ENPP1 Is Correlated With Insulin Resistance and Lipid Molecules in PCOS Rats

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Research

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Abstract

Background

Previous studies have shown that ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) may be an inhibitor of the insulin signalling pathway, and insulin resistance (IR) is believed to be the core mechanism in the pathophysiology of polycystic ovarian syndrome (PCOS). This study aimed to investigate the expression of ENPP1 in different tissues of PCOS rats and to analyse its potential role in the pathophysiology of PCOS.

Methods

Eighteen 23-day-old Sprague-Dawley rats were divided into the PCOS and control groups (n= 9/group). Serum, ovaries, skeletal muscle, and subcutaneous and visceral fat were collected after 20 days. Pathological examination, immunofluorescence and western blotting analyses were performed. Serum indicator levels were measured, including ENPP1, follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), monocyte chemoattractant protein-1 (MCP-1), fasting blood glucose (FBG), fasting insulin (FINS), free fatty acids (FFAs), adiponectin (ADP), leptin, and serum lipids.

Results

The levels of ENPP1, T, MCP-1, FBG, FINS, homeostasis model assessment of IR (HOMA-IR), FFAs, leptin, cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) were significantly higher in the PCOS group, while ADP and high-density lipoprotein cholesterol (HDL-C) were significantly lower than in the control group. Spearman's rank correlation analysis showed that ENPP1 was correlated with T, MCP-1, HOMA-IR, FFAs, leptin, serum lipids and ADP. The mRNA levels of ENPP1, BAX, and IRS1 were higher in the ovaries, skeletal muscle, subcutaneous fat, and visceral fat of PCOS rats, and the protein expression of ENPP1 was significantly higher in the ovaries.

Conclusion

ENPP1 is highly associated with IR and lipid metabolism-related molecules, which may promote pathophysiological changes in PCOS.

Background:

Polycystic ovarian syndrome (PCOS) is the most common endocrine condition in women of reproductive age, with a prevalence of 5 to 10%^[1]. Menstrual abnormalities, symptoms of androgen excess, and obesity are some of the clinical symptoms of PCOS. Apart from that, patients with PCOS also exhibit metabolic abnormalities, i.e., insulin resistance (IR), metabolic syndrome, hyperinsulinaemia, and dyslipidaemia^[2, 3]. According to reports, approximately 77% of patients with PCOS had IR, while 10% of those patients had type 2 diabetes (T2D)^[4]. In addition, the prevalence of metabolic syndrome in women

with PCOS was increased by 43%^[5]. Although the pathogenesis mechanism has not been well defined, IR is believed to be an important pathogenic factor^[6].

Ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1), also known as plasma cell membrane glycoprotein PC-1, is a type II transmembrane glycoprotein containing pyrophosphatase and phosphodiesterase enzymatic activity that is highly expressed in bone and cartilage^[7]. It plays a key role in phosphate balance central to bone mineralization by hydrolysing extracellular nucleotide triphosphates to produce pyrophosphate^[8]. Meanwhile, mutation of ENPP1 is related to generalized arterial calcification of infancy^[9]. ENPP1 is also considered to be a promising candidate for antitumour targets, as well as a new biomarker for aggressive tumours^[10]. Scientists postulated a role for ENPP1 in the insulin signalling pathway for the first time in 1995, concluding that overexpression of ENPP1 dramatically reduces insulin receptor function^[11]. ENPP1 binds to the insulin receptor β -subunit and affects IR signalling by blocking its autophosphorylation, thereby blocking the phosphorylation of insulin receptor substrate-1 and glucose transport, inhibiting the insulin signal transduction pathway, and contributing to the development of IR^[12–14]. Apart from that, ENPP1 also modulates obesity, T2D, and diabetic consequences by affecting IR function in many tissues^[15]. Therefore, in recent years, numerous studies have investigated the role of ENPP1 in T2D and suggested that its polymorphisms may be highly associated with T2D susceptibility and IR^[16–18].

Disturbance of lipid metabolism is one of the manifestations of PCOS. Studies have shown that overexpression of ENPP1 in adipocytes induces fatty liver, hyperlipidaemia, and dysglycaemia^[19]. In addition, genetic variants of the ENPP1 gene were associated with hypertriglyceridaemia in men and may contribute to the development of metabolic syndrome in this population^[20].

