Amending *in vitro* culture condition to overcome oxidative stress in assisted reproduction techniques (ART)

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ABSTRACT

In assisted reproduction techniques (ART) settings, reactive oxygen species (ROS) can be produced from endogenous and exogenous sources during *in vitro* manipulation. Endogenous sources of ROS include gametes and embryo, whereas exogenous sources are oxygen tension, light exposure, culture media, and the nature of some protocols, such as centrifugation or cryopreservation. Elevated ROS production can result in oxidative stress (OS), which is harmful to gametes and embryos, and reduces the procedure's outcomes. Therefore, addressing various aspects of the adverse effects of oxidative stress and its management is necessary.

Keywords: Reactive Oxygen Species; Oxidative stress; Assisted reproduction technique.

INTRODUCTION

Assisted reproductive technology (ART) is now increasingly available to infertile couples worldwide[1]. In addition to infertility treatment, ART can help patients undergoing cancer therapy with fertility preservation[2, 3]. In spite of the growth in ART and its advantages in human infertility, the current success rate of ART procedures is not optimum and needs improvement for better outcomes[4]. ART procedures, such as gamete and embryo manipulations, occur in *in vitro* environments that differ from *in vivo* conditions, and these differences can be harmful and reduce the success of procedures[5]. Thus, knowledge on the factors that negatively affect ART outcomes can help improve the success rates. Many studies have focused on the effects of *in vitro* conditions during ART procedures [6, 7].

Oxidative stress (OS), which is the imbalance between oxidants and antioxidants in cells, can be a consequence of *in vitro* conditions [8]. OS can occur with overproduction of oxidants, such as reactive oxygen species (ROS), or antioxidant system disturbance [9]. It has important roles in the pathophysiology of male infertility, and has been investigated in causes of female infertility [10, 11]. *In vitro* environments and the nature of ART manipulation and protocols can lead to elevated ROS generation and OS[7]. In addition to cells that produce ROS as a by-product of normal aerobic metabolism, *in vitro* sources of ROS can promote additional ROS, which may result in OS[8]. In this review, we highlight the effects of ROS on physiological reproductive events and then discuss the *in vitro* sources of ROS, effects of OS, and strategies for overcoming OS in ART.

ROS and OS

The oxidation–reduction (redox) status refers to the oxidizing/reducing agent ratio in cells. It is an important regulator in various cell metabolic functions[12]. In the redox reaction, electrons are transferred between two substrates, and the one that gains electrons is reduced, whereas the other substrate loses electrons and is oxidized[9]. Free radicals are molecules with a single unpaired electron in an outer electron layer, and are reactive oxidizing agents whereas antioxidants are reducing agents that can receive an electron from an oxidizing agent for redox balance in cells[13].

In biological systems, free radicals are categorized into two major groups of reactive oxidizing agents: ROS and reactive nitrogen species (RNS)[9].

ROS are by-products of normal metabolism and include oxygen-derived free radicals and non-free radicals in cells, such as superoxide anion, hydroxyl, peroxyl and alkoxyl radicals, and hydrogen peroxide[14]. Mitochondria, which consume O2 as an electron acceptor in the respiratory chain, is the major intracellular source of ROS, and complex I and III are major enzymes in the inner membrane of mitochondria that lead to ROS production[15]. In addition to mitochondria, other cellular organelles, such as peroxisomes and the endoplasmic reticulum, as well as enzyme systems (e.g., nicotinamide adenine dinucleotide phosphate-oxidase, xanthine oxidases, lipoxygenases, cyclooxygenase, and cytochrome P450 enzymes) can produce ROS[16-19]. ROS are by-products of normal metabolism, so cells have adapted to these agents with antioxidant defenses[14].

In living cells, the redox status is always regulated, and a balance exists between oxidant and antioxidant agents to maintain hemostasis[13]. OS is the result of the overproduction of oxidizing substrates rather than the antioxidant capacity of cells, thereby leading to cellular redox imbalance[14]. Hence, OS refers to a disturbance in balance between ROS production and antioxidant defenses.

ROS are potent oxidizing substrates with high reactivity because of the presence of an unpaired electron in the outer electron layer. ROS can attack all compounds of cells, such as lipids, proteins, carbohydrates, and nucleic acid molecules, and react and damage them[20].

