Evaluation of the follicular fluid thiol/disulfide balance among patients with poor ovarian response

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Objective: This study aimed to compare the thiol/disulfide balance, myeloperoxidase, and ischemia-modified albumin levels in the follicular fluid (FF) of poor ovarian response (POR) and normal ovarian response (NOR) women who received intracytoplasmic sperm injection (ICSI).

Methods: The study was performed between March 2021 and April 2022 at the Department of Obstetrics and Gynecology, Center for Reproductive Medicine, Ankara City Hospital. The study included 27 POR and 35 NOR women who underwent ICSI. FF was obtained after the controlled ovarian stimulation cycle. The FF thiol/disulfide balance was detected using spectrophotometric methods. A correlation analysis was conducted to determine whether these oxidative stress markers could contribute to predicting oocyte quality.

Results: Disulfide levels were significantly higher in the NOR group than in the POR group (p=0.014). The number of fertilized egg (2PN) oocytes was positively correlated with the total thiol level (r=0.258, p=0.046). The disulfide level was positively correlated with the anti-Müllerian hormone level (r=0.262, p=0.039) and the total number of retrieved oocytes (r=0.335, p=0.008).

Conclusion: The disulfide levels differed significantly between the NOR and POR groups. The statistically significant differences of fewer metaphase II oocytes and lower percentage of good-quality embryos in the NOR group compared to the POR group might have resulted from the NOR group’s elevated disulfide levels. The total thiol levels correlated with the total of 2PN oocytes. Future studies should examine the thiol/disulfide balance at assisted reproductive technology centers to predict which oocytes could be fertilized.

Keywords: Ischemia-modified albumin; Low ovarian response; Myeloperoxidase; Oocyte quality; Oxidative stress; Thiol/disulfide

Introduction

Although there is no obligation to select oocytes during the classical in vitro fertilization (IVF) process, it may become mandatory to select the best oocyte due to production limitations on excessive numbers of embryos, increased demand for oocyte freezing, and legal restrictions and religious concerns in some countries. Although oocyte morphology has been evaluated frequently in the past, it may not be correct to decide on insemination based on oocyte morphology alone [1]. Examining gene expression levels in granulosa cells and conducting a polar body biopsy have also been used for oocyte selection. However, these techniques are expensive and not easy to implement in clinical practice because they require extensive laboratory equipment.

Oocytes reside in the follicular fluid (FF), a metabolically active environment. The FF contains several factors, including steroids, polysaccharides, proteins, antioxidant enzymes, and trace elements, which together modulate the developmental capacity of the oocytes [2]. Some biochemical components of the FF affect oocyte maturity, fertilization potential, and embryo quality [3]. The oocytes obtained from low-ovarian-reserve patients who undergo IVF are often of low-quality, leading to high rates of cancellation and low rates of fertilization [4,5]. Among these few oocytes, it is essential to select one that can be fertilized. It is known that increased oxidative stress in FF negatively affects fertilization results [6,7]. However, poor ovarian response (POR) is not always accompanied by increased oxidative stress.
The thiol redox reaction is one of the main pathways in humans for coping with oxidative stress. The thiol contains a sulfhydryl group, and under oxidative stress, the thiol oxidizes to form reversible disulfide bridges (S-S). With the help of antioxidants, these disulfide bonds can also be reduced back to thiol groups [8]. Previously, only one arm of this balance could be evaluated by Ellman’s reagent [9]. However, with a method established by Erel and Neselioglu [8], it is now possible to assess the thiol/disulfide balance holistically. Myeloperoxidase (MPO) is an antioxidant enzyme found in neutrophil granules. It has been shown that MPO activity increases during the periovulatory phase and in response to physiological events such as increased estrogen [10,11]. In cases of severe endometriosis, MPO activity occurs at significantly higher levels in FF, and the use of antioxidant vitamins reduces FF MPO levels [12]. Although MPO reflects antimicrobial activity acutely, its chronic activation and production cause tissue damage and are associated with chronic vascular inflammatory diseases [13,14]. Ischemia-modified albumin (IMA) is a reliable biomarker for predicting oxidative stress. Its level increases in diseases accompanied by inflammation and oxidative stress [15].

