The Role of Oxidative Stress and Antioxidants in Assisted Reproduction

Sajal Gupta, Lucky Sekhon, Yesul Kim and Ashok Agarwal*

Center for Reproductive Medicine, Glickman Urological & Kidney Institute and Department of Obstetrics-Gynecology, Cleveland Clinic, Cleveland, OH, 44195, USA

Abstract: Aim: Oxidative stress contributes to the high rate of failure seen in assisted reproductive techniques in achieving fertilization and pregnancy. Many studies have been done to elucidate the sources of oxidative stress in the setting of assisted reproductive technology (ART) and interventions to overcome its negative influence on the outcome of IVF and ICSI. This article explores the utility of metabolomics as a novel, non-invasive method of accurately and efficiently quantifying oxidative stress. The aim of this study was to review the current literature on the effects of various interventions, including the use of antioxidants supplementation of IVF culture media and patients to improve fertilization and pregnancy rates in subfertile patients undergoing ART.

Methods: Review of recent publications through Pubmed and the Cochrane data base.

Results: Oxidative stress is correlated with negatives ART outcomes. Both exogenous and endogenous sources of reactive oxygen species during IVF/ICSI are well established in the literature. Compared to IVF, ICSI is known to minimize the exposure of gametes to endogenous sources of oxidative stress. Strategies to control exogenous sources of oxidative stress within the ART setting include reducing visible/near UV light exposure, the addition of metal chelators to culture media, maintenance of low oxygen tension in the environment and the use of antioxidant therapy. Antioxidant supplementation of culture media with vitamin C, vitamin E, and melatonin has been investigated and yielded conflicting results. Whereas oral antioxidant supplementation of male patients has been accepted and is currently practiced, there is a lack of consensus regarding the effectiveness of supplementation of vitamin C, vitamin E and melatonin in females undergoing ART.

Conclusion: There is a need for further investigation with randomized controlled studies to confirm the efficacy and safety of antioxidant supplementation of culture media and patients as well as the need to determine the dosage required to improve fertilization rates and pregnancy outcome with IVF/ICSI.

Keywords: In vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), infertility, oxidative stress, antioxidants, metabolomics.

INTRODUCTION

Infertility is a universal issue, affecting millions of men and women in both developed and developing areas of the world. Recent advances in the field of reproductive medicine may allow for a select group of infertile couples to conceive and bear offspring by utilizing assisted reproductive techniques (ART). However, any hope offered by these procedures is limited by persistently poor fertility outcomes. In 2003, a report by the Society for Assisted Reproductive Technology conceded that only 35% of ART transfer procedures result in a live birth delivery. A myriad of physiological and environmental factors are known to influence the success rates of ART in achieving fertilization and pregnancy culminating in living offspring. Reactive oxygen species (ROS) have been widely implicated in the literature to render a significant impact on these outcomes.

ART methods such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are designed with a view to emulate the process of natural, in vivo reproduction. However, the in vitro environment exposes gametes and embryos to an excess of reactive oxygen species not normally experienced during in vivo fertilization and pregnancy. Although reactive oxygen species are physiologically required for various biochemical pathways necessary for reproduction, their in vivo levels are tightly controlled by enzymatic antioxidants that scavenge and neutralize free radicals to maintain an optimal, physiologic oxygen tension in the male and female reproductive system.

In contrast, in vitro ART procedures are carried out without the protection of enzymatic antioxidants normally found in vivo, leading to unopposed, elevated levels of ROS, which have been shown to adversely affect gametes, gamete interaction, fertilization and pregnancy rates. Given the impact of redox status on ART outcomes, it is crucial to elucidate the mechanism by which ROS are generated, methods to accurately quantify ROS levels, and strategies to regulate the amount of ROS experienced to enhance embryo quality, promote single embryo transfer and reduce multiple pregnancy rates with ART.

Poor fertility outcomes with ART are particularly problematic when the high risk of failure in achieving reproduction is placed in an economic context. The high cost of ART

*Address correspondence to this author at the Center for Reproductive Medicine Glickman Urological & Kidney Institute and Department of Obstetrics & Gynecology, Cleveland Clinic, 9500 Euclid Avenue, Desk A19.1, Cleveland, OH 44195, USA; Tel: (216) 444-9485; Fax: (216) 445-6049, E-mail: agarwaa@ccf.org
coupled with relatively low success rates is especially unacceptable in the developing world, where resources are scarce and a high prevalence of infertility endures. Thus, it is imperative to investigate new strategies to improve the rate of successful outcomes in ART. The aim of this discussion is to clarify what is currently known regarding the role oxidative stress plays in ART and potential ways to alleviate the effects of oxidative stress, with specific interest in the use of antioxidants to optimize ART outcomes.

WHAT IS ART?

Assisted reproductive techniques can serve as an effective method to overcome a variety of causative factors of infertility such as pathology of the fallopian tubes, endometriosis, and male factor and unexplained infertility [1]. The primary focus of this review is centered on the effect of oxidative stress on the outcome of in vitro ART procedures and the potential role of antioxidants to protect against oxidative stress to increase the likelihood of achieving successful pregnancy with ART.

Intrauterine Insemination

Intrauterine insemination (IUI) is performed by threading a very thin, flexible catheter through the cervix and injecting washed spermatozoa into the uterus. This technique requires large numbers of forward-progressing motile spermatozoa [2]. Moreover, higher sperm counts increase the success rates of this procedure. Therefore, male infertility patients with a low number of spermatozoa are not suitable candidates for IUI. In addition, this method is not suitable for semen samples high in free radicals due to the presence of large number of leukocytes, cellular debris, and/or immature germ cells [2].

