Arch Gynecol Obstet (2016) 294:185–192
DOI 10.1007/s00404-016-4068-z

Altered FoxO3 expression and apoptosis in granulosa cells of women with polycystic ovary syndrome

Saideh Mikaeili[1] · Batool Hossein Rashidi[2] · Majid Safa[3,4] · Atefeh Najafi[1] · Aligholi Sobhani[1] · Ebrahim Asadi[1] · Mehdi Abbasi[1]

Received: 12 October 2015 / Accepted: 25 January 2016 / Published online: 18 March 2016

Abstract
Purpose To determine the level of apoptosis, and alteration of FoxO3 (forkhead box O3 transcription factor) expression and phosphorylation in human granulosa cells amongst polycystic ovary syndrome (PCOS) patients and control group.
Methods We recruited infertile women with PCOS ($n = 14$) and compared them with infertile women due to tubal blockage or male factor infertility ($n = 14$, controls). GnRH agonist and gonadotropins were used for ovarian stimulation. Follicular fluids from large follicles (>16 mm) were pooled and granulosa cells (GCs) were isolated using cell strainer methodology. Apoptosis of purified GCs was measured by flow cytometry using Annexin V and propidium iodide. Quantitative real-time PCR and western blotting were performed to assess alteration of FoxO3 expression and phosphorylation in GCs.
Results There were higher percentages of early and late apoptosis in GCs of PCOS patients than in the control group. FoxO3 mRNA level and total FoxO3 protein were significantly higher in PCOS group than in the control group. The ratio of p-FoxO3/total FoxO3 decreased significantly in PCOS than in the control group. It was inferred that unphosphorylated (active form) FoxO3 was higher in GCs of PCOS patients. Apoptosis was significantly and positively correlated with the total FoxO3 and negatively correlated with the p-FoxO3 protein levels in PCOS patients.
Conclusions Activation and overexpression of FoxO3 in granulosa cells of PCOS women correlated with higher apoptosis levels in these cells suggesting that FoxO3 may be a candidate for the higher apoptosis in granulosa cells from women with PCOS.

Keywords Apoptosis · Granulosa cells · FoxO3 · Polycystic ovary syndrome

✉ Mehdi Abbasi
abbasima@tums.ac.ir

[1] Department of Anatomy, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

[2] Vali-e-Asr Reproductive Health Research Centre, Tehran University of Medical Sciences, Tehran, Iran

[3] Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran

[4] Department of Hematology, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran

Introduction

Polycystic ovary syndrome (PCOS) is a complex metabolic syndrome and the principal ovarian factor that causes infertility in women of reproductive age [1]. Women with PCOS are unable to produce and release ovum due to a complex interaction of multiple hormonal imbalances such as hyperandrogenism, anti-Mullerian hormone excess, insulin resistance and gonadotropin abnormalities [2, 3]. One of the most common signs in PCOS is the presence of frequent follicular atresia which is known to be the result of apoptosis [4].

In the development of ovarian follicles, all stages of follicular atresia have been found to be closely related to the apoptosis of granulosa cells (GCs). In view of this, apoptosis of GCs has been considered as the main mechanism of follicular atresia [5]. Several factors can act as triggers of apoptosis in GCs including the depletion of cell survival factors [6].

Phosphatidylinositol 3-kinase (PI3K)-AKT signaling is the central anti-apoptotic intracellular signal transduction pathway initiated by survival factors such as hormones and/or growth factors. The PI3K-AKT pathway exerts its anti-apoptotic effect in part by phosphorylating the forkhead box O (FoxO) transcription factors. FoxO3 is a member of the FoxO transcription factor family and can be inactivated by PI3K-AKT through phosphorylation at threonine-32, serine-253, and serine-315. When survival factors are depleted, FoxO3 becomes dephosphorylated and translocates into nucleus resulting in transcription of pro-apoptotic factors, loss of mitochondrial membrane potential, cytochrome *c* release, and caspase activation [7–10].

Apoptosis of granulosa cells seems to have a negative effect on IVF outcomes. A higher incidence of apoptotic granulosa cells has been related to poor embryo development, and low fertilization and pregnancy rates [11]. PCOS patients undergoing IVF procedure produce a high number of oocytes but approximately, all of them have poor quality leading to poor outcomes [12, 13]. Thus, investigation of the mechanisms of high apoptosis in granulosa cells of PCOS patients is of great clinical importance.

The importance of FoxO3 has been demonstrated in rodent ovaries. For example, gene silencing of FoxO3 in mice resulted in premature follicular depletion and ovarian failure, suggesting an essential role of FoxO3 in follicular development [14]. However, little is known on the function of FoxO3 in human ovary, and there have not been any investigations on the role of this transcription factor in PCOS condition. For this reason, we investigated the changes in FoxO3 expression and phosphorylation in human granulosa cells. We hypothesized that changes in the expression and phosphorylation of FoxO3 in human granulosa cells can be a cause of higher rate of apoptosis in PCOS patients.

