

Review

Oxidative stress and its implications in female infertility – a clinician's perspective



Dr Ashok Agarwal is the Director of Research at the Centre for Advanced Research in Human Reproduction, Infertility, and Sexual Function, and the Director of the Clinical Andrology Laboratory and Reproductive Tissue Bank. He holds these positions at The Cleveland Clinic Foundation, where he is a Professor in the Lerner College of Medicine of Case Western Reserve University, and, since 1993, full staff in the Glickman Urological Institute, Departments of Obstetrics–Gynecology, Anatomic Pathology, and Immunology. Dr Agarwal has published extensively with over 225 original peer reviewed articles, 20 book chapters, and over 500 presentations at scientific meetings. His research is focused on studies of the role of oxidative stress, DNA integrity, and apoptosis in the pathophysiology of male and female reproduction.

Dr Ashok Agarwal

Ashok Agarwal¹, Sajal Gupta, Rakesh Sharma

Centre for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Department of Obstetrics–Gynecology and Glickman Urological Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Desk A19.1, Cleveland, OH 44195, USA

¹Correspondence: Fax: +1 216 4456049; e-mail: agarwaa@ccf.org

Abstract

Reactive oxygen species (ROS) have a role in the modulation of gamete quality and gamete interaction. Generation of ROS is inherent in spermatozoa and contaminating leukocytes. ROS influence spermatozoa, oocytes, embryos and their environment. Oxidative stress (OS) mediates peroxidative damage to the sperm membrane and induces nuclear DNA damage. ROS can modulate the fertilizing capabilities of the spermatozoa. There is extensive literature on OS and its role in male infertility and sperm DNA damage and its effects on assisted reproductive techniques. Evidence is accumulating on the role of ROS in female reproduction. Many animal and human studies have elucidated a role for ROS in oocyte development, maturation, follicular atresia, corpus luteum function and luteolysis. OS-mediated precipitation of pathologies in the female reproductive tract is similar to those involved in male infertility. OS influences the oocyte and embryo quality and thus the fertilization rates. ROS appears to play a significant role in the modulation of gamete interaction and also for successful fertilization to take place. ROS in culture media may impact post-fertilization development, i.e. cleavage rate, blastocyst yield and quality (indicators of assisted reproduction outcomes). OS is reported to affect both natural and assisted fertility. Antioxidant strategies should be able to intercept both extracellular and intracellular ROS. This review discusses the sources of ROS in media used in IVF–embryo transfer and strategies to overcome OS in oocyte in-vitro maturation, in-vitro culture and sperm preparation techniques.

Keywords: assisted reproduction, female infertility, oxidative stress, reactive oxygen species

Introduction

Oxidative stress (OS) affects the quality of gametes and the way in which they interact. Free radicals such as reactive oxygen species (ROS) influence oocytes, spermatozoa, and embryos and their environments. The microenvironments associated with follicular fluid, hydrosalpingeal fluid and peritoneal fluid have a direct bearing on oocyte quality, sperm–oocyte interaction, sperm-mediated oocyte activation, implantation, and early embryo development. OS affects early embryo development and implantation, which in turn affects pregnancy rate.

Infertility is a problem with a large magnitude. OS has been investigated as a causative factor. There is some evidence that OS

has a role in endometriosis as well as in tubal and peritoneal factor and unexplained infertility. This article focuses on evaluating the current evidence for the role of ROS in the normal functioning of the female reproductive tract and also on studies evaluating their role in infertility and assisted reproduction. The manner in which OS influences the successful outcomes of assisted reproduction is also discussed, along with strategies for overcoming OS in infertility and assisted reproduction.

Oxidative stress

Aerobic metabolism is associated with the generation of ROS (pro-oxidants) (Agarwal and Allamaneni, 2004a,b). ROS include the hydroxyl radicals, superoxide anion, and hydrogen

peroxide. There is a diverse range of antioxidants that limit the production of ROS, scavenge them, and repair cell damage. There is a complex interaction between the pro-oxidants and antioxidants that results in the maintenance of intracellular homeostasis. An imbalance between the pro-oxidants and antioxidants results in OS.

A complex interplay of cytokines, hormones, and other stressors results in the generation of free radicals. Free radicals further act through the modulation of gene expression and transcription factors (Harvey *et al.*, 2002; Dennery, 2004). Reactive oxygen species have a physiological and pathological role in the reproductive tract. They are key signal molecules modulating various reproductive functions such as oocyte maturation, folliculogenesis, tubal function and cyclical endometrial changes. Interventions designed to overcome OS *in vivo* and *in vitro* are also discussed in this paper.

Assisted reproductive techniques

There have been a number of dramatic developments in assisted reproduction since its introduction 25 years ago. A variety of causative factors for infertility can be indications for assisted reproduction, i.e. tubal factor infertility, endometriosis, male factor infertility and unexplained infertility (Braude and Rowell, 2003; Isaksson and Tiitinen, 2004). Assisted reproductive technologies offer excellent opportunities to infertile couples for achieving pregnancy.

A majority of the retrieved oocytes become fertilized, and up to 70% of these undergo the first three cleavage divisions during the first 3 days of culture. Less than 50% of the cleaved embryos undergo cavitation to form blastocysts by day 5 *in vitro* culture (Gardner *et al.*, 2000). Blastocyst development *in vitro* lags behind blastocyst development *in vivo*.

Free radicals are generated as a result of embryo metabolism. Generation of OS in *in vitro* culture media may have detrimental effects on post-fertilization development and assisted reproduction outcomes. There may be multiple sources of ROS in an IVF setting, including the oocytes, cumulus cell mass and spermatozoa used for insemination (Bedaiwy *et al.*, 2004). ROS generated in IVF media can cause detrimental effects on mitochondria (Comporti, 1989), DNA, RNA and sperm–oocyte fusion (Aitken *et al.*, 1993).

ROS and the follicle

Oxidative stress plays a role in the physiology of ovarian function. The follicular fluid microenvironment is a biological window into the quality of the oocyte and the subsequent embryo that is generated. Phagocytic macrophages, parenchymal steroidogenic cells and endothelial cells generate ROS in the ovaries (Halliwell and Gutteridge, 1988). Different investigators have found that ROS are involved in folliculogenesis, follicle maturation, ovulation and corpus luteal function (Tamate *et al.*, 1995; Sugino *et al.*, 1996; Jozwik *et al.*, 1999; Sabatini *et al.*, 1999). Cells involved in steroidogenesis such as theca cells, granulosa lutein cells, and hilus cells show stronger oxidative enzyme activity (Scully and Cohen, 1964). The expression of various biomarkers of OS has been demonstrated in normally cycling human ovaries (Shiotani *et al.*, 1991; Suzuki *et al.*,

1999). Expression of Cu–Zn superoxide dismutase (SOD) and Mn-SOD has been detected by immunohistochemical staining (Tamate *et al.*, 1995). The follicular fluid microenvironment is important for follicular maturation and granulosa cell functions. Reduced concentrations of glutathione peroxidase, a protective enzyme, may affect the fertilizing ability of the gametes (Paszowski *et al.*, 1995). Down-regulation of glutathione peroxidase in the follicular fluid is significantly associated with low fertilization rates. Smoking also significantly reduced glutathione peroxidase concentrations in the liquor folliculi. The mRNA expression of enzymatic antioxidants such as Cu–Zn-SOD, Mn-SOD, catalase, glutathione peroxidase and gamma-glutamyl synthetase were studied in human oocytes. Cu–Zn-SOD was particularly well expressed in the metaphase-II oocyte. Mn-SOD and glutathione peroxidase transcripts were expressed in the germinal vesicle stage and the metaphase II stage, suggesting that these enzymes are markers for oocyte maturation (El Mouatassim *et al.*, 1999). The expression of these enzymes indicates that the oocytes are exposed to OS and the enzymatic antioxidants act as catalysts in neutralizing the ROS. The expression of Cu-Zn-SOD and Mn-SOD is closely related to steroidogenesis in the human ovary (Suzuki *et al.*, 1999). The production of a viable oocyte is modulated by a complex interaction of endocrine, paracrine and autocrine factors, leading to follicular maturation, granulosa cell maturation, ovulation and luteinization.

