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Granulosa cells from immature follicles exhibit restricted glycolysis and reduced energy production: a dominant problem in polycystic ovary syndrome

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Abstract

Purpose We hypothesized that immature oocytes are associated with impaired energy production in surrounding granulosa cells (GCs) in polycystic ovary syndrome (PCOS). Thus, this study investigated mitochondrial function, determined expression of glycolytic regulatory enzymes, and measured ATP levels in GCs of PCOS patients.

Methods GCs were isolated from forty-five PCOS patients and 40 control women. Intracellular concentration of reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\psi_m$), the rate of glycolysis, total antioxidant capacity (TAC), activities of catalase (CAT) and superoxide dismutase (SOD), and ATP level were measured in GCs. The gene expression and protein levels of glycolytic enzymes (hexokinase, muscular phosphofructokinase, platelet derived phosphofructokinase, and muscular pyruvate kinase) were determined. Association of GC energy level with oocyte maturation was further validated by measuring glycolysis rate and ATP level in GCs isolated from mature and immature follicles from new set of fifteen PCOS patients and 10 controls.

Results PCOS patients showed higher ROS level, decreased TAC, reduced CAT and SOD activities, and lower $\Delta\psi_m$ together with reduced expression of key glycolytic enzymes. ATP concentration and biochemical pregnancy were lower in PCOS compared with control group. ATP levels were found to be significantly correlated with ROS and $\Delta\psi_m$ ($r = -0.724$ and $r = 0.487$, respectively). GCs isolated from immature follicles had significantly lower ATP levels and rate of glycolysis compared with the GCs separated from mature follicles in both PCOS patients and control.

Conclusion Declined energy due to the mitochondrial dysfunction and restrained glycolysis in GCs is associated with the immature oocytes and lower biochemical pregnancy in PCOS.

Keywords Granulosa cells · Glycolysis · In vitro fertilization · Polycystic ovary syndrome · Reactive oxygen species

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Background

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age, with a prevalence of 9–14%, depending on different diagnostic criteria [1], but prevalence increases to 20–40% in families with history of PCOS [2]. The success rate of in vitro fertilization (IVF) in patients with PCOS is low due to the poor quality of oocytes. Although the main cause of poor oocyte quality in PCOS is not yet fully understood, presumably granulosa cells (GCs) surrounding oocytes play important roles in the differentiation and maturation of oocytes by providing an energy source [3, 4]; thus, dysfunction of GCs may lead to a decline in the number of mature follicles [5–7]. Correlation of elevated reactive oxygen species (ROS) level with

investigated the correlation between the ROS content, mitochondrial membrane potential ($\Delta\psi_m$) as a characteristic of mitochondrial function, the rate of glycolysis, total antioxidant capacity, enzymatic activity of catalase and superoxide dismutase, and ATP level in granulosa cells of women with PCOS and determined the expression of key enzymes involved in glycolysis [hexokinase (HK¹), muscular phosphofructokinase (PFK_M), platelet-derived phosphofructokinase (PFK_P), and muscular pyruvate kinase (PK_M)] in granulosa cells.

Materials and methods

Study population- study design

In the first phase of recruitment of participants in this case-control study, a total of ninety women aged 20–38 years who admitted to Infertility Centre of Fatemeh Hospital and Omid Infertility Centre (Hamadan, Iran) as candidates for IVF procedure were enrolled during April to December 2019. Diagnosis of PCOS was carried out (in 40 women) by specialist according to the Rotterdam criteria [11]. In addition, forty-five healthy women with a normal ovulation cycle who showed no signs of hyperandrogenism and were candidates for IVF procedure due to their husbands' sterility were considered as control subjects.

The exclusion criteria were presence of prolactinoma, virilizing ovarian, adrenal tumors, congenital adrenal hyperplasia, Cushing syndrome, and those who were taking insulin-sensitizing drugs. After running all experiments and in the second phase of study, a new set of participants including fifteen PCOS patients and 10 control women were recruited to validate association of GCs energy level with oocyte maturation by measuring glycolysis rate and ATP level in GCs isolated from mature and immature follicles. The study was approved by Research Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1398.008 Hamadan-Iran). Objectives of the study were explained to the all subjects, and signed written consents were received from all

a number of female reproductive diseases including PCOS has been reported [8], and the inhibitory effect of increased ROS on glycolytic enzyme has recently been observed [9]. Interestingly, oocyte ATP level is directly correlated to the number of surrounding GCs and the rate of glucose uptake by GCs [10].

Therefore, it is postulated that the presence of higher number of immature follicles in PCOS might be due to the reduction in the expression of glycolysis pathway-regulatory enzymes in GCs. It is also believed that the higher level of ROS and lower ATP content together with mitochondrial dysfunction might be concurrent with the presence of immature follicles in PCOS. Thus, the present study for the first time

participants. All procedures performed in the study were in accordance with the ethical guidelines of the Declaration of Helsinki (1964; version 2013) and with the ethical standards of the National Iranian Research Committee.

Ovarian Stimulation Protocol

All participants received recombinant FSH and Cinnal-F® (CinnaGen Co., Tehran, Iran) and underwent serial ultrasonography to evaluate size of follicles. When the size of the dominant follicle reached 14 mm, Cetrotide antagonist (Merck, Darmstadt, Germany) was injected (0.50 µg daily), and once at least three adult follicles (14 mm in size) were observed in the ovaries; the recombinant Ovitrelle-hCG (Merck, Darmstadt, Germany) was administered. After 36 h, follicles were collected by transvaginal ultrasound-guided puncture, oocytes were isolated, and follicular fluid containing granulosa cells were collected.

Isolation of follicular fluid and granulosa cells

During IVF procedure and follicular puncture, after removing oocytes, follicular fluids were collected from all participants and transferred to sterile falcon tubes. Tubes were centrifuged at $1000 \times g$ for 5 min at 4°C, supernatants (follicular fluids) were separated, and the pellets containing GCs were recentrifuged after adding 10 ml of red blood cell lysing buffer (RLB) to each sample. The RLB washing step was repeated three times, and then granulosa cells were centrifuged with DMEM-F12 media at $500 \times g$ for 5 min and phosphate-buffered saline (PBS) at $300 \times g$ for 1 min.

Characterization of granulosa cells

To test viability of GCs, the isolated granulosa cells ($0.1-0.5 \times 10^5$ cells per each oocyte) were stained with trypan blue, but for characterization, GCs were first examined under differential interference contrast (DIC) microscopy and then stained by Giemsa to

differentiate GCs from red blood cells. The identity of GCs was then reconfirmed by flow cytometry using CD45 cell surface leukocyte marker to distinguish CD45-negative granulosa cells from leukocytes. Signals were captured at 488 nm and 635 nm, as excitation and emission wavelengths respectively, and the isolated GCs were counted and stored for further analysis.

Chances of IVF success

Follicles were examined by an embryologist, and those larger than or equal to 14 mm in diameter were considered as mature follicles. Then, oocytes were counted and after in vitro fertilization (IVF), and the number of embryos and their quality were evaluated. To estimate biochemical pregnancy, β -hCG level was measured 5 weeks after embryo transfer, and positive β -hCG result was considered as successful pregnancy.

Measurement of serum hormones

Electrochemiluminescence assays were carried out to investigate estradiol (E2), anti-Müllerian hormone (AMH), prolactin (PRL), luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and insulin levels in serum using Roche cobas e 811 Immunoassay Analyzer (Roche Diagnostics GmbH, Germany). The lower and upper limits of detection for E2 were 0 and 3000 pg/ml, respectively. The range of measurements were 0.01–23 ng/ml and 0.0470–470 ng/ml for AMH and PRL, respectively. LH and FSH measuring ranges were 0.1–200 mIU/ml, while a range of 0.000–100 µIU/ml was used for TSH, and lower and upper limits of detection for insulin were 0.20 and 1000 µIU/ml. Testosterone levels was measured using a TESTO (CLIA) chemiluminescence method (Mindray CL600, china) where the reportable range of TESTO kit was 0.1–16 ng/ml. The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated based on suggested formula.

Measurement of intracellular ROS level

For intracellular detection of ROS, a flow cytometry ROS assay kit (Cayman Chemicals, USA) containing 2',7'-dichlorofluorescein diacetate (DCFDA) and propidium iodide (PI) was used. N-acetyl-cysteine solution and pyocyanin working reagent were used as negative and positive controls, respectively. Briefly, 130 µl of ROS staining buffer was added to 20 µl of cellular deposition. Samples were then incubated in the dark for 30 min at 37°C. After centrifugation at 400 × g for 2 min, ROS staining solution was carefully discarded, and 100 µl of cell-based assay buffer was added to the samples. Signals were captured by Cyflow Space flow cytometer (Sysmex-Partec GmbH, Münster, Germany) using excitation and emission wavelengths at 488 nm and 520 nm, respectively.

Calorimetric assays for parameters of antioxidant system in GCs

Commercially available kits were used to determine total antioxidant capacity (TAC), catalase (CAT), and superoxide dismutase (SOD) enzyme activities (all from Navand Salamat Co., Iran) in granulosa cell lysates, according to the manufacturer's instructions. In addition, total protein concentrations were determined using commercially available Nadford kit (Navand Salamat Co., Iran) based on Bradford

Method,

Determination of total antioxidant capacity in GCs

A ready-to-use Naxifer Kit (Navand Salamat Co., Iran) was used to evaluate total antioxidant capacity (TAC) in granulosa cells. Naxifer kit measures antioxidant capacity of biomolecules in different biological specimens based on ferric reducing antioxidant power assay (FRAP) method and reduction ability of divalent iron and single electron transfer mechanism. Color

change resulting from the reaction was evaluated by a microplate reader at 593 nm, compared with standard curve, and finally TAC value was reported as mmol Fe²⁺/L.

