ART

Effect of follicular fluid oxidative stress parameters on intracytoplasmic sperm injection outcome

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Abstract
Objective. The purpose of this study was to evaluate the association between the follicular fluid (FF) reactive oxygen species (ROS) levels, total antioxidant capacity (TAC) and ROS-TAC score and pregnancy after intracytoplasmic sperm injection (ICSI).

Methods. A total of 138 consecutive women who had ICSI were included in this study. FF ROS and TAC were measured by enhanced chemiluminescence and colorimetric assay, respectively, and then the ROS-TAC score was calculated.

Results. Out of the 138 included patients, 42 (30%) achieved pregnancy after ICSI. Log ROS, TAC, and the ROS-TAC score were not significantly different across diagnoses. Pregnant cycles were associated with significantly lower ROS (P < 0.001), higher TAC (P < 0.001) and higher ROS-TAC scores (P < 0.001). After adjusting for age, there was a significant positive correlation between log ROS and the number of follicles on the day of HCG administration (correlation 0.20, 95% CI: 0.02, 0.39) as well as the number of oocytes retrieved (correlation 0.18, 0.001, 0.36) but not with TAC. Interestingly, in women with endometriosis, higher TAC levels and higher ROS-TAC scores were associated with a higher likelihood of finding normal oocytes (P = 0.005 and P = 0.002, respectively).

Conclusion. Higher FF TAC, higher FF ROS-TAC scores and lower FF ROS levels are associated with pregnancy after ICSI. Oxidative stress parameters may be markers of metabolic activity within the follicle.

Keywords: Follicular fluid, oxidative stress, assisted reproductive technology, intracytoplasmic sperm injection

Introduction
Oxidative stress (OS) has been shown to cause defective embryo development in vitro [1]. In addition, oxidative DNA injury has been proposed as a possible pathogenic mechanism for congenital anomalies. The question of OS induced DNA damage is of particular relevance during intracytoplasmic sperm injection (ICSI) as the exposure to in vitro conditions as well as the mechanical manipulation of both the ovum and sperm may increase OS [2]. Reactive oxygen species (ROS) may also originate from the embryo’s metabolism or culture conditions. The expression of various biomarkers of OS has been demonstrated in normal human ovaries during spontaneous menstrual cycles. Furthermore, a growing body of evidence suggests that certain levels of OS are essential for normal fertilization and ultimately, a fine balance between OS and antioxidants is crucial for successful reproductive outcome both in vivo and in vitro [3,4]. The presence of different OS biomarkers in the follicular fluid (FF) has been demonstrated and variations in their concentrations in response to ovulation have been demonstrated. In addition, a possible association between the variation in FF OS biomarkers and the outcome after assisted reproductive technology (ART) has been proposed [5–8]. The exact impact of FF OS on oocyte maturation, fertilization, and pregnancy is not fully understood [6].

OS is usually evaluated by measuring the levels of ROS and the total antioxidant capacity (TAC). In an effort to get a more accurate and comprehensive assessment of OS, we previously developed an overall OS score using both ROS and TAC with principal component analysis [9]. OS was evaluated by measuring ROS and TAC in the FF of patients undergoing ICSI and calculating an overall ROS-TAC score. We examined the ability of the individual ROS and TAC values versus the combined ROS-TAC score to predict pregnancy after ICSI.

Materials and methods
This prospective cohort study was approved by the Institutional Review Board of the Cleveland Clinic Foundation. Couples undergoing ICSI were invited to participate in the study. A total of 138 consecutive patients undergoing ICSI were enrolled in the study from January 2000 through December 2001. This cohort included couples with male infertility, endometriosis, tubal disease,
idiopathic infertility, ovulatory dysfunction, and combined male and female infertility.

**Interventions**

All patients underwent controlled ovarian hyperstimulation by pituitary desensitization with the gonadotropin-releasing hormone (GnRH) agonist leuprolide acetate followed by recombinant human follicle-stimulating hormone (FSH). An HCG injection was given to trigger the final stages of oocyte maturation. Serum estradiol levels and the number and size of the follicles were recorded on the day of HCG injection. FF was aspirated 36 h later using a standard transvaginal ultrasound-guided approach, and ICSI was performed after incubating the oocytes for an additional 4 h in culture. Fertilization was assessed 16–18 h later. Transabdominal ultrasound-guided embryo transfer was performed 3 or 5 days after oocyte retrieval. The number of embryos transferred was guided by the guidelines of the American Society for Reproductive Medicine (ASRM). Briefly, two embryos were transferred for women with age less than 35, three embryos for ages 36–39, and four plus above the age of 40. Equal number of embryos was transferred in the two groups.