To the best of our knowledge, only a few studies regarding ENPP1 in the pathophysiology of PCOS have been reported. In this study, a DHEA-induced rat PCOS model was established according to previous works^[21, 22]. We postulated that increased expression of ENPP1 in various tissues in PCOS rats was related to IR and abnormal levels of lipid metabolism-related molecules, which may promote the occurrence of PCOS.

Methods:

Animals

Eighteen female Sprague-Dawley adult rats (aged 23 days, weight 70–90 g) were purchased from the Laboratory Animal Center of Wuhan University (Wuhan, China). The rats were allowed to adapt to the environment for three days. All animals were grouped and housed in a room ($22 \pm 2^\circ\text{C}$, 12-h light/12-h dark cycle) with free access to food and water.

Rats were randomly divided into the PCOS group (n = 9) and the control group (n = 9). The animals in the PCOS group were subcutaneously injected with 6 mg/100 g DHEA (Aladdin Reagent Co., Ltd., Shanghai, China), which was dissolved in olive oil, once a day for 20 consecutive days. The animals in the control group received subcutaneous injections of equivalent olive oil alone.

The procedures involving rats and their care were carried out in accordance with the NIH guidelines (NIH Pub. No. 85–23, revised 1996). The experimenter obtained the certificate of qualification of laboratory animal professional technical examination (W20190028). Our study was approved by the Laboratory Animal Welfare & Ethics Committee of Renmin Hospital of Wuhan University (IACUC Issue No. WDRM 20200909).

Sample collection

After the last administration, all the rats fasted for 12 h. Then, all the rats were anaesthetized with isoflurane in the early morning of the following day. Immediately after opening the abdominal cavity, one ovary was removed and fixed in 4% paraformaldehyde, followed by paraffin embedding, and the other ovary was immediately stored at – 80°C. Then, the subcutaneous fat and visceral fat were separated. After cutting the leg skin, the skeletal muscles were separated. Two pieces of skeletal muscle, subcutaneous fat and visceral fat, each weighing approximately 2 g, were collected from each animal. One was immediately fixed in 4% paraformaldehyde, followed by paraffin embedding, and the other was immediately stored at – 80°C. After tissue collection was completed, the chest was opened. Blood was extracted from the heart, placed on ice for 20 minutes, and then separated by centrifugation at 3000 rpm for 20 minutes. The upper layer of serum was collected and stored at – 80°C.

Serum analysis

According to the manufacturer's instructions, commercial enzyme-linked immunosorbent assay (ELISA) kits (Bioswamp, Wuhan, China) were used to measure the serum concentrations of follicle-stimulating hormone (FSH) (RA20044), luteinizing hormone (LH) (RA20133), testosterone (T) (RA20653), fasting insulin (FINS) (RA20092), adiponectin (ADP) (RA20554), leptin (RA20489), free fatty acids (FFAs) (RA20313), and monocyte chemoattractant protein-1 (MCP-1) (RA20492). The serum concentration of ENPP1 was assayed using an ENPP1 ELISA kit (JYM1287Ra, Jiyinmei, Wuhan, China). Enzymatic colorimetric kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to determine the levels of serum fasting blood glucose (FBG) (F006-1-1), total cholesterol (TC) (A111-1-1), triglyceride (TG) (A110-1-1), high-density lipoprotein cholesterol (HDL-C) (A112-1-1), and low-density lipoprotein cholesterol (LDL-C) (A113-1-1). The homeostasis model assessment of IR (HOMA-IR) was calculated using the following formula: $HOMA-IR = [FBG (mmol/l) \times FINS (mU/l)] / 22.5$.

Haematoxylin-eosin (H&E) staining

Ovaries, skeletal muscles, subcutaneous fat, and visceral fat were embedded in paraffin, serially sliced into 4-µm-thick sections, and stained with H&E according to the operating instructions (Servicebio, Beijing, China). The above sections were observed under a microscope (NanoZoomer S360, Hamamatsu, Japan).

