Src family tyrosine kinase regulates acrosome reaction but not motility in porcine spermatozoa

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Abstract
During the capacitation process, spermatozoa acquire the ability to fertilize an oocyte, and upregulation of cAMP-dependent protein tyrosine phosphorylation occurs. Recently, Src family tyrosine kinase (SFK) has been involved in spermatozoa capacitation as a key PKA-dependent tyrosine kinase in several species. This work investigates the expression and role of SFK in porcine spermatozoa. SFK members Lyn and Yes are identified in porcine spermatozoa by western blotting as well as two proteins named SFK1 and SFK2 were also detected by their tyrosine 416 phosphorylation, a key residue for SFK activation. Spermatozoa with SFK1 and SFK2 increase their Y416 phosphorylation time-dependently under capacitating conditions compared with noncapacitating conditions. The specific SFK inhibitor SU6656 unaffected porcine spermatozoa motility or viability. Moreover, SFK inhibition in spermatozoa under capacitating conditions leads to a twofold increase in both nonstimulated and calcium-induced acrosome reaction. Our data show that capacitating conditions lead to a time-dependent increase in actin polymerization in boar spermatozoa and that long-term incubation with SFK inhibitor causes a reduction in the F-actin content. In summary, this work shows that the SFK members Lyn and Yes are expressed in porcine spermatozoa and that SFK1 and SFK2 are phosphorylated (activated) during capacitation. Our results point out the important role exerted by SFK in the acrosome reaction, likely mediated in part by its involvement in the actin polymerization process that accompanies capacitation, and rule out its involvement in porcine spermatozoa motility.

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Introduction
The main prerequisite for mammalian spermatozoa to bind and fertilize the oocyte is the process known as capacitation, which occurs in the female reproductive tract. It is widely accepted that after capacitation, spermatozoa have obtained the ability to fertilize an egg. The sperm capacitated state is correlated with different physiological and biochemical changes in these male germ cells, which include loss of cholesterol from plasma membrane, modifications in intracellular ion levels, increased membrane fluidity, hyperpolarization, hyperactivated motility, and increased intracellular protein tyrosine phosphorylation to render spermatozoa competent to recognize the oocyte and initiate fertilization (reviewed in Yanagimachi (1994)). The main sperm physiological processes are regulated by bicarbonate and Ca2+ ions that activate an atypical soluble adenylyl cyclase (SACY) present in spermatozoa, which catalyzes cAMP synthesis that allosterically activates protein kinase A. Activation of this kinase promotes phosphorylation of protein substrates in Ser/Thr residues, and in addition, PKA is an essential regulator of the protein tyrosine phosphorylation linked to spermatozoa function. However, the tyrosine kinase(s) responsible for the capacitation-associated increase in tyrosine phosphorylation has remained unknown. Recently, it has been proposed that Src family of protein tyrosine kinase (SFKs) mediates tyrosine phosphorylation in mouse (Baker et al. 2006) and human spermatozoa (Lawson et al. 2008, Mitchell et al. 2008, Varano et al. 2008). In mouse spermatozoa, another tyrosine kinase, c-Abl, has been involved together with Src (Baker et al. 2009).

The biological action of SFKs in mammalian somatic cells has been extensively studied and demonstrated to be pleiotropic. The prototypic member of SFK, c-Src, is activated through autophosphorylation of Y416 residue located in the activation loop, which induces a change to an open state (Roskoski 2005), such that phosphorylation of this residue correlates well with the

It has been shown that c-Src is expressed in mouse spermatozoa (Baker et al. 2006, Krapf et al. 2010) and that the specific inhibitor of c-Src kinase family, SU6656, blocks the capacitation-associated increase in protein tyrosine phosphorylation, as well as sperm motility (Krapf et al. 2010) and capacitation-associated hyperactivation (Baker et al. 2006). As this compound inhibits the kinase activity by competing with ATP, affects all members of the Src family, which we named SFK1 and SFK2, can be detected in porcine spermatozoa lysates when phosphorylated in the tyrosine 416 residue (Fig. 1C). As autophosphorylation of Y416 correlates well with SFK enzyme activity (Amini et al. 1986, Cartwright et al. 1989, Katagiri et al. 1989, Bagrodia et al. 1993, Kralisz & Cierniewski 2000), these results show that SFK members expressed in boar spermatogenesis.

