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Research Article

A histological and biochemical study of cumulus cells and the oocyte microenviroment in *in vitro* fertilization patients

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Abstract

Objectives: The aim of this study was to investigate the effect of chemical changes in the follicular fluid and histological changes in the cumulus cells of the oocyte microenvironment on the number of oocytes in infertile patients.

Methods: A total of 50 female patients aged 18-35 who presented at the Atatürk University Research Hospital Infertility Clinic and for infertility treatment were included. The patients were divided into 3 groups: Patients with fewer than 5 oocytes were classified as Group 1, patients with 5-20 oocytes comprised Group 2, and Group 3 was made up of patients with >20 oocytes. During the oocyte collection process, follicular fluid was aspirated from the follicles and the cumulus cells were collected. The follicular fluid was stored at -80°C for use in biochemical analysis of malondialdehyde (MDA), total antioxidant status (TAS), total oxidant status (TOS), superoxide dismutase (SOD), glutathione (GSH). Immunohistochemical staining was performed to examine caspase-3 and mechanistic target of rapamycin (mTOR) immunoreactivity at the stereological level.

Results: The MDA level and total oxidant capacity (TOC) in the follicular fluid were higher in Group 1 patients than in the other 2 groups, while the SOD was lower ($p<0.05$). In Group 2 patients, the MDA level and TOS were higher than those of Group 3, while the SOD level was lower ($p<0.05$). The total antioxidant capacity (TAC) and GSH levels did not vary significantly according to the number of oocytes ($p<0.05$). Immunohistochemical staining showed that mTOR and caspase-3 immunoreactivity were more intense in Group 1 than in the other groups.

Conclusion: The increase in mTOR expression may activate the caspase-3 pathway, which could lead to oxidative stress. The mTOR pathway may affect the oocyte count.

Keywords: Caspase-3, cumulus cell, follicular fluid, *in vitro* fertilization, mTOR

Infertility is a serious and important health problem that can cause psychological and social problems, and affects approximately 15% of couples around the world [1]. A female factor is responsible in approximately 50% of infertility cases, while 40% have a male factor cause. The rate of unexplained infertility is 10-15% [2]. An increase in the infertility rate has led to many changes in this field in recent years. One is the development of assisted reproductive techniques [3]. Simple and inexpensive techniques are now available to troubleshoot and diagnose problems in infertile couples, as well as more complex methods used at later points [4]. As in all biological processes, the chemical structure of the follicular fluid determines physiological character as well. Follicular fluid and cumulus cells have important roles in follicle development and oocyte maturation. Follicular fluid is a product of both the transfer of blood plasma constituents that cross the blood follicular barrier and of the secretory activity of oocyte and ganulosa cells [5]. Follicular wall permeability and the secretion capacity of cumulus cells lead to biochemical variability in the follicular fluid environment. This variability is closely related to steroid synthesis and oocyte de-

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velopment. The chemical content of the fluid includes sugars, hormones, proteins, reactive oxygen species (ROS), and antioxidants. Research of ROS has gained importance in recent years; however, studies thus far have more commonly examined ROS in males than females. The impact of ROS on female infertility is still a matter of debate. A balance between antioxidants and oxidants is necessary for cells to survive [6]. The information about the effect and precise mechanisms with respect to oocytes, embryo development, and pregnancy is still insufficient. Another important factor in the oocyte periphery is the cumulus oophorus complex cells, which are separated from other granulosa cells during luteinizing hormone surges in the ovulation phase. These cells are important to folliculogenesis, oocyte nucleation, and cytoplasm maturation [7]. Cumulus cells provide support and nutrients for oocytes, such as adenosine triphosphate (ATP) [8]. During oocyte maturation, both proliferation and cell death occur in the cumulus cells [9, 10]. There are arguments that this process affects many stages of oocyte maturation, embryo formation, and pregnancy. Autophagia in cumulus cells and apoptosis are considered the underlying mechanism of follicular atresia. The role of the mechanistic target of rapamycin (mTOR) remains unclear in the regulation of autophagy [11]. mTOR is a serine-threonine kinase enzyme with a molecular weight of 290 kDa [12]. This enzyme plays a crucial role in many tasks for the cell, such as translation and transcription regulation, glucose metabolism, actin cell organization, ribosome biogenesis, autophagy, stress response, and modulation of immune system cell activity. Studies have shown that mTOR is sensitive to follicle-stimulating hormone (FSH) and affects granulosa cell proliferation. The mTOR pathway can be expressed cytoplasmically in the mitotic filaments of the metaphase stage or in the contraction site close to this region during cytokinesis. This settlement emphasizes the importance of mTOR during division [13, 14]. The aim of this study was to investigate the relationship between the oxidative-antioxidant change in the follicular fluid content of infertile female patients referred to *in vitro* fertilization (IVF) centers, and the relationship between apoptosis and mTOR immunoreactivity in cumulus cells. The relationship between the number of oocytes in cumulus cells and follicular fluid content, apoptosis, and mTOR immunoreactivity was also examined.

