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Regulation of sperm function by reactive oxygen species

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Sperm capacitation can be increased by the addition of reactive oxygen species (ROS) and decreased by antioxidants. Broadly consistent results have been achieved with a wide variety of methods and across different species. Exposure to ROS increases protein tyrosine phosphorylation consequent on an increase in cAMP and activation of tyrosine kinase and inhibition of tyrosine phosphatase. The measurement of ROS production by sperm is complicated by contamination of suspensions by leukocytes, laying many studies open to doubt. In human sperm the observation that extracellular NADPH could support superoxide production detected with the chemiluminescent probe lucigenin and had physiological effects similar to hydrogen peroxide led to the suggestion that they contained NADPH oxidase activity to generate ROS to support capacitation. However, the realization that lucigenin can signal superoxide artefactually, combined with failure to detect superoxide production using spin trapping techniques or to detect NADPH oxidase components in mature sperm, and confirmation of old reports that NADPH solution contains substantial amounts of hydrogen peroxide due to autoxidation, have undermined this hypothesis. Although the presence of significant NADPH oxidase activity in mature human sperm now seems less likely, other observations continue to suggest that they can make ROS in some way. There is stronger evidence that animal sperm can make ROS although these may be mainly of mitochondrial origin.

Key words: capacitation/cyclicAMP/NADPH oxidase/reactive oxygen species/sperm

Introduction

Sperm are released from the testis with tightly supercoiled DNA which is transcriptionally inert (Sotolongo and Ward, 2000) but nevertheless they undergo profound changes in their functional capacity as they mature in the epididymis, capacitate in the female reproductive tract and fertilize. These changes must be achieved by modifications of existing proteins rather than changes in gene expression and can be modulated by signals from the sperm's environment or may occur spontaneously. For example, capacitation can be regulated by substances present in semen (Fraser and Adeoya-Osiguwa, 2001), by the female reproductive tract (Smith, 1998) or by progesterone and other substances secreted by the oocyte cumulus complex (Blackmore, 1993) but can also occur spontaneously under suitable conditions *in vitro*. This article reviews the evidence that reactive oxygen species (ROS) are among the messengers that influence sperm function during their journey from the testis to the oocyte and that they are intrinsic signals produced by sperm themselves. For reasons of space, the effects of reactive nitrogen species (RNS) will only be discussed where they interact with the effects of ROS.

Reactive oxygen species

The term ROS includes reduced forms of oxygen and their reaction products with other molecules. Some but not all are free radicals. Free radicals are highly chemically reactive because they contain unpaired electrons. Diatomic oxygen (O_2) contains two unpaired electrons but is relatively inert because these have parallel spins (Halliwell and Gutteridge, 1999). Successive reduction of oxygen to superoxide and peroxide removes the spin restriction so these molecules are more reactive than O_2 even though peroxide is not a free radical. The extremely reactive hydroxyl radical can be formed by reaction between superoxide and hydrogen peroxide or reduction of peroxide catalysed by ferrous ions. Superoxide reacts very avidly with nitric oxide to form peroxinitrite, and hydrogen peroxide can react with chloride to form hypochlorite (Figure 1). These products are among the most biologically important ROS but the term also includes singlet oxygen, ozone and organic peroxyl and alkoxyl radicals (Halliwell and Gutteridge, 1999).

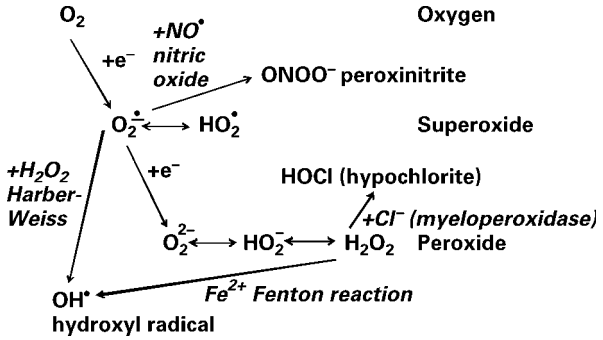

Figure 1. Derivation of reactive oxygen species from oxygen. Superoxide and peroxide can exist in charged and protonated forms, so their ability to cross membranes is pH dependent.

Biological roles of ROS

ROS can react with a wide range of biological molecules, notably unsaturated fatty acids, sulphydryl proteins and nucleic acids and are implicated in a large number of diseases, e.g. arthritis, artherosclerosis and degenerative diseases of ageing (Halliwell and Gutteridge, 1999). The first report that they could have harmful effects on sperm was published over 60 years ago (Macleod, 1943) and it is now generally accepted that ROS production in sperm suspensions, lipid peroxidation and DNA oxidation are associated with poor sperm function and subfertility (Aitken *et al.*, 1991, 1993; Sukcharoen *et al.*, 1995; Griveau and LeLannou, 1997a; Storey, 1997; Lopes *et al.*, 1998; Whittington *et al.*, 1999; Pasqualotto *et al.*, 2000; Shen and Ong, 2000; Agarwal *et al.*, 2003).

However, ROS also have physiological roles. They are produced by leukocytes as part of the phagocytic process to kill engulfed bacteria (Babior, 1999) but also in smaller amounts by other cell types to act as cell messengers. In the latter function they often act differently from traditional messengers that rely on conformational specificity to bind to sites on their receptor but without producing any covalent modification. By contrast, ROS often change the function of their targets through oxidation, usually of sulphydryl groups, and specificity is achieved through the chemical reactivity of the target. Such effects can be produced by the ROS itself or indirectly through oxidation of intracellular thiols, predominantly glutathione. These mechanisms offer novel possibilities of achieving specificity and cross-talk between different regulatory pathways (Cooper *et al.*, 2002).

ROS have been implicated in many pathways of cell regulation. These topics have been reviewed recently (Finkel, 1998; Hensley *et al.*, 2000; Thannickal and Fanburg, 2000; Droge, 2001; Chiarugi and Cirri, 2003; Finkel, 2003) and only some of the most recent original papers will be cited in the brief survey that follows. ROS can regulate gene expression, notably up-regulation of anti-oxidant proteins in the face of a higher level of oxidative stress. They are involved in the mechanism of 'oxygen sensors', e.g. in the carotid body (Jones *et al.*, 2000). They regulate cell adhesion and antibody production by leukocytes. Although not mandatory for apoptosis, they have pro-apoptotic effects. They can enhance the action of epidermal and platelet-derived growth factors, since hydrogen peroxide facilitates autophosphorylation of the receptor, so increasing its affinity for the agonist. Relatively high hydrogen peroxide concentrations ($\sim$1 mmol/l) or large oxidative shifts in thiol status can inhibit tyrosine phosphatases and so increase protein tyrosine phosphorylation triggered by tyrosine kinases. A reversible regulatory system is achieved by the cyclic oxidation and reduction of key sulphydryl groups involving a novel sulphenyl-amide (Salmeen *et al.*, 2003; van Montfort *et al.*, 2003). Relatively small concentrations of hydrogen peroxide or thiol status can activate the tyrosine kinase activity of the insulin receptor as well as intracellular kinases such as MAP kinase and protein kinase C. Oxidants can increase intracellular calcium concentration as well as acting through protein phosphorylation.

