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Endocrine activities modulated by adipose-mesenchymal stem cell in an animal model induced to polycystic ovary syndrome

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Abstract: Purpose - Animal models offer a useful way to study the reproductive and metabolic abnormalities, including polycystic ovary syndrome (PCOS). Mesenchymal stem cells (MSCs) have received increasing attention as a potential cell-based therapy and regenerative medicine, due to their effects in modulation of different molecular and biological pathways. The aims of the present work were to investigate modulation of the ovarian microenvironment by adipose-mesenchymal stem cells (AdMSCs) in an animal model induced to PCOS. Methods - Female rats were divided into control, polycystic ovary, and mesenchymal stem cell groups, evaluated at two different times after PCOS induction and injection of AdMSCs. Results - The polycystic ovary group showed changes in ovarian cycles, the presence of cysts in the ovaries, and hyperandrogenemia. In addition, changes in plasma insulin, glucose, leptin, and osteocalcin were observed in the polycystic ovary group. These metabolic changes were modulated by the injection of AdMSCs into the ovary. Data are presented for female rats in an animal model integrating PCOS with AdMSCs, together with the relationships among ovaries, bones, and adipocytes. Conclusion - The results suggested the existence of endocrine-metabolic-reproductive microenvironment relationships modulated by AdMSCs, which should help in guiding further investigations to clarify pathophysiological mechanisms that have not yet been fully elucidated.

Keywords: Ovary microenvironment. Mesenchymal stem cell. Biotechnology. Polycystic Ovary Syndrome. Animal model.

Introduction

The mechanisms underlying ovarian dysfunction in PCOS have not been definitively established. Hyperandrogenism is a major PCOS characteristic, with evidence indicating that it plays a key role in PCOS pathogenesis [1]. Genetic, physiological, social, and environmental causes appear to contribute to the condition. Among fertility disorders, PCOS is the most common endocrine disorder[2], affecting 5.6 to 21.3% of women of reproductive age worldwide[3], depending on the diagnostic criteria applied.

Stem cell paracrine signaling has been highlighted as an important mechanism underlying stem cell mediated tissue regeneration and immunomodulation, and as a potential cell-based therapy[4-7]. Mesenchymal stem cells derived from bone marrow, cord blood, and adipose tissue have been found to secrete a wide spectrum of growth factors, cytokines, and extracellular vesicles that enhance angiogenesis, neurogenesis, and wound healing [8-11]. The production of paracrine signals from transplanted MSCs can improve tissue regeneration and induce functional recovery of defective tissue by activation of endogenous cells in host tissue[9], including female reproductive organ defects leading to infertility, such as endometriosis, premature ovary failure, and PCOS [5].

Understanding the etiology of PCOS is important for identifying biomarkers and developing possible therapies. Although studies are being carried out in humans, there are important ethical restrictions. Therefore, studies with animal models are indispensable for understanding the pathophysiological mechanisms of this syndrome[12-19]. Animal models have been proposed that use steroid hormones and steroidogenic enzyme inhibitors for the development of a phenotype associated with PCOS[13, 20-21]. However, there is no animal model that is totally effective for studying PCOS, and the choice of a model must be guided by the objectives of the study.

A suitable animal model could provide a useful way to study the physiopathology of the characteristic reproductive and metabolic abnormalities associated with PCOS. So far, there is no consensus on the best animal model, which should ideally reproduce

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the key features associated with human PCOS [18,22]. Neonatal, peripubertal, and adult models demonstrate that activational action postnatal manipulations can generate sufficient POCS-like traits [23].

The aims of the present work were to investigate modulation of the ovarian microenvironment by AdMSCs in an animal model induced to PCOS. The results revealed reproductive and metabolic changes that were possibly mediated by mesenchymal stem cells.

Material and methods

Animals

Adult female Wistar Hannover rats (6 months old at the end of the experiments, *n* = 30) were obtained from the Ribeirão Preto Medical School (FMRP-USP, Ribeirão Preto, SP, Brazil). The animals were kept in the laboratory at the University of Araraquara (UNIARA, Araraquara, SP, Brazil), under controlled conditions of 22 ± 2 °C and 12-h light / dark cycles (lights on at 7:00 a.m.). Water and commercial chow diet were offered *ad libitum*. All the experimental procedures were approved by the Committee of Ethics in Animal Use (CEUA/UNIARA, n° 030/2016), following the norms of the National Council for Control of Animal Experimentation (CONCEA/MCTI, Brazil).

Polycystic ovary syndrome induction

The PCOS 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) [22]. Control animals received intramuscular injection of 0.2 mL of mineral oil. In addition to practicality and low cost, this PCOS model induced by EV showed important signs related to the syndrome, including hyperandrogenemia, irregular estrous cycles, and polycystic ovarian morphology.

Adipose-Mesenchymal stem cell injection

Female dog AdMSCs were used (Regenera Stem Cells®, Campinas, SP, Brazil). The cells were thawed in a water bath (37 °C), resuspended in 3 mL of defrosting medium (Regenera Stem Cells®, Campinas, SP, Brazil), centrifuged at 1000 rpm for 5 min, washed twice with 3 mL of washing solution (Regenera Stem Cells®, Campinas, SP, Brazil), centrifuged again, and resuspended in 1 mL of saline. The animals were anesthetized using intraperitoneal ketamine (75 mg/kg, Agener, SP, Brazil), and xylazine (10 mg/kg, Coopers, SP, Brazil), and laparotomy was performed to access the ovaries. The ovaries were exposed, and the AdMSCs were injected (1×10^6 cells / 0.2 mL of saline / ovary), using 1 mL syringes. The control group animals underwent the same surgical procedures, and 0.2 mL of saline was injected into each ovary. After surgery, the animals were treated with subcutaneous enrofloxacin (10 mg/kg, Bayer, SP, Brazil). The AdMSCs application was performed on the same day as the PCO induction.

Experimental protocol

After PCO induction and AdMSCs injection, the rats were divided into 6 groups (*n* = 5 / group), according to the time after induction (30 and 60 days), and the treatment (C, PCO, or MSC). The evaluation times were based on the time required for PCOS to appear[24, 25] and for observations of possible responses modulated by biologically factors secreted by the MSCs [26]. The groups were labeled as follows: C 30 and C 60 (control, C groups); PCO 30 and PCO 60 (polycystic ovary, PCO groups); MSC 30 and MSC 60 (adipose-mesenchymal stem cell, MSC groups).

