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Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings

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Oxidative stress is involved in the aetiology of defective embryo development. Reactive oxygen species (ROS) may originate from embryo metabolism and/or embryo surroundings. Embryo metabolism generates ROS via several enzymatic mechanisms. The relative contribution of each source seems different depending on the species, the stage of development, and the culture conditions. Several exogenous factors and culture conditions can enhance the production of ROS by embryos. ROS can alter most types of cellular molecules, and also induce development block and retardation. Multiple mechanisms of embryo protection against ROS exist, and these have complementary actions. External protection, present in follicular and tubal fluids, mainly comprises non-enzymatic antioxidants such as hypotaurine, taurine and ascorbic acid. Internal protection mainly comprises antioxidant enzymes: superoxide dismutase, glutathione peroxidase and γ.glutamylcysteine synthetase. Transcripts encoding for these enzymes are present in the oocyte, embryo and oviduct. It may be important that these transcripts are stored during oocyte maturation in order to allow the embryo to acquire the aptitude to develop. It is now common to add antioxidant compounds to culture media. Nevertheless, maintaining the pro-oxidant–antioxidant equilibrium in embryos through such supplementation is a complex problem. Further studies are necessary to limit oxidative stress during embryo culture.

Key words: antioxidant enzymes/embryo culture/non-enzymatic antioxidant defences/oocyte/reactive oxygen species

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Introduction

Even under basal conditions, aerobic metabolism entails the production of reactive oxygen species (ROS). ROS are formed during the intermediate steps of oxygen reduction: the superoxide anion radical ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH·), corresponding to the steps of reduction by one, two and three electrons, respectively. ROS, in combination with other atoms or molecules, can occur as alkoxyl (RO·) or peroxyl (ROO·) radicals, for example in lipids.

Furthermore, activation of molecular oxygen is catalysed by small molecules (e.g. xenobiotics) and iron complexes. Exogenous (and endogenous) low-molecular weight molecules can catalyse the electron transfer to oxygen (leading to the formation of $O_2^{-}\cdot$) from various reductases (e.g. NADPH reductase, NADH dehydrogenase, xanthine dehydrogenase, aldehyde oxidase). Such a redox cycle explains the toxic effects of several exogenous compounds (aromatic or nitro-heterocyclic derivatives, iron complexes). These compounds can also generate ROS via direct activation of NADPH oxidase.

H_2O_2, in conjunction with superoxide anion, can damage cells by allowing the most reactive metabolite, hydroxyl radicals, to form. This formation occurs, via superoxide dismutase (SOD) and the Haber–Weiss reaction (Figure 1), the latter reaction being greatly accelerated by catalytic amounts of metal salts (iron or copper) (Halliwell and Gutteridge, 1989).

In addition to $O_2^{-}\cdot$, H_2O_2 and OH·, the hydroperoxyl radical (HO_2), the conjugated acid of superoxide anion ($O_2^{-}\cdot + H^+ \longleftrightarrow HO_2$, pK_a = 4.7) plays a key role in the initiation of the lipid

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \text{ (Fenton reaction)}$$
$$Fe^{3+} + O_2^{-\bullet} \longrightarrow Fe^{2+} + O_2$$
$$\text{Net reaction} \quad H_2O_2 + O_2^{-\bullet} \longrightarrow O_2 + OH^- + OH^{\bullet}$$

Figure 1. The Haber–Weiss reaction.

peroxidation chain reaction in membrane lipids (Bielski *et al*., 1983; Alvarez and Storey, 1995). Nevertheless, the low value of pK_a demonstrates that at pH 7.2–7.4 (Dale *et al*., 1998), the HO_2 concentration is trivial.

This study will be limited to $O_2^{-}\cdot$, H_2O_2 and OH·, which are implicated in oxidative stress in gametes and embryos. Nitric oxide (NO), a free radical molecule that is produced by oocytes and embryos and seems to be a regulator of embryonic development and implantation, but is not implicated in oxidative damage, will not be investigated.

Production and deleterious effects of ROS

Production of ROS in oocytes and embryos

The metabolism of molecular oxygen is important in embryos (Magnusson *et al*., 1986; Houghton *et al.*, 1996; Thompson *et al*., 1996), the average oxygen consumption rate of bovine morulae and blastocysts being ~2 nl per embryo per hour (Overstrom *et al*., 1992). ROS production by embryos, which has been reported on many occasions, presents with three main characteristics: (i) the three main ROS, $O_2^{-}\cdot$, H_2O_2 and OH· are produced by embryos. For example, H_2O_2 and $O_2^{-}\cdot$ are produced by rabbit blastocysts, appearing between day 4 and day 5 post coitum and persisting for the remainder of the pre-implantation period (Manes and Lai, 1995); (ii) the level of this production is particularly important during in-vitro culture. An increased production of H_2O_2 has been measured in mouse embryos *in vitro* as compared with in-vivo-derived embryos (Goto *et al*., 1993); and (iii) the amount of ROS produced varies according to the stage of development. In mice, a rise in ROS production occurs on two occasions: the moment of fertilization; and the G_2/M phase of the second cell cycle (Nasr-Esfahani and Johnson, 1991).

ROS may originate either directly from gametes and embryos, or from their surroundings.

Endogenous sources of ROS

ROS can be produced by various metabolic pathways and enzymes, including mainly oxidative phosphorylation, NADPH oxidase and xanthine oxidase.

Oxidative phosphorylation (OXPHOS): A pre-implantation embryo generates adenosine triphosphate (ATP) via OXPHOS and glycolysis (Thompson *et al*., 2000). Inhibition of OXPHOS reduces ROS generation, and has a positive effect on in-vitro embryo development in both porcine (Machaty *et al*., 2000) and bovine (Thompson *et al*., 2000) species. In the mouse embryo, 70% of the oxygen is metabolized via OXPHOS at the blastocyst stage, and <30% at the 2- to 4-cell stages (Trimarchi *et al*., 2000).

Large amounts of glucose have deleterious effects on embryos *in vitro*. Embryos originating from diabetic rats show an increase of mRNA encoding for mitochondrial (Mn) -SOD, and a decrease in mRNA corresponding to catalase (Forsberg *et al*., 1996). These observations suggest that a mitochondrial origin of ROS, via OXPHOS, is quantitatively important when an embryo is in the presence of large amounts of glucose.

Nevertheless, mitochondrial inhibitors do not alter ROS production by mouse embryos (Nasr-Esfahani and Johnson, 1991). H_2O_2 production by rabbit blastocysts is cyanide-resistant, suggesting that it is not of mitochondrial origin (Manes and Lai, 1995). These observations suggest that in species such as mouse and rabbit, the embryo mitochondria are not the main source of ROS.

Oxidase activities are present in pre-implantation embryos (Filler and Lew, 1981), most notably NADPH oxidase and xanthine oxidase.

NADPH oxidase: Production of superoxide anion and H_2O_2 via NADPH oxidase has been described on a rabbit blastocyst surface (Manes and Lai, 1995). Also, incubation of 2-cell mouse embryos with an inhibitor of NADPH oxidase induces a concentration-dependent reduction in H_2O_2 production (Nasr-Esfahani and Johnson, 1991).

Xanthine oxidase: It has been suggested (Alexiou and Leese, 1992) that xanthine is the main end product of purine metabolism during pre-implantation development in the mouse. Inhibition of xanthine oxidase induces a decrease of ROS in embryos (Nasr-Esfahani and Johnson, 1991), while hypoxanthine causes a 2-cell block in mouse embryos (Loustradis *et al*., 1987), and purines inhibit the development of mouse embryo *in vitro* (Nureddin *et al*., 1990). Although no xanthine oxidase activity was observed in mouse blastocysts (Alexiou and Leese, 1994), hypoxanthine-mediated induction of 2-cell block can be overcome by adding compounds known to stimulate levels of cAMPi, suggesting that purine salvaging is involved in this artificial developmental block. The rise of ROS production in embryos at the time of 2-cell block may originate from degradation of purine nucleotides via xanthine oxidase. Consequently, $O_2^{-}\cdot$, and thereby H_2O_2, may be produced by the xanthine/xanthine oxidase system in mouse 2-cell embryos.

