Perfringolysin O as a useful tool to study human sperm physiology

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Objective: To evaluate perfringolysin O, a cholesterol-dependent pore-forming cytolysin, as a tool to study several aspects of human sperm physiology.
Design: Prospective study.
Setting: Basic research laboratory.
Patient(s): Human semen samples with normal parameters obtained from healthy donors.
Intervention(s): Interaction of recombinant perfringolysin O with human spermatozoa.
Main Outcome Measure(s): Assessment of perfringolysin O binding to spermatozoa, tests for acrosome and plasma membrane integrity, and acrosomal exocytosis assays.
Result(s): Perfringolysin O associated with human spermatozoa at 4°C. The binding was sensitive to changes in cholesterol concentrations and distribution occurring in the plasma membrane of these cells during capacitation. When perfringolysin O–treated sperm were incubated at 37°C, the plasma membrane became permeable, whereas the acrosome membrane remained intact. Permeabilized spermatozoa were able to respond to exocytic stimuli. The process was inhibited by proteins that interfere with membrane fusion, indicating that large molecules, including antibodies, were able to permeate into the spermatozoa.
Conclusion(s): PFO is a useful probe to assess changes in the amount and distribution of the active sterol fraction present in the sperm plasma membrane. The toxin can be used for the efficient and selective permeabilization of this membrane, rendering a flexible experimental model suitable for studying molecular processes occurring in the sperm cytoplasm. (Fertil Steril® 2013;99:99–106. ©2013 by American Society for Reproductive Medicine.)
Key Words: Perfringolysin O, cholesterol, acrosome reaction, human sperm, permeabilization

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Spermatozoon, the male gamete, is a highly differentiated cell capable of finding the female gamete, sorting through all the physical barriers that protect this immobile cell, and fusing with the oocyte to generate a diploid zygote (1). To perform these complex tasks, the spermatozoon undergoes dramatic morphological and functional changes during spermiogenesis, resulting in a very specialized cell capable of carrying out a limited set of functions with high efficiency. Sperm are excellent model cells to dissect fundamental processes such as flagellum-mediated cell motility (2), chemotaxis (3–5), and regulated exocytosis (6, 7). Therefore, unveiling the molecular mechanisms underlying these cellular functions in sperm is of great interest not only to address specific problems in reproductive biology but also to understand key processes in cell biology.

In mammals, ejaculated sperm are unable to respond properly to stimuli coming from the oocyte. They have to undergo a complex capacitation process in the female reproductive tract to acquire special functions that are important for fertilization, such as hyperactivated motility and the ability to respond to acrosome reaction inducers (8). Several important changes occur at the sperm's plasma membrane during capacitation, such as hyperpolarization, opening of voltage-gated calcium channels, loss of transbilayer phospholipid asymmetry (9), and efflux of cholesterol (for a review, see Flesch and Gadella, [10]). Cholesterol contents decrease up to 40% in various mammalian sperm capacitated in vitro (10). Our

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laboratory demonstrated that cholesterol efflux facilitates the exocytosis of the acrosome by favoring Rab3A membrane association (11). The regulation of cholesterol levels is important for the function of these cells. Hypercholesterolemia decreases male factor fertility in animal models, altering several sperm parameters (12, 13). Dyslipidemia also has an impact on human male factor fertility (14–16).

Acrosomal exocytosis is fundamental for gamete interactions and fusion (1). Sperm have a single, flat, and large secretory granule surrounding the anterior part of the nucleus. Similar to exocytic events occurring in other secretory cells, the plasma membrane and the granule membrane fuse during the acrosome reaction to release the granule contents. However, acrosomal exocytosis is special in several aspects. Upon stimulation, multiple fusion pores between the outer acrosomal membrane and the plasma membrane form, connecting the interior of the acrosome with the extracellular medium. By a still not well characterized process, these fusion pores expand, causing the fenestration of both membranes and the release of hybrid vesicles composed of patches of plasma membrane and outer acrosomal membrane (17).

A drawback for experiments with spermatozoa is the limited, almost null, transcriptional and translational activity of these cells. As a result, protein overexpression and knockdown are difficult and require the generation of transgenic animals, which is experimentally demanding for most species and prohibitive for human sperm. Controlled plasma membrane permeabilization has been used to gain access to the membrane fusion machinery required for exocytosis in several secretory cells (18, 19). We and others have resorted to this strategy to study the molecular aspects of sperm physiology (20–22).

