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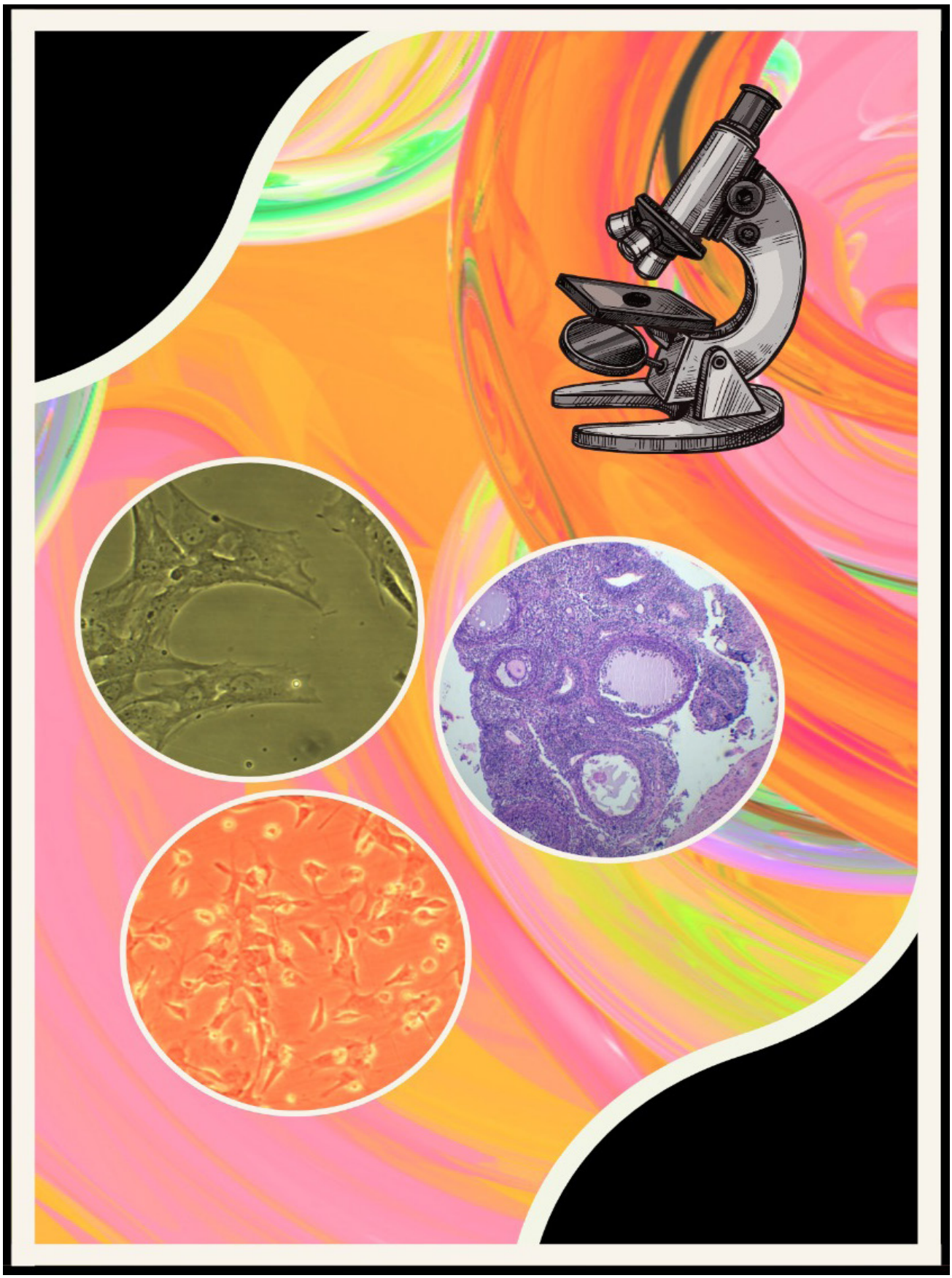

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Simultaneous alterations in ovaries and bone as a result of polycystic ovary syndrome

Bonfá, ALO.[1]; Alves, ED[1]; Fabrício, V[2,3]; Nonaka, KO[3]; Anselmo-Franci, JA[4]; Achcar, JA[1]; Montrezor, LH[1*]

**Corresponding author: E-mail address: lhmontrezor@uniara.edu.br*

Abstract: Polycystic ovary syndrome (PCOS) is one of the most widely recognized endocrine disorders affecting reproductive-age women. The etiopathogenesis and mechanisms of this syndrome remain unclear. Diagnosis requires two of the following: polycystic ovaries, oligo- or anovulation, and hyperandrogenism. Most women with PCOS display conditions such as metabolic abnormalities, diabetes, obesity, cardiovascular disease, and/or bone dysfunction. Considering the ethical limitations of human studies, animal and cell culture models that reflect some features of PCOS are important for investigation of this syndrome. The aim of the present work was to study some of the endocrine relationships between ovaries and bone tissue in a polycystic ovary syndrome animal model. The study was performed using an estradiol valerate PCOS-induced rat model (n = 30) and bone mesenchymal stem cell cultured from bone marrow of those animals. It was hypothesized that changes of the endocrine relationship between ovaries and bones could be observed in from in vivo animal model and in vitro cell culture assays. The ovarian morphological and endocrine changes seem to be correlated with endocrine, biophysical, and biomechanical changes in bone properties. Mesenchymal stem cells obtained from PCOS-induced rats, cultured for up to 21 days and differentiated into osteoblasts, presented lower viability and reduced mineralization of the extracellular matrix. Taken together, these results indicate important endocrine and structural effects of PCOS in ovaries and bones, contributing to part of the understanding of the pathophysiological mechanisms of PCOS.

Keywords: Polycystic Ovary Syndrome. Animal Model. Cell Culture. Bone. Osteocalcin.

