
The Effect of Follicular Fluid Reactive Oxygen Species on the Outcome of In Vitro Fertilization

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ABSTRACT: Objective-To examine the presence of reactive oxygen species [ROS] in the follicular fluid of women undergoing in vitro fertilization [IVF] and identify its role in pregnancy outcome;. Study Design-In this prospective study, ROS and total antioxidant capacity [TAC] levels were measured by the chemiluminescence method in the follicular fluid of 53 women. Age, number of oocytes recovered, percentage of oocytes fertilized, ROS and TAC levels were compared in women who did and did not become pregnant. **Results**-Patients who become pregnant had significantly higher log-transformed ROS levels (1.01 ± 0.14 , $P = 0.03$) than those who did not (0.69 ± 0.08). Women with endometriosis or male factor infertility who became pregnant had significantly higher ROS levels (1.44 ± 0.23 and 1.31 ± 0.19) than those who did not (0.60 ± 0.17 and 0.67 ± 0.16 ; $P < .006$ and $P < .01$) **Conclusion**-Follicular fluid ROS, at low concentrations, may be a potential marker for predicting success in IVF patients. *Int J Fertil 45[5]:314-320, 2000*

KEY WORDS: reactive oxygen species, total antioxidant capacity, follicular fluid, pregnancy

INTRODUCTION

OXYGEN RADICALS AND ASSOCIATED agents may serve as important mediators in tissue remodeling, hormone signaling, steroidogenesis, and germ cell function [1]. Reactive oxygen species [ROS] such as hydrogen peroxide, and free radicals, such as superoxide and hydroxyl radicals, are generally deleterious to tissue function when they overwhelm the antioxidant capacity. Thus, while controlled production of ROS is necessary for certain physiologic functions [2], higher levels of ROS can result in oxidative stress. Reactive oxygen species have been implicated in male infertility [3], immune responses [4,5], and inflammatory processes [3], and in the regulation of luteolysis in rats [6] and humans [7].

The cellular origin and nature of ROS in the human ovary are unknown. Multiple animal studies point to such potential sources as phagocytic leukocytes, parenchymal steroidogenic cell, and endothelial cells [8]. Macrophages and neutrophils reside in both follicles and corpora lutea [9]. In addition, several enzymes generate ROS. These include a unique plasma membrane NADPH oxidase in phagocytes, oxidases of mitochondrial, microsomal and peroxisomal origin, and cytosolic xanthine oxidase in the endothelial cells. One consequence of excess ROS production in the ovary is damage to plasma membranes through lipid peroxidation of polyunsaturated fatty acids [10].

The impact of ROS on embryo development has recently been examined [11]. Since human embryos are exposed to higher oxygen concentrations during in vitro culture than in the natural environment, they may be at greater risk of oxidative stress and subsequent poorer embryo quality. ROS have never been quantified in normal human follicles, though. Thus, the role of ROS, at the level of the oocyte, in female infertility and IVF outcome is unclear.

The follicular fluid environment surrounding the oocytes may play a critical role in fertilization and embryo development. Follicular growth is predictive of the pregnancy potential of the oocytes obtained from ovarian stimulation in IVF [12]. The oocyte resides in a metabolically active environment containing steroid hormones, growth factors, cytokines, granulosa cells, and leukocytes. It is not clear whether ROS are endogenous to this environment. In addition, the impact of follicular fluid ROS on oocyte maturation, fertilization, and pregnancy is unknown. An easily available source of follicular fluid is from patients participating in an IVF program. The objectives of this pilot study were to (1) ascertain the existence of ROS in follicular fluid, (2) quantify follicular fluid ROS and total antioxidant capacity (TAC) and (3) compare these levels in women who did and did not achieve pregnancy.

MATERIALS AND METHODS

The Institutional Review Board approved this study protocol. Between October 1997 and June 1998, 53 consecutive patients undergoing in vitro fertilization were enrolled. Clinical variables such as age, number of oocytes recovered, number of oocytes fertilized and IVF outcomes were recorded for each patient. The patient diagnoses were: tubal disease (n = 12), male factor (n = 15), endometriosis (n = 12), idiopathic infertility (n = 11), and "other" (n = 3, two with ovulatory dysfunction and one with pelvic adhesions).

