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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

SPINK3 for 1 h at 37 °C), fixed with 4% paraformaldehyde for 30 min. Excess of fixative was removed by washing with PBS by centrifugation (850 × *g*, 15 min). Immunoreaction was conducted according to reported protocols (Irwin et al., 1983). Briefly, sperm were smeared and dehydrated with absolute ethanol for 20 min, washed with PBS and blocked with 3% BSA and 0.1% Tween 20 in PBS for 30 min. Slides were incubated with a 1:50 dilution of anti -SPINK1 (Sigma, HPA027498) for 2 h at 37 °C, washed and incubated with a 1:200 dilution of anti-rabbit IgG FITC-conjugated (Sigma, F0382) for other 2 h. All incubations were done at room temperature in a humid chamber. After thoroughly washing with PBS, slides were mounted with PBS: glycerol (1:9). Bright field and fluorescence (480/525 nm), were observed and recorded with a Nikon Ds-Fi1 camera connected to a PC with analytic software (Nikon NIS Elements F) attached to an inverted microscope: Nikon Eclipse E200.

2.4. Experimental design 1. Analysis of SPINK3 effect to prevent cryo-damage

Before sperm had been frozen, an aliquot of the fresh ejaculate was incubated with sperm in a final concentration of 13.7 μM SPINK3. The cell population interacting with the protein was stored as described in the previous section. After 15 min post-incubation with SPINK3 an aliquot of treatment and control samples were taken and selected by a discontinuous Percoll gradient in PBS (90–45%). The pellet was collected, suspended in PBS at 10×10^6 sperm/mL and different assays (viability, motility and spontaneous acrosome reaction) were performed to ensure the innocuous effect of SPINK3 over the fresh spermatozoa.

Once thawed, and sperm selection performed, the effect of recombinant SPINK3 added to sperm before cryopreservation was analyzed by evaluating different physiological parameters (*spermatic parameters analyzed*).

2.5. Experiment design 2. Analysis of SPINK3 effect to minimized or repairing cryo-damage

Frozen semen samples without SPINK3 were thawed as describe above. Selected motile sperm suspension was incubated with SPINK3. A control without SPINK3 was performed simultaneously. Effects of the recombinant protein over post-thawed sperm were also evaluated throughout time by the parameters described below (*spermatic parameters analyzed*).

2.6. Spermatic parameters analyzed

2.6.1. Acrosome membrane integrity

Thawed selected spermatozoa were split and two sets of parameters were evaluated on each sample. On one set, the acrosomal damage caused by freezing/thawing was monitored at different times (0, 15, 30 and 45 min) after incubation of spermatozoa in PBS containing or not 13.7 μM SPINK3, according to each experimental design. Simultaneously on another set, the acrosomal loss induced by the addition of a calcium ionophore (A23187, Sigma C7522) was assessed. After a 15 min incubation period of spermatozoa in PBS with or without 13.7 μM SPINK3, 15 μM A23187 was added and aliquots were taken at different times after ionophore addition (0, 15, 30, 60 and 120 min). As control, the spermatozoa that were not induced with A23187 contained an equal proportion of DMSO. In both cases, cells were fixed with 4% paraformaldehyde solution in PBS for 1 h and washed with 0.1 M ammonium acetate buffer pH 9 (700 × *g* for 10 min). The acrosomal status was evaluated according to the Coomassie Brilliant Blue (CBB) G-250 staining technique (Bendahmane et al., 2002). Briefly, sperm were placed and smeared onto glass slides and stained with a 0.22% CBB G-250 solution prepared in 50% methanol and 10% acetic acid. The slides were washed with distilled water, dried, mounted in glycerol: PBS (9:1), and observed under a light microscope at 1000× magnification. A blue stain over the dorsal region of the sperm head was visualized in spermatozoa with intact acrosome, whereas no stain or a discontinuous pattern stain was observed in spermatozoa with reacted acrosome. Spermatozoa with intact acrosome was quantified as a percentage over 400 sperm cells per replicate ($n = 3$).