The catabolism of purines is accelerated before zygote genomic activation (ZGA): adenosine deaminase activity is maximal at that time. This period is marked by the activation of transcription by the embryonic genome and the inactivation or destruction of much of the pre-existing maternal mRNA. At this time, hypoxanthine phosphoribosyl transferase (HPRT) activity may be implicated in salvaging purine and limiting ROS generation. Inhibition of HPRT activity induces a conversion of hypoxanthine into xanthine via xanthine oxidase, and the production of superoxide anions. Glucose inhibits the HPRT activity in mouse embryo (Figure 2), and this inhibition induces developmental arrest in mouse (Downs and Dow, 1991). The generation of ROS in bovine embryos is partly caused by a high concentration of glucose in the medium (Iwata *et al*., 1998); purines inhibit mouse embryo development *in vitro* (Nureddin *et al*., 1990).

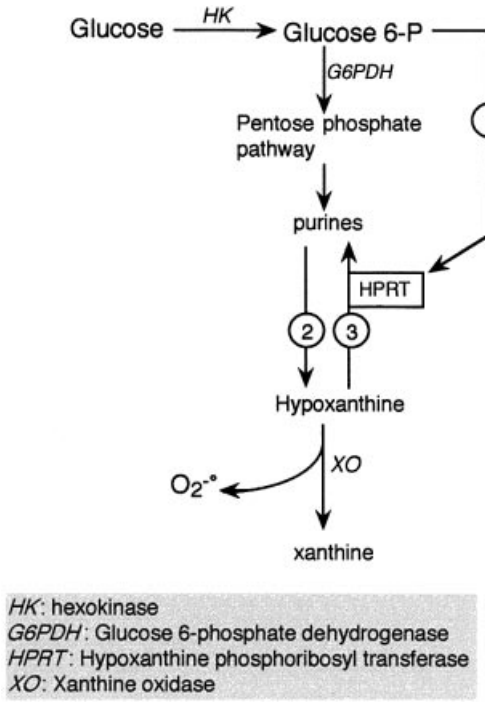

Figure 2. Interactions between glucose, purine metabolism and reactive oxygen species production. 1 Glucose inhibits the hypoxanthine phosphoribosyl transferase (HPRT) activity in the embryo and, consequently, induces an increase of hypoxanthine production. 2 Purine degradation. 3 Purine salvage.

Other oxidases: Other oxidases, such as glycolate oxidase, may be implicated in ROS production in embryos (Figure 3), since glycolate and glyoxylate have been shown to be synthesized by mouse embryos (Khatchadourian *et al.*, 1994).

OXPHOS, xanthine oxidase and NADPH oxidase seem to be the main sources of ROS in oocytes and embryos, though the relative contribution of each source seems different depending on the species and stage of development, and also possibly on culture conditions.

Exogenous factors inducing ROS generation

Several exogenous factors can enhance the production of ROS by embryos.

Oxygen concentration: Oxygen tension in the oviduct is approximately one-quarter to one-third of atmospheric tension (Mastroianni and Jones, 1965; Mass *et al*., 1976). In fact, at 37°C the oxygen concentration in the medium equilibrated with atmospheric oxygen is known to be 224 µmol/l (Jones, 1985), this being considerably higher than the physiological O_2 concentration within the cells, which is ~10 µmol/l. Oxidases are O_2-dependent, and their K_m values for O_2 are higher than the physiological O_2 concentration in the cells. Consequently, hyperoxic conditions may enhance this enzyme activity, resulting in an increase in the level of $O_2^{-}\cdot$ within cells.

ROS production in both mouse (Goto *et al*., 1993) and bovine (Nagao *et al*., 1994) embryos increases during culture at atmospheric oxygen concentration. In contrast, reducing the oxygen concentration enhances the embryo development of both mice (Goto *et al*., 1993) and ruminants, and allows the mouse 2-cell block to be overcome (Pabon *et al*., 1989). Furthermore, aerobic conditions may alter the defence mechanisms against oxidative stress: a depletion of the reduced glutathione (GSH) pool has been observed in spermatozoa incubated at atmospheric oxygen concentration (Bilodeau *et al*., 1999).

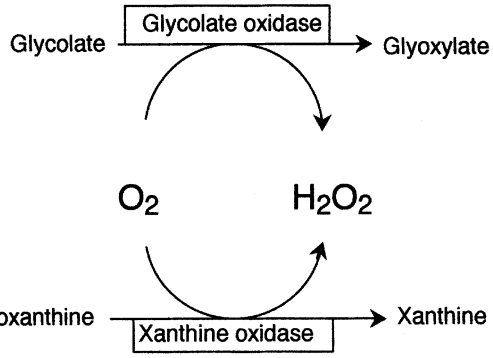

Figure 3. Two oxidases that may be involved in formation of H_2O_2 in the embryo.

Metallic cations: Traces of metallic cations such as Fe and Cu are inducers of ROS formation via the Fenton and Haber–Weiss reactions. Furthermore, iron can also act directly on lipids to magnify peroxidative damage once this has been initiated by free hydroxyl radicals. Traces of these cations are often present in water and/or chemical products used for culture media preparation, and may have deleterious effects on embryo development (Nasr-Esfahani *et al*., 1990b). By contrast, metallic ion chelators such as ethylenediamine tetra-acetic acid (EDTA) or transferrin overcome the developmental arrest *in vitro* (Nasr-Esfahani and Johnson, 1992; Nasr-Esfahani *et al*., 1992).

To our knowledge, no data are available about xenobiotics and oxidative stress in embryos. However, xenobiotic inducers of oxidative stress may be present in the air and/or water used for embryo culture.

Visible light: This is able to induce ROS production and cellular damage such as oxidation of bases and DNA strand break (Beehler *et al*., 1992). The excessive ROS production in embryos *in vitro* may also be explained by a transient exposure of embryos to visible light (Nakayama *et al*., 1994); an exposure of >5 min is sufficient to cause a major increase in H_2O_2 in mouse embryos (Goto *et al*., 1993).

Amine oxidase (EC 1.4.3.6): This enzyme catabolizes spermine and spermidine into hydrogen peroxide, aminoaldehydes and lower-order amines (Parchment *et al*., 1990). Serum, which may contain high levels of amine oxidase, is frequently added to culture media in animal embryo production. Furthermore, amine oxidases are released by dead spermatozoa (Shannon, 1978). This observation is a strong argument in favour of short insemination times (Quinn *et al*., 1998).

Spermatozoa: Another potential mechanism responsible for ROS-induced embryo damage in IVF could be related to the deleterious effect of ROS-producing spermatozoa during in-vitro insemination of oocytes. In-vitro incubation of oocytes with a critical number of ROS-producing spermatozoa that remain outside the oocyte could lead to oxidative damage of the oocytes or pronucleate embryos (Alvarez *et al*., 1996).

