

# Impact of oxidative stress on IVF

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Gametes and embryos are natural sources of free radicals. When manipulated *in vitro* during assisted reproductive techniques, these cells run the risk of generating and being exposed to supraphysiological levels of reactive oxygen species. It is therefore clear that free radicals and oxidative stress can have a significant impact on IVF outcome. This review summarizes the role of oxidative stress in the etiology and pathophysiology of human IVF, as well as considering different strategies and approaches to be followed to prevent the harmful effects of oxidative stress on IVF.

**KEYWORDS:** antioxidant • assisted reproductive technology • embryo • free radical • IVF • oocyte • oxidative stress • reactive oxygen species • spermatozoa

The term oxidative stress (OS) is generally applied when oxidants outnumber anti-oxidants [1], when peroxidation products develop [2] and when these phenomena cause pathological effects [3,4]. The imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage is known as OS [5]. All forms of life maintain a reducing environment within their cells. This reducing environment is preserved by enzymes that maintain the reduced state through a constant input of metabolic energy. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids and DNA [6]. The effects of OS depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe OS can cause cell death, and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis [7]. A particularly destructive aspect of OS is the production of ROS, which include free radicals and peroxides [8].

Various ROS play an important role in many physiologic functions, such as phagocytosis. Free radicals are also known as a necessary evil for intracellular signaling involved in the normal processes of cell proliferation, differentiation and migration [9–11]. In the

reproductive tract, free radicals also play a dual role and can modulate various reproductive functions. Physiological levels of ROS influence and mediate the gametes [12–14] and crucial reproductive processes, such as sperm–oocyte interaction [15], implantation and early embryo development [16]. An imbalance in the redox state can thus cause OS to develop, ultimately affecting successful pregnancy outcome [17–19].

Since the birth of the first IVF baby [20], assisted reproductive techniques (ART) have become the treatment of choice in many cases of male and female infertility [17]. These methods inevitably require manipulation of gametes and embryos *in vitro*, exposing these cells to additional OS [21]. Various factors, for example, the absence of cytokines/growth factors, pH shock, osmotic shock, temperature fluctuations, UV light damage and nutrient imbalance can influence the outcome of ART; however, OS has recently emerged as one of the most important factors negatively affecting ART outcome [5,22–24]. It has been hypothesized that this is predominantly due to a lack of *in vitro* gamete and embryo protection by oxygen radical scavengers [21,23,25].

The goal of this review is to discuss the possible sources of ROS that can lead to OS during ART, as well as the effects of OS on IVF outcome. Suggestions and possible solutions to curtail this necessary ROS evil to prevent OS in the IVF setting will also be presented.

### Free radicals, ROS & OS

A free radical is defined as any atom or molecule that possesses one or more unpaired electrons [26]. ROS are oxygen-derived free radicals that are formed during the intermediate steps of oxygen reduction [22]. Even under basal conditions, aerobic metabolism entails the production of ROS such as hydrogen peroxide ( $H_2O_2$ ), superoxide anion ( $O_2^-$ ) and the hydroxyl radical ( $OH^\cdot$ ) [18,22], while reactive nitrogen species such as nitric oxide are formed by the conversion of L-arginine to L-citrulline by nitric oxide synthase [27]. Due to their highly reactive nature, ROS can combine readily with other molecules, directly causing oxidation that can lead to structural and functional changes and, conversely, result in cellular damage [18,28,29]. Under normal physiological conditions, ROS must be neutralized continuously, while a small amount necessary to maintain normal cell function must be preserved. ROS can be inactivated by a defense system consisting of enzymes and antioxidants [21,30]. The most important source of reactive oxygen under normal conditions in aerobic organisms is probably the leakage of activated oxygen from mitochondria during normal oxidative respiration. In the event of excessive ROS production that exceeds the antioxidant defense mechanism of the cells, the result is OS and all of its accompanying effects.

### Sources of ROS/OS in the IVF setting

Despite the rapid advances in infertility treatments that have led to the development of new and improved techniques, the IVF setting still struggles to create microenvironments that emulate the physiological conditions of an *in vivo* system. Two of the main factors contributing to ROS accumulation *in vitro* are the absence of endogenous defense mechanisms and, second, exposure of the gametes and embryos to various manipulations/techniques, as well as an environment that can lead to the generation of OS. ROS, therefore, can originate from either internal sources (e.g., endogenous production by the gametes and embryos or exogenous factors inducing ROS generation) or external sources (e.g., IVF media) in the IVF set-up (Box 1).

### Internal sources of ROS

Just like any other cell, gametes and embryos have energy demands that are met by ATP through mitochondrial oxidative phosphorylation and glycolysis. Under normal physiological conditions these aerobic and anaerobic metabolism processes lead to the production of ROS. Various pathological conditions can influence excessive accumulation of ROS.

### Contribution of semen & spermatozoa to OS during IVF

It is well known that physiological levels of ROS are needed for the normal functioning of human spermatozoa, as the role of ROS was demonstrated in capacitation and acrosome

### Box 1. Sources of reactive oxygen species that can lead to oxidative stress during IVF.

#### Internal sources

- Spermatozoa
- Oocytes
- Embryo

#### External sources

- Media
- $O_2$  concentration
- Visible light
- Assisted reproductive technologies procedure
- Freeze–thawing

reaction [31]. In fact, human spermatozoa were the first cells in which the cellular generation of  $H_2O_2$  was indicated. There are basically three ways in which ROS can be generated in spermatozoa:

- The NADPH oxidase system at the level of the sperm plasma membrane;
- The NADH-dependent oxidoreductase (diphorase) system at mitochondrial level;
- The cytoplasmic cytochrome b5 reductase system [5,32], of which the mitochondrial system is the main source of ROS in spermatozoa from infertile men [33].

In a large proportion, if not in at least 50%, of all IVF cases, spermatozoa selected for ART originate from an environment experiencing OS [34]. In the ejaculate, morphologically abnormal spermatozoa and leukocytes (e.g., due to inflammatory processes *in vivo*) are the major sources of supraphysiologic ROS levels [17]. Impairment of spermatogenesis leads to morphologically abnormal spermatozoa. Increased levels of ROS in semen have been shown to be negatively correlated with normal morphology [35,36] and positively correlated to the sperm deformity index [9]. It has also been shown that, in cases of leukocytospermia, increases in extracellular ROS production are particularly evident, especially during infection when ROS production can increase 100-fold [33].

