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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Expression of pro-apoptotic and anti-apoptotic proteins in granulosa cells of women with diminished ovarian reserve

Sibel Bulgurcuoglu Kuran[1] · **Ayse Altun**[2] · **Burcin Karamustafaoglu Balci**[1] · **Ilknur Keskin**[2] · **Meryem Hocaoglu**[3]

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Abstract

Purpose To evaluate the expressions of caspase-3 and cytochrome c and heat shock protein 70 (Hsp70) in granulosa cells (GCs) from women with normal ovarian reserve (NOR) and diminished ovarian reserve (DOR) undergoing intracytoplasmic sperm injection (ICSI).

Methods GCs were collected from 117 infertile women during oocyte retrieval. Patients were classified into four groups as follows: DOR-COC score of 0, DOR-COC score of I, NOR-COC score of 0, and NOR-COC score of I. The caspase-3, cytochrome c, and Hsp70 analyses were performed immunohistochemically in GCs. The ICSI outcomes were evaluated prospectively.

Results The clinical pregnancy and live birth rates were higher in DOR-COC score of I (15, 30.6%; 14, 38.9%) and NOR-COC score of I (19, 38.77%; 19, 52.7%) groups, compared with DOR-COC score of 0 (12, 24.4%; 3, 6.1%) and NOR-COC score of 0 (3, 6.1%; 0%) groups ($p=0.0001$; 0.00002), respectively. Caspase-3 and cytochrome c expression levels were higher in DOR-COC score of 0 (23, 65.7%; 25, 71.4%) and NOR-COC score of 0 groups (19, 61.3%; 20, 64.5%), compared with DOR-COC score of I (8, 32%; 9, 36%) and NOR-COC score of I groups (7, 26.9%; 8, 30.8%) ($p=0.00297$; $p=0.002$), respectively. Lower expression levels of Hsp70 were found in DOR-COC score of 0 (11, 31.4%) and NOR-COC score of 0 groups (10, 32.3%), compared with DOR-COC score of I (16, 64%) and NOR-COC score of I groups (20, 76.9%) ($p=0.001$), respectively. Hsp70 expression levels were positively correlated with the number of day 3 good-quality embryo and negatively correlated with estradiol levels in the DOR group.

Conclusion Our data suggest that COC score of 0 is associated with increased expression levels of apoptotic proteins, decreased expression levels of anti-apoptotic protein, and poor ICSI clinical outcomes in women with and without DOR.

Keywords Diminished ovarian reserve · Caspase-3 · Cytochrome c · Heat shock protein 70 · Cumulus–oocyte complex · Apoptosis · In vitro fertilization

✉ Sibel Bulgurcuoglu Kuran
sbulgurcuoglu@yahoo.com

Ayse Altun
embaysealtun@gmail.com

Burcin Karamustafaoglu Balci
burcinkaramustafaoglu@yahoo.com

Ilknur Keskin
ilknurkeskin@medipol.edu.tr

Meryem Hocaoglu
dr.meryemtaskiran@gmail.com

[1] Department of Obstetrics and Gynecology School of Medicine, IVF Unit, Istanbul University, Istanbul, Turkey, TR 34452

[2] Department of Histology and Embryology, Medical Faculty, Istanbul Medipol University, Istanbul, Turkey

[3] Department of Obstetrics and Gynecology, Goztepe Prof. Dr. Suleyman Yalcin City Hospital Affiliated To Istanbul Medeniyet University, Istanbul, Turkey

Introduction

Diminished ovarian reserve (DOR) refers to a decline in the quantity and quality of oocytes [1]. It is related to the poor fertility outcomes even when the assisted reproductive techniques (ARTs) are used and remains a major clinical challenge in the field of fertility treatment, because it is characterized by poor ovarian response to ovarian stimulation for in vitro fertilization-embryo transfer, low pregnancy rates, and high rates of pregnancy loss [2–4]. However, the etiology and pathogenesis of DOR remain unclear in the majority of cases [5].

Apoptosis is defined as programmed cell death for homeostasis and is closely involved with most of the reproductive processes, including follicular atresia [6]. Granulosa cell (GC) apoptosis is a physiological phenomenon in follicle, can induce follicular atresia, and can cause a decrease in number of follicles [7, 8]. The studies, which investigate the follicular atresia, have focused increasingly on the molecular regulation of GC apoptosis [9]. Thus, resolving the mechanisms responsible for apoptosis of ovarian GC is crucial not only for analyzing the pathogenesis of follicular atresia but also for developing novel approaches to prevent premature ovarian insufficiency [10]. Limited data are suggestive of elevated apoptosis is shown in GCs collected from infertile women with DOR [11–14]. Conversely, a recent research found less apoptosis of GCs in women with DOR [8]. To date, it is still unclear that the effects of GC apoptosis on oocyte quality and response to ovarian stimulation.

On the other hand, successful fertilization, early embryo development, uterine implantation, and healthy pregnancy depend on the quality of oocytes, and morphological evaluation is one of the options used to predict quality levels. The morphological analysis of cumulus complex is one of the noninvasive methods conventionally utilized to determine competent oocytes [15]. Although at present there is little corroborated evidence to support a correlation with embryo developmental competence, cumulus–oocyte complex (COC) scoring provides an important tool for troubleshooting [16]. As far as we know, no previous research has investigated the apoptosis in GCs in relation with COC scoring. To address this gap, we decided to investigate pro-apoptotic and anti-apoptotic protein expressions in COC score of 0 and I from women with and without DOR undergoing vitro intracytoplasmic sperm injection (ICSI).

