REPRODUCTION
RESEARCH

# SPINK3 modulates mouse sperm physiology through the reduction of nitric oxide level independently of its trypsin inhibitory activity

L Zalazar, T E Saez Lancellotti[1], M Clementi[1], C Lombardo, L Lamattina, R De Castro, M W Fornés[1] and A Cesari

*Instituto de Investigaciones Biológicas (IIB), Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, CCT–Mar del Plata, CONICET, Funes 3250, 4th Floor, Mar del Plata 7600, Argentina and [1]Laboratorio de Investigaciones Andrológicas de Mendoza (LIAM, IHEM–CONICET), Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, CCT–Mendoza, CONICET, Mendoza, Argentina*

*Correspondence should be addressed to A Cesari; Email: acesari@mdp.edu.ar*

M W Fornés and A Cesari contributed equally to this work

## Abstract

**Serine protease inhibitor Kazal-type (SPINK3)/P12/PSTI-II is a small secretory protein from mouse seminal vesicle which contains a KAZAL domain and shows calcium ($Ca^{2+}$)-transport inhibitory (caltrin) activity. This molecule was obtained as a recombinant protein and its effect on capacitated sperm cells was examined. SPINK3 inhibited trypsin activity *in vitro* while the fusion protein GST-SPINK3 had no effect on this enzyme activity. The inactive GST-SPINK3 significantly reduced the percentage of spermatozoa positively stained for nitric oxide (NO) with the specific probe DAF-FM DA and NO concentration measured by Griess method in capacitated mouse sperm; the same effect was observed when sperm were capacitated under low $Ca^{2+}$ concentration, using either intracellular (BAPTA-AM) or extracellular $Ca^{2+}$ (EDTA) chelators. The percentage of sperm showing spontaneous and progesterone-induced acrosomal reaction was significantly lower in the presence of GST-SPINK3 compared to untreated capacitated spermatozoa. Interestingly, this decrease was overcome by the exogenous addition of the NO donors, sodium nitroprusside (SNP), and *S*-nitrosoglutathione (GSNO). Phosphorylation of sperm proteins in tyrosine residues was partially affected by GST-SPINK3, however, only GSNO was able to reverse this effect. Sperm progressive motility was not significantly diminished by GST-SPINK3 or BAPTA-AM but enhanced by the addition of SNP. This is the first report that demonstrates that SPINK3 modulates sperm physiology through a downstream reduction of endogenous NO concentration and independently of SPINK3 trypsin inhibitory activity.**

*Reproduction* (2012) **143** 281–295

## Introduction

In mammals, regulation of sperm physiology is mostly limited to posttranslational mechanisms controlled by signal transduction, since the transcription/translation level is restricted (Shima *et al*. 2004). Upon ejaculation, sperm are first capacitated and then they exhibit hyperactivated motility and undergo a physiological acrosome reaction (AR; Yanagimachi 1994). Capacitation can be described as an event naturally occurring within the female duct, associated with an increase in plasma membrane fluidity due to the efflux of cholesterol, removal of surface attached proteins, and redistribution of membrane phospholipids (Patrat *et al*. 2000). These changes in its membrane structure induce calcium ($Ca^{2+}$) influx, increases in intracellular pH (Phillips & Bedford 1988), intracellular cAMP concentration ([cAMP]i), and protein phosphorylation, as well as the production of reactive oxygen species such as nitric oxide (NO; Visconti *et al*. 2002, Aitken & Baker 2004, Harrison & Gadella 2005, O'Flaherty *et al*. 2006). The above-mentioned molecules are part of sperm signaling cascades involving numerous interconnected and also some redundant pathways which ensure acquisition of sperm fertilizing ability (Muratori *et al*. 2011).

Autocrine production of NO by sperm from the beginning of capacitation controls the increase in cAMP/cGMP and most of the known downstream serine, threonine, and tyrosine phosphorylation events, as well as tyrosine nitration of proteins; however high NO concentration blocks all sperm functions (Machado-Oliveira *et al*. 2008, de Lamirande *et al*. 2009, Roessner *et al*. 2010). NO is synthesized in mature sperm by the NO synthase (NOS), which exists as three


ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-11-0107
Online version via www.reproduction-online.org

isoforms – neural NOS (nNOS), endothelial NOS (eNOS), and inducible NOS – and localize in sperm head and midpiece (Meiser & Schulz 2003). The constitutively expressed nNOS and eNOS isoforms are $Ca^{2+}$/calmodulin (CaM) dependent (Herrero & Gagnon 2001). Thus, considering the activity of these isoforms, the time-related intracellular $Ca^{2+}$ increase occurring during capacitation should be necessary to induce NO production (de Lamirande *et al*. 2009, Muratori *et al*. 2011). The mechanisms involved in $Ca^{2+}$ regulation during capacitation, despite poorly known, are believed to be related to inhibition of $Ca^{2+}$ ATPase, increase in membrane permeability and $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores (Pons-Rejraji *et al*. 2009). Before capacitation, this $Ca^{2+}$ entry should be impaired.

A well-known $Ca^{2+}$-transport inhibitor I (caltrin; Chen *et al*. 1998, Luo *et al*. 2004), also known as P12 and pancreatic secretory trypsin inhibitor II, is a secretory serine protease inhibitor Kazal-type (SPINK3, NCBI ID: NP_033284.1) that is constitutively expressed in pancreas and its expression is androgen dependent in seminal vesicle and prostate of mice, rat and human (Mills *et al*. 1987, Novella *et al*. 1999). This protein, as other secretory proteins from the accessory glands, attaches to the sperm surface during sperm transit along the male duct and consequently, it is found on the surface of ejaculated sperm (Dematteis *et al*. 2008). Caltrin-like proteins are known to modulate $Ca^{2+}$ influx (Clark *et al*. 1993, Coronel *et al*. 1993). However, the physiological role, the mechanism by which they prevent intracellular $Ca^{2+}$ increase and the signaling pathways that they modulate are still unknown. In addition, although some of them have trypsin inhibitor activity (Lardy 2003), whether their caltrin function is related to its trypsin inhibitory activity remains to be demonstrated. Therefore, our first aim was to study the possible interaction between SPINK3 $Ca^{2+}$ uptake inhibition and the levels of NO in mouse sperm during capacitation. We tested whether trypsin inhibitor activity of SPINK3 was necessary to modulate sperm physiology. Finally, we determined the effect of SPINK3 in the presence or absence of NO donors on sperm AR, tyrosine phosphorylation, and motility.

## Results

### *Recombinant expression of active SPINK3*

To explore the role of SPINK3 in sperm physiology mouse recombinant SPINK3 was synthesized in *Escherichia coli* (*E. coli*) cells. The coding sequence of mouse mature SPINK3 (57 aminoacids corresponding to the C-terminus of the protein) was synthesized by RT-PCR from total mRNA of seminal vesicle (Fig. 1A) and this cDNA was cloned into the expression vector (pGEX-4T-3) as a fusion protein at the C-terminal region of glutathione *S*-transferase (GST) downstream a thrombin cleavage site (see Materials and Methods for details). Two protein bands were differentially expressed in *E. coli* cells harboring pGEX-4T-3-*spink3* after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 h which were enriched in the soluble fraction. The upper band showed the expected molecular size for GST-SPINK3 fusion protein (34 kDa; Fig. 1, lane 6). After purification (Fig. 1, lane 7), only the 34 kDa polypeptide was recovered which cross-reacted with anti-SPINK1 antibodies raised against the product of the human orthologue gene (Fig. 1, lane 8). This result suggests that the lower band may correspond to a degradation product of the recombinant protein unable to bind to the column. To obtain SPINK3, the GST-SPINK3 fusion protein was digested with thrombin (Fig. 1B, lanes 1 and 2) and purified as indicated in Materials and Methods (Fig. 1B, lane 3). Anti-SPINK1 cross-reacted with both the 34 and 8 kDa protein species obtained after thrombin cleavage (Fig. 1B, lane 2, Western blot) showing that the digestion was not exhaustive and confirming the identity of the recombinant proteins, GST-SPINK3 and SPINK3.

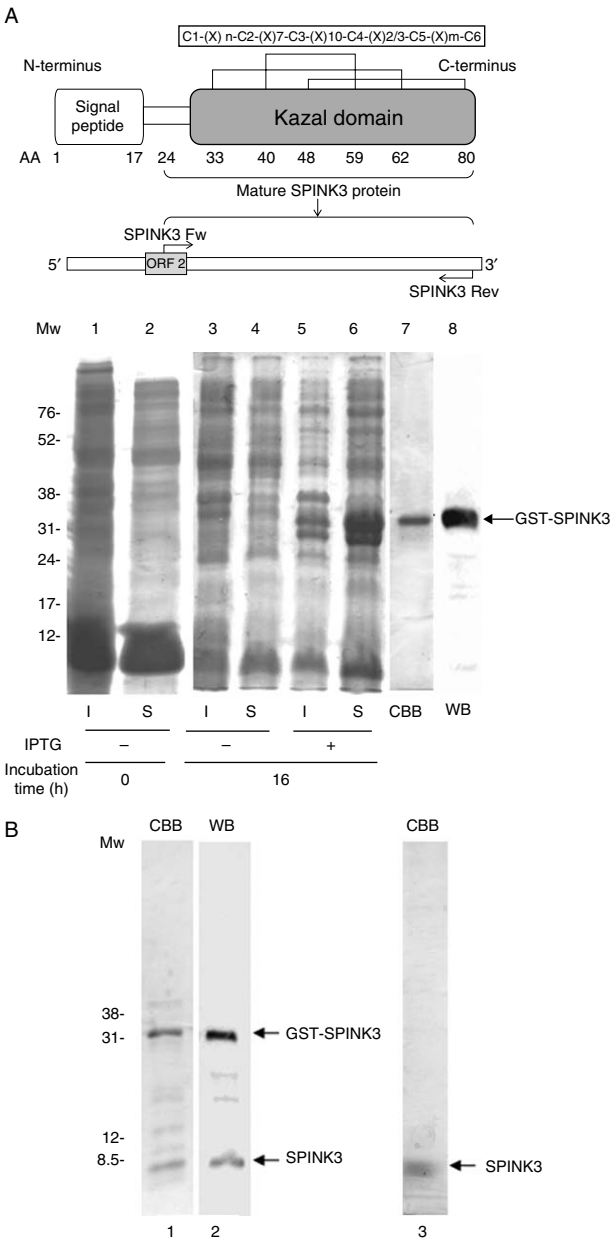
To assess whether the purified SPINK3 and GST-SPINK3 were active proteins, trypsin inhibitory activity was measured using two substrates, CBZ-Gly-Gly-Arg-4-aminomethylcoumarin (CGGR-MEC) and Boc-Val-Pro-Arg-4-aminomethylcoumarin (BVPR-MEC; Fig. 2). SPINK3 inhibited trypsin activity on both substrates ($94.80 \pm 4.73$ and $53.66 \pm 1.28$% on CGGR and BVPR respectively). The inhibitory activity of SPINK3 was similar to that of the soybean trypsin inhibitor (SBTI) on CGGR-MEC while it was less effective on BVPR-MEC. In contrast, GST-SPINK3 did not display protease inhibitory activity which was similar to that of the GST tag (control).

In order to separate serine protease inhibitor and caltrin SPINK3 activities the work was conducted with GST-SPINK3 because it lacks trypsin inhibitory activity.