In vitro Fertilization

(IVF) is another assisted reproductive technique in which sperm-oocyte interactions take place within culture media, leading to fertilization. Most IVF cycles utilize fertility drugs such as GnRH agonists and human menopausal gonadotropins to stimulate the ovaries to produce several mature eggs, in addition to the single egg normally produced each month. If ovarian stimulation is not done, the oocyte may simply be retrieved from the natural menstrual cycle, avoiding any risk of overstimulation and multiple pregnancy. The oocytes are collected into a specially prepared culture medium, which then is microscopically evaluated for maturity. Between 20,000-30,000 sperm are mixed with each oocyte in a drop of specially prepared culture medium and then incubated to ensure an optimal environment to facilitate fertilization. After fertilization is complete, the resulting embryos are qualitatively graded on the basis of morphology, and those chosen for transfer are loaded in a minute volume of medium into a transfer catheter. A catheter tip is advanced into the uterus and the embryos are expelled. The pregnancy rate increases with the number of embryos transferred.

This treatment is primarily for female-related infertility problems such as hydrosalphinges, damaged or inoperable fallopian tubes, endometriosis, and cervical mucus pathology. Idiopathic infertility, male infertility and immunologic infertility also respond well to IVF. A major disadvantage of IVF is the need for women to undergo ovarian stimulation with hCG or progesterone hormonal therapy to support the luteal phase, which can lead to ovarian hyperstimulation syndrome— the most common and potentially serious complication for conventional IVF treatment [3].

Intracytoplasmic Sperm Injection

Intracytoplasmic sperm injection (ICSI) is a laboratory procedure in which a single sperm is injected directly into an oocyte’s cytoplasm using a very fine needle. The process allows for oocyte fertilization regardless of the morphology and motility characteristics of the single spermatozoon injected [4]. This procedure is used in cases in which unsuccessful conventional IVF led to fertilization failure or in instances where a male-factor such as low sperm count is involved [4]. This procedure is also used for non-obstructive azoospermic patients, in which sperm may be retrieved using testicular sperm aspiration [5].

ICSI is particularly beneficial in infertile males with disordered sperm-zona pellucida interactions, in that it bypasses all of the preliminary penetration and fusion steps of fertilization. Despite these advantages, the injected sperm in ICSI are at an increased risk for having oxidatively damaged DNA which could adversely affect the outcome. ICSI circumvents the process of natural selection and may allow damaged spermatozoon to be directly injected into the oocyte, which can negatively impact embryo development. IVF is superior to ICSI with respect to avoiding this risk, as collateral peroxidative damage to the sperm plasma membrane will prevent fertilization by spermatozoa with DNA damage [2].

Similarities Between IVF and ICSI

Both IVF and ICSI are very costly procedures not covered by most health plans. They require special facilities and are subject to many regulatory restrictions. Although these procedures provide the possibility of a potential cure for infertile couples, there are many weaknesses and factors that often detract from successful reproductive outcomes. As in vitro procedures, the environment in which IVF and ICSI are conducted fails to mimic the intricate physiological conditions of an in vivo system. These methods are greatly affected by environmental factors without beneficial protective factors such as antioxidants provided by the in vivo environment. Measures aimed at minimizing differences between in vitro and physiologic in vivo conditions should be investigated and employed to maximize the efficiency of ART procedures. This article will review the role of oxidative stress in both conventional IVF and ICSI and approaches to optimize the chance of achieving successful pregnancy with these procedures.

WHAT IS OXIDATIVE STRESS?

Aerobic metabolism generates reactive oxygen species-hydroxyl radicals, superoxide anion, hydrogen peroxide, and nitric oxide. They are small and highly reactive due to unpaired valence shell electrons that are capable of initiating an uncontrolled cascade of chain reactions. Normally, this is regulated by antioxidants, which have the ability to scavenge and neutralize free radicals [2]. The controlled production of
such compounds is known to play a role in physiological reproductive processes such as hormone signaling, oocyte maturation, folliculogenesis, tubal function, ovarian steroidogenesis, cyclical endometrial changes, and germ cell function. However, at higher levels ROS may overwhelm antioxidant capacity, leading to oxidative stress. The high energy electrons of ROS are capable of modulating gene expression and transcription factors, with the ability to modify and damage DNA [2].

Intracellular homeostasis is achieved through a balance between pro-oxidant compounds and antioxidants. Antioxidants have the ability to oppose the effects of pro-oxidants by hindering ROS production, scavenging ROS, and repairing cell damage caused by ROS. Non-enzymatic antioxidants consist of vitamin C, taurine, hypotaurine, cysteamine, and glutathione. Enzymatic antioxidants include superoxide dismutase, catalase, and glutathione peroxidase. Within the body, enzymatic antioxidants protect and support gametes and embryos during fertilization and pregnancy from ROS-induced pathological changes. IVF and ICSI procedures lack these natural antioxidants and, therefore, expose gametes and embryos to a level of OS higher than that experienced in vivo during physiologic reproduction.

WHAT IS THE ROLE OF OXIDATIVE STRESS IN ART?

OS exerts toxic effects by altering cellular molecules such as lipids, proteins and nucleic acids. This can lead to an increase in membrane permeability, loss of membrane integrity, enzyme inactivation, structural damage to DNA, mitochondrial alterations, adenosine triphosphate depletion, and apoptosis. Free radicals are thought to act as a determinant in reproductive outcome due to their effects on oocytes, sperm, and embryos in their follicular fluid, tubal fluid, and peritoneal fluid microenvironments. Oocytes and embryos can be protected against OS by free radical-scavenging antioxidants that exist within the follicular and oviductal fluid [6]. However, this defense against OS is lost as sperm, oocytes, and embryos are removed from their natural microenvironments to be used for ART [6]. This loss of protection leads to increased exposure of gametes and embryos to OS, which can lead to impaired oocyte maturation and embryo development [7].

