

Impact of Oxidative Stress on Gametes and Embryos in an ART Laboratory

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INTRODUCTION

Free radicals are a group of highly reactive chemical molecules consisting of one or more unpaired electrons. Free radicals are transient ionic species with high chemical reactivity that are produced during oxidation of organic molecules. The interactions between free radicals and biomolecules result in oxidative biomolecular modifications (Halliwell and Gutteridge, 1990). Free radicals derived from oxygen metabolism are designated as Reactive Oxygen Species (ROS). ROS play an important positive role in many physiologic functions such as phagocytosis. However, since ROS are highly reactive, they initiate molecular function defects in spectator biomolecules as well (Clifford et al., 1997, Agarwal et al., 2004). Free radicals have a dual role in the reproductive tract and serve as key signal molecules modulating various reproductive functions. Free radicals can influence the oocytes, sperm, embryos in their microenvironments, e.g., follicular fluid, hydrosalpingeal fluid and peritoneal fluid. These microenvironments have a direct bearing on quality of oocytes, sperm oocyte interaction, implantation, and early embryo development. Thus, OS affects both early embryo development, as well as, implantation and both mechanisms determine successful pregnancy. The complex interplay of cytokines, hormones and other stressors influences

cellular generation of free radicals and maintenance of intracellular homeostasis. These free radicals further act through the modulation of many transcription factors and gene expression.

Oxidative stress (OS) has recently emerged as one of the most important factors negatively influencing assisted reproductive technique (ART) outcomes. Although assisted reproductive techniques are being utilized to treat couples with male and/or female factor infertility, fertility outcomes are still unsatisfactory (2004). The 2003 SART data reports that only 35% of the ART transfer procedures result in live birth delivery (Mayne et al., 2004). Poor ART fertility outcomes are hypothesized to occur due to a lack of in vitro oocyte and embryo protection by oxygen radical scavengers. In vivo, these scavengers are present in the female genital tract, as well as, in the microenvironment surrounding each embryo. In vitro, relatively high oxygen concentrations are observed accounting for a disturbance in the ROS/antioxidant balance (Guerin et al., 2001). Optimizing embryo quality by interventions to reduce the ROS effects can lead towards enhancing embryo quality, single embryo transfers and reduction in multiple pregnancy rates with ART.

ROS are hypothesized to have many possible origins. One source is a result of the embryo's

metabolic activity. Another origin of ROS generation is that from non-embryonic extraneous sources (Bedaiwy et al., 2004). A mitochondrial origin of reactive oxygen species via oxidative phosphorylation has been documented as well. Guerin, et al. discuss the idea that embryos exposed to high oxygen levels may suffer mitochondrial mRNA changes (Guerin et al., 2001). Nevertheless, in species such as mouse and rabbit, embryos, rather than their mitochondria, are the main source of ROS production (Manes, 2001). Indeed, small amounts of ROS are needed for fertilization (Gagnon et al., 1991) as well as the enhancement of sperm binding to the zona pellucida (de Lamirande et al., 1997), but high levels of ROS negatively affect embryo development and pregnancy rates (Goto et al., 1993). Due to the aforementioned negative effects of ROS and its resultant oxidative embryopathic stress, ROS control mechanisms need further investigation.

What are free radicals?

Free radicals are defined as a group of highly reactive unstable chemical molecules with one or more unpaired electrons (Portz et al., 1991). Free radicals may have a positive role in normal physiological function, but once present in excess, they induce pathologies. When free radicals acquire electrons from nucleic acids, lipids, proteins, carbohydrates, and any other nearby molecules, they became stable. Such binding and stabilization conversely causes a cascade reaction that results in cellular damage (Agarwal et al., 2005).

Reactive oxygen species are those free radicals specifically derived from oxygen metabolism such as superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH) (Agarwal et al., 2003). Reactive nitrogen species such as Nitric Oxide (NO) are formed by the conversion of L-arginine to L-citrulline by the nitric oxide synthase enzyme (Dong et al., 2001).