Caltrin activity of the recombinant fusion protein GST-SPINK3 was confirmed by measuring intracellular $Ca^{2+}$ concentration $[Ca^{2+}]i$ by the fluorometric probe fluo-3 acetoxymethyl (fluo-3 AM) ester. During capacitation, $[Ca^{2+}]i$ was increased by 79% compared to sperm incubated under noncapacitating conditions ($0.64 \pm 0.1$ and $3.15 \pm 0.6$ arbitrary units for control sperm under noncapacitating and capacitating conditions respectively; $P < 0.01$). The concentration used for the recombinant protein (2.8 μM) was capable to bind to the sperm surface (Fig. 3A) and promoted a 31% $Ca^{2+}$ uptake inhibition after 45 min of incubation under capacitating conditions ($3.15 \pm 0.6$ and $2.20 \pm 0.2$ arbitrary units, for control and GST-SPINK3-treated sperm under capacitating conditions respectively; $P < 0.05$) comparable to that of 50% previously reported by Chen *et al*. (1998) after 60 min for the purified native molecule. The reduction of $Ca^{2+}$ was mainly observed at the sperm head (Fig. 3B) as recently reported (Ou *et al*. 2012).

### *GST-SPINK3 treatment correlates with reduced NO production in capacitated sperm*

By different methodologies electron paramagnetic resonance (EPR) spectroscopy, fluorescent probes, and nitrite quantification) an increase in NO production was observed by us (Fig. 4C) and other authors in capacitated compared to noncapacitated sperm (Herrero *et al*. 2000, de Lamirande & Lamothe 2009, Rodriguez *et al*. 2011). In epididymal mouse sperm loaded with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) the distribution of NO changed from the head and midpiece to the midpiece upon capacitation (Fig. 4A, upper panels a and c), consistent with previously reported NOS relocalization (Herrero *et al*. 1996).




To investigate whether caltrin activity of SPINK3 affects sperm intracellular signaling by reducing NO concentration we examined the effect of the GST-SPINK3 on NO production in capacitated sperm by measuring the percentage of cells showing the signal of the fluorescent probe DAF-FM DA (Fig. 4B) and nitrite production (Fig. 4C) in sperm incubated with GST-SPINK3 during capacitation.

Both the percentage of capacitated sperm with NO signal and NO concentration were significantly diminished by the addition of GST-SPINK3 compared to untreated capacitated sperm or control with the same concentration of GST (Fig. 4B and C, black bars). No significant changes were observed by the effect of GST-SPINK3 on sperm incubated under noncapacitating conditions (Fig. 4B and C, grey bars). Whether SPINK3 caltrin activity was involved in the reduction of the number of cells with NO, a drop in $Ca^{2+}$ concentration should have similar effect as SPINK3. As expected, the treatment of capacitated sperm with a permeable (BAPTA-AM) or impermeable (EDTA) $Ca^{2+}$ chelators reduced the percentage of capacitated sperm with NO signal (Fig. 4B, right panel).

### *An NO donor reverses SPINK3 effects on AR*

Spontaneous and progesterone ($P_4$)-induced AR (Fig. 5A and B respectively) were assessed on capacitated sperm by evaluating acrosomal loss as evidenced by fading of the blue stained acrosome (Fig. 5, inset). Noncapacitated sperm were assessed as a control. As expected, when sperm were incubated under capacitating conditions, GST-SPINK3 significantly reduced the percentage of spontaneous AR compared to control sperm without

**Figure 1** Recombinant expression and purification of SPINK3. (A) The upper panel shows a schematic representation of SPINK3 structure. The positions of the primers used to amplify mature SPINK3 are indicated below the diagram of SPINK3. Note that most of the protein is occupied by the Kazal-type serine protease inhibitors family signature (Prosite entry PDOC00254, Lin *et al*. 2008) described in the box below. C, conserved cystein residues; the (X) followed by a number or a letter indicates conserved or variable number of aminoacid residues respectively. The lower panel shows the electrophoretic profile by SDS–PAGE of the soluble (S) and insoluble (I) fractions of *E. coli* cells harboring pGEX-4T-3-*spink3* before (lanes 1 and 2) and after 16 h in the absence (lanes 3 and 4) or presence of 0.1 mM IPTG (lanes 5 and 6). GST-SPINK3 was purified by Glutathione Sepharose 4B affinity chromatography, analyzed by electrophoresis stained with CBB R250 (lane 7) and identified by western blotting with anti-SPINK1 antibody (Western blot (WB), lane 8). (B) Electrophoretic pattern of thrombin cleavage product of GST from the fusion protein GST-SPINK3 (CBB, lane 1) and the corresponding western blotting developed with anti-SPINK1 (WB, lane 2). Cleaved protein was purified as described in the Materials and Methods section and analyzed by SDS–PAGE (CBB, lane 3). Protein bands were visualized by CBB staining except WB lanes. The position of molecular mass markers is indicated on the left. Arrows indicate the position of the recombinant proteins. A representative gel of three independent experiments is shown.

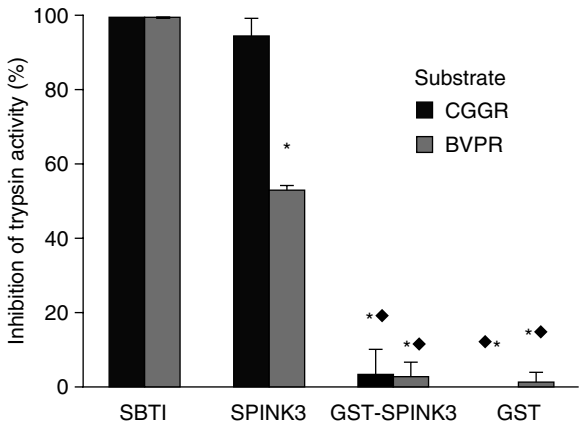



**Figure 2** Antitrypsin activity of recombinant SPINK3. Trypsin activity on CBZ-Gly-Gly-Arg-4-aminomethylcoumarin (CGGR-MEC) and Boc-Val-Pro-Arg-4-aminomethylcoumarin (BVPR-MEC) was assayed in the presence of 4 nM recombinant SPINK3, GST-SPINK3, and GST or 20 μg/μl SBTI, as controls. The activity was measured by the increment of fluorescence at 360/460 nm and the results were expressed as the mean ± S.E.M. of the percentage of inhibition of trypsin activity relative to that in the presence of SBTI (100%). The data represent the average of three independent assays. *Value different ($P<0.001$) from that observed in the treatment with SBTI. ◆Value different ($P<0.001$) from that observed in the treatment with SPINK3.

GST-SPINK3 (Fig. 5A), indicating that the recombinant protein affects AR as described for purified caltrin I (Dematteis *et al*. 2008). $P_4$-induced AR was also significantly affected by GST-SPINK3 compared to untreated capacitated sperm (Fig. 5B).

If endogenous NO was reduced as a consequence of the presence of GST-SPINK3, an NO donor added simultaneously with GST-SPINK3 should prevent the effect. This hypothesis was confirmed using two different NO donors: sodium nitroprusside (SNP) and *S*-nitrosoglutathione (GSNO). The concentration of SNP and GSNO used was sufficient to increase NO in sperm, independently of the presence of GST-SPINK3 (Fig. 4C). When sperm were incubated under capacitating conditions in the presence of SNP or GSNO plus GST-SPINK3 the percentages of spontaneous and $P_4$-induced AR sperm returned to the values of untreated capacitated sperm (Fig. 5A and B, black bars).

Sperm incubated under noncapacitating conditions had lower percentages of acrosomal loss than capacitated sperm and no effect was observed with any of the treatments (Fig. 5A and B, grey bars), indicating that nonphysiological AR percentages were very low, which is consistent with sperm viability that was invariable (around 82%) for all the treatments.

### *Effect of SPINK3 and NO on tyrosine phosphorylation*

When sperm were incubated under capacitating conditions, western blot analysis showed the characteristic increase in the 105 kDa band of hexokinase (Kalab *et al*. 1994) and the 82 kDa protein compared to noncapacitated sperm (Fig. 6, lanes 1 and 7, arrows). By this method we wanted to know whether GST-SPINK3 affects the signaling pathways activated by incubation of sperm in a capacitating media. It has been reported that rat caltrin I (Dematteis *et al*. 2008) and recombinant SPINK3 (Ou *et al*. 2012) had no effect on protein phosphorylation. Contrary to those reports in this work, even when capacitated sperm with GST-SPINK3 did not reestablish the pattern of tyrosine-phosphorylated proteins observed in untreated noncapacitating sperm, GST-SPINK3 reduced the intensity of the 82 and 105 kDa bands (Fig. 6, lane 8). This effect could not be abolished by SNP plus GST-SPINK3 (Fig. 6, lane 10) but was reversed by GSNO (Fig. 6, lane 12). Conversely, none of the treatments produced modifications in the electrophoretic pattern of tyrosine-phosphorylated proteins of noncapacitated sperm (Fig. 6, lanes 1–6), even NO donors.

### *Effect of SPINK3 and NO on sperm motility*

The effect of GST-SPINK3 on sperm motility was evaluated by quantifying total motility (TM) and progressive motility (PM) of epididymal sperm incubated for 30 min under noncapacitating or capacitating conditions with different reagents. After this time, sperm incubated under noncapacitating conditions were immotile in all treatments. The addition of GST-SPINK3 on capacitated sperm produced a nonsignificant reduction of sperm PM compared to untreated sperm at 30 min of capacitation, while the simultaneous addition of both SNP (100 μM) and GST-SPINK3 (2.8 μM) significantly enhanced sperm PM (Fig. 7). Surprisingly, GSNO (100 μM) added together with GST-SPINK3 did not produce any effect. As expected, the external $Ca^{2+}$ chelator, EDTA, produced a drastic effect showing no sperm with PM, while this parameter was unaffected by BAPTA-AM (Fig. 7). At this concentration, EDTA has no cytotoxic effect as denoted by the maintenance of cell viability (~82% for all treatments) and as previously reported (Lee *et al*. 1996, Okazaki *et al*. 2011). TM was maintained without variations for all the treatments, except with EDTA (Fig. 7). These results suggest that the enhancement of cell motility associated with the capacitation induced by BSA was not affected by GST-SPINK3.

## Discussion

SPINK3 is a secretory serine protease inhibitor expressed in the seminal vesicle of various mammals (Mills *et al*. 1987, Chen *et al*. 1998). It can be considered as a multifunctional protein since, in addition to its antitrypsin activity (Luo *et al*. 2004), inhibits $Ca^{2+}$ transport in sperm (Coronel *et al*. 1992, Dematteis *et al*. 2008). Because of this latter activity it has been named as caltrin protein.

