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The relationship between different granulosa cell morphologies and apoptosis in women with PCOS: Impact on clinical outcomes

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ABSTRACT

Objective: The microenvironment of the oocyte is composed of granulosa cells (GC), which play a vital role in the process of oogenesis. During Assisted Reproductive Technology (ART) treatment, the morphological characteristics of GC are evaluated. However, this evaluation does not provide definitive clarity. There is a limited amount of research on the underlying mechanisms of abnormal GC morphology, and it remains incompletely understood. Therefore, the aim of our study was to investigate apoptosis in GCs with varied morphologies in patients with different infertility etiologies undergoing intracytoplasmic sperm injection (ICSI). We performed an immunohistochemical analysis of caspase-3, cytochrome c, and heat shock protein-70 (HSP-70) expression levels, and evaluated their impact on assisted reproduction.

Patients and Methods: The study comprised 142 patients, including 65 with polycystic ovary syndrome (PCOS) and 77 with male factor (MF) infertility. The granulosa-oocyte complex was analyzed under a stereomicroscope and classified into dark granulosa cells (DGC) and light granulosa cells (LGC). Fertilization rates, embryonic development, and pregnancy outcomes following ICSI were evaluated. DGC exhibited reduced fertilization and pregnancy rates.

Results: Caspase-3 and cytochrome c expression levels were significantly elevated in the PCOS DGC (26, 86.6%; 25, 83.3%) and MF DGC (34, 77.2%; 32, 72.7%) groups compared to the PCOS LGC (8, 22.8%; 5, 14.2%) and MF LGC (4, 12.1%; 7, 21.2%) groups ($p = 0.001$; $p = 0.001$), respectively. In contrast, expression levels of HSP-70 were significantly lower in the PCOS DGC (6, 20%) and MF DGC (7, 15.9%) groups compared to the PCOS LGC (31, 88.5%) and MF LGC (32, 96.9%) groups ($p = 0.001$).

Conclusion: In the PCOS group, the number of good quality embryos on day 3 exhibited a positive correlation with HSP-70 expression levels and a negative correlation with cytochrome c expression levels. Our findings suggest that DGC morphology is a reflection of apoptosis, which impacts oocyte quality and, consequently, embryonic development, with PCOS contributing to the worsening of this condition.

Keywords: Granulosa cell, PCOS, Apoptosis, In vitro fertilization, Embryo development

1. INTRODUCTION

The World Health Organization (WHO) defines polycystic ovary syndrome (PCOS) as an endocrine and reproductive disorder impacting 8% to 13% of women of reproductive age. PCOS is characterized by the presence of at least two of the Rotterdam criteria: oligo-anovulation, clinical or biochemical signs of hyperandrogenism, and micropolycystic ovaries (indicated by an ovarian volume exceeding 10 ml and/or more than 12 follicles by the ovary) (Practice Committee of the American Society for Reproductive Medicine, 2013) [1]. Although the precise etiology of PCOS is not fully elucidated, it is widely regarded as a multifactorial disorder encompassing a range of genetic, metabolic, endocrine, and environmental abnormalities [2]. It leads to menstrual disorders, infertility, and obesity. Furthermore, it may also result in spontaneous abortion, gestational diabetes, hypertensive disorders during pregnancy, preterm birth, and potentially fetal growth abnormalities [3].

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Secondary oocytes are located within large antral follicles, which predominantly surrounded by granulosa cells (GCs) [4]. GCs play a crucial supportive role in physiological folliculogenesis through paracrine signaling and modifications of the oocyte microenvironment [5]. In patients with PCOS, the progression of multiple small antral follicles into larger dominant follicles is challenging. Additionally, the function of GCs is dysregulated in patients with PCOS. Abnormal proliferation of GCs is thought to be a contributing factor in the pathogenesis of PCOS [6]. GCs may exhibit morphological variations. Upon in vitro examination of the GC population, it is observed that cell complexes may present as dark, light, or exhibit more irregular distributions following maturation. The morphological variations among GCs and the underlying mechanisms remain inadequately understood [7].

Less than 1% of follicles progress to the ovulatory stage, with the majority undergoing a degenerative process known as atresia prior to ovulation [8]. Numerous studies have established that the primary cause of follicular atresia is the apoptosis of GCs [9,10]. Generally, GC apoptosis is recognized as a crucial factor in the initiation of follicular atresia, with this phenomenon becoming evident only when GC apoptosis surpasses a critical threshold. Recent findings have emphasized that preantral and antral follicular atresia may be mediated by the activation of distinct cell death pathways. It has been reported that antral follicular degeneration is triggered by extensive apoptosis of GCs, whereas preantral follicular atresia is predominantly driven by increased autophagy of GCs [11]. Therefore, GCs are essential in regulating the processes of both follicular selection and atresia. However, the apoptosis of follicular GCs is a complex process involving multiple factors, including hormones [12] cytokines, and oxidative stress (OS) [13].

Apoptosis is a physiological mechanism involved in the cellular turnover of GCs [14]. Elevated levels of apoptosis in GCs are a major contributor to follicular dysplasia and impaired oocyte maturation. Apoptosis and dysfunction of ovarian GCs have been implicated as key contributors to the pathogenesis of PCOS [15]. Consequently, apoptosis in GCs is correlated with poor oocyte quality and reduced success rates in in vitro fertilization (IVF) among patients with PCOS [6,16]. During apoptosis, proteins released from the mitochondrial intermembrane space, notably cytochrome c, are pivotal in the regulation of caspase activity [17]. Zheng et al., reported increased levels of cytochrome c in the GCs of patients with PCOS [18]. Caspase-3, a principal executioner of apoptosis, is essential for chromatin condensation, DNA fragmentation, nuclear collapse, and plasma membrane blebbing. Furthermore, caspase-3 is essential for distinct processes associated with cell disassembly and the formation of apoptotic bodies [19]. When apoptosis-related gene expressions were evaluated in GCs, an increase in caspase-3 expression was observed, which was associated with a reduced response to ovulation [20]. Another study reported a negative correlation between caspase-3 levels and embryo quality in patients with PCOS [21]. For this reason, the evaluation of apoptosis is considerable for Assisted Reproductive Technology (ART) outcomes.

Granulosa cells are highly sensitive to reactive oxygen species (ROS), and an excessive production of ROS can surpass the capacity of the antioxidant defense system, resulting in OS. This condition can lead to GC dysfunction and potentially induce apoptosis [22-24]. The pathological effects of OS-induced abnormal apoptosis and dysfunction in GCs can lead to various ovarian disorders, including premature ovarian failure (POF), PCOS, and endometriosis, ultimately impacting fertility [25]. It has also been observed that women with PCOS may have impaired antioxidant defense mechanisms, leading to an imbalance between ROS production and antioxidant defences, which results in increased OS [26]. Elevated levels of ROS can adversely affect GC function and impede follicle maturation, which may lead to anovulation [27]. Additionally, oxidative damage to the endometrial lining may hinder embryo implantation, thereby elucidating the commonly observed decrease in fertility among individuals with PCOS [28]. The heat shock protein-70 (HSP-70), an inducible member of the HSP family, shows a significant increase in expression in response to stress. However, it is known to be expressed at low levels under normal physiological conditions [29]. Alterations in the expression levels of HSP-70 molecules are associated with significant medical conditions, including endometriosis, endometrial neoplasms, and infertility [30,31]. HSP levels have been positively correlated with successful outcomes in ART. HSP-70 plays a protective role in cells by mitigating the effects of factors that induce apoptosis. Therefore, we assessed the levels of HSP-70 in GCs to elucidate its relationship with apoptosis and clinical parameters.

When scoring the GC complex, cell groups with a score of 0 exhibit apoptotic characteristics and a darkened morphology. Moreover, cell complexes with a lighter color and an opaque appearance are assigned a score of 1. Consequently, cell groups with a score of 0 can be termed dark granulosa cells (DGC), while those with a score of 1 can be referred to as light granulosa cells (LGC). In our previous study, we demonstrated a strong relationship between low scores (0) of the cumulus oocyte complex (COC) and elevated expression levels of apoptotic proteins, which are associated with poor intracytoplasmic sperm injection (ICSI) outcomes in women both with and without diminished ovarian reserve (DOR) [32]. We hypothesized that DGC in patients diagnosed with PCOS may negatively influence the development of good quality embryo and thus it may be considered as a marker of apoptosis. Therefore, in our present study, we evaluated the relationship between apoptosis of GCs and ICSI outcomes. We aim to investigate the development of embryos and pregnancy rates in PCOS patients with different GC morphologies, as determined through morphological evaluation.