Evidence that ROS modulate sperm function

Capacitation

Freshly ejaculated sperm cannot fertilize until they have spent some time in a suitable environment in order to capacitate. Capacitated sperm acquire the ability to exhibit hyperactivated motility and to undergo a physiological acrosome reaction (Yanagimachi, 1994). Capacitation is associated with a number of biochemical events, most notably an increase in protein tyrosine phosphorylation (Visconti and Kopf, 1998; Visconti *et al.*, 1998; Baldi *et al.*, 2000, 2002; Guraya, 2000; Breitbart, 2003). Although it can occur spontaneously under suitable conditions *in vitro*, *in vivo* its progress can be regulated by the female reproductive tract (Smith and Yanagimachi, 1989; Hunter *et al.*, 1998).

ROS have been reported to promote sperm capacitation in hamster, mouse, rat, bull, horse and human sperm. Capacitation has been assessed by the chlortetracycline (CTC) staining pattern, by the ability of the sperm to acrosome-react spontaneously or after stimulation with lysophosphatidyl choline, A23187 or progesterone, by the zona-free hamster oocyte test, by the prevalence of hyperactivation and by IVF. ROS have been generated by enzymes using xanthine/xanthine oxidase or glucose oxidase, by adding activated leukocytes, by direct addition of H_2O_2 or by addition of substances such as fetal cord serum ultrafiltrate that stimulate endogenous ROS production. NAD(P)H has also been used in this way but it remains uncertain whether it stimulates ROS production or acts as a source of H_2O_2 due to autoxidation (see below). ROS have been removed with the enzymes superoxide dismutase (SOD) and catalase or with various antioxidants. With few exceptions, adding ROS increased capacitation whereas removing them decreased it whatever methods were used. Some of the effects of ROS could be mimicked by other oxidizing agents, notably thiol oxidants. The studies that support these statements are summarized in Table I. They were done in many different laboratories by a variety of techniques and together provide convincing evidence that redox mechanisms promote sperm capacitation in several species.

Maturation

Sperm released from the testis are unable to exhibit progressive motility or to capacitate but acquire these abilities during their passage through the epididymis. These processes are referred to as maturation; other maturational changes include the completion of nuclear condensation and changes in the expression and distribution of molecules on the sperm surface (Cooper, 1995; Moore, 1996).

Table I. Effects of increasing or decreasing reactive oxygen species (ROS) on sperm capacitation

Reference	Species	ROS increased (+) or decreased (−)	Method	Events used to assess capacitation	Stimulated (+) or inhibited (−)
Bize *et al.* (1991)	Hamster	+	GO, H_2O_2	AR	+
		−	Catalase	AR	−
de Lamirande and Gagnon (1993a)	Human	+	X + XO	Hyp, LPC-induced AR	+
		−	SOD	Hyp	−
de Lamirande and Gagnon (1993b)	Human	+	X + XO, FCSU	Hyp, LPC-induced AR	+(blocked by SOD but not catalase)
de Lamirande *et al.* (1993)	Human	−	SOD	LPC-induced AR	−
de Lamirande and Gagnon (1993c)	Human	−	SOD	Hyp	−
Griveau *et al.* (1994)	Human	+	H_2O_2	Hyp, AR	+
		−	Catalase	Hyp, AR	−
Griveau *et al.* (1995)	Human	−	SOD, vitamin E	AR	− (no effect on hyperactivation)
Oehninger *et al.* (1995)	Human	+	H_2O_2	Spontaneous AR	+
				Hyp, stimulated AR, ZB	No effect
		−	Catalase	Progesterone effect on $[Ca^{2+}]_i$	+
Aitken *et al.* (1995)	Human	+	H_2O_2, GO, NADPH	HOPT, Tyr phos	+
		−	Catalase, thiols	HOPT, Tyr phos	− (catalase inhibited HOPT stimulation by ZP3 and progesterone)
Aitken *et al.* (1996b)	Human	+	NADPH	Progesterone-stimulated HOPT	+ (blocked by catalase or thiols but not SOD)
		−	Catalase, thiols	Progesterone-stimulated HOPT Tyr phos	−
Kodama *et al.* (1996)	Mouse	+	Lipox	IVF, ZB	+
Kuribayashi and Gagnon (1996)	Mouse	−	Catalase	Motility, IVF	No effect
			Thioredoxin	Blastocyst formation	+ (prevent toxic effects of H_2O_2 ?)
Leclerc *et al.* (1997)	Human	+	GO, H_2O_2, FCSU	LPC-induced AR Tyr phos	+
de Lamirande *et al.* (1997)	Mouse	+	X + XO	Hyp LPC-induced AR	+
O'Flaherty *et al.* (1997)	Bull (cryopreserved)	−	Vitamin E ± C, SOD	CTC	−
Leclerc *et al.* (1998)	Human	+	FCSU	LPC-induced AR, Tyr phos	+(probed relationship with cAMP and $[Ca^{2+}]_i$)
de Lamirande *et al.* (1998a)	Human	+	FCSU, progesterone, FFU, SP	LPC-induced AR, Tyr phos	+(body fluids but not NADPH stimulated superoxide production that initiated capacitation)
		−	SOD	LPC-induced AR, Tyr phos	−
de Lamirande *et al.* (1998b)	Human	+	FCSU, FFU, H_2O_2, XO	LPC-induced AR (post capacitation), Tyr phos	+(roles for superoxide and H_2O_2 effects greater in capacitated sperm)
		−	SOD, catalase	LPC-induced AR (post capacitation), Tyr phos	−
de Lamirande and Gagnon (1998)	Human		-SH alkylating or oxidizing agents	LPC-induced AR tyr phos	+(associated with superoxide production, inhibited by SOD)
			-S-S- reducing agents	LPC-induced AR Tyr phos	- (associated with decreased superoxide production)
Aitken *et al.* (1998a)	Human	+	NADPH	HOPT, tyr phos	+(blocked by catalase, diphenylene iodonium)
Cohen *et al.* (1998)	Mouse	+	630 nm laser light	Ca^{2+} transport, fertilization	+(light induced H_2O_2 production)
O'Flaherty *et al.* (1999)	Bull (cryopreserved)	+	X + XO	CTC	+
				AR	No effect
			H_2O_2	CTC	−
				AR	+
Hsu *et al.* (1999)	Rat	+	H_2O_2	A23187 or LPC-induced AR	+
Lin *et al.* (2000) (abstract only)	Mouse	+	XO, H_2O_2	Cap, AR, fertilization	+(ROS obviated need for glucose for optimal capacitation)
		−	Catalase	Cap, AR, fertilization	+(excess ROS harmful)
Herrero *et al.* (2001)	Human	+	Peroxynitrite	FF-induced AR	+(associated with tyrosine nitrosylation)
de Lamirande and Gagnon (2002)	Human	+	FCSU	LPC-induced AR	+(evidence for involvement of ERK)
Ecroyd *et al.* (2003)	Mouse	−	Catalase, DPI	Tyr phos	− (HCO_3^- required for ROS dependent capacitation)
			cAMP		No effect

