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Heterologous recombinant protein with decapacitating activity prevents and reverts cryodamage in ram sperm: An emerging biotechnological tool for cryobiology

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ABSTRACT

During the last decades fundamental and applied aspects of mammalian ram sperm cryopreservation have been increasingly explored by scientists and biotechnologists. Many works report modifications in the composition of the freezing extenders and explore the beneficial and detrimental effects of seminal plasma or seminal plasma components in cryopreservation. Seminal plasma is known to contain stabilizing proteins, thereby this is a good start point to study the maintenance of membrane stability based on the basic knowledge of sperm physiology. However, seminal plasma composition is variable among rams and also the introduction of exogenous seminal plasma or its fractions to commercial semen can be associated with the transmission of viral diseases. Our work shows that a mouse protein, called SPINK3 (Serine Protease Inhibitor Kazal type 3) with decapacitating activity interacts with heterologous ram sperm when it is produced as a recombinant molecule. By immunocytochemistry assays we demonstrate that this protein (naturally expressed by mouse seminal vesicle under androgenic control) binds to the apical portion of both fresh and frozen ram sperm, the same localization described in mouse homologous sperm. Furthermore, it significantly improves sperm progressive motility compared to non-treated samples when it is added to freezing extenders and to dilution media after thawing. On the contrary, addition of SPINK3 does not modify sperm viability. The percentage of sperm with intact acrosome after ionophore induction was also significantly higher in sperm frozen in the presence of SPINK3 compared to control samples and the addition of SPINK3 after thawing significantly reduced both induced and non induced acrosomal loss, indicating that heterologous SPINK3 might act as a calcium inhibitor transport as described in mouse. Based on our results SPINK3 may find a place as a desirable biotechnological tool to achieve a higher proportion of competent sperm to fertilize.

1. Introduction

The use of cryoprotectants as components of semen extenders has led to considerable improvements in the cryopreservation of spermatozoa. The development of cryopreservation protocols or their modifications

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generally arise by transfer from one species to another. However, spermatozoa of different species have specific cryobiological properties and varied degrees of sensitivity to experimental manipulation, cold shock (lipid phase transitions), freezing and osmotic tolerance (Holt, 2000). Moreover, high degrees of variability with respect to different individuals within a given species have been observed, and within a single individual among ejaculates.

On the other hand, the composition of the extenders is itself a variation factor when complex mixtures are added. This is the case of seminal plasma that has been reported to prevent/revert cold shock (Barrios et al., 2000; Perez-Pe et al., 2001, 2002; Barrios et al., 2005) and cryodamage (Maxwell et al., 1999; Bernardini et al., 2011; Ledesma et al., 2014) in ram sperm. However the results of the addition of seminal plasma to frozen/thawed sperm in order to improve the number of live and motile sperm are equivocal (Maxwell et al., 1999; Manjunath et al., 2002; Morrier et al., 2003; Cardozo et al., 2009; Leahy and de Graaf, 2012).