Nitric oxide (NO) is a local factor involved in the autocrine and paracrine modulation of ovarian folliculogenesis and steroidogenesis. At low concentrations (<1 $\mu\text{mol/l}$), the transduction effect of NO is mediated by activation of soluble guanyl cyclase and mediated through cyclic guanosine monophosphate (cGMP) (Rosselli *et al.*, 1998; Hanafy *et al.*, 2001). The role of NOS has been determined by NO synthase expression or by plasma nitrate concentrations. Studies suggest that concentrations of follicular NO increase in the secretory phase and peak at mid-cycle (Lee *et al.*, 2000). Low concentrations of NO in follicular fluid were associated with follicles containing mature oocytes that eventually became fertilized (Barrionuevo *et al.*, 2000). Follicular fluid NO concentrations have been negatively associated with embryo quality and rate of cleavage. Serum NO concentrations were elevated in patients with tubal factor or peritoneal factor infertility (Bedaiwy *et al.*, 2004). Higher concentrations of NO are associated with implantation failure, resulting in lower pregnancy rates. NO may induce apoptosis, resulting in embryo fragmentation.

Oxidative stress may affect theca-interstitial cells by inducing their proliferation and growth. This proliferation was demonstrated to be dose dependent *in vitro* in rat theca interstitial cells (Duleba *et al.*, 2004). Higher doses of OS inhibited the proliferation of theca-interstitial cells. Oxidative stress may also induce proliferation of ovarian mesenchymal cells in patients with polycystic ovarian syndrome. The effect OS has on both types of cells needs to be investigated further.

ROS and the corpus luteum

Antioxidant enzymes such as SOD are expressed cyclically in steroid-producing cells. ROS therefore plays a role in the formation of the corpus luteum and steroidogenesis.

Expression of Mn-SOD and Cu-Zn-SOD was positive in luteinized granulosa cells and theca cells. SOD has also been demonstrated in steroid-producing cells, implying that ROS has a role in steroidogenesis. Cu-Zn-SOD expression peaks from the early to mid-secretory phase, and this parallels progesterone production by the corpus luteum. Thus, Cu-Zn-SOD has a protective role in the maintenance of the corpus luteum (Sugino *et al.*, 1996). Mn-SOD expression peaks towards the late luteal phase, suggesting its role in luteal regression. Highest concentrations of OS markers were expressed in the corpora lutea in mid-cycle and were related to steroidogenic synthesis, which is maximal at this stage (Vega *et al.*, 1995). Cytokines influence the induction of Mn-SOD, which is considered to be the inducible form of SOD.

The endothelial form of NO synthase was the most abundant form of NO synthase expressed in the corpus luteum, and expression was highest in the late luteal phase (Friden *et al.*, 2000; Preutthipan *et al.*, 2004). Concentrations of prostaglandin (PG) F₂ α and prostaglandin PGE₂ increased significantly *in vitro* during the late luteal phase, suggesting a role in luteolysis. Elevated peroxidation concentrations in the follicle may be detrimental to oocyte maturation (Jozwik *et al.*, 1999). Concentrations of conjugated dienes, lipid hydroperoxides and thiobarbituric acid reactive substances in preovulatory follicular fluid were found to be significantly lower than the serum concentrations, suggesting a concentration gradient.

ROS and the endometrium

Expression of endothelial and inducible NO synthase (eNOS and iNOS) has been demonstrated in the human endometrium (Tseng *et al.*, 1996) and in endometrial vessels (Taguchi *et al.*, 2000). Endothelial NO synthase, originally identified in vascular endothelial cells, is also distributed in glandular surface epithelial cells in the human endometrium. NO also regulates the microvasculature of the endometrium and is important for the phenomenon of menstruation. Expression of eNOS mRNA has also been detected in the mid-secretory phase and late-secretory phase, suggesting a role in the decidualization of the endometrium. ROS brings about changes in the endometrium that prepare it for implantation.

Oxidative stress and infertility

Infertility affects about 6 million American women and their partners, or about 10% of the US population. Infertility is defined as 'the inability to conceive following 12 months of unprotected sexual intercourse, before an investigation is undertaken unless medical history and physical findings dictate earlier evaluation and treatment' (ASRM, 2004). Data from the National Survey for Family Growth (NSFG) indicate that the number of women with impaired fecundity has grown from 4.6 million in 1982 to 6.2 million in 1995, an increase of 26% (Abma *et al.*, 1997). Although the frequency and origin of different forms of infertility varies, 40–50% of cases are due to female causes (Duckitt, 2003), whereas 30% of cases are due to male causes. Unexplained infertility affects another 15% of couples (Eskandari, 2003). Although infertility is a common problem, treatment is sometimes inadequate because the aetiology is not fully understood (Agarwal and Allamaneni, 2004). The absolute number of couples seeking infertility services has increased

dramatically. Each year, approximately 1.3 million American couples receive medical advice or treatment (ASRM, 2004). Assisted reproductive techniques have allowed many infertile couples to realize their dream of having biological offspring.

Oxidative stress affects both natural and assisted fertility (Agarwal *et al.*, 2004b). Oxidative stress biomarkers have been found in various sites in the female reproductive tract, suggesting their role in various physiological functions. Other studies have suggested that ROS are involved in various causative factors of infertility, i.e. peritoneal factor, tubal factor, endometriosis and unexplained infertility. The scientific basis of unexplained infertility remains a challenge, and OS may have a role in its pathophysiology.

The role of OS in infertility is not completely ascertained. A number of studies have evaluated the role of OS in tubal factor infertility, endometriosis and peritoneal factor infertility (Ho *et al.*, 1997; Murphy *et al.*, 1998; Ota *et al.*, 1998; Dong *et al.*, 2001; Polak *et al.*, 2001; Van Langendonck *et al.*, 2002; Szczepanska *et al.*, 2003). The tubal and peritoneal microenvironments influence fertilization and early embryonic development. Elevated concentrations of ROS in these environments may have detrimental effects on the spermatozoa, oocytes, sperm oocyte interaction and embryos both in the Fallopian tube and the peritoneal cavity (Agarwal *et al.*, 2003). Activated macrophages have been implicated in the pathogenesis of endometriosis. These macrophages are the source of increased generation of ROS in the peritoneal environment associated with endometriosis.

