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Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization

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Capacitation is defined as the series of transformations that spermatozoa normally undergo during their migration through the female genital tract, in order to reach and bind to the zona pellucida, undergo the acrosome reaction, and fertilize the egg. During this process, extensive changes occur in all sperm compartments (head and flagellum; membrane, cytosol, cytoskeleton), factors originating from epididymal fluid and seminal plasma are lost or redistributed and membrane lipids and proteins are reorganized; ion fluxes induce biochemical modifications and controlled amounts of reactive oxygen species are generated; spermatozoa develop hyperactivated motility; and complex signal transduction mechanisms are initiated. The main purpose of capacitation is to ensure that spermatozoa reach the eggs at the appropriate time and in the appropriate state to fertilize these eggs, by finely controlling the rate of the changes necessary to prime spermatozoa and by activating all the mechanisms needed for the subsequent acrosome reaction. The reversibility of some of the mechanisms leading to sperm capacitation may therefore be a very important aspect of the fine regulation and perfect timing of this process.

Key words: cAMP/human spermatozoa/protein kinase/reactive oxygen species/sperm hyperactivation

Introduction

Spermatozoa are very peculiar cells because they undergo several important maturation steps throughout their life, resulting from their interaction with the different environments through which they migrate and the various functions that they are expected to accomplish. Furthermore, spermatozoa are compartmentalized cells, and the rate at which modifications occur within the various subcellular regions (acrosomal and post-acrosomal portions of the head, midpiece and distal portions of the flagellum) may vary widely. In the epididymis, spermatozoa acquire the potential for motility and fertility by the action of epithelial cell secretions and constituents of the luminal fluids. At ejaculation, they become motile and are 'conditioned' by seminal fluid constituents although they are not yet able to fertilize oocytes. During their migration through the female genital tract, spermatozoa undergo a series of controlled biochemical and membranous changes, globally termed capacitation, that enables them to reach and bind to the zona pellucida, undergo the acrosome reaction, penetrate the egg vestments and, finally, fuse with the oocyte.

Chang (1951) and Austin (1951) discovered that spermatozoa are unable to fertilize eggs unless they reside in the female genital tract for a specific period of time, and named 'capacitation' the ensemble of transformations these cells must undergo to become fertile. Since then, the physiological process of capacitation has been regarded as a maturation step, an activation or a 'switching on' process, a residential period in the female reproductive tract, a 'rubbing off' of the protective coat from sperm plasma membrane, a selection of a competent sperm population, etc. Furthermore, there is still controversy in the definition of capacitation because some investigators include the acrosome reaction in this process while others do not (Chang, 1984). However, results on the reversibility, prerequisites, and events associated with these two processes support the concept that they are clearly different maturational steps. Capacitation is a reversible phenomenon, which means that treatment of capacitated spermatozoa with specific substances or biological fluids, such as seminal plasma, reduces their ability to fertilize oocytes and therefore 'decapacitates' these cells; a continued incubation or a longer residence in the female genital tract is needed to overcome the inhibition and 'recapacitate' spermatozoa (Bedford and Chang, 1962). On the other hand, since the acrosome reaction is an exocytotic process, it cannot be stopped or reversed once it is induced (Yanagimachi, 1994). Furthermore, the specific ionic requirements (for example, the concentrations of bicarbonate and calcium; Fraser, 1995a) for the promotion and support of capacitation and acrosome reaction further segregate these two processes. In the present review, we will consider capacitation as the ensemble of transformations that spermatozoa must undergo after ejaculation up to the time they reach and bind to the zona pellucida. Special attention will also be given to sperm hyperactivation which is the specific, poorly progressive, and vigorous type of motility that is closely associated with capacitation.

Several experimental models have been used to study in-vivo sperm capacitation including hamsters (Smith *et al.*, 1987), mice (Demott and Suarez, 1992), rabbits (Suarez *et al.*,

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1983), sheep, cows and pigs (Hunter, 1987), etc. These investigations have the advantages of being more physiologically relevant because they closely follow sperm transport and modifications in their real space and time coordinates. However, because capacitation occurs internally within the female genital tract, these experiments have the disadvantages of being costly and difficult to perform. Furthermore, the very low numbers of spermatozoa that reach the oviduct and later the site of fertilization (Barratt and Cooke, 1991; Suarez *et al.*, 1992) do not allow quantitative measurements of sperm biochemical parameters (composition of cell membranes, enzyme activities, etc). Finally, for ethical reasons, there are strict experimental limitations for this type of study in humans. For these reasons, even though in-vitro systems carry inherent disadvantages being more or less similar to the in-vivo situation in terms of environmental conditions, time course of events, etc., such in-vitro models have been developed in many animal species such as mouse (Toyoda *et al.*, 1971; Neill and Olds-Clarke, 1987), hamster (Yanagimachi, 1982), rabbit (Young and Bodt, 1994), boar (Suarez *et al.*, 1992), bull (Parrish *et al.*, 1988), monkey (Wolf *et al.*, 1989; VandeVoort *et al.*, 1994) and man (Burkman, 1990), to study sperm capacitation, its requirements, characteristics and mechanisms.

In this review, we will attempt to summarize the present knowledge on sperm capacitation and hyperactivation. Because of the enormous amount of work done in this field of research, a special emphasis will be given to human spermatozoa whereas observations obtained on other species will be used mainly to support or complement these data. We will describe the methods used to evaluate sperm capacitation, in-vivo sperm capacitation, sperm hyperactivation, some of the events associated with sperm capacitation (ion fluxes, modifications of membrane lipids and antigens, generation of reactive oxygen species), and finally the regulation of sperm capacitation including the signal transduction mechanisms involved.

Measurement of capacitation

The ideal probe to assess sperm capacitation should not interfere with sperm physiology and should be easily available and specific for capacitated spermatozoa. Unfortunately, such an ideal compound does not exist. Most of the time, sperm capacitation is evaluated by changes in the expression and/or distribution of cell surface molecules, staining with chlortetracycline, or treatment of spermatozoa with substances known to induce the acrosome reaction preferentially in capacitated spermatozoa.

Redistribution, modification, removal or appearance of glycoproteins, surface sugars and sugar binding proteins, as well as other various antigens, occur during capacitation. Such modifications can be assessed by the use of specific antibodies, lectins, sugars conjugated to albumin, etc. (Cohen-Dayag and Eisenbach, 1994). The following two examples illustrate such modifications for which a correlation with sperm function has been proposed. Incubation of human spermatozoa under capacitating conditions was associated with the appearance of D-mannose binding lectins on the human sperm head, which was dependent on the reduction of the sterol (cholesterol mainly) content of cell membranes (Benoff *et al.*, 1993). Measurement of these D-mannose binding lectins might be physiologically relevant since these lectins are considered as putative zona pellucida recognition molecules on sperm membrane and a strong correlation was established between the transposition of these lectins to the plasma membrane and success in in-vitro fertilization (IVF) (Benoff *et al.*, 1993). There is also an increased expression, or an unmasking, of a cellular form of fibronectin on human sperm surface during capacitation (Fusi and Bronson, 1992). It was suggested that fibronectin is involved in sperm–oocyte interactions since anti-fibronectin antibodies greatly reduced the adhesion and penetration of zona-free hamster eggs by human spermatozoa (Fusi and Bronson, 1992; Hoshi *et al.*, 1994).

The evaluation of capacitation and acrosome reaction by changes in chlortetracycline fluorescence patterns was first performed with mouse spermatozoa (Ward and Storey, 1984) and was subsequently applied to human (Lee *et al.*, 1987; DasGupta *et al.*, 1993; Perry *et al.*, 1995), monkey (Kholkute *et al.*, 1990) and bull (Fraser *et al.*, 1995) spermatozoa. Chlortetracycline is an antibiotic, the fluorescence of which changes when it chelates membrane-associated divalent cations (mainly calcium) (Hallett *et al.*, 1972). This probe offers the advantage of measuring directly the percentages of non-capacitated, capacitated and acrosome-reacted spermatozoa in the same preparation. However, possibly due to the novelty of using chlortetracycline with human spermatozoa and/or the need of a special fluorescence filter (λ_{ex}: >515 nm; λ_{em}: 405 nm) for this assay, researchers often favoured other methods to measure human sperm capacitation.

Induction of the acrosome reaction with specific substances used at concentrations that allow discrimination between capacitated and non-capacitated spermatozoa was generally used to assess human sperm capacitation. The ideal and physiological agonist to induce the acrosome reaction is the zona pellucida glycoprotein (ZP3) that specifically binds to capacitated spermatozoa and provokes the acrosome reaction in these cells. Although solubilized zonae pellucidae and purified ZP3 have been used, these products are not easily available. Furthermore, even though human recombinant ZP3 induced the acrosome reaction in capacitated spermatozoa (Aitken *et al.*, 1995; Barratt and Hornby, 1995), such a product is not commercially available yet.

Progesterone can initiate the acrosome reaction in human spermatozoa *in vitro* and may be one of the substances that naturally induce this process (Meizel *et al.*, 1990; Cross, 1996). However, the use of progesterone is limited since it was also shown to capacitate human spermatozoa (Foresta *et al.*, 1992; Uhler *et al.*, 1992; Emiliozzi *et al.*, 1996).

The divalent cation ionophore A23187 has been widely used to induce the acrosome reaction in capacitated human spermatozoa. However, A23187 is potentially cytotoxic and the concentrations have to be carefully chosen according to the conditions of incubation. Although up to 10 μM A23187 was employed (Bielfeld *et al.*, 1991), concentrations of 1.25–2.5 μM (in the presence of albumin) were found more suitable to promote the acrosome reaction and cause maximal sperm/oocyte interaction and penetration without causing a

major drop in human sperm motility and viability (Aitken *et al.*, 1993).

Lysophosphatidylcholine (LPC), a membrane disturbing agent found at high concentrations in the region of the female genital tract where fertilization takes place (Grippo *et al.*, 1994), was also found to induce the acrosome reaction in capacitated guinea pig (Yanagimachi and Suzuki, 1985) and bull (Parrish *et al.*, 1988) spermatozoa. LPC was successfully used to assess human sperm capacitation (de Lamirande and Gagnon, 1993a,b, 1995a; Zini *et al.*, 1995) but, as for A23187, the concentrations of LPC and albumin in the incubation medium need to be carefully chosen to prevent potential toxic effects (Jarvi *et al.*, 1993). The differentiating effect of LPC on capacitated and non-capacitated spermatozoa obtained in bull spermatozoa (Parrish *et al.*, 1988) was reproduced in human spermatozoa (Figure 1a). The rate of LPC-induced acrosome reaction was higher in capacitated than in non-capacitated spermatozoa for all LPC concentrations tested, but motility and viability of spermatozoa were significantly affected when the incubation medium contained ⩾200 μM LPC. LPC appeared as reliable as A23187 to evaluate human sperm capacitation as indicated by the close correlation between the percentages of capacitation determined by the LPC- and the A23187-induced acrosome reaction (Figure 1b; Zini *et al.*, 1995).

There is presently no ideal method to measure human sperm capacitation. The methods that are currently available have their advantages and their limits. Therefore, they should be considered as tools to study sperm capacitation or compare fertile and infertile sperm samples while awaiting adequate recombinant human ZP3.

In-vivo capacitation

Spermatozoa are capacitated and become hyperactivated during their journey through the female reproductive tract. However, the exact location, time course and mechanisms regulating these events are still unknown. Data from in-vivo studies on humans are scarce in comparison with those of in-vitro studies and relate to experiments performed with cervical mucus, tissues recovered at the time of abdominal hysterectomy, or primary culture of cells originating from these tissues.

In the human, ejaculated spermatozoa are deposited in the vagina and must first penetrate the cervical mucus, in which they may lose some of their surface-adsorbed materials. Human cervical mucus induced sperm capacitation (Zinamen *et al.*, 1989) and hyperactivation (Zhu *et al.*, 1992, 1994) but not the acrosome reaction (Bielfield *et al.*, 1991), therefore preserving sperm function. However, spermatozoa that become capacitated in the cervical mucus may not be those that reach the fertilization site.

The very small numbers of human spermatozoa recovered from the uterus and the presence of neutrophils often invading this area (Barratt *et al.*, 1990; Barratt and Cooke, 1991) suggested that the uterus acts more as a conduit than as a reservoir for spermatozoa. However, the human endometrium may play a role in sperm maturation as it secretes a 54 kDa sialic acid-recognizing protein that specifically binds to the

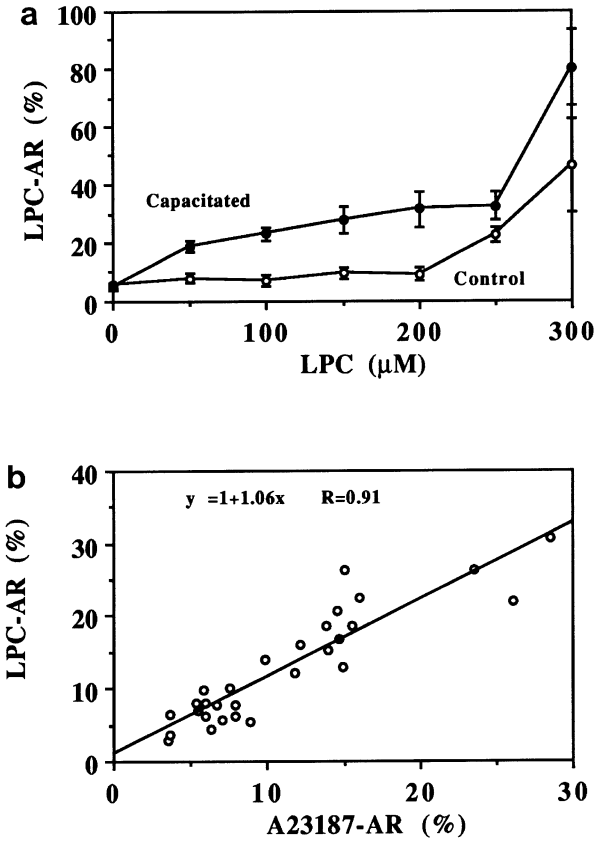

Figure 1. Lysophatidylcholine (LPC) as an inducer of the acrosome reaction in capacitated human spermatozoa. (**a**) Percoll-washed human spermatozoa were incubated at 37°C for 3.5 h in Ham's F-10 medium in the absence (control; empty circles) or presence (capacitated; filled circles) of fetal cord serum ultrafiltrate (7.5%, v/v; 3 kDa exclusion limit) (de Lamirande and Gagnon, 1993b, 1995). Bovine serum albumin (3 mg/ml) and various concentrations of LPC were then added and (30 min later) spermatozoa were washed in HEPES-balanced saline and fixed in ethanol. Sperm acrosomal status was evaluated using the fluorescein isothiocyanate conjugated–*Pisum sativum* agglutinin. Values are mean ± SEM of four experiments done with different sperm samples and on different days. The rate of LPC-induced acrosome reaction (LPC-AR) was higher ($P < 0.05$) in capacitated than in control spermatozoa for all LPC concentrations, but sperm motility and viability were significantly decreased in the presence of ⩾200 μM LPC. A 100 μM LPC concentration (in the presence of albumin) efficiently segregated capacitated from control spermatozoa without affecting cell motility or viability. (**b**) Human spermatozoa were incubated in various capacitating or non-capacitating conditions. Each sample was then divided in two portions and the acrosome reaction was triggered by LPC (100 μM) or A23187 (1 μM), both in the presence of bovine serum albumin (3 mg/ml). There is a highly significant ($P < 0.001$) correlation between the results obtained with the two inducers.

sperm head plasma membrane *in vitro* before, but not after, capacitation (Banerjee and Chowdhury, 1994). On the other hand, the strong binding of human spermatozoa to cultured epithelial cells from the Fallopian tube (Pacey *et al.*, 1995a,b) and the positive effects of these epithelial cells on sperm viability and motility (percentage and hyperactivation) (Kervancioglu *et al.*, 1994) indicated that sperm–epithelial interactions may be a major event during the residence of spermatozoa within the Fallopian tubes. Human spermatozoa

sequentially incubated with cervical mucus and tubal secretions *in vitro* expressed higher levels of hyperactivated motility but lower rates of acrosome reaction in the presence of follicular fluid in comparison with spermatozoa challenged with follicular fluid without prior incubation with tubal fluid (Zhu *et al.*, 1994). Thus, human oviductal fluid may serve to precondition spermatozoa, allowing the maintenance of motility and the progression of capacitation, but delaying the induction of the acrosome reaction until the appropriate time. Although the strong binding between spermatozoa and oviduct of farm animals was associated with the formation of a preovulatory sperm reservoir (Hunter, 1987), no such evidence has yet been obtained in humans (Williams *et al.*, 1993).