Pathological effects of OS include the loss of plasma membrane fluidity due to lipid peroxidation by ROS in spermatozoa. As a result, it decreases the phosphorylation of axonemal proteins and can lead to a decrease in vigor of motility and, ultimately, causes sperm immobilization [5]. Free radicals can also lead to DNA damage in spermatozoa in the form of modification of all bases, production of base-free sites, deletions, frameshifts, DNA cross-links, chromosomal rearrangements and single- and double-strand DNA breaks [37–39]. This DNA damage by ROS is implicated as one of the causes, if not the most important, of various adverse outcomes, including increased incidence of abortion, childhood cancers and dominant genetic diseases such as achondroplasia [40]. Elevated ROS levels are also negatively correlated with mitochondrial membrane potential. A decrease in mitochondrial membrane

potential is known to be one of the initiating events in the apoptosis cascade and it has been shown that high levels of seminal OS increase sperm apoptosis in patients with male factor infertility [41].

Taking this information into consideration in the IVF setting is of particular importance as it has very relevant clinical consequences – increased OS in the male germ cells has been associated with poor fertilization rates, impaired embryo development and higher rates of pregnancy loss [40]. With the use of intracytoplasmic sperm injection (ICSI) as a treatment modality, the risk that spermatozoa carrying damaged DNA may be injected directly into the oocyte is substantial, as the natural selection barrier has been bypassed [5,42].

### Contribution of oocytes & follicular fluid

It is hypothesized that physiological (low) levels of ROS may have a regulatory role in oocyte maturation, folliculogenesis, ovarian steroidogenesis, ovulation and luteolysis [25,43–45]. Speculation regarding the origin of the ROS still exists. In contrast to the male gamete and seminal plasma in which it is transported, inadequate data related to the oocyte environment inside the ovarian follicle exist [21]. As the preovulatory follicle grows into an antral follicle, it is filled with follicular fluid (FF) secreted from the follicular theca and granulosa cell layers. Despite aspirating FF during ART, it is often contaminated with blood or media, or mixing of FF from various follicles occurs. This hampers investigating the contributing role of FF to follicular development. In 2000, Attaran *et al.* first demonstrated the presence of ROS in FF of women undergoing ovarian hyperstimulation [14]. They suggested that low levels of FF ROS may be a potential marker for predicting success in IVF patients; these findings were supported and confirmed by Pasquallotto *et al.* in 2004 [23]. In 2005, Bedaiwy *et al.* also showed a negative correlation between FF ROS levels and pregnancy [46], as well as demonstrating a positive correlation between FF total antioxidant capacity (TAC) and pregnancy. Furthermore, lipid peroxidation is believed to be a good marker of metabolic activity within the follicle, and it is speculated that some amounts may be necessary to establish pregnancy [23].

The mRNA of the following ROS antioxidant enzymes was also found in bovine oocytes, irrespective of stage: mitochondrial manganese (Mn)–superoxide dismutase (SOD), cytosolic Cu/Zn SOD,  $\gamma$ -glutamyl-cysteine transferase, glutathione-peroxidase and sarcosine oxidase [47]. Oocyte maturity has been correlated with apoptosis levels (a marker of OS) in granulosa cells and cumulus cells. In the same study, Doppler imaging revealed a negative correlation between intraovarian artery vascular indices (resistive index and pulsatility index) and apoptosis level within granulosa cells. These indices are considered good indicators of follicular maturity and oxidation [48]. A decreased dissolved oxygen content of FF, attributed to poor vascularization, also reduces the oocyte's developmental potential; therefore, follicular oxygenation can act as a predictor of IVF success [49,50].

On the contrary, excessive ROS levels can be detrimental to oocytes in many ways. It can trigger disruption of the oocyte's cytoskeleton and is associated with altered microtubule function, chromosomal scattering and aneuploidy. Recently, Choi *et al.* demonstrated that OS causes alteration in metaphase II mouse oocyte spindle formation [51]. As the meiotic spindle is essential for the maintenance of chromosomal organization, disorganization of the meiotic spindles could result in chromosomal dispersion, failure of normal fertilization and termination of development, which is directly related to IVF outcome [52,53]. In 2002, Seino *et al.* reported more evidence suggesting a pathological role for higher ROS levels in IVF [54]. They have demonstrated that 8-hydroxy-2-deoxyguanosine (indicator of DNA damage due to OS) in granulosa cells is correlated with oocyte quality and, subsequently, embryo development in an IVF program.

### Mechanism of ROS generation by embryos

The embryo is a fast-developing organism with high energy needs that are met, as in other living aerobic cells, by generating ATP through mitochondrial oxidative phosphorylation and glycolysis. As the embryo develops from the zygote stage, its redox state is modulated by its ever-changing needs and metabolism. The embryo can produce ROS by several pathways, including oxidative phosphorylation, NADPH and xanthine oxidase systems [18], making it a major source of ROS. Excessive generation of ROS occurs at certain critical points due to increased energy demands, such as embryonic genome activation, embryonic compaction and hatching [36,55]. Interestingly, Goto *et al.* found that ROS production was increased in embryos cultured under *in vitro* conditions compared with those cultured *in vivo* [19]. At this stage it is still unclear whether the conditions and techniques employed during IVF are responsible for these increased levels of OS.

Pathological levels of ROS have been reported to have a negative impact on embryo quality and may also lead to early embryonic developmental block and retardation [5]. Bedaiwy *et al.* reported that slow development, high fragmentation and reduced formation of morphologically normal blastocysts are associated with increased levels of day 1 ROS during embryo culture, which ultimately leads to a lower clinical pregnancy rates [56]. Even during the first trimester, embryos grow best under low oxygen concentration, as it was reported that coelomic partial pressure of oxygen ( $pO_2$ , mean:  $21.0 \pm 1.14$  mmHg) does not change with advancing gestational age (weeks 7–10), whereas amniotic  $pO_2$  (mean:  $15.4 \pm 1.36$  mmHg) decreases rapidly between 11 and 16 weeks of gestation [57]. Low blastulation rates have also been reported by decreasing oxygen tension [58].

### External sources of ROS

There are numerous external sources that can lead to OS generation during IVF. Not only does the technique of ART itself contribute to ROS production, but also other external factors such

as oxygen concentration, visible light, amine oxidase, media and supplements/metallic ions, excess glucose, pollutants, freeze-thawing and the process of *in vitro* maturation of oocytes also play a role.

