

Chapter 2

Sources of ROS in ART

Reactive oxygen species (ROS) are molecules derived from oxygen, which occur as a by-product of cellular oxidative respiration. ROS are free radical molecules containing one or more unpaired electrons in their outer shell. They tend to remove an electron from surrounding molecules to complete their octet. As a ROS stabilizes itself with the addition of an electron, the molecule from which it removed the electron now becomes a free radical. In this manner, a self-propagating chain reaction that produces ROS is generated. As ROS goes on to react with other molecules, the molecule undergoes structural and functional modifications (Sharma and Agarwal 1996).

Commonly-occurring ROS in basal conditions, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^\cdot), are powerful oxidants that are found in low concentrations in the genital tract of both males and females (Guerin et al. 2001; Agarwal and Allamaneni 2004). In the electron transport chain, oxygen functions as the final electron acceptor. Most oxygen ions bind with hydrogen ions to form water molecules, but oxygen ions that do not undergo this reaction, are released from the mitochondria in the form of free radicals. As levels of ROS increase, the cell's antioxidant defences become overwhelmed and are inadequate in scavenging the unstable metabolites of oxygen, leading to a state of oxidative stress (OS) (Pasqualotto et al. 2004).

A subset of ROS is the nitrogen-containing compounds, known as reactive nitrogen species (RNS), whose formation is catalyzed by nitric oxide synthase enzymes (O'Bryan et al. 1998). Examples of RNS include nitric oxide $\cdot NO$, nitroxyl ion HNO , peroxyxynitrate anion $ONOO^-$ and nitrosyl-containing compounds. Physiological levels of RNS helps maintain normal sperm parameters and general reproductive functions, as well as stimulate the immune functions. However, pathological levels of RNS contribute towards a state of nitrosative stress, which negatively impacts sperm function and its fertilizing capacity, resulting in compromised male reproductive function (Doshi et al. 2012).

In the ART setting, the effect of OS is augmented by the lack of endogenous physiological defence mechanisms and the multiple potential sources of ROS, both

internally [endogenously from the gametes and exogenously from the environment and by manipulation of the gamete/embryo] and externally [*in vitro* factors such as oxygen partial pressure and culture media], during the ART procedure. As the *in vitro* setting during ART is unable to completely mimic physiological conditions *in vivo*, the gametes used in assisted reproduction are more susceptible to the detrimental effects of oxidative stress. This negatively impacts on the ART outcome (Lampiao 2012). Among the external factors that could potentially affect the gamete/embryo viability *in vitro* are cryopreservation/freezing-thaw procedure, visible light, the specific ART technique employed, pH and temperature, centrifugation, culture media, and most importantly, oxygen concentration or partial pressure. These exogenous ROS-inducing factors, along with endogenous sources of ROS are depicted in Fig. 2.1 and will be discussed in the following sections.

2.1 In Vivo Generation of ROS

2.1.1 In Vivo Generation of ROS in the Male

Spermatozoa use ATP as a source of energy, which is produced through mitochondrial oxidative phosphorylation and glycolysis. Spermatozoa physiologically undergo both aerobic and anaerobic metabolic processes, both of which contribute towards the production of ROS (du Plessis et al. 2008). Under physiological conditions, the primary source of ROS is the escape of activated oxygen from the mitochondria during oxidative respiration. Under typical conditions *in vivo*, ROS is neutralized continuously to maintain the required levels of ROS to facilitate the normal functioning of human spermatozoa. The low levels of ROS aid in the physiological processes that maintains male reproductive functions such as cell signalling, regulation of tight junctions, steroidogenesis, capacitation and acrosome reaction, sperm motility, and zona pellucida binding (de Lamirande and Gagnon 1993; Doshi et al. 2012).

Besides the ROS generated intrinsically from the plasma membrane and mitochondria of spermatozoa (Gavella and Lipovac 1992), other cells may also produce ROS in the male genital tract. Human spermatozoa generate O_2^- , which spontaneously dismutates to H_2O_2 (Alvarez et al. 1987).

Impaired spermatogenesis leads to the production of immature and morphologically-abnormal spermatozoa, which contribute to ROS in the ejaculate. A major extrinsic source of ROS in the human ejaculate is leukocytes. These may be present in the ejaculate due to *in vivo* inflammatory processes. Leukocytes physiologically produce about a 100-fold more ROS than spermatozoa (Plante et al. 1994; de Lamirande and Gagnon 1995). High levels of ROS produced during leukocytospermia, play a major role in infection, inflammation and cellular defence mechanisms against pathogens.

The highly reactive ROS combines easily with molecules causing cellular damage (de Lamirande and Gagnon 1995; Agarwal et al. 2005b). The plasma membrane of the spermatozoon contains a high amount of polyunsaturated fatty

