Letrozole vs estradiol valerate induced PCOS in rats: glycemic, oxidative and inflammatory status assessment

Alexandra Dăneasă¹, Cristina Cucolaş¹, Lavinia Manuela Lenghel², Diana Olteanu¹, Remus Orăsan¹ and Gabriela A Filip¹

Departments of ¹Physiology and ²Radiology, "Iuliu Hațieganu" University of Medicine and Pharmacy, Clinicilor Street, No. 1, Cluj-Napoca 400006, Romania

Correspondence should be addressed to G A Filip; Email: adrianafilip33@yahoo.com

Abstract

The objective of our study was to investigate glycemic, oxidative/antioxidative and inflammatory status in letrozole and estradiol valerate induced polycystic ovarian syndrome (PCOS) models. Sixty adult female Wistar rats were divided into four groups: L (0.2 mg letrozole/0.5 ml carboxymethyl cellulose (CMC), daily for 30 days), the control group C_L, EV (one i.m. injection of 5 mg EV/0.5 ml sesame oil) and its corresponding control group C_{EV}. After 30 days, ovarian morphology was assessed through ultrasound, serum free testosterone was determined, and an oral glucose tolerance test was performed. Blood, muscle, liver and periovarian adipose tissue (POAT) were collected for oxidative/antioxidative and inflammatory status evaluation. Free testosterone was increased only in the L group, while fasting glycemia was higher in the EV group. Both L and EV led to a significantly decreased level of muscle malondialdehyde (MDA) and liver glutathione peroxidase (GPx) activity, while in POAT, MDA level diminished and GPx activity increased. The only difference between the two protocols was in muscle, where after L administration, GPx activity was significantly lower. Implementation of both protocols resulted in an increased expression of pNFkB in muscle, liver and POAT. The expression of monocyte chemoattractant protein 1 (MCP1) increased in liver and POAT after L administration, while in the EV group, MCP1 and STAT3 decreased in POAT. Our study shows that both protocols are characterized by an inflammatory environment in the usually insulin resistant tissues of human PCOS, without generating oxidative stress. In addition, EV has mild metabolic effects and unexpected interference with MCP1 expression in POAT, which require further investigation.

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Introduction

Polycystic ovarian syndrome (PCOS) is the most common cause of anovulatory infertility (Broekmans *et al.* 2006), affecting 6–20% of reproductive age women (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). For its diagnosis, two out of the following criteria must be met: oligoovulation/anovulation, clinical/biochemical signs of hyperandrogenism and polycystic ovaries (Azziz *et al.* 2009). The definition is far from being exhaustive, the clinical presentation of women with PCOS being extremely variable, involving frequently abnormalities of lipid and glucose metabolism. The alteration of glucose metabolism results in hyperglycemic states such as impaired fasting glycemia, impaired glucose tolerance or both (Karakas *et al.* 2010). Moreover, oxidative stress (Sabuncu *et al.* 2001) and low-grade inflammation (Duleba & Dokras 2012) are emerging as factors in the pathogenesis of the syndrome. Women with PCOS have an increased oxidative status which is related to central obesity, age, blood pressure, serum glucose, insulin and triglyceride levels, and insulin resistance, correlated with alteration of the antioxidant status (Sabuncu *et al.* 2001). In addition,

markers of oxidative stress and inflammation are correlated with androgen level (González 2012).

Since the etiology of PCOS remains unclear, the use of animal models has offered valuable insights into the pathophysiology of the syndrome. However, as the array of pathological modifications observed in human PCOS widens, it becomes clear that the existing animal models should be characterized also from an inflammatory and oxidative/antioxidative point of view. This may lead to a better understanding of the underlying mechanisms of PCOS, which is essential for shifting the therapy from an incomplete, symptomatic approach to a curative one.

