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Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures

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Objective(s): To measure levels of lipid peroxidation (LPO) and total antioxidant capacity (TAC) in the follicular fluid from patients undergoing IVF and to examine the association among LPO and TAC and oocyte maturity, embryo quality, fertilization, cleavage, and pregnancy rates.

Design: Prospective study.

Setting: Tertiary care medical center.

Patient(s): Forty-one patients undergoing IVF.

Intervention(s): Clear follicular fluid specimens were collected at oocyte retrieval.

Main Outcome Measure(s): Follicular fluid LPO and TAC levels measured by the thiobarbituric acid and the chemiluminescence method, respectively.

Result(s): The mean LPO level was 0.95 μmol malonaldehyde/L, and the mean TAC level was 819.16 Trolox equivalents. No correlation was seen among LPO or TAC levels and oocyte maturity, fertilization, cleavage, and embryo quality. The pregnancy rate was 31.7%. Pregnant patients were significantly younger than the patients who did not become pregnant (30.53 ± 3.5 years vs. 34.32 ± 3.98 years). After adjusting for age, a positive correlation was seen between pregnancy rate and LPO and between pregnancy rate and TAC.

Conclusion(s): Both LPO and TAC levels were positively correlated with the pregnancy rate. Lipid peroxidation may be a good marker of metabolic activity within the follicle, and some amounts may be necessary to establish a pregnancy. (Fertil Steril® 2004;81:973–6. ©2004 by American Society for Reproductive Medicine.)

Key Words: Infertility, lipid peroxidation, oxidative stress, antioxidants, in vitro fertilization, follicular fluid

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Oxidative stress is involved in the etiology of defective embryo development (1, 2). Reactive oxygen species (ROS) may originate from the embryo metabolism and/or its surroundings. The clinical pregnancy rates for IVF/intracytoplasmic sperm injection (ICSI) have remained unchanged at 30%–40% depending on the diagnosis and the cause for this low success rate (3). Spermatozoa selected for assisted reproductive technologies (ART) most likely originate from an environment experiencing oxidative stress, and a high percentage of these sperm may have damaged DNA (4).

The question of oxidative stress–induced DNA damage is especially relevant during the ICSI procedure (5). However, the interaction between the spermatozoa and the oocytes may require certain levels of ROS (6). The follicular fluid environment surrounding the oocytes may play a critical role in fertilization and subsequent embryo development. The oocyte resides in a metabolically active environment consisting of steroid hormones, growth factors, cytokines, granulosa cells, and leukocytes.

The impact of follicular fluid oxidative stress on oocyte maturation, fertilization, and pregnancy is not clear. In bovine oocytes, the formation of ROS is reported to increase the developmental potential during in vitro maturation to produce embryos (6). In a preliminary study, we reported that low levels of ROS in the follicular fluid might be a potential marker for predicting success in IVF patients (7). Measuring levels of ROS in day 1 culture media may be an impor-

tant biochemical marker for early embryonic growth (2). It is therefore important to understand the impact of oxidative stress in the follicular fluid on pregnancy rates as it may assist physicians in predicting a woman's chances of becoming pregnant after ART.

Therefore, the objectives of our study were to measure the levels of two markers of oxidative stress—lipid peroxidation (LPO) and total antioxidant capacity (TAC)—in the follicular fluid of patients undergoing IVF and to examine the correlation among the oxidative stress markers (LPO and TAC) and oocyte maturity, embryo quality, fertilization, cleavage, and pregnancy rates.

MATERIALS AND METHODS

The patients were recruited from a fertility clinic in Allentown, Pennsylvania. All participants granted written informed consent. A total of 41 women undergoing IVF (n = 26) or ICSI (n = 15) participated in this study. The patients' mean age was 33 years (range, 24–44 years). The main indications for IVF were male factor infertility (n = 16, 39%), tubo-peritoneal factors (n = 17; 42%), idiopathic infertility (n = 3; 7%), and ovulatory factors (n = 5; 12%).