Recent research has focused to elucidate seminal plasma components responsible for the beneficial effect, specifically the proteins secreted by accessory glands (Desnoyers and Manjunath, 1992; Bergeron et al., 2005; Jobim et al., 2005; Maxwell et al., 2007; Dominguez et al., 2008; Bernardini et al., 2011). It is known that upon ejaculation, sperm is covered by proteins from the seminal plasma interacting with lipids and proteins from the plasma membrane, thus stabilizing the bilayer. Some of these proteins have been described to have decapacitating function (revised by Maxwell et al., 2007). Proteomics studies have demonstrated that there are many conserved proteins in seminal plasma of relevant domestic mammalian species that are related to sperm membrane stability (Druart et al., 2013). Two kinds of decapacitating proteins are secreted by accessory glands. One group called spermadhesins that includes BSPs (binder of sperm proteins) containing one or more fibronectin II domains. These proteins are known to bind to the sperm surface by interacting with the lipid bilayer thus stabilizing the membrane (Fernandez-Juan et al., 2006); Maxwell et al., 2007; Muino-Blanco et al., 2008). The other group is formed by proteins that interfere with signal molecules involved in the transduction pathway related to capacitation, such as SPINK3 (Serine Protease Inhibitor Kazal Type 3, also called “caltrin”) an inhibitor of calcium transport (Bi et al., 2009; Muratori et al., 2009; Zalazar et al., 2012). While the capacitation-like changes occurring during cryopreservation (cholesterol efflux, alteration in the protein composition of the surface, changes in the distribution of intramembranous particles and calcium influx) are influenced by changes in the proteins initially bound to the spermatozoa, the differences in the content of these decapacitating proteins among seminal plasmas might explain the differential cryotolerance of semen (Druart et al., 2013; Ledesma et al., 2015) In this sense, we hypothesized that a decapcitating protein from mouse may stabilize sperm membranes from other species and avoid/repair cryodamage. Moreover, the use of a recombinant protein with a known concentration may contribute to minimize extender variability. To the best of our knowledge, until now there is only a recent work (Holt et al., 2015) that evaluates the addition of a recombinant protein to the freezing extender and post thawing media, and the first to investigate the effect of an heterologous decapacitating recombinant protein.

The aim of this work was to study the ability of recombinant SPINK3 to prevent, minimize or repair the cryo-injuries of cryopreserved ram spermatozoa. For this purpose, the recombinant protein was added to the medium before sperm cryopreservation or once cells were thawed. Sperm populations after cryopreservation regarding to acrosomal membrane integrity, viability and motility were evaluated.

2. Materials and methods

2.1. Recombinant protein expression in Escherichia coli and purification

The cDNA encoding the SPINK3 mature form from *Mus musculus* (NCBI ID: NM 009258.5) was cloned into the pET-24b(+) (Novagen) expression vector. Overexpression of SPINK3 was performed in *E. coli* Rosetta cells (Novagen) and the recombinant protein was purified to apparent homogeneity by a HiTrap IMAC HP affinity chromatography as was described in a previous work (Assis et al., 2013). Purified recombinant protein was dialyzed against phosphate buffer saline (PBS, 10 mM phosphate buffer, 137 mM NaCl and 2.7 mM KCl pH 7.4).

2.2. Samples collection and freezing-thawing procedure

Sperm was collected from 6 mature (>2.5 years) Texel rams during the autumn by artificial vagina method (Marco-Jimenez et al., 2008). Males were maintained with an abstinence period of 48 h as stated by Ollero et al. (1996).

Two independent ejaculates with good wave motion (4; range 0–5), >60% total motile sperm and $>1 \times 10^9$ sperm/mL were pooled (3 independent pools were obtained). Samples were maintained at 37 °C and diluted (150×10^6 cells/mL) in one step with a TRIS–glucose–citric acid extender containing 300 mM Tris, 37.7 mM citric acid, 94.7 mM glucose, 20% (v/v) egg yolk, 5% (v/v) glycerol, sodium G penicillin (1×10^6 IU/L) and streptomycin sulfate (1 g/l). Pellets were cryopreserved according to each experimental design (see below). The extender volume was adjusted in such a way as to ensure that the addition of SPINK3 does not modify the final concentrations of the extender components. Briefly, diluted semen was cooled to 5–8 °C over 2 h, and then held at that temperature for another 2 h. Drops of 150 μl of diluted semen were dispensed in concavities of dry ice. After 10 min, the frozen pellets were plunged in liquid N_2 and stored until thawing (Bernardini et al., 2011).

Frozen sperm pellets were thawed in PBS in a water bath (37 °C) for 2 min. A motile sperm population was selected by a glass wool filtration and diluted to 10×10^6 cell/mL to avoid a possible seminal plasma effect.

2.3. Immunodetection of SPINK3 in ram sperm

Washed fresh or cryopreserved sperm were diluted to 5×10^6 cells/mL and incubated with 13.7 μM recombinant