The apoptotic cell death happens mainly through two different pathways: the extrinsic or death receptor pathway (type I cells), and the intrinsic or mitochondrial pathway (type II cells). Both pathways, intrinsic and extrinsic, activate caspase-3 which leads to death cell [17]. The expression of caspase-3 was found to be regulated by gonadotropin and may be upregulated as part of the apoptotic process in GCs [18, 19]. Cytochrome c is a signaling molecule essential for apoptotic cell death released from mitochondria to the cytosol. Cytosolic cytochrome c leads to the activation of caspase-3 and poly ADP-ribose polymerase (PARP), thus subsequently resulting in apoptosis [20, 21]. Moreover, heat shock protein 70 (Hsp70) is a 72-kDa stress protein that can block apoptosis that is induced by a variety of initiators. Protection by Hsp70 has been identified to happen downstream of cytochrome c release and in some cases downstream of caspase-3 activation and cleavage of caspase-3 substrates [22].

The present study was carried out to assess expressions of pro-apoptotic proteins caspase-3 and cytochrome c and anti-apoptotic protein Hsp70 in COC score of 0 and I from women with normal ovarian reserve (NOR) and DOR undergoing ICSI. Also, we sought to explore the relationship between GC apoptosis and ICSI outcomes in women with and without DOR.

Materials and methods

Study population

This is a prospective cohort study of women who attended the infertility clinic of Istanbul University School of Medicine (Istanbul, Turkey) to undergo the ICSI treatment between March 2019 and March 2020. The fresh granulosa cells were collected from 117 infertile Caucasian women of 20 to 40 years old, who undergo the ICSI. DOR was defined by using the Patient-Oriented Strategies Encompassing Individualized Oocyte Number (POSEIDON) criteria. POSEIDON groups 3 and 4 comprise patients with low anti-Müllerian hormone (AMH) (< 1.2 ng/mL) or low antral follicle count (AFC) (< 7) prior to the controlled ovarian hyperstimulation (COH) who are younger or older than 35 years, respectively [23]. The study population was classified into two groups. Group 1 consisted of women, who belong to POSEIDON groups 3 and 4 ($n = 60$), and group 2 consisted of women with normal ovarian reserve and had primary infertility due to the male factor ($n = 57$) [23, 24]. 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 demographic and clinical characteristics of all participants were recorded, including age, body mass index (BMI), serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), prolactin (PRL) levels measured

on menstrual day 3, total doses of gonadotrophins, total number of days of 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, clinical pregnancy, and live birth rates. Early pregnancy loss is defined as a nonviable, intrauterine pregnancy with either an empty gestational sac or a gestational sac containing an embryo or fetus without fetal heart activity within the first 12 6/7 weeks of gestation [25].

Stimulation protocol

All patients received the standard COH protocol, as defined below. The COH was started on the third day of the menstrual cycle. COH was performed with injectable gonadotropins using a GnRH antagonist protocol. Recombinant FSH (Gonal-F, EMD Serono, MA, USA) at a daily 150–375 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. 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 17 mm or more. Ultrasound-guided transvaginal oocyte retrieval took place 34–36 h after hCG administration.

Follicular fluid and granulosa cells collection

The follicular fluid (FF) samples were carefully gathered from follicles with a diameter of ≥ 18 mm, and transferred into medium (G-MOPS™, Sweden). Microscopic examination of the follicular aspirates was performed by the embryologist. The GCs were separated from collected COC. The cumulus–oocyte complexes were evaluated based on their morphological features with stereo microscope (SZX16/SZX10, Olympus). Then, COCs were scored as part of the morphological assessment. This is a binary score (0 or I), with a "good" COC (score of I) defined as having expanded cumulus and a radiating corona [16]. Furthermore, study subjects were reclassified into four groups according to COC scoring. Group A included 35 with DOR-COC score of 0, group B included 25 women with DOR-COC score of I, group C included 31 women with NOR-COC score of 0, and group D included 26 women with NOR-COC score of I. The cumulus GCs surrounding the remaining eggs were cut and were spread on poly-l-lysine-coated microscope slides. Immunohistochemical staining was applied to GCs that were air-dried and fixed to analyze apoptosis.

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. Fresh 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 until day 3 post fertilization. Embryo development was observed 2 and 3 days post-culture and embryos were graded as follows. Good-quality (grades 1 or 2) embryos were transferred according to the morphologic classification [16, 26].

Embryo transfer and luteal phase support

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 ß-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 detection of gestational sac and embryonic heart activity by transvaginal ultrasound embryonic heartbeat. The clinical outcomes per started ICSI cycle were determined, including positive serum ß-hCG levels, clinical pregnancy, and live birth (the birth of a neonate on or after 24 weeks' gestation).