OS and endometriosis

Many studies have reported both an increased number and activation of macrophages in the peritoneal cavity in patients with endometriosis (Szczepanska *et al.*, 2003). Macrophages may be the source of increased generation of ROS. Elevated levels of lipid peroxidation were also reported in peritoneal fluid of patients with endometriosis (Liu *et al.*, 2001). No significant difference was reported in the total antioxidant status or products of NO metabolism in the peritoneal fluid of patients with endometriosis (Ho *et al.*, 1997). NO synthase activity *in vitro* was higher in peritoneal macrophages isolated from women with endometriosis (Osborn *et al.*, 2002; Wu *et al.*, 2003). Expression of inducible NO synthase was higher in endometrial tissue from women with endometriosis than controls. In the peritoneal fluid, activated macrophages produce increased concentrations of cytokines and macrophage colony stimulating factor. Endothelial NO expression and SOD in the endometrium have been reported to increase in patients with endometriosis (Ota *et al.*, 1998, 1999).

SOD concentrations were significantly lower in the peritoneal fluid of women with endometriosis (Liu *et al.*, 2001; Szczepanska *et al.*, 2003). Contrary to these findings, other investigators found no difference in SOD concentrations in the peritoneal fluid from patients with endometriosis-associated with infertility, compared with women with idiopathic infertility. Bedaiwy *et al.* (2002) found evidence that serum interleukin (IL)-6 and peritoneal fluid tumour necrosis factor (TNF)- α concentrations could be used to distinguish patients with endometriosis from those without with a high

degree of sensitivity and specificity. ROS concentrations, on the other hand, were similar in peritoneal fluid of patients with endometriosis and disease-free controls (Bedaiwy and Falcone, 2003).

Evidence suggests that OS is present in the peritoneal cavity, and markers of increased lipid peroxidation, e.g. antibodies to the oxidized low-density lipoproteins, have been reported to be elevated in women with endometriosis (Murphy *et al.*, 1998; Shanti, 1999). Murphy *et al.* have demonstrated elevated modified lipoprotein complexes at the level of the endometrium. The literature lacks studies assessing a host of OS biomarkers in the peritoneal fluid and examining the individual antioxidants and the total antioxidant status. Future studies should therefore focus on using persistent biomarkers of OS to identify OS (Bedaiwy and Falcone, 2003).

OS and unexplained infertility

The pathophysiology of unexplained infertility remains a scientific challenge. Unexplained infertility may be caused by increased generation of ROS in the peritoneal cavity. ROS concentrations were higher in women undergoing laparoscopy for infertility evaluation than in women who underwent laparoscopic tubal ligation. Wang *et al.* (1997) found higher concentrations of ROS in women with idiopathic infertility in unprocessed and processed peritoneal fluid specimens, and these differences were statistically significant in the processed peritoneal fluid. Polak *et al.* found that the concentrations of antioxidants in patients with unexplained infertility were significantly lower than those in fertile patients (Polak *et al.*, 2001). Concentrations of malondialdehyde, a lipid peroxidation end product, in peritoneal fluid were higher in patients with unexplained infertility than in fertile women. Women with idiopathic infertility have reduced concentrations of antioxidants and increased ROS-induced lipid peroxidation damage resulting in infertility.

Clinical studies on OS and assisted reproduction

Two mitochondrial ATP synthesis enzymes, NADPH oxidase and xanthine oxidoreductase, are associated with the generation of ROS, mainly the superoxide radical. Oxygen is needed to generate energy for folliculogenesis and oocyte maturation, and ROS production is inherent in these processes. Early embryos from humans and mice have many similarities in in-vitro culture conditions. Both exhibit block in the activation of the embryonic genome in-vitro culture, and ROS may be instrumental in causing the embryo block. Current studies are focusing on the effects of growth factors, which are normally found in the Fallopian tubes and endometrium, to protect in-vitro cultured embryos from the detrimental effects of ROS. The factors being investigated are insulin-like growth factor I (IGF-I), and epidermal growth factor (EGF) in mouse embryos.

Lower levels of total antioxidant capacity (TAC) in the follicular fluid are predictive of decreased fertilization potential (Oyawoye *et al.*, 2003). Oyawoye's study was the first study to use ferric reducing antioxidant power assay to estimate TAC in follicular fluid. This study consisted of 303 follicular aspirates from 63

women. Lower levels of TAC were shown to be associated with increased viability of the embryos till the time of transfer. The fertilization potential decreased as TAC levels decrease.

Levels of SOD activity were higher in follicular fluid from oocytes that failed to fertilize (2.47 ± 0.41 IU/mg of protein) than in those that did [1.20 ± 0.09 ; ($P = 0.0001$) (Sabatini *et al.*, 1999)]. This may be due to the fact that Oyawoye *et al.* measured TAC, whereas Sabatini *et al.* measured SOD. A more comprehensive assessment of the redox status in follicle involves the assessment of both ROS and TAC levels in the follicular fluid. The ROS-TAC score, which utilizes principal component analysis of both ROS and TAC results, was found to be a better predictor of the overall OS affecting the spermatozoa (Sharma *et al.*, 1999).

ROS and TAC levels were measured by chemiluminescence assay (Attaran *et al.*, 2000). This study found that concentrations of ROS were lower in patients who did not become pregnant than in those who did. Thus, intrafollicular ROS concentrations may be used as a potential marker for predicting success with IVF. Further studies are required to determine normal TAC levels of the follicular fluid in unstimulated cycles.

The effects of follicular OS on oocyte maturation, fertilization and pregnancy were evaluated by Pasqualotto *et al.* (2004). In this study, 41 patients and 115 follicles aspirated for IVF or ICSI were examined. Patients who became pregnant had higher levels of lipid peroxidation (LPO) and TAC, although levels of LPO and TAC did not predict embryo quality. Pregnancy rates and levels of lipid peroxidation and TAC demonstrated a positive correlation.

An important determinant of IVF outcome is oocyte quality. DNA damage caused by OS can be reliably measured by 8-hydroxy-2-deoxyguanosine (Seino *et al.*, 2002). Higher concentrations of 8-hydroxy-2-deoxyguanosine were associated with lower fertilization rates and poor embryo quality (Seino *et al.*, 2002). High concentrations of 8-hydroxy-2-deoxyguanosine are also found in granulosa cells of patients with endometriosis, and this may impair the quality of oocytes.

Redox and early embryo development

Excessive generation of ROS may result from embryo metabolism involving xanthine oxidase pathway (Burton *et al.*, 2003). Physiological levels of redox are important for embryogenesis. Overgeneration of ROS can have detrimental effects on embryo development (Guerin *et al.*, 2001; Harvey *et al.*, 2002). In in-vitro conditions excessive generation of ROS leading to OS can compromise preimplantation embryo development. Specifically, the superoxide anion, hydrogen peroxide and hydroxyl radical can have detrimental effects on the embryo. ROS generation can result from oxidative phosphorylation occurring at the mitochondrial level when there is a leakage of electrons from the electron transport chain at the inner mitochondrial membranes. This electron gets transferred to the oxygen molecule, resulting in an unpaired electron in the orbit. ROS can also be produced by cytoplasmic NADPH-oxidase, cytochrome p450 enzymes and the xanthine oxidoreductase enzymes.

OS and abortion

Human reproduction is not a very efficient process. Before the end of the first trimester, 30–50% of conceptions result in abortions. Predominantly the loss occurs at the time of implantation. Fifteen to 20% of clinical pregnancies result in spontaneous abortions. Recurrent pregnancy loss (RPL) affects 0.5–3% of women in the reproductive age group. Fifty to 60% of RPL are idiopathic (Cramer and Wise, 2000). OS-induced damage has been postulated to have a role in spontaneous abortions, idiopathic RPL and fetal embryopathies.