The mechanisms regulating sperm–epithelial cell interactions and those by which tubal secretions act on spermatozoa are not well understood. In hamsters, a sialylated oligosaccharide similar to that found on fetuin is present on the epithelium of the oviduct and appeared both to be recognized by sperm surface components and to mediate sperm binding to this tissue (Demott *et al.*, 1995). Furthermore, the loss of specific fetuin- and sialic acid-recognizing lectins (27.5, 32, and 50 kDa) from hamster sperm membranes was temporally associated with the acquisition of hyperactivated motility and the release of spermatozoa from the oviductal epithelium (Demott *et al.*, 1995). In addition, there was a significant reduction in the binding to the oviduct when hamster or bull spermatozoa were capacitated (and hyperactivated) *in vitro* prior to their introduction into the oviduct or addition to oviductal epithelium explants (Smith and Yanagimachi, 1991; Lefebvre and Suarez, 1996). Whether the membrane modifications needed for the release of spermatozoa from the oviductal epithelium *in vivo* are performed by spermatozoa and/or epithelial cells and whether these membrane modifications play a role in the development of hyperactivation remains to be investigated.

The attachment of bull spermatozoa to the oviduct provoked important changes in protein (types and quantities) synthesis from epithelial cells (Ellington *et al.*, 1993). It was proposed that these newly secreted proteins play a role in sperm capacitation. Specific bovine oviductal proteins (72, 66, 39, 38, and 36 kDa) were found to bind strongly to spermatozoa and to allow long term maintenance of motility (Lapointe and Sirard, 1996). The binding of three of these proteins (39, 38, and 36 kDa) was calcium dependent, and it was hypothesized that they may stabilize cell membranes by binding strongly to spermatozoa in the isthmus where calcium concentration is higher (2.5 mM) and then promote capacitation by being slowly removed from spermatozoa in the ampulla where calcium concentration is lower (1.8 mM) (Lapointe and Sirard, 1996). In the human, proteins synthesized by the oviduct were found to bind and stabilize spermatozoa (Lippes and Wagh, 1989). On the other hand, oviductal fluid also contains proteins that could destabilize sperm membranes by promoting cholesterol efflux (Davis, 1982; Ehrenwald *et al.*, 1990; Ravnik *et al.*, 1990, 1995).

Small molecules, such as progesterone and lipids, present in oviductal fluid may also affect spermatozoa. Progesterone could stimulate sperm capacitation, possibly by initiating a calcium influx (Zhu *et al.*, 1994; Revelli *et al.*, 1994). In hamsters, spermatozoa incubated with progesterone at the concentration found in the oviduct (20 ng/ml) and for their normal period of residency in this tissue (5 h), had a higher rate of egg penetration than spermatozoa to which progesterone was added after capacitation and during coincubation with eggs (Libersky and Boatman, 1995). On the other hand, it is possible that the cholesterol present in the oviduct prevents a premature capacitation by stabilizing sperm membranes. In the bovine oviduct, the cholesterol to phospholipids ratio was highest in the fluid collected from the isthmus (sperm reservoir) at all times of the cycle, and lowest in the fluid obtained from the ampulla (site of fertilization) at the time of ovulation (Grippo *et al.*, 1994). Furthermore, the concentrations of membrane-disturbing lipids, such as lysophosphatidylcholine, were lower in the isthmic as compared to the ampullary fluid, again emphasizing the stabilizing and destabilizing character respectively, of these two regions of the oviduct (Grippo *et al.*, 1994).

Experiments performed in non-human species indicated that other factors such as oxygen concentration (Maas *et al.*, 1976) and temperature (Hunter and Nichol, 1986; Hunter, 1987) influence sperm capacitation in the oviduct. During the preovulatory period, the temperature of the isthmus was found to be lower, by 0.69°C, than that of the ampulla in mated pigs, a difference that was abolished at ovulation time when the temperature of the isthmus increased to reach that of the ampulla. Even though this difference in temperature is small, it may be sufficient to influence events associated with sperm capacitation.

The observations described above and summarized in Figure 2 demonstrate that, *in vivo*, sperm capacitation requires a fine balance between the actions of various promotors and inhibitors. Spermatozoa must be modified and become capacitated but at a controlled rate so that they reach the eggs at the appropriate time and in the appropriate physiological state to fertilize these eggs.

Hyperactivation

Description and measurement

Hyperactivation, which is the type of motility spermatozoa display at the site of fertilization, was qualified as non-progressive, vigorous, whiplash type, frantic, high amplitude, etc. and was observed in many species such as hamsters (Yanagimachi, 1982), mice (Neill and Olds-Clarke, 1987), pigs (Suarez *et al.*, 1992), rabbits (Young and Bodt, 1994), and humans (Robertson *et al.*, 1988; Burkman, 1990; Mortimer and Mortimer, 1990; Zhu *et al.*, 1994; Mortimer and Swan, 1995; Sukcharoen *et al.*, 1995). Although the motility patterns observed and the temporal relationship with capacitation may vary from one species to the other, sperm hyperactivation appears as an essential event of capacitation. Sperm hyperactivation is associated with an increased velocity, a decreased linearity, an increased amplitude of lateral head displacement, and whiplash movements of the flagellum. In humans, specific sperm motility patterns were described but there is no consensus yet on the classification of these sperm trajectories nor on the

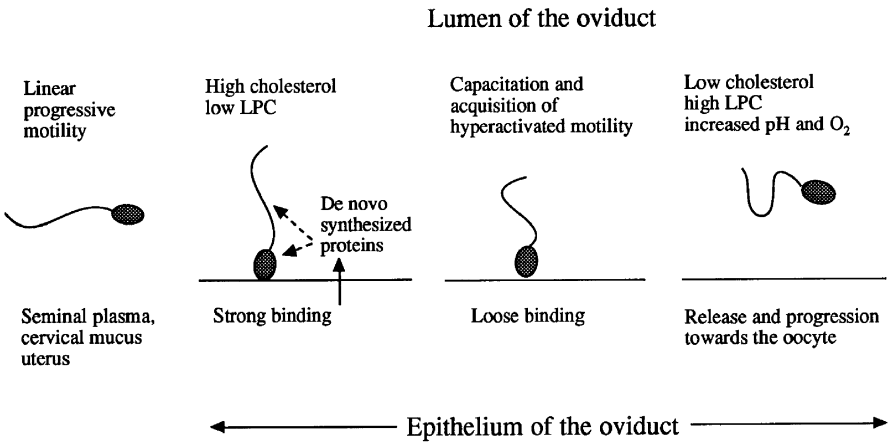

Figure 2. Schematic representation of in-vivo capacitation. Spermatozoa still coated with substances originating from the epididymis and the seminal plasma are not capacitated when they enter the oviduct and their motility is still expected to be linear and progressive. They strongly bind to the epithelial cells (possibly by recognition of sialylated oligosaccharides on the surface of these cells) of the oviduct, which respond by de novo synthesis of a variety of proteins. Specific oviductal proteins bind to spermatozoa and allow maintenance of motility and viability and it is to be expected that they play a role in the regulation of capacitation and its associated membrane changes. During sperm transit in the oviduct, there is a release of sialic acid-recognizing lectins and of decapacitating factors and the hydrolysis of sialic acid from sialoglycoconjugates on the sperm surface. Progesterone is present in the oviductal fluid but its capacitating abilities are probably counterbalanced by the presence of cholesterol or stabilizing proteins, such as oviductin. As ovulation occurs, physical and chemical changes (such as rises in temperature, oxygen, pH, etc.) in the environment may cause the capacitation to resume. The binding of spermatozoa to the epithelium weakens and the acquisition of hyperactivated motility allows spermatozoa to detach from the epithelium and progress further in the oviduct, to regions where the presence of lower concentrations of cholesterol and higher concentrations of lysophosphatidylcholine help in completing the capacitation process.

threshold criteria to identify hyperactivated spermatozoa. The 'transitional' trajectory which retains sufficient symmetry to permit forward sperm movement (Robertson *et al.*, 1988; Mortimer and Mortimer, 1990) shares similarities with the 'circling high-curvature' and the 'helical' motility patterns described by Burkman (1990) (Figure 3). The 'star spin' type of sperm motility (characterized mainly by very low linearity) (Figure 3) was observed by all these authors and appeared as a good predictor for the success of IVF (Sukcharoen *et al.*, 1995). Recently, the fractal analysis of sperm movement was found to give a good indication on the regularity of sperm trajectories and help in classifying hyperactivated spermatozoa (Mortimer *et al.*, 1996).

Results concerning the percentages of hyperactivation obtained with human spermatozoa, as well as the correlation between hyperactivation and fertilization, vary from one study to another. Differences in methodology could explain these discrepancies. For example, the use of a deeper measuring chamber gave a higher proportion of star spin, as compared to transitional, type of sperm motility (LeLannou *et al.*, 1992). The method for collection and analysis of images [total number, images/s, computer-assisted sperm analysis (CASA) system, etc.] also has a major influence on the data obtained (Robertson *et al.*, 1988; Mortimer and Mortimer, 1990; Mortimer and Swan, 1995; Sukcharoen *et al.*, 1995). Furthermore, hyperactivation is not a synchronous process in humans, which means that not all spermatozoa from a preparation display hyperactivated motility at the same time of incubation. Finally, human spermatozoa constantly switch from one pattern of hyperactivated motility to another or from non-hyperactivated to hyperactivated motility (Burkman, 1990; Murad *et al.*, 1992; de Lamirande and Gagnon, 1993b; Mortimer and Swan, 1995) (Figure 3). These observations emphasize the need for standardization of the measurement conditions.

Functions, requirements, and mechanisms

Hyperactivated motility may have multiple functions. *In vitro*, it allowed mouse and hamster spermatozoa to penetrate more efficiently media supplemented with Ficoll, methylcellulose or polyacrylamide, the viscoelasticities or densities of which were similar to those of the fluids from the female genital tract or zona pellucida (Suarez *et al.*, 1991; Suarez and Dai, 1992). *In vivo*, hyperactivated spermatozoa were also shown to more easily break free from the oviduct and progress along this tissue, as indicated by direct in-situ observations of oviducts from naturally-mated mice (Demott and Suarez, 1992) and from observation of human spermatozoa co-cultured with epithelial cells from Fallopian tubes (Pacey *et al.*, 1995a,b). Finally, Stauss *et al.* (1995) took advantage of the different bicarbonate requirements for the development of hyperactivation and capacitation in hamster spermatozoa (Boatman and Robin, 1991) to demonstrate that, although capacitation allows sperm binding to the zona pellucida and acrosome reaction, hyperactivation is needed for penetration of the zona pellucida. These data further stress that the acquisition of hyperactivated motility is an essential event of sperm capacitation to ensure successful fertilization.

Influx of bicarbonate ions appears as a major determinant in the development of hyperactivated motility. Most of the studies on this topic were performed with epididymal spermatozoa and the concentration of bicarbonate needed for mouse and hamster sperm hyperactivation (25 mM) was much higher than that needed to induce capacitation (2.9 mM) (Neill and Olds-Clarke, 1987; Boatman and Robin, 1991; Stauss *et al.*,

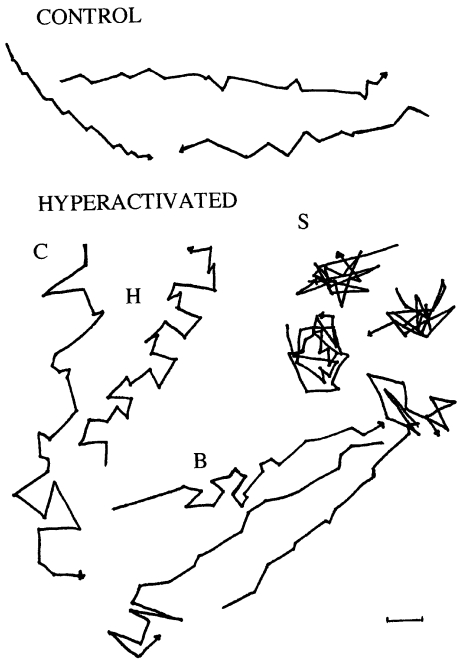

Figure 3. Representative sperm motility patterns of control and hyperactivated spermatozoa. Percoll-washed spermatozoa were incubated in Ham's F-10 medium alone (control, upper panel) or supplemented with fetal cord serum ultrafiltrate (7.5%, v/v) (hyperactivated, lower panel). They were then placed between Collodion-coated slides and coverslips (Murad *et al.*, 1992) and motility was analysed at 37°C. Tracks were obtained with the CellSoft© Research Module and corresponded to the displacement of sperm heads for 1 s. The circling high-curvature (C) and helical (H) motility patterns correspond to the transitional trajectories and retain a sufficient symmetry to permit forward sperm movement. The star spin (S) type of motility, characterized by very low linearity, was generally recognized and appeared as an important predictor of success in in-vitro fertilization. Human sperm hyperactivation is not constant and, even over the course of 1 s, spermatozoa change their motility pattern (biphasic, B). Bar = 10 μm.

1995). In humans, spermatozoa are in contact with a high concentration of bicarbonate at the time of ejaculation, when they are mixed with secretions from the seminal vesicles (Okamura *et al.*, 1986). Probably because of this pre-exposure to bicarbonate, the extracellular presence of these ions did not appear as an absolute requirement for in-vitro hyperactivation of ejaculated human spermatozoa (Murad *et al.*, 1992; de Lamirande and Gagnon, 1993a,b, 1995a). The absence or presence of bicarbonate (25 mM) in Ham's F-10 medium did not influence any motility parameters of human spermatozoa (E.de Lamirande and C.Gagnon, unpublished).

Although the calcium requirements for human sperm acrosome reaction were determined (Thomas and Meizel, 1989; Florman *et al.*, 1992), most of the studies on the need for this ion in sperm hyperactivation were performed in non-human species (reviewed in Fraser, 1995a). Due to their larger size, hamster spermatozoa were used to demonstrate an increase in intracellular calcium concentration during hyperactivation (from ~50 nM to 100–200 nM), which was lower than that observed during the acrosome reaction (300–400 nM) (Suarez and Dai, 1995). Furthermore, the calcium increase was greater in the flagellum during hyperactivation and in the head during the acrosome reaction (Suarez and Dai, 1995). Consistent with the known effect of calcium on the flagellar curvature (Lindemann and Goltz, 1988), Suarez *et al.* (1993) showed that, during hyperactivation, the calcium oscillations that occurred throughout whole spermatozoa correlated best with the flagellar beat cycle detected in the proximal flagellar midpiece, and were faster than calcium oscillations detected in any other cell types. One possible target for calcium during hyperactivation might be calcineurin, a calcium-dependent protein phosphatase that was suggested to be involved in flagellar beat asymmetry (Tash *et al.*, 1988).

Changes in the concentrations of various ions are probably only a part of the complex modifications which human spermatozoa undergo during hyperactivation. Thus, experiments performed with demembranated reactivated human spermatozoa, that were or were not previously hyperactivated, indicated that sperm hyperactivation is accompanied by interdependent changes at the cytosolic and axonemal levels (Murad *et al.*, 1992). Potential axonemal modifications may be related to phosphorylation of proteins, which is known to be involved in sperm motility (Tash, 1990; Leclerc and Gagnon, 1996). We recently observed that two proteins, called p105 and p81 (corresponding to their molecular masses of 105 and 81 kDa respectively), and located on sperm fibrous sheath, were progressively phosphorylated on tyrosine residues when human spermatozoa were incubated in capacitating conditions (Figure 4) (Leclerc *et al.*, 1996, 1997). This phosphorylation was significantly increased after 1 h of incubation and further increased for up to 3 h of incubation (Leclerc *et al.*, 1996, 1997), an interval of time that corresponds to the acquisition of hyperactivated motility in human spermatozoa incubated *in vitro* (Burkman, 1990).