Immunohistochemical analysis

The fixed granulose cells were rinsed with 70% alcohol and finally washed with fresh saline solution. The antigen retrieval was accomplished in the Decloaking chamber in a citrate buffer (pH 6.0). The endogenous peroxidase activity was blocked with 3% H_2O_2. Blocking reagent was applied to each slide followed by 5 min incubation at room temperature in a humid chamber. Sections were incubated for overnight at 4 °C with rabbit polyclonal caspase-3 antibody, cytochrome

c antibody, and Hsp70. The sections were biotinylated goat anti-rabbit antibodies. The slides were washed in PBS after which streptavidin peroxidase label reagent was applied. The colored product was incubated in 3, 3'-diaminobenzidine, tetrahydrochloride, dihydrate (DAB). The slides were counterstained with Mayer's hematoxylin. Immunohistochemical evaluation was done on light microscopic examination. The numbers of unstained and stained cells were counted.

Histologic score (H-SCORE)

The assessments of the immunohistochemical expressions of caspase-3, cytochrome c, and Hsp70 in GCs were achieved by utilizing H-SCORE. The relative intensity of specific immunohistochemical staining was scored as 0, negative; 1, weak positive; 2, moderate positive; and 3, strongly positive. H-score; $[\sum Pi\ (i+1)$, where i = intensity of staining with a value of (±), (+), (++), or (+++) (null/minimal, mild, moderate, or strong, respectively), and Pi is the percentage of GCs stained with each intensity, varying between 0 and 100% [27]. All immunohistochemical stained sections were evaluated by one experienced histologist (AA).

Statistical analyses

The statistical analyses were performed by using GraphPad Prism (ver. 7.00) and SPSS (ver. 25). Normality of the all data was tested by Shapiro–Wilk test. Comparisons of the differences in means for a continuous variable in the groups were performed by Mann–Whitney U test or Student's t-test according to test for the normality of the results. Comparisons of the three or more independent groups were performed by ANOVA for parametric and Kruskal–Wallis for the 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.

Results

Subject characteristics

All participants were Caucasian. BMI was not statistically significant among DOR-COC score of 0 (26.78 ± 0.80), DOR-COC score of I (24.36 ± 0.62), NOR-COC score of 0 (26.51 ± 0.77), and NOR-COC score of I (26.47 ± 0.94) groups ($p = 0.2057$). Demographic and baseline characteristics of the groups were similar between DOR and NOR groups in terms of age, E2 serum level on day 3 of the cycle, the number of oocytes retrieved, the number of D3 good-quality embryos, and clinical pregnancy rate ($p > 0.05$). Those with NOR had significantly more days of GnRH antagonist treatment (3.03 ± 0.20 vs 22. 2.44 ± 0.21, $p < 0.0343$), compared to the women with DOR. Demographic characteristics and ICSI outcome parameters of the DOR and NOR groups are listed in Table 1.

There was no significant difference in age, BMI, E2 level on day 3 of the cycle, the number of oocytes retrieved, the number of D3 good-quality embryos, and fertilization rate between the four groups. Total number of days of GnRH antagonist treatment was higher in NOR-COC score of I (3.567 ± 0.2687) and DOR-COC score of I (2.52 ± 0.3335) groups, compared with DOR-COC score of 0 (2.386 ± 0.2741) and NOR-COC score of 0 groups (2.581 ± 0.2695) ($p = 0.0249$), respectively. The number of poor-quality oocytes was found to be higher in DOR-COC score of 0 (6, 17.1%) and NOR-COC score of 0 (7, 22.7%) groups, compared with DOR-COC score of I (0, 0%) and NOR-COC score of I groups (2, 7.7%) ($p = 0.042$), respectively. In addition, the ICSI outcome parameters including clinical pregnancy, live birth, and early pregnancy loss rates were found to be statistically significant among the four groups ($p < 0.05$). The clinical pregnancy rate was higher in DOR-COC score of I (15, 30.6%) and NOR-COC score of I (19, 38.77%) groups, compared with DOR-COC score of 0 (12, 24.4%) and NOR-COC score of 0 groups (3, 6.1%) ($p = 0.0001$), respectively. Moreover, live birth rate was higher in DOR-COC score of I (14, 38.9%) and NOR-COC score of I groups (19, 52.7%), compared with DOR-COC score of 0 (3, 8.3%) and NOR-COC score of 0 groups (0%) ($p = 0.00002$), respectively. Conversely, early pregnancy loss rate was higher in DOR-COC score of 0 (9, 69.2%) and NOR-COC score of 0 groups (3, 23.07%), compared with DOR-COC score of I (1, 7.7%) and NOR-COC score of I groups (0%) ($p = 0.00297$), respectively. The demographic characteristics and ICSI outcome parameters of the four groups are presented in Table 2.