Placental OS has been proposed to have a role in the pathophysiology of miscarriage and pre-eclampsia. The outcomes of miscarriage and pre-eclampsia may be a continuum of the underlying pathophysiological process of placental OS. OS is known to modulate key transcription factors and expression of genes in the embryo (Dennery, 2004). Elevated concentrations of ROS can lead to detrimental effects in the embryo. Increased embryonic cytoplasmic fragmentation and apoptosis have been reported as a consequence of OS (Yang *et al.*, 1998). Aberrant apoptosis in the blastocyst can lead to embryonic death or congenital anomalies in the fetus or abortions.

Implantation is a very well orchestrated dialogue between the embryo and the uterine environment. A burst of OS occurs with the establishment of maternal circulation (Jauniaux *et al.*, 2000). Deficient trophoblastic invasion results in an impaired dialogue between the embryo and its environment and is associated with abortion. Spontaneous abortion is probably accompanied by a significant disruption of the pro-oxidant and antioxidant balance.

A recent study proposed that OS modulates the expression of cytokine receptors expressed in the placenta, cytotrophoblasts, vascular endothelial cells and smooth muscle cells (Banerjee *et al.*, 2004). Elevated concentrations of the antioxidant glutathione in pregnant patients with a history of recurrent abortion were associated with higher rates of miscarriage (Miller *et al.*, 2000). Glutathione depletion leads to the inhibition of T helper cell (TH) 1 type cytokine response, and elevated levels result in TH1 cytokine expression. The T helper cells can polarize to produce two kinds of cytokines TH1 and TH2 (T helper cell 2) and both are modulated by glutathione (Peterson *et al.*, 1998). Lin *et al.* (1993) studied murine pregnancy and found evidence of the production of TH2 type cytokines at the maternal–fetal interface. Expression of TH1 type cytokines is associated with a poor prognosis, including miscarriage.

A growing body of evidence suggests that the redox state has a critical role in modulating implantation and affecting preimplantation embryonic growth. The redox state can influence natural and assisted conception, and it is important to reduce generation of ROS and increase the scavenging capacity of in-vitro media with antioxidant supplementation.

Role of ROS in gamete interaction

ROS are involved in the physiology of sperm–oocyte interaction. Both spermatozoa and oocytes generate ROS. Certain concentrations of ROS are essential for the normal

functioning of spermatozoa and for sperm–oocyte interaction (Aitken *et al.*, 1989). The production of ROS by the gametes is balanced by the antioxidants. If the protective effect of the antioxidants is less than the ROS generated, it results in OS. Excessive concentrations of OS can impair the functions of spermatozoa. Excessive generation of ROS in the semen is associated with lower fertilization rates in conventional IVF (Krausz *et al.*, 1992; Sukcharoen *et al.*, 1996). Concentrations of OS vary greatly in infertile men. Physiological concentrations of OS may be essential for the sperm–acrosome reaction and sperm–oocyte interaction (Aitken *et al.*, 1989). Excessive generation of ROS can lead to DNA damage in spermatozoa (Kodama *et al.*, 1997; Lopes *et al.*, 1998). Severe OS can lead to infertility because of the negative impact on fusion events such as acrosome reaction and sperm–oocyte fusion (Aitken *et al.*, 2003).

Strategies to overcome ROS

Many factors influence the interaction of spermatozoa and oocytes *in vivo* and *in vitro*. The intrinsic quality of the spermatozoa and oocytes are important determinants of assisted reproduction outcomes. Development of mammalian embryos can be retarded in the presence of unfavourable media conditions. Blastocyst development *in vitro* always lags behind blastocyst development *in vivo* (Boni *et al.*, 1999; Viuff *et al.*, 1999). The culture conditions subsequent to fertilization affect the blastocyst number, quality and blastocyst hatching. ROS in culture media affects fertilization, embryo development and clinical pregnancy rates. Elevated concentrations of ROS in day 1 culture media were associated with lower pregnancy rates both with IVF and ICSI (Bedaiwy *et al.*, 2004).

Treatment strategies should focus on oral supplementation with antioxidants or supplementing IVF media with antioxidants to overcome OS. These strategies also depend on whether the patient is undergoing IVF or ICSI, since generation of the ROS in IVF and ICSI are from different sources. Antioxidant supplementation of culture media can improve embryo development and decrease apoptosis. IVF media are supplemented with antioxidants that protect spermatozoa against DNA damage.

Strategies to overcome OS in IVF culture media

The success rates in IVF are influenced by maternal age, number of oocytes retrieved and the quality of the embryos transferred. The quality of the embryos is influenced by extrinsic factors like culture media. The role of IVF media in generating ROS and its damaging effects are complex not completely understood. When ROS is generated, it can diffuse into the cells and damage lipids, proteins, nucleic acids, DNA and RNA. There are a large number of extrinsic factors that modulate OS that can influence successful outcomes of IVF–embryo transfer. These are oxygen concentration, ionizing radiation and concentrations of antioxidants (**Table 1**). Ethyl enediaminetetraacetic acid (EDTA), low oxygen tension, SOD and catalase are the antioxidants involved in overcoming OS (Orsi and Leese 2001).

The potential cellular sources of ROS with conventional IVF

are different from those with intracytoplasmic sperm injection (ICSI) (Bedaiwy *et al.*, 2004). In ICSI, the oocyte is devoid of any cumulus cells and therefore only the oocyte, injected spermatozoa and the injected culture medium are the potential sources of ROS. In contrast, in conventional IVF–embryo transfer, ROS may originate from multiple oocytes per dish, large number of cumulus cell mass and spermatozoa used for insemination.

Evidence suggests that media supplementation with antioxidants, disulphide reducing agents or divalent chelators of cations may be beneficial to embryos studied under in-vitro conditions (Guerin *et al.*, 2001). The mouse two-cell embryo block can be prevented by antioxidant supplementation (Nasr-Esfahani and Johnson 1991; Goto *et al.*, 1993). Co-incubation of mouse embryos that were exposed to exogenously induced ROS with vitamin C significantly increased blastocyst development rates (Wang *et al.*, 2002). Insulin-like growth factors I, II and epidermal growth factors have also been reported to have a positive effect on embryo development in mouse embryos that were exposed to exogenous OS (Kurzawa *et al.*, 2004). The addition of β -mercaptoethanol, a thiol protector, reduced concentrations of apoptosis and blastocyst degeneration in bovine blastocysts (Kitagawa *et al.*, 2004). This was also associated with increased synthesis of glutathione induced by beta-mercaptoethanol. The embryo in the first trimester grows under low oxygen concentration as seen in materno–fetal oxygen diffusion studies (Jauniaux *et al.*, 2003). In human embryos, the blastulation rate increased to 58.5% with low oxygen tension (5% O₂) and low illumination maintained throughout the period of embryo manipulation (Noda *et al.*, 1994). Removal of environmental pollutants via air filtration may protect in-vitro embryo growth. It has been recommended that low oxygen concentrations be used at all stages, such as insemination, fertilization and embryo culture (Catt and Henman, 2000). Decreased development of inner cell mass and reduced proportion of transferred blastocysts developing into embryos has also been reported (Karagenc *et al.*, 2004). Adoption of low oxygen concentration has been proposed as a standard for embryo culture, especially blastocyst production (Bavister, 2004).

