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Oxidative stress in an assisted reproductive techniques setting

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Objective: The manipulation of gametes and embryos in an in vitro environment when performing assisted reproductive techniques (ART) carries the risk of exposure of these cells to supraphysiological levels of reactive oxygen species (ROS). The main objective of this review is to provide ART personnel with all the necessary information regarding the development of oxidative stress in an ART setting, as well as the sources of ROS and the mechanisms of oxidative stress-induced damage during ART procedures. The impact of oxidative stress on ART outcome and the different strategies designed to prevent it are also discussed.

Design: Review of international scientific literature. A question-and-answer format was adopted in an attempt to convey comprehensive information in a simple yet focused manner.

Result(s): The pO₂ to which gametes and the embryo are normally exposed in vivo is significantly lower than in vitro. This results in increased production of ROS. Increase in levels of ROS without a concomitant rise in antioxidant defenses leads to oxidative stress. Lipid, protein, and DNA damage have all been associated with oxidative stress. This may ultimately result in suboptimal ART success rates.

Conclusion(s): Many modifiable conditions exist in an ART setting that may aid in reducing the toxic effects of ROS. (Fertil Steril® 2006;86:503–12. ©2006 by American Society for Reproductive Medicine.)

Key Words: Assisted reproduction, embryo, oocyte oxidative stress, reactive oxygen species

The presence of oxidant and antioxidant systems in various reproductive tissues has evoked great interest on the role of oxidative stress in human reproduction. Oxidative stress has been defined as an elevation in the steady-state levels of various reactive oxygen species (ROS) that exceeds the body's antioxidant defenses (1). This has been implicated in a number of different reproductive scenarios such as endometriosis, folliculogenesis, oocyte maturation, hydrosalpingeal fluid, necrozoospermia, asthenozoospermia, and sperm DNA damage. Oxidative stress can also be involved in the etiology of defective embryo development (2, 3). Furthermore, oxidative stress may play a role after embryo transfer. The posttransfer-preimplantation time could be detrimental to the implantation process itself. The presence of the transferred embryos in a microdrop of culture media hanging in

the endometrial cavity before the initiation of implantation could expose those embryos to the various components of the endometrial environment.

Assisted reproductive techniques (ARTs) have become the treatment of choice in many cases of male and female infertility. Despite numerous advances, the current success rates of these procedures remain unsatisfactory (4, 5). Knowledge of the factors that impact ART outcome may help increase success rates and, in turn, alleviate the socioeconomic burden imposed on patients and on public and private health institutions. Among the various factors that negatively affect ART outcome, oxidative stress has recently emerged as one of the most important ones (1, 6–8).

It is generally accepted that an in vitro setup can never mimic the exact physiological conditions of an in vivo system. Multiple factors impinge on an IVF setting leading to an increase in oxidative stress and suboptimal ART outcome. This review summarizes the latest evidence on the evolution of oxidative stress in an ART setting and should help identify those factors that contribute to oxidative stress

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in the ART laboratory. The necessary information on how to minimize and prevent oxidative stress is also provided.

DEVELOPMENT OF OXIDATIVE STRESS IN AN ART SETTING

How Does Oxidative Stress Generally Develop?

Reactive oxygen species are oxygen-derived molecules that act as powerful oxidants. Reactive oxygen species, such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^{\cdot}), are formed as intermediary products in low concentrations in the male and female genital tracts (6). Reactive oxygen species have the ability to react with any molecule and modify it oxidatively, resulting in structural and functional alterations (9). Reactive oxygen species are neutralized by an elaborate defense system consisting of enzymes such as catalase, superoxide dismutase, and glutathione peroxidase or reductase, and numerous non-enzymatic antioxidants such as vitamin C, vitamin E, vitamin A, pyruvate, glutathione, taurine, and hypotaurine (10). Under physiological conditions, ROS and antioxidants maintain a stable ratio. A shift toward ROS will give rise to oxidative stress.

Why Should We Expect the Development of Oxidative Stress during IVF?

