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Evaluation of antioxidant status and oxidative stress markers in follicular fluid for human in vitro fertilization outcome

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Abstract

Purpose: Antioxidant status and oxidative stress markers in human follicular fluid (FF) surrounding oocytes may be related to the outcomes of in vitro fertilization and embryo transfer (IVF-ET). Therefore, we herein examined the relationship between antioxidant status and oxidative stress markers in FF and the outcomes of IVF-ET.

Methods: One hundred and seventeen infertile women were included in this study. FF was obtained from mature follicles at the time of oocyte retrieval. The total antioxidant capacity (TAC) and total glutathione (GSH), vitamin C, and 8- hydroxy-2′-deoxyguanosine (8-OHdG) concentrations were measured.

Results: Total GSH levels were lower in patients who had a low fertilization rate after intracytoplasmic sperm injection (ICSI). In addition, 8-OHdG levels were higher in patients who had a low fertilization rate after ICSI and low rate of good quality blastocysts. Total GSH activity was lower in patients with endometriosis. No significant differences were noted in pregnancy outcomes.

Conclusions: Total GSH and 8-OHdG in human FF may be potential markers for fertilization in ART. Also, our findings may suggest that oxidative stress in women with infertility is associated with endometriosis.

KEYWORDS

eight-hydroxydeoxyguanosine, follicular fluid, glutathione, in vitro fertilization, oxidative stress

1 | INTRODUCTION

Assisted reproduction techniques (ART) are widely accepted procedures for the treatment of infertility. The success rate of in vitro fertilization and embryo transfer (IVF-ET) depends on a number of factors including maternal age, the cause of infertility, the quality of embryos, and lifestyle factors. Reactive oxygen species (ROS) have been shown to play multiple roles in female reproduction.[1,2] In female reproduction, oxidative stress is hypothesized to have a negative impact on, for example, folliculogenesis, embryo development, oocyte maturation, oocyte fertilization, endometriosis, polycystic ovary syndrome (PCOS), and unexplained infertility.[3,4]

The adverse effects of ROS are known to include DNA damage, lipid peroxidation, and protein damage. Besides these adverse effects, accumulating evidence has shown that controlled and adequate ROS concentrations exert physiological functions.[5] Oxidative stress is caused by an imbalance between ROS generation and antioxidant capacity. Low-molecular-weight antioxidants, such as antioxidative vitamins and glutathione (GSH), react with ROS, converting them to harmless compounds. GSH is the most abundant cellular thiol and provides the major antioxidant defense mechanism in all mammalian cells by neutralizing toxic peroxides.[6,7] Thus, adequate antioxidant concentrations may be essential for protecting oocytes and follicles from an excess of ROS. However, the relationship

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between the effects of ROS and activities of low-molecular-weight antioxidants on female reproduction has not yet been elucidated in detail.

In this study, we used antioxidant status (total antioxidant capacity (TAC), total GSH, vitamin C) and oxidative stress marker (8-hydroxy-2′-deoxyguanosine (8-OHdG)) to investigate the relationship between antioxidant status, oxidative stress markers, and outcomes of in vitro fertilization cycles. TAC is convenient for measuring the total potential of the antioxidant capacity, and the most important antioxidant is known to be GSH. High levels of GSH in mature oocytes have been shown to enhance the development of the male pronucleus at fertilization[8–10] and have also been implicated in promoting the development of preimplantation mouse embryos.[11] Vitamin C (ascorbic acid) plays an important physiological role in cells as a reducing ROS.[12,13] It has been detected in follicular fluid (FF), and its functions are multifaceted.[14–16] 8-OHdG is a sensitive indicator of DNA damage as a result of oxidative stress. Intrafollicular concentrations of 8-OHdG were previously found to be significantly higher in women with high rates of degenerate oocytes than in those with low rates.[17] Oxidative stress appears to affect multiple physiological processes from oocyte maturation to embryo development and pregnancy.