Introduction

Polycystic ovary syndrome (PCOS) is one of the commonest endocrine disorders in women of reproductive age, with worldwide prevalence from 2% to 27%, depending on the diagnostic criteria used[1-6]. Diagnosis of PCOS based on the Rotterdam Consensus criteria requires two of the following: polycystic ovaries, hyperandrogenism, and oligomenorrhea or amenorrhea. This reproductive endocrinopathy is associated with hyperandrogenism, hirsutism, polycystic or polyfollicular ovaries, oligo/anovulation, infertility, luteinizing hormone (LH) hypersecretion, increased levels of inflammatory markers, obesity, dyslipidemia, insulin resistance (IR), type 2 diabetes, metabolic syndrome, and bone abnormalities[5,7-16].

Androgen excess is a complex reproductive disorder also associated with IR, obesity, hyperlipidemia, hypertension, endothelial dysfunctions, and bone dysfunctions[17-20]. Androgen insufficiency and androgen excess both cause ovarian dysfunction. Lack of androgen in the ovary, specifically in granulosa cells, leads to ovarian insufficiency, while excess androgen is linked to PCOS. Excess androgen appears to be both a cause and a consequence of PCOS, in a vicious cycle[21]. Androgen receptors (AR) are expressed in all the bone cells, including osteoblasts, osteoclasts, and osteocytes[22], indicating the direct influence of androgen on bone. Bone mineral density (BMD) is higher in PCOS amenorrheic women than in non-PCOS amenorrheic women, while hyperandrogenic women with regular menses have been shown to have significantly higher BMD than either amenorrheic hyperandrogenic women or controls[23]. The excess of androgen in PCOS subjects affects the bone mass directly, as well as by the involvement of various other factors, such as insulin, glucose, and cytokines[19].

Assessment of the etiology of PCOS and evaluation of the long-term risks of PCOS, in relation to bone development and metabolic and reproductive diseases, requires the development of suitable animal models[24]. Information is limited concerning methods for establishing animal models of PCOS, although several animal models have been developed and studied for the human PCOS[24-29]. However, the etiology of PCOS is still unclear, due to its complex manifestation as a syndrome and the limitations of translational studies using animals.

In order to understand some of the endocrine relationships between ovaries and bone tissue in a polycystic ovary syndrome situation, an animal model with PCOS induced with estradiol valerate was used, considering a period of 60 days post-induction. In addition, bone mesenchymal stem cells were cultured for up to 21 days, with differentiation into osteoblasts, in order to evaluate whether endocrine effects *in vivo* could compromise cell viability and mineralization of the extracellular matrix.

MATERIAL AND METHODS

IN VIVO EXPERIMENTS

Animals

Thirty adult female Wistar rats (6-months old at the end of the experiments) were used. The animals were weighed at the beginning of the experiment (204.2 ± 11.04 g), housed in standard boxes (n = 5), and kept at the University of Araraquara (UNIARA, Araraquara, São Paulo State, Brazil), under controlled conditions of 22 ± 2 °C and 12-h light/dark cycles (lights on at 7:00 a.m.). Water and feed were offered *ad libitum*. All the experimental procedures were approved by the Committee of Ethics in Animal Use (CEUA/UNIARA protocol nº

[1] Department of Biological Science and Health – Biotechnology, University of Araraquara – UNIARA, Araraquara, SP, Brazil.
[2] Department Health Science, University Oeste Paulista – UNOESTE, Jaú, SP, Brazil .
[3] Biological Sciences and Health Center – UFSCar, São Carlos, SP, Brazil.
[4] Department of Morphology, Physiology and Basic Pathology – USP, Ribeirão Preto, SP, Brazil.

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018/2016), following the norms of the National Council for Control of Animal Experimentation (CONCEA/MCTI, Brazil).

Estrous cycle, body mass and induction of polycystic ovary

Estrous cycle analysis was performed daily for 4 weeks prior to induction of PCOS, in order to confirm the occurrence of normal and consecutive cycles. The rats that were used in the experiment had at least 4 normal and consecutive cycles[30–31]. The estrous cycle checks were continued daily until the end of the experimental periods.

Body mass analysis of the rats was performed on the day of arrival at the laboratory, on the day of induction of PCOS, weekly after the day of induction, and on the days of euthanasia.

The polycystic ovary induction was performed with a single dose of estradiol valerate (EV) (Sigma–Aldrich, MO, USA) dissolved in mineral oil (2.0 mg/0.2 mL/rat; intramuscular)[30]. Control (C) animals received intramuscular injection of 0.2 mL of mineral oil. After hormonal and mineral oil injections, the animals were divided into 6 groups (n = 5) that were evaluated after different periods: 30 days after induction (PCO 30 and C 30 groups), 45 days after induction (PCO 45 and C 45 groups), and 60 days after induction (PCO 60 and C 60 groups). The evaluation times were based on the time required for PCOS to appear[30–32]. The PCOS–induced rats showed important signs related to the syndrome, including hyperandrogenemia, irregular estrous cycles, and polycystic ovarian morphology.

Progesterone, testosterone, luteinizing hormone, alkaline phosphatase, and osteocalcin assays

Immediately after euthanasia, blood was collected by cardiac puncture and/or aortic arch, using heparinized syringes. The blood was centrifuged at 3000 rpm for 20 minutes to obtain the plasma, which was transferred to Eppendorf tubes and frozen at –20 °C for subsequent analyses of progesterone (P4), testosterone (T), luteinizing hormone, alkaline phosphatase (ALP), and osteocalcin (OCN). The plasma progesterone and testosterone concentrations were determined by double–antibody radioimmunoassay (RIA), using MAIA kits provided by Biochemistry Immunosystem (Bologna, Italy). The lower limits of detection for progesterone and testosterone were 0.02 ng/mL and 5.0 pg/mL, respectively. The intra–assay coefficients of variation were 7.5% for progesterone and 4% for testosterone. Plasma LH was assayed using specific kits provided by the National Hormone and Peptide Program (Harbor–UCLA, USA). The antiserum for LH was LH–S10, using RP3 as reference. The lower limit of detection was 0.04 ng/mL and the intra–assay coefficient of variation was 3.4%. Alkaline phosphatase activity was determined from measurements of the release of thymolphthalein from thymolphthalein monophosphate, using a commercial kit (Labtest Diagnóstica, Belo Horizonte, MG, Brazil). Aliquots of 50 µL of the culture medium were used. The absorbance was measured at 590 nm and the ALP activity was calculated based on the value for a standard. Osteocalcin was analyzed by electrochemiluminescence immunoassay, using a COBAS 6000 immunoassay analyzer (Roche Diagnostic, Germany). The inter–assay coefficient of variation was 4.8%.

