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Treatment with the synthetic PPARG ligand pioglitazone ameliorates early ovarian alterations induced by dehydroepiandrosterone in prepubertal rats

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Abstract

Background: Peroxisome proliferator-activated receptor gamma (PPARG) is a nuclear factor that may act on the early development of ovarian follicles and on follicular steroidogenesis. However, the exact mechanism of PPARG action remains unknown. We have previously found that androgen excess alters early ovarian function and the PPARG system. The aim of the present study was to evaluate whether PPARG activation (using the synthetic ligand pioglitazone (PGZ)) ameliorates the alterations in early ovarian function induced by androgen excess.

Methods: Female prepubertal rats were treated with equine chorionic gonadotropin (eCG) to induce folliculogenesis, together with dehydroepiandrosterone (DHEA) to induce hyperandrogenism and/or PGZ to evaluate PPARG activation. We assessed i) very early ovarian folliculogenesis, ii) PPARG activation, iii) ovarian steroidogenic enzymes, iv) the estradiol/testosterone ratio, v) the ovarian inflammatory status and vi) oxidative stress.

Results: PGZ prevented the inactivation of ovarian PPARG induced by androgen excess by increasing PPARG itself and the gene expression of PPARG-coactivator 1 alpha (PGC1A), and by decreasing the gene expression of nuclear co-repressor (NCOR). PGZ also prevented the altered ovarian steroidogenesis, pro-inflammatory status and oxidative stress induced by androgen excess.

Conclusions: Our findings suggest that PPARG activation plays important roles in modulating early ovarian function, and highlight the importance of understanding the role(s) of PPARG activation in the ovary, and the possible involvement in the treatment of ovarian pathologies, and/or the impact in regulating/improving fertility.

Key Words: PPARG, folliculogenesis, dehydroepiandrosterone, ovarian function, pioglitazone.

Introduction

Reproductive function is associated with energy balance [1, 2]. In the ovary, the nutritional status modulates ovarian function, [1] and several candidates have been involved as possible links between the nutritional status and the function of the different ovarian cells [3]. Some of these energy sensors are the peroxisome proliferator-activated receptors (PPARs), which are transcriptional factors belonging to the steroid receptor family. Currently, three different types of PPARs are recognized (PPARalpha,

PPARbeta/delta and PPARgamma) and described as key regulators of fatty acid and lipoprotein metabolism, glucose homeostasis, cellular proliferation/differentiation and the immune response [4, 5]. In the ovary, PPARgamma (PPARG) senses the nutritional status in the follicle [6] and may act in the development of follicles and their ability to support normal oocyte maturation (reviewed in [7]). The activation of PPARG by both endogenous and synthetic ligands modulates its transcriptional activity by increasing the recruitment of co-activators, such as PPARG co-activator 1 alpha (PGC1A) [8], and increasing the clearance of repressors such as the nuclear corepressor (NCOR), which down-regulates the transcriptional activity of PPARG [9]. In the ovary, upon activation, PPARG modulates ovarian steroidogenesis and cellular proliferation [6, 10], and its disruption leads to female subfertility [11]. However, the exact mechanism involved in PPARG activation remains unknown, particularly during early ovarian function.

We have previously shown that the administration of a follicle stimulating hormone (FSH) analog promotes early follicular development in prepubertal rats [12]. In this window of development, exposure to androgen excess induces alterations in the ovarian function and related endocrine parameters [12, 13]. Androgen excess disturbs ovarian follicular development, leading to chronic anovulation, as observed in polycystic ovary syndrome (PCOS), a condition in which impaired follicular growth lead to menstrual disturbances and anovulatory infertility [14, 15]. Moreover, evidence indicates that prenatal or pre-pubertal androgen excess may be involved in the pathophysiology of PCOS [16, 17]. It has been reported PPARG activators, such as pioglitazone (PGZ) and rosiglitazone, improves the androgenic status and ovarian function [15]. However, the clinical use of these activators goes beyond the knowledge of their mechanism of action.