ROS, such as superoxide ion, can attack lipids and oxidize unsaturated fatty acids and lead to the lipid peroxidation chain reaction, which can spread rapidly and affect other lipid molecules[21]. One of the end products of lipid peroxidation is malondialdehyde, which is a reactive, cytotoxic, and mutagenic agent[22]. Moreover, ROS can affect proteins by reacting with amino acids and peptide bonds, leading to changes in structure and enzyme activity[23, 24]. The bases and sugars in DNA are susceptible to ROS, and their oxidation with reactive species may lead to DNA mutation and scission[25, 26]. Therefore, if ROS production is not controlled, biomolecules will undergo severe damage and OS may develop. Finally, OS can lead to cellular apoptosis via mitochondria-dependent and mitochondria-independent pathways[27].

Physiological Roles of ROS in Reproduction

Uncontrolled ROS production results in OS and cellular damage. However, ROS have physiological roles in various signal transduction pathways[28]. ROS act as second messengers in cells, and regulate various cellular events involved in cell function, differentiation, and death[29, 30]. Low ROS concentrations are produced during cell stimulation with growth factors and cytokines, such as interlukine-6, interlukine-3, angiotensin II, platelet-derived growth factor, nerve growth factor, transforming growth factors, fibroblast growth factors[29]. Several non-receptor tyrosine kinases, such as Src kinases, can be activated by ROS, and protein tyrosine phosphatases can be inhibited by ROS[31, 32]. Furthermore, apoptosis as a physiologic event uses ROS as mediators in its pathways[27].

In reproductive physiology, ROS play important roles and have various functions that can regulate several reproductive events, which are briefly discussed in this paper.

In growing follicles, OS can occur because of the increase in steroidogenesis and cytochrome P_{450}, subsequently elevating ROS production[33, 34]. In follicular fluid (FF), ROS are present, and these reactive agents act as biomarkers of follicle metabolism[35]. Ketty Shkolnik et al., demonstrated that ROS in preovulatory follicles have an essential role in ovulation[36]. They showed that ROS are necessary for cumulus mucification and expansion, and concluded that ROS scavenging inhibits the follicle ovulatory responses to LH, and ROS production is an indispensable signaling event[36].

In the corpus luteum, ROS are produced during progesterone production and regulate steroid synthesis[37]. Such ROS production is controlled by antioxidants [e.g., superoxide dismutase (SOD)][38]. Elevated ROS in the absence of SOD can impair corpus luteum function[39]. In corpus luteum regression, a complex event, ROS also play key roles in apoptosis via various pathways[37].

ROS generation is associated with cell cycle progression and it improves the developmental competence of oocytes during maturation[40, 41]. Pandey et al. demonstrated that ROS transiently

increase in meiotic resumption from diplotene arrest, and high RNS amounts result in meiotic arrest[42]. Meanwhile, Tamura et al. showed that increased OS inhibits oocyte maturation, and melatonin as a free radical scavenger protects mouse oocytes from meiotic arrest[43]. Thus, the roles of ROS in oocyte maturation require further investigation to determine the exact roles of these reactive agents in meiotic progression.

ROS play an important role in ovulation, which is similar to inflammatory reactions. Shkolnik et al. examined the effects of ROS in mice ovulation[36]. They reported that ROS scavengers can reduce ovulation rates, and ROS are essential for cumulus expansion. They found that hydrogen peroxide can mimic the effects of LH on cumulus expansion and mucification. Moreover, progesterone production is impaired in the presence of antioxidants[36]. In follicle rupture, ROS is produced locally by inflammatory cells, such as macrophages, and may be involved in ovulation. They concluded that ovarian ROS production is an essential event for successful ovulation[36]. During sperm capacitation, several events occur, such as elevation in intracellular pH and calcium ion, controlled ROS generation, increase in membrane fluidity, and activation of signal transduction cascades, which result in the phosphorylation of various proteins[44].

de Lamirande et al. studied the dose-dependent effects of ROS on sperm motility and viability, and found that the lowest concentrations of ROS can promote hyperactivation[45]. They concluded that sperm capacitation might be driven by ROS. Their other studies showed that exogenous addition of ROS promotes sperm capacitation and antioxidants, such as SOD, and catalase can inhibit capacitation[46]. Their group reported the interaction between ROS and RNS during sperm capacitation, and these agents are physiologic actors in this process[46]. Also, ROS and RNS can modulate the signal transduction pathways that lead to Tyr phosphorylation of fibrous sheath proteins[47]. Therefore, The ROS content is essential for sperm capacitation by influencing the level of Tyr phosphorylation that allows the sperm acrosome reaction[48]. Hence, strong evidence on the important roles of ROS in sperm capacitation events exists.