2. PATIENTS and METHODS

Study Population

Between March 2023 and July 2024, this study was conducted at the Istanbul University, Istanbul Faculty of Medicine ART

Laboratory and included 142 patients undergoing 142 ICSI cycles. Fresh GCs were collected from 142 infertile women, aged 20 to 40 years, who underwent ICSI. Among the cases, 45.8% (n=65) were diagnosed with PCOS, while 54.2% (n=77) had male factor (MF) infertility. PCOS was diagnosed based on the Rotterdam criteria [33]. The PCOS group was selected based on the presence of at least two of the following criteria: biochemical and clinical hyperandrogenism, oligo-anovulation, and polycystic ovarian morphology. Women were excluded, if they had (1) any endocrine disease, genetic disease, autoimmune disease, or uterine abnormality; (2) history of cytotoxic chemotherapy and/or radiotherapy; (3) received pelvic irradiation; (4) had a history of ovarian cystectomy or oophorectomy; and (5) had an abnormal karyotype (chromosome polymorphisms were not excluded). The MF group was composed of patients who did not exhibit female factors (such as tubal factor, ovulatory factor, PCOS, hormonal causes, endometriosis, etc., which were exclusion criteria) and had semen parameters that were below the normal range according to WHO criteria (<16 x 10^6/ml sperm concentration, <32% progressive motile sperm, <4% morphologically normal spermatozoa). We included only patients with abnormal semen parameters; other MFs such as azoospermia, varicocele, endocrine or autoimmune disorders, genetic abnormalities, and urogenital infections were excluded. Since our primary study group consisted of PCOS patients with female factors, we established a control group composed of patients without female factors for comparative purposes.

The demographic and clinical characteristics of all participants were recorded, including age, body mass index (BMI), hormone levels [(luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E2), prolactin (PRL)] measured on menstrual day 3, total doses of gonadotrophins and gonadotropin-releasing hormone (GnRH) antagonist treatment, total number of oocytes retrieved, the number of mature (metaphase 2 or MII), immature (germinal vesicle (GV) and metaphase I (MI)), and poor-quality oocyte, normal fertilization rate, the number of good (GI), average (GII), and poor-quality (GIII) embryos on day 3 and clinical pregnancy rates.

All relevant materials were obtained in accordance with the Declaration of Helsinki and all procedures were approved by the Istanbul Medipol University Faculty of Medicine. The study was approved by Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee (approval number: E-10840098-202.3.02-2865 10/05/2024). Data and samples were obtained with the informed consent of all participants.

Stimulation Protocol

All patients received the standard controlled ovarian hyperstimulation (COH) protocol as defined below. The COH was started on the third day of the menstrual cycle and performed with injectable gonadotropins using a GnRH antagonist protocol. Recombinant FSH (Gonal-F, EMD Serono, MA, USA) at a daily dosage of 75–300 IU was started on the third day of the cycle. The women were monitored during gonadotropin stimulation for serial ultrasound evaluation of follicular development and endometrial thickness at a 1–3 day frequency. GnRH antagonist (Cetrotide 0.25 mg/day; Serono, Geneva, Switzerland) was initially administered when the leading follicle reached 12 to 13 mm in diameter and continued until human chorionic gonadotropin (hCG) injection day. To evaluate the risk of ovarian hyperstimulation syndrome (OHSS), E2 and progesterone levels were assessed in patients prior to the administration of hCG. A 6500 IU dose of hCG (Ovitrelle, Merck KGaA, Darmstadt, Germany) was injected to achieve follicular maturation when at least two follicles had reached a size of 16 mm or more.

Oocyte pick up (OPU)

Oocyte aspiration was performed by transvaginal ultrasound 35-36 hours after hCG administration. Labotect (Model 4014, Germany) device and double lumen (Swemed 14105, Sweden) OPU needles were used for aspiration. Follicles aspirated individually in sterile tubes (Falcon 2003, USA, France) using follicle fluids in GMOPS solution (Vitrolife, Sweden, EU) and GMOPS+HSA medium (Vitrolife, Sweden, EU) were examined under stereomicroscope in embryology laboratory (Olympus, SZH10 Japan). Cumulus-oocyte complexes (COCs) were transferred to a culture dish with medium and assessed under a stereomicroscope at 7X magnification. Intercellular connections were disrupted with the tip of the insulin needle, allowing the separation of the GCs surrounding the oocyte.

Morphological analyses of granulosa cells

In this study, the morphology of GCs was evaluated using a stereomicroscope. Dark and compact looking GCs were named as DGC; on the contrary light and expanded looking GCs were named as LGC (Figure 1). Afterwards, the study participants were reclassified into four distinct groups based on their scores. Group A consisted of patients with MF exhibiting DGC (n=44), Group B consisted of patients with MF exhibiting LGC (n=33), Group C consisted of women with PCOS exhibiting DGC (n=30), and Group D consisted of women with PCOS exhibiting LGC (n=35). The follicles from each patient were analyzed by their diameter, and those with a diameter of 18 mm or larger were collected. GCs that were entirely dark or entirely light were isolated and then spread onto microscope slides coated with poly-L-lysine.

Fertilization assessment and embryonic development

Oocytes were taken into bicarbonate containing culture medium (10% human serum albumin, G-IVF medium) (Vitrolife, Sweden). Subsequently, the denudation process was performed using enzymatic (Hyase 10 × Vitrolife, Sweden) and mechanical techniques. All ICSI procedures were performed 3 to 4 h after oocyte retrieval, then oocytes were individually cultured in 20 µL micro drops of culture medium and covered with paraffin oil in a humidified atmosphere with 6% CO^2 at 37 °C. 16-18 hours after microinjection, fertilization was checked under an inverted microscope. The presence of two pronuclei was considered as an indicator of fertilization and normally fertilized oocytes were transferred to new medium (G1, Vitrolife, Sweden). Embryo development was observed 2 and 3 days post-culture and

embryos were graded. Good-quality (grades 1 or 2) embryos were transferred according to the morphologic classification [18,28]. Embryo numbers and embryo transfer days were determined by considering the parameters of the patients. Morphological classification of the transferred embryos (cell number, fragmentation, multinucleation, cell size, etc.) was performed, and the embryo transfer process was completed on day 3 (Since embryo transfer is usually performed on the 3rd day in our center, patients with 4th and 5th day transfers were not included in the study. Therefore, blastocyst development was not evaluated.). Embryo transfer was not feasible for patients in the PCOS group who developed OHSS. As a result, these patients were excluded from the pregnancy evaluation.

Pregnancy – Clinical pregnancy

According to the Turkish legislation of elective single embryo transfer; for women aged less than 35 years and in their first two IVF attempts, one embryo was transferred. Two embryos were transferred to the patients who were ≥ 35 years old or had ≥ 2 failed IVF attempts. Luteal phase support was started on the oocyte retrieval day with vaginal progesterone gel (Crinone 8%; Actavis Pharma, Parsippany, NJ) and maintained until the 12th week of pregnancy if pregnancy is achieved.

Serum beta–human chorionic gonadotropin (β-hCG) levels were measured 12 days after embryo transfer to identify pregnancy. If the ß-hCG level was ≥ 5 mLU/mL, it was considered as positive ß-hCG. When the test results were positive, a second test was performed 2 days later. The clinical pregnancy was defined as the detection of a gestational sac and embryonic heart activity by transvaginal ultrasound embryonic heartbeat.

Immunohistochemical analysis

Air-dried GCs were treated with 4% paraformaldehyde (PFA) for fixation and washed with phosphate-buffered saline (PBS). After that, they were treated with 3% H_2O_2 dissolved in methanol to suppress endogenous peroxidase activity. For antigen retrieval, they were boiled with citrate buffer (pH 6.0). Blocking reagent was applied to each slide followed by 5 min incubation at room temperature in a humidified chamber. Sections were incubated with mouse anti-cytochrome c (A-8) monoclonal antibody (dilution 1:300; Santa Cruz, sc-13156), mouse anti-caspase-3 monoclonal antibody (dilution 1:200; Santa Cruz, sc-136219) and anti-HSP-70 / heat shock cognate 70 (Hsc70) mouse monoclonal antibody (dilution 1:250; Santa Cruz, sc-24) overnight at 4°C. Slides were incubated with biotinylated goat anti-mouse antibodies. Slides were washed in PBS after incubation with streptavidin peroxidase label reagent. Then incubated in 3, 3'-Diaminobenzidine, tetrahydrochloride, dihydrate (DAB). Slides were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and all coverslips were mounted using an aqueous mounting medium (Entellan®, Merck, Darmstadt, Germany).