**Follicular fluid preparation and detection of reactive oxygen species**

The FF samples were centrifuged at 300 × g for 7 min. The clear supernatant was divided into aliquots and frozen at −70°C. The frozen samples were thawed and analyzed. Samples from each patient group were always measured in parallel and in duplicate to avoid inter-assay variance. ROS was measured by an enhanced chemiluminescence assay using luminol as the probe. TAC was measured using the colorimetric assay (Randox Laboratories, Crumlin, UK). ROS-TAC score was calculated by principal components analysis described by Sharma et al. in 1999 [9], and is detailed below.

The ROS-TAC score was formulated using principal components analysis. The log of (ROS + 1) was used since ROS was right-skewed. First, ROS and TAC values were standardized to the male infertility group (‘the controls’) (i.e. by subtracting control mean and dividing by control SD), as below.

For log (ROS + 1):

\[
\text{Standardized ROS} = \frac{\log (\text{ROS} + 1) - 1.2342}{0.4938}
\]

For TAC:

\[
\text{Standardized TAC} = \frac{(\text{TAC} - 779.7731)}{323.5419}
\]

The first principal component provided the following linear equation:

\[
\text{Principal component} = (-0.707 \times \text{standardized ROS}) + (0.707 \times \text{standardized TAC}).
\]

To create an ROS-TAC score with mean (SD) of 50 (10) in controls, the score was transformed as ROS-TAC score = 50 + (Principal component × 8.80).

**Outcome definitions**

The primary endpoint of interest was pregnancy after ICSI. Secondary outcomes included the number of follicles on day of HCG injection and the number and quality of the retrieved oocytes. The embryo quality was evaluated 3–5 days before embryo transfer.

**Statistical analysis**

The relationship between diagnosis group and ROS, TAC, and the ROS-TAC score was assessed using the Kruskal–Wallis test. Demographic characteristics and OS scores were compared between patients who achieved pregnancy to those who did not using Wilcoxon rank-sum and Chi-square tests. OS measures were also compared according to smoking and alcohol consumption using Wilcoxon rank-sum tests.

The relationship between TAC, ROS, and the ROS-TAC score and estradiol level and number of follicles on day of HCG administration and number of oocytes retrieved were assessed using Spearman correlation; score-diagnosis interactions on these fertility measures were assessed using linear regression. We assessed the relationship of each score with the ratio of normal oocytes retrieved (events/trials) using generalized estimating equation models to account for within-subject correlation. The interaction between diagnosis and ROS, TAC, or ROS-TAC score and presence of a normal oocyte was assessed. Data were log-transformed to meet model assumptions.

Receiver operating characteristic (ROC) analysis was used to compare the predictive ability of ROS, TAC, and ROS-TAC on pregnancy. We compared area under the ROC curve (AUC) for the three scores using the method of DeLong. For each predictor we report the observed cut point corresponding to maximum sensitivity and specificity for detecting pregnancy. In all analyses, we adjusted for age, BMI, and parity if significantly associated with outcome. All tests used were two-sided with alpha 0.05. Bonferroni correction for multiple comparisons was used as appropriate. All statistical analyses were done using SAS version 8.2 (SAS Institute Inc, Cary, North Carolina).

**Results**

A cohort of 138 women who were scheduled for ICSI was prospectively enrolled in the study. The mean age was 33.8 ± 4.0 years and mean BMI was 24.1 ± 4.5 kg/m². Of the 138 included patients, 101 (75%) were nulliparous (primary infertility), 33 (25%) presented with secondary infertility, and 4 patients had missing information. Log ROS, TAC, and the ROS-TAC score were not significantly different across diagnoses. The mean estradiol level on day of HCG injection was 1949 ± 959 pg/mL (median 1863 pg/mL (IQR, 1173, 2609 pg/mL)), and the mean number of follicles per patient was 12.9 ± 6.7 (median 12 (IQR, 7, 16)).

Forty-two (30%) patients achieved pregnancy after one ICSI cycle. There were no significant differences in the baseline clinical characteristics between women who achieved pregnancy compared to those who did not (Table I). In addition, no differences were found between the groups in In Vitro Fertilization (IVF) cycle characteristics (Table II).