The role of oxidative stress has been documented in the pathogenesis of both male and female infertility (Sikka, 2001). Assisted reproductive technologies are being increasingly utilized and have

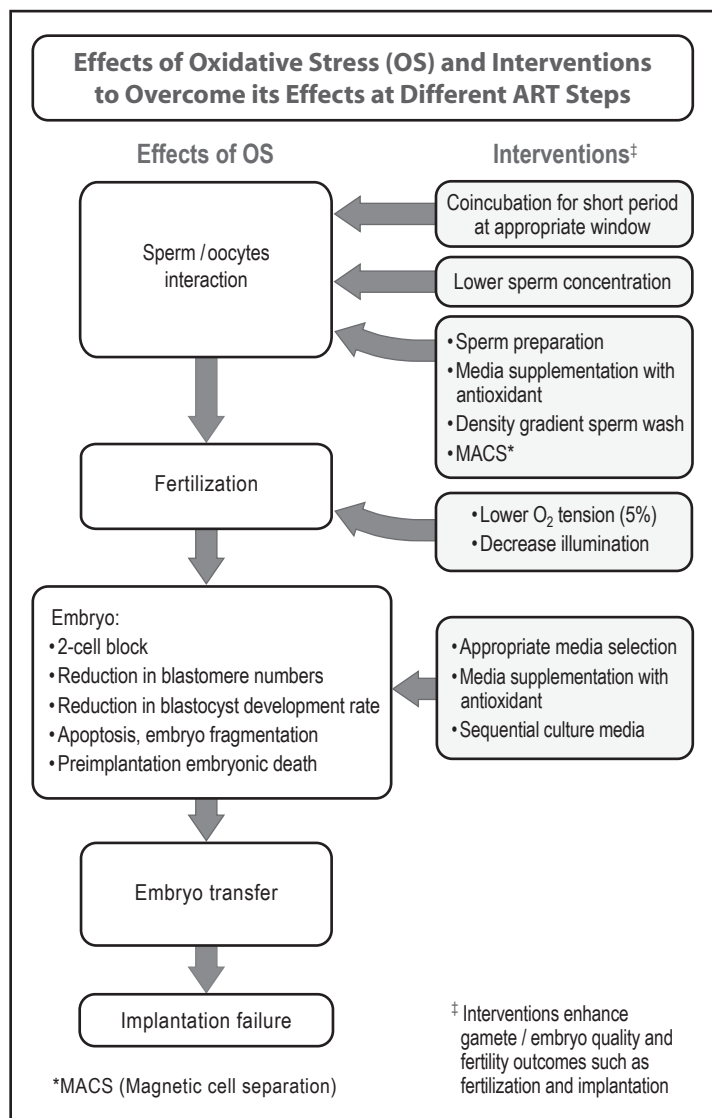


Figure 1.

benefited many infertile couples with fulfillment of their desire to have a biological child. Although low levels of ROS may aid in ART success (Esfandiari et al., 2005, Pasqualotto et al., 2004), elevated levels adversely affect ART outcomes.

What defense mechanisms exist to control ROS?

Overproduction of OS and the decrease in defense mechanisms against ROS lead to oxidative damage. To avoid the lethal effects of OS, oocytes and embryos are protected by antioxidants present in the follicular, tubal and peritoneal biological windows (Guerin et al., 2001). There are many ways to prevent oxidative stress (see Figure 1). These include

intercepting the formation of ROS, scavenging ROS by antioxidants, and utilizing reparative mechanisms already present in the body.

Antioxidants are any substance, not concentration specific, which may scientifically delay or prevent the oxidation of an oxidizable substrate (Halliwell, 1989). Agarwal, et al. reported that antioxidant defenses act via three mechanisms: prevention, interception and a reparative method (Agarwal et al., 2004). Antioxidants protect gametes and embryos from the lethal effects of oxidative stress through enzymatic and non enzymatic defense mechanisms (Guerin et al., 2001).

