrease in total DMPO/$^{\bullet}OH$ scavenger capacity.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet} \quad (1)$$

$$DMPO + HO^{\bullet} \rightarrow DMPO/^{\bullet}HO \quad (2)$$

In order to discard possible intrinsic effects of DHEA and metformin to scavenge, $^{\bullet}OH$ controls without ovarian homogenates were carried out in a cell-free medium. Metformin was studied in the following range of concentrations of 0.1–1000 µM, which include the pharmacological metformin concentration in human plasma (Yuen & Peh 1998) and DHEA was tested between 0.01 and 1000 µM, in order to include the mean serum DHEA concentration present in PCOS (Fridstrom *et al.* 1999).

Prostaglandin RIA

The capacity of the ovaries to synthesize and release $PGF_{2\alpha}$ and PGE was determined as previously reported (Elia *et al.* 2006). Briefly, each ovary (five ovaries per group for each PG) was incubated in Krebs-Ringer bicarbonate with 11.0 mmol/l glucose (pH 7) as external substrate 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 PG 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 PG RIA was performed. $PGF_{2\alpha}$ and PGE were quantified by using the specific rabbit antiserum from Sigma Chemical Co. Sensitivity was 10 pg/tube and cross-reactivity was <0.1% with other PGs. Cross-reactivity was 100% with $PGF_{2\alpha}$ and PGE respectively. Results are expressed as pg/mg protein.

Level of ovarian protein concentrations

Ovarian protein concentration was determined by the Bradford method (1976).

Statistical analysis

Statistical analyses were carried out by using the Instat program (GraphPAD software, San Diego, CA, USA). One-way 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.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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