Materials and methods

Subjects

The protocol was approved by the Ethics Committee of Tehran University of Medical Sciences, and informed consents were obtained from all of the participants. All participants were ≤40 years old. 14 control patients with no evidence of ovarian malfunction, where infertility has been ascribed to either tubal blockage or problem with the male partner were selected. Another group consisting of 14 PCOS patients was selected. PCOS was diagnosed according to the Rotterdam Consensus [15], i.e. the presence of two of the following three criteria: oligo- or anovulation, signs of clinical hyperandrogenism and/or biochemical signs of hyperandrogenism and polycystic ovaries on ultrasonography. Women with ovarian tumors, congenital adrenal hyperplasia, androgen secreting tumors, Cushing's syndrome and endometriosis were excluded.

Controlled ovarian hyperstimulation

All patients were subjected to the same controlled ovarian hyperstimulation (COH) for in vitro fertilization-embryo transfer. COH was achieved by long agonist protocol [16]. Briefly, Buserelin acetate (superfact, Aventis, Germany) was administrated in the midluteal phase of the preceding cycle for pituitary down-regulation. When down-regulation is achieved, ovarian stimulation was initiated with recombinant FSH (Gonal-F, Serono, Italy) on the third day of menstrual cycle. Dosages of gonadotropins were adjusted according to individual patient's response (serum estradiol levels and transvaginal ultrasonic measurements of the follicles). When at least three of the follicles reached diameters of 16–18 mm, ovulation was induced by the administration of 10,000 IU human chorionic gonadotropin (hCG) (Choriomon, IBSA Institut Biochimique S.A., Switzerland). 36 h after injection of hCG, transvaginal oocyte aspiration was performed with ultrasound-guided puncture. Follicle size was determined before oocyte retrieval under ultrasound, and follicular fluids from large follicles (>16 mm) were carefully collected. The size of large follicles was chosen similar to previous studies [17, 18].

Collection of GCs

Several human granulosa cell isolation techniques have been described in many studies, notwithstanding some of them could not clearly indicate the purity of GCs, and the protocol used in some of the studies also induced undesirable changes in GCs characteristics [19]. It has been established that cell strainer methodology is the most efficient method for isolation because a higher percentage of GCs with acceptable level of blood contamination are usually recovered [20]. We employed this method with some few modifications that ensured removal of blood contamination during GCs preparation.

The aspirates from large follicles (>16 mm) for each patient were pooled after oocyte retrieval and transported on ice to the laboratory. All samples were processed within 1 h after collection to avoid post-aspiration cell death. The aspirates were first filtered through a 40 μm cell strainer (BD Biosciences, CA, USA) which retained clusters of GCs. The strainer was then rinsed with phosphate buffered saline (PBS) to remove any remaining traces of blood contaminants, after which it was back washed with PBS containing 1 % BSA (Bovine serum albumin; Sigma, Louis, MO, USA) to collect GCs. The resulting suspension

was incubated for several minutes, and aspirated repeatedly through Pasteur pipettes to disperse the clusters of GCs. The suspension was then filtered through a 70 μm cell strainer (BD Biosciences) to remove unwanted undispersed material. The cell suspension was depleted of red blood cells (RBCs) by incubating the suspension with RBC lysis buffer (Sigma) according to the manufacturer's protocol. The cells were washed by centrifugation for 5 min at 1000*g*, and the supernatant was removed. The aliquots of cells were then subjected to the determination of the percentage of contaminating $CD45^+$ cells and GCs apoptosis by flow cytometry, and the remaining were frozen and stored at −80 °C until they were used for the further analysis.

Determination of the percentage of leukocytes by flow cytometry

Leukocytes ($CD45^+$ cells) from peripheral blood are the most abundant contaminating population in the follicular fluid, after the erythrocytes have been depleted (21). Therefore, the percentage of leukocytes, after granulosa cells' purification, was measured by flow cytometry method.

An antibody specific for human CD45, directly labelled with RPE-Cy5 (anti-CD45-R-Phycoerythrin-Cyanine5-conjugated, Dako, Denmark), was added at a dilution of 1:10. The other fraction of cell suspension was incubated with a similar concentration of human IgG1 isotype labelled with RPE-Cy5 (IgG1-RPE-Cy5, Dako, Denmark) as a negative control for CD45 antibodies and to establish a baseline for positive CD45 counting. The two fractions of cell suspensions were subsequently incubated on ice for 30 min in the dark, after which they were washed twice with PBS containing 2 % BSA to remove excess primary unlabelled antibodies. The pellets were resuspended in an appropriate fluid for flow cytometry. The percentage of $CD45^+$ population was determined using a FACSCalibur flow cytometer (BD Biosciences, CA, USA). The analysis was set to a maximum of 10,000 cells counted per sample.

Measurement of GCs apoptosis by flow cytometry

One of the earliest signs of apoptosis is a morphological change in the plasma membrane. This involves the translocation of the membrane phospholipid, phosphatidylserine (PS), after it has been reversed to the outer layer of the bilayer membrane in the process of apoptosis. Annexin V is a calcium-dependent phospholipid-binding protein that can specifically bind with high affinity to PS. Thus, the binding of Annexin V to cells with exposed PS provides a very sensitive method for detecting cellular apoptosis. Propidium iodide (PI) is a nucleic acid dye that cannot normally traverse the intact cell membrane, but in the later stages of apoptosis, it can stain the nucleus due to breaks in the cell membrane.