Pregnant cycles were associated with significantly lower FF ROS (P = 0.001), higher FF TAC (P < 0.001), and higher ROS-TAC score (P < 0.001) (Table II). ROS-TAC score was not different from TAC alone in predicting pregnancy (AUC of 0.84 versus 0.80, respectively, P = 0.13). However, the ROS-TAC score was significantly better than ROS level alone (AUC of 0.84 versus 0.68, respectively, P < 0.001), Figure 1. Cut points maximizing
sensitivity and specificity and the corresponding sensitivity and specificity estimates for detecting pregnancy are reported in Table III.

After adjusting for age, there was a significant but weak positive correlation between ROS levels and the number of follicles on the day of HCG administration (ρ = 0.20, 95% CI: 0.02, 0.39) as well as number of oocytes retrieved (ρ = 0.18, 95% CI: 0.01, 0.36) but not with TAC or ROS-TAC score. For both number of follicles and number of retrieved oocytes, no interactions were found between infertility diagnoses and OS measures. However, there was a significant interaction between infertility diagnosis and all three OS measures; ROS (P = 0.012), TAC (P = 0.002), and ROS-TAC score (P = 0.001). For women with endometriosis, a doubling in TAC score was associated with an odds ratio of 1.8 (95% CI: 1.2, 2.8, P = 0.005) for retrieving a normal oocyte, while a 50% increase in the ROS-TAC score gave an odds ratio of 1.7 (95% CI: 1.2, 2.3, P = 0.002).

Finally, we explored the relationship between both smoking and alcohol use on levels of OS in the FF in our cohort of women who underwent ICSI. Women who smoked did not have significantly different levels of ROS (P = 0.11), TAC (P = 0.16), or ROS-TAC scores (P = 0.78) compared to non-smokers. When compared to reported non-drinkers, women with occasional or moderate alcohol consumption (no heavy consumption was reported) did not have significantly different levels of ROS (P = 0.27), TAC (P = 0.12), or ROS-TAC scores (P = 0.17).

Discussion

The main finding in this study is that lower FF ROS, higher TAC, and higher ROS-TAC scores are significant

Table I. Characteristics of patients who achieved pregnancy compared to those who did not.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Pregnant cycles (n = 42)</th>
<th>Non-pregnant cycle (n = 96)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number patients/ICSI cycles</td>
<td>42/42</td>
<td>96/96</td>
<td>NA</td>
</tr>
<tr>
<td>Age 34.0 (31.0–36.0)</td>
<td>34.0 (31.0–37.0)</td>
<td>0.56f</td>
<td></td>
</tr>
<tr>
<td>BMI kg/m² 23.4 (21.6–26.7)</td>
<td>23.0 (20.9–26.4)</td>
<td>0.62f</td>
<td></td>
</tr>
<tr>
<td>Previous pregnancy</td>
<td>11 (26%)</td>
<td>22 (24%)</td>
<td>0.78f</td>
</tr>
<tr>
<td>Smoking 6 (14%)</td>
<td>15 (16%)</td>
<td>0.80f</td>
<td></td>
</tr>
<tr>
<td>Alcohol (occasional or moderate)</td>
<td>12 (29%)</td>
<td>32 (34%)</td>
<td>0.95f</td>
</tr>
<tr>
<td>Infertility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary 22 (59%)</td>
<td>46 (54%)</td>
<td>0.59f</td>
<td></td>
</tr>
<tr>
<td>Secondary 15 (41%)</td>
<td>39 (46%)</td>
<td>0.46f</td>
<td></td>
</tr>
<tr>
<td>Infertility factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male 11 (26%)</td>
<td>25 (26%)</td>
<td>0.4f</td>
<td></td>
</tr>
<tr>
<td>Anovulation 6 (14%)</td>
<td>8 (8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometriosis 6 (14%)</td>
<td>21 (22%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubal 5 (12%)</td>
<td>15 (16%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unexplained 13 (31%)</td>
<td>20 (21%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other 1 (2%)</td>
<td>7 (7%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA, not applicable, Categorical data are presented as number of patients (percentage of sample); continuous data, as median and interquartile range, 1Wilcoxon rank-sum test, 2χ² Test.