Non-enzymatic antioxidants such as taurine, hypotaurine, ascorbic acid, and vitamin E are present in the follicular, tubal and peritoneal fluids (Guerin and Menezo, 1995). A reduced form of the non-enzymatic antioxidant, glutathione (GSH), is the most important defense mechanism against ROS in oocytes and embryos. High concentrations of GSH protect embryos until the blastocyst stage. Depletion of glutathione is demonstrated to produce oxidative stress induced DNA damage in bovine embryos (Takahashi et al., 1993). Two cell stage and blastocyst stage embryos are able to recover their GSH levels after complete depletion within 45 minutes, indicating that glutathione is an important antioxidant (Gardiner et al., 1998).

Another antioxidant that performs in a non-enzymatic way is cysteine (CSH). CSH is known as a strong scavenger of the hydroxyl radical. Significant amounts of CSH have been detected in the follicular fluid of many animals (Guyader-Joly et al., 1998). CSH has a documented beneficial effect on embryo development and maturation when added to the culture media of bovine and porcine species (Grupe et al., 1995).

Some vitamin groups such as vitamins C and E are also antioxidants. Ascorbic acid is a potent antioxidant as it protects the cell from endogenous oxidative DNA damage (Fraga et al., 1991). It has been shown that ascorbate neutralizes hydroxyl, superoxide, and hydrogen peroxide radicals as

well as prevents sperm agglutination (Agarwal et al., 2004). Vitamin E is present within the cell membrane and neutralizes the hydroxyl group, superoxide anion, and hydroxyl radical. In addition, it prevents lipid oxidation of the plasma membrane (Wu et al., 1990).

Pentoxifylline is yet another substance that exhibits antioxidant activity. Pentoxifylline is a derivative of methylxanthine and acts as a non-specific inhibitor of phosphodiesterase (Stanic et al., 2002). Pentoxifylline has been shown to scavenge hydroxyl as well as superoxide radicals and inhibit their release (Bausero et al., 1998). Small doses of pentoxifylline have proven to be effective in their ability to scavenge ROS in cryopreserved sperm (Esteves et al., 1998). Pentoxifylline also demonstrates a beneficial effect in reducing ROS induced embryo damage and hence improves IVF outcomes (Zhang et al., 2005).

Superoxide dismutase (SOD) is present in what is considered the first enzymatic step that protects oocytes and embryos against oxygen free radicals (Li et al., 1993). SOD, located in the cytosol, and Manganese SOD, located in the mitochondria, can scavenge superoxide radicals. Both glutathione reductase (GR) and glutamyl cysteine synthetase (GCS) maintain high concentrations of GSH in mouse embryo cells.

Antioxidant enzymes are probably regulated at a pre-translational level since a good correlation between mRNA, proteins, and enzymatic levels has been documented (Forsberg et al., 1996). One of the embryo protective mechanisms specified against ROS depends on endogenous antioxidant enzymes stored as mRNA during oogenesis (El Mouatassim et al., 1999). Variations in mRNA synthesis during oocyte maturation may affect advanced embryo development (Guerin et al., 2001). In vitro set ups are unable to mimic the exact physiology of in-vivo conditions. In ART procedures, the physiological milieu may be simulated by a modification of the media as well as the techniques used. The complete ART system is susceptible at various steps to many

sources of ROS generation. These sources produce free radicals and accumulate excessive oxidative stress that later affects cells. Thus, in order to diminish oxidative stress on cells, both the internal and external sources need to be identified.

How does follicular fluid OS affect ART outcomes?

