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# Biological basis for human capacitation

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**More than 50 years ago Austin and Chang defined mammalian sperm capacitation as a period of time that sperm must reside in the female reproductive tract before they acquire the ability to fertilize oocytes. Since then numerous investigations have attempted to more clearly define the molecules and processes that are a part of capacitation. The data that have provided a more clear definition of capacitation were primarily derived from *in vitro* experiments. This is particularly true for studies on human sperm capacitation. While ethical constraints have limited an equal balance of *in vivo* studies there are those data that when coupled with some of the *in vitro* data allow for the formulation of a biological framework for human sperm capacitation *in vivo*. This review will put forth the biological basis for human capacitation.**

*Key words*: female tract/fertilization/*in vivo* capacitation/signal transduction sperm capacitation mechanisms

## Introduction

Austin (1951, 1952) and Chang (1951) independently described changes that are prerequisite for non-human mammalian spermatozoa to fertilize oocytes *in vivo*, described as the acquisition of ‘fertilizing capacity’. This acquisition process, termed ‘capacitation’ (Austin, 1952), occurred only after spermatozoa had spent a period of time in the female reproductive tract. In brief, and with only partial modification from Austin and Chang’s original descriptions, capacitation is characterized as a complex of structural and functional changes occurring in spermatozoa that: (1) begins after the removal of stabilizing factors acquired by spermatozoa while resident in seminal plasma; (2) proceeds throughout sperm transit in the female reproductive tract and (3) is considered to be complete when spermatozoa are able to respond to zona pellucida ligands by undergoing the acrosome reaction.

*In vivo* investigation of sperm capacitation has largely been limited to non-human mammalian models primarily for ethical reasons. This review will present a framework for the *in vivo* changes human spermatozoa may experience as they leave seminal plasma and make their way through the periovulatory reproductive tract by using (1) the relatively limited number of *in vivo* investigations, (2) the vast number of *in vitro* investigations and especially (3) those studies where biological factors emanating from the human female reproductive tract have been shown to influence capacitation. Figure 1 serves as an illustrative reference to accompany each section of this review in which capacitative changes in sperm are described as they transit out of seminal plasma and pass through each region of the female reproductive tract.

The normal human ejaculate contains many tens to hundreds of millions of motile spermatozoa. Clearly this incredible output far exceeds the number of sperm required for successful fertilization—that is, one sperm and one oocyte. However, in spite of this exorbitant number of motile cells not all of these and in fact very few will make it to journey’s end. Many cells will not pass into the cervical mucus. Of those that do penetrate, passage through and into the uterus will be possible only for those tens of thousands of sperm with vigorous progressive motility, morphological normality and a plasma membrane that is functioning appropriately in response to environmental conditions. The uterus acts to further the filtration process. For example, motility may diminish or fail for some sperm; others may undergo a premature and degenerative acrosome reaction rendering them inviable; other sperm may succumb to the deleterious effects of leukocyte-produced reactive oxygen molecules. At most, several hundred sperm will enter the oviducts. Sperm are known to attach to the oviductal epithelium with their release likely being mediated by molecular processes. In fact, some sperm may not be able to detach. Thus, the cumulus–oocyte–complex (COC), upon entry into the oviduct, will meet with no more than a hundred or so sperm, and that is likely to be an overestimate. The cumulus complex and zona serve as the final sperm filters such that perhaps 10 to 20 spermatozoa might reach the zona surface. The race to the finish will be governed by effective ligand–receptor interactions, functionally active signal transduction cascade pathways and—a bit of luck.

Having set the stage descriptively, it is important to outline several boundary parameters in the context of this review. First, is the theorem that capacitation and the acrosome reaction are

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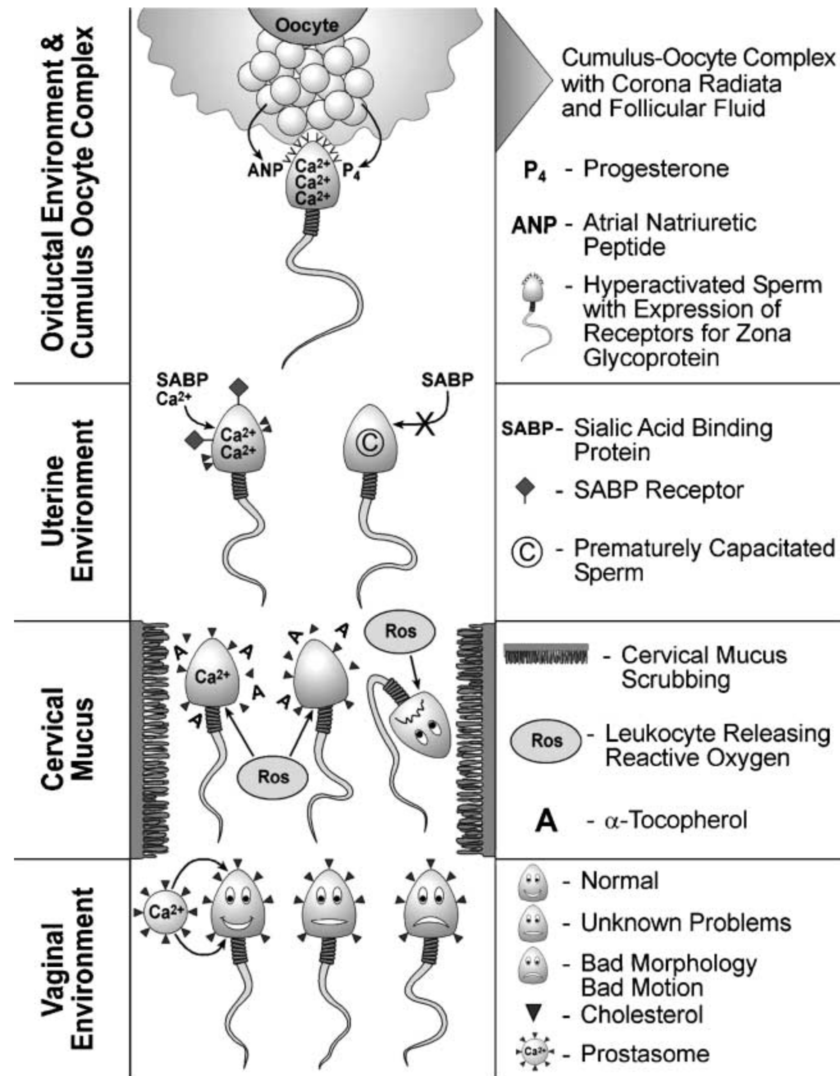