Materials and Methods

Patient selection

This study included female patients aged 18-35 years who presented at the IVF Center of Ataturk University Research Hospital in 2014 (January-December). A total of 50 female-factor infertility patients were included following evaluation of patient etiology. The patients included in the study were not using any drug therapy. The patients were divided into 3 groups according to the number of oocytes determined: Group 1 comprised patients with fewer than 5 oocytes, members of Group 2 had 5-20 oocytes, and patients with >20 oocytes were categorized in Group 3. Ethical approval for this research was granted by the Ataturk University Faculty of Medicine Clinical Research Ethics Committee (15.08.2017\7).

Sample collection and processing

Follicular aspiration was performed with a 10-mL plastic injector and an 18-gauge needle. Follicular fluid was drawn into a centrifuge tube. The selection of oocytes was performed using the method described by Brackett and Zuelke [15]. Oocytes were separated from the aspirated follicular fluid, the samples were centrifuged at 3000 g for 10 minutes, the supernatants were removed and placed into Eppendorf tubes (Isolab GmbH, Eschau, Germany), and the samples were stored at -80°C. The oocytes were then separated from the cumulus cells using thin-tip pipettes. The collected cumulus cells were stored at -80° in 3-(N-morpholino)propanesulfonic acid (MOPS) buffered medium (Vitrolife Sweden AB, Goteborg, Sweden).

Biochemical analysis

Measurement of oxidative stress markers of follicular fluid was performed in the Ataturk University Veterinary Faculty Biochemistry Department. Malondialdehyde (MDA), total antioxidant status (TAS), total oxidant status (TOS), superoxide dismutase (SOD), glutathione (GSH) were measured using methods described in the literature. All of the measurements were performed at room temperature [16].

Lipid peroxidation

To analyze lipid peroxidation (LPO), 1000 µL of homogenate was placed in a test tube, followed by 150 µL of purified water, 100 µL of 8% sodium dodecyl sulfate, 750 µL of 0.08% thiobarbituric acid, and 750 µL of 20% acetic acid and then vortexed. After allowing the homogenates to incubate at 100 °C for 60 minutes, 2.5 mL of n-butanol was added, and the spectrophotometric measurement was performed. The amount of red coloration at the end of the reaction was determined using 3-mL cuvettes at 535 nm, and the quantity of MDA in the samples was measured using the dilution coefficients and the standard plot of MDA. The quantity of LPO was described in µL. The measurement of each sample was repeated 3 times.

Total oxidant capacity

TOC measurement was based on spectrophotometer evaluation using the ferric thiocyanate method with xylenol orange. Iron ions take advantage of oxidative agents to oxidize ferric ions in an acidic environment. These ions are detected by the xylenol orange, which provides indirect measurement through peroxide content.

Total antioxidant capacity

TAC in the follicular fluid was measured using a total antioxidant status assay spectrophotometer. Tubes were filled with 3

mL of standard/serum/follicular fluid samples and then centrifuged with the subsequent supernatant diluted at a 1:10 ratio with sample buffer (reagent 1). After an initial measurement at 750 nm, it was stained by adding 150 µL of 2.2'-azino-di-(3-ethylbenzthiazoline sulphonate) radical solution (reagent 2). After incubation for 10 minutes, a second measurement was performed at 750 nm absorbance.

Superoxide dismutase

First, the follicular fluid was centrifuged. A 2450-µL mixture of 0.4 M sodium carbonate, 0.3 mM xanthine, 150 µM nitroblue tetrazolium, 0.6 mM ethylenediaminetetraacetic acid (EDTA), 1.2 g/L bovine serum albumin, 500 [mu] l of follicular fluid, and 50 [mu] l of xanthine oxidase sample was added to the spectrophotometer bath. The reaction was then terminated by the addition of a 1000 µL copper(II) chloride inhibitor solution. The amount of formazan in the reaction medium was read out at 440-460 nm using 3-mL quartz cuvettes. The SOD activity was described as mmol/minute/mg liquid. Each sample was measured 3 times.