Low ROS levels have been hypothesized to be necessary for ovulation to occur. ROS may have a regulatory role in oocyte maturation, folliculogenesis, ovarian steroidogenesis, and luteolysis. Markers of oxidative stress such as superoxide dismutase, Cu-Zn superoxide dismutase, Mn superoxide dismutase, glutathione peroxidase, γ -glutamyl synthetase, and lipid peroxides have been investigated by immunohistochemical localization, m-RNA expression, and a thiobarbituric acid method in normal cycling ovaries and in stimulated cycles (Esfandiari et al., 2005, Suzuki et al., 1999, Sugino et al., 2000). The expression of various OS biomarkers has been demonstrated in normally cycling human ovaries (Shiotani et al., 1991). All follicular stages have been examined for SOD expression including primordial, primary, preantral, non-dominant antral, dominant, and atretic (Suzuki et al., 1999). Oocyte maturity has been correlated to apoptosis levels in granulosa cells and cumulus cells; apoptosis being an indicator of oxidation. Color Doppler imaging revealed a negative correlation between the resistive index (RI) and the pulsatility index (PI) within granulosa cell apoptosis levels amongst patients undergoing IVF (Du et al., 2006). The vascular indices of RI and PI have been proposed to be good indicators of follicular maturity and oxidation. Decreased development potential of oocytes from poorly vascularised follicles has also been attributed to low intrafollicular oxygenation (Van Blerkom et al., 1997, Chui et al., 1997)

Oocyte quality is an important determining factor in the outcome of IVF/ET. High ROS levels have been shown to negatively impact the metaphase II meiotic spindle of mouse oocytes (see Figure 2) leading to aneuploidy and poor oocyte quality

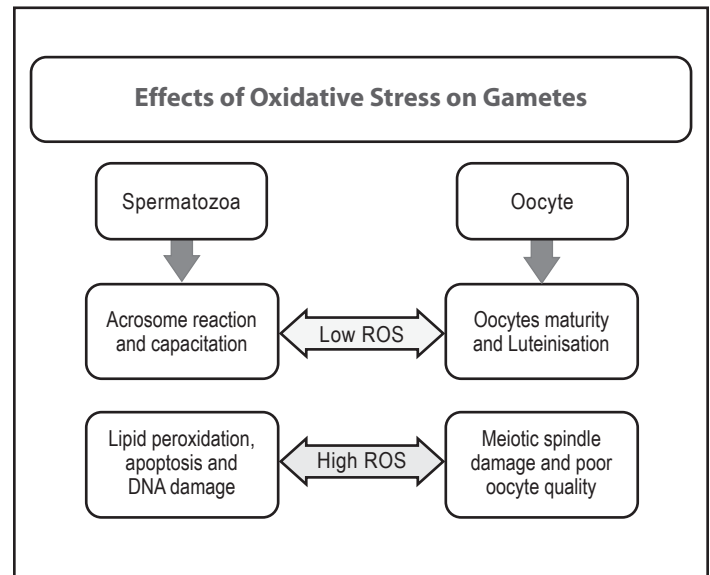


Figure 2.

(Choi et al., 2005). 8-hydroxy-2-deoxyguanosine follicular fluid levels are a reliable indicator of DNA damage caused by oxidative stress. Higher levels of 8 hydroxy 2-deoxyguanosine were associated with lower fertilization rates and poor embryo quality (Seino et al., 2002). Such high levels of OS biomarkers have also been documented in granulosa cells of patients with endometriosis. The high levels of ROS may be responsible for impairing the quality of developing oocytes.

The levels of three oxidative stress biomarkers including conjugated diene, lipid hydroperoxide, and thiobarbituric acid were determined in preovulatory follicles. A concentration gradient was noticed as levels of all three biomarkers were significantly lower in patient follicular fluid compared with serum levels (Jozwik et al., 1999). Intense peroxidation depletes a preovulatory follicle's potent antioxidant defenses (Jozwik et al., 1999). Glutathione peroxidase may also maintain low hydroperoxide levels inside the follicle and thus play an important role in gametogenesis as well as fertilization. Follicular fluid TAC levels and ROS-TAC scores were determined to correlate positively with pregnancy while ROS levels alone negatively correlated with pregnancy (Bedaiwy, 2005).

