

Female Infertility and Assisted Reproduction: Impact of Oxidative Stress

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Abstract: Oxidative stress (OS) occurs with an overabundance of reactive oxygen species (ROS) generation and the inability of scavengers, i.e. antioxidants, to neutralize excessive loads of ROS. OS has a role in the etiopathogenesis of many factors causing natural infertility. Infertility is a problem of great magnitude affecting 6 million American women. The etiologies of unexplained infertility and recurrent pregnancy loss remain unclear and present a scientific challenge. Oxidative stress may be a piece in this puzzle. Although investigation of the exact mechanisms by which OS causes pathological processes affecting female fertility is ongoing, research has clearly shown that the redox state affects gametes, their interactions, and the resultant embryo. OS has adverse effects on assisted fertility including IVF/ICSI and in-vitro maturation. This article addresses the role of OS in female infertility, the effect it has on assisted reproductive techniques, and OS prevention strategies including the use of in-vivo and in-vitro antioxidant supplementation.

INTRODUCTION

Reactive Oxygen Species, Oxidative Stress, and Antioxidants

The most stable form of an atom is its ground state where each outermost shell electron is paired with a complimentary electron. These electron pairs spin in opposite directions creating a balanced atom. Free radicals are independent atomic species with unpaired outer shell electrons. As covalent bonds are broken between molecules, lone pair electrons accompany each separated atom. Free radicals with oxygen centers are commonly known as reactive oxygen species (ROS). ROS have two unpaired electrons that are located in the outer shell of the atom. To achieve a ground state, free radicals commonly steal electrons from neighboring molecules. The stealing of a single electron results in the formation of a free radical containing one final unpaired electron. Consequently, electrons spinning antiparallel to this final unpaired electron are stolen from a bystander molecule which returns the unstable atom to its preferred ground state. Oxygen species are most commonly formed during the inner mitochondrial membrane's electron transport chain, specifically via the intermediate reduction states of complex I and complex III [1]. The electron transport chain utilizes oxygen to produce adenosine triphosphate (ATP), energy that a cell exploits to sustain its metabolic activity.

Reactive oxygen species function as either oxidants or reducers in response to their changing molecular environments. They also act distinctly when present in diverse tissue concentrations. For example, the presence of ROS in tissue at moderate levels indicates their function as normal cell signal responders. Once ROS are present in high concentrations, it is probable that their overabundance is a product of oxidative damage which triggers DNA damage and

increases cell apoptosis, also referred to as cellular death [2, 3]. Unlike other oxidants (e.g., molecular oxygen) ROS are unstable and aggressive molecules that are not capable of diffusing across biological membranes due to their polar nature. A number of ROS are commonly generated in the female reproductive tract including the hydroxyl radical (OH^\cdot), hydrogen peroxide (H_2O_2), and the superoxide anion radical ($\text{O}_2^\cdot^-$) [4].

The superoxide anion radical is a primary ROS of medium reactivity [5]. It is produced when an oxygen molecule gains one electron, consequently allowing the unstable atom to maintain an additional unpaired outer shell electron. Operating as a reductant, $\text{O}_2^\cdot^-$ converts to molecular oxygen; as an oxidant, it transforms into the peroxide dianion. When present at high concentrations, $\text{O}_2^\cdot^-$ supports cell component modifications and is susceptible to producing secondary ROS through metal-catalyzed or enzyme-catalyzed processes. The superoxide anion itself converts to H_2O_2 --a compound easily catalyzed by the enzyme superoxide dismutase (SOD) in the production of supplementary secondary ROS.

Universal chain reactions lead to the generation of the mitochondrial superoxide anion *in vivo*. This radical's rate of formation depends on oxygen flow through the mitochondria. Hydrogen peroxide is produced readily *in vivo* by assorted reactions as well. The hydrogen peroxide radical conversion is catalyzed by glutathione peroxidase during the alteration of glutathione to oxidized glutathione, converting H_2O_2 to H_2O . Hydrogen peroxide may also be converted to the hydroxyl radical via the Harber-Weiss reaction. Hydroxyl radicals are the most powerful free radicals due to their simultaneous short life spans and damaging properties. Hydroxyl radicals cause DNA damage by breaking nucleic acid strands via the modification of pyrimidines and purines [6].

