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Toxic effects of oxygen on human embryo development

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The toxic effects of oxygen on the embryos of various animal species are reviewed. Methodologies for assessing embryonic damage are discussed and possible ways of preventing the damage are explored. Three methods of potentially minimizing oxidative damage to human embryos were tested using gametes, zygotes, and embryos from a clinical IVF programme: (i) decreasing the oxygen tension in the gas phase used for culture during insemination, fertilization, and embryo growth; (ii) changing the formulation of culture media to include some components designed to protect against oxidative damage; and (iii) reducing the duration of insemination to minimize the effect of oxidative damage caused by spermatozoal metabolism. Fertilization, cleavage, embryo utilization, pregnancy, and embryo implantation rates were used to monitor these changes. Although all three methods gave an increase in success rates, there was still a dramatic decrease in success with patient age. It is suggested that, although the system of handling and culturing embryos can be optimized with respect to embryonic mitochondrial function, there are inherent age-related defects in oocytes and embryos that are still more fundamental than the environmental conditions of the embryo.

Key words: embryo/embryo culture/mitochondria/oxygen toxicity/reactive oxygen species

Introduction

The role of oxygen in embryonic metabolism and development is crucial and represents a balance between useful and harmful effect. Oxygen is consumed in oxidative phosphorylation and free radicals are generated from 'leakage' of high-energy electrons as they proceed down the electron transport chain. The free radicals that result are extremely reactive reducing agents and are dangerous to cellular biochemistry, including genomic integrity. This article reviews the gross effects of oxygen toxicity on human embryos *in vitro* and considers the ways in which optimizing environmental (culture) conditions might be expected to benefit mitochondrial function and embryo development.

The investigation of the effects of oxygen toxicity and methods to reduce this toxicity are confounded by various factors. The culture medium used for incubation can have a substantial effect in itself, making comparisons between laboratories difficult. The end-points measured can also be confounding. The growth of zygotes to blastocysts, used by

many researchers, might not detect effects on fertilization and, more importantly, on embryonic competency. Competency of embryos (i.e. their ability to implant and to develop into normal fetuses) is the end-point of choice, but is time-consuming to assess and requires an elaborate study design. Assessment of these end-points can be difficult enough with animal studies, but because of the inaccessibility of the material and obvious ethical constraints on experimental conditions, it becomes even more difficult to perform these studies with human gametes and embryos.

Several methods of potentially limiting the damage caused by oxygen have been assessed using clinical material processed from 1997 to 1999 at Sydney IVF and the results are presented with this review.

Oxygen concentration in the embryo's environment

Oxygen reaches the embryo by passive diffusion, the rate of which is controlled by various physicochemical factors. The most important of these are the oxygen tension in the gas phase, the solubility of oxygen in the medium, the boundary layer around the embryo, and the rate of diffusion through the cytoplasm. Each of these presents a potential barrier and offers the opportunity of being limiting. At present, it is not known which factor is the most limiting, but extrapolation from micro-organisms would suggest that the boundary layer around the embryo is crucial. Some calculations have suggested that diffusion of oxygen through a layer of oil is not rate limiting (Baltz and Biggers, 1991), but this has not been proven experimentally (McKiernan and Bavister, 1990).

The concentration gradient of oxygen is regulated by oxygen consumption during oxidative phosphorylation. This in turn is driven by availability of substrates and the integrity of the inner mitochondrial membrane-bound enzyme complexes. Embryos are pleiotrophic and will utilize any number of substrates, if these are available. Whilst this might be of benefit to the embryo, it can also lead to inappropriate metabolism (such as the Crabtree effect, whereby stimulation of glycolysis inhibits mitochondrial respiratory function) (Bavister, 1995), with negative consequences to the embryo's subsequent competency.