Optimal concentrations of individual amino acids, antioxidants, vitamins and energy sources in culture media were determined and applied to improve the embryo quality and achieve higher blastulation hatching rates with human embryos (Ali *et al.*, 2000). Different co-culture systems utilized for IVF are associated with improved embryo survival and increased pregnancy rates (Spandorfer *et al.*, 2004). The cells in co-culture produce enzymatic and non-enzymatic antioxidants and confer a protective effect on the embryos (Wang *et al.*, 2002). Repeated change of media and use of sequential culture systems may help reduce exposure to ROS. The metabolic requirements of the embryos change with the different stages of development (Harvey *et al.*, 2002). Culture media incubated with fragmented embryos showed a progressive decline in antioxidant capacity (Paszkowski and Clarke, 1996). Hence, preventing exposure to fragmented embryos and defective spermatozoa would help surmount OS.

Supplementation with vitamin C and E has also been investigated (Tarin *et al.*, 2002). Antioxidant supplementation with albumin, low molecular weight thiol and proteins (10% serum substitute

supplement) has been used for in-vitro oocyte maturation and embryo culture. When media was supplemented with vitamin E, the number of the bovine embryos that reached the expanded blastocyst stage increased (Olson and Seidel, 2000).

Strategies to overcome OS in spermatozoa

Spermatozoa utilized for assisted reproduction are likely to be exposed to OS, which can cause extensive DNA damage. The chances of selecting DNA-damaged spermatozoa are much higher with the ICSI procedure. Spermatozoa with DNA damage induced by ROS can result in impaired embryonic growth, early embryonic death and abortion. Zwitterion buffers such as 2-hydroxy ethyl-1-piperazineethane sulphonic acid (HEPES) were found to be the most potent protectors against DNA damage occurring in spermatozoa as determined by the plasmid relaxation assay, which is a measure of DNA damage (Ermilov *et al.*, 1999).

The conventional swim-up preparation technique used for sperm preparation in ejaculates with ROS can lead to sperm damage because of the close contact between the functional spermatozoa and the defective spermatozoa and leukocytes (Henkel and Schill, 2003). The techniques recommended for patients with elevated ROS concentrations in the ejaculate are density gradient centrifugation and glass wool filtration. The sperm preparation media can be supplemented with antioxidants such as pentoxifyline (Okada *et al.*, 1997), glutathione (Lenzi *et al.*, 1993; Griveau and Le Lannou, 1994), *N*-acetyl-cysteine (Oeda *et al.*, 1997; Comhaire *et al.*, 2000) and albumin (Twigg *et al.*, 1998) to scavenge the ROS.

Reports suggest that prolonged sperm–oocyte incubation time (16–20 h) increases generation of ROS. Two prospective randomized controlled studies have recommended shorter sperm–oocyte co-incubation time (Gianaroli *et al.*, 1996; Kattera and Chen, 2003). Co-incubation times of 1–2 h resulted in better quality embryos with significantly improved fertilization and implantation rates. Shorter coincubation times also produced better quality embryos and increased implantation and pregnancy rates (Gianaroli *et al.*, 1996; Quinn *et al.*, 1998; Kattera and Chen, 2003).

Antioxidants and the management of OS

Current evidence supports the use of systemic antioxidants for the management of selected cases of male infertility (Agarwal *et al.*, 2004). Antioxidants can also be used as media supplements for sperm preparation. Systemic supplementation with antioxidants may help overcome OS in female infertility as well. The duration and dose of antioxidants used in female infertility needs to be further investigated.

Systemic supplementation with vitamin C has been used in patients who are infertile, in those with luteal phase defects and in those who have experienced recurrent abortions. Supplementation with 400 mg of vitamin C improved ovulation induction rates with clomiphene (Igarashi, 1977). Vitamin C may play a role in fertilization (Wilson, 1973).

Table 1. Studies in animals and humans demonstrating the effects of antioxidant supplementation in IVF media.

Authors	Antioxidant supplementation	Effects of antioxidants
<i>Animal studies</i>		
Ali et al., 2003	Cysteine, N-acetyl-L-cysteine, catalase and superoxide dismutase (SOD)	Addition of cysteine resulted in significant improvement in morula and blastocyst development rates. IVF – addition of antioxidants catalase, SOD and NAC (N-acetyl L-cysteine) significantly reduced morula and blastocyst stage development rates in bovine embryos.
Iwata et al., 1998	SOD, catalase and mannitol	High concentration of glucose causes generation of reactive oxygen species (ROS). Low oxygen concentration significantly improved embryo development. Antioxidants SOD, catalase, and mannitol had no positive effects on embryo development.
Wang et al., 2002	Vitamin C and vitamin E	Vitamin C was more effective than vitamin E in reversing ROS-induced mouse embryotoxicity.
<i>Human studies</i>		
Tarin et al., 2002	Ascorbate (62.5 µmol/l) supplementation in HTF (human tubal fluid) medium	No significant effects of ascorbate supplementation on fertilization.
Noda et al., 1994	Low oxygen tension (5% O ₂) and low illumination	Higher blastulation rates
Lighten et al., 1998	Addition of human insulin-like growth factor-I ligand	Enhanced embryo survival and blastocyst formation
Ali et al., 2000	Antioxidants and chelators, i.e. catalase citric acid, desferroxamine, ethylenediaminetetraacetic acid, glutathione, pentoxifylline and probucol	Better quality embryos generated

In another study involving 65 women undergoing IVF and embryo transfer, the authors demonstrated that concentrations of ascorbic acid were higher in mature ovarian follicles than in the serum (Paszowski and Clarke, 1999). This suggested an active uptake of vitamin C by the follicle resulting in the sequestration of vitamin C. In patients undergoing IVF–embryo transfer, vitamin C supplementation was given during the period of hormonal stimulation, which resulted in higher follicular fluid concentrations of vitamin C (Crha et al., 2003). The reported pregnancy rates were higher in the supplemented group, though not statistically significant. Higher pregnancy rates, which reached significance, were reported amongst patients who were non-smokers and received supplementation.

A recent double-blind, placebo-controlled pilot study investigated a nutritional supplement containing L-arginine, vitamins and minerals. Three months of supplementation resulted in a trend towards an increase in mean mid-luteal progesterone concentrations and a significant increase in the number of days with basal temperatures >37°C. After 5 months of supplementation, the pregnancy rates were significantly higher in the supplemented group (33 versus 0%, $P < 0.01$) (Westphal et al., 2004).

A recent randomized controlled trial examined the effects of vitamin C supplementation (750 mg daily) in patients with

luteal phase defects. Ascorbic acid supplementation resulted in significantly higher serum progesterone concentrations (13.27 ± 0.63 versus 7.51 ± 0.22 ng/ml). Pregnancy rates were also significantly higher (25.0 versus 10.9%, $P < 0.0047$). Luteal phase defects decreased by 53% in the supplemented group whereas the defects spontaneously improved by 22% in the non-supplemented group (Henmi et al., 2003). Animal studies have shown that supplementation with antioxidants reduces age-related ovarian senescence and prevents decline in oocyte quality and numbers (Tarin et al., 1998).

Significantly reduced levels of SOD expression were found to be associated with first trimester miscarriage (Jenkins et al., 2000). Antioxidants help protect the embryo from damage caused by pro-oxidants, which thereby aids in the establishment of a successful pregnancy. Pregnancy is a state of OS, and increased concentrations of OS may be involved in the aetiopathogenesis of recurrent abortions. Antioxidant supplementation in patients with antiphospholipid syndrome and recurrent abortions is being investigated (Ames et al., 2000).