Previous studies examined various oxidative stress markers in FF.[18] FF is easily available during oocyte pick-up, is an optimal source of noninvasive biochemical markers, and provides an important microenvironment for the development of oocytes. It is reasonable to assume that some biochemical characteristics of FF surrounding the oocyte may play a critical role in oocyte quality and the subsequent potential to achieve fertilization, embryo development, and pregnancy. However, the relevance of these characteristics to oxidative stress markers in FF remains unclear because of equivocal conclusions.[19–25]

A large number of IVF clinics worldwide continue to select embryos for transfer based on their development rate and morphological features, as assessed by light microscopy.[26] However, some antioxidant status and oxidative stress markers in FF surrounding the oocyte may be related to the outcomes of IVF-ET. To provide support for this hypothesis, we herein examined the relationships between antioxidant status, oxidative stress markers, and age, infertility factors, and the outcomes of IVF-ET with the aim of developing and establishing more effective therapeutic strategies.

2 | MATERIALS AND METHODS

2.1 | Study population

This study used data obtained from 117 cycles from 117 patients in the IVF Japan group. Approval for this study was obtained from the local Ethics Committee of the IVF Japan group. Expressed informed consent was obtained from couples after ART treatments for the use of FF in this study. The outcomes of treatment with age, fertilization rates, rates of good quality embryos, blastocyst rates, and rates of good quality blastocysts were divided into lower and higher groups based on the data of our clinic.

2.2 | In vitro fertilization procedure and collection of FF

All patients were stimulated with a standard IVF protocol. Patients in the agonist group were administered the GnRH agonist, 600 μg buserelin (Suprecur® nasal solution 0.15%; Mochida Pharmaceutical, Tokyo, Japan) daily, and patients in the antagonist group were administered the GnRH antagonist, 0.25 mg ganirelix (GANIREST Subcutaneous 0.25 mg Syringe; MSD, Tokyo, Japan) daily. All patients were treated with injections of 300 IU recombinant FSH on the third day of the cycle (Gonal-F®; Merck Serono, Darmstadt, Germany). The same dose of the GnRH agonist or GnRH antagonist was continued until the day of the human chorionic gonadotropin (hCG) injection (human chorionic gonadotropin for injection; Fuji Pharma., Tokyo, Japan). Oocyte retrieval was performed by transvaginal aspiration 36 hours after the hCG injection. Individual dominant follicles (more than 18 mm) were completely aspirated with no flushing medium into polystyrene round-bottomed tubes (BD Falcon). After oocyte retrieval, FF samples without blood contamination were centrifuged and the clear supernatant obtained was stored at −80°C until assayed.

2.3 | Culture conditions, fertilization assessment, embryo assessment, and pregnancy test

All oocytes were inseminated in GM-HTF (Gynemed GmbH & Co. KG, Lensahn, Germany) or injected with sperm using the standard

TABLE 1 Baseline and IVF cycle characteristics of patients

Age (y)	35.9 ± 4.2
Infertility factors	
Endometriosis	35
PCOS	18
Tubal factors	9
Male factors	55
Protocols	
Agonist (long)	38
Agonist (short)	4
Antagonist	36
Serum estradiol (pg/mL)	2916.6 ± 1686.4
Average number of oocytes retrieved	13.1 ± 7.0
Insemination	
ICSI	79
cIVF	35
Split	3

Values are means ± SEM; ICSI, intracytoplasmic sperm injection; cIVF, conventional IVF; PCOS, Polycystic ovarian syndrome; Split, ICSI and cIVF in combination.

TABLE 2 Relationship between outcomes and follicular concentrations of antioxidant status and oxidative stress markers from lower and higher groups