**Figure 1.** Biological basis for human capacitation. Semen contains secretions from accessory glands, e.g. calcium-containing prostasomes from the prostate, and a heterogeneous sperm population comprised of normally functioning sperm, sperm of questionable function and dysfunctional sperm. Sperm migrate from the membrane-stabilizing, sterol-enriched seminal plasma and acidic vaginal environment into the cervical mucus. Sperm plasma membranes are scrubbed by the ultrastructural elements in the mucus, facilitating the removal from the plasma membrane of adsorbed molecules and sterols. Leukocytes infiltrate the cervical mucus coincident with sperm entry. Leukocytes produce reactive oxygen molecules that have a pro-capacitating influence on normally functioning sperm and a deleterious influence on dysfunctional sperm, facilitating the removal of the latter from the fertilizing sperm population. With the removal of poor quality and dysfunctional sperm, the widely heterogeneous sperm population that entered the cervical mucus has been made somewhat more homogenous upon exit from the mucus and entry into the uterine environment. While the time sperm spend resident in the uterus is likely to be brief, due in part to uterine contractions that propel sperm to the fundus, there is ample opportunity for additional and necessary changes to occur. The sperm plasma membrane is undergoing dynamic changes with the formation of ordered lipid microdomains and sterol removal, facilitated by uterine sterol sulphatase. Consequences of regionalization and removal of sterols are: (1) increased permeability to ions, such as $Ca^{2+}$ and (2) expression of receptors and binding of stimulatory ligands, such as sialic acid binding protein. The migrating sperm population becomes made more homogenous with the selection out of prematurely capacitating and dysfunctioning sperm. Upon arrival into the oviduct ipsilateral to the ovulatory follicle, sperm are introduced to an environment diverse in cellular and hormonal composition. With progression to the ampullary region of the oviduct, sperm detected the scent of the oocyte through the action of chemoattractant molecules, e.g. atrial natriuiretic peptide, secreted by the COC. Progesterone, adjacent and adsorbed to the cumulus, initiates inward $Ca^{2+}$ transients that brings sperm intracellular $Ca^{2+}$ concentrations to threshold levels for acrosome reaction stimulation by zona pellucida glycoproteins. The, perhaps, dozen sperm reaching the zona are likely to be very homogeneous relative to fully functional signal transduction mechanisms and motility characteristics necessary for fertilization. Thus, it is likely that the sperm that eventually fertilizes the oocyte has one attribute the others did not—luck.

separable and distinct processes. Most notably, the former is a reversible phenomenon while the latter is not. Any description of the acrosome reaction will relate only to the fact that an inducible acrosome reaction is the least disputed marker for indicating the completion of capacitation. Second, this paper will not itemize deficiencies in sperm function that are suspected of being causative of subfertility, as much of the existing literature data that have attempted to do so are derived after *in vitro* culture and for which the composition of media and protocols used for those investigations are as diverse as many of the corresponding results. Third, while mammalian sperm share unique similarities they also share unique differences. As such,

references will largely be restricted to those investigations using human spermatozoa. Reference to non-human species will be used sparingly, as inclusive reviews exist (e.g. Yanagimachi, 1994; de Lamirande *et al.*, 1997; Baldi *et al.*, 2000; Flesch and Gadella, 2000; Jha *et al.*, 2003; Hunter and Rodriguez-Martinez, 2004).

### *The biology of capacitation in vivo*

#### *Seminal plasma*

Seminal plasma has a direct effect on the immediate and future functional ability of sperm. It is beyond the scope of this review to address the topic in whole; however, there are some key points that are pertinent. Many of the factors described and/or characterized from seminal plasma have been shown to have an associative rather than a causative influence on capacitation. In addition, many of these factors have been evaluated in regards to their influence on the acrosome reaction (e.g. Han *et al.*, 1990; Drisdel *et al.*, 1995; Lopes *et al.*, 1998) or sperm–oocyte recognition (Chalabi *et al.*, 2002). There is one seminal plasma factor that has clearly been identified as having a regulatory influence on capacitation and that factor is cholesterol (Cross, 1998).

#### *Seminal plasma cholesterol*

Cholesterol is found in high abundance in seminal plasma (Grizard *et al.*, 1995; Cross, 1996; Arienti *et al.*, 1999). Cross (1993) reported that neat, 10% (v/v) and 5% (v/v) seminal plasma (seminal plasma v/v with Tyrode's medium) were effective at preventing sperm from acquiring acrosomal responsiveness to fusion promoting agonists. When sperm were incubated in 1% (v/v) seminal plasma, washed and resuspended in Tyrode's medium alone they acquired responsiveness to agonists in a time frame (~24 h) that mirrored that of sperm freshly isolated from the ejaculate and then incubated in seminal plasma-free Tyrode's medium. These data collectively demonstrate that one or more factors in seminal plasma keep sperm in a state of preparedness for capacitation.