Results

Identification of SFK in porcine spermatozoa

A western blotting analysis was performed to determine whether members of SFK are expressed in porcine spermatozoa. Two cross-reactive bands are present in boar spermatozoa lysates using anti-Lyn antibody (Fig. 1A, lanes 1, 2 and 3), whereas in porcine liver, lung, and brain, there is a unique band of 60 kDa (lanes 4, 5, and 6). The use of anti-Yes antibody reveals that this member of Src family is also expressed in porcine spermatozoa lysates (Fig. 1B), where two cross-reactive bands are also present, similar to data found in somatic cell types such as rat pancreatic acini (Fig. 1B, lane 4) and neuroblasts (Fig. 1B, lane 5 and 6). Western blot analysis with an antibody that specifically recognizes tyrosine phosphorylated at position 416 of SFK (anti-SFK-pY416) shows that at least two protein bands corresponding with the members of Src family, which we named SFK1 and SFK2, can be detected in porcine spermatozoa lysates when phosphorylated in the tyrosine 416 residue (Fig. 1C). As autophosphorylation of Y416 correlates well with SFK enzyme activity (Amini et al. 1986, Cartwright et al. 1989, Katagiri et al. 1989, Bagrodia et al. 1993, Kralisz & Cierniewski 2000), these results show that SFK members expressed in boar spermatogenesis.

In this work, we study the expression of Src family kinase members in porcine spermatozoa as well as regulation of their phosphorylation in Tyr416 as an assessment of their activity. We also investigate the role of Src family kinase in characteristic spermatozoa functional processes such as viability, motility, and acrosome reaction. Our results point out the important role exerted by SFK in the control of the acrosome reaction and rule out its involvement in porcine spermatozoa viability and motility.

Figure 1 Identification of SRC family kinase (SFK) members in porcine spermatozoa. Semen samples from several boars were lysed and run in a 10% SDS–PAGE, and western blotting was performed using anti-Lyn (A), anti-c-Yes (B), and anti-p-Y416 SFK (C) antibodies as described. Positive controls of different porcine tissues: liver, lung, and brain (A, lanes 4, 5 and 6), or somatic cell types such as rat pancreatic acini (B, lane 4) and neuroblasts (B, lane 5 and 6) were also included.

Figure 2 Enzymatic activation of SFK during porcine spermatozoa capacitation. Spermatozoa samples incubated under noncapacitating (TBM) or capacitating conditions (TCM) during 4 h were lysated and run in a 10% SDS–PAGE, and western blotting analysis was performed using anti-p-Y416 SFK or anti-actin as a protein loading control. Panel A shows a representative experiment for SFK1 and SFK2 tyrosine phosphorylation at the indicated times. Values shown at the bottom graphs in Panel B are mean ± S.E.M. of three independent experiments. Quantification of bands was performed by scanning densitometry. Asterisk shows statistical differences between samples incubated under capacitating conditions in TCM with those incubated in TBM at the same time point.
spermatozoa, SFK1 and SFK2, are slightly phosphorylated in Tyr416 under noncapacitating conditions (Tyrode’s basal medium (TBM)).

**Enzymatic activation of SFK during porcine spermatozoa capacitation**

In order to investigate the function of Src during the main processes of boar sperm activation, we first attempted to evaluate SFK activity during in vitro spermatozoa capacitation using the above-mentioned specific antibody directed against the phosphorylated tyrosine at 416, identifying the active kinase form of the SFK (Roskoski 2005). During the time-dependent process of capacitation (Tyrode’s complete medium (TCM)), there is a significant increase in phosphorylation of Tyr416 in both members, SFK1 and SFK2 (Fig. 2), when compared with noncapacitating medium (TBM).