Immunohistochemically stained cells were counted under light microscope. The staining intensities were evaluated by two different investigators. The relative intensity of specific immunohistochemical staining was scored as 0, negative; 1, weak positive; 2, moderate positive; and 3, strongly positive. The accuracy of the staining was confirmed immunohistochemically by antibody-free negative staining. Staining percentages were determined by counting a total of 100 cells in 10 different random microscopic fields in each patient. Power analysis was performed and the study was designed with a 90% power analysis.

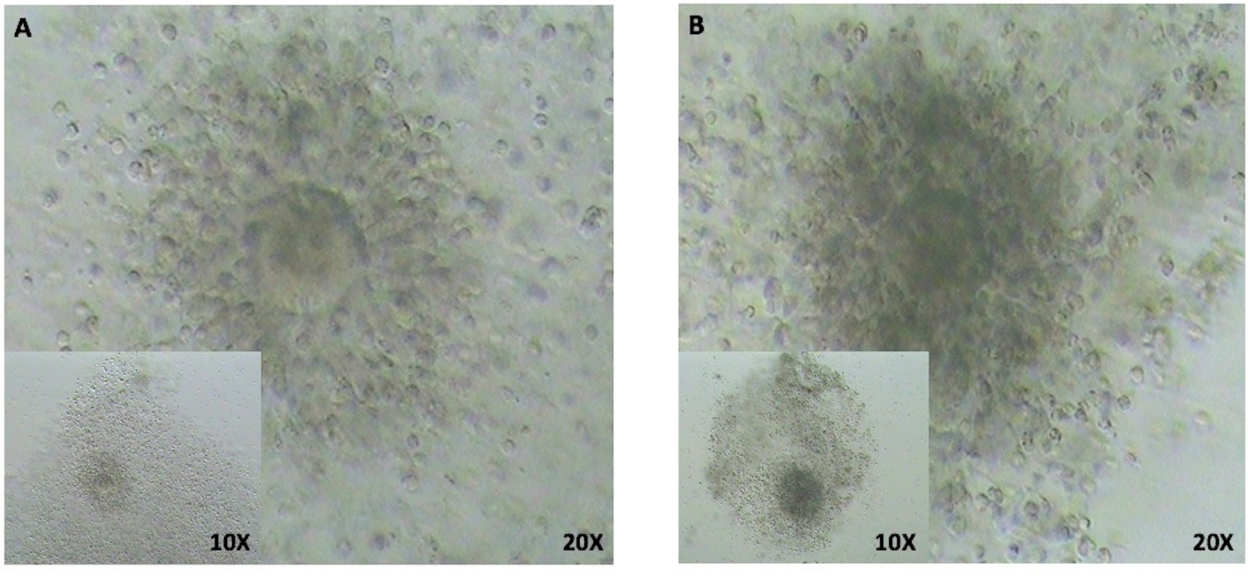


Figure 1. *Micrograph of light granulosa oocyte complex (A), micrograph of dark granulosa oocyte complex (B), inverted microscope.*

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Histologic score (H-SCORE)

The assessments of the immunohistochemical expressions in GCs was achieved by utilizing histological score (H-SCORE). H SCORE=∑P*i* (*i*+1). Here, *i* indicates the staining intensity (0=negative, 1=weak positive, 2=moderate positive, and 3=strongly positive) and P*i* is the percentage of the cells stained for each intensity. Apoptotic and OS indexes were calculated as the ratio of stained cells to all cells. All evaluations were performed by two histologists in a double-blind manner.

Statistical analysis

The statistical analyses were performed by using Graph – Pad Prism (ver. 7.00) and SPSS (ver. 25). Normality of all data was tested by Shapiro–Wilk test. Comparisons of the differences in means for a continuous variable between groups were performed using the Mann–Whitney U test or Student's t-test, according to the results of the normality test. Comparisons of the three or more independent groups were performed by ANOVA for parametric data and Kruskal–Wallis for non-parametric data. Pearson's chi-square test was used to measure the distributions of the parameters. Correlation of the parametric and non-parametric data was provided by the Pearson's correlation and Spearman's rank correlation coefficient. $p < 0.05$ value was accepted as statistically significant.

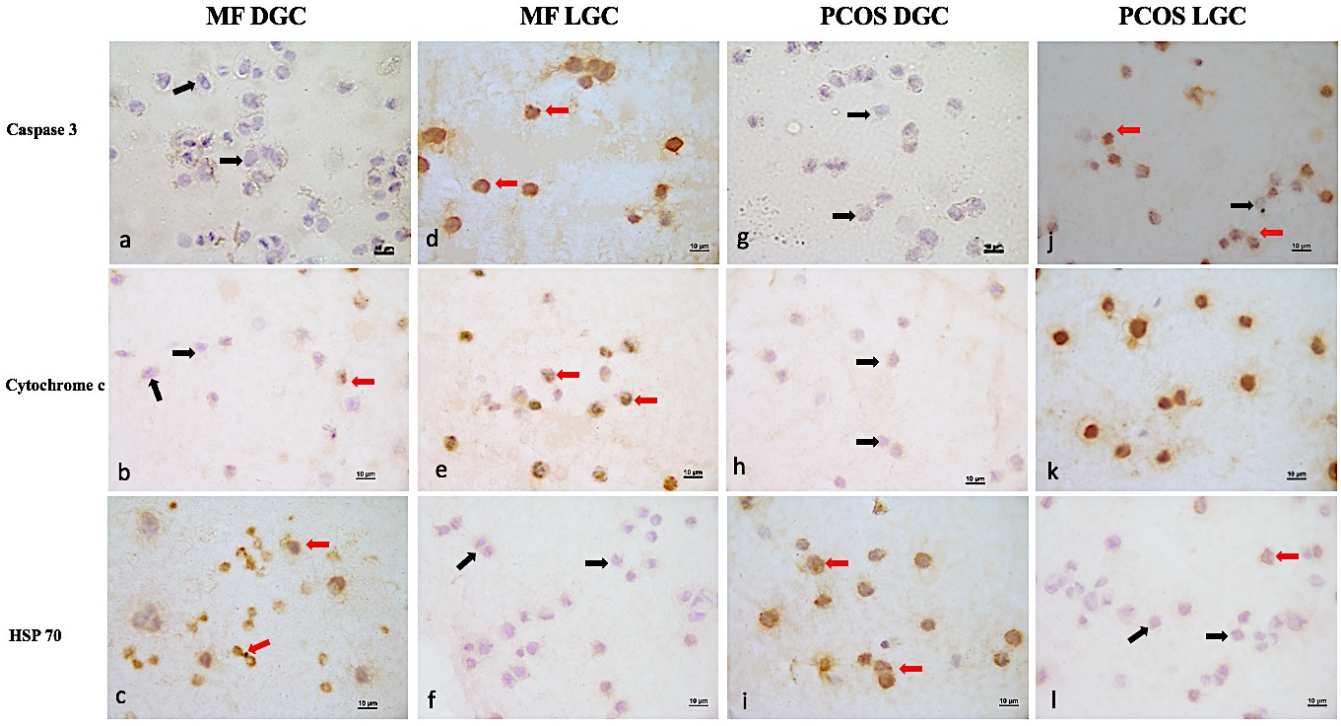


Figure 2. *Immunohistochemical (IHC) staining micrographs of granulosa cells (GCs) from women. The assessments of the immunohistochemical expressions of caspase-3, cytochrome c, and heat shock protein (Hsp70) in GCs were accomplished by utilizing H-SCORE: H-score=Pi (i+1), where i=the intensity of the staining (1=weak, 2=moderate, and 3=strong), and Pi was the percentage of cells stained at each intensity (0–100%). Red arrows indicate IHC-positively stained cells; black arrows indicate IHC-negatively stained cells. Low immunostaining of caspase 3, cytochrome c (a, b) and intense brown immunostaining of Hsp70 (c) were observed in GCs of male factor light granulosa cell-MF LGC group. Intense brown immunostaining of caspase-3, cytochrome-c (d, e) and low immunostaining of Hsp70 (f) were observed in GCs of male factor dark granulosa cells-MF DGC group. Low immunostaining of caspase-3, cytochrome-c (g, h) and intense brown immunostaining of Hsp70 (i) were observed in GCs of polycystic ovary syndrom light granulosa cell-PCOS LGC group. Intense brown immunostaining of caspase-3, cytochrome-c (j, k) and low immunostaining of Hsp70 (l) were observed in GCs of polycystic ovary syndrom dark granulosa cell-PCOS DGC group. At least 100 GCs were analyzed by light microscopy (×100). Scale bars are indicated (10 μm).*

