

ANTIOXIDANTS

The role of enzymatic antioxidants detected in the follicular fluid and semen of infertile couples undergoing assisted reproduction

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Abstract

The follicular fluid environment surrounding the oocytes may play a critical role in fertilization and subsequent embryo development. The goal of our study was to evaluate the oxidative stress markers in the semen, blood serum, and follicular fluid of couples undergoing Intracytoplasmic Sperm Injection (ICSI). Two hundred and eight infertile couples underwent ICSI and the levels of superoxide dismutase (SOD) and catalase and lipid peroxidation (LPO) were evaluated. Semen Catalase was highly correlated with fertilization and cleavage rates, but not with pregnancy rates. Fertilization and cleavage rates were correlated with the levels of SOD and Catalase in the follicular fluid. After adjusting for age, a negative correlation was detected between LPO levels in follicular fluid and pregnancy rates. Follicular fluid LPO levels may be a marker as a metabolic activity within the follicle need for establishing a pregnancy.

Keywords: Follicular fluid, oocyte, sperm antioxidants, oxidative stress

Introduction

Free Radicals are involved in the physiology of reproduction (Agarwal & Said, 2005) and may be defined as any molecule that has one or more unpaired electrons (Aitken, 1999). Oxygen radicals, such as the superoxide anion ($O_2^{\bullet-}$), the hydroxyl radical (OH^{\bullet}), hydrogen peroxide (H_2O_2) and hypochlorite radical ($OHCl^{\bullet}$) and the peroxy radical (ROO^{\bullet}), comprise the highly reactive group of oxygen species called reactive oxygen species (ROS) (Sharma et al., 1999; Pasqualotto et al., 2000). Also included in this group are the reactive nitrogen radicals, such as nitric oxide (NO^{\bullet}) and nitric dioxide (NO_2^{\bullet}) free radicals.

Oxidative stress is caused by an imbalance between the production of ROS and antioxidant capacity (Sharma et al., 1999; Pasqualotto et al., 2000; 2001). Although the controlled generation of ROS may have physiological functions as signaling molecules (second messengers) in many different cell types, their uncontrolled production is considered an important factor in embryo toxicity (Agarwal et al.,

2003, 2004, 2006). Lipid peroxidation (LPO) is a normal phenomenon that occurs continuously at low levels in all humans (Agarwal et al., 2005). Uncontrolled peroxidation can damage enzymes, lipids, proteins, and cell membranes, and results in cell injury and death (Agarwal & Said, 2005).

Studies have shown that 40–88% of nonselected infertile patients have high levels of seminal ROS (Pasqualotto et al., 2000; Agarwal & Said, 2005). The controlled generation of ROS in spermatozoa is associated with normal physiological functions (Sharma et al., 1999). Uncontrolled and excessive production of ROS, however, appears to have a significant role as one of the major factors leading to an infertile status (Agarwal et al., 2003, 2004). The role of ROS and antioxidants in relation to female reproductive function has, in contrast, received relatively little attention, although there is some evidence of both physiological and pathological effects (Attaran et al., 2000; Pasqualotto et al., 2004; Das et al., 2006; Oral et al., 2006).

One of the several consequences of ROS production in the ovary is damage to the plasma membranes

through LPO of polyunsaturated fatty acids (Pasqualotto et al., 2004; Wiener-Megnazi et al., 2004; Das et al., 2006). Studies on the role of ROS and LPO have been carried out in rat luteal cells during luteolysis (Loutradis et al., 1987; Jozwik et al., 1999; Agarwal et al., 2005). Both follicles and corpora lutea are also known to be subject to ischemic episodes, which could lead to excess ROS formation (Agarwal et al., 2003).

The follicular fluid environment surrounding the oocytes may also play a critical role in fertilization and subsequent embryo development (Ho et al., 1998; Yang et al., 1998; Carbone et al., 2003; Oyawoye et al., 2003). The oocyte resides in a metabolically active environment consisting of steroid hormones, growth factors, cytokines, granulosa cells, and leukocytes (Agarwal et al., 2004). 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 not clear. Das et al. (2006) suggested that high levels of ROS in follicular fluid obtained from women with tubal infertility tend to decrease the fertilization potential of oocytes. A higher percentage of embryo formation as well as good embryo quality has been reported to be associated with follicular fluid with ROS levels <100cps. This result contradicts that of Attaran et al. (2000) and Pasqualotto et al. (2004) where interference with pregnancy outcome by low ROS is suggested.