Estrous cycles and body mass

Estrous cycle analysis was performed daily for four weeks, prior to induction of PCO, to confirm the occurrence of normal and consecutive cycles. The animals used in the experiment had at least four consecutive regular 4-day cycles[12, 24]. The estrous cycles checks were continued daily until the end of the experimental period. Body mass analysis of the rats was performed on the day of arrival at the University Animal Facilities, on the day of induction of PCO and AdMSCs injection, weekly after the day of induction and injection, and on the day of euthanasia.

Gonadosomatic index and ovarian morphology

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): (ovarian mass / body mass) x 100. The ovaries were removed, properly cleaned, placed in histological cassettes, and kept for 24 hours in 4% formaldehyde (prepared from paraformaldehyde), in 0.1 M sodium phosphate buffer (pH 7.2), at room temperature, and subsequently in running water for another 2 hours to remove excess formaldehyde. The ovaries were then dehydrated in increasing concentrations of alcohol (70°, 80°, 90°, and 100° GL), diaphanized in xylol, infiltrated, and included in paraffin. Serial longitudinal cuts of 5 µm were made. After every 10 cuts, histological glass slides were mounted, stained with hematoxylin-eosin (HE), and analyzed by optical microscopy (Eclipse TS100, Nykon, Tokyo, Japan). The images were analyzed using ISCapture IS500 v.4.3.1 software (TUCSEN Photonics, Fujian, China). Cystic follicles were defined as those devoid

of oocyte and displaying a large antral cavity, a thin granulosa cell layer, and a thickened theca interna cell layer[12, 24].

Hormones and glucose assays

At the end of the experimental periods, the rats were euthanized using pentobarbital sodium (Merck, NJ, USA) at 3% (0.6 mL / 100 g, intramuscular), and a 5 mL blood sample was obtained from the heart into heparinized syringes. Plasma was separated by centrifugation at 3000 rpm for 20 min, at 4°C, and was stored at -20 °C for subsequent determination of progesterone (P4), testosterone (T), luteinizing hormone (LH), and follicle-stimulating hormone (FSH), by radioimmunoassay (RIA). Plasma P4 and T concentrations were determined by double-antibody RIA, using kits provided by Biochem Immunosystem (Bologna, Italy). The lower limits for detection of progesterone and testosterone were 0.03 and 4.8 ng/mL, respectively. The intra-assay coefficients of variation were 6.5% for P4 and 4.5% for T. Plasma LH and FSH were assayed using a kit provided by the National Hormone and Peptide Program (Harbor-UCLA, USA). The antiserum for LH was LH-S10, using RP3 as reference. The FSH primary anti-body was anti-rat FSH-S11, with FSH-RP2 as standard. The lower limits of detection for LH and FSH were 0.04 and 0.2 ng/mL, while the intra-assay coefficients of variation were 3.4% and 6.3%, respectively.

Plasma levels of leptin, osteocalcin (OCN), and insulin were determined using commercially available ELISA kits, following the instructions of the manufacturer (EMD Millipore Corporation, MA, USA). Glucose was determined using a drop of blood obtained by cardiac puncture. The blood was added to a test strip that was inserted into the measurement device (Accu-Chek® Performa, Roche, SP, Brazil), according to the manufacturer's instructions.

Statistical analysis

The results are reported as means ± SD. The data were analyzed using ANOVA (analysis of variance) to compare the means, while the Fisher test was used for multiple comparisons. Statistical analyses were performed using a software program (Sigma Stat, Systat Software, CA, USA). Significant statistical differences among the means of the treatment groups were considered for *P*-values < 0.05.

Results

Morphometric data

The morphometric data of body weight, ovarian weight, and gonadosomatic index are presented in Table 1. *30 days*: Higher body weight was observed for the C group, compared to the PCO and MSC groups. The GSI was higher for the PCO and MSC groups, compared to the C group. *60 days*: Higher body weights were obtained for the C and PCO groups, compared to the MSC group. Comparison of the values obtained at 30 and 60 days showed a reduction of body weight for the C group. For the C and MSC groups, the ovarian weight and GSI were lower at 60 days of the experiment, compared to 30 days.

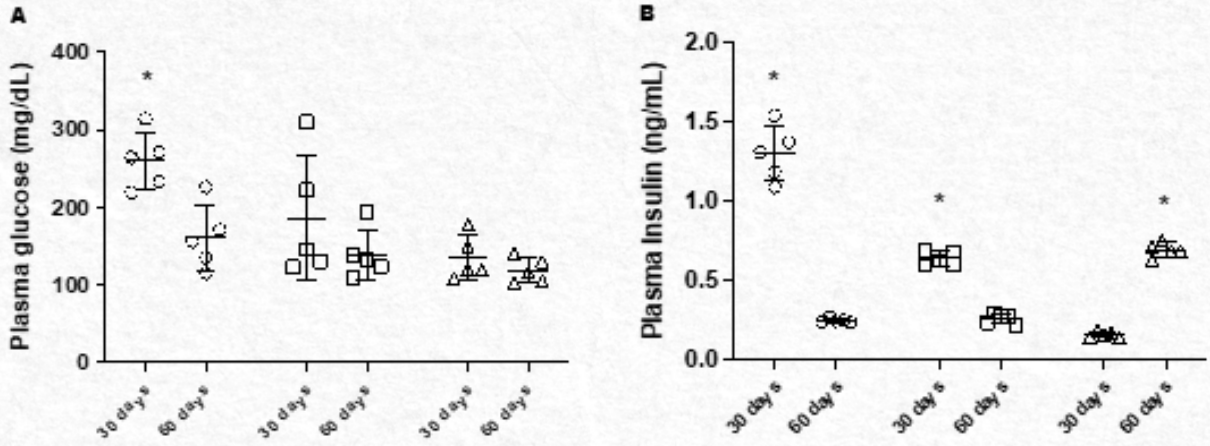
Table 1 - Body weight, ovary weight, and gonadosomatic index (GSI) for the control (C), polycystic ovary (PCO), and adipose-mesenchymal stem cell (MSC) groups, after 30 and 60 days of the *in vivo* experiments. The results are presented as mean ± SD (*n*=5). * Indicates statistically significant differences between means for different times in the same group. Different letters indicate statistically significant differences between groups for the same time (*P* < 0.05).