ImageJ software (Version 1.53a; NIH, Bethesda, MD, USA) was used to analyse the cross-sectional area (CSA) of skeletal muscles and the mean cell area of subcutaneous fat and visceral fat.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

mRNA levels of ENPP1, BCL2-associated X protein (BAX), and insulin receptor substrate 1 (IRS1) were extracted with TRIzol reagent (Accurate Biology, Changsha, China) from ovaries, skeletal muscle, subcutaneous fat, and visceral fat of rats. RNA was reverse transcribed into cDNA using an Evo M-MLV RT Mix Kit with gDNA Clean for qPCR (Accurate Biology, Changsha, China) according to the manufacturer's protocols. qRT-PCR detection of target genes was performed using the SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, Changsha, China) on a Bio-Rad system, and the specific primers are shown in Table 1. GAPDH served as an internal reference. The amplification conditions were as follows: an initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

Table 1

Primers of the genes used in qRT-PCR	
GAPDH-F	CGCTAACATCAAATGGGGTG
GAPDH-R	TTGCTGACAATCTTGAGGGAG
ENPP1-F	GAAAGACCACACTTTTACACTC
ENPP1-R	TTACAACCTGCCTTGTTCCATGCC
BAX-F	GATGGCCTCCTTTCCTACTTC
BAX-R	CTTCTTCCAGATGGTGAGTGAG
IRS1-F	GCCTGGAGTATTATGAGAACGAGAA
IRS1-R	GGGGATCGAGCGTTTGG

Immunofluorescence (IF) staining

Sections of ovaries were used for immunofluorescence staining following a previously described protocol^[23]. Briefly, paraffin sections were incubated with 10% goat serum in PBS for 30 min to block nonspecific binding of the antibody. Then, the sections were incubated with 1:800 rabbit anti-rat ENPP1 antibody (Bioss, Beijing, China) overnight at 4°C. After washing, the sections were incubated with 1:4000 goat anti-rabbit IgG (HRP) (Boster, Wuhan, China) at 37°C for 45 min and then incubated with 1:300 FITC (Servicebio, Beijing, China) for 10 min. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Servicebio, Beijing, China) at a dilution of 1:500 for 5 min. An Olympus laser scanning confocal microscope (BX53) was used for observation and photography.

Western blotting

Proteins of ovarian tissues were extracted in RIPA buffer in the presence of phosphatase and protease inhibitors. After separation by SDS-PAGE gels, protein samples were transferred onto PVDF membranes. After 5% nonfat milk was added, the membranes were blocked at room temperature. The membranes were incubated with 1:2000 rabbit anti-rat ENPP1 antibody (Bioss, Beijing, China) overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Servicebio, Beijing, China) at room temperature for 1 h. The protein bands were visualized by chemiluminescence reagent (Servicebio, Beijing, China) and analysed by ImageJ software.

Statistical analysis

All data were analysed using SPSS 26.0 statistical software (IBM, Armonk, NY, USA) and expressed as the mean $\pm$ standard deviation (SD). Differences between the two groups were compared using unpaired t-tests (data with normal distribution) or Mann–Whitney U tests (data with a skewed distribution). Spearman's rank correlation analysis was used to analyse the rank correlation between data. GraphPad Prism software was used to plot bar graphs. Differences between groups with $P < 0.05$ were considered significant.

Results:

Pathological observations

H&E staining showed reduced granulosa cell layers, increased theca cell layers, more follicular cysts and atretic follicles, fewer follicles at all levels, and fewer corpora lutea in the ovaries of the PCOS group compared with the control group (Fig. 1). The cross-sections of skeletal muscles showed a significantly larger CSA of skeletal muscle in PCOS rats than control rats (Fig. 2A, B). The mean area of visceral fat cells and subcutaneous fat cells was significantly greater in PCOS rats than in control rats (Fig. 2C, D, E, F).

Hormonal and biochemical indicators in serum

The serum levels of FSH, LH, LH/FSH, T, MCP-1, and ENPP1 in the PCOS group were significantly higher than those in the control group (Fig. 3). In addition, the PCOS group showed significantly higher levels of FBG, FINS and HOMA-IR index (Fig. 4). In addition, the PCOS group had significantly higher serum levels of FFAs, leptin, TC, TG, and LDL-C and significantly lower ADP and HDL-C levels than the control group (Fig. 5).