### Culture medium & supplements

The media used in the IVF setting can also contribute to ROS generation, and this can directly influence oocyte and embryo quality [17]. Depending on the composition of the commercially available culture media, some media can contain metallic ions (e.g.,  $\text{Fe}^{2+}$  and  $\text{Cu}^{2+}$ ) that can independently accelerate ROS generation within these cells by participating in the Fenton and Haber–Weiss reactions [18]. This occurs when these ions are incorporated into the cells during ART processing. Adding metal chelators is a possible solution to the adverse effects of these ions. Transferrin and ethylenediamine tetra-acetic acid are typical chelators with which the media can be supplemented [59,60]. Supplements are regularly added to the media for various reasons, but they may often increase the oxidant load, as is the case with serum containing amine oxidase, which leads to increased ROS ( $\text{H}_2\text{O}_2$ ) production. Proteins (e.g., thioredoxin) added to the media were able to reduce the apoptosis level and enhance hatching rates in mouse embryos, while the addition of glutathione and thioredoxin to the media were able to reduce the redox status of porcine embryos [61].

### Oxygen concentration

During IVF procedures, the  $\text{pO}_2$  in the culture medium is much higher than the  $\text{pO}_2$  at tissue level *in vivo*. It has been shown that at  $37^\circ\text{C}$  the  $\text{O}_2$  concentration in the medium equilibrated with atmospheric oxygen is 20-times higher than the physiological intracellular  $\text{O}_2$  concentration [62]. Similarly, it has also been estimated that, at the time of ovulation, the  $\text{pO}_2$  in the fallopian tubes of rhesus monkeys is three-times lower than atmospheric  $\text{pO}_2$  [63]. This richly oxygenated environment of the incubator can lead to generation of ROS and OS as it activates various oxidase enzymes in the cells. The general train of thought is that among the factors that impact on *in vitro* embryo development, oxygen atmosphere is considered to be a greater influence. Several experiments have been performed to investigate the effects of oxygen concentration in the IVF setup. Booth *et al.* showed that more porcine blastocysts were produced when incubated under low oxygen concentration (5%  $\text{O}_2$  + 5%  $\text{CO}_2$  + 90%  $\text{N}_2$ ) than when incubated under higher oxygen concentrations (air + 5%  $\text{CO}_2$ ) [64]. Similarly, Leoni *et al.* described that low oxygen (5 vs 20%  $\text{O}_2$ ) atmosphere during IVF affects positively the production of high-quality ovine blastocysts [65].

Recently however, Morimoto *et al.* demonstrated that follicles developed better under high oxygen conditions (100 vs 20%  $\text{O}_2$ ) in a human ovarian cortical tissue culture system [66]. This might be due to the fact that these developing follicles are

surrounded by cumulus cells and, therefore, the higher  $\text{pO}_2$  leads to improving  $\text{O}_2$  diffusion and better oxygenation of the developing follicles that are not denuded.

### Visible light

As visible light induces photodynamic stress, it can cause oxidative damage to unsaturated lipids and cholesterol in membranes [67]. This can lead to ROS production and DNA damage [68]. Speculation still exists related to the duration of exposure needed for generation of pathological ROS levels. Some researchers advocate that transient exposure can lead to OS while others report that more than 5 min of light exposure can lead to significant increases in  $\text{H}_2\text{O}_2$  levels and, subsequently, increasing numbers of laboratories are using fluorescent light filters [19,69].

### ART techniques/nature of procedure

During ART the gametes are manipulated and prepared, while the type of fertilization procedure also varies. This contributes to the cellular sources of ROS in conventional IVF being different from those of ICSI [17]. Spermatozoa are normally centrifuged during preparation techniques. Centrifugation has been shown to increase ROS production and OS in male gametes [70]. During conventional IVF, the oocytes, cumulus cell mass and spermatozoa can all generate and contribute to the ROS levels in the media. During preparation for ICSI, oocytes are denuded and stripped of their cumulus cells, while incubation time is also relatively shorter. ICSI also avoids sperm–oocyte co-incubation and thereby prevents exposure of the oocyte to ROS producing defective spermatozoa with the accompanying possibility of ROS-induced damage. The problem, however, is that during sperm introduction some medium is injected directly into the egg, thereby increasing the risk of maternal DNA damage by ROS present in the culture media [5].

### Cryopreservation/freezing–thawing

IVF treatment often requires cryopreservation, with subsequent thawing of gametes, embryos and ovarian tissue. Cryopreservation of spermatozoa causes these cells to lose their antioxidant defense systems [72]; it has been shown that levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. This led to the reduction of glutathione concentrations by 78% and SOD activity by 50% [73], thus enhancing membrane lipid peroxidation susceptibility due to ROS [74]. This strongly suggests that OS occurs during and/or after the cycle of freeze–thaw. Oocyte cryopreservation is still a novel and not very well established technique, often leading to cytogenetic, cellular and developmental consequences, as well as DNA instability [71]. The freeze–thawing process seems to leave the female gametes even more vulnerable to the detrimental effects of damage brought about by ROS.

Cryopreservation of ovarian tissue is largely experimental and used more regularly as a fertility-preservation strategy, and different cryopreservation protocols, such as vitrification, are being explored. Rahimi *et al.* showed that both ROS development and apoptosis levels were decreased in tissue undergoing rapid cooling when compared with tissue exposed to slower cooling techniques after thawing [71].

### Gamete natural defense mechanisms against OS

Spermatozoa, oocytes and embryos possess natural antioxidant defense mechanisms against OS. According to Agarwal *et al.*, antioxidants can protect cells against OS via three mechanisms: prevention, interception and a reparative method [9]. Antioxidants can be divided into two main categories:

- Enzymatic (e.g., SOD, catalase and glutathione peroxidase/reductase [GPX/GRD])
- Nonenzymatic (e.g., vitamin C, vitamin E, vitamin A, albumin, transferrin, glutathione and pyruvate [75,76])

It is important that the TAC of the gametes and embryos does not decrease as this defense mechanism protects them from the lethal effects of OS.

Due to the size and small volume of cytoplasm, as well as the low concentration of scavenging enzymes, spermatozoa have limited antioxidant defense properties. Human sperm mainly contain enzymatic antioxidants; this includes SOD and GPX/GPR, which are mainly present in the midpiece. A few nonenzymatic antioxidants, such as vitamin E, vitamin A, haptoglobin, transferrin and ceruloplasmin, are present in the plasma membrane of spermatozoa and act as preventative antioxidants. Of utmost importance in protecting spermatozoa against OS is the role of antioxidants in the seminal plasma. Seminal plasma contains both enzymatic antioxidants, as well as an array of nonenzymatic antioxidants (e.g., ascorbate, urate, vitamin E, vitamin A, pyruvate, glutathione, albumin, ubiquinol, taurine and hypotaurine) [76].