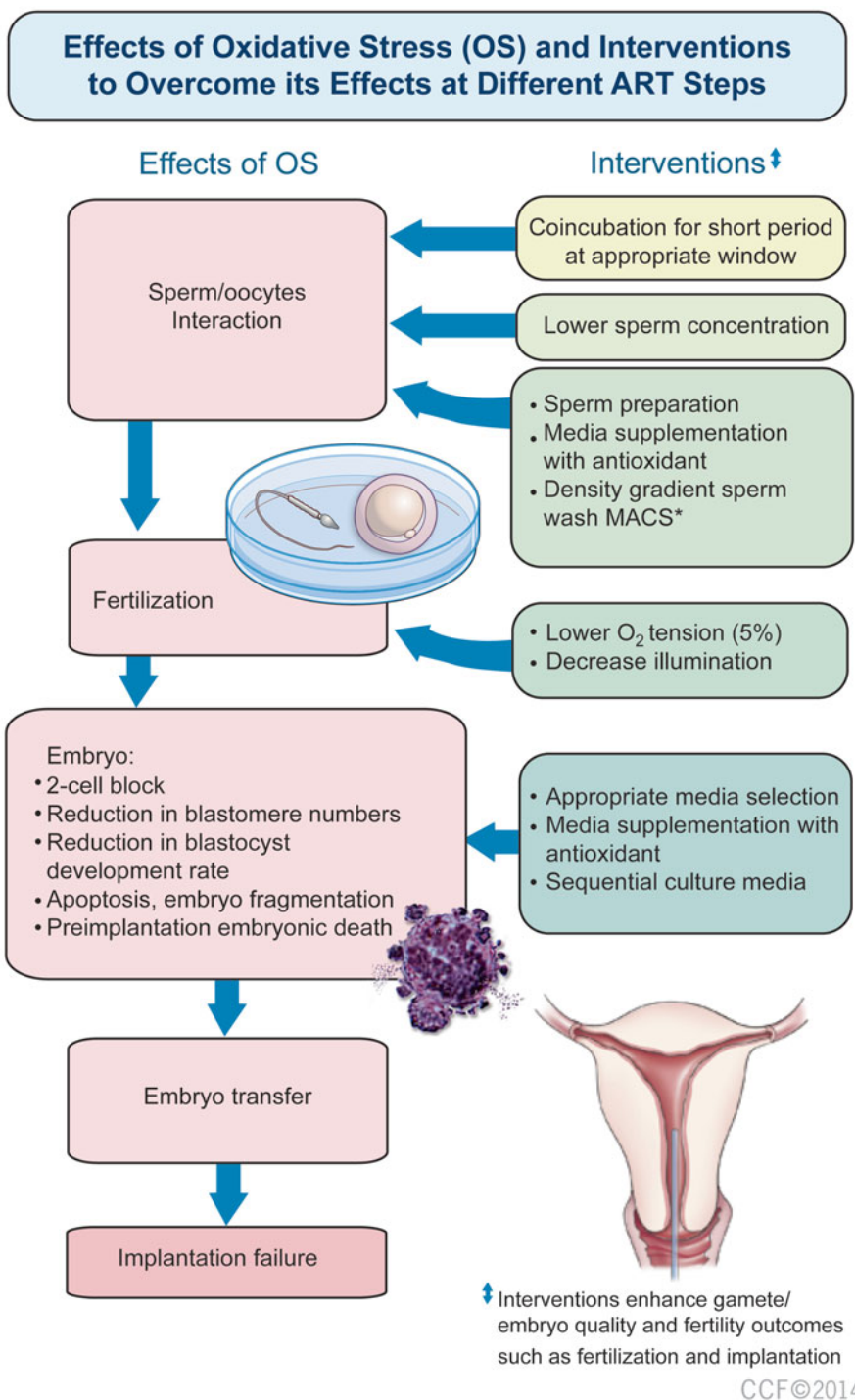


Fig. 2.1 Sources of reactive oxygen species in assisted reproductive technology
 *MACS magnetic cell separation

acids (PUFAs), making it very susceptible to elevated ROS levels during OS. The double bonds of membrane lipids are easily oxidized by ROS, causing a decrease in membrane fluidity. Besides the lipid membranes, OS also affects the structural proteins and nucleic acids of the sperm. As a result of this lipid peroxidation, the motility of the spermatozoon is compromised and could ultimately lead to immobilization of spermatozoa (Agarwal et al. 2003). OS leads to DNA fragmentation and damage in the nucleus of spermatozoa. Typically this include single- or double-strand breaks, DNA crosslinks, chromosomal rearrangements as well as deletions (Aitken and Krausz 2001). Damage to the sperm DNA by the increased levels of ROS is implicated in adverse outcomes such as higher incidence of abortion and childhood cancers (Baker and Aitken 2005). High ROS levels also cause a decrease in the mitochondrial membrane potential, which is an initiating event in the apoptosis cascade (Wang et al. 2003).

The consequences of high ROS levels are especially significant in infertile patients seeking assisted reproduction as sperm selected from these patients are very likely to have already been exposed to OS (Saleh et al. 2003). Increased OS in spermatozoa have a negative impact on ART outcome, with poor fertilization rates, embryo development and pregnancy rates (Baker and Aitken 2005).

The pro-oxidant activities of ROS are quenched by circulating antioxidants in order to prevent the detrimental effects of OS on the male gamete. Antioxidants function to maintain low, physiological concentrations of ROS in the cell and act as a natural defence mechanism against OS. Antioxidants can either be enzymatic (e.g. superoxide dismutase SOD, catalase, glutathione peroxidase GPx and glutathione reductase GR) or non-enzymatic (e.g., vitamins C, E and A, glutathione, pyruvate, taurine and hypotaurine, albumin and transferrin) (Sharma et al. 2004; Sikka 2004).

The mid-piece of the human sperm contains mainly enzymatic antioxidants (SOD, GPx and GR) and the plasma membrane contains a few non-enzymatic antioxidants (vitamins E and A, transferrin); while the seminal plasma contains both enzymatic and non-enzymatic antioxidants (Agarwal and Prabakaran 2005). These antioxidants contribute towards the total antioxidant capacity of the gamete. Under normal conditions, seminal plasma has adequate antioxidant mechanisms to minimize ROS action (Ford et al. 1997). However, during preparation of the sperm for the assisted reproductive technique, the sperm is separated from the seminal plasma, which decreases the spermatozoa's natural defence mechanisms.

With time, the limited antioxidant capacity of the spermatozoa declines as the aging sperm has reduced GPx and SOD activity (Sikka 2004). While ROS is present in sperm from both fertile and infertile men, more infertile men tend to have excessive levels of ROS levels compared to fertile men. Elevated ROS production compromises the structural integrity and functional capacity of the sperm, causing lipid peroxidation, DNA fragmentation and apoptosis. Elevated RNS levels can also cause lipid peroxidation, DNA damage, inhibition of steroidogenesis, increased caspase activity ultimately leading to apoptosis. Furthermore, RNS negatively impacts sperm parameters by reducing motility and viability, causing abnormal morphology, decreasing capacitation and the acrosome reaction, as well as sperm-oocyte fusion (Doshi et al. 2012).

In summary, spermatozoa have increased susceptibility to OS, as there is a lack of cytoplasm in the mature sperm compared to somatic cells, and therefore its poorer antioxidant capacity tends to render the spermatozoa at greater vulnerability to OS.

2.1.2 In Vivo Generation of ROS in Females

The role of ROS in female infertility has been of significant interest and research over the last decade. The presence of ROS in the fluids and organs involved in female reproductive processes has been well documented. In the female, the sources that generate ROS *in vivo* are mainly the follicular fluid, fallopian tube and uterine environment (Pasqualotto et al. 2004; Bedaiwy et al. 2004; Guerin et al. 2001). At these locations, ROS appear to have a physiological role in oocyte maturation, ovarian steroidogenesis and ovulation, implantation and formation of fluid-filled cavity, blastocyst, luteolysis and luteal maintenance in pregnancy. Moreover, a limited amount of lipid peroxidation relevant to ROS in follicular fluid was found obligatory for establishing pregnancy in human IVF cycles (Agarwal et al. 2005a).