2.6.2. Subjective motility

Once sperm were thawed, they were incubated with or without 13.7 μM SPINK3 for 45 min in PBS containing 1.5 mg/mL fructose. An aliquot of each sample (at 0, 15 and 45 min) was placed onto slides and observed under a light microscope (400×) on a warm surface (37 °C). Motility was recorded and then each individually sperm path was evaluated. At least 200–300 cells per replicate (five to eight fields) were counted, followed and classified individually according to the World Health Organization (WHO) manual criteria as follows:

Pattern A (fast progressive): spermatozoa that swim forward fast on a straight line.
Pattern B (slow progressive): spermatozoa that swim forward slowly on a straight line.
Pattern C (non-progressive): spermatozoa with moving tails, either they do not change their location (local motility only) or move on a curved/crooked line (non-linear motility).
Pattern D (immotile): spermatozoa that do not move in the slightest.

2.6.3. Viability

Viability was evaluated by a modified eosin–nigrosine staining technique (Mortimer, 1994). Briefly, an aliquot of each treatment was mixed during 10 s with a same volume of eosin–nigrosine solution (Campbell et al., 1956) and then smeared onto a pre-warmed microscope slide and air-dried. At least 400 cells were evaluated per slide using light microscopy (400×). Viability was analyzed after freezing/thawing under the different treatments at 0, 15, 30 and 45 min of incubation.

2.7. Data analysis

Experimental data obtained from each ram sperm sample (a pool of two ejaculates from independent animals per replicate) were studied repeatedly through time

(0, 15, 30 and 45 min) by general linear mixed-effect models (GLMM). This analysis is particularly useful when data are not independent (Pinheiro and Bates, 2000). Therefore, mixed-effect models were used in order to include sperm sample as random effect and thus accounting for within-sample correlation in all models (Littell et al., 2000).

Since data were expressed as percentages (proportions), models had a binomial distribution. Information-Theoretic procedures were performed to get the best model, computing AICc (Akaike Information Criterion for small samples) (Johnson and Omland, 2004; Symonds and Moussalli, 2011). A 95% confidence interval was also calculated for parameters estimated in each best model. GLMM analysis was performed by Open Access Software R (RCoreTeam, 2011), with the "nlme" package (Pinheiro et al., 2013).

3. Results

Interaction between SPINK3 and fresh or cryopreserved spermatozoa was confirmed by an immunocytochemistry assay (Fig. 1). Localization of SPINK3 was detected on the acrosomal region, similar to the reported localization of this protein in mouse sperm (Zalazar et al., 2012).

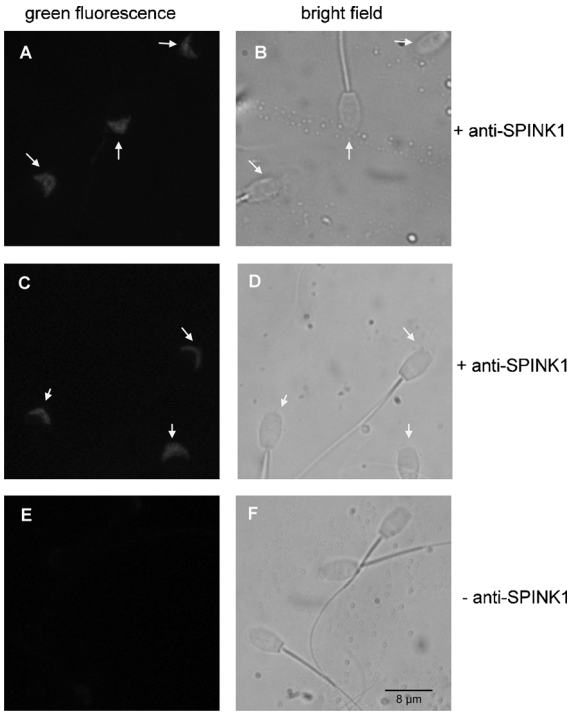

Fig. 1. Binding of recombinant heterologous SPINK3 to acrosomal region of ram sperm. Fresh (A, B) or cryopreserved (C, F) sperm were selected as described in M&M and incubated with the recombinant protein SPINK3 and fixed. After washing, sperm were incubated with (A–D) or without (E, F) anti-SPINK1 antibody and developed by anti-rabbit IgG FITC-conjugated. The fluorescent images (A, C and E) show the specific binding of SPINK3 to the cells distributed on the dorsal surface of the head of sperm cells (arrows). Bright field images are shown at the right (B, D and F). Slides were examined with a Nikon fluorescent microscope under magnification = 1000×. A representative picture out of three experiments is shown. Bar = 8 μm.