The effects of oxidative stress in an ART setting may be amplified due to the lack of physiological defense mechanisms available and due to the number of potential sources of ROS at play. Oxidative stress has been implicated in the etiology of infertility. The role of oxidative stress in the pathogenesis of male factor infertility has been documented in numerous studies (10, 11). Sperm damage induced by oxidative stress includes membrane and DNA damage leading to necrozoospermia, asthenozoospermia, and DNA fragmentation. Use in ART of spermatozoa that have been damaged during maturation in the seminiferous tubules epithelium, sperm transport through the epididymis, or during sperm processing in the ART lab may result in altered oocyte and/or embryo development. In the context of female infertility, oxidative stress has been poorly characterized (6). Nevertheless, oxidative stress indices in the female reproductive system have been demonstrated to correlate with fertility. Certain ROS levels and selenium-dependent glutathione peroxidase activity in follicular fluid have been positively correlated with pregnancy rates (PRs) (12, 13). Other markers of oxidative stress in follicular fluid such as lipid peroxidation, total antioxidant capacity, and superoxide dismutase activity are also strongly correlated with oocyte fertilization and pregnancy rates following IVF (7, 14, 15). In addition, 8-hydroxy-2'-deoxyguanosine—an important oxidative stress marker in granulosa cells—displays a negative correlation with embryo quality following IVF (16).

Can Human Gametes Counteract Oxidative Stress?

Human gametes possess natural antioxidant defenses. A decrease in their total antioxidant capacity (TAC) may lead

to oxidative stress. The environment surrounding the oocyte and embryo contains nonenzymatic antioxidants such as vitamin C, glutathione, hypotaurine, and taurine, which protect the embryo from external sources of ROS (17). The levels of these antioxidants may be indicative of the extent of oxidative stress. Although TAC levels do not differ in the follicular fluid whether the follicle contains an oocyte or not, they are significantly higher in fluid from follicles where the oocyte successfully fertilizes (14). However, TAC levels were also reported to be significantly lower in the follicular fluid from follicles where the resulting embryo survived until transfer (14), which indicates that the role of TAC in folliculogenesis and early embryonic development remains controversial.

On the other hand, spermatozoa have limited antioxidant defenses because their cytoplasm contains low concentrations of scavenging enzymes. Moreover, they are particularly susceptible to oxidative stress-induced damage due to the high content of polyunsaturated fatty acids in their membranes (18). Nevertheless, the sperm midpiece contains superoxide dismutase and glutathione peroxidase as well as α -glutamyl transpeptidase, which regulates the glutathione content of the oocyte, thus providing protection against oxidative stress (19).

In the male reproductive tract, somatic and germ cells are maintained within a delicate balance of oxidants and antioxidants. Minimal levels of oxidants are needed for the physiological regulation of processes such as capacitation and acrosome reaction (20); however, if these levels increase above a critical threshold, they result in structural and functional damage, which may lead to motility loss, premature acrosomal reaction, lipid peroxidation, apoptosis, and DNA damage (21–25). Antioxidants can protect against some of these events (26–28).

REACTIVE OXYGEN SPECIES PRODUCTION IN AN ART SETTING

How Are ROS Produced during ART?

Reactive oxygen species can be produced either intracellularly, originating from gametes, or extracellularly from environmental factors. A potential source of ROS in the ART media is its generation during the preparation of semen due to the activation of ROS production by immature spermatozoa by centrifugation, the absence of the antioxidant-rich seminal plasma, or contamination by leukocytes. Moreover, spermatozoa selected for ART most likely originate from an environment experiencing oxidative stress, and a large percentage of these sperm may already have DNA damage before semen processing (29). Similarly, oocytes and embryos contribute to the increase in ROS levels because of their metabolism and the lack of the protective antioxidant mechanisms present in their natural habitat (2, 30). The external environment that surrounds the ART procedure also plays an important role in the development of oxidative stress. The most important external factor that may affect

gamete and embryo viability *in vitro* is pO_2 . Cells cultured *in vitro* are exposed to a relative “hyperoxic” environment compared with the *in vivo* conditions. In addition to pO_2 , other physicochemical environmental factors may also affect gamete and embryo development *in vitro*. Therefore, the external environment in the ART procedure can be a potential source of oxidative stress.