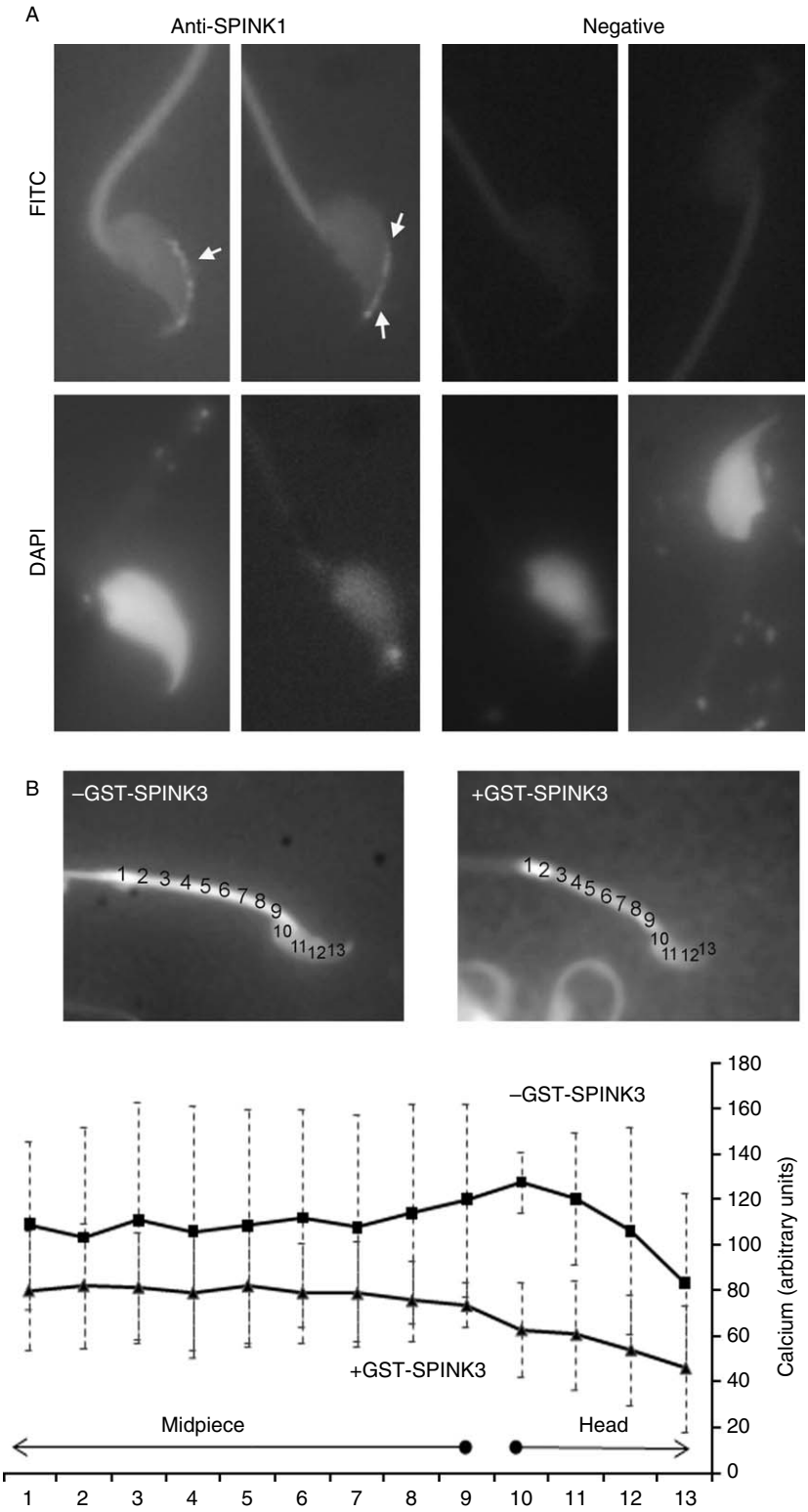



**Figure 3** Binding to sperm and caltrin effect of recombinant GST-SPINK3. Epididymal sperm were incubated with or without the recombinant protein, GST-SPINK3. (A) For immunostaining, sperm was incubated with anti-SPINK1 antibody and developed by anti-rabbit IgG FITC-conjugated. The fluorescence shows the specific binding of GST-SPINK3 to epididymal spermatozoa distributed on the dorsal surface of the head of sperm cells (arrows). DAPI staining was performed to localize the sperm nuclei as a control. Slides were examined with a Nikon fluorescent microscope under magnification = 1000×. A representative picture out of three experiments is shown. (B) Reduction of $[Ca^{2+}]i$ in the mouse sperm head by GST-SPINK3 was assessed using the fluo-3 AM. Spermatozoa loaded with the probe were incubated under capacitating conditions with or without GST-SPINK3 for 60 min at 37 °C and then observed under fluorescence microscope under magnification = 1000× (upper panel). Fluorescence was quantified at different points of individual sperm cells (denoted as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13) were analyzed using ImageJ Software and expressed as arbitrary units (lower panel). Each data point is the mean ± S.D. of three independent determinations of different cells.

This work demonstrates that incubation of capacitated mouse sperm with recombinant GST-SPINK3 modulates sperm physiology through a downstream reduction of endogenous NO concentration that is independent of SPINK3 antitrypsin activity. To the best of our knowledge, this is the first report that links SPINK3 activity to a signaling pathway involving NO.

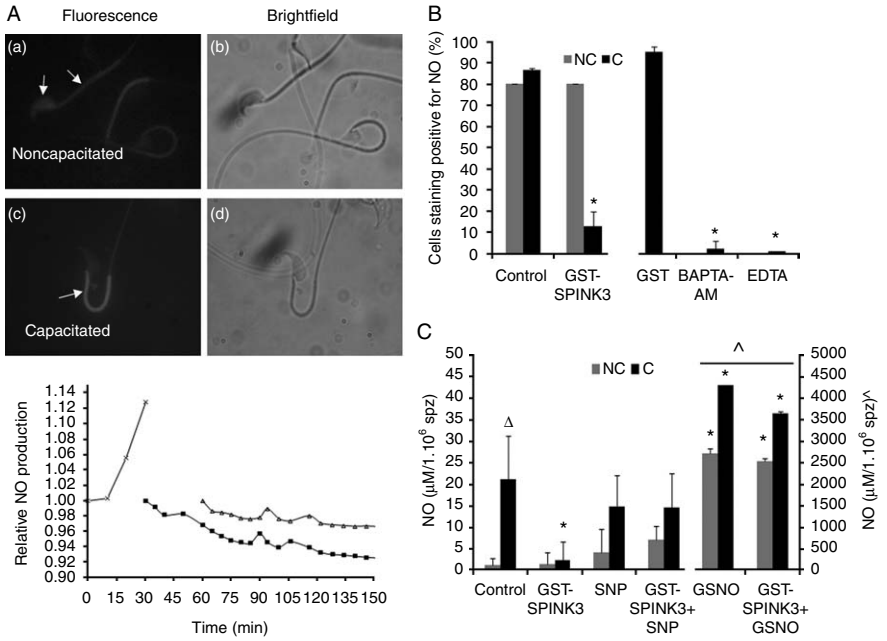



**Figure 4** Effect of GST-SPINK3 on the endogenous NO production in capacitated mouse sperm. (A) Localization of NO in sperm cells (upper panels). Fluorescence microscopy assay was used to observe the distribution of NO signal on sperm (right panels). Motile sperm from cauda epididymis were incubated in the dark under noncapacitating or capacitating conditions with 10 μM of the specific NO fluorescent probe DAF-FM DA. The presence of NO in the spermatozoa (a) head and midpiece or only in the (c) midpiece is indicated (arrows). Slides were observed using a fluorescence microscope or brightfield (b and d) under magnification = 1000×. A representative picture of two independent experiments is shown. NO detection with DAF-FM DA added at different times during capacitation (lower panel). DAF-FM DA was added at: the beginning of capacitation (cross), 30 min (black square) or 60 min (white triangle) after transferring spermatozoa to capacitating conditions. The fluorescence intensity measurement (excitation 480 nm; emission 525 nm) was carried out using a fluorescence plate reader at 37 °C and values were normalized to the measurement at the time of addition of DAF-FM DA. Note that only when sperm were loaded with the probe at the beginning of capacitation the increase in NO could be detected. (B) Percentage of cells with NO fluorescent signal. Motile sperm from cauda epididymis were incubated under noncapacitating or capacitating conditions with or without GST-SPINK3, two $Ca^{2+}$ chelators (EDTA and BAPTA-AM), or GST, as indicated. Sperm were loaded with10 μM DAF-FM DA from the beginning of capacitation. Slides were observed using a fluorescence microscope with magnification = 1000×. For each treatment, spermatozoa with fluorescence either in the head and midpiece (noncapacitated) or in the midpiece (capacitated) were quantified and expressed as percentage of total cells observed ($n$ = 100 per replica). Data are expressed as the mean ± S.E.M. of two independent samples. (C) Quantification of NO level by Griess method. NO sperm concentration was estimated by measuring nitrite levels using the Griess reagent in noncapacitated or capacitated sperm in the presence of GST-SPINK3, SNP or both (left panel); or alternatively in the presence of GSNO or GST-SPINK3 plus GSNO (right panel). Data are expressed as the mean ± S.E.M. of three independent samples. *Value different ($P$<0.05) from that observed in untreated spermatozoa (control) for each condition. $^{\Delta}$Control capacitated sperm differed from control noncapacitated sperm ($P$<0.05).

## Is SPINK3 antitrypsin activity necessary to modulate sperm function?

SPINK3 binds to the spermatozoa surface by means of an unknown receptor. It can inhibit trypsin *in vitro* (Fig. 2) as shown with purified P12 (Lai *et al*. 1994, Winnica *et al*. 2000). It also inhibits acrosin (Winnica *et al*. 2000), but the target protease in sperm is still unknown. Recently, a membrane-bound serine protease, TESPL, has been proposed to be the SPINK3 anchoring protein because interaction between these proteins has been demonstrated by yeast two-hybrid assay (Ou *et al*. 2010). However, it is known that the ability of SPINK3 to bind sperm and its trypsin inhibitory activity are not linked. Single-site mutations in the recombinant mouse protein P12 (SPINK3) demonstrated that aminoacid residue R19 was essential for protease inhibition and D22 and/or Y21 were responsible for the binding of P12 to sperm (Luo *et al*. 2004, Lin *et al*. 2006). Accordingly, in this work we showed that a modified recombinant SPINK3 protein that lacks trypsin inhibitory activity (GST-SPINK3) can bind sperm, reduce intracellular $Ca^{2+}$ concentration, and modulate AR and protein phosphorylation, while it has no effect on sperm motility. All these data taken together show that the serine protease inhibitory activity of SPINK3 might not be necessary in sperm signaling which is consistent with the results obtained by Ou *et al*. (2012) with the recombinant SPINK3 carrying the point mutation R19L.

Remarkably, SPINK3 is not the only serine protease inhibitor from male glands that modulates sperm physiology. In guinea pig, two different caltrin molecules purified from the seminal vesicle secretion are equally effective inhibitors of sperm $Ca^{2+}$ uptake, although

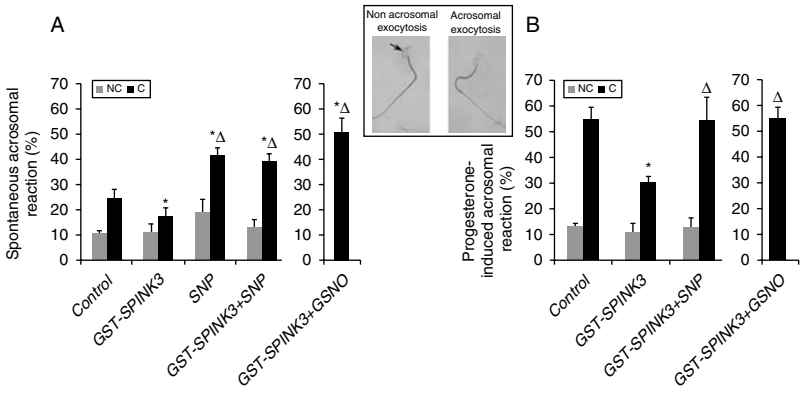



**Figure 5** Effect of GST-SPINK3 and NO on (A) spontaneous and (B) progesterone-induced acrosomal exocytosis. Cauda epididymis motile mice sperm were incubated under noncapacitating or capacitating conditions in the presence or absence of GST-SPINK3 with or without the NO donors SNP and GSNO. For progesterone-induced acrosomal exocytosis, sperm were incubated with 10 μM progesterone after capacitation. Sperm cells were stained with CBB G-250 and then spermatozoa with acrosomal loss were quantified and expressed as a percentage as total sperm ($n=200$ per replica). Inset: A blue stain over the sperm head dorsal and/or ventral surface was visualized in spermatozoa with intact acrosome (arrow), whereas no stain was observed in spermatozoa with reacted acrosome. Magnification=1000×. Data are expressed as the mean±S.E.M. of three and four independent experiments for spontaneous and $P_4$-induced AR respectively. *Value different ($P<0.05$) from that observed for untreated spermatozoa for each condition. $^{\Delta}$Value different ($P<0.05$) from that observed for spermatozoa treated with GST-SPINK3 for each condition.

caltrin I binds to the head of epididymal sperm and prevents AR during *in vitro* capacitation (Winnica *et al.* 2000); while caltrin II binds to the principal portion of the tail and delays sperm hyperactivation (Coronel & Lardy 1992). Caltrin II has no structural homology with caltrin I (Winnica *et al.* 2000) even though its gene conserves a whey acidic protein (WAP) domain, which is present in serine protease inhibitors (Furutani *et al.* 2004).