Before freezing, it was essential to know about sample quality and whether the dose selected of SPINK3 had a harmful effect over ram sperm. Thus, cell viability was tested and no significant differences were detected between SPINK3 treatment and the control (without SPINK3) after 15 min of incubation with fresh sperm (control: 74.59% ± 5.17; SPINK3: 76.34% ± 13.21). Similar results were observed when acrosome membrane integrity (control: 93.94% ± 2.62; SPINK3: 91.93% ± 5.25), total motility (control: 70.83% ± 13.57; SPINK3: 72.50% ± 12.55) and progressive motility (control = SPINK3: 87.50% ± 5.00) were evaluated (Supp. Table 1). Indeed, these data confirmed that the protein do not adversely affect the fresh sperm parameters studied.

3.1. Analysis of SPINK3 effect to prevent cryo-injuries

Evaluation of selected sperm at time zero after thawing showed that sperm (previously frozen in the presence of SPINK3) presented a significantly greater percentage of fast progressive motile cells than the control (without protein) (Fig. 2A, Supp. Table 2). In addition, a lower percentage of immotile sperm than the control was observed (Fig. 2D, Supp. Table 2). Neither slow progressive nor non-progressive percentages were altered by the addition of SPINK3 (Fig. 2B and C, Supp. Table 2). In this way, results demonstrate that freezing sperm in the presence of SPINK3 improves the fast progressive motile sperm percentage reducing the number of immotile cells at time zero of thawing. Nevertheless, no significant improvement was observed by SPINK3 addition when viable cells or acrosome status were analyzed (Fig. 3, Supp. Table 2).

Thereafter, we studied whether this protective effect of SPINK3 observed at time zero of thawing would be maintained or further improve the motility and membrane stability throughout time after thawing. Treatment with SPINK3 maintained the percentage of fast progressive sperm higher than control (Fig. 2A, Supp. Tables 3 and 4) at each time analyzed. It also decreased slow progressive and immotile percentages respect to control (Fig. 2B and D, respectively, Supp. Tables 3 and 4). The percentage of non-progressive sperm with SPINK3 treatment was lower than the control throughout the time studied (Fig. 2C, Supp. Table 3). Surprisingly, when the acrosomal integrity was evaluated, in the presence of A23187, SPINK3 improved the percentage of intact acrosomes throughout the time compared with the control. Nevertheless, there was not detected an improvement without ionophore (Fig. 3, Supp. Tables 3 and 4). Moreover, no differences between control and treatment were observed in the percentages of viable cells (Supp. Table 3).

3.2. Analysis of SPINK3 effect to minimize or repair cryo-injuries

SPINK3 added to sperm once thawed, lead to a significant improvement in the mean percentage of fast progressive motile sperm throughout time (Fig. 4A, Supp.