ROS and ART

In the ART setting, *in vivo* simulation is incomplete, and these differences can have detrimental effects on gametes, embryo, and outcomes of ART procedures. OS can be produced during various ART protocols, and exert destructive effects[6, 7]. In sperm preparation, the nature of protocols, centrifugation, and presence of immature or dead sperms and leukocytes can lead to ROS production and OS, as well as reduce *in vitro* sperm function. *In vitro* incubation of human sperm causes time-dependent motility loss, which is partly a consequence of mitochondrial ROS generation and OS and the absence of sufficient *in vitro* antioxidant defense[49]. Lampiao et al. indicated that centrifugation as a promoter for ROS production can reduce sperm motility and viability[50]. A positive correlation was observed between the OS markers and DNA damage in semen, which affects the pregnancy rate and spontaneous abortion[51]. During *in vitro* maturation (IVM) of oocytes, oxidative status alteration can occur and reduce the successfulness of the technique[7]. OS can alter meiotic spindle structure and chromosome alignment in mouse oocytes[52]. Zhang et al. indicated that OS can time- and dose-dependently induce apoptosis in female gametes[53]. Furthermore, OS influences meiotic progression, DNA damage, apoptosis, and gene expression during oocyte IVM[7].

Various reports have shown a conflicting correlation between OS markers in FF and pregnancy in women undergoing IVF. Pasqualotto et al. observed a positive correlation between FF TAC and pregnancy[54]. Das et al. also demonstrated that high levels of ROS in the FF can reduce the fertilization rates of oocytes[55]. Meanwhile, Appasamy et al. reported that no relationship exists between FF TAC and pregnancy outcomes[56]. In 2014, Pekel et al. demonstrated that FF OS is significantly decreased in infertile women undergoing IVF[57]. Recently, Lee et al. determined that high FF ROS levels are associated with poor embryo quality in ICSI cycles, and increased ROS in culture media increase embryo fragmentation and reduce the embryo implantation rate[58].The following section presents the main factors involved in the elevation of ROS production and OS in the ART setting.

Innate Sources of ROS

As an endogenous agent, sperm can time-dependently produce ROS in culture media[59].

In IVF for infertile patients, oocyte and sperm are incubated for several hours, which can allow sperm to produce ROS in culture media. Thus, a short sperm–oocyte incubation time may result in better outcomes[60]. In 2009, Enkhmaa et al. showed that sperm in culture media can produce ROS, and a short incubation time can reduce ROS generation[59]. They concluded that prolonged incubation of sperm and oocyte has a negative effect on the survival and development of embryos resulting from sperm ROS production in culture media. In 2013, a meta-analysis reported that short-term incubation is associated with an improvement in pregnancy rates and clinical outcomes in comparison with conventional incubation times[60].

Oocytes in antral follicle are surrounded by FF, which is a microenvironment consisting of growth factors, steroid hormones, ROS, antioxidants, and various mediators. ROS production is a consequence of aerobic metabolism in follicles, and it plays important roles in follicle physiology[61].

Studies have presented conflicting evidence for the positive or negative correlation between ROS in FF and outcomes of ART. Appasamy et al. reported the positive effects of FF ROS on steroid production in follicles and on the success of IVF cycles[56]. Other studies also evaluated the effects of FF ROS and oocyte competence for ART, and found positive roles of ROS[54, 62]. By contrast, several reports showed no correlation between them. In 2011, Fujimoto suggested that lipid peroxidation and antioxidant activities have no association with embryo quality[63]. Singha et al. demonstrated that reactive agents have high levels in women with endometriosis, and such agents are associated with low embryo quality and low pregnancy rates after IVF[64]. Therefore, the roles of follicular ROS and outcomes of ART are controversial.

ROS can time-dependently accumulate in post-ovulatory oocytes *in vitro*, and such OS can result in aging of these oocytes[65]. Lord et al. found a window for fertilization, which can be widened by reduction in ROS, in oocytes[65]. In 2015, Alvarez et al. showed that endogenous ROS decrease during porcine IVM but increase in the presence of exogenous ROS[66]. Hence, intracellular ROS levels in oocytes are controlled because OS can cause damage and apoptosis.