Oocyte quality is thought to act as the main determinant of IVF outcomes [8]. Generally, higher quality oocytes develop into healthy, high quality embryos, ultimately resulting in a higher birth rate [8]. The compound 8-OHdG acts as a sensitive marker of OS-induced DNA damage in granulosa cells during ovulation. A study by Seino et al. demonstrated an association between increased levels of 8-OHdG and decreased fertilization rates and embryo quality. Thus, increased OS in the granulosa cell environment that surrounds oocytes adversely affects the oocytes themselves, leading to lower success rates for IVF [8]. Wang et al. conducted a study exposing mouse embryos to PMA-activated leukocyte supernatant which generated ROS. The findings of the study relate increased OS levels with a decreased blastocyst development rate [6]. Thus, the literature implicates OS and its deleterious effects in low fertilization rates with IVF and ICSI. Therefore, a thorough analysis of the endogenous and exogenous factors that contribute to the oxidative stress faced by gametes and embryos during IVF and ICSI is necessary to appropriately manipulate the in vitro culture medium to minimize oxidative stress and improve ART outcomes.

WHAT IS THE ORIGIN OF ROS IN ART?

Endogenous Factors Affecting Both IVF and ICSI: Embryo Development

ROS play a significant physiological role in the modulation of gamete interaction and successful fertilization. Oocyte metabolism generates ROS. This, coupled with the lack of protective antioxidant mechanisms that normally would be found in vivo [9], results in an overall increase in OS in the in vitro environment. Preimplantation embryonic development is accompanied by a change in preference in energy metabolism pathways [10]. Oxidative phosphorylation is utilized throughout the preimplantation embryo development period. Increased energy demand is met by a significant shift from dependence on oxidative phosphorylation to glycolysis to generate ATP [7]. Oxygen is needed for oxidative phosphorylation to convert ADP to ATP, which is required for the processes of folliculogenesis and oocyte maturation. This use of oxygen results in ROS production, which in excess has a toxic influence on embryo development [7]. ROS are primarily produced at the inner mitochondrial membrane where electrons leak from the electron transport chain and are transferred to the oxygen molecule, resulting in oxygen with an extra unpaired electron [10, 11]. In addition, cytoplasmic or membrane-bound NADPH-oxidase, cytochrome p450 enzymes and the xanthine-xanthine oxidase systems [10] are also capable of generating an excess of ROS.

Endogenous Factors that Differ Between IVF and ICSI

There are differences between the potential sources of ROS derived from conventional IVF and ICSI [12] (Fig. 1). For instance, oocytes used in ICSI are initially denuded of their cumulus cells so that the only possible source of ROS is within the culture media environment, the oocyte, and the injected spermatozoa [9]. With IVF, however, ROS may arise from the multiple oocytes per dish, the large cumulus cell mass, and from the spermatozoa used for insemination. Unlike IVF, ICSI avoids contact time between the sperm and oocyte, thus minimizing the opportunity for ROS generation by defective spermatozoa [9, 13]. Lower sperm concentrations in the culture media are correlated with an improvement in fertilization, implantation, and pregnancy rates and with yielding higher quality embryos [14]. Thus the ICSI procedure, which utilizes single spermatozoa at a time, minimizes OS development from the gametes themselves.

Exogenous Factors Affecting Both IVF and ICSI

The external environment and culture media surrounding embryos during both IVF and ICSI are believed to significantly influence the outcomes of these procedures. IVF and ICSI do not significantly differ with respect to the level of exposure of sperm, oocytes, and embryos to ROS [8]—aside from the fact that the incubation time interval is shorter in the ICSI procedure, decreasing the amount of exposure of
the culture media to external environmental factors [9, 13]. An incubation time limited to 1-2 hours has been associated with better ART outcomes. With IVF and ICSI, ROS may arise from the male or female gamete, the embryo, or indirectly from the external environment, which includes cumulus cells, leukocytes, and the culture media.

As fertilization and embryo development in vivo are known to occur in an environment of low oxygen tension, it is hypothesized that OS experienced in the in vitro setting is a major contributor to the persistently high failure rate of ART. Therefore, ART outcomes should improve if in vivo conditions are emulated by avoiding the various factors that promote ROS production.

**OXYGEN CONCENTRATION**

ROS may be derived from exogenous factors that impact both IVF and ICSI. This effect may be more pronounced in IVF due to longer incubation time intervals leading to increased exposure to environmental oxygen concentration. A hyperoxic environment promotes enzyme activity, which generates an increased level of the superoxide radical. The growth of a first trimester embryo in vivo has been demonstrated to take place in a microenvironment with low oxygen tension [15]. This leads to the assertion that a low oxygen concentration is necessary during ART, which attempts to mimic the conditions of in vivo fertilization and pregnancy [15].

Studies have shown that embryos cultured in an environment with atmospheric oxygen concentration and exposed to increased levels of hydrogen peroxide radicals resulted in DNA fragmentation of approximately 20% of embryos, compared with DNA damage seen in 5% of embryos grown in an environment with low oxygen tension [16]. Reports of successful blastocyst development with embryos cultured in a low oxygen tension environment [16] imply that the oxygen concentration experienced in the in vitro setting plays an influential role in the outcome of ART procedures. A study by Noda et al. confirmed the advantage of lower oxygen concentrations in the in vitro environment by measuring OS levels encountered when using a new IVF culture system with low oxygen tension and a low level of illumination [17]. This manipulation of the culture media resulted in high blastulation rates and enhanced human embryo development [17]. Another study found that, in addition to improved blastocyst outcomes in low-oxygen environments (5%) versus high-oxygen concentrations (19%), human embryos cultured in low oxygen tension are associated with an increased live birth rate compared with embryos incubated under the influence of high oxygen concentrations [18].