Determination of catalase (CAT) activity in GCs

To assess catalase (CAT) activity Nactaz™ Catalase Activity Assay Kit (Navand Salamat Co., Iran) was used. Approximately 1 × 10⁶ granulosa cells were homogenized in 1 ml of lysing buffer and centrifuged at 400 × g for 10 min. Supernatants were separated and used for the assessment of enzyme activity. Detection was based on the peroxidative activity of catalase enzyme present in the sample. Briefly, in the presence of hydrogen peroxide and methanol, enzymatic activity of catalase generates formaldehyde which reacts with a dye to form a chromogen detectable at 500 nm. Enzyme activity was finally reported as nmol/min. mg protein.

Determination of superoxide dismutase (SOD) activity in GCs

A Nasdox™–Superoxide Dismutase Assay Kit (Navand Salamat Co., Iran) was used to evaluate SOD activity in granulosa cells. Briefly, 1 × 10⁶ cells were centrifuged at 400 × g for 2 min, and supernatants were discarded. The precipitated cells were then rinsed twice with PBS and mixed with 0.5 ml of lysing buffer, vortexed for 10 min, centrifuged at 12,000 × g for 5 min, and supernatants were collected for SOD assay. SOD activity was measured through pyrogallol autooxidation, a process highly dependent on superoxide, which is a substrate for SOD. Hence, in the presence of SOD, pyrogallol autooxidation was inhibited, and the SOD activity was indirectly assayed at 420 nm. A calibration curve was performed with purified SOD as a standard, and the results were represented as U per mg of protein.

Assessment of the mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential ($\Delta\psi_m$) was measured using a Cayman mitochondrial membrane potential detection kit (Cayman Chemicals, USA)

where the 2,2',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide reagent (JC-1) may emit green or red fluorescence based on the $\Delta\psi_m$ level. Briefly, 100 μ l of JC-1 staining solution was added to the cells and mixed gently. Samples were incubated in a CO₂ incubator at 37 °C for 30 min and centrifuged at 400 \times g at room temperature for 5 min to carefully separate the supernatant. Next, 1 ml of assay buffer was added to the samples, and centrifugation was repeated. Finally, after addition of 100 μ l of assay buffer, fluorescence intensities were measured with the excitation and emission at 488 nm and 530 nm, respectively. Images were captured using an Olympus fluorescent microscope (Olympus, Tokyo, Japan), and results were quantified using ImageJ-1.82 Software (<https://imagej.nih.gov/ij/>).

RT- qPCR for gene expression assay

Gene expression levels of HK1, PFK_P, PFK_M, and PK_M in granulosa cells were determined by RT-qPCR using RealQ Plus Master Mix Green (Ampliqon, Odense, Denmark) on a Roche LightCyclerVR⁹⁶ System (Roche Life Science, Sandhofer, Germany). Briefly, total RNA was isolated, quantified using NanoDrop™ (Thermo Fisher Scientific-USA), and its quality and integrity were confirmed by 1% agarose gel electrophoresis. cDNA was synthesized by reverse transcription of 100 ng of total RNA using HyperScript Reverse Transcriptase cDNA Kit (GeneAll Biotechnology Co., Seoul, Korea). Gene-specific primers were designed using AlleleID[®] software (Premier Biosoft Corporation, USA). The specificities of designed primers to the corresponding genes were reconfirmed by online primer blast software (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Genespecific forward: 5'-TGG CTG CGG TTG TGG ATA AG-3' and reverse: 5'-TCA GAC AGG AGG AAG GAC AC-3' primers were used for HK1; a set of forward 5'-GGG GAA

GTA CTT GGA AGA GA-3' and reverse: 5'-GCA GGT GTC GGT

GAT AGT G-3' primer was used for PFK_P gene; a pair of forward 5'-CGTGCT ACA GTC TCC AAC AA-3' and reverse: 5'-GTA GCC ACC CAT AGT CTC AA-3' primers was applied for PFK_M; and 5'-AGC CTC AAG TCA CTC CAC AGA-3' and 5'-AGGGAA G ATGCC A CG GTA CAG-3' primers were used as forward and reverse primers, respectively, for PK_M gene. β -actin was considered as housekeeping gene with a primer set of forward 5'-CAC CAA CTG GGA CGA CAT -3' and reverse 5'-ACAGCC TGG ATA GCA ACG -3'. Finally, the relative mRNA expression levels were calculated as $2^{-\Delta Ct}$ compared with the expression of β -actin.

Western blotting for protein assay

To specifically quantify target proteins, total protein contents of granulosa cells were first measured in all samples using Bicinchoninic Acid Protein Assay Kit (Thermo Scientific, USA). Briefly 1 \times 10⁶ granulosa cells were homogenized using lysis buffer containing 500 μ l of RIPA buffer (Thermo Scientific, USA) and 1 μ l of the protease inhibitor (SigmaAldrich, USA). The cell homogenates were placed on a 4 °C shaker for 2 h and then centrifuged at 12,000 \times g for 20 min. The supernatants containing cell soluble proteins were transferred to new microtubes, and protein concentrations were measured using BCA protein assay kit with bovine serum albumin as reference protein.

For Western blotting, 20 μ g of total protein from each sample was separated by electrophoresis on SDS-PAGE gel, and proteins were transferred from the gel onto the polyvinylidene fluoride membranes. After blocking with Tris-buffered saline-Tween containing 0.5% lean milk for 2 h, membranes were incubated with primary antibodies against HK1 (Abcam, USA, ab100699), PFK_P (Abcam, USA, ab186132), PFK_M (Abcam, USA, ab237040), PK_M (Abcam, USA, ab137791), and β -actin (Abcam, USA, ab12227) for 12 h. Membranes were then washed three times with TBST buffer for 10 min and incubated with the secondary antibody (Abcam, USA,

ab191866) for 1 h. Next, membranes were stained with enhanced chemiluminescence (Bio-Rad, Germany), and the protein bands were exposed to the film in the darkness. Finally, the relative protein expression levels were quantified by ImageJ-1.92 software and normalized to β -actin as control.

Cell-based glycolysis assay

L-lactate as the end product of cellular glycolysis was measured with colorimetric method in granulosa cells using Cayman Glycolysis Cell-Based Assay Kit (Cayman Chemicals, USA). Briefly, lactate dehydrogenase catalyzed the reaction between NAD^+ and lactate, yielding pyruvate and NADH. The NADH was directly reduced by a tetrazolium salt to a colored formazan with maximum absorbance at 490 nm. Then, the absorbance was read using a spectrophotometer, and the concentration (mM) of L-lactate in each sample was obtained using a standard curve.

Determination of ATP content in granulosa cells

The ATP content of granulosa cell lysates were measured using ZellBio ATP-Detection Assay Kit (ZellBio GmbH, Deutschland, Germany), according to the manufacture's instruction. Method was based on the biotin double antibody sandwich technology for the assessment of the human adenosine triphosphate (ATP). Cells were homogenized in 100 μl of lysing buffer, sonicated, and centrifuged for 10 min at $2000 \times g$. After transferring supernatants to separate wells, assay buffer and substrates were mixed with the samples. Finally, the absorbances were recorded at 450 nm, and concentrations of ATP were obtained using a standard curve and expressed as ng/ml.

Flow cytofluorometric analysis for detection of apoptosis

To detect apoptosis in granulosa cells, flow cytofluorometric analysis was carried out using Phosphatidyl Serine Detection Kit (IQ Products, The Netherlands) which contains Annexin V-FITC and propidium iodide (PI). The Annexin V corresponding signal provides a very sensitive method for detecting cellular apoptosis, while PI is used to detect necrotic or late apoptotic cells. Detection was based on the fact that phosphatidylserine (PS) becomes exposed on the outside of the cell membrane in the dying cells during early stage of apoptosis where the exposed phosphatidylserine can be detected by PS-specific binding proteins. Briefly, GCs were washed with PBS buffer and centrifuged. After adding binding buffer on GCs sediment, Annexin V-FITC and PI was added and samples incubated for 10 min at 4 °C in the dark. After overnight incubation of cells with dexamethasone to induce apoptosis, apoptotic cells excluded PI and expressed phosphatidylserine. Necrotic or dead cells were permeable to PI which associates with nuclear DNA and is visible as red fluorescence. Samples were read by BD FACSCalibur™ Flow Cytometer (BD Biosciences, USA).

Validation of association between GCs energy level and oocyte maturation

To reconfirm the hypothesis that GCs of PCOS patients have lower energy level than corresponding cells in control women, 30 independent participants (10 women with PCOS and 10 control subjects) were recruited in the second phase of the study. Separation of follicles was carried out by a gynecologist under ultrasonography and based on the size of the follicles. Follicles with a diameter of 18 mm or larger were considered as mature follicles, whereas smaller follicles were classified as immature. Mature and immature follicles were collected in separate tubes and delivered to the embryology laboratory. Then, granulosa cells were isolated from mature and immature follicles of control individuals (CMF and CIMF, respectively), and from mature or immature follicles of PCOS patients (PMF and PIMF,

respectively). Finally, the rate of glycolysis and ATP content were determined in isolated GCs of each group. Lower energy level in GCs is associated with the presence of immature follicles in PCOS patients.