Does Oxidative Stress Play Any Role in the Early Stages of Oocyte Development and Subsequent Oocyte Quality?

The developmental competence of the oocyte is believed to be of utmost complexity. Factors controlling this process may date back to when the germ cells migrated from the yolk sac endoderm to the genital ridge. Evidence exists that oxidative stress may be one of the factors that controls background follicular atresia, initial recruitment of the primordial follicular cohort, subsequent growth of follicles, and selection and dominance of the one destined for ovulation.

In a cow animal model, Lonergan et al. (31) used real-time quantitative polymerase chain reaction (RT-PCR) to compare the relative abundance of a cohort of oxidative stress related gene transcripts in oocytes at different stages of development. They studied oocytes derived from 2–6 mm follicles, preovulatory follicles retrieved just before the LH surge and matured *in vitro*, or preovulatory follicles retrieved just before ovulation. The relative mRNA expression of mitochondrial Mn-superoxide dismutase (MnSOD), cytosolic Cu/Zn superoxide dismutase (Cu/ZnSOD), gamma-glutamyl-cysteine transferase, glutathione peroxidase, and sarcosine oxidase was evaluated. They found that irrespective of the oocyte stage, transcripts of all five enzymes were found. Interestingly, the cytoplasmic Cu/ZnSOD transcripts were expressed in significantly higher levels in *in vitro* maturation (IVM) oocytes. In addition, mitochondrial MnSOD was expressed in higher levels in oocytes derived from smaller follicles, suggesting that mitochondrial defenses vary according to the stage of oocyte development (31).

This finding was consistent with a prior observation by El Moutassim et al. (32), who reported that the transcripts for some of these antioxidant genes are present in immature oocytes, whereas other totally different transcripts are present in mature oocytes. On the other hand, transcripts of MnSOD were detected in germinal vesicle oocytes but not metaphase-II oocytes (33). From these observations, it is clear that the oocyte defense mechanism against ROS varies according to its own developmental stage. The integrity of the antioxidant defenses within the different stages of oocyte development may contribute significantly to the overall quality of the oocytes.

Further support for this mechanism comes from the fact that various toxicants can indirectly influence oocyte quality by changing the follicular fluid environment. It was found that intrafollicular exposure to cigarette smoking metabolites was associated with a significant increase in follicular lipid peroxidation. This was, in turn, accompanied by a significant

decrease in the local antioxidative potential. Consequently, oxidative stress imbalance may be responsible for impaired folliculogenesis in female smokers (34). Another mechanism by which smoking can impair oocyte quality via increased levels of oxidative stress is by depleting levels of the antioxidant β -carotene. In one study, it was reported that fertilization rates were significantly lower in smokers with lower follicular fluid β -carotene levels (35).

Is There Any Relationship between Oxidative Stress and In Vitro Maturation of Oocytes?

The role of ROS in oocyte IVM is still controversial. In a study where the cumulus-oocyte complexes (COCs) were matured in TCM-199 + 10% steer serum for 24 hours at 39°C in 5% CO_2 :95% humidified air, SOD, glutathione peroxidase, and catalase activities were measured spectrophotometrically. It was found that no increase in ROS production per COC occurred, but ROS levels per cumulus cell were lower under standard IVM conditions. Moreover, the presence or absence of the surrounding cumulus cells did not affect ROS levels in IVM oocytes. The activities of the three aforementioned enzymes were significantly lower in denuded oocytes compared with cumulus-intact oocytes. Therefore, these antioxidant enzymes could scavenge ROS during IVM (36). The same study proved that cumulus cells are responsible for providing pyruvate and/or lactate as oxidative substrates to be used by the bovine oocyte during IVM, and this supply would be regulated by the lactic dehydrogenase activity in these cells (37).