	BODY WEIGHT (g)		OVARY WEIGHT (g)		GSI (%)	
GROUPS	30 days	60 days	30 days	60 days	30 days	60 days
C	514.4 ± 14.1 ^{a*}	477.6 ± 14.7 ^{a}	0.065 ± 0.008 *	0.041 ± 0.01	0.049 ± 0.001 ^{a*}	0.008 ± 0.003
PCO	460.4 ± 30.7 ^{b}	446 ± 22.7 ^{a}	0.073 ± 0.03	0.041 ± 0.01	0.015 ± 0.007 ^{b}	0.009 ± 0.003
MSC	391.4 ± 26.8 ^{c}	404.8 ± 25.4 ^{b}	0.06 ± 0.01 *	0.038 ± 0.006	0.015 ± 0.002 ^{b*}	0.009 ± 0.001

Plasma glucose and insulin

The variations of plasma glucose and insulin are shown in Figure 1. *30 days*: There were decreases of blood glucose in the PCO and MSC groups, compared to the C group (Fig. 1A). In addition, there were reductions of plasma insulin in the PCO and MSC groups, compared to the C group (Fig. 1B). *60 days*: Plasma insulin was higher in the MSC group, compared to the PCO and C groups (Fig. 1B). The C group showed a reduction of blood glucose at 60 days of the experiment, compared to 30 days. The C and PCO groups showed decreased of plasma insulin at 60 days, compared to 30 days. Finally, the MSC group showed an increase of plasma insulin at 60 days, compared to 30 days.

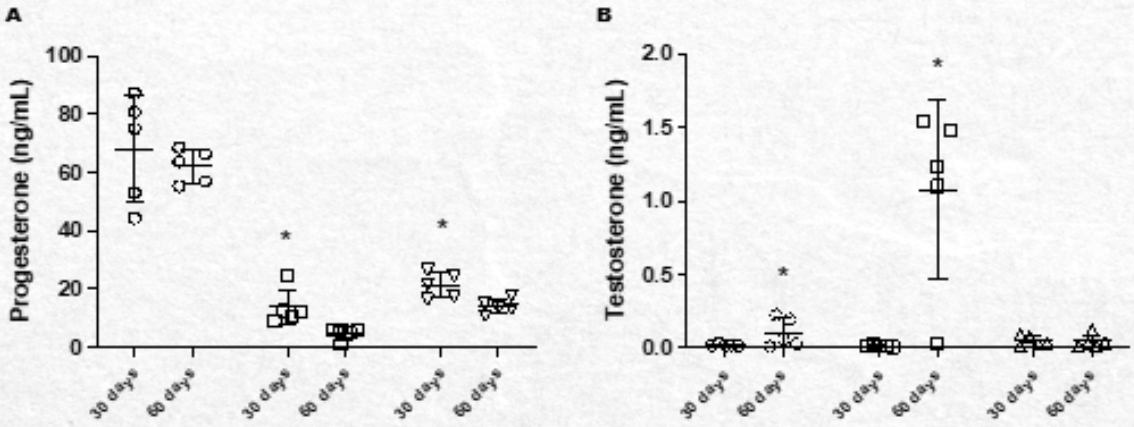
Figure 1 - Plasma glucose (A) and insulin (B) for the control (C - circles), polycystic ovary (PCO - squares), and adipose-mesenchymal stem cell (MSC - triangles) groups, after 30 and 60 days of the in vivo experiments. The results are presented as mean ± SD (n=5). * Indicates statistically significant differences (P < 0.05).



Plasma progesterone and testosterone

Plasma progesterone and testosterone are shown in Figure 2. *30 days*: There were reductions of plasma P4 in the MSC and PCO groups, compared to the C group (Fig. 2A). *60 days*: Plasma P4 showed a similar pattern to that at 30 days., with reductions in the MSC and PCO groups, compared to the C group (Fig. 2A). Higher plasma T was observed for the PCO group, compared to the C and MSC groups (Fig. 2B). The comparison between 30 and 60 days showed a reduction of plasma P4 in the PCO and MSC groups at 60 days, compared to 30 days. There was an increase of plasma T in the PCO and C groups at 60 days, compared to 30 days.

Figure 2 - Plasma progesterone (A) and testosterone (B) for the control (C - circles), polycystic ovary (PCO - squares), and adipose-mesenchymal stem cell (MSC - triangles) groups, after 30 and 60 days of the *in vivo* experiments. The results are presented as mean ± SD (*n*=5). * Indicates statistically significant differences (*P* < 0.05).



Plasma gonadotropins

The plasma variations of pituitary gonadotropins and the LH/FSH ratio are shown in Table 2. *30 days*: The LH concentration was higher for the C group, compared to the MSC group. The FSH concentration was higher for the C group, compared to the PCO and MSC groups. The LH/FSH ratio was higher for the MSC group, compared to the PCO and C groups. *60 days*: The LH concentration was higher for the MSC and PCO groups, compared to the C group. The FSH concentration was higher for the MSC group, compared to the PCO and C groups. The LH/FSH ratio was higher for the C group, compared to the PCO and the MSC groups. For the PCO and MSC groups, LH was higher at 60 days than at 30 days. For the C group, FSH was higher at 30 days than at 60 days, while for the MSC group, FSH was higher at 60 days than at 30 days. Finally, for the C and PCO groups, the LH/FSH ratio was higher at 60 days than at 30 days, while for the MSC group, it was higher at 30 days than at 60 days.

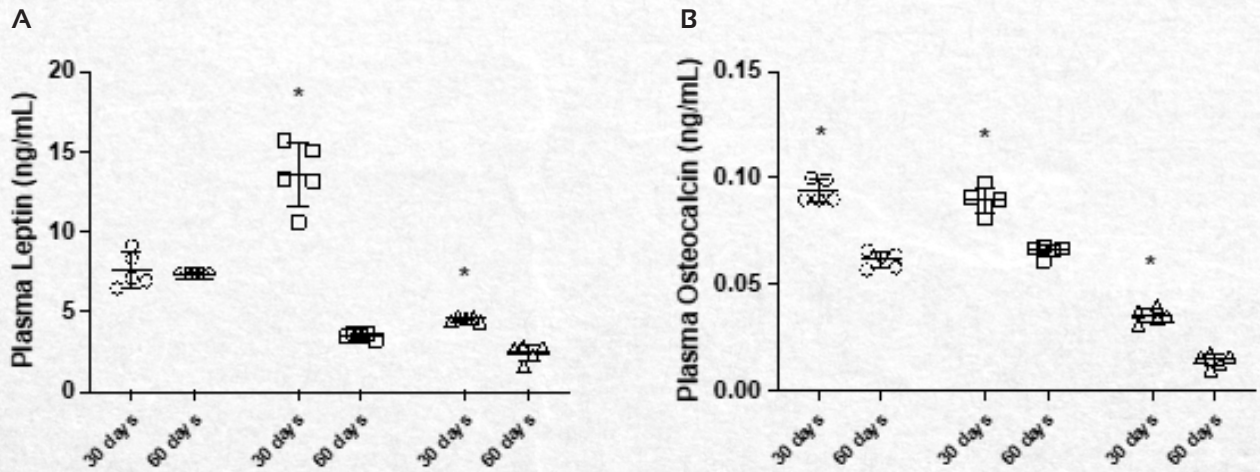
Table 2 - Luteinizing hormone (LH), follicle-stimulating hormone (FSH), and LH/FSH ratio values for the control (C), polycystic ovary (PCO), and adipose-mesenchymal stem cell (MSC) groups, after 30 and 60 days of the *in vivo* experiments. The results are presented as mean ± SD (*n*=5). *Indicates statistically significant differences between the times for the same group. Different letters indicate statistically significant differences between the groups at the same time ($P < 0.05$).