Our murine model consists in a very short-term treatment of immature female animals with two hormones: equine chorionic gonadotropin (eCG), an efficient interspecies inducer of follicular recruitment [18], and dehydroepiandrosterone (DHEA), which promotes a hyperandrogenic condition [12]. Our previous results showed that DHEA treatment induces alterations in the very early ovarian function, along with a down-regulation of the PPARG system [12, 19]. Based on these previous findings, in the present study, we aimed to investigate whether the activation of the PPARG system, by the synthetic ligand PGZ, was able to prevent the alterations induced by androgen treatment. Specifically, we studied the role of PGZ in preventing the adverse effects of androgen excess on i) very early ovarian folliculogenesis, ii) PPARG activation, iii) ovarian steroidogenesis, iv) the estradiol2 (E2)/testosterone (T) ratio, v) Ovarian inflammatory status and vi) oxidative stress.

Materials and Methods

Animal model

Sprague-Dawley prepubertal female rats (22-25 days of age) were housed under controlled temperature and illumination and allowed free access to food and water. All procedures were conducted in accordance with The National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). We used prepubertal rats to avoid previous estrous cycles that may interfere with the results, and our choice is supported by the fact that prepubertal rats are widely used to study the effect of androgen excess administration on ovarian function [20-22]. In this model, we have previously assayed the effect of a single dose of eCG on prepubertal rats and we found that eCG treatment yield the peak of progesterone at 8h, after this time serum hormone decreases [19]. This finding is in agreement with other authors whose reported an early effect of eCG on ovarian steroidogenesis [23]. In fact,

we have previously found an acute effect of both eCG and DHEA in early stage of follicular development, as previously reported [12, 19]. A group of 25 rats were injected intraperitoneally (*ip*) with 25 IU of eCG (Novormon, Syntex SA) in saline solution (eCG group). eCG+DHEA or DHEA group consisted of 25 rats injected with eCG plus a sc injection of 60 mg/kg body weight of DHEA (Sigma-Aldrich, USA) in sesame oil. A third group of 25 rats were injected with eCG together with DHEA and then orally administered with 1 mg/kg body weight of PGZ (ELEA, Buenos Aires, Argentina) in water (eCG+DHEA+PGZ). Although the current treatments with thiazoldineniones are prolonged, Cox *et al* showed that the bioavailability in plasma after a single dose is rapidly seen within 24 hours [24], therefore we decided to test the effect of PGZ at 8h. To assess any effect of PGZ *per se*, a fourth group of 25 rats were ip injected with eCG together with orally administration of PGZ (eCG+PGZ). After 8 h of treatments, rats were anesthetized with CO_2 and killed by decapitation. Ovarian tissue was removed and; a) immediately fixed in 4% (w/v) paraformaldehyde for morphological studies, or b) stored at -80°C for subsequent ovarian assays. Also, trunk blood was collected and serum was separated by centrifugation, and stored at -80°C for subsequent oxidative stress and sexual hormones assays.

Ovarian morphology

Histological serial sections were obtained as described before [12]. Sections were analyzed independently by three of the authors, and ovarian follicles were classified and quantified as primordial (PF), primary (PrF), secondary (SF), and antral (AF). PF were classified as those formed by an oocyte surrounded by a flattened layer of pre-granulosa cells; PrF were those with at least one cuboidal layer of granulosa cells (GCs); SF were those with more than one layer of cuboidal GCs and an incipient layer of theca cells (TCs); and AFs were those with the antrum and the oocyte with the

surrounded zona pellucida, and a basal lamina between GCs and TCs. Follicular atresia was also quantified, and atretic follicles were defined as follicles with >5% of the GCs having pyknotic nuclei.

qPCR analysis

mRNA levels in ovarian tissue were measured by qPCR as described before [12].We evaluated PPARG, NCOR, PGC1A, steroidogenic acute regulatory protein (STAR), cytochrome P450-17A1 (CYP17), 3β-hydroxysteroid dehydrogenase (3BHSD), 17β-hydroxysteroid dehydrogenase (17BHSD), aromatase (CYP19), tumor necrosis factor alpha (TNFA) and cyclooxygenase 2 (COX2). The 2(-DeltaDelta CT) method was used to analyze the relative changes in gene expression. Results are expressed as arbitrary units, and the RPL32 gene was used as a reference. Primers are shown in Table 1.

WB analysis

Protein levels were measured by WB as described before [12]. Diluted primary antibodies of (1/100) PPARG (H-100, sc-7196), (1/200) STAR (FL-285, sc-25806) and (1/100) COX2 (H-62, sc-7951) (Santa Cruz Biotechnology, Inc., USA) were used. Data of protein loading was normalized by applying the protein βActin (1/500) (Sigma Co.). The experiment was independently repeated three times. Results are expressed as arbitrary units.