**METALLIC CATIONS**

In the setting of ART, metallic cations may promote ROS generation and act as an exogenous source of OS. According to the Haber-Weiss reaction, traces of metallic ions such as iron or copper in the culture medium may lead to increased ROS generation [19]. The binding of metallic ions by metal chelators such as ethylenediamine tetra-acetic acid (EDTA) or transferrin can inhibit the ability of metals to react and produce ROS, suggesting their potential use within the embryo culture media to block the induction of ROS by metals present in the in vitro setting. Use of the iron chelator transferrin within the culture media has been shown to decrease lipid peroxidation and the formation of hydroxy radicals [20]. Minimizing the production of highly toxic hydroxy radicals is protective against free-radical-mediated damage to oocytes and preimplantation embryos and can lead to improved fertilization and pregnancy rates [20].

**ILLUMINATION**

Visible light acts as an exogenous source of OS in that it promotes ROS production, causing cellular damage through the oxidation of DNA bases and DNA strand breaks [21]. The findings of a study by Beehler et al. implicated UV
radiation in ROS-induced DNA base modifications. Furthermore, Takenaka et al. showed that smaller amounts of short-wavelength visible light are advantageous in ART as they expose oocytes and embryos to lower levels of OS than cool white light [22]. These results suggest that minimizing the exposure of oocytes, zygotes, and embryos to visible light and near-UV light will serve to better mimic *in vivo* conditions, thereby yielding more successful ART outcomes [22].

**SPERMATOZOA**

Sperm quality is a crucial determinant of the potential for successful fertilization and pregnancy achieved by ART [23]. At physiologic levels, ROS facilitates normal sperm function such as the acrosome reaction, oocyte fusion, and capacitation. Intracellularly, sperm generate ROS at the level of their plasma membrane and mitochondria. Leukocytes act as a source of OS by generating extracellular ROS in prostatic and seminal vesicle secretions [23, 24]. Leukocytes can cause ROS-induced damage when seminal plasma is removed during sperm preparation for assisted reproduction. Seminal leukocytes stimulate ROS production by spermatozoa.

Male germ cells are extremely vulnerable to OS as the sperm membrane is rich in unsaturated fatty acids [23] and lacks the capacity for DNA repair [25]. Human spermatozoa generate superoxide radicals that can cause lipid peroxidation, which occurs in a self-propagating manner. This is associated with a decrease in membrane fluidity and reduced activity of membrane and ion channels which impairs spermatozoa fertilization capacity. In addition to these effects, OS arising from spermatozoa induce oxidative damage to the oocyte and its DNA, reducing the likelihood of successful fertilization [25]. A study conducted by Hammadeh et al. demonstrated that metaphase oocytes incubated with DNA-damaged spermatozoa during IVF were associated with high rates of failed fertilization, defective embryo development, implantation failure and early abortion [26]. Therefore, utilizing sperm preparation techniques that minimize ROS production within seminal fluid and by spermatozoa themselves is likely to improve the success of IVF and ICSI procedures.

Swim-up preparation is a sperm preparation technique in which a semen sample is centrifuged to form pellets that are coated with culture medium. The most motile spermatozoa will swim up into the culture medium, and they are selected for use in the IVF procedure. This technique is not appropriate for semen samples with excessive leukocytes or immature or damaged spermatozoa [9] as close contact with defective spermatozoa and leukocytes [11] will lead to ROS-induced damage of functional spermatozoa. It is hypothesized that the risk of OS-induced damage may be reduced by incorporating the use of antioxidants in the swim-up technique [27]. ROS levels have been shown to be significantly lower in sperm suspensions washed with antioxidants than in the control group without antioxidant protection. Antioxidant supplementation was seen to significantly decrease DNA fragmentation rates in spermatozoa, whereas it had no effect on lipid peroxidation. These results suggest that antioxidant supplementation may serve a beneficial role in sperm preparation to improve ART outcomes by enhancing the overall functional parameters of spermatozoa through reducing levels of oxidative stress [27].

Density-gradient centrifugation is a sperm preparation technique used to isolate mature, leukocyte-free spermatozoa [9]. This method employs centrifugation to separate fractions of spermatozoa based on motility, size, and density [3]. The process of centrifugation can activate seminal leukocytes and disturb the redox balance within a semen sample, with the potential to adversely affect sperm function [28]. A study by Shekarriz et al. demonstrated that even a single-step centrifugation for a short period could significantly increase ROS formation in human semen [28]. Increased levels of ROS are also thought to arise during this type of sperm preparation technique due to mechanical perturbation of the sperm plasma membrane. Thus, minimizing centrifugation time minimizes the formation of excess ROS and may ensure the use of high quality sperm in ART [28].

In general, the literature confirms an inverse correlation between ROS levels and sperm functional characteristics, including density, motility, and morphology. Oxidative stress is related to DNA fragmentation of spermatozoa that is sub-optimal for use in IVF or ICSI [26]. Thus, strategies used to prepare sperm and decrease ROS levels in a semen sample may allow for an increased chance of successful fertilization and pregnancy with ART.

**HOW ARE ROS LEVELS MEASURED?**

**Follicular Fluid**

Oxidative stress is thought to occur within the follicular fluid of women undergoing IVF and ICSI [25]. The follicular fluid microenvironment plays an integral role in shaping the quality of the oocyte, which is a major determinant of successful ART outcomes [1, 25]. The results of a study by Van Blerkom et al. demonstrate that hypoxic conditions, as evidenced by low intrafollicular oxygenation, are related to an increased likelihood of oocyte cytoplasmic defects such as impaired cleavage and abnormal chromosomal segregation [29]. The degree of OS can be quantified by assessing biomarkers of lipid peroxidation and the total antioxidant capacity (TAC) within follicular fluid [25]. The compound malondialdehyde (MDA) is a by-product of lipid peroxide decomposition and can be used to measure ROS levels. Pasqualotto et al. discovered that the mean MDA levels of pregnant women were significantly lower than the mean MDA levels seen in non-pregnant control subjects [30]. In addition to its role as a biomarker for OS, MDA levels may be a predictive marker of ART outcome. Follicular fluid of oocytes that were later successfully fertilized exhibited an increase in TAC [1]. Thus, lower TAC, which would lead to a redox imbalance with overwhelming levels of unopposed ROS, can be related to decreased fertilization potential as an adverse effect of OS.

