Prostasomes, post-testicular sperm maturation and fertility

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1. ABSTRACT

Prostasomes are known as extracellular organelles originating from the epithelial cells of the prostate and detected in its secretions where they are mixed with sperm cells after ejaculation. They were first described in men but are also present in the semen of all mammals studied. Since their characterization by Gunnar Ronquist in the late 1970's studies have focused on different aspects which primarily include their molecular composition and structure, and secondly their ability to interact or even fuse with cells, particularly spermatozoa. They have the ability to bring molecules such as lipids or proteins to spermatozoa during their journey to the oocyte, and their role thus seem to be a sort of a "reservoir" that sperm may use depending on the surrounding conditions. Other properties have been suggested but this review will focus on the properties, acquired by sperm cells *via* prostasomes, that may influence fertility outcome.

2. INTRODUCTION

The fate of a mammalian spermatozoon appears very simple: swimming up the female genital tract to meet, recognize, and fertilize the oocyte. This journey is, however, a little more intricate than it appears, due to the particular nature of the male gamete. This cell has highly compacted DNA, in order to protect the paternal genetic material, and this does not allow transcriptional activity (1). To meet the swimming obligation, the cell is polarized with its flagellum representing approximately 80% of its ultrastructure, and with the necessity to carry very little cytoplasm. These characteristics make the sperm a very vulnerable cell without a defense program to counteract environmental stresses (2). Furthermore, the different maturational steps that a spermatozoon undergoes after its production in the testis, globally named post-testicular maturation, cannot be performed by the usual pathways of gene transcription activation. Consequently, the post-testicular protection and modifications of the male gametes are entirely dependent on their direct environment, starting in the epididymal lumen and terminating in the vicinity of the female gamete in the upper genital tract (3). In this issue, the multiple vesicle-like particles that sperm encounter during their journey are discussed in relation to their roles in male fertility. This review will shed light on the roles played by one type of extracellular vesicles, encountered by sperm cells after ejaculation, produced by the prostate and termed prostasomes.

3. PROSTASOMES: WHAT ARE THEY?

Prostasomes were isolated for the first time in 1978 after ultracentrifugation of human seminal plasma devoid of spermatozoa and cellular debris (4). Their production, storage and secretion are related to the prostate epithelial cells' activity, prostasomes being mixed with epididymal spermatozoa and seminal vesicles
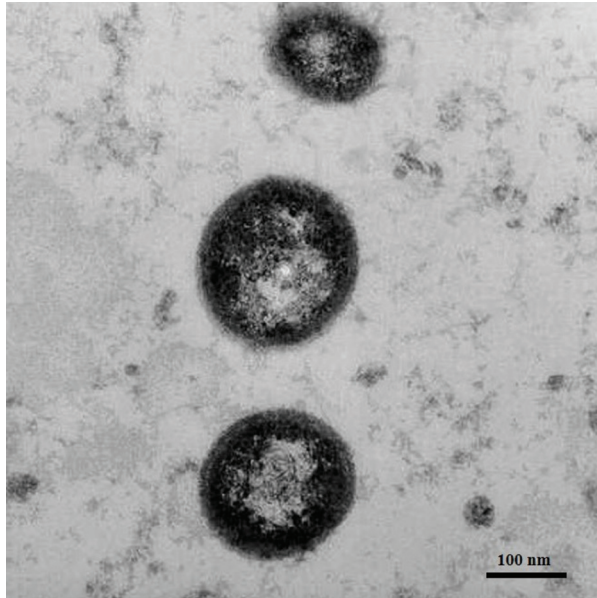
Figure 1. Electron photomicrographs of prostasomes purified from human semen. Three prostasomes appear as electron dense material and show their size heterogeneity. The original photo was kindly provided by Dr Kemeny and Dr Guy (Centre Hospitalier Universitaire de Clermont-Ferrand, 63000 France). Reproduced with permission from Journal of Andrology, ref # (65).