All 41 patients underwent controlled ovarian hyperstimulation, which consisted of pituitary desensitization with the gonadotropin-releasing hormone agonist leuprolide acetate followed by recombinant human FSH. When at least two follicles reached 20 mm, an hCG injection was given. Follicular fluid was aspirated 36 hours later using a standard transvaginal ultrasound-guided approach.

Care was taken to completely aspirate each follicle within one tube. Each follicle was aspirated separately, and the follicular fluid was not mixed with the culture media. Follicular fluid from follicles containing more than one oocyte or no eggs was excluded from the analysis. Specimens that were contaminated with blood were discarded.

The samples were taken to a laboratory and centrifuged at 300 × *g* for 7 minutes, and the clear supernatant was divided into aliquots and frozen at –70°C. After the oocyte retrieval, oocyte classification was performed (8). Fertilization results were assessed 16–18 hours after IVF/ICSI. Cleavage of fertilized oocytes was assessed 24 hours later.

Embryo quality was assessed before ET (8), and ET was performed approximately 72 hours after oocyte retrieval. Luteal phase support was provided in the form of vaginal gel containing P. Clinical pregnancy was defined as the presence of an intrauterine embryo with cardiac activity on transvaginal ultrasound.

A 100-μL aliquot of the sample was used to measure the TAC using the enhanced chemiluminescence assay (7), and results were expressed as molar Trolox equivalents. Similarly, a 50-μL aliquot of the follicular fluid was used to measure the levels of LPO using the thiobarbituric acid method (9). The malonaldehyde (MDA) concentration in the sample was calculated by comparing it with the optical density produced by the MDA standard, and results were expressed as μmol MDA/L of follicular fluid.

TABLE 1

Measures of oxidative stress in the follicular fluid of 41 infertile women and results of IVF.

Variable	Measurement
LPO in follicular fluid (μmol MDA/L)[a]	0.95 ± 0.64
TAC in follicular fluid (Trolox equivalent)[a]	819.16 ± 232.65
Mature oocytes retrieved/per patient (mean)	
Pregnant	14 ± 4.37
Nonpregnant	11.67 ± 4.43
Mean fertilization rate per patient (%)	73.7
Mean cleavage rate per patient (%)	96.2
Mean no. of embryos transferred/per patient	3.7
Embryo quality (%)	
Grade 1	41.9
Grade 2	30.9

Note: Values are mean ± SD unless otherwise specified. LPO = lipid peroxidation; MDA = malonaldehyde; TAC = total antioxidant capacity.
[a] Based on measurements taken from 115 follicles.

Pasqualotto. LPO and TAC levels in follicular fluid. Fertil Steril 2004.

Student's *t*-test and Fisher's exact test were used to compare the continuous variables (age, number of oocytes retrieved, fertilization rate, number of oocytes transferred, and mean LPO and TAC levels) between the women who became pregnant and those who did not. Spearman's correlation was used to compare oocyte maturity, embryo quality, fertilization and cleavage rates, and pregnancy rate with LPO and TAC levels. The statistical analysis used the multivariate generalized estimating equation method to determine whether age, number of oocytes retrieved, fertilization rate, or number of oocytes transferred were significantly related to pregnancy success. Age adjustment was done to minimize the differences in the variables being examined. $P<.05$ was considered statistically significant. All analyses were calculated with the SAS statistical software package (version 8.1; SAS Institute Inc., Cary, NC). Results are reported as mean (±SD) unless otherwise indicated.

RESULTS

In this study, 115 follicles from 41 women undergoing IVF were analyzed. Table 1 reports the results of the LPO and TAC measurements as well as those for the remaining variables. The follicular fluid volume from each follicle (mean ± SE) was 4.56 ± 0.24 mL in women who became pregnant and 3.71 ± 0.31 mL in those who did not.

