Prenatal hyperandrogenism and lipid profile during different age stages: an experimental study

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Objective: The present study investigates the effect of prenatal hyperandrogenization on lipid metabolism and oxidant/antioxidant balance.
Design: Experimental study.
Setting: Research institute.
Animal(s): Pregnant Sprague Dawley rats were subcutaneously injected with 2 mg free T between days 16 and 19 of pregnancy, and controls (C) received vehicle (0.1 mL of sesame oil). Prenatally hyperandrogenized female offspring (T2) had a condition that resembles polycystic ovary (PCO). Animals were weighed and killed at 21 and 60 days of age (N = 15 rats/group).
Intervention(s): Ovarian tissue and truncal blood were obtained from the C and T2 groups.
Main Outcome Measure(s): Circulating lipid profile (total cholesterol, high-density lipoprotein [HDL], low-density lipoprotein [LDL] cholesterol, and triglycerides) was quantified by colorimetric-enzymatic methods. Ovarian oxidative stress was evaluated by quantifying lipid peroxidation and glutathione content by spectofotometric assays. Ovarian fat content was evaluated by Red Oil staining and ovarian messenger RNA (mRNA) expression of peroxisome proliferator-activated receptor gamma (PPAR-γ) by real-time polymerase chain reaction (PCR).
Result(s): At 60 days of age, 100% of group C rats and 20% of group T2 rats ovulated. At 21 days of age the T2 rats displayed lower body weight than C rats; however, at 60 days of age T2 and C rats showed similar body weights. The lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides) was altered in the anovulatory and ovulatory phenotype of the T2 group, but the levels were higher in the anovulatory phenotype. Lipid peroxidation of rats at 21 and 60 days of age from T2 was similar to C but the antioxidant glutathione level was decreased in 21-day-old rats compared with C rats. The lipid content of ovarian tissue, determined by Red Oil staining, was higher in the T2 than in the C group. The mRNA expression of ovarian PPAR-γ, quantified by real time PCR, decreased in anovulatory rats at 60 days of age from T2 compared to C rats.
Conclusion(s): Our findings reveal the importance of evaluating the complete lipid profile, especially at early stages of life after the prenatal hyperandrogenism condition. In addition, we demonstrated that the antioxidant-reduced glutathione would represent a good marker of oxidative stress as it is altered before lipid peroxidation. Prenatal hyperandrogenization also alters the gene expression of PPAR-γ in rats. Here we demonstrated for the first time that abnormalities in PPAR-γ and lipid profile were higher in rats showing an anovulatory phenotype than those displaying an ovulatory phenotype. (Fertil Steril® 2013;99:551–7. ©2013 by American Society for Reproductive Medicine.)
Key Words: Prenatal hyperandrogenization, lipid profile, peroxisome proliferator-activated receptor gamma, ovarian oxidative stress

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Polycystic ovary syndrome (PCOS) is a common reproductive disorder that affects women in their reproductive ages (1). It is characterized by hyperandrogenism, ovarian cysts, and/or oligo-ovulation or anovulation (1) and is associated with insulin resistance, obesity, metabolic abnormalities, increased cardiovascular risk, and type 2 diabetes (2). Although several studies have been

carried out in human samples and animal models during the past decade, its etiology remains unknown. One of the current theories emphasizes that adverse intrauterine conditions coupled with genetic and/or environmental factors are related to the development of PCOS during the adult life (3–5). It has been reported that prenatal exposure to androgens reduces fetal growth (4–9) and may induce PCOS in the offspring (10, 11). Daughters of women with PCOS display PCOS-like antecedents as early as 2 months of age (12). In rhesus monkey dams and their offspring, Abbott et al. (13) demonstrated that fetal androgen excess may result in transient hyperglycemic episodes in the intrauterine environment, enough to induce relative increases in the pancreatic function of prenatally hyperandrogenized infants. In addition, fetal programming, associated with prenatal hyperandrogenism, is related to hyperinsulinemia, dyslipidemia, insulin resistance, cardiovascular disease (CVD), and the development of PCOS during adolescence or adult life (5, 14–16). Daughters of women with PCOS display higher prevalence of small-for-gestational age babies than those of healthy women (17). However, some studies do not agree with the theory that intrauterine environment is related to fetal development. In that context, Bjercke et al. (18) found no significant difference in the weight at birth of daughters of women with PCOS with or without insulin resistance. In addition, it has been described that pregnant women with and without PCOS show no difference in perinatal outcome (19–22). In addition, Legro et al. (23) demonstrated no association between weight at birth and reproductive and metabolic abnormalities in women with PCOS. In a previous study of the endocrine and metabolic disturbances during adult life induced by fetal programming, we found that the level of T prenatally administered in pregnant rats is directly related to the PCOS phenotype displayed in the offspring (24). We have also found that higher doses of T induce a more severe insulin resistance, a proinflammatory status in the ovarian tissue, and anovulatory estrous cycles (24). Considering that in patients with PCOS, an adverse growth in utero is related to enhanced CVD during both adolescence and adult life (25–28) and that a pro-oxidant status is related to central obesity, age, blood pressure, insulin resistance, and CVD (5, 12, 14, 29), we were interested in studying whether prenatal hyperandrogenization impacts on lipid metabolism and oxidative stress during both the prepubertal and the pubertal life.