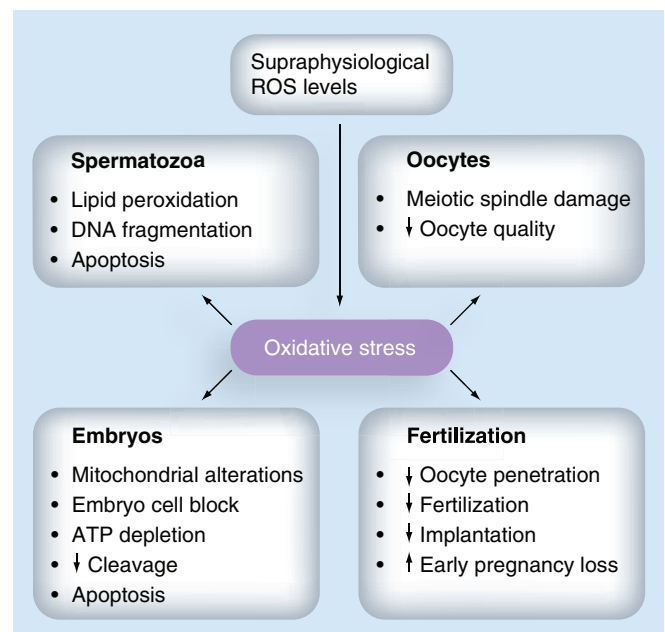
Oocytes and FF also have defense mechanisms against ROS. Nonenzymatic antioxidants, such as taurine, hypotaurine, ascorbic acid, vitamin E and cysteine (strong scavenger of hydroxyl radical) are present in FF [18,77]. Glutathione is the most important defense mechanism against ROS in oocytes and embryos [78,79]. Its depletion is associated with disrupted microtubule formation [80]. SOD was shown to be present in normal cycling human ovaries [81] and is considered the first enzymatic step that protects oocytes and embryos against ROS [82]. SOD is present in the cytosol, while Mn-SOD, located in the mitochondria, can scavenge superoxide radicals. El Mouatassim *et al.* reported that Cu-Zn-SOD, Mn-SOD, GPX and  $\gamma$ -glutamyl cysteine synthetase (GCS) are expressed in human oocytes [83]. However, transcripts corresponding to GPX and Mn-SOD have not been detected in human oocytes at the germinal vesicle stage. This suggests that the oocyte's defense against ROS varies according to developmental stage.

The same enzymes were also studied in a bovine oocyte model at various stages of oocyte development. It was found that the cytoplasmic Cu-Zn-SOD transcripts were expressed in significantly higher levels in *in vitro* maturation oocytes, while mitochondrial Mn-SOD was expressed in higher levels in oocytes derived from smaller follicles. This suggests that mitochondrial defense varies according to the stage of oocyte development [47].

It must be remembered that spermatozoa are separated from seminal plasma and oocytes removed from FF during IVF, thereby removing gametes from environments that help to protect them from the detrimental effects of ROS. Oocytes are being denuded in preparation for ICSI, further decreasing the natural defense mechanisms as provided by the cumulus cells. Cetica *et al.* reported that enzymatic units were much lower (SOD: 37%; GPX: 25%; catalase: 11%) in denuded oocytes with respect to cumulus-oocyte complexes [84].

### Effect of OS on IVF success/outcome

In the ART set-up, the physiological milieu is simulated by modification of the media, as well as the technique used. Data suggest that embryo-maternal communication exists in the preimplantation period, with the female reproductive tract providing the optimal microenvironment conducive to fertilization and the development of embryos [85]. However, an *in vitro* set-up can never mimic the exact physiology of an *in vivo* condition and, therefore, generation of ROS is inevitable. In the IVF set-up this leads to OS with various detrimental consequences (FIGURE 1).



**Figure 1. Effects of pathological levels of reactive oxygen species during IVF.**

ROS: Reactive oxygen species.

### Effect of OS on embryo quality, development & fragmentation

Embryos cultured *in vitro* are exposed to amplified amounts of OS. This is due to a lack of antioxidants present in tubal fluid, which act as a natural physiological defense against OS, as well as the presence of potential sources that can generate ROS in embryo culture media.

Reactive oxygen species, such as  $O_2^-$ , are very unstable and can react easily with and alter most types of cellular molecules, such as lipids, proteins and nucleic acids (DNA).  $H_2O_2$  is a more stable ROS member and can readily diffuse and pass through cell membranes. Increased ROS levels can also affect mitochondria. These deleterious effects appear to be mediated, at least in part, by deregulation of the apoptotic cascade [86]. Effects of ROS on sperm DNA have been established and correlated to poor ART outcomes [87], and the use of spermatozoa with abnormal DNA during ART procedures leads to poor embryo quality [16]. The consequences of increased ROS on the embryo include mitochondrial alterations, cell block, ATP depletion and apoptosis.

Oxidative stress has been shown to induce mitochondrial damage [88]. Mitochondrial DNA is especially susceptible to mutation because of its lack of histones, which incidentally quench ROS. Defective embryo mitochondrial DNA may induce metabolic dysfunction and, consequently, disturb embryo development. The consequences of these alterations may be numerous and include embryo development retardation and arrest, metabolic dysfunctions and possibly apoptosis [18].

The activation of embryonic genome expression occurs at the four- to eight-cell stage in human embryos [89]. This suggests that the paternal genome may not be effective until that stage and also that OS-induced sperm damage seems to be of importance in the later stages of embryonic development [90]. Oocyte maturation and embryo development are also affected due to increased ROS or decreased antioxidant defenses [91,92].