Total antioxidant capacity and some oxidative stress markers were previously examined in different patient groups undergoing artificial reproductive techniques. However, to the best of our knowledge, previous investigations have not focused on ovarian response in evaluating the thiol/disulfide balance, IMA, and MPO levels in the FF of patient groups with idiopathic POR and their correlations with intracytoplasmic sperm injection (ICSI) outcomes and success rate [13,15-18].

This study aimed to evaluate the presence of oxidative stress markers in the FF of patients with idiopathic POR; to evaluate the correlations of these markers, such as thiol/disulfide, MPO, and IMA, which can predict ICSI success, with clinical and laboratory results; and to compare the markers with those of patients with normal ovarian response (NOR).

**Methods**

Ethical permission for this research was given by the Ethical Review Board of Ankara City Hospital (E1/089/2019). For inclusion in our study, we considered the infertile women who were eligible for ICSI and who received treatment between March 2021 and April 2022 at the Department of Obstetrics and Gynecology, Center for Reproductive Medicine, Ankara City Hospital. Only the eligible patients who volunteered were included, and informed consent was received from all. In total, our study’s scope consisted of 27 women with a poor response (<5 oocytes retrieved) and 35 women with a normal response (≥5 oocytes retrieved) to ovarian stimulation. A second group with normal response was normoovulatory; these patients received ICSI due to male factor infertility.

Our study excluded heavy smokers; patients with thyroid, diabetes mellitus, renal, or hepatic diseases; and those with polycystic ovary syndrome, unexplained infertility, hypothalamic amenorrhea, systemic inflammation, or severe endometriosis. Those using vitamin supplements, antioxidants, or medications that could affect their oxidative stress marker levels were also excluded from the study. In the POR group, only cases with idiopathic causes were included, and patients with severe endometrioma or iatrogenic factors such as prior ovarian surgery, chemotherapy, radiotherapy history, and familial cases of POR were excluded.

All participants in this study underwent controlled ovarian stimulation according to routine gonadotropin-releasing hormone (GnRH) antagonist protocols. The POR participants were given 225 to 300 IU/day of human menopausal gonadotropin (Menogon, Ferring; or Merional, IBSA), and the control group’s members (normal responders) were given 150 to 187 IU/day of recombinant follicle-stimulating hormone (Gonal F, Merck Serono; or Puregon, MSD Organon). The doses were introduced subcutaneously from day 2 or day 3 of a spontaneous menstrual cycle. Next, the GnRH antagonist, either Ganirelix (Orgalutran; MSD) or Cetrorelix (Cetrotide; Merck Serono), was administered by subcutaneous injection (0.25 mg/ day) starting from the day of the stimulation cycle when the follicle size first reached 13 to 14 mm or when the serum estradiol concentration was 350 to 400 pg/mL. These treatments were continued until the day when the human chorionic gonadotropin (HCG) was administered. Oocyte retrieval was performed transvaginally 35 to 36 hours after HCG was administered, when ≥2 follicles became 18 mm in diameter.

During the oocyte pickup procedure, FF was aspirated from the largest one or two ovarian follicles, each ≥18 mm. Care was taken to avoid contamination with the flushing medium. Only FF samples with macroscopic clearance, as an indication of no blood contamination, were considered in this study. After oocyte isolation, the FF was placed into a 15-mL polypropylene centrifuge tube with a conical bottom, centrifuged at 2,500 ×g for 10 minutes in order to remove the cells and insoluble particles, and stored at −80°C until the assay. After denudation, the entire ICSI procedure was performed by the same embryologist. Pronuclear scoring was performed 16 to 18 hours after the procedure. The rate of fertilization was calculated by the ratio of fertilized egg fertilized oocytes to metaphase II (MII) oocytes. Embryo quality was assessed before the embryo transfer.