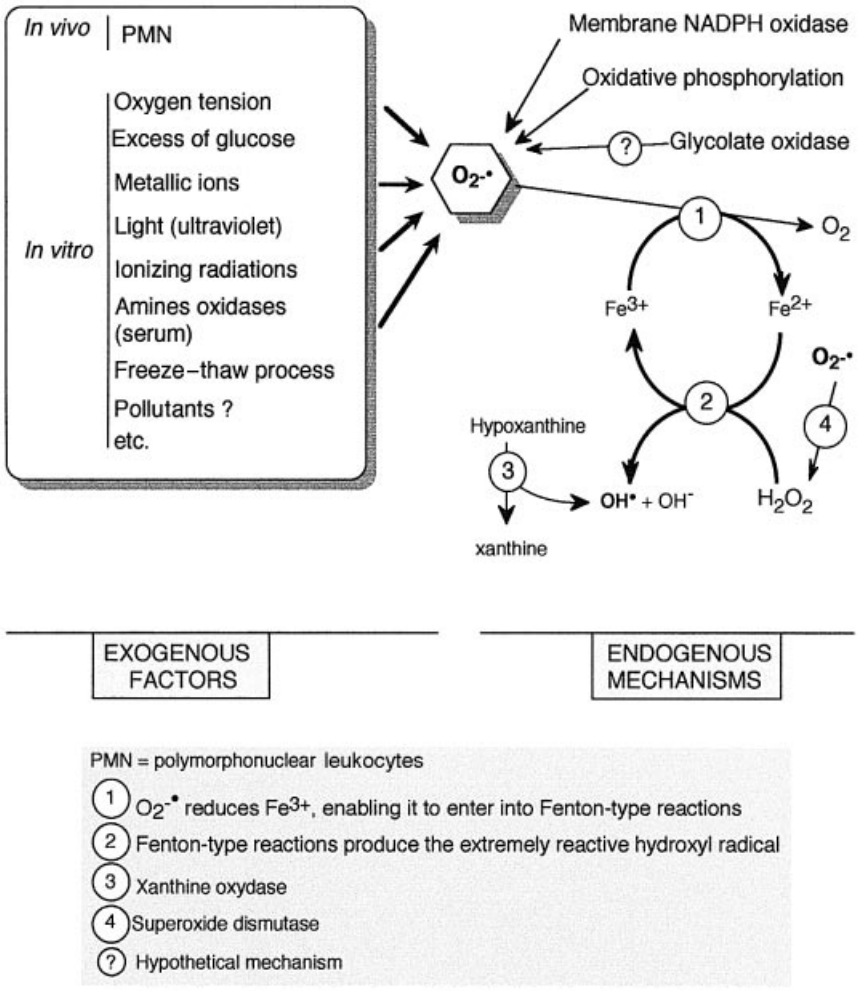

Figure 4. Schematic representation of the main origins of reactive oxygen species in oocytes and embryos.

Numerous endogenous and exogenous conditions can induce oxidative stress on embryos. For example, ROS play a key role in diabetes-induced embryo toxicity (Ornoy *et al*., 1996); another report has suggested the occurrence of age-associated oxidative stress in oocytes and embryos (Tarín, 1996).

The main sources of ROS in embryos are presented in Figure 4.

Deleterious effects of ROS on embryos

Oxidative stress seems responsible for numerous types of embryo damage. ROS such as $O_2^{-}\cdot$ are able to diffuse and pass through cell membranes and alter most types of cellular molecules such as lipids, proteins and nucleic acids. The consequences are multiple, and include mitochondrial alterations, embryo cell block, ATP depletion and apoptosis (programmed cell death).

Lipids

ROS induce lipid peroxidations with related effects on cell division, metabolite transport and mitochondrial dysfunction. The 2-cell block observed in mouse embryos is associated with a rise in lipid peroxides (Nasr-Esfahani *et al*, 1990a; Noda *et al*., 1991).

Proteins

ROS can induce protein sulphydryl oxidation and disulphide formation. With oxidative stress, the rates of disulphide bonds and mixed disulphide formations increase within the cell. As a consequence, inactivation of enzymes such as glyceraldehyde 3-phosphate dehydrogenase (G_3PDH) can occur (Halliwell and Gutteridge, 1989).

DNA

ROS induce nuclear DNA strand breaks (Munné and Estop, 1991). A four-fold increase in the nuclear DNA fragmentation rate was observed after spermatozoa were exposed to ROS (Lopes *et al*., 1998). Such nuclear DNA lesions are involved in embryo development arrest observed *in vitro.*

Mitochondrial alterations

Oxidative stress induces mitochondrial damage (Richter *et al*., 1985; Kowaltowski and Vercesi, 1999). Mitochondrial DNA (mtDNA) is especially susceptible to mutation because of its lack of histones which normally quench ROS. During oxidative stress,

mtDNA mutation are four-fold more frequent than nuclear DNA mutations. mtDNA encodes essential enzymes of OXPHOS machinery (Taanman, 1999). Defective embryo mtDNA may induce metabolic dysfunction and, consequently, disturb embryo development; arrest of embryo development observed *in vitro* is associated with dysfunction of mitochondria.

The consequences of these alterations are multiple, and include embryo development retardation and arrest, metabolic dysfunctions and possibly also apoptosis.

Embryo developmental blocks

ROS have been implicated in the impaired development of mammalian embryos *in vitro* (Johnson and Nasr-Esfahani, 1994). The 2-cell embryo block observed in mouse embryos is associated with a rise in ROS (Nasr-Esfahani *et al*, 1990a; Noda *et al*., 1991), the rise being observed only after culture *in vitro.* No such effect is observed in embryos collected *in vivo* (Noda *et al*., 1991). Deleterious effects of ROS during oocyte maturation may alter embryo development (Blondin *et al*., 1997).

ATP depletion

ATP depletion occurs in cells via inactivation of G_3PDH and/or inactivation of glycolytic and mitochondrial pathways (Hyslop *et al*., 1988). Oxidative stress induces consumption of reducing equivalents such as GSH. Glutathione reductase (GR) activity allows the GSH endogenous pool to be maintained. GR is NADPH-dependent and the main source of NADPH in the monophosphate shunt (pentose phosphate pathway). Consequently, oxidative stress, via competitive consumption of reducing equivalents, can interfere with important metabolic functions and divert glucose from other pathways by inducing the monophosphate shunt.

Apoptosis and fragmentation of embryos

Accumulation of superoxide radicals and a decline in SOD levels are involved in apoptotic cell death, whereas antioxidants including SOD can inhibit apoptosis. H_2O_2 is a mediator of apoptosis in blastocysts (Pierce *et al*., 1991). The appearance of cytoplasmic fragments in blastocysts seems to be related to apoptosis (Yang and Rajamahendran, 1999). A direct relationship was also observed between increased H_2O_2 concentration and apoptosis in human fragmented embryos (Yang *et al*., 1998). Fragmentation in mouse and human embryos occurs just before the time of in-vitro block; such fragmentation may be a mechanism to regulate the nucleocytoplasmic ratio in blastomeres, and/or a protective mechanism against damage induced by oxidative stress.

Freeze–thaw survival

In assisted reproduction technology (ART), the freeze–thaw process makes cells more sensitive to ROS. DNA instability is observed in mouse oocytes after cryopreservation. Furthermore, the freeze–thaw process reduces GSH concentrations by 78% and SOD activity by 50% in bovine spermatozoa (Bilodeau *et al*., 1999). The increase in lipid peroxidation observed after sperm cryopreservation is due to a loss of SOD activity (Alvarez and Storey, 1992), which strongly suggests that oxidative stress occurs during and/or after the cycle of freeze–thaw. This may partly explain the observed deleterious effect of cryopreservation on gamete/embryo viability. Modifications of membrane lipids related to ROS and the resulting spatial modifications of membrane structures may clearly lead to cryodamage.

Nevertheless, it should be borne in mind that ROS play a physiological role in gametes and embryos. They are implicated in the control of capacitation, acrosomal reaction (de Lamirande *et al*., 1998) and fertilization (Miesel *et al*., 1993) processes. Furthermore, ROS are probably implicated in regulating the speed of pre-implantation embryo development (Yamashita *et al*., 1997).

Embryos may also have different sensitivities to ROS at different developmental stages. For example, it has been observed (Morales *et al*., 1999) that 9- to 16-cell bovine embryos are more resistant to exogenous H_2O_2 than zygotes and blastocysts. These different sensitivities are due to variations in defence mechanism thresholds.

Defence mechanisms against ROS

Several defence mechanisms are present in both embryos and their surroundings. *In vivo*, oocytes and embryos seem to be protected against oxidative stress by oxygen scavengers present in follicular and oviductal fluids.

