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Spermatozoa in the sperm-peak-fraction of the boar ejaculate show a lower flow of Ca^{2+} under capacitation conditions post-thaw which might account for their higher membrane stability after cryopreservation

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Short title: Boar sperm sensitivity to Ca^{2+} -ionophore challenge differs between ejaculate portions

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Abstract

Boar spermatozoa collected in the ejaculate sperm peak-portion (P1, first 10 mL of the sperm-rich fraction, SRF), had shown a higher resilience to freezing and thawing compared to spermatozoa from the rest of the ejaculate (2nd portion of the SRF plus the post-sperm-rich fraction, PSRF), even when using a simplified freezing technique, as long as spermatozoa were incubated in their own seminal plasma (SP). This experiment studied the stability of P1- and SRF-P1 boar spermatozoa frozen in MiniFlatPacks (MFP), post-thaw, using flow cytometry. Since spermatozoa from either portion showed similar cryosurvival and low proportions of unstable membranes (<3%, annexin-V/propidium iodide staining), and only a tendency for SRF-P1 live spermatozoa to depict acrosome exocytosis (FITC-PNA/PI/H33342); they were explored for Ca^{2+} contents using a Fluo-4 probe under *in vitro* capacitating conditions (mBO+medium), as well they were tested for their ability to sustain a short Ca^{2+}-ionophore (A23187) *in vitro* challenge. The proportions of live spermatozoa depicting high Ca^{2+} -levels were initially <2% but increased over incubation time, particularly in SRF-P1($P<0.05$), while proportions of live spermatozoa with low Ca^{2+}-levels were basically constant over incubation time (~11-14%), for either portion. Incubation in capacitation medium did not modify the proportions of low- Ca^{2+} but dramatically increased the proportions of high- Ca^{2+} spermatozoa ($P<0.001$) already after 15 min exposure, highest for SRF-P1 spermatozoa. While the proportion of live spermatozoa with intact acrosome was significantly decreased among SRF-P1 ($P<0.001$), that of P1-spermatozoa remained unchanged, probably owing to the lowest relative content of cytosolic Ca^{2+}. The results suggest that spermatozoa in the P1-portion are more resilient to express acrosome exocytosis post-thaw compared to those bathing in the rest of the SRF-fraction when cryopreserved using a simplified technique, in MFPs.

Key words: boar, calcium, membrane stability, sperm, SRF.

Introduction

Cryopreservation of boar semen has been considered a sub-optimal technology (Rodriguez-Martinez, 2003). Since about 50% (or more) of the spermatozoa usually succumb the procedures when freezing large AI-doses (with high sperm numbers), few doses can be prepared per ejaculate. Moreover, conventional freezing takes time, usually more than 8 hours, requiring expensive refrigerated centrifuges. Both these aspects makes freezing costly, in terms of boar and operator use as well as equipment. Last but not least, fertility post-AI is lower than for liquid semen, mostly because the surviving spermatozoa show signs of (membrane) instability, and a short lifespan (Rodriguez-Martinez, 2000).

New procedures have recently been presented that (i) shorten the time required for freezing, (ii) waive the need of heavy equipment, (iii) freeze concentrated small samples (with good cryosurvival and acceptable fertility when using deep intrauterine AI), thus leading to a better use of a single ejaculate (Wongtawan et al., 2006; Saravia et al., 2009). Behind this development is a long series of studies of the differences among fractions and portions within fraction of the boar ejaculate, particularly regarding the impact different seminal plasma (SP) components play on sperm survival (Rodriguez-Martinez et al., 2008). Such studies have identified that the spermatozoa bathing in the concentrated sperm peak-fraction of the ejaculate (the 1st 10 mL of the sperm rich fraction, SRF, also called P1) show, due to a higher contents of epididymal fluid, low bicarbonate contents and a series of particular proteins present in certain concentrations in the surrounding fluid; a higher resilience to storage and processing (Rodriguez-Martinez et al., 2009; Saravia et al., 2009), compared to spermatozoa present in the rest of the ejaculate, where the SP has another composition. Use of the P1 peak semen portion for freezing (for gene banking, or AI) simultaneously allows the routine use of the rest of the ejaculate for liquid semen, without modifying the routines of the semen companies.