An oxidant-antioxidant balance exists *in vivo*. Antioxidants are ROS scavengers responsible for neutralizing oxidants and include α -tocopherol (vitamin E), β -carotene, ascorbate (vitamin C), and glutathione as well as enzymes such as SOD, glutathione peroxidase, and catalase [7]. When

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the balance of antioxidants and oxidants is disturbed in favor of the oxidants, oxidative stress (OS) occurs.

Female Infertility and Assisted Reproduction Techniques

Enough evidence is available to suggest that excess oxidant production and the resultant OS may cause female infertility. According to the American Society of Reproductive Medicine, infertility is the inability to attain pregnancy after engaging in regular unprotected sexual intercourse for at least one year [8]. Infertility is a common condition affecting approximately 13%-14% of all couples. Varied causes of infertility amongst men and women exist; therefore, every case is an entity in itself. Female factors are responsible for 40%-50% of infertility cases while the others are due to male causes as well as combined female/male causes and unexplained infertility [9].

As of 2003, female infertility (age-dependent) affected 7% to 28% of women [10]. A demand for infertility services has increased substantially over the past decade due to the prevalence of infertility in the general population. Although infertility represents a small facet of the broad intellectual scope of reproductive endocrinology, patients seeking infertility treatment represent the mainstay of a clinical reproductive endocrinologist's practice. The presence of oxidant and antioxidant systems in various reproductive tissues has evoked great interest in the role of OS in human reproduction [4].

OXIDATIVE STRESS IN FEMALE INFERTILITY

Oxidative stress is hypothesized to physiologically and pathologically contribute negatively to a number of reproductive processes including folliculogenesis, oocyte maturation, sperm DNA damage, necrozoospermia, asthenospermia, endometriosis, and the etiology of defective embryo development [11-15]. Oxidative stress often damages the cellular membrane, retards embryo development [16], and induces cellular apoptosis which can yield fragmented embryos with a limited ability to implant [17]. An association between OS and infertility is hypothesized to exist, but a true cause and effect relationship has yet to be established. The role of OS in various infertile groups including women with tubal or peritoneal factor infertility and endometriosis has been evaluated, but the cumulative studies do not yield a definitive conclusion regarding OS, infertility, and the relationship between each factor [18-20]. Women with endometriosis often have elevated macrophage levels that may produce excessive ROS in the peritoneal environment [21]. Increased ROS levels in the tubal and peritoneal environments negatively alter fertilization capabilities and embryonic development [16].

According to Szczepanska *et al.*, endometriosis patients exhibit increased macrophage activation in the peritoneal cavity [22]. Activated mononuclear phagocytes produce cytokines, growth factors, and various other proinflammatory mediators, which further disturb the body's internal antioxidant/pro-oxidant balance [21, 23]. The peritoneal fluid of endometriosis patients has also been shown to have lower concentrations of SOD than patients with idiopathic infertility [22]. Others argue that levels of SOD in the peritoneal fluid of endometriosis patient are neither increased nor decreased.

Unexplained infertility is more ambiguous than other types of infertility. Unexplained infertility can result from increased ROS production in the peritoneal cavity. Samples of peritoneal fluid from women with idiopathic infertility often are characterized by increased ROS levels [24]. Furthermore, antioxidant levels in patients with unexplained infertility are significantly lower than those of healthy controls [20]. It has been proposed that the imbalance between antioxidants and ROS, favoring the latter, is responsible for increased OS levels that induce infertility.

OXIDATIVE STRESS IN ASSISTED REPRODUCTION TECHNIQUES

Generation of ROS during Assisted Reproduction Techniques

Reactive oxygen species may originate directly from gametes and embryos (endogenous sources) as well as their surroundings (exogenous sources). Several exogenous factors identified in culture media enhance embryo production of ROS: oxygen concentration, the presence of metallic cations, and visible light exposure. Spermatozoa may also aid in the production of ROS during assisted reproduction techniques.