Depending on their fragmentation percentage and number of cells, the cleavage rate ranked the embryos from 1 to 4. While grade 1 represented 6 to 8 symmetrical and equal-volume cells that did not show any fragmentation, grade 4 described embryos having >50% fragmentation. Grades 1 and 2 were characterized as good-quality...
embryos, and grades 3 and 4 were characterized as low-quality.

To score blastocyst quality, we used a previously established system based on the inner cell mass (ICM) development, blastocyst expansion, and trophoderm (TE) view [19]. The degree of expansion was categorized into six classes ranging from non-expanded embryos to blastocysts fully hatched from the zona. We evaluated the ICM development and TE of the embryos that had expansion scores ≥3.

Blastocyst quality was categorized into four classes. The ICM grade was designated as B when it was loosely grouped and had several cells. TE grade B was defined as when the TE had a few cells comprising a loose epithelium. The embryos graded AA, AB, BA, and BB were considered good quality, while CA, AC, CB, BC, and CC were categorized as poor quality [20,21].

Embryo transfer was achieved 2 to 5 days after oocyte collection. We verified the implantation presence by assessing the serum β-HCG levels 14 days after embryo transfer. The presence of a clinical pregnancy was determined by the existence of a gestational sac detected by transvaginal ultrasonography. The live birth rate per embryo transfer cycle was defined as the ratio of the number of live births to the number of cycles in which embryos were transferred.

The tests for thiol/disulfide homeostasis were performed by an automated spectrophotometry method as previously described by Erel and Neselioglu [8]. To summarize, first, sodium borohydride was used to reduce the disulfide bonds into free functional thiol groups. Second, the unused sodium borohydride was eliminated by using formaldehyde to prevent the reduction of 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB). Next, we detected all thiol groups, including the reduced and native thiol, by a reaction with DTNB. Meanwhile, the amount of dynamic disulfide was calculated by taking half the difference between the native and total thiol. Lastly, after the amounts of native thiol, total thiol, and disulfide were measured, the native thiol/total thiol percent ratios (SH/SH+SS), disulfide/total thiol percent ratios (SS/SH+SS), and disulfide/native thiol percent ratios (SS/SH) were calculated [8].

We determined the serum MPO activity through a modification of the o-dianisidine method [22], which is based on kinetic measurement at 460 nm with the rate of formation of a yellowish orange product from the oxidation of o-dianisidine with MPO in the presence of hydrogen peroxide (H₂O₂). One unit of MPO was defined as what would degrade 1 μmol of H₂O₂/min at 25 °C. A molar extinction coefficient of 1.13×10⁶ of oxidized o-dianisidine was used for the calculation. MPO activity was measured in units per liter of serum.

We measured IMA levels using venous blood samples within 1 hour of admittance. We stored the specimens for 30 minutes at room temperature and then centrifuged them at 3,500 rpm for 5 minutes. Next, we transferred the samples to Eppendorf tubes (Eppendorf SE) and stored them at –80 °C until analysis occurred. To detect the presence of IMA, we used the albumin cobalt binding test. The test was accomplished by adding 50 mL 0.1% cobalt (II) chloride (CoCl₂·6H₂O) (Sigma-Aldrich Chemie GmbH) to the patient’s serum. After mixing, 10 minutes of incubation were allowed for albumin cobalt binding, and then we added 50 mL of 1.5 mg/mL dithiothreitol. After mixing followed by 2 minutes of incubation, we added 1.0 mL of a 0.9% sodium chloride solution to diminish the binding capacity. We prepared the blank in a similar way with distilled water instead of dithiothreitol. Using a spectrophotometer, we measured the absorbance of samples at 470 nm. The results were presented in absorbance units [23].

SPSS version 21 (IBM Corp.) was used for all statistical analyses. The Shapiro-Wilk test was used to evaluate the normality assumption. Data are presented as mean±standard deviation or median (inter-quartile range) for continuous variables, depending on the normality of the data distribution. The independent sample t-test was performed to analyze normally distributed variables. Whenever the normality assumption was not met, we used the Mann-Whitney U test. Spearman or Pearson correlation coefficients were calculated to assess the relationships between continuous variables. A p-value of <0.05 was considered to indicate statistical significance.