	LH (ng/mL)		FSH (ng/mL)		LH/FSH ratio	
GROUPS	**30 days**	**60 days**	**30 days**	**60 days**	**30 days**	**60 days**
C	0.334 ± 0.06 ^{a}	0.366 ± 0.05 ^{b}	4.078 ± 0.63 ^{a*}	0.293 ± 0.58 ^{c}	0.082 ± 0.018 ^{b}	2.33 ± 0.88 ^{a*}
PCO	0.231 ± 0.06 ^{a,b}	1.017 ± 0.28 ^{a*}	2.994 ± 0.92 ^{b}	2.35 ± 0.29 ^{b}	0.09 ± 0.052 ^{b}	0.43 ± 0.092 ^{b*}
MSC	0.179 ± 0.03 ^{b}	1.062 ± 0.13 ^{a*}	0.612 ± 0.44 ^{c}	4.716 ± 0.4 ^{a*}	0.422 ± 0.085 ^{a*}	0.228 ± 0.046 ^{c}

Plasma leptin and osteocalcin

Figure 3 presents the results for plasma leptin and osteocalcin. *30 days*: Plasma leptin was higher for the PCO group, compared to the C and MSC groups (Fig 3A). There was no significant difference in plasma osteocalcin between the C and PCO groups, but both values were higher than for the MSC group (Fig. 3B). *60 days*: There were reductions of plasma leptin in the PCO and MSC groups, compared to the C group (Fig. 3A). There was no significant difference in plasma osteocalcin between the C and PCO groups, but both values were higher than for the MSC group (Fig. 3B). In the PCO and MSC groups, leptin was lower at 60 days, compared to 30 days. In all the groups, osteocalcin was reduced at 60 days, compared to the 30 days.

Figure 3 - Plasma leptin (A) and osteocalcin (B) for the control (C - circles), polycystic ovary (PCO - squares), and adipose-mesenchymal stem cell (MSC - triangles) groups, after 30 and 60 days of the in vivo experiments. The results are presented as mean ± SD (n=5). *Indicates statistically significant differences ($P < 0.05$).



Ovarian morphology

Figure 4 shows images of the ovaries from all the groups for experimental times of both 30 and 60 day. At 30 days, the presence of follicles and corpus luteum could be seen for all the groups. Currently, there was no presence of ovarian cysts (Fig. 4A-F). After 60 days, there were ovarian cysts in the PCO group (Fig. 4I-J), while these cysts were absent in groups C (Fig. 4 G-H) and MSC (Fig. 4 K-L). In the latter groups, there were healthy follicles and corpus luteum. At 60 days, the numbers of healthy follicles were higher in the C and MSC groups, compared to the PCO group. On the other hand, the number of follicular cysts was higher in the PCO group, compared to groups C and MSC (Table 3).

Figure 4 - Histological photomicrographs of sections of the rat ovaries after 30 days (A-B: control group (C); C-D: polycystic ovary group (PCO); E-F: adipose-mesenchymal stem cell group (MSC)) and 60 days (G-H: control group (C); I-J: polycystic ovary group (PCO); K-L: adipose-mesenchymal stem cell group (MSC)) of the *in vivo* experiments. Red arrows indicate follicles. CL: corpus luteum; C: follicular cysts. Sections stained using hematoxylin-eosin; 100 x magnification; bars = 20 µm.

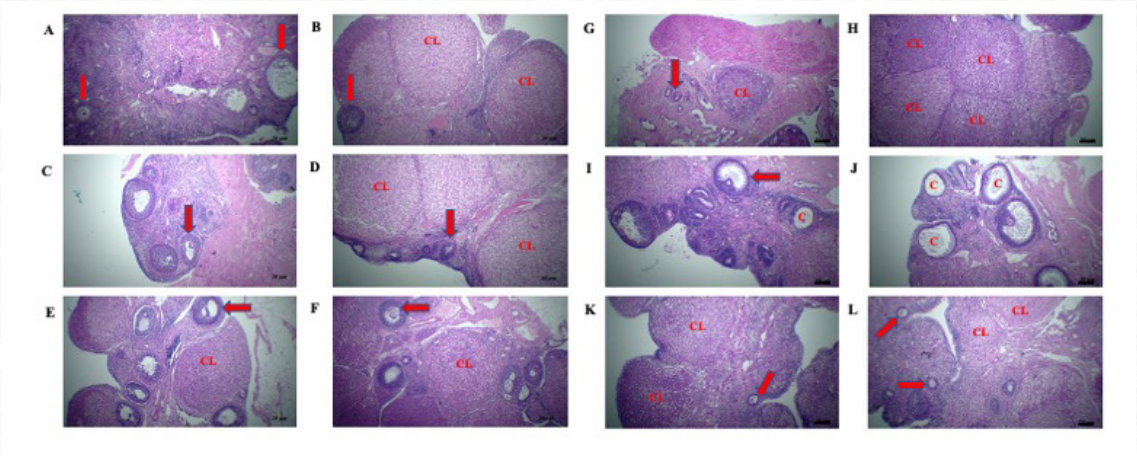


Table 3 - Number of follicles, corpus luteum, and follicular cysts from control (C 30 and C 60 days), polycystic ovary (PCO 30 and PCO 60 days), and adipose-mesenchymal stem cell (MSC 30 days, and MSC 60 days) rats. Data are shown as the mean ± SEM (*n*=5). *Indicates statistically significant differences between means for different times in the same group. Different letters indicate statistically significant differences between groups for the same time ($P < 0.05$).