Of the 53 patients enrolled in the study, 45 patients initially underwent pituitary desensitization with the gonadotropin releasing hormone agonist Lupron (TAP Pharmaceuticals, Abbott Park, IL) followed by recombinant human follicle stimulating hormone (FSH, Gonal F, Serono Laboratories, Randolph, MA). Lupron and FSH were begun concomitantly on day 3 of the cycle in three patients; FSH alone was utilized in five patients. Serial pelvic ultrasonograms and estradiol levels were obtained throughout the stimulation to individualize the amount of FSH necessary. Human chorionic

gonadotropin (hcg) (Profasi, Serono) 10,000 IU was administered when at least two follicles had attained a minimum mean diameter of 20 mm.

Patients underwent oocyte retrieval 36 hours after hCG by transvaginal ultrasound-guided follicular aspiration. Care was taken to aspirate completely each follicle within one tube. Patients with only female-factor infertility had conventional IVF with 100,000 motile spermatozoa [13]; patients with only malefactor infertility underwent intracytoplasmic sperm injection (ICSI). Embryo transfers were scheduled three days after oocyte retrieval. A maximum of two or three embryos of best quality were transferred into the patient's uterus. Clinical pregnancy was defined as the presence of an intrauterine embryo with cardiac activity on transvaginal ultrasonography.

Extreme care was taken to minimize the time between retrieval of the oocytes and processing of the specimen to reduce variability in the results. Oocytes were removed from the aspirated follicular fluid immediately, and the remaining sample was processed immediately for ROS. Specimens that were contaminated with blood or were not straw colored were discarded, since vigorous oocyte retrieval can cause trauma and may alter the ROS content. The presence of white blood cells, especially the neutrophils, was detected by the myeloperoxidase (MPO) test [14]. Multiple follicular fluid samples from each patient were individually prepared for ROS measurement. All measurements were made on the freshly aspirated follicular fluid, using a Berthold luminometer (Autolumat LB 953, Wallac Inc., Gaithersburg, MD). To determine if the ROS production was due to the cellular component of the follicular fluid, each sample was split into two aliquots. One was used without any further processing (unprocessed), and the second aliquot was prepared by centrifugation at 300g for seven minutes (processed). Aliquots of 400 μ L of both unprocessed and processed specimens were prepared in duplicate along with the blank and control. Levels of ROS were determined by chemiluminescence assay using luminol as a probe [14]. Luminol is a very sensitive probe, and reacts with a variety of reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, and superoxide ion. It is capable of measuring both extracellular and intracellular ROS [15]. Great care was taken in performing the assay in the dark. After the addition of 10 μ L of luminol (5 mM), measurements were recorded for 15 minutes in integration mode, and the results were expressed as 10^4 counted photons per minute (cmp).

Aliquots of the processed follicular fluid were stored at -20°C. At the time of analysis, each sample was thawed at room temperature and immediately assessed for measurement of the nonenzymatic total antioxidant capacity. Follicular fluid was diluted 1:10 with deionized water and filtered through a 0.2- μ m millipore filter (Alliance Health Care, McGaw Park, IL). Total antioxidant capacity was measured in the follicular fluid using the enhanced chemiluminescence assay [16]. Signal reagent was prepared with chemiluminescence kit (Amersham Life Science, Buckinghamshire, U.K.). Twenty microliters of horseradish peroxidase-linked immunoglobulin (HRP-linked Ig; Americium Life Science) was added to 4.98 mL of deionized water. This was further diluted 1:1 to give a working solution with the desired luminescence output (3×10^7 cpm). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble α -tocopherol analogue, was used as a standard (50-150 μ M).

With the luminometer set in the kinetic mode, 100 μ L of signal reagent and 100 μ L of HRP-linked Ig were added to 700 μ L of deionized water and mixed. The solution was equilibrated to the desired level of chemiluminescence output (between 2 and 3×10^7 CPM) for 100 seconds. One hundred microliters of the prepared follicular fluid was added immediately to the signal reagent and HRP, and the chemiluminescence was measured. Suppression of chemiluminescence and the time from the addition of follicular fluid to 10% recovery of the initial chemiluminescence were recorded. Antioxidant capacity was expressed as molar Trolox equivalents.