To further investigate the nature of interaction between seminal plasma and sperm, Cross (1993) tested for reversibility of capacitation. He added seminal plasma (7% v/v) to sperm incubated for 6 h in Tyrode's medium. Surprisingly, the sperm acrosome-reacted and in high percentage when simultaneously treated with progesterone. If added sequentially, and separated by only 5 min, the stimulatory effect was significantly dampened. Further, when progesterone was added after sperm had first been incubated in Tyrode's followed by incubation with the seminal plasma solution for 2 h or more a very minimal stimulatory response was detected and approached control levels. $Ca^{2+}$ quenching via albumin, sperm plasma membrane homeostasis regulatory mechanisms and cholesterol loading of sperm plasma membranes are likely explanations for these latter findings.

The influence of seminal plasma on sperm stimulated a closer examination of the biological mechanism(s) behind the inhibitory effect. Cross (1996) confirmed that seminal plasma cholesterol is the predominant inhibitor of capacitation. He subjected seminal plasma to a sequence of purification and isolation steps, which included treatment with organic solvents, absorption, gas and thin layer chromatography, and gas spectrometry, to identify cholesterol as the principle inhibitory factor in seminal plasma. In that same investigation, Cross went further to demonstrate a dose-dependent inhibition of sperm acrosomal responsiveness to progesterone and that the inhibitory effect was highly related with the amount of cholesterol in the seminal plasma. Further, the carefully determined $ID_{50}$ dose in this investigation was reassuringly similar to that found in his 1993 report.

The above series of simply elegant experiments clearly demonstrate the critical regulatory role of seminal plasma cholesterol (and also desmosterol; see Cross, 2003) on the initiation and promotion of capacitation.

The seemingly conflicting results (Cross, 1993) of seminal plasma inhibition and transient albeit stimulatory effect of the seminal plasma/progesterone combination on capacitated sperm merits review. One possible explanation is that the extraction procedure used in the experiments for seminal plasma may have stimulated iatrogenic biochemical reactions that contributed to a pathologic acrosome reaction. Equally plausible is that the stimulatory reaction may have occurred because of one or more components in the seminal plasma.

#### *Prostasomes*

Prostasomes are calcium-storing, cholesterol-enriched vesicles secreted by the prostate that are contributed to seminal plasma (Arienti *et al.*, 2004). While prostasomes have not yet been shown to have a regulatory role on capacitation the potential for such a role, based on literature data, offers sufficient impetus to briefly introduce the subject. Arienti *et al.* (1997) showed that prostasomes, upon fusion with sperm, deliver calcium to the cytosolic space. The seminal plasma extraction procedure used by Cross (1993) could have concentrated prostasomes into the seminal plasma test pool. The prostasomes, whether lysed or not, would effectively contribute to increased calcium availability in the form of either free or stored calcium, respectively.

Progesterone is known to promote $Ca^{2+}$ influx and, if sperm are capacitated, the acrosome reaction. In the capacitation reversibility experiments, Cross (1993) used progesterone to test for acrosomal responsiveness. Arienti *et al.* (2001) showed that the intracellular calcium response of sperm to progesterone is potentiated if prostasomes are first fused with the sperm. Thus, the combination of increased $Ca^{2+}$ availability and mechanisms in place to rapidly transport calcium into sperm may have been sufficient for acrosome reaction stimulation. Supportively, Palmerini *et al.* (2003) have recently shown that the fusion of prostasomes with human sperm followed by treatment with progesterone stimulates the acrosome reaction.

Of biological significance, the mildly acidic (pH 5–6) conditions necessary to promote fusion of prostasomes (Arienti *et al.*, 1997; Carlini *et al.*, 1997) with sperm are remarkably similar to vaginal pH at the periovulatory period (pH 4.5–6). Thus, a compelling scenario is that upon deposit of the ejaculate into the acidic environment of the vagina, prostasomes become incorporated into sperm, priming them with intracellular $Ca^{2+}$ for their ultimate encounter with the progesterone-rich COC. In addition, the possibility for some level of cholesterol loading of sperm membranes via prostasomes could be beneficial to

stabilize the plasma membrane to protect against an ionically leaky membrane and premature acquisition of acrosomal responsiveness, i.e. premature capacitation. This theory merits more intensive investigation.

*Fertilization promoting peptide*

A tripeptide, similar to thyrotrophin-releasing hormone, called fertilization promoting peptide (FPP) is present in human seminal plasma (Cockle *et al.*, 1994). FPP stimulates capacitation in sperm within 1 h of incubation, lacks a dose-dependent effect on capacitation and the percentage of capacitated cells in FPP-treated sperm versus controls are indistinct after 22 h (Green *et al.*, 1996). The mechanism of FPP action on human sperm has not been clarified. However, experiments with mouse sperm demonstrate that FPP (1) acts via g-protein coupled receptors, (2) to stimulate membrane bound adenylate cyclase and (3) thereby regulating cAMP production positively in non-capacitated sperm and negatively in capacitated sperm (Fraser *et al.*, 2005). While the binding kinetics and half-life of response to FPP have not been defined in the context of IVF, it is compelling to imagine FPP to be a sperm capacitation regulatory molecule.

*Cervical mucus*

Native seminal plasma positively contributes to the ability of sperm to migrate from the ejaculate into the cervical mucus (Overstreet *et al.*, 1980). Sperm begin to migrate out of seminal plasma and into mucus almost immediately (~90 s) after deposition of the ejaculate into the vagina (Sobrero and McLeod, 1962). Results from evaluating sperm after migration into cervical mucus clearly show that cervical mucus modifies sperm in a way that sustains and promotes their subsequent ability to penetrate zona-free hamster oocytes as well as human zonae, and to be responsive to agonists of the acrosome reaction.