Expression profile of caspase-3, cytochrome c, and Hsp70 in the study groups

The expression levels of caspase-3 were higher in DOR-COC score of 0 (23, 65.7%) and NOR-COC score of 0 groups (19, 61.3%), compared with DOR-COC score of I (8, 32%) and NOR-COC score of I groups (7, 26.9%) ($p = 0.00297$), respectively. Similarly, cytochrome c expression levels were higher in DOR-COC score of 0 (25, 71.4%) and NOR-COC score of 0 groups (20, 64.5%), compared with DOR-COC score of I (9, 36%) and NOR-COC score of I groups (8, 30.8%) ($p = 0.002$), respectively. As expected, we observed significantly lower expression levels of Hsp70 in DOR-COC score of 0 (11, 31.4%) and NOR-COC score of 0 groups (10, 32.3%), compared with DOR-COC score of I (16, 64%) and

Table 1 Demographic characteristics and intracytoplasmic sperm injection (ICSI) outcome parameters of diminished ovarian reserve (DOR) and normal ovarian reserve (NOR) groups using Student's *t*, Mann–Whitney *U*, and Pearson's chi-square tests

Variables	DOR ($n=60$)	NOR ($n=57$)	p
Age (year)[a]	31.95 ± 4.4	31.32 ± 4.7	0.4532
BMI (kg/m^2)[b]	25.77 ± 0.55	26.49 ± 0.59	0.3182
FSH (IU/mL)[bc]	7.246 ± 0.28	7.055 ± 0.23	0.4329
LH (IU/mL)[bc]	5.82 ± 0.40	6.99 ± 0.77	0.4605
Estradiol-E2 (pg/mL)[bc]	48.04 ± 5.2	43.95 ± 2.8	0.9616
Prolactin (ng/mL)[bc]	18.51 ± 1.31	20.31 ± 1.79	0.8184
Total dose of gonadotropins (IUs)[b]	2193 ± 109.8	2141 ± 124.7	0.4035
Total number of days of GnRH antagonist treatment[b]	2.44 ± 0.21	3.03 ± 0.20	0.0343*
Number of oocytes retrieved[b]	8.25 ± 0.60	7.63 ± 0.53	0.4907
MII oocyte ≤ 5[d]	29 (52.7)	26 (47.2)	0.686
MII oocyte 6–10[d]	28 (52.8)	25 (47.1)	0.680
MII oocyte ≥ 11[d]	3 (25)	6 (75)	0.317
MI oocyte[b]	1.68 ± 0.27	1.10 ± 0.17	0.3487
Number of poor-quality oocytes[b]	0.116 ± 0.048	0.228 ± 0.079	0.3238
Fertilization rate[b]	78.28 ± 9.87	61.18 ± 3.45	0.1021
Number of D3 good-quality embryos (GI)[b]	2.42 ± 0.38	1.45 ± 0.16	0.2150
Number of D3 average-quality embryos (GII)[b]	1.517 ± 0.22	1.649 ± 0.19	0.3413
Number of D3 poor-quality embryos (GIII)[b]	0.68 ± 0.143	0.61 ± 0.169	0.3714
Number of arrested embryos[b]	0.18 ± 0.074	0.19 ± 0.052	0.3803
Clinical pregnancy[d]	22 (45.8)	26 (54.2)	0.564
Live birth[d]	19 (52.8)	17 (47.2)	0.739
Early pregnancy loss[d]	3 (25)	9 (75)	0.083

Abbreviations: *DOR*, diminished ovarian reserve; *NOR*, normal ovarian reserve; *SE*, standard error; *BMI*, body mass index; *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone; *GnRH*, gonadotropin-releasing hormone

Data are presented as either means ± standard error or number (%)

[a]Student's *t* test was used

[b]Mann-Whitney *U* test was used

[c]Day 3 of the cycle, before stimulation

[d]Pearson's chi-square test was used

*Significant at $p<0.05$

NOR-COC score of I groups (20, 76.9%) ($p=0.001$), respectively (Table 3, Fig. 1). Caspase-3, cytochrome c, and Hsp70 expression profiling in relation to the ICSI outcomes are presented in Table 4. Women with early pregnancy loss had significantly lower expression of Hsp70 (32 ± 2.082), compared to caspase-3 (65.43 ± 2.287) and cytochrome c expression levels (68.57 ± 2.034) ($p=<0.0001$), respectively.

Correlation analysis between expression levels of caspase-3, cytochrome c, and Hsp70 and clinical parameters

The correlation analyses were performed to verify the associations of clinical parameters and caspase-3, cytochrome c, and Hsp70 expression levels. We observed that the expression levels of caspase-3 were positively correlated with serum level of E2 ($r=0.580$; $p=0.012$) and negatively correlated with serum PRL level ($r=-0.671$; $p=0.002$) in the DOR group. It was found that the expression levels of cytochrome c were positively correlated with serum E2 level ($r=0.696$; $p=0.001$) and BMI ($r=0.766$; $p=0.0002$), and negatively correlated with serum PRL level ($r=-0.742$; $p=0.0004$) and the number of D3 good-quality embryos ($r=-0.602$; $p=0.0140$) in the DOR group. Moreover, the correlation analyses revealed that Hsp70 expression levels were positively correlated with serum PRL level ($r=0.725$; $p=0.001$) and the number of D3 good-quality embryos ($r=0.596$; $p=0.015$) and negatively correlated with BMI ($r=-0.775$; $p=0.0001$) and serum level of E2 ($r=-0.630$; $p=0.005$) in the DOR group (Table 5). Expression levels of caspase-3 ($r=-0.544$; $p=0.029$) and cytochrome c ($r=-0.582$; $p=0.018$) were negatively correlated with the number of D3 average-quality embryos in the NOR group (Table 6).