PCOS experimental models have been developed on various animals, such as rodents (reviewed by Shi & Vine 2012, Walters *et al.* 2012, McNeilly & Colin Duncan 2013, Maliqueo *et al.* 2014), sheep (reviewed by Padmanabhan & Veiga-Lopez 2013) or rhesus monkeys (reviewed by Abbott *et al.* 2013). Rhesus monkeys and sheep models provide promising perspectives and obvious advantages related to their intrinsic physiological characteristics which resemble those of humans. However, the rodents are the most commonly used because they offer a practical way to study PCOS and they

are also cost-effective. Two widely used models for PCOS study, the ones induced by letrozole (L) and estradiol valerate (EV) respectively were concerned especially with morphological, endocrinological and/or metabolic aspects, while the study of oxidative stress and inflammation was not enough investigated. Letrozole is a third generation aromatase inhibitor that blocks the conversion of testosterone to estrogen, thus inducing hyperandrogenism. Estradiol valerate is a long-acting estrogen which acts by disturbing hypothalamic gonadotrophin-releasing hormone secretion, which in turns interferes with the secretion and storage of LH (Simard *et al.* 1987).

The aim of our study was to investigate glycemic, oxidative/antioxidative inflammatory status in the above described murine experimental models. These investigations included tissues involved in maintaining glucose homeostasis such as liver and muscle, with a special focus on the periovarian adipose tissue (POAT).

Materials and methods

Reactives

The Bradford protein assay kit, SDS-PAGE gels, PVDF membranes, SDS Laemli sample buffer, Tris-glycine buffers, nonfat dry milk, Tween 20 were purchased from Bio-Rad and kit for free testosterone from MyBioSource (San Diego, CA, USA). Antibodies against phosphorylated Ser 311 of nuclear factor kappa-light-chain-enhancer 88 of activated B cells p65 subunit (pNFKB), NFKB p65, secondary corresponding HRP linked antibodies, anti-human TRP1 antibody-conjugated with Alexa Fluor 488, were from Santa Cruz Biotechnology. Monocyte chemoattractant protein 1 (MCP1) antibody was purchased from Linaris Biologische Produkte (Mannheim, Germany), and anti-GAPDH from Trevigen-Biotechnology (Gaithersburg, MD, USA). Letrozole and estradiol valerate were purchased from Sigma-Aldrich Chemicals GmbH.

Animals

Sixty female Wistar rats, 16–18 weeks of age, weighing 170–200 g, were obtained from the Animal Department of 'Iuliu Hațieganu' University of Medicine and Pharmacy, Cluj-Napoca, Romania. For acclimatization, the female rats were housed in the Physiology Department's Biobase a week before and throughout the experiment at a controlled temperature of 22–24 °C, having a 12 h light:12 h darkness cycle. The rats had access to pellet food and tap water *ad libitum*. All experiments were performed according to the approved animal care protocols of the Ethics Committee on Animal Welfare of the 'Iuliu Hațieganu' University in accordance with European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Council of Europe no. 123, Strasbourg 1985.

Experimental design

Letrozole protocol

Thirty female Wistar rats were divided in two groups: the rats in the control group C_L ($n=8$) received 0.5 ml carboxymethyl

cellulose (CMC) 0.5%, while the rats in the L group ($n=22$) received 0.2 mg letrozole dissolved in 0.5 ml CMC 0.5%. Administration of the substances was done daily, for 30 days through gavage.

Estradiol valerate protocol

Thirty female Wistar rats were divided into two groups: the rats in the control group C_{EV} ($n=8$) received a single i.m. injection of 0.5 ml sesame oil, while the rats in the EV group ($n=22$) received a single i.m. injection of 5 mg EV dissolved in 0.5 ml sesame oil.

The doses were chosen based on previous studies in which letrozole or estradiol valerate was used to induce PCOS (Stener-Victorin *et al.* 2000, Kafali *et al.* 2004). At the end of the experiment (days 30–32), under anesthesia with 90 mg/kg ketamine and 10 mg/kg xylazine injected intraperitoneally, the ovarian morphology was assessed through ultrasound; an oral glucose tolerance test (OGTT) was performed; the rats were sacrificed and blood, liver, muscle, and POAT were taken for analysis.

Ultrasound evaluation

At the end of the experiment, an ultrasound examination was performed in the L and EV groups in order to locate and evaluate the morphologic aspect of the ovaries. We used the Ultrasonix Sono Touch Series ultrasound diagnostic system with a linear transducer, 1.5 cm aperture, wide frequency selection range (8–40 MHz), 40 MHz operating frequency, depth of focus <1 cm, spatial resolution <0.1 mm. Transversal sections were performed in order to localize and measure the width and length of every ovary and also to measure the maximum size of every endoovarian fluid formation observed.