**SFK inhibition enhances spontaneous acrosome reaction in porcine spermatozoa**

We next investigated whether the SFK plays a role in the spontaneous acrosome reaction by preincubating porcine spermatozoa with the specific SFK inhibitor SU6656 (Blake et al. 2000) and measuring the acrosome-reacted spermatozoa by flow cytometry. SU6656 compound is a competitive inhibitor of ATP binding to SFK and we have chosen 10 μM SU6656 as this concentration has been used previously in mouse spermatozoa (Baker et al. 2006). In spermatozoa incubated under noncapacitating conditions, SU6656 is able to slightly but significantly increase the spontaneous acrosome reaction (Fig. 3). Incubation of spermatozoa with SU6656 in a capacitating medium (TCM) causes a significant twofold increase in the number of acrosome-reacted spermatozoa, reaching a maximum of 33% of PNA-positive and PI-negative spermatozoa at 60 min (Fig. 3).

**SFK inhibition enhances calcium-stimulated acrosome reaction in porcine spermatozoa**

In order to investigate the function of SFK in calcium-stimulated acrosome reaction, we have incubated porcine spermatozoa at different times in TCM at 38.5 °C with the calcium ionophore A23187 (1 μM) in the absence or presence of SU6656 (10 μM) and measured the acrosome-reacted spermatozoa by flow cytometry. Calcium ionophore leads to a clear and significant increase in the percentage of acrosome-reacted spermatozoa after 60 min of treatment (Fig. 4). Preincubation with the SFK inhibitor causes a statistically significant increase in the percentage of acrosome-reacted spermatozoa in the

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Figure 3 SFK inhibition enhances spontaneous acrosome reaction in porcine spermatozoa. Spermatozoa samples were incubated under noncapacitating (TBM) or capacitating conditions (TCM) in the presence or absence of the SFK inhibitor SU6656 (10 μM) for 4 h. Flow cytometry was then performed using FITC-PNA as a specific marker for spermatozoa acrosome status and propidium iodide (PI) as a marker for cell death. Results are expressed as the percentage of PNA-positive and PI-negative spermatozoa ± s.e.m. (n=6). Asterisk shows statistical differences of samples incubated with or without SU6656 at the same time point.

Figure 4 SFK inhibition enhances ionophore-induced acrosome reaction in porcine spermatozoa. Spermatozoa were incubated under capacitating conditions (TCM) with the ionophore A23187 (1 μM) in the presence or absence of the SFK inhibitor SU6656 (10 μM) for 4 h. Flow cytometry was then performed using FITC-PNA as a specific marker for spermatozoa acrosome status and propidium iodide (PI) as a marker for cell death. Results are expressed as the percentage of PNA-positive and PI-negative spermatozoa ± s.e.m. (n=4). Asterisk shows statistical differences of samples incubated with or without SU6656 at the same time point.
presence of the ionophore after 30 min treatment (Fig. 4). Moreover, inhibition of SFK significantly increases the maximum of ionophore-stimulated acrosome-reacted spermatozoa, 58.45 ± 4.4 vs 39.01 ± 1.0 respectively.

**Effect of SFK inhibition on F-actin content in porcine spermatozoa**

The incubation of boar spermatozoa in a capacitating medium induces a clear increase in the percentage of spermatozoa highly stained with FITC–phalloidin that indicates a higher F-actin content (or higher polymerization of actin) in a time-dependent manner (Fig. 5B, solid bars). As shown in Fig. 5B, the maximum effect of actin polymerization is observed at 4 h in TCM, where 61.98% ± 2.47 of spermatozoa have high fluorescence intensity compared with 23.9% ± 2.34 of spermatozoa at time 0. Our data confirm that the induction of acrosome reaction to capacitated spermatozoa (2 h in TCM) by adding calcium ionophore A23187 (10 μM) for 30 min clearly decreases the F-actin spermatozoa content to a basal level detected at time 0 (Fig. 5A). The preincubation of spermatozoa with SFK inhibitor causes a decrease in the percentage of spermatozoa with high F-actin content (striped bars in Fig. 5B), which is visible at 2 h and statistically significant at 4 h of incubation in TCM (47.50 ± 3.02% in the presence of SU6656 vs 61.98 ± 2.47% in the absence of SU6656).