Glutathione

Samples were centrifuged at 3000 g for 10 minutes, and the level of GSH in the supernatant was measured. First, 1500 µL of measuring solution (0.2 mM EDTA, 200 mM tris hydrochloride, pH=8) was added to the test tubes followed by 500 µL supernatant, 100 µL 5.5'-dithio-bis (2-nitrobenzoic acid), and 7900 [mu] l of methanol, and the sample vortexed by pipetting. Incubation was performed for 30 minutes at 37°C and then measurements were taken by spectrophotometer. The quantity of yellow coloring after the reaction was read at 412 nm through 3-mL quartz cuvettes and the GSH was measured using the dilution coefficients as a basis and the standard graphic of the GSH stock mixture. The GSH level of the samples was described as µmol/L. Each sample was measured 3 times.

Immunohistochemistry

Cumulus cells obtained from the COC collected from patients after oocyte aspiration were used for immunohistochemical analysis [17]. Cumulus cells were removed from the COC after incubation for 2-3 hours following oocyte retrieval. Cumulus cells were collected in medium (G-MOPS, Vitrolife Sweden AB, Goteborg, Sweden) during the procedure. Next, medium (G-IVF PLUS, Vitrolife Sweden AB, Goteborg, Sweden) was added and disintegrated with an insulin injector. The supernatant was then removed by centrifugation at 2000 rpm for 5 minutes and the cells in the pellet were dried by spreading them on the coverslip with a pipette tip.

Smear preparations were stained with indirect immunohistochemistry to examine the caspase-3 and mTOR proteins in the cumulus cells at the light microscopic level.

Cell preparation for immunocytochemistry

The samples were kept in a mixture of acetone and absolute alcohol at -20 C° for 30 minutes and washed 3 times for 5 minutes with phosphate buffered saline (PBS). The slides were then treated with Triton X for 10 minutes. The slides were washed with PBS 3 times for 5 minutes. Next, 3% hydrogen peroxide (Hydrogen Peroxide Assay Kit ab102500; Abcam plc, Cambridge, England) drops were placed on the samples and allowed to stand for 20 minutes. The slides were washed again with PBS 3 times for 5 minutes. The primary antibodies (Anti-Cleaved Caspase-3 antibody ab2302; Anti-mTOR antibody ab2732; Abcam plc, Cambridge, England) were added at a dilution of 1/100, and the suspension was allowed to stand for 1 hour. The slides were washed with PBS 3 times for 5 minutes. Subsequently, drops of biotinylated secondary antibodies (ab1227; Abcam plc, Cambridge, England) were added to the preparation and it was incubated again for 30 minutes at room temperature. The slides were washed once again with PBS 3 times for 5 minutes. 3.3'-diaminobenzidine solution (DAB substrate kit ab64238; Abcam plc, Cambridge, England) was added and it was left for about 5 minutes. Once a brown color was observed, the slides were washed under running water. Hematoxylin treatment of 45 seconds was followed by another 5-minute wash with pure water. The sections were passed through an alcohol and xylol series. The painted slides were then examined under a light microscope (DM6200; Leica Camera AG, Wetzlar, Germany) and photographed with an Olympus DP20 camera (Olympus Corp., Tokyo, Japan). Apoptosis criteria (condensation, half-moon view, fragmentation) were evaluated under the microscope. The mTOR criteria were evaluated according to cytoplasmic staining. A primary antibody was not used in the tissues prepared for negative control, but the other steps were the same. In terms of cell immunoreactivity, at least 3 non-overlapping areas were evaluated in photographs taken from an average of 3 histological sections randomly selected from each group: 0 score for no positive cells, 1 if the ratio of positive stained cells was <1/100, 2 if between 1/100 and 1/10, 3 if between 1/10 and 1/3, 4 if between 1/3 and 2/3, and 5 if > 2/3. A score was then calculated expressing the average density of positive cells: a value of 0 when there was no staining, 1+ for weak staining, 2+ for moderate staining, and 3+ in the presence of significant staining. After the distribution and density scores were added, a total score between 0 and 8 was obtained. Staining values between 0 and 2 were judged negative and those between 3 and 8 were evaluated as positive.