In a study designed to determine the level of  $H_2O_2$  concentration within embryos and the morphological features of cell damage induced by  $H_2O_2$ , 31 fragmented embryos, 15 nonfragmented embryos and 16 unfertilized oocytes were evaluated [86]. The intraembryonic  $H_2O_2$  concentration was measured using 2',7'-dichlorodihydrofluorescein diacetate by fluorescence imaging. DNA fragmentation was assessed by transmission electron microscopy and an *in situ* apoptosis detection kit. It was reported that the relative  $H_2O_2$  concentration was significantly higher in the fragmented embryos ( $p < 0.05$ ) than in the nonfragmented embryos and unfertilized oocytes ( $72.21 \pm 9.62$  vs  $31.30 \pm 3.50$ ). In addition, electron microscopy confirmed that apoptosis was observed only in the fragmented embryos. This study clearly showed that a direct relationship exists between increased ROS concentration and apoptosis. Further support of this finding comes from the observation that 5%  $O_2$  decreases the relative concentration of  $H_2O_2$  and results in improved embryo

development in terms of quantity and quality in a mouse model [93]. A direct relationship was observed between increased  $H_2O_2$  concentration and apoptosis in human fragmented embryos [85]. It was also mentioned that  $H_2O_2$  is a mediator of apoptosis in blastocysts [94].

Another study demonstrated that development to the blastocyst stage was higher in embryos cultured under 5%  $O_2$  vs 20%  $O_2$ , (36.3 vs 22.5%;  $p < 0.05$ ), the  $H_2O_2$  content as a ROS was lower (2 vs 111 pixels;  $p < 0.05$ ); and fragmentation of DNA in eight- to 16-cell stage embryos (estimated by COMET/single-cell gel electrophoresis assay) resulted in a shorter ( $p < 0.05$ ) DNA tail (36 vs 141  $\mu m$ ) [94]. Therefore, it can be concluded that low  $O_2$  concentration during *in vitro* culture of embryos decreases the  $H_2O_2$  content and, therefore, reduces DNA fragmentation and improves developmental competence [95].

Given the fact that condensed chromatin, degraded DNA (as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling), 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 [95]. 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 delayed embryo development, reduced cell number and, ultimately, preimplantation embryo death [96].

The *in vitro* culture environment differs from *in vivo* conditions in that the  $O_2$  concentration is higher. In such a condition, mouse embryos show a higher ROS concentration in simple culture media [97]. Studies also suggest that oxygen toxicity is closely related to developmental blockage on embryos cultured *in vitro*. In a study carried out by Goto *et al.*, mouse pronuclear stage embryos from the oviduct were cultured for a specified time under various conditions in a medium to which a fluorescent dye was added [98]. Following washing of embryos, the fluorescence emissions of  $H_2O_2$ -dependent oxidative product in embryos were measured. Fluorescent emissions were lowest in embryos cultured under 5%  $O_2$  and highest under 40%  $O_2$  and these emissions increased with the time of exposure to visible light.

Oxygen radicals are involved in the *in vitro* block phenomenon of embryo development. Goto *et al.* also proposed that one of the target molecules damaged by oxygen radicals may be the thiol group of proteins as it is oxidized readily [19]. This proposition was further strengthened when they demonstrated that culture of mouse pronuclear embryos recovered 17 h after human chorionic gonadotrophin administration in the presence of thioredoxin (200  $\mu g/ml$ ) significantly increased the blastulation rate (75.3%) when compared with the culture control system (8.9%).

In a study to show the effect of  $O_2$  concentration on embryo development, cell numbers and gene expression, day 5 compacting bovine embryos were cultured under different  $O_2$  tensions (2, 7 and 20%). The *in vitro* embryos

responded to variations in  $O_2$  concentration by altering gene expression. Glucose transporter (GLUT)1 expression was higher at 2%  $O_2$  concentration while hypoxia-inducible factor mRNA expression was unaltered at any oxygen concentration. Oxygen concentration also significantly altered the inner cell mass cell proportion at the blastocyst stage [99].

In another study attempting to define the stage of block in development of the embryo due to OS *in vitro*, the rise in  $H_2O_2$  was minimal in *in vivo*-derived mouse embryos in their transition from the unfertilized stage towards the eight-cell stage [97]. By contrast, when embryos spent some time *in vitro* before being incubated with the fluorochrome dichlorofluorescein diacetate, a marked rise in green fluorescent signal was observed, which reflected increased conversion of 2',7'-dichlorodihydrofluorescein to DCF by oxidation. They also reported that this increase is restricted to G2/M phase of the second cell cycle, but is not evident before this nor after entry to the four-cell stage is completed, nor during the passage through the four-cell stage to eight-cell stage. They concluded that this was not a consequence of total time spent *in vitro*, but reflects an interaction between *in vitro* conditions and the stage of the cell cycle. The increase in  $H_2O_2$  production *in vitro* takes place during the atypically long G2 period of the second cell cycle. This period coincides with that of the two-cell block and the potential rise in damaging free radicals generated from the  $H_2O_2$ . A further study also demonstrated that the rise in ROS is dependent on the activation of the oocyte [100]. These researchers demonstrated that only fertilized oocytes demonstrated a rise in  $H_2O_2$ , whereas unfertilized oocytes showed no such rise. They also showed that a rise in  $H_2O_2$  occurs regardless of activation mechanism (sperm activation vs parthenogenesis).

In human IVF, only a few oocytes develop into good-quality embryos, depending on the incubation conditions and the quality of the ovum and the spermatozoon. Embryo quality is also highly female and couple dependent. The rest of the embryos show abnormal morphology due to unequal cell division or cell fragmentation [101]. It was also concluded that morphologically abnormal embryos showed some morphometrical differences, and embryos that successfully implanted and progressed to birth showed a higher coefficient of diversity between sister blastomeres. Fragmented embryos have a limited developmental potential and rarely result in implantation [102,103].

Depletion in ATP occurs via inactivation of glyceraldehyde 3-phosphate dehydrogenase and/or inactivation of glycolytic and mitochondrial pathways [104]. OS induces consumption of reducing equivalents such as glutathione. Glutathione reductase activity allows the glutathione endogenous pool to be maintained. Glutathione reductase is NADPH-dependent and is the main source of NADPH in the monophosphate shunt. Consequently, OS, via competitive consumption of reducing equivalents, can interfere with important metabolic functions and divert glucose from other pathways by inducing the monophosphate shunt [18].

### Effect on fertilization & pregnancy rates

In a previous study reported by our group, we showed that both lipid peroxidation and TAC levels were positively correlated with pregnancy rate [23]. Of the 41 women who underwent IVF, 13 got pregnant, spontaneous abortion occurred in one, and the take-home pregnancy rate (12 of 41) was 29.2%. After adjusting for age, the patients who did not become pregnant had significantly lower levels of lipid peroxidation ( $p = 0.022$ ) and TAC ( $p = 0.018$ ) compared with the patients who became pregnant. However, no correlation with lipid peroxidation or TAC levels was shown by fertilization rates and embryo cleavage rates.