Gonadosomatic index and bone biomechanical parameters

The animals were weighed before euthanasia. Subsequently, the ovaries were removed, cleaned, and weighed. The values obtained were used to determine the gonadosomatic index (GSI), as follows: (ovarian mass/body mass) x 100.

Immediately after euthanasia, the femoral bones were removed and dissected to remove the muscle and soft tissue. The isolated bone material was maintained in 0.9% saline solution, at –20 °C, for subsequent analysis. The right femurs were removed and cleaned for determination of BMD and bone mineral content (BMC), according to the Archimedes Principle[33]. The biomechanical parameters of the right femurs were obtained by the three–point bending test, using a universal test machine (Model 4444, INSTRON) and a load cell with maximum capacity of 100 kgf, at a speed of 5 mm/min[34]. The test results were recorded in graphical form using Instron Series IX software, generating load versus displacement curves. Analysis of the curve provided the following parameters: maximum load, maximum load at fracture, and stiffness.

Ex Vivo Experiments

Mesenchymal stem cells

Mesenchymal stem cells were obtained from the left femurs of the control and PCOS females (30, 45, and 60 days), with isolation according to the Wang protocol[35]. Briefly, after euthanasia, the femurs were removed from the animals, dissected, and transferred to Falcon tubes containing DMEM (Dulbecco's Modified Eagle Medium) supplemented with $NaHCO_3$, L–glutamine, 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL). Under aseptic conditions, the femur proximal and distal epiphyses were sectioned with surgical pliers. The cells were extracted by flushing the bone marrow using a syringe (20 mL) and needle (25 x 8). The cell suspension obtained was centrifuged (10000 rpm for 4 minutes at 4 °C), the supernatant was discarded, and the cells were resuspended in culture medium. The cell pools (for each experimental time: 30, 45, and 60 days) were prepared in culture bottles, with one pool obtained

from the control animals and another from the PCOS animals. The culture bottles were kept under controlled conditions (37 °C, 5% CO_2, and 95% atmospheric air), until the cells reached 70–80% confluence (approximately 10 days). The culture medium was changed every 48 h.

Mesenchymal stem cell differentiation

After reaching cell confluency in the bottles, the cells were transferred to 24–well culture plates, at a density of 3 x 10^4 cells/mL/well, with osteogenic differentiation medium[36] (ascorbic acid, dexamethasone, and β–glycerophosphate – Sigma–Aldrich, St. Louis, MO, USA) added to the control medium (DMEM). The culture plates for the control and the PCOS groups (for each experimental time: 30, 45, and 60 days) were kept (in triplicate for each experimental time) under conditions of 37 °C, 5% CO_2, and 95% atmospheric air for 7, 14, and 21 days after addition of the osteogenic medium. The culture medium was changed every 72 h and it was frozen at –20 °C for subsequent analysis of alkaline phosphatase.

Cell metabolic activity

Cell metabolic activity was assessed after 7, 14, and 21 days of culture, using the colorimetric assay involving the reduction of 3– (4,5–dimethylthiazol–2–yl)–2,5– diphenyltetrazolium bromide (MTT) (Sigma–Aldrich, St. Louis, MO, USA), with formation of formazan (a blue crystalline product), by the action of mitochondrial dehydrogenase in viable cells. The quantity of the product is directly proportional to the blue coloration, enabling estimation of the number of mitochondria and, consequently, the number of viable cells in the culture, hence providing an indirect measure of cell metabolic activity. After removal of the culture medium from the wells, 50 μL of MTT (0.5 mg/mL) was added to each well and the plate was incubated for 4 h at 37 °C, under 5% CO_2. Subsequently, 100 μL of acid isopropanol was added to each well, in order to fully solubilize the precipitate formed, and the absorbance of the solution was measured at 570 nm[37], using a microplate reader (Polaris, Celer Biotecnologia, Belo Horizonte, MG, Brazil).

Mineralization nodes analysis

The method described by Gregory et al.[38] was used to detect extracellular matrix mineralization. Briefly, after 7, 14, and 21 days of culture, the plate wells containing osteogenic cells were washed with cold phosphate–buffered saline (PBS) (Sigma–Aldrich, St. Louis, MO, USA), filled with formaldehyde (10% v/v) (Merck, Kenilworth, NJ, USA), and left for 30 min. The wells were then washed with deionized water, followed by addition of 1 mL of Alizarin Red solution (Sigma–Aldrich, St. Louis, MO, USA). After 30 min, the excess Alizarin Red solution was removed, the wells were washed with water, and the plates were kept at room temperature for the wells to dry. Quantification of extracellular matrix mineralization was performed by adding 450 μL of acetic acid solution (10% v/v) to each well that had been previously stained with Alizarin Red. The plates were kept on a shaker for 30 min, at room temperature, after which 400 μL aliquots of the contents of the wells were transferred to Eppendorf tubes, followed by addition of 150 μL of ammonium hydroxide (NH_4OH). Finally, the contents of the Eppendorf tubes were transferred to 96–well plates and the absorbances were measured at 405 nm with a microplate reader.

Statistical analysis

The results are reported as mean ± SEM. The data were analyzed using ANOVA (analysis of variance), with one classification for six independent groups of measures, and a Fisher test was used for multiple comparisons. The statistical analyses were performed with SigmaStat software (Systat Software, Point Richmond, CA, USA). Statistically significant differences among the means for the treatment groups considered a p–values < 0.05. Logarithmic data transformation was used to satisfy the assumptions of the ANOVA model (normality and constant variance of the errors). These assumptions were confirmed using residual plots.