Oxidative damage may result from overproduction and/or decreased clearance of ROS by the scavenging mechanisms. Three strategies avoid oxidative stress by prevention against ROS formation, interception (by antioxidants) and repair.

Metal chelation is a major means of controlling lipid peroxidation and DNA fragmentation, and metal-binding proteins such as transferrin are of central importance in the control of potential radical-generating reactions. After albumin, transferrin is the most abundant protein present in tubal fluid.

Antioxidants have been defined as ‘any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate’ (Halliwell and Gutteridge, 1989). This definition includes compounds of a non-enzymatic as well as an enzymatic nature.

Non-enzymatic antioxidants

Numerous compounds have antioxidant functions, including vitamins A, C and E, and also pyruvate. Sulphur compounds are of particular interest, and include GSH, hypotaurine, taurine and cysteamine (CSH) (Figures 5 and 6).

GSH

GSH appears to be the main non-enzymatic defence system against ROS in embryos (Takahashi *et al*., 1993; Gardiner and Reed, 1994, 1995a; Gardiner *et al*., 1998). GSH plays a role in reducing the environment in oocytes and embryos, and is also the substrate of glutathione peroxidase (GPX), the main antioxidant enzyme. Oocytes contain unusually high concentrations of GSH, especially in pigs (Yoshida *et al*., 1993). The concentration of GSH in bovine embryos is highly correlated to early development and viability after freezing (Takahashi *et al*., 1993), while in hamsters the GSH concentration is higher in mature than in immature oocytes (Perreault *et al*., 1988). Synthesis of GSH during oocyte maturation has been reported in the oocytes of mouse, hamster, pig and cattle (Yoshida *et al*., 1993). This

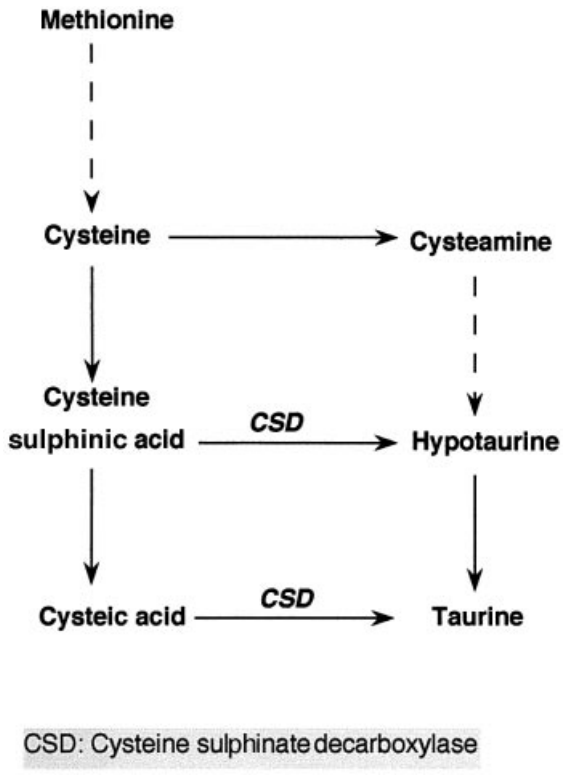

Figure 5. Biosynthetic pathways of cysteamine, hypotaurine and taurine.

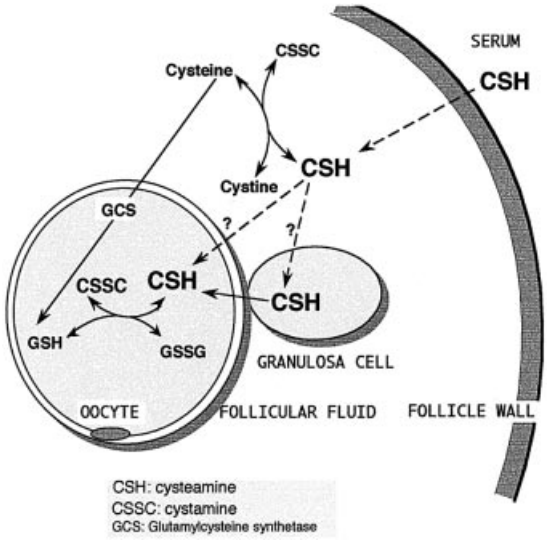

Figure 6. Metabolism of cysteamine in the oocyte and its surroundings: hypothetical pathways.

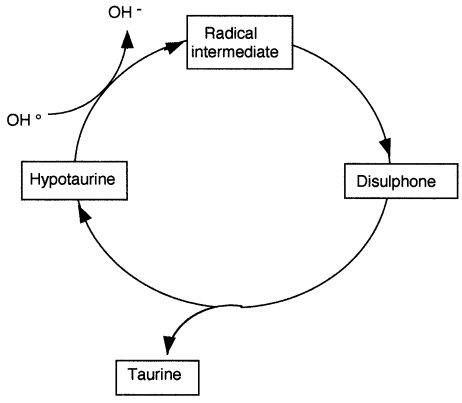

Figure 7. Hydroxyl radical trap in the proposed route of oxidation of hypotaurine to taurine (modified from Fellman *et al*., 1987).

increase in GSH content provides oocytes with large GSH stores available for decondensation of the sperm nucleus, and also protects the embryo against ROS until the blastocyst stage (de Matos *et al*., 1995). Cumulus cells were found to synthesize large amounts of GSH in the hamster (Zuelke and Perreault, 1994) and pig (Funahashi and Day, 1995), while porcine oocytes without cumulus cells utilize cysteine directly for GSH synthesis. DNA damage has been observed in embryos when GSH synthesis is inhibited; depletion of GSH causes an increase in H_2O_2 concentrations as well as DNA lesions in bovine embryos (Takahashi *et al*., 1993). In contrast, an improvement in bovine embryo production *in vitro* by GSH supplementation has been observed (Luvoni *et al*., 1996).

CSH

CSH protects cells against ionizing radiation, and is known as a scavenger of OH· (Zheng *et al*., 1988). Significant amounts of CSH have been detected in the follicular fluid of the cow, sow, goat and dog (Guyader-Joly *et al*., 1998). CSH may contribute to maintaining the redox status in oocytes and the high ratio of GSH/GSSG (reduced glutathione/oxidized glutathione) which is important for GPX function (Figure 6). CSH enhances the in-vitro development of porcine oocytes that have been matured and fertilized *in vitro* (Grupen *et al*., 1995). Addition of CSH and cysteine to the maturation medium also increases GSH synthesis in bovine oocytes during in-vitro maturation (de Matos *et al*., 1995). Cysteine may contribute to the maintenance of GSH and CSH endogenous pools in oocytes (Guyader-Joly *et al*., 1998). The metabolism of CSH in oocytes is presented in Figure 6.

When CSH was added to the maturation medium, a beneficial effect on subsequent embryo development was observed in bovine (de Matos *et al*., 1995) and porcine (Grupen *et al*., 1995) species. However, our results indicate that supplementing the maturation or culture medium with identical concentrations of CSH did not induce any improvement of bovine embryo development in our in-vitro culture system (Guyader-Joly *et al*., 1998).

CSH may be converted into hypotaurine via free radical scavenging (Harvey *et al*., 1995).

Taurine and hypotaurine

Large amounts of taurine and hypotaurine are present in gametes and in the embryo environment in all species examined (Guérin and Ménézo, 1995). Furthermore, these compounds are synthesized and secreted by oviduct epithelial cells (Guérin *et al*., 1995a). Hypotaurine can neutralize hydroxyl radicals (Figure 7) and prevent sperm lipid peroxidation (Alvarez and Storey, 1983), this antioxidative function being effective even at a low concentration (Aruoma *et al*., 1988). In this way, hypotaurine in genital fluids may have important antioxidant functions for gametes and embryos.