Elevated ROS levels generated exogenously or endogenously influence the gametes, gamete interaction, fertilization and pregnancy rates with IVF/ICSI. Oxidative insult to embryos can lead to 2-cell block, embryonic arrest or even embryonic demise [25, 26]. Higher levels of ROS in the follicular fluid and semen are associated with poor fertility outcomes with assisted reproduction [27, 28]. In a meta-analysis from our group, ROS in semen have been reported to significantly effect the fertilization rate with IVF [29]. Measurement of ROS levels may help counsel patients on their adverse effects.

Embryo development depends on a variety of factors such as embryo density, gas phase, and incubation volume. Gas in the culture media is an important aspect to consider as it balances neutral and ionic bicarbonate concentrations. The addition of gaseous carbon dioxide (CO₂) is necessary to maintain a gaseous balance. Oxygen (O₂) tension in healthy oviducts and uteri is normally 2% - 8% in the presence of gametes [30]. Elevated O₂ levels (>20%) may create unfavorable conditions that produce excess free oxygen radicals. These radicals may then damage lipids, DNA, and proteins as well as block embryo development. Studies have monitored oxygen tension in the oviducts and uteri of the rhesus monkey, hamster, and rabbit. Oxygen tension ranges from 8.7% in the rabbit oviduct to 1.5% in the monkey uterus [30]. The growth of human embryos in low oxygen concentrations can mimic physiological conditions. In a study of 106 patients, embryos were cultured in the presence of either 20% O₂ (atmospheric condition) or 5% O₂ (physiological condition). Improved embryo morphology was evident in the embryos cultured in a physiological gas phase (i.e. 5% O₂), but the study failed to demonstrate a significant difference in fertilization and pregnancy rates. It may be logical to grow an embryo in the physiological conditions of the mammalian reproductive tract characterized by 2% - 8% oxygen concentrations. In addition, aerobic conditions may alter defense mechanisms

against OS by reducing glutathione. This has been observed in spermatozoa when incubated at atmospheric concentrations [31].

Iron can directly act on lipids and magnify peroxidative damage once initiation by free hydroxyl radicals has begun. Traces of iron and copper are often present in water used during culture media preparation [32]. Limiting trace amounts of these metals is ideal. Exposing embryos to visible light for more than 5 minutes can also increase production of ROS [33]. Visible light can induce ROS production and cellular damage by oxidation of bases, resulting in DNA strand breaks [34].

Reactive oxygen species are most commonly produced by endogenous factors [11]. A pre-implantation embryo generates ATP via oxidative phosphorylation and glycolysis. Inhibition of oxidative phosphorylation results in reduced production of ROS, which has been shown to improve embryo development in both porcine and bovine species [35]. Glucose, in excessive quantities, exhibits deleterious effects on embryos *in vitro*. Forsberg *et al.* showed that embryos originating from diabetic rats were characterized by an increase in mRNA encoding for mitochondrial SOD (Mn-SOD) and a decrease in mRNA corresponding to catalase [36]. NADPH and xanthine oxidase are the oxidative enzymes present in pre-implantation embryos [37]. The incubation of 2-cell mouse embryos with an NADPH oxidase inhibitor results in the reduction of H₂O₂ production.

Follicular Fluid OS

Oocytes surrounded by follicular fluid and granulosa cells create the microenvironment of a maturing oocyte prior to ovulation and act as predictors of ART outcomes such as fertilization, embryo cleavage, and pregnancy rates. In a recent study, follicular fluid and ejaculate were assessed for glutathione (GSH), cysteine (Cys), homocysteine (Hcy), and cysteinylglycine (CGS) in 156 couples undergoing ART [28]. 67.9% of the patients had intracytoplasmic sperm injection (ICSI) and 32.1% had *in vitro* fertilization (IVF) performed. The two main fertility outcomes measured were: number of fertilized oocytes and cleaved embryos. Higher levels of homocysteine in follicular fluid were reported in patients with endometriosis compared with women having idiopathic infertility. Increased levels of homocysteine lead to poor embryo quality on day 3, a significant finding in both the groups.