Each month, a cohort of oocytes develops and matures in the ovary. However, meiosis I will resume only in the dominant oocyte. This process could be threatened by an increase in ROS. Conversely, high levels of ROS could be inhibited by antioxidants. This implies an intricate association between ROS and antioxidants in the ovary (Behrman et al. 2001; Agarwal et al. 2012). Inside the ovary, the follicular fluid environment enclosing the oocyte plays an important role in the fertilization process. Once fertilized, follicular fluid (along with the fallopian tube and endometrium), plays an important role in subsequent embryo development. During follicular maturation, oocytes are well-sheltered against lethal injury due to OS by antioxidants such as catalase, superoxide dismutase, glutathione transferase GST, paraoxonase PON, heat shock protein 27 and protein isomerase (Ambekar et al. 2013). Additionally, there are other antioxidants present such as vitamin E, carotene, ascorbate, cysteamine, taurine, hypotaurine, transferrin, thioredoxin, and dithiothreitol. The imbalance between these antioxidants and pro-oxidants in female infertility has been postulated to alter gene expression and impair adenosine triphosphate (ATP) generation (Agarwal et al. 2005a), the latter of which can affect ovulation, oocyte quality, fertilisation, embryo development and implantation (Agarwal et al. 2006a; Revelli et al. 2009; Das et al. 2006; Pasqualotto et al. 2009). An excess of free radicals also play a key role in gynaecological problems such as polycystic ovarian syndrome (PCOS), endometriosis and tubal factor infertility (Pasqualotto et al. 2009). Numerous studies discussed in this section have demonstrated the relationship between these disorders, OS and successful ART outcome.

The oocyte, granulosa and surrounding cells, such as the endothelial and thecal cells constitute the follicular fluid environment. It also contains phagocytic macrophages, parenchymal steroidogenic and endothelial cells that generate ovarian ROS (Halliwell and Gutteridge 1988; Agarwal et al. 2005a). The composition of follicular

fluid is a key feature for predicting a successful ART outcome in females (Agarwal et al. 2005a). While large amounts of ROS in the follicular fluid pose a serious threat to the success of assisted reproduction, a limited amount of ROS was found to be obligatory for establishing pregnancy in human IVF cycles (Oral et al. 2006). A summary of the experimental studies investigating the relationship between follicular fluid and ART outcome is portrayed in Table 2.1.

In assisted reproduction, the assessment of ROS and RNS in the follicular fluid of women undergoing IVF is usually obtained by aspiration of follicular fluid from each follicle during the oocyte retrieval process. Follicular fluid samples are then centrifuged and evaluated, most commonly by the chemiluminescence assay using luminol (other methods include nitroblue tetrazolium staining, thiobarbituric acid-reacting substances (TBARS), and flow cytometry) (Askoxylaki et al. 2013). In a study by (Jana et al. 2010), the upper cut-off limit for ROS levels, beyond which viable embryo formation is not favourable, was observed to be approximately 107 counted photons per second (cps)/40 μ L of follicular fluid. This cut-off level (previously determined in infertile women with tubal factor), was validated next in women with PCOS and endometriosis, in which they were shown to adversely affect oocyte and embryo development as well as pregnancy outcome. ROS values above this threshold value were shown to hinder oocyte quality, maturation, fertilization, and embryo formation. Conversely, significantly lowered levels of ROS (<100 cps) were linked with good embryo quality. Similar were also reported the lower and upper ROS values as 41 and 150 cps respectively (Das et al. 2006).

A prospective study by Attaran et al. (2000) reported that ROS levels were significantly lower in the follicular fluid of patients (with tubal disease, endometriosis and idiopathic infertility) who failed to establish pregnancy as compared to those who did (although the specific cut off values were not described). These results were in accordance with Pasqualotto's study, in which patients who became pregnant had higher lipid peroxidation levels and total antioxidant capacity (TAC) (Pasqualotto et al. 2004). Despite the fact that an imbalance of pro-oxidants and antioxidants can cause a disturbance in natural female reproductive tendencies, however these results indicate that physiological levels of ROS within the follicular fluid is essential for different phases of oocyte development and maturation. However, the exact function of ROS in the follicular fluid remains unknown (Pasqualotto et al. 2004; Oyawoye et al. 2003; Jana et al. 2010).

In a study of 63 women undergoing IVF, a total of 303 follicular aspirates were analysed using ferric reducing antioxidant power (FRAP) assay for baseline TAC (Oyawoye et al. 2003). The study revealed that TAC levels were significantly higher in follicular fluid where the oocyte was successfully fertilized. In a more recent study, the association between follicular fluid, ROS levels, TAC, ROS-TAC score and pregnancy following ICSI were evaluated in 138 women (Bedaiwy et al. 2012). Results of this study illustrated that pregnancy cycles were associated with significantly lower ROS and higher TAC. Interestingly, their study found that TAC levels were higher in women with endometriosis. Their ROS-TAC scores were also higher and were associated with a greater probability of having normal oocytes. In these women, elevated TAC levels, higher ROS-TAC scores and lower ROS levels in their follicular fluid were allied with a successful pregnancy after ICSI (Bedaiwy et al. 2012).

Table 2.1 Clinical parameters of studies examining reactive oxygen species in follicular fluid in infertile women

Study	Sample collected	Patient population/characteristics	Factors measured in the sample collected	Outcomes measured
Attaran et al. (2000)	FF	Patients undergoing ovarian stimulation for ART: women with tubal disease, male factor, endometriosis, idiopathic infertility, ovulatory dysfunction, pelvic adhesions	OS markers: 1. ROS 2. TAC	Age, number of oocytes recovered, percentage of oocytes fertilized, achievement of pregnancy
Barriouneuo et al. (2000)	FF	Patients undergoing IVF	1. Nitric oxide metabolites nitrate/nitrite (NO_3/NO_2) 2. IL-1 β levels	Oocyte maturation, fertilization rate, embryo cleavage rate
Oyawoye et al. (2003)	FF	Patients undergoing IVF-ET treatment	1. TAC	Oocyte retrieval, fertilization rate, embryo viability
Pasqualotto et al. (2004)	FF	Patients undergoing IVF: male factor infertility, tubo-peritoneal factors, idiopathic infertility, ovulatory factors	OS markers: 1. LPO 2. TAC	Oocyte maturity, fertilization rate, cleavage rate embryo quality, and pregnancy rate
Das et al. (2006)	FF	Patients undergoing IVF-ET treatment by controlled ovarian stimulation (long protocol): tubal factor infertility	OS markers: 1. ROS 2. LPO	Oocyte quality and fertilization potential, embryo quality
Chattopadhyay et al. (2010)	FF	PCOS	OS markers: 1. ROS 2. LPO 3. TAC	Meiotic spindle formation, fertilization rate, number of good quality embryos, clinical pregnancy rates
Jana et al. (2010)	FF	Patients undergoing IVF-ET by controlled ovarian stimulation: tubal factor infertility, endometriosis, PCOS	OS markers: 1. ROS 2. LPO 3. DNA fragmentation 4. TAC	Oocyte quality, fertilization rate, embryo quality

(continued)

Table 2.1 (continued)

