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Modulation of Sperm Function during Sperm Transport in the Female

Heriberto RODRÍGUEZ-MARTÍNEZ

Division of Comparative Reproduction, Obstetrics and Udder Health, Department of Clinical Sciences, Faculty of Veterinary Medicine & Animal Science, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden

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Abstract

In the cow, sow and mare, a functional tubal sperm reservoir is established before ovulation to ensure availability of suitable numbers of viable spermatozoa for fertilization. Although identification of subpopulations reaching this reservoir has been attempted, it is still unclear whether this recruitment is programmed or fortituous. Those spermatozoa not reaching the oviduct are generally destroyed by phagocytosis of invading leukocytes. While the type of ejaculate differs in these species, seminal plasma proteins and/or the spermatozoa appear to act as leukocyte chemoattractant both *in vitro* and *in vivo*. Those spermatozoa in the sperm reservoir not only escape phagocytosis or rejection by the female immune system but sustain viability and potential fertilizing capacity by not capacitating or acrosome-reacting while residing in the oviduct. Sperm numbers in the reservoir diminish gradually in relation to ovulation, spermatozoa being continuously redistributed towards the upper isthmus. *In vitro*, only uncapacitated spermatozoa bind to epithelial explants, suggesting that the reservoir milieu modulates sperm capacitation. *In vivo*, most viable spermatozoa during preovulatory spontaneous standing oestrus are uncapacitated, with capacitation significantly increasing after ovulation. In either species, there seems to be different components of the oviductal fluid effecting capacitation, and bicarbonate appears to be common denominator for the membrane destabilizing changes that encompasses the first stages of the process. Such effects can be blocked or even reversed by co-incubation with isthmic fluid or specific glycosaminoglycans such as hyaluronan. Although the pattern of response to *in vitro* induction of sperm capacitation is similar for all spermatozoa, the capacity of response and its speed is very individual. Such diverse individual response to capacitation would not only confirm capacitation does not occur massively in the reservoir but clearly insures full sperm viability before ovulation and the presence of spermatozoa at different stages of capacitation in the upper oviduct, thus maximizing the chances of normal fertilization.

Introduction

Sperm transport in the female genitalia follows a general pattern in domestic animals with spermatozoa being sequentially exposed to various genital environments, before encountering the oocytes and participating in fertilization. While the transport is usually rapid through the cervix and uterus, depending on the place of sperm deposition among species, most of the lengthy phases of sperm transport occur in the oviduct. The mammalian oviduct provides suitable environment for sperm transport, storage and capacitation, oocyte pick-up, transport and maturation, fertilization and ultimately, early embryonic cleavage (Hunter & Rodriguez-Martinez 2004). During their tubal permanence, spermatozoa bathe in the isthmic secretion in contact with oviduct-specific proteins, enzymes, glyco- and lipoproteins and become eventually associated, to various degrees, to the epithelial lining (Smith, 1998). Secretion flow, ciliary beating and myosalpinx contractions all play a major role in the formation and mixing of a tubal fluid where gametes meet. Oviductal fluid (ODF) differs from blood plasma in terms of ionic composition, pH, osmolarity and macromolecular content (Leese 1988), originating from selective transudation (mostly at the ampullar segment) and, a minor part, as secretion from the lining epithelium (particularly the isthmus segment) thus establishing regional differences in composition that might relate to the process of gamete preparation for fertilization. The present paper reviews aspects of the interactions

between spermatozoa and the surrounding tubal milieu in relation to the modulation of capacitation and fertilization.