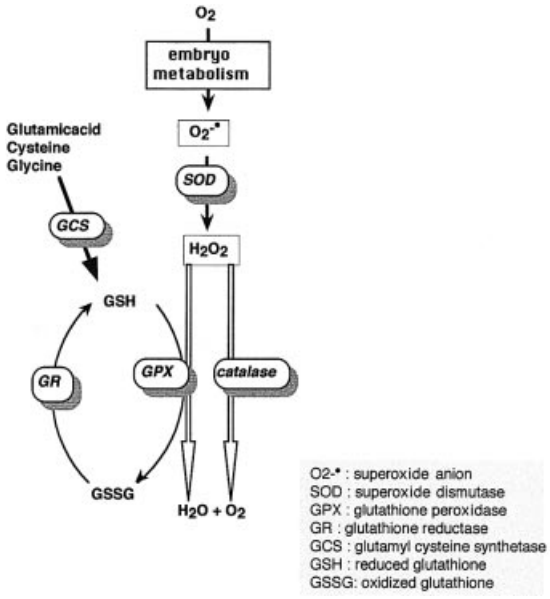

Figure 8. Schematic representation of enzymatic antioxidant defences in oocytes and embryos.

It is important to emphasize that the by-product of hypotaurine, after free radical scavenging, is taurine (Figure 7). Taurine has indirect antioxidant effects: it contributes to limiting the deleterious effects of ROS by neutralizing cytotoxic aldehydes, the end-products of the peroxidation cascade reaction (Ogasawara *et al*., 1993). Mammalian embryos are capable of taurine uptake (Van Winkle and Dickinson, 1995). The protective effect of taurine on cells is effective at a low concentration (0.3 µmol/l). CSH, hypotaurine and taurine might have a sequential and complementary action on gametes and embryos.

Adding hypotaurine to a culture medium has beneficial effects on embryo development in hamster (Barnett and Bavister, 1992), mouse (Dumoulin *et al*., 1992), pig (Reed *et al*., 1992), cow (Guyader-Joly *et al*., 1998) and human (Devreker and Hardy, 1997). For taurine, the results are contradictory: it has been observed that supplementing taurine may have beneficial effects on embryo development (Dumoulin *et al*., 1992; Li *et al*., 1993), though others have found this not to be the case (Devreker and Hardy, 1997).

Ascorbate

Ascorbate is a potent direct antioxidant: the stable modification of lipoproteins by dehydroascorbate increases resistance to metal ion-induced oxidation. Ascorbic acid protects against endogenous oxidative DNA damage (Fraga *et al*., 1991). Furthermore, ascorbate, at physiological concentrations, induces the release of hypotaurine and taurine by oviduct epithelial cells (Guérin *et al*., 1995b). The liquid present in the ampullar section of the oviduct at the time of fertilization is a mixture of tubal and follicular fluids (Hansen *et al*., 1991). It is possible that large amounts of ascorbate, present in follicular fluid (Paszkowski and Clarke, 1999), induce taurine and hypotaurine release in oviductal fluid at the time of ovulation. However, in the presence of transition metals, ascorbate shows a pro-oxidative activity (by converting ferric into ferrous ions, thereby catalysing the Fenton reaction).

Other vitamins

Vitamin E is a natural antioxidant, and semen quality appears to be correlated with vitamin E concentration in spermatozoa (Cerolini *et al*., 1997). Significant amounts of vitamins E and A are present in the ovary or follicular fluid (Schweigert and Zucker, 1988). Vitamin E inhibits NADPH oxidase-mediated generation of superoxide anion. These lipid-soluble antioxidants protect membranes against oxidative stress (Pascoe *et al*., 1987).

Pyruvate

Pyruvate plays a pivotal role in both primary energy metabolism and the redox potential. A possible function for pyruvate in protecting embryos against oxidative stress was suggested (O'Fallon and Wright, 1995). Pyruvate may be secreted by some cells to function as an extracellular antioxidant (O'Donnell-Tormey *et al*., 1987). Pyruvate prevents peroxide-induced injury of in-vitro-cultured bovine embryos (Morales *et al*., 1999) and, when associated with lactate by being added to the culture medium, prevents the effects of ROS on human spermatozoa (de Lamirande and Gagnon, 1992). Pyruvate can be decarboxylated in the presence of H_2O_2 to produce acetate, carbon dioxide and water.

Other antioxidant compounds

These are present in the embryo environment *in vivo*. Transferrin can neutralize iron ions, while albumin prevents lipid peroxidation in spermatozoa (Alvarez and Storey, 1983) by trapping numerous compounds such as lipid peroxides.

Interrelations between these different antioxidants are important. Depletion of vitamin E is an early event in cell injury with the subsequent loss of GSH (Pascoe *et al*., 1987), but vitamin E is rapidly replenished through depletion of vitamin C.

Enzymatic defence mechanisms

Several antioxidant enzymes protect oocytes and embryos against peroxidative damage by SOD, catalase or GPX (Li *et al*., 1993). Copper, Zinc Superoxide Dismutase (Cu,Zn-SOD, located in the cytosol) and Manganese-Superoxide Dismutase (Mn-SOD located in the mitochondria) allow superoxide radicals to be scavenged. Both SODs belong to a first enzymatic step that protects cells against toxic oxygen radicals. H_2O_2, the by-product of SOD action, is eliminated either by catalase or by GPX. The latter enzyme has direct and indirect antioxidant actions: lipid hydroperoxides and H_2O_2 are reduced via GPX since catalase is effective only against H_2O_2. This suggests that GPX has a pivotal role in cell antioxidant protection. On the basis of glutathione reductase (GR) and glutamyl cysteine synthetase (GCS), high GSH concentrations can be maintained in cells (Figure 8). After having induced a depletion of GSH in mouse embryos, it was observed (Gardiner and Reed, 1995b) that 2-cell-stage and blastocyst-stage embryos were able to recover their GSH concentrations within 45 min. These results suggest that there is a major protective role for GR in embryos.

Transcripts coding Cu,Zn-SOD are present in oocytes at all stages of maturation, especially in the human (El Mouatassim *et al*., 1999), suggesting that this enzyme plays a crucial role in

protecting embryos against oxygen toxicity *in vivo* as well as *in vitro.* Their high expression in the mouse blastocyst is further proof of this role (El Mouatassim *et al*., 1999).

GCS and Cu,Zn-SOD transcripts are present in human and mouse oocytes at germinal vesicle (GV) and metaphase II (MII) stages (El Mouatassim *et al*., 1999). GPX and Mn-SOD transcripts are detected in mouse and human MII oocytes. In human, they seem to be present at the GV stage but in a deadenylated form. Therefore, a readenylation process may be implicated in regulating the expression of these transcripts (Paynton and Bachvarova, 1994). Final modifications of mRNA polyadenylation to regulate further expression/translation have been described in the mouse and cow (Brevini-Gandolfi *et al*., 1999). Mn-SOD was not detected in cow oocytes and embryos (Harvey *et al*., 1995), but may be either absent or present in a deadenylated form.