Other serine protease inhibitors, like SERPINE2 (nexin-1) from seminal vesicle and epididymal protease inhibitor (EPPIN), bind to uncapacitated sperm and are believed to inhibit capacitation. SERPINE2 inhibits both plasminogen activator and thrombin, and also inhibits *in vitro* BSA-induced tyrosine phosphorylation of sperm under capacitating conditions by unknown mechanisms (Lu *et al.* 2011). EPPIN, characterized by both WAP-type and Kunitz-type consensus sequences (Wang *et al.* 2005, 2008, O'Rand *et al.* 2007), acts a decapacitating factor by modulating the activity of PSA in semenogelin, which inhibits human sperm capacitation (de Lamirande *et al.* 2001). However, it has been found that anti-EPPIN antibodies significantly inhibited the human sperm AR induced by A23187, reduced intracellular $Ca^{2+}$ concentration, and did not change tyrosine phosphorylation of sperm proteins (Zhang *et al.* 2010).

Among all these molecules described as decapacitating factors and also serine protease inhibitors, it is clear that EPPIN and SERPINE2 have a role outside the sperm. This might also be the case for SPINK3 antitrypsin activity.

## SPINK3 caltrin activity modulates sperm physiology through a downstream NO reduction

In fact, SPINK3 expressed as a GST-fusion protein reduced NO production, spontaneous and $P_4$-induced AR in mice epididymal sperm capacitated *in vitro* but did not completely affect sperm protein phosphorylation in tyrosine residues and progressive motility.

Extracellular $Ca^{2+}$ is needed for capacitation and NO production (de Lamirande *et al.* 2009). It has been reported that NO donors cause an elevation of human sperm $[Ca^{2+}]i$ coming from $Ca^{2+}$ internal stores by sperm protein *S*-nitrosylation (Machado-Oliveira *et al.* 2008). However, SPINK3 cannot cross the sperm plasma membrane, it binds to the sperm surface during ejaculation and comes off during AR (Dematteis *et al.* 2008), so one could speculate that the reduction of $[Ca^{2+}]i$ observed in sperm upon treatment with GST-SPINK3, and also reported for caltrin I (San Agustin *et al.* 1987, Dematteis *et al.* 2008), should act upstream of NO. This speculation is consistent with the reduction of NO observed by us and other authors in capacitated sperm treated with either GST-SPINK3 (Fig. 4) or $Ca^{2+}$ chelators (Fig. 4; de Lamirande *et al.* 2009). GST-SPINK3 caltrin effect might inhibit the $Ca^{2+}$-/CaM-dependent brain nNOS, as reported for bovine caltrin (Schaad *et al.* 1996); yet, under capacitating standard conditions, extracellular $Ca^{2+}$ uptake would activate NOS, increasing NO levels and continuing through a signaling pathway that finally modulates different sperm processes (Fig. 8).

It has been reported that, without external triggers, active NO production by NOS was observed in most mature human spermatozoa (Roessner *et al.* 2010). This is in agreement with our observation of a strong NO fluorescent signal in all the sperm obtained from cauda epididymis when loaded with DAF-FM DA, where sperm are already mature. Also, the redistribution of NO that we observed upon capacitation from the head and midpiece to the neck is in accordance with the NOS relocalization in mouse and human sperm, observed by

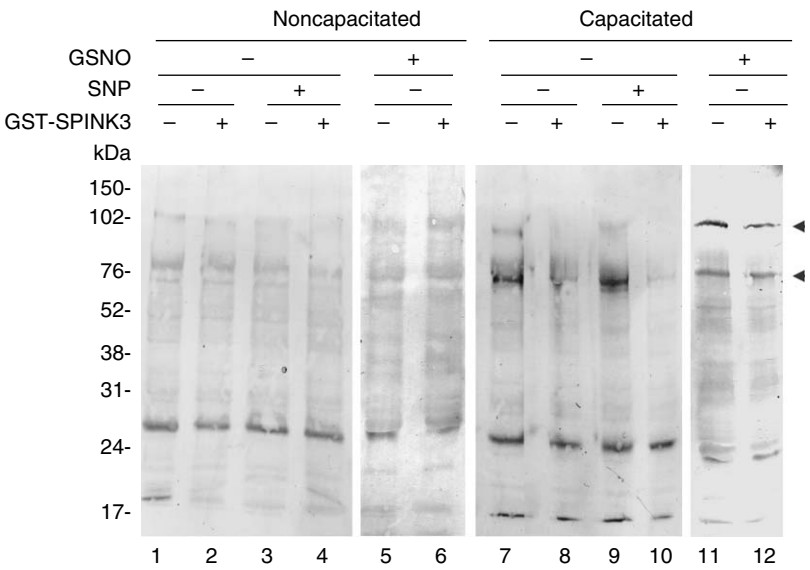



**Figure 6** Effect of GST-SPINK3 and NO on tyrosine phosphorylation of sperm proteins. Cauda epididymis motile mice sperm were incubated under noncapacitating or capacitating conditions in the presence or absence of GST-SPINK3 and with or without the NO donors, SNP, and GSNO. Tyrosine phosphorylation was assessed by western blot analysis of total sperm proteins using a monoclonal anti-phosphotyrosine antibody. Lanes 1, 2, 3, 4, 5 and 6: proteins from noncapacitated sperm; lanes 7, 8, 9, 10, 11 and 12: proteins from capacitated sperm; lanes 2 and 8: proteins from sperm treated with GST-SPINK3; lanes 3 and 9: proteins from sperm treated with SNP; lanes 4 and 10: proteins from sperm treated with GST-SPINK3 and SNP; lanes 5 and 11: proteins from sperm treated with GSNO; and lanes 6 and 12: proteins from sperm treated with GST-SPINK3 and GSNO. The positions of molecular mass markers (kDa) are indicated at the left. The immunoreactive bands that change during capacitation are indicated by arrows.

immunofluorescence using antibodies that cannot distinguish among NOS forms (Herrero *et al*. 1996). However, de Lamirande & Lamothe (2009) observed, using the same probe, an increase of NO signal on the head in human sperm during capacitation. This discrepancy should be attributed to a specie-dependent behavior of NO or alternatively, to differences in the methodology, since our experiments did not include fixation of sperm neither we used an antifading agent. Probably, NO production at the sperm head is quenched due to the lack of an antifading agent, so we were able to detect the relocalization of the NOS to the midpiece where NO is being produced, which might occur later after the initiation of capacitation. For this reason we used the Griess reaction to detect total NO.

What is the effect that SPINK3 has on the signaling pathways activated during capacitation? Capacitation is a $Ca^{2+}$-dependent process and is known to be prevented by intercellular and extracellular chelators (Leclerc *et al*. 1998), so one is prompt to hypothesize that SPINK3 should inhibit capacitation. However, previous work with the native rat protein or the recombinant protein showed no effect on protein phosphorylation (Dematteis *et al*. 2008, Ou *et al*. 2012). In this work, the change in sperm to capacitating medium triggered the typical increase of tyrosine phosphorylation of sperm proteins of 105 and 81 kDa observed when the assay is performed without the phosphatase inhibitor orthovanadate (Leclerc *et al*. 1996, Herrero *et al*. 1999, Kulanand & Shivaji 2001), coincidently with a detectable endogenous NO production (see Figs 4 and 6).

It has been reported that phosphorylation in tyrosine, threonine, and glutamine residues of sperm proteins in human sperm is triggered by NO donors and inhibited by NO scavengers (Herrero *et al*. 1999, Herrero & Gagnon 2001, Thundathil *et al*. 2003). Moreover, NO is believed to modulate the increase in cAMP and cGMP that leads to protein phosphorylation (Machado-Oliveira *et al*. 2008, de Lamirande *et al*. 2009, Roessner *et al*. 2010). In particular, Thundathil *et al*. (2003) demonstrated the increment of the double phosphorylation (P-Thr-Glu-Tyr-P) in p80/105 proteins of human sperm under noncapacitating conditions (containing $CaCl_2$) by the addition of spermine NONOate (Thundathil *et al*. 2003). In contrast, we were able to observe an increase in

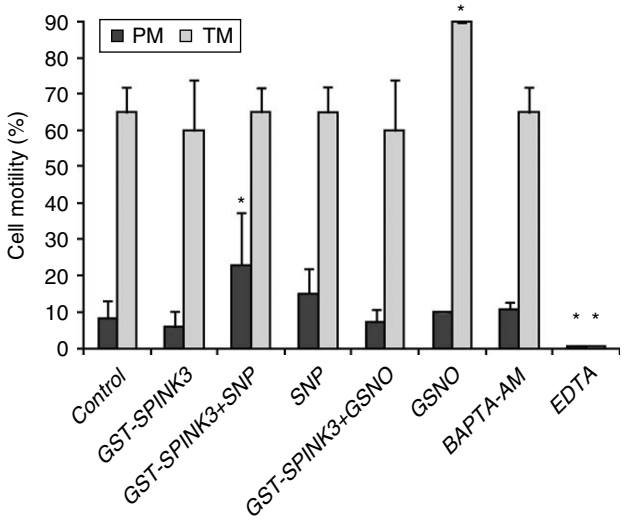



**Figure 7** Effect of GST-SPINK3 and NO on mice sperm motility. Cauda epididymis motile mice sperm were incubated under noncapacitating (data not shown) or capacitating conditions in the presence of two $Ca^{2+}$ chelators (EDTA and BAPTA-AM) or in the presence or absence of GST-SPINK3 with or without SNP or GSNO, as indicated. Spermatozoa showing any kind of motility (TM) or only PM were quantified after 30 min incubation under brightfield microscope as percentage of total quantified sperm ($n=200$ per replica). Data are expressed as the mean ± S.E.M. of three independent samples. *Value different ($P<0.05$) from that observed for control spermatozoa under each condition.