The levels of such metabolites (e.g., glucose, fatty acids, and amino acids) and hormones (e.g., adiponectin, insulin leptin, and ghrelin) regulate fertility (30). Peroxisome proliferator-activated receptors (PPARs) are a family of transcriptional nuclear factors that regulate gene expression (31, 32). Particularly, the activation of PPAR-γ modulates the synthesis of steroid hormones in the granulosa cells (GC) (33), and the disruption in the ovary leads to female subfertility (34). We have reported that acute hyperandrogenization decreases the protein and gene expression of ovarian PPAR-γ and induces oxidative stress and apoptosis in ovaries from prepubertal rats (35). We also found that the protein expression of ovarian PPAR-γ of prenatally hyperandrogenized rats depends on the level of T injected (24). These data led us to investigate whether prenatal hyperandrogenism modulates ovarian gene PPAR-γ expression during the prepubertal and the pubertal life.

MATERIALS AND METHODS

Animals and Experimental Design

The animal model consisted of virgin female rats of the Sprague Dawley strain mated with fertile males of the same strain. Three females and one male were housed in each cage under controlled conditions of light (12 hours light, 12 hours dark) and temperature (23°–25°C). Animals received food and water ad libitum. Day 1 of pregnancy was defined as the morning in which spermatozoa were observed in the vaginal fluid. Between days 16 and 19 of pregnancy, rats were hyperandrogenized as described previously (36). Briefly, pregnant rats (N = 90) received SC injections of 2 mg of free T (T-1500; Sigma) dissolved in 100 μL of sesame oil from days 16–19 of pregnancy (T2 group). The doses of T used result in circulating T levels that are similar to those of male rats (37). The control group (N = 90) was SC injected with 100 μL of sesame oil. Under the conditions of our animal facilities, spontaneous term labor occurs on day 22 of gestation. Pups were culled from litters to equalize group sizes (10 pups per mother). Females were separated from males at 21 days of age and randomly chosen. Animals were allowed free access to Purina rat chow (Cooperación, Argentina) and water. All procedures involving animals were conducted in accordance with the Animal Care and Use Committee of Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. The Ethic Committee of the School of Medicine of University of Buenos Aires approved the present study. To study whether treatments altered the estrous cycle, it was determined by vaginal smears taken daily from ages 45–60 days. As in previous studies (24), when vaginal smears showed that rats stayed at diestrous, we considered that these animals belonged to the anovulatory phenotype, whereas ovulatory phenotype showed vaginal smears corresponding to different stages of the cycle.

To examine how prenatal hyperandrogenization alters lipid metabolism and/or the oxidant–antioxidant balance, offspring from animals of the control and T2 groups were weighed and then killed at 21 and 60 days of age. A total of 60 rats (15 rats per group, and a total of 4 groups: control at 21 days, T2 at 21 days, control at 60 days, and T2 at 60 days of age) were anesthetized with carbon dioxide and killed by decapitation. Trunk blood was collected and serum was separated by centrifugation at 1,000 $\times$ g for 15 minutes and stored at −80°C.