Flow cytometry was performed according to the apoptosis detection kit (IQ Co, The Netherlands) procedures. Cells were washed with calcium buffer and then centrifuged at 1000*g* for 5 min. 1×10^6 cells were collected and 10 μl of the FITC-Annexin V mix was added to 100 μl cell suspension. The cell suspension was incubated for 20 min on ice in the dark and then washed with calcium buffer, after which they were centrifuged at 1000*g* for 5 min. 10 μl propidium iodide was added and the cell suspension was incubated again for at least 10 min on ice. Flow cytometry was performed using a FACSCalibur flow cytometer; a maximum of 10,000 cells were counted per sample.

Flow cytometry, using the Annexin V/PI double staining method, was used to generate an apoptotic cell scatter plot. The scatter plot was composed of four subgroups as follows: living cells were Annexin V^-/PI^- (in the lower left quadrant), necrotic cells were Annexin V^-/PI^+ (in the upper left quadrant), early apoptotic cells were Annexin V^+/PI^- (in the lower right quadrant) and late apoptotic cells were Annexin V^+/PI^+ (in the upper right quadrant).

Detection of FoxO3 expression by quantitative real-time PCR

Total RNA was isolated from GCs using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purity and concentration of RNA were determined by NanoDrop2000 Spectrophotometer (Thermoscientific, Inc., USA). One microgram RNA was used to prepare cDNA using Transcriptor First Strand cDNA Syntesis Kit (Roche, Mannheim, Germany). The cDNA samples were diluted in DNase and RNase free water at a proportion of 1:3 before further analysis. Quantitative real-time PCR (qRT-PCR) was performed using RealQ plus 2× Master Mix Green (Ampliqon, Odense, Denmark) in the Rotor Gene 6000 Real-Time PCR System (Corbett Research, Hilden, Germany). Reactions were performed in a reaction mix containing 1 μl forward primer, 1 μl reverse primer, 7.5 μl premix with SYBR Green, 1.5 μl cDNA template, and 4 μl of nuclease-free water in a total volume of 15 μl. Thermal cycling conditions included an initial activation step at 95 °C for 10 min followed by 40 cycles including a denaturation step at 95 °C for 5 s, and a combined annealing and elongation step at 60 °C for 30 s. Each experiment was repeated three times and values were normalized to β-actin, a housekeeping gene, to control the amount of the input cDNA. The relative expression of each mRNA was calculated by Δ threshold cycle (C_t) method, in which C_t is a value obtained

by subtracting the C_t value of housekeeping gene from the C_t value of the target mRNA, and the amount of target mRNA relative to housekeeping gene mRNA was expressed as $2^{-\Delta\Delta Ct}$. Relative expression level of FoxO3 was normalized to β-actin. The oligonucleotide sequences of primers used for detecting the mRNA expressions were as follows:

FoxO3 (forward primer, 5′-TGT TGG TTT GAA CGT GGG GA-3′; reverse primer, 5′-GTT TGA GGG TCT GCT TTG CC-3′).

β-actin (forward primer, 5′-GAT CAT TGC TCC TCC TGA GC-3′; reverse primer; 5′-ACT CCT GCT TGC TGA TCC AC-3′).

Detection of p-FoxO3 and total FoxO3 proteins by western blotting

Total protein from GCs was extracted using RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma, Louis, MO, USA). The cell lysate was centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatant was collected. Total protein of the supernatant was quantified using the Bradford protein assay, and 15 μg was separated by 10 % sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). The resolved proteins were then transferred from the gels to nitrocellulose membranes (Hybond-ECL, Amersham Corp). The membranes were incubated in blocking buffer (5 % nonfat dry milk in TBST buffer) for 1 h and probed overnight at 4 °C with specific primary antibodies (Cell Signaling Technology Inc, Danvers, MA, USA) against phospho-FoxO3 (p-FoxO3, Ser253, 1:1000), total FoxO3 (1:1000) and β-actin (1:1000). After washing five times in TBST buffer, the membranes were incubated with horse radish peroxidase (HRP)-conjugated secondary antibody, goat anti-rabbit IgG (1:2000, Cell Signaling) for 1 h at room temperature. The immunoreactive proteins were then visualized with a chemiluminescence detection system (Amersham ECL Advance Kit, GE Healthcare) according to the manufacturer's protocol. Band densities were measured using UVIdoc image analysis system (UVIdoc, Houston, TX, USA). The band densities for total FoxO3 and p-FoxO3 were normalized to β-actin (internal control). Each experiment was repeated in triplicate.

Statistical analysis

All experiments were performed three times and data were expressed as the mean ± SEM. All the data in this study were analysed using the Independent-Sample *T* Test with SPSS 22.0 software (SPSS, Inc., Chicago, USA). Pearson's correlation coefficient was applied to investigate the correlation between early and late apoptosis levels and total FoxO3 and p-FoxO3 and the ratio of p-FoxO3/total FoxO3 in GCs from PCOS and control groups. $P < 0.05$ was considered to be statistically significant.