Study	Sample collected	Patient population/characteristics	Factors measured in the sample collected	Outcomes measured
Borowiecka et al. (2012)	FF	Patients undergoing IVF	Lipid and protein peroxidation markers:	Pregnancy rates
			1. TBARS	
			2. Protein carbonyl	
Bedaawy et al. (2012)	FF	Patients who had ICSI: couples with male infertility, endometriosis, tubal disease, idiopathic infertility, ovulatory dysfunction, combined male and female infertility	3. Thiol groups	ROS-TAC score, number of follicles, number of oocytes retrieved, oocyte quality, pregnancy rate
			OS markers:	
			1. ROS	
Otsuki et al. (2012)	FF	Infertile patients	2. TAC	Oocyte viability
			1. Redox state	
			2. Albumin	
Rajani et al. (2012)	FF	Patients undergoing ICSI-ET: with endometriosis, PCOS, tubal infertility (control)	1. ROS levels	Presence of meiotic spindle, number of oocytes retrieved, mature MII oocytes, fertilization rate, good embryo formation rate, pregnancy rate
			OS markers:	
			1. ROS	
Singh et al. (2013)	FF	Patients undergoing IVF: endometriosis and tubal factor (controls)	2. NO (nitrite/nitrate)	Oocyte quality, embryo quality, pregnancy rate
			3. LPO	
			4. TAC	
			5. Antioxidant (enzymatic and non-enzymatic) levels	

Liu et al. (2013)	FF	Patients undergoing IVF-ET: endometriosis, tubal factor infertility	OS markers: 1. ROS 2. SOD 3. Vitamin E	Oocyte quality, fertilization rate
Seino et al. (2002)	GC	Patients undergoing IVF-ET treatment and ICSI: endometriosis, male factor, tubal factor, unknown	1. 8-OHdG expression in granulosa cells	Oocyte quality, fertilization rate, embryo quality (good embryo rate)
Liu and Li (2010)	GC	Patients undergoing IVF-ET: tubal factor infertility	1. MDA 2. SOD 3. Apoptosis 4. Good embryo rate	Number of retrieved oocytes, oocyte maturity, embryo quality, fertilization, cleavage
Karuputhula et al. (2013)	GC	Patients undergoing IVF-ET: endometriosis, PCOS, tubal factor infertility	OS markers: 1. ROS 2. MMP 3. DNA fragmentation 4. Apoptosis	GC characteristics: Fertilization rate, number of oocytes retrieved, oocyte quality, good quality embryo formation rate, pregnancy outcome
Polak et al. (2001b)	PF, plasma samples	Infertile women with minimal or mild endometriosis, unexplained infertility, PCOS, tubal infertility	1. 4-HNE levels 2. MDA levels (lipid peroxide levels)	Oxidative stress/free radicals activity
Bedaawy et al. (2004)	Culture media	Patients undergoing IVF/ICSI: male factor, anovulation, endometriosis, tubal factor, unexplained and other factors	1. ROS in culture media	Fertilization rate, cleavage rate, fragmentation, embryonic fragmentation levels, blastocyst formation rate

Sample types: 4-HNE 4-hydroxynonenal, 8-OHdG 8-hydroxy-2'-deoxyguanosine, ART assisted reproductive technology, CoQ₁₀ Coenzyme Q₁₀, DNA deoxyribonucleic acid, EDTA ethylenediaminetetraacetic acid, FF follicular fluid, GC granulosa cells, hCG human chorionic gonadotrophin, ICSI intracytoplasmic sperm injection, ICSI-ET intracytoplasmic sperm injection and embryo transfer, IL-1 β Interleukin-1 beta, IVF in vitro fertilization, IVF-ET in vitro fertilization and embryo transfer, LPO lipid peroxidation, MDA malondialdehyde, MII metaphase II, MMP mitochondrial membrane potential, NO nitric oxide, OS oxidative stress, PCOS polycystic ovarian syndrome, PF peritoneal fluid, ROS reactive oxygen species, SOD superoxide dismutase, TAC total antioxidant capacity, TBARS thiobarbituric acid reactive substances

In a much earlier study, Polak's group demonstrated the total antioxidant status of peritoneal fluid in infertile women (Polak et al. 2001a). The peritoneal fluid was obtained from infertile women distressing from minimal or mild endometriosis, unexplained infertility, tubal infertility and a few fertile women. The results of this study demonstrated a significantly lower antioxidant status in peritoneal fluid obtained from women with unexplained infertility.

Oocyte quality is another important determinant of IVF outcome. The follicular fluid aspirated during oocyte retrieval from women with endometriosis and tubal infertility undergoing IVF was measured using spectroscopy and HPLC (Singh et al. 2013). Increased levels of ROS and NO in endometriosis and tubal infertility were found to correlate with poor oocyte and embryo quality. Further, increased levels of ROS, nitric oxide, lipid peroxidation, cadmium and lead were seen in women who failed to become pregnant compared to those who did.

Borowiecka et al. (2012) evaluated the levels of lipid and protein peroxidation markers (TBARS, protein carbonyl, and thiol groups) in the follicular fluid of patients undergoing IVF. The OS markers were compared between the pregnancy positive and pregnancy negative patient groups. Results demonstrate that the mean follicular fluid TBARS level of non-pregnant women was significantly higher than that found in pregnant women. These findings suggested that elevated follicular fluid lipid and protein peroxidation levels may have a negative impact on IVF outcome and also supported the idea that increased levels of OS markers in follicular fluid may play an important role in fertility.

The association between malondialdehyde (MDA), superoxide dismutase (SOD) and incidence of apoptosis of granulosa cells in follicular fluid was examined in women with tubal factor infertility (Liu and Li 2010). The level of MDA and the activity of the SOD were measured by TBARS using a chemiluminescence methods, respectively. It was discovered that non-pregnant patients showed significantly higher MDA levels, higher incidence of apoptosis and lower SOD levels in the granulosa cells with lower good-embryo rate as compared to the pregnant patients. In general, OS induced apoptosis in granulosa cells and subsequently lowered oocyte quality, leading to poor outcome of IVF-ET. The levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in granulosa cells were detected in Seino's study (Seino et al. 2002). The outcome of their study suggested that OS in granulosa cells reduced fertilization rates and consequently reduced embryo quality. They also found that the quality of oocytes of endometriosis patients was reduced by the presence of 8-OHdG. The outcome of this study was in agreement with that of Karuputhula's group. A ~20- and 100-fold increase in granulosa cells ROS generation and MMP, was seen in PCOS patients as compared to tubal factor patients. Significant apoptosis was also evident in PCOS and endometriosis patients. IVF outcome parameters comprise fertilization rate, formation rate of good quality embryo, and pregnancy rates, and these were badly affected in endometriosis patients (Karuputhula et al. 2013).