Clinical relevance of sperm hyperactivation

Several studies (Burkman, 1990; Chan *et al.*, 1992; Wang *et al.*, 1993; Sukcharoen *et al.*, 1995), reporting a clear correlation between the level of human sperm hyperactivation and the success of IVF, emphasize the physiological relevance of this process. However, *in vivo*, this phenomenon should be well synchronized with other events, such as ovulation, and should take place at the right time and location. Premature hyperactivation of spermatozoa in semen was observed in a significant proportion (22/120; 18%) of infertile men whose spermiograms were otherwise considered normal according to the World Health Organization standards, but not in semen of fertile volunteers (de Lamirande and Gagnon, 1993c,d). In addition, mouse spermatozoa that were hyperactivated *in vitro* prior to insemination fertilized significantly fewer oocytes than spermatozoa that were not hyperactivated (Olds-Clarke and Wivell, 1992; Olds-Clarke and Sego, 1992). Furthermore, the number of inseminated hamster spermatozoa entering the utero–tubal junction was reduced when hyperactivated spermatozoa were used (Shalgi *et al.*, 1992). Together, these observations indicate that premature sperm hyperactivation impairs sperm transport through the female reproductive tract.

In conclusion, sperm hyperactivation appears as a major event temporally associated with capacitation. These two

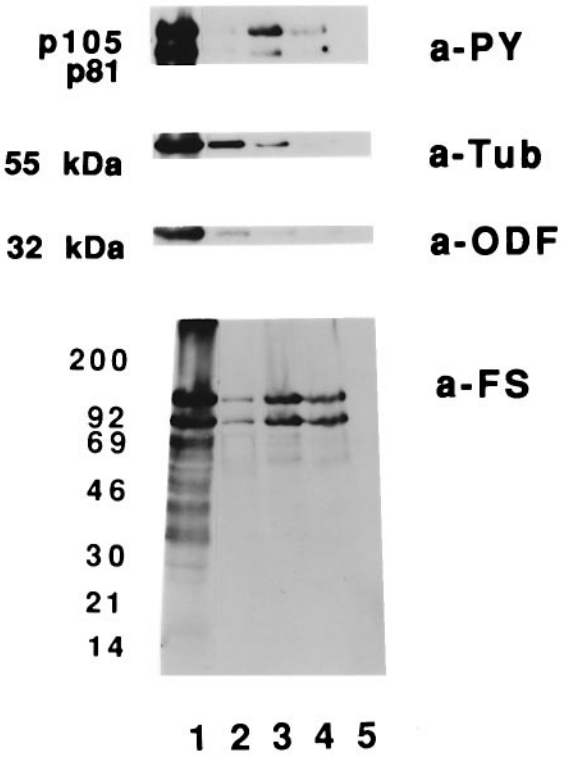

Figure 4. Localization of the two main tyrosine-phosphorylated proteins in human spermatozoa. The purification of sperm fibrous sheath was performed as described by Oko (1988). Briefly, Percoll-washed spermatozoa were incubated at room temperature in the presence of 5 M urea and 25 mM dithiothreitol until sperm heads were dislocated from flagellae. An equal volume of HEPES-buffered saline was added to the suspension, which was then separated by centrifugation (10 000 *g*, 10 min) on a discontinuous 45/90% Percoll gradient. The presence of the two major phosphotyrosine-containing proteins was assessed in whole spermatozoa (lane 1), soluble fraction (top of the Percoll gradient, lane 2), particulate fraction that did not enter the 45% Percoll layer (lane 3), particulate fraction that migrated to the 45%/90% Percoll interface (lane 4), and pellet (lane 5), using a monoclonal anti-phosphotyrosine antibody (a-PY, top panel). The same fractions were also tested for the presence of axonemal proteins (with a monoclonal anti-tubulin antibody, a-Tub) and for sperm outer dense fibres (ODF) and fibrous sheath (FS) (with polyclonal antibodies, a-ODF and a-FS, respectively) (generously donated by Dr Richard Oko, Queen's University, Ontario, Canada). The 32 kDa protein is the major protein reacting with the anti-ODF antibody. The position of the molecular mass markers is shown on the left. Note the good correlation in the molecular masses and the location (Percoll fraction) between p105 and p81 and the two major fibrous sheath proteins.

processes are reversible but the conditions required for their development may not be always identical, which could suggest that hyperactivation is not a part of capacitation. However, in-vivo experiments demonstrated that hyperactivation develops during sperm capacitation in the female genital tract and allows spermatozoa to detach from the epithelium of the oviduct and progress towards the egg. Furthermore, the fact that capacitated spermatozoa can bind to the zona pellucida and induce the acrosome reaction, but cannot penetrate the zona pellucida unless they are hyperactivated, stresses the importance of hyperactivation in the capacitation process.

Ions and sperm capacitation

Sodium, potassium, and bicarbonate

The requirements for sodium (Na^+) and potassium (K^+) ions in sperm capacitation have not been studied extensively. Very low concentrations of sodium ions appeared to be needed for guinea pig sperm capacitation (Hyne *et al.*, 1984) and the small sodium influx that occurred during mouse sperm capacitation is thought to be regulated by a membrane Na^+,K^+ATPase (Fraser *et al.*, 1993). Capacitation of mouse and bull spermatozoa was associated with an increased permeability to potassium ions that would be responsible for the observed membrane hyperpolarization (Zeng *et al.*, 1995). The present data are however insufficient to support a definitive role of these two ions in sperm capacitation.

As stated above, human spermatozoa acquire the bicarbonate (HCO_3^-) they need from seminal plasma at the time of ejaculation (Okamura *et al.*, 1986). Furthermore, the high concentration of bicarbonate present in the fluids of the female genital tract (Maas *et al.*, 1977) is sufficient to maintain a high concentration of this ion inside the cell. We successfully induced human sperm capacitation in Ham's F-10 medium devoid of bicarbonate (de Lamirande and Gagnon, 1993a,b, 1995a) and addition of bicarbonate to this medium did not further increase the level of capacitation observed (Leclerc *et al.*, 1996), indicating that the bicarbonate influx that took place in seminal plasma was sufficient to support sperm function. One of the functions of bicarbonate ions is probably to allow the rise in intracellular pH that is associated with sperm capacitation (Fraser, 1995a,b; Vredenburg-Wilberg and Parrish, 1995). In bull spermatozoa, heparin-induced capacitation was associated with a significant increase in intracellular pH in both the head and midpiece of the cell. The increase in pH was, on average, relatively small (0.2 units) but frequency distribution analysis of sperm intracellular pH revealed that a subpopulation of spermatozoa (possibly the capacitating ones) was more affected with pH rises of up to 0.6 units (Vredenburg-Wilberg and Parrish, 1995). On the other hand, experiments performed with hamster (Boatman and Robbins, 1991) and pig (Suzuki *et al.*, 1994) spermatozoa suggested that bicarbonate did not only function as a buffer to increase the intracellular pH, but also affected sperm function, possibly by stimulating the adenylyl cyclase (Okamura *et al.*, 1985).

Calcium

There is, to our knowledge, only one report on the time course of the calcium influx associated with human sperm capacitation. The intracellular free calcium concentration of human spermatozoa incubated in capacitating conditions rose from 70 to 250 nM during the first 2 h of incubation and did not significantly increase thereafter (Baldi *et al.*, 1991). The addition of progesterone to the medium caused a faster and greater influx of calcium (up to 350 nM) in spermatozoa, but also an induction of the acrosome reaction (Baldi *et al.*, 1991). It is worth noting that the calcium concentrations in uncapacitated, capacitated and acrosome reacting human spermatozoa are similar to those of hamster spermatozoa under similar conditions (Suarez and Dai, 1995; see section on

hyperactivation). These results suggest that calcium concentration increases stepwise during capacitation and acrosome reaction and that different mechanisms regulating calcium influx may be involved in these two phenomena.

Three mechanisms have been proposed for the regulation of calcium influx in spermatozoa and all could be involved in the capacitation event. Voltage-dependent calcium channels capable of allowing Ca^{2+} influx are possible candidates but they were associated more with the acrosome reaction than with capacitation (Florman *et al.*, 1992). On the other hand, experiments performed with Ca^{2+}-ATPase inhibitors (e.g. quercitin) and calmodulin antagonists (e.g. W-7) provided indirect evidence for the presence and involvement of a Ca^{2+}-ATPase that pumps calcium out of the cell in guinea pig (Roldan and Fleming, 1989), human (DasGupta *et al.*, 1994), and bull (Fraser *et al.*, 1995) spermatozoa. The fact that mouse spermatozoa, incubated in a medium supplemented with a low concentration of glucose (5.56 μM) to decrease intracellular ATP concentration, became capacitated faster than those incubated in the presence of a standard glucose concentration (5.56 mM) substantiated the hypothesis that the activity of a membrane Ca^{2+}-ATPase was involved in the regulation of the calcium influx during sperm capacitation (Adeoya-Osigua and Fraser, 1993; DasGupta *et al.*, 1994). It was suggested that decapacitation factors adsorbed on the sperm membrane activate a Ca^{2+}-ATPase and prevent the net increase in intracellular calcium concentration and premature capacitation; thus, the loss of these decapacitation factors during incubation would cause a decrease of Ca^{2+}-ATPase activity and, consequently, a rise in intracellular calcium (Adeoya-Osiguwa and Fraser, 1993, 1994). Indeed, a decapacitating factor of epididymal origin was recently shown to stimulate a calcium-dependent ATPase activity in membranes isolated from heads, but not from tails, of mouse spermatozoa (Fraser, 1995b; Adeoya-Osiguwa and Fraser, 1996). This decapacitating factor inhibited sperm capacitation and fertility but not hyperactivation, further suggesting that different mechanisms may regulate intracellular ionic composition in sperm head and tail (Fraser, 1995b; Adeoya-Osiguwa and Fraser, 1996). Lipids such as lysophosphatidylcholine (inhibitor) and lysophosphatidylserine (activator), and modifications of disulphide bridges were also proposed as regulators of Ca^{2+}-ATPase activity, as observed in guinea pig spermatozoa (Roldan and Fleming, 1989). The third candidate possibly responsible for calcium influx is an Na^{+}(out)–Ca^{2+}(in) exchanger. From experiments performed in bull spermatozoa, it was hypothesized that the activity of this exchanger would be regulated by caltrin, a low molecular weight protein originating from seminal plasma that binds to spermatozoa and is released during capacitation (Rufo *et al.*, 1984). Both the Na^{+}–Ca^{2+} exchanger and the Ca^{2+}-ATPase could be involved in the capacitation-associated calcium influx but there is not yet direct evidence for their involvement in this process.

The mechanism of calcium action during capacitation remains to be delineated but, as for bicarbonate, it could stimulate adenylyl cyclase activity (Gross *et al.*, 1987; Parinaud and Milhet, 1996). Other enzymes present in spermatozoa, such as phospholipase A_2 and protein kinase C, are also dependent on the presence of calcium and, although their role in sperm acrosome reaction was recognized (Yanagimachi, 1994; Doherty *et al.*, 1995), their involvement in sperm capacitation is not established yet. Calcium action could also be by direct interaction with phospholipids, modifying their physical state, phase separation behaviour and consequently membrane fluidity (Holt, 1995).

Zinc

Zinc (Zn^{2+}) is present at high concentrations in human seminal plasma, appears as a membrane stabilizing compound for spermatozoa, and therefore could be considered as a decapacitation factor (Andrews *et al.*, 1994). A study by Riffo *et al.* (1992) indicated that addition of 100 μM zinc to the incubation medium prevented human sperm capacitation and/or acrosome reaction (no differentiation of these two sperm functions was made). In a preliminary study, we observed that 50 μM zinc decreased hyperactivation and capacitation of human spermatozoa without affecting the percentages of cell motility and viability (E.de Lamirande and C.Gagnon, unpublished). Zinc also inhibited mouse and hamster sperm capacitation while the acrosome reaction was unaffected (Aonuma *et al.*, 1981; Andrews *et al.*, 1994). In hamsters, this inhibitory effect of zinc was progressively eliminated by supplementing the incubation medium with higher albumin concentration or zinc chelators such as D-penicillamine, L-histidine, or L-cysteine (Andrews and Bavister, 1989). The use of a zinc-specific fluorochrome allowed the demonstration that zinc is removed from hamster spermatozoa (mainly acrosomal and post-acrosomal regions) in a time-dependent fashion during in-vitro capacitation (Andrews *et al.*, 1994). However, although zinc removal appeared essential, it was not sufficient by itself to induce sperm capacitation and may be part of a process that causes membrane destabilization and/or triggers other membrane-related events associated with this process (Andrews *et al.*, 1994).

Membrane events

As emphasized in the previous sections, major membrane modifications have been observed during sperm capacitation. Membrane coating materials originating from the epididymis (Boué *et al.*, 1992, 1994, 1996) and seminal plasma (Oliphant, 1976; Audhya *et al.*, 1987; Thérien *et al.*, 1995; Cross, 1996) are removed or altered, membrane antigens are relocalized (Fusi and Bronson, 1992; Fusi *et al.*, 1992; Benoff *et al.*, 1993; Cohen-Dayag and Eisenbach, 1994), mechanisms responsible for ion fluxes are activated or inhibited (see section above), and membrane fluidity increases (Wolf *et al.*, 1986) due to changes in lipid composition (Langlais *et al.*, 1988; Benoff, 1993). It is not possible in the context of this review to cite all the numerous studies that have dealt with the membrane modifications related to sperm capacitation and we will present only a few selected ones.

Membrane lipids

In contrast to what is observed in mammalian somatic cells, a high proportion of lipids and proteins from sperm membranes

is not free to diffuse and it was suggested that relocalization of membrane proteins is in many cases controlled by lipid fluidity (Wolf and Cardullo, 1991). Although few studies on the levels of phospholipids were published (e.g. Snider and Clegg, 1975; Llanos and Meizel, 1983; Harrison and Gadella, 1995), much more attention has been directed towards cholesterol in sperm membrane and its fate during capacitation. The cholesterol present in sperm membranes was shown to limit ion permeability, protein insertion and mobility in phospholipid bilayers, as well as to rigidify and stabilize membranes (Parks and Ehrenwald, 1990), therefore preventing some of the phenomena associated with sperm capacitation. Cholesteryl sulphate, which was also found in human spermatozoa, was thought to endow greater stability and rigidity to sperm membranes than cholesterol itself (Langlais *et al.*, 1981). It was hypothesized that its desulphation in the female reproductive tract and its subsequent release from the membrane would dramatically change the biophysical properties of the sperm membrane towards a more fusogenic state (Langlais *et al.*, 1981, Holt, 1995). On the other hand, it was also suggested that cholesteryl sulphate, in conjunction with calcium, could destabilize membrane in preparation for membrane fusion during the acrosome reaction (Holt, 1995).

Cholesterol efflux and/or redistribution are expected to allow destabilization of sperm membranes and induction of capacitation. Indeed, incubation of washed spermatozoa with albumin (as 'sink' to adsorb cholesterol) or with follicular or oviduct fluids (which contain 'lipid transfer proteins') favours cholesterol efflux and sperm capacitation (Davis, 1982; Langlais *et al.*, 1988; Ehrenwald *et al.*, 1990; Parks and Ehrenwald, 1990; Ravnik *et al.*, 1990). Suzuki and Yanagimachi (1989) observed that sterols were abundant and evenly distributed in the plasma membrane over the acrosome in non-capacitated hamster spermatozoa but that their density was sharply reduced after capacitation. Conversely, prevention of cholesterol efflux inhibited or delayed sperm capacitation. For example, a recent study emphasized that the presence of high concentrations of cholesterol in seminal plasma, which is expected to maintain a high cholesterol concentration in sperm membranes, may be a more important factor for the inhibition of capacitation (Cross, 1996) than other factors such as antifertility proteins (Audhya *et al.*, 1987; Han *et al.*, 1990), spermine (Rubenstein and Breitbart, 1991), zinc (Riffo *et al.*, 1992; Andrews *et al.*, 1994), etc. that were previously thought to be responsible for the decapacitating activity of seminal plasma.