Results

Assessment of GCs purification

It has been proposed that CD45 can be used as a marker to distinguish granulosa cells (CD45 negative) from leukocytes (CD45 positive). We analysed granulosa cells using flow cytometry on the basis of their negativity for CD45 antigen. Flow cytometry analysis showed that the amount of $CD45^+$ cells, after purification method, was always less than 4 % (data not shown).

Measurement of apoptosis in GCs

The result obtained with Annexin V/PI detection in flow cytometry is shown in Fig. 1. The average level of early apoptosis was 17.36 ± 0.48 in the PCOS group and 4.31 ± 0.34 in the control group ($P < 0.05$), and late apoptosis level was 21.22 ± 0.56 in PCOS vs. 12.06 ± 0.43 in the control group ($P < 0.05$). The results indicated that early and late apoptotic levels in human granulosa cells were significantly higher in the PCOS group.

FoxO3 mRNA expression in GCs

Relative expression of FoxO3/β-actin was 3.55 ± 0.88 in PCOS patients and 1.21 ± 0.26 in controls. As shown in Fig. 2, FoxO3 mRNA expression was significantly higher in GCs of women with PCOS than in the control group ($P < 0.05$).

Phosphorylated FoxO3 and total FoxO3 levels in GCs

Western blot assay was performed for the detection phosphorylated FoxO3 (p-FoxO3) at the preferential phosphorylation site (Ser253) by Akt as well as the expression of total FoxO3 (Fig. 3). Expression of total FoxO3 protein was significantly increased in PCOS than in the control group (9.57 ± 0.12 vs. 6.47 ± 0.03, respectively, $P < 0.05$), but the expression of p-FoxO3 (inactive form) showed no significant difference between the two groups (3.04 ± 0.11 vs. 3.13 ± 0.33, $P > 0.05$). As shown in Fig. 3b, the ratio of p-FoxO3/total FoxO3 was decreased significantly in PCOS than in the control group (0.31 ± 0.01 vs. 0.47 ± 0.04, $P < 0.05$). Therefore, the above data indicated that unphosphorylated (active form) FoxO3 was higher in GCs of PCOS patients.

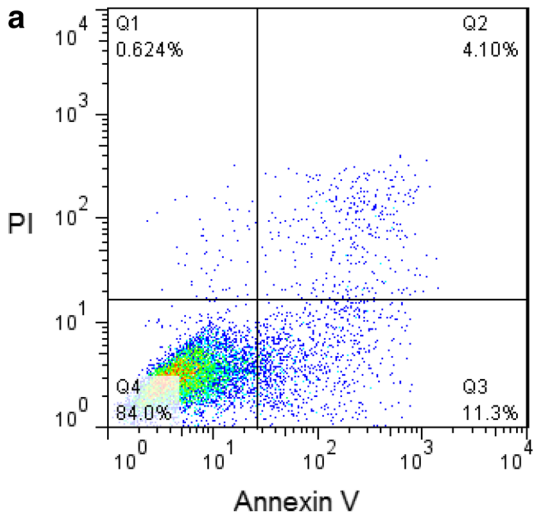

Fig. 1 Early apoptotic cells (quadrant Q3) and late apoptotic cells (quadrant Q2) can be distinguished from necrotic (quadrant Q1) and vital cells (quadrant Q4). **a** Results of flow cytometric analysis of granulosa cells of one control group. **b** Results of flow cytometric analysis of granulosa cells of one PCOS group

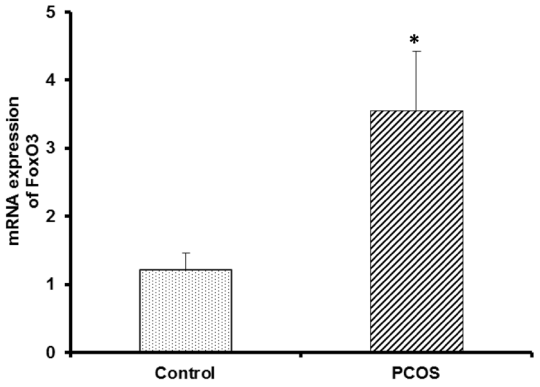

Fig. 2 Quantification of FoxO3 mRNA was determined by qRT-PCR. Relative expression of FoxO3 was normalized to β-actin. *Bars* represents mean ± SEM. *Asterisk* represent significant difference from control

Correlation between total and phosphorylated FoxO3 and apoptosis in GCs

As shown in Fig. 4, total FoxO3 was significantly correlated with early and late apoptosis ($r = 0.571$, 0.586, $P < 0.05$), and p-FoxO3 was also significantly and negatively correlated with late apoptosis ($r = -0.46$, $P < 0.5$) in the control group. There was no significant correlation between p-FoxO3 and early apoptosis in the control group ($r = -0.349$, $P > 0.05$). Total FoxO3 was significantly correlated with early and late apoptosis ($r = 0.473$, 0.489, $P < 0.05$), and p-FoxO3 was also significantly and negatively correlated with early and late apoptosis ($r = -0.604$, -0.524, $P < 0.5$) in the PCOS group. There was no significant correlation between the ratio of p-FoxO3/total FoxO3 and early and late apoptosis in the controls ($r = -0.088$, -0.326, $P > 0.05$) and PCOS group ($r = -0.422$, 0.292, $P > 0.05$).