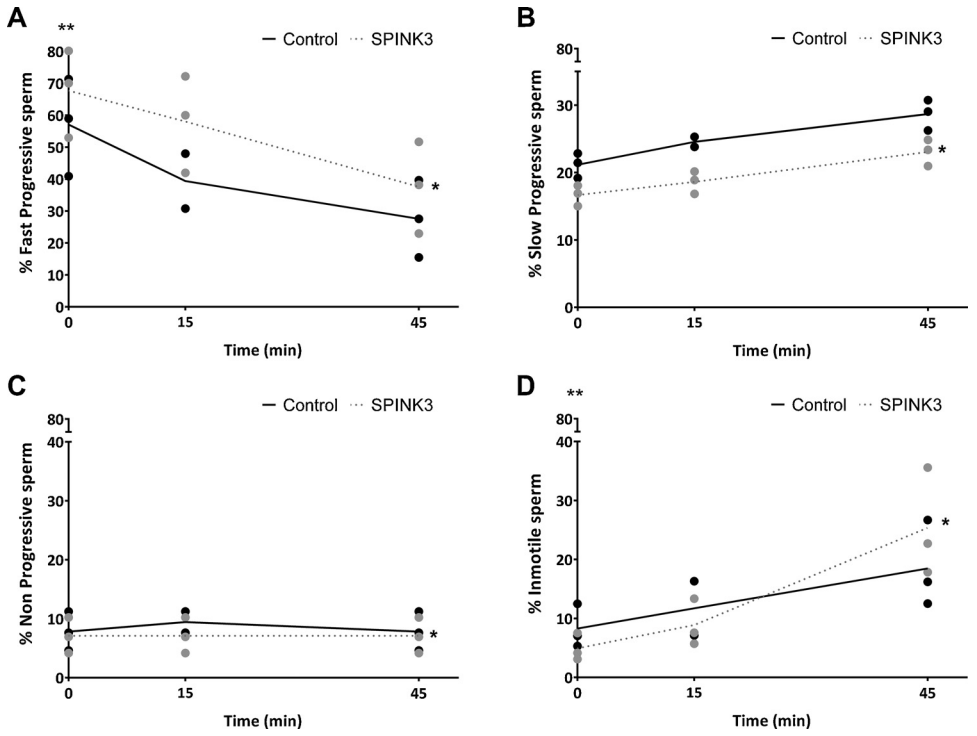

Fig. 2. Post-thawing motility patterns analysis of sperm frozen in the presence of SPINK3. Each graph represents the percentage of spermatozoa with a certain motility pattern (A, B, C, D) over total cells analyzed ($n = 400$, 5–8 different fields analyzed per sample) at 0, 15 and 45 min after thawing followed by sperm glass-wool selection. Lines represent the mean percentage estimated from GLMM analysis for each treatment. Asterisk (*) represents significant differences compared to the control (Supp. Tables 3 and 4). Double asterisk (**) represents significant differences analyzed for a particular time (Supp. Table 2). Black dots: control samples. Grey dots: SPINK3 treatment.

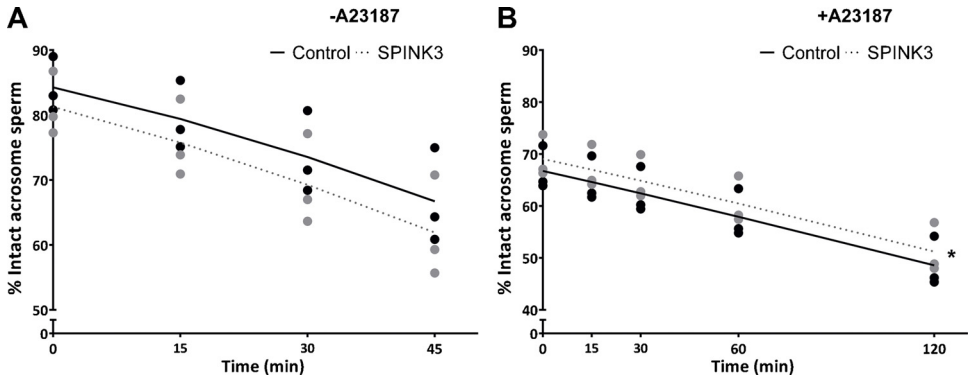

Fig. 3. Effect of SPINK3 addition to sperm before freezing on spontaneous (A) or A23187-ionophore induced acrosome loss (B). Each graph represents the percentage of spermatozoa with intact acrosome detected ($n = 400$) throughout time. Evaluation was begun once sperm was thawed, selected (T0) and incubated with or without A23187-ionophore. Acrosomal status was evaluated according to the Coomassie Brilliant Blue G-250 staining technique. Each line represents the mean percentage estimated from the GLMM analyses. Asterisk (*) represents significant improvement compared to the control (Supp. Tables 3 and 4). Black dots: Control samples. Grey dots: SPINK3 treatment.