The sample size necessary to obtain statistical significance was calculated. Reactive oxygen species values were log transformed ($\log [1 + \text{ROS}]$) to normalize the data distribution. Comparisons between pregnant and non pregnant women were calculated using Student's t-test or the Wilcoxon rank-sum test for continuous variables, and Fisher's exact tests for categorical variables. Patients with multiple ROS measures were included in analyses to determine if intra-patient ROS variance differed significantly between the pregnant and non pregnant women by Student's t-test. Comparisons among various diagnoses were performed with a one-way ANOVA and Fisher's exact tests for continuous (i.e., ROS and TAC) and categorical (i.e., pregnancy rates) variables, respectively.

A P value of <0.05 was considered statistically significant; summary statistics are presented as mean \pm standard error. Data were analyzed with the SAS statistical software package (SAS Institute, Cary, NC).

RESULTS

A total of 539 oocytes were recovered. The pregnant and non pregnant patients did not differ significantly in age, total number of oocytes retrieved, or percentage of oocytes fertilized (Table I). Individual follicular fluid measurements of ROS were conducted. However, since individual oocytes were not co-incubated with spermatozoa, it was difficult to establish individual variations in follicular fluid specimens for any particular egg that was fertilized. ROS and TAC values were averaged for all the oocytes recovered from a given patient. Processed follicular fluid ROS levels were negligible, indicating that ROS production was primarily a cellular event. Since the myeloperoxidase test was negative for all the samples tested for neutrophils ($<1 \times 10^6$ /mL), ROS production was primarily from granulosa cells. Of the 53 patients, 34.0% (18/53) became pregnant. Patients who did not become pregnant had significantly lower unprocessed ROS levels (0.69 ± 0.08) than did patients who became pregnant (1.01 ± 0.14) ($P < 0.03$). However, no significant differences were seen in the TAC of the pregnant and non pregnant patients (Table I). Forty patients (40/53, 75%) exhibited a normal ovarian response, defined as a peak estradiol level greater than 1000 pg/mL and retrieval of 4 or more oocytes. ROS values were not significantly different between poor and normal ovarian responders (0.83 ± 0.08 and 0.7 ± 0.14) ($P = 0.44$).

When patients were stratified by diagnosis, significantly higher ROS levels were seen in patients with endometriosis or male factor infertility who achieved pregnancy (Table II). However, the TAC levels were not significantly different between the pregnant and non pregnant patients in any of the diagnosis groups (Table II). When the variability of multiple: ROS values per patient was compared between pregnant and non pregnant patients, no significant differences were observed in the intra-patient variability of ROS values between the two groups ($P = 0.94$).

TABLE I
Comparison of unprocessed ROS, TAC, and other variables in pregnant and non-pregnant patients.

Patient Group	Number of Patients	Age (years)	Oocytes Recovered	Oocytes Fertilized	Log [ROS + 1]	TAC (Trolox equiv.)
Non pregnant	35	35.43 ± 0.61	9.03 ± 0.76	5.31 ± 0.60	0.69 ± 0.08	722.15 ± .48.76
Pregnant	18	33.72 ± 1.02	12.39 ± 1.88	7.41 ± 1.27	1.01 ± 0.14	819.22 ± 54.23
P		0.13	0.24	0.2	0.03	0.12

Values are mean ± S.E.

P < .05 is considered significant.

ROS = reactive oxygen species; TAC = total antioxidant capacity.

DISCUSSION

Reactive oxygen species are produced by the ovary and are regulated enzymatically to generate products necessary for ovulation and luteolysis [17]. We have shown for the first time the presence of ROS in follicular fluid of women undergoing ovarian stimulation for assisted reproductive procedures. These levels were 10- to 100-fold lower than ROS levels found in semen, peritoneal fluid of endometriosis patients [15], or serum of preeclamptic patients [19].

Follicular fluid ROS levels may represent physiologic ranges of ROS necessary for the normal development of the oocyte and subsequent embryo. Lack of a reference value in normal healthy women (unstimulated ovarian cycles) makes it difficult to determine if the ROS levels observed in the follicular fluid are in the physiological or pathological range. As in many other systems, a physiologic amount of ROS may be indicative of healthy developing oocytes, whereas excessively high levels may be indicative of oxidative stress.