Antioxidant status and oxidative stress markers	Age (y)			Fertilization rate of ICSI (%)			Fertilization rate of cIVF (%)		
	Lower group <40 (n = 92)	Higher group ≥40 (n = 25)	*P*	Lower group <75% (n = 19)	Higher group ≥75% (n = 62)	*P*	Lower group <75% (n = 18)	Higher group ≥75% (n = 19)	*P*
TAC (μmol/L)	1149.7 ± 221.5	1221.1 ± 350.1	0.35	1090.3 ± 193.0	1176.8 ± 249.3	0.13	1297.4 ± 368.7	1128.3 ± 114.4	0.10
Total GSH (μmol/L)	0.331 ± 0.40	0.386 ± 0.36	0.52	0.204 ± 0.23	0.403 ± 0.49	<0.05	0.291 ± 0.27	0.330 ± 0.37	0.71
Vitamin C (mg/L)	22.3 ± 5.4	22.8 ± 5.3	0.69	21.6 ± 5.7	23.6 ± 5.0	0.19	21.1 ± 5.6	20.2 ± 4.2	0.61
8-OHdG (ng/mL)	0.92 ± 0.41	0.98 ± 0.33	0.41	1.10 ± 0.35	0.88 ± 0.38	<0.05	1.02 ± 0.42	0.81 ± 0.41	0.14
Antioxidant status and oxidative stress markers	**Rate of good quality embryos (%)**			**Blastocyst rate (%)**			**Rate of good quality blastocysts (%)**		
	Lower group <60% (n = 45)	Higher group ≥60% (n = 55)	*P*	Lower group <40% (n = 27)	Higher group ≥40% (n = 61)	*P*	Lower group <40% (n = 22)	Higher group ≥40% (n = 21)	*P*
TAC (μmol/L)	1169.6 ± 333.8	1125.1 ± 198.6	0.42	1175.3 ± 352.5	1132.2 ± 227.5	0.56	1113.7 ± 248.8	1154.3 ± 202.3	0.44
Total GSH (μmol/L)	0.353 ± 0.39	0.340 ± 0.42	0.88	0.294 ± 0.39	0.368 ± 0.43	0.42	0.379 ± 0.51	0.306 ± 0.29	0.40
Vitamin C (mg/L)	22.4 ± 5.9	22.4 ± 5.0	0.97	22.0 ± 5.3	22.5 ± 5.2	0.68	22.5 ± 5.5	22.2 ± 4.9	0.83
8-OHdG (ng/mL)	0.98 ± 0.35	0.86 ± 0.38	0.15	0.96 ± 0.36	0.87 ± 0.41	0.37	0.97 ± 0.36	0.74 ± 0.42	<0.05

Values are means ± SEM; GSH, glutathione; TAC, Total antioxidant capacity; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

intracytoplasmic sperm injection (ICSI) technique. Fertilization was confirmed by the presence of two pronuclei and extrusion of the second polar body. Fertilized oocytes were cultured in groups of a maximum of 4 oocytes in 1 mL of global® total™ (LifeGlobal, Ontario, Canada) until day 5. The embryos were classified according to the criteria proposed by Veeck.[27] A good quality embryo was defined as one that had reached the four-cell stage on day 2, reached the seven-cell stage on day 3, and had less than 20% of its volume filled with fragments. The blastocysts were classified according to the criteria proposed by Gardner.[28] A good quality blastocyst was defined as being in a full blastocyst stage and did not include an inner cell mass or trophectoderm with very few cells. A pregnancy test was performed two weeks after embryo transfer. Pregnancy was confirmed when fetal heart activity was detected on transvaginal ultrasound four weeks after embryo transfer.

2.4 | Measurement of antioxidant status and oxidative stress markers in FF

TAC was measured in FF using a test kit for Potential Antioxidant (Nikken Seil Co., Ltd., Shizuoka, Japan). This assay evaluated Cu^{+} levels derived by the reduction in Cu^{++} from the action of antioxidants present in the sample. GSH was measured using a glutathione assay kit (Northwest Life Science Specialities, Vancouver, WA, USA) based on the manufacturer's instructions. FF samples were deproteinized aliquots mixed with metaphosphoric acid. Samples with DTNB reagent and GSH reductase reagent were added to the wells of the microplate. NADPH reagent was added to all wells, and absorbance values were read at 405 nm. Vitamin C was measured using a Vitamin C colorimetric assay kit (Immundiagnostik AG, Bensheim, Germany) based on the manufacturer's instructions. Vitamin C exists as ascorbic acid as well as its oxidized form, dehydroascorbate in FF. As both forms are biologically active, they were both measured. 8-OHdG was measured using a New 8-OHdG Check ELISA (Nikken SEIL Co. Ltd, Shizuoka, Japan) based on the manufacturer's instructions. Each FF (50 μL) sample was filtered using an ultrafilter (cutoff molecular weight of 10 kDa) and used in duplicate assays.