Free testosterone assay

Serum free testosterone level was determined by using an ELISA kit purchased from MyBioSource. The minimum rat detectable free testosterone was 0.05 ng/ml. The assay was performed according to the manufacturer's instructions.

OGTT protocol

OGTT test was performed on all rats using an Accu Chek Active glucometer (Roche Diagnostics Ltd). The rats were fasted overnight for 12 h before the fasting glycemia was measured by using blood samples from the tail vein. Each rat received per os (orally or through gavage) 0.5 ml solution containing 400 mg glucose. After 2 h, the glycemia was again assessed.

Oxidative/antioxidative status assessment

Muscle, liver and POAT preparation for oxidative stress parameters determination

Liver and muscle tissue samples were homogenized with a Polytron homogenizer (Brinkman Kinematica, Lucern, Switzerland) for 3 min on ice in phosphate buffered saline (pH 7.4) added at a ratio of 1:4 (w/v). The suspension was centrifuged for 5 min at 3000 g and 4 °C in order to prepare the cytosolic fraction.

POAT was homogenized using a Polytron homogenizer (Brinkman Kinematica, Switzerland). Minced tissue was mixed with a HNTG lysis buffer solution which contained 5.96 g HEPES (50 mM); 4.38 g NaCl (150 mM); 50 ml glycerol (10%); 5 ml triton-X-100 (1%). The homogenate was transferred in 2 ml Eppendorf tubes and was rotated at 4 °C for 30 min. The obtained lysate was centrifuged at 18 000 *g* at 4 °C for 30 min, resulting three layers. For analysis, only the middle layer was used. Protein concentration was determined using the Bradford assay (Noble & Bailey 2009).

Oxidative stress assay

Oxidative stress analysis included the determination of malondialdehyde (MDA), reduced/oxidized glutathione ratio (GSH/GSSG) and glutathione peroxidase (GPx) activity in muscle, liver and POAT. In serum, MDA and GSH/GSSG were also determined.

MDA, the marker most frequently used for lipid peroxidation, was determined by the fluorimetric method with 2-thiobarbituric acid (TBA) as previously described (Conti *et al.* 1991). Tissue homogenates were heated in a boiling water bath for 1 h in 75 mM K₂HPO₄, pH=3, containing 10 mM TBA. After cooling, the solution was extracted with 3 ml *n*-butanol in 0.6 ml TBA. MDA was determined spectrofluorimetrically in the organic phase using a synchronous technique with excitation at 534 nm and emission at 548 nm. MDA is reported as nmol/mg protein.

GSH and GSSG were measured fluorimetrically using *O*-phtalaldehyde (Hu 1994). Samples were treated with trichloroacetic acid (10%) and centrifuged. A solution of *O*-phtalaldehyde (1 mg/ml in methanol) was added to supernatants diluted with sodium phosphate buffer 0.1 M/EDTA 5 mM, pH 8.0. After 15 min, the fluorescence was recorded (350 nm excitation and 420 nm emission). GSH and GSSG concentrations were determined using a standard curve and expressed as nmoles/mg protein in tissue homogenate, and as nmoles/ml in serum.

GPx activity was determined with Flohe & Gunzler (1984) method, with some modifications. The reaction mixture consisting 1 mM GSH, 0.24 U/ml glutathione reductase and 0.15 mM NADPH (final concentrations) in 50 mM phosphate buffer (pH 7.0) was incubated at 37 °C for 5 min with the appropriate amounts of tissue homogenates. The assay was initiated with a 12 mM *t*-butyl hydroperoxide solution. The decrease in absorbance at 340 nm was recorded for 3 min. GPx activity was expressed as U/g protein in muscle and liver, and as U/mg protein in POAT.

