Reactive oxygen species and sperm physiology

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Although high concentrations of reactive oxygen species (ROS) cause sperm pathology (ATP depletion leading to insufficient axonemal phosphorylation, lipid peroxidation and loss of motility and viability), recent evidence demonstrates that low and controlled concentrations of these ROS play an important role in sperm physiology. Reactive oxygen species, such as the superoxide anion, hydrogen peroxide and nitric oxide, induce sperm hyperactivation, capacitation or the acrosome reaction in vitro. The ROS involved in these processes may vary depending on experimental conditions, but all the evidence converges to describe these events as ‘oxidative’ or ‘redox regulated’. Human sperm capacitation and acrosome reaction are associated with extracellular production of a superoxide anion that is thought to originate from a membrane ‘oxidase’. The enzymes responsible for tyrosine phosphorylation–dephosphorylation of sperm proteins are possible targets for ROS since mild oxidative conditions cause increases in protein tyrosine phosphorylation and acrosome reaction. The lipid peroxidation resulting from low concentrations of ROS promotes binding to the zona pellucida and may trigger the release of unesterified fatty acids from the sperm plasma membrane. The fine balance between ROS production and scavenging, as well as the right timing and site for ROS production are of paramount importance for acquisition of fertilizing ability.

Like all cells living under aerobic conditions, spermatozoa produce reactive oxygen species (ROS), mostly originating from normal metabolic activity. Human spermatozoa generate the superoxide anion (O$_2^-$) (Aitken and Clarkson, 1987; Alvarez et al., 1987) which spontaneously or enzymatically dismutates to hydrogen peroxide (H$_2$O$_2$) (Halliwell and Gutteridge, 1989). Owing to its low reactivity and short half-life (1 ms), O$_2^-$ is not very harmful, although reaction with its targets can produce more toxic species, such as thyl radicals (RS•) (Halliwell and Gutteridge, 1989). However, H$_2$O$_2$ is relatively stable and has a higher oxidant potential, and, being uncharged, can freely cross cell membranes (Halliwell and Gutteridge, 1989). The very low concentrations of iron present in almost any solutions are sufficient to catalyse the formation of the hydroxyl radical (•OH) from O$_2^-$ and H$_2$O$_2$; this ROS reacts with virtually any cell component, the toxic effects observed being limited only by its very short half-life (1ns) (Halliwell and Gutteridge, 1989).

Spermatozoa and seminal plasma contain a battery of ROS scavengers, including enzymes such as superoxide dismutase (SOD) (Nissen and Kreyssel, 1983; Alvarez et al., 1987), catalase (Jeulin et al., 1989) and the glutathione peroxidase/reductase system (Alvarez and Storey, 1989), and also a variety of substances with SOD-like or catalase-like activities (Zini et al., 1993), such as α-tocopherol, ascorbic acid, glutathione (Halliwell and Gutteridge, 1989), pyruvate (de Lamirande and Gagnon, 1992b), taurine, hypotaurine and albumin (Alvarez and Storey, 1983).

The balance between ROS generation and scavenging, as well as the moment and the location at which spermatozoa come into contact with ROS determine the effects observed. Excessive ROS generation that overcomes the ROS scavenging ability of human spermatozoa appears to be related to male infertility (Iwasaki and Gagnon, 1992). High concentrations of ROS produced by spermatozoa themselves (Aitken and Clarkson, 1987; Alvarez et al., 1987) or by the combinations of xanthine plus xanthine oxidase (Aitken et al., 1993) or of iron (Fe$^{2+}$) plus ascorbic acid (Aitken et al., 1989) induce the formation of toxic lipid peroxides (Jones et al., 1979; Windsor et al., 1993) and compromise sperm viability. Lower concentrations of ROS cause a reversible loss of sperm motility that is due to intracellular ATP depletion and insufficient axonemal protein phosphorylation (de Lamirande and Gagnon, 1992a,b). Finally, concentrations of ROS low enough not to affect the percentage of sperm motility over a 6 h incubation cause a reduction in the potential of spermatozoa to acquire hyperactivated motility and to undergo the acrosome reaction when challenged with the calcium ionophore A23187 (Griveau et al., 1995a). H$_2$O$_2$ is the primary ROS responsible for the loss of sperm functions since catalase, which selectively degrades this ROS, is the only scavenger to confer complete protection to spermatozoa (de Lamirande and Gagnon, 1992a,b; Aitken et al., 1993; Griveau et al., 1995a).