The aim of the present work was to characterize perfringolysin O (PFO) as a useful tool to study several aspects of sperm physiology. PFO is a pore-forming toxin secreted by *Clostridium perfringens* (23). It belongs to the family of cholesterol-dependent cytolysins (24). After binding to sterol-containing membranes via the C-terminal domain (25), PFO oligomerizes forming a large prepore complex composed of up to 50 monomers (26) and inserts a transmembrane β-barrel of approximately 25 nm in diameter (27). Our goal was to explore the possibility of using the cholesterol-binding and pore-forming properties of this protein to investigate changes in cholesterol availability during capacitation and to study the molecular mechanism of acrosomal exocytosis.

MATERIALS AND METHODS

Additional Materials and Methods information can be found in Supplemental Materials (available online).

Recombinant PFO

The plasmid pAH11 encoding His_6-tagged native PFO (28) was transformed into *Escherichia coli* strain BL21 (DE3) pLysS. Protein synthesis was induced 2.5 hours at 37°C with 1 mM isopropyl beta-D-1-thiogalactopyranoside (IPTG). Bacteria were harvested and lysed by sonication, and PFO was purified under native conditions by affinity chromatography on Ni-NTA-agarose. PFO preparations (about 1 μM) were divided in 15 μL aliquots and stored at −80°C in elution buffer (250 mM imidazole, 100 mM NaCl, 50 mM Tris-HCl, pH 8). Under these storage conditions, PFO was stable and fully active for at least 6 months.

Sperm

Human semen samples were provided by masturbation from 15 healthy volunteer donors. The informed consent signed by the donors and the protocol for semen sample handling were approved by the Ethics Committee of the School of Medicine, National University of Cuyo. At least 120 sperm samples were used to perform this work. After sample liquefaction (20–30 minutes at 37°C), highly motile sperm were recovered by swim-up separation for 1.5 hours in human tubal fluid media (HTF, as formulated by Irvine Scientific) at 37°C in an atmosphere of 5% CO_2/95% air. The sperm suspension was diluted with HTF to 7×10^6 sperm/mL and incubated under the same conditions for 2–3 hours. When capacitated sperm were required, HTF was supplemented with 5 mg/mL bovine serum albumin (BSA) during the swim-up and the postdilution incubation.

PFO Binding to Sperm Plasma Membrane

Capacitated and noncapacitated spermatozoa were washed twice, resuspended in ice-cold phosphate-buffered saline (PBS) containing 25 nM PFO, and incubated for 15 minutes at 4°C. After washing twice with PBS to remove the free toxin, the cells were either fixed for immunofluorescence assays or solubilized for Western blot analysis (details in Supplemental Materials).

Plasma Membrane Permeabilization with PFO

Capacitated spermatozoa were washed twice, resuspended in ice-cold PBS containing 25 nM PFO, and incubated for 15 minutes at 4°C. After washing twice with PBS to remove the unbound toxin, the cells were resuspended (7×10^6/mL) in ice-cold HB-EGTA buffer (250 mM sucrose, 0.5 mM EGTA, 20 mM Hepes-K, pH 7) containing (or not) 2 mM dithiothreitol (DTT). After incubation at 37°C for different times (15 minutes when not indicated otherwise), permeabilization was assessed using 0.1% eosin Y or 500 nM propidium iodide (both in HB-EGTA).

RESULTS

PFO Bound Differentially to Capacitated and Noncapacitated Sperm Cells

Sperm capacitation in mammals requires an efflux of cholesterol from the plasma membrane. We tested whether PFO was able to detect the difference in cholesterol content between noncapacitated and capacitated sperm. For this purpose, the pore-forming property of the toxin was inhibited by incubating spermatozoa with PFO at low temperature (4°C) in the absence of reducing agents. Under these conditions, the toxin associated with both capacitated and noncapacitated sperm as assessed by Western blot analysis. The binding was more efficient for noncapacitated sperm as would be expected owing to the higher cholesterol contents of these cells (Fig. 1A,