Cholesterol efflux presumably causes an increase in membrane fluidity, a cell parameter that can be determined by lipid diffusion measurements. Wolf *et al.* (1986) observed that the diffusion of lipids in mouse spermatozoa was compart-

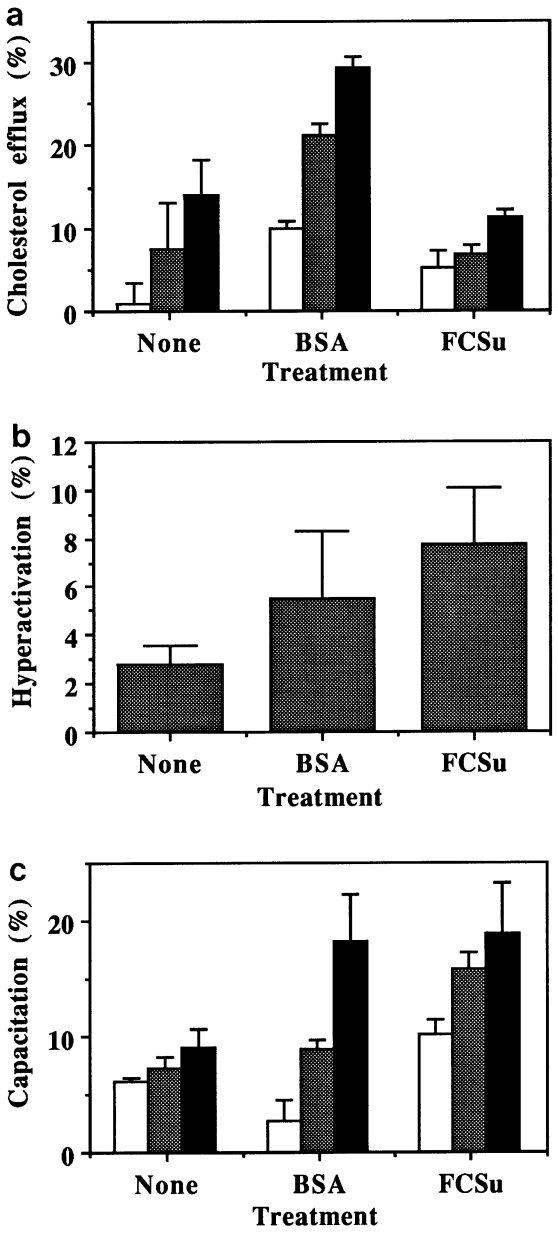

Figure 5. Cholesterol efflux from human spermatozoa during capacitation. Percoll-washed human spermatozoa resuspended in HEPES-buffered saline were labelled with [^{3}H]cholesterol (46.5 Ci/mmol, emulsified in HEPES-buffered saline by sonication) for 1 h at 37°C. Spermatozoa were then washed and resuspended at 20×10^6 cells/ml in Ham's F-10 medium alone (none) or supplemented with bovine serum albumin (BSA, 3 mg/ml) or fetal cord serum ultrafiltrate (7.5%, v/v) (FCSu). (**a**) Cholesterol efflux was measured after 1 (white bars), 3 (grey bars), and 6 (black bars) h of incubation by the radioactivity remaining on spermatozoa. (**b**) Sperm hyperactivation was measured after 3 h use of the CellSoft© computer assisted semen analyser as described by de Lamirande and Gagnon (1993a,b). (**c**) Sperm capacitation was determined after 1 (white bars), 3 (grey bars), and 6 (black bars) h of incubation by the lysophosphatidylcholine-induced acrosome reaction as described in Figure 1. Values are mean ± SEM of four experiments performed with sperm samples from different volunteers. The time-dependent cholesterol efflux of spermatozoa incubated in the presence of albumin is significantly higher ($P<0.05$) than that of spermatozoa treated with fetal cord serum ultrafiltrate at all times measured even though these two conditions allowed sperm hyperactivation and capacitation. On the other hand, the cholesterol efflux of non-capacitating spermatozoa (medium alone) was similar to that of spermatozoa treated with fetal cord serum ultrafiltrate, even though the levels of hyperactivation and capacitation obtained with these two treatments were significantly ($P<0.001$) different.

mentalized and occurred faster in the flagellum than the head in all cases studied. Sperm capacitation (as measured by hyperactivation) was associated with an increased lipid diffusion rate in membranes of the acrosome, midpiece, and flagellum but not in those of the post-acrosomal region of spermatozoa. Furthermore, the diffusing lipid fraction of the acrosomal membrane decreased during sperm capacitation (Wolf *et al.*, 1986). These physical changes are consistent with the functional changes, acquisition of hyperactivated motility and of fertilizing ability that occur in capacitating spermatozoa. Membrane fluidity could also be influenced by the increase in temperature that occurs in the isthmus of the oviduct at the time of ovulation (pig; Hunter and Nichol, 1986) (see section on in-vivo capacitation).

The requirement for cholesterol efflux for sperm capacitation, as well as its causative role in this process, is still debated (Parks and Ehrenwald, 1990). Cholesterol efflux appeared as prerequisite for the exposition of specific antigens such as head-specific mannose-ligand proteins (one of the putative zona pellucida recognition molecules) that is transposed from subplasma membrane sites to an integral membrane position during human sperm capacitation (Benoff *et al.*, 1993; Benoff, 1993). On the other hand, preliminary results from our laboratory indicated that, *in vitro*, cholesterol efflux can occur to some extent even in the absence of albumin or lipid transfer proteins and that capacitation can be induced even if only a small proportion of cholesterol is released from human spermatozoa (Figure 5). Albumin and fetal cord serum ultrafiltrate induced similar rates of human sperm hyperactivation and capacitation but the cholesterol efflux was much higher when albumin was present in the medium. On the other hand, the cholesterol efflux was similar in spermatozoa incubated in the absence or presence of fetal cord serum ultrafiltrate, but sperm hyperactivation and capacitation were observed only in the latter case. Therefore, if cholesterol efflux is essential for sperm hyperactivation and capacitation, it did not appear to be by itself sufficient. It is also possible that the release of only a small proportion of the total cholesterol is required during these processes.

Membrane antigens

Many membrane antigens are relocalized during sperm capacitation (Cohen-Dayag and Ehrenwald, 1994) but their functional link to this process has been assessed in only a few cases. For example, a correlation was established between the expression of cell adhesion molecules, such as fibronectin (Fusi and Bronson, 1992; Hoshi *et al.*, 1994), vitronectin and laminin (Fusi *et al.*, 1992), during human or hamster sperm capacitation and the subsequent ability to bind zona-free hamster eggs.

The binding and/or localization of other factors, originating from seminal plasma or epididymal fluid, are also modified during sperm capacitation. For example, bovine seminal plasma proteins from a family globally called BSP, were found to bind phosphatidylcholine molecules of sperm membrane and to facilitate heparin-induced capacitation (Thérien *et al.*, 1995). Furthermore, these bovine proteins shared antigenic determinants with phosphatidylcholine-binding proteins found in the seminal plasma of different species, including human (Leblond *et al.*, 1993). It was hypothesized that BSP would be released during capacitation and help this process by extracting cholesterol and phospholipids from the sperm membrane (Thérien *et al.*, 1995). A tripeptide similar to thyrotrophin releasing hormone, present in seminal plasma (prostatic origin), was also found to stimulate the capacitation and fertilizing ability of human spermatozoa *in vitro* (Greene *et al.*, 1996). The surface localization of another protein, called P34H (P26h in hamsters), on human spermatozoa demonstrates the complexity of the transformations these cells must undergo. Boué *et al.* (1996) observed that P34H is secreted within the epididymal fluid, progressively and strongly binds to the acrosomal cap of spermatozoa during their transit from the caput to the cauda of the epididymis, is then internalized (or masked) at the time of ejaculation, reappears during capacitation, and is finally eliminated after the acrosome reaction. Antibodies to P34H inhibited human sperm–zona interactions without affecting hyperactivation, capacitation, or zona free hamster egg penetration suggesting that P34H may also be a potential sperm receptor for the zona pellucida (Boué *et al.*, 1994). In addition, the amount of P34H, as well as the degree of binding to homologous zona pellucida, was greatly reduced in spermatozoa of 9 out of 16 infertile men as compared to what was observed in fertile men, further emphasizing the importance of this epididymal factor in the acquisition of the fertility potential (Boué and Sullivan, 1996).

Other membrane modifications

Capacitation is also associated with a decrease in surface-negative charge that may be a consequence either of removal of sialic acid from the surface sialylglycoconjugates (Lasalle and Testart, 1994) or of redistribution of these sialylglycoconjugates (Focarelli *et al.*, 1995). Furthermore, experiments performed in bulls and mice indicated that sperm membrane becomes hyperpolarized during capacitation (Zeng *et al.*, 1995). The decrease in membrane potential (from ~–30 mV in non-capacitated spermatozoa, to ~–60 mV in capacitated spermatozoa) appeared to be due to an enhanced permeability to potassium ions (Zeng *et al.*, 1995) and appeared essential for the subsequent zona pellucida-induced acrosome reaction.

It is presently not known which of the changes described above induces sperm capacitation, in what order they proceed and which ones are mere consequences of this process. These studies, and those not cited because of lack of space, suggest that there are probably complex interdependent relationships between these membrane events in the induction of sperm capacitation.

Reactive oxygen species

Induction of sperm hyperactivation and capacitation

Spermatozoa incubated under aerobic conditions generate reactive oxygen species (ROS) such as the superoxide anion ($O_2^{\cdot -}$) (Aitken and Clarkson, 1987; Alvarez *et al.*, 1987) which spontaneously dismutates to hydrogen peroxide (H_2O_2). Whereas high concentrations of ROS, and mainly hydrogen

peroxide, were shown to have detrimental effects on human spermatozoa (Aitken and Clarkson, 1987; Alvarez *et al.*, 1987; de Lamirande and Gagnon, 1995b), there is now evidence that very low and controlled concentrations of ROS are involved in sperm acquisition of fertilizing ability (de Lamirande *et al.*, 1995b, 1996).

Human spermatozoa incubated in the presence of a superoxide-generating system (xanthine + xanthine oxidase + catalase) developed higher levels of hyperactivation and capacitation than spermatozoa incubated in Ham's F-10 medium alone, an effect that was prevented by the presence of superoxide dismutase (SOD) (de Lamirande and Gagnon, 1993a,b). These results demonstrated that exogenously added superoxide anion induced human sperm hyperactivation and capacitation (de Lamirande and Gagnon, 1993a,b). The relevance of this finding was confirmed when human sperm capacitation induced by various biological fluids, such as fetal cord serum and follicular fluid (whole, dialysed, or ultrafiltrate), was always prevented by the presence of SOD, even though these fluids did not produce detectable concentrations of superoxide anion by themselves (de Lamirande and Gagnon, 1993b; de Lamirande *et al.*, 1993). Interestingly, the level of sperm capacitation induced by the various biological fluids was inversely proportional to the potential of these fluids to scavenge the superoxide anion (de Lamirande *et al.*, 1993). It was then proposed that the triggering of capacitation originated from various stimulators from the biological fluids (an 'on' mechanism) but that its regulation would be through the superoxide scavenging activity of the fluids, which is an inhibiting system (an 'off' mechanism).

The superoxide anion involved in capacitation originated from spermatozoa themselves (de Lamirande and Gagnon, 1995a). Furthermore, the fact that superoxide dismutase (a cell impermeant protein) prevented sperm capacitation indicated that the superoxide anion was generated at the level of the plasma membrane and acted at the external surface of the cell (de Lamirande and Gagnon, 1995a). The superoxide production of human spermatozoa started immediately at the beginning of incubation under capacitating conditions (Ham's F-10 medium supplemented with fetal cord serum ultrafiltrate), reached a plateau 15–25 min later, and was sustained for $>$4 h (de Lamirande and Gagnon, 1995a). On the other hand, sperm hyperactivation peaked after 1–3 h of incubation and capacitation increased progressively over 6 h of incubation, indicating that generation of superoxide anion is one of the first events for the induction and development of these sperm functions (de Lamirande and Gagnon, 1995a).

The involvement of ROS in sperm capacitation is not limited to the superoxide anion and human spermatozoa. Hydrogen peroxide may also be involved in these processes since catalase reduced, and hydrogen peroxide accelerated, the hyperactivation and capacitation of human spermatozoa (Griveau *et al.*, 1994; Leclerc *et al.*, 1997). No direct measurement of hydrogen peroxide generation was performed on capacitating spermatozoa and it can be postulated that this ROS originated from the dismutation of the superoxide anion known to be produced by capacitating human spermatozoa (de Lamirande and Gagnon, 1995a). The concentrations of exogenously added hydrogen peroxide (50 μM) (Griveau *et al.*, 1994; Leclerc *et al.*, 1997) or superoxide anion (0.5 mM generated over a 30 min period) (de Lamirande and Gagnon, 1993a, b) needed to induce human sperm hyperactivation and capacitation *in vitro* exceed by 2–3 orders of magnitude that produced in capacitating sperm suspensions (de Lamirande and Gagnon, 1995a). Therefore, it is possible that the generation of low concentrations of ROS by spermatozoa would be sufficient to induce sperm hyperactivation and capacitation because it occurs in close proximity to the target site on the cell surface. In addition, ROS generated in the immediate environment of the target molecule on sperm surface could reach much higher local concentrations than those effectively measured in whole cell suspensions.

Low concentrations of nitric oxide ($NO^{\cdot}$) also promoted human sperm capacitation (Zini *et al.*, 1995). Surprisingly, catalase prevented the effects of nitric oxide, indicating a complex mechanism of action involving hydrogen peroxide (Zini *et al.*, 1995). The fact that no nitric oxide synthase (NOS) activity was detected in human spermatozoa (capacitated or not) (Zini *et al.*, 1995) suggested that, if nitric oxide plays a role in sperm function *in vivo*, it must originate from the female reproductive tract. Even though the NOS found in the female genital tract was until now associated with modulation of smooth muscle relaxation (Sladek *et al.*, 1993), it is possible that this enzyme is also present in the epithelium of the oviduct.

In hamsters, catalase, but not SOD, reduced sperm capacitation whereas hydrogen peroxide (generated by the combination glucose + glucose oxidase) promoted it, suggesting a role for hydrogen peroxide in sperm capacitation (Bize *et al.*, 1991). In mice, the increases in hyperactivation (110 $\pm$ 20%) and capacitation (46 $\pm$ 4%) that were observed after treatment of spermatozoa with xanthine + xanthine oxidase were totally prevented in the presence of SOD or catalase, indicating that, in this case, both the superoxide anion and hydrogen peroxide were needed for these processes (de Lamirande *et al.*, 1996). There is still no direct evidence for the production of ROS in these two models.

The studies presented above tend to demonstrate the oxidative nature of the capacitation process. During in-vivo capacitation spermatozoa may not be the only source of ROS. Fluids and cells from the female reproductive tract may also produce ROS or promote the formation of ROS by spermatozoa. Indeed, the concentration of oxygen in these fluids rises sharply at the time of ovulation (Maas *et al.*, 1976) further emphasizing the physiological relevance of oxygen and ROS in sperm functions.

Mechanisms of action and origin of ROS

At the present time, very little is known concerning the target for ROS that is involved in sperm capacitation. It should be a molecule or type of molecule that can be oxidized by both the superoxide anion and by hydrogen peroxide since these two ROS induced human sperm capacitation (de Lamirande and Gagnon, 1993a,b, 1995a; Griveau *et al.*, 1994; Leclerc *et al.*, 1997). In addition, this target should be located on the external side of sperm plasma membrane because the superoxide anion

produced by capacitating spermatozoa is released extracellularly and because cell impermeant ROS scavengers such as the superoxide anion and catalase prevented ROS effects (de Lamirande and Gagnon, 1993a,b, 1995a; Griveau *et al.*, 1994; Leclerc *et al.*, 1997). Finally, the reversibility of sperm capacitation could suggest that the ROS target may be subject to oxidation/reduction cycles, which would allow another method of regulating this process. ROS may directly or indirectly affect the enzymes responsible for protein phosphorylation. Indeed, human sperm capacitation was associated with an increase in tyrosine phosphorylation of two proteins, p105 and p81 (see section on hyperactivation), which was abolished in the presence of SOD or catalase (Leclerc *et al.*, 1997). Conversely, incubation of spermatozoa with ROS (xanthine + xanthine oxidase, hydrogen peroxide, or glucose + glucose oxidase) caused significant increases in sperm capacitation and tyrosine phosphorylation of p105 and p81 (Leclerc *et al.*, 1997). Furthermore, the ability of human spermatozoa to respond to progesterone appeared to depend on their level of tyrosine phosphorylation (Aitken *et al.*, 1996). Protein tyrosine phosphorylation was shown to be subject to redox regulation in other cells and organelles (Bauskin *et al.*, 1991; Fialkow *et al.*, 1993) and in sperm acrosome reaction (Aitken *et al.*, 1995). These data strongly suggest the involvement of ROS in signal transduction mechanisms leading to sperm capacitation.