Table 2 Demographic characteristics and intracytoplasmic sperm injection (ICSI) outcome parameters of the study groups using one-way analysis of variance (ANOVA) with Tukey's multiple comparison, Kruskal–Wallis with Dunn's multiple comparison, and Pearson's chi-square tests

Variables	DOR-COC score of 0 ($n=35$)	DOR-COC score of I ($n=25$)	NOR-COC score of 0 ($n=31$)	NOR-COC score of I ($n=26$)	p
Age (year)[a]	31.23 ± 0.7627	32.96 ± 0.8195	32.32 ± 0.8877	30.12 ± 0.829	0.1096
BMI (kg/m^2)[b]	26.78 ± 0.8007	24.36 ± 0.6201	26.51 ± 0.7723	26.47 ± 0.9459	0.2057
FSH (IU/mL)[ac]	6.386 ± 0.351	7.82 ± 0.4558	7.118 ± 0.3476	6.978 ± 0.2939	0.2703
LH (IU/mL)[bc]	6.145 ± 0.6195	5.381 ± 0.4343	7.048 ± 0.9759	6.922 ± 1.263	0.5167
Estradiol-E2 (pg/mL)[bc]	57.3 ± 8.471	35.44 ± 3.021	44.66 ± 4.077	43.11 ± 3.87	0.0590
Prolactin (ng/mL)[bc]	15.13 ± 0.9577	23.23 ± 2.604	18.32 ± 1.638	22.69 ± 3.394	0.1015
Total dose of gonadotropins (IUs)[b]	2138 ± 151.7	2387 ± 153.8	2154 ± 145.1	2125 ± 215.1	0.48
Total number of days of GnRH antagonist treatment[b]	2.386 ± 0.2741	2.52 ± 0.3335	2.581 ± 0.2695	3.567 ± 0.2687	0.0249*
Number of oocytes retrieved[a]	8.429 ± 0.8434	8 ± 0.8679	7.548 ± 0.6844	7.731 ± 0.8639	0.8660
MII oocyte ≤ 5[d]	18 (31.03)	11 (19)	16 (27.6)	13 (22.4)	0.572
MII oocyte 6–10[d]	12 (25)	12 (25)	15 (31.25)	9 (18.75)	0.682
MII oocyte ≥ 11[d]	5 (45.45)	2 (18.2)	3 (27.3)	1 (9.1)	0.364
MI oocyte[b]	1.743 ± 0.3463	1.6 ± 0.4619	1.097 ± 0.2289	1.115 ± 0.2736	0.6540
Number of poor-quality oocytes[d]	6 (17.1)	0	7 (22.7)	2 (7.7)	0.042*
Fertilization rate[b]	66.86 ± 4.574	94.28 ± 22.7	60.68 ± 4.936	61.77 ± 4.883	0.2826
Number of D3 good-quality embryos (GI)[b]	2.171 ± 0.54	2.64 ± 0.4722	1.29 ± 0.2281	1.654 ± 0.2477	0.1754
Number of D3 average-quality embryos (GII)[b]	1.571 ± 0.3235	1.44 ± 0.3059	1.452 ± 0.2167	1.885 ± 0.3297	0.6996
Number of D3 poor-quality embryos (GIII)[b]	0.6286 ± 0.2012	0.76 ± 0.2023	0.7097 ± 0.2672	0.5 ± 0.1941	0.6191
Number of arrested embryos[b]	0.2647 ± 0.1217	0.08 ± 0.05538	0.1935 ± 0.07213	0.1923 ± 0.07882	0.6624
Clinical pregnancy[d]	12 (24.4)	15 (30.6)	3 (6.1)	19 (38.77)	0.0001*
Live birth[d]	3 (8.3)	14 (38.9)	0	19 (52.7)	0.00002*
Early pregnancy loss[d]	9 (69.2)	1 (7.7)	3 (23.07)	0	0.00297*

Cumulus–oocyte complexes (COC) scoring was done. This is a binary score (0 or 1), with a "good" COC (score of 1) defined as having expanded cumulus and a radiating corona

Abbreviations: *DOR*, diminished ovarian reserve; *NOR*, normal ovarian reserve; *COC*, cumulus–oocyte complexes; *SE*, standard error; *BMI*, body mass index; *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone; *GnRH*, gonadotropin-releasing hormone

Data are presented as either means ± standard error or number (%)

[a]One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used

[b]Kruskal-Wallis with Dunn's multiple comparison test was used

[c]Day 3 of the cycle, before stimulation

[d]Pearson's chi-square test was used

*Significant at $p<0.05$

Discussion

Comparison with existing literature

During follicle development, critical cross-talk between the oocyte and surrounding GCs is crucial for generating a fertilizable oocyte and regulating ovarian functions [28]. Investigating the mechanisms within the apoptosis pathway in GCs obtained from infertile women with DOR may contribute to a better understanding of the underlying molecular etiology of DOR that is still poorly understood. Jiang et al. identified decreased sigma-1 receptor which is an endoplasmic reticulum stress (ERS)-associated membrane chaperone protein expression is accompanied by increased apoptosis rate in the GCs of women with DOR [29]. A recent study by Wei et al. revealed that reduced microRNA-221-3p expression may upregulate Forkhead box O1 (FOXO1) expression and hence contribute to the pathogenesis of DOR by promoting apoptosis of GCs [30]. In contrast, Regan et al. did not find an increase in GC apoptosis in the large (> 23 mm) preovulatory follicles of older patients which had a poor ovarian reserve. Also, younger patients showed a ~ sevenfold higher level