Results

Table 1 summarizes the baseline clinical and laboratory characteristics of the participants by ovarian response. Compared to the NOR group, the basal follicle-stimulating hormone level (p=0.006) was statistically significantly higher in the POR group, and the anti-Müllerian hormone (AMH) (p<0.001), antral follicle count (p=0.014), peak estradiol level (p<0.001), and total retrieved oocyte number (p<0.001) were statistically significantly lower in the POR group.

Table 2 shows oxidative stress marker levels by ovarian response. In the participants’ FF samples, we compared (1) native thiol, (2) total thiol, (3) disulfide, (4) SS/SH, (5) SS/SH+SS, and (6) SH/SH+SS. Among those, a significant difference was found only for the disulfide levels, which were significantly higher in the NOR group than in the POR group (p=0.014).

Transfers could be performed for 44 participants. β-HCG positivity was detected in 16 participants (36.3%). Among these, the transfer for one participant (2.2%) resulted in a biochemical abortus, and another one resulted in an ectopic pregnancy (2.2%). Clinical pregnancy was detected in 14 participants (31.8%).

Table 3 represents the outcomes of assisted reproduction cycles in POR and NOR participants. The MII oocyte and good-quality embryo percentages were significantly higher in the POR group than in the NOR group (p=0.022 and p=0.001, respectively).

We also performed a correlation analysis. A positive correlation
Table 1. Baseline clinical and laboratory characteristics of participants by ovarian response

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal ovarian response (n = 35)</th>
<th>Poor ovarian response (n = 27)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>30.54 ± 4.08</td>
<td>32.85 ± 5.48</td>
<td>0.073</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.4 (22.0–27.0)</td>
<td>26.7 (22.5–29.2)</td>
<td>0.244</td>
</tr>
<tr>
<td>Endometrial thickness (mm)</td>
<td>9.87 ± 2.20</td>
<td>9.93 ± 2.11</td>
<td>0.914</td>
</tr>
<tr>
<td>AMH (ng/mL)</td>
<td>1.96 (0.97–3.66)</td>
<td>0.65 (0.28–1.41)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Antral follicle count</td>
<td>9.43 ± 4.09</td>
<td>6.81 ± 3.93</td>
<td>0.014</td>
</tr>
<tr>
<td>Basal FSH level (IU/L)</td>
<td>8.2 (6.7–9.9)</td>
<td>10 (8.8–11.5)</td>
<td>0.006</td>
</tr>
<tr>
<td>Total gonadotropin dose (IU)</td>
<td>1,683 (1,350–2,475)</td>
<td>2,100 (1,685–2,550)</td>
<td>0.116</td>
</tr>
<tr>
<td>Peak E2 level (pg/mL)</td>
<td>1617.89 ± 867.26</td>
<td>773.44 ± 401.95</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Retrieved oocyte number</td>
<td>8 (6–11)</td>
<td>3 (1.5–3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sperm concentration (10⁶/mL)</td>
<td>15 (7–50.4)</td>
<td>50 (20–92)</td>
<td>0.057</td>
</tr>
<tr>
<td>Motility (A+B) (%)</td>
<td>15 (2–38)</td>
<td>30 (15–43)</td>
<td>0.119</td>
</tr>
<tr>
<td>Morphology (% normal)</td>
<td>3 (0–7)</td>
<td>6 (2–8)</td>
<td>0.128</td>
</tr>
<tr>
<td>TMSC (10⁶/ejaculate)</td>
<td>2.4 (0.3–19.6)</td>
<td>15.5 (0.96–42)</td>
<td>0.063</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation or median (interquartile range).

BMI, body mass index; AMH, anti-Müllerian hormone; FSH, follicle-stimulating hormone; E2, estradiol; TMSC, total motile sperm count.