Gould (Gould *et al.*, 1984) evaluated functional characteristics of sperm isolated from periovulatory cervical mucus aspirates at various time intervals after donor sperm insemination (a cervical cap was used to introduce the ejaculate to the external os) and after incubation in Biggers–Whitten–Whittingham (BWW) medium supplemented with 35 mg/ml human serum albumin (HSA). Sperm binding (100%) to isolated human zona was evident by 1 h and a similar level of binding ability (96%) was maintained throughout the 80 h experimental time course. The percentage of sperm that successfully penetrated (~30%) through the zona and into the perivitelline space was maintained throughout experimentation but was greatly reduced relative to those zona-bound sperm (96%). This large difference suggests that successful penetration into the perivitelline space was because those sperm had reached a more advanced state of capacitation, i.e. plasma membrane alterations, receptor expression and fusogenicity, than those sperm still remaining bound to the outer zona surface.

Several investigators have demonstrated evidence in support of an apparent priming effect of cervical mucus on capacitation. Lambert *et al.* (1985) compared donor sperm isolated from mucus (48–56 h post-insemination using a cervical cap) with a fraction of the same donor sperm isolated from seminal plasma and examined their ability to penetrate human zonae and zona-free hamster oocytes while cultured in BWW medium supplemented with 3 mg/ml bovine serum albumin (BSA). Sperm isolated from mucus showed high percentages of human zona and zona-free hamster oocyte penetration, 71 and 42%, respectively. In stark contrast, sperm isolated from seminal plasma and incubated for 2 h were unable to penetrate in either of the biological assays. The same biological assays were done using phosphate-buffered saline without protein (BSA) as culture medium and incubation was extended to 4 h. In the absence of BSA, penetration of human zonae and zona-free oocytes by mucus-isolated sperm was diminished, 40 and 5%, respectively. Again, sperm isolated from semen failed to penetrate in both assays. Thus, cervical mucus is demonstrated to have a capacitating influence that is augmented in the presence of serum albumin.

Barros *et al.* (1988) aspirated non-inseminated periovulatory cervical mucus from women over a 7 day period (starting on day 10 of the menstrual cycle). Husband semen was divided into two aliquots; to isolate sperm, one aliquot of semen was exposed (1 h incubation) to partner's mucus that had been loaded into flat capillary tubes and sperm from the other semen aliquot were isolated using a swim-up method (1 h incubation). The mucus and swim-up isolated sperm were incubated separately for 3 h in medium containing 35 mg/ml HSA, then introduced to zona-free hamster oocytes and incubated for an additional 5–6 h. The average percentage of oocytes penetrated by sperm aspirated from mucus cycle day 10–13 was approximately 25%, control sperm penetration was approximately 50%. This penetration rate of sperm isolated from mucus approximates that of Lambert *et al.* (1985) albeit the former value is lower. A conclusion that can be made is that cervical mucus modifies sperm but not to the point where they are prematurely activated. Important to note is that there was considerable variability in penetration rate results between couples both in control (range 5–100% penetration) and experimental groups (range 0–100% penetration), regardless of the day of mucus aspiration. This may in part be reflective of the undetermined fertility status of couples recruited for the study.

Using donor sperm and the same insemination method as Gould *et al.* (1984), Zinaman *et al.* (1989) aspirated cervical mucus from different women over time (1 h–3 days) and evaluated acrosomal status before and after mucus migration, and then after post-mucus migration *in vitro* culture (1 and 6 h) followed by acrosome reaction inducibility using human follicular fluid (20% v/v). Acrosome reaction status of pre and post-mucus migration sperm was equivalent and very low (1–5%), regardless of duration of residence in cervical mucus. Further, 1 h *in vitro* incubation of post-migration sperm in BWW containing 3 mg/ml BSA, regardless of days of residence in mucus, did not increase the percentage of the population that was acrosomally responsive to follicular fluid challenge; the percentage of acrosome reaction being very low and no different than the non-incubated treatment groups. It was not until post-migration sperm had been *in vitro* incubated for 6 h that a significant percentage of the sperm acquired acrosomal responsiveness. The same result profile was detected for sperm that had migrated through mucus under *in vitro* conditions. Sperm not exposed to mucus required 18 h of additional *in vitro* incubation in order for a percentage of the population to attain a similar level of acrosomal responsiveness as mucus isolated sperm. Several

observations can be made from these results. First, regardless of duration of residence in cervical mucus, sperm appear to be held in static state in regards to viability ($>80\%$) and acrosome reaction sensitivity. Second, *in vitro* incubation ($\geq 6$ h) is required before a significant proportion of the sperm become acrosomally responsive to follicular fluid (or human zona). Finally, sperm not exposed to cervical mucus and incubated in Tyrode's medium containing, or not, albumin require a much longer exposure incubation time (24 h) before they show similar acrosome reactivity.

The results from the several studies outlined above can be interpreted to be that periovulatory cervical mucus initiates or primes spermatozoa as a part of the capacitation process but it is not the only contributing factor in that process. If indeed cervical mucus does initiate or prime sperm as a part of the capacitation process then the question is how—what might be the possible mechanism(s)?

*Cervical mucus ultrastructure*

Yudin *et al.* (1989) evaluated the fine structure of human ovulatory cervical mucus and noted that the ultrastructural elements of the mucus were small, somewhat fibrous and ribbon-like in appearance. Further, the mucus was found to be very viscoelastic and there appeared to be a compacted microstructure at the fringes of the mucus, which they suggested provided greater resistance to penetration by sperm. When sperm were added to the mucus their motion was largely restricted to two dimensions, with flagellar beat being confined distally. Thus, one can envisage sperm as sliding and squeezing in-between a complex of elastic fibrous strands using the benefit of small amplitude flagellar motion to facilitate forward passage.