In fact, the key question of oxidative stress-induced DNA damage is especially relevant during the intracytoplasmic sperm injection (ICSI) procedure although the interaction between the spermatozoa and the oocytes may require certain levels of ROS (Alvarez, 2003; Agarwal et al., 2005, 2004; Eskenazi et al., 2005). Since a relationship between markers of oxidative stress and both gametes (sperm and oocyte) is lacking in the literature, the goal of our study was to evaluate superoxide dismutase (SOD), catalase and LPO levels in the semen, serum and follicular fluid of couples undergoing assisted reproduction techniques.

Materials and methods

Subjects

Review Board and patient consent was obtained. Between January 2006 and December 2008, 208 consecutive patients undergoing *in vitro* fertilization (IVF) with ICSI were enrolled. Of these 208 patients, 113 underwent IVF/ICSI because of female factor infertility, 76 because of male factor infertility and 19 due to idiopathic infertility. The main indications for IVF were endometriosis ($n=73$; 35.1%), patients who underwent a varicocele, and

remained infertile and had semen abnormalities ($n=38$; 18.3%), tubal disease ($n=20$; 9.62%), and idiopathic infertility ($n=19$; 9.13%). Clinical variables such as age, number of oocytes recovered, and IVF outcomes (fertilization, cleavage, and pregnancy) were recorded for each patient.

In vitro fertilization, intracytoplasmic sperm injection, and embryo transfer

All 208 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, Puregon, Organon Laboratories, Randolph MA). Serial pelvic ultrasonograms and estradiol levels were obtained throughout the stimulation to individualize the amount of FSH necessary. Human chorionic gonadotropin (hCG) (Ovidrel, Serono, Randolph, MA) was administered when at least two follicles had attained a minimum mean diameter of 20 mm.

Patients underwent oocyte retrieval 36 h after hCG by transvaginal ultrasound guided follicular aspiration. 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 oocytes was excluded from the analysis. Specimens that were contaminated with blood were discarded. Prior measurements were obtained of the remaining amounts of fluid in the needle and found to be negligible.

The samples were taken to a laboratory and centrifuged at 300g for 7 min, and the clear supernatant was divided into aliquots and frozen at -70°C . All patients underwent ICSI. Embryo transfers were scheduled 3 days after oocyte retrieval. A maximum of three best quality embryos were transferred into the patient's uterus. Clinical pregnancy was defined as the presence of an intrauterine embryo with cardiac activity on transvaginal ultrasound.

Superoxide dismutase and catalase

Levels of SOD and Catalase for each patient represent the average of three readings.

SOD and Catalase levels were determined with a spectrophotometer. The activity of the SOD was based on the adrenocromo concentration, resulting from adrenaline oxidation by the superoxide radical (Misra & Fridovich, 1972). One Unit of SOD was defined as the volume (μl) of the enzyme able to inhibit 50% of the reaction resulting in adrenocromo and results were expressed as USOD/g. Catalase

activity was determined by the rate of hydrogen peroxide consumed (Maehly & Chance, 1954). Values are expressed as micromoles of H₂O₂ consumed per milligram of protein and expressed by UCAT/mg.

Lipid peroxidation

A 50 µl aliquot of the follicular fluid was used to measure the levels of LPO using the thiobarbituric acid method (Wang et al., 1997). The malondialdehyde (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.

Statistical analysis

The variables were log transformed to normalize the data distribution and analysis of variance was used to compare the mean values. A *P* value of <0.05 was considered statistically significant, and summary statistics are presented as mean ± standard error. Data were analyzed with the SAS statistical software package (SAS Institute, Cary, NC).

Results

Semen Catalase was highly correlated with fertilization ($r=0.11$; $p=0.01$) and cleavage rates ($r=0.23$; $p<0.001$), but not with pregnancy rates ($r=0.01$; $p=0.89$) (Table I). Semen SOD did not correlate with fertilization, cleavage, or pregnancy rates.

The presence of SOD, Catalase, and LPO levels were determined in 932 follicles from 208 patients. Of those, 781 (83.8%) were mature. The mean and the standard deviation for SOD (4.45 ± 1.4) and Catalase (1.7 ± 0.28) in the follicular fluid were higher than the levels in blood serum (3.6 ± 0.5 ; $p=0.04$, and 1.1 ± 0.13 ; $p=0.03$, respectively).