Correlation of serum ENPP1 with hormones and molecules

Correlations between ENPP1 and the HOMA-IR, hormones and lipid levels were observed (Tables 2, 3). The results of Spearman's rank correlation analysis showed that ENPP1 had a strong positive correlation

with T, HOMA-IR, MCP-1, FFAs, leptin, TC, TG, and LDL-C. In addition, ENPP1 also showed a strong negative correlation with ADP. However, there was no significant correlation between ENPP1 and HDL-C.

Table 2
Spearman rank analysis for the correlation of ENPP1 with hormones and indicators related to glucose metabolism

		ENPP1	FSH	LH	T	FBG	FINS	HOMA-IR
FSH	r	.796**						
	P	.000						
LH	r	.713**	.818**					
	P	.001	.000					
T	r	.785**	.719**	.738**				
	P	.000	.001	.000				
FBG	r	.193	.193	.257	.430			
	P	.443	.443	.303	.075			
FINS	r	.662**	.690**	.765**	.732**	.349		
	P	.003	.002	.000	.001	.156		
HOMA-IR	r	.577*	.540*	.593**	.686**	-.227	.847**	
	P	.012	.021	.009	.002	.365	.000	
MCP-1	r	.773**	.839**	.792**	.684**	.359	.527*	.503*
	P	.000	.000	.000	.002	.143	.025	.034

Spearman rank's analysis for the correlation of ENPP1 with HOMA-IR and hormones. * P < 0.05, ** P < 0.01.

Table 3

Spearman rank analysis for the correlation of ENPP1 with indicators related to lipid metabolism

		ENPP1	FFA	ADP	Leptin	TC	TG	HDL-C
FFA	r	.550*						
	P	.018						
ADP	r	-.655**	-.453					
	P	.003	.059					
Leptin	r	.688**	.839**	.686**				
	P	.002	.000	.002				
TC	r	.779**	.699**	-.589*	.695**			
	P	.000	.001	.010	.001			
TG	r	.680**	.545*	-.666**	.630**	.702**		
	P	.002	.019	.003	.005	.001		
HDL-C	r	-.451	-.482*	.597**	-.620**	-.497*	.701**	
	P	.060	.043	.009	.006	.036	.001	
LDL-C	r	.755**	.602**	-.462	.642**	.901**	.664**	.767**
	P	.000	.008	.054	.004	.000	3.000	.000

Spearman rank's analysis for the correlation of ENPP1 with lipid levels. * $P < 0.05$, ** $P < 0.01$.

Expression of mRNAs in ovaries, skeletal muscles, visceral fat, and subcutaneous fat

In the ovaries and skeletal muscle, ENPP1 mRNA, BAX mRNA, and IRS1 mRNA were significantly higher in the PCOS group than in the control group ($P < 0.05$) (Fig. 6A, B). In visceral fat, ENPP1 mRNA was significantly higher in the PCOS group ($P < 0.05$). Although BAX mRNA and IRS1 mRNA levels were higher in the PCOS group, no significant difference was found (Fig. 6C). In addition, in subcutaneous fat, ENPP1 mRNA and BAX mRNA were significantly higher in the PCOS group than in the control group ($P < 0.05$). Although IRS1 mRNA was higher in the PCOS group, the difference was not significant (Fig. 6D).

Expression of ENPP1 in ovarian tissues

IF staining of ovaries showed that ENPP1 was mainly expressed in the cytoplasm and cell membrane (Fig. 7A). In addition, western blotting analysis showed that the expression level of ENPP1 was significantly higher in the ovaries of the PCOS group than in the control group ($P < 0.05$) (Fig. 7B, C).

Discussion:

In this study, DHEA-treated rats were used to simulate endocrine disorders and ovarian pathological changes in PCOS. The ovaries of PCOS rats showed polycystic changes. The CSA of skeletal muscle and the mean cell area of visceral fat and subcutaneous fat were significantly greater in PCOS rats than in control rats. We found that ENPP1 was expressed at higher levels in the ovaries, skeletal muscles, visceral fat, and subcutaneous fat of PCOS rats than in control rats. The serum levels of ENPP1, FSH, LH, T, FBG, FINS, MCP-1, FFAs, leptin, TC, TG, and LDL-C were significantly higher, and the levels of ADP and HDL-C were significantly lower in the PCOS group than in the control group. In addition, the concentration of ENPP1 was highly correlated with the levels of T, HOMA-IR, MCP-1, FFAs, ADP, leptin, TC, TG, and LDL-C.