secretions at the time of ejaculation. The composition of seminal fluid is species-dependent and vesicles originating from secretory glands other than the prostate may be present, making data obtained for one species not always accurate in another one (5). Morphologically, prostasomes are sub-micron vesicles, with their size generally ranging from 30 to 500 nm (6), and surrounded by multi-lamellar lipoprotein bilayers (Figure 1 and reviewed by (7)). They are members of a large group of vesicles that mammalian cells secrete in their normal physiological state and referred to as extracellular vesicles. This group is formed mainly by two different types of particles, the exosomes and the microvesicles. Prostasomes are released by epithelial prostatic cells in a process involving the fusion of multi-vesicular bodies of late endosomal origin (storage vesicles), containing the prostasomes, with the plasma membrane, making them related to exosomes (8).

Concerning their structure and composition, prostasomes have already been described as very rigid membranous structures using biophysical detection methods such as electron spin resonance (9), and this is due to several aspects related to their lipid composition. First, they have a high cholesterol/phospholipids (chol/PL) ratio which is an indirect indicator of membrane rigidity. In red blood cells or spermatozoa, this ratio was measured at values around 0.6.5 (10) whereas it was determined at 2.0 in human prostasomes (10) and 1.7 in stallion prostasomes (11). Second, the relative proportion

of different phospholipids is also unusual in prostasomes with a high representation of sphingomyelin (SM) which accounts for around 50% of the total phospholipids, when other types such as phosphatidylethanolamine (PE) or phosphatidylcholine (PC) are mostly represented in somatic cells. Third, the fatty acid composition is also unique with high proportions of stearic and palmitic acids (saturated) or oleic acid (mono-unsaturated) (12). More recently, it was shown that the population of prostasomes was not homogeneous but was rather composed of two differentially characterized subpopulations. These subpopulations could be detected by using seminal fluid from vasectomized men as biological material, thus ensuring the absence of vesicles originating from the testis or the epididymis. Briefly, the two isolated subpopulations were different in size and protein composition (13). The lipid composition was further examined in these two subpopulations, using a LC/MS lipidomic approach: this study confirmed the high proportion of cholesterol, sphingomyelin and mono-unsaturated fatty acids. The two subpopulations were different in their proportion of SM and of hexosylceramides (derivatives of SM), SM being more abundant in larger prostasomes compared to smaller ones, whereas the opposite situation was noticed for hexosylceramides (14). These authors discuss the relationships that may exist between the differences in the lipid composition and the formation pathways of these two types of prostasomes. However, experimental evidence is required to validate the proposed hypotheses.

The protein composition of prostasomes is of great interest as the proteins can potentially be transferred to the male gametes and ultimately play a role in the fertilization process. Several proteomic analyses were performed on human prostasomes, revealing the presence of 139 (15) or more recently 440 proteins (16). A study focused on lipid rafts of prostasomes also very recently revealed a complex protein composition of these membrane domains (17), probably in relation with their functional roles in male gametes, a point that will be discussed later. The proteomic characterization of prostate cancer metastasis-derived prostasomes has been reported (18), with the focus of determining a putative role of prostasomes in the interaction between prostate and prostate-derived tumor cells and their environment. The objective of this review is not to discuss in detail the proteins that have been characterized, but to emphasize important ones in the biological processes, as shown below. For detailed analysis, the reader is referred to the above-mentioned publications.

Prostasomes have been shown to possess many different biological functions, which were recently summarized in an excellent review by Aalberts *et al.* (8). The roles played by prostasomes are essentially the results of their interaction and/or fusion with surrounding cells. We will first focus on the consequences of their interaction with sperm cells regarding fertility, and then

review their actions on other cells that have an impact on the reproductive outcome.

4. INTERACTION BETWEEN PROSTASOMES AND SPERMATOZOA – ROLES ON THE SPERM FERTILIZING ABILITY

As previously mentioned, sperm post-testicular maturation relies on the direct environment surrounding these cells. The molecular structure and composition of prostasomes have long been supposed to be intimately related to their functions. However, the way they interact with male gametes is still not completely understood and this point deserves to be developed in order to better grasp the consequences of these interactions on male fertility.