Tables 3 and 4). Moreover, the percentage of immotile cells treated with SPINK3 was significantly lower than control ones in the same times evaluated (Fig. 4D, Supp. Tables 3 and 4). As expected, and similarly to the analysis of SPINK3 added prior to freezing also showed, total sperm motility decreased in both treatment and control throughout time (Fig. 4, Supp. Table 3). Since GLMM analyses of slow progressive and non-progressive motile sperm only included the explanatory variable time, no differences between control and SPINK3 treatment were found (Fig. 4B and C, Supp. Table 3).

Post-thawed sperm treated with SPINK3 had a higher percentage of intact acrosomes through time compared to control in both spontaneous and A23187-ionophore-induced acrosome loss experiments (Fig. 5A and B, Supp. Tables 3 and 4). Again, no differences were observed in the percentage of viable cells between control and treatment (Supp. Table 3).

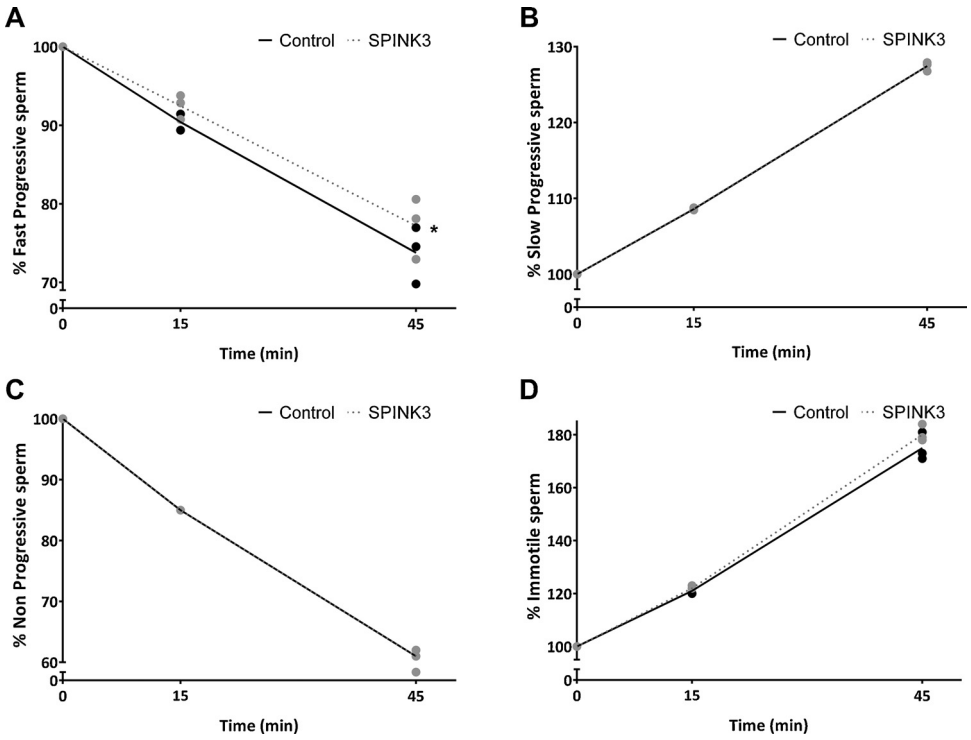

Fig. 4. Motility patterns analysis of sperm incubated with SPINK3 after thawing. Each graph represents the percentage of spermatozoa with a certain motility pattern (A–D) over the total cells analyzed ($n = 400$, 5–8 different fields analyzed per sample) at 0, 15 and 45 min sperm post-thawing and selection by glass-wool. Lines represent the mean percentage estimated from the GLMM analysis for each treatment asterisk (*) represents significant differences compared to the control (Supp. Tables 3 and 4). Black dots: control samples (without protein). Grey dots: SPINK3 treatment.