2.5 | Statistical analysis

Descriptive statistics and the Student's *t* test to compare groups were performed using Stat view Version 5.0 (SAS Institute Inc, Cary, NC, USA).

3 | RESULTS

One hundred and seventeen patients underwent 117 cycles of ART during the study duration. Of those, 79 were ICSI cycles, 35 were conventional IVF cycles, and three were split cycles. The baseline and IVF cycle characteristics of our patients are shown in Table 1. Thirty-five women with a diagnosis of endometriosis, 18 with the polycystic ovarian syndrome, 9 with tubal factors, and 55 with male factors underwent IVF treatments.

The relationship between antioxidant status and oxidative stress markers and the outcomes of IVF-ET are presented in Table 2. Total GSH levels were lower in patients who had a low fertilization rate after ICSI (0.204 ± 0.23 μmol/L vs 0.403 ± 0.49 μmol/L; $P < 0.05$). In addition, 8-OHdG levels were higher in patients who had a low fertilization rate after ICSI (1.10 ± 0.35 ng/mL vs 0.88 ± 0.38 ng/mL; $P < 0.05$) and low rate of good quality blastocysts (0.97 ± 0.36 ng/mL vs 0.74 ± 0.42 ng/mL; $P < 0.05$). However, no significant difference

TABLE 3 Follicular concentrations of antioxidant status and oxidative stress markers in pregnancy

Antioxidant status and oxidative stress markers	Pregnant (n = 34)	Not pregnant (n = 30)	*P*
TAC (μmol/L)	1168.1 ± 323.9	1185.1 ± 214.5	0.81
Total GSH (μmol/L)	0.356 ± 0.42	0.402 ± 0.41	0.66
Vitamin C (mg/L)	23.9 ± 4.6	22.4 ± 5.5	0.25
8-OHdG (ng/mL)	0.849 ± 0.41	0.997 ± 0.34	0.13

Values are means ± SEM; GSH, glutathione; TAC, Total antioxidant capacity; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

TABLE 4 Effects of different infertility factors on follicular concentrations of antioxidant status and oxidative stress markers

Antioxidant status and oxidative stress markers	Infertility factors			
	Endometriosis (n = 35)	PCOS (n = 18)	Tubal factors (n = 9)	Male factors (n = 55)
TAC (μmol/L)	1210.7 ± 311.2	1108.9 ± 207.3	1223.5 ± 238.6	1144.7 ± 225.7
Total GSH (μmol/L)	0.238 ± 0.25[a]	0.279 ± 0.25[ab]	0.392 ± 0.53[ab]	0.441 ± 0.47[b]
Vitamin C (mg/L)	21.2 ± 4.9	22.5 ± 6.2	22.3 ± 4.0	23.1 ± 5.4
8-OHdG (ng/mL)	0.99 ± 0.36	1.02 ± 0.50	0.79 ± 0.41	0.89 ± 0.47

Values are means ± SEM; GSH, glutathione; PCOS, Polycystic ovarian syndrome; TAC, Total antioxidant capacity; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.
Values with different superscripts in the same row are significantly different ($P < 0.05$).

was observed in age, the rate of good quality embryos, or the blastocyst rate.

The relationship between antioxidant status, oxidative stress markers, and pregnancy outcomes are shown in Table 3. No significant differences were observed in pregnancy outcomes. These results indicated the absence of a relationship between antioxidant status, oxidative stress markers in FF, and pregnancy outcomes.

The relationship between antioxidant status, oxidative stress markers, and infertility factors is shown in Table 4. Total GSH activity was lower in patients with endometriosis than in patients whose infertility was attributed to male factors (0.238 ± 0.25 μmol/L vs 0.441 ± 0.47 μmol/L; $P < 0.05$).