Intimate contact between the sperm and the ultrastructural elements may quite possibly serve as a kind of sperm membrane scrubber to help in the removal of adsorbed molecules acquired during transit through and storage in the epididymis and while resident in seminal plasma. Evidence to support mucus-mediated sperm plasma membrane scrubbing comes from Feki *et al.* (2004). These investigators showed that after sperm migration through ovulatory cervical mucus the sperm plasma membrane undergoes remodelling in the form of cholesterol and glycerophospholipid removal. After transit through mucus, sperm membrane cholesterol is reduced in half and cervical mucus acquires cholesterol. Albumin, a known receptor for cholesterol, is present in cervical mucus but at the periovulatory period it alone is not present in sufficient concentration to explain the almost 2-fold decrease in sperm membrane cholesterol.

Rosselli *et al.* (1990) labelled plasma membranes of human spermatozoa with cationized ferritin and then exposed them to a mucus filled column. The sperm isolated post-migration were devoid of the ferritinized sperm coat. These data combined with those of Feki *et al.* (2004) stimulate the question of whether one or more of the numerous enzymes present in mucus might help to facilitate the change in membrane lipid content and/or if the ultrastructural elements in mucus might participate in mechanically stripping molecules, e.g. lipid and/or protein, from the plasma membrane. Regardless, there must be some mechanism at play that facilitates the significant cholesterol exchange and membrane scrubbing.

*Cervical mucus and sperm plasma membrane modifications*

An additional plasma membrane modification discovered by Feki *et al.* (2004) was a significant cervical mucus-mediated decrease in vitamin E ($\alpha$-tocopherol) from the sperm plasma membrane. Vitamin E is an effective protectant against oxidation-induced damage to membrane lipids. It would seem that cervical mucus directly contributes to a situation in which sperm membranes are made susceptible to oxidation and potential damage. Reactive oxygen species (ROS), produced largely by leukocytes but also by sperm, have been found to have a 'double-edged sword' effect on sperm (see review, Ford, 2004). On the one hand ROS are extremely harmful but on the other hand they exert a beneficial effect on capacitation (see review, Ford, 2004). Immature and dysfunctional sperm have been shown to be more negatively affected by ROS's and are producers of ROS's themselves (Aitken *et al.*, 1989; Ford, 2004). Leukocytes, major ROS producers, infiltrate cervical mucus coincident with the arrival of sperm in the mucus and are found in uterine flushings post-coitus/insemination (Thompson *et al.*, 1992; Williams *et al.*, 1993a).

One can imagine then that vitamin E removal from the plasma membrane is a potentially essential component of the mucus-mediated priming/initiation of capacitation. Vitamin E removal from immature and poorly functioning sperm would make those sperm more vulnerable to oxidative damage by endogenous and leukocyte-produced ROS's, such exposure rendering those sperm inviable and effectively removed from the migrating, functional cohort of sperm. Removal of vitamin E from plasma membranes of normal, functional sperm could potentially facilitate the beneficial ROS-mediated capacitation.

*The uterine environment*

After the passage of sperm through the cervical environment there is a considerable distance for sperm to travel in which they will encounter a varied cellular and hormonal environment before encountering the COC. Regrettably the impact of those changing surroundings on the cohort of sperm taking part in that odyssey remains largely obscure and for understandable reasons. There are studies published that have investigated the *in vitro* influence of the different cell types and hormones from the aforementioned regions in the female tract on sperm function and they merit review as a part of establishing a framework for human capacitation *in vivo*.

*The uterus and facilitated sperm transport*

If one accepts that capacitation is initiated after passage through cervical mucus, with calcium loading of sperm as part of that experience, then it seems reasonable that transport of these newly 'charged' cells to the Fallopian tubes should be expedited and occur without any significant delay. The following evidence fortifies this hypothesis. First, in addition to sperm's inherent motility, facilitative transport of sperm occurs via peristaltic contractions initiating in the cervical region of the uterus and propagating to the fundal region with increasing frequency and intensity as the follicular phase of the cycle progresses towards ovulation (Kunz *et al.*, 1997). Further, the oviducts participate in the facilitative transport of sperm, only during the follicular phase of the cycle and with transport predominantly directed to

the tube on the same side as the ovary containing the dominant follicle (Wildt *et al.*, 1998). Second, a number of investigations on sperm transport have documented the presence of sperm in the oviducts as soon as ~10 min post-coitus or after artificial vaginal insemination (Rubenstein *et al.*, 1951; Settlage *et al.*, 1973; Ahlgren, 1975). These data, when taken together, would suggest that the actively sperm-propulsive uterine environment is not likely a significant resource for pro-capacitative contributions or, for that matter, anti-capacitative contributions (Bastias *et al.*, 1993; Lai *et al.*, 1996; Guerin *et al.*, 1997).

*The uterus and sperm membrane cholesterol removal*

With the aforementioned having been proposed for the uterine environment, perhaps it would be premature to simply dismiss the uterus as having no capacitating influence. For example, *in vitro* studies have shown that the sperm plasma membrane undergoes modifications in content that are contributory to capacitation (for review, Flesch and Gadella, 2000). The most investigated and significant membrane content change is the loss of membrane cholesterol. Removal of cholesterol from the plasma membrane is thought to promote an increase in membrane fluidity that is a prerequisite for subsequent membrane fusion, i.e. acrosome reaction. Zarintash and Cross (1996) elaborated on the form of cholesterol that is lost from the plasma membrane. They quantified the unesterified cholesterol content in the membranes, and after a 24 h *in vitro* incubation period they detected a 29% reduction of that molecule. The percentage reduction in cholesterol directly corresponded with the percentage of sperm that had acquired acrosomal responsiveness after progesterone stimulation. When a dose–response addition of cholesterol to the culture medium was done two significant findings were obtained. First, increasing doses of cholesterol to the medium had a corresponding inhibitory effect on cholesterol loss from the plasma membranes ($ED_{50} = 406$ nM). Second, the sensitivity of sperm to progesterone challenge directly paralleled inhibition of plasma membrane cholesterol loss ($ED_{50} = 388$ nM). Finally, addition of dilute seminal plasma (1:150 v/v) had the same inhibitory effect on both plasma membrane cholesterol removal and responsiveness to progesterone.