Table I. *Comparing MF DGC, MF LGC, PCOS DGC and PCOS LGC parameters between groups*

	MF DGC (n=44)	MF LGC (n=33)	PCOS DGC (n=30)	PCOS LGC (n=35)	[a]p-value
	Median (Q1, Q3)	Median (Q1, Q3)	Median (Q1, Q3)	Median (Q1, Q3)	
Age (year)[a] Ort±SD	34.37±4.58	33.75±4.54	30.9±4.85	32.05±4.18	**0.194**
Total dose of gonadotropins (IUs)[b]	2025±122.2	1800 ±108.1	2530 ± 131.3	2039 ±120.1	**0.05***
Total number of days of GnRH Antagonist treatment[b]	3.1 ±0.164	2.96±0.133	3.5±0.212	3.2 ± 0.174	**0.230**
FSH(IU/mL)[ac]	6.65±0.317	6.8± 0.3608	9.80 ± 0.5402	6.4± 0.309	**0.044***
LH (IU/mL)[bc]	5.91±0. 445	5.2±0.396	5.13±0.329	5.29±0.401	**0.336**
Estradiol-E2 (pg/mL)[bc]	36 ± 3.101	36±3.10	39.6±3.72	42.5±3.84	**0.070**
Prolactin-PRL(ng/mL)[bc]	15.15±0.967	15.7±1.021	23.2±3.12	18.9 ± 1.642	**0.049***
BMI(kg/m2)[b]	25 ± 0.723	25±0.723	23.7±0.621	24.1± 0.687	**0.34**
Total oocyte count[a]	7±0.634	6 ± 0.534	13.2 ± 1.32	14.1 ± 1.453	**<0.001***
MII oocyte £ 5[a]	6 (8)	6 (8)	9.8 (10.7)	10.3 (12.1)	**<0.001***
MII oocyte[3] 6[d]	1 (9.2)	1 (9.2)	1.4 (10, 5)	2.3 (20, 4)	**0.05***
MI oocyte [b]	1.3±0.312	1.3±0.312	2.24±0.643	2.22±0.640	**0.05***
Fertilization rate[b]	58.5±4.654	70 ±7.53	78 ± 6.480	83.3±25.86	**0.041***
Number of D3 good-quality embryos (GI)[b]	1 ±0.20	1± 0.20	1.6 ± 0.46	3.4 ± 1.13	**0.038***
Number of D3 average-quality embryos (GII)[b]	1.2 ± 0.264	1.22 ± 0.268(0, 3)	1.42± 0.312	1.36 ± 0.299	**0.055**
Number of D3 poor-quality embryos (GIII)[b]	0.68± 0.201	0.71± 0.211	0.5 ± 0.192	0.62 ± 0.198	**0.430**
	n (%)	n (%)	n (%)	n (%)	
Clinical Pregnancy[d]					[b]**<0.001***
Yes	5 (11.4)	22 (66.7)	10 (40)	15(55)	
No	29 (65.9)	11 (33.3)	15 (60)	12(44)	

[a]*Oneway ANOVA test&post hoc Bonferroni test.* [b]*Kruskal-Wallis test, results are presented as median (first quarter, third quarter).* [c]*Day 3 of the cycle, before stimulation.* [d]*Pearson's chi-square test. *Significant at p<0.05.*

MF DGC: Male factor dark granulosa cells, MF LGC, Male factor light granulosa cells, PCOS DGC: Polycystic ovary syndrome dark granulosa cells, PCOS LGC: Polycystic ovary syndrome light granulosa cells, GnRH: Gonadotropin-releasing hormone, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, E2:Estradiol, PRL:Prolactin, BMI: Body mass index, MII: Oocyte, metaphase II, MI: Oocyte, metaphase I

Table II. *Comparison of the caspase-3, cytochrome c and HSP-70 stained cell numbers between the groups*

	MF DGC (n=44)	MF LGC (n=33)	PCOS DGC (n=30)	PCOS LGC (n=35)	[a]p-value
	n(%)	n(%)	n (%)	n (%)	
Caspase-3 (A)	34 (77.2)	4 (12.1)	26 (86.6)	8 (22.8)	**0.001****
Cytochrome c (B)	32 (72.7)	7 (21.2)	25 (83.3)	5 (14.2)	**0.001****
HSP-70 (C)	7 (15.9)	32 (96.9)	6 (20)	31 (88.5)	**0.001****
(A+B)/ C	9.4	0.3	8.5	0.41	**0.001****

[a]*Pearson's chi-square test.**Significant at p<0.01.*

MF DGC: Male factor dark granulosa cells, MF LGC: Male factor light granulosa cells, PCOS DGC: Polycystic ovary syndrome dark granulosa cells, PCOS LGC: Polycystic ovary syndrome light granulosa cells, HSP-70: Heat shock protein-70

Table III. *Association between caspase-3, cytochrome c and HSP-70 expression levels and clinical pregnancy outcomes*

Variables	Caspase-3	Cytochrome c	HSP-70	p-value
Clinical pregnancy	29±2.5	31±4.01	72±1.3	**<0.001***

*Data are presented as either means ± standard error. Pearson's chi-square test was used. *Significant at p<0.05. HSP-70: Heat shock protein-70*

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3. RESULTS

Subject characteristics

Demographic and basic clinical features in the study population are shown in Table I. Demographic and baseline characteristics showed no significant differences among four groups in terms of age, BMI, total antagonist dose, LH and E2 hormone levels, and the number of day 3 GI, II, and III embryos ($p > 0.05$). Total gonadotropin dose was higher in the PCOS DGC group (2530 ± 131.3) compared with the MF DGC (2025±122.2), MF LGC (1800 ±108.1) and PCOS LGC (2039 ±120.1) groups ($p = 0.05$). FSH serum level was higher in the PCOS DGC group (9.80 ± 0.5402) compared with the MF DGC (6.65±0.317), MF LGC (6.8± 0.3608) and PCOS LGC (6.4± 0.309) groups ($p = 0.044$). PRL serum levels were higher in the PCOS DGC (23.2±3.12) and PCOS LGC (18.9 ± 1.642) groups, compared with the MF DGC (15.15±0.967) and MF LGC (15.7±1.021) groups ($p = 0.049$). Total oocyte count was found to be higher in the PCOS DGC (13.2 ± 1.32) and PCOS LGC (14.1 ± 1.453) groups, when compared with the MF DGC (7±0.634) and MF LGC (6 ± 0.534) groups ($p<0.001$). Also, MI, MII and GIV oocyte counts showed a significant difference between the PCOS and MF groups (MI: $p<0.001$, MII: $p=0.05$, GIV: $p=0.05$ respectively). Fertilization rates were higher in the PCOS LGC (83.3±25.86) and PCOS DGC (78 ± 6.480) groups when compared with the MF DGC (58.5±4.654) group ($p= 0.041$). On Day 3, GI embryos in the MF DGC (1 ± 0.20) and MF LGC (1 ± 0.20) groups were significantly lower compared to those in the PCOS DGC (1.6 ± 0.46) and PCOS LGC (3.4 ± 1.13) groups ($p = 0.038$). Clinical pregnancy rates exhibited a statistically significant difference among the four groups ($p <0.001$). The MF DGC group had the lowest clinical pregnancy rate, at 11%.

Expression profile of cytochrome c, caspase-3, and HSP-70

The differences between immunohistochemical staining and non-staining of the cells were illustrated in Figure 2, based on the analysis of the immunohistochemical results. Expressions of the early apoptosis marker cytochrome c differed among the four groups. DGCs demonstrated significantly higher cytochrome c expression levels compared to LGCs ($p <0.01$). The PCOS DGC group (83.3%) had the highest rate and showed a significant difference when compared to the MF DGC (72.7%), MF LGC (21.2%), and PCOS LGC (14.2%) groups.

The apoptotic marker caspase-3 showed a statistically significant difference between the DGC and LGC groups ($p<0.01$). DGCs exhibited elevated levels of caspase-3, with MF DGCs showing 77.2% expression and PCOS DGCs showing 86.6% expression. The comparison between the PCOS and MF groups did not reveal a statistically significant difference ($p>0.05$).