Table II. Parameters of controlled ovarian hyperstimulation, ROS, TAC, and the score in pregnant and non-pregnant cycles.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pregnant cycles (n = 42)</th>
<th>Non-pregnant cycle (n = 96)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days of stimulation</td>
<td>9 (8, 10)</td>
<td>9 (8, 10)</td>
<td>0.99</td>
</tr>
<tr>
<td>Total FSH used (IU)</td>
<td>2700 (2025, 3150)</td>
<td>2963 (1950, 3300)</td>
<td>0.46</td>
</tr>
<tr>
<td>Estradiol (pg/mL) day of HCG administration</td>
<td>1966 (1259, 2895)</td>
<td>1810 (1078, 2549)</td>
<td>0.28</td>
</tr>
<tr>
<td>Number of follicles</td>
<td>11.0 (8.0, 16.0)</td>
<td>12.0 (7.0, 16.0)</td>
<td>0.84</td>
</tr>
<tr>
<td>Number of oocytes retrieved</td>
<td>10.0 (7.0, 15.0)</td>
<td>10.0 (7.0, 16.0)</td>
<td>0.94</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>65.5 (50.0, 74.5)</td>
<td>57.1 (40.0, 71.4)</td>
<td>0.27</td>
</tr>
<tr>
<td>ROS (cpm)</td>
<td>7.4 (2.8, 24.5)</td>
<td>33.7 (6.4, 54.1)</td>
<td>0.01</td>
</tr>
<tr>
<td>Log ROS + 1</td>
<td>0.9 (0.6, 1.4)</td>
<td>1.5 (0.9, 1.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>TAC</td>
<td>1115.0 (811.3, 1790.0)</td>
<td>593.8 (540.9, 722.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ROS-TAC score</td>
<td>60.5 (54.8, 76.0)</td>
<td>43.6 (40.2, 53.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Multiple pregnancy (%)</td>
<td>10 (24%)</td>
<td>–</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not applicable, Categorical data are presented as number of patients (percentage of sample); continuous data, as median and interquartile range, 1Wilcoxon rank-sum test.
evaluate whether the interactions we observed represent a stratified analyses based on the cause of infertility. When an interaction was significant, data was presented as different types of infertility, we evaluated potential interactions and reduce potential bias associated with the inclusion of pregnancy outcome is the infertility diagnosis. To evaluate using logistic regression. Another known predictor of theoretically affected by smoking and alcohol use. We addition, pregnancy after ICSI is known to be affected by high ROS and lower TAC in smokers [21–25] and consumption had no significantly different levels of ROS, while ROS-TAC level appears to be a significantly better predictor of achieving pregnancy than the ROS level alone. Nevertheless, the ROS-TAC score is recommended as it represents the overall OS stress milieu [1,11,12].

FF OS measures including ROS, TAC, and ROS-TAC score also correlated with the number of follicle retrieved and the number of retrieved oocytes. All of these associations were found to be independent of the infertility diagnosis and are thus generalizable to all cases of ICSI. The only exception to the above is that women with endometriosis had a significantly greater likelihood of finding normal oocytes if OS was lower, as indicated by a higher TAC and ROS-TAC score. This finding is in agreement with previous data from our group and others which suggested that OS may play an important role in the pathogenesis of infertility in women with endometriosis [13–20]. There is ample evidence from basic research and clinical studies supporting high ROS and lower TAC in smokers [21–25] and alcohol users [26]. No previous study has evaluated the impact of smoking or alcohol consumption on FF ROS and TAC. Levels of ROS, TAC, and ROS-TAC scores were not significantly different between women who smoked and those who did not. When compared to women who did not use alcohol, women with occasional or moderate alcohol consumption had no significantly different levels of ROS, TAC, and ROS-TAC scores. This might be explained by the high variability in the ROS and TAC levels. These conflicting results might be attributed to the small number of women in subgroups of alcohol consumption along with possibility of underestimation of alcohol intake in women who undergo infertility treatment.

The current study is limited by the use of pooled FF for the assays. Assessment of the OS parameters in the FF of individual follicles may have provided more precise measurement of the intraovarian ROS-TAC balance. In addition, pregnancy after ICSI is known to be affected by many confounders including maternal age, previous pregnancy, and BMI; and the levels of ROS can be theoretically affected by smoking and alcohol use. We adjusted for potential confounding due to these factors using logistic regression. Another known predictor of pregnancy outcome is the infertility diagnosis. To evaluate and reduce potential bias associated with the inclusion of different types of infertility, we evaluated potential interactions between the OS scores and the infertility diagnosis. When an interaction was significant, data was presented as stratified analyses based on the cause of infertility. However, we caution that further research is needed to evaluate whether the interactions we observed represent a true phenomenon or simply a function of our data set.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**References**