Another study which was performed on patients with PCOS, examined the meiotic spindle in oocytes along with ROS levels in follicular fluid of women (Rajani et al. 2012). It was demonstrated that women with endometriosis had low ROS levels and good spindle imaging results suggesting a possible role of endometrial receptivity

accounting for lower pregnancy rates in these women. Poor oocyte quality, as reflected by higher mean ROS levels and low number of oocytes with spindle visualization, could be the factor impeding pregnancy in women with PCOS as compared to women with tubal block (Rajani et al. 2012). Another group examined that the effect of follicular fluid OS on the formation of meiotic spindle in oocytes and the outcome in women with PCOS (Chattopadhyay et al. 2010). Oocytes were examined to visualize for the meiotic spindle using a PolScope (a polarised light microscope). From the results of this study, it was observed that OS was responsible for the absence of the meiotic spindle which was significantly found in the groups with low fertilization rate, reduced number of good quality embryos and clinical pregnancy. Otsuki's group studied the influence of the redox state of follicular fluid on the viability of aspirated human oocytes (Otsuki et al. 2012). The redox state of the follicular fluid and serum, at the time of oocyte retrieval, was analysed by high performance liquid chromatography. Their results showed that the redox state of follicular fluid that contained degenerated oocytes had a significantly higher oxidised state compared with fluids that capitulated normal oocytes. A prospective case-control study was conducted in endometriosis patients who underwent IVF-embryo transfer IVF-ET. The expression and the role of OS markers in serum and follicular fluid of the patients were investigated. The results of the study reported significantly higher levels of ROS in both serum and follicular fluid. Mature oocyte and fertilization rates were also significantly lower than in the control group (Liu et al. 2013).

Nitric oxide concentrations in follicular fluid have been negatively associated with low pregnancy rates. It was found that serum nitric oxide concentrations in patients with tubal factor or peritoneal factor infertility negatively correlated. They reported that higher concentrations of nitric oxide are associated with implantation failure, which then result in lower pregnancy rates (Bedaiwy et al. 2004).

In summary, the findings of the studies discussed in this section highlight the importance for the detection of ROS in the female reproductive system for a variety of reasons and indicate how ROS levels can also be used to determine the link between gynaecological diseases (PCOS, endometriosis, tubal factor) and OS.

2.2 In Vitro Generation of ROS in ART

2.2.1 Cryopreservation

Cryopreservation is a process whereby cells and whole tissues are conserved by cooling to sub-zero temperatures (-196°C) (Di Santo et al. 2012). Recent advances in assisted reproduction and embryology have made cryopreservation an appropriate method for long-term storage of human reproductive cells, embryos and gonadal tissues to preserve and protect fecundity in cases of infertility, malignancy (Woods et al. 2004) and in some non-malignant treatments (such as diabetes and autoimmune disorders that may lead to testicular injury) (Anger et al. 2003). Nonetheless, despite highly optimised protocols appear to improve cell viability, the extreme

stress of freezing and thawing treatments can modify the structure and integrity of the sperm plasma membrane (mainly composed of phospholipids and cholesterol) (Giraud et al. 2000; Di Santo et al. 2012).

Various reports studied the relationship between cryopreservation and antioxidant defence system. The occurrence of DNA fragmentation in testicular sperm of men with obstructive azoospermia, such as in vasectomised males, those with blocked or missing ducts, and those with an absence of the vas deferens was investigated (Dalzell et al. 2004). The study showed a significant increase in DNA fragmentation at 24 h after incubation of fresh testicular sperm from men with obstructive azoospermia. Frozen-thawed sperm DNA was found to be significantly more damaged than fresh testicular sperm DNA. Even after 4 and 24 h post-thaw incubation, sperm DNA continued to become more damaged compared to fresh sperm DNA. These findings were further confirmed by Thomson's study, which reported increased levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) (a biomarker of oxidative stress), indicating an increase in sperm DNA fragmentation due to cryopreservation of human semen (Thomson et al. 2009). Furthermore the effect of cryopreservation on motility and viability was evaluated on spermatozoa of men struggling with infertility. The results in these men showed a significant decrease in sperm motility and viability post-cryostorage. In addition, cryopreservation/thaw significantly increased sperm DNA fragmentation and DNA oxidative damage (Zribi et al. 2010).

In human oocytes, cryopreservation frequently leads to developmental arrest during early cleavage stages and display aberrant patterns of cytokinesis (cytoplasmic division of a cell at the end of mitosis or meiosis, bringing about the separation into two daughter cells) (Van Blerkom and Davis 1994). Various studies (Gualtieri et al. 2009; Jones et al. 2004) revealed significant reduction of mitochondrial potential in slow cooled human oocytes resulted from changes in redox status of the cell due to cryopreservation. However, a study by Chen et al. (2012) evaluated the impact of vitrification on mitochondrial membrane potential (MMP) in human metaphase II (MII) oocytes, and the changes of mitochondrial membrane potential in thawed MII oocytes. It was observed that in the vitrification/thawing process, the mitochondrial membrane potential of MII oocytes could have temporary dynamic changes within a couple of hours post-thaw but would recover fully after 4 h of culture. In another study, the effect of different vitrification protocols on ROS were evaluated in human ovarian tissues, which were exposed to different vitrification solutions (Rahimi et al. 2003). The intracellular redox state levels were measured using the fluorescent dye dichlorodihydrofluorescein diacetate. After vitrification and warming, apoptotic cells imaging was monitored by anti-caspase-3 immunolabelling. The results showed that a slower cooling of tissue resulted in significantly elevated ROS levels and apoptosis after warming (Rahimi et al. 2003).

Based on the outcome of multiple studies, there appears to be a clear picture where cryopreservation is responsible for OS during ART. The higher incidence of studies regarding the male gamete may likely confirm that spermatozoa are more vulnerable to redox alterations than oocytes during cryopreservation. This difference in sensitivity to OS is due to the higher susceptibility of spermatozoa to lipid peroxidation and to the limited amount of ROS scavengers available, as the antioxidant defences are localised mainly in the seminal plasma (Bozhedomov et al. 2009).

2.2.2 Visible Light

Visible light, also known as visible spectrum, is the portion of the electromagnetic spectrum that is visible to the human eye, having a wavelength in the range of 400–700 nanometres (nm)—between the infrared light with longer wavelengths and the ultraviolet light with shorter wavelengths (Buser et al. 1992) (Fig. 2.1). In an ART laboratory, oocytes, zygotes and embryos which are kept in an artificial medium for assisted fertilization procedures such as IVF and ICSI, are exposed to daylight or artificial light for variable periods before the embryo is transferred to females.