Discussion

Oocyte retrieval by transvaginal ultrasound-guided aspiration causes contamination of follicular fluid with blood component such as WBCs (15–17 %) and RBCs [22]. Because of high secretory activity of WBCs, these cells can strongly interfere with the function of GCs [21]. Therefore, efficient granulosa isolation protocol is essential for accurate interpretation of data obtained from experiments.

In this study, we evaluated the purity of recovered GCs and acquired GCs with acceptable level of purity (≥96 %). Apoptosis in granulosa cells can be activated by factors such as oxidative stress [23], androgens [24], and gonadotropin releasing hormone and its analogue [25]. PCOS patients have multiple endocrine disorders such as oxidative stress [26], hyperandrogenemia [27] and disturbance of LH/FSH ratio [28] which can cause high level of apoptosis in GCs.

In this research, apoptosis in GCs was examined by flow cytometry. Flow cytometry has a number of advantages when employed for the detection of apoptosis including its high precision, detection of early phases of apoptosis and ability to quickly analyse a large number of samples [29]. We found that the percentage of apoptosis in granulosa cells was significantly higher in PCOS than in the control group. Further, in accordance with our results, some studies have shown that the percentage of apoptosis in granulosa cells of ovaries from PCOS rats was significantly higher than the control rats although they measured apoptosis in tissue sections of ovaries and by different method (TUNEL

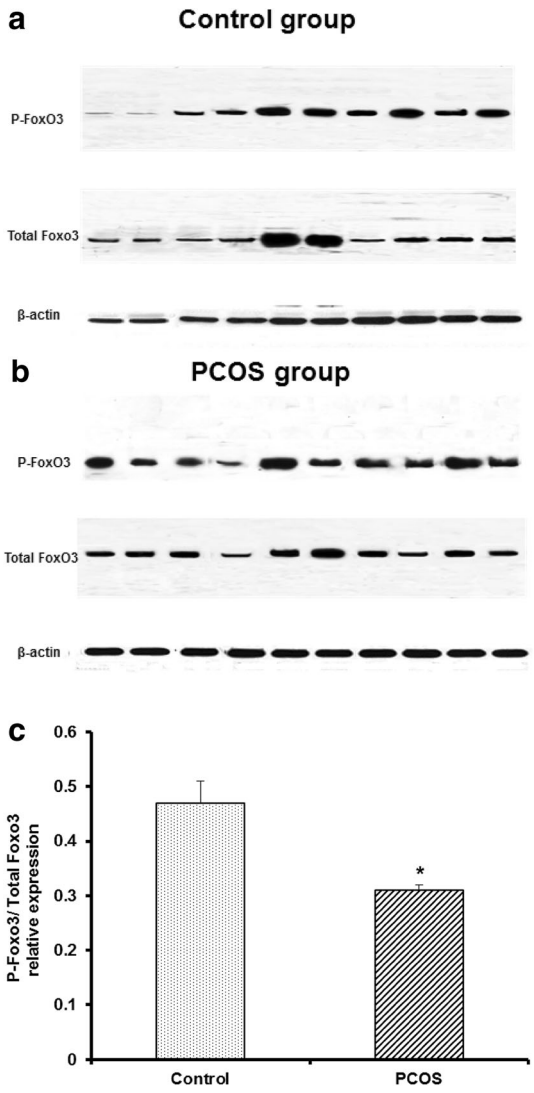

Fig. 3 P-FoxO3, total FoxO3 and β-actin expression was assessed by western blotting for control group (**a**) and PCOS group (**b**). The β-actin was regarded as internal control in both groups. **c** The graph demonstrates the ratio of the densitometric analysis of p-FoxO3 proteins normalized against its total forms in PCOS and control groups. *Bars* represent mean ± SEM. *Asterisk* represents significant difference from control

assay) [4, 30]. Moreover, another study showed high levels of apoptosis in GCs amongst PCOS women using GCs prepared from follicular fluid of FSH-primed ovaries [31].

In contrast, some studies have reported that there are fewer number of apoptotic granulosa cells in PCOS patients than in normo-ovulatory women [32, 33]. This discrepancy between our results and theirs may be due to the fact that they prepared granulosa cells using follicular fluid from small follicles and from unstimulated ovaries, whilst we used granulosa cells of large follicles and our patients were subjected to controlled ovarian hyperstimulation protocol.