TABLE II
Unprocessed ROS and TAC levels in the follicular fluid of patients with various diagnoses.

Diagnosis	Patients Pregnant	Patients Non Pregnant	ROS [log (ROS+1)] Pregnant	ROS [Log (ROS+1)] Non-pregnant	ROS [Log(ROS+1)] P	TAC Pregnant	TAC Non-pregnant	TAC P
Tubal disease (n = 12)	4	8	0.61 ± 0.32	0.70 ± 0.18	0.79	631.5 ± 173.9	737.3 ± 110.0	0.68
Male factor (n = 15)	6	9	1.31 ± 0.19	0.67 ± 0.16	0.01	884.6 ± 100.4	688.3 ± 69.1	0.15
Endometriosis (n = 12)	4	8	1.44 ± 0.23	0.60 ± 0.17	0	646.1 ± 122.9	811.1 ± 86.9	0.28
Idiopathic infertility (n = 11)	3	8	0.58 ± 0.27	0.62 ± 0.17	0.89	850.2 ± 142.0	739.3 ± 86.9	0.52
Others (n = 3)	1	2	0.5	1.34 ± 0.33	NA*	1,239.2	573.3 ± 173.9	NA*

Values are mean ± S.E.

P < .05 was considered significant by Student's t-test.

*Sample size does not provide adequate comparison.

ROS = reactive oxygen species; TAC = total antioxidant capacity.

Support for a role of ROS in oocyte maturation was provided by Doyle et al [20]. The LH analogue chorionic gonadotropin induced oocyte maturation, and this effect was accompanied by an increase in follicular lipid peroxidation. A relationship between ROS and ovulation was demonstrated by Miyazuki et al [21], and the role of prostaglandin PGF_{2a} as a prerequisite for ovulation was emphasized. In the sea urchin, there is an obligatory role of hydrogen peroxide in the fertilization

process [22]. Preliminary results suggest that activated oxygen species may play a physiological role in mediating successful sperm-zona interaction [23].

The source of ROS in the follicular fluid is unknown. A metabolically active system such as the preovulatory follicle is likely to have multiple sources of ROS production, and would be susceptible to oxidative stress. The follicular fluid environment is composed of the

oocyte, granulosa cells, and surrounding cells, such as endothelial and thecal cells. Since the theca interna is quite vascularized there is the potential for transudation of factors from the circulation into the follicular fluid [24]. In addition, the follicular fluid is known to contain cytokines, neutrophils and macrophages, all which can produce oxygen free radicals. Within the ovary are mechanisms for detoxification of and protection against ROS. These include enzymes such as catalase and superoxide dismutase, antioxidants such as vitamins E and C [25], the peroxidase cofactor reduced glutathione [26], and the carotenoid lutein [27].

The role of oxidative stress in female infertility is unclear. It is plausible that abnormally low amounts of ROS in follicular fluid may indicate subnormal oocyte quality. We noted significantly lower ROS levels in the group that did not achieve pregnancy versus the group that did. This difference could not be attributed to poor versus a normal response to ovarian stimulation. The distribution of ROS levels in individual specimens from each patient was highly variable in both groups. Thus, intrafollicular ROS levels may be utilized as a potential marker for predicting success with IVF, since they are indicators of an individual follicle's status versus the entire ovary.

Our results are supported by a recent preliminary report, which compared the lipid peroxidation (LPO) and total antioxidant levels in the follicular fluid and sera of patients undergoing IVF following ovarian stimulation [28]. The authors demonstrated a positive correlation between the LPO concentration in the follicular fluid and the pregnancy rates ($r=0.32$, $P=0.007$) and attributed this association to the intense metabolism of the developing follicle. Although we did not correlate ROS levels with the maturity of the oocyte, the ROS levels measured in the follicular aspirate of women who achieved pregnancy in this study may be reflective of healthier cells.