As mentioned previously, sperm DNA damage can be induced by OS during IVF. If sperm DNA damage impairs embryo development, it is logical to suggest that subsequent implantation will also be adversely affected. This has been demonstrated by Duran *et al.* who showed that DNA fragmentation has been found to correlate negatively with pregnancy [105].

Oxidative stress-induced DNA damage may also lead to early pregnancy loss. Of 6077 ICSI and 8975 IVF cycles reported in 1997 in the USA, there were 17–18% abortions compared with 10–12% in the general population [106]. In another study, day 1 ROS levels were negatively correlated with percentage of embryos with high cell number at day 3 ( $p = 0.01$ ), and day 1 ROS levels were significantly related to increased embryonic fragmentation at day 3 ( $p = 0.03$ ) [107]. However, the researchers could not determine such a relationship in IVF cycles. They concluded that ROS level in day 1 culture media is an important marker for early embryonic growth and has a strong relationship with early embryonic development, particularly in the cleavage rate and increased embryo fragmentation.

Sperm are highly packed in the epididymis, which facilitates damage by oxygen radicals by nature of the close contact between mature and immature sperm [35,108]. A similar mechanism occurs in the pellet of centrifuged semen where sperm are also highly packed. In a recent study, Greco *et al.* reported that DNA fragmentation, as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling, in ejaculated sperm was significantly higher than that found in testicular sperm from the same males (23.6 vs 4.8%;  $p < 0.001$ ) [109]. Pregnancy rates obtained with testicular sperm were higher than those obtained with ejaculated sperm (44.4 vs 5.6%;  $p < 0.001$ ).

In a similar study, Steele *et al.* found that the level of DNA fragmentation in epididymal sperm was significantly higher than that of testicular sperm obtained from the same patients. In another study, researchers reported that administration of 1 g/day of vitamins E and C for 2 months to males with high levels of DNA fragmentation in semen reduced sperm DNA fragmentation to levels comparable to those observed in testicular sperm and significantly increased pregnancy rates after ICSI [110]. The studies mentioned above underscore the significance of DNA fragmentation in ART outcome.

An idea of the detrimental effects of ROS on sperm penetration and fertilization ability can be gained from a study in which ROS exposure was shown to reduce ( $p < 0.001$ ) the rates of oocyte penetration significantly (control:  $56\% \pm 4$  SEM; ROS:  $16 \pm 2\text{--}23\% \pm 7$  SEM) [91]. The researchers also concluded that improper oocyte maturation may be reflected in abnormal embryo development.

The effect of ROS on sperm DNA has been established and correlated with poor ART outcomes [34,111,112]. Poor embryo quality results in cases of ART using sperm with abnormal DNA [37,113].

DNA damage in the sperm during ART may correlate with poor fertilization rates. In a study involving 143 IVF samples, Sun *et al.* reported a significant negative association between the percentage of sperm with DNA fragmentation and fertilization rate ( $p = 0.008$ ) and embryo cleavage rate ( $p = 0.01$ ) [114].

### Effect of OS on implantation

Successful implantation requires an appropriate interplay between the embryo and endometrium. First, the embryo should be of good quality; in addition, the endometrium must be receptive. Any interference with either of the two may lead to implantation failure. Although ample evidence exists to suggest that OS can jeopardize embryonic health, it is not clear how much of a role, if any, OS plays in jeopardizing endometrial receptivity.

A recent study compared gene expression profiles of pre-receptive (2 days after LH surge) versus receptive (7 days after LH surge) endometria obtained from the same proven fertile women ( $n = 5$ ) in the same menstrual cycle. Endometrial biopsies were analyzed using a DNA microarray chip containing approximately 12,000 genes, and approximately 211 regulated genes were found. mRNA quantification by real-time quantitative fluorescent PCR of three upregulated genes (glutathione peroxidase 3 [*GPx-3*]; claudin-4 and solute carrier family 1 member 1 [*SLC1A1*]) were incorporated for validation of array data. 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 mid- 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 [115].

### Effect on offspring

Assisted reproductive techniques have been implicated in some cases of malformations and defects in the offspring. Of the many causes that may lead to adverse effects on the offspring, OS and OS-induced DNA damage are the most commonly implicated.

Free radicals in particular cause detrimental effects to sperm structure and function depending on their nature and concentration [116] and also inflict damage to mitochondrial and genomic DNA [39]. OS inflicts DNA damage by inducing strand breaks and oxidative base damage in human spermatozoa [117,118]. Such susceptibility to OS is explained by the lack of DNA repair mechanisms and antioxidants in spermatozoa [119,120]. DNA damage in male gametes, in turn, has been associated with impaired preimplantation development and increased abortion and elevated disease levels in offspring [121,122].

Hansen *et al.* reported a compilation of data from the registries in Western Australia involving 301 infants conceived with ICSI, 837 infants conceived with IVF and 4000 naturally conceived controls, and found the incidence of major birth defects to be more than twofold higher for ICSI and IVF groups (8.6 and 9%) compared with normal controls (4.2%) [123]. They also showed an increased incidence of chromosomal abnormalities in the ICSI group (1% for all infants and 1.6% for singletons only) compared with IVF (0.7% for all infants and 0.6% for singletons only; the difference not statistically significant) and normal controls (0.2% for all infants and 0.2% for singletons only;  $p < 0.05$ ).

Another group reported karyotype analysis performed for prenatal diagnosis in a total of 2139 pregnancies conceived with ICSI in summarized data from seven studies [124,125]. They showed a slight but significant increase in *de novo* sex chromosomal aneuploidy and structural autosomal abnormalities compared with the general population, 0.6 versus 0.2% and 0.4 versus 0.07%, respectively.

Defects in patterns of DNA methylation, which is a heritable epigenetic modification, could be a mechanism by which paternal DNA damage might lead to disorders of pregnancy and development. IVF relies on the manipulation and culture of gametes and embryos at times when epigenetic programs are being acquired and modified. Epigenetic marks, especially DNA methylation, are unstable and can be altered by culture conditions [126,127]. Recently, six studies have reported two imprinting disorders, Beckwith–Weidman syndrome [128–130] and Angelman syndrome [131,132] in association with ART. The maternal allele is affected in these syndromes because some aspect of the assisted reproductive procedure has disturbed methylation of the maternal genome in the oocyte or early embryo [128]. However, studies in animal models have emphasized the possible impact of aberrant paternally imprinted genes on development [133].