How do free radicals affect spermatozoa?

Excessive ROS levels lead to an increase in lipid peroxidation of the sperm plasma membrane and result in a loss of sperm membrane fluidity, essential for sperm motility and sperm-oocyte fusion (Said et al., 2006). High levels of seminal oxidative stress may induce sperm DNA damage (Agarwal et al., 2003) and may also increase sperm apoptosis in patients with male factor infertility (Wang et al., 2003). Increased ROS production by spermatozoa is associated with a decreased Mitochondrial Membrane Potential (MMP). A MMP decrease alters mitochondrial function (Wang et al., 2003). Elevated ROS levels are negatively correlated with sperm of normal morphology and borderline morphology as well. They are positively correlated with sperm containing amorphous heads, damaged acrosomes, midpiece defects, cytoplasmic droplets, and tail defects. The sperm deformity index (SDI), calculated by dividing the total number of deformities observed by the number of sperm evaluated, is also positively correlated with ROS levels (Agarwal et al., 2004). The standard SDI is helpful in identifying infertile males with high ROS levels and oxidative stress induced sperm DNA damage in the infertility clinic (Said et al., 2005).

As mentioned, the ability of spermatozoa to generate ROS is negatively correlated with semen quality (Sharma et al., 2004). Oxidative stress may affect sperm function by decreasing acrosin activity in patients with leucocytospermia or abnormal semen parameters (Zalata et al., 2004). The levels of ROS in normal donor neat semen specimens is lower compared with washed spermatozoa, therefore, semen processing itself may be a causative factor of ROS generation (Allamaneni et al., 2005). The measurement of ROS levels in semen before IVF may be useful in predicting IVF outcomes as ROS is hypothesized to affect fertilization rates post-IVF (Agarwal et al., 2005).

What are the effects of changing energy needs of blastocyst on OS levels?

Pre-implantation embryos are not a static entity as demonstrated by sequential culture. Embryos pass through many hurdles during their developmental process and have ever-changing needs. Pre-implantation embryonic development is associated with a change in preference of energy metabolism pathways. Embryos possess inherent energy requirements that are met by ATP generation from oxidative phosphorylation and glycolysis. Blastocyst development is accompanied by a shift in pathway of ATP generation from oxidative phosphorylation to an increasing dependence on ATP generation from glycolysis. Increased glucose uptake in the post compaction stage meets the increased energy demands of an embryo. Blastocyst development from the two-cell stage embryo is modulated by the ratio of pyruvate to lactate in the culture medium as this in turn affects the intracellular pyruvate to lactate ratio. The redox state of the embryo is modulated by embryonic metabolism and changing energy needs of the embryo. Excessive generation of ROS occurs at certain critical points accompanied by increased energy demands such as embryonic genome activation, embryonic compaction, and hatching (Ollero et al., 2001, Gott et al., 1990). Minimal levels of reactive oxygen species may play a role during the critical points of embryogenesis. Excessive levels of ROS have adverse impacts on embryo quality and competence (Guerin et al., 2001, Agarwal et al., 2003, Warren et al., 1987). The literature reports that a reduction in OS levels leads to better ART outcomes (Buhimschi et al., 2000, Machaty et al., 2001).

A majority of retrieved mature oocytes fertilize, but of these only up to 70% undergo the first three cleavage divisions during the first three days in culture (Lane et al., 2002). Less than 50% of the cleaved embryos undergo cavitation and proceed to blastocyst formation by day five in culture (Lighten et al., 1998, Racowsky et al., 2000). Similarly,

only approximately 30% of day three embryos will progress to develop into morphologically normal blastocysts.