The ejaculate

The bull ejaculates a single fraction of rather highly concentrated spermatozoa in a single jet of about 5 mL (range 2-10 mL) after secreting a pre-sperm secretion from the bulbourethral glands that cleanse the urethra. Such ejaculate contains a mean of 1 billion spermatozoa/mL bathing in a mixture of the cauda epidydimal fluid and secretions from the ampullae deferens, the prostate rich in electrolytes and carbohydrates and a basic protein-rich secretion from the seminal vesicles. Most of these proteins adhere to the spermatozoa (so-called spermoadhesins) of which a large part are able to bind to glycosaminoglycans (GAGs) such as heparin sulphate and heparin. Some proteins, including the heparin-binding proteins and osteopontin have been related to the fertility of the males (Killian et al. 1993). Other males, such as the stallion or the boar, ejaculate distinct fractions, with spermatozoa being located in a second-third fraction (the so-called sperm-rich) coincidental with the different jets ejected by ejaculatory thursts. Also here, the seminal plasma contains a large amount of proteins that interact with spermatozoa. In the stallion, the mean total number of spermatozoa ascends to 9 billion, suspended in a seminal plasma fluid that contains as much as 14 different types of major proteins (Frazer & Bucci 1996). Although stallion seminal plasma has its own typical protein profile in SDS-PAGE-gels, the relative protein amounts varies between stallions (Kareskoski et al. 2005). Calvete et al. (1994) isolated eight types of horse seminal plasma proteins (HSP-1 – HSP-8) by reverse phase HPLC. All the identified proteins had a low molecular mass of 14-30 kDa, and all of the proteins except HSP-4 were also peripherally bound to the sperm surface. The major proteins in stallion seminal plasma, HSP-1 and HSP-2, are heparin-binding proteins. HSP-3 is a member of the cysteine-rich secretory protein (CRISP) family, and it could be involved in membrane-fusion processes or act as an anti-infectious agent in seminal plasma (Calvete et al. 1997, Magdaleno et al. 1997). HSP-7 is a zona-pellucida-binding protein that is the sole member of the spermadhesin protein family in the stallion (Reinert et al. 1996).

The boar ejaculates also a high number of spermatozoa (mean 60 billion) in a large volume of seminal plasma (200 to 250 mL) mainly (95-98%) built by the secretions of the accessory sexual glands whose secretion is verted in a sequential manner, providing a series of easily recognizable fractions that can be separated during manual collection of a boar (Einarsson 1971, Lavon & Boursnell 1975). These fractions are usually called *presperm* (dominated by the secretion of the urethral and bulbourethral glands, as well as the prostate), *sperm-rich* (SRF, in which the vast majority of spermatozoa are present and where the emitted epididymal fluid in which they originally bathe is diluted with a fluid derived from the seminal vesicles and the prostate), and *post-sperm-rich* (PSRF), in which few spermatozoa are present and the fluid is primarily derived from the increasing secretion of the seminal vesicles, the prostate and, by the end of the ejaculation, the bulbourethral glands. The latter produces the tapioca-like floccula that coagulates the seminal plasma, as seen shortly after the ejaculate is collected in a receptacle, and that serves *in vivo* to retain the ejaculate *in utero*, thus minimizing retrograde flow through the cervix. The spermatozoa are therefore ejaculated with a maximum concentration in the first portion of the SRF, decreasing thereafter in number along this fraction to virtually disappear by the PSRF. The secretion of the seminal vesicles, albeit present at the moment at which spermatozoa are first emitted, increases in volume toward the PSRF.

More than 90% of the seminal plasma proteins in boars belong to the spermadhesin protein family comprising five members: AQN-1, AQN-3, AWN, PSP-I, and PSP-II, with different biological activities, depending on their sequence, glycosylation or aggregation state, and ability to bind heparin (Calvete et al. 1995a-c). The remaining components are proteins of low molecular mass (5-10 kDa) of which the acrosin inhibitor is the best identified. Although AQN-1, AQN-3, and AWN are sperm-coating molecules that can bind heparin, they seem to stabilize the plasma membrane over the acrosome but are mainly released during capacitation (Dostàlova et al. 1994, Calvete et al 1997); the other two proteins, PSP-I and PSP-II (Kwok et al. 1993), account for >50% of the total proteins, forming a non-heparin-binding heterodimer of glycosylated spermadhesins (Calvete et al. 1996) that displays immunostimulatory

activity *in vitro* by binding to porcine lymphocytes (Yang et al. 1998) and modulating their activity *in vitro* (Leshin et al. 1998). Since the various seminal plasma proteins originate from the testis, the epididymides, or the sexual accessory glands, they are present in the entire ejaculate but their relative concentration therefore varies with the different fractions of the boar ejaculate the lowest concentrations of spermadhesins being recorded by the end of the pre-sperm fraction, and particularly by the first portion of the SRF, compared to samples collected in the PSRF, where the seminal vesicles deliver both heparin-binding proteins (HBP) and PSPI/II (Rodriguez-Martinez et al. 2005).