Table I *Continued*

Reference	Species	ROS increased (+) or decreased (−)	Method	Events used to assess capacitation	Stimulated (+) or inhibited (−)
Baumber *et al.* (2003)	Horse	+	X + XO, NADPH	Progesterone-induced AR Tyr phos	+(effects decreased by SOD or catalase)
O'Flaherty *et al.* (2003)	Bull (cryopreserved)	+	X + XO + catalase	CTC/LPC-induced AR (post capacitation)	+(heparin promoted capacitation associated with ROS production)
		–	SOD	CTC/LPC-induced AR (post capacitation)	–
Villegas *et al.* (2003)	Human	+	Leukocytes	CTC	+(reversible by seminal plasma)
Brener *et al.* (2003)	Ram, bull	+	H_2O_2	Tyr phos Actin polymerization	+(but did not stimulate AR in absence of heparin)
Rivlin *et al.* (2004)	Bull	+	H_2O_2, NADPH	Tyr phos	+(activates adenyl cyclase)
		–	Catalase	Tyr phos	–

AR = acrosome reaction; CTC = chlortetracycline staining pattern indicating capacitation; DPI = diphenylene iodonium; FCSU = fetal cord serum ultrafiltrate; FF(U) = follicular fluid (ultrafiltrate); GO = glucose oxidase; HOPT = hamster oocyte penetration test; Hyp = hyperactivated motility; lipox = lipid peroxidation induced with Fe^{2+}/ascorbate; LPC = lysophosphatidyl choline; SOD = superoxide dismutase; SP = seminal plasma; Tyr phos = protein tyrosine phosphorylation; X + XO = xanthine + xanthine oxidase; ZB = zona binding

It has been proposed that ROS play a role in the regulation of maturation. Phospholipid hydroperoxide glutathione peroxidase (GPX4) can utilise the thiol groups in nuclear proteins as an alternative reductant to glutathione. Generation of lipid peroxides by ROS might provide a substrate for GPX4 to drive the oxidation of these proteins and to facilitate nuclear condensation (Aitken and Vernet, 1998). ROS and might also be involved in motility initiation by enhancing cAMP synthesis and protein phosphorylation at ejaculation (Aitken, 2000).

Cell signalling pathways

There is a broad measure of agreement that the potentiation of capacitation by ROS is associated with increased protein tyrosine phosphorylation and shares features with the cAMP-dependent capacitation pathway. The main targets are proteins with a mol. wt of ~100 kDa. Aitken *et al.* (1995) identified a protein of 116 kDa whereas Leclerc *et al.* (1997) identified proteins migrating with mol. wt of 105 and 81 kDa. It is likely that the bands are complex and that two components are fibrous sheath proteins related to protein kinase A anchoring protein and its precursor (Carrera *et al.*, 1996; Ficarro *et al.*, 2003).

Physiological concentrations of ROS have been proposed to enhance sperm capacitation by increasing cAMP synthesis and by inhibiting protein tyrosine phosphatases whilst activating tyrosine kinase (Figure 2). Evidence that they increase intracellular cAMP concentrations includes the observations that addition of a phosphodiesterase inhibitor or of dibutyryl cAMP had a similar potency to stimulating ROS production by addition of NADPH, in promoting human sperm fusion with zona-free hamster oocytes (although there was some additive effect when both cAMP and NADPH were both added) and that increasing cAMP or adding NADPH produced a similar pattern of tyrosine phosphorylation that could be blocked by the protein kinase A inhibitor H89 (Aitken *et al.*, 1998a). Exposure to superoxide increased sperm cAMP concentration (Zhang and Zheng, 1996) and exposure to NADPH produced a larger increase in intracellular cAMP than the phosphodiesterase inhibitor pentoxifylline (Aitken *et al.*, 1998a). In the latter study, neither pentoxifylline nor dibutyryl cAMP had any effect on ROS production. By contrast, Leclerc *et al.* (1998) argued that increasing intracellular Ca^{2+} activity during capacitation provoked an increase in cAMP concentration that stimulated superoxide production which in turn induced protein tyrosine phosphorylation. It has been proposed that in maturing rat sperm, superoxide stimulates tyrosine kinase indirectly by raising the cAMP concentration whereas H_2O_2 directly stimulates tyrosine kinase and inhibits tyrosine phosphatase (Lewis and Aitken, 2001), and this is broadly consistent with conclusions from human sperm (de Lamirande *et al.*, 1998b). However, 50 μmol/l H_2O_2 increased the cAMP concentration in bull sperm by stimulating adenyl cyclase activity and increased tyrosine phosphorylation in a similar way to capacitating agents such as heparin. ROS activation of adenyl cyclase has been reported in cultured rat embryo thoracic aorta cells (Tan *et al.*, 1995) and in rat adipocytes (Raimondi *et al.*, 2000) although in the latter study inhibition of phosphodiesterases could be an alternative explanation. In sperm, one intriguing possibility is that the bicarbonate-sensitive soluble adenylyl cyclase (sAC) is activated by H_2O_2 as well as bicarbonate (Rivlin *et al.*, 2004). sAC is expressed much more strongly in male germ cells and sperm than in other tissues and exists as two functional splice variants (Buck *et al.*, 1999; Sinclair *et al.*, 2000; Jaiswal and Conti, 2001). It is essential for fertility in male mice (Esposito *et al.*, 2004). The 469 amino acid variant of rat sAC contains 12 cysteines of which eight are in catalytic domains (Buck *et al.*, 1999) so it is likely to be open to redox regulation.