An embryo, during the first trimester, grows best under low oxygen concentrations as documented in materno-fetal oxygen diffusion studies (Jauniaux et al., 2003). In human embryos, elevated blastulation rates have been reported by decreasing the oxygen tension (5% O₂) and maintaining low illumination levels throughout the embryo manipulation period (Noda et al., 1994). Oxidative phosphorylation, NADPH oxidase and xanthine oxidase are predominant sources of ROS generation in oocytes and embryos.

Oxidative phosphorylation is a process necessary for the generation of ATP in order to meet embryo energy requirements and it results in ROS production. In the inner mitochondrial membrane, electron leakage occurs during the electron transport chain allowing various spontaneous molecular interactions to occur. Specifically, excess electrons transfer to oxygen molecules and result in an unpaired electron located in oxygen's outer orbital.

Reactive oxygen species induced embryopathic stress is important in the etiology of defective embryo development. High O₂ concentrations during in-vitro cultures lead to an increase in hydrogen peroxide (H₂O₂) levels, DNA fragmentation, and reduction in embryo development competency. ROS such as H₂O₂ are responsible for programmed cell death, also known as apoptosis, and may cause the failure of blastocyst development and preimplantation embryo death.

Many factors influence the interaction between sperm and oocytes in vivo and in vitro. The intrinsic qualities of sperm and oocytes are important determinants of ART outcomes as has been revealed in various literature reports and meta-analyses. Development of mammalian embryos can be retarded in the presence of unfavorable media conditions. Hence, blastocyst development in vitro has been documented to lag behind blastocyst development in vivo (Boni et al., 1999, Viuff et al., 1999).

What is the clinical significance of ROS estimation in the culture media?

Culture conditions subsequent to fertilization affect blastocyst number, quality, and hatching. Suboptimal culture conditions can result in increased media generation of ROS. In culture media, the ROS affect fertilization, embryo development, and clinical pregnancy rates. Elevated concentrations of ROS in day-1 culture media are associated with lower pregnancy rates in both IVF and ICSI. However, in ICSI cycles, higher day-1 ROS levels are associated with reduced fertilization, cleavage, and blastocyst development rates (Bedaiwy et al., 2004). In both IVF and ICSI cycles, decreased embryonic fragmentation, enhanced cleavage rate, and increased blastocyst development rate have been reported to be partially related to day 1 total antioxidant capacity (TAC) in the culture media. This relationship may be a cause and effect relationship. Thus, elevated ROS are negatively correlated with embryo quality, whereas TAC levels in culture media are positively correlated with embryonic quality and competence.

How do OS levels affect IVF and ICSI?

The common cellular sources of ROS in IVF and ICSI are oocytes, cumulus cells, and spermatozoa. Cumulus cells are a potential source of ROS solely in IVF. Tanghe, et al. documented that luminol-dependent chemiluminescence revealed a higher ROS load in conditioned medium of cumulus-enclosed oocytes (CEOs) than in that of CDOs after sperm-oocyte co-incubation (P<0.05) (Tanghe et al., 2003). However, this bovine in-vitro study also discussed the fact that certain levels of ROS produced by cumulus cells may help spermatozoal fertilizing capability. It has also been proposed that cumulus cells may protect the oocyte against OS and may also lead to enhanced fertilization and cleavage rates (Fatehi et al., 2005). A gradual increase in ROS generation has been reported from the early embryonic to the late morula stages in bovine ICSI embryos and studied in in-vitro culture (Dalvit et al.,

2005). Levels of ROS generation may vary with IVF and ICSI at different stages of embryo development.

Can modifying sperm preparation protocols overcome OS?

Spermatozoa utilized for ART are likely to experience OS exposure that may result in extensive DNA damage. Morphologically abnormal spermatozoa and the seminal leucocytes are the main sources of the ROS. It is documented that human oocytes have a narrow optimal insemination window (Ermilov et al., 1999). Studies also show that in-vitro insemination in mice, when delayed by 2 hours, reduces blastocyst development (Edgar et al., 1987). Sperm preparation techniques that remove the spermatozoa affected by oxidative stress and apoptotic sperm are of vital importance. Appropriate timing of insemination and shortening the exposure of oocyte to sperm may be important modalities in reducing oxidative stress levels in the ART setting.