Embryonic cells, such as other aerobic cells, utilize oxygen for oxidative phosphorylation in mitochondria. Thus, developing embryo can generate ROS as a by-product of its metabolism[67]. Prior to the morula stage, metabolism is low and accompanied with blastocyst formation, followed by an increase in metabolism and glycolysis[68]. Moreover, oxygen consumption increases from the morula to blastocyst stage in mouse embryos[68]. Lopes et al. demonstrated that oxygen consumption increases at the time of fertilization which in turn lead to increase in ROS production [69]. In addition to oxygen consumption in mitochondria for oxidative phosphorylation, other enzymes that consume oxygen and produce reactive species are also present. NADPH and xanthine oxidase systems are examples of these non-mitochondrial systems that can generate ROS[68].

Extrinsic Sources of ROS

In addition to endogenous sources of ROS, exogenous factors are also responsible for elevated ROS in ART settings.If we attempt to reduce the effects of these factors, ART outcomes will increase.

Culture media can trigger ROS generation depending on its composition[70]. The presence of riboflavin, nucleotides, and metal ions, as well as light and oxygen exposure, can lead to ROS production in culture media[8]. Martín-Romero et al. reported that different cell-free IVF media can produce ROS at different rates, and showed that culture media can damage oocytes by ROS generation depending on their composition[70]. They concluded that some culture media produce more ROS than other media, and should be replaced or supplemented with antioxidant agents. Serum used as a supplement in culture media can amplify ROS generation by oxidase activities, such as amine oxidase[71]. Therefore, culture media and supplementation can function as ROS generators *in vitro* and cause damage to gametes and embryo.

The oxygen concentration in the female reproductive tract is lower than 3%–9% under conventional *in vitro* settings[72, 73].

Noda et al. observed beneficial effects of lower oxygen tension *in vitro* using a novel IVF culture system with low levels of oxygen tension and illumination[74]. These changes in *in vitro*condition resulted in high blastulation rates and improved human embryo development. Some investigations indicated, embryos cultured in an environment with 20% oxygen tension result in poor outcomes, compared with embryos cultured in an environment with low oxygen concentration[75-77].It was found that low oxygen tension in culture media improves the rates of IVF and embryo production [78, 79]. The influence of O2 concentration is a controversial topic in various species and different conditions because some studies showed positive effects of highoxygen concentrations, such as in IVM of porcine oocytes[80].

Light exposure is one of the physical factors that can promote ROS generation *invitro*, and its effects cannot be ignored. Lavi et al. examined the induction of ROS production in several cell types such as sperm by visible light exposure, and reported that visible light can initiate a photochemical reaction and promote ROS production in these cells. Also, it has been shown that H_2O_2 production increases in cultured mouse embryos depending on the time of exposure of visible light [81]. Takenaka showed that short-wavelength visible light, which is commonly used in laboratories, is more harmful than cool white light in exposed oocytes and embryos because of increased ROS generation[82]. Visible light exposure, such as solar radiation to skin cells, can also lead to ROS production *in vivo*[83]. Flavins are photosensitizers in cells that can lead to light-induced ROS production[84]. It was demonstrated that ,various light sources can reduce the motility and fertilization rates of ram sperm by ROS production[85]. Therefore, various findings support the hypothesis that visible light exposure can induce ROS generation *in vitro*.

Although cryopreservation has several indications in ART and is a useful option for infertility management, physical and chemical stress during cryopreservation can damage cells and reduce outcomes of ART techniques. ROS generation and antioxidant system impairment are two of the harmful effects of cryopreservation[86, 87].Ovarian tissue cryopreservation protocols and oocyte vitrification can cause ROS generation during freeze-thaw processes. Cryopreservation of granulosa cells can also result in OS and apoptosis[88]. We demonstrated that the lower maturation and fertilization rates of GV oocytes from vitrified ovaries may be due to changes in their mitochondrial function and distribution which in turn lead to increase the ROS generation [89]. Osmotic stress in cryopreservation can lead to ROS production and OS, and this observation was reported by McCarthy et al. in spermatozoa[90]. In the cryopreservation of ovarian follicles, our group demonstrated that vitrification can increase ROS generation, in which ROS levels in vitrified mouse preantral follicles were higher than those in fresh groups[91]. Thus, ROS generation induced by cryopreservation.