Evidence for linkage between OS-induced DNA damage in the male germ cell line and abnormalities in the developing embryo or child can be found in the wealth of data indicating that powerful associations exist between childhood disease and paternal occupation [119,134]. It is therefore speculated that the use of ICSI as a therapeutic technique can only exacerbate the problem. Since DNA damage in the male germ line is associated with an increased incidence of childhood cancer,



it is possible that the children of ICSI conceptions will be vulnerable to this disease. Childhood cancer may not be the only consequence of conceptions involving DNA-damaged spermatozoa. It is also possible that double-strand sDNA breakage induced by OS results in infertility in the male offspring as a consequence of irreparable deletions on the long arm of the Y chromosome [119,134]

### Suggestions & solutions to moderate/curb OS in the IVF setup

With OS an important factor contributing to poor IVF outcome, preventing the development of, or moderating, OS is essential. A discussion of possible strategies to curb OS follows.

#### Sperm preparation protocols

In many cases, spermatozoa selected for ART are of poor quality and originate from an OS environment. They are further exposed to even more OS during ART. As a consequence, this may cause DNA damage, ultimately resulting in impaired embryonic development, early embryonic death and abortion. As discussed previously, leukocytospermia and morphologically abnormal spermatozoa are the main sources of ROS. Removing viable sperm from ROS-producing sperm and leukocytes as soon as possible is very important, as is minimizing the ROS-inducing effect of sperm preparation techniques.

Various techniques are employed during sperm preparation, with swim-up, one-step wash, density gradient centrifugation and glass wool filtration currently the most commonly used. Henkel and Schill [135] recommend double-density gradient centrifugation and glass wool filtration [136] as these techniques separate the mature spermatozoa from the immature and damaged spermatozoa and leukocytes, as opposed to the swim-up method where these cells are all pelleted and mature sperm are directly exposed to these pathological, ROS-generating cells.

Reducing exposure of spermatozoa to centrifugation, as well as supplementation of media with antioxidants, are other strategies being pursued. Magnetic-activated cell separation is a technique that can separate leukocytes from spermatozoa by paramagnetic microbeads targeted against CD-14, -15, -16 or -45. Similarly, it too can be employed to separate apoptotic cells from normal cells on the basis of annexin V targeting [137]. Sperm preparation that combines magnetic-activated cell separation with double-density centrifugation provides spermatozoa of higher quality in terms of motility, viability and apoptosis indices compared with other conventional sperm preparation methods [137–140], thereby eliminating unnecessary centrifugation steps leading to ROS production. Various antioxidants have also been successfully added during sperm preparation in order to scavenge ROS, for example, pentoxifyline [141], glutathione [142], N-acetyl-cysteine [143] and albumin [37].

#### Antioxidants

Both *in vivo* and *in vitro* antioxidant supplementations are possibilities to minimize OS during IVF. Various antioxidants and scavengers can be added to the media during IVF procedures (see Media) and at optimal concentrations can curtail the development of a state of imbalance in oxidants and antioxidants. A considerable body of evidence indicates that supplementation of culture medium with antioxidants, vitamins C and E, amino acids, ROS scavengers, disulphide reducing agents and divalent chelators of cations can reduce OS and be beneficial to embryo survival and blastulation rates in animal studies [21]. Furthermore, Zhang reported that pentoxifylline can significantly reduce the embryotoxic effects of H<sub>2</sub>O<sub>2</sub> on mouse two-cell embryos [144]. Fewer human studies have been performed, but *in vitro* supplementation of vitamin E prevented loss of motility due to ROS originating from leukocytes [145]. Lane also reported that the addition of ascorbate during cryopreservation could reduce the levels of H<sub>2</sub>O<sub>2</sub> and prevent OS in mammalian embryos [146].

Supplemental intake of vitamins A, C or E has been tried in an attempt to enhance reproductive function with reasonable success in farm and experimental animals [21,147]. The case for oral supplementation in males is more speculative and depends on whether it can actually increase antioxidant levels in the reproductive tract and gametes themselves [21,148]. Some trials have reported that oral administration of antioxidants may improve sperm quality in heavy smokers [149] and male factor infertility patients [22,150,151], while others could not find any benefits or changes in semen parameters of subfertile men treated with vitamins C and E [152]. Trials investigating antioxidant supplementation in females are few and lack power, but the results look promising, especially with vitamin C and E supplementation [29]. Recently it has also been reported that melatonin supplementation showed promising results in protecting oocytes against free radical damage, thereby improving fertilization rates [153].

Considering the etiology of infertility in various patients, antioxidants may not be effective. Cocuzza and Agarwal recently suggested that therapeutics against each specific etiological cause of elevated ROS should be attempted once the primary cause of infertility has been treated [154].

#### Procedures

Considerable evidence is pointing to the fact that exposure of gametes and embryos to high concentrations of O<sub>2</sub> during IVF treatment leads to decreased blastocyst formation and reduction of pO<sub>2</sub> from 20 to 5% can prevent ROS formation *in vitro* [64,155]. Reducing the handling and centrifugation of gametes and insemination time during conventional IVF, as well as exposure to ROS-generating media, can also result in improving both embryo yield and quality [153,157], while lowering sperm number during insemination can contribute to preventing excess ROS generation.

A further strategy to overcome OS is to reduce the sperm–oocyte incubation time. Several studies over recent years have shown that by reducing the co-incubation time of gametes to less than 2 h prevents increased ROS production, resulting in better embryo quality with significantly improved implantation rates [157,158]. Performing all of these techniques under minimal exposure to visible light is also advisable.

### Media

The media used in an ART set-up is supposed to mimic the physiological milieu; however, it can never mimic the exact physiology of an *in vivo* condition. Therefore, including ingredients in the media to meet the changing needs and provide a stable environment for the gametes and embryos, as well as maintaining a stable antioxidant–pro-oxidant balance is very important. Currently, IVF media used for bovine and mouse embryo culture are supplemented with antioxidants such as vitamins C and E, taurine, hypotaurine, thiols, SOD,  $\beta$ -mercaptoethanol and cysteine with great success. As serum can protect embryos from OS, serum supplementation to the media is also starting to be used; however, it has been shown recently that serum-free embryo culture medium improves *in vitro* survival of bovine blastocysts to vitrification [159].