In another study designed to examine the protective effect of cumulus cells on ROS-induced damage during IVM of porcine oocytes, it was found that exposure of cumulus-denuded oocytes (DOs) to ROS resulted in meiotic arrest and an increase of degenerated oocytes. These degenerated DOs underwent apoptosis, as evidenced by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling-positive reaction within their germinal vesicles and caspase-3 activation. The investigators suggested that cumulus cells have a critical role in protecting oocytes against oxidative stress-induced apoptosis through the enhancement of glutathione content in oocytes during the IVM process (38). Recently, the same group found that follicular fluid addition to the IVM media is essential for protecting the oocytes from oxidative stress through a higher level of radical scavenging activity elicited from SOD isoenzymes, resulting in the enhancement of cytoplasmic maturation and developmental competence postfertilization (39).

What Are the Mechanisms of ROS Generation by Embryos?

Similar to other living aerobic cells, the embryo and the oocyte are major sources of ROS because they use oxygen to produce energy through mitochondrial oxidative phosphorylation. They produce ROS by several pathways, namely, oxidative phosphorylation, nicotinamide adenine dinucleotide phosphate oxidase, and xanthine oxidase systems (3). It is noteworthy that ROS production is increased within em-

bryos cultured in vitro as compared with those in vivo (2). Whether this increase is due to intrinsic changes in embryo metabolism or the result of a higher pO_2 or other environmental factors remains to be elucidated. Furthermore, it is unclear whether embryonic micromanipulations have an effect on the overall oxidative stress balance. The effects of techniques such as assisted hatching, fragment removal, and embryonic biopsy on the metabolism of the respective embryos needs further assessment.

How Can the Ejaculate Contribute to Oxidative Stress during ART?

Morphologically abnormal spermatozoa and leukocytes are major sources of high ROS levels in human ejaculates. Sperm intracellular mechanisms may generate ROS at the level of plasma membrane (40) and mitochondria (41). Human spermatozoa generate $O_2^{\cdot-}$ (42), which spontaneously or enzymatically dismutates to H_2O_2 . In the presence of metal ions (iron), $O_2^{\cdot-}$ and H_2O_2 produce OH^- . Extracellular production of ROS by leukocytes is present in the secretions of the prostatic and seminal vesicles secretions (43, 44). An increase in extracellular ROS production is particularly evident in cases of leukocytospermia. Activated leukocytes during inflammation and infection are capable of producing 100-fold higher amounts of ROS than nonactivated leukocytes (45).

Oxidative stress-induced sperm damage not only can affect the oocyte after penetration; the defective sperm that surround the oocyte may also affect oocyte and embryo development in vitro. Dead spermatozoa liberate the enzyme amine oxidase, which acts on spermine and spermidine to produce H_2O_2 and other amine compounds (46). Alvarez et al. (47) reported that PRs in conventional IVF were significantly higher in cycles that used sperm with stress test scores >0.75 . These scores were calculated by dividing the percentage of sperm motility after incubation at $40^\circ C$ for 4 hours by the percentage of motility before incubation in the actual sample used for IVF. Stress test scores >0.75 were indicative of high sperm survival and lower lipid peroxidation rates. The investigators concluded that the higher PRs observed in cycles using sperm samples with scores >0.75 could be due to two main mechanisms: [1] the probability that the spermatozoon that fertilized the oocyte had intact membranes and DNA is higher in samples with stress test scores >0.75 , and [2] the production of ROS and other toxic factors into the IVF culture media by the remaining sperm that surround the oocyte during in vitro incubation would be lower in samples with scores $>75\%$. The accumulation of these toxic factors could be avoided by either performing intracytoplasmic sperm injection (ICSI) or by shortening the time of sperm contact with the oocyte during incubation in IVF, as also suggested by Gianaroli et al. (48).

What Is the Contribution of the Laboratory Environment to Increased Oxidative Stress?

Many sources of ROS exist in an ART laboratory, and all can increase ROS generation by the embryo. Incubators used in the

ART lab derive approximately 95% of their ambient air from the laboratory room (49). Therefore, as indicated previously, differences in pO_2 in vivo vs. in vitro conditions could be one of the leading causes of increased oxidative stress in an ART setting. The pO_2 in the fallopian tubes of rabbits and rhesus monkeys at the time of ovulation has been estimated to be one-third of the atmospheric oxygen tension at the time of ovulation (50, 51). In addition, the pO_2 in the media at room temperature is 20-fold higher compared with intracellular pO_2 . This higher pO_2 activates various oxidase enzyme systems in the cells and contributes to increased ROS generation.