	FOLLICLE		CORPUS LUTEUM		FOLLICULAR CYST	
GROUPS	**30 days**	**60 days**	**30 days**	**60 days**	**30 days**	**60 days**
C	10 ± 1 ^{a}	9 ± 0.7 ^{a}	6.1 ± 0.8 ^{a}	5.8 ± 0.6 ^{a}	0.28 ± 0.3 ^{b}	0.28 ± 0.3 ^{c}
PCO	3.1 ± 1.2 ^{b*}	2.3 ± 0.6 ^{b}	3.9 ± 0.7 ^{b*}	2.1 ± 0.8 ^{b}	4 ± 0.9 ^{a}	7 ± 1.1 ^{a*}
MSC	6.4 ± 0.8 ^{b}	8 ± 0.9 ^{a}	6 ± 0.6 ^{a}	4.8 ± 0.9 ^{a}	0.3 ± 0.2 ^{b}	0.4 ± 0.1 ^{b}

Discussion

Rodents and sheep induced to PCOS like women with PCOS have increased ovarian volume and multifollicular ovarian morphology. The body weight of animals induced to PCOS can increase or decrease, depending on the animal model and the PCOS induction method[12, 22, 27-29]. In the present study, the PCO and MSC groups had lower body mass and higher GSI, compared to the control group. Body weight analyses were performed considering weight gain between the two experimental times, with the greatest weight gain 60 days after PCOS induction being observed for the PCO group. In addition, the plasma levels of insulin, leptin, osteocalcin, and testosterone may have caused important metabolic changes that resulted in body weight and GSI modifications. Women with high T values exhibit increased body mass index, central adiposity, and insulin resistance[30]. Abnormal metabolic conditions could result from a sustained positive energy balance, when the energy intake is higher than the energy expenditure, and exacerbated body weight gain is associated with irregular fat accumulation, which leads to obesity[30-31] in women with PCOS[30, 32-33], and in animals induced to PCOS [13]. Overweight/obese women with PCOS are at increased risk of developing lipotoxicity due the excess fat free acid into non-adipose cells, including the muscle, liver,

pancreas, and ovaries[30].

Women with PCOS seem to have a level of peripheral insulin resistance that is much like that of women with type 2 diabetes, which is characterized by a 35-40% decrease in insulin-mediated glucose uptake, and insulin resistance might contribute to hyperandrogenism and gonadotropin abnormalities[30, 33]. Insulin resistance and glucose intolerance have also been described in animal models induced by PCOS[2, 13, 20]. The present results showed that up to 30 days after PCOS induction, plasma glucose and insulin levels were normal, with lower concentrations for the MSC group. However, 60 days after PCOS induction, all the groups showed high blood glucose, with only the MSC group showing functional relationships between insulin and glucose levels. MSCs transplantation has been found to reduce glucose levels in diabetic rats[34]. In human, it has been found to attenuate ß cell dysfunction by reversing ß cell differentiation in an IL-1Ra-mediated manner, in response to the elevated expression of proinflammatory cytokines[35]. Soluble factors, extracellular vesicles, and miRNAs secreted by mesenchymal stroma cells can reach many organs, modulating their functions[8]. Therefore, the results suggested that the injection of AdMSCs into the ovaries could have restored follicular steroidogenic activity, especially T synthesis, which modulated plasma glucose and insulin levels. In addition, chemical signals from the AdMSCs injected into the ovaries could have reached the pancreas, modulating insulin secretion.

Animals induced to PCOS develop anovulatory cycles[2, 36-37]. However, the causes for such cyclical changes are variable and depend on the treatment used[38]. In the present study, the PCO group showed changes in ovarian cycles, with the cyclicity returning to normal about 45 days after AdMSCs injection. The observation of ovarian changes, such as the presence of cysts in the ovaries of the PCO group and changes in plasma levels of P4, T, and pituitary gonadotropins, suggested that such cycle changes were probably mainly due to ovarian steroidogenic changes that altered the secretion of pituitary gonadotropins.

The plasma P4 concentrations were lower for the PCO group, compared to the control group, indicative of lower ovarian synthesis due to reduced follicular activity, fewer ovulations, and a reduced quantity of corpus luteum. The plasma P4 concentration in the MSC group was higher than in the PCO group, but lower than in the control group. It is interesting to note that in the MSC group, the ovary morphology did not show cysts, only follicles and corpus luteum. The results suggested that the higher P4 levels in the MSC group were related to the return of ovarian activities such as folliculogenesis, ovulation, and corpus luteum activity. MSC can both reducing nitric oxide synthase reducing fibrose and can also exert their healing effects by donating mitochondria to target cells, as an important mechanism in apoptosis prevention and metabolic damage[7]. In this way, since follicular steroidogenesis occurs in the mitochondrial matrix, of theca and granulosa cells, it appeared that paracrine effects on ovarian steroidogenesis could be driven by AdMSCs.

Rats and mice induced to PCOS present hyperandrogenism, with endocrine, reproductive, and metabolic characteristics like those found in women with PCOS, including adipocyte hypertrophy, insulin intolerance, increase in the size of the ovaries, and anovulatory cycles[28, 39]. In the present work, the MSC group presented plasma T levels like those of the control group. The plasma T levels, ovarian morphology with the presence of cysts, and absence of cyclicity indicated that this animal model was suitable for the intended purpose, especially at 60 days after induction of PCOS, providing a model for reproductive studies related to this syndrome. It is interesting to note that after PCOS induction and AdMSCs injection, plasma T levels did not change in the MSC group, compared to the control group. In addition, the ovary morphology of the MCS group showed a reduction in the number of follicular cysts, together with increases of the number of follicles and corpus luteum, compared to the PCO group, suggesting that the AdMSCs injection strategy would be appropriate for use in studies employing animal models to investigate ovarian dynamics. This potential is based on ovarian actions that are modulated by the factors secreted by the MCSs, including transforming growth factor (TGF), insulin growth factor (IGF-1), hepatocyte growth factor (HGF), interleukin (IL), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF)[7-8,40], and as previously mentioned, MSC can donate mitochondria to target cell, and they can reduce ovary fibrosis and apoptosis activities. However, future studies will be needed for molecular investigations.