The expression of the anti-apoptotic HSP-70 protein was inversely correlated with the levels of caspase-3 and cytochrome-c proteins. The LGC groups demonstrated significantly higher expression levels compared to the DGC groups ($p<0.01$). Furthermore, the HSP-70 level in the MF LGC group was significantly elevated compared to the PCOS LGC group ($p=0.0053$; $p<0.01$). No significant difference was observed between the MF DGC and PCOS DGC groups ($p>0.05$). Expression levels of the four groups are presented in Table II. The ratio of HSP-70 to caspase-3 and cytochrome c was calculated after immunohistochemical staining. Apoptotic/antiapoptotic ratio of the PCOS DGC and MF DGC groups was significantly higher than the PCOS LGC and MF LGC groups ($p=0.001$). PCOS LGC and MF LGC comparison showed no significant difference, as did the comparison between PCOS DGC and MF DGC ($p >0.05$). The expression profiles of cytochrome c, caspase-3, and HSP-70 in relation to ICSI outcomes are presented in Table III. Decrease in apoptotic proteins caspase-3 and cytochrome c levels and increase in anti-apoptotic protein HSP-70 levels showed a positive correlation with clinical pregnancy rates regardless of groups ($p<0.001$). The results confirm our hypothesis.

Correlation of caspase-3, cytochrome c, and HSP-70 expression levels and clinical parameters

Correlation analyses were performed to examine the associations between clinical parameters and the expression levels of caspase-3, cytochrome c, and HSP-70. We observed a positive correlation between serum E2 levels and the expression of caspase-3 ($r =0.584$; $p =0.015$) and cytochrome c ($r =0.701$; $p =0.004$) in the PCOS group. In contrast, a negative correlation was observed in the expression of HSP-70 ($r =-0.618$; $p =0.0043$). In the PCOS group, PRL levels demonstrated a negative correlation with the expression of caspase-3 ($r =-0.641$; $p =0.002$) and cytochrome c ($r =-0.732$; $p =0.003$), whereas HSP-70 expression ($r =0.711$; $p =0.001$) exhibited a positive correlation. Furthermore, analysis of BMI revealed a positive correlation with cytochrome c expression ($r =0.698$; $p =0.002$) and a negative correlation with HSP-70 expression. Notably, in the PCOS group, the number of day 3 GI embryos showed a positive correlation with HSP-70 expression ($r =0.580$; $p =0.022$) and a negative correlation with cytochrome c expression ($r =-0.598$; $p =0.0149$) (Table IV). The results indicated that apoptosis has a negative impact on the developmental competency of good-quality embryos in the PCOS group.

In the MF group, a correlation was observed only between the number of day 3 GII embryos and protein expression levels. Caspase-3 ($r =-0.424$; $p =0.06$) and cytochrome c ($r =-0.516$; $p =0.028$) levels exhibited a negative correlation with the number of day 3 GII embryos (Table V).

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Marmara Med J 2026;39(1): 6-18

Table IV. *Correlation coefficients for caspase-3, cytochrome c, and heat shock protein (Hsp-70) expression levels and clinical parameters in the diminished polycystic ovary syndrome-(PCOS) group using Pearson's correlation and Spearman's rank correlation coefficient*

Parameters	Caspase- 3		Cytochrome c		Hsp-70	
	r	p-value	r	p-value	r	p-value
Age (year)a	0.016	0.910	-0.232	0.366	-0.311	0.218
Total dose of gonadotropins (IUs)b	0.189	0.522	0.346	0.324	0.198	0.643
Total number of days of GnRH antagonist treatmentb	0.019	0.954	-0.052	0.744	-0.166	0.568
FSH (IU/mL)bc	0.09	0.762	-0.194	0.910	0.072	0.0915
LH (IU/mL)bc	0.071	0.780	0.190	0.461	-0.070	0.816
E2 (pg/mL)bc	0.584	0.015	0.701	0.004	-0.618	0.0043
PRL (ng/mL)bc	-0.641	0.002	-0.732	0.003	0.711	0.001
BMI (KG/M2)b	0.261	0.632	0.698	0.002	-0.813	0.0001
Number of oocytes retrieved	0.0062	0.983	-0.041	0.895	0.083	0.692
MII oocyte £ 5a	0.230	0.640	-0.073	0.910	0.018	0.819
MII oocyte³ 6b	-0.193	0.752	-0.181	0.786	-0.332	0.516
MI oocyte b	-0.173	0.510	-0.114	0.651	0.331	0.172
Fertilization rateb	0.408	0.211	0.320	0.310	0.322	0.220
Number of D3 good-quality embryos (GI)b	-0.330	0.213	-0.598	0.0149	0.580	0.022
Number of D3 average-quality embryos (GII)b	-0.120	0.640	-0.016	0.948	0.164	0.517
Number of D3 poor-quality embryos (GIII)b	-0.189	0.486	-0.163	0.549	0.154	0.588
Number of arrested embryosb	0.248	0.321	0.210	0.590	-0.290	0.270

[a]Pearson's correlation coefficient was used. [b]Spearman's rank correlation coefficient was used. [c]Day 3 of the cycle before stimulation. Significant at p<0.05. GnRH: Gonadotropin-releasing hormone, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, E2: Estradiol, PRL: Prolactin, BMI: Body mass index. MII: Oocyte, metaphase II, MI: Oocyte, metaphase I

Table V. *Correlation coefficients for caspase-3, cytochrome c, and heat shock protein (Hsp70) expression levels and clinical parameters in the diminished male factor-MF group using Pearson's correlation and Spearman's rank correlation coefficient.*

Parameters	Caspase- 3		Cytochrome c		Hsp-70	
	r	p-value	r	p-value	r	p-value
Age (year)a	-0.18	0.420	-0.231	0.472	0.380	0.148
Total dose of gonadotropins (IUs)b	-0.029	0.912	-0.146	0.620	0.148	0.587
Total number of days of GnRH antagonist treatmentb	-0.219	0.254	-0.152	0.064	0.461	0.120
FSH (IU/mL)bc	0.492	0.072	0.392	0.110	-0.371	0.119
LH (IU/mL)bc	-0.379	0.180	-0.090	0.763	-0.272	0.316
E2 (pg/mL)bc	0.054	0.917	-0.171	0.704	0.143	0.438
PRL (ng/mL)bc	-0.141	0.662	-0.132	0.483	-0.111	0.551
BMI (KG/M2)b	-0.284	0.336	-0.068	0.762	0.443	0.086
Number of oocytes retrieved	-0.262	0.382	-0.417	0.185	0.208	0.362
MII oocyte £ 5a	-0.563	0.164	-0.573	0.1491	0.518	0.287
MII oocyte³ 6b	-0.619	0.172	-0.718	0.078	0.532	0.251
MI oocyte b	-0.216	0.425	-0.154	0.551	-0.033	0.892
Fertilization rateb	-0.438	0.071	-0.350	0.131	0.234	0.361
Number of D3 good-quality embryos (GI)b	-0.333	0.176	-0.358	0.192	0.282	0.322
Number of D3 average-quality embryos (GII)b	-0.424	0.061	-0.516	0.028	0.416	0.056
Number of D3 poor-quality embryos (GIII)b	0.179	0.477	0.063	0.844	0.043	0.918
Number of arrested embryosb	0.078	0.672	0.213	0.459	-0.081	0.775

[a]Pearson's correlation coefficient was used. [b]Spearman's rank correlation coefficient was used. [c]Day 3 of the cycle before stimulation. Significant at p<0.05. GnRH: Gonadotropin-releasing hormone, FSH: Follicle-stimulating hormone, LH:, Luteinizing hormone, E2: Estradiol, PRL: Prolactin, BMI: Body mass index, MII: Oocyte, metaphase II, MI: Oocyte, metaphase I

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4. DISCUSSION

In this study, we demonstrated that GC morphology is closely associated with apoptotic and anti-apoptotic protein expression patterns, which in turn significantly influence ICSI outcomes. DGC exhibited increased expression of cytochrome c and caspase-3, whereas LGC showed higher HSP-70 expression, correlating with improved embryo quality and clinical pregnancy rates.