Prenatal Hyperandrogenization and Lipid Profile

To determine the systemic lipid profile, total cholesterol, high-density lipoprotein (HDL), and triglycerides were quantified by colorimetric-enzymatic methods (Weiner Lab). The chromophoric product was measured at 505 nm for cholesterol, at 600 nm for HDL, and at 490 nm for triglycerides. Low-density protein (LDL) cholesterol was estimated indirectly by the following formula: LDL = Total cholesterol−HDL + Triglycerides/5 (38).

Prenatal Hyperandrogenization and Ovarian Oxidative Stress

To assess the effect of prenatal hyperandrogenization in the ovarian oxidant–antioxidant balance, we next evaluated the lipid peroxidation index and the content of the antioxidant glutathione. The amount of malondialdehyde formed from the breakdown of polyunsaturated fatty acids is taken as an index of the peroxidation reaction. The method used (39) quantifies malondialdehyde as the product of lipid peroxidation that reacts with trichloracetic acid–thiobarbituric acid. Reduced glutathione (GSH) was quantified by the measurement of acid-soluble thiol, which reacts with Ellman's reagent (a sulfhydryl reagent, 5,5′-dithiobis-(2 nitrobenzoic acid); Sigma) (40).

Prenatal Hyperandrogenization and Ovarian Lipid Content

To evaluate the lipid accumulation in ovarian tissue, lipid droplets were visualized by the oil red-O staining. Ovarian tissue (five ovaries per group were randomly chosen) was immediately frozen and then, 4-μm step sections were mounted at 50-μm intervals onto microscope slides according to the method described by Woodruff et al. (41). Then oil red-O and hematoxylin staining quantification was carried out by using the Image Pro Plus software (Image Processing and Analysis in Java, www.imagej.nih.gov/ij/).

Prenatal Hyperandrogenization and Ovarian PPARγ Gene Expression

To determine whether prenatal hyperandrogenism alters the gene expression of PPAR-γ, messenger RNA (mRNA) levels were measured by real time polymerase chain reaction (PCR) analysis. Total mRNA of ovarian tissue from control and prenatal hyperandrogenized rats at 21 and 60 days of age was extracted using RNAzol RT (MRC gene, Molecular Research Center) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from mRNA by using specific primers (sense 5′-TTT TCA AGG GTG CCA GTT TC-3′; antisense 5′- GAG GCC AGC ATG GTG TAG AT-3′) and the ribosomal L32 gene as reference gene. The amplified products were quantified by fluorescence using the Rotor Gene 6000 Corbette (Corbette Life Science).

Statistical Analysis

Statistical analyses were carried out by using the Instant program (GraphPad software). Student's t test was used to compare two columns. Analysis of variance (ANOVA) followed by Newman-Keuls test were used to compare more than two columns. Statistical significance was considered as $P<.05$.

RESULTS

Prenatal Hyperandrogenization on Metabolism

The treatment with T did not modify the spontaneous term labor, the female-to-male offspring ratio, or the number of pups per litter. As found in our previous experiments, rats from the T2 group showed increased serum T levels, insulin resistance, and ovarian cysts compared with controls (24).

To assess whether prenatal hyperandrogenization affected fetal development, we determined the body weight at 21 and 60 days of age. We assume that prenatal hyperandrogenization induces an adverse intrauterine condition, as it diminished the body weight at 21 days of age (control, 40 ± 1 g; T2, 33 ± 2 g; $P<.01$) compared with controls. This adverse effect was compensate when animals were 60 days of age, as no significant differences between groups were found (control, 185 ± 16 g; T2, 188 ± 16 g).

Prenatal Hyperandrogenization and Estrous Cycle

With respect to the sexual cycle, we found that 98% ± 2% of the 60-day-old rats showed either regular or irregular estrous cycles (4–6 days). In contrast, only 20% ± 3% of rats from T2 showed regular or irregular estrous cycles. Vaginal smears from the rats from the T2 group, which did not ovulate, showed that these rats stayed at the diestrous stage.

