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Investigation of oxidative stress status in cumulus cells in patıents with in vitro fertilization

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Background/aim: The negative impact of oxidative stress on oocytes obtained from in vitro fertilization (IVF) patients is a challenge for the optimization of live birth rates. In this study, it is aimed to investigate whether oxidant/antioxidant parameters have a predictive value in terms of determining the count and quality of oocytes.

Materials and methods: Catalase (CAT), glutathione-S-transferase (GST), arylesterase (ARE) enzyme activities, and malondialdehyde (MDA) levels were analysed in cumulus cells of poor responder (n = 28, oocyte count ≤ 4), normo responder (n = 48, 5 ≤ oocyte count ≤ 14), and high responder (n = 26, oocyte count ≥ 15) patient groups continuing IVF treatment.

Results: The cumulus cell GST enzyme activity were statistically significantly increased in the high responders group compared to the poor responder and the normo responder's groups ($p < 0.001$ and $p = 0.002$, respectively). The cumulus cell MDA levels were significantly decreased in the high responder group compared to the poor responder group ($p = 0.008$). The cumulus cell CAT ($p = 0.175$) and ARE ($p = 0.124$) enzyme activities were examined but no statistically significant difference found between the groups.

Conclusion: The significant increase in GST enzyme activity and significant decrease in MDA levels in the high responder group indicate that oxidative stress has an effect oocyte status and quality.

Key words: In vitro fertilization, cumulus cell, catalase, glutathione-S-transferase, arylesterase, malondialdehyde

1. Introduction

Infertility is defined as inability to conceive naturally after one year of regular unprotected sexual intercourse [1]. Tubal factor, endometriosis, cervical factor, pelvic adhesion, male factor, and unexplained infertility are among the main causes of infertility [2]. Assisted reproductive techniques are used for infertile couples to have a healthy baby. In vitro fertilization (IVF), which is the most widely used method among assisted reproductive techniques, increases the chance of infertile individuals to havechildren. The count and quality of embryos transferred are among the factors affecting IVF success rates [3].

Cumulus cells and oocyte together form the cumulus-oocyte complex located in the antral follicle in contact with the follicular fluid [4]. Cumulus cells protect the oocyte from oxidative stress damage during growth and maturation and play an important role in protecting oocytes against apoptosis [5].

Despite major advances in assisted reproductive techniques, poor oocyte quality continues to be a problem for infertility in women [6]. Reactive oxygen species (ROS) produced in healthy follicles during physiological processes are important for oocyte maturation. Despite their essential role, excessive ROS production can damage the follicle and negatively affect oocyte maturation [4]. It has been suggested that understanding how cumulus cells are affected by conditions that cause increased oxidative stress and the effect of this situation on oocyte quality may affect clinical practices during infertility management [7].

CAT, GST, and ARE enzymes are among the enzymatic antioxidants that are effective in defense against oxidative stress in organisms [8–10]. CAT is an enzyme that protects cells from the toxic effects of hydrogen peroxide (H_2O_2) [8]. GSTs are a family of enzymes that catalyze the conjugation of reduced glutathione (GSH) to a wide variety of substrates and generally cause detoxification

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[11]. Paraoxonase (PON) is an ester hydrolase with both paraoxonase and ARE activity [12]. Under oxidative stress, lipid peroxidation occurs not only in low-density lipoproteins (LDL) but also in high-density lipoproteins (HDL) [13]. It has been reported that paraoxonase1 (PON1) protects both LDL and HDL from oxidation [14] and is effective in embryogenesis [15].

The most common ROS in the organism is the lipid radical formed by the removal of a hydrogen from the allyl group of unsaturated fatty acids [16]. When reactive oxygen is in excess, it causes peroxidation of lipids in the membrane, causing impairment of permeability and thus intracellular ion imbalance [17]. Thiobarbituric acid reagents such as MDA are the most well-known aldehyde form of lipid peroxides and arise as a result of the oxygenation of arachidonic acid or non-enzymatic oxidative degradation of polyunsaturated fatty acids [18].