Statistical analysis

Data analysis was performed using SPSS software version 22.0 (SPSS Inc., Chicago, USA) and GraphPad Prism software version 8.0 (GraphPad Software, USA). Kolmogorov–Smirnov test, Mann–Whitney *U* test, Student's *t* test, and chi-square test were used as appropriate. Spearman's and Pearson's correlation coefficients were used to examine the relationship between variables. Data was expressed as mean \pm SEM, and $p < 0.05$ was considered as statistically significant difference. For second phase of the study, a twoway ANOVA analysis followed by Kruskal–Wallis test was performed to validate the difference of energy level between GCs isolated from PCOS patients and control women as well as those isolated from mature and immature follicles.

Results

Characteristics of participants and outcomes of IVF

Demographic characteristics, hormonal status, and outcome of IVF procedures in participants from the both phases of the study are shown in Table 1. Data analysis showed that there was no significant difference between participants in maternal age and duration of infertility. Women with PCOS had higher body mass index (BMI) than control subjects. FSH hormone levels did not differ between groups; however, a ratio of LH to FSH, E2, PRL, and AMH were significantly higher in women with PCOS compared to the control group ($p < 0.001$). The number of retrieved oocytes and the number of metaphase II-oocytes (MII) were significantly higher in PCOS patients (Table 1). Although more embryos

were obtained from PCOS patients, the ratio of available embryos to MII was significantly lower in women with PCOS. Follow-up of embryo transfer results confirmed significantly lower successful fertilization rate in PCOS patients (12.1%) compared with control women (34.0%) based on the positive β -hCG test results.

Characterization of GCs

Examination of GCs under differential interference contrast (DIC) microscopy showed typical morphology of granulosa cells displaying highly granular cytoplasm representative of lipid droplets around the nucleus (Fig. 1a). Upon staining of GCs with Giemsa, large central spherical nucleus were observed (Fig. 1b) which distinguished GCs from nucleusfree RBCs. The identity of GCs was further reconfirmed by discriminating of GCs from leukocytes using flow cytometry where granulosa cells were recognized as CD45-negative cells compared with CD45-positive leukocytes. Over 80% of the cells were observed below the threshold line indicating the absence of CD45 marker in majority of cells (Fig. 1c).

Reduction of antioxidant potency of granulosa cells in PCOS

Based on flow cytometry analysis, a lower percentage of the granulosa cells containing detectable ROS level (Fig. 2a)

phase of the study. Significant differences ($p < 0.05$) are shown in Bold

was detected in control subjects compared to the relatively higher dye-positive (DCFH+) GCs which were observed in PCOS patients (Fig. 2b).

Accordingly, the mean level of ROS in granulosa cells of PCOS patients was found significantly higher ($0.21 \pm 0.07\%$) than that of the control women ($0.08 \pm 0.01\%$), and the ROS content was almost 81% greater in PCOS compared with control group (Fig. 2c).

Variable	Participants of first phase of study			Participants of second phase of study		
	Control (n=45)	PCOS (n=45)	p value	Control* (n=15)	PCOS* (n=15)	p value
Age (year)	30.84 ± 0.527	31.20 ± 0.492	NS	29.866 ± 0.792	30.400 ± 0.558	
Duration of infertility (year)	4.88 ± 0.29	5.48 ± 0.29	NS	5.000 ± 0.414	5.333 ± 0.503	
BMI (kg/m ²)	24.29 ± 0.365	27.10 ± 0.548	0.01	23.542 ± 0.514	26.692 ± 0.852	0.009
FSH (IU/L)	5.04 ± 0.449	5.40				
LH (IU/L)	4.30 ± 0.456	7.79				
LH/FSH	0.85 ± 0.058	1.76				
E2 (pg/ml)	34.69 ± 1.721	52.65				
AMH (ng/ml)	1.69 ± 0.117	4.95				
PRL (ng/ml)	11.28 ± 0.791	17.82				
TSH (μU/ml)	1.41 ± 0.096	1.84				
Testosterone (ng/ml)	0.64 ± 0.037	0.95				
FBS (mg/dl)	79.96 ± 1.248	81.49				
Insulin (μU/ml)	5.82 ± 0.348	12.51				
HOMA-IR	1.19 ± 0.079	2.57				
Retrieved oocytes (N) ^a	7.67 ± 0.95	14.71				
Immature follicles	1.86 ± 0.185	3.55				
Number of oocytes in MI	0.65 ± 0.14	0.46				
Number of oocytes in MII	5.17 ± 0.61	11.17				
Available embryos	3.79 ± 0.50	6.91				
Available embryos/MI	0.79 ± 0.06	0.59 ± 0.04	0.014	0.884 ± 0.100	0.646 ± 0.111	0.016
Biochemical pregnancy (%)	34.5%	12.1%	0.036	72.7%	18.2%	0.028

Data are presented as mean ± SEM. AMH, anti-Müllerian hormone; BMI, body mass index; E2, estradiol; FBS, fasting blood sugar; FSH, follicle-stimulating hormone; HOMA-IR, index of insulin resistance, LH, luteinizing hormone, MI and MII, metaphase I and II; N, indicates the

number of oocytes; PRL, prolactin; TSH, thyroid-stimulating hormone; NS, not significant. Asterisk symbol (*) represents participants from 1st phase of study

In addition, as data tabulated in Table 1, PCOS patients showed significantly lower TAC and lower CAT and SOD activities. While over 11% greater ROS content was detected in PCOS patients, these patients had 42.5% lower TAC compared with control women. Patients had also nearly 36% and 53% lesser CAT and SOD activities, respectively, in their granulosa cells (Table 1).

Increased intracellular ROS level was accompanied with the lower Δψ_m

Analysis of Δψ_m showed that in PCOS women, the JC-1 predominantly is monomer that yields green fluorescence representing low Δψ_m (Fig. 2a), whereas in control subjects, the aggregated dye yielded a red color indicative of high Δψ_m. The Δψ_m was found nearly 50% lower in PCOS patients (Fig. 2b) compared with the control

group ($p < 0.001$). Using Spearman correlation analysis, a strong inverse correlation was observed (Fig. 2c) between the amount of ROS and Δψ_m ($r = -0.829$, $p < 0.001$). Moreover, logistic regression analysis showed that for every increased unit in ROS level in granulosa cells, 90% confidence interval (90% CI) for the risk of developing PCOS increases by 4 unit [Exp(B) = 4.045, $p = 0.003$].

Downregulation of key enzymes of glycolysis in PCOS

Our findings showed that the relative gene expression levels of HK1, PFK_P, PFK_M, and PK_M enzymes were significantly lower in the PCOS patient compared with the control group. Likewise, significant reductions were observed in the protein levels of the key enzymes involved in glycolysis in women with PCOS indicating concurrent alterations