Inflammatory markers assessment

Muscle, liver and POAT preparation for WB

Samples of snap-frozen tissue samples were homogenized in lysis buffer containing Igepal-nonidet 1% (Sigma-Aldrich Chemicals GmbH), 1% protease inhibitor complex (Sigma-Aldrich Chemicals GmbH) in PBS for 1 h, on ice. Cell extracts were spun at 14 000 *g* for 30 min at 4 °C. Supernatant was collected and 50 µl were used to determine the protein content by the Bradford method. Lysates were mixed 1:2 (v/v) with

Laemli sample buffer (Bio-Rad) containing 2-mercaptoethanol and the proteins were denaturized at 95 °C for 10 min. The protein concentration in each sample was 4 mg/ml.

Western blot analysis of NFKB, pNFKB and MCP1

Lysates (20 µg protein/lane) were separated by electrophoresis. Samples were separated by 12% SDS-PAGE gels and transferred to PVDF membranes using Bio-Rad Mini-protein system (Bio-Rad). Blots were blocked for 1 h at room temperature and then were incubated with the primary antibodies against NFKB, pNFKB, MCP1 and GAPDH 1:200, overnight at 4 °C. After washing with PBS-T, the blots were incubated with specific secondary antibodies for 90 min at room temperature. The proteins were visualized and detected using Supersignal West Femto Chemiluminiscent substrate (Thermo Fisher Scientific, Rockford, IL, USA), and were quantified using Image Lab analysis software (Bio-Rad); GAPDH was used as protein loading control.

ELISA analysis

TNFα and STAT3 were determined in the POAT, which was prepared as was described for the WB analysis. The TNFα and STAT3 immunoassay kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA) and from Life Technologies respectively and were used according to manufacturer's instructions.

For the ELISA analysis (free testosterone, TNFα and STAT3) and for the investigation of OS parameters the number of analyzed samples was determined by using the 'resource equation' (Mead 1990), while the WB analysis was performed in triplicate.

Statistical analysis

Obtained data were analyzed with GraphPad Prism Software, version 5.0 (San Diego, CA, USA) by using unpaired *t*-tests. D'Agostino & Pearson omnibus test was used to check the normality of data. All values in text and figures were expressed as mean ± s.d., with a limit of statistical significance of *P* < 0.05.

Results

Ultrasound evaluation

At the end of the experiment, ultrasound assessment of the ovaries was performed in the L and EV groups. In the L group, mean ovarian dimensions (length/width) were 4.68 ± 0.39/2.85 ± 0.56 mm and no cystic modifications were observed (Fig. 1A). In the EV group, the mean ovarian dimensions were 2.63 ± 0.34/1.68 ± 0.36 mm with transonic images of 0.73/1.51 mm in average (Fig. 1B). Both length and width of the ovaries were higher in the L group as compared with EV group (*P* < 0.001 and *P* < 0.01 respectively).

Figure 1 Ultrasound assessment of the ovaries at the end of the experiment. In the L group increased dimensions of the ovaries were observed (A), while in the EV group transonic images were encountered (B).

Free testosterone assay

Serum free testosterone was significantly higher in the L group as compared with C_L (1.48 ± 0.38 ng/ml vs 0.58 ± 0.03 ng/ml, $P < 0.05$), while after EV administration, free testosterone level was not significantly modified in the EV group as compared with C_{EV} (0.85 ± 0.15 ng/ml vs 0.98 ± 0.25 ng/ml, $P > 0.05$) (Fig. 2A).

Oral glucose tolerance test

In the L experiment, there were no significant changes between groups, neither regarding fasting glycemia (65.75 ± 11.76 mg/dl in the L group compared with 71.50 ± 10.20 mg/dl in the C_L group; $P > 0.05$) nor 2-h glycemia (97.57 ± 6.53 mg/dl after L administration compared with 102.6 ± 15.6 mg/dl in the corresponding control group; $P > 0.05$) (Fig. 2B). Fasting glycemia was significantly increased in the EV group as compared with C_{EV} (105.2 ± 9.13 mg/dl vs 75.75 ± 8.73 mg/dl, $P < 0.001$), but no significant difference was observed regarding 2-h glycemia (99.73 ± 18.32 mg/dl compared with 110.4 ± 22.09 mg/dl in the C_{EV} group; $P > 0.05$, Fig. 2C).