Androgens are well known to increase muscle strength and mass, and muscle size is positively correlated with serum androgen levels in women with PCOS^[24]. The DHEA rats in this study mimicked the increased androgens in PCOS patients, and it led to an increase in skeletal muscle fibre CSA, which represents muscle hypertrophy. In addition, excess androgens can also lead to visceral obesity, i.e., hypertrophy of visceral and subcutaneous fat cells, as verified in this study, as well as dyslipidaemia. It is believed that androgen excess leads to PCOS exhibiting IR and visceral obesity and further promotes androgen secretion from the ovaries and adrenal glands, thus fostering a vicious cycle of PCOS^[25].

The abnormal expression of indicators in the serum of PCOS rats reflected the possible existence of IR and lipid metabolism disorders in PCOS. The success of the modelling was verified by a significant increase in T in the serum of PCOS rats. Serum MCP-1 was significantly higher in PCOS rats than in controls, further confirming the pathogenesis of PCOS from the viewpoint of the presence of a chronic low-grade inflammatory basis^[22]. FFAs have a close relationship with IR because FFAs are involved in the production of lipid metabolites, proinflammatory cytokines (TNF- α , IL-1 β , IL-6, MCP-1), and cellular stress, such as oxidative and endoplasmic reticulum stress^[26]. The above processes may cause the development of IR. In addition, ADP and leptin were reported to be associated with IR, independent of obesity^[27]. Moreover, it was reported that PCOS patients had considerably higher TG/HDL-C, TC/HDL-C, and LDL-C/HDL-C ratios than age-matched healthy women and a strong positive connection with HOMA-IR^[28]. In this study, Spearman's rank correlation analysis showed a strong correlation between ENPP1 and almost all of the detected indicators, which suggested that ENPP1 was highly correlated with IR and lipid metabolism in PCOS.

ENPP1 plays a role in the female reproductive system. A case-control study reported that the ENPP1 level in echo-guided aspirated fluids of endometriomas was considerably greater than that in the control group^[29]. ENPP1 was also shown to be closely linked to ovarian cancer patient prognosis, and ENPP1 levels rose in ovarian cancer patients, suggesting that ENPP1 may promote migration^[30, 31]. Some scientists have proposed a possible mechanism of action of ENPP1 in IR. A study suggested that when the missense variant K121 of the ENPP1 gene occurred, in which lysine (K) was replaced by glutamine (Q), it predisposed patients to IR^[32]. ENPP1 carrying the Q121 variant had increased physical interaction

with the insulin receptor at the cell membrane and became a stronger inhibitor of insulin signalling than ENPP1 carrying K121. This further affected insulin-stimulated receptor autophosphorylation and subsequent stimulation of IRS1 phosphorylation, PI3-kinase activation, and glycogen synthesis. Although there are only a few reports about ENPP1 in PCOS, it has been shown that the K121Q variant in the ENPP1 gene is significantly linked to PCOS risk^[33].

ENPP1 is significantly upregulated in cancerous tissues, may be an initiator of malignant transformation of normal epithelial cells, and increases cell proliferation, migration, and invasion^[7, 34]. Compared with the control group, we found that ENPP1 levels were significantly higher in both the ovaries and serum of PCOS rats. In addition, IF staining of ovaries showed that ENPP1 was mainly expressed in the cytoplasm and cell membrane. ENPP1 was more highly expressed in the granulosa cells of PCOS rats, suggesting that elevated ENPP1 was associated with higher proliferation rates and lower apoptosis rates in ovarian granulosa cells of PCOS rats.