In a study designed to examine the role of follicular fluid in oocyte quality and embryo formation, ROS and lipid peroxidation (LPO) levels were found to be higher in unfertilized oocytes and in embryos of poor quality. 208 follicular fluid samples from 78 women with tubal-factor infertility undergoing IVF were examined for ROS and LPO. The oocyte maturity did not vary with changing levels of ROS and LPO [27]. In order to better understand these findings, the role of antioxidant activity in follicular fluid must be further assessed.

ROS Production in the Spermatozoa

Spermatozoa are also responsible for ROS-induced damage to embryos. Morphologically abnormal spermatozoa and seminal leucocytes are the main sources of ROS.

Inefficient spermatogenesis in men determines the presence of immature sperm, which produce high oxygen radical levels. Reactive oxygen species act on a sperm's DNA by altering its structure and resulting in poor ART outcomes [38]. The utilization of spermatozoa with abnormal DNA in ART may result in poor embryo quality and, ultimately, poor ART outcomes. A positive correlation between sperm containing amorphous heads, damaged acrosomes, midpiece defects, tail defects and ROS has been identified suggesting that ROS affect sperm morphology [38].

The sperm deformity index (SDI) is helpful in identifying infertile men; it divides the total number of sperm deformities by the number of sperm evaluated. Increased SDI is positively correlated with OS-induced male infertility [39]. Acrosin activity in patients with leukocytospermia is reduced due to OS affecting sperm function [40]. Such studies have demonstrated the importance of measuring ROS levels in female patients undergoing ART.

The Influence of ROS and Total Antioxidant Capacity in IVF and ICSI Cycles

Women who have undergone IVF have a tendency toward higher levels of ROS in their follicular fluid than those who conceive naturally [41,42]. Bedaiwy *et al.* assessed day 1 culture media ROS effects on fertilization rates, cleavage rates, fragmentation, and blastocyst formation after prolonged culture [42]. The authors compared conventional IVF with ICSI in order to determine which technique produced higher ROS levels. The results showed that slow development, high fragmentation, and reduced formation of morphologically normal blastocysts were associated with increased levels of Day 1 ROS. This study did not show any significant difference in fertilization rates and blastocyst development rates in IVF cycles, but significant reduction in these outcomes was reported in ICSI cycles with higher Day-1 ROS levels.

Bedaiwy *et al.* extended their study to measure the total antioxidant capacity (TAC) in IVF and ICSI cycles [43]. In this study, an increased TAC level correlated with higher fertilization and blastocyst development rates and decreased embryo fragmentation in both IVF and ICSI cycles. Elevated day 1 TAC was associated with increased pregnancy rates in ICSI, but not in conventional IVF cycles.

Often, increased levels of OS seem to be responsible for oocyte, sperm, and embryo damage. As superoxide ions penetrate cell membranes and alter cellular components such as lipids, proteins and nucleic acids, the observation of OS-induced damage is common. Mitochondrial alterations, ATP depletion, embryo cell block and apoptosis accompany high levels of OS. The literature has identified hydrogen peroxide as a mediator of apoptosis in blastocysts. A direct correlation has been observed between increased H₂O₂ concentrations and apoptosis in human fragmented embryos [44].

OXIDATIVE STRESS AND ART OUTCOMES

The damage caused by OS to oocytes, spermatozoa, and embryos may be due to the superoxide ion. As mentioned previously, the superoxide ion [O₂⁻] is a type of ROS that penetrates cellular membranes and alters intracellular molecules such as lipids, proteins and nucleic acids. This can