However, several enterprises do not collect the entire ejaculate (fractionated or not) but the SRF, particularly for freezing. Therefore, it is of utmost importance to determine whether the P1-portion would differ in terms of freezability with the rest of the SRF, in case a similar approach as the above ought to be made, or to determine if one of the portions still prevails in freezability. For that reason, a recent experiment compared the post-thaw survival of boar spermatozoa from the P1 vs the SRF-P1, processed using a simplified technique and MFPs, in terms of sperm kinematics, acrosome and plasma membrane integrity and stability (Siqueira et al., 2011). Sperm motility post-thaw reached 50%, without differences between portions of the SRF, but with clear inter-boar variation. Neither plasma membrane

nor acrosome integrity differed (ns) between SRF-portions, thus indicating that either portion could be frozen with the simplified method. However, SRF-P1 showed a tendency for live spermatozoa to depict acrosome exocytosis, with large variation among boars. Acrosome exocytosis is an irreversible phenomenon that can occur during sperm manipulation, leading to cell "infertility". However, such phenomenon, if occurring in live cells, might relate to spermatozoa having a higher degree of membrane instability, visible only when special conditions appear. One of these conditions could be the exposure to "capacitation-like" environments which, by inducing changes in the spermatozoon, as mobilization of Ca^{2+}, would eventually lead to an undesirable acrosome exocytosis. Challenges of this kind can be done mimicking capacitation conditions such as incubation in specific media (as mBO+, Brackett and Oliphant, 1975) where Ca^{2+} mobilization is progressive over time, or through brief exposure to a Ca^{2+}-chelator, such as an ionophore, which surpasses capacitation-like changes and leads to a quicker exocytosis. Ca^{2+}-changes and the endpoint acrosome exocytosis can be followed by way of flow cytometry, which advantageously examines thousands of cells in a relatively short period (Piehler et al., 2006).

The present experiment compared the stability of P1- and SRF-P1 boar spermatozoa frozen in MFP, post-thaw, using flow cytometry. Since spermatozoa from either portion showed similar cryosurvival and low proportions of unstable membranes (<3%, Annexin-V), albeit with SRF-P1 having a tendency for live spermatozoa depicting acrosome exocytosis (FITC-PNA/PI/H33342); they were further explored for Ca^{2+} contents using a Fluo-4 probe under *in vitro* capacitating conditions (mBO+ medium), as well they were tested for their ability to sustain a short Ca^{2+}-ionophore (A23187) *in vitro* challenge.

Material and methods

Frozen semen (4 to 5 ejaculates) derived from four mature boars (1.5-4 years old, one Swedish Landrace, two Swedish Yorkshire, and one Norwegian Landrace, see Siqueira et al., 2011) was used. The experimental protocol for the freezing of semen had previously been approved by the Ethical Committee for Experimentation with Animals, Uppsala, Sweden. The semen, collected as the first 10 mL of the SRF (P1) and the rest of the SRF (SRF-P1, 30-40 mL), was processed using a simplified freezing method (Saravia et al., 2009) in MFPs, each containing ~ 500 $\times 10^6$ spermatozoa and stored in liquid N_2 (-196° C) until analysed. The MFPs were thawed in circulating water at 35° C for 20s (five MFPs per ejaculate).

All sperm analyses were performed using an LSR flow cytometer (FC, Becton Dickinson, San José, CA, USA) at room temperature, following established protocols.