Table 3 Expression profile of caspase-3, cytochrome c, and heat shock protein (Hsp70) in the study groups using Pearson's chi-square test

	DOR-COC score of 0 ($n=35$)	DOR-COC score of I ($n=25$)	NOR-COC score of 0 ($n=31$)	NOR-COC score of I ($n=26$)	p
Caspase-3	23 (65.7)	8 (32.0)	19 (61.3)	7 (26.9)	0.003*
Cytochrome c	25 (71.4)	9 (36.0)	20 (64.5)	8 (30.8)	0.002*
Hsp70	11 (31.4)	16 (64.0)	10 (32.3)	20 (76.9)	0.001*

Abbreviations: *DOR*, diminished ovarian reserve; *NOR*, normal ovarian reserve; *COC*, cumulus–oocyte complexes; *Hsp*, heat shock protein

Cumulus–oocyte complexes (COC) scoring was done. This is a binary score (0 or 1), with a "good" COC (score of 1) defined as having expanded cumulus and a radiating corona

Data are presented as number (%). Pearson's chi-square test was used

*Significant at $p<0.05$

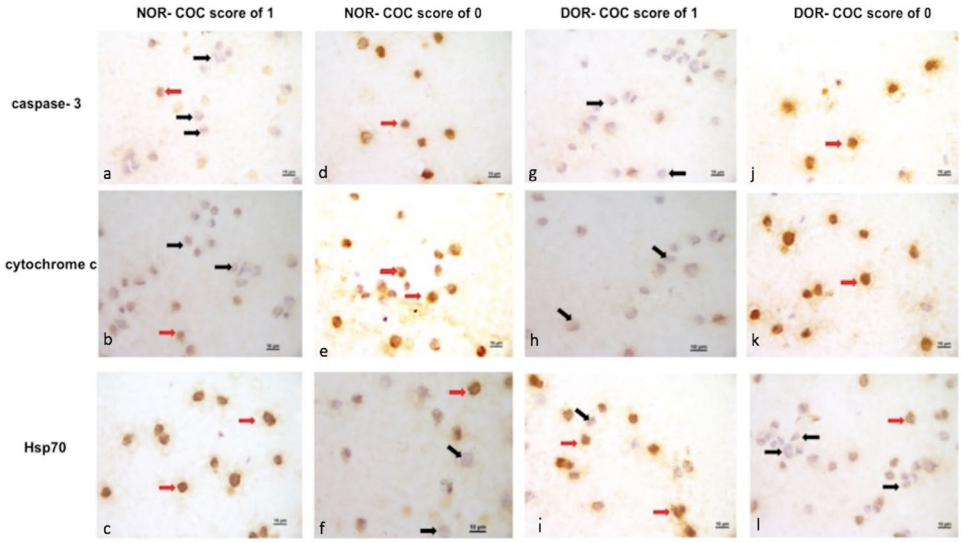

Fig. 1 Immunohistochemical (IHC) staining micrographs of granulosa cells (GCs) from women with diminished ovarian reserve (DOR)-cumulus–oocyte complexes (COC) score of 0, DOR-COC score of I, normal ovarian reserve (NOR)-COC score of 0, and NOR-COC score of I. The assessments of the immunohistochemical 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 NOR-COC score of I group. Intense brown immunostaining of caspase-3, cytochrome-c (**d**, **e**) and low immunostaining of Hsp70 (**f**) were observed in GCs of NOR-COC score of 0 group. Low immunostaining of caspase-3, cytochrome-c (**g**, **h**) and intense brown immunostaining of Hsp70 (**i**) were observed in GCs of DOR-COC score of I group. Intense brown immunostaining of caspase-3, cytochrome-c (**j**, **k**) and low immunostaining of Hsp70 (**l**) were observed in GCs of DOR-COC score of 0 group. At least 100 GCs were analyzed by light microscopy (×100). Scale bars are indicated (10 μm)

Table 4 Caspase-3, cytochrome c, and heat shock protein (Hsp70) expression profiling in relation to intracytoplasmic sperm injection (ICSI) outcomes using Pearson's chi-square test

Variables	Caspase-3	Cytochrome c	Hsp70	p
Clinical pregnancy	52.04 ± 3.2	55.63 ± 3.14	61.83 ± 4.09	0.0018*
Live birth	32.4 ± 6.022	34 ± 4.05	77.82 ± 1.025	< 0.0001*
Early pregnancy loss	65.43 ± 2.287	68.57 ± 2.034	32 ± 2.082	< 0.0001*

Abbreviation: *Hsp70*, heat shock protein 70

Data are presented as either means ± standard error. Pearson's chi-square test was used

*Significant at $p<0.05$

Table 5 Correlation coefficients for caspase-3, cytochrome c, and heat shock protein (Hsp70) expression levels and clinical parameters in the diminished ovarian reserve (DOR) group using Pearson's correlation and Spearman's rank correlation coefficient