Antioxidants against unexpected excessive ROS

In addition to strategies that attempt to reduce the external factors responsible for elevated ROS production such as light and oxygen pressure, we can use antioxidant agents for protecting cells against OS during ART. Various studies presented the positive effects of different antioxidant systems on the outcomes of ART procedures [91-93].Cells utilize a series of antioxidants as reducing agents for protection against free radicals and a balance redox status. Antioxidant systems can be categorized into enzymatic and non-enzymatic[9].

Catalase is an enzyme responsible for the decomposition of H2O2 to H2O and oxygen. Catalase has a protective role against DNA damage induced by OS in spermatozoa[94]. Chi et al. indicated that catalase can reduce ROS production during sperm preparation, and increase the acrosome reaction rate[95]. Moubasher and co-workers found that catalase supplementation during sperm cryopreservation has a positive effect on the vitality, motility, and DNA integrity of sperms after thawing[96].Superoxide dismutase (SOD) is another enzyme responsible for catalyzing the dismutation of superoxide ion into hydrogen peroxide. SOD has two isoforms, namely, SOD 1 and 2. Several studies reported reduced SOD activity in infertile men[97, 98]. Shiva et al. showed that impaired SOD activity is associated with reduced count and motility of human sperms, and concluded that declined SOD activity may be involved in poor semen quality[99]. Another study found a similar

correlation between SOD activity and semen parameters, and reported that SOD activity is reduced in infertile men compared with that in healthy sperm donors[97].

Glutathione peroxidase (GPX) family in cells is involved in H2O2 decomposition by glutathione oxidation. Meseguer et al. reported that GPX mRNA expression in sperm is negatively correlated with the *in vitro* development of embryos at days 3 and 5[100]. Seminal plasma GPX has a positive correlation with concentration, motility, and morphology of sperm[101]. It can also predict sperm quality, but IVF outcomes are unaffected[101].

Vitamin C or ascorbic acid is a cofactor for several enzymes and has antioxidant activity. Ascorbic acid can reduce DNA damage after sperm cryopreservation in infertile men[102]. Li et al. reported that cryodamage is a result of elevated ROS generation during sperm cryopreservation, and ascorbic acid can decrease ROS levels and improve sperm quality[103]. Ascorbic acid, at certain concentrations, can facilitate meiotic maturation of porcine cumulus-free oocytes in culture media[104]. It can also diminish the apoptosis of cumulus oocyte complexes cultured in serum-free medium[105]. However, ascorbic acid supplementation in the luteal phase has no positive effect on women undergoing IVF/embryo transfer (ET)[106].

Vitamin E is a lipid-soluble antioxidant that has several forms, including tocopherols and tocotrienols. Zhou et al. showed vitamin E can reduce the destructive effects of induced OS in rat testis[107]. In 2011, Moslemi et al. demonstrated that vitamin E combined with selenium can improve sperm quality in men with asthenoteratospermia[108]. Vitamin E can reduce ROS production in culture media and improve the developmental parameters of porcine embryos [109]. Wang et al. indicated that vitamin E supplementation medium can increase the blastocyst rate of embryos exposed to ROS[110].

Vitamin B refers to a family containing water-soluble vitamin members. It plays important roles in metabolism. Lynn et al. demonstrated that non-methyltetrahydrofolate levels are higher in male smokers than those in males who do not smoke, and negatively correlated with sperm density and total count[111]. Several studies confirmed that folic acid and zinc oxide increase sperm counts in subfertile and fertile men[112]. However, Boxmeer et al. showed an inverse correlation between folate semen plasma and DNA damage in fertile men[113]. Lambrot et al. reported that low paternal dietary folate can change sperm epigenetics, and these alterations are associated with genes involved in the development of chronic diseases[114]. Papaleo and co-workers showed that folic acid and myo-inositol can reduce Germinal Vesicle and degenerated oocytes at the day of ovum pick-up[115].