A study by Wiener-Megnazi et al. used a novel thermochemiluminescence assay to assess the degree of OS in follicular fluid [31]. Increased levels of OS within follicular fluid were significantly related to poor fertilization rates and post-fertilization outcomes, manifested as decreased blastocyst cleavage and development, and a lower likelihood for completion of a successful pregnancy [31].
Culture Fluid

Within the culture fluid, the TAC levels of Day 1 culture media are postulated to serve as a biochemical marker of protection against OS in the early stages of embryo development [25]. Day 1 TAC levels have been shown to significantly correlate with increased clinical pregnancy rates in ICSI cycles [25]. Bedaiwy et al. analyzed the effect of ROS levels on early human embryonic development in IVF and ICSI culture media on Day 1 post-insemination [12]. The findings implicate increased Day 1 ROS levels in decreased development rates, higher degrees of fragmentation, and reduced formation of morphologically normal blastocysts [12]. The results also showed that decreased fertilization outcomes in ICSI were significantly related to increased Day 1 ROS levels in the culture fluid. However, this apparent relationship was not exhibited by the conventional IVF cycles studied. Another study by Bedaiwy et al. used TAC levels in Day 1 culture media as a biochemical marker of OS to demonstrate an association between increased Day 1 ROS levels and slower development, higher fragmentation rates, and reduced formation of morphologically normal blastocysts [13]. Day 1 ROS and TAC levels within culture media serve as useful biomarkers of OS and can be utilized to quantify the degree of OS present in the setting of IVF and ICSI and relate OS to embryonic development parameters and thus ART outcomes.

Metabolomic Profiling to Assess ROS

In the past, evaluation of ROS and OS was based entirely on biochemical methods that are inconvenient and labor-intensive. Efforts have continued to determine a more efficient way of identifying and measuring biomarkers that accurately quantify and correlate OS to clinical reproductive outcomes. OS may be assessed on a molecular level using metabolomics- the systematic study of metabolites as small-molecule biomarkers that contribute to the functional phenotype of a cell, tissue, or organism [32]. The metabolome refers to the complete inventory of small molecules, metabolic intermediates such as amino acids, lipids and nucleotides, ATP, hormones, other signalling molecules, and secondary metabolites [33]. Low-molecular weight metabolites represent end products of cell regulatory processes, revealing the response of biological systems to a variety of genetic, nutrient, or environmental influences [34]. Therefore, the metabolome represents the end product of gene expression, illustrating the interaction of environmental conditions with physiology [35] (Fig. 2).

Metabolomic profiling is a rapid, non-invasive method of measuring OS markers such as -CH, -NH, -OH and ROH. OS is reflected by the CH:ROH ratio. Gas chromatography, high performance liquid chromatography, or capillary electrophoresis is used to separate biomarkers, while methods of spectrometry are used to identify and quantify them [36]. Metabolomic profiling has been investigated for its use in quantifying the degree of OS during ART and identifying those gametes and embryos most likely to contribute to successful implantation and pregnancy.

Currently, the morphology and cleavage rate of embryos are used as a predictive measure of implantation potential in IVF. Morphological assessment consists of observing the developmental pattern of embryos during culture, fragmentation, inclusion bodies, cell number, morphology of the inner cell mass and trophoectoderm, and blastocoele expansion at the blastocyst stage [37]. However, these parameters may not accurately reflect functional status, as normal-looking gametes and embryos can still harbour genetic or epigenetic defects such as ROS-induced damage [38]. The inability to precisely determine the reproductive potential of gametes and embryos contributes to the high failure rate in IVF. To minimize the risk of implantation failure, multiple embryos often are transferred simultaneously, leading to an increased incidence of multiple pregnancies [35]. Metabolomic profiling used in conjunction with morphological assessment of gametes and embryos may serve to maintain or increase overall pregnancy rates while decreasing the unfavorable occurrence of multiple gestations [35].

Fig. (2). The use of metabolomic profiling to quantify oxidative stress.
A quantitative, objective assessment of gametes retrieved for use in IVF may provide a prognostic indication of IVF outcome and clarification of the reasons for success or failure of the procedure. Metabolomic profiling can be used to assess the impact of OS on sperm function and fertilization capacity. A study by Agarwal et al. demonstrates the existence of unique spectral signatures of semen samples that indicate statistically significant differences in the levels of oxidative stress among men with various conditions known to be associated with altered redox status including varicocele, idiopathic male factor infertility, and vasectomy reversal. The various degrees of OS were evidenced by unique changes in the ratios of –CH to ROH. Significant differences in –CH, -NH, and –OH concentrations were observed among the groups of subjects [39]. These findings assert that metabolomic analysis is a rapid, non-invasive diagnostic method that can be used to assess semen for abnormalities related to reactive oxygen species.

The metabolome of an oocyte is thought to be ‘fingerprinted’ by its interaction with its IVF culture medium and exogenous factors such as environmental oxidative stress [40]. Metabolomic profiles of follicular fluid samples have demonstrated a significant relationship between high levels of OS in follicular fluid and decreased oocyte viability and poor embryo quality [41]. A study by Nagy et al. correlated the metabolome of oocytes with corresponding embryo fertilization, development, and viability and demonstrated the ability of NIR spectroscopy to predict fertility potential as early as the pre-fertilization stage of oocytes. Metabolomic analysis of the spent culture media of oocytes was shown to predict embryo development at Day 3 and Day 5, with the potential to indicate embryo viability. NIR analysis was shown to assess the metabolomic status of oocytes with high sensitivity and significant correlation with healthy embryo morphology and high implantation potential [40].