ROS were shown to affect various cell mechanisms that could be expected to exist in spermatozoa and be involved in capacitation. For example, a significant increase in phospholipase A_2 activity and in ATP-dependent calcium uptake was observed *in vivo* in plasma membranes from ovaries following the generation of superoxide anion (Sawada and Carlson, 1991). Furthermore, adenylyl cyclase of a murine vascular smooth muscle cell line was activated in a dose-dependent fashion after treatment with hydrogen peroxide (Tan *et al.*, 1995). Protein kinase C activity (Tahler *et al.*, 1993) and calcium pumps (Grover *et al.*, 1992) were also modified by oxidant stress. Finally, the activity of protein tyrosine kinases and phosphatases, the two types of enzymes regulating the level of tyrosine phosphorylation of proteins, are both known to be susceptible to redox regulation. Oxidants caused an increase of tyrosine kinase Lkt (Bauskin *et al.*, 1991) activity in lymphocytes whereas hydrogen peroxide inhibited tyrosine phosphatases by oxidizing the essential sulphydryl groups present at the active site (Hecht and Zick, 1992). This last observation is potentially important since the presence of reducing agents (such as dithiothreitol) prevented the induction of human sperm capacitation whereas the presence of oxidants targeted at sulphydryl groups (such as phenylarsine oxide and vanadate) promoted tyrosine phosphorylation of p105 and p81 (Leclerc *et al.*, 1997).

The enzymatic system responsible for the generation of the superoxide anion in human spermatozoa and linked to sperm hyperactivation and capacitation remains unknown. Due to some similarities between events occurring during sperm capacitation (calcium influx, increase in intracellular cAMP, protein phosphorylation, etc.) (Yanagimachi, 1994) and the oxidative burst of neutrophils (Morel *et al.*, 1991), we postulated that spermatozoa possess an oxidase at the level of the plasma membrane that would be activated at the time of capacitation (de Lamirande and Gagnon, 1995a; Leclerc *et al.*, 1997). The four inhibitors of neutrophil NADPH oxidase (Thelen *et al.*, 1993) that were tested prevented sperm hyperactivation and capacitation, and three of them significantly decreased the superoxide production related to sperm capacitation (Table I). Two of these inhibitors, diphenyliodonium and diphenyleneiodonium, were also tested for their effect on tyrosine phosphorylation of sperm proteins and were found to significantly reduce this phenomenon (Figure 6 and Leclerc *et al.*, 1997). Therefore, the oxidase of human spermatozoa would share some similarities with that of neutrophils. However, considering that the amounts of superoxide anion produced by spermatozoa during capacitation are more than three orders of magnitude lower than those of activated neutrophils (de Lamirande and Gagnon, 1995a), we can expect that the sperm oxidase would also be quite different from the NADPH oxidase of neutrophils. In addition, signal transduction mechanisms appear to be different in neutrophils and spermatozoa; whereas the tyrosine phosphorylation of specific proteins was a prerequisite for the oxidative burst of neutrophils (Fialkow *et al.*, 1993), it appeared to be a consequence of ROS production in spermatozoa (Leclerc *et al.*, 1997). The exact location, mechanism of activation, and co-factors (NADPH, NADH or others) of the sperm oxidase remain to be determined.

Table I. Effect of NADPH oxidase inhibitors on human sperm hyperactivation, capacitation and superoxide production

Inhibitor	Hyperactivation (% of control)	Capacitation (% of control)	Superoxide production (% of control)
None	100	100	100
Diphenyliodonium	21 ± 6	5 ± 1	32 ± 5
Diphenyleneiodonium	27 ± 4	16 ± 2	112 ± 16
Cibacron blue	61 ± 9	3 ± 1	66 ± 4
Lapachol	18 ± 4	32 ± 5	68 ± 11

Percoll-washed human spermatozoa were incubated in Ham's F-10 medium supplemented or not with fetal cord serum ultrafiltrate (7.5%, v/v), and in the absence (none) or presence of NADPH oxidase inhibitors (100 μM). Sperm hyperactivation was measured after 1 h of incubation using the CellSoft™ system (Murad *et al.*, 1992) and sperm capacitation was determined after 3.5 h of incubation by the lysophosphatidylcholine-induced acrosome reaction as described in Figure 1. The superoxide anion production was measured by chemiluminescence as described by de Lamirande and Gagnon (1995a). Values are expressed as a percentage of the hyperactivation, capacitation and superoxide production measured in the presence of fetal cord serum ultrafiltrate. Values are mean ± SEM of four to six experiments performed with semen samples from different volunteers. Values obtained with the various NADPH oxidase inhibitors are all significantly different ($P < 0.05$) from those of capacitating spermatozoa except that for the superoxide production of spermatozoa treated with diphenyleneiodonium.

Clinical relevance of ROS in sperm hyperactivation

The needs for a fine balance between ROS production and scavenging, as well as the right timing for ROS production, are of paramount importance for sperm functions. As stated above, spontaneous sperm hyperactivation was observed in 18% (22/120) of semen samples from infertile patients whose spermiograms were considered normal according to the World Health Organization criteria (de Lamirande and Gagnon,

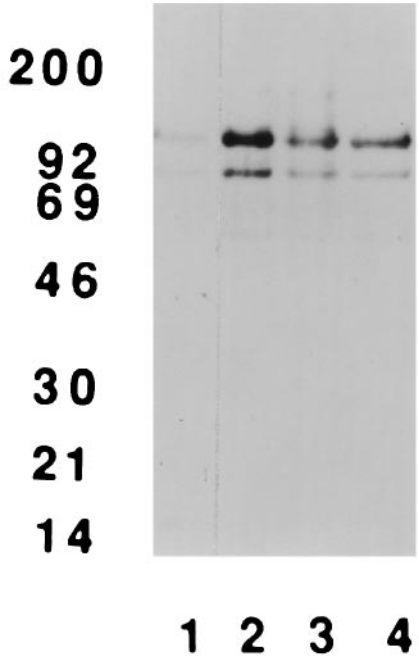

Figure 6. Effect of NADPH oxidase inhibitors on tyrosine phosphorylation of proteins in capacitating human spermatozoa. Percoll-washed human spermatozoa were incubated for 2.5 h at 37°C in Ham's F-10 medium alone (no capacitation, lane 1) or Ham's F-10 + 10% fetal cord serum ultrafiltrate in the absence (capacitation, lane 2) or presence of 0.1 mM diphenyliodonium (lane 3), or diphenyleneiodonium (lane 4) (inhibition of capacitation). Total sperm proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose. Phosphotyrosine-containing proteins were detected on Western blots using a monoclonal antiphosphotyrosine antibody (Leclerc *et al.*, 1996a,b). The position of the molecular mass markers (kDa) is shown on the left. Results are from one experiment representative of four others. Human sperm capacitation was associated with a marked increase in tyrosine phosphorylation of two proteins, p105 and p81. NADPH oxidase inhibitors caused a significant reduction both in this phosphorylation and in sperm capacitation (Table I).

1993c,d). Interestingly, this phenomenon was associated with a decrease in the superoxide scavenging capacity of the seminal plasma (by 37%) and spermatozoa (by 40%) of these patients as compared to those of seminal plasma and spermatozoa from normal men (de Lamirande and Gagnon, 1993c,d). It was postulated that spermatozoa from these patients prematurely initiated superoxide production and that the resulting imbalance between the superoxide production and scavenging led to a net superoxide release from spermatozoa which caused a premature sperm hyperactivation (de Lamirande and Gagnon, 1993c,d).

Cellular controls of sperm capacitation

Whereas signal transduction mechanisms involved in sperm acrosome reaction have been studied extensively (Yanagimachi, 1994; Doherty *et al.*, 1995), relatively little information is available on the controls of sperm capacitation. At the present time, there is evidence for the involvement of calcium, ROS, cAMP, protein kinases and protein phosphatases (for serine/threonine and tyrosine residues of proteins) in human sperm capacitation. The relationships that exist between these modulators are not well established and are probably complex since they can influence and interact with each other in multiple ways.

Adenylyl cyclase and cAMP

Calcium influx and generation of ROS appear as the earliest events of sperm capacitation since they are initiated immediately at the beginning of incubation under capacitating conditions (Baldi *et al.*, 1991; de Lamirande and Gagnon, 1995a). These two processes may be responsible for the physiological activation of adenylyl cyclase which might play a role in the development of human sperm capacitation. Indeed, substances that contribute to the rise in intracellular concentrations of cAMP, such as forskolin (an adenylyl cyclase activator), dibutyryl cAMP (a cell permeant analogue of cAMP), as well as caffeine and isobutylmethylxanthine (phosphodiesterase inhibitors), stimulated human sperm capacitation and protein tyrosine phosphorylation of two proteins, p105 and p81 (Leclerc *et al.*, 1997). Furthermore, progesterone was recently shown to stimulate calcium influx in human spermatozoa and consequently to cause increases in intracellular cAMP concentrations, protein tyrosine phosphorylation and rate of capacitation (Emiliozzi *et al.*, 1996; Parinaud and Milhet, 1996).

The complexity of the mechanisms by which cAMP is produced and acts is well illustrated by results obtained with monkey spermatozoa. In this species, caffeine increased the number of spermatozoa binding to the zona pellucida, whereas dibutyryl cAMP induced higher rates of sperm acrosome reaction; however, the combination of both substances was needed for the development of hyperactivated motility (VandeVoort *et al.*, 1994). Even though caffeine and dibutyryl cAMP are both recognized as agents that cause an increase in intracellular cAMP concentration, it appeared that they may have other mechanisms of action and/or that the increase in cAMP concentrations in different compartments of spermatozoa may have different effects (VandeVoort *et al.*, 1994).

The fact that mouse spermatozoa incubated in a medium devoid of calcium but supplemented with cell permeant cAMP analogues were able to undergo capacitation (Visconti *et al.*, 1995a,b) corroborated the proposition that calcium influx would directly or indirectly lead to activation of adenylyl cyclase. Conversely, the induction of human sperm capacitation and its associated tyrosine phosphorylation by isobutylmethylxanthine was not inhibited by superoxide dismutase (Figure 7), suggesting that the intracellular increase in cAMP occurred downstream of ROS production. It is therefore possible that ROS take part in activation of sperm adenylyl cyclase as demonstrated in other cellular systems (Tahler *et al.*, 1993).

Protein kinases

The increase in adenylyl cyclase activity results in an increase in cAMP, which has been implicated in the initiation and maintenance of sperm motility (Tash, 1990) and capacitation (Yanagimachi, 1994; Leclerc *et al.*, 1996, 1997). Most of the

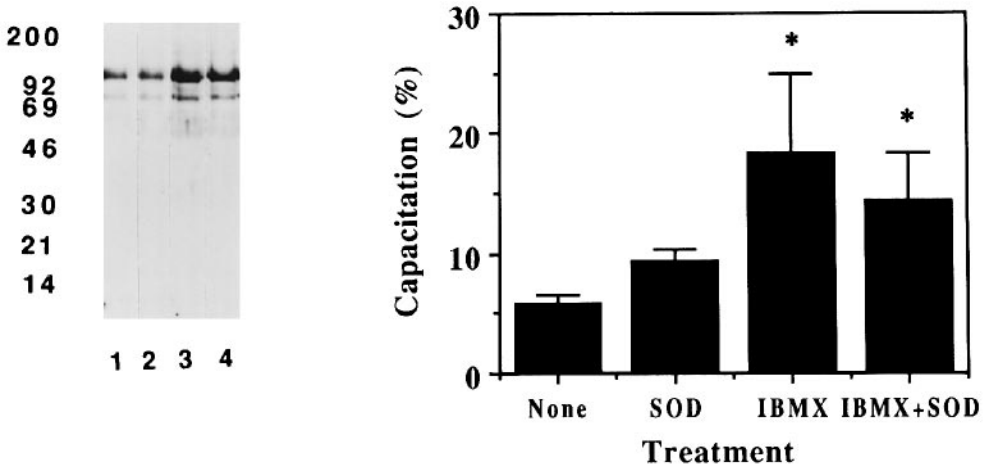

Figure 7. Effect of superoxide dismutase (SOD) and isobutylmethylxanthine on sperm capacitation and protein tyrosine phosphorylation. Percoll-washed human spermatozoa were incubated at 37°C in Ham's F-10 medium alone (lane 1), or supplemented with superoxide dismutase (SOD, 0.1 mg/ml) (lane 2), isobutylmethylxanthine (IBMX, 0.5 mM) (lane 3) or both of these substances (lane 4). Protein tyrosine phosphorylation (left panel) was evaluated after 2.5 h of incubation, as described in Figure 4. The position of the molecular mass markers (kDa) is shown on the left. Results are from one experiment representative of five others. Sperm capacitation (right panel) was determined after 4 h of incubation, by the LPC-induced acrosome reaction as described in Figure 1. Values are mean ± SEM of six experiments performed with different sperm samples. *Value significantly higher than those obtained with no supplementation of Ham's F-10 (none) or SOD. IBMX-induced sperm capacitation and protein tyrosine phosphorylation were not prevented by the presence of SOD.

actions of cAMP are mediated through activation of protein kinases A (PKA), the presence of which has been reported in spermatozoa (Garbers *et al.*, 1973). In mice (Visconti *et al.*, 1995a,b) and humans (Leclerc *et al.*, 1996), inhibitors of PKA (H-89 and Rp-cAMPS) blocked sperm capacitation and the associated protein tyrosine phosphorylation, indicating that PKA takes part in these processes. Treatment with dibutyryl cGMP also accelerated mouse sperm capacitation (Furuya *et al.*, 1992), suggesting the possible involvement of guanylyl cyclase and cGMP. However, the guanylyl cyclase/cGMP system is known more for its role in sperm acrosome reaction (Santos-Sacchi and Gordon, 1980; Yanagimachi, 1994), and its involvement and mechanism of action in sperm capacitation remain to be confirmed.

The enhancing effect of phorbol myristate acetate (PMA), an activator of protein kinase C (PKC), on the phosphorylation of numerous human sperm proteins (78, 71, 57.5, and 56 kDa) and on mouse and human sperm capacitation, as well as the reversal of these effects by staurosporine or H-7 (non-specific inhibitors of protein kinases) could indicate the involvement of PKC in capacitation (Furuya *et al.*, 1992; 1993b). Indeed, various forms of PKC were found to be compartmentalized in human spermatozoa, PKCα and $PKC\beta_{II}$ being located in the equatorial segment of the sperm head and $PKC\beta_{I}$ and PKCε in the principal piece of the flagellum (Naor *et al.*, 1995). However, the stimulation of human sperm capacitation (Furuya *et al.*, 1993b) and acrosome reaction (Naor *et al.*, 1995) by PMA were not calcium dependent, which did not favour the hypothesis of an activation of PKC. It was recently observed that the stimulation of human sperm capacitation and tyrosine phosphorylation by PMA was bicarbonate-dependent (Leclerc *et al.*, 1996). Therefore, it is possible that, as observed in hamster spermatozoa (Visconti *et al.*, 1990), PMA caused a bicarbonate-dependent increase in cAMP concentration. The mechanism by which this stimulation would occur is not yet known but cAMP/PKA- and PKC-dependent pathways appear to be interrelated and ultimately converge in the induction of the acrosome reaction in human spermatozoa (Doherty *et al.*, 1995).