Statistical analysis

Normal distribution of the data was assessed using the Shapiro-Wilk test. To assess the relationship between the oxidative stress parameters and the number of eggs in the follicular fluid, data with a normal distribution were evaluated using analysis of variance and non-normally distributed data were evaluated with the Kruskal-Wallis test. The statistical analysis of all immunohistochemical parameters was performed using

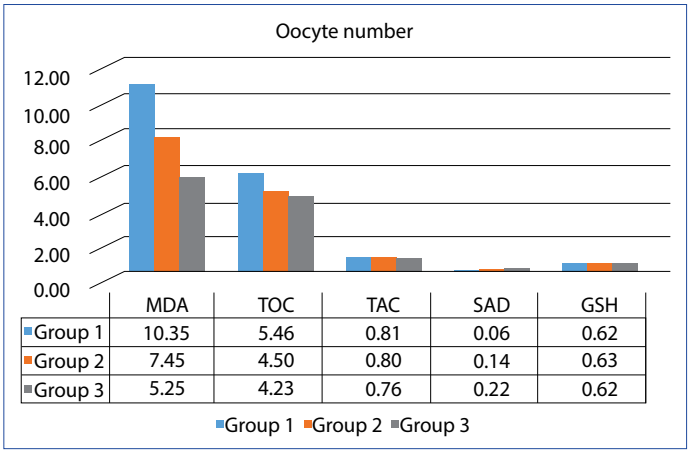

Figure 1. Graph of the results of follicular fluid biochemical analysis in all groups. Group 1: oocyte count <5, Group 2: oocyte count 5-20, Group 3: oocyte count >20.

GSH: Glutathione; MDA: Malondialdehyde; SOD: Superoxide dismutase; TAC: Total antioxidant capacity; TOC: Total oxidant capacity.

one-way analysis of variance followed by Duncan's multiple range test. IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA) was used to analyze the findings and the results were expressed as mean, SD, median, minimum, and maximum. The statistical significance level applied was $p<0.05$.

Results

Biochemistry

MDA and TOC values differed in Group 1, Group 2, and Group 3, as shown in Figure 1. Group 1 patient results were statistically significantly higher than the other 2 groups ($p<0.05$). The MDA and TOC levels of Group 2 were statistically greater than those of Group 3 ($p<0.05$). When TAC levels were compared, no significant difference was observed between the groups ($p>0.05$). The level of SOD, an antioxidant enzyme, was different between the groups: SOD activity in Group 1 was significantly lower than that seen in the other 2 groups and the Group 2 SOD level was lower than that of Group 3 ($p<0.05$) (Fig. 1). The GSH level demonstrated no significant difference between groups ($p>0.05$).

Immunohistochemistry

Caspase-3, one of the final mediators of apoptosis, was immunohistochemically stained in the cumulus cells. Cells with active caspase-3 reactivity were stained brown with DAB chromogen. More immune cell reactivity was observed in Group 1 patients than in the other groups ($p<0.05$; Fig. 2). There was also a significant difference between Group 2 and Group 3 ($p<0.05$; Fig. 2).

mTOR immunoreactivity examination revealed differences between the groups. Immunopositivity was more prevalent in Group 1 patients. ($p<0.05$; Fig. 3). There was also a significant difference between Group 2 and Group 3 ($p<0.05$). Immunohistochemical staining of Caspase-3 (Fig. 4) and mTOR (Fig. 5) in cumulus cells is shown.

Discussion

An increase in the number of couples with fertility problems has led to greater curiosity about reproduction and IVF treatment [18]. In recent years, biomolecules in the follicle fluid that could predict IVF success have been a subject of research. The

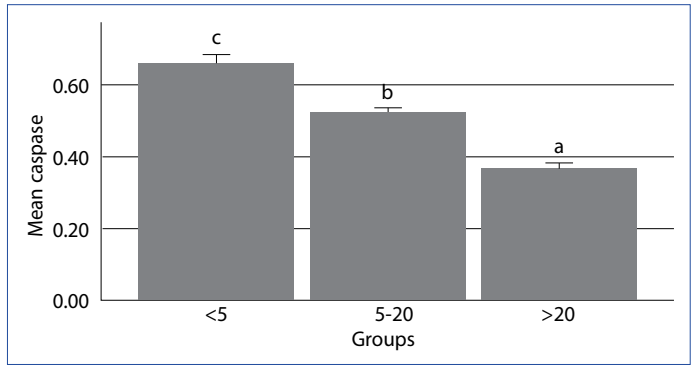

Figure 2. Immunohistochemical staining of caspase-3 in cumulus cells. Data are presented as mean±SD.