Results

in vivo experiments

Body mass, ovary mass, and GSI

There were no significant differences of body mass between the control and PCO animals (Figs. 1A, 1C, and 1E). For the 30–day groups (Fig. 1B), the left ovary mass was higher for the control animals (0.0727 ± 0.0228 g), compared to the PCO animals (0.0454 ± 0.0158 g). For the 45–day groups (Fig. 1D), the right ovary mass was lower for the PCO group (0.0346 ± 0.0054 g), compared to the control group (0.054 ± 0.012 g). For the 60–day groups (Fig. 1F), the masses of both ovaries were higher for the PCO group (right: 0.0552 ± 0.0059 g; left: 0.0554 ± 0.0035 g), compared to the control group (right: 0.0482 ± 0.002 g; left: 0.046 ± 0.0051 g).

Table 1 presents the gonadosomatic index results. The GSI values for the 30–day PCO groups (0.023 ± 0.0015)

and the 45–day PCO group (0.014 ± 0.0006) were lower than for the corresponding control groups (30 days: 0.029 ± 0.0021; 45 days: 0.021 ± 0.0015). For the 60–day groups, the GSI was higher for the PCO group (0.024 ± 0.0016), compared to the control group (0.019 ± 0.0009). An effect of time on GSI was also observed. For the control groups, GSI was higher for the 30–day group, with 60–day group presenting the lowest GSI. Among the PCO groups, the lowest GSI was observed for the 45–day group.

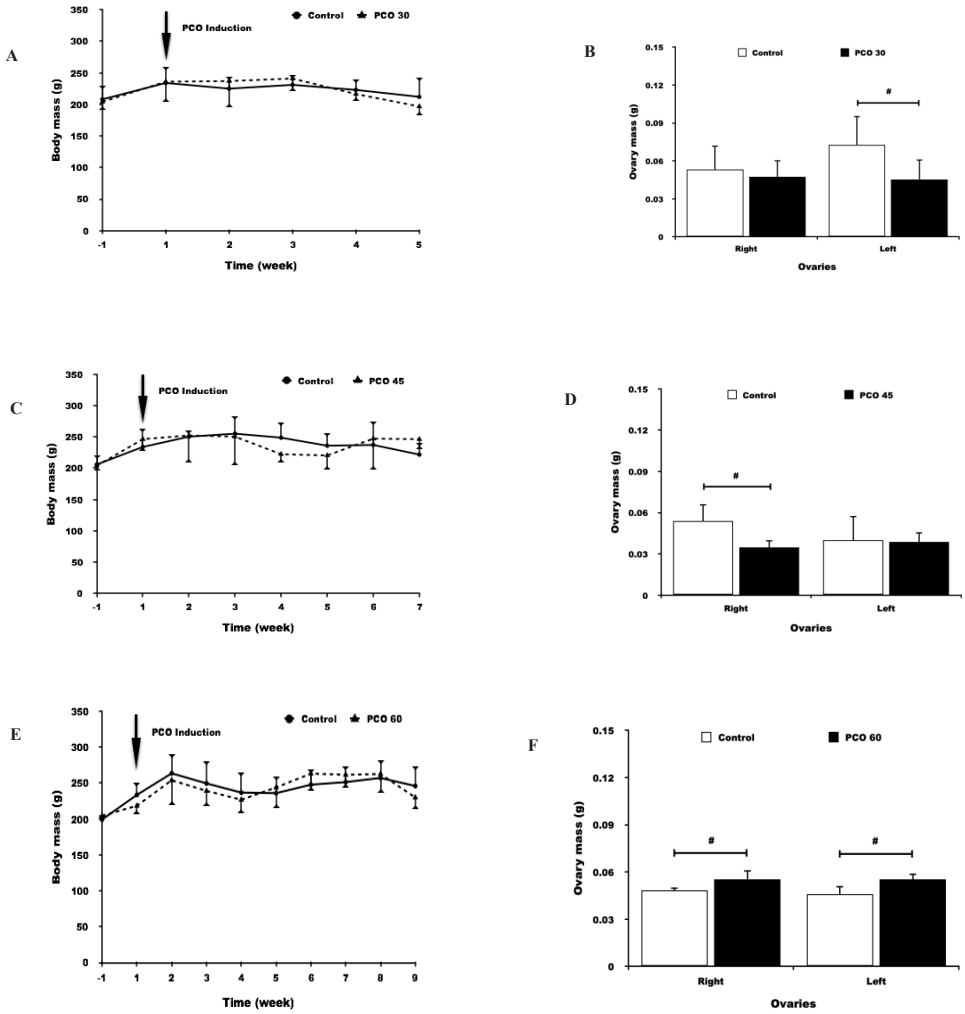

Figure 1. Mean biometric data variations for the control and PCO rats. 30–day groups: body mass (A), and ovary mass (B); 45–day groups: body mass (C), and ovary mass (D); 60–day groups: body mass (E), and ovary mass (F). The data are shown as mean ± SEM (n = 5). # Significant difference (p–values < 0.05).

Table 1. Gonadosomatic index (GSI), alkaline phosphatase (ALP), progesterone (P4), testosterone (T), luteinizing hormone (LH), and osteocalcin (OCN) values for the control and PCO groups (30, 45, and 60 days) of female adult rats. The data are shown as mean ± SEM (n = 5 per group). Different superscript lower case letters indicate significant differences (p–values < 0.05).