**SFK inhibition does not affect motility of porcine spermatozoa**

To study the physiological role of SFK family in porcine spermatozoa motility, these male germ cells were preincubated for 30 min with the inhibitor SU6656 under capacitating and/or noncapacitating conditions, and sperm motility parameters were evaluated by computer-assisted sperm analysis (CASA) system as described. The inhibition of SFK with SU6656 has no effect in any motility parameter evaluated under our experimental conditions (Table 1).

**SFK inhibition does not affect the viability of porcine spermatozoa**

As some results of this work are based on a pharmacological approach, we ensured that the concentration and/or time used for SFK inhibitor SU6656 are not inducing unspecific effects that might cause a loss in porcine germ cell viability. We measured spermatozoa viability by flow cytometry using IP as a probe over 240 min, and data show that there are not statistical differences between spermatozoa samples treated with or without SU6656, under both capacitating and noncapacitating conditions at any time (Table 2).
Porcine spermatozoa were washed and incubated for 1 h at 38.5 °C in a noncapacitating medium (TBM) or in a capacitating medium (TCM) in the presence or absence of the SFK inhibitor SU6656 (10 μM). After incubation, motility parameters were evaluated in the sperm samples by CASA and values shown are expressed as mean ± S.E.M. (n = 6). Within a row, values with different superscripts are statistically different from each other, so that for (a and b), P < 0.05.

**Discussion**

The expression of Src family members in germ cells has been studied during mammalian spermatogenesis in the rat (Nishio et al. 1995) and human (Lawson et al. 2008). In addition, the presence of Src family members has been described in spermatozoa of rat (Zhao et al. 1990), mouse (Baker et al. 2006, Krapf et al. 2010), and human (Leclerc & Goupil 2002, Kumar & Meizel 2005, Lawson et al. 2008, Mitchell et al. 2008, Varano et al. 2008). Moreover, this is the first work that demonstrates the protein expression of SFK members in porcine spermatozoa, including c-yes, c-lyn, and two active isoforms, named SFK1 and SFK2, detected by their Tyr416 phosphorylation.

Our results demonstrate that SFK is not involved in the regulation of spermatozoa motility in boar, which is in agreement with the previous results using the same SFK inhibitor SU6656 in human spermatozoa (Varano et al. 2008). By contrast, a different result is obtained in mouse spermatozoa, where SU6656 significantly inhibits all motility parameters (Krapf et al. 2010) and is able to suppress the sperm capacitation-associated hyperactivation (Baker et al. 2006). Some of the discrepancies may be explained by the different roles supported by several apparent isoforms of Src in the different sperm species studied, in addition to their intracellular localization.

Previous studies on somatic cells such as T-cells, carcinoma cell lines, embryo fibroblasts, and platelets have demonstrated that upon activation, Src undergoes a process of autophosphorylation at the Tyr residue 416 (Amini et al. 1986, Cartwright et al. 1989, Katagiri et al. 1989, Bagrodia et al. 1993, Kralisz & Cierniewski 2000).
filaments and therefore allowing actin polymerization to occur. Moreover, before acrosome reaction, gelsolin is activated by Tyr dephosphorylation and allows the necessary F-actin depolimerization (Finkelstein et al. 2010). Based on these data, one might expect in porcine spermatozoa that SFK activity would cause Tyr phosphorylation-induced gelsolin inactivation and F-actin polymerization during the capacitation process. The effect of SFK inhibition in porcine spermatozoa would cause gelsolin to become activated and F-actin dispersed, a necessary requirement for acrosome reaction to occur. Unfortunately, commercially available antibodies against gelsolin used by us during this work were not able to recognize the protein in pig, and therefore, we were unable to confirm the gelsolin hypothesis. However, our data demonstrate that during capacitation of boar spermatozoa, when SFK is activated, a time-dependent increase in F-actin content is clearly detected. Moreover, inhibition of SFK during capacitation causes a decrease in the F-actin content in boar spermatozoa. These results support the mentioned idea that SFK plays a role in actin polymerization during spermatozoa capacitation and that its inhibition contributes to F-actin dispersion, which is necessary for the acrosome reaction to occur. In this regard, our results suggest that SFK is involved in the regulation of acrosome reaction that occurs in boar spermatozoa based on the following reasons: a) during capacitation, the physiological and previous process of the sperm acrosome reaction that occurs in parallel to actin polymerization, SFK activity is stimulated. b) SFK inhibitor leads to a decrease in the actin polymerization that occurs during capacitation of boar spermatozoa. c) SFK inhibitor is able to increase the acrosome reaction in the absence of external agonist under capacitating conditions. d) SFK inhibition leads to a further increase in the calcium-induced acrosome reaction under capacitating conditions.