Apoptosis in granulosa cells is an intricate process that requires extrinsic regulators as well as intrinsic factors such as FoxO3 transcription factor [34]. When survival factors are depleted, activation of FoxO3 (loss of post-translational phosphorylation of FoxO3) can induce apoptosis in GCs by up-regulating pro-apoptotic factors such as FAS ligand (cell death ligand), BCL2L11(pro-apototic member of BCL2 family) in human granulosa tumor-like cell line [35] and TRAIL (pro-apoptotic member of TNF-family) in pig granulosa cells [36]. While FoxO3 is highly expressed in most mammalian tissues, the most remarkable disorder observed in ovaries following FoxO3 gene knockdown, suggesting that, FoxO3 has a major role in ovarian function [14]. Immunohistochemical experiment on porcine ovaries has shown that FoxO3 staining in granulosa layer was more than oocytes, showing FoxO3 has more critical roles in granulosa cells [35].

In another study, the authors reported that expression of mRNA for FoxO3 was higher in porcine granulosa cells during follicular atresia. Using TUNEL method and total FoxO3 protein staining, they showed that FoxO3 protein was highly expressed in granulosa layers of atretic follicles [35]. In accordance with the above findings, our study showed that FoxO3 mRNA and total protein levels were significantly higher in GCs from PCOS group. The ratio of p-FoxO3/total FoxO3 was significantly lower in GCs from PCOS group than in the control group, suggesting that active form of FoxO3 was more in GCs from PCOS patient. Our data showed that apoptosis in granulosa cells from PCOS patients was significantly and positively correlated with the total FoxO3 and negatively correlated with the phosphorylated FoxO3 protein levels. These findings suggest that higher levels of FoxO3 (total FoxO3 and active form) may be involved in the increased apoptosis in GCs from women with PCOS.

To our knowledge, this is the first report which has determined the frequency of FoxO3 expression and activation in human granulosa cells amongst PCOS patients. However, the question of what causes the overexpression of FoxO3 and its activation in GCs of PCOS patients was not answered. Considering the importance of apoptosis in granulosa cells during follicular atresia, further studies on the factors that up-regulate and activate FoxO3 in GCs of PCOS patients remain to be examined in the future.

In conclusion, this study showed that activation and overexpression of FoxO3 in granulosa cells of PCOS women correlated with higher apoptosis levels in these cells suggesting that FoxO3 may be a candidate for the apoptosis in granulosa cells from women with PCOS.

Acknowledgments The present study was funded by Deputy Ministry for Research, Tehran University of Medical Sciences; Grant No. 91-03-30-19007. We would like to thank Dr. Ensieh Tehraninejad