Hypergonadism due to LH excess, observed in women with PCOS, is also a characteristic in monkeys[30, 37, 41], sheep[42], and rats[43] induced to PCOS. Such an endocrine change may reduce the sensitivity of the neuroendocrine system to negative feedback from ovarian steroids, especially lower sensitivity to testosterone[2]. Follicular granulosa cells from women with PCOS demonstrated abnormal responses in E2 secretion to gonadotropin stimuli[44]. It has been reported that MSCs improve ovarian function and assist ovarian functional recovery, but it remains unclear whether this effect is achieved

by the differentiation of MSCs into oocytes or by supporting steroidogenesis of the follicular and stromal cells[5, 36, 40, 45]. The results showed that the control group present normal feedback between plasma T levels and pituitary gonadotropins. However, the PCO group showed an increase of plasma LH at 60 days, despite high plasma T concentrations, suggesting lower sensitivity of the pituitary to T. This corroborated the vicious cycle of hyperandrogenemia, ovarian cysts, follicular hyperthecosis, increased LH, greater stimulus for T synthesis by theca cells, increased plasma T, and more cysts. On the other hand, there was an apparent restoration of feedback mechanisms between plasma T and LH in the MSC group, again suggesting paracrine modulation of the ovarian steroidogenesis microenvironment by AdMSCs.

Circulating leptin levels have been positively correlated with body fat, independent of PCOS, according to some studies[46-47], but not others[48-49]. Leptin directly affects steroidogenesis of thecal cells, and normally fluctuating concentrations of leptin in blood may be important in communicating the metabolic status of the animal to the reproductive system[50]. A low dose of leptin was found to increase P4 accumulation by luteinized porcine granulosa cells *in vitro*, whereas a high dose was inhibitory[51]. Leptin deficiency in female mice was associated with impaired folliculogenesis and increased follicular atresia[52]. The results showed that the highest leptin level occurred 30 days after PCOS induction, which could have inhibited ovarian steroidogenesis, since plasma levels of P4 and T were very low. In addition, folliculogenesis could also have been affected by leptin, because the ovarian morphology analyses showed the highest number of ovarian cysts in the PCO group. However, the MSC group showed a reduction in leptin levels, compared to the PCO group. As mentioned earlier, the reduction in leptin levels, the increase in P4 levels, and reduction in the number of ovarian cysts suggested that the injection of AdMSCs into the ovaries could have modulated ovarian activities such as steroidogenesis and folliculogenesis.

Osteocalcin, a polypeptide secreted by osteoblasts, is found at high concentrations in the bone extracellular matrix, and it also possesses several characteristics of a hormone[53]. Studies indicate that osteocalcin can regulate fat mass, insulin secretion, male fertility, and energy expenditure, according to multiple mechanisms, although fasting indices are not always well correlate with insulin resistance[52-53]. The results of the present study revealed metabolic changes that could be associated with OCN, especially in the MSC group. In this group, the lowest OCN concentration could be related to reductions of body weight, ovary weight, and GSI. In other work, osteocalcin decreased fat mass and serum triglycerides levels, with the effect of osteocalcin on fat mass possibly being indirect and secondary to another physiological action[52]. This was corroborated by the present results, with such changes probably being a consequence of the integrated effects of OCN, leptin, and insulin, especially for the 60-day time. However, future studies are needed to clarify these relationships. Osteocalcin can control glucose metabolism by directly affecting pancreatic islet biology, as well as insulin synthesis and secretion. Different to the present results, Hinoi et al.[54], found that insulin secretion was increased by OCN, due to its ability to increase cytosol calcium levels. In the animal model used in the present work, the injection of AdMSCs induced decreases of plasma OCN and leptin, increase of insulin, and decreases of body weight, ovary weight, and GSI, compared to the control and PCO groups. Soluble factors secreted by MSCs include TGF, IGF-1, HGF, IL, and PDGF [8], which can modulate hepatic, adipocyte, pancreas, skeletal muscle, and bone metabolism, affecting insulin synthesis and secretion, glucose metabolism, and body weight. In addition, as PCOS patients and rodent models are characterized by hyperandrogenism, it may not be surprising that OCN levels may change in PCOS[53], and results present here showed the relationship among OCN and testosterone were modulated by AdMSCs, compared to the PCO group.

In conclusion, the results revealed endocrine-metabolic-reproductive functions modulated by AdMSCs in the ovaries. However, despite the results showing changes among the P4, T, leptin, insulin, and OCN levels, and the LH:FSH ratio, it was not possible to determine whether the AdMSCs modulated ovarian activities directly or indirectly. The action of the AdMSCs injected into the ovaries could be direct, with modulation of the ovarian functions involving the hypothalamic-pituitary axis, or indirect, by the effects of leptin, osteocalcin, and insulin on the ovarian dynamics. The findings described here are interesting, because they present data from female rats, in an animal model integrating PCOS with AdMSCs, considering their effects and the relationships among ovaries, bones, and adipocytes. The results suggested the existence of endocrine-metabolic-reproductive microenvironment relationships modulated by AdMSCs, which could assist in guiding further investigations to clarify pathophysiological mechanisms that have not yet been fully elucidated.

Declarations

Conflict of Interest

The authors declare that there are no competing interests. The authors have read the journal's policy on disclosure of potential conflicts of interest and agreed with the journal's authorship statement.

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Ethical Approval

All the experimental procedures were approved by the Committee of Ethics in Animal Use (CEUA/UNIARA, n° 030/2016), following the norms of the National Council for Control of Animal Experimentation (CONCEA/MCTI, Brazil).

Author's contributions

Luís Henrique Montrezor: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. Eduardo Donato Alves: Methodology, Investigation, Writing - review & editing. Luíz Guilherme Dércore Benevenuto: Methodology, Investigation. Janete Aparecida Anselmo-Franci: Methodology, Writing - review & editing. Edilson Ervolino: Methodology. Bruna Pereira de Morais: Methodology, Writing – review & editing. Michele Andrade de Barros: Methodology. Jorge Alberto Achcar: Formal analysis, Writing - review & editing.

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References

[1]. Aflatounian A, Eduwards MC, Paris VR, Bertoldo MJ, Desai R, Glichrist RB. Ledger WL, Handelsman DJ, Walters KA. Androgen signaling pathways driving reproductive and metabolic phenotypes in a PCOS mouse model. J Endocrinol. 2020;245(3):381-395.

[2].Padmanabhan V, Veiga-Lopez A. Sheep models of polycystic ovary syndrome phenotype. Mol Cell Endocrinol. 2013;373:8-20.

[3].Doretto L, Mari FC, Chaves AC. Polycystic ovary syndrome and psychotic disorders. Front Psychiatry. 2020;11:543.