Although, in male reproduction, ROS are known mostly for their detrimental effects on sperm functions, there is now increasing evidence to suggest that, as has been observed in other cell types (for example, neutrophils) (Fialkow et al., 1993) or organelles (for example, endoplasmic reticulum) (Bauskin et al., 1991), very low and controlled concentrations of ROS participate in signal transduction mechanisms. Sperm capacitation and acrosome reaction are complex processes also regulated by signal...
Hyperactivation and capacitation

The first experimental evidence for involvement of ROS in human sperm physiology comes from the observation that spermatozoa exposed to O$_2^•-$ (xanthine plus xanthine oxidase plus catalase) show higher hyperactivation and capacitation than do those treated with fetal cord serum (a capacitation inducer) or Ham’s F-10 medium alone, and that SOD prevents these effects (de Lamirande and Gagnon, 1993a). Sperm capacitation induced by biological fluids, such as fetal cord serum or follicular fluid (whole or their dialysed or ultrafiltrate counterparts), is also prevented by SOD which further supports the biological relevance of these findings (de Lamirande and Gagnon, 1993b,c). Furthermore, the different rates of sperm capacitation induced by biological fluids are inversely correlated with the potential of these fluids to scavenge O$_2^•-$ (de Lamirande et al., 1994).

Human spermatozoa incubated under capacitating conditions (Ham’s F-10 supplemented with fetal cord serum ultrafiltrate) produce higher concentrations of intra- and extracellular O$_2^•-$ than do control spermatozoa (medium alone) (de Lamirande and Gagnon, 1995a,b). The increase in intracellular O$_2^•-$ concentration (detected by FACScan measurements using dihydrorhodamine) is not prevented by SOD and therefore may reflect an increased metabolic activity rather than a direct involvement in sperm capacitation (de Lamirande and Gagnon, 1995b). However, the increased extracellular O$_2^•-$ production (detected by chemiluminescence measurements with a Cypridina luciferin analogue) that starts immediately at the beginning of incubation and lasts for more than 4 h is essential for the manifestation of sperm capacitation (de Lamirande and Gagnon, 1995a).

The involvement of ROS in the acquisition of fertilizing ability may not be limited to O$_2^•-$ or to human spermatozoa. Addition of catalase to human spermatozoa incubated in B2 Menezo medium reduces both the hyperactivation (by 43%) and the A23187-induced acrosome reaction (by 46%) without affecting the percentage of motile or viable cells (Griveau et al., 1994). Conversely, a low concentration of H$_2$O$_2$ (50 µmol l$^{-1}$) accelerates the development of sperm hyperactivation (by 37%) and capacitation (by 43%) after incubation for 3 h (Griveau et al., 1994). This same concentration of H$_2$O$_2$ induces hyperactivation and capacitation of human spermatozoa incubated in Ham’s F-10 medium (P. Leclerc, E. de Lamirande and C. Gagnon, unpublished). Since there is no direct evidence yet for a genuine production of H$_2$O$_2$ by capacitating human spermatozoa, we can hypothesize that H$_2$O$_2$ may originate from the dismutation of O$_2^•-$ generated by spermatozoa (de Lamirande and Gagnon, 1995a). The concentration of H$_2$O$_2$ (50 µmol l$^{-1}$) needed to induce sperm capacitation (Griveau et al., 1994) corresponds to a concentration of ROS far in excess of that measured in capacitating sperm suspensions (de Lamirande and Gagnon, 1995a), which would suggest that H$_2$O$_2$ is perhaps not as efficient as O$_2^•-$ in supporting capacitation, or that ROS generated in the immediate environment of the target molecule on the sperm surface reach much higher concentrations than those effectively measured in whole cell suspensions.