4.1. Interaction process

A strong hydrophobic interaction between mouse cauda epididymal spermatozoa and prostasomes was first described by Ronquist *et al.* (1996), who noted that the interaction was inhibited after treatment of the prostasomes with anti-prostasomes monoclonal antibodies. The first evidence of fusion was evoked in 1997 when prostasomes were loaded with self-quenching octadecyl Rhodamine B Chloride (R_{18}), the relief of the quenching after interaction with spermatozoa being a sign of lipid mixing (19). These data were completed with fluorescence microscopy and flow cytometry demonstrating that the fusion occurred at acidic pH (around 4-5) and in a time lapse of 10 min. However, one could argue that lipid exchange did not necessarily involve fusion and that the R_{18} probe may have been exchanged via a different method. The process seemed to be quite specific to prostasomes because when the same experiments were performed using liposomes made from rat liver lipid extracts, it did not involve a protein moiety and occurred at neutral pH (19). Furthermore, the hypothesis of proteins helping the fusion process on the surface of both sperm and prostasomes was supported by the fact that they both needed to be treated with pronase to abolish the fusion. It thus appeared that both lipid and protein compositions were involved in the sperm-prostasomes interaction dynamics. The same group demonstrated that under the same conditions, the fusion of prostasomes with sperm triggered a decrease in sperm membrane fluidity, the extent of which was correlated to the prostasomes to sperm ratio (10). The fusion may have a role in the regulation of the intracellular calcium concentration (($Ca^{2+})_i$) and thus on capacitation and acrosome reaction, which will be developed below.

Even though these works strongly suggested a fusion step, no "clear-cut" demonstration was provided, the observed changes might be due to lipid exchanges without fusion. This kind of interaction was already proposed for protein acquisition by epididymal spermatozoa interacting with epididymosomes (as earlier reviewed (3)).

4.2. Modulation of sperm motility

Sperm motility is one of the parameters that may affect fertility. It is taken into account during the clinical examination of a sperm sample and may have a possible influence on the choice of the technique used in an assisted reproductive technology protocol. Sperm are kept quiescent in the reservoir of the *cauda epididymidis* and become highly motile in the female genital tract after ejaculation. The enhancement of sperm progressive motility was one of the first functions attributed to prostasomes (20), and confirmed by others (21-23). The mechanism of action, proposed by these different authors, was attributed to the modification of the Ca^{2+} concentration in the sperm microenvironment. This hypothesis was supported by the fact that the fusion of prostasomes with spermatozoa triggered a transient increase in their ($Ca^{2+})_i$ (24). This is also consistent with the description that prostasomes contain calcium inside their vesicular structure (25). However, no convincing data was published concerning the precise action mechanism at a molecular level until recently. In 2011, a study showed that prostasomes bring essential Ca^{2+} signaling actors related to sperm motility after progesterone induction (26). This work studied the Ca^{2+} signaling pathways occurring before and after fusion of sperm to prostasomes and showed that the transfer of an ADPR-cyclase (Adenosine DiPhosphoRibose), i.e., CD38, from prostasomes to the midpiece of human sperm was the starting point of cyclic-ADPR production and elicited a response to progesterone by initiating a Ca^{2+} signaling pathway. This acquisition was pH-dependent, with a higher efficiency at acidic pH (5.0. and 6.0.), supporting the possible prostasomes to sperm fusion in these conditions. The acquired Ca^{2+} signaling actors were determined by comparing non-fused sperm to prostasome-fused sperm, using western blots on determined targets. Prostasome-fused sperm were shown to be enriched in progesterone receptors, ryanodine receptors, V-ATPase A1 and SPCA1 (secretory pathway Ca^{2+} ATPase 1). All these proteins were acquired on the midpiece by spermatozoa, as shown by immunofluorescence. The acquisition of these new actors had great consequences on sperm motility as analyzed by Computer Assisted Sperm Analysis (CASA): the effect of progesterone on the percentage of motile sperm, the percentage of hyperactivated sperm and on several motility parameters was abolished when 8-Bromo-cADP-Ribose, an antagonist of the cADP receptors, was co-incubated, thus indicating that an ADPR-cyclase in prostasomes was transferred to spermatozoa, produced cADPR and stimulated sperm motility. Calcium fluxes in sperm are crucial for the regulation of essential processes that are capacitation and acrosome reaction (see below for details). Progesterone-induced calcium influx is characterized by a biphasic increase in ($Ca^{2+})_i$. Parks *et al.* found that prostasomes-sperm fusion is fundamental for the long-term sustained response and is mediated by