Fertilization rate was correlated SOD in follicular fluid ($r=0.12$; $p=0.01$) (Figure 1), Catalase in follicular fluid ($r=0.1$; $p=0.03$), Catalase in serum

($r=0.12$; $p=0.03$), and negatively correlated with follicular fluid LPO levels ($r=-0.16$; $p<0.001$).

Cleavage rates were correlated with SOD in the follicular fluid ($r=0.1$; $p=0.03$), Catalase in the follicular fluid ($r=0.14$; $p=0.01$), serum SOD levels ($r=0.14$; $p=0.02$), and negatively correlated with LPO levels in follicular fluid ($r=-0.19$; $p=0.001$).

After adjusting for age, a negative correlation was detected between the LPO levels in follicular fluid ($r=-0.21$; $p<0.001$) (Figure 2) and in blood serum ($r=-0.17$; $p=0.01$) (Figure 3) with the pregnancy rates.

Discussion

The controlled generation of ROS has a physiological role in spermatozoal functions such as hyperactivation, capacitation, and acrosome reaction (Aitken, 1999; Sharma et al., 1999; Pasqualotto et al., 2000; 2001; Agarwal & Said, 2005; Agarwal et al., 2006). Increased levels of ROS have been

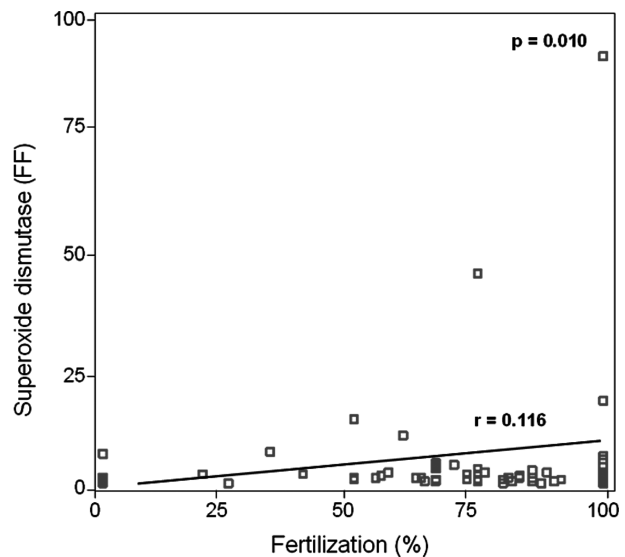


Figure 1. Correlation between superoxide dismutase in the follicular fluid and fertilization rates.

Table I. SOD, Catalase and LPO levels (mean ± SD) in patients undergoing assisted reproductive treatments.

Variables (mean ± SD)	Fertilization	Cleavage	Pregnancy
Semen SOD (USOD/g) 4.12 ± 1.21	$r=0.05$; $p=0.32$	$r=0.04$; $p=0.38$	$r=0.05$; $p=0.31$
Semen Catalase (UCAT/mg) 1.34 ± 0.53	$r=0.11$; $p=0.01$	$r=0.23$; $p<0.001$	$r=0.01$; $p=0.89$
FF SOD (USOD/g) 4.45 ± 1.41	$r=0.12$; $p=0.01$	$r=0.1$; $p=0.03$	$r=0.02$; $p=0.66$
FF Catalase (UCAT/mg) 1.73 ± 0.28	$r=0.1$; $p=0.03$	$r=0.14$; $p=0.01$	$r=-0.08$; $p=0.08$
FF LPO (µmol MDA/l) 2.36 ± 0.15	$r=-0.16$; $p<0.001$	$r=-0.19$; $p<0.001$	$r=-0.21$; $p<0.001$
Serum SOD (USOD/g) 3.62 ± 0.53	$r=-0.01$; $p=0.85$	$r=0.14$; $p=0.02$	$r=-0.01$; $p=0.86$
Serum catalase (UCAT/mg) 1.12 ± 0.13	$r=0.12$; $p=0.03$	$r=-0.07$; $p=0.21$	$r=0.01$; $p=0.84$
Serum LPO (µmol MDA/l) 4.25 ± 0.47	$r=-0.15$; $p=0.78$	$r=0.27$; $p=0.18$	$r=-0.17$; $p=0.01$