Sperm transport in the female genital tract

The site of sperm deposition differs among species. In the bovine, semen is deposited in the cranial segment of the vagina, close to the cervical opening during mating. However, when artificial insemination (AI) is used, spermatozoa are deposited intra-utero, waiving the cervical barrier. In swine, spermatozoa are -during mating or during conventional AI- deposited in the narrow cervical canal, thus entering the uterine cavity rather quickly. In the equine species, the stallion (or the operator during AI) deposits the spermatozoa directly in the uterine cavity, owing to the large opening provided by the cervix. Despite these differences in sperm deposition, the process of sperm transport through the female genitalia is comparable in these three exemplified species and therefore can be divided in three phases: a) a rapid trans-uterine transport immediately after semen deposition, b) the colonization of a sperm reservoir in the lower oviduct, and c) a slow release from the reservoir towards the site of fertilization (ampullary-isthmic junction, AIJ), in relation to ovulation (Barrat & Cooke 1991).

Are there subpopulations among the ejaculated spermatozoa?

The spermatozoa present in a given ejaculate are part of an aliquot stored, for different periods, in the cauda epididymides. They represent, therefore, a heterogenous population of cells released by tubuli seminiferi that underwent sperm maturation along the ductus epididymidis at various intervals. Although highly seeked, identification of sup-populations has been restricted to morphological and functional attributes separating abnormal from normal (e.g. potentially fertile) spermatozoa. Determination of which spermatozoa actually participate in the fertilization process among those potentially fertile has yet not being possible. On the other hand, the ability of spermatozoa to interact with different seminal plasma proteins has indicated that some spermadhesins are able, when present in very low concentrations, to maintain the viability and fertilizing ability of boar spermatozoa (Vazquez et al. 2001, Centurión et al. 2003, Caballero et al. 2004a, 2005) with significant variation among boars (Caballero et al. 2004b).

Once ejaculated, a certain proportion pass the cervix-uterus (bovine) or the uterus (horse, pig), while the majority of the spermatozoa are rapidly eliminated from the genital tract, either by way of retrograde flow or by intrauterine (Einarsson 1985, Rodriguez-Martinez et al. 2005), with the exception of a small subpopulation of spermatozoa that is rapidly (in minutes [Hunter 1981]) transported by the myometrial contractions towards the uterotubal junction (UTJ) during the so-called *rapid phase* of the process of sperm transport in the female internal genital tract and that colonizes the sperm reservoir in the oviduct (rev by Rodriguez-Martinez et al. 2001, 2005).

Our own studies have tested the hypothesis that there are sperm subpopulations in the boar ejaculate, one of which first colonizes the sperm reservoir during natural mating (Rodriguez-Martinez et al 2005), where spermatozoa from the first portion of the SRF made up the bulk of spermatozoa in the oviductal sperm reservoir under the experimental conditions used, when fractionated sperm deposition was mimicked. Spermatozoa from this particular first portion of the SRF sustain better handling in the laboratory, such as storage at room temperature, cooling, or freezing-thawing, than the spermatozoa of the rest of the ejaculate (Sellés et al. 2001, Peña et al. 2003, 2005a-b). It seems, therefore, that a window of opportunity exists for a particular, albeit fortuitous, sperm subpopulation (e.g., bathing in a particular SP fraction) that not only presents the best viability but also escapes leukocyte phagocytosis. Interestingly, the seminal plasma present in the above-mentioned first portion of the SRF differs significantly in its relative contents of total protein and, particularly, in protein compositions compared to the rest of

the ejaculate. This first SRF-portion of the seminal plasma is characterized by major components of 5, 7 (acrosin inhibitor) and 10 kDa as well as a relative low amount of the glycosylated heterodimer PSP-I/PSP-II. The first named are not present in the later fractions of the ejaculate where instead we found high concentrations of spermadhesins (including the glycosylated heterodimer PSP-I/PSP-II) (Rodriguez-Martinez et al. 2005).