Table 2. Oxidative stress markers by ovarian response

<table>
<thead>
<tr>
<th>Oxidative stress markers</th>
<th>Normal ovarian response (n = 35)</th>
<th>Poor ovarian response (n = 27)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native thiol (µmol/L)</td>
<td>381.1 (331.9–408.4)</td>
<td>328.9 (284.8–405.8)</td>
<td>0.162</td>
</tr>
<tr>
<td>Total thiol (µmol/L)</td>
<td>417.9 (368.4–444.1)</td>
<td>369.6 (317.5–433.1)</td>
<td>0.107</td>
</tr>
<tr>
<td>Disulfide (µmol/L)</td>
<td>17.9 (14.95–21.2)</td>
<td>14.4 (13.4–17.95)</td>
<td>0.014</td>
</tr>
<tr>
<td>Disulfide/native thiol</td>
<td>5.09 ± 1.19</td>
<td>4.72 ± 0.99</td>
<td>0.194</td>
</tr>
<tr>
<td>Disulfide/total thiol</td>
<td>4.60 ± 0.97</td>
<td>4.30 ± 0.82</td>
<td>0.201</td>
</tr>
<tr>
<td>Native thiol/total thiol</td>
<td>90.8 ± 1.94</td>
<td>91.4 ± 1.64</td>
<td>0.200</td>
</tr>
<tr>
<td>IMA FF (ABSU)</td>
<td>0.78 (0.66–0.92)</td>
<td>0.68 (0.62–0.74)</td>
<td>0.065</td>
</tr>
<tr>
<td>MPO FF (U/L)</td>
<td>140.75 (127.80–177.85)</td>
<td>156.33 (92.69–176.48)</td>
<td>0.556</td>
</tr>
</tbody>
</table>

Values are presented as median (interquartile range) or mean±standard deviation.

IMA, ischemia-modified albumin; FF, follicular fluid; ABSU, absorbance units; MPO, myeloperoxidase.

Table 3. Outcomes of assisted reproductive cycles by ovarian response

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal ovarian response (n = 35)</th>
<th>Poor ovarian response (n = 27)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metaphase II number (%)</td>
<td>212/299 (70.9)</td>
<td>58/69 (84.0)</td>
<td>0.022</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>41/56 (73.2)</td>
<td>133/212 (62.7)</td>
<td>0.143</td>
</tr>
<tr>
<td>Cleavage rate (%)</td>
<td>100 (0–100)</td>
<td>100 (100–100)</td>
<td>0.253</td>
</tr>
<tr>
<td>Good-quality embryo (%)</td>
<td>54/116 (46.5)</td>
<td>27/35 (77.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>Clinical pregnancy rate per embryo transfer (%)</td>
<td>8/27 (29.6)</td>
<td>6/17 (35.2)</td>
<td>0.694</td>
</tr>
<tr>
<td>Live birth rate per embryo transfer cycle</td>
<td>100 (100–100)</td>
<td>100 (50–100)</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Values are presented as frequency (percentage) or median (range).

was identified between the total thiol level and the number of fertilized egg (2PN) oocytes ($r=0.258, p=0.046$). Furthermore, the disulfide level was found to be positively correlated with the AMH level ($r=0.262, p=0.039$) and total number of retrieved oocytes ($r=0.335, p=0.008$).

**Discussion**

In our study, the FF disulfide level was found to be significantly higher in participants with NOR than those with POR. The number of mature oocytes, their fertilization potential, and embryo quality are...
known to be affected by the components of the FF [6]. Although the thiol/disulfide balance is frequently cited as an oxidative stress marker in the literature [24,25], due to the nature of SH and S-S bond characteristics, it is subject to change due to various environmental factors. Compared to other molecular biomarkers, they represent ambiguous indicators in terms of oxidative stress. Strictly speaking, these measurements are rather rudimentary to characterize oxidative stress in the body. The fact that MPO and IMA levels in the FF did not differ significantly between our study’s two groups also supports this idea. Therefore, based on these data, it can be said only that some environmental toxins were found to be significantly higher in the NOR group’s FF. Assuming that patients with idiopathic POR have oxidative stress markers may not always be correct due to the indirect nature of the thiol/disulfide balance.