lead to mitochondrial alterations, ATP depletion, embryo cell block and eventually apoptosis [44]. High levels of ROS act negatively on the metaphase 2 meiotic spindle of mouse oocytes. The transcripts of Mn-SOD were detected in germinal vesicle oocytes, but not in metaphase 2 oocytes [45]. High levels of ROS may thus be responsible for impairing the quality of developing oocytes. It is clear that oocyte defense mechanisms vary according to its developmental stage. Poor defense mechanisms may yield poor embryo quality and aneuploidy (refer Fig. 1). Reactive oxygen species lead to 2-cell embryo block due to xanthine, the product of purine metabolism. It has also been demonstrated that ROS production decreases with the addition of hypoxanthine[46]. The changing energy needs of developing embryos induce the 2-cell block. ATP is the main source of energy for an embryo, but a shift in metabolic pathways from oxidative phosphorylation to glycolysis occurs due to increased energy requirements. Inhibition of oxidative phosphorylation reduces ROS generation and positively affects *in vitro* embryo development in porcine and bovine species [47]. Hydrogen peroxide is a mediator of apoptosis

in blastocysts, and a direct correlation has also been observed between increased H_2O_2 concentrations and apoptosis in human fragmented embryos [44].

TREATMENT

Assessing Levels of ROS and OS

Prior to the treatment of female infertility, ROS levels should be assessed. By estimating ROS levels, it may be possible to identify the cause(s) of infertility, especially in cases of idiopathic infertility [4]. The only known method for directly measuring ROS levels in the body is electron spin resonance (ESR) [4]. As a spectroscopic technique, ESR identifies molecular species containing unpaired valence shell electrons via the excitation of electron spins.

However, the most commonly used method for assessing ROS is the measurement of stable peroxidation product concentrations. Flow cytometry and chemiluminescence measure peroxidation products generated via the interaction of a reagent and ROS *in vitro*. Light-sensitive luminol, used in the chemiluminescence assay, detects and measures the