The cAMP-dependent phosphorylation of human sperm proteins appeared as a prerequisite for protein tyrosine phosphorylation, a phenomenon often associated with capacitation. Naz *et al.* (1991) observed tyrosine phosphorylation of detergent-solubilized human sperm membrane proteins in four molecular weight regions (94, 46, 25, and 12 kDa), the importance of which varied according to the state of capacitation. The 94 kDa and the 12 kDa proteins were respectively 30% more and 12% less phosphorylated in capacitated than in non-capacitated spermatozoa. Immunofluorescence observations on methanol-fixed human spermatozoa indicated a progressive change in the localization of tyrosine phosphorylated proteins from the flagellum to the acrosomal region during capacitation (Naz *et al.*, 1991). Other researchers observed a time-dependent increase in tyrosine phosphorylation of proteins of ~97 and 75 kDa (Luconi *et al.*, 1995) and 94 kDa (Emiliozzi *et al.*, 1996) during human sperm capacitation but did not attempt to localize these proteins. More recently, Leclerc *et al.* (1996, 1997) observed that tyrosine phosphorylation of two proteins, p105 and p81, was higher in capacitating than in non-capacitating spermatozoa. This phosphorylation was time-dependent and significantly increased as early as 1 h after the beginning of the incubation. The localization of p105 and p81 in the fibrous sheath of the sperm flagellum (Figure 4) (Leclerc *et al.*, 1997) is suggestive of their possible role in the control of sperm motility.

The increased degree of protein phosphorylation observed during sperm capacitation may result from a stimulation of kinases and/or an inhibition of phosphatases but the relative role of these enzymes is not yet known. Human spermatozoa contain type 1 and type 2B protein phosphatases (Tash *et al.*,

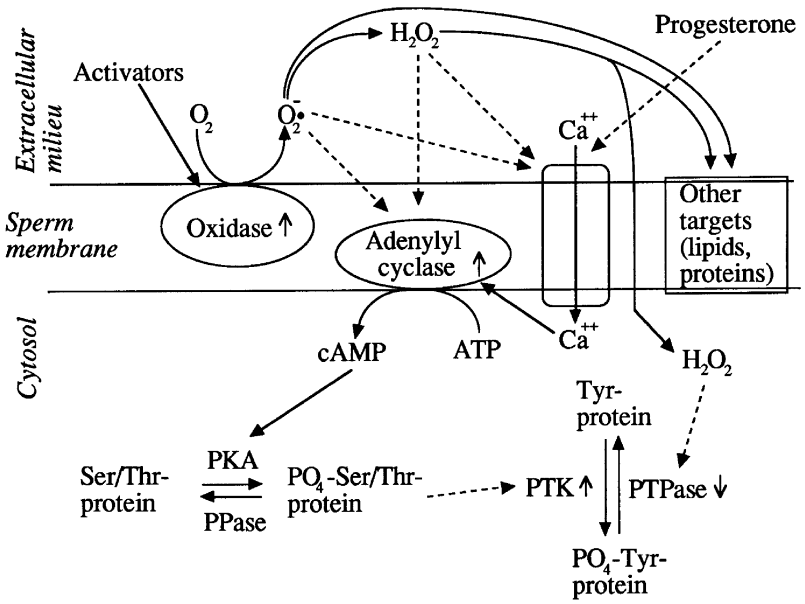

Figure 8. Hypothetical signalling pathways during human sperm capacitation. ROS generation and calcium influx appear as the first cellular events of capacitation. At the beginning of the capacitation period, a yet unidentified biological effector would activate an oxidase at the level of the sperm plasma membrane, which would result in an immediate and sustained production of the superoxide anion ($O_2^{\cdot -}$). This ROS or its dismutation product, hydrogen peroxide (H_2O_2), could activate sperm adenylyl cyclase as observed in other cell systems or promote calcium influx through inactivation of calcium ATPases or calcium channels (dashed lines indicate hypothetical phenomenon). It is also possible that ROS transform lipids, therefore promoting their extraction from the plasma membrane. In addition ROS could oxidize proteins and modify their structure and functions. Calcium influx, which could possibly be induced by the action of progesterone on specific receptor or inactivation of calcium ATPases, also occurs immediately at the beginning of the capacitation period, and reaches its maximum 2 h later. Calcium could activate the adenylyl cyclase as well as many other enzymes, such as protein kinase C and phospholipases, for which there is no evidence yet for their involvement in sperm capacitation. Stimulation of adenylyl cyclase activity would lead to an increased formation of cAMP. As a consequence of the production of this second messenger, protein kinase A (PKA) would be activated and cause an increase in the serine/threonine phosphorylation of numerous proteins therefore modulating their functions to elicit an overall cellular response. The increase in Ser/Thr protein phosphorylation could also be caused by an inhibition of protein phosphatase activity. Protein tyrosine kinases (PTK) are potential targets for PKA or their phosphorylated substrates; the consequent increase in PTK activity would result in the phosphorylation of various proteins, the location (fibrous sheath, membrane; flagellum, head) of which would vary with the degree of capacitation achieved. Moreover, the increase in protein phosphotyrosine content could be due to inhibition of protein tyrosine phosphatases (PTPases), since these enzymes are very sensitive to oxidation.

1988; Tash and Bracho, 1994; Smith *et al.*, 1996). Calyculin A and okadaic acid (inhibitors of type 1 and 2A phosphatases) promoted human sperm capacitation (Furuya *et al.*, 1993a; Leclerc *et al.*, 1996) but only calyculin A caused an increase of p105 and p81 tyrosine phosphorylation (Leclerc *et al.*, 1996). The fact that these inhibitors block serine/threonine (Ser/Thr) protein phosphorylation suggests that Ser/Thr phosphorylated proteins are involved (possibly through cAMP/PKA regulation) in sperm capacitation and in tyrosine phosphorylation of p105 and p81. Whether such Ser/Thr phosphorylated proteins are themselves tyrosine kinases or phosphatases or other regulators involved in the tyrosine phosphorylation pathway remains to be established. Substances such as phenylarsine oxide, vanadate, and hydrogen peroxide that can oxidize the cysteine residues present at the active site of phosphotyrosine protein phosphatases (Garcia-Morales *et al.*, 1990; Hecht and Zick., 1992) increased protein tyrosine phosphorylation in human spermatozoa (Leclerc *et al.*, 1997). However, the correlation between sperm capacitation and tyrosine phosphorylation is not perfect, which suggests that, although a cAMP-dependent phosphorylation of proteins appears as a common intermediate step, there are probably divergent pathways regulating tyrosine phosphorylation of proteins, capacitation, and motility in human spermatozoa.

Considering the variety and complexity of mechanisms described above (summarized in Figure 8), the possibility of cross talk between these processes, as well as the paramount importance of sperm capacitation in the fertilizing process, it is tempting to speculate that more than one pathway may be available to ensure the success of this event. In other words, the redundancy which is observed in so many other cell types would ensure that if the main pathway fails, capacitation can still proceed.

Capacitation as a priming and regulatory step for the acrosome reaction

The factors mentioned above, calcium influx, ROS generation, increase in adenylyl cyclase activity and protein phosphorylation, are also involved in the induction of the acrosome reaction (Yanagimachi, 1994; Aitken *et al.*, 1995; Doherty *et al.*, 1995; Leclerc and Kopf, 1995). It is therefore possible that sperm capacitation may be considered as a 'priming' event during which the cellular systems are brought to the specific level of activation that will be needed for the acrosome reaction to

proceed upon exposure to the proper stimulus. This would imply the presence of inhibitory mechanisms to keep the system in this reversible state of activation, thus preventing a premature acrosome reaction. It could also suggest the need for a very specific stimulus for the acrosome reaction and/or the recruitment of one or several regulatory mechanisms not involved in sperm capacitation. A very good example of the stepwise nature of sperm capacitation is illustrated by results obtained by Suarez and Dai (1995) on calcium influx in hamster spermatozoa. During capacitation, the intracellular calcium concentration increased to a specific value that remained constant until the induction of the acrosome reaction, at which time there was a second calcium influx. Presently, no time sequence of events has yet been established for the concentrations of cAMP, rate of ROS generation, degree of protein phosphorylation, etc. attained during sperm capacitation and acrosome reaction. These data would provide essential information on the mechanisms that are common or different in these two cellular processes. Furthermore, they would help to pinpoint the factors (or mechanisms) responsible for maintaining spermatozoa in the capacitated ('primed') state, as well as those that are specific for the second step of activation, that is, the acrosome reaction. Important intermediates in signal transduction mechanisms, such as phospholipases, G-proteins, phosphatidylinositol, ion channels, etc. have been shown to be involved in sperm acrosome reaction but their potential role in sperm capacitation has not yet been studied.

Conclusion

Capacitation of spermatozoa, which is essential for the acquisition of fertilizing ability, is a complex process that involves major cellular modifications. Interestingly, changes occur both in sperm head and flagellum, and in multiple cellular compartments (membrane, cytosol, cytoskeleton) of spermatozoa. The coordinated loss of specific factors originating from epididymal fluid or seminal plasma is associated with extensive reorganization of membranes and initiation of signal transduction mechanisms that cause spermatozoa to become capacitated or 'primed'. In this context, the reversibility of some of the mechanisms leading to sperm capacitation may be one of the most important factors that allow the perfect timing of this process. We can postulate that an equilibrium exists in the exchanges between spermatozoa and the surrounding fluids (for example the binding and removal of decapacitating factors and the cholesterol exchange between lipids of the cell membrane and cholesterol and proteins of the extracellular milieu) to keep them capacitated but unable to spontaneously acrosome react before they reach the egg.

Sperm capacitation is temporally associated with the acquisition of hyperactivated motility. The different requirements for these two processes (e.g. bicarbonate and calcium concentrations) could suggest that hyperactivation is not a part of capacitation. However, *in vivo*, spermatozoa are exposed to concentrations of these ions that are sufficient to support the development of both processes. Sperm hyperactivation develops during capacitation in the female genital tract and allows spermatozoa to detach from the epithelium of the oviduct and progress towards the egg. Furthermore, capacitation allows sperm binding to the zona pellucida and acrosome reaction, but hyperactivated motility is essential for penetration of the zona pellucida, demonstrating that real (physiological) capacitation cannot proceed without hyperactivation.

Many questions remain to be addressed and elucidated in the understanding of events leading to sperm capacitation. One main field of interest concerns in-vivo capacitation. The improvement of tissue culture systems (type of co-culture, temperature, oxygen concentration, etc.) could help to reproduce in-vivo conditions and result in a physiologically more relevant process of human sperm capacitation *in vitro*. These studies could help to elucidate how spermatozoa and oviduct interact with each other, and to identify the real inducers and the mechanisms regulating sperm capacitation, etc.. On the other hand, more research should also be undertaken to understand the signal transduction mechanisms leading to sperm capacitation and what makes them different (in type, concentration, or activity of a messenger, in cellular localization, etc.) from those involved in the acrosome reaction. The knowledge obtained in these two areas of research could provide better conditions for IVF or treatment of spermatozoa to improve their fertilizing ability.

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References

Adeoya-Osiguwa, S. and Fraser, L.R. (1993) A biphasic pattern of $^{45}Ca^{2+}$ uptake by mouse spermatozoa *in vitro* correlates with changing functional potential. *J. Reprod. Fertil.*, **99,** 187–194.

Adeoya-Osiguwa, S. and Fraser, L.R. (1994) Evidence for Ca^{2+}-dependent ATPase activity, modulated by decapacitating factors and calmodulin, in mouse spermatozoa. *J. Reprod. Fertil.*, (Abstr. Ser.), **14,** 9–10.

Adeoya-Osiguwa, S. and Fraser, L.R. (1996) Evidence for Ca^{2+}-dependent ATPase activity, stimulated by decapacitation factor and calmodulin, in mouse sperm. *Mol. Reprod. Dev.*, **44,** 111–120.

Aitken, R.J. and Clarkson, J.S. (1987) Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fertil.*, **81,** 459–469.

Aitken, R.J., Buckingham, D.W. and Fang, H.G. (1993) Analysis of the response of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. *J. Androl.*, **14,** 132–141.

Aitken, R.J., Paterson, M., Fisher, H. *et al.* (1995) Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J. Cell Sci.*, **180,** 2017–2025.

Aitken, R.J., Buckingham, D.W. and Harkiss, D. *et al.* (1996) The extragenomic action of progesterone on human spermatozoa is influenced by the redox regulated changes in tyrosine phosphorylation during capacitation. *Mol. Cell. Endocrinol.*, **117,** 83–93.

Alvarez, J.G., Touchtone, J.C., Blasco, L. and Storey B.T. (1987) Spontaneous lipid peroxidation and and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as a major enzyme protectant against oxygen toxicity. *J. Androl.*, **8,** 338–348.

Andrews, J.C. and Bavister, B.D. (1989) Capacitation of hamster spermatozoa with the divalent cation chelators D-penicillamine, L-histidine, and L-cysteine in a protein-free culture medium. *Gamete Res.*, **23,** 159–170.

Andrews, J.C., Noland, J.P., Hammerstedt, R.H. and Bavister, B.D. (1994) Role of zinc in hamster sperm capacitation. *Biol. Reprod.*, **51,** 1238–1247.

Aonuma, S., Okabe, M., Kawaguchi, M. and Kishi, Y. (1981) Zinc effects on mouse spermatozoa and *in vitro* fertilization. *J. Reprod. Fertil.*, **63,** 463–466.

Audhya, T., Reddy, J. and Zaneveld, L.J.D. (1987) Purification and partial chemical characterization of a glycoprotein with antifertility activity from human seminal plasma. *Biol. Reprod*., **36,** 511–521.

Austin, C.R. (1951) Observations on the penetration of the sperm into the mammalian egg. *Aust. J. Sci. Res.* B, **4,** 581–596.

Baldi, E., Casana, R., Falsetti, C. *et al.* (1991) Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J. Androl.*, **12,** 323–330.

Banerjee, M. and Chowdhury, M. (1994) Purification and characterization of a sperm-binding glycoprotein from human endometrium. *Hum. Reprod.*, **9,** 1497–1504.

Barratt, C.L.R. and Cooke, I.D. (1991) Sperm transport in the human female reproductive tract – a dynamic interaction. *Int. J. Androl.*, **14,** 394–411.

Barratt, C.L.R. and Hornby, D.A. (1995) Induction of the human acrosome reaction by rhuZP3. In Fénichel, P. and Parinaud, J. (eds), *Human Sperm Acrosome Reaction.* Colloque INSERM no. 236. John Libbey Eurotext Ltd., Paris, France, pp. 105–122.

Barratt, C.L.R., Bolton, A.E. and Cooke, I.D. (1990) Functional significance of white blood cells in the male and female reproductive tract. *Hum. Reprod*., **5,** 639–648.

Bauskin, A.R., Alkalai, I. and Ben-Neriah, Y. (1991) Redox regulation of tyrosine kinase in the endoplasmic reticulum. *Cell*, **56,** 685–696.

Bedford, J.M. and Chang, M.C. (1962) Removal of decapacitation factor from seminal plasma by high speed centrifugation. *Am. J. Physiol.*, **202,** 179–181.

Benoff, S. (1993) Preliminaries to fertilization. The role of cholesterol during capacitation of human spermatozoa. *Hum. Reprod*., **12,** 2001–2008.

Benoff, S., Hurley, I., Cooper, G.W. *et al.* (1993) Head-specific mannose-ligand receptor expression in human spermatozoa is dependent on capacitation-associated membrane cholesterol loss. *Hum. Reprod.*, **8,** 2141–2154.

Bielfeld, P., Jeyendran, R.S. and Zanaveld, L.J.D. (1991) Human spermatozoa do not undergo the acrosome reaction during storage in the cervix. *Int. J. Fertil.*, **36,** 302–306.

Bize, I., Santander, G., Cabello, P. *et al.* (1991) Hydrogen peroxide is involved in hamster sperm capacitation *in vitro*. *Biol. Reprod*., **44,** 398–403.

Boatman, D.E. and Robbins, R.S. (1991) Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. *Biol. Reprod.*, **44,** 806–813.

Boué, F. and Sullivan, R. (1996) Cases of human infertility are associated with the absence of P34H, an epididymal sperm antigen. *Biol. Reprod*., **54,** 1018–1024.

Boué, F., Lasalle, B., Duquenne, C. *et al.* (1992) Human sperm proteins from testicular and epididymal origin that participate in fertilization: modulation of sperm binding to zona-free hamster oocytes, using monoclonal antibodies. *Mol. Reprod. Dev.*, **33,** 470–480.

Boué, F., Bérubé, B., de Lamirande, E. *et al.* (1994) Human sperm-zona pellucida interaction is inhibited by antiserum against a hamster sperm protein. *Biol. Reprod*., **51,** 577–587.

Boué, F., Blais, J. and Sullivan, R. (1996) Surface localization of P34H, an epididymal protein, during maturation, capacitation and acrosome reaction of human spermatozoa. *Biol. Reprod*., **54,** 1009–1017.