Estradiol and testosterone radioimmunoassays.

E2 and T levels were determined by specific RIAs as described before [25]. E2 sensitivity was 5–10 pg/tube andT sensitivity was 25–1600 pg/tube. The intra-assay and inter-assay variations of T were 7.5 and 15.1% respectively, and the cross-reaction between T and DHEA was <0.01 pg. Results are expressed as pg/ml serum.

PGE radioimmunoassay

PGE content was determined by RIA as previously reported [26]. Sensitivities of these assays were 10 pg/tube for PGE. The cross-reactivity of PGE2α was 100% with PGE1 and <0.1% with other prostaglandins. Results are expressed as pg/μg protein. Protein concentration was determined by the Bradford assay (Bio-Rad).

Determination of lipid peroxidation

The method used in the present study, as described before [26], quantifies serum MDA as the product of lipid peroxidation that reacts with trichloroacetic acid–thiobarbituric acid–HCl (TCA-TBA-HCL) (Sigma), yielding a red compound that absorbs at 535 nm. Results are expressed as content of MDA (nanomoles MDA formed/ml serum).

Determination of glutathione content in serum

Glutathione (GSH) was quantified in serum as previously described [27]. The reduced form of GSH comprises the bulk of cellular protein sulfhydryl groups. Results are expressed as μM GSH.

Statistical analysis

Statistical analyses were carried out with the GraphPad Prism 5.0 (GraphPad software, San Diego, USA). Data were analyzed by 2-way ANOVA to assess the effects and interactions of 2 independent variables, and multiple comparisons were achieved using the Bonferroni *post-hoc* test. Statistical significance was defined at $p<0.05$

Results

PGZ prevented the down-regulation of the ovarian PPARG system induced by DHEA

PGZ (eCG+DHEA+PGZ) partially prevented the decrease in both mRNA and protein levels of PPARG induced by DHEA treatment (Fig. 1 A-B). Note: we quantified the two observed bands of the corresponding WB, corresponding to PPARG1 and

PPARG2 isoforms, according to the supplier. PGZ also prevented the decrease in the mRNA levels of PCG1A and partially prevented the increase in the mRNA levels of NCOR, induced by DHEA treatment (Fig. 1C-D). No significant effects of PGZ *per se* (eCG+PGZ) were found on PPARG, PGC1A or NCOR levels, when comparing to the eCG group (Fig. 1 A-D).

PGZ treatment and ovarian morphology

No preovulatory follicles or corpora lutea were observed in any of the treatment groups, an expected result considering the short age of the prepubertal rats. No differences in total ovarian weight were found between groups (data not shown).

The percentage of PF was higher whereas that of PrF was lower in the DHEA treatment group than in the eCG group (Fig. 2A). PGZ partially prevented the alterations caused by the DHEA treatment, since the percentages of PF and PrF were similar to those in the DHEA treatment and eCG groups (Fig. 2A). In the PGZ *per se* group, we found no differences in the percentages of SF and AF in any of the treatment groups (Fig. 2A), or in any follicle class, comparing to the eCG group (Fig. 2A). The percentage of atresia was similar in all the treatment groups (Fig. 2B).

The activation of the PPARG system prevented the alterations induced by DHEA in the levels of ovarian steroidogenic enzymes

PGZ prevented the DHEA-induced increase in both the mRNA and protein levels of STAR (Fig. 3A-B), and the DHEA-induced increase in the mRNA levels of CYP17 (Fig. 3C), 3BHSD (Fig. 3D) and 17BHSD (Fig. 3E). To evaluate the balance in the synthesis of E2 and T, we assessed the mRNA levels of the aromatase CYP19. We found that PGZ prevented the DHEA-induced decrease in the mRNA levels of CYP19. The PGZ *per se* group showed a decrease in CYP17 mRNA levels, with no differences

in the levels of STAR, 3β-HSD, 17β-HSD or CYP19, comparing to the eCG group (Fig. 3C-F).

The activation of the PPARG system prevented the alterations caused by DHEA in the E2/T ratio

PGZ increased the serum levels of E2 and decreased those of T (Fig. 4A and B) as compared with the DHEA treatment group, thus leading the E2/T ratio to levels similar to those of the eCG group (Fig. 4C). PGZ *per se* did not alter systemic E2, T or E2/T ratio as compared with the eCG group (Fig. 4A-B).