Over the years, enormous consideration has been given to the role of visible light in the production of ROS in animals (Takenaka et al. 2007; Moshkdanian et al. 2011). However, the effects of light have not been studied as extensively in humans. Light is either measured as units of intensity (lux) or by the level of irradiation (W/m^2). According to an older study, rabbit oocytes when exposed to strong “cool white” fluorescent light of 3,250 lux for 20–30 min at 37 °C developed into normal near-term fetuses. However, this observation does not mean that visible light is undisruptive to the gametes and embryos of humans (Bedford and Dobrenis 1989).

In an IVF laboratory, light is generated by microscopes, fluorescent lighting and indirect sunlight. When gametes and embryos are exposed to light, it is absorbed by intracellular chromophores, which includes plasma membrane NADPH oxidase system consisting of flavoproteins and cytochrome b (Edwards and Silva 2001; Eichler et al. 2005). These chromophores are photosensitizers—which means they are able to absorb light and then transfer the energy to nearby oxygen molecules. Electrons in the shell get excited and shift to a shorter-lived singlet state. These excited electrons then change their spin and produce a longer-lived triplet state. As it returns to the ground state, it releases energy. This energy is then transferred to oxygen to generate ROS (singlet oxygen and free radicals) that mediate cellular toxicity (Girotti 2001). This mechanism of cellular toxicity was also described earlier (Hockberger et al. 1999). It was shown that violet-blue light (445–455 nm), irradiated from an inverted fluorescence microscope, initiated photoreduction of flavins. These activate flavin-containing oxidases in mitochondria and peroxisomes, resulting in production of H_2O_2 in human foreskin keratinocytes (HK cells).

The intensity and spectral composition of light reaching embryos during IVF was also investigated, and it was detected that microscopes, at a setting appropriate for embryo inspection, produced light at 2,500–5,000 lux. Meanwhile, light from other sources like natural room lighting and sunlight were more than tenfold lower, at 200–400 lux and had little effect on cultured embryos (Ottosen et al. 2007). It was suggested that microscope light exposure is a more hazardous source of light for oocyte and embryo viability compared to ambient light. The light intensity and wavelengths during embryo manipulation are important factors to maintain pre-implantation embryos viability *in vitro*.

The effect of visible light on human sperm motility and hyperactivation was demonstrated in a recent study (Shahar et al. 2011). The group evaluated the pathways mediating these effects. In their experiment, they irradiated human spermatozoa for 3 min with 40 mW/cm^2 visible light (400–800 nm with maximum energy at

600 nm) and suggested that ROS was produced during incubation, and that the production was enhanced after 1–3 min of light irradiation.

Over the years, attention has been focused towards the light microscopes used in IVF laboratories. Scientists recommend reducing the inspection light to as low as possible and maintaining the exposure time to as short as possible. In addition, microscope filters (optical filters) mounted on the microscope are used in a variety of microscopy applications for increasing contrast, obstructing ambient light, and eliminating harmful ultraviolet or infrared light (green filters block wavelengths below 500 nm). Moreover, to reduce ultraviolet (UV) lightwaves which are emitted from fluorescent lights, the fluorescent light could be substituted with yellow lights or by positioning UV light protectors over the fluorescent tubes (Ottosen et al. 2007).

2.2.3 ROS from Gamete Manipulation/ART Technique

In an *in vitro* environment, handling or manipulating the gametes and embryos during ART brings forth a risk of exposing these cells to higher than physiological levels of levels ROS (Lampiao 2012).

During ART procedures, the gametes are manipulated and prepared for various fertilization procedures such as IVF and ICSI. During conventional IVF, the selected samples of spermatozoa (concentration in millions) and oocytes are combined in the fertilisation medium in a petri dish and inspected for fertilisation after 16–20 h of incubation. During this period, ROS are generated from sources like oocytes, the cumulus cell mass and spermatozoa in culture medium. On the other hand, during the ICSI procedure, the cumulus cells are stripped from around the oocyte and a single sperm is injected directly into an oocyte before incubation in culture medium. Thus in an ICSI procedure, the oocyte and spermatozoa are the only potential sources of ROS. Furthermore, this procedure involves a shorter incubation time, which decreases the exposure of gametes to external environmental factors (Agarwal et al. 2006b).

However, since most ICSI cycles are done due to poor sperm characterises (which is not the case in an IVF procedure), therefore, it is commonplace to find higher rates of spermatozoa with greater DNA fragmentation levels in ICSI compared to IVF cycles (Benchai et al. 2003). In addition, it was hypothesised that during the IVF procedure, human zona pellucida has the capacity to select against sperm with aneuploidy, which was supported by a theory that IVF steps leads to a ‘natural selection of spermatozoa’ (Van Dyk et al. 2000). The spermatozoon that is chosen for fertilization will have normal morphology and be exceedingly motile with intact DNA (Benchai et al. 2003). Also with ICSI, in case of very poor sperm characteristics, the choice of spermatozoa to be injected is done using a very imperfect criteria and where it is tough to choose one normal motile sperm, so the risk of injecting spermatozoa with ROS and impaired DNA is high (Gandini et al. 2000; Irvine et al. 2000). It was also assumed that during the ICSI procedure, the selection of a motile and morphologically normal spermatozoa is operator-dependant. Therefore this spermatozoon has additional chances of having intact DNA, but at the same time a spermatozoa can be considered as ‘normal’ and still have damaged DNA (Host et al. 2000).

Elevated ROS levels were observed in the follicular fluid of women, whereby high ROS levels were prominent in those undergoing ICSI cycles, but not in IVF (Lee et al. 2012). It was demonstrated that the ICSI procedure might induce stress or shearing force on the plasma membrane of the oocyte, while the integrity of the plasma membrane has been considered as the origin of the deleterious effects of OS on fertilization, cleavage, or even implantation (Agarwal et al. 2003; Guerin et al. 2001). Consequently, ICSI manipulation might impair the developmental potential of oocytes after OS injury within the follicular fluid. The results indicate that ROS levels in follicular fluid may have a negative effect on the oocytes and its subsequent development, causing it to be affected by the insemination procedure (Agarwal et al. 2003). Furthermore, it has been recommended that the oocyte-handling time during ART procedures (denuding, ICSI, during media transfer) should be kept at a minimum and that incubation time of oocytes in the culture medium be managed well. This is to enhance oocyte quality and consequently, the success rate of the IVF cycle. For example, exposure time to pro-oxidant media (such as IVF medium) (even before insemination) should be kept at a minimum, as it would be helpful to conserve the quality of oocytes (Martin-Romero et al. 2008).