It is well established that a moderate increase in intracellular calcium concentration induced by the addition of ionophore to a capacitating medium produces a significant increase in the percentage of acrosome-reacted spermatozoa. In addition, it has been described in human spermatozoa that the activation of c-SRC requires calcium (Lawson et al. 2008). Our data show that SFK inhibition in a capacitating medium in the presence of ionophore induces a significant increase in the percentage of acrosome-reacted spermatozoa in boar, which would support the possible inhibitory role of SFK in the nonstimulated acrosome reaction by agonists. Recently, it has been demonstrated that different SFK inhibitors block the acrosome reaction induced by different agonists such as lysophosphatidic acid or angiotensin II in bull (Etkovitz et al. 2009), whereas in humans, the SFK inhibitor SU6656 abrogates progesterone-induced acrosome reaction (Varano et al. 2008) by interfering with the Ca2+ response elicited by progesterone. As mentioned, all these results are based on the effect of SFK inhibitors in the acrosome reaction experimentally achieved by different stimuli that induce intracellular signals through SFK but also through many other signaling pathways in sperm. In this sense, it is widely accepted that the acrosome reaction is a sperm function orchestrated by the simultaneous actions of different transduction pathways. Therefore, and besides the different specie studied in this work, we think that the comparison of the effect of SFK inhibition in the nonstimulated and agonist-induced acrosome reaction under capacitating conditions is unwarranted.

In addition, as SFK has been shown to regulate Ser/Thr phosphatases in other cell types (Chen et al. 1992, 1994), we cannot obviate a possible effect of SFK inhibitor regulating Ser/Thr phosphatases in porcine spermatozoa, as Krapf et al. (2010) recently demonstrated in mouse sperm.

We ruled out the possibility that SU6656 concentration might be affecting other intracellular targets in porcine spermatozoa by confirming that viability is not compromised after 4 h treatment of spermatozoa with SU6656 under the same conditions.

Based on our results, we hypothesize that SFK activation during porcine spermatozoa capacitation leads to inhibition of the acrosome reaction, likely due in part to the involvement of SFK in actin polymerization. This could explain that after inhibition of SFK under capacitating conditions, the F-actin content partially decreases and the number of acrosome-reacted spermatozoa increases by twofold under any conditions, in the presence or in the absence of calcium ionophore. As defined by Yanagimachi (1994), the acrosome reaction may also take place spontaneously in the absence of external stimulus in vitro in a capacitating medium. Thus, we hypothesize that SFK activity is important for repressing the sperm acrosome reaction that occurs under capacitating conditions without external agonists. In most species, spermatozoa that have undergone the spontaneous acrosome reaction have lost receptor(s) for recognizing and binding to the zona pellucida and are therefore unable to bind to a zona-intact egg and consequently they are not capable to fertilize the egg (Yanagimachi 1994).

Our hypothesis points out that SFK activity, by regulating the actin polymerization process during spermatozoa capacitation, might be necessary to avoid the spontaneous acrosome reaction that might exist in capacitated porcine spermatozoa and could represent a regulatory mechanism that inhibits acrosome reaction to ensure that the population of capacitated spermatozoa remains capacitated until physiological agonists induce the acrosome reaction.