Burkman, L.J. (1990) Hyperactivated motility of human spermatozoa during *in vitro* capacitation and implications for fertility. In Gagnon,C. (ed.), *Controls of Sperm Motlity: Biological and Clinical Aspects.* CRC Press, Boca Raton, FL, USA, pp. 303–329.

Chan, P.J., Prough, S.G., Hening, I. and Tredway, D.R. (1992) Predictive value of sperm hyperactivation measurement based on the dilution effect method in clinical *in vitro* fertilization. *Int. J. Fertil.*, **37,** 373–377.

Chang, M.C. (1951) Fertilizing capacity of spermatozoa deposited in Fallopian tubes. *Nature*, **168,** 997–998.

Chang, M.C. (1984) The meaning of sperm capacitation. *J. Androl.*, **5,** 45–50.

Cohen-Dayag, A. and Eisenbach, M. (1994) Potential assays for sperm capacitation in mammals. *Am. J. Physiol.*, **267,** C1167–C1176.

Cross, N.L. (1996) Human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist, progesterone: cholesterol is the major inhibitor. *Biol. Reprod*., **54,** 138–145.

DasGupta, S., Mills, C.L. and Fraser, L.R. (1993) Ca^{2+}-related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. *J. Reprod. Fertil.*, **99,** 135–143.

DasGupta, S., Mills, C.L. and Fraser, L.R. (1994) A possible role for Ca^{2+}-ATPase in human sperm capacitation. *J. Reprod. Fertil*., **102,** 107–116.

Davis, B.K. (1982) Uterine fluid proteins bind sperm cholesterol during capacitation in the rabbit. *Experientia*, **38,** 1063–1064.

de Lamirande, E. and Gagnon, C. (1993a) A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *Int. J. Androl*., **16,** 21–25.

de Lamirande, E. and Gagnon, C. (1993b) Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Rad. Biol. Med*., **14,** 157–166.

de Lamirande, E. and Gagnon, C. (1993c) Human sperm hyperactivation in whole semen and its association with low superoxide scavenging capacity in seminal plasma. *Fertil. Steril*., **59,** 1291–1295.

de Lamirande, E. and Gagnon, C. (1993d) Human sperm hyperactivation in whole semen and its association with low superoxide scavenging capacity in seminal plasma and spermatozoa. *Fertil. Steril*., **59** (Suppl.), 221.

de Lamirande, E. and Gagnon, C. (1995a) Capacitation-associated production of superoxide anion by human spermatozoa. *Free Rad. Biol. Med.* **18,** 487–495.

de Lamirande, E. and Gagnon, C. (1995b) Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. *Hum. Reprod*., **10** (Suppl. 1), 15–21.

de Lamirande, E., Eiley, D. and Gagnon, C. (1993) Inverse relationship between the induction of human sperm capacitation and spontaneous acrosome reaction by various biological fluids and the superoxide scavenging capacity of these fluids. *Int. J. Androl.*, **16,** 258–263.

de Lamirande, E. Jiang, H., Zini, A. *et al.* (1997) Reactive oxygen species and sperm physiology. *Rev. Reprod*., **2,** 48–54.

Demott, R.P. and Suarez, S.S. (1992) Hyperactivated sperm progress in the mouse oviduct. *Biol. Reprod.*, **46,** 779–785.

Demott, R.P., Lefebvre, R. and Suarez, S.S. (1995) Carbohydrates mediate the adherence of hamster sperm to oviductal epithelium. *Biol. Reprod.*, **52,** 1395–1403.

Doherty, C.M., Tarchala, S.M., Radwanska, E. and DeJonge, C.J. (1995) Characterization of two second messenger pathways and their interaction in eliciting the human sperm acrosome reaction. *J. Androl.*, **16,** 36–46.

Ehrenwald, E., Foote, R.H. and Parks, J.E. (1990) Bovine oviductal fluid components and their potential role in sperm cholesterol efflux. *Mol. Reprod. Dev.*, **25,** 195–204.

Ellington, J.E., Ignotz, G.G., Meyer-Wallen, V.N. and Currie, W.B. (1993) *De novo* protein synthesis by bovine uterine tube (oviduct) epithelial cells changes during co-culture with bull spermatozoa. *Biol. Reprod*., **48,** 851–856.

Emiliozzi, C., Cordonier, H., Guérin, J.F. *et al.* (1996) Effects of progesterone on human spermatozoa prepared for *in vitro* fertilization. *Int. J. Androl.*, **19,** 39–47.

Fialkow, L., Chan, C.K., Grinstein, S. and Downey, G.P. (1993) Regulation of tyrosine phosphorylation in neutrophils by the NADPH oxidase. *J. Biol. Chem*., **268,** 17131–17137.

Florman, H.M., Corron, M.E., Kim, T.D.H. and Babcock, D.F. (1992) Activation of voltage-dependent calcium channels of mammaliam sperm is required for zona-induced acrosomal exocytosis. *Dev. Biol.*, **152,** 304–314.

Focarelli, R., Giuffrida, A. and Rosati, F. (1995) Changes in the sialylglycoconjugate distribution on the human sperm surface during in-vitro capacitation: Partial purification of a 20 kDa sialylglycoprotein of capacitated spermatozoa. *Mol. Hum. Reprod*., **1,** see *Hum. Reprod.*, **10,** 2755–2759.

Foresta, C., Rossato, M., Mioni, R. and Zorzi, M. (1992) Progesterone induces capacitation in human spermatozoa. *Andrologia*, **24,** 33–35.

Fraser, L.R. (1995a) Ionic control of sperm function. *Reprod. Fertil. Dev.*, **7,** 905–925.

Fraser, L.R. (1995b) Mechanisms regulating capacitation and the acrosome reaction. In Fénichel, P. and Parinaud, J. (eds), *Human Sperm Acrosome Reaction.* Colloque INSERM no. 236. John Libbey Eurotext Ltd., Paris, France, pp. 17–33.

Fraser, L.R., Umar, G. and Sayed, S. (1993) Na^{+} requiring mechanisms modulate capacitation and acrosomal exocytosis in mouse spermatozoa. *J. Reprod. Fertil*., **97,** 539–549.

Fraser, L.R., Abeydeera, L.R. and Niwa, K. (1995) Ca^{2+}-regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. *Mol. Reprod. Dev.*, **40,** 233–241.

Furuya, S., Endo, Y., Oba, M. *et al.* (1992) Effects of modulators of protein kinases and phosphatases on mouse sperm capacitation. *J. Assist. Reprod. Genetics*, **9,** 391–399.

Furuya, S., Endo, Y., Osumi, K. *et al.* (1993a) Calyculin A, protein phosphatase inhibitor, enhances capacitation of human sperm. *Fertil. Steril*., **59,** 216–222.

Furuya, S., Endo, Y., Osumi, K. *et al.* (1993b) Effect of modulators of protein kinase C on human sperm capacitation. *Fertil. Steril*., **59,** 1285–1290.

Fusi, F.M. and Bronson, R.A. (1992) Sperm surface fibronectin. Expression following capacitation. *J. Androl.*, **13,** 28–35.

Fusi, F.M., Lorenzetti, I., Vignali, M. and Bronson, R.A. (1992) Sperm surface proteins after capacitation. Expression of vitronectin on the spermatozoan head and laminin on the sperm tail. *J. Androl.*, **13,** 488–497.

Garbers, D.L., First, N.L. and Lardy, H.A. (1973) Properties of adenosine 3′5′-monophosphate-dependent protein kinases isolated from bovine epididymal spermatozoa. *J. Biol. Chem.*, **248,** 875–879.

Garcia-Morales, P., Minami, Y., Luong, E. *et al.* (1990) Tyrosine phosphorylation in T cells is regulated is regulated by phosphatase activity: studies with phenylarsine oxide. *Proc. Natl. Acad. Sci. USA*, **87,** 9255–9259.

Green, C.M., Cockle, S.M., Watson, P.F. and Fraser, L.R. (1996) Fertilization promoting peptide, a tripeptide similar to thyrotrophin-releasing hormone, stimulates the capacitation and fertilizing ability of human spermatozoa *in vitro*. *Hum. Reprod.*, **11,** 830–836.

Grippo, A.A., Anderson, S.H., Chapman, D.A. *et al.* (1994) Cholesterol, phospholipidid and phospholipase activity of ampullary and isthmic fluid from the bovine oviduct. *J. Reprod. Fertil.*, **102,** 87–93.

Griveau, J.F., Renard, P. and LeLannou, D. (1994) An *in vitro* promoting role for hydrogen peroxide in human sperm capacitation. *Int. J. Androl.*, **17,** 300–307.

Gross, M.K., Toscano, D.G. and Toscano, W.A.Jr. (1987) Calmodulin-mediated adenylate cyclase from mammalian sperm. *J. Biol. Chem.*, **262,** 8672–8676.

Grover, A.K., Samson, S.E. and Fomin, V.P. (1992) Peroxide inactivates calcium pumps in pig coronary artery. *Am. J. Physiol.*, **263,** H537-H543.

Hallett, M., Schneider, A.S. and Carbone, E. (1972) Tetracycline fluorescence as calcium-probe for nerve membrane with some studies using erythrocyte ghosts. *J. Membr. Biol.*, **10,** 31–44.

Han, H., Mack, S., DeJonge, C. and Zaneveld, L. (1990) Inhibition of the human sperm acrosome reaction by a high molecular weight factor from human seminal plasma. *Fertil. Steril.*, **54,** 1177–1179.

Harrison, R.A.P. and Gadella, B.M. (1995) Membrane changes during sperm capacitation. In Fénichel, P. and Parinaud, J. (eds), *Human Sperm Acrosome Reaction.* Colloque INSERM no. 236. John Libbey Eurotext Ltd., pp. 45–65.

Hecht, D. and Zick, Y. (1992) Selective inhibition of protein tyrosine phosphatase activities by H_2O_2 and vanadate *in vitro*. *Biochem. Biophys. Res. Comm.*, **188,** 773–779.

Holt, W.V. (1995) The sperm plasma membrane. In Fénichel, P. and Parinaud, J. (eds), *Human Sperm Acrosome Reaction.* Colloque INSERM no. 236. John Libbey Eurotext Ltd., pp. 1–16.

Hoshi, K., Sasaki, H., Yanagida, K. *et al.* (1994) Localization of fibronectin on the surface of human spermatozoa and relation to the sperm-egg interaction. *Fertil. Steril.*, **61,** 542–547.

Hunter, R.H.F. (1987) Periovulatory physiology of the oviduct, with special reference to progression, storage and capacitation of spermatozoa. In Mohri, H. (ed.), *New Horizons in Sperm Cell Research*. Japan Sci. Soc. Press, Tokyo/Gordon and Breach Sci. Publ., New York, pp. 31–45.

Hunter, R.H.F. and Nichol, R. (1986) A preovulatory temperature gradient between the isthmus and ampulla of pig oviducts during the phase of sperm storage. *J. Reprod. Fertil.*, **77,** 599–606.

Hyne, R.V., Higginson, R.E., Kohlman, D. and Lopata, A. (1984) Sodium requirement for capacitation and membrane fusion during the guinea-pig sperm acrosome reaction. *J. Reprod. Fertil.*, **70,** 83–94.

Jarvi, K., Roberts, K.D., Langlais, J. and Gagnon, C. (1993) Effect of platelet-activating factor, lyso-platelet activating factor, and lysophosphatidylcholine on sperm motion: importance of albumin for motility stimulation. *Fertil. Steril.*, **59,** 1266–1275.

Kervancioglu, M.E., Djahanbackhch, O. and Aitken, R.J. (1994) Epithelial cell coculture and the induction of sperm capacitation. *Fertil. Steril.*, **61,** 1103–1108.

Kholkute, S.D., Lian, Y., Roudebush, W.E. and Dukelow, W.R. (1990) Capacitation and the acrosome reaction in squirrel monkey (Samiri sciureus) spermatozoa evaluated by the chlortetracycline fluorescence assay. *Am. J. Primatol.* **20,** 115–125.

Langlais, J., Zollinger, M., Plante, L. *et al.* (1981) Localization of cholesteryl sulfate in human spermatozoa in support of a hypothesis for the mechanism of capacitation. *Proc. Natl. Acad. Sci. USA*, **78,** 7266–7270.

Langlais, J., Kan, F.W.K., Granger, L. *et al.* (1988) Identification of sterol acceptors that stimulate cholesterol efflux from human spermatozoa during *in vitro* capacitation. *Gamete Res.*, **20,** 185–201.

Lapointe, S. and Sirard, M.-A. (1996) Importance of calcium for the binding of oviductal fluid proteins to the membranes of bovine spermatozoa. *Mol. Reprod. Dev.*, **44,** 234–240.

Lasalle, B. and Testart, J. (1994) Human zona pellucida recognition associated with the removal of sialic acid from human sperm surface. *J. Reprod. Fertil.*, **101,** 703–711.

Leblond, E., Desnoyers, L. and Manjunath, P. (1993) Phosphorylcholine-binding proteins from the seminal fluids of different species share antigenic determinants with the major proteins of bovine seminal plasma. *Mol. Reprod. Dev.*, **34,** 443–449.

Leclerc, P. and Kopf, G.J. (1995) Mouse sperm adenylyl cyclase: general properties and regulation by the zona pellucida. *Biol. Reprod.*, **52,** 1227–1233.

Leclerc, P. and Gagnon, C. (1996) Phosphorylation of Triton X-100 soluble and insoluble protein substrates in a demembranated/reactivated human sperm model. *Mol. Reprod. Dev.*, **44,** 200–211.

Leclerc, P., de Lamirande, E. and Gagnon, C. (1996) cAMP-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol. Reprod.*, **55,** 684–692.

Leclerc, P., de Lamirande, E. and Gagnon, C. (1997) Regulation of protein tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. *Free Rad. Biol. Med.*, **22,** 643–656.

Lee, M.A., Trucco, G.S., Bechtol, K.B. *et al.* (1987) Capacitation and acrosome reaction of human spermatozoa monitored by a chlortetracycline fluorescence assay. *Fertil. Steril.*, **48,** 649–658.

Lefebvre, R. and Suarez, S.S. (1996) Effect of capacitation on bull sperm binding to homologous oviductal epithelium. *Biol. Reprod.*, **54,** 575–582.

LeLannou, D., Griveau, J.F., Le Pichon, J.P. and Quero, J.C. (1992) Effect of chamber depth on the motion pattern of human spermatozoa in semen or in capacitating medium. *Hum. Reprod.*, **7,** 1417–1421.

Libersky, E.A. and Boatmen, D.E. (1995) Effects of progesterone on *in vitro* sperm capacitation and egg penetration in the golden hamster. *Biol. Reprod.*, **53,** 483–487.

Lindemann, C.B. and Goltz, J.S. (1988) Calcium regulation of flagellar curvature and swimming pattern in Triton X-100-extracted rat sperm. *Cell Motil. Cytoskeleton*, **10,** 420–431.

Lippes, J. and Wagh, P.V. (1989) Human oviductal fluid (hOV) proteins. IV. Evidence for hOF proteins binding to human sperm. *Fertil. Steril.*, **51,** 89–94.

Llanos, M.N. and Meizel, S. (1983) Phospholipid methylation increases during sperm capacitation of golden hamster sperm *in vitro*. *Biol. Reprod.*, **28,** 1043–1051.

Luconi, M., Bonaccorsi, L., Krausz, C. *et al.* (1995) Stimulation of protein phosphorylation by platelet-activating factor and progesterone in human spermatozoa. *Mol. Cell. Endocrinol.*, **108,** 35–42.

Maas, D.H.A., Storey, B.T. and Mastroianni, L. (1976) Oxygen tension in the oviduct of the Rhesus monkey (Macaca mulatta). *Fertil. Steril.*, **27,** 1312–1318.

Maas, D.H.A., Storey, B.T. and Mastroianni, L. (1977) Hydrogen ion and carbon dioxide content of the oviductal fluid of the Rhesus monkey (*Macaca mulatta*). *Fertil. Steril.*, **28,** 281–285.

Meizel, S., Pillai, M., Diaz-Perez, E. and Thomas, P. (1990) Initiation of the human sperm acrosome reaction by components of follicular fluid and cumulus secretions including steroids. In Bavister, B.D., Cummins, J. and Roldan, E.R.S. (eds), *Fertilization in Mammals*. Serono Symposia, Norwell, MA, USA, pp. 205–222.