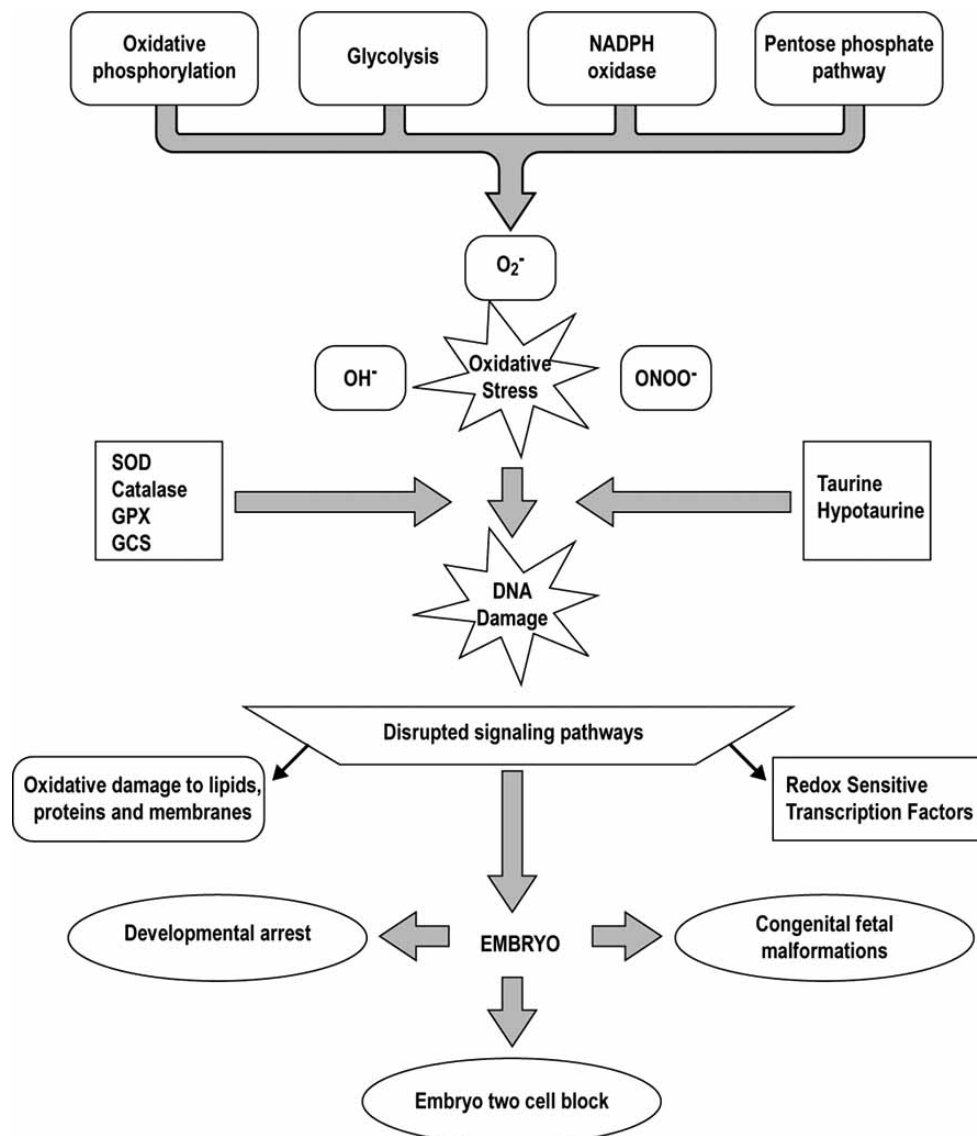


Fig. (1). Impact of oxidative stress on embryogenesis.

overall intracellular as well as extracellular ROS concentrations [48]. Intracellular ROS radicals can be measured individually using flow cytometry.

The thiobarbituric acid assay estimates levels of stable endogenous products such as malondialdehyde, whereas other methods measure DNA oxidation markers and oxidized proteins [4]. Total antioxidant capacity is commonly used to estimate OS levels in a system [12, 49]. Elevated OS and ROS levels as well as antioxidant concentrations in peritoneal fluid and serum have also been demonstrated in unexplained infertility, tubal infertility, and endometriosis patients [20, 50].

Antioxidant Supplementation

The use of both natural and synthetic antioxidants in treating female infertility patients is currently under investigation. Antioxidant mimetic molecules are also in the developmental stages and include phenolic, porphyrinic, and peptidyl structures of Zn, Cu and Mn complexes that mimic SOD. Synthetic mimetics administered orally catalyze superoxide anion dismutation *in vivo*. Furthermore, H₂O₂ is decomposed and prevents oxidative tissue damage [51]. *In vivo*, antioxidants are produced to supply the body's defense system with oxidant neutralizers. When OS is present, antioxidants prevent further formation of ROS and help repair the already present damage induced by ROS [4].

Antioxidants are classified into two categories: enzymatic antioxidants and non-enzymatic antioxidants. Of the enzymatic antioxidants, SOD prevents the destruction of cellular molecules. It is the first enzyme present to defend molecules from superoxide radicals [7]. In addition, catalase, glutathione reductase, and glutathione peroxidase reduce H₂O₂ into its reactants--water and alcohol--and decompose SOD, neutralizing possible ROS reactivity [5]. Non-enzymatic antioxidants including Vitamins A, C, and E, zinc, glutathione, beta-carotene, and carotene are dietary supplements (the synthetic antioxidants) that aid the female body's oxidant defense system [16]. Vitamins A, C, and E prevent the oxidative chain from proliferating prior to the alteration of neighboring molecules by being oxidized into harmless compounds [5]. Peroxidation is increased by metallic ions; therefore, metal-binding proteins--including transferrin and albumin--prevent the proliferation of the peroxidative process [4]. Glutathione is localized in tubal fluid and the oocyte itself. This non-enzymatic antioxidant promotes zygote development [52].

Culture Media Alteration

Optimizing culture media conditions leads to a reduction of OS, thus increasing high quality embryo yield. Optimization is possible by supplementing culture media with various antioxidant compounds and metal chelators including Vitamin C, Vitamin E, albumin, thiol, antioxidant enzymes and hypotaurine. Supplementing sperm media with vitamins C and E reduces ROS production by decreasing H₂O₂ concentrations [53]. Oxidative damage to polyunsaturated fatty acids increases when concentrations of ascorbic acid are low. With the addition of reduced glutathione (GSH), a positive effect is observed in *in vitro* fertilization of bovine oocytes [54]. The addition of beta-mercaptoethanol, a disulphide reducing agent, and cysteine to culture media has

increased *in vitro* maturation of bovine embryos to the blastocyst stage. Cysteine also has a positive effect on *in vitro* oocyte maturation in porcine [55]. N-acetyl cysteine, more stable than cysteine and GSH, has been successfully used in oocyte/embryo culture media [56].