C-Jun amino-terminal kinase (JNK)-IRS1-BAX is a signalling pathway reflecting IR, glucose homeostasis and apoptosis^[35]. Since JNK requires testing for its phosphorylation and cannot be detected by qRT-PCR, we only tested two downstream indicators, namely, IRS1 and BAX. IRS1 is a major protein involved in insulin signalling that activates several signalling pathways involved in the regulation of glucose uptake, protein synthesis and gene expression^[35]. BAX is a key proapoptotic protein of the BCL-2 family and a major regulator of the mitochondrial pathway^[36]. In the ovaries and skeletal muscle, the mRNA levels of ENPP1, BAX, and IRS1 were higher in the PCOS group than in the control group, and the level of ENPP1 mRNA was significantly correlated with BAX mRNA, IRS1 mRNA, and the HOMA-IR index. In subcutaneous fat and visceral fat, the level of ENPP1 mRNA was higher in the PCOS rats than in the control group, and it was strongly positively correlated with the HOMA-IR index. This finding suggested that ENPP1 was highly associated with apoptosis of ovarian granulosa cells and the insulin pathway in the ovary. Meanwhile, it may also be involved in the process of IR in skeletal muscle, subcutaneous fat, and visceral fat, thus changing the general state of PCOS rats.

The strength of this study is that we systematically explored the expression of ENPP1 in different tissues of PCOS rats and its correlation with IR and lipid metabolism-related molecules, which has not been thoroughly investigated. However, one of the limitations of our study is that we have only preliminarily discussed the correlation between ENPP1, IR and lipid metabolism-related molecules. The mechanism of ENPP1 in PCOS has not yet been determined. Second, the rats modelled by DHEA cannot fully simulate all the physiological characteristics of PCOS patients. Therefore, our next research plan is to collect the blood and follicular fluid of PCOS patients, analyse the expression of ENPP1 in human specimens and regulate the expression of ENPP1 in a human granulosa-like tumour cell line to further explore the role of ENPP1 in pathology and pathological processes.

Conclusion:

ENPP1 expression was significantly higher in the ovaries, skeletal muscle, subcutaneous fat, and serum of PCOS rats. The abnormal expression of ENPP1 was highly correlated with insulin resistance and lipid metabolism-related molecules, suggesting that ENPP1 may play an important role in the process of insulin resistance in PCOS.

Abbreviations:

PCOS, polycystic ovarian syndrome; IR, insulin resistance; T2D, type 2 diabetes; ENPP1, ectonucleotide pyrophosphatase phosphodiesterase 1; CSA, cross-sectional area; FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone; FINS, fasting insulin; ADP, adiponectin; FFAs, free fatty acids; MCP-1, monocyte chemoattractant protein-1; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; H&E, haematoxylin-eosin; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; IF, immunofluorescence; JNK, c-Jun amino-terminal kinases; BAX, BCL2-associated X; IRS1, insulin receptor substrate 1; DAPI, 4',6-diamidino-2-phenylindole; SD, standard deviation.

Declarations:

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Author contribution statement

GXW and JY led the entire project. JX evaluated all data. JX, YQY and ZY designed and performed the experiments. JX wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Figures

Figure 1

Comparison of rat ovarian structure (H&E staining, ×4 magnification). The ovaries of the control group showed different stages of the corpus luteum and follicles. The granulosa cells were arranged in order with intact morphology, mostly with 6-8 layers. The PCOS group showed an increase in cystic follicles.

Follicles and corpus luteum at different developmental stages were rare. The number of granulosa cells decreased to 2 to 3 layers, the follicular cell layer became thicker, and the follicular membrane cells proliferated. PCOS, polycystic ovarian syndrome. Scale bar 500 μm .

Figure 2

Pathological examination images of skeletal muscle, visceral fat, and subcutaneous fat in control and PCOS groups (H&E staining). (A) skeletal muscle cross-sections images of two groups ($\times 200$ magnification, scale bar 100 μm); (B) CSA of skeletal muscles; (C) visceral fat images of two groups ($\times 400$ magnification, scale bar 50 μm); (D) Mean area of visceral fat cells; (E) subcutaneous fat images of control and PCOS groups ($\times 400$ magnification, scale bar 50 μm); (F) Mean area of subcutaneous fat cells. PCOS group showed significantly greater CSA of skeletal muscle than control group. Besides, the mean cell areas of visceral fat and subcutaneous fat were significantly greater in PCOS group than control group. Data were analysed using unpaired t-tests. Values represent the mean $\pm$ SD. PCOS, polycystic ovarian syndrome; CSA, cross-sectional area. SD, standard deviation. **: $P < 0.001$.