Visible light is another environmental factor that may lead to increased ROS production. It induces photodynamic stress, leading to oxidative damage of unsaturated lipids and sterols within the membranes (52). In support of this hypothesis, transient exposure to visible light was sufficient to cause a major increase in H_2O_2 in mouse embryos (2).

Can the Culture Media Used in ART Contribute to Oxidative Stress?

Specific commercial culture media can generate ROS depending on their composition. Metallic ions such as Fe^{2+} and Cu^{2+} are reportedly present in culture media and have the potential of accelerating ROS generation within the cell by participating in Fenton and Haber-Weiss reactions (3). Media additives can also act as ROS inducers. Serum albumin is an important additive due to its powerful antioxidant properties (53). Controversially, serum preparations, which are commonly added to culture media, contain high levels of amine oxidase, which leads to an increase in H_2O_2 production (54).

Does the Source of Oxidative Stress in ART Depend on the Nature of the Procedure?

Yes. The potential cellular sources of ROS in conventional IVF are different from those of ICSI. In conventional IVF, ROS in the culture media may originate from the oocytes (4–5 oocytes per dish), cumulus cell mass (thousands of cells), and the spermatozoa used for insemination. On the other hand, the cumulus cells are not a potential source of ROS in ICSI because the incubation is initiated after denuding the oocytes from their entire cumulus cells. In addition, during ICSI, the incubation time is shorter, thus decreasing exposure to external environmental factors such as high pO_2 . The contact time between sperm and the oocyte is also minimized, reducing ROS-induced damage by ROS-producing defective spermatozoa. On the other hand, ROS levels are not significantly different in culture media surrounding the embryos in a conventional IVF setting than in an ICSI setting under group culture conditions (55).

OXIDATIVE STRESS AND CRYOPRESERVATION OF GAMETES, EMBRYOS, AND OVARIAN TISSUE Does Cryopreservation-Thawing Further Increase Oxidative Stress?

Cryopreservation enhances lipid peroxidation, as ROS-induced membrane lipid damage was demonstrated in frozen

spermatozoa (56). Moreover, cryopreserved spermatozoa tend to lose their limited antioxidant defenses (57). In a recent study, Dalzell et al. (58) reported that aerobic incubation of testicular sperm results in a significant increase in DNA fragmentation. The DNA fragmentation was higher in cryopreserved sperm than in fresh testicular sperm, and it was maximal after 4 hours of incubation. Therefore, care must be taken to avoid incubating cryopreserved and fresh testicular sperm for prolonged periods of time before ICSI is performed.

In human oocytes, cryopreservation frequently leads to developmental arrest during early cleavage stages as well as to aberrant patterns of cytokinesis (59). Thus, it appears that the cryopreservation-thawing process renders cells more susceptible to ROS-induced damage. Improved cryopreservation protocols and optimization of cryoprotectants can improve cryosurvival of gametes.

Does Cryopreservation of Ovarian Tissue Contribute to Oxidative Stress?

Ovarian tissue cryopreservation with subsequent transplantation is becoming increasingly popular as a fertility preservation strategy in female cancer survivors (55). Two pregnancies were reported following reimplantation of thawed ovarian tissue in a spontaneous cycle (60) and after an IVF treatment cycle (61).

The effect of different vitrification protocols on ROS and apoptosis in human ovarian tissue was recently evaluated by exposure to different vitrification solutions. The intracellular redox state level was measured using a fluorescent dye (dichlorodihydrofluorescein diacetate). The apoptotic cells were monitored by anticaspase-3 immunolabelling after vitrification and warming. It was found that tissue that was cooled very rapidly displayed no statistically significant increase in either tissue ROS levels or the number of apoptotic cells after warming. Conversely, a slower cooling resulted in significantly elevated ROS levels and apoptosis after warming (62).