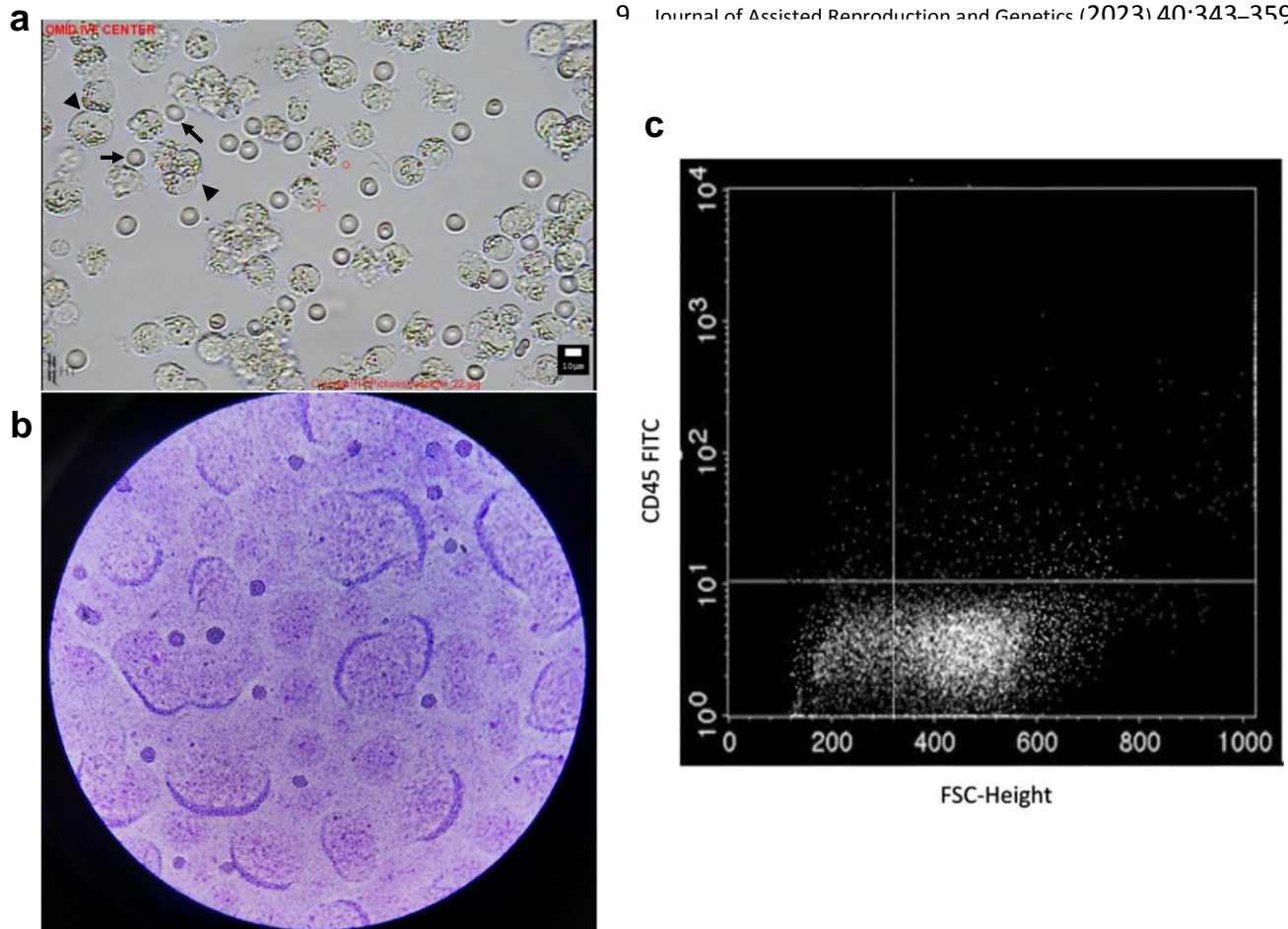


Fig. 1 Characterization of granulosa cells obtained from PCOS (a) which is recognizable from red blood cells (b) and (c) as recognized as CD45-negative cells in flow cytometry. DIC microscopy showed typical morphology of granulosa cells displaying highly granular cytoplasm representative of lipid droplets around the nucleus. Giemsa staining confirmed the presence of a large nucleus in granulosa cells differentiating them from RBCs. In flow cytometry, most of the cells were observed below the threshold line indicating the absence of leukocyte-specific CD45 marker on the cell surface

patients and control women as determined by a differential interference contrast (DIC) microscopy, b stained with Giemsa, and c as recognized as CD45-negative cells in flow cytometry. DIC microscopy showed typical morphology of granulosa cells displaying highly granular cytoplasm representative of lipid droplets around the nucleus. Giemsa staining confirmed the presence of a large nucleus in granulosa cells differentiating them from RBCs. In flow cytometry, most of the cells were

Table 2 Parameters of antioxidant system in granulosa cells

Variable	Control	PCOS	<i>p</i> value
ROS	28.77 ± 2.112	52.11 ± 0.871	≤ 0.01
TAC	0.699 ± 0.196	0.373 ± 0.013	≤ 0.01
CAT	1.064 ± 0.058	0.677 ± 0.027	≤ 0.01
SOD	1.291 ± 0.078	0.606 ± 0.021	≤ 0.01

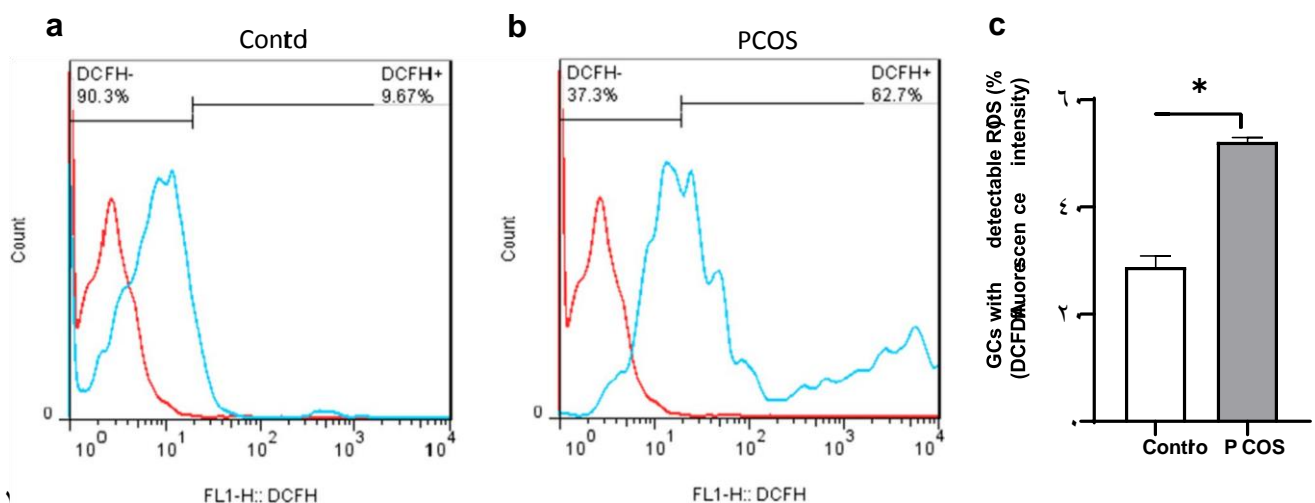


Fig. 2 Determination of intracellular ROS levels in granulosa cells. (a) and (b) show the percentage of granulosa cells containing detectable level of ROS. The percentage of granulosa cells containing detectable levels of ROS was determined by flow cytometry in GCs obtained from control and PCOS women. Data is represented as mean and standard deviation. Asterisk (*) indicates significant difference between groups

Data are presented as mean \pm SEM. *CAT*, catalase; *ROS*, radical oxygen species; *SOD*, superoxide dismutase; *TAC*, total antioxidant capacity

glycolysis rate (Fig. 5a), as determined by the concentration of L-lactate (1.93 ± 0.10 mM vs 3.61 ± 1.08 mM) and in the energy production (38.21 ± 0.87 ng/ml vs 47.98 ± 1.13 ng/ml), as measured by the ATP level in PCOS patients compared with control (Fig. 5b). Over 46% reduction in glycolysis rate and more than 20% decline in ATP production were detected in PCOS patients compared with control women.

Concerted alterations leading to the decreased production of energy in PCOS

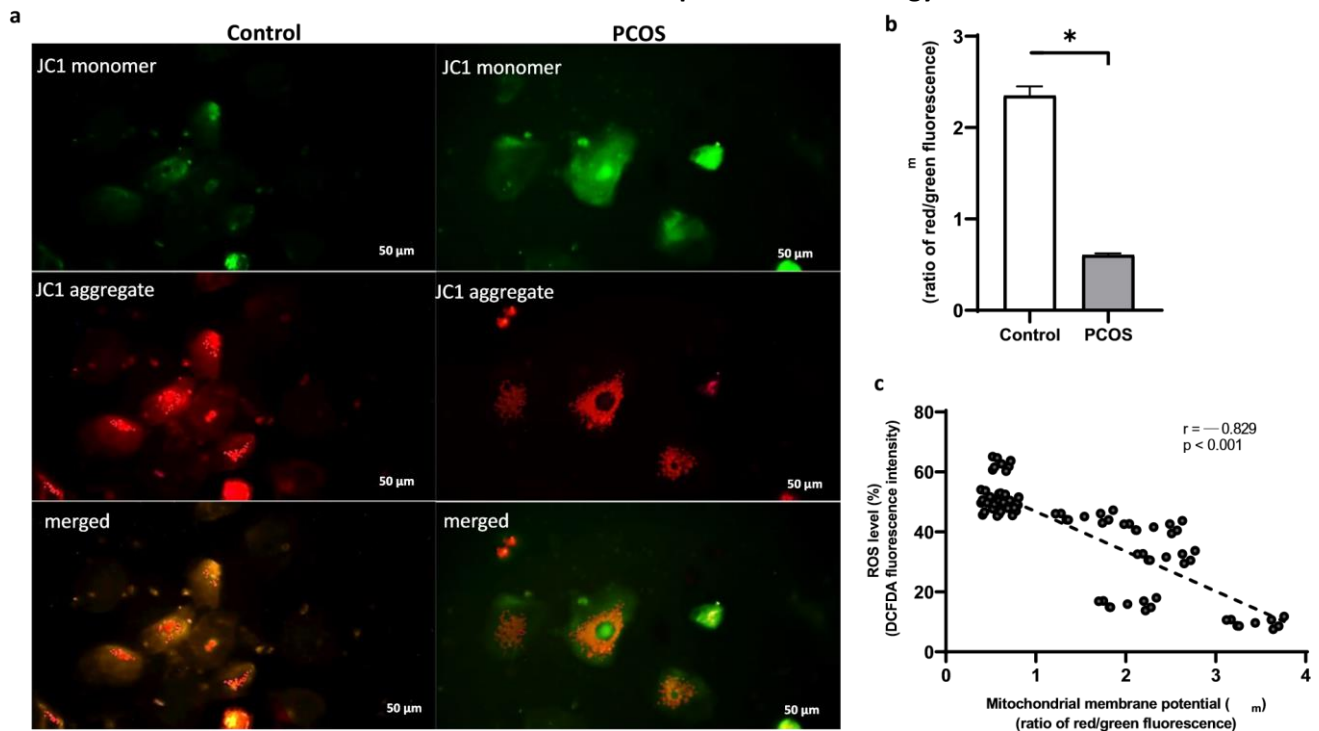


Fig. 3 Determination of mitochondrial membrane potential ($\Delta\psi_m$) and its correlation with intracellular ROS levels in GCs obtained from control subjects ($n = 10$) and women with PCOS ($n = 10$). JC-1 mitochondrial membrane potential assay was performed, and green fluorescence indicating of JC-1 monomer and red fluorescence representing JC-1 aggregates were captured. Intensities were merged to obtain green/red fluorescence ratio corresponding to the $\Delta\psi_m$ levels