Embryo metabolomic profiles also have been shown to vary according to embryo implantation potential. A study by Scott et al. used metabolomic profiling to calculate a viability index for 41 spent media samples from 19 patients with known reproductive potential. Higher viability indices were observed in both Day 3 and Day 5 embryos with proven reproductive potential than in those that failed to implant. An overall diagnostic accuracy of 80.5% in predicting delivery or a failed implantation demonstrates the significant relationship between the reproductive potential of embryos and modifications of their culture media [42]. Sell et al. used a multivariate analysis approach to compare the spectral profiles between embryos that resulted in live births with those that failed to implant. Markers of OS discriminated between the two study populations and were most predictive of pregnancy outcome. CH:ROH content was significantly lower in the culture media of embryos that progressed to pregnancy than in the culture media of embryos that failed to implant [43]. Therefore, in vitro cultured embryos with high reproductive potential may alter their environment differently compared with embryos that do not result in pregnancy [44].

A similar investigation by Agarwal et al. used metabolomic profiling to identify the OS biomarkers R-OH, CH, OH, and NH in 228 embryo media, 72 follicular fluid, and 133 seminal plasma samples. The discarded culture media, follicular fluid, and seminal plasma were shown to consistently produce unique metabolomic OS profiles, which correlated well with pregnancy versus non-pregnancy outcomes [45].

In addition to indices of redox status, metabolomic profiling of amino acids may be helpful determining the functional status of embryos. During the IVF process, embryo culture media is often supplemented with mixtures of amino acids that influence blastocyst formation [46]. A study by Houghton et al. found that competent preimplantation embryos that develop into blastocysts demonstrate a lower rate of amino acid turnover than embryos that did not progress to the blastocyst stage. These results suggest that embryos with higher metabolic activity are more likely to arrest, contributing to failed outcomes in ART [47]. Brison et al. reported an association between decreased glycine and leucine and increased asparagine levels in the culture media with increased clinical pregnancy and live birth rates. Lower levels of amino acid metabolism in embryos were correlated with increased viability [48]. Cryopreserved embryos used in successful IVF procedures also have been shown to exhibit lower rates of amino acid metabolism compared with those that failed to implant [49]. Therefore, the use of metabolomic profiling to measure parameters such as CH:ROH and amino acid turnover may allow for better discernment when evaluating and selecting embryos for transfer in IVF. These methods of objectively quantifying embryo viability and implantation potential may minimize the number of embryos needed to be transferred, thereby decreasing the incidence of multiple infant births and improving overall pregnancy outcomes.

In addition to predicting the potential of gametes and embryos to give rise to favorable reproductive outcomes, metabolomic profiling of the uterine endometrial lining may indicate the degree of endometrial receptivity to blastocyst implantation, providing the opportunity to optimally time embryo transfer in ART [32]. Although the results of many studies suggest various ways in which metabolomic profiling may help to improve ART outcomes, this method of evaluating the functional status and potential of gametes and embryos has yet to be standardized and requires validation by further studies.

WHAT IS THE ROLE OF ANTIOXIDANTS IN IVF AND ICSI?

The culture media used in IVF and ICSI can be a source of ROS generation during ART procedures [50]. The in vitro environment exposes gametes to ROS in excess of what they would normally face in vivo [50]. An excess of ROS has the ability to damage lipids, proteins, nucleic acids, DNA, and RNA [25]. Thus, IVF protocols must be revised to incorporate strategies that decrease or prevent the generation of ROS [50].

Javiet et al. established that culture media riddled with a toxic level of OS compromises oocyte and embryo integrity. Because the success rates of IVF are influenced by the quality of the embryos transferred, antioxidant supplementation...
to neutralize the effects of ROS on oocyte quality may have a beneficial effect on ART outcome [25]. Repeated changes of the culture media and the use of sequential culture systems may help to minimize the ROS generated from within the culture media itself [11]. The use of supplemental antioxidants to modulate ROS levels in the culture media is hypothesized to promote an ideal environment for pre-implanted embryos produced by ART [25]. Ongoing trials continue to investigate the role for oral antioxidant supplementation in both infertile men and women with the aim of optimizing the success rates of IVF and ICSI procedures.

**Antioxidant Supplementation Within the Culture Media**

The success of ART depends on how closely the *in vitro* setting mimics *in vivo* conditions. Therefore, it is crucial to have physiological concentrations of individual amino acids, antioxidants, vitamins, and energy sources within the culture media of embryos to maximize blastulation hatching rates [51]. However, the literature presents conflicting reports regarding the validity of using any one specific antioxidant therapy to improve ART outcomes. However, given the strong evidence that OS plays a pathogenic role in decreasing the success rates of IVF and ICSI, the potential for the use of antioxidants to control OS in the *in vitro* setting is a promising concept that warrants further investigation in the field of reproductive medicine (Fig. 3).

Wang *et al.* elucidated the effects of adding the antioxidant vitamins C and E to culture media in ART. The results of this study demonstrated that these antioxidants led to increased rates of blastocyst development, by way of counteracting the embryotoxic effects of ROS [6]. Embryo culture supplementation with vitamin C was seen to exert a protective effect on embryo development in culture media containing an ROS-generating PMA-activated leukocyte supernatant [52]. Furthermore, the findings of Wang *et al.* suggest that the beneficial, ROS-neutralizing effect of vitamin C supplementation in culture media may be superior to the effects seen with vitamin E supplementation or simultaneous supplementation with both vitamins C and E [6]. The idea that there is a critical composition and dosage of antioxidant supplementation, below or beyond which the full potential protective benefit may not be realized, is confirmed by the findings of Choi *et al.* [53]. This study showed that vitamin C administered at a higher dosage than needed is capable of inducing damage. Moderate dosages, however, were seen to reduce oxidative damage in the culture media, with no detrimental effect on mouse oocyte quality [53].