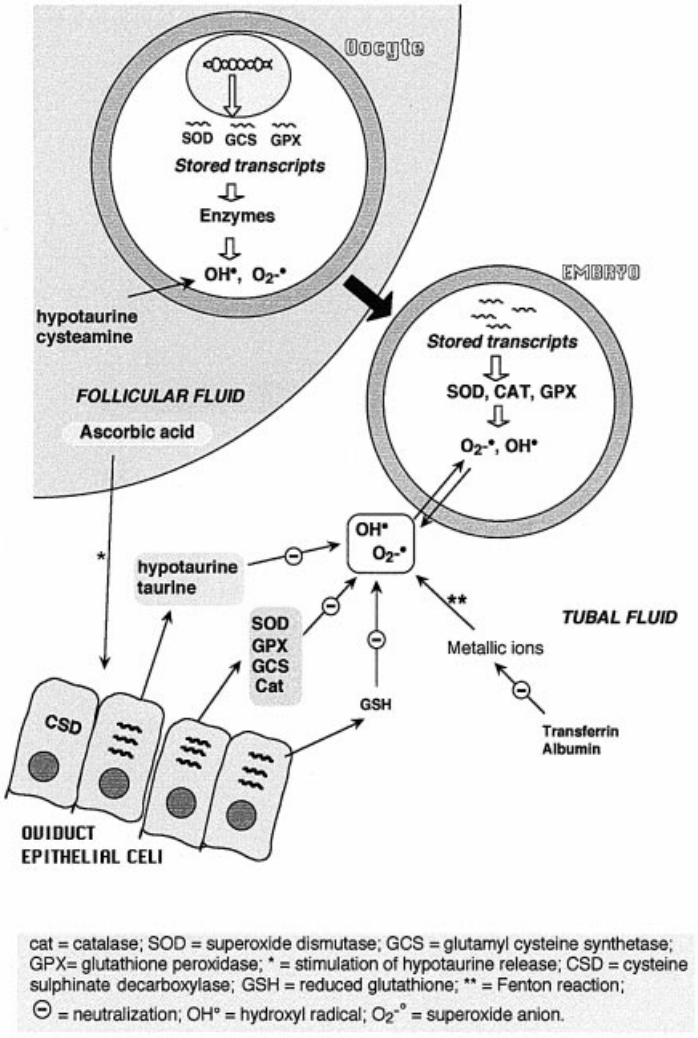

Figure 9. Schematic representation of some complementary antioxidant protections for oocytes and embryos.

Catalase transcripts seem to be absent in MII oocytes but are present in mouse blastocysts (El Mouatassim *et al*., 1999); this protection may be effective only after ZGA (Harvey *et al*., 1995). Catalase activity has been demonstrated in bovine, porcine and human oviductal fluids (Lapointe *et al*., 1998). Moreover, catalase binds to spermatozoa (Lapointe *et al*., 1998), suggesting a potential survival function of female reproductive tract antioxidant enzymes bound to gametes and embryos.

A good correlation has been observed between mRNA, proteins and enzyme activity levels (Forsberg *et al*., 1996), suggesting that the antioxidant enzymes (SOD, catalase, GPX) are regulated primarily at the pre-translational level. Thus, the observed transcripts are probably translated and produce active enzymes. These observations suggest that embryo protection against ROS depends, in part, upon an endogenous pool of antioxidant enzymes (Harvey *et al.*, 1995), stored as mRNA in the oocyte during oogenesis. The total amount of maternal transcripts decreases during embryo development (Pikó and Clegg, 1982), until ZGA. Variations in maternal mRNA synthesis or accumulation during oocyte maturation may affect in-vitro development of the embryo until ZGA. A drop below a critical threshold may lead to developmental arrest.

The expression of many genes can be up- or down-regulated by ROS. The antioxidant enzyme gene expression is stimulated by oxidative stress (Maître *et al.*, 1993). The intracellular redox potential can modulate the activity of some transcription factors, and in this way ROS may activate the antioxidant defence genes (Schultz, 1993).

Embryo 2-cell block is associated with an oxidative burst, suggesting that the embryo may be particularly vulnerable to ROS at this stage. At this time, the embryo *in vivo* is present in the oviduct which provides various radical scavengers (Guérin and Ménézo, 1995). The presence of four out of five

Table I. Expression profiles of antioxidant enzymes in human and mouse oocytes, embryo and oviduct

Species	Stage	GCS	GPX	Cu-Zn-SOD	Mn-SOD	Catalase
Human	GV	+	–	+	–	–
	MII	+	+	+	+	–
	Oviduct	–	+	+	–	+
Mouse	GV	+	+	+	+	–
	MII	+	+	+	+	–
	Blastocyst	+	+	+	+	+
	Oviduct	+	+	+	+	+

GCS = glutamylcysteine synthetase; GPX = glutathione peroxidase; GV = germinal vesicle oocyte stage; MII = metaphase II oocyte stage; SOD = superoxide dismutase. (El Mouatassim *et al*., 1999).

Table II. Overview of some antioxidant defences in the mammalian embryo and its surroundings

System	Localization	Remarks
Non-enzymatic		
Ascorbate (vitamin C)	Follicular fluid	Diverse antioxidant functions
Glutathione (GSH)	Tubal fluid Oocyte/Embryo	Diverse antioxidant functions
Proteins		
Transferrin	Tubal fluid	Metal chelation
Albumin	Tubal fluid	Trapping activity (lipid peroxides)
Cysteamine	Follicular fluid	Precursor of hypotaurine
Hypotaurine	Tubal fluid	OH· neutralization
	Follicular fluid	Precursor of taurine
Taurine	Tubal fluid Follicular fluid	Neutralization of cytotoxic aldehydes
Antioxidant amino acids[a]	Tubal fluid Follicular fluid	
α-Tocopherol/β-carotene	Ovary or follicular fluid	Radical chain-breaking
Enzymatic (direct)		
Superoxide dismutase	Cytoplasmic (Cu,Zn-SOD) Mitochondrial (Mn-SOD)	O_2^{-}· neutralization
Catalase	Tubal fluid	Removal of hydrogen peroxide
GSH peroxidase		Reduction of hydrogen peroxide and organic (lipid) peroxides
Enzymatic (indirect)		
Glutamylcysteine synthetase	Oocyte/Embryo	Maintaining GSH concentrations
GSSG reductase		Maintaining GSH concentrations
NADPH supply		NADPH for GSSG reductase
Repair systems	DNA repair in oocytes	Oxidized protein turnover? Oxidized phospholipid turnover?

[a]Tryptophan, cysteine, tyronine, homocysteine.

antioxidant enzymes transcripts at the GV/MII stages in mouse oocytes and MII stage in human oocytes (El Mouatassim *et al*., 1999) suggests that these defence mechanisms are important for further embryo development, whatever the species (Table I).

Different gene expression patterns of the antioxidant enzymes between human, bovine and murine species may reflect the variations in the ability of embryos to develop *in vivo* and *in vitro*. The more complete panel of transcripts encoding for antioxidant enzymes in mouse oocytes and embryos when compared with humans can be correlated with a better ability of mouse embryo to develop *in vitro*.

The high reactivity of some ROS (e.g. OH·) first requires that activation of the defence mechanisms occurs promptly after ROS generation. Just as ROS can originate in different cell compartments, cells possess antioxidant defences in every site of production. For example, in embryos, superoxide radicals can originate from the mitochondria as well as the cytosol and membranes. Consequently, SOD is present in oocyte/embryo mitochondria (Mn-SOD) and cytoplasm (Cu,Zn SOD). Embryos seem to be protected from OH· damage by the conversion of superoxide anions into H_2O_2, and its removal by the activities of catalase in peroxisomes or GPX in the cytosol and mitochondria. Together with Cu,Zn-SOD, these enzymes clearly have complementary roles.

The intracellular redox status can modulate the activity of antioxidant transcription factors. Unfavourable embryo culture conditions may result in alteration of embryo metabolism and intracellular production of ROS, which may in turn affect antioxidant enzyme gene expression.

It is evident that the embryo is protected by a wide range of antioxidant systems working together. Metal catalysts of oxidative reactions are removed in embryo environments by metal-binding molecules (transferrin, albumin). SOD, GPX and catalase within the embryo remove superoxides and peroxides before they react with metal catalysts to form more reactive species. Finally, peroxidative chain reactions initiated by ROS that escaped enzymatic degradation are terminated by chain-breaking antioxidants, including water-soluble ascorbate, GSH, hypotaurine and lipid-soluble vitamin E. When peroxidative damage occurs despite these mechanisms, the cytotoxic aldehydes (the end-products of peroxidation reactions) are neutralized by compounds such as taurine. Therefore, multiple and complementary protection mechanisms seem to be present in the embryo and its environment.