Prenatal Hyperandrogenization and Lipid Profile

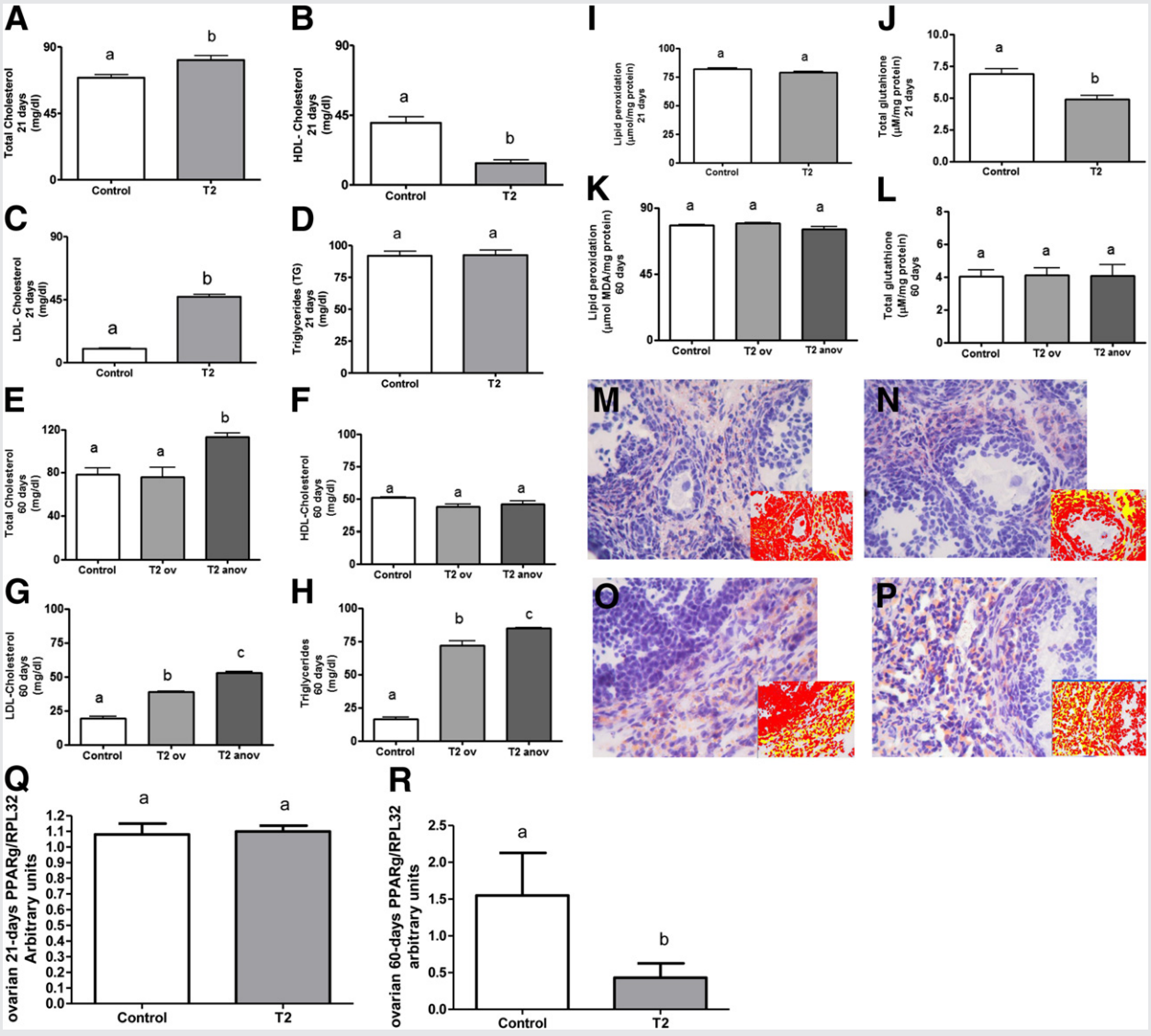
At 21 days of age, rats from the T2 group showed increased total cholesterol and LDL cholesterol levels (Fig. 1A; $P<.05$ and Fig. 1C; $P<.001$, respectively), decreased HDL cholesterol levels (Fig. 1B; $P<.0001$) and no changes in triglycerides (Fig. 1D) compared with controls. At 60 days of age, rats from the T2 group showed increased total cholesterol in the anovulatory T2 phenotype (Fig. 1E; $P<.001$) and no changes in HDL cholesterol compared with controls (Fig. 1F). Both the ovulatory and anovulatory T2 phenotype showed increased LDL cholesterol (Fig. 1G; $P<.001$) and triglycerides (Fig. 1H; $P<.001$) compared with controls. In addition, rats from the anovulatory T2 phenotype showed higher LDL cholesterol and triglyceride levels than those of the ovulatory T2 phenotype (Fig. 1G and H b vs. c; $P<.001$, respectively).