Energy production in granulosa cells declined in PCOS

Concurrent with the decrease in mRNA and protein levels of key enzymes of glycolysis, significant reduction was also occurred in

and quantified with ImageJ software (a). The mean $\Delta\psi_m$ values were compared in control subjects and women with PCOS (b), and the correlation of $\Delta\psi_m$ values with intracellular ROS levels was obtained with Spearman's correlation analysis (c). Data is represented as mean \pm SE, and asterisk (*) indicated significant difference between groups ($p < 0.001$)

As indicated in Table 3, ATP concentration showed a significant correlation with mitochondrial function, and higher ATP concentrations were found associated with greater $\Delta\psi_m$ ($r = 0.887$, $p < 0.001$), whereas the higher levels of ROS were associated with lower ATP contents ($r = -0.724$, $p < 0.001$). In

addition, glycolysis rate showed a significant direct correlation ($r = 0.88$, $p < 0.001$) with ATP level. Interestingly, unlike negative correlation of ROS, direct associations were observed between ATP, glycolysis rate, and $\Delta\psi_m$ with the ratio of the number of available embryos to the number of metaphase-II oocytes (available embryos/MII) confirming that GCs with normal

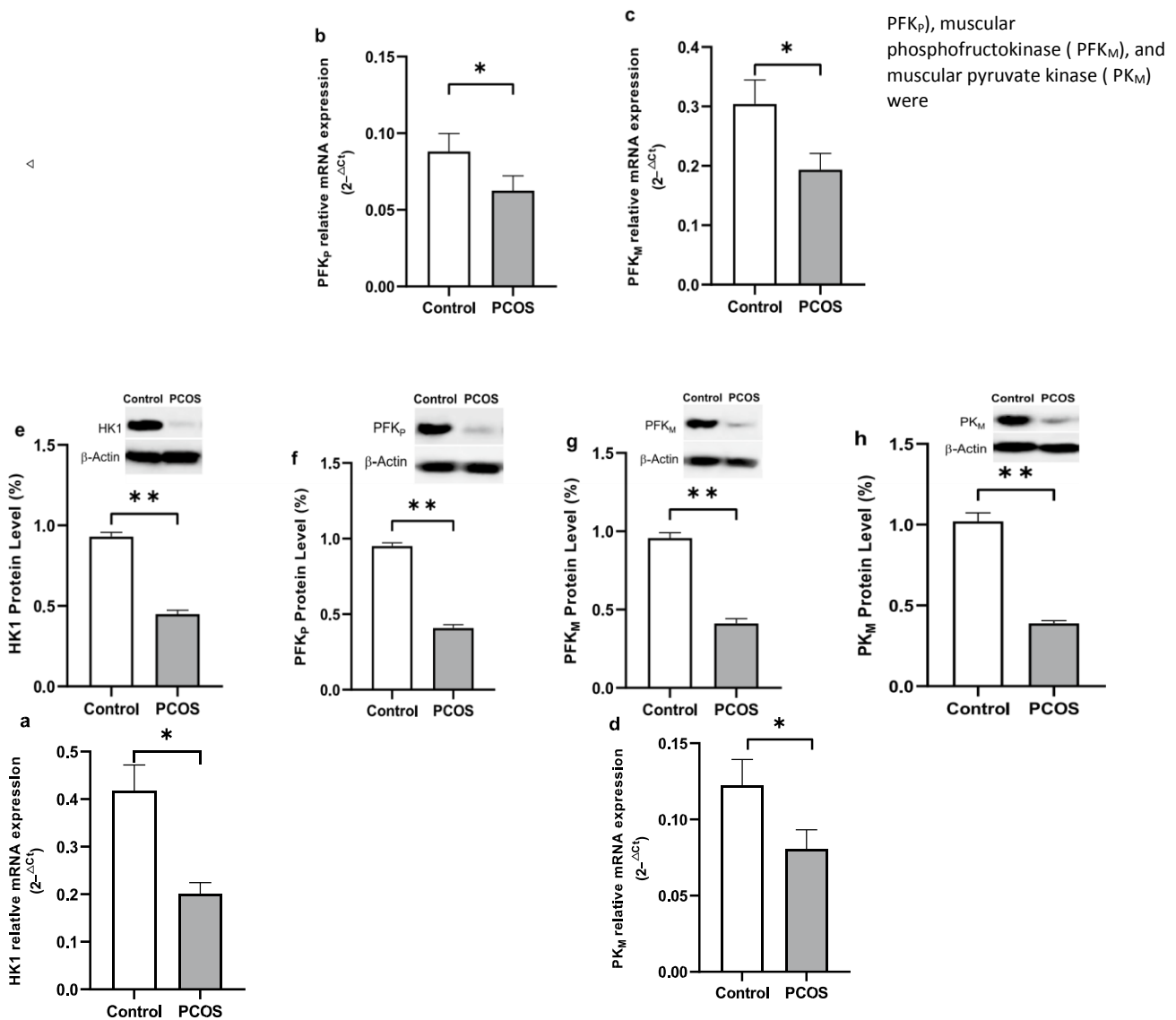


Fig. 4 Determination of the expression of key regulatory enzymes involved in glycolysis. The mRNA expression of hexokinase (HK¹), platelet-derived phosphofructokinase (

mitochondrial function and higher energy level eventuate in the higher number of conceived embryos, whereas deleterious effects of oxidative stress lower the number of available embryos/MII.

Although there were no significant correlations between the relative gene expression levels of key glycolytic enzymes and cellular ROS level, protein levels of all enzymes were found negatively

determined with qRT-PCR, whereas Western blotting technique was used to determine protein levels of the enzymes (e–h). Data is represented as mean \pm SE, and asterisk “*” and “***” indicate $p < 0.05$ and $p < 0.001$, respectively

correlated with ROS while were positively correlated with glycolysis rate and $\Delta\psi_m$, as shown in Table 4.

PCOS patients had higher levels of apoptotic granulosa cells

Flow cytometry analysis for detection of apoptosis in a number of samples from PCOS patients and control women showed significantly higher number of

apoptotic granulosa cells in PCOS patients compared with control women (12.30% vs 0.99%, respectively), as shown in Fig. 1.

Lower energy production in granulosa cells is associated with the presence of immature follicles

To investigate whether the lower energy level in GCs is associated with the presence of immature follicles, the rate of glycolysis and the ATP content once again were measured in granulosa cells isolated from a new set of PCOS patients (PCOS*) and control women (Control*) which showed significantly lower rate of glycolysis and ATP level in PCOS patients, as expected (Fig. 4a, b). Then, GCs were separated from mature and immature follicles both in control women (CMF and CIMF) and PCOS patients (PMF and PIMF), and the rate of glycolysis and ATP level were evaluated in isolated GCs. Data analysis showed that in both control subjects and PCOS patients, the granulosa cells which were isolated from immature follicles had the lower rate of glycolysis and lesser ATP level compared with those of cells separated from mature follicles. Therefore, as shown in Fig. 4c and d, a lower rate of glycolysis and ATP content was observed in CIMF and PIMF compared with CMF and PMF.

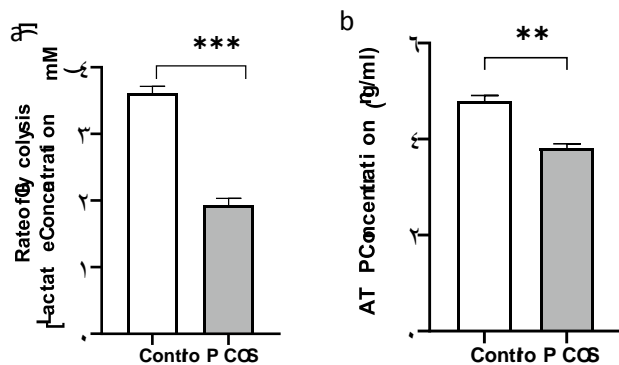


Fig. 5 Determination of the rate of glycolysis (a) and ATP level (b) in GCs. Granulosa cells were isolated from PCOS patients and control women. The rate of glycolysis in granulosa cells were assessed based on the concentration of lactate, whereas the ATP content of GCs was measured as marker of energy level. Data is represented as mean \pm SE, and “***” indicates $p < 0.001$

In addition, a significantly lower rate of glycolysis ($p < 0.001$) was observed in GCs obtained from mature (PMF) follicles in PCOS patients compared with corresponding CMF groups in control subjects. Likewise, GCs separated from immature follicles in patients (PIMF) had also markedly reduced rate of glycolysis than immature control group (CIMF). Interestingly, GCs surrounding mature oocytes in PCOS (PMF) had almost similar rate ($p = 0.424$) of glycolysis to the GCs isolated from immature follicles in control women (CIMF) which somehow explains lower functionality of mature follicles in PCOS patients (Fig. 4c).

As shown in Fig. 4d, GCs isolated from immature follicles in PCOS patients (PIMF) had lower ATP content compared with that of mature follicles in patients (PMF), but no significant difference was found in ATP levels between

Table 3 Correlation of ATP and glycolysis rate with $\Delta\psi_m$ and ROS, as obtained in the first phase of the study

Variables	ATP (ng/ml)	Glycolysis (mM)	$\Delta\psi_m$	ROS (%)
Available embryos/MII	0.236 0.030	0.278 0.013	0.200 0.026	— 0.222 0.046
ATP (ng/ml)	—	0.483 0.001	0.487 0.001	— 0.624 0.001
Glycolysis (mM)	—	—	0.610 0.001	— 0.066 0.001
$\Delta\psi_m$	—	—	—	— 0.829 0.001

In each cell, the number in the first row represents Spearman's correlation coefficient, and the number in the second row indicates p value. $\Delta\psi_m$, mitochondrial membrane potential; ROS, reactive oxygen species

GCs separated from immature follicles in control (CIMF) individuals and women with PCOS (PIMF).