ROS-dependent tyrosine phosphorylation was blocked by H89 whereas this inhibitor had no effect on tyrosine phosphorylation

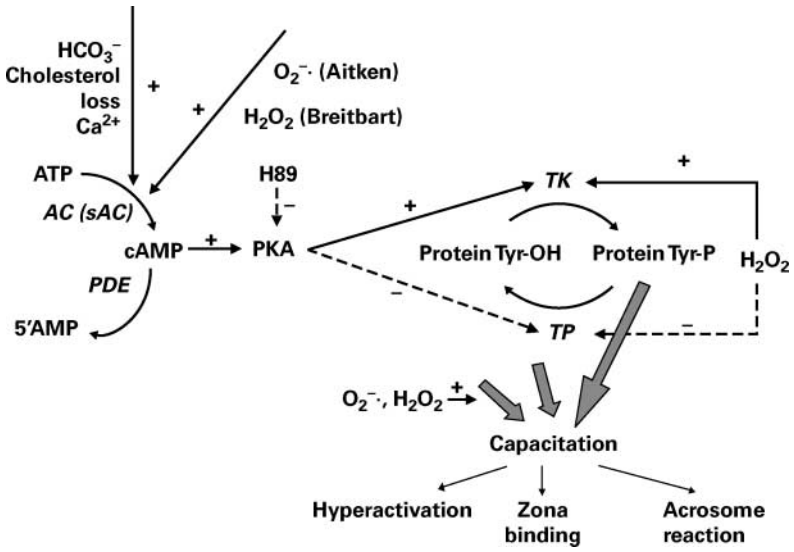

Figure 2. Postulated effects of reactive oxygen species (ROS) on intracellular signalling during sperm capacitation. Note that this diagram combines information from different species. Not all components may exist in a given species and the relative importance of different components may vary. ROS act alongside other factors including bicarbonate, loss of membrane cholesterol and increasing intracellular Ca^{2+} activity to increase intracellular cAMP. Lewis and Aitken (2001) propose that superoxide is the species responsible for activating adenyl cyclase (rat) whereas Rivlin *et al.* (2004) suggest that H_2O_2 is responsible and can substitute for bicarbonate in activating the cyclase (bull). It is possible that the target is the soluble adenylyl cyclase (sAC) cAMP is metabolised by phosphodiesterase (PDE). Increased cAMP activates protein kinase A (PKA) that in turn activates tyrosine kinase (TK) and inhibits tyrosine phosphatase (TP) though unknown mechanisms. The involvement of PKA has been confirmed with the inhibitor H89. Hydrogen peroxide directly activates TK and inhibits TP. The increase in tyrosine phosphorylation produced by these changes is the major driving force for capacitation (largest grey arrow) but other redox-sensitive pathways (smaller grey arrows) may be involved because SOD can block endpoints of capacitation but not tyrosine phosphorylation in sperm treated with sulphydryl reagents (de Lamirande and Gagnon, 1998). It is possible that different functional endpoints of capacitation may be controlled by different pathways; thus, nitric oxide synthase inhibitors inhibit human sperm fusion with the oolemma but have no effect on zona binding (Francavilla *et al.*, 2000).

induced with the tyrosine phosphatase inhibitor vanadate (Rivlin *et al.*, 2004). Although these results must be interpreted with caution, since vanadate is a very non-specific inhibitor, they suggest that activation of the kinase pathway is an important component of the effect of ROS on protein phosphorylation. The cAMP-dependent increase in protein tyrosine phosphorylation may be catalysed at least in part by the tyrosine kinase c-*yes* (Leclerc and Goupil, 2002). An extracellular signal-regulated kinase (ERK) pathway has been implicated in the regulation of capacitation upstream of tyrosine phosphorylation (de Lamirande and Gagnon, 2002). Double phosphorylation of the threonine-glutamine-tyrosine motif characteristic of ERK1/2 activation is regulated by nitric oxide (Thundathil *et al.*, 2003). Stimulation of this event by fetal cord serum ultrafitrate was blocked by the nitric oxide synthase inhibitor N^G-nitro-L-argenine methyl ester (L-NAME) but not SOD or catalase. However, superoxide did influence phosphorylation of the Thr-Glu-Tyr motif in proteins of lighter mol. wt (16–33 kDa) and regulated phosphorylation of some insoluble ERK1/2 substrates in parallel with the ERK pathway (de Lamirande and Gagnon, 2002). It is also likely that superoxide may react with nitric oxide to form peroxinitrite, which can induce tyrosine nitration of sperm proteins as well as tyrosine phosphorylation (Herrero *et al.*, 2001).

It is possible that the effects of ROS are transduced through changes in the redox state of protein sulphydryl groups. Addition of reduced sulphydryl compounds can block capacitation and antagonize the effects of ROS. Addition of compounds that react with sulphydryl groups promoted capacitation; this effect was concomitant with increased superoxide production and was blocked by SOD, although SOD did not affect changes in protein tyrosine phosphorylation (de Lamirande and Gagnon, 1998). By contrast, sulphydryl reducing agents impeded capacitation and decreased superoxide production and protein tyrosine phosphorylation (Aitken *et al.*, 1996b; de Lamirande and Gagnon, 1998). Capacitation was associated with rapid (10–15 min) changes in the sulphydryl content of $\geq$10 proteins; five exhibiting an increase and five a decrease. The changes were reversed after 30–120 min. The changes were prevented by SOD and catalase but were similar whether induced by fetal cord serum ultrafiltrate, follicular fluid ultrafiltrate or progesterone (de Lamirande and Gagnon, 2003). The increasing availability of proteomic technologies may assist the identification of target proteins.

Conclusions on the regulation of sperm function by ROS

There is convincing evidence that ROS can modulate sperm capacitation *in vitro*. Capacitation judged by a variety of endpoints can be accelerated by addition of ROS in different ways and retarded by trapping ROS in various ways. Similar results can be achieved with sperm from different species. However, many questions remain unanswered about the mechanism of action. What are the roles of the different ROS family members? What are the ROS receptors in the sperm cell? Do ROS activate sperm adenylyl cyclase and, if so, how? Other questions concern the source of the ROS: are they generated by the sperm themselves and, if so, how is their production controlled; or are they derived from

the environment? The remainder of this review will be concerned with the latter problem.

Source of ROS involved in regulation of sperm function

Pathways of ROS production in cells

In cells, ROS can be produced by intracellular oxidases and peroxidases, e.g. xanthine oxidase, or as an alternate product by other enzymes such as cytochrome p450 and nitric oxide synthase; but perhaps the main source in most cells is leakage of electrons from the electron transport chain (Chance *et al.*, 1979; Halliwell and Gutteridge, 1999). ROS produced by these means are usually regarded as toxic by-products of other metabolic processes and are implicated in the aetiology of disease and ageing (Raha and Robinson, 2000) although mitochondrial ROS production can be controlled and is involved in cell regulation including the initiation of apoptosis (Brookes *et al.*, 2002).