While membrane cholesterol loss is believed to increase membrane fluidity, and thus capacitative state, recent *in vitro* data suggest that a sterol removal-dependent decrease in lipid order may more likely be the sterol-mediated mechanism responsible for initiating and promoting capacitation (Cross, 2003). When lipid fluidity was experimentally enhanced there was no concordant increase in capacitative state; suggesting that formation of ordered lipid microdomains are more important than bulk lipid fluidity (Cross, 2003). Thus, alterations in sperm membrane characteristics attributable to cholesterol removal and that correspondingly contribute to acquisition of acrosomal responsiveness remains to be clarified.

Sterol sulphatase is present in the female reproductive tract and with activity in the endometrium 10-times higher than that in the oviducts (Lalumiere *et al.*, 1976; Hobkirk 1985). It is likely then that sperm plasma membrane sterols are a substrate for uterine sterol sulphatase activity. Thus, the uterine environment may serve as a critical site for additional, necessary sperm plasma membrane cholesterol removal and the promulgation of capacitation.

The uterus may serve as a location for other notable sperm plasma membrane modifications. A 54 kDa sialic acid-binding protein (SABP) in fluid secreted by human endometrial cells was isolated and purified (Banerjee and Chowdhury, 1994). Estradiol was found to regulate both the synthesis and secretion of SABP (Sen *et al.*, 2001). Labelled SABP was found to bind to the head region of non-capacitated sperm (Banerjee and Chowdhury, 1994). SABP was subsequently shown to bind $Ca^{2+}$ and to facilitate $Ca^{2+}$ influx into sperm (Banerjee and Chowdhury, 1995); an increase in intracellular $Ca^{2+}$ is integral to both capacitation and the acrosome reaction. Indeed a 25 kDa receptor for SABP has been localized on the surface of spermatozoa in the head region (Banerjee and Chowdhury, 1997). The binding of SABP to the receptor was dependent upon the sperm being non-capacitated. Precapacitated or desialylated sperm failed to bind SABP. Lastly, SABP stimulated the release from sperm plasma membranes of labelled sialoglycoconjugates. Sialoglycoconjugates confer a negative charge to the sperm plasma membrane surface and a decrease in net negative charge has been associated with capacitation. Collectively these data offer compelling evidence for a possible significant uterine role in human sperm capacitation. This is an area ripe for investigation.

*The oviductal environment*

The significance of the oviductal environment in human reproduction cannot be overstated, as it serves as the passageway for gamete and embryo transport and early embryo development (see review, Leese *et al.*, 2001; Croxatto, 2002). Reinforcing the findings of Wildt *et al.* (1998), Williams *et al.* (1993b) provided direct evidence for a greater number of sperm in the oviduct ipsilateral to the ovulatory follicle in comparison to the contralateral oviduct. However, that observation was only true for the ampullary region, there was no difference in sperm numbers between tubes in the isthmic region. This finding would suggest a possible preferential attraction of sperm not only to the ovulatory side but, even more so, to the site for encounter with the COC.

The oviduct has been suggested to serve as a potential sperm reservoir (Baillie *et al.*, 1997) because sperm bind to oviductal explants *in vitro* (Morales *et al.*, 1996; Baillie *et al.*, 1997). However, this issue is not without controversy as surgically excised oviducts post-insemination contained sperm but none of the sperm were bound to the epithelium (Williams *et al.*, 1993b). In other *in vitro* oviductal cell culture experiments, the quality of sperm bound to oviductal epithelium was superior to those sperm still free-swimming, as reflected by sperm DNA fragmentation, morphology and other sperm functional parameters (Ellington *et al.*, 1999). Further, *in vitro* studies have shown that culture with oviductal cells enhances sperm survival and motility (Kervancioglu *et al.*, 1994; Ellington *et al.*, 1998; Kervancioglu *et al.*, 2000; Yao *et al.*, 2000).

No consistent stimulatory effect of oviductal cell culture on capacitation has been demonstrated. In two studies (Kervancioglu *et al.*, 1994, 2000) it was concluded that co-culture of sperm and oviductal cells stimulates capacitation; the marker for capacitation being the onset of hyperactivation. Studies using the acrosome reaction as the end-point of capacitation are conflicting, wherein two studies (Yao *et al.*, 1999a,b) reported a stabilizing effect on the sperm acrosome after oviductal cell culture while

another study reported a significant stimulation of the acrosome reaction after co-culture (Ellington *et al.*, 1999). In contrast, Murray and Smith (1997) demonstrated that sperm evaluated after co-culture with apical plasma membranes from human oviductal epithelia were retarded in their transition from non-capacitated to the capacitated state. In an effort to approximate *in vivo* sperm transport and environmentally-induced functional changes, Zhu *et al.* (1994) exposed sperm sequentially to human cervical mucus, oviductal and follicular fluids. They found a diminished acrosome reaction response to follicular fluid after culture in oviductal fluid as compared to no pre-culture. While sperm motility and kinematics appear to be positively influenced by oviductal cell co-culture, sperm capacitation, as assayed by acrosomal responsivity, is apparently far less positively affected.