Although there are some discrepancies between species about the physiological function of SFK in spermatozoa, which may be explained by the different expression of SFK members, by their relative cellular roles and by their different intracellular localization, we think that our work points out an important role of SFK in the regulation of the sperm acrosome reaction.
In summary, we demonstrate in this work that i) porcine germ cells express several members of SFK, including Lyn, Yes, SFK1, and SFK2; ii) SFK1 and SFK2 are activated during porcine spermatozoa capacitation; iii) SFK inhibition increases nonstimulated and calcium-induced acrosome reaction; iv) SFK inhibition causes a decrease in the F-actin content in capacitated spermatozoa. In addition, this study suggests that SFK is not involved in the biochemical control of neither motility nor viability in porcine spermatozoa. However, during sperm capacitation, activated SFK plays an inhibitory role mediated, at least partially, through the control of actin polymerization, in the regulation of one of the most relevant functions of male germ cells: the acrosome reaction.

Materials and Methods

Chemicals, equipments, and sources

Live/dead spermatozoa viability kit was purchased from Molecular Probes (Leiden, The Netherlands). Complete, EDTA-free, protease inhibitor cocktail was from Roche Diagnostics. Tris/glycine/SDS buffer (10×) and Tris/glycine buffer (10×) were from Bio-Rad (Richmond, CA, USA). Hyperfilm ECL was from GE Healthcare Europe (Freiburg, Germany). Enhanced chemiluminescence detection reagents, HRP-conjugated anti-mouse IgG, and HRP-conjugated anti-rabbit IgG were from Pierce (Rockford, IL, USA). Nitrocellulose membranes were from Schleicher & Schuell, BioScience (Keene, NH, USA). FITC-PNA and anti-actin antibody were from Sigma–Aldrich; anti-Yes antibody was from BD Transduction Laboratories (Franklin Lakes, NJ, USA); anti-pY416 SFK was from Cell Signaling (Beverly, CA, USA); anti-Lyn antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); SU6656 and A23187 from Calbiochem (La Jolla, CA, USA); and FITC–phalloidin from Life Technologies.

Media

TCM was used as spermatozoa capacitating medium (Aparicio et al. 2005) and consisted of 96 mM NaCl, 4.7 mM KCl, 0.4 mM MgSO4, 0.3 mM Na2HPO4, 5.5 mM glucose, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 1 mM CaCl2, 10 mM NaHCO3, 20 mM Hepes (pH 7.45), and 3 mg/ml BSA. TCM was equilibrated with 95% O2 and 5% CO2. A variant of the TCM medium, which is a noncapacitating osmolarity of 290–310 mOsm/kg.

Collection and preparation of porcine semen

Commercial semen doses from Iberian boars of proven fertility and routinely used for artificial insemination were used. Semen doses were obtained from Semen Porcino Andalucia (Sevilla, Spain) and consisted in 80 ml of a commercial extender (MR-A, Kubus, Madrid, Spain) containing 30×10⁸ spermatozoa per dose. All doses were stored for 12 h at 17 °C before use and, in order to minimize individual variation between boars in each experiment, semen from up to four animals was pooled using doses from a minimum of eight boars in different combinations. Semen pool was centrifuged for 3 min at 2000 g and washed with TBM. Samples of 1.5 ml containing 100×10⁸ spermatozoa per milliliter were incubated at 38.5 °C in TCM, with or without ionophore A23187 (1 or 10 μM), or TBM for different times. In experiments involving the SFK inhibitor SU6656, spermatozoa were preincubated with 10 μM SU6656 for 30 min at 38.5 °C. In order to minimize possible experimental variations, every experiment with different treatments was performed in the same semen pool. When necessary, a control with the final concentration of the solvent (dimethyl sulfoxide (DMSO) 0.1%) was included.