Sperm membrane architecture and destabilization were evaluated by staining with annexin-V/propidium iodide (PI). Annexin V is a calcium-dependent probe that is used for tracking externalization of phosphatidylserine (PS) in the sperm membrane. Since PS is normally located exclusively at the inner face of the lipid bilayer, such externalization monitors early changes in membrane stability and intactness, detectable earlier than when PI is used. The staining was performed as described by Saravia et al. (2009), using an Annexin-V-fluorescein isothiocianate (FITC) apoptosis detection kit I (Pharmigen, San Diego, CA, USA) as previously reported (Peña et al., 2003). Frozen semen samples were thawed and an aliquot of 10 µL was extended with 90 µl of binding buffer. To 3 µL of this suspension, 90 µL of binding buffer, 3.5 µL Annexin-V, 10µL of PI and 2 µL Hoechst 33342 (500µg/ml) were added. The samples were incubated in the dark at room temperature for 15 min. 400 µL of binding buffer were added before the analysis. Analysis was performed with a BD LSR flow cytometer (BD Biosciences, San Jose, CA). Cells were gated using Hoechst and scatter properties to gate out debris. From each spermatozoa, forward light scatter (FSC), orthogonal light scatter (SSC), A-FITC fluorescence (FL1), and PI fluorescence (FL3) were evaluated using Cellquest version 3.3 (Becton Dickinson). For the gated cells, the percentages of viable spermatozoa with a stable plasmalemma [Annexin-V (AN)-negative/PI-negative (PI–)], spermatozoa with an unstable yet intact plasma membrane (AN+/PI–), and membrane-damaged cells (AN–/PI+), as well as double positive (AN+/PI+) cells were evaluated based on quadrants determined from single-stained and unstained control samples.

Cytosolic Ca^{2+} content was measured by using fluorescent probe Fluo-4 (Molecular Probes™, Invitrogen, Eugene, Oregon, USA) as previously described (Piehler et al., 2006) with slight

modification. Fluo-4 stock solution was prepared as: 91 µL DMSO (Fluka AG, Buchs, Switzerland) to 50 µg Fluo-4. After that, 91 µL of 20% Pluronic F127 (Molecular Probes^{TM}, Invitrogen, Eugene, Oregon, USA) in DMSO (Fluka AG, Buchs, Switzerland) were dissolved and finally added with Fluo-4. Concentration of stock solution was 250 µM. A 25 µL of sperm sample was added to 375 µL of control medium (mBO- medium, without bicarbonate, Ca^{2+}, caffeine or BSA, 300 mOsm hereafter named as mBO- or non capacitating medium). After adding 4 µL of Fluo-4 stock solution to the sperm suspension, samples were incubated for 30 min at 37°C in a cell culture incubator. After the completion of incubation, 2 mL of control medium was added and samples were centrifuged for 10 min at 300 *g* at 20 $^{\circ}$C. The supernatant was removed, again resuspended with 2 mL of control medium and centrifuged for 10 min at 300 *g* at 20 $^{\circ}$C. After that the supernatant was removed again and 2 mL mBO- medium was added and separated in two different vials centrifuged again, supernatant removed and added 400 µL of previously gassed control or mBO+ medium (Brackett-Oliphant medium, Brackett and Oliphant, 1975) composed of 37 mM $NaHCO_3$, 2.25 mM $CaCl_2$, 2 mM caffeine and 0.5% bovine serum albumin, 300 mOSM. Into the samples, 2.5 µL of PI, 20 µl of Hoechst 33342 were added and incubated for 15 min at 37 $^{\circ}$C. Samples were analyzed and the procedure was repeated after 30 min and 60 min. Data from at least 10, 000 spermatozoa were analyzed per sample. The 15 min mbo- sample was used as a control for defining the border between cells with high and low calcium content.

Acrosome intactness was assessed with the acrosome-specific peanut agglutinin conjugated with fluorescein isothiocyanate (FITC-PNA, Sigma Chemical Co., St. Louis, MO) in conjugation with PI and Hoechst 33342 (Molecular Probes, Eugene, OR) according to the method described by Spjuth et al. (2007). Briefly, spermatozoa were challenged with Ca-ionophore A23187 (Sigma, Chem. MO, USA, Cat C7522). The basic medium for this study was Beltsville thawing solution (BTS, IMV, L'Aigle, France) composed of glucose, 205 mM; Nacl, 20.39 mM; KCl, 5.4 mM; $NaHCO_3$, 15.01 mM; and ethylenediaminetetraacitic acid (EDTA), 3.35 mM. Sperm sample of 0.5 µL was added to 500 µL of medium for acrosome exocytosis (10 mL BTS, 31.5 µL calcium chloride 2.25 mM and 60 µL calcium ionophore A23187 1.67 mM)], stained with PI (2.5 µL), HO342 (1 µL) and FITC PNA (5 µL), incubated for 10 min at 37°C, and analyzed by flow cytometry. The ionophore A23187 was initially dissolved in dimethyl sulphoxide (DMSO, Fluka AG, Buchs, Switzerland) and stored as a 5-mM stock solution at −20 ◦C. The FITC-PNA fluorescence was detected at 515-545 nm fluorescence detector 1 (FL1). Working solutions were obtained by dilution of the stock solution with phosphate buffered saline