PR-cADPR-RyR, whereas the short response relies on the activation of CatSper channels (27-29) located on the principal piece, independent of prostasomes fusion.

Other necessary elements for intracellular calcium regulation have recently been shown to be brought to human spermatozoa by prostasomes (30). The plasma membrane Ca^{2+}-ATPase 4 (PMCA4), a major Ca^{2+} efflux pump in murine spermatozoa, is delivered to human sperm *in vitro* when co-incubated with prostasomes. PMCA4 is localized on the neck and midpiece of sperm, a region known to contain a high amount of lipid rafts (31) and confirmed by the fact that PMCA4 was detected in these rafts in the murine male gamete (32). PMCA4 in prostasomes is tightly associated to nitric oxide synthases (NOS), particularly endothelial NOS (eNOS) and neuronal NOS (nNOS), as shown by co-immunoprecipitation methods. The interactions between these proteins were shown to be stronger when $(Ca^{2+})_i$ was high, as during capacitation. It is thus proposed that, under conditions where high $(Ca^{2+})_i$ is present, PMCA4 could limit the action of NOSs and thus the production of peroxynitrite derivatives. *Pmca4$^{-/-}$* male mice are infertile due to asthenozoospermia, most likely related to the toxic effects of high peroxynitrite levels when NOSs are freely active as they are not sequestrated by PMCA4 (33, 34). The acquisition of PMCA4 by sperm during post-testicular maturational steps seems to be crucial for motility regulation and fertility, as it is a main regulator of $(Ca^{2+})_i$. This protein is transferred in the first place in the epididymis, as shown in mice (35), and then by prostasomes (30). In the proteomic study published recently (17), PMCA3 was detected in the lipid rafts isolated from prostasomes. However, among the 4 peptides related to PMCA4 identified in this work, 2 have 100% identity with sequences found in PMCA4. These overall results suggest a particular mechanism of transfer from vesicles to spermatozoa, involving lipid rafts, as it was previously demonstrated in the bovine epididymis (36).

4.3. Modulation of capacitation, acrosome reaction and fertilizing ability

The process of sperm capacitation was described in the early 1950's as a necessity for the male gametes to reside for a certain amount of time in the female genital tract in order to acquire the ability to fertilize an oocyte (37, 38). The molecular mechanisms underlying this process, although extensively studied since then, are still not completely understood (see (39) for a review). Cholesterol loss from the sperm plasma membrane was described as an early event in capacitating mammalian spermatozoa (40). Considering the lipid composition of prostasomes, and particularly their high content in cholesterol, it could be speculated that they may interfere with this process, which was demonstrated in 1997 (41), as pre-incubation of sperm with prostasomes inhibited progesterone-induced acrosome reaction, normally occurring in capacitated sperm only. This inhibition was not associated with fusion, a point that is not in accordance with the previously mentioned observations.

Capacitation is dependent on a cAMP intracellular signaling pathway triggered by membrane dynamics changes and also by bicarbonate and calcium entry in the spermatozoa. It was recently proposed that prostasomes may interfere with this pathway (42). Indeed, they were shown to inhibit the appearance of final markers of the capacitation step, i.e. phosphotyrosines, after 3h of incubation, confirming a previous study (43). Surprisingly, prostasomes have the ability to increase sperm intracellular cAMP (which may be related to their own content in cAMP) but this increase may not be related to the capacitation-triggered pathway, a point discussed later. The inhibition of tyrosine phosphorylation was in accordance with the decrease of the chlortetracycline staining profiles related to capacitated sperm and also with an increase of sperm membrane integrity measured by permeability to propidium iodide. Even though no molecular mechanism was shown to support these findings, this study brought new information on the influence of prostasomes on the kinetics of the capacitation process, showing that they had the ability to interact and probably exchange molecules (cholesterol being a very likely candidate) in capacitating conditions, which is in an alkaline environment and not at acidic pH. This observation is in accordance with the data reported by Cross and Mahasreshti (41) and mentioned above.