Bize et al. (1991) proposed that H$_2$O$_2$ is involved in hamster sperm capacitation. Catalase, but not SOD, strongly (by 88%) reduces the rate of acrosome reaction of spermatozoa incubated for 5 h in the presence of adrenaline, a substance known to stimulate this process, but also to generate H$_2$O$_2$ when kept in aerobic conditions. Furthermore, addition of H$_2$O$_2$, either directly or through enzymatic generation by the combination of glucose and glucose oxidase, stimulates the acrosome reaction by 85–150%, depending on the time of observation (Bize et al., 1991). Finally, the reduction in the rate of acrosome reaction is less important if catalase is added after a delay than when added initially, suggesting that H$_2$O$_2$ is involved in hamster sperm capacitation rather than acrosome reaction. There is no direct evidence for the production of ROS by capacitating hamster spermatozoa, but it is conceivable that, in vivo, H$_2$O$_2$ is generated as such or that it originates from the dismutation of O$_2^•-$, or even that spermatozoa are subjected to ROS generated by the fluids or the cells of the female reproductive tract.

In mice, the combination of xanthine plus xanthine oxidase causes a significant increase in sperm hyperactivation and capacitation (Fig. 1; H. Jiang, E. de Lamirande and C. Gagnon, unpublished). Individually, SOD or catalase completely prevent these effects, whereas together they decrease capacitation to rates even lower than those observed in control spermatozoa. These results suggest that, under these conditions, both O$_2^•-$ and H$_2$O$_2$ may be needed to promote mouse sperm...
hyperactivation and capacitation. Whether these two ROS act together or sequentially, and on the same or different targets remains to be elucidated.

Nitric oxide (NO•) is a free radical of relatively long half-life (7 s) that inhibits human sperm motility at high concentrations (Zini et al., 1995). However, low concentrations of NO•, slowly released from agents such as diethyline NONOate and spermine-NONOate (0.1 mmol l-1), induce a 85-100% increase in human sperm capacitation (as measured by the lysosphosphatidylincholined induced acrosome reaction) without affecting sperm motility (Zini et al., 1995). Catalase blocks NO• induced capacitation which suggests a complex mechanism of action involving H2O2. Human spermatozoa from healthy volunteers do not possess any detectable NO• synthase activity and even high concentrations of an inhibitor of this enzyme do not block sperm capacitation (Zini et al., 1995). Therefore, we may hypothesize that, if NO• plays a role in sperm capacitation in vivo, NO• must originate from the female reproductive tract, which contains NO• synthase activity (Sladek et al., 1993). As it was observed for other ROS, there may be species differences, and experiments performed using high concentrations (5-20 mmol l-1) of a NO• synthase inhibitor suggest that NO• may be involved in hamster sperm hyperactivation (Yeoman, 1994).

The studies presented above converge to demonstrate that sperm capacitation is an oxidative process; low concentrations of ROS exogenously added or minute amounts generated by spermatozoa are needed to trigger this phenomenon in vivo. The involvement of a specific ROS may depend on the incubation conditions (for example, medium, inducer) and on the species of spermatozoa. Furthermore, fluids or cells from the female reproductive tract may also produce ROS or promote the formation of ROS by spermatozoa. The fact that the concentration of oxygen in these fluids remains low except at the time of ovulation (Maas et al., 1976) further substantiates the physiological relevance of oxygen and its metabolites in sperm functions.

Binding to the zona pellucida and acrosome reaction

The concept that mild oxidative conditions promote or are even required for binding of spermatozoa to the zona pellucida and acrosome reaction are recent. Lipid peroxidation is generally associated with decreased sperm function and viability (Jones et al., 1979; Aitken et al., 1989, 1993; Griveau et al., 1995a), but has also a significant enhancing effect on the ability of spermatozoa to bind both homologous and heterologous zonae pellucidae (Aitken et al., 1989). Kodama et al. (1996) demonstrated that treatment of mouse spermatozoa with low concentrations of iron (Fe2+, 0.4 mmol l-1) plus ascorbic acid (2 mmol l-1) in the presence of albumin (10 mg l-1) induces a 4.6-fold increase in their content of thiobarbituric acid reactive substances (a measure of lipid peroxidation) and improves their fertilizing potential by 50%. This increased formation of two-cell embryos is not due to a change in sperm motility parameters (including hyperactivation) or capacitation but appears related to a marked increase in binding of spermatozoa to mouse zona pellucida.