Fate of the spermatozoa in the female genital tract

As already mentioned, the spermatozoa (and the seminal plasma that surrounds them) that do not colonize but are retained in the uterine cavity are eliminated, both by vaginal reflux and by phagocytosis by leukocytes that migrate from the endometrium to the uterine lumen and, partially, by macrophages in the endometrial epithelium, phenomena best studied in pigs (Viring & Einarsson 1981, Einarsson 1985), but recorded in most species, including the bovine (Cobb & Watson 1995) and the horse (Kotilainen et al. 1994, Tunón et al. 2000). The entry of semen into the uterine cavity provokes, by means that are not yet known in detail, a massive invasion of leukocytes (mostly PMN) into the uterine lumen. These PMN migrate from the lamina propria, subjacent to the lining epithelium, where they accumulate after extravasation, presumably as a result of the high levels of estrogens that dominate the proestrus in the sow (Lovell & Getty 1968). Interesting to note is the fact that the PMN do not reach the uterine lumen immediately after semen deposition, at least not during the first 10 min. A massive presence of PMN is first detected 30 min after semen deposition (Lovell & Getty 1968), increasing in a sustained manner for the following 2 to 3 h (Viring & Einarsson 1981). Through this primary leukocytic reaction (phagocytosis), the majority of the ejaculated spermatozoa and the proteins surrounding them, both considered foreign by the female, are eliminated from a uterine lumen that should be cleansed and prepared to host and nurture the early embryos; in the pig, these can already reach the uterus 48 h after ovulation. Several factors have been implicated as mediators of the PMN recruitment toward the uterine lumen, including the uterine distension *per se* (Matthjis et al 2003), the spermatozoa (Kotilainen et al. 1994, Rozeboom et al. 1999), or the seminal plasma (Claus 1990, Bischof et al. 1994, Hadjisavas et al. 1994).

The spermadhesin PSP-I/PSP-II and its isolated subunits can induce migration of PMN *in vitro* and *in vivo* in rodents (Assreuy et al. 2002, 2003) as well as in pigs *in vivo* (Rodriguez-Martinez 2005), at doses five-fold lower than those present in the boar ejaculate. Associated the data presented thus far, spermatozoa that would temporarily (and fortuitously) be present in the fist portion of the SRF of pigs would benefit, under *in vivo* conditions from the absence of signal substances (such as PSP-I/II) at levels needed for stimulation of leukocyte migration to the uterine lumen (and the resulting sperm phagocytosis). Entry of the rest of the ejaculate into the utero, having higher levels of the heterodimer, would stimulate PMN migration and eliminate spermatozoa from the lumen. Such a period of latency for PMN migration to the uterine lumen (<30 min) suggests that there may be a window of opportunity for a certain subpopulation of ejaculated spermatozoa to traverse the uterine lumen during the first phase of sperm transport, without risking phagocytosis. Such sperm phagocytosis would start when a relevant number of spermatozoa had already colonized the sperm reservoir of the oviduct.

The oviduct and sperm transport

The mammalian oviduct is anatomically divided into three main segments; e.g. the isthmus, ampulla and infundibulum, counted from the ad-uterine to the ovarian end. Connecting areas are also described, i.e. the uterotubal (UTJ) and the ampullary-isthmic (AIJ) junctions, as well as a terminal section connected to the ovarian fimbriae and bursa in the abdominal opening (ostium; Beck & Boots 1974). The histoarchitecture is very simple, with a non-glandular mucosa (endosalpinx), covered by a lining epithelium composed of non-ciliated (secretory) and ciliated cells, an underlying double-layered smooth muscle (myosalpinx) and a covering serosa (mesosalpinx) continuous with the peritoneal covering. While the thickness of the internal, circular smooth muscle becomes thinner, the longitudinal mucosal plicae gain complexity (with secondary and tertiary plicae) and the number of ciliated cells increases dramatically towards the ostium (Rodriguez-Martinez et al. 2001). This histoarchitecture defines the presence of tubal compartments each one with a specific