[4]Spallanzani RG. Visceral adipose tissue mesenchymal stroma cells in the intersection of immunology and metabolism. Am J Physiol Endocrinol Metab. 2021;320:E512-E519.

[5]Esfandyari S, Chugh RM, Park H, Hobeika E, Ulin M, Al-Hendy A. Mesenchymal stem cells as a Bio-organ for treatment of female infertility. Cells. 2020;9:2253.

[6]Song N, Scholtemeijer M, Shah K. Mesenchymal stem cell immunomodulation: mechanisms and therapeutic potential. Trends Pharmacol Sci. 2020;41(9):653-664.

[7]Gomez-Salazar M, Gonzales-Galofre ZN, Casamitijana J, Crisan M, James AW, Péault B. 2020 Five decades later, are mesenchymal stem cells still relevant? Front Bioeng Biotechnol. 2003;8:148.

[8]Maacha S, Sidahmed H, Jacob S, Gentilcore G, Calzone R, Grivel JC, Cugno C. Paracrine mechanisms of mesenchymal stromal cells in angiogenesis. Stem Cell Inter. 2020;9:4356359.

[9]Yang K, Oark HJ, Han S, Lee J, Ko E, Kim J, Lee JS, Yu JH, Song KY, Cheong E, Cho SR, Chung S, Cho SW. Recapitulation of in vivo-like paracrine signals of human mesenchymal stem cells for functional neuronal differentiation of human neural stem cells in a 3D microfluidic system. Biomaterials. 2015;63:177-188.

[10]Segers VF, Lee RT. Stem-cell therapy for cardiac disease. Nature. 2008;451:937-942.

[11]Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. Plos One. 2008;3:e1886.

[12]Alves ED, Bonfá ALO, Pigatto GR, Anselmo-Franci JA, Achcar JA, Parizotto NA, Montrezor LH. Photobiomodulation can improve ovarian activity in polycystic ovary syndrome-induced rats. J Photochem Photobiol B Biol. 2019;194:6-13.

[13]Ryu Y, Kim SW, Kim YY, Ku SY. Animal Models for Human Polycystic Ovary Syndrome (PCOS) Focused on the Use of Indirect Hormonal Perturbations: A Review of the Literature. Int J Mol Sci. 2019;20(11):1-20.

[14]Kuang HY, Li Y, Zhang HP, Legro RS, Stener-Victorin E. Data do not support effectiveness of acupuncture for improving live birth rate in women with polycystic ovary syndrome. Chinese J Integ Med. 2018;24(5):399-400.

[15]Caldwell AS, Caldwell ASL, Edwards MC, Desai R, Jimenez H, Gilchrist RB, Handelsman DJ, Walters KA. Neuroendocrine androgen action is a key extraovarian mediator in the development of polycystic ovary syndrome. Proc Natl Acad Sci USA. 2017;114:3334–3343.

[16]Krishnan A, Muthusami S. Hormonal alterations in PCOS and its influence on bone metabolism. J Endocrinol. 2017;232(2):99-113.

[17]Marcondes RR, Maliqueo M, Fornes R, Benrick A, Hu M, Ivarsson N, Carlström M, Cushman SW, Stenkula KG, Maciel GAR, Stener-Victorin E. Exercise differentially affects metabolic functions and white adipose tissue in female letrozole- and dihydrotestosterone-induced mouse models of polycystic ovary syndrome. Mol Cel Endocrinol. 2017;448:66-76.

[18]Walters KA, Allan CM, Handelsman DJ. Rodent models for human polycystic ovary syndrome. Biol Reprod. 2012;86(5):1-12.

[19]Shi D, Vine DF. Animal model of polycystic ovary syndrome: a focused review of rodent models in relationship to clinical phenotypes and cardiometabolic risk. Fert Steril. 2012;98(1):185-193.

[20]Walters KA, Rodriguez PV, Aflatounain A, Handelsman DJ. Androgens and ovarian function: translation from basic discovery research of clinical impact. J Endocrinol. 2019;242(2):R23-R50.

[21]Wickenheisser JK, Nelson-DeGrave VL, McAllister JM. Dysregulation of cytochrome P450 17alpha-hydroxylase messenger ribonucleic acid stability in theca cells isolated from women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2005;90(3):1720-1727.

[22]Paixão L, Ramos RB, Lavarda A, Morsh DM, Spritzer PM. Animal model of hyperandrogenism and ovarian morphology changes as features of polycystic ovary syndrome: a systematic review. Reprod Biol Endocrinol. 2017;15:12.

[23]Stener-Victorin E, Padmanabhan V, Walters KA, Campbell RE, Bernick A, Giacobini P, Dumesic DA, Abbott DH. Animal models to understand the etiology and pathophysiology of polycystic ovary syndrome. Endocr Rev. 2020;41:538-576.

[24]Brawer JR, Munoz M, Farookhi R. Development of the polycystic ovarian condition (PCO) in the estradiol valerate-treated rat. Biol Reprod. 1986;35(3):647-655.

[25]Montrezor LH, Carvalho D, Dias MB, Anselmo-Franci JA, Bícego KC, Gargaglioni LH. Hypoxic and hypercapnic ventilatory responses in rats with polycystic ovaries. Resp Physiol Neurobiol. 2015;217:17–24.

[26]Li C, Li G, Liu M, Zhou T, Zhou H. Paracrine effect of inflammatory cytokine-activated bone marrow mesenchymal stem cell and its role in osteoblast function. J Biosci Bioeng. 2016;121(2):213-219.

[27]Ma Y, Andrisse S, Chen Y, Childress S, Xue P, Wang Z, Jones D, Ko CM, Wu S. Androgen receptor in the ovary theca cells plays a critical role in androgen-induced reproductive dysfunction. Endocrinol. 2017;158(1):98–108.

[28]Caldwell AS, Middleton LJ, Jimenez H, Desai R, Mcmahon AC, Allan CM, Handelsman CJ, Walters KA. Characterization of reproductive, metabolic, and endocrine features of polycystic ovary syndrome in female hyperandrogenic mouse models. Endocrinol. 2014;155:3146-3159.

[29]Van Houten EI, Kramer P, Mcluskey A, Karels B, Themmen AP, Visser JA. Reproductive and metabolic phenotype of a mouse model of PCOS. Endocrinol. 2012;153(6):2861–2869.