The integrity of the mitochondrial membrane complexes is crucial to oxidative phosphorylation because they channel the high-energy electrons produced. Failure to protect the complexes from the products has drastic results: the energetic electrons are inappropriately transferred to other molecules and free radicals are generated (Trounce, 2000). These free radicals then damage the complexes and the mitochondrial (mt) DNA, causing a catastrophic cascade (Shigenaga *et al.*, 1994). The generation of reactive oxygen species would be expected to occur more abundantly as more oxygen is available, because the stimulation of oxidative phosphorylation will give rise to a surfeit of high-energy electrons. Therefore, at least in theory, preventing excessive oxygen tension ought to be of special importance for ensuring good mitochondrial health in embryos.

Effects of free radicals derived from oxygen

Inadvertent transfer of electrons to molecular oxygen yields the superoxide radical (O_2^-), a highly reducing reactive oxygen species that is usually detoxified by superoxide dismutase to produce hydrogen peroxide, which in turn is degraded by catalases and peroxidases to non-toxic products. Peroxide that escapes detoxification can react with iron or copper ions to form the highly toxic hydroxyl radical (OH^o), which is short of an electron in comparison to hydroxide ion (OH^-), and is therefore

a powerful oxidizing agent, ready to oxidize target molecules by taking electrons. This oxidation load then has cellular consequences.

The main area of inappropriate oxidative and reductive damage will be at the site of production, the mitochondrion. Reactive oxygen species will react with DNA, protein and lipid (Miquel and Fleming, 1986). Mitochondrial DNA is particularly sensitive to damage, as it has no protective histone proteins and minimal repair systems. This can result in an increased mutation rate and hence damage to the RNA transcripts or to the DNA itself. Having multiple copies of the mitochondrial DNA ought to mitigate this to a certain extent in somatic cells, but the mitochondria of mammalian embryonic cells are thought to have a low mtDNA copy number (Pikó and Matsumoto, 1987; Pikó and Taylor, 1987).

Mutated DNA will synthesize abnormal proteins (if any at all) and, because some key oxidative phosphorylation enzymes are encoded by the mitochondrial DNA, the potential of electron loss (and hence free radical production) is substantial, causing further damage. Proteins themselves can also be the targets for free radicals, resulting in conformational changes and loss of function.

Lipids can be affected in a similar fashion. The oxidation of lipids in membranes by the hydroxyl-free radical changes physico–chemical properties, e.g. eutectic points. This can have important affects on membrane stability and permeability, thereby affecting cellular processes, including the integrity of the electron transport chain.

Leakage of oxygen free radicals from mitochondria can also affect compounds in the cytoplasm. The ratio of glutathione to glutathione disulphide can be disturbed, and this in turn can affect free calcium concentrations (Orrenius *et al.*, 1992), thereby causing various potentially catastrophic biochemical cascades.

All of the molecular events can contribute to the cellular expression of oxygen toxicity. Various cytoplasmic aberrations have been reported (Tarín, 1996), including aggregation of cytoskeleton components and endoplasmic reticulum condensates, resulting in the formation of inclusion bodies. These can have adverse effects on embryonic development. A lack of elasticity in the plasmalemmae of spermatozoa and oocytes may also affect fertilization, a process that is reliant on membrane fluidity. Microtubules and membrane fluidity are essential to embryo cleavage and disruption of these can give rise to arrested embryos, a fairly common event in clinical embryology. In addition, embryonic fragmentation has been associated with embryos that have undergone oxidative damage (Noda *et al.*, 1994). Fragmentation is a complex phenomenon and appears to involve mechanisms similar to apoptosis, which in itself involves free radicals (Endich *et al.*, 1996). Finally, and most importantly from a clinical embryological view, the competency of the embryo can be assumed to be affected by oxidative damage that is not immediately apparent in the earlier stages of development.

Endogenous oxygen free radical protection

Ovulated oocytes and early embryos are rich in protective enzymes, particularly superoxide dismutase (El Mouatassim *et al.*, 1999), and so to some extent are protected against the harmful effects of reactive oxygen species. Although there is no direct evidence for the existence of endogenous free radical 'sinks' in embryos, such mechanisms ought to protect them against the effects of inappropriate free radical generation from normal oxidative phosphorylation. It is these mechanisms that may be overwhelmed during inappropriate handling and culture of human embryos in the laboratory.