function, providing the best environment to sustain and regulate gamete preparation, fertilization and the first steps of zygote development while transported through the tubal lumen (Boatman 1997). Those spermatozoa that ascended the uterus in the first phase of sperm transport colonise rapidly (minutes to 1-2 hours, second phase of sperm transport) the UTJs and the adjacent tubal segment in very reduced, albeit significant numbers (from thousands to 1-2 10^9 spermatozoa) compared to the original sperm population contained in the AI-dose or ejaculate, thus depending on the species. This segment marks the building up of a pre-ovulatory sperm reservoir as a consequence of several concerted factors, both mechanical and biochemical, and whose functionality is theoretically prolonged in pigs up to 30 h from onset of estrus (rev by Rodriguez-Martinez et al. 2001, 2005). These sperm numbers in the reservoir remain basically unchanged during the pre-ovulatory period for up to 18 hours in the cow (Hunter & Wilmut 1984, Hawk 1987) or 24 hours in the pig (Hunter 1995a, Mburu et al. 1996), immersed in the tubal fluid or contacting the lining epithelium. Most spermatozoa present in the pre-ovulatory sperm reservoir remain viable and potentially fertile (rev by Rodriguez-Martinez et al. 2005) until they ascend to the upper tubal segments either shortly before ovulation (Hunter & Wilmut 1984, Hunter 1995a) or as a continuous stream during the peri-ovulatory period (Larsson & Larsson 1985; Mburu et al. 1997).

Composition of the tubal fluid

The intraluminal ODF is, as stated above, created by secretion from the epithelium and by transudation from the blood through the lamina propria (Leese et al. 2001) varying in volume and composition with the stage of the oestrous cycle (Carlson et al. 1970, Buhi 2002, Rodriguez-Martinez et al. 2001). The ODF of bovine, equine and porcine species contains, among other compounds, GAGs either non-sulphated (hyaluronan) or sulphated (S-GAGs, eg chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate and heparin) (Lee & Ax 1984, Varner et al. 1991, Tienthai et al. 2000, Bergqvist et al. 2005, Bergqvist & Rodriguez-Martinez 2005). The mean concentrations of total S-GAGs in tubal fluid differ between species; being larger in cows (Bergqvist & Rodriguez-Martinez 2005) than pigs (Tienthai et al., 2001). However, in either species levels and variations in concentrations are different between isthmus and ampulla and vary also in relation to the moment of the cycle. While in the cow the concentrations in the ampulla are significantly higher than in isthmus, the situation is reversed in pigs, probably owing to the larger secretory capacity of the latter. S-GAG-levels increase significantly in isthmus during preovulatory oestrus, to decrease towards metaoestrus. While not differing between sides in pigs, the concentration is higher in the "ovulatory" side in cows, compared to the contralateral oviduct. Regarding hyaluronan, this non-sulphated GAG is present in the ODF in both species, without segmental differences, but with a tendency to increase during standing oestrus, highest around ovulation. Absolute concentrations were significantly higher in pigs compared to cows. Both HA synthases, HA-binding proteins and specific membrane receptors are present in the epithelial lining, particularly in the sperm reservoir (Tienthai et al. 2001, 2003a-b, Bergqvist et al. 2005), where mucus accumulate pre-ovulation (Rodriguez-Martinez et al. 1998a-b, Johansson et al. 2000). These GAGs seem to be beneficial for sperm survival and capacitation (Rodriguez-Martinez et al. 2001, 2005).

Modulation of sperm capacitation in the oviduct

Sperm capacitation is a gradual, essential event pre-requisite for fertilization that takes place *in vivo* during the sequential exposure of spermatozoa to the different compartments of the female genital tract that occurs during sperm transport (Yanagimachi 1994). It can also be mimicked during incubation of spermatozoa *in vitro*, although our knowledge of the different steps of the process are yet to be fully unveiled. Capacitated spermatozoa are endowed with a number of abilities, including release from the sperm reservoir, penetration of the cumulus layers, and binding to the ZP, that permit the occurrence of the acrosome reaction (reviewed by Rodriguez-Martinez et al. 2001). In order to reach this status in ejaculated spermatozoa, bound proteins from the cauda epididymidis and the seminal plasma SP are removed from the sperm surface, particularly over the acrosomal region. When this sperm surface domain is exposed, it becomes accessible to lipid-binding components of the female intra-luminal fluids either in the uterus, but mainly from the oviduct.