[30]Dumesic DA, Padmanabhan V, Chazenbalk GD, Abbott DH. Polycystic ovary syndrome as a plausible evolutionary outcome of metabolic adaptation. Reprod Biol Endocrinol. 2022;20:12.

[31]Oliveira M, Mathias LS, Rodrigues BM, Mariani BG, Graceli JB, De Sibio MT, Olimpio RMC, Moretto FCF, Deprá IC, Nogueira CR. The roles of triiodothyronine and irisin in improving the lipid profile and directing the browning of human adipose subcutaneous cells. Mol Cell Endocrinol. 2020;506:110744.

[32]Amirkhanloo F, Esmaeilzadeh S, Mirabi P, Abedini A, Amiri M, Saghebi R, Golsorkhtabaramiri M. Comparison of Foeniculum Vulgare versus metformin on insulin resistance and anthropometric indices of women with polycystic ovary, an open-label controlled trial study. Obesity Medicine. 2022;31:100401.

[33]Norman RJ, Dewailly D, Legro RS, Hickey TE. Polycystic ovary syndrome. The Lancet. 2007;370:685-597.

[34]Domouky AM, Hegab AS, Al-Shahat A, Raafat N. Mesenchymal stem cells and differentiated insulin producing cell are new horizons for pancreatic regeneration in type I diabetes mellitus. Int J Biochem Cell Biol. 2017;87:77-85.

[35]Wang L, Liu T, Liang R, Wang G, Liu Y, Zou J, Liu N, Zhang B, Liu Y, Ding X, Cai S, Wang Z, Xu X, Ricordi C, Wang S, Shen Z. Mesenchymal stem cells ameliorate ß cell dysfunction of human type 2 diabetic islet by reversing ß cell dedifferentiation. EBioMedicine. 2020;51:102615.

[36]Xie Q, Xiong XL, Xiao N, He K, Chen M, Peng J, Su X, Mei H, Dai Y, Wei D, Lin, G, Cheng L. Mesenchymal stem cells alleviate DHEA-induced polycystic ovary syndrome (PCOS) by inhibiting inflammation in mice. Stem Cells Inter. 2019;9782373.

[37]Abbott DH, Barnett DK, Bruns CM, Dumesic SA. Androgen excess fetal programming of female reproduction: a developmental aetiology for polycystic ovary syndrome? Hum Reprod Update. 2005;11:357-374.

[38]Sullivan SD, Moenter SM. Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common fertility disorder. Proc Natl Acad Sci USA. 2004;101:7129-7134.

[39]Kawa IA, Masood A, Ganie MA, Fatima Q, Jeelani H, Manzoor S, Rizvi SM, Muzamil M, Rashid F. Bisphenol A (BPA) acts as an endocrine disruptor in women with polycystic ovary syndrome: hormonal and metabolic evaluation. Obesity Medicine. 2019;14:100090.

[40]Malard PF, Peixer MAS, Grazia JG, Brunel HSS, Feres LF, Villarroel CL, Siqueira LGB, Dode MAN, Pogue R, Viana JHM, Carvalho JL. Intraovarian injection of mesenchymal stem cells improve oocyte yield and in vitro embryo production in a bovine model of fertility

loss. Scientific Reports. 2020;10:8018.

[41]Dumesic DA, Abbott DH, Eisner JR, Goy RW. Prenatal exposure of female rhesus monkey to testosterone propionate increases serum luteinizing hormone levels in adulthood. Fertil Steril. 1997;67:155-163.

[42]Sharma TP, Herkimer C, West C, Ye W, Birch R, Robinson JE, Foster DL, Padmanabhan V. Fetal programming: prenatal androgen disrupts positive feedback actions of estradiol but does not affect timing of puberty in female sheep. Biol Reprod. 2020;66:924-933.

[43]Wu XY, Li ZL, Wu CY, Liu Y-M, Lin H, Wang S-H, Xiao W-F. Endocrine traits of polycystic ovary syndrome in prenatally androgenized female Sprague-Dawley rats. Endocr J. 2010;57:201-209.

[44]Harrison TNH, Chang RJ. Ovarian response to follicle-stimulating hormone in women with polycystic ovary syndrome is diminished compared to ovulatory controls. Clin Endocrinol. 2020;97:310-318.

[45]Zhao YX, Chen SR, Su PP, Huang FH, Shi YC, Shi QY, Lin S. Using mesenchymal stem cells to treat female infertility: an update on female reproductive diseases. Stem Cells Int. 2019;9071720.

[46]Tan BK, Chen J, Hu J, Amar O, Mattu HS, Adya R, Patel VH, Ramanjaneya M, Lehnert H, Randeva HS. Metformin increases the novel adipokine caronectin/CTRP3 in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2013;98:E1891-900.

[47]Plati E, Kouskouni E, Malamitsi-Puchner A, Boutsikou M, Kaparos G, Baka S. Visfatin and leptina levels in women with polycystic ovaries undergoing ovarian stimulation. Fertil Steril. 2010;94,:451-1456.

[48]Barber TM, Franks S. Adipocyte biology in polycystic ovary syndrome. Mol Cell Endocrinol. 2013;373:68-76.

[49]Svendsen PF, Christiansen M, Hedley PL, Nilas L, Pedersen SB, Madsbad S. Adipose expression of adipocytokines in women with polycystic ovary syndrome. Fertil Steril. 2012;98:235-41.

[50]Swain JE, Dunn RL, McConnell D, Gonzales-Martinez J, Smith GD. Direct effects of leptin on mouse reproductive function: regulation of follicular, oocyte, and embryo development. Biol Reprod. 2004;71(5):1446-1452.

[51]Ruiz-Cortés ZT, Martel-Kennes Y, Gévry NY, Downey BR, Palin M-F, Murphy BD. Biphasic effects of leptin in porcine granulosa cells. Biol Reprod. 2003;68(3):789-796.

[52]Ferron M, Lacombe J. Regulation of energy metabolism by the skeleton: osteocalcin and beyond. Arch Biochem Bioph. 2014;561:137-146.

[53]Shan C, Yue J, Liu W. Broadening the role of osteocalcin in the hypothalamic-pituitary-gonadal axis. J Edocrinol. 2021;249(2):R43-R51.

[54]Hinoi E, Gao N, Jung DY, Yadav V, Yoshizawa T, Myers Jr MG, Chua Jr SC, Kim JK, Kaestner KH, Karsenty G. The sympathetic tone mediates leptin's inhibition of insulin secretion by modulating osteocalcin bioactivity. J Cell Biol. 2008;183:1235-1242.