Conclusions

Assisted reproductive technologies are being increasingly used to help infertile couples realize their dream of having a biological

child. Various clinical studies have documented the role of ROS in assisted reproduction. Strategies have been designed to surmount OS based on the understanding of the effects of ROS on various stages of fertilization such as early cleavage, blastocyst development and hatching and embryo development. It is important to identify the source of excessive generation of ROS, whether it is defective spermatozoa, spermatozoa with DNA damage, leukocytospermia, oocytes or the in-vitro media itself.

Management options for overcoming OS include in-vivo and in-vitro antioxidant supplementation. Supplementing in-vitro media with optimum concentrations of antioxidants, amino acids and vitamins helps scavenge ROS. In IVF, low oxygen concentration and low illumination should be maintained throughout the period of embryo manipulation. In addition, the media should be repeatedly changed and sequential culture media systems should be used. Both measures will help prevent the oocytes and embryos from being exposed to defective spermatozoa and fragmented embryos, and therefore to ROS. Antioxidant supplementation, disulphide reducing agents and divalent chelators of cations have beneficial effects on embryos under in-vitro conditions. Apoptosis and blastocyst degeneration can be improved by adding betamercaptoethanol to in-vitro culture media, which increases blastocyst development rates (Kitagawa *et al.*, 2004). Sperm preparation techniques reduce the amount of functional spermatozoa that are exposed to defective spermatozoa and leukocytes, and their use should be advocated. Sperm preparation media can be supplemented with antioxidants like pentoxifyline, glutathione, *N*-acetylcysteine and albumin to neutralize free radicals. It is important that reproductive endocrinologists be aware of the deleterious effects of ROS on natural and assisted fertility.

References

- Abma JC, Chandra A, Mosher WD *et al.* 1997 Fertility, family planning, and women's health: new data from the 1995 National Survey of Family Growth. *Vital and Health Statistics* **23**, 1–114.
- Agarwal A, Allamaneni SS 2004 Role of free radicals in female reproductive diseases and assisted reproduction. *Reproductive BioMedicine Online* **9**, 338–347.
- Agarwal A, Allamaneni SS, Said TM 2004a Chemiluminescence technique for measuring reactive oxygen species. *Reproductive BioMedicine Online* **9**, 466–468.
- Agarwal A, Nallella KP, Allamaneni SS *et al.* 2004b Role of antioxidants in treatment of male infertility: an overview of the literature. *Human Reproduction* **8**, 616–627.
- Agarwal A, Saleh RA, Bedaiwy MA 2003 Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertility and Sterility* **79**, 829–843.
- Aitken RJ, Baker MA, Sawyer D 2003 Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reproductive BioMedicine Online* **7**, 65–70.
- Aitken RJ, Harkiss D, Buckingham D 1993 Relationship between iron-catalysed lipid peroxidation potential and human sperm function. *Journal of Reproduction and Fertility* **98**, 257–265.
- Aitken RJ, Clarkson JS, Fishel S 1989 Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biology of Reproduction* **41**, 183–197.
- Ali AA, Bilodeau JF, Sirard MA 2003 Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. *Theriogenology* **59**, 939–949.
- Ali J, Shahata MA, Al-Natsha SD 2000 Formulation of a protein-free medium for human assisted reproduction. *Human Reproduction* **15**, 145–156.
- Ames PR, Tommasino C, Alves J *et al.* 2000 Antioxidant susceptibility of pathogenic pathways in subjects with antiphospholipid antibodies: a pilot study. *Lupus* **9**, 688–695.
- ASRM 2004 Definition of 'infertility'. *Fertility and Sterility* **82** (suppl. 1), S206.
- Attaran M, Pasqualotto E, Falcone T *et al.* 2000 The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. *International Journal of Fertility and Women's Medicine* **45**, 314–320.
- Banerjee S, Smallwood A, Moorhead J *et al.* 2004 Placental expression of IFN- γ and its receptor IFN- γ R2 fail to switch from early hypoxic to late normotensive development in pre-eclampsia. *Journal of Clinical Endocrinology Metabolism* **90**, 944–952.
- Barrionuevo MJ, Schwandt RA, Rao, PS *et al.* 2000 Nitric oxide (NO) and interleukin-1 β (IL-1 β) in follicular fluid and their correlation with fertilization and embryo cleavage. *American Journal of Reproductive Immunology* **44**, 359–364.
- Bavister B 2004 Oxygen concentration and preimplantation development. *Reproductive BioMedicine Online* **9**, 484–486.
- Bedaiwy MA, Falcone T 2003 Peritoneal fluid environment in endometriosis. Clinicopathological implications. *Minerva Ginecologica* **55**, 333–345.
- Bedaiwy MA, Falcone T, Mohamed MS *et al.* 2004 Differential growth of human embryos in vitro: role of reactive oxygen species. *Fertility and Sterility* **82**, 593–600.
- Bedaiwy MA, Falcone T, Sharma RK *et al.* 2002 Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial. *Human Reproduction* **17**, 426–431.
- Boni R, Tosti E, Roviello S *et al.* 1999 Intercellular communication in in vivo- and in vitro-produced bovine embryos. *Biology of Reproduction* **61**, 1050–1055.
- Braude P, Rowell P 2003 Assisted conception. II – in vitro fertilisation and intracytoplasmic sperm injection. *British Medical Journal* **327**, 852–855.
- Burton GJ, Hempstock J, Jauniaux E 2003 Oxygen, early embryonic metabolism and free radical-mediated embryopathies. *Human Reproduction* **6**, 84–96.
- Catt JW, Henman M 2000 Toxic effects of oxygen on human embryo development. *Human Reproduction* **15** (suppl. 2), 199–206.
- Comhaire FH, Christophe AB, Zalata AA *et al.* 2000 The effects of combined conventional treatment, oral antioxidants and essential fatty acids on sperm biology in subfertile men. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **63**, 159–165.
- Comporti M 1989 Three models of free radical-induced cell injury. *Chemical Biological Interaction* **72**, 1–56.
- Cramer DW, Wise LA 2000 The epidemiology of recurrent pregnancy loss. *Seminars in Reproductive Medicine* **18**, 331–339.
- Crha I, Hrubá D, Ventruba P *et al.* 2003 Ascorbic acid and infertility treatment. *Central European Journal of Public Health* **11**, 63–67.
- Dennery PA 2004 Role of redox in fetal development and neonatal diseases. *Antioxidant and Redox Signal* **6**, 147–153.
- Dong M, Shi Y, Cheng Q *et al.* 2001 Increased nitric oxide in peritoneal fluid from women with idiopathic infertility and endometriosis. *Journal of Reproductive Medicine* **46**, 887–891.
- Duckitt K 2003 Infertility and subfertility. *Clinical Evidence* **11**, 2427–2458.
- Duleba AJ, Foyouzi N, Karaca M *et al.* 2004 Proliferation of ovarian theca-interstitial cells is modulated by antioxidants and oxidative stress. *Human Reproduction* **19**, 1519–1524.
- El Moutassim S, Guerin P, Menezo Y 1999 Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. *Molecular Human Reproduction* **5**, 720–725.
- Ermilov A, Diamond MP, Sacco AG *et al.* 1999 Culture media and their components differ in their ability to scavenge reactive oxygen species in the plasmid relaxation assay. *Fertility and Sterility* **72**, 154–157.
- Eskandari N 2003 Infertility. In: Cherney A, Nathan L (eds), *Current Obstetric and Gynecologic Diagnosis and Treatment*, 9th edn. McGraw-Hill, New York: Chapter 53, pp. 979–1000.