These in turn are able to remove cholesterol from the sperm plasma membrane, thus enhancing membrane fluidity, which in turn causes lipid scrambling, and initiates further capacitation changes such as the uptake of extracellular Ca^{++}, tyrosine phosphorylation and the reorganization of the sperm membrane (Töpfer-Petersen et al. 2002, Tardif et al. 2003). During capacitation, intracellular pH and Ca^{++} rise, adenylate cyclase is activated, resulting in a rise in cAMP levels, and specific proteins (including extracellular signal-regulated cyclases) are then tyrosine phosphorylated (Tardif et al. 2003). In parallel, lipid redistribution in the plasma membrane and membrane destabilization result in a more fusogenic membrane with the exposure, and perhaps also the hiding, of specific receptors (Jaiswal et al. 1999). Therefore, sperm capacitation can be triggered *in vitro* by specific signals such as certain GAGs or changes in pH or Ca^{++} (Rodriguez-Martinez et al. 1998), clearly imitating events occurring *in vivo*. In the pig, horse and bovine, bicarbonate (a stimulator of adenyl cyclase) appears to be the effector molecule that is able to trigger the lipid scrambling seen in the lipid bilayer of the plasma membrane, and thus it is considered to be one of the earliest signs of capacitation (Harrison 1996, 1997, Harrison et al. 1996, Gadella & Harrison 2000, Harrison & Gadella 2005). All these modifications of the fluidity of the sperm membrane precede specific changes in Ca^{++} movement and of motility patterns (such as the associated hyperactivated motility), that predispose to the acrosome reaction and the penetration of the ZP during fertilisation.

Capacitation lasts for different times depending on the ability of the environment to cleanse the surface of the spermatozoa, and the exposure of these to environments which enable the sequence of events listed above, to occur. Since spermatozoa are exposed to uterine and tubal fluids, these ought to be able to regulate the speed of the process *in vivo*. Capacitation can be elicited more rapidly, for instance, if boar spermatozoa are deposited directly in the caudal isthmic portion of the oviduct, rather than in the UTJ or the upper ampulla (Hunter & Rodriguez-Martinez 2004). Not only in pigs, but also in all other species of mammals studied, the sperm reservoir in the oviduct appears to retard, rather than promote sperm capacitation (Smith & Nothnick 1997), apparently intending the extension of the viability and fertilizing capacity of those spermatozoa retained in this environment (Murray & Smith 1997, Rodriguez-Martinez et al. 2005). When spermatozoa leave this segment, either continuously or following a signal during ovulation, the suppressive effect of the sperm reservoir disappears or spermatozoa are confronted to a more favourable environment, triggering capacitation. In either case, and in all species studied thus far, capacitation is to be considered a peri-ovulatory process (Rodriguez-Martinez et al. 2001).

Our own experimental evidence, obtained *in vivo* by way of the flushing of specific tubal segments at well-defined stages of standing estrus (pre-, peri- or immediately post-spontaneous ovulation), indicate that the majority of boar spermatozoa retained in the SR during the period from 10-8 h before ovulation to 8-10 h after ovulation maintain a stable plasmalemma and are therefore not to be considered as undergoing capacitation (monitored as the significant increase in lipid scrambling at the membrane level by flow cytometry of Merocyanine 540/Yo-Pro-1-loaded spermatozoa (Rodriguez-Martinez et al. 2001), except for a significant increase (on the order of 10%) in the percentage of sperm capacitation after ovulation (Tienthai et al. 2004).

In following studies in the bovine, spermatozoa were exposed to bovine oviductal fluid surgically collected *in vivo*, to different glycosaminoglycans (GAGs) as well as to bicarbonate-enriched media. Following different exposure length, the spermatozoa were stained either with Chlortetracycline (CTC) or loaded with Merocyanine 450-Yo-Pro-1, and evaluated with epi-fluorecent light microscopy or flow cytometry, respectively for events related to sperm capacitation (Bergqvist et al. 2005, unpublished). When extended, but not chilled bull spermatozoa were exposed between 30 min and 2 hours to oviductal fluid (ODF), from either the isthmic or the ampullar regions and collected either during standing oestrus or at the day of ovulation, there were significant increases ($p<0.05$) in capacitation as measured by Merocyanine and CTC, indicating factors present in the ODF can trigger sperm capacitation. When exposing bull spermatozoa to the different GAGs known to be present in the ODF of the cyclic cow, hyaluronic acid was the only GAG that seemed to cause a slight capacitation, as detected by CTC, with a significant increase in B-pattern sper-