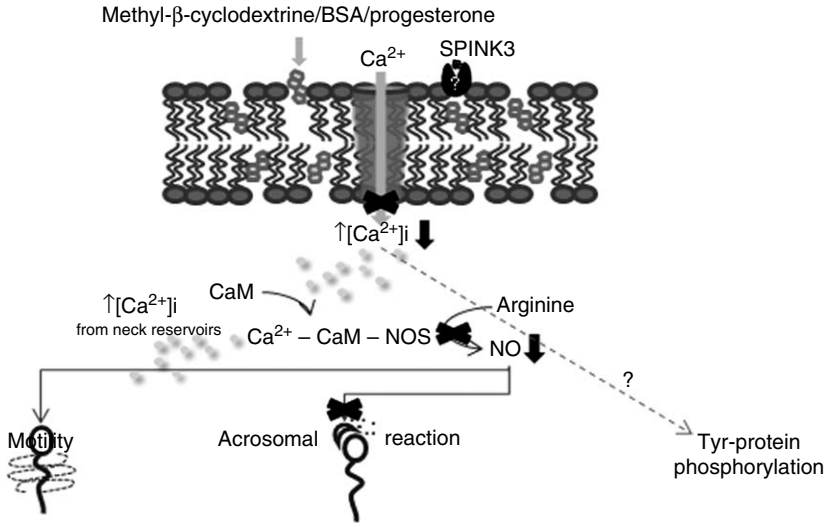



**Figure 8** Schematic representation of the effect of GST-SPINK3. Cholesterol efflux induced *in vitro* by BSA or methyl-β-cyclodextrine (Choi & Toyoda 1998) and *in vivo* by progesterone (Costello *et al.* 2009) and female tract neighborhood promotes the remodeling of the plasma membrane and triggers signaling pathways leading to different sperm processes that account for sperm fertilizing ability. Extracellular $Ca^{2+}$ influx might induce $Ca^{2+}$/CaM-dependent NOS activity, increasing NO production. SPINK3 attaches to the sperm surface by unknown receptors (Dematteis *et al.* 2008) and reduces intracellular $Ca^{2+}$ concentration by acting at the level of the plasma membrane (Breitbart *et al.* 1990), consequently a reduction of NO is observed (symbolized as ⋈). Under capacitating conditions this binding produces the inhibition of the AR that could be overcome by NO donors. SPINK3 also produce a modification in the protein phosphorylation pattern that is also reversed by a strong NO donor, however it was not sufficient to induce protein phosphorylation itself, suggesting a possible control of protein kinases directly by $Ca^{2+}$ (dashed line). SPINK3 did not affect sperm motility but it was enhanced by SNP suggesting that other $Ca^{2+}$ reservoirs are involved (long arrow). The blocked points targeted by SPINK3 in the pathway are indicated by dark black. Caltrin activity can be considered a checkpoint that turns on or off signaling pathways and thus maintaining sperm in a latent state until it reaches the ovum.

tyrosine-phosphorylated proteins caused by either SNP or GSNO merely in sperm incubated with capacitating medium but no changes were provoked by these donors in HM media devoid of bicarbonate, $Ca^{2+}$, and BSA (Fig. 6).

In addition, under capacitating conditions, SNP did not reverse the effect of GST-SPINK3 on tyrosine phosphorylation, however, the addition of GSNO indeed abolished the effect of GST-SPINK3, which can be explained by the higher levels of NO released by this donor under the assayed conditions (Fig. 4). Whether GSNO effect is through an NO-dependent activation of adenylate or guanylate cyclase or through protein *S*-nitrosylation cannot be concluded.

In view of these results it can be assumed that NO is not sufficient to mediate protein tyrosine phosphorylation, and maybe it can be directly mediated by $Ca^{2+}$ as also proposed by Muratori *et al.* (2011). Most of the tyrosine-phosphorylated proteins identified are flagellar proteins, therefore this process might be related to hyperactivation but not with AR.

The effect of SPINK3 on AR might be more clearly related to a reduction in endogenous NO concentration produced by the $Ca^{2+}$ transport inhibiting effect of SPINK3. In agreement with previous results of purified caltrin I from rat (Dematteis *et al.* 2008) and recombinant SPINK3 (Ou *et al.* 2012), we observed an inhibition of both spontaneous and $P_4$-induced AR mediated by GST-SPINK3, while viability remained invariable. However, the physiological significance of the effect over induced AR is uncertain. It has recently been proposed that SPINK3 does not detach from the sperm surface during capacitation but might be removed by the trypsin-like activity in the uterine fluid of estrous females before the sperm meets the ovum (Ou *et al.* 2012), still, $Ca^{2+}$ concentrations in this region are low (Abou-haila & Tulsiani 2009).

According to our hypothesis, both NO donors completely reversed the effect of GST-SPINK3, demonstrating that NO was necessary for triggering AR as previously reported (Herrero & Gagnon 2001). The signaling pathway that mediates AR via NO probably involves the activation of a soluble guanylate cyclase since incubation of bovine sperm with SNP caused an increase in cGMP levels during AR, although it was observed in mouse sperm that cGMP levels were not affected by NOS inhibitors (Herrero & Gagnon 2001). Acrosomal stores of $Ca^{2+}$ have been also proposed to be activated by cGMP (Costello *et al.* 2009) as part of the pathway. Accordingly, thapsigargin, a molecule that raises cytosolic $Ca^{2+}$ concentration by blocking the ability of the cell to pump $Ca^{2+}$ into the acrosome, was sufficient to prompt AR in the presence of EGTA (Herrick *et al.* 2005).

Motility was slightly affected by the addition of GST-SPINK3 to capacitating sperm, according to previous

results with purified rat caltrin I, where no effect was observed (Dematteis *et al*. 2008). In this sense, it is noteworthy that $Ca^{2+}$ reduction caused by GST-SPINK3 in capacitating sperm was mainly circumscribed to the head region, consistent with other results (Ou *et al*. 2012), and explaining the low percentage of reduction observed when fluorescence was measured in the sperm suspension loaded with the probe fluo-3 AM (Fig. 3). Besides, a distinct effect was observed in our experiments with both $Ca^{2+}$ chelators: a complete impairment of motility caused by EDTA, as reported (Lee *et al*. 1996) contrary to a lack of effect of BAPTA-AM (Fig. 7), consistent with the observation that BAPTA-AM did not reduce $Ca^{2+}$-specific labeling with fluo-3 AM in sperm midpiece (De Blas *et al*. 2002).

In particular, $Ca^{2+}$ is essential for the development of sperm motility, and its intracellular levels are tightly regulated by several $Ca^{2+}$ channels (Muratori *et al*. 2011). Signaling pathway that controls flagellar movement is thought to be an NO-dependent mechanism independent of cAMP and cGMP (Machado-Oliveira *et al*. 2008) and dependent of midpiece stores of $Ca^{2+}$ (Costello *et al*. 2009). On the contrary, it was reported that NO donors are known to stimulate sperm motility by an increased synthesis of cGMP (Miraglia *et al*. 2011), however there are some discrepancies regarding the effect of NO donors on motility depending on the doses utilized (Herrero *et al*. 1999). Surprisingly, in this work SNP plus GST-SPINK3 but not SNP alone or either GSNO enhanced sperm PM, probably due to the differences in NO concentration released under these conditions (Figs 4 and 7).

It was also reported that application of thapsigargin caused elevation of $Ca^{2+}$ in the neck region and hyperactivation in bovine sperm, these effects being independent of $Ca^{2+}$ from the outside (Costello *et al*. 2009).

Still, several questions remain unanswered and require further investigation: what does relocalization of NO to the midpiece during capacitation means?; is $Ca^{2+}$ compartmentalization the fork in the road that converts a signal to different intracellular pathways? To address these questions real-time imaging should be necessary to understand the timing of these transient signals using intracellular $Ca^{2+}$ chelators and/or ionophores that regulate different $Ca^{2+}$ channels.

## Conclusion

Recombinant SPINK3 modulates sperm physiology through a downstream reduction of endogenous NO concentration. The effect of SPINK3 can be reversed by the exogenous addition of NO and is independent of SPINK3 antitrypsin activity. However, the effect on sperm protein phosphorylation was not dramatic and almost no effect was observed by SPINK3 on sperm motility.

It can be speculated that SPINK3, a secretory molecule from seminal vesicle, has two different activities: a serine protease inhibitory activity that has not yet been completely related to sperm physiology and caltrin activity that can be considered a checkpoint that turns on or off signaling pathways and thus maintaining sperm in a latent state until it reaches the ovum.

## Materials and Methods

### *Reagents*

Tryptone and yeast extract for growth medium was purchased from Oxoid (Basingstoke, Hampshire, UK); Agarose from Genbiotech (Buenos Aires, Argentina); M-MLV Reverse Transcriptase from Promega; Taq polymerase from PB-L (Buenos Aires, Argentina); Quiaquick Gel Extraction and Miniprep kits (Qiagen); random primers DNA labeling system and DNA fluorescent dyes (SYBR Safe and SYBR Gold) from Invitrogen. Restriction enzymes were purchased from Fermentas Inc (Ontario, Canada). DAF-FM DA was purchased from Invitrogen, BAPTA-AM from Calbiochem (Merck, Darmstadt, Germany), Griess colorimetric kit form Promega, SNP from Merck, secondary antibodies, ipegal CA630, and DAPI from Sigma, fluo-3 AM (Molecular Probes F1242; Invitrogen) and 0.05% Silwet L-77 surfactant (Arysta Life Science, Tokyo, Japan). All other chemicals and reagents were of analytical grade and obtained either from Merck or Sigma–Aldrich.

### *Animals*

BalbC and CF-1 mice (*Mus musculus* (*M. musculus*)) were maintained at 22 °C with a photoperiod of 1200 h light: 1200 h darkness, food, and water *ad libitum*. Sexually mature (2–3 months old) male mice were killed by cervical dislocation and epididymides and seminal vesicles were immediately removed. All procedures were in agreement with the Local Ethics Committee of the National University of Mar del Plata and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf). Epididymides were processed immediately as described below (Sperm peparation and capacitation) and seminal vesicles were sectioned into 0.5 $cm^3$ pieces, immersed in RNAlater (Ambion), and stored at −80 °C until used.

### *Molecular cloning and heterologous synthesis of recombinant SPINK3 and GST-SPINK3*

Total RNA was extracted from mice seminal vesicles (300 mg) using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Tissue was disrupted by homogenization in an IKAWERKE T10 basic Ultra-Turrax using an S10N-8G tool for 2 min at 4 °C. First strand cDNA was synthesized by RT with M-MLV Reverse Transcriptase (Promega) according to the manufacturer's instructions. The reaction mix (25 µl) contained the following: 5 µg RNA, 2 µg/µl oligo ($dT_{15}$) primer, 0.5 mM dNTPs, and 0.2 U enzyme. A DNA fragment corresponding to mature SPINK3 (nucleotides 158–331 that encoded a polypeptide of 34 kDa starting from residue 24 to the stop codon, Fig. 1) was obtained by PCR amplification of single-stranded cDNA using primers designed on the basis of SPINK3 mRNA

sequence from *M. musculus* (NCBI ID: NM_009258.5). To facilitate cloning of SPINK3 cDNA into the expression vector, the restriction sites BamH1 and XhoI were added to the 5′ end nucleotide sequences of the SPINK3-Fw (5′-CGC GGA TCC GCT AAG GTG ACT GGA-3′) and SPINK3-Rev (5′-CCG CTC GAG TCA GCA AGG CCC ACC-3′) primers respectively. The PCR reaction was carried out in a mixture (20 µl) containing 1× buffer, 1.5 mM $Cl_2Mg$, 0.2 mM dNTPs mix, 1 µM primers, 3 µl cDNA as template, and 2.5 U Taq DNA polymerase. The PCR amplification protocol was as follows: 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 59 °C for 1 min, and primer extension at 72 °C for 7 min. The PCR product of the expected size (192 bp) was agarose gel-purified according to the MinElute purification kit (Qiagen) and its identity was confirmed by DNA sequence analysis.