The activation of the PPARG system prevented the ovarian pro-inflammatory status induced by DHEA

PGZ prevented the DHEA-induced increase in mRNA levels of TNFA, an early inflammatory marker (Fig. 5A), and both the mRNA and protein levels of COX2 (Fig. 5B-C). Moreover, PGZ decreased the levels of PGE, comparing to the DHEA treatment, to values even lower than those observed in the eCG group (Fig. 5B-D). In addition, PGZ *per se* did not alter the protein or mRNA levels of COX2, mRNA levels of TNFA, or PGE content, comparing with the eCG group (Fig. 5C).

The activation of the PPARG system exerted a protective effect against DHEA-induced oxidative stress

PGZ prevented the DHEA-induced alterations in the systemic oxidant/antioxidant balance evaluated by MDA and GSH (Fig. 6A-B). We found no effect *per se* of PGZ on serum MDA or GSH levels, as compared with those of the eCG group (Fig. 6A and B).

Discussion

In previous studies, we found that androgens alter early ovarian function, impair follicular steroidogenesis, establish an ovarian pro-inflammatory and pro-oxidant

status, and decrease the activation of PPARG in the ovary [12, 19]. In agreement with these results, it has been shown an association between PPARG and androgen excess disorders like PCOS [28-30].

Here, we showed that PGZ activated PPARG through the modulation of PPARG itself and the gene expression of co-regulators PGC1A and NCOR. These data are in agreement with previous findings showing that PGZ increases the gene expression of the co-activator PGC1A [31] and promotes the clearance of NCOR [9]. Despite the complexity of the transcriptional mechanism of PPARG [7], this is the first time that PGZ has been shown to activate PPARG through its own expression and the gene expression of PGC1A and NCOR, during early ovarian function.

It has been found that gonadotropins, together with intraovarian regulators, have a stimulatory effect on early folliculogenesis [32]. Thus, we induced early ovarian development in prepubertal rats by means of an eCG injection. In that context, when follicular stimulation was induced in the presence of androgen excess, the percentage of PF increased while that of PrF decreased. These data are in agreement with the evidence that androgens are involved in early follicular recruitment [32, 33]. Furthermore, we did not find an altered percentage of atretic follicles in the presence of androgen excess, in agreement with Vendola *et al* [33], and we suggest that, at least in the short term, androgens are not atretogenic. These findings are consistent with that observed in women with PCOS [34], who show prolonged survival of preantral follicles with respect to normal-cycling women, in which preantral follicles either grow rapidly to become dominant follicles or collapse in atresia. However, if the exposure to androgen excess continued, follicles with abnormal growth would eventually collapse back into the ovarian stroma, leading to the stromal hypertrophy typical of PCOS and chronic testosterone treatment [35]. Here, we found, for the first time, that PPARG activation

prevented the alterations induced by the excess of androgens, since PGZ restored the percentages of both PF and PrF to gonadotropin-induced values. These findings support the notion that PPARG plays a role in the early stages of follicular development [6, 7].

PPARG system modulates the expression of genes involved in follicular development, ovulation, oocyte maturation, and corpus luteum development [6, 10]; however, this is the first time that it is shown that PPARG activation prevents the deregulation of steroidogenic pathway enzymes induced by androgen excess. In fact, PPARG activation prevented the alteration in the estrogens/androgens ratio, by means of modulating the gene expression of steroidogenic enzymes, especially by increasing the gene expression of aromatase CYP19, the enzyme that synthesizes E2 from T. The consequence of this action is the restoration of the E2/T ratio to gonadotropin-induced values. An abnormal E2/T ratio contributes to the poor oocyte quality observed in prenatally hyperandrogenized female rhesus monkeys, sheep and rats [17] and also in women with PCOS [36]. A balanced E2/T ratio is essential during follicular development to promote the dominant follicle [37] and this could explain why women with PCOS are unable to produce an ovulatory follicle. Moreover, the re-establishment of the E2/T ratio by PPARG activation could also explain the prevention of the alterations in PF and PrF described above.

In normal conditions, a pro-inflammatory status is established just before ovulation occurs, which correlates with a down-regulation of the PPARG system [7]. In addition, the gene expression of COX2, the rate limiting enzyme of PG synthesis, is low in the early stages of folliculogenesis and is high prior to ovulation [38]. Here we showed that activation of PPARG decreased the pro-inflammatory status induced by androgen excess (by the decrease in the gene and protein expression of COX2, PGE synthesis and the gene expression of TNFA). These findings suggest that activation of

PPARG exerts an anti-inflammatory effect, as shown by other authors [39, 40]. Moreover, an anti-inflammatory status results favorable during the early stages of ovarian folliculogenesis [41]. In summary, the modulation of the inflammatory process by PGZ induces a favorable early ovarian environment necessary for the establishment of the future dominant follicle.