The effect of the nonenzymatic antioxidant, ascorbic acid in protecting ovarian tissue from apoptosis caused by ischemia was also evaluated (63). Fresh and frozen and thawed cortical sections of bovine ovaries were incubated with or without ascorbic acid for variable durations. Oxygen consumption rates, lactate dehydrogenase concentrations, apoptosis rates determined by TUNEL assay, and DNA fragmentation analysis were performed. It was found that oxygen consumption rates were correlated inversely with the duration of incubation. The tissues exposed to ascorbic acid displayed significantly decreased apoptosis in ovarian cortex stromal cells after 24 hours of incubation. Although ovarian tissue cryopreservation technology is developing, it appears that the oxidative stress status is modulated by the cryopreservation protocol. Moreover, oxidative stress may mediate ischemia-reperfusion injury in these tissues following reimplantation.

EFFECT OF OXIDATIVE STRESS ON ART How Can Oxidative Stress Affect Gametes and the Embryo?

Increased ROS levels can affect cell membranes, DNA, and mitochondria. These deleterious effects appear to be mediated, at least in part, by deregulation of the apoptotic cascade (63). Effects of ROS on sperm DNA have been established and correlated to poor ART outcomes (64). Subsequently, the use of spermatozoa with abnormal DNA during ART procedures leads to poor embryo quality (65). Oocyte maturation and embryo development are also affected due to increased ROS or decreased antioxidant defenses (66, 67). This may be attributed to the ability of oxidative stress to hamper the activity of the enzymes of energy generation within the embryo. Increased levels of ROS can inactivate glyceraldehyde-3 phosphate dehydrogenase and thus reduce adenosine triphosphate (ATP) generation (68). Oxidative stress can lead to mitochondrial DNA disruption due to lack of histones, which also decreases the ATP content (69–71). Nevertheless, low levels of ROS are needed for normal activity in the ovarian follicle (12, 72) and for normal sperm-oocyte interaction and sperm capacitation (73).

How Does Oxidative Stress Lead to Embryonic Fragmentation?

In a study designed to determine the level of H₂O₂ concentration within embryos and the morphological features of cell damage induced by H₂O₂, 31 fragmented embryos, 15 nonfragmented embryos, and 16 unfertilized oocytes were evaluated (63). Intraembryonic H₂O₂ concentration was measured using 2',7'-dichlorodihydrofluorescein diacetate by Quanti cell 500 fluorescence imaging (Applied Imaging Co., Sunderland, UK). Transmission electron microscopy and an in situ apoptosis detection kit were used to evaluate DNA fragmentation. The H₂O₂ concentration was significantly higher in the fragmented embryos than in the nonfragmented embryos and unfertilized oocytes. Interestingly, apoptosis was observed only in the fragmented embryos, as confirmed by electron microscopy. From this human study, it is clear that a direct relationship exists between increased ROS concentration and apoptosis (63). Further support of this finding comes from the observation that 5% O₂ decreases the relative concentration of H₂O₂ and results in improved embryo development in terms of quantity and quality in a mouse animal model (74). Therefore, a low O₂ concentration during in vitro culture of embryos decreases the H₂O₂ content and, therefore, reduces DNA fragmentation and improves developmental competence (75).

Given the fact that condensed chromatin, degraded DNA (as measured by TUNEL), cell corpses, and apoptotic bodies are found in fragmented human embryos, this strongly suggests that programmed cell death is triggered at a stage before blastocyst formation (76). In addition, it indicates that ROS could be responsible, at least in part, for failure of blastocyst formation in extended in vitro culture and could account for preimplantation embryo death (76).

What Is the Effect of Oxidative Stress on ART Outcome?

Oxidative stress can originate from the early steps of ART involving the oocyte, sperm, and embryo, as well as later on in the endometrial environment following embryo transfer. Recent studies suggest that DNA fragmentation in sperm is induced, for the most part, during sperm transport through the seminiferous tubules and the epididymis (77–79). This could be mediated by ROS produced by immature sperm and by the nitric oxide-producing epithelial cells that line the epididymis.

The fact that sperm are highly packed in the epididymis facilitates damage by oxygen radicals because their lifespan is on the order of nanoseconds to microseconds, and, therefore, mature and immature sperm must be in close contact for ROS damage to take place. A similar mechanism occurs in the pellet of centrifuged semen where sperm would also be highly packed.