The investigations of Olson *et al.* determined that vitamin E supplementation in culture media increased the number of bovine embryos that reached the fully expanded blastocyst stage [52]. This effect is thought to be mediated by the role vitamin E plays in protecting unsaturated membrane fatty acids. The peroxidation of these membrane lipids can lead to structural damage and affect its membrane function. Furthermore, the results showed that vitamin E supplementation alone improved conditions for embryonic development more than that seen when vitamin E and C were employed in combination.

Supplementing the culture media with melatonin, a powerful hormonal agent known to possess the ability to neutralize ROS, also has been shown to improve the efficiency of *in vitro* embryo production in experiments using buffalos [54]. This has led to studies investigating the merits of oral melatonin supplementation to improve fertility.

L-carnitine also has been evaluated for its potential use as a supplement in embryo culture medium and the effects of this supplementation on developing mouse embryos [55]. L-carnitine is known to possess antioxidant properties, consisting of free radical scavenging and metal-chelating properties [55]. Specifically, this compound has the ability to neutralize the embryotoxic effects of exogenous production of oxidative stress by hydrogen peroxide [55]. Embryo culture medium supplementation with L-carnitine has been shown to bring about a significant improvement in the blastocyst development rate compared with a control group without antioxidant supplementation. At moderate concentrations, L-carnitine is has been proven effective in blocking the effect of ROS, resulting in a decreased level of DNA damage [55].

---

**Fig. (3).** Interventions to control OS during IVF/ICSI.
**Oral Antioxidant Supplementation**

Studies investigating the benefits of oral antioxidant therapy in male and female patients undergoing IVF or ICSI procedures have yielded inconsistent data and conflicting reports.

**FEMALE**

Oral antioxidant supplementation in female patients undergoing conventional IVF or ICSI has been evaluated in terms of the effects on fertilization rates and pregnancy outcome.

Melatonin, a free radical scavenger that acts within the mitochondria to decrease protein damage, improve electron transport chain activity, and reduce mitochondrial DNA damage [56], has been shown to protect against the toxic effects of oxidative stress on oocyte maturation, thus improving oocyte quality and fertilization rates [56]. Tamura et al. used the biomarker 8-hydroxy-2-deoxyguanosine to evaluate the association between OS in follicular fluid and poor oocyte quality. Orally administered melatonin was seen to improve both the redox status within follicular fluid and oocyte quality [56]. In addition to confirming the toxic effects of OS on oocyte maturation, the study supported the idea that oral melatonin supplementation can be used to bring about a significant reduction in the number of degenerate oocytes and increase the number of fertilized embryos [56].

Despite the findings of Tamura et al., clinical trials have failed to demonstrate a specific regimen and dosage of antioxidant supplementation that will provide a definitive increase in ART success rates. Antioxidant supplementation with ascorbic acid has long been hypothesized to have a favorable influence on ART procedures for female factor infertility. A significant correlation is known to exist between the level of ascorbic acid in a woman’s blood serum and follicular fluid, with the follicles having a higher concentration of ascorbic acid [54]. Crha et al. conducted an investigation in which a group of women supplemented with vitamin C during the period of hormonal treatment in IVF had a statistically insignificant increase in the ability to achieve pregnancy compared with the control group that did not receive oral antioxidant supplementation [57].

Based on the premise that ascorbic acid confers an improvement in fertility and ART outcomes, Griesinger et al. evaluated the impact of ascorbic acid at different doses on women undergoing IVF procedures [58]. In contrast to the findings of Crha et al., the results of this study failed to reveal clinical evidence of any beneficial effect of ascorbic acid on IVF [58]. Furthermore, Tarin et al. found that the oral administration of pharmacological doses of vitamins C and E on mice had no effect on time to pregnancy, age of cessation of female reproductive life or pregnancy rate [59].

**MALE**

In contrast to the accepted management of female infertility, oral antioxidant supplementation in the management and treatment of infertile men is widely accepted and practiced. A study by Geva et al. demonstrated that oral antioxidant supplementation with vitamin E led to a significant increase in fertilization rates in IVF for male factor infertility [60]. The results of this study provide evidence for the potential of vitamin E therapy to improve fertilization rates of fertile normospermic patients with low fertilization rates after one month of treatment. Greco et al. demonstrated a significantly reduced percentage of DNA-fragmented spermatozoa in a study group treated with oral vitamins C and E compared with control group subjects who were not given oral antioxidant supplements [61]. However, this study failed to show any improvement in pregnancy rates with oral antioxidant therapy. Another study by Greco et al., which examined the beneficial effect of oral antioxidant treatment in cases of DNA-damaged sperm utilized for ICSI, revealed no differences in fertilization and cleavage rates or in embryo morphology with vitamin C and E antioxidant treatment. However, a marked improvement in the number of clinical pregnancies and implantation rates was observed in the antioxidant treatment group [62]. Vitamin E itself has been hypothesized to protect against the loss of sperm motility by lipid peroxidation and improve sperm motility and increase the possibility of successful fertilization with sperm from asthenospermic patients [63]. Furthermore, Suleiman et al. showed a significant decrease in ROS-induced sperm and improvements in spontaneous pregnancy rates during the next six months with oral vitamin E supplementation [63].

Tremellen et al. investigated Menevit (Bayer Health Care, Pymble, NSW, Australia), an oral antioxidant treatment used by male patients, and found no differences in quality of resultant embryos between the antioxidant-treated and control groups. However, supplementation was related to significant improvements in implantation rates and pregnancy outcomes. Therefore, treating infertile males with the oral antioxidant Menevit may serve to improve pregnancy rates by optimizing factors related to the amount of OS experienced during the course of IVF/ICSI treatment [64].