Systemic oxidative stress may be related to the ovarian pro-inflammatory status found in this study in the presence of androgen excess, since an increase of TNFA, COX2 and PGE is involved in the generation of systemic free radicals and subsequent oxidative stress [42]. Here, we demonstrated that PPARG activation was able to prevent the circulating oxidative stress, in agreement with other authors, who have indicated that PPARG can exert a protective effect against oxidative stress [40], decreasing the generation of free radicals [43] and increasing the antioxidant metabolite GSH [44].

One question that remains to be answered is the effect of PGZ *per se* treatment. It has been described thiazoldineniones effects in the ovary independent of PPARG activation [7]. In the present study, we found activation of ovarian PPARG by PGZ only in the presence of androgen excess, and not in the PGZ *per se* treatment. The only PGZ *per se* effect was the down-regulation of the gene expression of steroidogenic enzyme CYP17. The same effect of PGZ independent of PPARG activation has been observed in a human model of adrenal steroidogenesis, where PGZ, and not rosiglitazone, downregulated CYP17 expression [45]. Further studies are necessary to discern the mechanism of action of PGZ dependent and independent of PPARG activation in the early ovarian function. In addition, it is important to point out that the PPARs have been shown to coordinately regulate the expression of genes including those that control fatty acid and lipoprotein metabolism, glucose homeostasis, cellular proliferation/ differentiation and the immune response [4, 5]. Consequently, PPARs play important

roles in the treatment of endocrine disorders: PPARG ligands are effective in treating insulin resistance and T2DM patients, PPARalpha agonists provides anti-dyslipidemic and anti-atherosclerotic outcomes, and recent findings indicate that PPARdelta ligands have beneficial effects on circulating lipids and obesity (reviewed in [46-48]).

Taken together, our results demonstrate that the activation of PPARG by PGZ prevents the alterations caused by androgen excess in the early ovarian function. Moreover, this is the first study showing evidence of this very early molecular mechanism of PPARG activation and reveals the importance of its activation during the early stages of follicular development. However, the precise role of PPARG in the ovary in both physiological and pathological conditions remains to be fully elucidated.

Declarations of interest: none

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Short title: PPARG activation and ovarian function in DHEA-treated rats

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Legends and tables

Figure 1: Expression PPARG, PGC1 and NCOR. Relative levels of: mRNA (A) and proteins (B) for PPARG. A representative WB of PPARG is shown in (B).

Relative levels of: mRNA for PGC1A (C) and NCOR (D). The columns represent the mean values ± SEM. (N=5 replicates per group). *p<0.05 **p<0.01 compared with the eCG group. #p< 0.05 comparing eCG+DHEA *vs.* eCG+DHEA+PGZ groups. NS: not significant.

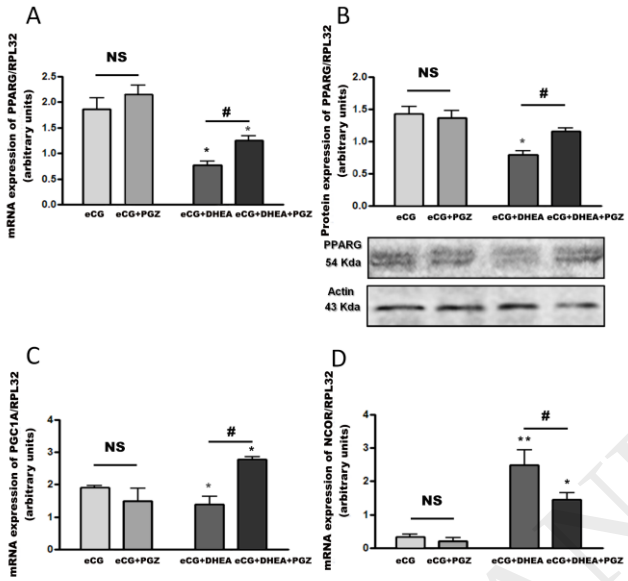
Figure 2: Ovarian morphology. A) Percentages of the different types of ovarian follicles present in the ovaries. Statistical differences were assessed between treatments within each class of follicle (C) Percentage of atretic follicles present in ovaries. The columns represent the mean values ± SEM. (N= 5 replicates per group). *p< 0.05 compared with the eCG group. NS: not significant.