The correlation of the disulfide level with the AMH level and total number of retrieved oocytes may indicate that the levels of these toxins increase as the number of oocytes collected increases. The increases in total and native thiol levels were not statistically significant in the NOR group, yet they may have occurred through compensation. Again, although the total oocyte count, MII total, and number of good-quality embryos were significantly higher in the NOR group than the POR group, the percentages of MII and good-quality embryos were significantly lower among NOR participants than POR participants. This may be due to the harmful effects of disulfide, which may have accumulated as an environmental toxin. In fact, the disulfide level was found to be significantly higher in the NOR group than in the POR group. Due to the harmful effects of disulfide, the oocyte maturity and embryo quality were affected among the participants with good responses. However, since the two groups showed no significant differences in pregnancy rate or live birth rate, it is not possible to claim that disulfide is a meaningful marker for use in clinical practice for IVF procedures. Özdemir et al. [17] evaluated the thiol/disulfide balance in the serum of 62 women, including women with POR, unexplained infertility, and infertility due to male factors but with NOR. Nevertheless, they did not find any differences among the groups regarding disulfide, native thiol, and total thiol levels. As in our study, Özdemir et al. [17] found disulfide levels to be lower in the group with POR. Although the difference was not statistically significant, this may have been due to the small number of patients in each group. In contrast, Erdogan et al. [26] determined that in patients with POR (Poseidon category 3), antioxidant system markers and both FF and serum levels of native and total thiol were all significantly decreased. The different result in our study may arise from our study’s categorization of participants according to ovarian response, and the fact that the participants with idiopathic POR were a very heterogeneous group [26].

In a study of patient groups with and without polycystic ovary syndrome, Tola et al. [18] found that the fertilization rate, calculated by dividing the number of fertilized oocytes by the MII total, was correlated with native thiol and total thiol. They also showed that total thiol and native thiol are variables that predict fertilization. Similarly, in our study, the total thiol measurement was found to be correlated with the number of 2PN oocytes. Because the FF total thiol level is positively correlated with the 2PN oocyte number, it may be possible to predict noninvasively which oocytes could be fertilized in an embryological laboratory and to work cost-effectively.

It was determined by Kilic et al. [27] that the disulfide level, disulfide/thiol, and disulfide/total thiol levels were lower in patients with psoriasis than in the control group. It is thought that the thiol/disulfide balance is associated with antioxidant status in proliferative diseases. In our study, although the participants with POR had significantly different disulfide levels consistent with having proliferative diseases, no significant difference was found between the SS/SH and SS/total SH ratios. For this reason, our results were not in accordance with the patterns of thiol/disulfide balance described for degenerative or proliferative diseases. However, this may be due to the small number of participants.

Although the thiol/disulfide balance is interpreted in many studies as an oxidative stress marker and is associated with disease pathogenesis, we suggest considering the thiol/disulfide balance from a different perspective, based on identifying higher disulfide levels in the NOR group than the POR group, similar to the results in earlier studies. Our findings indicate that increased oxidative stress was not accompanied by idiopathic POR. Additionally, although the disulfide values showed significant differences between groups, we found that a marker could not be used to predict ICSI results because the groups had no significant differences in pregnancy rate or live birth rate. Further studies with larger numbers of participants are needed for definitive results. Through an evaluation of the thiol/disulfide balance in the FF of different infertile patient groups with a larger number of participants, the cut-off values that can predict oocyte quality could be determined. In this way, it may be possible to predict oocyte quality, fertilization ability, and embryo quality from FF samples in a noninvasive and cost-effective manner.

**Conflict of interest**

No potential conflict of interest relevant to this article was reported.

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Author contributions

Conceptualization: ET, BE. Methodology: ET, SN. Formal analysis: ET, BE, SN. Data curation: ET. Project administration: ET, NKY. Visualization: ET, NKY, ÖMT. Writing-original draft: ET, BE. Writing-review & editing: NKY, ÖMT. Approval of final manuscript: ET, BE, SN, NKY, ÖMT.

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