2.2.4 pH and Temperature

The *in vitro* environment is stressful for gametes and embryos as the temperature and pH tends to fluctuate. The measure of acidity or basicity of a solution is defined as pH, or hydrogen ion concentration. Intracellular pH is a vital aspect of cell homeostasis, and is controlled by membrane potential and osmolarity. The key intracellular processes are highly susceptible to pH, including protein synthesis, metabolism, mitochondrial function and cytoskeletal regulation (Will et al. 2011). In culture media, pH is an important variable that influences motility and sperm binding, oocyte maturation and embryo development, though confounding factors such as bicarbonate and CO₂ levels exist (Will et al. 2011; Bagger et al. 1987).

Animal studies have suggested that even a little increase in external pH (pHe) during minor manipulations outside the incubator can significantly obstruct sperm function (Marquez and Suarez 2007), alter organelle localization (Squirrell et al. 2001; Will et al. 2011), impact the development of mouse blastocyst and hatching, and alter gene expression profiles (Huntriss and Picton 2008). Hamster embryo studies show that even very slight deviations of internal pH (pHi) for short periods of time, from a set point of 7.21 (slightly raised 7.42 or lowered 6.87 pHi), can greatly impact the developing embryo. Philips and his group employed the pH-sensitive fluorescent dye BCECF to evaluate the pHi in human oocytes and demonstrated that the pHi values for oocytes and embryos changes during various developmental stages: such as at GV-intact MI 7.04, MII 7.03 and at Cleavage stage 6.98 pHi (Phillips et al. 2000).

In IVF, the most common buffer used in media is sodium bicarbonate (Will et al. 2011). In addition, the HEPES media buffer has regularly been seen to be a safe and effective buffer for the storing and handling of spermatozoa compared to other types

of buffers. CO₂ and pH have an inverse relationship; as CO₂ concentration decreases, pH increases. The media pH may be maintained provided that the levels of CO₂ remain constant in the incubators; nevertheless, this may be a challenge due to frequent openings/closings of incubator doors in order to observe the cell and while performing manipulations at room atmosphere (Will et al. 2011).

In case of procedures performed in room atmosphere, such as gamete collection, ICSI, cryopreservation, and embryo transfer, labs may choose to use handling media with reduced levels of bicarbonate and include another pH buffer to maintain pH outside the incubator. Sperm medium with added bicarbonate helps with the recovery of motile sperm (Mehta and Sigman 2014). Likewise, temperature is another factor that has an important role in pH and pH buffering. pH and pKa values decrease when the temperature increases (Ferguson et al. 1980). Temperature is measured and maintained by the incubator's thermostat and the incubator's heating system. In an IVF lab, all incubator temperatures are set at 37 °C in order to mimic *in vivo* conditions (Ferguson et al. 1980). Based on the physical properties of these membranes and membrane-associated processes, they may be more sensitive to temperature stress (Davidson and Schiestl 2001). Temperature stress may impair mitochondrial functions and induce oxidative damage, causing lipid peroxidation (Larkindale and Knight 2002).

2.2.5 Centrifugation

Sperm centrifugation is regularly done during semen processing and is a common step used for sperm preparation in ART (Agarwal et al. 2006c). The centrifugation process separates sperm cells from the seminal plasma, and motile sperm from non-motile or dead sperm and cell debris. Some of the commonly used sperm preparation techniques during ART that include the centrifugation step(s) are the double wash sperm swim-up technique, and the double density gradient separation. For example, simple washing (which removes only the seminal plasma) and swim up technique (which involves the further removal cellular debris and non-motile sperm) involve centrifugation at 300–400 × g for about 10 min, while discontinuous (density) gradient involves centrifugation twice, once at <500 × g for 20 min and then again at 300 × g for 5–10 min. These techniques aid in the selection of sperm with enhanced motility and are more viable. Besides these advantages, the removal of spermatozoa from seminal plasma is important in assisted reproduction, as this step eliminates the seminal plasma that contains leukocytes, a source of ROS. Furthermore, in severely oligospermic semen samples, centrifugation increases the chances for selecting better quality sperm, while in azoospermic semen samples, centrifugation increases the chances of identifying the rare sperm, if any (Sharma et al. 1997).

However, the centrifugation process itself generates ROS (Alvarez et al. 1993; Agarwal et al. 1994; Lampiao et al. 2010). The sperm membrane is mainly made up of polyunsaturated fatty acids (PUFAs) and is therefore particularly susceptible to

damage by ROS. High ROS concentrations could lead to lipid peroxidation of sperm plasma membrane, causing the loss of membrane fluidity, which impairs sperm motility. As the concentration of progressively motile sperm is indicative of success of ART outcome/pregnancy, poor sperm motility augurs an unfavourable ART outcome. In addition, the excessive presence of ROS causes DNA damage, which translates to poor embryo development.

Moreover, the g force, time (Shekarriz et al. 1995) and temperature employed during the process of centrifugation, influences the amount of ROS produced and thereby affects the quality of the sperm that has been processed. Centrifugation speeds greater than $500\times g$ and continuous centrifugation for longer than 5–7 min was found to compromise sperm quality (Sharma et al. 1997). In another study, both 10 and 30 min of sperm centrifugation was found to compromise sperm quality and viability, with the centrifugation time of 30 min being more detrimental to sperm quality compared to 10 min (Lampiao et al. 2010).

Longer centrifugation time increases the temperature during centrifugation, which affects the quality of sperm, despite the initial quality of the sample (Henkel and Schill 2003). The increased temperature during centrifugation also affects sperm motility, which could compromise ART outcome.

Therefore, sperm preparation techniques in ART should ideally exclude the centrifugation step altogether, or should at least avoid the use of prolonged periods of centrifugation (Lampiao et al. 2010). In protocols that require sperm centrifugation, the addition of antioxidants or other ROS scavengers prior to centrifugation may help quench the ROS produced due to the centrifugation process, and yield processed sperm with less damage (Lampiao et al. 2010). For example, pentoxifylline which is added to the sperm preparation to stimulate sperm motility, also quenches the ROS produced by spermatozoa (McKinney et al. 1996).

2.2.6 Culture Media

There are various types of media that may be used to culture human oocytes and pre-implantation embryos during the IVF and ICSI procedures. ART laboratories have a variety of commercially-available culture media to choose from, and these media may be composed of different types of ingredients, depending on the manufacturer. However, the type of media that is eventually used is crucial as culture media used has an important direct bearing on the quality of the embryo produced and subsequently, the success rate of the IVF procedure (Agarwal et al. 2006b). Some media may contain metallic ions such as iron (Fe^{2+}) and copper (Cu^{2+}). These ions can incorporate into the gametes or the developing embryo during culture, leading to the Fenton and Haber-Weiss reactions to occur, which generates ROS (Guerin et al. 2001). To prevent these reactions from taking place and reduce the formation of ROS, common metal chelators such as transferrin and ethylenediamine tetra-acetic acid (EDTA) can be added to the media (Nasr-Esfahani and Johnson 1992; Orsi and Leese 2001).