Polycystic ovary syndrome is the leading cause of endocrine infertility in women, with significant heterogeneity and complexity. It is characterised by increased ovarian androgen production, anovulation, and, as previously noted, infertility [34,35]. A significant amount of research has examined the effects of hormones on PCOS [36,37]. E2 regulates follicle transformation by promoting the increase in Follicle-Stimulating Hormone Receptor (FSHR) expression and the acquisition of LH receptors (LHR), enabling follicles to undergo cyclic recruitment and participate in the cohort of selectable follicles [38]. Based on our findings, serum E2 levels exhibited a positive correlation with the expressions of caspase-3 and cytochrome c in the PCOS group. This indicates that increased levels of apoptotic proteins may be influenced by serum E2 concentrations. PRL levels have been demonstrated to have a positive association with sex hormones, including estradiol and total testosterone [39]. Saei et al., reported that PRL levels in patients diagnosed with PCOS according to the Rotterdam criteria were significantly higher than those in non-PCOS participants [40]. According to our findings, the PCOS group demonstrated higher PRL levels than the MF group. Additionally, we found that PRL levels were negatively correlated with the expressions of caspase-3 and cytochrome c in the PCOS group. Moreover, this study revealed a positive correlation between BMI and the apoptotic protein cytochrome c. It is known that PCOS has significant clinical implications and can contribute to health issues associated with adipose tissue accumulation, including obesity, insulin resistance, metabolic syndrome, and type 2 diabetes [41]. Based on this data, we consider obesity and related insulin resistance to potentially induce apoptosis in GCs in patients with PCOS. Recent studies have emphasized the involvement of the gut microbiome in PCOS [42]. Altering dietary habits, which can subsequently affect the gut microbiome and decrease insulin resistance, may be beneficial in reducing apoptosis in GCs.

The quality of oocytes is influenced by their microenvironment [43]. The microenvironment plays a crucial role in cell signaling and morphological assessments, with apoptosis serving as a significant indicator in this context [44]. Dysregulation of the hypothalamic-pituitary-ovarian axis in women with PCOS results in impaired ovarian function, leading to the premature arrest of developing follicles and the accumulation of small, immature follicles [45]. Excessive production of anti-Müllerian hormone (AMH) by small follicles inhibits FSH-induced aromatase activity, impeding follicular development and subsequently leading to ovarian dysfunction [46]. Thus, aggravating the follicular arrest. Throughout follicular development, 99% of follicles undergo destruction due to follicular atresia [47]. The relationship between apoptosis and follicular atresia is very clear. Many studies have employed the incidence of apoptosis as a criterion for evaluating oocyte quality and development [48-50]. In a study, researchers revealed species-specific differences in the apoptotic activity of theca interna and GCs concerning follicular atresia, utilizing the TUNEL assay on pig and bovine ovaries [51]. Han et al. noted that, alongside apoptosis in GCs, various factors, such as the morphology of the cumulus-oocyte complex, should be assessed in conjunction with oocyte quality. Peng observed that the level of apoptosis in GCs was markedly elevated in patients with PCOS [52]. In this study, we demonstrated that the expression of the key apoptotic protein caspase-3 and the cytochrome c released from the mitochondria in association with caspase-3 differed among GCs of varying morphology. Elevated apoptosis in GCs, which are morphologically classified as dark and of poor quality, has been associated with unfavorable clinical outcomes. Our findings indicate that apoptotic DGC may negatively impact ART success in patients.

When we assessed the total and MII oocyte counts, the MF group exhibited lower values compared to the PCOS group. The observed difference was statistically significant and attributed to the presence of PCOS. It is well-established that, due to excessive initial recruitment and prolonged follicular survival [53], polycystic ovaries exhibit a higher follicular density and a greater number of follicles compared to normal ovaries [54]. A large-scale study reported that women with PCOS had an increased number of retrieved oocytes, as well as higher clinical pregnancy rates and live birth rates, until the age of 40 compared with control subjects [46]. In this study, the fertilization rate observed in the MF group was significantly lower than that in the PCOS group. However, it is well-established that early embryonic development in the MF group is diminished due to the effects of sperm, independent of the influence of GCs. This represents one of the limitations of our study. Studies have demonstrated that sperm quality can significantly influence fertilization rates, embryo morphology, and implantation rates in both conventional IVF and ICSI procedures [55,56]. Liao et al., found that the concentration and motility of sperm were strongly correlated with the rates of two pronuclei (2PN) embryos, good-quality embryos on D3, and blastocysts and high-quality embryos on D5/6 [57]. Furthermore, total oocyte count and MII oocyte count may be associated with age. In their study on the rates of apoptotic cumulus cells (CCs) and blastocyst formation, Corn et al., found that both apoptotic cell rates and blastocyst formation rates were lower in female patients older than 35 years [58]. Furthermore, a poor oocyte response is expected in DGC patients who receive elevated total gonadotropin doses in the context of PCOS. This consequently leads to poor embryonic development. Abundant ovarian reserve in women with PCOS has a corresponding ovarian response in COH during IVF cycles [59]. If OHSS occurs, embryo transfer cannot be performed in these patients. Therefore, we did not evaluate the pregnancy rates for all individuals with PCOS.

Oxidative stress may inhibit oogenesis promoted by follicles [60]. Under conditions of OS (physical, chemical, toxicity, etc.) protein misfolding can occur. A repair mechanism may

be triggered to rectify these misfolded proteins; however, continuous production of misfolded proteins leads to apoptosis and ultimately results in cell destruction [61]. In women with PCOS, OS is exacerbated by the overproduction of ROS. OS markers are elevated in the follicular fluid and serum of women with PCOS, which may be one of the reasons for the poor quality of oocytes [38,62]. Elevated markers of OS in the follicular fluid of oocytes contribute to abnormal follicle growth and maturation, resulting in poor-quality oocytes or embryos, which ultimately leads to infertility. In women with PCOS, both CCs and oocytes display dysregulation in oxidative phosphorylation and metabolic pathways [63]. Heat shock proteins, which play a role in OS, belong to a multifunctional protein family and are produced in response to stress conditions such as extreme temperature fluctuations, anoxia, imbalances between ROS metabolites and glycolysis level, as well as aging. HSP-70, a member of the HSP family, plays a crucial role in protein regulation, including preventing protein aggregation and facilitating the transport of proteins across cellular membranes [64]. HSP-70 plays a critical role in protecting GCs in PCOS by modulating apoptotic pathways. It achieves this by adjusting the Bax/Bcl-2 ratio and suppressing caspase-3 activation, thereby reducing oxidative damage and mitigating apoptosis triggered by cellular stress [65]. Furthermore, HSP-70 supports steroidogenesis, particularly by enhancing progesterone synthesis in GCs. This function is especially vital in PCOS, where hormonal dysregulation frequently disrupts normal steroid hormone production, compromising follicular development and reproductive health [66]. In our study, we observed decrease in HSP-70 expression DGC subgroups, regardless of PCOS and MF groups. Although other studies have shown that PCOS pathology affects HSP-70 expression, we detected an increase or decrease according to the morphology of the GCs rather than the PCOS relationship. We hypothesize that the decline in HSP-70 levels is associated with the initiation of the apoptotic process in cells exposed to OS conditions. In our study, we observed that the apoptotic index increased in these dark cell groups, leading the cells to undergo programmed cell death. Additionally, there was a decrease in the function of HSP-70, which is involved in the repair mechanism that regulates this in case of cellular stress. We wanted to draw attention to this.

In patients with PCOS, an impaired oocyte microenvironment is associated with atretic morphological characteristics, while dark coloration of oocytes is indicative of poor embryonic development and lower pregnancy outcomes. Follicle cells may exhibit deficiencies in protein repair mechanisms as a result of OS and the increased demands of protein synthesis during the process of folliculogenesis. Since DGCs surrounding the oocyte microenvironment can induce apoptosis and OS, the condition of DGCs, irrespective of the indication for treatment, can lead to impaired embryonic development, low pregnancy rates and thus IVF failure. Therefore, pro-apoptotic caspase-3 and cytochrome c expressions lead to cell apoptosis [67,68]. These conditions in the developing oocyte microenvironment lead to poor oocyte quality and poor IVF results [69]. Cellular death mechanisms in DGCs may be an explanation for infertility in patients. Further studies to show the relationship between apoptosis, OS and infertility may clarify these findings.

Our study has limitations in terms of providing more accurate results. We need to increase the number of patients; this will provide us with clearer results allowing us to have statistical confidence. Assessment of apoptotic and OS states may require alternative methods to support the findings. We believe that if these limitations of our study are addressed, the robustness of the data will increase diagnostic support for infertility treatment. We believe that molecular diagnosis will guide the treatment process of infertile couples. Furthermore, quantification of the expression levels of the antibodies studied, such as biochemical, genetic and western blot, may help to confirm the immunohistochemical results of our study. We think that examination of dark and light granulosa cells under electron microscope in patients with PCOS may elucidate the source of the problem.

Compliance with Ethical Standards

Ethical approval: All relevant materials were obtained in accordance with the Declaration of Helsinki and all procedures were approved by the Istanbul Medipol University Faculty of Medicine. The study was approved by Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee (approval number: E-10840098-202.3.02-2865 10/05/2024). Data and samples were obtained with the informed consent of all participants.

Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions: AAA: Designed the study and revised the manuscript, BK and KNU: Provided expert opinions and drafted the manuscript, AAA, IK an OD: Assisted with data collection, statistical analyses, and editing. All authors contributed to the manuscript and approved the submitted version.

REFERENCES

[1] Practice Committee of the American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss: a committee opinion. Fertil Steril 2013;99:63–67. doı: 10.1016/j.fertnstert.2012.09.023

[2] Franks S, Mccarthy MI, Hardy K. Development of polycystic ovary syndrome: Involvement of Genetic and Environmental Factors Int J Androl 2006;29:278-85. doi: 10.1111/j.1365-2605.2005.00623.x.

[3] Mirza FG, Tahlak MA, Rjeili RB, et al. Polycystic ovarian syndrome (PCOS): Does the challenge end at conception? Int J Environ Res Public Health 2022;12;19:14914. doi: 10.3390/ijerph192214914.

[4] Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction 2010;139:685-95. doi: 10.1530/REP-09-0345.

[5] Tu J, Cheung AH, Chan CL, Chan WY. The role of microRNAs in ovarian granulosa cells in health and disease. Front Endocrinol 2019;10:174. doi: 10.3389/fendo.2019.00174.

[6] Yildiz BO, Bozdag G, Yapici Z, Esinler I, Yarali H. Prevalence, phenotype and cardiometabolic risk of polycystic ovary syndrome under different diagnostic criteria. Hum Reprod 2012; 27:3067-73. doi: 10.1093/humrep/des232.

[7] Regan SLP, Knight PG, Yovich JL, Leung Y, Arfuso F, Dharmarajan A. Granulosa cell apoptosis in the ovarian follicle—a changing view. Front Endocrinol 2018;9:61. doi: 10.3389/fendo.2018.00061.

[8] Evans AC. Characteristics of ovarian follicle development in domestic animals. Reprod Domest Anim 2003;38:240-6. doi: 10.1046/j.1439-0531.2003.00439.x.

[9] Tilly JL. Apoptosis and ovarian function. Endocr Rev 1993;14:175-99. doi: 10.1530/ror.0.0010162.

[10] Hsueh AJW, Billig H, Tsafriri A. Ovarian follicle atresia: a hormonally controlled apoptotic process. Endocr Rev 1994;15:707-24. doi: 10.1210/edrv-15-6-707.

[11] Meng L, Jan SZ, Hamer G, et al. Preantral follicular atresia occurs mainly through autophagy, while antral follicles degenerate mostly through apoptosis. Biol Reprod 2018;1;99:853-63. doi: 10.1093/biolre/ioy116.

[12] Erbaş O, Akman L, Yavaşoğlu A, Terek MC, Akman T, Taskiran D. Oxytocin improves follicular reserve in a cisplatin-induced gonadotoxicity model in rats. Biomed Res Int 2014;2014:703691. doi: 10.1155/2014/703691.

[13] Stanley AJ, Sivakumar KK, Nithy KT, et al. Postnatal exposure to chromium through mother's milk accelerates follicular atresia in F1 offspring through increased oxidative stress and depletion of antioxidant enzymes. Free Radic Biol Med 2013;61:179-96. doi: 10.1016/j.freeradbiomed.2013.02.006.

[14] Li Y, Zheng Q, Sun D, et al. Dehydroepiandrosterone stimulates inflammation and impairs ovarian functions of polycystic ovary syndrome. J Cell Physiol 2019;234:7435-47. doi: 10.1002/jcp.27501.

[15] An R, Wang X, Yang L, et al. Polystyrene microplastics cause granulosa cells apoptosis and fibrosis in ovary through oxidative stress in rats. Toxicology 2021;449:152665. doi: 10.1016/j.tox.2020.152665.

[16] Lai Q, Xiang W, Li Q, et al. Oxidative stress in granulosa cells contributes to poor oocyte quality and IVF-ET outcomes in women with polycystic ovary syndrome. Front Med 2018;12:518-24. doi: 10.1007/s11684-017-0575-y.

[17] Ow YP, Green DR, Hao Z, Mak TW. Cytochrome c: functions beyond respiration. Nat Rev Mol Cell Biol 2008;9:532-42. doi: 10.1038/nrm2434.

[18] Zheng B, Meng J, Zhu Y, Ding M, Zhang Y, Zhou J. Melatonin enhances SIRT1 to ameliorate mitochondrial membrane damage by activating PDK1/Akt in granulosa cells of PCOS. J Ovarian Res 2021;14:152. doi: 10.1186/s13048.021.00912-y.

[19] Lamkanfi M, Kanneganti T-D. Caspase-7: a protease involved in apoptosis and inflammation. J Biochem Cell Biol 2010;42:21-4. doi: 10.1016/j.biocel.2009.09.013.

[20] Lobach VN, Casalechi M, Dela Cruz C, Pereira MT, Del Puerto HL, Reis FM. Caspase-3 gene expression in human luteinized granulosa cells is inversely correlated with the number of oocytes retrieved after controlled ovarian stimulation. Hum Fertil (Camb) 2019;22:33-8.doi: 10.1080/14647273.2017.1356474.

[21] Salehi E, Aflatoonian R, Moeini A, et al. Apoptotic biomarkers in cumulus cells in relation to embryo quality in polycystic ovary syndrome. Arch Gynecol Obstet 2017;296:1219-27. doi: 10.1007/s00404-017-4523-5.

[22] Yang H, Xie Y, Yang D, Ren D. Oxidative stress-induced apoptosis in granulosa cells involves JNK, p53 and puma. Oncotarget 2017;8:25310-22. doi: 10.18632/oncotarget.15813.

[23] Devine PJ, Perreault SD, Luderer U. Roles of reactive oxygen species and antioxidants in ovarian toxicity. Biol Reprod 2012;86:27. doi: 10.1095/biolreprod.111.095224.

[24] Luderer U. Ovarian toxicity from reactive oxygen species. Vitam Horm 2014;94:99-127. doi: 10.1016/B978-0-12-800095-3.00004-3.

[25] Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: A review. Reprod Biol Endocrinol 2012;10:49. doi: 10.1186/1477-7827-10-49.

[26] Gao Y, Zou Y, Wu G, Zheng L. Oxidative stress and mitochondrial dysfunction of granulosa cells in polycystic ovarian syndrome. Front Med 2023;10:1193749. 10.3389/fmed.2023.1193749.

[27] Uçkan K, Demir H, Turan K, Sarıkaya E, Demir C. Role of oxidative stress in obese and Nonbesse PCOS patient. Int J Clin Pract 2022; 9;2022:4579831. doi:10.1155/2022/4579831.

[28] Murri PM, Luque-Ramírez M, Insenser M, Ojeda-Ojeda M, Escobar-Morreale HF. Circulating markers of oxidative stress and polycystic ovary syndrome (PCOS): a systematic review and meta-analysis. Hum Reprod Update 2013;19:268-88. doi: 10.1093/humupd/dms059.

[29] Kurashova NA, Madaeva IM, Kolesnikova LI. Expression of HSP70 heat-shock proteins under oxidative stress. Adv Gerontol 2020; 10: 20-5. doi:10.1134/S2079057020010099

[30] Grigorian IYu, Linkova NS, Polyakova VO, et al. Signaling molecules of the endometrium: gerontological and general pathological aspects, Adv Gerontol 2016;6: 36-43. doi. org/10.1134/S207.905.7016010045

[31] Cedenho AP, Lima SB, Cenedeze MA, Spaine DM, Ortiz V, Oehninger S. Oligozoospermia and heat-shock protein expression in ejaculated spermatozoa. Hum Reprod 2006;21:1791-4. doi: 10.1093/humrep/del055.

[32] Kuran SB, Altun A, Balci BK, Keskin I, Hocaoglu M. Expression of pro-apoptotic and anti-apoptotic proteins in granulosa cells of women with diminished ovarian reserve . J Assist Reprod Genet 2022;39:765-775. doi: 10.1007/s10815-022-02422-2.

[33] Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Hum Reprod 2004;19:41-7. doi: 10.1093/humrep/deh098.

[34] Teede HJ, Misso ML, Costello MF, et al. Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. Hum. Reprod 2018;33:1602-18. doi: 10.1016/j.fertnstert.2018.05.004.

[35] Bozdag G, Mumusoglu S, Zengin D, Karabulut E, Yildiz BO. The prevalence and phenotypic features of polycystic ovary syndrome: A systematic review and meta-analysis. Hum Reprod 2016;31:2841-55. doi: 10.1093/humrep/dew218.