Glutathione and glutathione disulphide provide the potential for a free radical sink but,

as they play a central role in various cellular events such as sperm head decondensation, their concentrations within an embryo may be critical. The fatty acid composition of membranes is important for structural integrity and cellular dynamics, but there may also be less 'important' fatty acids that are incorporated for protective purposes.

Although protective mechanisms may exist, the best way to reduce oxidative damage no doubt is to prevent the generation of inappropriate or excessive oxygen free radicals in the first place. Before embarking on complicated experimental and indirect inferences about optimal oxygen tensions for embryos *in vitro*, it might be expedient to base laboratory practice on determinations of actual reproductive tract oxygen tension in physiological circumstances, and then mimic it in culture. Direct measurements of oxygen tension show that it can vary between 2–6% in the oviduct and uterus, depending on the species (Fischer and Bavister 1993). Care should be taken when inferring that this is the best oxygen tension for the embryo, as the estimation is gross and ignores any possible microenvironment that the embryos experience in these tracts, but nonetheless the difference between these empirically determined natural conditions and the 20% oxygen tension used in many human IVF culture sytems is very striking.

Embryo culture systems that reduce oxygen toxicity

There are several ways in which culture systems can be manipulated to reduce the oxygen tension that the embryo experiences. The following experiments were performed at Sydney IVF over several years and represent sequential attempts to improve the environment of the embryo. The first change was a reduction of oxygen in the gas phase, followed by the addition of antioxidants to the culture medium, and the most recent change has been to decrease sperm exposure time. Our present pregnancy rates, after having adopted all these changes, are then summarized.

Reduction of oxygen in the gas phase

Oxygen reduction can be achieved by using special gas mixes provided by a bottled gas supplier. It is relatively expensive, but most studies have used containers that are gassed inside normal incubators. Studies in mice have shown that reducing the oxygen tension can improve embryo development and quality (Whitten, 1971; Pabon, 1989; Umoaka *et al.*, 1992), although other studies have not shown such positive effects (e.g. Nasr-Esfahani *et al.*, 1992). There can be problems with experimental design and choice of both the medium to be used and the strain of mouse. Beneficial effects have, however, also been found in the hamster (McKiernan and Bavister, 1990), the rat (Kishi *et al.*, 1991), the rabbit (Li and Foote, 1993), the cow (Fukui *et al.*, 1991), the sheep (Thompson *et al.*, 1990) and the goat (Batt *et al.*, 1991). There is also direct empirical evidence that low oxygen can benefit human embryos in culture (Noda *et al.*, 1994).

A new type of incubator has been developed to allow gamete and embryo culture in a stable environment of choice (MINC incubator; Cook IVF, Eight Mile Plains, Queensland, Australia). The clinical data presented in Table I represent the effect of a change to the new incubators, i.e. a change from a 6% CO_2-in-air to a 6% CO_2/5% O_2/balance nitrogen gas phase. No differences were found in fertilization rate nor in the embryo utilization rate (defined as the percentage of embryos either transferred or cryopreserved from the total zygotes produced). Both the pregnancy rate and the implantation rate were increased, however, consistent with a substantial beneficial effect from the reduced oxygen tension.

Table I. Effects of gas phase during culture on clinical IVF outcomes in a controlled trial. There was a significant improvement in outcome with low oxygen tension

	5% CO_2-in-air	6% CO_2/5% O_2/89% N_2
Oocyte retrievals (*n*)	128	133
Fertilization rate of all recovered oocytes (%)	57	62
Utilization rate (see text) (%)	75	74
Clinical pregnancies per transfer (1–3 embryos) (%)	19*	32**
Implantation rate per embryo transferred (%)	10*	14**

Patients were alternated between the two treatments on a fortnightly basis. This was repeated four times. (A retrospective analysis of patient ages and aetiology showed no significant differences between treatments.) The mean number of embryos transferred was not significantly different between treatments. Quinn's human tubal fluid (HTF) medium and a sperm exposure time of 18 h were used. Source: Sydney IVF data.
Figures within rows with different superscripts differed significantly ($P < 0.02$, χ^2 test).