Some cells have other mechanisms to produce ROS for physiological purposes. Phagocytic leukocytes contain a NADPH oxidase which secretes superoxide into the phagocytic vesicle during the 'killing reaction'. The 'core' of this enzyme, termed gp91*phox*, contains a flavoprotein and a cytochrome b_{558} component and is activated by combining with regulatory subunits (Segal and Abo, 1993; Babior *et al.*, 1997; Babior, 1999). It has become clear that gp91*phox* is a member of a NOX enzyme family widely distributed in other cell types and involved in generating ROS for regulatory and other purposes (Lambeth *et al.*, 2000). There is current controversy about whether sperm contain NADPH oxidase activity of this sort. Another potential source of ROS production is reduction of oxygen by plasma membrane redox systems (PMRS) designed to transport reducing power across the cell plasma membrane (de Grey, 2003)

Here, I review the evidence that mammalian sperm produce ROS and contain NADPH oxidase activity and discuss some of the technical problems that complicate the investigation of these questions. A sceptical view has been adopted to encourage readers to consider the quality of the evidence critically before accepting that sperm produce ROS by a given mechanism.

The problem of leukocyte contamination

Sperm preparations usually contain some contaminating leukocytes. Activated leukocytes are prolific sources of ROS (Babior *et al.*, 1997) and leukocytes are the predominant source of ROS in crude suspensions of human sperm (Aitken and West, 1990; Aitken *et al.*, 1992b, 1994a; Kessopoulou *et al.*, 1992; Whittington and Ford, 1999) and the ROS they produce can explain the long-recognized toxicity of oxygen towards human sperm (Macleod, 1943; Whittington and Ford, 1998; Whittington *et al.*, 1999). As discussed in greater detail below, human sperm themselves make relatively small amounts of ROS, if any. Consequently sensitive detection methods have to be used and these can detect ROS production from very few leukocytes, e.g. electron paramagnetic resonance spectroscopy with the spin trap DEPMPO can detect superoxide produced by as few as 2000 activated leukocytes/ml. Experiments with sperm often use sperm concentrations of 20×10^6/ml. Therefore the presence of one activated leukocyte per 20 000 sperm would produce a detectable amount of ROS. Luminescent detection methods are more sensitive still, implying that a yet lower level of leukocyte contamination might be significant (Ford, 2003). Fortunately leukocytes can be detected very sensitively and specifically by immunocytochemistry (Kessopoulou *et al.*, 1992) or by challenging the suspension with the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine (NFMLP) whilst measuring ROS production with the luminol–horseradish peroxidase (HRP) system. Phagocytes have NFMLP receptors and respond with a large increase in ROS production whereas sperm do not (Krausz *et al.*, 1992). Leukocytes can be removed from sperm suspensions by using magnetic beads coated with antibodies against the common leukocyte antigen CD45 often combined with anti-CD15 for greater effectiveness against neutrophils and monocytes (Aitken *et al.*, 1996a; Richer and Ford, 2001). Unless leukocytes are removed in this way and their absence is confirmed by an NFMLP challenge test or by immunocytochemistry then there is a risk that any ROS detected are produced by leukocytes and claims that they are derived from sperm have to be treated with caution.

Detection of ROS production by sperm

The first indication that human sperm might make ROS was the demonstration by Macleod (1943) that catalase could protect sperm against the harmful effects of oxygen. In retrospect this was probably due to the presence of leukocytes in the sperm suspension. Other early work demonstrated that bull, boar and ram sperm could produce H_2O_2 through an oxidase acting on L-aromatic amino acids (Tosic and Walton, 1950).

Holland and Storey (1981) demonstrated that rabbit sperm produced H_2O_2 by three routes, a cytoplasmic system that required low mol. wt co-factors and rotenone-sensitive and -insensitive mitochondrial pathways. This is likely to be due to the dismutation of superoxide since a later paper demonstrated that rabbit sperm produce superoxide much of which is degraded by SOD (Holland *et al.*, 1982). Mouse sperm also produced superoxide much of which was converted to H_2O_2 by SOD (Alvarez and Storey, 1984). These studies used acetylated cytochrome C to detect superoxide and acetylated cytochrome C oxidation by cytochrome C peroxidase to detect H_2O_2. These are reliable methods but although likely to be less than for human ejaculated sperm the impact of leukocyte contamination on these results is unclear. Similar techniques were used to demonstrate the production of superoxide and H_2O_2 by human sperm (Alvarez *et al.*, 1987) but this is more likely to reflect contamination by leukocytes.

Aitken and Clarkson (1987) used the chemiluminescent probe, luminol, to demonstrate ROS production by human sperm. ROS production was associated with defective sperm function. It was greater in less dense fractions from Percoll gradients and was increased by centrifugation (Aitken and Clarkson, 1988; Aitken *et al.*, 1989a,b). Similar results were reported by Gagnon's group (Iwasaki and Gagnon, 1992). It became clear that much of the ROS detected in these experiments was produced by leukocytes as discussed above. Nevertheless a number of papers detected ROS production in sperm suspensions where no leukocytes could be detected or that had been depleted of

leukocytes with Dynabeads (Aitken and West, 1990; Aitken *et al.*, 1994b, 1996a; Whittington and Ford, 1999). ROS production by these fractions could be stimulated by phorbol esters but not by NfMLP (Krausz *et al.*, 1992, 1994). Sperm in such fractions were characterized by a high cytoplasmic volume associated with a high content of cytoplasmic enzymes including creatine kinase and glucose-6-phosphate dehydrogenase (Aitken *et al.*, 1994c; Gomez *et al.*, 1996; Gil-Guzman *et al.*, 2001). It was proposed that the presence of excess glucose 6-phosphate dehydrogenase allowed the production of greater amounts of NADPH that drove superoxide production by an NADPH oxidase-like enzyme located in the sperm plasma membrane (Aitken *et al.*, 1994c; Gomez *et al.*, 1996). In these experiments, ROS was detected with a peroxidase-enhanced luminol assay that detects mainly extracellular H_2O_2, which may derive from the dismutation of superoxide by SOD (Aitken *et al.*, 1992a).