[36] Krishnan A, Muthusami S. Hormonal alterations in PCOS and its influence on bone metabolism. J Endocrinol 2017;232:R99-R113. doi: 10.1530/JOE-16-0405.

[37] Dunaif A. Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. N Engl J Med 2016;375:54-64. doi: 10.1056/NEJMra1413510.

[38] Dumesic DA, Meldrum DR, Katz-Jaffe MG, Krisher RL, Schoolcraft WB. Oocyte environment: follicular fluid and cumulus cells are critical for oocyte health. Fertil Steril 2015;103:303-16. doi: 10.1016/j.fertnstert.2014.11.015.

[39] Glintborg D, Altinok M, Mumm H, Buch K, Ravn P, Andersen M. Prolactin is associated with metabolic risk and cortisol in 1007 women with polycystic ovary syndrome. Hum Reprod 2014;29:1773-9. doi: 10.1093/humrep/deu133.

[40] Saei Ghare Naz M, Mousavi M, Mahboobifard F, Niknam A, Ramezani Tehrani F. A meta-analysis of observational studies on prolactin levels in women with polycystic ovary syndrome. Diagnostics (Basel) 2022;23;12:2924. doi: 10.3390/diagnostics12122924.

[41] Rudnicka E, Suchta K, Grymowicz M, et al. Chronic low grade inflammation in pathogenesis of PCOS. Int J Mol Sci 2021;6;22:3789. doi: 10.3390/ijms22073789.

[42] Giampaolino P, Foreste V, Di Filippo C, et al. Microbiome and PCOS: State-of-art and future aspects. Int J Mol Sci 2021;19;22:2048. doi: 10.3390/ijms22042048.

[43] Wit AA, Wurth YA, Kruip TA. Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. J Anim Sci 2000; 78: 1277-83. doi: 10.2527/2000.7851277x.

[44] Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH. Cumulus cell apoptosis as an indicator to predict the quality of oocytes and the outcome of IVF-ET. J Ass Reprod Gen 2001; 18: 490-8. doi: 10.1023/a:1016649026353.

[45] Qiao J, Feng HL. Extra – and intra-ovarian factors in polycystic ovary syndrome: Impact on oocyte maturation and embryo developmental competence. Hum Reprod Update 2011; 17:17-33. doi: 10.1093/humupd/dmq032.

[46] Kalra SK, Ratcliffe SJ, Dokras A. Is the fertile window extended in women with polycystic ovary syndrome? Utilizing the society for assisted reproductive technology registry to assess the impact of reproductive aging on live-birth rate. Fertil Steril 2013; 100:208-213. doi: 10.1016/j.fertnstert.2013.02.055

[47] Inoue N, Matsuda F, Goto Y, Manabe N. Role of cell-death ligand-receptor system of granulosa cells in selective follicular atresia in porcine ovary. J Reprod Dev 2011; 57: 169-75. doi: 10.1262/jrd.10-198e.

[48] Matsuda-Minehata F, Inoue N, Goto Y, Manabe N. The regulation of ovarian granulosa cell death by pro – and anti-apoptotic molecules. J Reprod Dev 2006;52: 695-705. doi: 10.1262/jrd.18069.

[49] Han ZB, Lan GC, Wu YG, et al. Interactive effect of granulosa cell apoptosis, follicle size, cumulus-oocyte complex morphology, and cumulus expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system. Reprod 2006;132: 749-58. doi: 10.1530/REP-06-0055.

[50] Amopour H, Mohammad AK, Mohiti MJ, Solaimani M. Rate of cumulus cell apoptosis from fertilized and unfertilized oocytes and acid phosphatase levels in follicular fluids after intracytoplasmic sperm injection. Iran Biomed J 2005; 9: 163-7.

[51] Nakayama M, Manabe N, Nishihara S, Miyamoto H. Species-specific differences in apoptotic cell localization in granulosa and theca interna cells during follicular atresia in porcine and bovine ovaries. J Reprod Develop 2000; 46: 147-56. doi: 10.1262/jrd.46.147.

[52] Peng SL, Wu QF, Xie Q, Tan J, Shu KY. PATL2 regulated the apoptosis of ovarian granulosa cells in patients with PCOS. Gynecol Endocrinol 2021;37:629–34. doi: 10.1080/09513590.2021.1928066.

[53] Das M, Djahanbakhch O, Hacihanefioglu B, et al. Granulosa cell survival and proliferation are altered in polycystic ovary syndrome. J Clin Endocrinol Metab 2008;93(3:881-7. doi: 10.1210/jc.2007-1650.

[54] Webber LJ, Stubbs S, Stark J, et al. Formation and early development of follicles in the polycystic ovary. Lancet 2003; 362(9389):1017-21. doi: 10.1016/s0140-6736(03)14410-8.

[55] Tesarik J, Mendoza C, Greco E. Paternal effects acting during the first cell cycle of human preimplantation development after ICSI. Hum Reprod 2002;17:184-9 doi: 10.1093/humrep/17.1.184.

[56] Barroso G, Valdespin C, Vega E, et al. Developmental sperm contributions: fertilization and beyond. Fertil Steril 2009;92: 835-48. doi: 10.1016/j.fertnstert.2009.06.030.

[57] Liao QY, Huang B, Zhang SJ, et al. Influence of different quality sperm on early embryo morphokinetic parameters and cleavage patterns: A retrospective time-lapse study. Curr Med Sci 2020;40:960-7. doi: 10.1007/s11596.020.2272-3.

[58] Corn CM, Hauser-Kronberger C, Moser M, Tews G, Ebner T. Predictive value of cumulus cell apoptosis with regard to blastocyst development of corresponding gametes. Fertil Steril 2005;84: 627-33. doi: 10.1016/j.fertnstert.2005.03.061.

[59] Heijnen EM, Eijkemans MJ, Hughes EG, Laven JS, Macklon NS. Fauser BC. A meta-analysis of outcomes of conventional

IVF in women with polycystic ovary syndrome. Hum Reprod Update 2006; 12:13-21. doi: 10.1093/humupd/dmi036.

[60] Ding XZ, Fernandez-Prada CM, Bhattacharjee AK, Hoover DL. Over-expression of hsp-70 inhibits bacterial lipopolysaccharide-induced production of cytokines in human monocyte-derived macrophages. Cytokine 2001;21;16:210-9. doi: 10.1006/cyto.2001.0959.

[61] Hartl FU, Martin J, Neupert W. Protein folding in the cell: the role of molecular chaperones Hsp70 and Hsp60. Annu Rev Biophys Biomol Struct 1992; 21:293-322. doi: 10.1146/annurev.bb.21.060192.001453.

[62] Liu Y, Yu Z, Zhao S, et al. Oxidative stress markers in the follicular fluid of patients with polycystic ovary syndrome correlate with a decrease in embryo quality. J Assist Reprod Genet 2021;38:471-7. doi: 10.1007/s10815.020.02014-y.

[63] Li J, Chen H, Gou M, et al. Molecular features of polycystic ovary syndrome revealed by transcriptome analysis of oocytes and cumulus cells. Front Cell Dev Biol 2021;9. doi: 10.3389/fcell.2021.735684.

[64] Ulrich Hartl F, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. Nature 2011; 20;475(7356):324-32. doi: 10.1038/nature10317.

[65] Khan A, Dou J, Wang Y, et al. Evaluation of heat stress effects on cellular and transcriptional adaptation of bovine granulosa cells. J Anim Sci Biotechnol 2020;18;11:25. doi: 10.1186/s40104-019-0408-8

[66] Khan MZ, Khan A, Chen W, Chai W, Wang C. Advancements in genetic biomarkers and exogenous antioxidant supplementation for safeguarding mammalian cells against heat-induced oxidative stress and apoptosis. Antioxidants (Basel) 2024;20;13:258. doi: 10.3390/antiox13030258.

[67] Matos L, Stevenson D, Gomes F, Silva-Carvalho JL, Almeida H. Superoxide dismutase expression in human cumulus oophorus cells. Mol Hum Reprod 2009;411-9. doi: 10.1093/molehr/gap034.

[68] Fencwick MA, Hurst PR. 2002. Immunohistochemical localization of active caspase-3 in the mouse ovary: growth and atresia of small follicles. Reprod 2009;124:659-65. doi: 10.1530/rep.0.1240659.

[69] Daniel A, Dumesic MD, David R, Meldrum MD, Mandy G, Katz-Jaffe PD. Oocyte environment: follicular fluid and cumulus cells are critical for oocyte health. Fertil Steril 2015; 303-16. doi: 10.1016/j.fertnstert.2014.11.015.