	Control			PCO		
	30 days	**45 days**	**60 days**	**30 days**	**45 days**	**60 days**
GSI (%)	0.029 ± 0.002 [a]	0.0021 ± 0.0015 [b]	0.019 ± 0.0009 [c]	0.023 ± 0.0015 [b]	0.014 ± 0.006 [c]	0.024 ± 0.0016 [b]
ALP (IU/L)	60 ± 18.31 [b]	81.11 ± 23.58 [b]	70.66 ± 29.52 [b]	139.74 ± 27.09 [a]	114 ± 28.04 [a]	107.45 ± 20.97 [a]
P4 (ng/mL)	38.22 ± 28.53	17.27 ± 8.768	26.16 ± 14.21	4.154 ± 4.549	17.98 ± 14.74	23.13 ± 21.33
T (pg/mL)	6.732 ± 2.5788 [b]	28.23 ± 16.51 [a]	24.51 ± 7.26 [a]	23.86 ± 11.028 [a]	19.81 ± 9.47 [a]	48.79 ± 30.28 [a]
LH (ng/mL)	0.806 ± 0.376 [a]	1.146 ± 1.036 [a]	0.5652 ± 0.1825 [a]	1.137 ± 0.842 [a]	0.2116 ± 0.1025 [b]	0.638 ± 0.508 [ab]
OCN (ng/mL)	21 ± 2.13 [a]	19.25 ± 1.31 [a]	17.5 ± 2.01 [a]	14.25 ± 1.01 [b]	17 ± 1.98 [ab]	14.5 ± 1.2 [b]

Femurs biomechanical parameters

For the 60–day groups, the maximum load was higher for the control group (0.2312 ± 0.0039 kN), compared to the PCO group (0.1886 ± 0.0326 kN). The maximum load showed time dependent effect and was higher for the 60–day group, compared to the 30–day group (0.1827 ± 0.013 kN) and the 45–day group (0.1943 ± 0.0142 kN) (Fig. 2A). At 60 days, the maximum load at the fracture point was higher for the control group (0.2152 ± 0.0439 kN) than for the PCO group (0.1147 ± 0.0588 kN). For both groups, the maximum load at the fracture point was time dependent.

In the case of the control groups, a higher value was obtained for the 60–day group (0.2152 ± 0.0439 kN), compared to the 30–day group (0.1579 ± 0.016 kN) and the 45–day group (0.177 ± 0.0193 kN). The value for the 60–day PCO group (0.1147 ± 0.0588 kN) was lower than for the 30–day PCO group (0.1707 ± 0.037 kN) and the PCO 45–day group (0.1754 ± 0.0297 kN) (Fig. 2C).

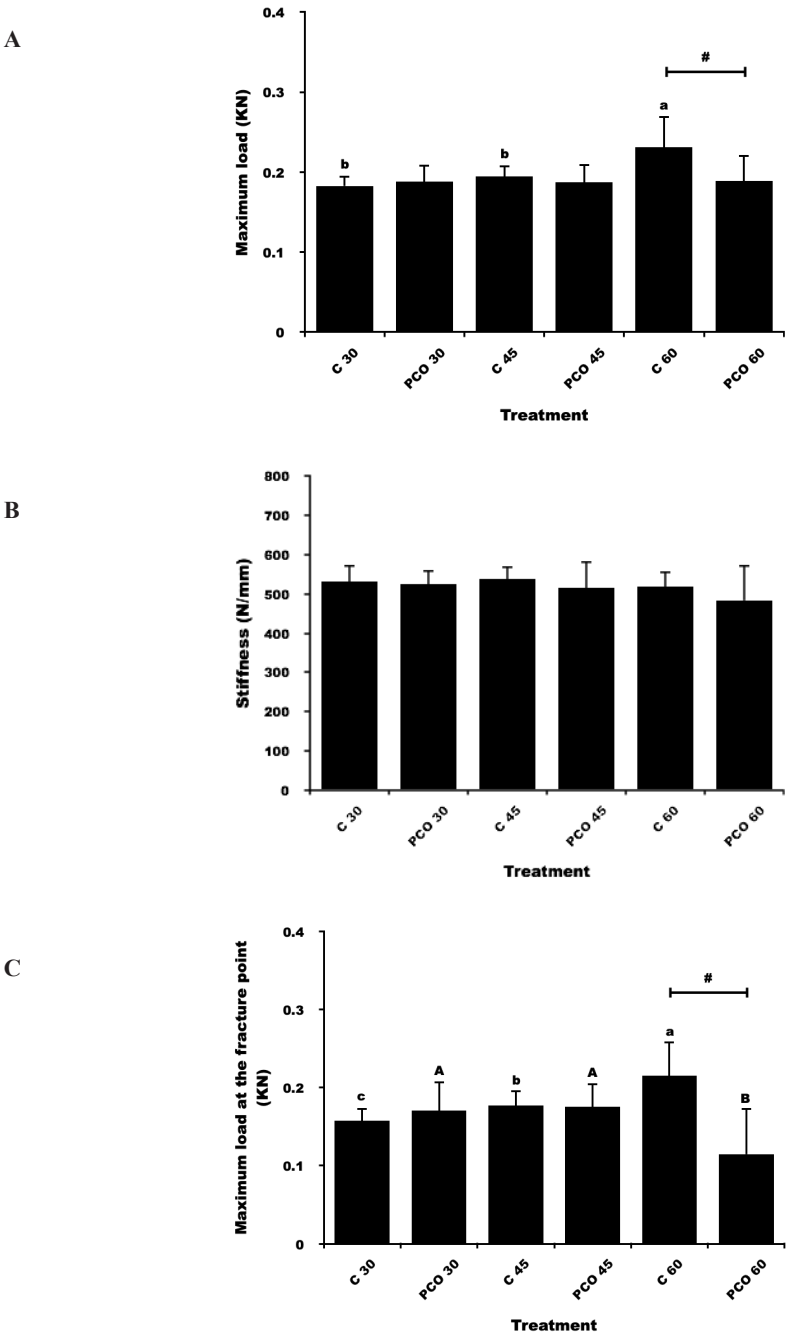

Figure 2. Biomechanical parameters for the femurs of the rats in the control (C 30, C 45, and C 60), and polycystic ovary (PCO 30, PCO 45, and PCO 60) groups: (A) maximum load; (B) stiffness; (C) maximum load at the fracture point. The data are shown as mean ± SEM (n = 5). Different lower case letters indicate differences between the control groups. Different capital letters indicate differences between the PCO groups. # Difference between the control and PCO rats (p–values < 0.05).