Meneco et al. [65] correlated oral vitamin intake with reduced levels of ROS-induced DNA fragmentation, leading to improved fertility. However, in addition to better semen quality, oral antioxidant supplementation also was associated with increased sperm decondensation, which may prevent IVF/ICSI success [25]. A study by Rolf et al. failed to demonstrate any improvement in semen parameters or survival rates of sperm from men with asthenozoospermia or moderate oligoasthenozoospermia who were supplemented with oral vitamin C and E [66]. Although considerable data suggest a possible benefit from vitamin C and E supplementation on ART outcomes, several investigations have yielded conflicting findings. This lack of consensus in the literature complicates the debate as to whether oral antioxidant supplementation has a definitive role in optimizing ART outcomes.

**CONCLUSION**

Despite the many advances in ART, the issue of using antioxidant therapy to alleviate the burden of infertility by improving IVF and ICSI procedures and their outcomes continues to be the subject of much debate. Although data from the existing literature fail to provide any definite conclusions regarding whether specific antioxidant supplementation of infertility patients and culture media used in ART will
increase successful ART outcomes, significant evidence suggests that it has the potential to combat oxidative stress, a known contributor to ART failure. There is no doubt that there is an underlying link between OS and difficulty in achieving fertilization and eventual pregnancy with IVF and ICSI. Further controlled evaluations using large sample size populations are needed to arrive at a consensus regarding the use of antioxidant supplementation in the culture media and oral administration of these compounds in both male and female patients undergoing ART.

EXPERT COMMENTARY

The purpose of this article was to discuss the role played by oxidative stress in assisted reproductive technologies such as IVF and ICSI. Controlled levels of OS are known to serve a physiological role in the various biochemical pathways that comprise human reproduction. However ART procedures lack the protection of the natural antioxidant defense of the female reproductive tract, the microenvironment where fertilization normally takes place. Therefore, gametes used in ART procedures are highly susceptible to damage from overwhelming and unopposed degrees of oxidative stress that may arise endogenously from the gametes themselves and from exogenous influences within the laboratory environment such as oxygen tension, light, and metallic cations. Increased OS has been shown to hinder sperm-oocyte interaction and adversely affect fertility and successful pregnancy rates. Given the evidence that implicates oxidative stress in producing poor ART outcomes, elucidating the precise mechanisms by which ROS arise in the ART environment, current methods to accurately quantify OS, and potential strategies to modulate the amount of OS experienced by gametes within both the human reproductive tract and the in vitro culture media must be a top priority.

The field of metabolomics has given rise to novel techniques to more accurately measure oxidative stress. Oxidative stress quantification may allow for the selection of the most viable gametes with the least OS-induced damage to be used in ART to optimize fertility outcomes and ensure greater efficiency. ART outcomes also may be improved by modulating the amount of OS experienced by gametes and embryos in vitro by supplementing the culture media with protective antioxidants. Furthermore, increased levels of oxidative stress that may be seen in vivo in both the male and female partner participating in ART may be treated by oral antioxidant supplementation. The use of oral antioxidants to treat male factor causes of infertility are well-established and in clinical use. However, the safety and efficacy of oral antioxidant supplementation to treat female infertility requires further investigation.

FIVE YEAR VIEW

Success rates in ART are influenced by maternal age, number of oocytes retrieved, and the quality of the embryos transferred. Embryo quality is influenced by extrinsic factors like culture media. A large number of extrinsic factors that modulate OS can influence successful outcomes of IVF and embryo transfer including oxygen concentration, ionizing radiation, and levels of the antioxidants EDTA, SOD, and catalase. Evidence suggests that media supplementation with antioxidants, disulphide reducing agents, or divalent chelators of cations may be beneficial to gametes/embryos. Repeated change of media and use of sequential culture systems may help reduce exposure to ROS. Oocytes also can be protected from oxidant-induced early apoptosis by supplementing media with vitamin E and C. In addition to strategies to modulate OS to reduce the chance of fertilization and pregnancy failure, more accurate methods to assess the degree of oxidative stress may allow for selection of healthy gametes and embryos for use in IVF or ICSI. Optimizing the procedure in this way may serve to reduce patient time and costs associated with the need for repeat use of assisted reproductive technologies. The significance of improved IVF/ICSI outcomes would be enormous to the many infertile couples who seek assisted reproduction due to infertility issues.

KEY ISSUES

- IVF and ICSI are assisted reproductive techniques that achieve fertilization by circumventing various anatomic, mechanical and functional obstacles to fertility in vivo.
- Oxidative stress arises when high levels of ROS overwhelm antioxidant capacity, resulting in modified gene expression and transcription factors and damaged DNA. ART procedures are susceptible to increased OS as gametes lack the natural antioxidant defence seen during in vivo reproduction in the male and female reproductive tract.
- Endogenous sources of oxidative stress in ART include the multiple oocytes per dish, cumulus cell mass, and spermatozoa used for insemination in IUI or incubation with oocytes in IVF. ICSI avoids excess ROS that may arise from defective spermatozoa in IVF.
- Exogenous sources of oxidative stress in ART include oxygen concentration, metallic cations, and illumination.
- In the past, studies quantified OS by inconvenient and labor-intensive biochemical methods to measure ROS and antioxidant status within follicular fluid and culture fluid. Metabolomic profiling is a faster, more accurate method of quantifying OS during ART and may be used to identify gametes and embryos more likely to contribute to successful implantation and pregnancy.
- The success of ART is increased when the in vitro culture setting mimics in vivo conditions. Therefore, the use of the following antioxidants in culture media may improve outcome: vitamin C, vitamin E, melatonin, and L-carnitine.
- Various studies on the benefits of oral antioxidant supplementation in male and female patients undergoing ART procedures have yielded inconsistent and conflicting reports, and further research is required.

REFERENCES

The Role of Oxidative Stress and Antioxidants in Assisted Reproduction

Current Women’s Health Reviews, 2010, Vol. 6, No. 3