This clearly suggests that the sperm-prostasome interactions may be different depending on the environment, a fact highly supporting their role as protectors and as "reservoirs" for sperm cells. The other possibility is that different subpopulations of prostasomes, bearing different properties, may fuse at different time points during the sperm journey. Aalberts *et al.* recently showed in the stallion that a subpopulation of 60 nm purified prostasomes could bind to live sperm at a pH of 7.5 and higher, supporting this second hypothesis (44). This will however need to be confirmed in humans, as stallion semen is deposited directly in the female uterus and thus sperm do not encounter an acidic environment such as the vaginal pH. Also, they do not have to go through the cervical mucus, an event that could interfere with sperm-prostasomes interactions. However, different subpopulations of prostasomes have already been described in humans, with different lipid composition, and may thus have different interacting properties with spermatozoa (14). Others have also reported the existence of different prostasomes subpopulations with one having comparable size (around 60nm) to the stallion subpopulation (13, 45).

Capacitation primes spermatozoa in order to undergo the acrosome reaction (AR) after binding to the glycoprotein ZP3 of the oocyte's zona pellucida. Progesterone in the vicinity of the oocyte, released

by cumulus cells, also has the ability to trigger the AR. Cholesterol and prostasomes have long been identified as inhibitors of the progesterone-induced AR in humans (41, 46). However, several reports show that prostasome-to-sperm fusion could stimulate the progesterone-induced AR in humans (26, 47) and the spontaneous AR in pig (48). AR is also a Ca^{2+}-dependent process, these discrepancies may come from experimental design, as the stimulatory action of prostasomes were shown under acidic pH conditions, favoring fusion. Indeed, the transfer of the Ca^{2+}-signaling elements occurring at such pH (26) may positively influence progesterone-induced AR whereas it is the opposite at physiological pH because only cholesterol transfer occurs in these conditions. This is in accordance with the recent hypothesis concerning the kinetics of prostasome action proposed by Aalberts *et al.* (44): prostasomes could regulate sperm function by first binding to the male gametes, and then fusing. Thus, the observed effects would be highly dependent on the environment. In humans, early fusion events could occur in the vagina, with a favoring-acidic pH, but the roles of these putative early events are not clear. Prostasomes could then remain adsorbed on spermatozoa that would get through the cervical mucus and delay capacitation by bringing cholesterol to the sperm cells, maintaining their fertilizing ability. The stimulation of progesterone-induced AR could be the result of fusion in the intercellular spaces of the cumulus, allowing sperm acquisition of the Ca^{2+}-signaling actors necessary for late capacitation events and the AR. Although speculative at this point, this hypothesis seems to be very plausible (8, 49).

Prostasomes thus have an influence on fertility outcome. The Ca^{2+}-signaling actors acquired by sperm cells after fusion to prostasomes were shown to favor the fertilization rate in mice during *in vitro* fertilization studies, as non-fused sperm had a fertilization rate of about 30% compared to fused-sperm (26). In this study, the authors also demonstrated using intra-uterine insemination that female mice inseminated with prostasomes-fused sperm had around 10% of the oocytes recovered going to two-cell embryo whereas non-fused spermatozoa did not show any fertilization. However, it is clear that *in vitro* fertilization can be achieved with epididymis-retrieved spermatozoa that were never in contact with prostasomes. Prostasomes are not essential for fertilization but, *in vivo*, they seem to have several accessory roles that help the fertilization process by maintaining spermatozoa in a "steady-state" until they reach the site of fertilization in the upper female genital tract. Once again, mechanisms may differ between species based on the site of semen deposition.