ROS are also involved in the process of acrosome reaction. In human spermatozoa capacitated in BWW medium supplemented with bovine serum albumin, catalase prevents the A23187-induced acrosome reaction, and the binding and penetration of zona-free hamster oocyte, whereas H2O2 (50 µmol l-1) stimulates these processes (Aitken et al., 1995). There is, however, no direct evidence yet for the generation of this ROS by spermatozoa during the A23187-induced acrosome reaction. On the other hand, Griveau et al. (1995b) reported that human spermatozoa capacitated in B2 Menezo medium have a four- to fivefold increase in production of O2• during the A23187-induced acrosome reaction and that SOD blocks this process. Finally, when the membrane disturbing agent lysosphosphatidylincholine is used to induce the acrosome reaction in spermatozoa capacitated in Ham’s F-10 supplemented with fetal cord serum, catalase (C. Tsang, E. de Lamirande and C. Gagnon, unpublished), but not SOD (de Lamirande and Gagnon, 1993a), prevents the acrosome reaction, indicating that, under these conditions, H2O2 is the ROS involved.

As was the case for capacitation, the results cited above differ as to the ROS involved in the acrosome reaction. Differences in the methods used to capacitate spermatozoa and to induce the acrosome reaction could again explain these discrepancies. However, the important message is that capacitation and the acrosome reaction are oxidative processes that involve H2O2, O2• or both, and that these ROS are probably generated by spermatozoa.

Reactive oxygen species generating system in spermatozoa

The enzymatic system responsible for the production of O2• by spermatozoa and linked to sperm hyperactivation, capacitation and acrosome reaction is currently unknown. Many of the events related to these processes (for example increase in cAMP concentration, stimulation of protein kinases and phospholipases, calcium influx) (Yanagimachi, 1994) also occur during NADPH oxidase activation in neutrophils (Morel et al., 1991). The data accumulated so far suggest the presence of an oxidase on the sperm plasma membrane and its activation during capacitation (de Lamirande and Gagnon, 1993b, 1995a) and acrosome reaction (Aitken et al., 1995). Other types of cell such as fibroblasts (Meier et al., 1991) and endothelial cells (Matsubara and Ziff, 1986) generate O2• under specific conditions and in the presence of NADH, NADPH or both factors. The fact that a high concentration of phorbol myristate acetate (10 µmol l-1) stimulates the acrosome reaction and O2• production of capacitated spermatozoa to rates similar to those due to A23187 (Griveau et al., 1995b) suggests that a protein kinase C may be involved in the activation of this hypothetical oxidase. The location, as well as requirements for activation, cofactors (NADH, NADPH, or others) of this oxidase, and its similarities with the NADPH oxidase of neutrophils, remain to be established. The amounts of O2• generated by capacitating spermatozoa are at least three orders of magnitude lower (on a per cell basis) than those of activated neutrophils (de Lamirande and Gagnon, 1995a), suggesting that the sperm oxidase may be quite different from that of neutrophils.

Mechanisms of action of reactive oxygen species

In many of the experiments cited above, the asynchrony between the time of ROS generation and the time required for the manifestation of their effects must be emphasized. Capacitation
studies provide a very good example of this asynchrony since exogenously added ROS (xanthine plus xanthine oxidase plus catalase or H₂O₂) are present in the sperm suspension only for the first 30–60 min of incubation, but sperm capacitation occurs over a much longer period (Bize et al., 1991; de Lamirande and Gagnon, 1993a,b; Griveau et al., 1994). Furthermore, even though endogenous O₂•- production by capacitating human spermatozoa starts at the beginning of the incubation period, sperm hyperactivation peaks 1–3 h later and capacitation progressively increases over the 6 h incubation (de Lamirande and Gagnon, 1995). These data suggest that ROS may be some of the first mediators that initiate a cascade of reactions leading to acquisition of fertilizing ability. Extracellular, but not intracellular, production of O₂•- by spermatozoa is important for sperm capacitation (de Lamirande and Gagnon, 1995) and acrosome reaction (Griveau et al., 1995). Lipids and components of signal transduction mechanisms are potential candidates for ROS attack.