When analyzing lipid ratio as markers of atherogenic index, CVD, and obesity, we found that both the total cholesterol-to-HDL cholesterol and triglycerides-to-HDL cholesterol ratios were increased in the hyperandrogenized 60-day-old rats with respect to controls. In addition, the total cholesterol-to-HDL cholesterol ratio (marker of atherogenic index and CVD) was higher in the anovulatory (2.28 ± 0.09) than in the ovulatory (2.20 ± 0.04) hyperandrogenized rats with respect to controls (1.56 ± 0.18). We also found that the triglycerides-to-HDL cholesterol ratio (marker of obesity) was higher in the anovulatory (1.87 ± 0.04) than in the ovulatory (1.57 ± 0.17) hyperandrogenized rats with respect to controls (0.32 ± 0.19).

Prenatal Hyperandrogenization and Ovarian Oxidative Stress

We found that the amount of serum malondialdehyde from rats at 21 and 60 days of age from the T2 group was similar to that of controls (Fig. 1I and K, respectively). The GSH levels in rats from the T2 group at 21 days of age were decreased (Fig. 1J; $P<.001$), whereas those in rats at 60 days of age showed no significant differences compared with controls (Fig. 1L).

FIGURE 1



Lipid profile from rats in the control (C) and prenatally hyperandrogenized rats (T2) at different ages. Serum levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides corresponding to C and T2 rats at 21 days of age (A, B, C, D, respectively) and corresponding to C and T2 rats at 60 days of age (E, F, G, H, respectively). Each column represents the mean ± SEM from 10 different animals. a versus b, $P<.05$ (A); a versus b, $P<.001$ (B, C) by Student's *t* test; a versus b, $P<.001$ (E, G, H); b versus c, $P<.001$ (G, H) by analysis of variance (ANOVA) test. Ovarian oxidant–antioxidant balance of control and prenatally hyperandrogenized (T2) rats. (I) Lipid peroxidation and (J) total glutathione for rats at 21 days of birth. (K) Lipid peroxidation and (L) total glutathione for rats at 60 days of birth. Each column represents the mean ± SEM from 10 different animals. a versus b, $P<.001$ (J) by Student's *t* test. (K, L) Data analyzed by ANOVA test. A representative Oil Red-O (*red*) and hematoxylin staining of ovarian tissue from control (M) and T2 rats (N) at 21 days of birth and from control (O) and T2 rats (P) at 60 days of birth (×400). The cytoplasmic lipid droplets are shown in red. Each photo shows the detail of the corresponding quantification by Image Pro Plus software; in yellow cytoplasmic lipid droplets. Messenger RNA expression of the peroxisome proliferator-activated receptor gamma (PPAR-γ) in ovarian tissue from C and T2 rats at 21 days of birth (Q) and 60 days of birth (R); a versus b, $P<.001$ by Student's *t*-test. Graphs represent the amplified products quantified by fluorescence by real-time polymerase chain reaction (PCR). Each column represents the mean ± SEM from 10 different animals.

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Prenatal Hyperandrogenization and Ovarian Lipid Content

Lipid droplets were localized in the cytoplasm of stromal and theca cells (Fig. 1M–1P). Ovarian tissue from 21-day-old T2 rats displayed an increase of 39% in lipid content compared with controls (Fig. 1M and N), whereas ovarian tissue from 60-day-old T2 rats showed an increase of 83% in lipid content compared with controls (Fig. 1O and P).

Prenatal Hyperandrogenization and Ovarian PPAR-γ Gene Expression

Figure 1Q shows that PPAR-γ was not modified by prenatal hyperandrogenization of ovaries from 21-day-old rats but decreased in ovaries from 60-day-old rats showing the anovulatory phenotype (Fig. 1R; $P<.05$). Prenatal hyperandrogenization did not modify the gene expression of PPAR-γ from the ovulatory phenotype (data not shown).