Morel, F., Doussiere, J. and Vignais, P.V. (1991) The superoxide-generating oxidase of phagocytic cells: physiological, molecular and pathological aspects. *Eur. J. Biochem.*, **201,** 523–546.

Mortimer, S.T. and Mortimer, D. (1990) Kinematics of human spermatozoa incubated under capacitating conditions. *J.Androl.*, **11,** 195–203.

Mortimer, S.T. and Swan, M.A. (1995) Variable kinematics of capacitating human spermatozoa. *Hum. Reprod.*, **10,** 3178–3182.

Mortimer, S.T., Swan, M.A. and Mortimer, D. (1996) Fractal analysis of capacitating human spermatozoa. *Hum. Reprod.*, **11,** 1049–1054.

Murad, C., de Lamirande, E. and Gagnon, C. (1992) Hyperactivated motility is coupled with interdependent modifications at axonemal and cytosolic levels in human spermatozoa. *J. Androl.*, **13,** 323–331.

Naor, Z., Rotem, R. and Kalina, M. (1995) Role of protein kinase C in human sperm acrosome reaction. In Fénichel, P. and Parinaud, J. (eds), *Human Sperm Acrosome Reaction.* Colloque INSERM no. 236. John Libbey Eurotext Ltd., pp. 217–223.

Naz, R.K., Ahmed, K. and Kumar, R. (1991) Role of membrane tyrosine proteins in human spermatozoal functions. *J. Cell Sci.*, **99,** 157–165.

Neill, J.M. and Olds-Clarke, P. (1987) A computer-assisted assay for mouse sperm hyperactivation demonstrate that bicarbonate but not bovine serum albumin is required. *Gamete Res.*, **18,** 121–140.

Okamura, N., Tajima, Y., Soejima, E. *et al.* (1985) Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *J. Biol. Chem.*, **260,** 9699–9705.

Okamura, N., Tajima, Y., Ishikawa, H. *et al.* (1986) Lowered levels of bicarbonate in seminal plasma cause the poor sperm motility in human infertile patients. *Fertil. Steril.*, **45,** 265–272.

Oko,R. (1988) Comparative analysis of proteins from the fibrous sheath and outer dense fibers of rat spermatozoa. *Biol. Reprod.* **39,** 169–182.

Oliphant, G. (1976) Removal of sperm-bound seminal plasma components as a prerequisite for induction of the rabbit acrosome reaction. *Fertil. Steril.*, **27,** 28–38.

Olds-Clarke, P. and Sego, R (1992) Calcium alters capacitation and progressive motility of uterine spermatozoa from +/+ and congenic $t^{w32/+}$ mice. *Biol. Reprod.*, **47,** 629–635.

Olds-Clarke, P. and Wivell, W. (1992) Impaired transport and fertilization *in vivo* of calcium-treated spermatozoa from +/+ and congenic $t^{w32/+}$ mice. *Biol. Reprod.*, **47,** 621–628.

Pacey, A.A., Davies, N., Warren, M.A. *et al.* (1995a) Hyperactivation may assist human spermatozoa to detach from intimate association with the endosalpinx. *Hum. Reprod.*, **10,** 2603–2609.

Pacey, A.A., Hill, C.J., Scudamore, I.W. *et al.* (1995b) The interaction *in vitro* of human spermatozoa with epithelial cells from the human uterine (Fallopian) tube. *Hum. Reprod.*, **10,** 360–366.

Parinaud, J. and Milhet, P. (1996) Progesterone induces a Ca^{++}-dependent 3′,5′-cyclic adenosine monophosphate increase in human sperm. *J. Clin. Endocrinol. Metab*., **81,** 1357–1360.

Parks, J.E. and Ehrenwald, E. (1990) Cholesterol efflux from mammalian sperm and its potential role in capacitation. In Gagnon, C. (ed.), *Controls of Sperm Motility: Biological and Clinical Aspects.* CRC Press, Boca Raton, FL, USA, pp. 155–167.

Parrish, J.J., Susko-Parrish, J., Winer, M.A. and First, N.L. (1988) Capacitation of bovine sperm by heparin. *Biol. Reprod.*, **38,** 1171–1188.

Perry, R.L., Naneeni, M., Barratt, C.L.R. *et al.* (1995) A time course study of capacitation and the acrosome reaction in human spermatozoa using a revised chlortetracycline pattern classification. *Fertil. Steril.*, **64,** 150–159.

Ravnik, S.E., Zarutskie, P.W. and Muller, C.H. (1990) Lipid transfer activity in human follicular fluid: relation to human sperm capacitation. *J. Androl.*, **11,** 216–226.

Ravnik, S.E., Albers, J.J. and Muller, C.H. (1995) Stimulation of human sperm capacitation by purified lipid transfer protein. *J. Exp. Biol.*, **272,** 78–83.

Revelli, A., Modotti, M., Piffaretti-Yanez, A. *et al.* (1994) Steroid receptors in human spermatozoa. *Hum. Reprod.*, **9,** 760–766.

Riffo, M., Leiva, S. and Astudillo, J. (1992) Effect of zinc on human sperm motility and the acrosome reaction. *Int. J. Androl*., **15,** 229–237.

Robertson, L., Wolf, D.P. and Tash, J.S. (1988) Temporal changes in motility parameters related to acrosomal status: identification and characterization of populations of hyperactivated spermatozoa. *Biol. Reprod.*, **39,** 797–805.

Roldan, E.R.S. and Flemming, A.D. (1989) Is a Ca^{2+}-ATPase involved in Ca^{2+} regulation during capacitation and the acrosome reaction of guinea-pig spermatozoa? *J. Reprod. Fertil.*, **85,** 297–308.

Rubinstein, S. and Breitbart, H. (1991) Role of spermine in mammalian sperm capacitation and acrosome reaction. *Biochem. J*., **278,** 25–28.

Rufo, G.A., Schoff, P.K. and Lardy, H.A. (1984) Regulation of calcium content in bovine spermatozoa. *J. Biol. Chem.*, **259,** 2547–2552.

Santos-Sacchi, J. and Gordon, M. (1980) Induction of the acrosome reaction in guinea pig spermatozoa by cGMP analogues. *J. Cell Biol.*, **85,** 789–803.

Sawada, M. and Carlson, J.C. (1991) Rapid plasma changes in superoxide radical formation, fluidity, and phospholipase A_2 activity in the corpus luteum of the rat during induction of luteolysis. *Endocrinology,* **129,** 2992–2998.

Shalgi, R., Smith, T.T. and Yanagimachi, R. (1992) A quantitative comparison of the passage of capacitated and uncapacitated hamster spermatozoa through the uterotubal junction. *Biol. Reprod.* **46,** 419–424.

Sladek, M.S., Regenstein, A.C., Lykins, D. and Roberts, J.M. (1993) Nitric oxide synthetase activity in pregnant rabbit uterus decreases on the last day of pregnancy. *Am. J. Gynecol*., **169,** 1285–1291.

Smith, G.D., Wolf, D.P., Trautman, K.C. *et al.* (1996) Primate sperm contain protein phosphatase 1, a biochemical mediator of motility. *Biol. Reprod*., **54,** 719–727.

Smith, T.T. and Yanagimachi, R. (1991) Attachment and release of spermatozoa from the caudal isthmus of the hamster oviduct. *J. Reprod. Fertil.*, **91,** 567–573.

Smith, T.T., Koyanagi, F. and Yanagimachi, R. (1987) Distribution and number of spermatozoa in the oviduct of the golden hamster after mating and artificial insemination. *Biol. Reprod.*, **37,** 225–234.

Snider, D.R. and Clegg, E.D. (1975) Alterations of phospholipids in porcine spermatozoa during *in vivo* uterus and oviduct incubation. *J. Anim. Sci*., **40,** 269–274.

Stauss, C.R., Votta, T.J. and Suarez, S.S. (1995) Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. *Biol. Reprod.* **53,** 1280–1285.

Suarez, S.S. and Dai, X. (1992) Hyperactivation enhanced mouse sperm capacity for penetrating viscoelastic media. *Biol. Reprod.*, **46,** 686–691.

Suarez, S.S. and Dai, X. (1995) Intracellular calcium reaches different levels of elevation in hyperactivated and acrosome reacted hamster sperm. *Mol. Reprod. Dev.*, **42,** 325–333.

Suarez, S.S., Katz, D.F. and Overstreet, W. (1983) Movement characteristics and acrosomal status of rabbit spermatozoa recovered at the site and time of fertilization. *Biol. Reprod.*, **29,** 1127–1287.

Suarez, S.S., Katz, D.F., Owen, D.H. *et al.* (1991) Evidence for the function of hyperactivated motility in sperm. *Biol. Reprod.*, **44,** 375–381.

Suarez, S.S., Dai, X., Demott, R.P. *et al.* (1992) Movement characteristics of boar sperm obtained from the oviduct or hyperactivated *in vitro*. *J. Androl.*, **13,** 75–80.

Suarez, S.S., Varosi, S.M. and Dai, X. (1993) Intracellular calcium increases with hyperactivation in intact, moving hamster sperm and oscillates with the flagellar beat cycle. *Proc. Natl. Acad. Sci. USA,* **90,** 4660–4664.

Sukcharoen, N., Keith, J., Irvine, D.S. and Aitken, R.J. (1995) Definition of the optimal criteria for identifying hyperactivated human spermatozoa at 25 Hz using in-vitro fertilization as a functional end-point. *Hum. Reprod.*, **10,** 2928–2937.

Suzuki, F. and Yanagimachi, R. (1989) Changes in the distribution of intra-membranous particles and Filipin-reactive membrane sterols during *in vitro* capacitation of golden hamster spermatozoa. *Gamete Res*., **23,** 335–347.

Suzuki, K., Ebihara, M., Nagai, T. *et al.* (1994) Importance of bicarbonate/CO_2 for fertilization of pig oocytes *in vitro* and synergism with caffeine. *Reprod. Fertil. Dev.*, **6,** 221–227.

Taher, M.M., Garcia, J.G.N. and Natarajan, V. (1993) Hydroperoxide-induced diacylglycerol formation and protein kinase C activation in vascular endothelial cells. *Arch. Biochem. Biophys*., **303,** 260–266.

Tan, C.M., Xenoyannis, S. and Feldman, R.D. (1995) Oxidant stress enhances adenylyl cyclase activation. *Circ. Res*., **77,** 710–717.

Tash, J.S. (1990) Role of cAMP, calcium and protein phosphorylation in sperm motility. In Gagnon, C. (ed.), *Controls of Sperm Motlity: Biological and Clinical Aspects.* CRC Press, Boca Raton, FL, USA, pp. 229–241.

Tash, J. and Bracho, G.E. (1994) Regulation of sperm motility: emerging evidence for a major role for protein phosphatases. *J. Androl*., **15,** 505–509.

Tash, J.S., Krinks, M., Leiser, M. and Orr, G.A. (1988) Identification, characterization, and functional correlation of calmodulin dependent protein phosphatase in sperm. *J. Cell Biol.*, **106,** 1625–1629.

Thelen, M., Dewal, B. and Baggiolini, M. (1993) Neutrophils signal transduction and activation of the respiratory burst. *Physiol. Rev.*, **73,** 797–821.

Therien, I., Bleau, G. and Manjunath, P. (1995) Phosphatidylcholine-binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin. *Biol. Reprod*., **52,** 1372–1379.

Thomas, P. and Meizel, S. (1989) Phosphatidylinositol 4,5-biphosphate hydrolysis in human sperm stimulated follicular fluid or progesterone is dependent upon Ca^{2+} influx. *Biochem. J.*, **264,** 539–546.

Toyoda, Y., Yokohama, M. and Hoshi, T. (1971) Studies on the fertilization of mouse eggs *in vitro*. *Jpn. J. Anim. Reprod*., **16,** 147–157.

Uhler, M.L., Leung, A., Chan, S.Y.W. and Wang, C. (1992) Direct effects of progesterone and antiprogesterone on human sperm hyperactivated motility and acrosome reaction. *Fertil. Steril.*, **58,** 1191–1198.

VandeVoort, C.A., Tollner, T.L. and Overstreet, J.W. (1994) Separate effects of caffeine and dbcAMP on macaque sperm motility and interaction with the zona pellucida. *Mol. Reprod. Dev.*, **37,** 299–304.

Visconti, P.E., Muschietti, J.P., Flawia, M.M. and Tezón, J.G. (1990) Bicarbonate dependence of cAMP accumulation induced by phorbol esters in hamster spermatozoa. *Biochim. Biophys. Acta*, **1044,** 231–236.

Visconti, P.E., Bailey, J.L., Moore, G.D. *et al.* (1995a) Capacitation in mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development*, **121,** 1129–1137.

Visconti, P.E., Moore, G.D., Bailey, J.L. *et al.* (1995b) Capacitation in mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development*, **121,** 1139–1150.

Vredenburg-Wilberg, W.L. and Parrish, J.J. (1995) Intracellular pH of bovine sperm increases during capacitation. *Mol. Reprod. Dev.*, **40,** 490–502.

Wang, C., Lee, G.S., Leung, A. *et al.* (1993) Human sperm hyperactivation and acrosome reaction and their relationships to human *in vitro* fertilization. *Fertil. Steril.*, **59,** 1221–1227.

Ward, C.R. and Storey, B.T. (1984) Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. *Dev. Biol.*, **104,** 287–296.

William, M., Hill, C.L., Scudamore, I.W. *et al.* (1993) Sperm numbers and distribution within the human Fallopian tube around ovulation. *Hum. Reprod.*, **8,** 2019–2026.

Wolf, D.E. and Gadulo, R.A. (1991) Physical properties of the mammalian sperm membrane. In Bacetti, B. (ed.), *Comparative Spermatology 20 Years After.* Raven Press, New York, pp. 599–604.

Wolf, D.E., Hagopian, S.S. and Ishijima, S. (1986) Changes in sperm plasma membrane lipid diffusibility after hyperactivation during *in vitro* capacitation in the mouse. *J. Cell Biol.*, **102,** 1372–1377.

Wolf, D.P., VandeVoort, C.A., Meyer-Haas, G.R. *et al.* (1989) *In vitro* fertilization and embryo transfer in the Rhesus monkey. *Biol. Reprod.*, **41,** 335–346.

Yanagimachi, R. (1982) Requirement of extracellular calcium ions for various stages of fertilization and fertilization related phenomena in the hamster. *Gamete Res.*, **5,** 323–344.

Yanagimachi, R. (1994) Mammalian fertilization. In Knobil, E. and Neill, J.D. (eds), *The Physiology of Reproduction.* 2nd edn. Raven Press Ltd., New York, pp. 189–317.

Yanagimachi, R. and Suzuki, F. (1985) A further study of the lysolecithin-mediated acrosome reaction of guinea pig spermatozoa. *Gamete Res.*, **11,** 29–40.

Young, R.J. and Bodt, B.A. (1994) Development of computer directed methods for the identification of hyperactivated motion using motion patterns developed by rabbit sperm during incubation under capacitating conditions. *J. Androl.*, **15,** 362–377.

Zeng, Y., Clark, E.N. and Florman, H.M. (1995) Sperm membrane potential: Hyperpolarization during capacitation regulates zona pellucida-dependent acrosomal secretion. *Dev. Biol.*, **171,** 554–563.

Zhu, J.J., Barratt, C.L.R. and Cooke, I.D. (1992) Effect of human cervical mucus on human sperm motion and hyperactivation *in vitro*. *Hum. Reprod.* **7,** 1402–1406.

Zhu, J.J., Barratt, C.L.R., Lippes, J. *et al.* (1994) The sequential effect of human cervical mucus, oviductal fluid, and follicular fluid on sperm function. *Fertil. Steril.*, **61,** 1129–1135.

Zinamen, M., Drobnis, E.Z., Morales, P. *et al.* (1989) The physiology of sperm recovered from the human uterine cervix: acrosomal status and response to inducers of the acrosome reaction. *Biol. Reprod.*, **41,** 790–797.

Zini, A., de Lamirande, E. and Gagnon, C. (1995) Low levels of nitric oxide promote human sperm capacitation *in vitro*. *J. Androl.*, **16,** 424–431.

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