It is well recognized that, owing to its low reactivity, O₂•- rarely induces lipid peroxidation unless it is produced or solubilized in the hydrophobic membrane interior or is present in its protonated form (HO₂••) (Halliwell and Gutteridge, 1989). Nevertheless, Griveau et al. (1995b) report that direct addition of potassium superoxide (0.5 mmol l⁻¹) to capacitated spermatozoa causes a 50% increase in the unesterified fatty acid content of these cells, an effect that is, however, not sufficient to induce the acrosome reaction. The fact that α-tocopherol causes a dose-dependent decrease in the rate of acrosome reaction further indicates that mild oxidation of membrane lipids may play a role in this process (Griveau et al., 1995). Even though the amount of superoxide used in these experiments is two orders of magnitude higher than that produced by spermatozoa during the acrosome reaction, and that 10 mmol α-tocopherol l⁻¹ is needed to decrease the rate of acrosome reaction by 50%, it is proposed that the essential role for O₂•- in the induction of human sperm acrosome reaction is in triggering an increase in membrane fluidity through the release of unesterfied fatty acids (Griveau et al., 1995). The requirement of lipid oxidation for capacitation and acrosome reaction may vary between species, since agents such as adrenaline and albumin accelerate capacitation in hamster spermatozoa (Bize et al., 1991) while being at the same time inhibitors of lipid peroxidation (Alvarez and Storey, 1983).

Sperm capacitation and acrosome reaction involve tyrosine phosphorylation of specific proteins (Naz et al., 1991; Tesarik et al., 1993), a post-translational modification of proteins subjected to redox regulation in many types of cell and system (Bauskin, 1991; Fialkow et al., 1993). When spermatozoa are incubated in conditions that promote capacitation and superoxide production, there is an increase in tyrosine phosphorylation of two major human sperm proteins of 81 and 105 kDa (Leclerc et al., 1996). However, tyrosine phosphorylation of different proteins (82, 116, 133, 159, 220 and 222 kDa) occurs during the acrosome reaction induced by A23187 (Aitken et al., 1995). This last effect is inhibited by catalase but reproduced in spermatozoa challenged with low concentrations of H₂O₂ or other oxidizing agents (Aitken et al., 1995).

Tyrosine kinases and phosphatases, the two types of enzyme involved in the regulation of tyrosine phosphorylation, are susceptible to redox regulation. For example, the activity of the tyrosine kinase Ltk present in the endoplasmic reticulum of

Table 1. Proposed roles of reactive oxygen species (ROS) in sperm functions

<table>
<thead>
<tr>
<th>ROS</th>
<th>Sperm function</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide anion</td>
<td>Hyperactivation</td>
<td>Human</td>
<td>de Lamirande and Gagnon, 1993a, b, 1995; de Lamirande et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Capacitation</td>
<td>Human</td>
<td>de Lamirande and Gagnon, 1993a, b, 1995; de Lamirande et al., 1993</td>
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<tr>
<td>A23187-induced</td>
<td>Hyperactivation</td>
<td>Human</td>
<td>Griveau et al., 1995</td>
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<tr>
<td>acrosome reaction</td>
<td>Capacitation</td>
<td>Human</td>
<td>Griveau et al., 1994</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>Hyperactivation</td>
<td>Human</td>
<td>Bize et al., 1991</td>
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<tr>
<td></td>
<td>Capacitation</td>
<td>Human</td>
<td>Griveau et al., 1994</td>
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<tr>
<td>A23187-induced</td>
<td>Hyperactivation</td>
<td>Human</td>
<td>Aitken et al., 1995</td>
</tr>
<tr>
<td>acrosome reaction</td>
<td>Capacitation</td>
<td>Human</td>
<td>Aitken et al., 1995</td>
</tr>
<tr>
<td>LPC-induced</td>
<td>Hyperactivation</td>
<td>Human</td>
<td>C. Tsang, E. de Lamirande and C. Gagnon, unpublished</td>
</tr>
<tr>
<td>acrosome reaction</td>
<td>Capacitation</td>
<td>Human</td>
<td>Aitken et al., 1995</td>
</tr>
<tr>
<td>Zona pellucida</td>
<td>Hyperactivation</td>
<td>Hamster</td>
<td>Yeoman, 1994</td>
</tr>
<tr>
<td>binding and penetration</td>
<td>Capacitation</td>
<td>Human</td>
<td>Zini et al., 1995</td>
</tr>
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E. de Lamirande et al.