Oxidative/antioxidative status assessment

For the oxidative/antioxidative status assessment MDA level, GSH/GSSG ratio and GPx activity were evaluated

in muscle, liver and in POAT. In addition, MDA level and GSH/GSSG ratio were assessed in serum.

In muscle, MDA level was 0.20 ± 0.03 nmol/mg protein in the L group and 0.26 ± 0.03 nmol/mg protein in the C_L group ($P < 0.05$, Fig. 3A) and 0.17 ± 0.02 nmol/mg protein in the EV group and 0.30 ± 0.05 nmol/mg protein in the C_{EV} group ($P < 0.001$, Fig. 3A). The GSH/GSSG ratio was not significantly modified by either L or EV administration (Fig. 3B). GPx activity was significantly decreased in the L group as compared with C_L (28.08 ± 3.12 U/g protein vs 44.8 ± 9.83 U/g protein, $P < 0.05$, Fig. 3C).

In liver, MDA level and GSH/GSSG ratio were not significantly modified by either L or EV administration (Fig. 4A and B). The activity of GPx was significantly diminished in the L group as compared with C_L (161.4 ± 16.46 U/g protein vs 188.3 ± 3.87 U/g protein, $P < 0.05$, Fig. 4C) and in the EV group as compared with C_{EV} (190.4 ± 11.33 U/g protein vs 309.2 ± 22.38 U/g protein, $P < 0.001$, Fig. 4C).

In POAT, MDA level was significantly decreased in the L group as compared with C_L (1.05 ± 0.16 nmol/mg protein vs 2.04 ± 0.59 nmol/mg protein, $P < 0.01$, Fig. 5A) and in the EV group as compared with C_{EV} (0.77 ± 0.3 nmol/mg protein vs 1.46 ± 0.56 nmol/mg protein, $P < 0.05$, Fig. 5A). The GSH/GSSG ratio was not significantly modified in either experiments (Fig. 5B), while the activity of GPx enhanced in the L group as compared with C_L (28.94 ± 3.3 U/mg protein vs 21.68 ± 2.05 U/mg protein, $P < 0.01$, Fig. 5C) and in the EV group as compared with C_{EV} (31.16 ± 6.33 U/mg protein vs 23.5 ± 3.95 U/mg protein, $P < 0.05$, Fig. 5C).

In serum, neither MDA level nor GSH/GSSG ratio was significantly modified (Fig. 6A and B). In the L group was observed a decreasing tendency of GSH/GSSG ratio as compared with C_L ($P = 0.0509$, Fig. 6B).

Inflammatory markers assessment

This evaluation included the determination of NFkB, pNFkB and MCP1 expression in muscle, liver and POAT. Moreover, in POAT, the levels of TNF α and STAT3 were quantified.

Figure 2 Serum free testosterone level and OGTT results at the end of the experiment. (A) The serum free testosterone level was increased in the L group as compared with C_L ($*P < 0.05$), while no significant changes were noted in the EV experiment. (B) In the L experiment there were no significant differences regarding neither fasting glycemia (G0h) nor 2-h glycemia (G2h). (C) In the EV experiment G0h was significantly increased in the EV group as compared with the C_{EV} ($***P < 0.001$).

Figure 3 Oxidative stress parameters determined in muscle. (A) The level of MDA decreased in the L group as compared with C_L (* $P < 0.05$) and in the EV group as compared with C_{EV} (** $P < 0.001$). (B) The GSH/GSSG ratio was not significantly modified. (C) The GPx activity decreased in the L group as compared with C_L (* $P < 0.05$) and was not significantly modified in the EV group as compared with C_{EV} .

In muscle, the expression of pNFKB increased significantly in both L (0.52 ± 0.008 pNFKB/GAPDH) and EV (0.39 ± 0.01 pNFKB/GAPDH) groups as compared with their corresponding controls: 0.33 ± 0.02 pNFKB/GAPDH in C_L , and 0.23 ± 0.01 pNFKB/GAPDH in C_{EV} respectively (both $P < 0.001$, Fig. 7C). NFKB and MCP1 expression was not significantly modified (Fig. 7B and D).