In a recent study, Greco et al. (78) reported that DNA fragmentation in ejaculated sperm, as measured by TUNEL in a selected group of oligozoospermic and normozoospermic males, was significantly higher than that found in testicular sperm from these same males (23.6% vs. 4.8%, $P < .001$). Pregnancy rates obtained with testicular sperm were significantly higher than those obtained with ejaculated sperm (44.4% vs. 5.6%, $P < .001$) (78). More recently, these investigators also found that administration of 1 g/d of vitamins E and C for 2 months to males with high levels of DNA fragmentation in semen [1] reduced sperm DNA fragmentation to levels comparable with those observed in testicular sperm, and [2] significantly increased PRs after ICSI (78, 80).

In a similar study, Steele et al. (81) found that the level of DNA fragmentation in epididymal sperm was significantly higher than that of testicular sperm obtained from the same patients. All these findings support the hypothesis that ROS induces DNA fragmentation during sperm transport through the seminiferous tubules and epididymis, and that this is one of the main mechanisms for DNA damage in sperm. These results also underscore the significance of DNA fragmentation in ART outcome.

Embryo development and the effect of oxidative stress have been thoroughly studied both in animals and human systems. The energy demands of the developing embryo are high; the embryo derives energy by generating ATP through oxidative phosphorylation and glycolysis (82). Oxidative phosphorylation can result in an increase in ROS production. A reduction in ROS production could significantly improve ART outcome (82, 83). Current reports implicate oxidative stress in the etiology of defective embryo development (3). Differential growth patterns have been correlated to ROS levels on days 1 to 6 of embryo culture (55). The same study demonstrated increased embryonic fragmentation and low cleavage in ICSI cycles with increased ROS levels on day 1. In addition, atmospheric oxygen was found to be involved in

the two-cell block phenomenon in mouse embryos apparently due to the lack of antioxidants, which are physiologically present in the oviduct (84).

How Does Oxidative Stress Lead to Failure of Implantation?

Successful implantation requires an appropriate interaction between the embryo and the endometrium. The embryo should be of good quality and the endometrium receptive. Interference with any of these two prerequisites may lead to implantation failure. From the preceding discussion, it is clear that ample evidence exists to suggest that oxidative stress can jeopardize embryonic health. However, it is not clear how much of a role, if any, oxidative stress plays in jeopardizing endometrial receptivity. A recent study compared gene expression profiles of pre-receptive (2 days after the LH surge) vs. receptive (7 days after the LH surge) endometria obtained from the same proven fertile woman in the same menstrual cycle. Endometrial biopsies were analyzed using a DNA chip containing approximately 12,000 genes. Approximately 211 regulated genes were found. Validation of array data was accomplished by mRNA quantification by real-time quantitative fluorescent PCR (Q-PCR) of three up-regulated genes (glutathione peroxidase 3 [GPx-3], claudin-4, and solute carrier family 1 member 1 [SLC1A1]). Human claudin-4 peaked specifically during the implantation window, whereas GPx-3 and SLC1A1 displayed the highest expression in the late secretory phase. In situ hybridization experiments demonstrated that GPx-3 and SLC1A1 expression was restricted to glandular and luminal epithelial cells during the midluteal and late luteal phases. This important experiment highlights the fact that GPx-3 may not be associated with the earliest stages of implantation, but it takes over later in the process (85).

STRATEGIES TO REDUCE OXIDATIVE STRESS IN THE ART LABORATORY

Which Culture Media Are Less Likely to Show Increased ROS Levels?

During IVM of mouse oocytes, chromosomal abnormalities can be significantly reduced by adding antioxidants to the culture media (86). Therefore, several antioxidants, such as ascorbic acid, urate, isoflavones, taurine (53), hypotaurine (53), genistein, and α -tocopherol, can be used as culture media supplements to reduce the risk of oxidative stress and subsequent DNA damage (10). Albumin may be substituted by multiple components such as amino acids, antioxidants and chelators, osmolytes, vitamins, and alternative energy sources (87). Vitamin E supplementation also results in inhibition of lipid peroxidation (88). The adverse effects of metallic ions, which are usually present in culture media, can be reversed by addition of metal chelators. The addition of ethylene diamine tetraacetic acid or transferrin overcomes the developmental arrests (30).