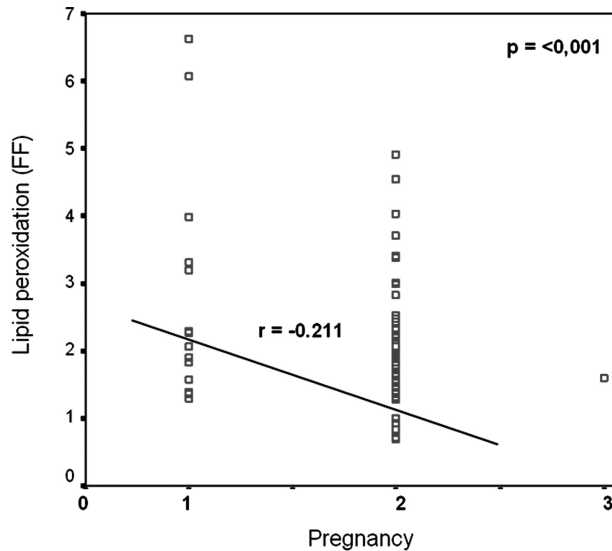


Figure 2. Correlation between lipid peroxidation in the follicular fluid and pregnancy rates.

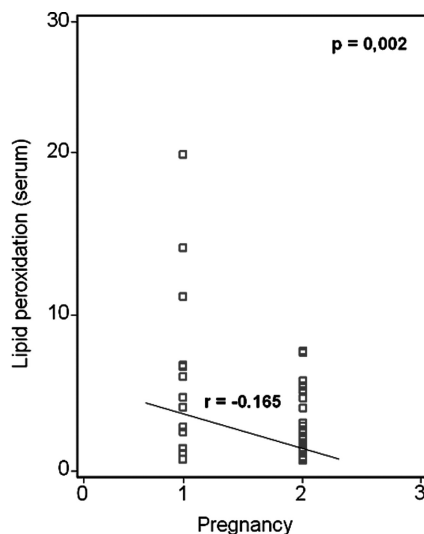


Figure 3. Correlation between lipid peroxidation in the serum and pregnancy rates.

found in the semen of infertile men (Sharma et al., 1999; Pasqualotto et al., 2001). ROS can cause an increase in DNA fragmentation, and pretreatment with antioxidants that dispose, scavenge, and suppress the formation of ROS, can reduce DNA damage (Pasqualotto et al., 2000; Agarwal et al., 2004; Eskenazi et al., 2005). In addition, it has been demonstrated that infertile men empirically treated with antioxidants may exhibit improved semen characteristics, increasing the pregnancy rates following IVF (Eskenazi et al., 2005).

Interestingly, in our study, we did not find a correlation between antioxidants and oocyte fertilization, cleavage, and pregnancy rates. Also, we did not find a correlation between LPO levels and oocyte

fertilization, cleavage, and pregnancy rates. We think that these markers of oxidative stress may contribute to a woman's ability to establish a pregnancy following sexual intercourse and that after semen processing for ICSI these enzymatic markers present in the seminal plasma do not interfere with oocyte fertilization and pregnancy rates.

ROS are produced by the ovary and are regulated enzymatically to generate products necessary for ovulation and luteolysis (Sabatini et al., 1999; Agarwal et al., 2004). Follicular fluid ROS levels may represent physiological ranges of ROS necessary for the normal development of the oocyte and subsequent embryo (Attaran et al., 2000; Agarwal et al., 2003, 2006; Pasqualotto et al., 2004). Unfortunately, the lack of a reference value in normal healthy women (unstimulated ovarian cycles) makes it difficult to determine if the ROS levels observed in follicular fluid are in the physiological or pathological range. As in many other systems, a physiological amount of ROS may be indicative of healthy developing oocytes, whereas excessively high levels may be indicative of oxidative stress (Das et al., 2006).

In the human, Paszkowski and Clarke (1996) found decreased levels of SeGPx in follicular fluid of women with unexplained infertility. In the same study, SeGPx levels were higher in follicles yielding oocytes that subsequently were successfully fertilized compared with those from follicles whose oocytes failed to fertilize. Yang et al. (1998) found higher levels of hydrogen peroxide in fragmented compared with nonfragmented embryos and unfertilized oocytes, whereas others (Paszkowski et al., 1995) reported increased antioxidant consumption (suggesting increased ROS activity) during incubation of poor quality embryos. On the other hand, Attaran et al. (2000) reported a beneficial role of ROS, with higher levels in follicular fluid in IVF conception cycles compared with nonconception cycles. The authors did not, however, find any association between total antioxidant capacity (TAC) and conception.