*The oviductal environment, follicular fluid and motility regulation*

The cumulus oophorus surrounding the oocyte is a rich source for steroids and other factors acquired while bathed in the fluid-filled environment of the ovulatory follicle. It is reasonable to conclude that, in addition to the follicular fluid-soaked COC, some fraction of the fluid itself also passes into the ampulla of the oviduct. Atrial natriuretic peptide (ANP) has been identified in follicular fluid (Sundsfjord *et al.*, 1989); the lowest ANP concentrations were detected in follicles containing no oocyte, moderate ANP concentrations in follicles that contained oocytes and the highest ANP concentrations detected were found in follicles from which oocytes subsequently became fertilized *in vitro* (Anderson *et al.*, 1994). A receptor for ANP has been identified on the plasma membrane of human sperm (Silvestroni *et al.*, 1992). ANP appears to activate a guanylyl cyclase-dependent pathway (Anderson *et al.*, 1995). ANP has been shown to directly influence sperm swimming speed and, more importantly, to act as a chemoattractant *in vitro* (Zamir *et al.*, 1993; Anderson *et al.*, 1995). Equally compelling is that the chemoattractant response was dependent on the capacitation state of the sperm, and also with sperm decapacitation (Cohen-Dayag *et al.*, 1995). Other chemoattractant factors in follicular fluid and/or the COC may exist that attract sperm; however, that remains to be elucidated. A recent report suggests that the oocyte may also have a chemoattractant role (Sun *et al.*, 2004). Thus, the roadmap to facilitate sperm transport to the oocyte may be clearly detailed in specific chemoattractant signals.

Hyperactivation describes changes in sperm swimming characteristics that are considered to be associated with capacitation and immediately preceding but perhaps not dissociated from the acrosome reaction. While hyperactivation occurs during capacitation the two processes are apparently regulated by separable mechanisms (Ho and Suarez, 2001). This review will not detail hyperactivation, as numerous excellent critical descriptions exist (e.g. Mortimer *et al.*, 1997; Kay and Robertson, 1998; Ho and Suarez, 2001). Based on the current literature data, it is suggested that chemotactic responsivity be considered as an integral component of capacitation; a process distinct from hyperactivation.

*Follicular fluid, progesterone and calcium transport*

The sperm plasma membrane, like that of other cell types, is responsible for the selective trafficking of information between intra and extracellular spaces (Flesch and Gadella, 2000). Lipid–protein interactions can cause changes in membrane permeability. More specifically, membrane lipids appear to modify the properties of channels that, under normal conditions, maintain an electrochemical balance. Progesterone is a major steroid component of ovulatory follicular fluid and is also secreted by cumulus cells. The predominant *in vitro* influence of progesterone on sperm is to stimulate a biphasic $Ca^{2+}$ influx (Kirkman-Brown *et al.*, 2000) and acrosome reaction (for review, see Blackmore, 1993). Recently, Harper *et al.* (2004) created *in vitro* conditions to mimic the cumulus-produced progesterone gradient that human sperm might encounter *in vivo* prior to contacting the oocyte. They found that biologically relevant progesterone concentration gradients stimulated a slow persistent inward $Ca^{2+}$ transient that subsequently triggered head-associated intracellular $Ca^{2+}$ oscillations in approximately 34% of the population, which corresponds to the percentage of spermatozoa that subsequently demonstrate acrosomal responsivity to human zonae (e.g. Bielfeld *et al.*, 1994). Whether the oscillations can be attributed to influx of calcium or mobilization of $Ca^{2+}$ from intracellular stores remains to be clarified. It is notable that under the conditions of the experiment, a typical acrosome reaction response to progesterone was not detected and a modulation of flagellar beat was detected. Thus, it will be of great interest to determine if the progesterone-stimulated $Ca^{2+}$ response results of Harper *et al.* (2004) will become clarified to more clearly define the *in vivo* role of progesterone and calcium in human capacitation.

*The cumulus oophorus*

The COC is just that complex. As stated earlier, the COC is a rich source for progesterone and one or more sperm chemoattractants. Proteins secreted by cumulus cells have been shown to stimulate conversion of proacrosin to acrosin and the acrosome reaction (Tesarik *et al.*, 1988). These same investigators showed that a protein isolated from the intercellular matrix was stimulatory of the acrosome reaction and hyperactivation (Tesarik *et al.*, 1990). Carrell *et al.* (1993) detected similar acrosome reaction stimulation by cumulus cells, with mature oocyte-containing cumulus having the greatest stimulatory effect. Incubation of sperm in media that had been used to culture human cumulus cells for 3, 5 and 7 h had no stimulatory effect on the ability of the sperm to subsequently bind to human zonae, suggesting no stimulation or facilitation of the acrosome reaction (Hong *et al.*, 2003). In contrast, Hong *et al.* (2004) showed subsequently, using a capillary-cumulus model, that a greater percentage of the sperm population contained within the cumulus were capacitated, acrosome reacted and were able to bind to human zonae. Due to the constraints and superficiality of *in vitro* conditions, stimulation of the acrosome reaction by the cumulus would seem counter-intuitive because available evidence supports that the acrosome reaction in humans takes place on the zona, as stimulated by zona glycoprotein. However, perhaps the cumulus acts as a checkpoint to select out those sperm that have prematurely reached an advanced state of excitability.

The results above suggest that direct contact of sperm with the cumulus mass and more specifically the intercellular matrix has a stimulatory influence on sperm. Cumulus cells are held together primarily by hyaluronate (hyaluronic acid). Hyaluronidase activity by the sperm plasma membrane protein

PH-20 acting on its hyaluronate substrate facilitates sperm penetration of the cumulus matrix (Lin *et al.*, 1994). More specifically, using mouse sperm as a model, PH-20 enables the passage of acrosome intact sperm through the cumulus mass (Lin *et al.*, 1994). Using an *in vitro* system, Huszar *et al.* (2003) have shown that hyaluronic acid selectively binds spermatogenically and genomically mature, viable and acrosome intact human sperm.