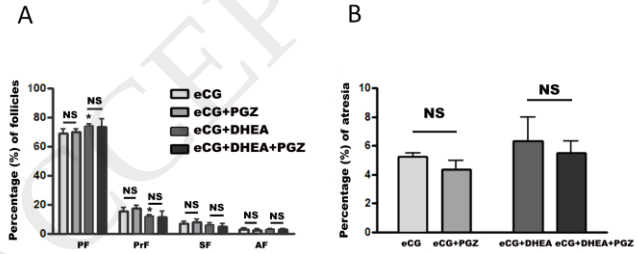
Figure 3: Expression of ovarian steroidogenic enzymes. Relative levels of: mRNA (A) and proteins (B) of STAR. A representative WB of STAR is shown in (B). Relative levels of mRNA for CYP17 (C), 3BHSD (D), 17BHSD (E) and CYP19 (F). The columns represent the mean values ± SEM. (N=5 replicates per group). *p< 0.05 **p<0.01 and ***p<0.001, compared with the eCG group. $^{\#}p$<0.05, $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001, comparing eCG+DHEA *vs.* eCG+DHEA+PGZ groups. NS: not significant.

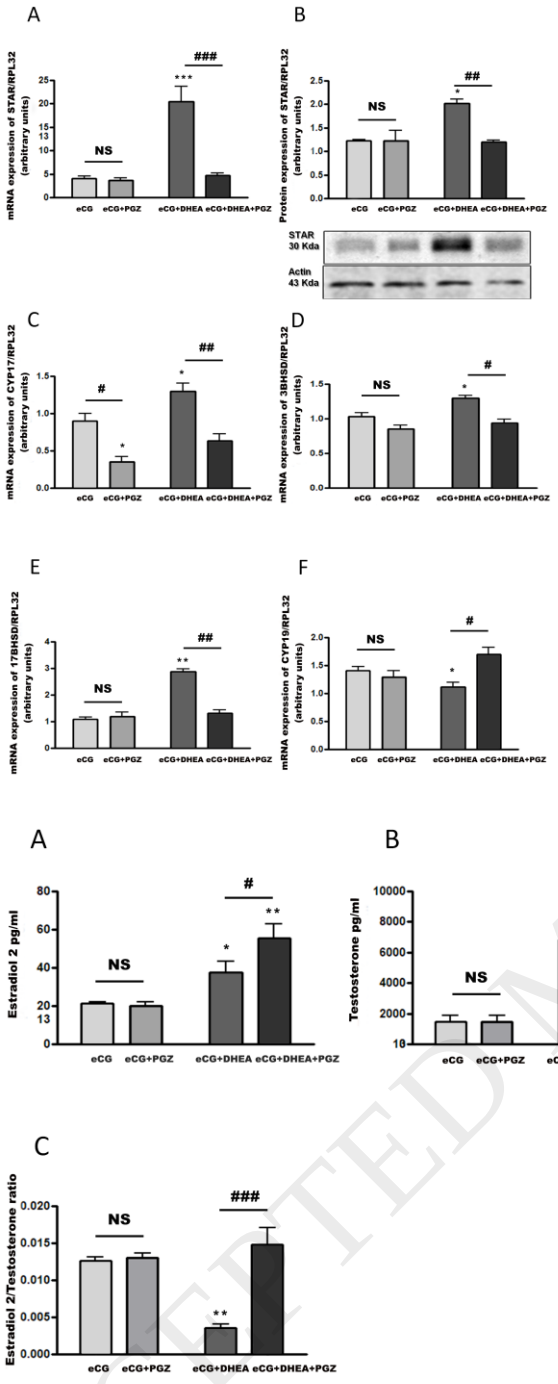
Figure 4: T, E2 and E2/T ratio. Serum levels of T (A) and E2 (B). E2/T ratio is shown in (C). Each column represents the mean values ± SE.M. (N=5 replicates per group) *p<0.05 and **p<0.01, compared with the eCG group. #p<0.05, $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001, comparing eCG+DHEA *vs*. eCG+DHEA+PGZ groups. NS: not significant.