In line with this information, in our study, CAT, GST, ARE activities, and MDA levels were analyzed in cumulus cells of poor responder, normo responder and high responder patients under IVF treatment. It was aimed to investigate whether these parameters have a predictive value in terms of determining the count and quality of oocytes.

2. Material and methods

2.1. Patient selection

Ethical committee approval for this study was obtained by Gazi University Clinical Research Ethical Committee with the decision dated 23.12.2019 (Decision No:271).

102 female patients who received infertility treatment with IVF after examination and routine laboratory tests in the Gazi University, Faculty of Medicine, Department of Gynecology and Obstetrics IVF Center and private Nova Art IVF Center between December 2019 and December 2020 were included in the study. The causes of infertility of the patients included in the study were male factor, female factor (tubal factor, anovulation and endometriosis), unexplained infertility, or a combination of male and female factors. Patients under 23 and over 40 years of age and fertile patients with diabetes were not included in the study. CAT, GST, ARE enzyme activities, and MDA levels in cumulus cells were analyzed by spectrophotometric method in Gazi University, Faculty of Medicine, Department of Medical Biochemistry Research Laboratory.

Patient groups were classified as poor responder, normo responder, and high responder according to the count of oocytes taken on the day of OPU (oocyte retrieval). The 28 poor responder (oocyte count ≤ 4), 48 normo responder (5 ≤ oocyte count ≤ 14), and 26 high responders (oocyte count ≥ 15) groups of patientswere included in the study.

2.2. Sample collection

In antagonist cycles, after the ultrasonographic evaluation of the ovaries showed that ovarian cysts were not found, the treatment was initiated on the 3rd day of the cycle and serum progesterone levels were found to be < 1 ng/mL. The stimulation protocol included 150–300 IU/day of gonadotrophins, either recombinant (Gonal F, Merck Serono, Turkey or Puregon, Merck Sharp Dohme, Turkey) or in combination with hMG (Menogon, Erkim, Turkey, or Merional, IBSA, Turkey). When two or more follicles with a diameter of > 18mm and accompanying follicles were > 14 mm and there was an adequate E2 response, patients received human chorionic gonadotropin (hCG; Ovidrell 250, Merck Serono, Turkey). Oocytes were harvested 36 h after hCG injection. All patients in the study were treated with intracytoplasmic sperm injection (ICSI).

The collected cumulus-oocyte complexes were placed in dishes with culture medium (G-MOPS, VitroLife, Sweden); wherein the cumulus cells were mechanically dissected using two 26 G x 1/2 needles, one to hold the oocyte and the other to examine the cumulus cells surrounding the oocyte. Cumulus cells from each patient were collected in a single eppendorf tube. Each of the samples were washed twice with 0.5 mL of phosphate buffered saline (PBS) (Caprıcorn Scıentıfic, Bulbecco's PBS). It was placed in a 1 mL eppendorf tube, 0.5 mL of isotonic saline was added on it and the samples were kept at –80 °C until analysis.

2.3. Biochemical analysis

Cumulus cells were taken from –80 °C and left to dissolve on ice-water mix. Afterwards, the cumulus cells were homogenized (with Braun Melsungen homogenizator) for 3 min.

Analysis of oxidant/antioxidant parameters was performed manually without using commercial kits. CAT, GST, ARE enzyme activities and MDA levels were studied according to the methods of Aebi et al. [19], Habig et al. [20], Clement et al. [21], and Van Ye et al. [22] respectively. Shimadzu UV-1601 (Kyoto, Japan) spectrophotometer device was used in all analyzes. The results obtained in the enzyme activities determination are given as IU/L, and the results obtained in the MDA analysis are given as nmol/mL.

Determination of CAT enzyme activity: The absorbance decrease observed at 240 nm wavelength together with the degradation rate to H_2O and O_2 by CAT of H_2O_2 added to the experimental medium was measured spectrophotometrically. Phosphate buffer (50 mM, pH: 7) and 30% H_2O_2 were used as reagents.