Aitken *et al.* (1995) demonstrated that externally added NADPH could promote sperm capacitation in a similar way to hydrogen peroxide, as judged by the zona-free hamster oocyte test and tyrosine phosphorylation. Superoxide production measured by lucigenin chemiluminesence could be supported by externally added NADPH or NADH and was inhibited by flavoprotein inhibitors in a similar way to the leukocyte NADPH oxidase. Chemiluminescence was not affected by mitochondrial or diaphorase inhibitors. NADPH oxidase activity was localized in a membrane fraction. It was also possible to detect superoxide production in response to high NADPH concentrations by SOD-sensitive cytochrome C reduction, although the effect of inhibitors was not tested in this system (Aitken *et al.*, 1997; Griveau and LeLannou, 1997b). It was proposed that superoxide production by the NADPH oxidase supported a novel cAMP-dependent pathway of tyrosine phosphorylation required for capacitation (Aitken *et al.*, 1998a). Addition of increasing concentrations of NADPH to human sperm suspensions at first mimicked the pro-capacitation effects of H_2O_2 but as the concentration exceeded 5–10 mmol/l caused lipid peroxidation and DNA oxidation similar to the pathological effects of higher concentrations of H_2O_2 (Aitken *et al.*, 1998b; Twigg *et al.*, 1998a,b). In these experiments leukocytes were removed with Dynabeads and their absence confirmed by a NFMLP challenge. These observations are consistent with the presence of NADPH oxidase-like activity in the human sperm plasma membrane. Further support for this idea came from the demonstration that the gene for the NADPH oxidase family member NOX5 is expressed in human primary spermatocytes (Banfi *et al.*, 2001) although the presence of the enzyme has never been confirmed in mature sperm.

However, experiments using the alternative luminescent probe 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo [1,2a]pyrazin-3-one (MCLA) failed to demonstrate an increase in superoxide production in response to NADPH, although stimulation of superoxide production was observed in response to biological fluid ultrafiltrates (De Lamirande *et al.*, 1998a). The discrepancy might be explained by the properties of the lucigenin detection system. The detection of superoxide by lucigenin requires its reduction to a lucigenin cation radical. This reaction can be catalysed by many intracellular dehydrogenases. The lucigenin cation radical can react with superoxide to form an unstable dioxetane intermediate that decomposes to produce two molecules of *N*-methylacridone, one of which is in an excited state and emits a photon upon its return to the ground state. Unfortunately the lucigenin cation radical can also react with molecular oxygen to form superoxide and regenerate lucigenin. The superoxide can react with other lucigenin cation radicals to produce light emission. Thus lucigenin can both produce and signal superoxide in the absence of any physiological production (Figure 3a) (Liochev and Fridovitch, 1997, 1998). Richer and Ford (2001) demonstrated that such redox cycling occurred in human sperm since NADPH consumption was increased by the addition of lucigenin (Figure 3b). They were unable to detect ROS production by human sperm using EPR spectroscopy and the spin trap DEPMPO or with the sensitive H_2O_2 probe

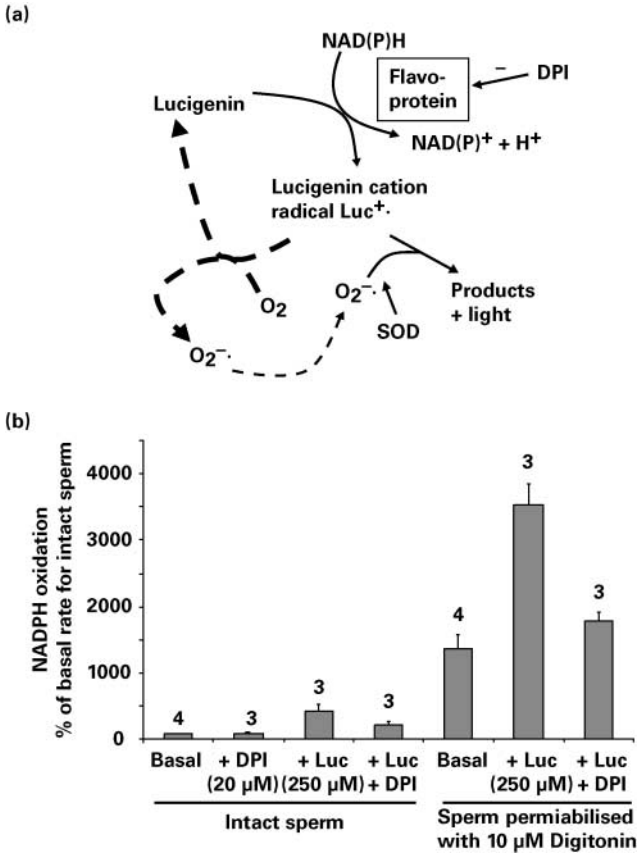

Figure 3. (a) Redox cycling of lucigenin. The detection mechanism requires lucigenin to be reduced to the lucigenin cation radical $Luc^{+}\cdot$. This can be catalysed by cellular flavoprotein dehydrogenases that can be inhibited by diphenylene iodonium (DPI). $Luc^{+}\cdot$ can react with superoxide ($O_2^{-}\cdot$) to form unstable products that decompose with the emission of light. However, $Luc^{+}\cdot$ can also react with molecular oxygen (O_2) to produce superoxide and regenerate lucigenin, and this is the favoured reaction. The $O_2^{-}\cdot$ so formed can react with further $Luc^{+}\cdot$ to produce unstable products and light. Thus lucigenin can participate in a redox cycle that can both produce and signal in the absence of any physiological source (Liochev and Fridovitch, 1997). The operation of such a cycle implies that addition of lucigenin would increase consumption of NAD(P)H and oxygen by sperm. **(b)** As predicted by the redox cycling scheme outlined in a, addition of 250 μmol/l lucigenin to suspensions of intact or permeablized human sperm caused a large increase in the rate of NADPH oxidation. The increase but not the basal rate was effectively inhibited by 25 μmol/l DPI. Data are means + SEM for the number of replicates shown above the bars. SOD = superoxide dismutase. (Modified from Richer and Ford, 2001, with permission.)

'Amplex red' and they were unable to detect consistently SOD-sensitive cytochome C reduction. In samples where cytochome C reduction was detected it was not sensitive to diphenylene iodonium, an effective although non-specific inhibitor of NADPH oxidase (Hancock and Jones, 1987). They could not detect the cytochrome b558 component of NADPH oxidase. Likewise, Armstrong *et al.* (2002) failed to detect ROS production by human sperm using spin trapping with DEPMPO, MCLA chemiluminesence or nitroblue tetrazolium reduction, although all techniques readily detected ROS production by leukocytes and they too were unable to detect cytochrome b558 in sperm. Superoxide production by rat sperm has been measured by EPR spin trapping (Kumar *et al.*, 1991) and there is one report that it has been measured by this method in human sperm (Zhang and Zheng, 1996) but it is uncertain if leukocytes were removed effectively from the sperm suspensions. For comparison, most investigators who have demonstrated positive effects of H_2O_2 on sperm have used concentrations of $\geq 10\,\mu$mol/l (see above) and such amounts should be easy to detect.