The source of ROS in the follicular fluid is unknown (Agarwal et al., 2003, 2006). The follicular fluid environment is composed of the oocyte, granulosa cells, and surrounding cells such as endothelial and thecal cells (Carbone et al., 2003). Because the theca interna is quite vascularized there is the potential for transudation of factors from the circulation into the follicular fluid (Agarwal et al., 2003, 2006). Preantral oocytes are metabolically active and synthesize RNAs such as epidermal growth factor (Agarwal et al., 2003, 2005, 2006). A metabolically active system is likely to produce ROS and be susceptible to oxidative stress (Agarwal et al., 2003, 2006). In addition, follicular fluid is known to contain cytokines, neutrophils, and

macrophages, all of which can produce oxygen free radicals (Pasqualotto et al., 2004). Within the ovary are mechanisms for detoxification and protection against ROS (Attaran et al., 2000; Pasqualotto et al., 2004).

The role of oxidative stress in female infertility is unclear. It is possible that abnormal amounts of ROS in follicular fluid may indicate subnormal oocyte quality (Agarwal et al., 2003; 2006). Oyawoye et al. (2003) found no evidence of any relationship between follicular antioxidant activity and the presence of oocytes in follicular fluid, with baseline TAC and antioxidant consumption. Also, the authors found that lower levels of TAC predicted decreased fertilization potential. Paszkowski et al. (1995) demonstrated that lower levels of SeGPx were associated with failed fertilization in the human. This, however, conflicts with the results of a study by Sabatini et al. (1999) in which higher levels of SOD activity were present in fluid from follicles whose oocytes did not fertilize compared with follicular fluid whose oocytes did fertilize. These results also conflict with the findings of Paszkowski and Clarke (1996) who found that increased ROS activity was associated with impaired embryo development. The reasons for this discrepancy are also not clear. It appears that ROS may have different effects at different stages of embryo development. The results demonstrated that women who became pregnant had higher levels of ROS than those who did not, leading the authors to suggest that ROS, at low concentrations, may be a potential marker for predicting the success of IVF patients (Sabatini et al., 1999; Pasqualotto et al., 2004). On the other hand, Ho et al. (1998) in genetic manipulation studies in mice, found that mice made deficient in SOD had both decreased litter size and decreased number of litters per month (Oyawoye et al., 2003). In our study, we observed a good correlation between the follicular fluid enzymatic antioxidants evaluated (SOD and Catalase) with fertilization and cleavage rates, but not with the pregnancy rates. Therefore, these markers may reflect oocyte quality and the initial embryo quality, but may not reflect the most important clinical outcome: the pregnancy rate.

There are no clinical studies demonstrating the presence of follicular fluid LPO in normal healthy women in unstimulated cycles. Thus, the LPO levels measured in normal women exposed to high-dose gonadotrophins 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 LPO levels. Antioxidants scavenge ROS to protect the environment from oxidative damage (Schweigert et al., 2000; Agarwal et al., 2004). In a very elegant study, it has been demonstrated that oxidative stress causes toxic effects on

oocyte maturation and melatonin protects oocytes from oxidative stress. Melatonin is likely to improve oocyte quality and fertilization rates (Tamura et al., 2008). Also, oxidative stress has an impact on the production of granulosa cell steroid hormones, in particular E(2), which is an important predictor of ovarian response. The positive association between E(2) present in follicular fluid and TAC suggests that E(2) may have a possible role in the ovarian antioxidant-oxidant balance (Appasamy et al., 2008). In the male, depressed levels of total antioxidants are seen in the semen of infertile patients with elevated LPO levels (Pasqualotto et al., 2000; 2001).

Several limitations of our study must be considered. First, the follicular fluid LPO levels from women undergoing superovulation may differ from those in women experiencing unstimulated cycles. Second, while LPO values were obtained from individual follicles, the reported LPO levels represent the mean of several follicular fluid specimens from each patient. Third, the pregnancy outcome was not linked to the results of a single oocyte. However, the LPO levels had a high negative correlation with pregnancy rates. Because all the oocytes from a given patient were coincubated, we could not determine the fertilization status of any individual oocyte to correlate it with its corresponding LPO level. Future studies will attempt to compare a single follicular fluid LPO level with fertilization of the follicle's oocyte, subsequent embryo development and the resulting pregnancy.

In summary, the role of ROS in relation to human reproduction remains unclear. Further studies to clarify their physiological and pathological roles and their relationship to female reproduction should be undertaken, as they could lead to the development of novel strategies for fertility regulation in the human.

Acknowledgement

Support: PQ 10/2005, processo 300670/2005-2 Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Oxidative stress in male infertility, prostate and testicular cancer, Process no: 409651/2006-0 Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

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