The DNA fragment corresponding to SPINK3 was cloned into pGEX-4T-3 expression vector (GE) downstream the GST coding sequence and a thrombin cleavage site to facilitate the purification of the recombinant protein. The cDNA encoding mature SPINK3 and the pGEX-4T-3 expression vector were digested with XhoI and BamHI (Fermentas) following the manufacturer's instructions and ligated with T4 DNA Ligase (Promega) according to standard protocols. The generated construct, pGEX-4T-3-*spink3* which encoded GST-SPINK3, was then amplified in *E. coli* DH5α competent cells and the positive clones were confirmed by DNA sequencing.

For overexpression of GST-SPINK3, *E. coli* Rosetta cells (Novagen, Merck, Darmstadt, Germany) were transformed with the pGEX-4T-3-*spink3* recombinant plasmid. *E. coli* cells containing pGEX-4T-3-*spink3* were grown overnight at 37 °C in Luria Bertani (LB) medium (0.5% yeast extract, 1% tryptone, and 0.5% NaCl) supplemented with 100 µg/ml ampicillin and 20 µg/ml chloramphenicol. This culture was used to inoculate (1/100) fresh LB medium (50 ml) containing ampicillin/chloramphenicol and grown at 37 °C under continuous shaking until the optical density at 600 nm ($OD_{600}$) of the culture reached 0.4, then shifted to 18 °C and incubated until the $OD_{600}$ was ~0.6. Synthesis of the recombinant protein was induced in the cells harboring pGEX-4T-3-*spink3* by the addition of 0.1 mM IPTG for 16 h at 18 °C.

Bacteria were harvested by centrifugation (1700 ***g*** for 8 min), suspended in PBS (ICN tablet 2810305; 10 mM phosphate buffer (pH 7.4), 137 mM NaCl, and 2.7 mM KCl), containing 1 mg/ml lysozyme, 0.2% (v/v) Triton X-100, and 0.5 mM PMSF and lysed by sonication (6×30 s, 40 W, Cole Parmer 4710 (London, UK)). The soluble (S) and insoluble (I) cell lysate fractions were obtained by centrifugation at 12 000 ***g*** for 25 min at 4 °C and then analyzed by SDS–PAGE (15% w/v polyacrylamide gel; Laemmli *et al*. 1970).

Glutathione Sepharose 4B affinity chromatography (G2879; Molecular Probes) was employed for batch purification of GST-SPINK3 from the soluble fraction of induced cells. The column was equilibrated and washed with PBS and eluted with 8 mM reduced glutathione in 50 mM Tris–HCl (pH 8).

Cleavage of GST from the fusion protein was carried out by two different protocols: a) in-slurry digestion: cleavage was performed during purification by incubating the slurry containing GST-SPINK3 bound to the matrix with thrombin (T6634; Sigma; 1 U/50 µg recombinant protein), in buffer T (0.1 M NaCl, 2.5 mM $CaCl_2$, and 50 mM Tris–HCl (pH 8)) at 18 °C for 16 h with gentle agitation. Then, SPINK3 was collected by washing with PBS and released GST was eluted with reduced glutathione. b) After-elution digestion: GST-SPINK3 bound to the matrix was eluted with reduced glutathione and then incubated with thrombin under the conditions previously described.

Protein digests from either protocol (a or b) were fractionated on a 15% polyacrylamide gel, negatively stained with zinc–imidazole and eluted according to Hardy & Castellanos-Serra (2004). Briefly, the gel was soaked for 30 s in double-distilled water, incubated for 15 min in 0.2 M imidazole, 0.1% (w/v) SDS, rinsed with water, and developed for 1 min in 0.2 M zinc sulfate. Proteins, visualized as clear bands on a dark background, were excised from the gel, washed by incubation (2×10 min) in PBS containing 100 mM EDTA (pH 7.4) followed by PBS (2×5 min). Elution was performed by crushing the gel to microparticles suspended in 200 µl PBS, vortexing the suspension for a few seconds and incubating for 20 min to allow passive diffusion of intact biomolecules and proteins were collected by centrifugation.

Efficient digestion of GST-SPINK3 by using protocol (a) was not successful, therefore, as a source of recombinant protein for the following experiments, GST-SPINK3 was purified from the column, SPINK3 without GST was obtained using protocol (b) followed by gel purification, and GST (control) was obtained by protocol (a) followed by gel purification.

These proteins were concentrated by ultrafiltration in YM-3000 membranes (Amicon, Millipore, MA, USA) and analyzed by SDS–PAGE. The identity of SPINK3 was confirmed by western blotting.

### *Identification of recombinant proteins by western blotting*

Proteins were fractionated on 15% (w/v) polyacrylamide gels and transferred onto PVDF membranes for 2 h, 75 mA in a TE 70 PWR (GE). Membranes were incubated overnight in blocking solution (3% fish gelatin Sigma G7765 in buffer TBS–T (25 mM Tris, 0.192 mM glycine, and 0.01% Tween 20)) at 4 °C. Incubation with primary antibody was performed for 1 h at 37 °C with anti-SPINK1 (1:4500, HPA02749; Sigma) in blocking solution. After washing with TBS–T, membranes were incubated for 40 min with anti-rabbit IgG-biotin conjugated (1:1000, B6648; Sigma–Aldrich) and streptavidin–peroxidase (1:1000, E8386; Sigma–Aldrich) in blocking solution. Development of the membranes was performed by incubation with one volume of solution A (0.1 M Tris (pH 8.5), 2.5 mM luminol, and 0.4 mM coumaric acid) plus one volume of solution B (0.1 M Tris–HCl (pH 8.5), and 0.018% $H_2O_2$) for 1 min, washed, and analyzed in an LAS-4000 (Fujifilm, Tokyo, Japan).

The expressed SPINK3 sequence has a 67% identity (82% similarity) with the immunogenic sequence of the antibody.

### *Determination of trypsin inhibitory activity*

Antitrypsin activity was assessed fluorometrically using two synthetic trypsin-specific substrates: CGGR-MEC and BVPR-MEC. Reaction mixtures containing 0.2 mM substrate,

50 mM phosphate buffer (pH 7.8), and 0.31 U trypsin (T1426; Sigma) preincubated with 4 nM recombinant SPINK3, GST-SPINK3 or GST, and 20 μg/μl SBTI. Trypsin inhibitory activity was determined by measuring the increment of fluorescence at 360/460 nm at 37 °C for 30 min. The results were expressed as inhibition percentage of trypsin activity.

### Immunodetection of GST-SPINK3 in sperm

Washed epididymal sperm were diluted to $5\times10^6$ cells/ml and incubated with 2.8 μM GST-SPINK3 for 1 h at 37 °C. Then, sperm were washed with PBS by centrifugation (850 ***g***, 15 min), fixed with 4% paraformaldehyde for 30 min. Excess of fixative was removed by washing twice. 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 for 30 min. Slides were incubated with a 1:50 dilution of anti-SPINK1 (HPA027498; Sigma) for 2 h at 37 °C, washed and incubated with a 1:200 dilution of anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated (F0382; Sigma) for other 2 h. All incubations were done at room temperature in a humidified chamber. Samples were also stained by 1:1000 4′-6-diamidino-2-phenylindole DAPI diacetate (D9564; Sigma). After thoroughly washing with PBS, slides were mounted with PBS:glycerol (1:9) and the sperm fluorescence were observed under 360/460 nm for DAPI and 480/525 nm for FITC, and recorded with a Hamamatsu C4742-95 camera connected to a computer with analytic software (MetaMorph, Molecular Devices, CA, USA) attached to an inverted microscope: Nikon Eclipse T2000.

### Sperm preparation and capacitation

Cauda epididymides were immersed in 2 ml HM medium (modified Krebs Ringer bicarbonate medium; Visconti *et al.* 1995; 25 mM Hepes, 109 mM NaCl, 14.77 mM KCl, 1.19 mM $MgSO_4$, 1 mg/ml glucose, 21.18 mM sodium lactate, 1.19 mM $KH_2PO_4$, plus 1 mM sodium pyruvate (pH 7.4)) contained by culture dishes on a warm plate at 37 °C. The tissues were minced with scissors to allow the sperm dispersion into the media. After 10 min tissue debris were removed and aliquots (100 μl) of the sperm suspension were transferred to 1.5 ml polypropylene tubes carefully underlying a layer of 500 μl fresh media.

Samples were incubated in a 5% $CO_2$, 37 °C incubator (precision) for 30 min to perform a swim-up technique for sperm separation (Ren *et al.* 2004). The supernatants (300 μl) containing the most motile sperm were removed from the top of each tube, collected in a separate tube and concentration was evaluated in a Neubauer chamber. Sperm were pelleted by centrifugation (0.7 ***g*** for 10 min) and suspended in HM or HMB (25 mM Hepes, 109 mM NaCl, 14.77 mM KCl, 1.19 mM $MgSO_4$, 1 mg/ml glucose, 21.18 mM sodium lactate, 1.19 mM $KH_2PO_4$ (pH 7.4) plus 1 mM sodium pyruvate, 1.7 mM $CaCl_2$, 0.8 mM methyl-β-cyclodextrin, and 25 mM $NaHCO_3$) medium, as indicated in assay conditions. Unless indicated, methyl-β-cyclodextrin was used as a replacement of 3% BSA for capacitation, since the latter may affect the fluorescence in the DAF-FM DA assay. The use of methyl-β-cyclodextrine has been already reported (Choi & Toyoda 1998). Sperm concentration was adjusted to the concentration indicated for each assay.

Sperm incubations were carried out with HM or HMB medium with or without different reagents (Assays Conditions) at 37 °C and 5% $CO_2$ for 1 h.

### Measurement of intracellular $Ca^{2+}$ in sperm

Cauda epididymides were immersed in 1 ml HM medium (without $CaCl_2$). After 10 min, sperm aliquots were transferred to 1.5 ml polypropylene tubes and incubated in the presence of 10 μM fluo-3 AM (Molecular Probes F1242; Invitrogen) and 0.05% Silwet L-77 (Arysta Life Science) as a surfactant in a 5% $CO_2$ at 37 °C for 30 min. After washing by centrifugation ($3\times$ 0.7 ***g***, 5 min), spermatozoa were suspended to a concentration of $20\times10^6$ cells/ml in HM or HMB medium in the presence or absence of 2.8 μM GST-SPINK3. After a 60-min incubation, an aliquot of each treatment was placed onto a slide and observed under fluorescence microscope (excitation 480 nm; emission 525 nm) at 1000× magnification; spermatozoa showing fluorescence were captured with a Nikon DS-Fi1 camera connected to a computer with analytic software (Nis-Elements F 3.0, Nikon) attached to an inverted microscope Nikon Eclipse E200. Signal intensity was quantified at different sections of single sperm by image analysis using ImageJ 1.43 free software (National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/). $Ca^{2+}$ fluorescence intensity (excitation 460 nm; emission 525 nm) in the whole sperm suspension was measured immediately using an Ascent Microplate Fluorometer (Thermo Fisher Scientific, USA) at 37 °C for 45 min.

### NO production in sperm

#### *Determination of nitrite concentration by Griess method*

After incubation of spermatozoa under noncapacitating or capacitating conditions, aliquots of the sperm suspensions containing $30\times10^6$ sperm/ml were centrifuged (9700 ***g*** for 15 min) and NO production by sperm under different conditions (Assay Conditions) was estimated by assessing the levels of nitrate ($NO_3^-$) and nitrite ($NO_2^-$) in the culture media (supernatant), using a colorimetric kit (G2930; Promega) based on Griess reaction. Nitrite concentration was normalized to $1\times10^6$ sperm.