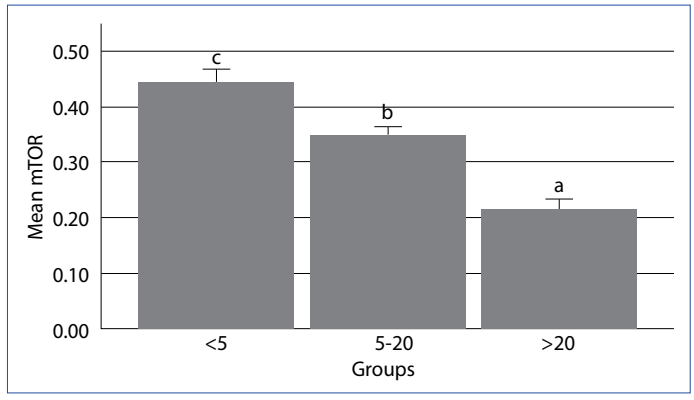

Figure 3. Immunohistochemical staining of mTOR in cumulus cells. Data are presented as mean±SD.

mTOR: Mechanistic target of rapamycin.

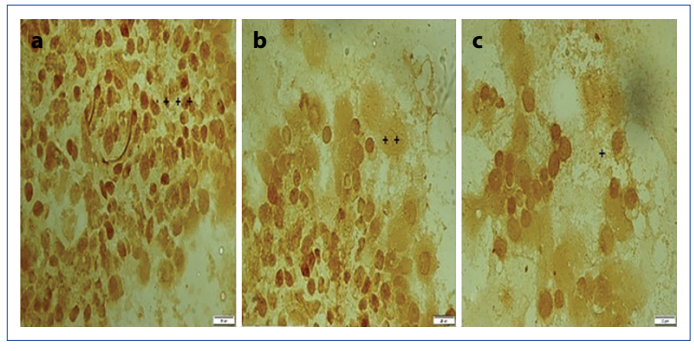

Figure 4. Caspase-3 immunoreactivity seen in cumulus cells in the smear preparation. (a) Group 1, (b) Group 2, (c) Group 3 (x40, Bar 20µm).

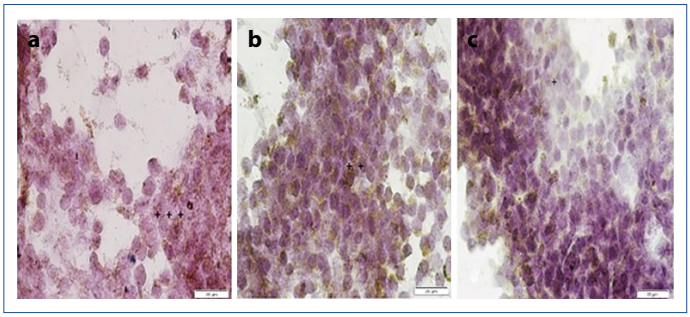

Figure 5. mTOR immunoreactivity observed in the cumulus cells in the smear preparation. (a) Group 1, (b) Group 2, (c) Group 3 (x40, Bar 20µm).

ability to recognize these biomolecules and understand the correlations between their functions are relevant not only to the quality of the embryo and the oocyte but also to the development of new fertility methods and estimating the reproductive success. Modifying the specific quantity of molecules in the follicular fluid can affect oocyte development, number, myotic maturation, fertilization, embryo quality, and the potential for pregnancy [19, 20].

The biochemical composition of follicle fluid includes proteins, sugars, ROS, antioxidants, and hormones. In recent years, the relationship between infertility and ROS and antioxidants has drawn substantial attention. Oxidative stress has been associated with embryo development, mitochondrial changes, decreased MDA level, and blastomere count and there is great interest in the relationship between oxidant-antioxidant parameters such as MDA, TOC, SOD, GSH, TAC, and IVF results [21].

In this study, the oxidant-antioxidant levels in follicular fluid of patients treated with intracytoplasmic sperm injection were evaluated biochemically and the caspase-3 and mTOR pathway in cumulus cells were examined immunohistochemically. Unlike many previous studies, this research examined the number of oocytes in patients aged 35 and younger.