If we accept the results that purified sperm suspensions do not make superoxide in response to added NADPH, how can we explain that addition of NAD(P)H causes effects similar to the addition of H_2O_2? The answer may lie in the fact that traces of H_2O_2 are always present in NAD(P)H solutions due to autoxidation. This process is faster in acidic media (Halliwell, 1978; Halliwell and Gutteridge, 1999) but occurs at a substantial rate in Biggers–Whitten–Whittingham (BWW) medium at pH 7.4. The generation of H_2O_2 in 0–20 mmol/l solutions of NADPH in BWW buffer is shown in Figure 4. In 20 mmol/l NADPH the H_2O_2 concentration exceeded 80 μmol/l. These amounts are nearly sufficient to account for the oxidative effects of NADPH on human sperm based on comparing effects of NADPH and H_2O_2 on sperm motility and DNA oxidation

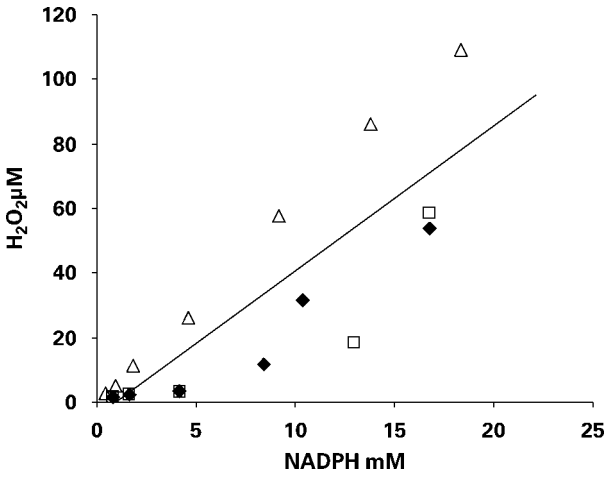

Figure 4. Hydrogen peroxide in NADPH solutions. Three batches of 20 mmol/l NADPH were prepared in Biggers–Whitten–Whitingham (BWW) buffer and were diluted to the concentrations shown. Each batch is denoted by a different symbol. Samples (200 μl) of the solutions were added to cuvettes containing 20 μmol/l Amplex Red, 2 IU horseradish peroxidase, 15 μg superoxide dismutase in 1.875 ml BWW buffer. Cuvettes were incubated at 37°C and fluorescence (excitation 570 nm, emission 585 nm) was measured 3 h after preparation of the original H_2O_2 solution. Concentrations of H_2O_2 standards and of NADPH solutions were calibrated spectrophotometrically.

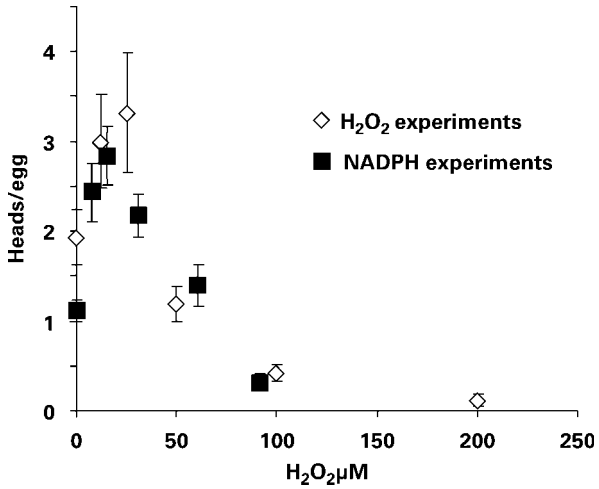

Figure 5. Effect of H_2O_2 on the penetration of zona-free hamster oocytes by human sperm. Graphs are based on data in Aitken *et al.* (1998b). H_2O_2 experiments were re-plotted without re-calculation. NADPH experiments were plotted after calculating the H_2O_2 concentration in the NADPH solutions used by Aitken *et al.* based on the regression line shown in Figure 4.

(Aitken *et al.*, 1998b) (Figure 5). However, the system is quite complex: the data in Figure 4 show the total accumulation of H_2O_2 measured by trapping it by reaction with the detection reagent Amplex Red; H_2O_2 undergoes spontaneous dismutation and the rate of this increases in solutions whose pH is >7, therefore at any given time the concentration of H_2O_2 may be considerably less than shown in Figure 4. A further factor is that on the one hand H_2O_2 will be actively metabolized by sperm whereas on the other hand any peroxidases present in sperm will increase the rate of H_2O_2 formation from NADPH. Therefore it will be difficult to prove conclusively that the spontaneous oxidation of NADPH to form H_2O_2 can explain the pro-oxidative effects of NADPH on sperm. However, this hypothesis appears to account for the published observations and removes the need to postulate NADPH oxidase activity to account for the biological effects of high extracellular NADPH concentrations on sperm.

Aitken *et al.* (2003) now envisage that human sperm contain multiple forms of redox activity that include a plasma membrane redox system (PMRS) (de Grey, 2003) capable of reducing the tetrazolium compound 2-(4-iodophenyl)-3–4-(nitrophenyl)-5–2,4-disulphophenyl)-2*H*-tetrazolium (WST-1) in the presence of the intermediate electron acceptor phenazine methosulphate (PMS) or extracellular NADH, a diaphorase-like enzyme catalysing the reduction of WST-1 by NADPH, a different enzyme capable of eliciting lucigenin chemiluminesence in the presence of NADPH and finally a calcium-sensitive system tentatively identified as NOX5 that can generate a luminescent signal from luminol–HRP.