Another potential antioxidant mechanism in the early embryo could reside in its own metabolism. That is, ATP production in the early embryo relies mostly on glycolysis, with <30% being produced via OXPHOS (Trimarchi *et al*., 2000). This suggests

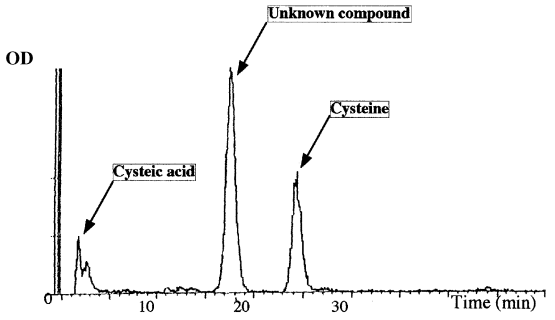

Figure 10. Cation-exchange chromatogram of cysteine after 10 min incubation in M16 medium. Conditions of analysis: amino acid analyser Beckman 6300; international standard: glucosaminic acid; aminoacids were colorimetrically detected by ninhidrin. Coloration ratio at two wavelengths (570 and 440 nm) permitted identification of cysteine and cysteic acid.

that the more 'anaerobic' metabolism of the early embryo could help to minimize the production of ROS by the mitochondria, and thus reduce the risk of oxidative stress.

Some complementary antioxidant defences protecting oocyte and embryos are represented in Figure 9 and Table II.

Repair systems in oxidative stress

The importance of repair systems for ROS-induced cellular damage has only recently been recognized. Both embryos and spermatozoa possess repair mechanisms to counteract the toxic effects of ROS.

Sperm motility can spontaneously reinitiate after ROS treatment (de Lamirande and Gagnon, 1992). Newly fertilized eggs are capable of repairing the damaged DNA of spermatozoa (Matsuda and Tobari, 1989). Experimental evidence in different in-vivo and in-vitro systems clearly indicates that the vertebrate oocyte is capable of repairing endogenous and exogenous DNA damaged as a result of meiotic recombination, the action of UV-irradiation, or the effects of mutagenic chemicals (Ashwood-Smith and Edwards, 1996). Enzymatic repair activity in oocyte extracts *in vitro* has been observed, and in-vivo repair of damaged spermatozoa or injected DNA is known to occur in oocytes (Ashwood-Smith and Edwards, 1996). Restitution of radiation-damaged DNA has been observed in mouse pre-implantation embryos.

By transferring the nucleus of H_2O_2-treated mouse zygotes into enucleated cytoplasm, it was observed that the pronuclei of oxidative-damaged embryos can be rescued partly by transferring them into normal cytoplasm (Liu and Keefe, 1999). Therefore, repair mechanisms of nuclear oxidative damage seem to be present in embryo cytoplasm. Nevertheless, it is possible that the nuclei of the H_2O_2-treated zygotes were not damaged at all, i.e. in relation to the nucleus state and/or the cell cycle stage.

We have seen above that the inability of mtDNA to auto-repair leads to a progressive decline in mitochondrial function. In embryos, this decline may be overcome by a rapid turnover of mitochondria (Barritt *et al*., 1999).

Consequences of in-vitro embryo production techniques

The importance of protecting pre-implantation embryos from oxidative damage *in vitro* is being increasingly recognized. To optimise in-vitro embryo production, oxidative stress must be controlled during in-vitro culture.

Oocytes and embryos must be protected by reducing oxygen concentration in the gaseous environment, by a radical buffering system produced by co-cultured cells, and/or by adding supplements to the culture medium.

Gaseous environment

The reduction of O_2 concentrations from ~20% to 5% during the in-vitro culture of embryos has been shown to improve embryonic development in a variety of species (Quinn and Harlow, 1978; Thompson *et al*., 1990; Batt *et al*., 1991; Li and Foote, 1993; Watson *et al*., 1994; Berthelot and Terqui, 1996; Dumoulin *et al*., 1999; Lim *et al*., 1999; Callesen *et al*., 2000). In one report (Callesen *et al*., 2000), significantly more day-7 pig blastocysts were observed in cultures incubated uner 5% CO_2/5% O_2/90% N_2 than under 5% CO_2 in air. The effect is not immediate, as no difference was observed concerning cleavage rates. This observation concerns only in-vitro-produced embryos and not in-vivo obtained ones. It has also been suggested (Callesen *et al*., 2000) that in-vivo-flushed embryos have a much greater developmental potential and are less easily perturbed by a sub-optimal culture environment than those that have probably been inadequately matured *in vitro*.

Filtering the air used in CO_2 incubators might also permit the elimination of atmospheric pollutants.

Co-culture systems

The rate of blastocyst production is increased by using co-culture (Ménézo *et al*., 1990). Furthermore, the blastocysts obtained are more able to support cryopreservation process. Co-cultured cells (oviduct epithelial cells) secrete GSH (Ouhibi *et al*., 1989), hypotaurine and taurine (Guérin and Ménézo, 1995; Guérin *et al*., 1995b), and contain transcript encoding for the main antioxidant enzymes (El Mouatassim *et al*., 2000). Co-culture techniques probably allow the equilibrium between antioxidants and pro-oxidants in the culture medium to be maintained. Chemically defined media seem less able to protect embryos against oxidative stress.

The beneficial effect of co-cultured cells may partly occur due to both the reduction of O_2 concentrations in the culture medium, and to the detoxification of highly diffusable ROS by the antioxidant systems present in these cells (e.g. SOD, GPX). In addition, under increased oxidative stress conditions, expression of some of these antioxidant enzymes can be induced.

Light

It may be beneficial to reduce the time of embryo exposure to visible light, and to manipulate embryos only under inactinic light (red filters can stop UV radiations).

Cryopreservation

Cryopreservation of spermatozoa reduces the antioxidant defences of these cells (Bilodeau *et al*., 1999). As a consequence, the addition of antioxidant compounds in media used for gamete/embryo cryopreservation compounds is now commonly used.

Addition of antioxidant compounds to culture media

Today, media are often supplemented with metal chelators and various antioxidant compounds (Iwata *et al*., 1998). The redox potential of culture media is very important (–0.1 mV for B_2

medium). A slightly reducing medium permits embryo development, probably by limiting the peroxidation process, and compounds such as ascorbate, cysteine or GSH contribute to maintaining this redox potential. The culture medium must have a radical-trapping antioxidant ability. Various antioxidant compounds can contribute to this trapping ability, including vitamin C, vitamin E, albumin and hypotaurine; among these compounds, albumin appears to be one of the most active (Wayner and Burton, 1987).

Antioxidant enzymes

Supplementing culture media with catalase or Cu,Zn-SOD results in an increase in the rate of blastocyst formation in rabbit (Li *et al*., 1993), mouse (Nonogaki *et al*., 1992) and bovine (Lauria *et al*., 1994) species. SOD is effective in overcoming the mouse 2-cell block (Noda *et al*., 1991). An additive effect of low oxygen concentration and SOD on mouse embryo development has been observed (Umaoka *et al*., 1992), though adding antioxidant enzymes is relatively expensive and, in the mouse, does not necessarily overcome developmental retardation (Payne *et al*., 1992).

Metal chelators

These have beneficial effects on embryo development. Divalent cations chelators such as apotransferrin, D-penicillamine, L-histidine, L-cysteine and EDTA prevent Fe^{2+} ions from acting as catalysts of oxidation to produce OH·, and help to overcome the mouse embryo's 2-cell block. EDTA was reported to inhibit enzymatic and non-enzymatic oxidation of GSH. Furthermore, EDTA may inhibit xanthine oxidase (an Fe-dependent enzyme) which is probably the main source of ROS in mouse embryos *in vitro*. Penicillamine (cuprimine) prevents copper ions from acting as a catalyst in the Haber–Weiss reaction, and is frequently added to bovine IVF and embryo culture media.