Parameters	Caspase-3		Cytochrome c		Hsp70	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value
Age (year)[a]	−0.222	0.376	0.028	0.913	−0.328	0.215
BMI (kg/m^2)[b]	0.166	0.539	**0.766**	**0.0002**	**−0.775**	**0.0001**
FSH (IU/mL)[bc]	−0.225	0.922	0.070	0.782	0.006	0.980
LH (IU/mL)[bc]	0.070	0.783	0.186	0.459	−0.062	0.807
Estradiol-E2 (pg/mL)[bc]	**0.580**	**0.012**	**0.696**	**0.001**	**−0.630**	**0.005**
Prolactin (ng/mL)[bc]	**−0.671**	**0.002**	**−0.742**	**0.0004**	**0.725**	**0.001**
Total dose of gonadotropins (IUs)[b]	0.199	0.639	0.248	0.322	−0.182	0.469
Total number of days of GnRH antagonist treatment[b]	−0.052	0.837	0.017	0.948	−0.148	0.557
Number of oocytes retrieved[b]	0.006	0.980	−0.035	0.890	0.092	0.717
MII oocyte ≤ 5[a]	0.202	0.632	−0.065	0.878	0.000	1.000
MII oocyte ≥ 6[b]	−0.169	0.749	−0.169	0.749	−0.338	0.512
MI oocyte[b]	−0.163	0.518	−0.112	0.659	0.335	0.175
Number of poor-quality oocytes[a]	0.428	0.076	0.312	0.207	−0.329	0.183
Fertilization rate[b]	0.316	0.201	0.302	0.310	0.326	0.218
Number of D3 good-quality embryos (GI)[b]	−0.327	0.217	**−0.602**	**0.0140**	**0.596**	**0.015**
Number of D3 average-quality embryos (GII)[b]	−0.108	0.670	−0.014	0.958	0.162	0.520
Number of D3 poor-quality embryos (GIII)[b]	−0.190	0.481	−0.158	0.558	0.141	0.602
Number of arrested embryos[b]	0.246	0.324	0.121	0.633	−0.270	0.278

Abbreviations: *DOR*, diminished ovarian reserve; *BMI*, body mass index; *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone; *GnRH*, gonadotropin-releasing hormone; *MI oocyte*, metaphase I; *MII*, metaphase II

[a]Pearson's correlation coefficient was used

[b]Spearman's rank correlation coefficient was used

[c]Day 3 of the cycle before stimulation

of apoptosis in follicles of the same size class (> 23 mm) [8]. They concluded that dysregulation of receptor expression in the older patient may suppress the mitogenic growth rate in healthy follicles indicated by reduced GC apoptosis [8]. Unique to the present study, our data clearly show that expression levels of caspase-3 and cytochrome c were higher in DOR-COC score of 0 and NOR-COC score of 0 groups, compared with DOR-COC score of I and NOR-COC score of I groups, respectively. As expected, we found significantly lower expression levels of Hsp70 in DOR-COC score of 0 and NOR-COC score of 0 groups, compared with DOR-COC score of 1 and NOR-COC score of I groups, respectively. These data suggest that "good" COCs (score of I) are associated not only with lower levels of pro-apoptotic proteins but also higher anti-apoptotic protein levels in both DOR and NOR groups. Thus, apoptosis which is shown in GCs is defined as morphologically dark (score of 0) and poor-quality [31]. Emerging evidence suggests that increased GC apoptosis is typical of older patients and is related to oocyte quality [32]. Our study population is not stratified by age. Thus, we did not observe significant difference in age between the four groups. Consistent with previous literature, we found that the number of poor-quality oocytes was found to be higher in DOR-COC score of 0 and NOR-COC score of 0 groups, compared with DOR-COC score of I and NOR-COC score of I groups, respectively.

On the other hand, we observed no significant difference in clinical pregnancy, live birth, and early pregnancy loss rates between the DOR and NOR groups. However, our results revealed that clinical pregnancy and live birth rates were higher in DOR-COC score of I and NOR-COC score of I groups, compared to DOR-COC score of 0 and NOR-COC score of 0 groups, respectively. In addition, early pregnancy loss rate was higher in DOR-COC score of 0 and NOR-COC score of 0 groups, compared to DOR-COC score of I and NOR-COC score of I groups, respectively. We speculate that having COC score of 0 could have a marked impact on clinical outcomes of ICSI regardless of whether the women with DOR or not, as shown by the results of higher pro-apoptotic and lower anti-apoptotic protein expression levels in our research. Benifla et al. suggested that women with lower percentages of GC apoptosis had higher pregnancy rates [33]. Previous studies have shown that a rise in the apoptotic rate of cumulus GCs, a reduction in intracellular junction, and a decrease and even disappearance of microvilli may cause low maturation and abnormal morphology of oocytes, which

Table 6 Correlation coefficients for caspase-3, cytochrome c, and heat shock protein (Hsp70) expression levels and clinical parameters in the normal ovarian reserve (NOR) group using Pearson's correlation and Spearman's rank correlation coefficient