Lack of association between serum testosterone and cellular energy

To investigate whether the present findings are independent of or because of hyperandrogenism, Spearman's correlation analysis was performed, and association of testosterone level with the rate of glycolysis, ATP level, mitochondrial membrane potential ($\Delta\psi$), and ROS content in granulosa cells was investigated. As shown in Table 6, serum testosterone showed negative correlations with all these parameters in all participants, whereas no association was observed between these variables and testosterone exclusively in control women or in PCOS patients.

In the same way, evaluation of association between serum testosterone and insulin and insulin resistance index (HOMA-IR) showed that testosterone level is significantly correlated with both insulin concentration and insulin resistance in all participants (Table 6). However, after separately analyzing of Spearman's correlation test in control and PCOS groups, no association of testosterone with insulin and HOMA-IR was observed (Table 6).

Discussion

It is believed that PCOS with 10% prevalence has the highest contribution in chronic anovulation [12]. Despite the high number of follicles obtained in PCOS, the chances of successful IVF are low due to the poor quality of oocytes [13]. A prominent and pivotal player in attainment of highquality oocytes is energy. Glycolytic pathway in granulosa cells (GCs) is needed to provide required energy for follicle maturation and development [14, 15]. The necessary metabolic mediators of Krebs cycle (the predominant energy generator pathway in oocytes) are also provided in GCs [16, 17]. Therefore, GCs play key roles in maintaining optimal ATP levels in oocyte [18]. Thus, it was hypothesized that in PCOS, disruption of energy production in GCs may reduce oocyte quality. Accordingly, for the first time, the present study simultaneously evaluated

mitochondrial function, glycolysis rate, ATP concentration, and the expression of key glycolysis enzymes both at the mRNA and protein levels and examined ROS level as well as activity of antioxidant enzymes CAT and SOD in granulosa cells.

Our results showed the presence of higher amounts of ROS concurrent with the lower TAC, reduced antioxidant enzymes activities (CAT and SOD), and lower $\Delta\psi_m$ in granulosa cells of women with PCOS. In addition, the higher number of apoptotic granulosa cells was observed in PCOS patients compared with control women. The results

Table 4 Correlation of energy, ROS, and $\Delta\psi_m$ with the expression of key enzymes in granulosa cells of PCOS patients and control subjects

Variables	HK ¹ gene	HK ¹ protein	PFK _P gene	PFK _P protein	PFK _M gene	PFK _M protein	PK _M gene	PK _M protein
ATP	-0.096 0.378	0.492 <0.001	0.016 0.879	0.496 <0.001	-0.000 0.704	0.740 <0.001	0.008 0.087	0.494 <0.001
Glycolysis	0.240 0.020	0.080 <0.001	0.131 0.218	0.009 <0.001	0.148 0.163	0.710 <0.001	0.167 0.116	0.036 <0.001
$\Delta\psi_m$	0.270 0.010	0.790 <0.001	0.173 0.102	0.794 <0.001	0.320 0.002	0.769 <0.001	0.110 0.281	0.722 <0.001
ROS	-0.112 0.290	- 0.799 <0.001	-0.147 0.166	-0.806 <0.001	-0.147 0.166	- 0.727 <0.001	-0.096 0.370	-0.742 <0.001

$\Delta\psi_m$, mitochondrial membrane potential; HK¹, hexokinase-1; PFK_M, muscular phosphofructokinase; PFK_P, platelet phosphofructokinase; PK_M, muscular pyruvate kinase; ROS, reactive oxygen species. Significant differences ($p < 0.05$) are shown in Bold

also showed that the expression of key enzymes of glycolysis (HK¹, PFK_P, PFK_M, and PK_M) significantly reduced in PCOS. Accordingly, a marked decrease in glycolysis rate followed by depleted ATP levels was observed in granulosa cells in PCOS patients compared with control subjects. Association of reduced rate of glycolysis and declined ATP level with PCOS was further reconfirmed in an independent set of participants which showed GCs isolated from mature (PMF) or immature (PIMF) follicles of PCOS patients have lesser energy production than corresponding cells in control women.

In line with the results of the present study, an elevated level of ROS has already been reported in granulosa cells of PCOS where higher level of ROS affected oocyte quality and reduced the positive results of in vitro fertilization-embryo transfer (IVF-ET) in women with PCOS [19]. Although the exact mechanism of ROS production in granulosa cells in PCOS has not been yet elucidated [19, 20], it was shown that excessive accumulation of ROS may affect efficiency of GCs leading to the female infertility [21]. Since in the present study high level of ROS together with the low ATP content showed negative correlation with the ratio of the number of available embryos to the number of metaphase-II oocytes, it is possible that the increased level of ROS

in GCs affected the quality of oocyte and aggravated IVF outcome.

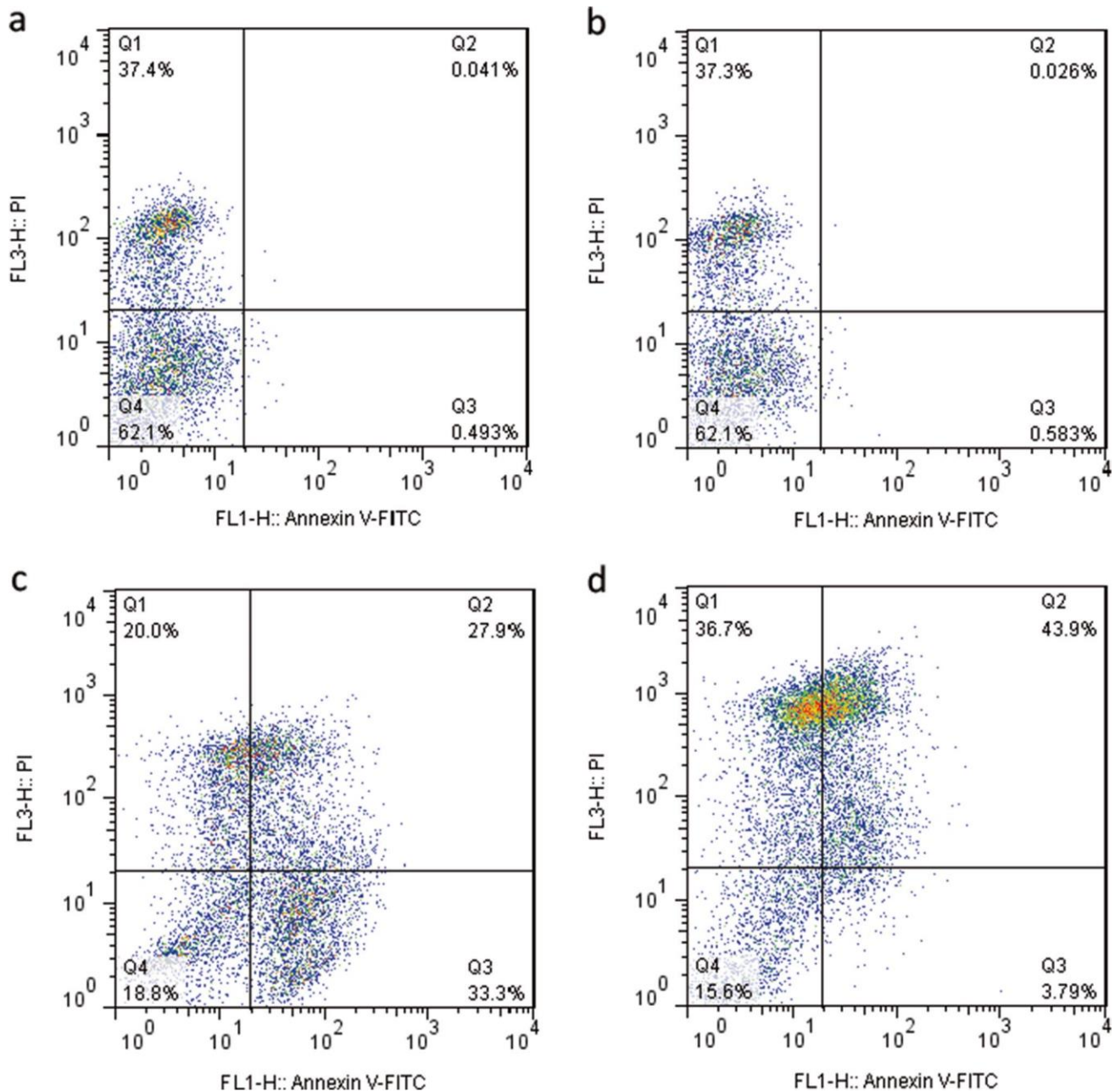
The importance of mitochondrial health in women fertility has previously been reported [14, 22]. Several lines of evidence suggest that mitochondrial dysfunction may have important implication on oocyte quality and may preclude oocyte maturation by virtue of inducing of ROS generation and lowering ATP production [23, 24]. In the same way, the increment in ROS and decline in $\Delta\psi_m$, were observed in the present study. These observations which confirmed mitochondrial dysfunction in granulosa cells of PCOS patients may explain the nearly two-fold lower biochemical pregnancy in these patients.