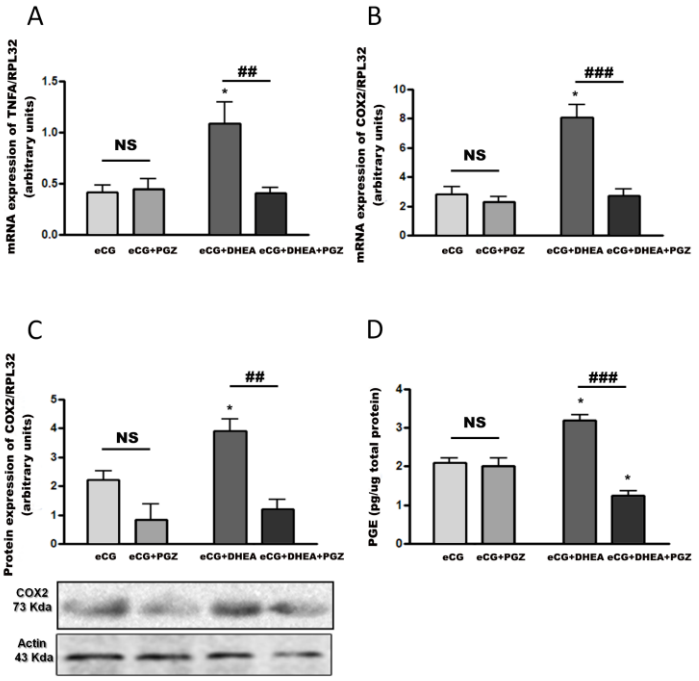
Figure 5: Expression of ovarian inflammatory markers. (A) Relative levels of mRNA of cyclooxygenase 2 (COX2). (B) A representative WB of COX2 (D) Relative levels of COX2 protein (E) Ovarian content of prostaglandin E (PGE). Each column represents the mean values ± SEM (N=5 replicates per group) *p<0.05 compared with the eCG group. $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001, comparing eCG+DHEA *vs.* eCG+DHEA+PGZ groups. NS: not significant.

Figure 6: Oxidative stress in serum. Levels of MDA (A) and GSH (B). Each column represents the mean values ± SEM. (N=5 replicates per group) *p<0.05 and **p<0.01, compared with the eCG group. $^{\#}p$<0.05 comparing eCG+DHEA *vs*. eCG+DHEA+PGZ groups. NS: not significant.









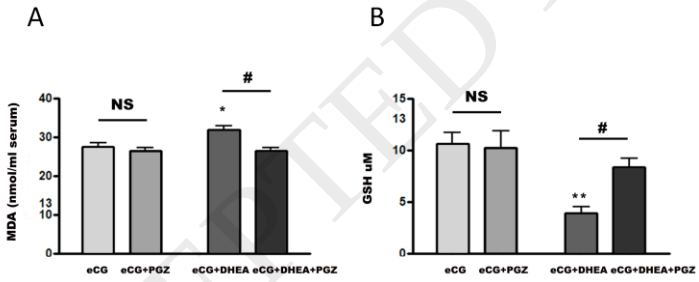


Table 1: List of primers used in qPCR

Gene	Primer Forward (5' – 3')	Primer Reverse (5' – 3')
Pparg	TTTTCAAGGGTGCCAGTTTC	GAGGCCAGCATGGTGTAGAT
Ncor	TATCGGAGCCATCTTCCCAC	ACTTGGGTATCCTGGGGTTG
Pgc1a	AATGCAGCGGTCTTAGCACT	GTGTGAGGAGGGTCATCGTT
Star	GCAGGGGGATTTCTGAATTT	GTCTCCGTCTCTGTGGCTTC
Cyp17a	TCTCATTACACCCACGCAGA	CGGGGCAGTTGTTTATCATC
3bhsd	GACACCCCTCACCAAAGCTA	TTGTAAAATGGACGCAGCAG
17bhsd	TCTCATTACACCCACGCAGA	CGGGGCAGTTGTTTATCATC
Cyp19a	CCTGGCAAGCACTCCTTATC	CCACGTCTCTCAGCGAAAAT
Tnfa	TCCCAGAAAAGCAAGCAACC	TAGACAGAAGAGCGTGGTGG
Cox2	ATGAGTACCGCAAACGCTTC	CCCCAAAGATAGCATCTGGA
Rpl32	TGGTCCACAATGTCAAGG	CAAAACAGGCACACAAGC