In liver, pNFKB expression was significantly higher in the L group (0.68 ± 0.02 pNFKB/GAPDH) as compared with C_L (0.21 ± 0.01 pNFKB/GAPDH, $P < 0.001$, Fig. 8C). Likewise, the expression of MCP1 increased in the L group (1.23 ± 0.11 MCP1/GAPDH) as compared with control C_L (0.8 ± 0.04 MCP1/GAPDH, $P < 0.05$, Fig. 8D). In the EV group, the expression of NFKB (0.46 ± 0.02 NFKB/GAPDH) diminished significantly as compared with C_{EV} (0.60 ± 0.01 NFKB/GAPDH, $P < 0.05$, Fig. 8B), while pNFKB increased at statistically significant levels (0.36 ± 0.01 pNFKB/GAPDH vs 0.1 ± 0.01 pNFKB/GAPDH in C_{EV} group, $P < 0.001$, Fig. 8C).

In the POAT, the expression of NFKB was higher in the L group (1.22 ± 0.06 NFKB/GAPDH) as compared with C_L (0.57 ± 0.12 NFKB/GAPDH, $P < 0.01$, Fig. 9B). A significant increase was also observed regarding pNFKB expression: 2.83 ± 0.11 pNFKB/GAPDH in the L group vs 1.99 ± 0.09 pNFKB/GAPDH in the C_L group ($P < 0.001$, Fig. 9C) and MCP1 expression: 0.89 ± 0.01 MCP1/GAPDH in the L group as compared with 0.62 ± 0.01 MCP1/GAPDH in the C_L group ($P < 0.001$, Fig. 9D). In the EV group, the levels of NFKB (1.1 ± 0.05 NFKB/GAPDH) was higher as compared with C_{EV} (0.6 ± 0.03 NFKB/GAPDH, $P < 0.001$, Fig. 9B). A similar increase was observed regarding pNFKB level: 1.18 ± 0.1 pNFKB/GAPDH in the EV group vs 0.68 ± 0.04 pNFKB/GAPDH in the C_{EV} group ($P < 0.01$, Fig. 9C). The level of MCP1 was significantly lower in the EV group (2.16 ± 0.16 MCP1/GAPDH) as compared with C_{EV} (2.82 ± 0.22

MCP1/GAPDH, $P < 0.05$, Fig. 9D). TNF α secretion was not significantly modified in either L or EV group (Fig. 10A), while STAT3 level was lower in the EV group (4.82 ± 1.02 U/mg protein) as compared with C_{EV} (11.03 ± 1.92 U/mg protein, $P < 0.01$, Fig. 10B).

Discussion

Since PCOS is associated with glucose metabolism abnormalities and the role of inflammation and oxidative stress emerge as factors in the pathogenesis of the syndrome, our study adds new information to the characteristics of the letrozole and estradiol valerate induced PCOS in rats.

As usually observed in these murine models (reviewed by Maliqueo *et al.* 2014b), the free testosterone level was increased after letrozole administration, but was not significantly modified in the EV group at the end of the experiment. Our results show that administration of L or EV has different effects on the glucose metabolism. Both protocols caused inflammation in the examined tissues, but influenced differently the expression of the pro-inflammatory cytokine MCP1. However, neither L nor EV has generated oxidative stress. In addition, the ultrasound assessment of ovaries revealed increased dimensions of the ovaries in the L group as compared with the EV group, result which is comparable with other histopathological findings (Lim *et al.* 2011). Also, transonic images were visualized after EV administration, in agreement with our previous ultrasonographic and histopathological results (Dăneasă *et al.* 2014).

Glucose metabolism parameters should be systematically evaluated in PCOS models since they are involved in the long-term complications of the syndrome. While L had no significant effect from this perspective, in the EV group we observed an increased level of fasting glycemia. This result was consistent with

Figure 4 Oxidative stress parameters determined in liver. The level of MDA (A) and the GSH/GSSG ratio (B) were not significantly modified. (C) The GPx activity diminished significantly in the L group as compared with C_L (* $P < 0.05$) and in the EV group as compared with C_{EV} (** $P < 0.001$).