Agarwal *et al.* point out that culture media and conditions have evolved from monoculture to co-culture and currently to sequential culture media for the purpose of overcoming OS [25]. Multiple cell types have been used for the purpose of co-culture and thus the elimination of potentially harmful substances, such as heavy metals and ammonium and free radical formation, thereby detoxifying the culture medium. In general, the sequential culture is composed of two different media designed to meet the metabolic requirements throughout embryo development [160].

### Conclusions

Oxidative stress is a real threat to gametes and embryos *in vitro* as these cells are removed from their natural environments that afford them defense mechanisms against ROS. Consequently, OS has a significant impact on IVF outcome. The origin of ROS imbalance and, therefore, OS can be due to internal or external sources. This ROS imbalance can lead to poor fertilization, implantation and pregnancy rates.

Various strategies can be followed to curb and overcome OS in the IVF setting. IVF protocols should be revisited and steps implemented to not only reduce ROS generation, but also scavenge excessive ROS levels. Gametes and embryos should be handled as little as possible and minimally exposed to high oxygen concentrations and visible light to prevent ROS production. Modifying sperm selection methods, reducing sperm number and sperm–oocyte co-incubation time can also reduce OS. Prophylactic oral antioxidant treatments, as well as culture media supplementation with antioxidants, can ensure the delivery of better quality gametes and benefit embryo development. Simply adding antioxidants is not sufficient. The appropriate

antioxidant compounds and their concentrations need to be determined, and this remains a field of interest. Research still needs to be performed that will minimize OS during ART and focus on creating media as close as possible to the physiological milieu *in vitro* to improve effectiveness and outcome. Despite the relative success with IVF treatments, OS is unavoidable. A better understanding of the exact mode of action would help in devising more effective IVF strategies leading to improved and more cost-effective results for patients.

### Expert commentary

The purpose of this article was to highlight the implications of OS during IVF as IVF has become the treatment option of choice for the benefit of infertile couples. The involvement of OS during IVF may be implicated not only in failure of IVF cycles but also in causing irreparable damage to the offspring.

Oxidative stress in IVF may result from internal or external sources. Internal sources include the spermatozoa, gametes and the embryos. More importantly, the external sources of ROS production have plagued the procedure for a long time. Usage of culture media containing  $\text{Fe}^{2+}$  and  $\text{Cu}^{2+}$ , high oxygen concentration in the IVF microenvironment, photodynamic stress due to over-exposure to light, and techniques such as centrifugation and cryopreservation have singly and collectively led to significant amounts of OS during IVF.

The review also highlights the implications of OS on IVF outcome. Not only is the embryo quality jeopardized, but also fragmentation and developmental blocks occur. In addition, poor fertilization rates and pregnancy rates have also been reported due to OS during IVF treatment. The reports of major birth defects in offspring from IVF cycles affected by OS are disturbing. OS has also been implicated in imprinting diseases, abortions, sex chromosome aneuploidy and childhood cancers. This calls for the development of techniques and protocols that will reduce the development and implications of OS during IVF. Methods by which this can be achieved include the following:

- Developing better sperm preparation protocols. Magnetic-activated cell separation and density gradient methods have shown significantly better results than swim-up;
- Using antioxidants such as vitamins A, C and E *in vivo* and *in vitro*;
- Lowering  $\text{O}_2$  concentration during the procedure, reducing sperm–oocyte incubation time, and reducing exposure to light during the handling of embryos and gametes;
- Incorporating substances such as antioxidants and serum in the media in an attempt to simulate the physiological environment as closely as possible.

Hopefully, with the incorporation of one or a combination of these preventative actions, higher success rates with IVF cycles will be achieved. This will not only lessen the economic burden of a failed IVF cycle on a family, but it will also help to lessen their psychological trauma and grief.

### Five-year view

Despite the fact that IVF technique has developed dramatically since its inception, the presence of excessive ROS with the subsequent development of OS during IVF has been a major factor that negatively influences its success. More research is required to identify the exact origin of OS during IVF to generate a larger body of knowledge to serve as a reference for future research.

Sperm preparation techniques must be tailored and new ones developed to reduce and phase out centrifugation as a step, thereby eliminating excessive ROS generation by spermatozoa. New techniques may be developed in the future to identify a single suitable spermatozoon for selection during ICSI. The use of ROS viability and DNA integrity probes that are not harmful to cells will be of utmost importance. Measurement of ROS levels in semen before IVF may also be used to determine specific treatment of the sample as well as predict IVF outcome, as ROS is hypothesized to affect fertilization rates post-IVF [110]. As these techniques emerge, the WHO will need to revise and update its recommendations for semen evaluation and sperm preparation.

A promising area is the field of antioxidant supplementation. *In vivo* supplementation must be refined in order for these antioxidants to specifically target the gonads and reproductive tract and produce gametes of better quality, undamaged by OS. *In vitro* antioxidant supplementation goes hand

in hand with the development of a culture media that will support IVF and assist in minimizing OS effects on both gametes and embryos.

New IVF protocols are likely to evolve that will not only see the emergence of new ART techniques, but also lead to the identification of optimal O<sub>2</sub> concentration and sperm–oocyte co-incubation times. This will aid in the decrease of ROS generation.

The environment in an IVF laboratory should also be optimized to minimize ROS development. This can be done by creating more awareness regarding the use of light filters, as well as making use of alternative light sources that will prevent ROS generation. In addition, efficient quality-control measures should be incorporated in the IVF setting. Care should be taken that safety is never compromised during the search for better and more cost-effective techniques and protocols. Ultimately, curbing OS will benefit patients seeking IVF treatment.

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### Key issues

- Oxidative stress (OS) is the development of an imbalance between oxidants and antioxidants, leading to pathological effects in the biological system.
- IVF settings fail to simulate physiological milieu and may lead to OS.
- During IVF, OS may result from internal and external sources. Internal sources include spermatozoa, oocytes and embryo; and external sources include IVF media, O<sub>2</sub> concentration, visible light, assisted reproductive techniques procedure and freeze–thawing.
- OS may lead to embryo developmental block, ATP depletion, fragmentation and apoptosis in embryos.
- OS leads to poor sperm penetration, fertilization, implantation and pregnancy rates.
- Increases in abortion and disease rates in the offspring due to OS have been reported.
- Improved sperm preparation protocols (magnetic-activated cell separation, density gradient centrifugation plus glass wool filtration), antioxidants and the use of low O<sub>2</sub> concentrations during IVF may be helpful to reduce OS in IVF.
- Further research is needed to minimize OS in IVF by focusing on media and culture conditions that provide an environment as close to the physiological environment as possible and developing better ART methodologies for sperm preparation.

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