Coenzyme Q10, which is also known as ubiquinone, is a lipid-soluble component that participates in the electron transfer chain in the inner membrane of mitochondria. It has antioxidant properties in cells. In a prospective study in 2012, coenzyme Q10 supplementation was reported to improve semen quality, thereby leading to increasing pregnancy rates in infertile men with idiopathic oligo-astheno-teratozoospermia[116]. Talevi et al. also demonstrated that coenzyme Q10 can prevent the decrease in motility during sperm incubation *in vitro*, and it exerts protective effects on lipid peroxidation and DNA fragmentation[117]. In a double-blind randomized trial study, coenzyme Q10 was found to improve the sperm kinetic features in men with idiopathic asthenozoospermia[118]. Coenzyme Q10 is present in FF of human follicles. Moreover, it is positively correlated with oocyte maturation, and improves embryo quality in women undergoing IVF/ET[119].**Also** we observed that Coenzyme Q10 can improve developmental parameters of vitrified-warmed preantral follicles via increasingthe TAC during IVM of follicles [120].

Alpha lipoic acid (ALA)is a sulfur-containing cofactor that is involved in several multi-enzyme complexes, such as pyruvate dehydrogenase. Lipoic acid and its reduced form dihydrolipoic acid have antioxidant properties, such as ROS scavenging, metal chelation, and antioxidant recycling[121]. Ibrahim et al. observed that lipoic acid administration can improve motility and decrease DNA damage in *in vitro* incubation of spermatozoa[122]. In 2006, lipoic acid was reported to reverse the cyclophosphamide (CP) effects on epididymal sperm characteristics, antioxidant status, and DNA damage, and improve sperm quality in CP-administered rats[123]. Zhang

et al. indicated that ALA supplementation in IVM medium can improve the maturation rate and developmental competence of oocytes after somatic cell nucleus transfer[124]. Our group recently investigated the effects of ALA on follicle *in vitro* culture, and we observed that ALA can decrease ROS elevation and increase total antioxidant capacity during IVM of follicles[91, 125]. We also reported that ALA can improve the developmental competence of *in vitro* matured cryopreserved follicles and subsequently derived oocytes[92, 93]. In another study, we found no significant effects of ALA on oocyte IVM and developmental competence of mature oocytes[126].

Trace elements can have antioxidant properties, and several studies have investigated the antioxidant effects of trace elements, such as zinc, selenium, and carnitine, on sperm quality. Abedelahi et al. showed that sodium selenite improves the IVM of ovarian follicles and developmental competence of released oocytes[127]. They also indicated in other study that these improved effects of sodium selenite are mediated by a reduction in ROS generation and elevation in antioxidant capacity [86]. The antioxidant roles of melatonin, which is a hormone secreted by the pineal gland and regulates various physiological events in the body were discussed previously [128-131]. In this regards, the study of de-Plessis et al., melatonin is concluded to have positive effects on human spermatozoa, and it can neutralize NO radical[128]. However, Cheuqueman et al. found no positive effects on sperm function[129]. Jang et al. reported that melatonin can improve sperm motility, viability, survival rates, and membrane integrity of boar semen during *in vitro* storage, and increase the developmental capacity of IVM/IVF porcine embryo[130].

Espino et al. showed the positive effects of melatonin on oxidative damage and apoptosis in human sperm, and they, as well as Ortiz et al., suggested that supplementation of sperm preparation media with melatonin can improve ART outcomes[131, 132]. The beneficial effects of melatonin on *in vitro* oocyte maturation and embryo development of animal models have been reported. Kim et al. demonstrated that melatonin is present in human follicles, and has a positive effect on oocyte maturation[133].

They also showed that supplementation of IVM medium with melatonin can improve the cytoplasmic maturation of immature oocytes and subsequent clinical outcomes in women with poly cystic ovarian syndrome (PCOS). In other studies, melatonin administration has been found to improve oocyte quality, embryo quality, and pregnancy rates in women undergoing IVF[134, 135].

In one cohort study, melatonin and myo-inositol treatment reportedly improved ovarian stimulation and pregnancy rates in infertile women with poor oocyte quality [136].

CONCLUSION

In conclusion, Effects of ROS should be considered during ART protocols. Therefore, employing some strategies that can reduce harmful physical factors, such as oxygen tension, exposure time, or type of light source during *in vitro* ART procedures are useful in overcoming the OS-derived state from these factors in clinical laboratories.

Moreover, the selection of optimum culture media, antioxidants or metal chelators, and improvement in protocols, which can result in OS, should be considered to minimize ROS overproduction during ART protocols and improve ART outcomes.

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