Figure 5 Oxidative stress parameters determined in POAT. (A) The level of MDA was significantly decreased in the L group as compared with C_L (** $P < 0.01$) and in the EV group as compared with C_{EV} (* $P < 0.05$). (B) The GSH/GSSG ratio was not significantly modified. (C) GPx activity was significantly increased in the L group as compared with C_L (** $P < 0.01$) and in the EV group as compared with C_{EV} (* $P < 0.05$).

our previous study (Dăneasă *et al.* 2014), but contrary to the results obtained in literature (Stener-Victorin *et al.* 2005, Dikmen *et al.* 2012). Slightly elevated glycemic levels are able to promote mitochondrial reactive oxygen species (ROS) production (Nishikawa *et al.* 2000). A drawback of our study was that the blood and the tissues were not collected in a fasting state, thus the full extent of oxidative/antioxidative modifications could not be quantified. This would have been particularly important in the EV group, which presented increased levels of fasting glycemia.

However, an unexpected finding was that neither protocol generated oxidative stress. On the contrary, the levels of MDA, marker of ROS attack on membrane lipids, were lower in muscle and POAT or were not significantly modified in liver and serum. Dikmen *et al.* investigated glycemic and oxidative/antioxidative status in an EV-induced PCOS rat model and found similar results in serum. The authors hypothesize that this was probably due either to compensatory mechanisms activated by the oxidative stimuli or to the antioxidative role of sesame oil used as vehicle (Dikmen *et al.* 2012). However, in our study, in the L group in which CMC was used as vehicle, the MDA level was also lower, so the first hypothesis is more plausible.

Based only on the oxidative stress parameters evaluated in this study, we cannot establish a link with the inflammatory environment observed in muscle, liver and POAT. Both L and EV led to an increase expression of pNFKB at these sites, which are usually involved in insulin resistance in human PCOS. NFKB controls the synthesis of cytokines involved in inflammation and can be activated through phosphorylation by various stimuli, among which ROS, hyperglycemia and

hyperandrogenism. This results in a subsequent increase in pro-inflammatory cytokine production, such as TNF α , which mediates insulin-resistance (González *et al.* 2006, 2012). Indeed, in our study the level of TNF α was increased in the POAT after administration of both L and EV, but not sufficiently to be considered statistically significant. Another study confirms the increased mRNA expression of TNF α in the parametrial adipose tissue of a transgenic PCOS mouse model induced by letrozole (Kauffman *et al.* 2015). TNF α stimulates the hyperplasia of androgen producing theca cells which promotes hyperandrogenism (Spaczynski *et al.* 1999). Also, in the long run, inflammation can interfere with insulin signaling, which is an important step in the development of metabolic disturbances. Thus, the pro-inflammatory molecules became targets for various therapeutical interventions. Concomitant administration of carvedilol or semelil (Angipars) with letrozole decreased the level of inflammatory biomarkers, among which TNF α ; OS markers were diminished, while the antioxidant capacity was preserved. Moreover, the range of beneficial effects extended to ovarian morphology and physiology (Rezvanfar *et al.* 2015).

Figure 6 Oxidative stress parameters determined in serum. The MDA level (A) and the GSH/GSSG ratio (B) were not significantly modified.

Figure 7 Inflammatory parameters determined in muscle. (A) Western blot analysis of NFKB, pNFKB and MCP1, with GAPDH as loading control protein. The expression of NFKB (B) was not significantly modified while the active form pNFKB (C) increased significantly in L group as compared with C_L (*** $P < 0.001$) and in the EV group as compared with C_{EV} (*** $P < 0.001$). The expression of MCP1 (D) was not significantly changed.

Figure 8 Inflammatory parameters determined in liver. (A) Western blot analysis of NFKB, pNFKB and MCP1, with GAPDH as loading control protein. The expression of NFKB (B) was not significantly modified in the L group as compared with C_L and was diminished in the EV group as compared with C_{EV} (* $P < 0.05$). The expression of pNFKB (C) was significantly increased in L group as compared with C_L (** $P < 0.001$) and in the EV group as compared with C_{EV} (** $P < 0.001$). MCP1 (D) expression was augmented in the L group as compared with C_L (* $P < 0.05$) and was not significantly changed in the EV group as compared with C_{EV}.