Albumin traps ROS and various products by absorbing peroxidase. Albumin also negatively effects the embryo by transporting absorbed peroxides from the serum to other cellular locations [57]. Thioredoxin promotes *in vitro* embryo development by repairing post-oxidative alterations in the sulphhydryl groups [58]. The divalent cation chelators--apotransferin, D-penicillamine, L-histidine, L-cysteine and EDTA--help to overcome the 2-cell embryo block by preventing the formation of Fe²⁺ that act as catalysts of oxidation. EDTA aids in overcoming the developmental arrest of embryos *in vitro* as discussed by Nasr-Esfahani *et al.*, indicating its beneficial effects on embryo development [59].

Semen Preparation Techniques

A limited number of methods are recommended to reduce ROS production during semen preparation for ART. The swim-up technique, the one-step wash and density gradient centrifugation are the most commonly used methods in spermatozoal ART preparation [60]. Density gradient centrifugation isolates leukocyte-free mature spermatozoa [61]. It is therefore more advantageous to use than the swim-up and one-step wash techniques when increased leukocyte levels and immature sperm are present in a sample.

Magnetic cell separation (MACS) is a simple system used to separate large quantities of cells [62]. Hipler *et al.* demonstrated that MACS can extract leukocytes from sperm suspensions when specialized micro beads are used that target CD 14, 15 and 16 cells [63].

Gaseous Environment Changes and Incubation Period Alterations

Quinn and Harlow have shown that the reduction of O₂ concentrations from 20% to 5% improves embryonic development in the *in vitro* culture of embryos. In a mouse model, the 2-cell block is prevented during *in vitro* development of the embryo via the aforementioned pO₂ reduction from 20% to 5% [64].

It has also been indicated that prolonged sperm and oocyte incubation (>16 to 20 hours) increases the generation of ROS. Kattera and Chen conducted a prospective trial showing that shorter interaction times between the sperm and oocyte resulted in better quality embryos and also increased implantation and pregnancy rates [65].

CONCLUSION

Oxidative stress has a significant impact on female fertility and ART outcomes. The production of ROS is high in female reproductive tissue due to active metabolism and steroidogenesis. The role of OS in various infertile groups including women with tubal- and peritoneal-factor infertility and/or endometriosis has been analyzed, but the cumulative studies do not yield a definitive conclusion regarding OS, infertility, and the relationship between each factor. The role of antioxidants in female infertility is being investigated.

Antioxidants may be advised when a specific etiology cannot be defined as in idiopathic infertility. Treatment strategies using antioxidant supplementation to reduce OS need additional investigation via randomized controlled trials. The generation of ROS occurs even with normal embryonic metabolism, although specific defense systems are present at each site of ROS generation. The sources of ROS generation in ART are both internal and external. Gametes and embryos are the main internal causes. Exposure to light, high oxygen concentrations, traces of transitional elements, disturbed concentrations of metabolic substrates, and possible xenobiotics contribute to the external sources of ROS generation. *In vitro* production and manipulation of gametes as well as embryos increase ROS production and decrease antioxidant defenses. Efficient strategies and interventions should be adopted for ART sperm preparation. Such interventions may include the optimization of oocyte-sperm interactions, a decreased number of sperm used during insemination and more common density gradient centrifugation for ART preparation of sperm with elevated ROS. It is imperative to recognize an embryo's needs during reproduction as well as to aid the cell's defense mechanisms with antioxidant supplementation in order to improve the overall outcomes of ART. Additional studies are required to examine the ROS content and production in commercial media as well as to evaluate the use of antioxidants in an ART setting to achieve higher live birth rates.

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