Parameters	Caspase-3		Cytochrome c		Hsp70	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value
Age (year)[a]	−0.220	0.414	−0.198	0.462	0.403	0.122
BMI (kg/m^2)[b]	−0.263	0.324	−0.096	0.724	0.445	0.084
FSH (IU/mL)[bc]	0.466	0.069	0.355	0.177	−0.353	0.180
LH (IU/mL)[bc]	−0.397	0.128	−0.093	0.732	0.245	0.361
Estradiol-E2 (pg/mL)[bc]	0.019	0.943	−0.101	0.711	0.151	0.578
Prolactin (ng/mL)[bc]	−0.122	0.653	−0.191	0.479	−0.178	0.510
Total dose of gonadotropins (IUs)[b]	−0.032	0.907	−0.126	0.642	0.146	0.590
Total number of days of GnRH antagonist treatment[b]	−0.291	0.274	−0.475	0.063	0.402	0.123
Number of oocytes retrieved[b]	−0.227	0.398	−0.407	0.118	0.265	0.321
MII oocyte ≤ 5[a]	−0.593	0.121	−0.570	0.141	0.548	0.160
MII oocyte ≥ 6[b]	−0.667	0.140	−0.765	0.076	0.559	0.249
MI oocyte[b]	−0.207	0.441	−0.170	0.529	−0.047	0.863
Number of poor-quality oocytes[a]	0.248	0.354	0.173	0.523	−0.182	0.501
Fertilization rate[b]	−0.432	0.095	−0.392	0.133	0.253	0.344
Number of D3 good-quality embryos (GI)[b]	−0.320	0.195	−0.335	0.174	0.250	0.318
Number of D3 average-quality embryos (GII)[b]	**−0.544**	**0.029**	**−0.582**	**0.018**	0.475	0.063
Number of D3 poor-quality embryos (GIII)[b]	0.175	0.487	0.092	0.717	0.029	0.910
Number of arrested embryos[b]	0.088	0.747	0.211	0.433	−0.070	0.798

Abbreviations: *NOR*, normal ovarian reserve; *BMI*, body mass index; *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone; *GnRH*, gonadotropin-releasing hormone; *MI oocyte*, metaphase I; *MII*, metaphase II

[a]Pearson's correlation coefficient was used

[b]Spearman's rank correlation coefficient was used

[c]Day 3 of the cycle before stimulation

affects oocyte development, thereby affecting IVF or ICSI pregnancy outcomes [34, 35]. Interestingly, we did not find significant difference in good-quality embryo between the four groups. But, also, we found that cytochrome c expression levels were negatively correlated with the number of D3 good-quality embryos in the DOR group and expression levels of caspase-3 and cytochrome c were negatively correlated with the number of D3 average-quality embryos in the NOR group. Lee et al. demonstrated the higher incidence of cumulus cell apoptosis in unfertilized oocytes than in fertilized oocytes, but the frequency of apoptosis in cumulus cells was not associated to embryo quality [6]. However, the underlying mechanisms by which pro-apoptotic and anti-apoptotic protein expressions in GC affect ICSI outcomes are not fully understood.

Strengths and limitations

It is notable that the investigation of the microenvironment that surrounds the oocyte — GCs, cumulus cells, and follicular fluid — and their correlation with clinical parameters is extremely important and may finally allow scientists and clinicians to harness better protocols of ovarian stimulation for infertility treatments [36, 37]. Thus, this article is pioneering in that it demonstrates the relationship between apoptosis and COC scoring and their correlations with ICSI outcomes. Since apoptosis in the GCs is predominantly via caspase-dependent signaling pathways, we studied the expression levels of caspase-3 and caspase-3-related cytochrome c which are released from the endoplasmic reticulum [32].

We are aware of the limitations of the present study. We used primary antibodies that recognize full-length caspases instead of cleaved caspases, which are the final executioners of apoptosis [38]. Second, the relatively average size of women was classified according to COC scoring.

Open questions and future research

We analyzed cumulus cells in the follicular fluid aspirate rather than pure mural GCs. Besides that, Fan et al. for the first time demonstrated that total mural GC apoptosis (but not total cumulus cell apoptosis) is increased in women with DOR, correlated with worse ovarian response, and with fewer oocyte and embryo numbers [14]. It would be of great interest to investigate GC apoptosis in different GC subpopulations from women with and without DOR.

Previous studies indicate that the incidence of apoptosis in GCs was higher in women treated with gonadotropin-releasing hormone agonist (GnRHa) than in natural cycles or cycles without GnRHa [39, 40]. In addition, it was shown that the GnRH agonist leuprolide acetate produced an increase in the percentage of apoptotic cells and GnRH antagonist blocked the apoptotic effect produced by LA [41]. Further studies are necessary in order to compare GC apoptosis in relation with COC scoring in different types of treatment cycle.

Conclusion

The present study is, to our knowledge, the first to investigate pro-apoptotic and anti-apoptotic markers in COC score of 0 and I from women with and without DOR. Our present findings indicate that COC score of 0 is associated with increased expression levels of apoptotic proteins and poor ICSI clinical outcomes in the women with and without DOR. It is necessary to conduct more histopathological and genetic/molecular studies to explore the functional roles of the different biomarkers related with apoptosis in COC score of 0 and I from women undergoing ICSI.

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Declarations

Ethics approval Ethical approval for the present study was obtained from Ethics Committee of Istanbul Medipol University (E-10840098–772.02–1134). All participants wrote and signed informed consent the start of the investigation. All procedures performed in this study were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest The authors declare no competing interests.

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