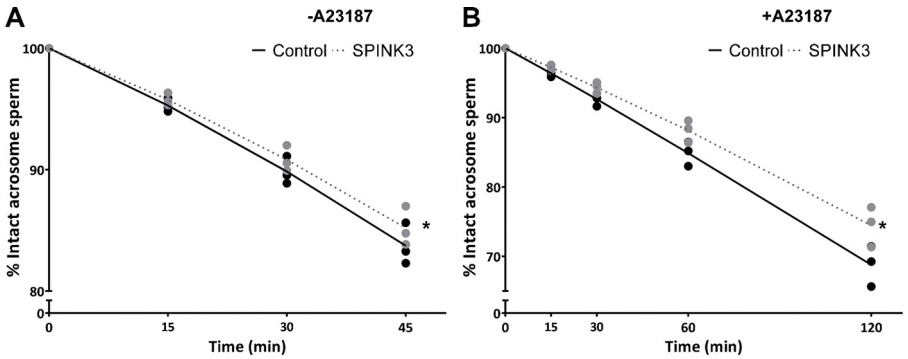

Fig. 5. Acrosomal membrane status of sperm incubated with SPINK3 after thawing. Evaluation of spontaneous (A) or A23187-ionophore induced (B) acrosome loss was performed according to the Coomassie Brilliant Blue G-250 staining technique. Each graph represents the percentage of spermatozoa with intact acrosome detected ($n = 400$) throughout time. Each line represents the mean percentage estimated from the GLMM analyses. Asterisk (*) represents significant differences compared to the control (Supp. Tables 3 and 4). Black dots: control samples (without protein). Grey dots: SPINK3 treatment.

In this way, SPINK3 was able to improve significantly acrosome membrane stability as well as motility patterns from cryopreserved sperm through the time evaluated just as results indicate.

4. Discussion

In the present study, we evaluated the potential benefits of a heterologous decapacitating recombinant protein (SPINK3) for the cryopreservation of ejaculated ram spermatozoa. We evaluated both the ability of the protein to "prevent" and to "reverse" cryodamage using a dose comparable to the one previously reported in mouse and guinea pig sperm (Dematteis et al., 2008).

Under experimental conditions, the addition of this recombinant protein to the freezing extender improved sperm progressive motility, upon thawing and further selection. The differences in the sperm motility due to the improved initial stage were maintained along the evaluated period of time (45 min) and the percentage of sperm with intact acrosome was enhanced under the effect of a calcium ionophore (A23187). On the other side, SPINK3

added after thawing enhanced both sperm motility and acrosomal integrity along the time compared to non-treated samples.

The addition of a recombinant heterologous protein with decapacitating activity to sperm has no precedents, however it has been largely demonstrated that the addition of seminal plasma proteins before or after cold shock prevents and reverts membrane damage in ram sperm (revised by Muino-Blanco et al., 2008). In addition, seminal plasma added after thawing was probed to improve ram sperm parameters (Maxwell et al., 1999; Dominguez et al., 2008). Although the composition of seminal plasma is variable among individuals and among species, it is consensus that it includes decapacitating proteins secreted by accessory glands that are key modulators of sperm functionality (Luna et al., 2015, Fernandez-Juan et al., 2006, Maxwell et al., 2007, Muino-Blanco et al., 2008). Despite their differences in primary sequence, orthologous decapacitating proteins are found in all mammalian seminal plasma that share origin and function. It was shown in recent works that the orthologous of SPINK3 was detected in ram seminal plasma by a proteomic approach (Soleilhavoup et al., 2014) and that this protein is more abundant in rams with high freezing resilient spermatozoa (Rickard et al., 2015). These reports support our results that increasing the endogenous SPINK3-like content by an exogenous addition, might stabilize sperm plasma membrane through SPINK3-surface interaction. As a consequence of this stabilization, it is possible to explain why the percentage of fast motile sperm increase and the acrosomal status of the sperm population is improved.