- Friden BE, Runesson E, Hahlin M *et al.* 2000 Evidence for nitric oxide acting as a luteolytic factor in the human corpus luteum. *Molecular Human Reproduction* **6**, 397–403.
- Gardner DK, Lane M, Stevens J *et al.* 2000 Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertility and Sterility* **73**, 1155–1158.
- Gianaroli L, Fiorentino A, Magli MC *et al.* 1996 Prolonged sperm-oocyte exposure and high sperm concentration affect human embryo viability and pregnancy rate. *Human Reproduction* **11**, 2507–2511.
- Goto Y, Noda Y, Mori T *et al.* 1993 Increased generation of reactive oxygen species in embryos cultured in vitro. *Free Radicals in Biology and Medicine* **15**, 69–75.
- Griveau JF, Le Lannou D 1994 Effects of antioxidants on human sperm preparation techniques. *International Journal of Andrology* **17**, 225–231.
- Guerin P, El Moutassim S, Menezo Y 2001 Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reproduction Update* **7**, 175–189.
- Halliwell B, Gutteridge JM 1988 Free radicals and antioxidant protection: mechanisms and significance in toxicology and disease. *Human Toxicology* **7**, 7–13.
- Hanafy KA, Krumenacker JS, Murad F 2001 NO, nitrotyrosine, and cyclic GMP in signal transduction. *Medical Science Monitor* **7**, 801–819.
- Harvey AJ, Kind KL, Thompson JG 2002 REDOX regulation of early embryo development. *Reproduction* **123**, 479–486.
- Henkel RR, Schill WB 2003 Sperm preparation for assisted reproduction. *Reproductive Biology and Endocrinology* **1**, 108.
- Henmi H, Endo T, Kitajima Y *et al.* 2003 Effects of ascorbic acid supplementation on serum progesterone levels in patients with a luteal phase defect. *Fertility and Sterility* **80**, 459–461.
- Ho HN, Wu MY, Chen SU *et al.* 1997 Total antioxidant status and nitric oxide do not increase in peritoneal fluids from women with endometriosis. *Human Reproduction* **12**, 2810–2815.
- Igarashi M 1977 Augmentative effect of ascorbic acid upon induction of human ovulation in clomiphene-ineffective anovulatory women. *International Journal of Fertility* **22**, 168–173.
- Isaksson R, Tiitinen A 2004 Present concept of unexplained infertility. *Gynecologic Endocrinology* **18**, 278–290.
- Iwata H, Akamatsu S, Minami N *et al.* 1998 Effects of antioxidants on the development of bovine IVF/IVF embryos in various concentrations of glucose. *Theriogenology* **50**, 365–375.
- Jauniaux E, Gulbis B, Burton GJ 2003 Physiological implications of the maternal-fetal oxygen gradient in human early pregnancy. *Reproductive BioMedicine Online* **7**, 250–253.
- Jauniaux E, Watson AL, Hempstock J *et al.* 2000 Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. *American Journal of Pathology* **157**, 2111–2122.
- Jenkins C, Wilson R, Roberts J *et al.* 2000 Antioxidants: their role in pregnancy and miscarriage. *Antioxidant and Redox Signal* **2**, 623–628.
- Jozwik M, Wolczynski S, Szamatowicz M 1999 Oxidative stress markers in preovulatory follicular fluid in humans. *Molecular Human Reproduction* **5**, 409–413.
- Karagenc L, Sertkaya Z, Ciray N *et al.* 2004 Impact of oxygen concentration on embryonic development of mouse zygotes. *Human Reproduction* **9**, 409–417.
- Kattera S, Chen C 2003 Short cocubation of gametes in in vitro fertilization improves implantation and pregnancy rates: a prospective, randomized, controlled study. *Fertility and Sterility* **80**, 1017–1021.
- Kitagawa, Y, Suzuki, K, Yoneda A *et al.* 2004 Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. *Theriogenology* **62**, 1186–1197.
- Kodama H, Yamaguchi R, Fukuda J *et al.* 1997 Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertility and Sterility* **68**, 519–524.
- Krausz C, West K, Buckingham D *et al.* 1992 Development of a technique for monitoring the contamination of human semen samples with leukocytes. *Fertility and Sterility* **57**, 1317–1325.
- Kurzawa R, Glabowski W, Baczkowski T *et al.* 2004 Growth factors protect in vitro cultured embryos from the consequences of oxidative stress. *Zygote* **12**, 231–240.
- Lee KS, Joo BS, Na YJ *et al.* 2000 Relationships between concentrations of tumor necrosis factor-alpha and nitric oxide in follicular fluid and oocyte quality. *Journal of Assisted Reproduction and Genetics* **17**, 222–228.
- Lenzi A, Culasso F, Gandini L *et al.* 1993 Placebo-controlled, double-blind, crossover trial of glutathione therapy in male infertility. *Human Reproduction* **8**, 1657–1662.
- Lighten AD, Moore GE, Winston RM *et al.* 1998 Routine addition of human insulin-like growth factor-I ligand could benefit clinical in vitro fertilization culture. *Human Reproduction* **13**, 3144–3150.
- Lin H, Mosmann TR, Guilbert L *et al.* 1993 Synthesis of T helper 2-type cytokines at the maternal-fetal interface. *Journal of Immunology* **151**, 4562–4573.
- Liu Y, Luo L, Zhao H 2001 Levels of lipid peroxides and superoxide dismutase in peritoneal fluid of patients with endometriosis. *Journal of Tongji Medical University* **21**, 166–167.
- Lopes S, Jurisicova A, Sun J G *et al.* 1998 Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. *Human Reproduction* **13**, 896–900.
- Miller H, Wilson R, Jenkins C *et al.* 2000 Glutathione levels and miscarriage. *Fertility and Sterility* **74**, 1257–1258.
- Murphy AA, Palinski W, Rankin S *et al.* 1998 Macrophage scavenger receptor(s) and oxidatively modified proteins in endometriosis. *Fertility and Sterility* **69**, 1085–1091.
- Nasr-Esfahani MM, Johnson MH 1991 The origin of reactive oxygen species in mouse embryos cultured in vitro. *Development* **113**, 551–560.
- Noda Y, Goto Y, Umaoka Y *et al.* 1994 Culture of human embryos in alpha modification of Eagle's medium under low oxygen tension and low illumination. *Fertility and Sterility* **62**, 1022–1027.
- Oeda T, Henkel R, Ohmori H *et al.* 1997 Scavenging effect of N-acetyl-L-cysteine against reactive oxygen species in human semen: a possible therapeutic modality for male factor infertility? *Andrologia* **29**, 125–131.
- Okada, H, Tatsumi, N, Kanzaki M *et al.* 1997 Formation of reactive oxygen species by spermatozoa from asthenospermic patients: response to treatment with pentoxifylline. *Journal of Urology* **157**, 2140–2146.
- Olson SE, Seidel GE Jr 2000 Culture of in vitro-produced bovine embryos with vitamin E improves development in vitro and after transfer to recipients. *Biology of Reproduction* **62**, 248–252.
- Orsi NM, Leese HJ 2001 Protection against reactive oxygen species during mouse preimplantation embryo development: role of EDTA, oxygen tension, catalase, superoxide dismutase and pyruvate. *Molecular Reproductive Development* **59**, 44–53.
- Osborn BH, Haney AF, Misukonis MA *et al.* 2002 Inducible nitric oxide synthase expression by peritoneal macrophages in endometriosis-associated infertility. *Fertility and Sterility* **77**, 46–51.
- Ota H, Igarashi S, Hatazawa J *et al.* 1999 Endometriosis and free radicals. *Gynecologic and Obstetric Investigation* **48** (suppl. 1), 29–35.
- Ota H, Igarashi S, Hatazawa J *et al.* 1998 Endothelial nitric oxide synthase in the endometrium during the menstrual cycle in patients with endometriosis and adenomyosis. *Fertility and Sterility* **69**, 303–308.
- Oyawoye O, Abdel Gadir A, Garner A *et al.* 2003 Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. *Human Reproduction* **18**, 2270–2274.
- Pasqualotto EB, Agarwal A, Sharma RK *et al.* 2004 Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. *Fertility and Sterility* **81**, 973–976.
- Paszowski T, Clarke RN 1999 The Graafian follicle is a site of L-ascorbate accumulation. *Journal of Assisted Reproduction and*