Western blotting

Spermatozoa samples after different treatments were centrifuged for 15 s at 7000 g, washed with PBS supplemented with 0.2 mM Na3VO4, and then lysated in a lysis buffer consisting of 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 1 mM EGTA, 0.4 mM EDTA, protease inhibitors cocktail (complete, EDTA-free), and 0.2 mM Na2VO4 by sonication for 5 s at 4 °C. After 30 min, samples were then centrifuged for 15 min at 10 000 g (4 °C) and the supernatant was used for analysis of protein concentration. Proteins from porcine spermatozoa lysates were resolved by 10% SDS–PAGE and electrotransferred to nitrocellulose membranes. Western blotting was performed as described previously (Aparicio et al. 2003) using anti-pY416 SFK (1:1000), anti c-Yes (1:1000), and anti c-Lyn (1:1000) as primary antibodies.

Spermatozoa motility evaluation by CASA system

Sperm motility analysis is based on the examination of 25 consecutive digitalized images obtained from a single field using a 10× negative phase-contrast objective. Images were taken with a time lapse of 1 s. The setting parameters for the ISAS program (Projectes i Serveis R+D, SL; Buriol, Spain) were as follows: spermatozoa with an average path velocity (VAP) <10 μm/s were considered immobile, while spermatozoa with a VAP >10 μm/s were considered motile. Spermatozoa with a VAP between 10–15 and 16–45 μm/s were considered as low and medium speed, respectively, whereas those with a VAP >45 μm/s were considered as rapid speed spermatozoa. Male germ cells deviated <10% from a straight line were designated linear motile.

Evaluation of the number of acrosome-reacted spermatozoa by flow cytometry

Flow cytometry analysis was performed using a Coulter EPICS XL flow cytometer (Beckman Coulter Ltd., Brea, CA, USA) equipped with a 15 mW argon laser with excitation at 488 nm. Side- and forward-light scatter parameters were used to identify the spermatozoa and 10 000 sperm cells per sample were collected at low sample pressure. Light-scatter data were collected in a linear mode while fluorescence data were collected in a logarithmic mode. The acrosome status of spermatozoa by flow cytometry.
spermatozoa was examined by incubating samples for 5 min with 2 µg/ml FITC-PNA, as a specific marker for spermatozoa acrosome status, and 6 µmol/l propidium iodide (PI), as a marker for cell death (Waterhouse et al. 2004). Results are expressed as the percentage of PNA-positive and PI-negative spermatozoa ± S.E.M.

**Evaluation of the F-actin content in spermatozoa by flow cytometry**

After incubation in TCM in the presence or absence of the SFK inhibitor SU6656, spermatozoa were fixed in 4% formaldehyde in PBS for 20 min, washed and placed in 0.25% Triton X-100 in PBS for 10 min, washed three times at 5 min intervals, and then incubated with FITC–phalloidin (0.33 µM in methanol) for 60 min at 38.5 °C diluted with 400 µl of HEPES-buffered saline solution before analysis by flow cytometry. Results are expressed as the average percentage of spermatozoa showing high fluorescence intensity of FITC–phalloidin (indicating a higher F-actin content) ± S.E.M. (n = 5).

**Analysis of spermatozoa viability by flow cytometry**

As described previously (Aparicio et al. 2007), fluorescent staining using the live/dead spermatozoa viability kit was performed to assess porcine spermatozoa viability. Briefly, 5 µl SYBR 14 (2 mM) and 10 µl PI (5 mM) were added to 500 µl diluted spermatozoa sample (50X 10⁶ spermatozoa per ml) in HEPES-buffered saline solution (10 mM HEPES and 150 mM NaCl, pH 7.4) and incubated for 15 min at 38.5 °C. After incubation, 10,000 cells were analyzed on a Coulter EPICS XL flow cytometer using EXPO 2 Analysis Software. Results are expressed as the percentage of SYBR14-positive and PI-negative spermatozoa ± S.E.M.

**Statistical analysis**

The mean and S.E.M. were calculated for descriptive statistics. When appropriate for multiple comparisons, we have used ANOVA with the Scheffe test for comparisons between treatments. To analyze the percentage of motile and rapid spermatozoa, we used the Pearson’s χ² test. All analyses were performed using SPSS v11.0 for MacOs X Software (SPSS, Inc., Chicago, IL, USA). The level of significance was set at P < 0.05.

**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.

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