Culture medium components

Various additions can be made to the culture medium either to reduce the production of free radicals or to scavenge them after production. Heavy metal ions have been shown to promote free radical production, and the addition of chelating molecules can be beneficial (Bavister, 1995). Chelators include EDTA, EGTA, vitamins E and C, amino acids and proteins. It is noteworthy that EDTA does have an activity that is not associated with its chelation properties, acting as an inhibitor of glycolysis (Gardner and Lane, 1996). More complex factors such as human serum can be added, acting non-specifically (in the case of albumin) or specifically (in the case of transferrin, which in the reduced form is a specific free radical inhibitor) (Nasr-Esfahani and Johnson 1992). Co-culture, i.e. the culturing of embryos on (usually) monolayers of foreign cells, has been used as a clinical expedient in several laboratories and can mask many of the harmful effects of an inappropriately chosen culture medium; there are many good reasons for regarding co-culture as a suboptimal solution to defective culture methods and these have been discussed elsewhere (for review see Bavister, 1995).

Enzymes, e.g. superoxide dismutase, catalase, and thioredoxin, can be added to the culture medium. Some of these have been shown to have a beneficial effect on embryonic development to the blastocyst stage (Noda *et al.*, 1991; Nonozaki *et al.*, 1991; Umoaka *et al.*, 1992), but because the site of free radical production is not accessible by these enzymes their protective properties against endogenous free radicals is likely to be limited.

Table II. Effects of adding antioxidants to the medium during culture on clinical IVF outcomes, in a controlled trial. There is a significant improvement in outcomes with antioxidant protection

	Standard medium (HTF)	Antioxidant medium (SIVF medium)
Oocyte retrievals (*n*)	143	138
Fertilization rate of all recovered oocytes (%)	56	61
Utilization rate (see text) (%)	62*	72**
Clinical pregnancies per transfer (1–3 embryos) (%)	23*	31**
Implantation rate per embryo transferred (%)	11*	13**

HTF = Quinn's human tubal fluid.
Patients were alternated between the two treatments (see notes to Table I) and the mean number of embryos transferred did not differ significantly between treatments. Low oxygen levels in the gas phase and a sperm exposure time of 18 h were used. Source: Sydney IVF data. Figures within rows with different superscripts differed significantly ($P < 0.02$, χ^2 test).

In a developing medium designed to be optimal for all stages of gamete and embryo culture, one of the additions made to the media suite developed at Sydney IVF was the inclusion of antioxidants. Table II shows that there are some beneficial results of changing

from a medium with no oxidation protection to one that has several levels of protection included.

Minimum free radical exposure during insemination

Perhaps one of the most obvious ways of reducing oxidative damage in IVF clinics is to reduce the exposure of gametes and embryos to environments that contain free radicals or that allow their generation. Reducing or eliminating exposure of gametes and embryos to air during handling can be beneficial (Noda *et al.*, 1994). The single greatest concentration of exogenous reactive oxygen species that gametes and embryos are subject to is likely to derive from the spermatozoa that are used to inseminate the oocytes *in vitro*. It is still customary in most IVF laboratories to use relatively long insemination times (e.g. 16–18 h). Such long exposure to spermatozoa can increase chances of oxidative damage (Aitken *et al.*, 1991), and therefore a number of laboratories have investigated reducing the exposure time. The results have been conflicting, partially due to difficulties with experimental design and the use of different media (Giannaroli *et al.*, 1996; Quinn *et al.*, 1998).