#### *NO detected with the fluorescent probe DAF-FM DA*

To measure the NO production in sperm cells, the pH insensitive NO indicator, DAF-FM DA cell permeate, was used. Inside the cell, DAF-FM DA is cleaved by esterases to generate intracellular DAF-FM, which is then oxidized by NO to a triazole product accompanied by increased fluorescence. The probe, 10 μM DAF-FM DA, was added either at the beginning, at 30 min, or at 60 min of incubation of sperm in a 96 well microplate under capacitating conditions in HMB medium (100 μl containing $20\times10^6$ sperm/ml) to evaluate the best time for its addition. NO fluorescence intensity (excitation 480 nm; emission 525 nm) was measured using an Ascent Microplate Fluorometer (Thermo Fisher Scientific) at 37 °C for 230 h. The NO production was the highest during the first

30 min of capacitation (Fig. 4A, lower panel), so the addition of the probe at the beginning of capacitation was the condition adopted for the experiment of the NO production under a fluorescent microscope.

To detect NO production under fluorescence microscope, 10 μM DAF-FM DA were added to motile sperm cells under different conditions (Assays Conditions). Following the incubation period, samples were washed with HM medium, incubated for 5 min and free DAF-FM DA washed by centrifugation (3×0.7 ***g***, 5 min). After washing, spermatozoa were suspended to a concentration of $5\times10^6$ sperm/ml in HM medium.

For detection of NO, slides were prepared by placing an aliquot of the sperm suspension on a glass slide and adding a cover slip mounted with glycerol: PBS (9:1). The presence of NO was detected under a fluorescent microscope 490/535 nm (Nikon Eclipse E200) at 1000× magnification by two independent observers. In each treatment, spermatozoa showing fluorescence were quantified as percentage of total sperm evaluated.

### Evaluation of spontaneous and $P_4$-induced AR

For this assay CF-1 mice were used due to their higher percentages of AR. Incubation was conducted under non-capacitating or capacitating conditions under different treatments (Assays Conditions) for 1 h at 37 °C in an atmosphere of 5% $CO_2$. Spontaneous AR was evaluated after this incubation period. For progesterone-induced AR, sperm incubated under each treatment were incubated with 10 μM $P_4$ for 15 min after capacitation. In both cases, AR was stopped by centrifugation (850 ***g*** for 10 min) and pelleted cells were fixed with 4% paraformaldehyde solution in PBS for 2 h. 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, smeared in glycerol: PBS (9:1), and observed under a light microscope at 1000× magnification by two independent observers. A blue stain over the sperm head dorsal and/or ventral edge was visualized in spermatozoa with intact acrosome, whereas no stain was observed in spermatozoa with reacted acrosome. Spermatozoa with acrosomal loss were quantified as a percentage over 200 sperm cells per replicate.

### Determination of sperm viability

Sperm viability was evaluated by trypan blue staining technique. After incubation, an aliquot of each sperm treatment (see Assays Conditions) was placed onto a glass slide with an equal volume of 0.2% trypan blue and immediately observed under a light microscope at 400× magnification by two independent observers and the percentage of light blue colored spermatozoa (dead) was recorded out of 100 cells.

### Determination of protein tyrosine phosphorylation

Sperm proteins corresponding to $0.75\times10^6$ sperm cells incubated under different conditions (Assays Conditions) were disrupted by boiling for 5 min with sample buffer (12 mM Tris–HCl (pH 6.2), 5% glycerol, 0.4% SDS, 0.02% bromophenol blue, and 100 mM dithiothreitol) and separated by electrophoresis on 12% SDS–PAGE. Then, the proteins were electrotransferred onto PVDF membranes in towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol) using a TE 70 PWRtransfer equipment (Amersham) at 80 mA for 2 h. Nonspecific binding sites on the membrane were blocked with 3% skimmed milk in Tris–buffered-saline (10 mM Tris–HCl (pH 7.51), 0.15 M NaCl, 0.1% Ipegal CA630–Sigma I3021). Afterward, the membrane was incubated overnight with 1:5000 anti-phosphotyrosine (MP, PY20 691371). The membrane was washed, incubated with 1:5000 anti-mouse IgG labeled with alkaline phosphatase (A3562 Sigma), and developed with 0.33 mg/ml nitro-blue tetrazolium and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphatase buffer (0.1 M Tris (pH 9.5); 0.1 M NaCl, and 5 mM $MgCl_2$).

### Evaluation of progressive sperm motility

Cauda epididymides were immersed in 1 ml HM medium contained in culture dishes on a warm plate at 37 °C as described above. After 10 min the sperm suspension was transferred to HMB medium (containing 3 mg/ml BSA instead of methyl-β-cyclodextrine) and distributed for the different treatments (Assays Conditions) at 37 °C and 5% $CO_2$. Samples presenting ≤35% PM and/or ≤70% TM at time zero were discarded. Motility was evaluated at 0 and 30 min after initiation of capacitation. An aliquot of each treatment was placed onto slides and observed under a light microscope at 400× magnification on a warm surface at 37 °C by two independent observers. Spermatozoa showing PM in different microscopic fields were quantified as percentage of total sperm evaluated ($n$=200 per replicate).

### Assays conditions

All the assays (immunocytochemistry, NO detection, $Ca^{2+}$ uptake, tyrosine phosphorylation, and motility) were performed using spermatozoa from BalbC mice, except for AR where CF-1 mice were employed. Viability was evaluated in sperm from both strains under all the treatments.

Motile mice sperm were incubated under noncapacitating (HM) or capacitating (HMB) media with or without either 2.8 μM GST-SPINK3, 2.8 μM GST, 100 μM SNP, 100 μM GSNO, and/or two $Ca^{2+}$ chelators (10 mM EDTA (pH 7.5) and 25 μM BAPTA-AM) as indicated in each assay. Unless specified, the reagents were added at the beginning of capacitation. At the concentrations assayed, no detrimental effect over sperm viability (~82%) was observed with none of the reagents. All treatments were incubated at 37 °C in an atmosphere of 5% $CO_2$/95% air for the times indicated.

### Statistical analysis

All data in the text and figures were expressed as means ± S.E.M. Statistical analysis was carried out using the Student's *t*-test for paired data (two-tailed), after having normalized the data by

arcsine transformation. Statistical significant differences were determined at $P<0.05$, unless indicated.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Funding

This work was supported by CONICET (PIP 112-220801-00232 and PIP 1783), ANPCyT (PICT2005 15-32925 and PICT2007 1244).

## Acknowledgements

We gratefully thank Dr Carlos Coronel for the supply of first aliquot of purified caltrin I from rat which was used for the setup of the assays. We also thank Dra. Cecilia Terrile for the supply of valuable reagents and vectors and all the Lab #8 team for their precious guidelines related to NO.

## References

**Abou-haila A & Tulsiani DR** 2009 Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. *Archives of Biochemistry and Biophysics* **485** 72–81. (doi:10.1016/j.abb.2009.02.003)

**Aitken RJ & Baker MA** 2004 Oxidative stress and male reproductive biology. *Reproduction, Fertility, and Development* **16** 581–588. (doi:10.1071/RD03089)

**Bendahmane M, Zeng HT & Tulsiani DR** 2002 Assessment of acrosomal status in rat spermatozoa: studies on carbohydrate and non-carbohydrate agonists. *Archives of Biochemistry and Biophysics* **404** 38–47. (doi:10.1016/S0003-9861(02)00278-3)

**Breitbart H, Wehbie RS, San Agustin J & Lardy HA** 1990 Inhibition by caltrin of calcium transport into spermatozoa, liver and heart mitochondria. *Biochimica et Biophysica Acta* **1022** 27–32. (doi:10.1016/0005-2736(90)90396-6)

**Chen LY, Lin YH, Lai ML & Chen YH** 1998 Developmental profile of a caltrin-like protease inhibitor, P12, in mouse seminal vesicle and characterization of its binding sites on sperm surface. *Biology of Reproduction* **59** 1498–1505. (doi:10.1095/biolreprod59.6.1498)

**Choi YH & Toyoda Y** 1998 Cyclodextrin removes cholesterol from mouse sperm and induces capacitation in a protein-free medium. *Biology of Reproduction* **59** 1328–1333. (doi:10.1095/biolreprod59.6.1328)

**Clark EN, Corron ME & Florman HM** 1993 Caltrin, the calcium transport regulatory peptide of spermatozoa, modulates acrosomal exocytosis in response to the egg's zona pellucida. *Journal of Biological Chemistry* **268** 5309–5316.

**Coronel CE & Lardy HA** 1992 Functional properties of caltrin proteins from seminal vesicle of the guinea pig. *Molecular Reproduction and Development* **33** 74–80. (doi:10.1002/mrd.1080330111)

**Coronel CE, Winnica DE, Novella ML & Lardy HA** 1992 Purification, structure, and characterization of caltrin proteins from seminal vesicle of the rat and mouse. *Journal of Biological Chemistry* **267** 20909–20915.

**Coronel CE, Novella ML, Winnica DE & Lardy HA** 1993 Isolation and characterization of a 54-kilodalton precursor of caltrin, the calcium transport inhibitor protein from seminal vesicles of the rat. *Biology of Reproduction* **48** 1326–1333. (doi:10.1095/biolreprod48.6.1326)

**Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, Barratt C, Kirkman-Brown J & Publicover S** 2009 $Ca^{2+}$-stores in sperm: their identities and functions. *Reproduction* **138** 425–437. (doi:10.1530/REP-09-0134)

**De Blas G, Michaut M, Trevino CL, Tomes CN, Yunes R, Darszon A & Mayorga LS** 2002 The intraacrosomal calcium pool plays a direct role in acrosomal exocytosis. *Journal of Biological Chemistry* **277** 49326–49331. (doi:10.1074/jbc.M208587200)

**Dematteis A, Miranda SD, Novella ML, Maldonado C, Ponce RH, Maldera JA, Cuasnicu PS & Coronel CE** 2008 Rat caltrin protein modulates the acrosomal exocytosis during sperm capacitation. *Biology of Reproduction* **79** 493–500. (doi:10.1095/biolreprod.107.067538)

**Furutani Y, Kato A, Kawai R, Fibriani A, Kojima S & Hirose S** 2004 Androgen-dependent expression, gene structure, and molecular evolution of guinea pig caltrin II, a WAP-motif protein. *Biology of Reproduction* **71** 1583–1590. (doi:10.1095/biolreprod.104.028993)

**Hardy E & Castellanos-Serra LR** 2004 "Reverse-staining" of biomolecules in electrophoresis gels: analytical and micropreparative applications. *Analytical Biochemistry* **321** 1–13. (doi:10.1016/j.ab.2004.02.017)

**Harrison RA & Gadella BM** 2005 Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology* **63** 342–351. (doi:10.1016/j.theriogenology.2004.09.016)

**Herrero MB & Gagnon C** 2001 Nitric oxide: a novel mediator of sperm function. *Journal of Andrology* **22** 349–356.

**Herrero MB, Perez Martinez S, Viggiano JM, Polak JM & de Gimeno MF** 1996 Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. *Reproduction, Fertility, and Development* **8** 931–934. (doi:10.1071/RD9960931)

**Herrero MB, de Lamirande E & Gagnon C** 1999 Nitric oxide regulates human sperm capacitation and protein-tyrosine phosphorylation *in vitro*. *Biology of Reproduction* **61** 575–581. (doi:10.1095/biolreprod61.3.575)

**Herrero M, Chatterjee S, Lefievre L, de Lamirande E & Gagnon C** 2000 Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. *Free Radical Biology & Medicine* **29** 522–536. (doi:10.1016/S0891-5849(00)00339-7)

**Herrick B, Schweissinger DL, Kim SW, Bayan KR, Mann S & Cardullo RA** 2005 The acrosomal vesicle of mouse sperm is a calcium store. *Journal of Cellular Physiology* **202** 663–671. (doi:10.1002/jcp.20172)

**Irwin M, Nicholson N, Haywood JT & Poirier GR** 1983 Immunofluorescent localization of a murine seminal vesicle proteinase inhibitor. *Biology of Reproduction* **28** 1201–1206. (doi:10.1095/biolreprod28.5.1201)

**Kalab P, Visconti P, Leclerc P & Kopf GS** 1994 p95, the major phosphotyrosine-containing protein in mouse spermatozoa, is a hexokinase with unique properties. *Journal of Biological Chemistry* **269** 3810–3817.