The capacitation-like changes occurring during cryopreservation are clearly not true capacitation. However, the role of seminal plasma in the stabilization of sperm membranes has been focused on the effect of decapacitating proteins initially bound to the spermatozoa (Maxwell et al., 2007; Bernardini et al., 2011; Ledesma et al., 2014, Luna et al., 2015). In fact, in this study we demonstrated that exogenous SPINK3 also binds to the ram sperm head in the same region as in mouse sperm, supporting the idea that this heterologous protein would have a conserved function and hence, stabilize ram sperm membranes.

Despite of the above mentioned evidences for the presence of the endogenous SPINK3-like protein in ram seminal plasma, there are no reports about its physiological role in this specie. In mice, SPINK3 (Serine Protease Inhibitor Kazal Type 3) is a multifunctional protein secreted under androgenic control by the seminal vesicle that binds to the spermatozoa apical portion of the head (Chen et al., 1998; Luo et al., 2004). The ligands to which SPINK3 attaches are still unknown but we have preliminary evidence that suggest that there are proteic and lipidic targets of sperm head for this protein (non published results). Upon attachment, SPINK3 modulates the calcium increase and downregulates the signal transduction pathway leading to capacitation (Zalazar et al., 2012) until it detaches from the sperm in the uterus (Ou et al., 2012).

SPINK3 effectively regulated the cell response to a calcium ionophore, as previously demonstrated in the mouse for this protein and for a similar molecule named SPINKL (Lin et al., 2008; Zalazar et al., 2012). These results suggest that in ram spermatozoa, recombinant SPINK3 might act by reducing sperm permeability to Ca^{+2}, a requisite to undergo acrosome reaction induced by A23187. Conversely, when added after thawing, SPINK3 was able to control both induced and non-induced acrosomal loss, probably due to the absence of seminal plasma and extender that might allow a different stoichiometric interaction between SPINK3 and surface ligands resulting on a membrane protective effect.

The dose selected of SPINK3 for these experiments had no beneficial nor a harmful effect over ram sperm viability and motility. This is not surprising whether we accept that SPINK3 regulates calcium permeability that is directly linked to acrosome reaction and motility but not to cell viability. Moreover, our previous experimental work evidenced that this amount of recombinant SPINK3 was capable to change tyrosine phosphorylation pattern of mouse sperm protein, one of the first molecular signals of capacitation in response to calcium influx (Zalazar et al., 2012).

In spite of the recognized decapacitating effect of the homologous seminal plasma in cryopreservation (Okazaki and Shimada, 2012) that is known to content endogenous SPINK3 protein (Rickard et al., 2015, Soleilhavoup et al., 2014), it has to be considered that dilution of semen in the freezing extender and the membrane destabilization during freezing/thawing reduces the concentration of decapacitating proteins. Therefore, attenuating any positive effect it may be having on sperm membranes. In this regard, the advantages of the addition of a recombinant protein in the extender or after thawing is not only to reinforce the concentration of decapacitating proteins of the seminal plasma but also it has an added value: the concentration of the peptide in the media is controlled and constant. Furthermore, the exogenous peptide does not compete with endogenous molecules and its application almost reduces the risks of transmission of viral diseases that is associated with the use of seminal plasma from infected donor rams.

It is worth mentioning, that the use of a recombinant heterologous decapacitating protein in freezing/thawing processes is a novel approach in the direction of generating defined media for semen handling.

Most of the efforts being made to improve cryopreserved ram semen quality are focused on cryoprotectants and/or on the elimination or addition of seminal plasma fractions, without enough satisfied results. Remarkably, our findings demonstrate that a recombinant heterologous protein is able to protect, prevent and/or diminish some of the deleterious effects produced over ram sperm when it is frozen/thawed. It is promising that the combined addition of this molecule in the extender and after thawing could be used complementing formulations presently used.

Further studies to evaluate the freezing ability of the treated semen are required to gain insight on the improvement effects of this recombinant protein over sperm. SPINK3 would find a place as a virtuous biotechnological tool to achieve a higher proportion of competent sperm and thus, higher potentially rates of fertilization when cryopreserved ram sperm is used for insemination practice. If

we contribute to this insight, the goal of the present study will be accomplished.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.anireprosci.2015.11.007.

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