Plasma levels of alkaline phosphatase and osteocalcin

Increases in plasma alkaline phosphatase concentrations (Table 1) were observed for all the PCO groups (30–day: 139.74 ± 27.09 IU/L; 45–day: 114 ± 28.04 IU/L; and 60–day: 107.45 ± 20.97 IU/L), compared to the corresponding control groups (30–day: 60 ± 18.31 IU/L; 45–day: 81.11 ± 23.58 IU/L; 60–day: 70.66 ± 29.52 IU/L).

The plasma osteocalcin concentration (Table 1) was lower for the 30–day PCO group (14.25 ± 1.01 ng/mL), compared to the 30–day control group (21 ± 2.13 ng/mL). The 60–day PCO group presented a lower osteocalcin concentration (14.5 ± 1.2 ng/mL) than the 60–day control group (17.5 ± 2.01 ng/mL).

Plasma progesterone, testosterone, and luteinizing hormone concentrations

The variations of progesterone, testosterone, and luteinizing hormone are shown in Table 1. There were no significant differences in plasma progesterone concentrations, despite important biological variations of the P4. Lower plasma T concentration was observed for the 30–day control group (6.732 ± 2.578 pg/mL), compared to the other groups. Significantly lower plasma LH was found for the 45–day PCO group (0.2116 ± 0.1025 ng/mL), compared to the other experimental groups, with the exception of the 60–day PCO group (0.638 ± 0.508 ng/mL).

IN VITRO EXPERIMENTS

Cellular metabolic activity

The 30–day PCO group showed lower cellular metabolic activity, compared to the 30–day control group, for all culture times: 7 days (C: 0.2423 ± 0.008; PCO: 0.166 ± 0.007), 14 days (C: 0.2413 ± 0.009; PCO: 0.1586 ± 0.006), and 21 days (C: 0.2870 ± 0.01; PCO: 0.1473 ± 0.016). Among the control groups, the highest cellular metabolic activity was observed with 21 days of culture (0.2870 ± 0.01) (Fig. 3A).

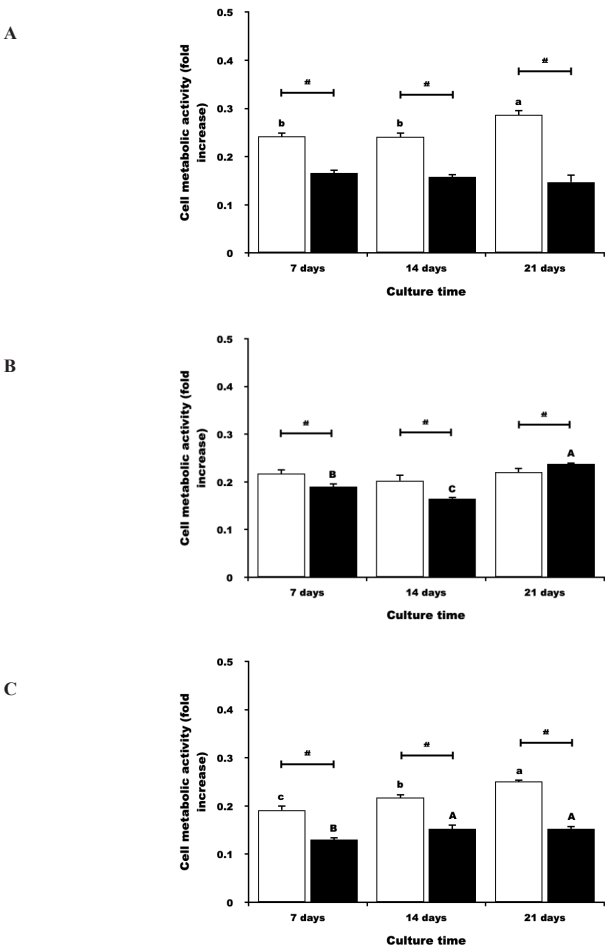

Figure 3. Cellular metabolic activity determined by reduction of methylthiazolyldiphenyl–tetrazolium bromide (MTT), with absorbance at a wavelength of 570 nm. Cells were cultured for 7, 14, and 21 days. (A) 30–day groups, (B) 45–day groups, and (C) 60–day groups. The white and black columns correspond to the control and PCO groups, respectively. The data are shown as mean ± SEM (n = 5). Different lower case letters indicate differences between control groups. Different capital letters indicate differences between PCO groups. # Difference between control and PCO rats (p–values < 0.05).

The cellular metabolic activity of the 45–day PCO group was lower than that of the control group for culture time of 7 days (C: 0.2173 ± 0.009; PCO: 0.19 ± 0.007) and 14 days (C: 0.2026 ± 0.013; PCO: 0.165 ± 0.004). For the culture time of 21 days, the 45–day PCO group presented higher metabolic activity (0.2376 ± 0.006) than the 45–day control group (0.2206 ± 0.009). Among the PCO groups, the highest metabolic activity was observed with 21 days of culture (0.2376 ± 0.006) (Fig. 3B).

The cellular metabolic activity of the 60–day PCO group was lower than that of the control group, for all the culture times analyzed: 7 days (C: 0.1916 ± 0.01; PCO: 0.1303 ± 0.005), 14 days (C: 0.2176 ± 0.007; PCO: 0.1526 ± 0.009), and 21 days (C: 0.2513 ± 0.004; PCO: 0.1530 ± 0.006) (Fig. 3C). Among the 60–day control groups, the highest cellular metabolic activity was observed with culturing for 21 days (0.2513 ± 0.004). Among the 60–day PCO groups, the highest cellular metabolic activities were observed with culturing for14 days (0.1526 ± 0.009) and 21 days (0.1530 ± 0.006) (Fig. 3C).

Organic mineralization

When the mesenchymal stem cells were differentiated into osteoblasts kept in culture for 21 days, the 30–day control group showed higher mineralization at 14 days of culture (3.27 ± 0.114) and lower mineralization at 21 days of culture (0.233 ± 0.0294). Similar results were observed for the 30–day PCO group, with higher mineralization at 14 days of culture (3.1193 ± 0.1156) and lower mineralization at 21 days (0.218 ± 0.0234) (Fig. 4A).