We studied 500 IVF cycles at Sydney IVF in which a long sperm exposure time (18 h) was alternated on a monthly basis with a short exposure time (1 h). The outcome measures used were fertilization, cleavage, embryo utilization (defined above), pregnancy, and implantation rates. To investigate a potential effect of oxidative damage on embryos that were cryopreserved and subsequently thawed, the frozen embryo transfer cycles resulting from embryos derived in this study were also monitored. The results are given in Tables III and IV.

Short duration of insemination (limiting exposure of fertilized oocytes to free radical-producing spermatozoa) had no effect on fertilization, utilization, pregnancy, and implantation rates for fresh embryos transfers. There was, however, a small increase in cleavage rates, which were significantly higher ($P < 0.02$) for the oocytes exposed to spermatozoa for the shorter time. There was also no effect of duration of sperm exposure on embryonic

Table III. Comparison between the effects of long (18 h) and short (1 h) exposure of oocytes to spermatozoa *in vitro* on clinical IVF outcomes in a controlled trial. No improvement was observed with shorter exposure time

	Sperm exposure (18 h)	Sperm exposure (1 h)
Oocyte retrievals (*n*)	251	251
Fertilization rate of all recovered oocytes (%)	62	61
Cleavage rate (%)	95	97
Utilization rate (see text) (%)	78	78
Clinical pregnancies per transfer (1–3 embryos) (%)	33	35
Implantation rate per embryo transferred (%)	19	22

Patients were alternated between treatments (see notes to Table I) and the mean number of embryos transferred did not differ significantly between treatments. Low oxygen levels in the gas phase and M91 media were used. Source: Sydney IVF data.

Table IV. Comparison between the effects of long (18 h) and short (1 h) exposure of oocytes to spermatozoa *in vitro* on clinical IVF outcome among supplementary embryos cryostored and later transferred. There was a significant improvement in outcomes with short sperm exposure, believed to reflect shorter exposure of oocytes to reactive oxygen species

	Sperm exposure (18 h)	Sperm exposure (1 h)
Cryostored embryo transfers (1–3 embryos per transfer) (*n*)	100	100
Pregnancy rate (HCG detection) (%)	21	25
Clinical pregnancy rate (fetal heart detection) (%)	12*	21*
Implantation rate per embryo transferred (%)	7**	14**

*$P < 0.03$, one-tailed Fisher test; **$P < 0.02$, χ^2 test. See also notes to Table III.

Table V. Summary of present clinical pregnancy rates at different female ages using low-oxygen-tension, stage-specific medium (M91) with antioxidant protection, and short sperm-exposure times

Female age (years)	≤37	38–39	≥40
Embryo transfers (1–3 embryos per transfer) (*n*)	496	85	137
Clinical pregnancy rate (fetal heart detection) (%)	40	29	11
Implantation rate per embryo transferred (%)	25	17	7

Source: Sydney IVF data, 1997 and 1998.

survival after thawing and no effect on (biochemical) pregnancy rate using cryopreserved embryos. However, there was a clinically substantial and statistically significant reduced rate of early pregnancy loss and an increase in implantation rates with thawed embryos derived from short sperm exposure, reflecting the subtleties and eventual potential benefits of fine tuning the microenvironment of the oocyte.

These clinical outcomes are consistent with a subtle beneficial effect of short exposure to spermatozoa in IVF circumstances. It is a matter of conjecture as to whether the reason for the improvement is due to reduction of oxidative damage, but the evidence warrants more basic research to investigate underlying mechanisms.

Conclusions and future perspectives

The task of the laboratory embryologist is to be sure that each embryo reaches its genetic potential through as sound an in-vitro environment as possible. This is likely to mean safeguarding presumed mitochondrial function through, particularly, the prevention of further damage to mitochondrial genomes and metabolism caused by the excessive production of reactive oxygen species. As maternal age increases, there is a major decrease in both pregnancy and implantation rates, as shown in Table V. However, the significant potential for improving IVF results when embryos are at their developmental limits through the effects of maternal age is also revealed.

More research is needed on the mechanisms underlying oxidative damage and the best ways of avoiding this damage; significant further improvements can be expected, at least for embryos with a normal complement of chromosomes.

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