Taken together the results above suggest that (1) progesterone accompanying the COC and also produced by the cumulus cells is a likely initiator of the acrosome reaction in those transiting capacitated sperm, (2) transit of sperm through the matrix requires motion characteristics that can be defined as hyperactivation, with that process being locally stimulated and (3) the intercellular matrix of the cumulus may act similarly as cervical mucus to selectively filter sperm with more normal morphology. The nature of the relationship between sperm cellular and nuclear maturity and the ability of hyaluronic acid to select those sperm remains to be clarified using intact cumulus masses.

At this stage in the fertilization process there are probably no more than tens of sperm that have reached and begun to penetrate through the cumulus. It is also likely that this sperm population is heterogeneous relative to expression and receptivity of receptors for zona glycoprotein and functionality of signal transduction mechanisms that will ultimately participate in the zona-induced acrosome reaction, zona penetration and fertilization. Upon contact with the zona there may only be slightly more than a handful of sperm that completely fulfil the preceding elements; perhaps chance is the final determinant for which of these is the fertilizing sperm.

## Summary

In 1677 Antoni van Leeuwenhoek observed human sperm through a primitive microscope and wrote, ‘a human being will originate from an animalcule in the sperm’. He got it half right. Roughly three centuries later Austin and Chang first described requirements necessary for sperm to fertilize oocytes. They independently concluded that the capacity of sperm for fertilization, i.e. capacitation, was acquired only after a period of residence in the female reproductive tract. Since the reports by Austin and Chang there have been many subsequent but primarily *in vitro* investigations into molecules and processes suspected of regulating capacitation. The goal of this review was to construct a biological framework for *in vivo* human sperm capacitation.

Human sperm capacitation can readily be accomplished *in vitro* provided culture conditions facilitate and support membrane composition alterations and signal transduction pathway activation similar to those occurring *in vivo*. Using *in vivo* data as a template, several critical components can be identified that must be present in order for capacitation to be initiated and optimized. First, sperm must be removed from the seminal factors that act to stabilize sperm plasma membranes. Historically such techniques included dilution of semen with media followed by isolation of sperm via centrifugation. Today, density gradient centrifugation is the method of choice and one can liken the process to the passage of sperm through cervical mucus. Second, the media must contain a sterol-acceptor molecule, e.g. serum albumin or cyclodextrin. Third, the media used to culture sperm must contain an ionic composition that is supportive of sperm homeostasis and facilitative of signal transduction processes. For example, $Ca^{2+}$ influx has been demonstrated to occur in sperm after exposure to factors emanating from the female reproductive tract. *In vitro* experiments have established culture media requirements for $Ca^{2+}$ concentrations that are supportive of specific sperm functions, including capacitation (e.g. Marin-Briggiler *et al.*, 2003). However, it is not likely that $Ca^{2+}$ is the only essential ion in culture media that facilitates capacitation; for example, $HCO_3^-$ is rapidly being identified as an ion critical to the capacitation process (see for review, Gadella and van Gestel, 2004). Determining the potential significance for the role of any molecule in capacitation is made very difficult when trying to extrapolate from the *in vitro* to the *in vivo* environment.

There has been wide variation in the *in vitro* culture conditions, e.g. incubation time and media composition, under which experiments investigating capacitation have been conducted. For example, a capacitation interval of 3 h versus 24 h may yield vastly different results concerning intracellular events or membrane structural and permeability changes—yet the literature is rife with such dramatic differences. In addition, there has been considerable heterogeneity in the media composition used in studies on capacitation, yet often times the marker(s) being used as indicative of capacitation are the same. Highlighting the implications of the aforementioned are recent data (FL Moseley, L Lefièvre, CLR Barratt, personal communication) showing that human sperm capacitation was differentially influenced by incubation time and medium composition. One medium, used in IVF, stimulated a more rapid acquisition of capacitation than another medium traditionally used for human sperm capacitation. Tyrosine phosphorylation, a proposed marker for capacitation (Naz and Rajesh, 2004), was enhanced in sperm cultured in both media but the IVF medium stimulated a faster onset. Sperm hyperactivation, another proposed marker for capacitation, was only increased after culture in the IVF medium. With these markers of capacitation being in conflict one is left to question, which set of *in vitro* capacitating conditions and what markers are more closely reflective of those occurring during capacitation *in vivo*? Regrettably there is no clear answer. As a consequence it becomes difficult to clearly and unequivocally define one or another process as truly being integral to capacitation.

In reviewing the literature data it was remarkable to note the disproportionately limited number of references that could be found in which *in vivo* events contributing to human sperm capacitation were described relative to *in vitro* investigations. In fact, a considerable portion of what is purported to be essential for human sperm capacitation has been derived from *in vitro* observations. This is not meant to be dismissive of *in vitro* data and their relevance but rather to emphasize that (1) more *in vivo* data are needed; (2) more experiments are needed using *in vivo* biologicals, e.g. cervical mucus; (3) changes occurring or not in the different sperm compartments should be distinguishable and (4) conclusions about molecules and processes involved in capacitation based on *in vitro* data should largely be considered only in that context until such a time when *in vivo* verification can be made.

It is now half a century since the reports of Austin and Chang and even in this era of genomics, proteomics and microarrays, we still know very little about how humans make more of

themselves. The tripartite recommendations made by Barratt and Cooke (1991) are highly relevant: (1) more experimentation on sperm recovered after *in vivo* transport, (2) better *in vitro* analysis of sperm function after exposure to female tract fluids and cells and (3) optimized co-culture systems. Experiments employing conditions such as these will help to clarify some of the still enigmatic aspects of human sperm capacitation.

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