Although it is now common to supplement culture media with antioxidants, maintaining a prooxidant-antioxidant

equilibrium in embryos is a complex problem. Further studies are necessary to limit oxidative stress during embryo culture (3).

What Are the Preferred Methods for Sperm Preparation in ART?

A constitutive, highly inefficient spermiogenesis in men determines the presence of immature sperm subsets in semen that produce high levels of oxygen radicals and that, to a variable degree, can induce iatrogenic damage during semen preparation. This could lead to permeabilization of the plasma membrane (and subsequent necroasthenozoospermia), damage to the acrosome, centrioles, phospholipase C_{ζ} (which is the putative oocyte activation factor), and DNA fragmentation. This damage has been characterized as iatrogenic sperm dysfunction.

To avoid this type of damage, a limited number of methods are recommended for semen preparation. Currently, density gradient centrifugation, swim-up, and one-step wash are the most commonly used techniques for sperm preparation. Morphologically abnormal spermatozoa and seminal leukocytes are considered the main sources of ROS production in semen (18). Ejaculates with increased ROS production due to the presence of excessive leukocytes or immature or damaged spermatozoa should not be separated by means of conventional swim-up or one-step wash (89). In these techniques, all the ejaculate cellular content is forced to the bottom pellet; in turn, leukocytes and abnormal sperm come in contact with mature spermatozoa and cause damage to them. On the other hand, double density gradient centrifugation has great potential in isolating mature, leukocyte-free spermatozoa (90). To avoid leukocyte contamination, several procedures have been suggested on the basis of leukocyte-specific surface markers. Magnetic cell separation (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) is a simple and fast sorting system for the separation of large numbers of cells (91). The leukocytes can be isolated and excluded from sperm suspensions by means of MACS using paramagnetic microbeads targeted against CD 14, 15, 16, or 45 (92).

What Procedures Should Be Used to Minimize ROS Production?

In an ART setup, the physiological milieu can be simulated by modifying the protocol used. This would require the use of revised protocols in aspects such as choice of media, additives in the media, and reductions in oocyte-handling time and exposure to media that generate ROS. It has been suggested that minimal exposure of zygotes to atmospheric oxygen concentration is critical for optimal development past the 8–16 cell stage (93). The reduction of pO_2 from 20% to 5% in the mouse model enhances embryo development in vitro and prevents the two-cell block (2, 94–97). Shorter insemination times during IVF are preferred because defective spermatozoa can release ROS and also spermine and spermidine, which increases H_2O_2 production and sub-

sequent oxidative damage (98). Reduction in the sperm-oocyte incubation time is recommended to minimize the development of oxidative stress. Limited incubation times (1–2 hours) and lower sperm concentrations reportedly result in an improvement in fertilization, implantation, and PRs, as well as higher embryo quality (48, 99).

CONCLUSIONS

Oxidative stress plays a significant role in the outcome of ART. Many factors contribute to an increase in oxidative stress during ART procedures. These factors originate mainly from the external environment where the procedure is performed. The human gametes themselves act as a source of ROS. Moreover, the IVF setting removes cells from their natural habitat, which is endowed with enzymatic and nonenzymatic antioxidants. Utmost care should be taken to avoid inducing excessive ROS production. Efficient technical and quality control measures should be adopted for sperm preparation. The laboratory environment should be optimized, and proper culture media should be selected. Additional studies are still needed to evaluate the use of antioxidants in an ART setting to achieve higher live-birth rates. In addition, commercial media should be further evaluated concerning ROS content and production.

Although gamete damage induced by ROS is difficult to demonstrate in an ART setting because [1] the oocyte can repair, to some extent, DNA damage, and [2] ROS levels are not normally measured, one of the principles that should guide any medical practice is *primum non nocere*, as the old Hippocratic aphorism states. The personnel responsible for ART laboratories should avoid, when possible, the use of procedures that are known to be deleterious, especially when safer procedures can be used.

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