However, adding supplements to the media could end up increasing the oxidant load. For example, media additives such as serum albumin which contains elevated levels of amine oxidase leads to a higher generation of hydrogen peroxide, a form of ROS (Shannon 1978; du Plessis et al. 2008). The rate with which the culture media generates ROS varies according to its composition (Jana et al. 2010). OS within the culture media could partially deplete the GSH content in the oocyte, which could disrupt oocyte fertilization and viability (Martin-Romero et al. 2008).

Supplementation of culture media with antioxidants such as ascorbic acid, alpha-tocopherol, taurine, hypotaurine, isoflavones reduced the risk of OS and its subsequent adverse effects on the gamete (Sikka 2004; Alvarez and Storey 1983). Lipid peroxidation due to the presence of ROS can be deterred using vitamin E supplementation (Jain et al. 2000). In mouse embryos, the addition of thioredoxin to the media reduced apoptosis rates and enhanced hatching rates, while in porcine embryos, supplementation of media with glutathione and thioredoxin reduced the redox status (Ozawa et al. 2006).

In the *in vitro* fertilization procedure, sperm from the male partner interacts with the oocyte from the female partner in culture media, leading to fertilization. In ICSI, sperm prepared in culture media is injected directly into the cytoplasm of the oocyte. As the sperm is injected into the oocyte, there is an additional risk of transferring some of the ROS that is present in the culture media along with the sperm into the oocyte—which would have further detrimental effects on the oocyte's DNA material (Shen et al. 2003).

The consequences of ROS on gametes and early embryos have been experimentally-investigated, and multiple authors have reported increased levels of intracellular ROS during the various stages of embryo development (Hu et al. 2001; Bedaiwy et al. 2004).

Bedaiwy and his group examined the association of ROS levels in the culture media on the first day (day 1 ROS) after ICSI (Bedaiwy et al. 2004). During this experiment, fertilization as well as early cultures were done in human tubal fluid supplemented with 5 % serum substitute and levels of ROS were monitored by a chemiluminescence method using a luminol probe. The results illustrated that high ROS levels in culture media on day 1 caused low blastocyst rate, low fertilization rate, low cleavage rate, and high embryonic fragmentation in ICSI cases but not in those of conventional IVF. However, lower pregnancy rates were observed in both IVF and ICSI cycles with high day 1 ROS levels in the culture media. The same group followed up the experiment on examining ROS levels in day 3 culture media to the outcome of ICSI (Bedaiwy et al. 2010). The results confirmed that increased levels of ROS production in day 3 embryo culture media have detrimental effects on the embryo growth parameters as well as clinical pregnancy rates.

From the studies discussed, we can conclude that in order to improve sperm quality, embryo development as well as clinical pregnancy rates, short co-incubation time for gametes should be clinically applied in an attempt to improve ART outcome. Furthermore, supplementing of the culture media with required antioxidants to help scavenge excessive production of ROS in culture media is beneficial.

In addition, the design of the culture media used should be such that the media takes into consideration the role of ROS generation and eliminate their contribution without changing the metabolic requirements of gametes and embryos.

2.2.7 Oxygen Concentration

Gametes/embryos are exposed to oxygen tension during procedures in assisted reproduction such as insemination, fertilization and embryo growth (Catt and Henman 2000). The development of the human pre-implantation embryo *in vitro* is influenced by the atmospheric oxygen partial pressure and dissolved oxygen concentration in the culture medium. In ART laboratories, cells are commonly cultured *in vitro* in an atmospheric oxygen concentration of 20 % (160 mmHg) or an incubator environment of ~20 % oxygen and 5 % carbon dioxide. However, under physiological conditions *in vivo* in the oviduct and uterus, embryos are exposed to much lower oxygen concentrations of 2–8 % or 19–70 mmHg (Calzi et al. 2012). At 37 °C, the oxygen concentration in the medium equilibrated with atmospheric oxygen was found to be 20-times higher than physiological intracellular oxygen concentration (Jones 1985). Thus, the use of dissolved oxygen levels in the culture media *in vitro* at ~5 %, which is adopted by ART laboratories nowadays, is more comparable to the oxygen concentration at the tissue level *in vivo*.

Oxygen plays an essential role in cell growth and differentiation, but in an IVF setting, the presence of high concentrations of oxygen during incubation activates various cellular oxidase enzymes. This in turn increases the generation of ROS—leading to a state of OS (Cohen et al. 1997). Thus, the gametes and media in the ART laboratory setting should be protected from exposure to high partial pressures of oxygen in order to minimize the production of ROS during IVF procedures. When compared to embryos cultured under atmospheric oxygen conditions, those cultured at 5 % oxygen yielded better developed embryos and higher pregnancy rates (even among the poor responders), during IVF and ICSI cycles (Kovacic and Vlaisavljevic 2008; Kovacic et al. 2010). Interestingly a meta-analysis of seven randomized controlled trials (RCTs) comparing the effects of oocyte/embryo culturing at low (~5 %) and atmospheric (~20 %) oxygen concentration on assisted reproduction outcomes such as fertilisation, implantation and ongoing pregnancy rates showed no significant difference between the two groups (Gomes-Sobrinho et al. 2011). Embryos transferred on days 2 or 3 had similar implantation rates regardless of the oxygen tension used (~5 % vs. ~20 %) during culture, but for embryos transferred on day 5 or 6 (blastocyst stage), the implantation rates of embryos cultured at ~5 % oxygen were significantly higher than embryos cultured at ~20 % oxygen. However, the ongoing pregnancy rates did not differ significantly despite the different oxygen tensions used (~5 % or ~20 %) or the day of transfer (days 2/3 or days 5/6) (Gomes-Sobrinho et al. 2011).

Results of a recent Cochrane systematic review (involving 7 studies, 2,422 participants) and meta-analysis (involving 4 studies, 1,382 participants) showed that embryos cultured in low (5 %) oxygen concentrations developed better and were therefore of improved quality. This resulted in higher ongoing and clinical pregnancies rates and improved live birth rates. Thus compared to embryo culture in atmospheric (~20 %) oxygen concentrations, the culture of preimplantation embryos under low (~5 %) oxygen concentrations improves IVF/ICSI success rates and results in the birth of healthier babies (Bontekoe et al. 2012).

To strengthen these positive results, larger, well-designed randomized controlled trials are required. Results from these type of studies would provide more conclusive evidence on the impact of low oxygen culture on IVF outcome. In general, incubation of spermatozoa is usually done at 37 °C in an atmosphere of 5 % CO₂ for at least 1–2 h before ICSI or conventional insemination. In addition, these types of preparation and incubation conditions could affect the DNA integrity of ejaculated human spermatozoa.

Strategies to Ameliorate Oxidative Stress During
Assisted Reproduction

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