Determination of GST enzyme activity: The GST activity method is based on measuring the absorbance changes at 340 nm due to the formation of the GSH-1-chloro 2,4-dinitrobenzene (GSH-CDNB) complex.

Phosphate buffer (100 mM, pH: 6.5) and CDNB (25 mM) and GSH (50 mM) were used as reagents.

Determination of ARE enzyme activity: phenylacetate was used as a substrate for ARE activity measurement and the ARE activity was determined by measuring the absorbance at 270 nm of the generated phenol. Tris-HCl buffer (100 mM, pH: 8), $CaCl_2$ (2 mM), and phenyl acetate solution (1 mM) were used as reagents.

Determination of MDA levels: 1,1,3,3,-tetraethoxy propane (TEP) was used as the MDA standard solution. A pink pigment is formed as a result of the reaction of MDA with thiobarbituric acid (TBA). This pigment, which is used to determine the MDA levels, gives a maximum absorbance at 532 nm.

2.4. Statistical analyses

SPSS v. 22.0 (SPSS Inc. Chicago, USA) program was used for statistical analysis of research data. Continuous variables are presented with mean ± standard deviation and median (min-max). The conformity of continuous variables to normal distribution was evaluated using visual (histogram and probability graphs) and analytical methods (Kolmogorov–Smirnov tests) and the data observed not to be distributed normally. The Mann–Whitney U test was used for the comparison analysis between two independent groups and the Kruskal–Wallis test (post hoc test: Bonferroni corrected Mann–Whitney U test, the significance level was used for this test $p < 0.017$) was used for the comparison analysis between the 3 groups in the data that did not conform to normal distribution. In this study, the statistical significance level was accepted as $p < 0.05$.

3. Results

It was observed that the average age decreased significantly in the normo responder and high responder groups compared to the poor responder group ($p < 0.001$, $p < 0.001$, respectively) (Table 1). There was no significant difference between the groups in terms of body mass index (BMI) levels ($p = 0.118$) (Table 1).

Cumulus cell GST enzyme activity levels were statistically significantly increased in the high responder group compared to the poor responder group ($p < 0.001$) and compared to the normo responder group ($p = 0.002$) (Table 2). Cumulus cell MDA levels were significantly decreased in the high responder group compared to the poor responder group ($p = 0.008$) (Table 2). Regarding the cumulus cell CAT and ARE enzyme activities, there was no statistically significant difference between the groups (respectively, $p = 0.175$, $p = 0.124$). (Table 2).

4. Discussion

It is suggested that oxidative stress and antioxidants in the female reproductive system may be associated with conditions that limit the success of assisted reproductive techniques. Although there is some evidence regarding the role of oxidative stress in the pathophysiology and treatment of infertility and IVF, the available data are conflicting [23,24]. ROS is produced during the normal physiological functions of the ovaries [25]. The low concentrations of ROS play a role in the acquisition of developmental competence of oocytes and in the regulation of pre-implantation embryo development rate. The deterioration of the balance between ROS and antioxidants in favor of ROS increase may result in increased oxidative damage and apoptosis in cells, causing impairment of cellular functions, pathological disorders in processes such as oocyte maturation, ovulation, fertilization, implantation and embryo development, and finally, formation of infertility and reduction of pregnancy outcome with IVF [23,25–28].

Table 1. The values of age and BMI of the groups.

	Poor responder	Normo responder	High responder	
	(n = 28)	(n = 48)	(n = 26)	P[1]
	mean ± SD	mean ± SD	mean ± SD	
	median (min-max)	median (min-max)	median (min-max)	
Age	37.5 ± 4.89	32.50 ± 6.21	29.58 ± 4.76	< 0.001[2]
	39.5 (23–40)	33.5 (23–40)	29 (23–40)	
BMI	26.22 ± 5.06	23.50 ± 3.70	24.47 ± 4.97	0.118
	25.5 (17–34)	23 (19–36)	24 (19–36)	

BMI: body mass index.
[1]Kruskal–Wallis test.
[2]Bonferronni corrected Mann–Whitney U test: poor responder vs. normo responder $p < 0.001$; poor responder vs. high responder $p < 0.001$; for age.