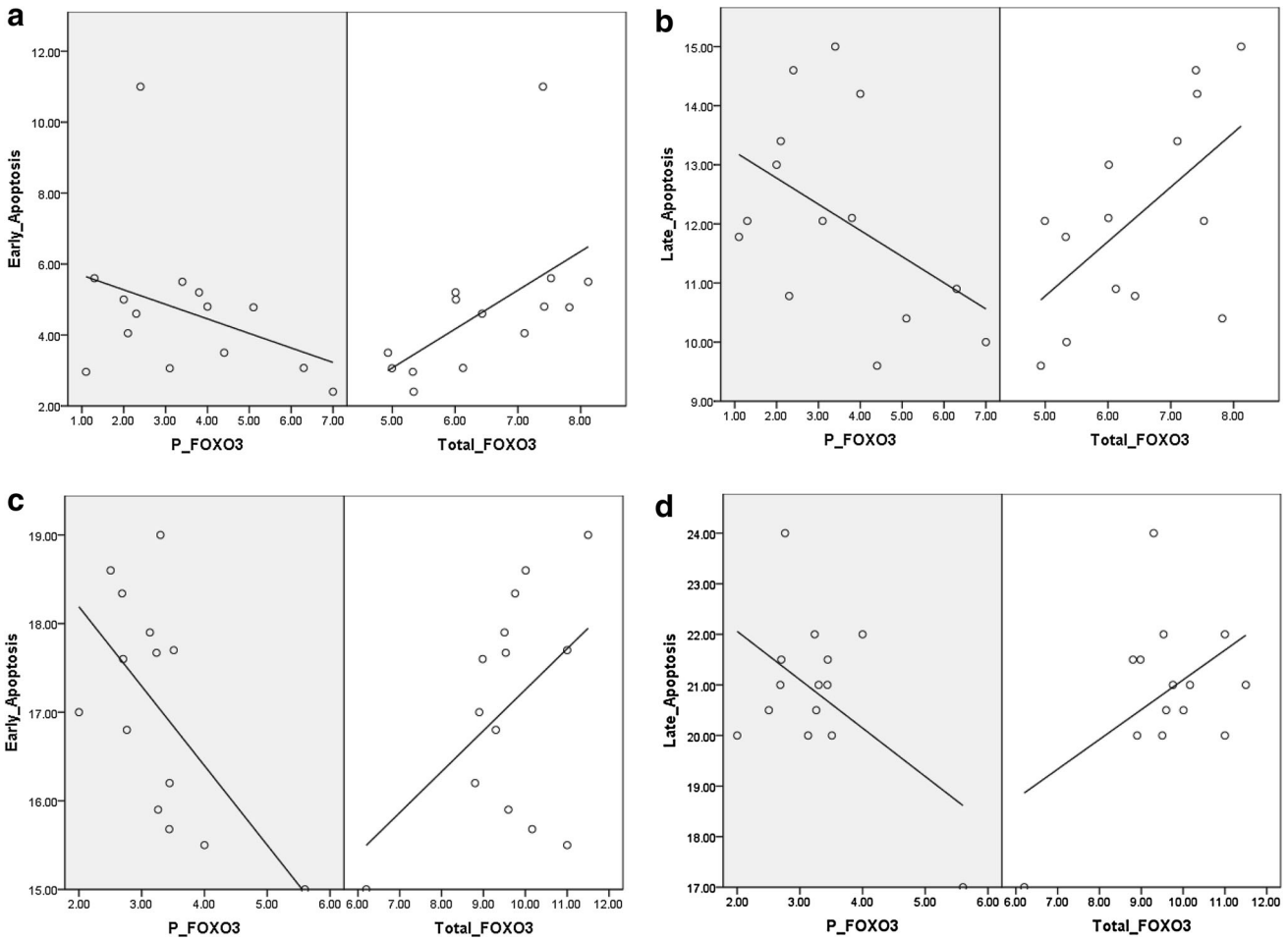

Fig. 4 Correlation between p-FoxO3 and total FoxO3 and early and late apoptosis in both groups. **a**, **b** Total FoxO3 was significantly correlated with early and late apoptosis ($r = 0.571$, 0.586, $P < 0.05$), and p-FoxO3 was also significantly and negatively correlated with late apoptosis ($r = -0.46$, $P < 0.5$) in the control group. There was no significant correlation between p-FoxO3 and early apoptosis in the control group ($r = -0.349$, $P > 0.05$). **c**, **d** Total FoxO3 was significantly correlated with early and late apoptosis ($r = 0.473$, 0.489, $P < 0.05$), and p-FoxO3 was also significantly and negatively correlated with early and late apoptosis ($r = -0.604$, -0.524, $P < 0.5$) in the PCOS group

(Vali-e-Asr Reproductive Health Research Centre, Tehran University of Medical Sciences, Tehran, Iran) and Parisa Hayat (Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran) for their kind help with the present research.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests associated with this publication.

References

1. Ehrmann DA (2005) Polycystic ovary syndrome. New Engl J Med 352:1223–1236
2. Moran L, Teede H (2009) Metabolic features of the reproductive phenotypes of polycystic ovary syndrome. Hum Reprod Update 15:477–488
3. Köninger A, Koch L, Edimiris P, Enekwe A, Nagarajah J, Kasimir-Bauer S, Kimmig R, Strowitzki T, Schmidt B (2014) Anti-Mullerian Hormone: an indicator for the severity of polycystic ovarian syndrome. Arch Gynecol Obstet 290:1023–1030
4. Honnma H, Endo T, Henmi H, Nagasawa K, Baba T, Yamazaki K, Kitajima Y, Hayashi T, Manase K, Saito T (2006) Altered expression of Fas/Fas ligand/caspase 8 and membrane type 1-matrix metalloproteinase in atretic follicles within dehydroepiandrosterone-induced polycystic ovaries in rats. Apoptosis 11:1525–1533
5. Yu YS, Sui HS, Han ZB, Wei L, Luo MJ, Tan JH (2004) Apoptosis in granulosa cells during follicular atresia: relationship with steroids and insulin-like growth factors. Cell Res 14:341–346
6. Maeda A, Inoue N, Matsuda-Minehata F, Goto Y, Cheng Y, Manabe N (2007) The role of interleukin-6 in the regulation of granulosa cell apoptosis during follicular atresia in pig ovaries. J Reprod Dev 53:481–490
7. Dijkers PF, Birkenkamp KU, Lam EW-F, Thomas NSB, Lammers J-WJ, Koenderman L, Coffer PJ (2002) FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal protein kinase B—enhanced cell survival through maintenance of mitochondrial integrity. J Cell Biol 156:531–542