DISCUSSION

Murine models have emerged as important tools to study PCOS. Particularly, for prenatal hyperandrogenism, rats are considered as one of the best animal models because they allow the study of fetal programming (12, 14, 24, 42, 43). In the present study, we demonstrated that prenatally hyperandrogenized rats displayed decreased body weight at 21 days of age but the body weight was similar to those of controls at 60 days of age. These data suggest that prenatal hyperandrogenism is correlated with an adverse intrauterine environment, which in turn, develops a compensatory growth mechanism. This finding is in agreement with previous reports (10, 11, 13, 24). In addition, it has been recently demonstrated that maternal metabolic disruption explains why offspring of hyperandrogenized rhesus monkey dams exhibit insulin resistance as a sequela of a transient hyperglycemic gestational environment, rather than of a fetal androgen excess per se (13, 44, 45).

It is well established that an adverse intrauterine environment has consequences during the adult life (5–16). However, there is still controversy on which are the altered parameters, and an early good marker has not been found (19–24). The strong association of dyslipidemia with PCOS, insulin resistance, and enhanced cardiovascular risk has led to the need to assess the lipid profile. Current diagnostic guidelines of cardiovascular risk of PCOS patients have turned from the presence of metabolic syndrome to the search for lipid markers (46). In that context, it has been recently described that in Mediterranean women with PCOS, the prevalence of metabolic syndrome is low, whereas other markers, such as increased LDL cholesterol levels are more prevalent (46). It has thus been concluded that a more comprehensive lipid evaluation is needed. In the present work, we unexpectedly found that prenatal hyperandrogenization alters the lipid profile of prepubertal rats even when the body weight is decreased with respect to controls. This finding, which is in agreement with that recently reported (47), demonstrates the importance of assessing the lipid profile in the population at risk, such as daughters of women with PCOS, infant girls small for their gestational age, and/or with low weight at birth. We also demonstrated that in hyperandrogenized rats that the lipid profile worsens with age and is stronger in the anovulatory than in the ovulatory phenotype. These data are related to the insulin resistance–lipid profile–PCOS phenotype link (48). The total cholesterol-to-HDL cholesterol ratio is associated with both the atherogenic index and CVD (49), whereas increased triglyceride levels and the triglyceride-to-HDL cholesterol ratio, associated with obesity, hyperglycemia, and insulin resistance, are considered markers for metabolic syndrome (50). Our results reveal that there are altered markers of atherogenic index and CVD both in the ovulatory and the anovulatory phenotypes, but that they are higher in the anovulatory than in the ovulatory phenotype. In addition, we demonstrated that the lipid content was also increased in ovarian tissue from prenatally hyperandrogenized rats.

The oxidative metabolism, and thus, the generation of reactive oxygen species is essential for physiological processes (e.g., cellular activations, second messengers). However, the overproduction of reactive oxygen species induces damage to molecules and structures. Oxidative stress is involved in the pathogenesis of insulin resistance and hyperandrogenism in PCOS (51–54), and it has been reported that women with PCOS present increased oxidative stress (29, 55) and/or decreased total antioxidant status (56). However, some studies have failed to identify a pro-oxidant status in women with PCOS (57, 58) and no differences in the levels of serum markers of oxidative stress are found when comparing nonobese infertile patients with PCOS and controls (59). Protection against oxidative stress in cells is provided by enzymes, metabolites (as GSH), or antioxidant vitamins, and it has been reported that the oxidant–antioxidant balance is endocrine regulated (39, 60). In the present study, we demonstrated that lipid peroxidation in prenatally hyperandrogenized rats at 21 days of age was not modified, whereas serum GSH levels were decreased compared with controls. These data suggest that the antioxidant GSH would be controlling lipid peroxidation and that this is why the lipid peroxidation index was not modified and GSH was decreased. These results are in agreement both with our previous findings regarding the fact that prenatal hyperandrogenization does not modify lipid peroxidation of ovarian tissues at the expense of the consumption of GSH (24) as well as with those reported in other tissues (61–65).

One of the main finding reported in this study is that prenatal hyperandrogenization decreases the gene expression of PPAR-γ in rats displaying the anovulatory phenotype with respect to controls and the ovulatory phenotype. These data point out the importance of the PPAR-γ system in the modulation of ovarian functions. In addition, our findings reveal the importance of evaluating the complete lipid profile, especially at early stages of life. In addition, we demonstrated that the antioxidant GSH would represent a good marker of oxidative stress as it is altered before lipid peroxidation. Further experiments are being designed to study whether prenatal hyperandrogenism alters metabolic and endocrine disturbances during the adult life.

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