Table 2. The values of CAT, GST, ARE enzyme activities and MDA levels of the groups.

	Poor responder	Normo responder	High responder	
	(n = 28)	(n = 48)	(n = 26)	P[1]
	mean ± SD	mean ± SD	mean ± SD	
	median (min-max)	median (min-max)	median (min-max)	
CAT (IU/L)	162.61 ± 23.80	80.73 ± 16.48	63.79 ± 17.65	0.175
	51.24 (7.32–680.76)	21.96 (7.32–897.00)	25.62 (7.32–322.08)	
GST (IU/L)	0.13 ± 0.09	0.15 ± 0.09	0.22 ± 0.12	< 0.001[3]
	0.11 (0.01–0.42)	0.13 (0.01–0.47)	0.21 (0.03–0.64)	
ARE (IU/L)	0.06 ± 0.04	0.05 ± 0.04	0.07 ± 0.05	0.124
	0.04 (0.00–0.32)	0.04 (0.01–0.20)	0.07 (0.01–0.19)	
MDA (nmol/mL)	2.56 ± 0.18	2.38 ± 0.38	2.27 ± 0.41	0.023[4]
	2.55 (2.28–3.01)	2.43 (1.38–3.06)	2.38 (1.33–2.67)	

CAT: catalase; GST: glutathione-S-transferase; ARE: arylesterase; MDA: malondialdehyde.
[1]Kruskal–Wallis test.
[3]Bonferronni corrected Mann–Whitney U test: poor responder vs. high responder $p < 0.001$; normo responder vs. high responder $p = 0.002$; for GST.
[4]Bonferronni corrected Mann–Whitney U test: poor responder vs. high responder $p = 0.008$; for MDA.

It has been suggested that oxidative stress is one of the main causes of age-related cellular and molecular damage [29]. The balance between reactive oxygen species and antioxidants in young and healthy individuals deteriorates in favor of ROS due to the dysfunction of antioxidants with aging [30]. The accumulation of damage caused by ROS produced by mitochondria during daily biological metabolism is one of the mechanisms behind the age-related decline in oocyte quality [31]. In our study, in accordance with the literature, age was found to be significantly lower in the normo responder and high responder groups compared to the poor responder group.

In organisms, superoxide dismutase (SOD), one of the enzymes that help cells to repair the damage caused by ROS, acts as an antioxidant by removing the superoxide anion in the dismutation reaction and creating H_2O_2 and molecular oxygen. Further detoxification to H_2O and O_2 is catalyzed by both CAT and glutathione peroxidase to remove H_2O_2 [28,32,33]. CAT inhibition may cause chromosome defects in the oocyte nucleus, such as chromosome misalignment and deoxyribonucleic acid (DNA) damage [25]. In the study of Carbone et al. [29], CAT levels were found to be lower in the follicular fluid of elderly women compared to young women. In the study of Nuñez-Calonge et al. [34], CAT mRNAs were found to be higher in the cumulus cells of normo responder women compared to poor responder women. On the other hand, it is suggested that serum follicle-stimulating hormone (FSH) levels that gradually increase with aging increase CAT activity [25,30]. It was observed that CAT activity increased after FSH stimulation and this increase was higher in granulosa cells of large follicles compared to medium and small follicles [35]. In our study, age significantly decreased in the normo responder and high responder groups compared to the poor responder group, and parallel to this, a non-significant statistically decrease in CAT activity was observed in the cumulus cells of the other groups compared to the poor responder group. The higher CAT activity in the poor responder group may be attributed to the increase in oxidative stress exposure and the need for antioxidant levels as the age increases, depending on ROS produced during metabolism, lifestyle and environmental conditions. At the same time, as it is known that the increase in FSH with the advancement of age increases CAT activity, FSH is thought to be a factor for increased CAT activity in the poor responder group depending our study finding.