Of the 41 women, 13 (31.7%) became pregnant after IVF. Spontaneous abortion occurred in one of 13 (7.3%) pregnant women. The remaining 12 pregnancies remained healthy until birth, and the take home baby rate (12 of 41) was

TABLE 2

Comparison between women who became pregnant and those who did not after undergoing IVF/intracytoplasmic sperm injection.

Variable	No. of patients	Age (years)	No. of oocytes retrieved	Fertilization rate (%)	No. of embryos transferred	LPO (μmol/MDA)	TAC (Trolox equivalents)
Pregnant	13	30.53 ± 3.50	14 ± 4.37	75.81 ± 23.45	3.84 ± 0.37	1.31 ± 0.64	1,054.21 ± 188.99
Nonpregnant	28	34.32 ± 3.98	11.67 ± 4.43	72.71 ± 17.14	3.71 ± 0.59	0.7 ± 0.39	684.6 ± 202.9
P	—	.006	.097	.634	.389	.02	.02

Note: Values are mean ± SD. $P<.05$ was considered significant using multivariate logistic regression. LPO = lipid peroxidation; MDA = malonaldehyde; TAC = total antioxidant capacity.

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29.2%. The patients who became pregnant were significantly younger than patients who did not (Table 2). After adjusting for age, the patients who did not become pregnant had significantly lower levels of LPO and TAC compared with the patients who became pregnant. The mean percentage of mature oocytes retrieved from patients who underwent IVF was comparable to those undergoing ICSI (86.1% and 76.7%, respectively).

The correlations of different parameters with LPO and TAC are shown in Table 3. Patient age, oocyte maturity (both IVF and ICSI), and fertilization or cleavage rate did not show any correlation with levels of LPO or TAC levels. A positive correlation was seen between the grade 2 embryos and the TAC levels ($r = .37$; $P=.044$). After adjusting for age, a positive correlation was seen between the pregnancy rate and LPO ($P=.022$) and the pregnancy rate and TAC ($P=.018$).

TABLE 3

Correlation between various parameters and LPO and TAC levels.

	LPO (μmol/L MDA)		TAC (Trolox equivalents)	
Variable	Spearman's correlation	*P*	Spearman's correlation	*P*
Age (years)	−0.16	.316	−0.22	.241
Oocyte maturity:				
IVF:				
Grade 1	0.167	.414	−0.253	.282
Grade 2	0.32	.106	0.324	.094
Grade 3	−0.107	.603	0.246	.297
Intracytoplasmic sperm injection:				
Prophase 1	0.252	.365	0.20	.579
Metaphase 1	0.197	.481	−0.455	.187
Metaphase 2	−0.193	.491	0.213	.555
Embryo quality				
Grade 1	0.017	.915	0.115	.544
Grade 2	0.113	.480	0.370	.044
Grade 3	−0.115	.475	−0.264	.159
Grade 4	−0.305	.052	−0.255	.174
Grade 5	0.166	.301	−0.289	.121
Fertilization rate (%)	−0.097	.547	0.056	.770
Cleavage rate (%)	0.10	.535	−0.10	.598
Pregnancy rate (%)	0.381	.022	0.523	.018

Note: LPO = lipid peroxidation; MDA = malonaldehyde; TAC = total antioxidant capacity. $P<.05$ was considered statistically significant.

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DISCUSSION

The objectives of the present study were to measure levels of LPO and TAC in the follicular fluid of patients undergoing IVF and to examine the correlation between the two oxidative stress markers (LPO and TAC) with oocyte maturity, embryo quality, fertilization, and cleavage and pregnancy rates. The mean LPO concentration (0.95 μmol MDA/L) is similar to that in the study published by Jozwik et al. (10). The mean TAC concentration (819.16 Trolox equivalents) is similar to those reported in the seminal plasma (11) and higher than the concentrations reported in embryo culture medium and the serum of infertile patients undergoing IVF (12). These concentrations are similar to the ones described in our earlier study for a similar group of patients (7). The presence of TAC in higher amounts in the follicular fluid than in the serum corroborates the hypothesis that germinal cells have a potent antioxidant defense mechanism. Multiple and complementary protection mechanisms seem to be present in the embryo and its surrounding environment.