The chances of using DNA-damaged spermatozoa are much higher in the ICSI procedure. Spermatozoa with DNA damage induced by ROS may result in impaired embryonic growth, early embryonic death, or abortion. The currently employed sperm preparation techniques such as double density gradient centrifugation separate the leucocytes and immature /damaged spermatozoa (Chen and Bongso, 1999, Henkel and Schill, 2003)). The swim-up and the one-step wash sperm preparation techniques result in the cellular content such as leucocytes and the abnormal spermatozoa gravitate to the bottom pellet (Henkel and Schill, 2003). This leads to contamination and contact of the normal spermatozoa with the ROS producing leucocytes and abnormal spermatozoa. The conventional swim-up preparation technique used for sperm preparation in ejaculates with ROS can lead to sperm damage due to the interaction of functional spermatozoa, defective sperm, and leukocytes (Henkel and Schill, 2003). The techniques recommended for patients with elevated ejaculate ROS levels are density

gradient centrifugation and glass wool filtration. The sperm preparation media may be supplemented with antioxidants such as pentoxifyline (Okada et al., 1997), glutathione (Lenzi et al., 1993, Griveau and Le Lannou, 1994), N-acetyl-cysteine (Zalata et al., 2004, Oeda et al., 1997) and albumin (Twigg et al., 1998) to scavenge ROS.

Does sperm oocyte interaction time impact the generation of OS?

Reports suggest that prolonged sperm-oocyte incubation (16-20 hours) increases the generation of ROS. Two prospective randomized controlled studies have recommended shorter sperm-oocyte co-incubation times (Kattera and Chen, 2003, Gianaroli et al., 1996). A co-incubation of 1-2 hours results in better quality embryos with significantly improved fertilization and implantation rates. Shorter co-incubation time also produces better quality embryos and increases implantation and pregnancy rates (Kattera and Chen, 2003, Gianaroli et al., 1996, Quinn and Harlow, 1978)

What is the appropriate media for OS prevention?

The media chosen for in-vitro culture of the human embryos should have potential ingredients to meet changing nutritional and metabolic requirements and provide a stable environment for the embryos. It is imperative to maintain the antioxidant-proxidant balance in the in-vitro culture media as the post fertilization culture milieu has significant influence on cleavage rates and blastocyst development. Supplementation of culture media with antioxidants such as β -mercaptoethanol, cysteine, vitamins C and E, taurine, hypotaurine, thiols, and superoxide dismutase has beneficial effects on mouse and bovine embryo cultures. Supplementation with serum is widely practiced as it protects embryos from the pathogenic effects of oxidative stress.

The evolution of culture media development has led from the monoculture to co-culture and presently to sequential culture media for overcoming OS and improving blastocyst development rates.

Conclusions

Oxidative stress has a significant impact on the fertility outcomes of ART. The sources of reactive oxygen species generation in ART may be internal or external. The internal causes being the gametes themselves, gamete interaction, and the embryos. It is important to optimize techniques and protocols to overcome OS in ART settings. Strategies and interventions include optimizing oocyte sperm interactions, lowering sperm numbers utilized for insemination, and inseminating during the appropriate window. Recognition of suitable embryo culture needs and media supplementation with antioxidants is important. Successful implementation of quality control and quality assurance measures in the laboratory ensures quality of the procedures and helps prevent suboptimal conditions, which may lead to increased ROS levels. Further studies should examine the ROS content and production in commercial media and study the effects of embryo manipulation and generation of ROS. Revised protocols to minimize ROS effects will help improve cost effectiveness and outcomes of the assisted reproductive technologies. ■

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