The organic mineralization of the 45–day groups is shown in Fig. 4B. The control group showed the highest mineralization at 14 days of culture (0.8038 ± 0.0647) and the lowest mineralization at 21 days (0.2493 ± 0.0345). The PCO group showed similar behavior, with the highest mineralization at 14 days of culture (0.717 ± 0.0123) and the lowest mineralization at 21 days (0.23 ± 0.0193).

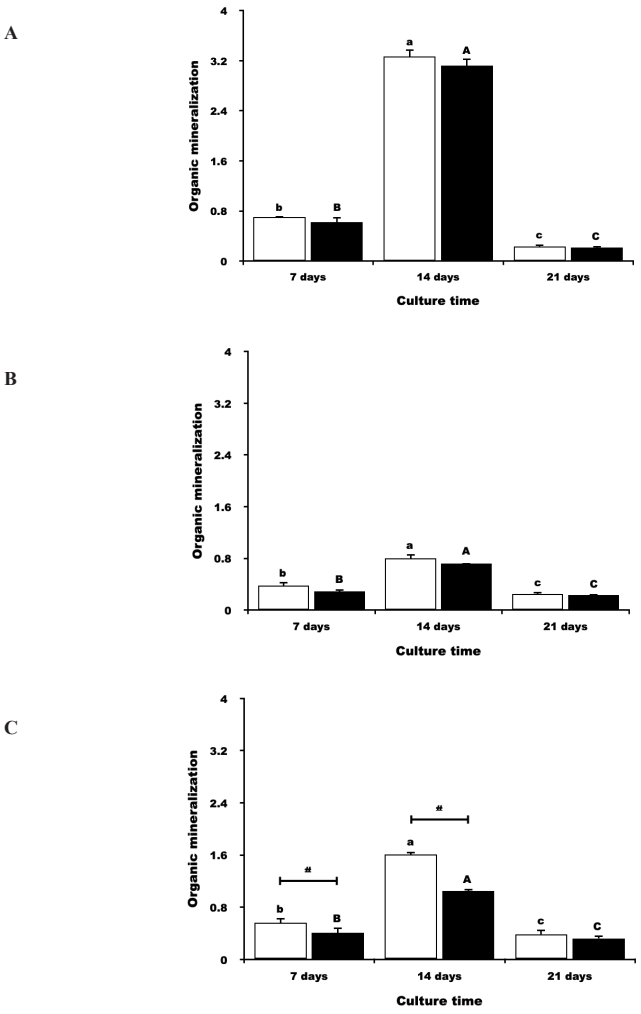

Figure 4. Organic mineralization of osteoblasts cultured for 7, 14, and 21 days: (A) 30–day PCO group; (B) 45–day PCO group; (C) 60–day PCO group. The white and black columns correspond to the control and PCO groups, respectively. The data are shown as mean ± SEM (n = 5). Different lower case letters indicate differences between control groups. Different capital letters indicate differences between PCO groups. # Indicates difference between control and PCO rats (p–values < 0.05).

Fig. 4C shows the results for organic mineralization of the cells of the 60–day groups. The control group showed the highest mineralization at 14 days of culture (1.6107 ± 0.0413) and the lowest mineralization at 21 days of culture (0.3855 ± 0.0696). The PCO group also showed higher mineralization at 14 days of culture (1.0512 ± 0.0329) and lower mineralization at 21 days of culture (0.3227 ± 0.0467). Comparison of the control and PCO groups showed that the PCO group presented lower organic mineralization (7 days: 0.4108 ± 0.0769; 14 days: 1.0512 ± 0.0329) than the control groups (7 days: 0.5615 ± 0.0749; 14–days: 1.6107 ± 0.0413).

Discussion

The results obtained in the present study using a model of adult Wistar rats induced to PCOS with a single dose of EV showed ovarian and estrous cycle changes, bone property modifications in vivo and in vitro, and alterations of ALP, OCN, and T in the plasma. Other studies have employed PCOS animal models[24,29,39], with differences among the findings for PCOS rats being due to factors such as hormone type and dosage, route of administration, timing and duration of exposure, phase of the estrous cycle, and animal ages at the time of the study[5].

Androgens and AR play important roles in normal follicular development and female fertility. Evidence of the involvement of androgens in folliculogenesis has been found using in vivo animal models[26,40]. The synthesis of androgens is under the control of luteinizing hormone from the pituitary and depends on ovarian cell steroidogenic acute regulatory protein (STAR), cholesterol side–chain cleavage cytochrome P450 (CYP11A1), 17μ–hydroxylase (CYP17), and 3μ–hydroxysteroid dehydrogenase (HSD3B)[26,41].

Women with PCOS have abnormalities in estrogen and androgen metabolism[14,19,23,42]. Hyperandrogenism is a typical feature of PCOS and often manifests as disturbed folliculogenesis, reduced fertility, and adverse effects on oocyte developmental competence[43]. The results obtained in the present work demonstrated that T levels were elevated in the 60–day animals induced to PCOS, compared to the control animals. It has been reported that the hormone effect in the female reproductive system is strongly influenced by the time of exposure[5,10,21,44]. In the present case, the animal model employed had suitable hyperandrogenic characteristics for the duration of the assays performed. In addition to hyperandrogenism, the rats showed important ovarian alterations, such as reduction of ovarian mass 45 days after induction and increase of ovarian mass at 60 days, which could have been due to a greater number of ovarian cysts. No alteration of body weight was observed, although there was a reduction of GSI of the PCOS animals up to 45 days after EV treatment, followed by an increase at 60 days, suggesting morphological and steroidogenic ovarian alterations. At 60 days after PCOS induction, the treated animals showed increases of GSI and production of T. This was suggestive of a vicious cycle, with the endocrine alteration causing ovary morphological alteration, consequently altering hormone synthesis, which could alter T synthesis, and so on.