LPO is one of the most important oxidative stress markers. MDA, a marker of LPO, can damage the integrity of the cell. MDA is an easily identifiable and widely used parameter for measuring the level of peroxidation [22].

Previous research has shown that the MDA level in serum and follicular fluid is significantly higher in patients with endometriosis compared with patients with male infertility [23]. Another study demonstrated that there was no statistically significant relationship between MDA level in follicular fluid and pregnancy rates [24]. Other reported findings include an observation that the MDA level of patients with endometriosis was higher than that of patients with unexplained infertility [25]. Pasqualotto et al. [26] did not find a correlation between MDA level and embryo quality and pregnancy. In a study conducted by Karakaya et al. [27], they found that the MDA level among patients older than 35 years of age in those with an oocyte count of <5 compared with normal (5-20) and higher (>20) count groups. In our study, Group 1 patients with a poor overian-reserve had a higher MDA level than Group 2 and Group 3 ($p<0.05$). The MDA level of Group 2 was significantly higher than that of Group 3 ($p<0.05$).

Our results suggest that high LPO in the follicle fluid may have an effect on the oocyte count. The increase in ROS in the cell is based on an increased mitochondria requirement for ATP, which may increase MDA. ROS imbalance may be a result of decreased folliculogenesis, rather than a physiological effect. Similar logic may explain our finding of a reduced number of oocytes and a high MDA level.

TOC and TAC measurements may include oxidant and antioxidant species that are not completely known. TOC and TAC analysis can be useful to assess individual antioxidant and oxidant enzymes [28].

In particular, the ratio of TOC to TAC may play a role in determining oxidative stress. TAC influences the protection of cellular components [29]. It has been reported in the literature that TAC was associated with endometriosis in patients with unexplained infertility and tubal factors [30]. Oyawoye et al. [31] found that TAC was decreased when oocytes were fertilized, but without statistical significance in comparison with viable embryos. A low TAC appears to be an indicator of low fertilization potential.

Our results yielded no statistical significance between TAC measured in follicular fluid and oocyte count ($p>0.05$). While the TOC was observed to be higher in Group 1 ($p<0.05$), there was no statistical significance between the other 2 groups ($p>0.05$). This result suggests that the increased TOC may be related to an increased ROS level.

Ovarian aging is thought to be associated with increased oxidative stress. Antioxidants in the follicle fluid eliminate ROS to protect the oocyte and embryonic oxidative balance [32, 33]. SOD plays a protective role for the cell by suppressing hydrogen peroxide. The SOD enzyme has been shown to be important for germinal vesicle oocytes and metaphase II oocytes at the stage of oocyte maturation. It has been demonstrated in animal studies that increased SOD may inhibit HCG-induced ovulation and that overexpression may be associated with follicular atresia [34, 35]. The SOD level of patients with unexplained infertility was higher than that of infertile male patients. Similarly, the SOD level of patients with polycystic ovary syndrome (PCOS) and endometriosis was higher than that of controls [16]. Sabatini et al. [36] reported that they observed positive correlations between SOD activity and oocyte fertilization capacity. Carbone et al. [32] found that the SOD level was higher in older women. Karakaya et al. [27] noted that the SOD level was normally low in patients with overian-reserve. Pekel et al. [16] reported that the SOD level was higher in infertile female patients than in controls. A decrease in GSH and an increase in SOD have also been seen in older patients [31].

Our results were consistent with the literature [27]. The SOD activity of Group 1 was found to be statistically lower than the other 2 groups ($p<0.05$) and the SOD value in Group 2 was also significantly lower than that of Group 3 ($p<0.05$). This is

thought to be due to the decrease in the amount of SOD with increased LPO.

Antioxidants in the follicle fluid balance their oxidants and protect the oocytes. GSH is an endogenous source of antioxidants. The GSH antioxidant is an important scavenger that contributes to oocyte cytoplasmic maturation and culture media for embryo formation in the dissolution of disulfide bonds during localization of the pronucleus in the embryo [37].

There is little work in the literature related to GSH and IVF. Ebisch et al. [38] found no correlation between GSH and follicle size. In another study, there was no difference in GSH levels of pregnant and non-pregnant women [18]. When we compared our study groups, there was no statistically significant relationship between follicular fluid GSH level and the number of oocytes.