Thiol compounds

GSH supplementation has been recommended for overcoming the 2-cell block and improving the development of 1-cell stage mouse embryos (Nasr-Esfahani *et al*., 1992), and also improves bovine embryo production *in vitro* (Luvoni *et al*., 1996). A positive effect of GSH supplementation was observed on the in-vitro fertilization of bovine oocytes, together with a dose-dependent increase in GSH concentration in the oocytes (Kim *et al*., 1999). Inclusion of β-mercaptoethanol (β-ME) and CSH in culture media facilitates the development of in-vitro maturation (IVM)/IVF bovine embryos to the blastocyst stage without any feeder cells (Otoi *et al*., 2000). This improvement was accompanied by increased intracellular GSH concentrations (Takahashi *et al*., 1993). Supplementing the IVM medium with CSH, β-ME, cysteine and cystine induces bovine GSH synthesis (de Matos and Furnus, 2000). Furthermore, buthionine sulphoximine—a specific inhibitor of GSH synthesis—can neutralize the promoting effect of thiol compounds on the development and GSH synthesis of bovine embryos (Takahashi *et al*., 1993). Cysteine has a positive effect on oocyte maturation *in vitro* in the pig (Yoshida *et al*., 1993). These compounds are reported to promote cell viability by a direct antioxidant action, and are also reported to increase intracellular GSH in oocytes and embryos (Takahashi *et al*., 1993). Nevertheless, we observed that cysteine is rapidly oxidized after a short time of incubation in a culture medium (Figure 10). Supplementing with thiol protectors such as β-ME avoids cysteine oxidation. *N*-Acetylcysteine (NAC), which has also been used successfully in oocyte/embryo culture media (Issel *et al*., 1988), is more stable than cysteine and GSH. Depletion of sulphur amino acids can induce a depletion of the endogenous pool of GSH in oocytes and embryos; thus, in order to maintain this pool, thiol compounds are added to the maturation media, especially in pig embryo culture (Sawai *et al*., 1997).

In our culture conditions, no positive effect of CSH supplementation was observed on oocyte maturation and blastocyst development (Guyader-Joly *et al*., 1998). At a CSH concentration of 250 µmol/l, a negative effect was observed on the rate of obtaining blastocysts. This suggests that an excess of thiol or reductor compounds may be deleterious, and that the redox equilibrium is important for oocyte maturation and embryo development. Altering thiol-redox status in embryos may result in cell cycle arrest, apoptosis, and/or cell death (Liu *et al*., 1999).

Hypotaurine

This has direct and indirect (via conversion into taurine) beneficial effects, and seems to be a good candidate for addition to culture media for oocyte/embryo culture. Indeed, hypotaurine is now frequently added to bovine embryo culture media.

Proteins

The positive effect of protein (usually serum albumin) supplementation has been demonstrated. Albumin participates in the antioxidant defence of embryos by trapping ROS and/or end-products of peroxidation by virtue of its peroxyl-absorbing capacity. Nevertheless, albumin may have negative effects on embryo by transporting deleterious compounds such as peroxides originating from the serum (Ménézo and Khatchadourian, 1986).

Thioredoxin, a small protein, plays a defensive role against oxidative stress and induces the release of 2-cell block in mice, probably by reducing protein disulphide (Natsuyama *et al*., 1992). In mouse embryos, thioredoxin is also thought to promote development *in vitro* by facilitating the repair of oxidative changes of sulphydryl groups (Natsuyama *et al*., 1992).

Vitamins

Supplementing culture media with antioxidant vitamins such as ascorbate and vitamin E has been proposed. Vitamin C has been used at various concentrations (Tarín *et al*., 1994). H_2O_2-induced ROS production is significantly reduced in a dose-dependent manner by supplementing sperm preparation media with vitamin C plus vitamin E (Donnelly *et al*., 1999).

In order to avoid oxidative stress during oocyte and embryo culture, it is important to limit ROS generation on the one hand, and to facilitate ROS neutralization on the other hand. It is also important to eliminate the damaged embryos during the culture process, as these may produce ROS (as do damaged spermatozoa). Modifying the embryo environment by reducing atmospheric oxygen, adding antioxidant and metal-binding compounds or manipulating embryos under inactinic light may be required in order to establish a suitable culture system. Supplementing culture media with antioxidant compounds is a complex problem, and simply adding ROS scavengers or enzymes such as SOD and catalase may not be fruitful. Indeed, it might even be deleterious

to add a single antioxidant to the medium as it can exert pro-oxidant activity. Both ascorbate and alpha-tocopherol supplementation combined with a sperm preparation medium can induce DNA damage and intensify the damage induced by H_2O_2 (Donnelly *et al*., 1999). Oxidative damage to polyunsaturated fatty acids (lipid peroxidation) is enhanced by low concentrations of ascorbic acid. CSH, cysteine and NAC could induce deleterious effects by reducing the disulphide bonds of proteins, leading to denaturation or inactivation. However, such an effect is not observed with hypotaurine or taurine.

In a culture without a radical buffering system, as in chemically defined media, some antioxidant compounds have a short half-life (Tarín *et al*., 1994). Replacement of the medium, and sequential media systems, could facilitate preservation of the redox potential.

Conclusions

ROS are generated during normal embryo metabolism, and the high reactivity of some ROS (e.g. OH·) requires first, that the activation of defence mechanisms occurs promptly after ROS generation, and second that the defence systems be present at the site of ROS generation. ROS must be continuously inactivated in embryos, and this explains why the protective mechanisms of embryos against ROS are multiple in nature and appear redundant. The prevention against ROS formation and ROS destruction seems to be linked both to internal protection and external protection through the oviductal environment. It is evident that internal embryo protection is mainly represented by antioxidant enzymes, and the transcripts encoding for these enzymes are stored in oocytes during the final stages of maturation. The aptitude of embryos to develop *in vitro* seems to depend on the quality of this storage.

ROS production is particularly important during in-vitro culture: the recovery and culture of oocytes/embryos involves their exposure to light, high oxygen concentrations, traces of transitional elements, disturbed concentrations of metabolic substrates, and possibly xenobiotics. Consequently, the in-vitro production and manipulation of gametes and embryos favours ROS generation in embryos, and may partly explain the low rate of obtaining viable embryo in many species. The rise in H_2O_2 concentrations observed in embryos cultured *in vitro* is considered to be a response to culture conditions. In-vitro production and manipulation of gametes and embryos both increases ROS generation and decreases antioxidant defences.

The avoidance of oxidative stress during oocyte and embryo culture is a complex problem. Simply adding the necessary ROS scavengers is not sufficient, as the choice of antioxidant compounds to be used, and their concentrations, are difficult to ascertain. Transferrin, penicillamine, hypotaurine and taurine are often added to culture media because positive effects on embryo development have been observed, without any negative impact. Albumin is a very effective trapping compound, although animal proteins are no longer used in humans for safety reasons. Other compounds such as thiols and vitamins must also be used with care as they too can have a negative impact on the embryo. An excess of antioxidant compounds (via increasing reducing status in the medium) may have deleterious effects on the embryo. An equilibrium between antioxidants and pro-oxidants must be preserved in cells (Aitken and Fisher, 1994), and this is clearly more difficult to preserve during in-vitro culture. For example, chemical compounds and/or water may be contaminated with traces of metallic ions, while albumin can be associated with peroxidized fatty acids that have deleterious effects on embryos. Co-culture with somatic cells most likely facilitates the preservation of such an equilibrium.

These difficulties explain the contradictory results observed previously in the literature: a compound can have beneficial effects on embryo development in a defined medium, but no effect (or even deleterious effects) in a different medium. Furthermore, one compound (e.g. EDTA) can have beneficial effects on the early development stages, but negative effects after ZGA.

It is clear that culture systems constitute limited conditions for embryo development, and that oxidative stress is unavoidable. Improving the knowledge of the antioxidant mechanisms present in the embryo and its surroundings will help improve the culture media used to obtain embryos *in vitro*. Assessing antioxidant defences in oocytes and embryos could help in developing a method to evaluate oocyte and/or embryo quality.

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