Follicular atresia is a complex and multifactorial apoptotic process that depends on apoptosis of granulosa cells[43,45–47]. During cyclic recruitment, atresia is promoted by androgens[48–50]. A single dihydrotestosterone (DHT) injection in hypophysectomized immature female rats was often seen to result in decreased ovarian weight, which was associated with the stimulation of follicular atresia and the reduction of healthy follicles of all types[51]. In the present study, all the rats of the PCO groups showed estrous cycle changes, with irregularities such as the maintenance of a phase of the estrous cycle (for example, diestrus) for several weeks. Such alterations, observed using daily vaginal smears, were in agreement with the morphological alterations of the ovaries. All the animals of the PCO groups showed reductions of the numbers of secondary and tertiary follicles, as well as corpora lutea, while there were increases of the numbers of ovarian cysts and atretic follicles (data not shown), similar to our previous observations[15,31]. These results suggested that the ovarian alterations related to the increase in T production, due to possible enzymatic alterations, were the probable cause of the ovarian morphological changes that altered the estrous cycles in the rats induced to PCOS by EV. In PCOS women, the activities of the CYP17 and HSD3B enzymes were found to increase by more than 500% and 1000%, respectively[19,52]. In addition, elevated plasma T levels did not alter the pituitary activity for LH secretion, since the plasma levels of this gonadotrophin were in agreement with the negative feedback performed by T on the pituitary, suggesting that the animal model used did not present alteration of the hypothalamic–pituitary axis.

The bone remodeling cycle begins with alterations of the extracellular matrix due to stimuli that may be mechanical, electrical, hormonal, and magnetic, which are converted into molecular signals and messages (such as nitrous oxide and prostaglandins, changes in electrical charge, and plasma membrane alterations involving calcium release). Normal plasma concentrations of estrogens and androgens have been shown to increase bone mass in men, women, and animals[5,24,53–54]. Androgen exposure enhances osteoblast differentiation and the synthesis of extracellular matrix proteins such as type 1 collagen, osteocalcin, and osteonectin, in addition to stimulating mineralization[19]. Hence, normal concentrations of androgen have an important role in regulating bone matrix production and mineralization. Sawalha and Kovats[55] demonstrated that different androgens can modulate activity in different parts of the bones.

Osteocalcin, which can act in extracellular matrix mineralization, has been used as a serum marker of osteoblastic

bone formation in both clinical and basic research[56]. Higher BMD has been observed in PCOS amenorrheic women than in non–PCOS amenorrheic patients, while hyperandrogenic women with regular menses exhibited significantly higher BMD than controls or hyperandrogenic patients[19,23]. There is conflicting information concerning the actions of OCN in the reproductive system. Osteocalcin can regulate male fertility, while not affecting female reproduction[57–58]. Testosterone was found to be positively correlated with serum osteocalcin, indirectly supporting the participation of serum osteocalcin in sex hormone regulation[59]. The results of the present work revealed changes in bone activity, both in vivo and in vitro. After 60 days following induction to PCOS, there were decreased of the maximum load at the fracture point and the plasma osteocalcin concentration. At the same time, there was the highest plasma concentrations of T. These observations suggested that testosterone plays a crucial role in mediating bone mass and osteocalcin levels in adult rats induced to PCOS by EV. On the other hand, the increase in circulating osteocalcin levels could provide a further stimulus for increase of plasma T, maintaining a vicious cycle between the bone and the ovaries, in this animal model, as suggested in studies indicating the existence of an independent bone–osteocalcin–gonadal axis[60–61].

Alkaline phosphatase is a marker of bone formation and bone turnover and is used in the evaluation of skeletal status[62–63]. Elevated serum ALP is correlated with low bone mineral density and greater structural damage. Significant associations between ALP and bone mineral density, after controlling for other variables, suggested that ALP might interacts with other factors, leading to alteration of bone metabolism[63]. In contrast, no relationship was found between ALP and bone density in elderly men, suggesting that ALP was not useful for monitoring bone integrity[64], while no correlations were found between levels of osteocalcin and bone alkaline phosphatase in healthy and postmenopausal osteoporotic women[65]. The results of the present study showed that plasma ALP levels were elevated in PCOS–induced animals. In addition, the animals induced to PCOS presented greater bone fragility, as shown by the reductions of both maximum load and maximum load at the fracture point. Taken together with the plasma T and osteocalcin levels, the data suggested that in this EV–induced PCOS animal model, the hormone changes led to impaired bone metabolism.

Cells are substrate–smart and will use any fuel available during tissue culture[66]. The artificial environment in which in vitro studies are conducted can provide valuable insights. The osteogenic differentiation potential of cultured bone mesenchymal stem cells changes with the age of the donor and the treatment applied both in vivo and in vitro by means of cellular subculture. The expression of growth factors and increased bone matrix production was found to favor osteogenesis, improving the structural and mechanical properties of bone in exercised aged animals[67–68]. The mesenchymal stem cells used in the present study were obtained from the femurs bone marrow of adult rats. The cells remained viable throughout the in vitro experimental period and differentiated into osteoblasts. However, the cells obtained from the rats induced to PCOS exhibited decreased potential for osteoblastic differentiation and reduced viability, compared to the control animals. In this way, the bone alterations observed using in vivo studies can be explained, at least in part, by using in vitro results. The evidence suggests that lower osteoblast viability and mineralization are likely to among the mechanisms contributing to higher risk of fractures in women with PCOS.

In conclusion, the use of animal models and culture cells studies, such as those described in the present work are required for evaluation of different aspects of the etiology and pathophysiology of PCOS.

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Author contributions

L.H.M. and A.L.O.B. conceived and designed the research. A.L.O.B., E.D.A., and V.F. developed the experiments. K.O.N. and J.A.A.F. performed the bone and hormonal assay experiments, respectively. J.A.A. performed the statistical analyses of the data. L.H.M., A.L.O.B., E.D.A., V.F., K.O.N., J.A.A.F., and J.A.A. discussed the results and contributed to the final manuscript.

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