Figure 3

Concentrations of serum hormone detected by ELISA kits (mean $\pm$ SD, n=9 per group). (A) Serum FSH levels; (B) Serum LH levels; (C) Serum LH/FSH levels; (D) Serum T levels; (E) Serum MCP-1 levels; (F) Serum ENPP1 levels. The serum levels of FSH, LH, LH/FSH, T, MCP-1 and ENPP1 in PCOS group were significantly higher than those in the control group. Data were analysed using unpaired t-tests. Values represent the mean $\pm$ SD. FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone; MCP-1, monocyte chemoattractant protein-1; ENPP1, ectonucleotide pyrophosphatase phosphodiesterase 1; PCOS, polycystic ovarian syndrome; SD, standard deviation. *: P < 0.05; **: P < 0.001.

Figure 4

Levels of FBG, FINS and HOMA-IR index (mean $\pm$ SD, n=9 per group). (A) Serum FBG levels; (B) Serum FINS levels. (C) levels of HOMA-IR index. PCOS group showed significantly higher levels of serum FBG and FINS than control group. Besides, the HOMA-IR index of PCOS group was significantly higher than that of control group. Data were analysed using unpaired t-tests. Values represent the mean $\pm$ SD. FBG, fasting blood glucose; FINS, fasting insulin; HOMA-IR, homeostasis model assessment of insulin resistance; PCOS, polycystic ovarian syndrome; SD, standard deviation. *: P < 0.05; **: P < 0.001.

Figure 5

Serum levels of lipid-metabolism related molecules (mean $\pm$ SD, n=9 per group). (A) Serum FFAs levels; (B) Serum ADP levels; (C) Serum leptin levels; (D) Serum TC levels; (E) Serum TG levels; (F) Serum HDL-C

levels; (G) Serum LDL-C levels. PCOS group showed significantly higher levels of FFAs, leptin, TC, TG, LDL-C and significantly lower levels of ADP and HDL-C than those in control group. Data were analysed using unpaired t-tests. Values represent the mean $\pm$ SD. FFAs, free fatty acids; ADP, adiponectin; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. PCOS, polycystic ovarian syndrome; SD, standard deviation. *: $P < 0.05$; **: $P < 0.001$.

Figure 6

MRNA expression levels of ENPP1, BAX, IRS1 in ovarian tissues, skeletal muscles, visceral fat, and subcutaneous fat (mean $\pm$ SD, $n=5$ per group). (A) MRNA levels in the ovary; The mRNA expression levels of ENPP1, BAX and IRS1 in the ovarian tissues of PCOS group were significantly higher than those in control group. (B) MRNA levels in the skeletal muscle; The mRNA expression levels of ENPP1, BAX and IRS1 in the skeletal muscles of PCOS group were significantly higher than those in control group. (C) MRNA levels in the visceral fat; The mRNA expression levels of ENPP1 in the visceral fat of PCOS group was significantly higher than control group. MRNA levels of BAX and IRS1 were higher in PCOS group, but the differences were not significant. (D) MRNA levels in the subcutaneous fat; The mRNA expression levels of ENPP1 and BAX in the subcutaneous fat of PCOS group were significantly higher than those in the control group. Level of IRS1 was higher in PCOS group, but the difference was not significant. Data were analysed using unpaired t-tests. Values represent the mean $\pm$ SD; SD, standard deviation; ENPP1,

ectonucleotide pyrophosphatase phosphodiesterase 1; BAX, BCL2-associated X; IRS1, insulin receptor substrate 1; PCOS, polycystic ovarian syndrome; SD, standard deviation. *: $P < 0.05$; **: $P < 0.001$.

Figure 7

Expression of ENPP1 protein in ovarian tissues using IF and western blotting analysis. (A) The IF images of ENPP1 expression in ovarian tissues ($\times 200$ magnification, scale bar $100\ \mu\text{m}$). Green color (FITC) and blue color (DAPI) represents the results of ENPP1 in the ovarian tissues. ENPP1 was mainly expressed in the cytoplasm and cell membrane. (B) Representative western blotting bands. (C) Bar graph represents the level of ENPP1, normalized to the total amount of GAPDH protein (mean $\pm$ SD, $n=5$ per group). Protein expression level of ENPP1 in the PCOS group was significantly higher than control group. IF, immunofluorescence; DAPI, 4',6-diamidino-2-phenylindole; SD, standard deviation; PCOS, polycystic ovarian syndrome. **: $P < 0.001$.