In our study, patients who became pregnant had higher levels of LPO than those who did not. Therefore, we conclude that elevated levels of LPO in the follicular fluid do not impair oocyte and embryo development and quality. We found no correlation between LPO and TAC levels and oocyte maturity, embryo quality, fertilization, and cleavage. However, LPO and TAC levels were lower in patients who did not become pregnant than in patients who did. Lipid peroxidation is an index of oxidative stress and is inversely correlated with the individual antioxidant levels as well as the total antioxidant levels as demonstrated in women with endometriosis (13). The positive correlation of LPO and TAC to pregnancy rate is not difficult to explain. While the

relation is positive, the MDA levels are significantly lower (<1–2 μmol concentration) compared with the elevated levels seen in the peritoneal fluid of infertile patients with endometriosis (9–140 μmol) (13) or hydrosalpingeal fluid (>5,000 μmol) (14). In addition, higher levels of antioxidants observed in the patients who became pregnant may also be able to act as a buffer and counteract the toxic effects of elevated LPO levels.

In a study by Paszkowski and Clarke (12), incubation of poor-quality embryos was associated with a decline in TAC levels, which were significantly higher than those observed in the embryos of "good" and "fair" quality. However, in our study, we did not observe any correlation between the levels of LPO and TAC measured in the follicular fluid and embryo quality.

Considering the detrimental effects of ROS on cell integrity, one would expect a negative correlation between levels of LPO and IVF outcome (pregnancy). One recent study (15) found that the alignment of the chromosomes at the equatorial position was significantly improved in metaphase II oocytes obtained from follicle culture with high oxygen levels. The investigators showed that the maturation rate was significantly reduced when oxygen tension was reduced to 5%. Their study supports the notion that a certain amount of oxygen is critical during the preantral follicle culture, particularly during resumption of oocyte maturation when spindle assembly and chromosome alignment occur (15).

Recent results from our laboratory have shown a positive correlation between levels of ROS in the hydrosalpingeal fluid and blastocyst development (14). In that study, two-cell mouse embryos were incubated with different concentrations of hydrosalpingeal fluid aspirated at laparoscopic salpingectomy, and ROS and LPO were measured. It was suggested that the detection of ROS at low concentrations might be a marker of normal tubal secretory function (14). In the current study, we found a positive correlation between LPO and TAC levels and pregnancy status.

Previous publications from our group have demonstrated that low levels of ROS in follicular fluid and hydrosalpingeal fluid may be a potential marker for predicting success in IVF patients and may be a marker of normal tubal secretory function (2). We assume that follicles with lower levels of oxygen available (in hypoxia) could contain lower levels of LPO and TAC, but at the same time they would have a smaller chance of establishing a pregnancy because of the hypoxemic status. We believe that this may be associated with the intense metabolism of the developing follicle.

A major limitation of our study was that individual follicles were not available, so the outcome reflects the overall effect of pooled follicles and oocytes. With the widespread use of oocyte donor programs, it would be interesting to compare our findings with individual oocytes or in oocyte donors (normal fertile women). Another possibility would be to correlate the follicular fluid levels of LPO and TAC from individual follicles with the results of preimplantation genetic diagnosis and the subsequent embryo formed from a specific oocyte.

In conclusion, our findings suggest that levels of LPO and TAC in patients who become pregnant through IVF can serve as markers for an obligatory minimal metabolic activity within the preovulatory follicle that is necessary to establish a pregnancy. More studies are needed before we can definitively show that LPO and TAC are beneficial in small amounts and may play an important role in reproductive function, whereas high levels of both ROS and LPO mediate a wide range of pathological conditions that affect fertility potential. Strategies should aim to reduce oxidative stress and maintain ROS and LPO levels at the amounts necessary to maintain normal healthy functional cells.

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