The transcription of pro-inflammatory cytokine MCP1 is also regulated through NFKB pathway. MCP1 is involved in atherogenesis, thus in the long-term complications of PCOS (Niu & Kolattukudy 2009). In our study, the subsequent step following higher levels of pNFKB would have been a parallel increase of MCP1. However, this was not observed in all the cases, suggesting that MCP1 expression is not exclusively dependent on NFKB pathway and that it is tissue specific. The increased expression of MCP1 in POAT after letrozole administration is consistent with the results obtained

Figure 9 Inflammatory parameters determined in POAT. (A) Western blot analysis of NFKB, pNFKB and MCP1, with GAPDH as loading control protein. The expression of NFKB (B) increased significantly in L group as compared with C_L (** $P < 0.01$) and in the EV group as compared with C_{EV} (** $P < 0.001$). The expression of pNFKB (C) increased significantly in L group as compared with C_L (** $P < 0.001$) and in the EV group as compared with C_{EV} (** $P < 0.01$). The expression of MCP1 (D) enhanced in the L group as compared with C_L (** $P < 0.001$) and significantly decreased in the EV group as compared with C_{EV} (* $P < 0.05$).

in the parametrial adipose tissue in another study (Kauffman *et al.* 2015), but our findings in the POAT after EV administration raised some questions: Why MCP1 was significantly decreased, even though the expressions of NFKB and pNFKB were elevated? Was it possible that EV administration as part of the induction protocol to interfere with MCP1 release in POAT? Some studies revealed that estradiol administration slowed down wound healing process in parallel with the decrease of local MCP1 (Plackett *et al.* 2015). By contrast, its administration amplified the inflammatory cytokine production in temporomandibular joint, but in this second study, the expression of MCP1 was not determined (Kou *et al.* 2011).

If indeed EV administration had anti-inflammatory effects, why NFKB and pNFKB were not subsequently decreased? Which other factors are involved in the expression of MCP1 in the POAT following estradiol administration? It is known that STAT3 is both an important transcription factor that controls adipose tissue (Richard & Stephens 2014) and reproductive functions (Robker *et al.* 2014). Also, its interaction with the NFKB inflammatory pathway was documented (Sarközi *et al.* 2015). Therefore STAT3 was analyzed in POAT and the same descending trend was observed, which could indicate a possible role in MCP1 release at this site. Because adipose tissue is a highly active metabolic organ, the study should be extended to other possible candidates known for their interaction with NFKB such as Sp1 (Ping *et al.* 2000) or AP1 (Fujioka *et al.* 2004). Jun N-terminal kinase (JNK), which is known as 'Stress Activated Protein Kinase', is an important component of AP1 transcription factor. The ovarian alterations observed in the EV-induced PCOS rat model were reduced after the inhibition of JNK. The obtained results were assumed to be a consequence of the diminished inflammatory response and fibrosis observed in the treated group (Bulut *et al.* 2015).

As a conclusion, we observed that both protocols induced an inflammatory environment in the tissues involved in insulin resistance, without generating OS. However, we believe that the EV-induced PCOS, which is used primarily to investigate ovarian morphology, can be appropriate for the study of early glucose metabolism

Figure 10 TNF α and STAT3 levels in POAT determined through ELISA. (A) The level of TNF α was not significantly modified. (B) STAT3 level was not significantly modified in the L group as compared with C_L and decreased significantly in the EV group as compared with C_{EV} (** $P < 0.01$).

modifications encountered in PCOS. Moreover, the intriguing results found in the POAT raised new questions about the intricate pathways involved in inflammatory cytokines expression, which need further exploration. Last but not least, EV-induced PCOS protocol is easier and more accessible to implement.

Critical analysis of various animal models available has had an indisputable value in the current understanding of PCOS. Deciphering the mechanisms involved in its pathogenesis is important since it is the most frequent cause of anovulatory infertility. Moreover, PCOS is more than a reproductive disorder and the long-term cardiovascular and metabolic risks should be seriously taken into consideration. Inflammation and oxidative stress are relatively new aspects observed in PCOS, thus is important to investigate these models from this perspective, with the purpose of highlighting pathogenetic mechanisms, which ultimately could lead to the development of curative treatments.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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