Collectively, from the results of the present study, it can be postulated that the increased ROS and decreased $\Delta\psi_m$ were responsible for mitochondrial dysfunction. Since mitochondrion involves in providing energy required for the maturation of oocytes, impairment in its function may brought about lower biochemical pregnancy. Our results showed that $\Delta\psi_m$ was significantly lower in the PCOS group and negatively correlated with increased intracellular ROS levels. There was also a weak positive correlation between $\Delta\psi_m$ and available embryos/MII. In this regard, previous studies have shown that $\Delta\psi_m$, an important marker of

mitochondrial activity, generally decreases in response to oxidative stress [46]. Considering the decreased $\Delta\psi_m$ of GCs and reduced numbers of total or mature oocytes retrieved in the aging group, GCs are likely involved in energy facilitating oocyte development [46, 47]; however, studies are still scant.

A high level of insulin and increased level of androgen, elevated oxidative stress together with mitochondrial dysfunction, were observed in PCOS patients in this study. These observations can be explained by the fact that insulin resistance (IR) and hyperandrogenism are closely related and their occurrence are indispensable for mitochondrial dysfunction [48]. Androgen overexposure causes IR by inducing oxidative stress elevation and may account for the high occurrence of IR in patients with PCOS. Together, ovarian hyperandrogenism, mitochondrial dysfunction, and hyperinsulinemia can interfere with follicular activation, survival, growth, and selection in women with PCOS [48]. The reduction in mitochondrial membrane potential (MMP) is indicative of abnormal mitochondrial function and representative of increased ROS production. On the other hand, oxidative stress (elevated ROS level) enhances the activity of ovarian steroid-producing enzymes and leads to hyperandrogenism. Therefore, mitochondrial dysfunction (e.g., the reduction in MMP) in PCOS patients is related to damage to oocyte development potential. Considering reduced mitochondrial biogenesis rate, mitochondrial DNA content, and MMP in the cumulus/granulosa cells of PCOS patients [48] and taking to account the overall lower level of glycolysis and impaired ATP production, it is concluded that insufficient

Fig. 6 Detection of apoptosis in control women (**a** and **b**) and PCOS patients (**c** and **d**) using flow cytometry. In each flow cytogram, Q_1 quadrant shows Necrotic cells which are PI positive and Annexin negative, whereas late apoptotic cells which are PI and Annexin positive are seen in Q_3 quadrant. Apoptotic cells which are PI negative and Annexin positive are present in Q_2 , and quadrant Q_4 (Q_4) represents



viable cells which are negative to both probes

energy supply by GCs for follicular development would be the primary factor mediating PCOS-related follicular dysplasia [29].

Due to the presence of high testosterone level in patients, PCOS-associated hyperandrogenism has recently attracted much attention to reveal how likely manifestations of PCOS are independent of or because of hyperandrogenism. Therefore, in the

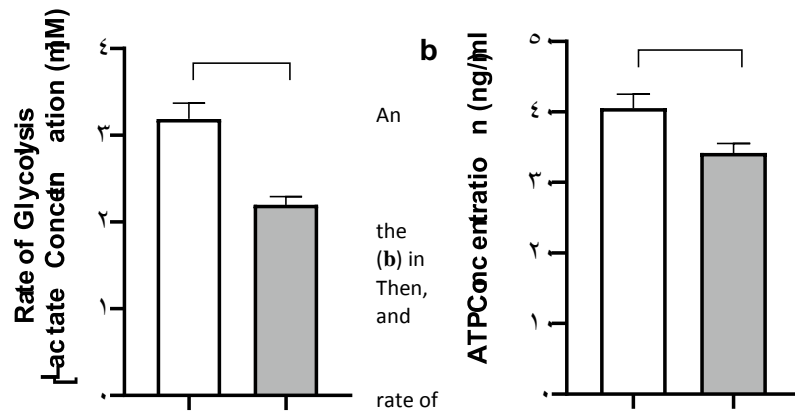
present study, Spearman's correlation analysis was performed to evaluate association of hyperandrogenism with the rate of glycolysis, ATP level, $\Delta\psi$, and ROS content in granulosa cells. Negative correlations of testosterone with these variables in all participants (control and PCOS) suggested that elevation of testosterone is somehow occurs concurrent by the decline in mitochondrial membrane potential and energy level. However,

serum testosterone showed any association with ATP level and the rate of glycolysis neither

energy level in GCs with the Fig. 7

Association of lower **a** ******

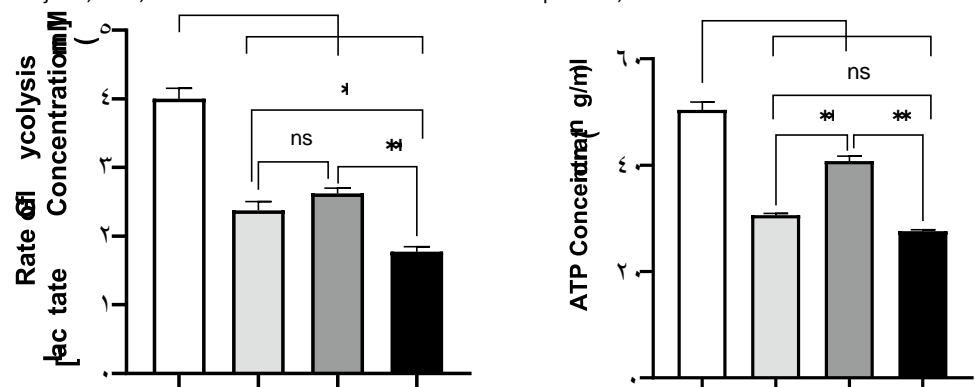
******presence of immature follicles. independent set of PCOS patients (PCOS*) and control subjects (Control*) were recruited in the second phase of study to determine rate of glycolysis (a) and ATP level GCs isolated from the participants. GCs were separated from mature immature follicles both in control women (CMF and CIMF) and PCOS patients (PMF and PIMF), and the glycolysis (c) and



ATP level (d) was evaluated in **Control* PCOS* Control* PCOS*** each group. CMF, GCs isolated from mature follicles in control subjects; CIMF, GCs isolated

from immature follicles in ******control subjects; PMF, GCs isolated from mature follicles in PCOS patients; PIMF: GCs isolated from immature follicles in PCOS patients. Data is represented as mean \pm SE, and asterisk “*” and “**” indicate $p < .05$ and $p < .01$, respectively

CMF CIMF PMF
PIMF CMF
CIMF PMF
PIMF



in control women nor in PCOS patients. It had also no significant correlations with $\Delta\psi$ or ROS content in granulosa cells in neither of groups.

Although it was not plausible to find strong associations in control subjects due to their lower levels of testosterone, lack of any significant association in PCOS group was more surprising finding. Therefore, based on the lack of association of hyperandrogenism with aforementioned variables in PCOS patients, it is believed that serum testosterone may

not be a proper criterion as follicular fluid hormonal panel in representing follicle-microenvironment interaction in PCOS. In addition, the lack of association observed between serum testosterone and cellular energy level suggests that hyperandrogenism probably does not have direct effect on GC energy level and thus the rate of glycolysis might be the main contributor to the impaired maturation of oocytes in PCOS.

In the same way, serum testosterone showed a significant positive correlation with both insulin concentration and

Table 5 Correlation of serum testosterone level with the rate of glycolysis, ATP level, $\Delta\psi_m$, ROS content, insulin concentration, and HOMA-IR index in granulosa cells, as determined in all participants, control women, and PCOS patients

	Glycolysis (mM)	ATP (ng/ml)	$\Delta\psi$	ROS (%)	Insulin (μ U/ml)	HOMA-IR
All participants	-.0408 0.001	-.0423 0.001	0.001	-.0070 0.001	0.447 0.001	0.001
Control	0.001 NS	-.0111 NS	-.0146 NS	-.0119 NS	0.077 NS	0.121 NS
PCOS	0.066 NS	0.03 NS	0.127 NS	-.033 NS	0.034 NS	-.019 NS

In each cell, the number in the first row represents Spearman's correlation coefficient, and the number in the second row indicates *p* value. $\Delta\psi_m$, mitochondrial membrane potential; ROS, reactive oxygen species.

Significant differences ($p < 0.05$) are shown in Bold

insulin resistance in all participants (Table 6) suggesting that insulin resistance (IR) and hyperandrogenism are closely related. However, after separately analyzing of Spearman's correlation test in control and PCOS groups, no association of testosterone with insulin and HOMA-IR was observed (Table 6). Based on these contentious findings, we think that a larger number of samples may be required to observe association of testosterone with manifestations of PCOS.

The role of the glycolysis pathway and mitochondria in ATP production on follicular maturation and differentiation of granulosa cells during follicular development have been reported [30–32]. The glycolysis pathway in GCs is an important source of energy (and metabolites) required for oocytes to undergo maturation process from metaphase-I to metaphase-II [14, 30]. Maturation from the early-antral follicles to antral follicles requires proliferation of GCs, possibly by activating the glycolysis pathway [33]. In accordance with these reports, reduced expression of PFKp, lower $\Delta\psi_m$, declined ATP content, and higher ROS level have previously been observed in cultured GCs of women with PCOS [6].

Similarly, reduced HK1, PFK, and PKM2 gene expression together with the instantaneous lower ATP have been reported in PCOS rat model [7]. Concordant with these cell culture and animal model studies, our results accentuated the presence of impaired energy production in GCs of women with PCOS patients by showing significant decrease in the expression of key glycolytic enzymes, a marked reduction in the rate of glycolysis, and deprivation of ATP.