Different subpopulations of leukocytes have been detected in the follicular fluid of women with endometriosis, tubal factor, and idiopathic infertility [29]. It is possible that these differences affect oocyte maturation and ROS generation. We were unable to detect differences in the intrafollicular ROS or TAC levels of endometriosis, tubal factor, idiopathic or malefactor infertility patients. However, patients with endometriosis or malefactor infertility who became pregnant had significantly greater ROS levels than did

those who did not become pregnant. This may be explained by the fact that in these two groups the outcome of IVF is dependent primarily on the ovaries, and follicular fluid ROS levels may be indicative of a better-quality oocyte. But in tubal-Factor infertility, factors other than the ovary, such as existence of a hydrosalpinx have an impact on subsequent pregnancy. Therefore, differing ROS levels may not be expected in the pregnant versus non pregnant groups.

There are no clinical studies demonstrating follicular fluid ROS in normal healthy women in unstimulated cycles. Thus, the ROS measured in normal women exposed to high-dose gonadotropins are a reflection of a response to an exogenous factor. It may be possible that some normal women have a different response to gonadotropins leading to altered ROS levels.

Antioxidants scavenge ROS to protect the environment from oxidative damage. In the male, depressed levels of total antioxidants are seen in the semen of infertile patients with elevated ROS [3,30]. The normal TAC level of follicular fluid is unknown. Lack of a significant alteration of TAC in our study may be due to several factors. Luteinizing hormone administration doubles vitamin E levels within 24 hours in vivo (17). Since all of our patients had received hCG, it is likely that the TAC measurement is a reflection of this change. In addition, the intrafollicular TAC levels may not be different due to exposure of our patients to gonadotropins and a multivitamin containing antioxidants (vitamins A, C, and E). Finally, it is possible that TAC is a poor indicator of follicular health and subsequent pregnancy.

Several limitations of our study must be discussed. First, the follicular fluid ROS levels from women undergoing superovulation may differ from those in women experiencing unstimulated cycles. Second, while ROS values were obtained from individual follicles, the reported ROS represents the mean of several follicular fluid specimens from each patient. Third, the pregnancy outcome was not linked to the results of a single oocyte and its ROS or TAC level. Because all the oocytes from a given patient were co-incubated, we could not determine the fertilization status of any individual oocyte to correlate it with its corresponding ROS level. Future studies will attempt to associate a single follicular fluid ROS value with fertilization of the oocyte from that follicle, subsequent embryo development, and the resulting pregnancy.

In conclusion, the follicular fluid of women undergoing ovarian stimulation contains ROS. While their exact role is unclear, follicular fluid ROS levels may be a marker for an obligatory minimum metabolic activity within the follicle, necessary for establishment of a pregnancy.

ACKNOWLEDGMENTS

We would like to thank Kelvin Fry for his help in collecting follicular fluid specimens and David R. Nelson for his assistance in the statistical analysis of the manuscript.