lymphocytes increases upon exposure to oxidants (Bauskin et al., 1991). However, oxidation of two specific sulfhydryl groups at the active site of tyrosine phosphatases by \( \text{H}_2\text{O}_2 \) inactivates these enzymes (Hecht and Zick, 1992). This mechanism is potentially important since reducing conditions caused by the presence of agents such as dithiothreitol prevent the acrosome reaction in guinea-pig (Fleming et al., 1982), hamster (Bize et al., 1991) and human (Aitken et al., 1995) spermatozoa. It remains to be established whether the increase in protein tyrosine phosphorylation observed during sperm capacitation and acrosome reaction results from a decrease in phosphatase or an increase in kinase activities.

Tyrosine phosphorylation of proteins is the only component of the signal transduction mechanism for which a redox regulation linked to sperm acrosome reaction has been demonstrated (Aitken et al., 1995). It is, however, possible that, as observed in various systems, oxidative conditions activate or inhibit other enzymes or mechanisms essential for sperm capacitation and acrosome reaction. For example, in pig coronary artery, micromolar concentrations of \( \text{H}_2\text{O}_2 \) inactivate calcium pumps (Grover et al., 1992), and, in endothelial cells, oxidants and lipid hydroperoxides modulate the activity of protein kinase C and the formation of diacylglycerol (Taher et al., 1993). It is therefore conceivable that more than one component of signal transduction pathways is modified by oxidation so that sperm capacitation and acrosome reaction proceed.

**The balance between reactive oxygen species production and scavenging**

If the fine balance between the production and scavenging of ROS as well as the adequate timing for ROS production are not respected, normal sperm functions may be compromised. Spontaneous sperm hyperactivation occurs in 18% (22/120)
of semen samples from patients whose spermograms are considered ‘normal’ according to the World Health Organization criteria (de Lamirande and Gagnon, 1993c). The O2−* scavenging capacity of seminal plasma and spermatozoa from these patients are, respectively, 37% and 40% lower than those found in seminal plasma and spermatozoa from normal men (de Lamirande and Gagnon, 1993c,d). This spontaneous hyperactivation in semen could impair sperm transport along the lower female reproductive tract, as observed in mice (Olds-Clarke and Wivel, 1992), or lead to a premature capacitation.

Mild and even undetectable oxidative stress for extensive periods could alter the condensation state of sperm DNA, an effect that would not be visible before fertilization. Sperm maturation in the epididymis is characterized by a gradual oxidation of thiol groups, which, in the sperm head, results in the extensive disulfide formation in protamines that maintains chromatin compaction and protects DNA from physical or chemical damage (Rousseau and Rousseau-Prevost, 1995). Spermatozoa from oligospermic men are significantly less reduced by dithiothreitol than are those from fertile men (Rufas et al., 1991), and the extreme DNA stability that ensues could prevent glutathione-induced DNA decondensation inside the oocyte (Rodriguez et al., 1985). These results support the hypothesis that exposure of spermatozoa to prolonged mild oxidation or premature ageing in vitro could lead to overoxidation of thiol groups and ‘hypercondensation’ of DNA (Rufas et al., 1991).

Conclusion

Recent experimental evidence, summarized in Table 1 and Figure 2, demonstrates that, although excessive concentrations of ROS cause sperm pathology, low and controlled concentrations of the same ROS play an important role in sperm physiology and, more precisely, in the acquisition of fertilizing ability. The ROS involved in sperm hyperactivation, capacitation and acrosome reaction in vitro may vary depending on experimental conditions but all data converge to define these processes as ‘oxidative’ or ‘redox regulated’. Spermatozoa themselves appear to be the source of ROS but contribution of fluids and cells from the female genital tract is also possible, although not yet demonstrated. The fine balance between ROS production and scavenging as well as the right timing for ROS production are of paramount importance for the acquisition of fertilizing ability by spermatozoa.

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