Recent work on animal sperm has followed a similar line to that the human but with the added complication that there is a more significant contribution from the mitochondria. Epididymal sperm from rodents generated lucigenin chemiluminesence but the spontaneous rate was lower in sperm from guinea-pigs and hamsters compared to mice and rats. In a given species,

there was no difference in spontaneous chemiluminesence between different regions of the epididymis, but addition of extracellular NADPH caused a big increase in chemiluminesence with the greatest effect in caput sperm. Among rat immature germ cells the highest NADPH-dependent lucigenin chemiluminesence was seen in pachytene spermatocytes. By contrast, spontaneous H_2O_2 production measured with luminol–HRP was highest in cauda sperm and did not vary between rat germ cells at different stages of development (Fisher and Aitken, 1997; Lewis and Aitken, 2001). It was postulated that the lucigenin activity reflected the ability of the sperm to generate peroxides to act as hydrogen acceptors during nuclear condensation, whereas the ability to produce H_2O_2 reflected acquisition of the ability to capacitate (Aitken and Vernet, 1998) and these ideas were elaborated into a model incorporating ROS in motility activation and capacitation (Aitken, 2000). The generation of lucigenin-dependent chemiluminesence could be divided into a mitochondrial component due to electron leakage at complexes 1 and 2 of the respiratory chain and a contribution from a putative plasma membrane NADPH oxidase that was inhibited by a cytosolic component (Vernet *et al.*, 2001). We have since confirmed that rat sperm produce hydrogen peroxide using the Amplex Red assay and have shown that this is increased by substrates such as succinate or glycerol-1-phosphate that feed into complex 2 of the mitochondrial electron transport chain (M.Adlam and W.C.L.Ford, unpublished observations). Mouse sperm from the cauda epididymis produced a chemiluminescent signal from luminol–HRP that increased slowly from the start of the incubation to reach a plateau value proportional to the sperm concentration. This signal was inhibited by catalase, SOD and diphenylene iodonium and by omitting bicarbonate from the medium. It was not seen with caput sperm (Ecroyd *et al.*, 2003). A similar activity in rat sperm has been ascribed to formation of peroxinitrite. Activity was blocked by inhibitors of plasma membrane redox systems and of nitric oxide synthase and by scavengers of peroxinitrite. These inhibitors also suppressed the ability of the sperm to undergo the acrosome reaction, which suggests that the redox activity is physiologically important (Aitken *et al.*, 2004). Recently Aitken's group have demonstrated that the NAPDH oxidase activity reported in caput epididymal rat sperm could be accounted for by a cytochrome p450 reductase located in epithelial cells contaminating the sperm suspensions (Baker *et al.*, 2004). This undermines most of the evidence supporting NADPH oxidase activity in rodent sperm and focuses attention on the mitochondria as the main source of ROS production by sperm from these species.

Equine sperm produced H_2O_2, measured with an 'Amplex Red' assay; activity was increased by permiabilizing the cells by freezing and thawing or by adding NADPH. Activity was stimulated by A23187 but not phorbol esters or NFMLP and was greater in sperm from the low density region of a polyvinylpyrrolidone-coated silica gradient (Ball *et al.*, 2001). Bovine sperm produced increased amounts of H_2O_2 (measured with a *p*-hydroxyphenylacetic acid–HRP system) after capacitation by addition of heparin, although superoxide appeared to be the species involved in cell regulation (O'Flaherty *et al.*, 2003). Further work is required to elucidate the source of ROS production in sperm from these species.

Conclusions

There is robust evidence that ROS can promote sperm capacitation. One mechanism is by increasing protein tyrosine phosphorylation with a pattern similar to that produced by cAMP, though this cannot explain all the effects of ROS. ROS may stimulate the sperm sAC but further evidence is needed to confirm this. It remains unclear which ROS are involved but superoxide and peroxide may each have their own specific effects and the role of other ROS, notably peroxinitrite, are only beginning to be investigated. It is also unclear how the promotion of capacitation by ROS fits into the pattern of events in natural fertilization or IVF.

By contrast, the question of whether sperm can produce ROS is steeped in controversy. Evidence for NADPH oxidase activity looks increasingly uncertain. Lucigenin is an unreliable probe for superoxide and NADPH-dependent superoxide production has never been convincingly demonstrated by other methods. The pro-oxidative effects of NADPH may well be explained by the spontaneous reduction of oxygen to H_2O_2 in NADPH solutions. Although NOX gene expression has been demonstrated in human spermatocytes there is no immunological evidence of NOX protein components in mature sperm and at least two groups have recorded their failure to detect the cytochrome b558 component of *gp*91*phox*. It now appears that NADPH-dependent lucigenin chemiluminesence in rodent epididymal sperm is produced by a cytochrome p450 reductase present in contaminating epididymal epithelial cells (Baker *et al.*, 2004). The mechanism in human sperm remains to be elucidated but it is clear that the plasma membrane of these sperm contains multiple redox enzyme activities.

There is stronger evidence that animal sperm make ROS. The possibility of leukocyte contamination always has to be borne in mind but is less likely be a serious problem than for human sperm. Much of this activity may be accounted for by the mitochondria, at least in rodents. Human sperm have relatively few mitochondria and these appear to be less metabolically active than in most animal species (Ford and Rees, 1990) but it is surprising that no superoxide production from this source has been reported.

Some evidence that suspensions of human sperm produce ROS remains. Superoxide was detected with the probe MCLA after stimulation with biological fluids such as fetal cord serum ultrafiltrate but not NADPH (De Lamirande and Gagnon, 1995; De Lamirande *et al.*, 1998a). However, although these experiments used sperm from healthy donors after purification on a 95% Percoll gradient, no further steps were taken to remove leukocytes or to test for their presence. Luminol–HRP chemiluminesence was generated by some leukocyte free sperm fractions (Aitken *et al.*, 1994b). We also have to account for why removing ROS from sperm suspensions with SOD and/or catalase promotes capacitation (see Table I).

Much of the confusion that surrounds ROS production by sperm arises from the assays that have been used. Chemiluminescent assays can be extremely sensitive but are difficult to calibrate in molar units. This makes it difficult to compare results between different laboratories or to judge the physiological or pathological implications of the data. Assays that allow ROS production to be measured in molar units are to be

preferred. As well as carrying routine controls with SOD and catalase, researchers should confirm that the probe they choose to use does not perturb the system in other ways, e.g. by increasing substrate utilization. Above all, they must ensure that their sperm preparations are free from leukocytes.

Finally we should remember that only relatively small numbers of sperm reach the isthmal region of the Fallopian tube where capacitation begins *in vivo*, and that this environment is rich in antioxidant enzymes (Lapointe *et al.*, 1998; El Mouatassim *et al.*, 2000; Kaneko *et al.*, 2001).Given that the effect of ROS on sperm capacitation can be blocked by SOD and/or catalase, they must be presented extracellularly to exert their effects. If sperm produce only small amounts of ROS, could they generate sufficient to achieve the 10–100 μmol/l concentrations apparently required to promote capacitation? Alternative sources should be considered; one possibility might be that they are generated by the cumulus mass surrounding the oocyte. The observation that cumulus of bovine oocytes enhances fertilization in an oxygen-dependent manner (Tanghe *et al.*, 2003) is consistent with this idea and it seems logical that the oocyte should greet the approaching sperm with a battery of messengers to signal them to complete the process of capacitation and prepare to fertilize.

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