**Kulanand J & Shivaji S** 2001 Capacitation-associated changes in protein tyrosine phosphorylation, hyperactivation and acrosome reaction in hamster spermatozoa. *Andrologia* **33** 95–104. (doi:10.1046/j.1439-0272.2001.00410.x)

**Laemmli UK, Beguin F & Gujer-Kellenberger G** 1970 A factor preventing the major head protein of bacteriophage $T_4$ from random aggregation. *Journal of Molecular Biology* **47** 69–85. (doi:10.1016/0022-2836(70)90402-X)

**Lai ML, Li SH & Chen YH** 1994 Purification and biochemical characterization of a recombinant mouse seminal vesicle trypsin inhibitor produced in *Escherichia coli*. *Protein Expression and Purification* **5** 22–26. (doi:10.1006/prep.1994.1003)

**de Lamirande E & Lamothe G** 2009 Reactive oxygen-induced reactive oxygen formation during human sperm capacitation. *Free Radical Biology & Medicine* **46** 502–510. (doi:10.1016/j.freeradbiomed.2008.11.004)

**de Lamirande E, Yoshida K, Yoshiike TM, Iwamoto T & Gagnon C** 2001 Semenogelin, the main protein of semen coagulum, inhibits human sperm capacitation by interfering with the superoxide anion generated during this process. *Journal of Andrology* **22** 672–679.

**de Lamirande E, Lamothe G & Villemure M** 2009 Control of superoxide and nitric oxide formation during human sperm capacitation. *Free Radical Biology & Medicine* **46** 1420–1427. (doi:10.1016/j.freeradbiomed.2009.02.022)

**Lardy H** 2003 Happily at work. *Journal of Biological Chemistry* **278** 3499–3509. (doi:10.1074/jbc.X200003200)

**Leclerc P, de Lamirande E & Gagnon C** 1996 Cyclic adenosine 3′,5′monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biology of Reproduction* **55** 684–692. (doi:10.1095/biolreprod55.3.684)

**Leclerc P, de Lamirande E & Gagnon C** 1998 Interaction between $Ca^{2+}$, cyclic 3′,5′ adenosine monophosphate, the superoxide anion, and tyrosine phosphorylation pathways in the regulation of human sperm capacitation. *Journal of Andrology* **19** 434–443.

**Lee CH, Anderson M & Chien YW** 1996 Characterization of *in-vitro* spermicidal activity of chelating agent against human sperm. *Journal of Pharmacological Sciences* **85** 649–654. (doi:10.1021/js9501573)

**Lin HJ, Luo CW, Wang CY & Chen YH** 2006 Epitope topology and removal of mouse acrosomal plasma membrane by P12-targeted immunoaggregation. *Biochemical and Biophysical Research Communications* **349** 284–288. (doi:10.1016/j.bbrc.2006.08.045)

**Lin MH, Lee RK, Hwu YM, Lu CH, Chu SL, Chen YJ, Chang WC & Li SH** 2008 SPINKL, a Kazal-type serine protease inhibitor-like protein purified from mouse seminal vesicle fluid, is able to inhibit sperm capacitation. *Reproduction* **136** 559–571. (doi:10.1530/REP-07-0375)

**Lu CH, Lee RK, Hwu YM, Chu SL, Chen YJ, Chang WC, Lin SP & Li SH** 2011 SERPINE2, a serine protease inhibitor extensively expressed in adult male mouse reproductive tissues, may serve as a murine sperm decapacitation factor. *Biology of Reproduction* **84** 514–525. (doi:10.1095/biolreprod.110.085100)

**Luo CW, Lin HJ, Gopinath SC & Chen YH** 2004 Distinction of sperm-binding site and reactive site for trypsin inhibition on p12 secreted from the accessory sex glands of male mice. *Biology of Reproduction* **70** 965–971. (doi:10.1095/biolreprod.103.020552)

**Machado-Oliveira G, Lefievre L, Ford C, Herrero MB, Barratt C, Connolly TJ, Nash K, Morales-Garcia A, Kirkman-Brown J & Publicover S** 2008 Mobilisation of $Ca^{2+}$ stores and flagellar regulation in human sperm by *S*-nitrosylation: a role for NO synthesised in the female reproductive tract. *Development* **135** 3677–3686. (doi:10.1242/dev.024521)

**Meiser H & Schulz R** 2003 Detection and localization of two constitutive NOS isoforms in bull spermatozoa. *Anatomia, Histologia, Embryologia* **32** 321–325. (doi:10.1111/j.1439-0264.2003.00459.x)

**Mills JS, Needham M & Parker MG** 1987 A secretory protease inhibitor requires androgens for its expression in male sex accessory tissues but is expressed constitutively in pancreas. *EMBO Journal* **6** 3711–3717.

**Miraglia E, De Angelis F, Gazzano E, Hassanpour H, Bertagna A, Aldieri E, Revelli A & Ghigo D** 2011 Nitric oxide stimulates human sperm motility via activation of the cyclic GMP/protein kinase G signaling pathway. *Reproduction* **141** 47–54. (doi:10.1530/REP-10-0151)

**Muratori M, Marchiani S, Tamburrino L, Forti G, Luconi M & Baldi E** 2011 Markers of human sperm functions in the ICSI era. *Frontiers in Bioscience* **16** 1344–1363. (doi:10.2741/3793)

**Novella ML, Maldonado C, Aoki A & Coronel CE** 1999 Androgen-dependent synthesis/secretion of caltrin, calcium transport inhibitor protein of mammalian seminal vesicle. *Archives of Andrology* **43** 1–12. (doi:10.1080/014850199262689)

**O'Flaherty C, de Lamirande E & Gagnon C** 2006 Reactive oxygen species modulate independent protein phosphorylation pathways during human sperm capacitation. *Free Radical Biology & Medicine* **40** 1045–1055. (doi:10.1016/j.freeradbiomed.2005.10.055)

**Okazaki T, Yoshida S, Teshima H & Shimada M** 2011 The addition of calcium ion chelator, EGTA to thawing solution improves fertilizing ability in frozen-thawed boar sperm. *Animal Science Journal* **82** 412–419. (doi:10.1111/j.1740-0929.2010.00856.x)

**O'Rand MG, Widgren EE, Wang Z & Richardson RT** 2007 Eppin: an epididymal protease inhibitor and a target for male contraception. *Society of Reproduction and Fertility Supplement* **63** 445–453.

**Ou CM, Lin SR, Lin HJ, Luo CW & Chen YH** 2010 Exclusive expression of a membrane-bound Spink3-interacting serine protease-like protein TESPL in mouse testis. *Journal of Cellular Biochemistry* **110** 620–629. (doi:10.1002/jcb.22571)

**Ou CM, Tang JB, Huang MS, Sudhakar Gandhi PS, Geetha S, Li SH & Chen YH** 2012 The mode of reproductive-derived Spink (serine protease inhibitor Kazal-type) action in the modulation of mammalian sperm activity. *International Journal of Andrology* **35** 52–62. (doi:10.1111/j.1365-2605.2011.01159.x)

**Patrat C, Serres C & Jouannet P** 2000 Induction of a sodium ion influx by progesterone in human spermatozoa. *Biology of Reproduction* **62** 1380–1386. (doi:10.1095/biolreprod62.5.1380)

**Phillips DM & Bedford JM** 1988 Sperm–sperm association in the Loris epididymis. *Gamete Research* **18** 17–25. (doi:10.1002/mrd.1120180104)

**Pons-Rejraji H, Bailey JL & Leclerc P** 2009 Modulation of bovine sperm signalling pathways: correlation between intracellular parameters and sperm capacitation and acrosome exocytosis. *Reproduction, Fertility, and Development* **21** 511–524. (doi:10.1071/RD07169)

**Ren SS, Sun GH, Ku CH, Chen DC & Wu GJ** 2004 Comparison of four methods for sperm preparation for IUI. *Archives of Andrology* **50** 139–143. (doi:10.1080/01485010490425566)

**Rodriguez PC, Valdez LB, Zaobornyj T, Boveris A & Beconi MT** 2011 Nitric oxide and superoxide anion production during heparin-induced capacitation in cryopreserved bovine spermatozoa. *Reproduction in Domestic Animals* **46** 74–81. (doi:10.1111/j.1439-0531.2010.01583.x)

**Roessner C, Paasch U, Glander HJ & Grunewald S** 2010 Activity of nitric oxide synthase in mature and immature human spermatozoa. *Andrologia* **42** 132–137. (doi:10.1111/j.1439-0272.2009.01005.x)

**San Agustin JT, Hughes P & Lardy HA** 1987 Properties and function of caltrin, the calcium-transport inhibitor of bull seminal plasma. *FASEB Journal* **1** 60–66.

**Schaad NC, Zhang XQ, Campana A & Schorderet-Slatkine S** 1996 Human seminal plasma inhibits brain nitric oxide synthase activity. *Human Reproduction* **11** 561–565.

**Shima JE, McLean DJ, McCarrey JR & Griswold MD** 2004 The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. *Biology of Reproduction* **71** 319–330. (doi:10.1095/biolreprod.103.026880)

**Thundathil J, de Lamirande E & Gagnon C** 2003 Nitric oxide regulates the phosphorylation of the threonine–glutamine–tyrosine motif in proteins of human spermatozoa during capacitation. *Biology of Reproduction* **68** 1291–1298. (doi:10.1095/biolreprod.102.008276)

**Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P & Kopf GS** 1995 Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* **121** 1139–1150.

**Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S & Diekman AB** 2002 Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *Journal of Reproductive Immunology* **53** 133–150. (doi:10.1016/S0165-0378(01)00103-6)

**Wang Z, Widgren EE, Sivashanmugam P, O'Rand MG & Richardson RT** 2005 Association of eppin with semenogelin on human spermatozoa. *Biology of Reproduction* **72** 1064–1070. (doi:10.1095/biolreprod.104.036483)

**Wang ZJ, Zhang W, Feng NH, Song NH, Wu HF & Sui YG** 2008 Molecular mechanism of epididymal protease inhibitor modulating the liquefaction of human semen. *Asian Journal of Andrology* **10** 770–775. (doi:10.1111/j.1745-7262.2008.00393.x)

**Winnica DE, Novella ML, Dematteis A & Coronel CE** 2000 Trypsin/acrosin inhibitor activity of rat and guinea pig caltrin proteins. Structural and functional studies. *Biology of Reproduction* **63** 42–48. (doi:10.1095/biolreprod63.1.42)

**Yanagimachi R** 1994 Mammalian fertilisation. In *The Physiology of Reproduction*, pp 189–317. Eds E Knobil& J Neill. New York: Raven Press.

**Zhang J, Ding X, Bian Z, Xia Y, Lu C, Wang S, Song L & Wang X** 2010 The effect of anti-eppin antibodies on ionophore A23187-induced calcium influx and acrosome reaction of human spermatozoa. *Human Reproduction* **25** 29–36. (doi:10.1093/humrep/dep356)

Received 4 April 2011
First decision 3 May 2011
Revised manuscript received 20 December 2011
Accepted 6 January 2012