GSTs consist of a large family of enzymes that protect cells from oxidative damage, lipid peroxidation of cell membranes, and toxic compounds [4]. In addition to the functions of GSTs such as peroxidase, isomerase and catalyzing the conjugation of genotoxins to GSH, they also have effects on protecting cells against H_2O_2-induced cell death by inhibiting Jun N-terminal kinase, which plays a role in cellular apoptosis pathway [30]. Although it is suggested that GST activities are affected by age and there is a decrease in GST activities with increasing age, the results are contradictory. The GST alpha isoenzyme in

the follicular fluid of young and older women receiving IVF treatment did not differ in the two groups. GST Pi isoenzyme has been found to be higher in young women compared to older women [29]. In the study of Meijide et al. [36], it was shown that GST activity in both donors and patients with fertility problems was significantly lower in mature oocytes compared to small oocytes. In our study, while age decreased in the high responder group compared to the other groups, GST activity increased significantly. This increase suggests that GST activity may have a protective role for the apoptosis.

In the IVF, antioxidant levels of follicular fluid decrease and lipid peroxidation levels increase [14]. It is suggested that lipid peroxidation is strongly associated with oocyte fertilization and pregnancy rates after IVF [37]. The fact that oocytes have a lipid-rich membrane increases the risk of lipid peroxidation and exposure to the harmful effects of ROS [38]. MDA is a byproduct of lipid peroxide decomposition used to track the degree of peroxidative damage in cells [38]. In the study of Kumar et al. [39], MDA levels in follicular fluid were found to be significantly higher in women with negative IVF results compared to women with positive IVF results. In the study of Karakaya et al. [40], MDA levels were found to be higher in the poor responder group compared with the normo responder and high responder groups in the follicular fluid of patients who underwent controlled ovarian stimulation. In this study, in parallel with these studies, MDA levels in cumulus cells increased in the poor responder group compared to the other groups. This situation may bereleated to the fact that cells become more prone to lipid peroxidation with increasing age.

PON1 is an HDL-dependent antioxidant enzyme with paraoxonase and ARE activity, which is found in serum and follicular fluid and protects the cell membrane and circulating lipids against oxidative damage [14,15]. Repeated exposure to high ROS produced by folliculogenesis during repetitive ovulation cycles may induce oxidative stress and may also cause a decrease in oocyte quality, implantation frequency and pregnancy rates [31]. Nuñez-Calonge et al. [34] found a correlation between the expression of genes that regulate oxidative stress and the response to apoptosis and ovarian stimulation; in this case, they noted that an increase in ovarian oxidative stress could reduce the count of oocytes obtained after ovarian stimulation. In the study of Okyay et al. [14], serum PON1, and ARE activities were found significantly lower on the day of OPU compared to before ovarian stimulation, while lipid hydroperoxide (LOOH) levels were found to be significantly higher. Besides, Angelucci et al. [41] described PON1 with relatively high amounts of antioxidant activity in the follicular fluid of normovulatory women. They noted that these enzymes probably help to protect the follicle from oxidative stress during maturation [41]. In a study conducted with subfertile female patients and fertile women (oocyte donors), follicular fluid PON1 paraoxonase and ARE activity showed a positive correlation with the count of oocytes taken from donors [42]. In our study, an increase in arylesterase activity was observed in the cumulus cells of the high responder group compared to the poor and normo responder groups, but this increase was not statistically significant. However, in our study, the significant decrease in MDA levels in the cumulus cells of the high responder group compared to the poor responder group suggests that the ARE activity may be effective in reducing lipid peroxidation and this situation affects the count and quality of oocytes obtained. In addition, the low number of patients may have been effective in the lack of statistical significance.

5. Conclusion

In our current study, although a decrease in CAT enzyme activity was observed in cumulus cells in the high responder group compared to other groups, we think that the increase in GST and ARE enzyme activities was effective in the decrease observed in MDA levels and protection against oxidative stress. In the high responder group, an increase in response to ovarian stimulation was observed with a decrease in age compared to the other groups. We suggest that the effects of oxidant/antioxidant parameters in the response of IVF patients to ovarian stimulation should be supported with larger patient groups in terms of predictive values.

Conflict of interest

The authors declare no conflict of interest.

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