8. Stahl M, Dijkers PF, Kops GJ, Lens SM, Coffer PJ, Burgering BM, Medema RH (2002) The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. J Immunol 168:5024–5031
9. Accili D, Arden KC (2004) FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell 117:421–426
10. Obexer P, Geiger K, Ambros P, Meister B, Ausserlechner M (2007) FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells. Cell Death Differ 14:534–547
11. Suh CS, Jee BC, Choi YM, Kim JG, Lee JY, Moon SY, Kim SH (2002) Prognostic implication of apoptosis in human luteinized granulosa cells during IVF–ET. J Assist Reprod Gen 19:209–214
12. Urman B, Tiras B, Yakin K (2004) Assisted reproduction in the treatment of polycystic ovarian syndrome. Reprod Biomed Online 8:419–430
13. Iavazzo C, Vitoratos N (2010) Polycystic ovarian syndrome and pregnancy outcome. Arch Gynecol Obstet 282:235–239
14. Castrillon DH, Miao L, Kollipara R, Horner JW, DePinho RA (2003) Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science 301:215–218
15. Rotterdam ESHRE/ASRM sponsored consensus workshop group (2004) Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod 19:41–47
16. Nikolettos N, Kupker W, Al-Hasani S, Demirel L, Schöpper B, Sturm R, Diedrich K (2000) ICSI outcome in patients of 40 years age and over: a retrospective analysis. Eur J Obstet Gyn R B 91:177–182
17. Catteau-Jonard S, Jamin SP, Leclerc A, Gonzalès J, Dewailly D, di Clemente N (2008) Anti-Mullerian hormone, its receptor, FSH receptor, and androgen receptor genes are overexpressed by granulosa cells from stimulated follicles in women with polycystic ovary syndrome. J Clin Endocrinol Metab 93:4456–4461
18. Smith LP, Nierstenhoefer M, Yoo SW, Penzias AS, Tobiasch E, Usheva A (2009) The bile acid synthesis pathway is present and functional in the human ovary. PLoS One 4:e7333
19. Wolffe AP, Tata JR (1984) Primary culture, cellular stress and differentiated function. Febs L 176:8–15
20. Ferrero H, Delgado-Rosas F, Garcia-Pascual CM, Monterde M, Zimmermann RC, Simón C, Pellicer A, Gómez R (2012) Efficiency and purity provided by the existing methods for the isolation of luteinized granulosa cells: a comparative study. Hum Reprod 27:1781–1789
21. Fedorcsák P, Ráki M, Storeng R (2007) Characterization and depletion of leukocytes from cells isolated from the pre-ovulatory ovarian follicle. Hum Reprod 22:989–994
22. Figenschau Y, Sundsfjord J, Yousef M, Fuskevåg O, Sveinbjörnsson B, Bertheussen K (1997) A simplified serum-free method for preparation and cultivation of human granulosa-luteal cells. Hum Reprod 12:523–531
23. Al-Gubory KH, Fowler PA, Garrel C (2010) The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol 42:1634–1650
24. Billig H, Furuta I, Hsueh A (1993) Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinol 133:2204–2212
25. Kaneko T, Saito H, Takahashi T, Ohta N, Saito T, Hiroi M (2000) Effects of controlled ovarian hyperstimulation on oocyte quality in terms of the incidence of apoptotic granulosa cells. J Assist Reprod Gen 17:580–585
26. Karuputhula NB, Chattopadhyay R, Chakravarty B, Chaudhury K (2013) Oxidative status in granulosa cells of infertile women undergoing IVF. Syst Biol Reprod Med 59:91–98
27. Nisenblat V, Norman RJ (2009) Androgens and polycystic ovary syndrome. Curr Opin Endocrinol Diabetes Obes 16:224–231
28. Balen AH, Tan SL, Jacobs HS (1993) Hypersecretion of luteinising hormone: a significant cause of infertility and miscarriage. BJOG 100:1082–1089
29. Nakahara K, Saito H, Saito T, Ito M, Ohta N, Takahashi T, Hiroi M (1997) The incidence of apoptotic bodies in membrana granulosa can predict prognosis of ova from patients participating in in vitro fertilization programs. Fertil Steril 68:312–317
30. Zhang J, Zhu G, Wang X, Xu B, Hu L (2007) Apoptosis and expression of protein TRAIL in granulosa cells of rats with polycystic ovarian syndrome. J Huazhong Univ Sci Technol 27:311–314
31. Onalan G, Selam B, Baran Y, Cincik M, Onalan R, Gündüz U, Ural AU, Pabuccu R (2005) Serum and follicular fluid levels of soluble Fas, soluble Fas ligand and apoptosis of luteinized granulosa cells in PCOS patients undergoing IVF. Hum Reprod 20:2391–2395
32. Das M, Djahanbakhch O, Hacihanefioglu B, Saridogan E, Ikram M, Ghali L, Raveendran M, Storey A (2008) Granulosa cell survival and proliferation are altered in polycystic ovary syndrome. J Clin Endocrinol Metab 93:881–887
33. Almahbobi G, Anderiesz C, Hutchinson P, McFarlane JR, Wood C, Trounson AO (1996) Functional integrity of granulosa cells from polycystic ovaries. Clin Endocrinol 44:571–580
34. Wang X-L, Wu Y, Tan L-B, Tian Z, Liu J-H, Zhu D-S, Zeng S-M (2012) Follicle-stimulating hormone regulates pro-apoptotic protein Bcl-2-interacting mediator of cell death-extra long (BimEL)-induced porcine granulosa cell apoptosis. J Biol Chem 287:10166–10177
35. Matsuda F, Inoue N, Maeda A, Cheng Y, Sai T, Gonda H, Goto Y, Sakamaki K, Manabe N (2011) Expression and function of apoptosis initiator FOXO3 in granulosa cells during follicular atresia in pig ovaries. J Reprod Develop 57:151–158
36. Inoue N, Maeda A, Matsuda-Minehata F, Fukuta K, Manabe N (2006) Expression and localization of Fas ligand and Fas during atresia in porcine ovarian follicles. J Reprod Develop 52:723–730