REFERENCES

- Riley JCM, Behrman HR: In vivo generation of hydrogen peroxide in the rat corpus luteum during luteolysis. *Endocrinology* 128:1749-1753, 1991.
- deLarnirande E, Gagnon C: A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *Int J Androl* 16:21-25, 1993.
- Sharma RK, Agarwal A: Role of reactive oxygen species in male infertility. *Urology* 48:835-850, 1996.
- Babior BIM, Curnutte IT, Okamura N: The respiratory burst oxidase of the human neutrophil, in Halliwell B (ed): *Oxygen Radicals and Tissue Injury*. FASEB, Bethesda, MD, p 43, 1987.
- Farber TL, Kyle ME, Coleman JB: Biology of disease. Mechanisms of cell injury by activated oxygen species *Lab Invest* 62:670-679, 1990.
- Behrman H, Preston S: Luteolytic actions of peroxide in rat ovarian cells. *Endocrinology* 128:2958-2966 1991.
- Endo T, Aten RF, Wang F, et al: Coordinate induction and activation of metallo-proteins and ascorbate depletion in structural luteolysis. *Endocrinology* 133:690-698, 1993.
- Halliwell B, Gutteridge JMC: *Free Radicals in Biology and Medicine*. Oxford; Oxford University Press, 1989.
- Bagavandoss P, Kunkel S, Wiggins R; et al: Tumor necrosis factor- α (TNF- α) production and localization of macrophages and T lymphocytes in the rabbit corpus luteum. *Endocrinology* 122:1185, 1988.
- Sawada M, Carlson JC: Superoxide radical production in plasma samples from regressing corpora lutea. *Can J Physiol Pharmacol* 67:465-471, 1989.
- Paszkowski T, Clarke RN: Generation of reactive oxygen species by human preovulatory granulosa cells. *Ginekol Pol* 67:990-1107, 1996.
- Tourney I, Devroey P, Camus M, et al: Comparison of in vitro fertilization in male and tubal infertility; a 3 year survey. *Hum Reprod* 7:218-222, 1992.
- Miller KF, Falcons T, Goldberg J, et al: Previous Fertilization failure with conventional in vitro fertilization is associated with poor outcome of intracytoplasmic sperm injection. *Fertil Steril* 41:242-245, 1998.
- Shekarriz M, Sharma RK, Thomas AJ, Jr, et al: Positive myeloperoxidase staining (Endtz test) as an indicator of excessive reactive oxygen species formation in semen. *J Assist Reprod Genet* 12:70-74, 1995.
- Aitken RJ, Clarkson JS: Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 81:159-169, 1987.
- Kolettis PN, Sharma RK, Pasqualotto FF, et al: Effect of seminal oxidative stress on fertility after vasectomy reversal. *Fertil Steril* 71:249-255, 1999.
- Aten RF, Duarte KM, Behrman HR: Regulation of ovarian antioxidant vitamins, reduced glutathione, and lipid peroxidation by luteinizing hormone and prostaglandin $F_{2\alpha}$. *Biol Reprod* 46:401-407, 1992.
- Wang Y, Sharma RK, Falcone T, et al: Importance of reactive oxygen species in the peritoneal fluid of women with endometriosis or idiopathic infertility. *Fertil Steril* 68:826-830, 1997.
- Hubel CA: Dyslipidemia, iron, and oxidative stress in preeclampsia: assessment of maternal and fetoplacental interactions. *Semin Reprod Endocrinol* 16:75-92, 1998.
- Doyle MB, Prestoin SL, Gatzuili E, Behrman HR: Oxygen radicals and antioxidants in follicular and oocyte function (Abstract). Society of Gynecologic Investigation, 1990.
- Miyazaki T, Sueoka K, Dharmnarajan A, et al: Effect of inhibition of oxygen free radical on ovulation and progesterone production by the in-vitro perfused rabbit ovary. *J Reprod Fertil* 91:207-212, 1991.
- Shapiro BM: The control of oxidant stress at fertilization. *Science* 252:523-536, 1991.
- Mliesel R, Drzejczak PJ, Kurpisz M: Oxidative stress during the interaction of gametes. *Biol Reprod* 49:918-923, 1993

24. Tarrin J: Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Mol Hum Reprod* 2:717-724, 1996.
25. Laloraya MI, Kumar GP, Laloraya M: Changes in the levels of superoxide anion radical and superoxide dismutase during the estrous cycle of *Rattus norvegicus* and induction of superoxide dismutase in rat ovary by lutropin. *Biochem Biophys Res Commun* 157:146-153, 1988.
26. Mattison DR, Shiromizu K, Pendergrass JA, et al: Ontogeny of ovarian glutathione and sensitivity to primordial oocyte destruction by cyclophosphamide. *Pediatr Pharm* 3:49-55, 1983.
27. Chew BP, Holpuch DM, O'Fallon TV: Vitamin A and beta carotene in bovine and porcine plasma, liver, corpora lutea, and follicular fluid. *J Dairy Sci* 67:1316-1322, 1984.
28. Jozwik M, Jr, Wolczynski S; Jozwik VI, Szamatowicz IM: Annual Meeting of the American Society for Reproductive Medicine, Abstract #P.090, 1998
29. Lachpelle MH, Hemmings R, Roy DC, et al: Flow cytometric evaluation of leukocyte subpopulations in the follicular fluids of infertile patients. *Fertil Steril* 65:1135-1140, 1996.
30. Hendin BN, Kolettis PN, Sharma RK, et al: Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *J Urol* 161:1831-1834, 1999.

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