- Genetics* **16**, 41–45.
- Paszkowski T, Clarke RN 1996 Antioxidative capacity of preimplantation embryo culture medium declines following the incubation of poor quality embryos. *Human Reproduction* **11**, 2493–2495.
- Paszkowski T, Traub AI, Robinson SY *et al.* 1995 Selenium dependent glutathione peroxidase activity in human follicular fluid. *Clinica Chimica Acta* **236**, 173–180.
- Peterson JD, Herzenberg LA, Vasquez K *et al.* 1998 Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proceedings of the National Academy of Sciences of the USA* **95**, 3071–3076.
- Polak G, Koziol-Montewka M, Gogacz M *et al.* 2001 Total antioxidant status of peritoneal fluid in infertile women. *European Journal of Obstetrics, Gynecology and Reproductive Biology* **94**, 261–263.
- Preuthippan S, Chen SH, Tilly JL *et al.* 2004 Inhibition of nitric oxide synthesis potentiates apoptosis in the rabbit corpus luteum. *Reproductive BioMedicine Online* **9**, 264–270.
- Quinn P, Lydic ML, Ho M *et al.* 1998 Confirmation of the beneficial effects of brief coincubation of gametes in human in vitro fertilization. *Fertility and Sterility* **69**, 399–402.
- Rosselli M, Keller PJ, Dubey RK 1998 Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Human Reproduction Update* **4**, 3–24.
- Sabatini L, Wilson C, Lower A *et al.* 1999 Superoxide dismutase activity in human follicular fluid after controlled ovarian hyperstimulation in women undergoing in vitro fertilization. *Fertility and Sterility* **72**, 1027–1034.
- Scully RE, Cohen RB 1964 Oxidative-enzyme activity in normal and pathologic human ovaries. *Obstetrics and Gynecology* **24**, 667–681.
- Seino T, Saito H, Kaneko T *et al.* 2002 Eight-hydroxy-2'-deoxyguanosine in granulosa cells is correlated with the quality of oocytes and embryos in an in vitro fertilization-embryo transfer program. *Fertility and Sterility* **77**, 1184–1190.
- Shanti A, Santanam N, Morales AJ *et al.* 1999 Autoantibodies to markers of oxidative stress are elevated in women with endometriosis. *Fertility and Sterility* **71**, 1115–1118.
- Sharma RK, Pasqualotto FF, Nelson DR *et al.* 1999 The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Human Reproduction* **14**, 2801–2807.
- Shiotani M, Noda Y, Narimoto K *et al.* 1991 Immunohistochemical localization of superoxide dismutase in the human ovary. *Human Reproduction* **6**, 1349–1353.
- Spandorfer SD, Pascal P, Parks J *et al.* 2004 Autologous endometrial coculture in patients with IVF failure: outcome of the first 1,030 cases. *Journal of Reproductive Medicine* **49**, 463–467.
- Sugino N, Takiguchi S, Ono M *et al.* 1996 Nitric oxide concentrations in the follicular fluid and apoptosis of granulosa cells in human follicles. *Human Reproduction* **11**, 2484–2487.
- Sukcharoen N, Keith J, Irvine DS *et al.* 1996 Prediction of the in-vitro fertilization (IVF) potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF. *Human Reproduction* **11**, 1030–1034.
- Suzuki T, Sugino N, Fukaya T *et al.* 1999 Superoxide dismutase in normal cycling human ovaries: immunohistochemical localization and characterization. *Fertility and Sterility* **72**, 720–726.
- Szczepanska M, Kozlik J, Skrzypczak J *et al.* 2003 Oxidative stress may be a piece in the endometriosis puzzle. *Fertility and Sterility* **79**, 1288–1293.
- Taguchi M, Alfer J, Chwalisz K *et al.* 2000 Endothelial nitric oxide synthase is differently expressed in human endometrial vessels during the menstrual cycle. *Molecular Human Reproduction* **6**, 185–190.
- Tamate K, Sengoku K, Ishikawa M 1995 The role of superoxide dismutase in the human ovary and Fallopian tube. *Journal of Obstetrics and Gynaecology* **21**, 401–409.
- Tarin JJ, Perez-Albala S, Pertusa JF *et al.* 2002 Oral administration of pharmacological doses of vitamins C and E reduces reproductive fitness and impairs the ovarian and uterine functions of female mice. *Theriogenology* **57**, 1539–1550.
- Tarin JJ, Vendrell FJ, Ten J *et al.* 1998 Antioxidant therapy counteracts the disturbing effects of diamide and maternal ageing on meiotic division and chromosomal segregation in mouse oocytes. *Molecular Human Reproduction* **4**, 281–288.
- Tseng L, Zhang J, Peresleni T *et al.* 1996 Cyclic expression of endothelial nitric oxide synthase mRNA in the epithelial glands of human endometrium. *Journal of the Society for Gynecologic Investigation* **3**, 33–38.
- Twigg JP, Irvine DS, Aitken RJ 1998 Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Human Reproduction* **13**, 1864–1871.
- Van Langendonck A, Casanas-Roux F, Donnez J 2002 Oxidative stress and peritoneal endometriosis. *Fertility and Sterility* **77**, 861–870.
- Vega M, Carrasco I, Castillo T *et al.* 1995 Functional luteolysis in response to hydrogen peroxide in human luteal cells. *Journal of Endocrinology* **147**, 177–182.
- Viuff D, Rickords L, Offenberg H *et al.* 1999 A high proportion of bovine blastocysts produced in vitro are mixoploid. *Biology of Reproduction* **60**, 1273–1278.
- Wang X, Falcone T, Attaran M *et al.* 2002 Vitamin C and vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. *Fertility and Sterility* **78**, 1272–1277.
- Wang Y, Sharma RK, Falcone T *et al.* 1997 Importance of reactive oxygen species in the peritoneal fluid of women with endometriosis or idiopathic infertility. *Fertility and Sterility* **68**, 826–830.
- Westphal LM, Polan ML, Trant AS *et al.* 2004 A nutritional supplement for improving fertility in women: a pilot study. *Journal of Reproductive Medicine* **49**, 289–293.
- Wilson CW 1973 Letter: Vitamin C and fertility. *Lancet* **2**, 859–860.
- Wu MY, Chao KH, Yang JH *et al.* 2003 Nitric oxide synthesis is increased in the endometrial tissue of women with endometriosis. *Human Reproduction* **18**, 2668–2671.
- Yang HW, Hwang KJ, Kwon HC *et al.* 1998 Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Human Reproduction* **13**, 998–1002.

Received 11 May 2005; refereed 16 June 2005; accepted 7 July 2005.