Based on previous reports, in granulosa cells of PCOS mouse model, resveratrol was able to increase lactate and ATP levels, decrease pyruvate level, restore the glycolytic process, and regulate the levels of enzymes involved in the glycolysis pathway, LDHA, HK2, and PKM2. Its antioxidant function repaired ovarian damage in PCOS mice by restoring glycolytic activity and provided potential strategy for PCOS treatment [34]. Similarly, in the present study, we showed lower energy production, downregulated key enzymes of glycolysis pathway at the both gene and protein levels, reduced mitochondrial membrane potential, and elevated oxidative stress in granulosa cells of PCOS patients. Correlation of glycolysis rate and ATP level of granulosa cells with the number of embryos from metaphase-II oocytes were also observed, and an association between antioxidant level of GCs and the number of embryos obtained from metaphase-II oocytes was also found. Together, these observations provided evidences that activating of glycolysis pathway and strengthening of antioxidant potency of GCs (e.g., by dietary supplementation) might be effective strategies in provoking follicular development and maturation and lowering the risk of occurrence of PCOS. In addition, as proposed by Xiaodan Zhang et al., enhancement of glycolysis in

granulosa cells may activate primordial follicles and can be considered as potential implications for the treatment of clinical infertility [30].

A low level of lactate production has previously been detected in granulosa cells of PCOS subjects with chronic anovulation [36]. Likewise, a decreased lactate level as the final product of glycolysis was found in PCOS granulosa cells in the present study. Considering the important role of GCs in supplying energy and pyruvate for the growth and maturation of follicles, it can be concluded that the decrease in ATP and lactate in these cells might be responsible for impaired maturation of follicles and the presence of a large number of immature follicles in these women.

GCs play key roles in maintaining optimal ATP levels in oocyte [18] by providing necessary metabolic mediators required for oocyte Krebs cycle [16, 17]. However, based on our observations in granulosa cells of PCOS patients, downregulation of glycolysis enzymes, reduced rate of glycolysis, and therefore decreased production of these metabolic mediators more likely impede supportive role of GCs. In addition, it has very recently been reported that complex I, as the first member of mitochondrial respiratory chain, is assembled and fully functional in maturing (stage III) and mature oocytes and is absent in early immature oocytes [37]. Therefore, it is possible that the lack of adequate support from GCs together with the absence of complex I in immature oocytes eventuates in a lower energy level and hinders follicular maturation process and results in lower fertility rate, as observed in this study in PCOS patients.

A review in the literature showed that epigenetic dysregulation of genes in granulosa cells may be involved in the development of PCOS [38]. Also, multiomics analysis has revealed that differentially expressed long noncoding RNAs (lncRNAs), microRNAs (miRNAs), and methylated regions-associated genes between PCOS patients and control subjects were commonly associated with pathways such as glycolysis and gluconeogenesis [39]. In addition, Jianping Cao et al. in a transcriptome sequencing (RNA-seq) analysis on follicular fluid exosomal miRNAs have confirmed that the differentially expressed miR-143-3p and miR-100-5p might be regulatory factors of glycolysis [29]. They have also concluded that miRNAs control the expression of key glycolytic enzymes, including hexokinase- γ (HK γ), pyruvate kinase muscle isozyme M γ (PKM γ), and lactate dehydrogenase A (LDHA) to regulate glycolysis in cultured granulosa cell line [29]. Thus, it is supposed that the difference in glycolysis rate and lower expression of key enzymes of glycolysis in PCOS patients, as observed in the present study, are probably due to the epigenetic changes, differential methylation of the genes, and differentially expressed miRNAs which are regulating glycolysis in granulosa cells.

Apart from methylation, a widespread lysine acetylation of proteins was also detected in granulosa cells of PCOS patients compared with control women in a proteomics study.

Interestingly, over 10 enzymes from metabolic pathways of glycolysis and TCA cycle were found differentially hypo- or hyper-acetylated in PCOS [40]. Although several proteomics, metabolomics, and transcriptome studies which have been carried out during last few years showed differential expression patterns of genes, proteins, and miRNAs between PCOS subjects and control women [29, 38–43], the involvement of enzymes and proteins from glycolysis pathway was the main finding of almost all studies. Therefore, by taking to account of the lower energy production and downregulated key enzymes of glycolysis, as observed in the present study, we believed that further studies are needed to deeply investigate epigenetic changes of HK γ , PFK $_M$, PFK $_P$, and PKM in granulosa cells. Probable therapeutic

strategies should also be evaluated by targeting miRNAs that specifically regulate expression of these enzymes.

Association of reduced rate of glycolysis and declined ATP level in GCs with the presence of immature follicles in PCOS was further reconfirmed in an independent set of participants. Data analysis showed that (i) overall, the rate of glycolysis and ATP level in GCs of PCOS patients were significantly lower compared with control women, (ii) the ATP level and the rate of glycolysis were remarkably lower in GCs isolated from mature follicle of PCOS patients (PMF) compared with those cells isolated from mature follicles (CMF) of control subjects, (iii) the granulosa cells isolated from immature follicles of whether PCOS patients (PIMF) or control subjects (CIMF) have lower rate of glycolysis and much lesser energy content than cells isolated from mature follicles in corresponding subjects (PMF and CMF, respectively), and (iv) ATP level did not differ in immature cells obtained from control individuals (CIMF) and PCOS patients (PIMF). Therefore, it can be concluded that first, lower energy level in GCs might be correlated with restricted maturation of oocytes, as seen in both controls and PCOS. Second, since even GCs from mature follicles in PCOS patients have lower energy than those cells isolated from mature follicles in control, it is assumed that low ATP level of GCs in PCOS may contribute, albeit to some extent, to the impaired fertilization in these patients. Third, despite the fact that GCs surrounding mature follicles in control women had significantly higher energy level compared with PCOS patients, the energy level of granulosa cells obtained from immature follicles in control women is almost as low as that of PCOS patients. Therefore, this gives the impression that lower energy level is correlated to the presence of immature follicles (regardless of being fertile women or suffering from PCOS). Together, this conclusion can be drawn that the rate of fertility in women (both healthy and PCOS) is mostly dependent on the energy level in GCs surrounding mature follicles.

The present study is the first study so far which investigated energy level in GCs of mature and immature follicles in PCOS. Although we showed a non-difference in ATP level between immature follicles from the control and PCOS patients, our findings are preliminary, and further studies are needed. A question that needs to be answered is that if the impaired glycolysis and lower expression of key glycolysis pathway are involved in lower ATP level in PCOS (which are not observed in controls), what factors are responsible for the low ATP level in immature follicles of control women?

In the present study, the amount of ROS was inversely correlated to the expression of key glycolysis enzymes in PCOS. Taken to account all these observations, it can be inferred that since oocytes need the glycolytic activity of their surrounding GCs for maturation, perhaps increased ROS levels in GCs impaired glycolysis, reduced energy levels, and eventually disrupted GCs function giving rise to the presence of a myriad of immature oocytes in PCOS. This conclusion was further strengthened by observing the lesser available embryos/MII and significantly lower biochemical pregnancy in PCOS patients.

Although physiological levels of ROS are required in female reproductive pathways for healthy development of oocytes, ovulation fertilization, and better IVF outcomes [40], higher ROS levels greatly increase cell apoptosis, affect oocyte quality, and decrease positive in vitro fertilization embryo transfer outcomes in women with PCOS [19]. Similarly, here, we showed significant increase in apoptotic GCs in PCOS patients compared with control women which suggests presence of higher levels of ROS in the cells. Therefore, the low biochemical pregnancy rate, as observed in PCOS patients, to some extent might be the result of the toxic effects induced by the high level of ROS.

In the present study, all experiments were performed on isolated GCs without further culturing of the GCs in culture media to avoid any possible confounding effect on cell morphology or on the results of experiments. Another strength of the present study is simultaneous measurement of the expression of all key enzymes of glycolysis pathway at the both gene and protein levels in GCs. We have further validated the results by repeating the measurement of glycolysis rate and the level of ATP in granulosa cells in the new set of PCOS patients and control subjects.

The present study showed simultaneously higher ROS level, lower antioxidant capacity of the cells, decreased glycolysis rate, downregulation of glycolytic enzymes, and mitochondrial dysfunction. In addition, reduced ATP levels concurrent with the lower biochemical pregnancy were observed in PCOS patients. However, due to the limitations of our study, we believe that measurement of the activity of key glycolytic enzymes and determination of other enzymatic and non-enzymatic cellular antioxidant agents may further delineate molecular abnormality of GCs in PCOS patients. Since oocyte maturation is affected by metabolic impacts of granulosa and theca cells and involves communication between the cumulus-oocyte complexes, performing of a multi-faceted study covering metabolic correlations of oocytes with all surrounding cell types is also needed to reveal maturation process in more depth. Finally, outstanding question that should be addressed by future studies is whether evoking of glycolysis in granulosa cells may foster maturation of oocytes in women with PCOS.

Conclusion

In conclusion, the results of this study confirmed the role of glycolysis pathway and mitochondrial function of GCs in oocyte maturation. Therefore, it is assumed that empowerment of GCs to provide adequate and sustainable energy level or overcoming intracellular oxidative stress may be envisioned as therapeutic strategies to increase the success of IVF results in patients with PCOS.

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Author contribution IK and SM contributed to the conceptualization and designing of the study. MSF and IA contributed in provision of materials, collaborated in patient enrolment, and managed sample collection. SM carried out all laboratory works and IK, HT, and JK supervised the experiments. SM and EA collected the data. IK, SM, and EA performed statistical analysis. IK and SM wrote the first draft of the manuscript, and all authors reviewed the manuscript. IK edited the manuscript, and the revised version was approved by all authors. IK supervised the study.

Data availability The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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