4 | DISCUSSION

In the present study, total GSH levels were lower in patients who had low fertilization rates after ICSI. Previous studies proposed that GSH plays important roles in oocyte spindle function and pronucleus development,[29–31] in maintaining the biological value of female germ cells, and in the fertilization process.[32] In other words, the adverse effects of oxidative stress on oocytes may mainly occur in FF. We also demonstrated that 8-OHdG levels were higher in patients with low fertilization rates and low rates of good quality blastocysts. Oocytes within ovarian follicles begin meiosis during embryogenesis, but then arrest at prophase of meiosis I until luteinizing hormone is released. During this period of meiotic maturation, oocytes accumulate molecules of mRNA, proteins, and lipids as well as oxidative stress.[33] TAC enables the evaluation of not only hydrophilic antioxidants, including vitamin C and glutathione, but also hydrophobic antioxidants, such as vitamin E. However, no correlations were observed between TAC and IVF-ET outcomes in this study. Jana et al demonstrated that significantly decreased TAC in FF correlated with poor oocyte and embryo qualities and a low fertilization rate.[34] Oyawoye et al showed that the decline in TAC was lower when oocytes were fertilized and higher in association with embryo viability.[23] Furthermore, TAC showed a positive correlation with embryo quality in IVF,[24] which, in turn, showed that higher potential antioxidants may be markers of mature follicles, leading to the growth of high-quality oocytes. However, no reliable main effect of antioxidants has been detected to date. In other words, the measurement of TAC cannot identify the most important antioxidant. Hence, these results suggest the importance of measuring individual antioxidant status or oxidative stress markers such as GSH or 8-OHdG.

In the present study, no correlations were observed in antioxidant status and oxidative stress markers between the pregnant and not pregnant groups. Pasqualotto et al demonstrated that patients who did not become pregnant had significantly lower levels of lipid peroxidation (LPO) and TAC than those who became pregnant.[24] Therefore, the higher levels of antioxidants observed in patients who became pregnant may also act as a buffer and counteract the adverse effects of ROS levels. ROS are often regarded as the unwanted products of biological oxidation. On the other hand, the production of small amounts of ROS may act as physiological signaling pathways in the embryo.[35] Further studies are needed to confirm that low-molecular-weight antioxidants are beneficial in small amounts and may play an important role in ART.

Our results showed that total GSH activity was lower in patients with endometriosis. Andrade AZ et al reported a positive association between infertility related to endometriosis, an advanced disease stage, and elevated serum hydroxyperoxide levels, suggesting an increase in the production of ROS in women with endometriosis.[36] Murphy et al also suggested that endometriosis is a disease originating from or associated with oxidative stress.[37] They indicated that the presence of elements such as macrophages, iron, or environmental contaminants disrupts the balance between ROS and antioxidants, leading to oxidative stress and endometriosis. Taken together, these findings suggest that oxidative stress in women with infertility is associated with endometriosis. Antioxidant supplements may have potential therapeutic uses in the treatment of chronic inflammatory diseases like endometriosis.

Our results might indicate that total GSH and 8-OHdG in human FF have potential as markers for fertilization in ART. An analysis of FF antioxidant stress markers may provide information for the development of more effective therapeutic strategies. Antioxidant supplementation may effectively increase GSH levels and decrease 8-OHdG levels, which may lead to good IVF-ET outcomes. However, this study was not designed to investigate the association of individual follicular fluid status with the quality of the corresponding oocyte and embryo. Further investigations are needed to evaluate the importance of measuring the activities of antioxidants in FF, including measurement of individual follicles, other oxidative stress markers or the clinical characteristics of patients.

ETHICAL APPROVAL

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Approval for this study was obtained from the local Ethics Committee of the IVF Japan group. This article does not contain any study with animal participants that has been performed by any of the authors.

CONFLICTS OF INTEREST

Takuji Nishihara, Kazuya Matsumoto, Yoshihiko Hosoi, and Yoshiharu Morimoto declare that they have no conflict of interest.

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