matozoa ($p=0.012$) compared to the negative controls. The only GAG that gave a significant increase in the Merocyanine high fluorescence sperm population was dermatan sulphate ($p=0.035$). Such effects were somewhat puzzling regarding the S-GAGs, since heparin is routinely used to induce sperm capacitation of bull spermatozoa *in vitro*. However, these results were obtained using an exposure to specific substances one by one, loosing eventual synergistic effects or dose response-interactive effects that ought to be the ones reflected by the ODF. Hyaluronan has been able to induce similar early changes in boar spermatozoa (B-pattern of CTC, for instance) but without leading to the acrosome reaction (Rodriguez-Martinez et al. 1997). That hyaluronan was able, *in vitro*, to induce capacitation (monitored via Merocyanine or CTC) but without eliciting the acrosome reaction has comparative interest. The presence of sperm reservoir fluid or hyaluronan during the incubation of boar spermatozoa flushed from the sperm reservoirs during pre-ovulation has prevented the induction of sperm capacitation after exposure to bicarbonate *in vitro* (Tienthai et al., 2004). These data indicate, albeit indirectly, that the SR fluid before ovulation maintains sperm viability without causing sperm capacitation, perhaps because of its HA content. When the same treatment was used with spermatozoa collected from the SR after ovulation, however, the presence of HA increased the rate of sperm capacitation, suggesting the temporal nature of the effect.

When exposed to a bicarbonate-enriched medium for 30 min, the number of bull spermatozoa depicting a higher degree of lipid disorder in the plasma membrane (Merocyanine with high fluorescence, Mero high) increased significantly ($p<0.0001$), compared to before bicarbonate addition and independent of the treatment before the exposure (including pre-exposure to GAGs, both sulphated or non-sulphated. There was no significant difference in the number of spermatozoa depicting B-pattern when bicarbonate was added, compared to negative controls, but an increase in the proportion of spermatozoa with AR-pattern (acrosome reacted spermatozoa) was registered ($p<0.0001$). As well, exposure to solubilised homologous ZP proteins significantly increased the proportion of acrosome-reacted spermatozoa ($p=0.016$). These results indicate that bicarbonate is also the effector molecule for bovine spermatozoa, as it has been the case for boar or stallion spermatozoa (Harrison & Gadella 2005). Moreover, it points out that bull spermatozoa exposed to a hyaluronan-rich medium can undergo sperm capacitation if further exposed to a low concentration of bicarbonate (30 mM).

The results with bull spermatozoa were, moreover, fitting with previous studies where Tienthai et al. (2004) were able to initiate the process of capacitation in boar spermatozoa, retrieved from the sperm reservoir, when exposed *in vitro* in a medium containing the effector bicarbonate (HCO_3^-) at concentrations similar to those recorded *in vivo* in the AIJ/ampulla segment of the peri-ovulatory pig oviduct (e.g., 33–35 mM/L (Rodriguez-Martinez et al. 1998). Taken together, the results suggest that the immersion of boar or bull spermatozoa in homologous ODF is not, *per se*, able to induce sperm capacitation unless the exposure is done with peri-ovulatory ODF. The triggering of the process of membrane destabilization that capacitation implies, does not seem to occur until spermatozoa are exposed to a specific effector, such as bicarbonate that leads the spermatozoa to acrosome exocytosis. Taking into consideration that the bicarbonate levels used were on the same order of magnitude as those in the AIJ, it seems possible, although speculative, that the progression of individual spermatozoa out of the sperm reservoir (as an expression of the innate heterogeneity of the ejaculate) is sufficient to induce capacitation when adequate levels of the effector are encountered outside of the sperm reservoir area has been proven for boar spermatozoa (Rodriguez et al. 2005).

A progressive and continuous release of spermatozoa from the sperm reservoirs in the oviducts, already occurring before ovulation (albeit increasing, but not massively, after ovulation) may be related to the gradual induction of capacitation following exposure to the fluid of the upper tubal segments. In this case, the numbers of capacitated spermatozoa at one particular time would be low, and because the capacitated state in the spermatozoon is transient and eventually leads to cell death due to its irreversibility *in vivo*, they would exocytose their acrosome contents and die if they are not near an oocyte. Following this hypothesis (Rodriguez-Martinez et al. 2005), there should be a continuous replacement of capacitated, short-lived spermatozoa leading to low sperm numbers per area

at any one time, albeit ensuring the availability of capacitated spermatozoa for such an extended time that could cover the very long interval between sperm deposition and ovulation.

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