5. INTERACTION BETWEEN PROSTASOMES AND OTHER CELLS

Among the different functions ascribed to prostasomes, interactions with other cell types have been shown, particularly with cells of the immune system, but also with bacterial pathogens. The roles of these interactions will be discussed in regards to the possible consequences on fertility.

5.1. Modulation of the female immune response

The first evidences of an interaction of prostasomes with immune cells were based on their activity as inhibitors of lympholiferation (50, 51). Prostasomes could also act on macrophages (51), inhibiting their ability to phagocytize latex particles and to produce reactive oxygen species (ROS) used to destroy the englobed material (52, 53). In these studies, the activity of the neutrophil NADPH-oxidase-producing ROS was decreased in association with a decrease in plasma membrane fluidity as shown using an electron spin resonance method. This suggested that cholesterol transfer could also be involved, likely in the lower female genital tract. Sperm carrying prostasomes in the female genital tract may thus have the ability to reduce their phagocytosis by resident macrophages or neutrophils of the mucus (54), favoring their survival and thus fertility.

Prostasomes contain several molecules with anti-complement activity: CD55 (Decay Accelerating Factor, inhibitor of the complement cascade), CD46 (Membrane Cofactor Protein, an inhibitory complement receptor) and CD59 (protectin, an inhibitor of the membrane attack complex). Prostasomes were shown to be able to transfer CD59 to spermatozoa *in vitro*, as well as to red blood cells or fibroblasts (55, 56). The active protein is transferred to red blood cells as it confers protection against complement mediated hemolysis (57). CD46 is a transmembrane protein which is also efficiently transferred to red blood cells, suggesting a complex molecular mechanism underlying the transfer as both GPI-anchored proteins (CD59) and transmembrane proteins (CD46) can be transferred (55). More recently, another immunomodulatory protein, galectin-3, has been detected in human prostasomes (58). The galectin-3 ligand, known as Mac-2 binding protein (M2BP), is also present on prostasomes and could also play a role in immune modulation by acting more specifically on monocytes and macrophages (59).

Altogether, these different immunomodulators, by interacting with different cell types, could favor sperm survival in the female genital tract and consequently have a positive effect on fertility outcome.

5.2. Other functions

Even though this is not the focus of this review, it is necessary to mention that prostasomes have other functions, such as an antibacterial activity (60) and an association with nucleic acids (61). The latter is quite interesting as the DNA carried is insensitive to enzymatic degradation, suggesting that it is contained inside the

prostasomal matrix (62). The DNA present was reported to be composed of random fragments coming from the entire genome and could be transferred to spermatozoa, probably by the previously proposed fusion mechanism occurring at acidic pH, but the physiological role of this transferred DNA still needs to be elucidated (63). Nucleic acid transfer to spermatozoa *via* exosome-like particles seems to have an important physiological role in the post-testicular maturation of the male gametes, as it was recently shown that epididymosomes also carry nucleic acids, i.e. miRNAs, throughout the organ, possibly as a communication tool between different parts of the organ and also to maturing sperm (64).

6. CONCLUSION

Prostasomes have the ability to modify spermatozoa after ejaculation, during their journey to the oocyte. Their mechanism(s) of action is (are) still very poorly understood but they seem to act as a sort of "reservoir" which can bring different properties to spermatozoa depending on the surrounding environment. Spermatozoa also encounter other types of extracellular microvesicles secreted first by the epididymis and those later in their journey by the female genital tract, topics that are covered in this issue. The interrelations between all these subgroups will certainly be interesting to investigate, even though very challenging on a technical side, but will bring new insights on the cellular aspects of gamete and reproductive biology. Lately, research on exosomes has gained intense interest due to the effectiveness of this new communication mode to modify the physiology of a target cell that can be physically very far from the exosome-secreting cell. Diagnostic and pharmaceutical applications will certainly emerge from this new knowledge and some may be related to post-testicular sperm maturation and fertility.

7. ACKNOWLEDGEMENTS

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