Cumulus cells, which have bidirectional paracrine-type nexus connections to oocytes, also have a major effect on oocyte development [39]. The COC plays a critical role in oocyte development. Cumulus cells provide oocytes with nutrients for ATP synthesis. Therefore, cumulus cells are an important factor in mitochondrial changes. Protection against apoptosis in cumulus cells induced by oxidative stress may have a major therapeutic effect in the treatment of reproductive aging [40]. No detailed method has yet been found to assess oocyte quality; generally, evaluations are morphological. However, morphology is not sufficient, and it has been proposed that the proportion of cumulus cell apoptosis could be a potential marker in determining IVF success. It has also been suggested that the rate of apoptosis and cleavage of cumulus cells may be important in predicting oocyte quality, number, and pregnancy rates [41, 42].

Apoptosis is one of the basic mechanisms of regression after follicular atresia and ovulation in mammals. Apoptosis causes fragmentation in the embryo and limits potential implantation, which leads to poor fertilization results [43, 44]. Studies have reported that the amount of apoptosis in cumulus cells may affect IVF outcomes [4, 45]. It has also been noted that the rate of apoptosis in the cumulus cells of fertilized patients was lower than those without fertilization. No correlation between embryo quality and zona pellucida thickness and apoptosis of cumulus cells has been seen. An age-matched study showed an increase in apoptotic cell density in patients 35 years and older [46]. Lee et al. [10] reported that apoptosis in granulosa cells was useful in understanding overian-reserve. It was observed that the rate of apoptosis was greater in patients over 40 years of age. In our study, the caspase-3 concentration in cumulus cells was investigated using indirect immunohistochemical methods. Apoptotic immunoreactivity was found to be greater in Group 1 than in the other groups. Similarly, more intense reactivity was observed in Group 2 staining than in Group 3. These results suggest that the cause of apoptosis in Group 1 may be increased MDA. However, further studies are needed for additional clarification.

The role of mTOR in cumulus cell apoptosis remains uncertain. mTOR affects energy balance, oocyte development, mitosis, and meiosis [47]. mTOR influences oocyte development by playing an active role in the proliferation of cumulus cells. mTOR is necessary to maintain oocyte development competence. mTOR cumulus cells proliferate and serve to protect the integrity of the transcription of cumulus cells [48]. m-TOR plays an important role in the coordinated functioning of oocyte and cumulus cells [49]. Previous immunohistochemistry studies of dehydroepiandrosterone-treated PCOS mice have revealed mTOR in granulosa cells and found greater positive cell density in patients with PCOS. This suggests that mTOR may be responsible for increased granulosa cells [12]. In another study, apoptosis and ROS were found to be low in patients treated with rapamycin [50]. Rapamycin contributes to blastocyst recovery, and mTOR and ROS have been shown to be related to aging. Rapamycin therapy reduces protein synthesis of mTOR. Rapamycin modulates mTOR by reducing reactive oxygen and apoptosis [48, 50].

In our study, mTOR immunoreactivity was more concentrated in the smears from Group 1 patients. When the other groups were compared, it was seen that Group 2 staining was more intense than that of Group 3.

In conclusion, our microscopic findings indicated that apoptosis and mTOR expression in cumulus cells may be related. We observed that mTOR contributed to an increase in caspase-3 activity through oxidative stress. Our results can be used as a marker to predict outcome parameters in IVF clinics. In IVF cycles, the number and quality of oocytes does not depend on apoptosis alone, or on oxidative stress alone. To further evaluate the relationship between these criteria, additional studies with large and homogeneous patient groups are needed, as well as research using other standard methods. Finally, we add that the high cost of infertility treatment promotes the use of antioxidants and mTOR inhibitors in preventive therapy.

Conflict of Interest: The authors do not have any conflict of interest in the manuscript.

Ethics Committee Approval: The study was approved by the Ataturk University Faculty of Medicine Clinical Research Ethics Committee (approval number and date: 7-15.08.2017).

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Authorship Contributions: Concept – N.E.; Design – N.E., T.D., O.O.A.; Supervision – N.E., T.D.; Funding – N.E., K.K., M.H.; Materials – N.E., T.D.; Data collection &/or processing – N.E., T.D.; Analysis and/or interpretation – K.K., M.H., N.E., T.D., O.O.A.; Literature search – N.E., T.D.; Writing – N.E., T.D.; Critical review – N.E., O.O.A., K.K., M.H., T.D.

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