(PBS) before use. The final DMSO concentration was less than 0.2% in the test tubes. Non-sperm events were gated out of the analysis and evaluation was performed on scatter properties only. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Spermatozoa were grouped as i) live acrosome-intact (PI-negative & FITC-PNA-positive), ii) dead acrosome-intact (PI-positive & FITC-PNA-negative), iii) dead acrosome-exocytosed (PI-positive & FITC-PNA-positive) and iv) live acrosome-exocytosed (PI-negative & FITC-PNA-positive) spermatozoa.

To check the membrane stability of cryopreserved spermatozoa in two different portions of sperm-rich fraction (P1 and SRF-P1), spermatozoa were suspended in BTS medium. To evaluate acrosome integrity, spermatozoa were suspended in two different conditions. Control spermatozoa were incubated in BTS, while to induce acrosome exocytosis, BTS medium was supplemented with calcium chloride and calcium ionophore A23187. The cytosolic Ca^{2+} was determined in both capacitating medium (mBO+) and non capacitating medium (mBO-).

Data (as mean values) for sperm parameters (using arcsine transformation where data did not have a normal distribution) were examined by analysis of variance (ANOVA) using the General Linear Models (GLM) procedure of the SPSS statistical package, version 17.0 (SPSS Inc., Chicago, USA). The statistical model included the fixed effects of male, portion within the SRF fraction (P1 & SRF-P1) and their interaction. This model also considered effect of time, medium and their interaction in the calcium content study. No significant interactions were found. When overall significance was found, *t* student test was used for comparing two groups and Student-Newman-Keuls test for comparing three groups. The results are presented as mean ± SEM and the level of significance was set at $P < 0.05$.

Results

Differences in boar sperm plasma membrane stability and integrity between two different portions of the sperm-rich fraction are shown in Table 1. In either portion of semen, about 50% of the spermatozoa sustained a stable plasma membrane post-thaw. It was also evident that a tendency ($P<0.052$) for membrane instability was seen in the spermatozoa of the SRF-P1 portion. There was no appreciable difference between P1 and SRF-P1 neither for spermatozoa with compromised plasma membrane or for those alive with an intact plasma membrane.

The dynamics of sperm cytosolic Ca^{2+} ion in different incubating media is shown in Table 2. The changes in the percentage of spermatozoa with decreased Ca^{2+} ion content in different incubation medium progressed in a time-dependent manner in both P1 and SRF-P1 ($P<0.05$). In the mBO+ medium there was a gradual decline in high Ca^{2+} spermatozoa, while a gradual increase in the control medium was observed. Interestingly enough, spermatozoa with high Ca^{2+} of SRF-P1 were much higher than those of spermatozoa in the P1 portion, irrespective of culture media. A constant proportion of spermatozoa with low Ca^{2+} was maintained in both the P1 and SRF-P1 spermatozoa, though there was a tendency ($P<0.058$) of increasing proportions of SRF-P1 spermatozoa with low Ca^{2+} among controls. Proportions of PI-positive spermatozoa in the mBO+ medium were lower than in control medium. While the percentage of PI-positive spermatozoa in mBO+ medium increased over time, a reverse scenario was present in control medium in both P1 and SRF-P1.

The data of acrosome exocytosis post-thaw are shown in Table 3. The sensitivity of frozen-thawed spermatozoa from these P1 and SRF-P1 portions to respond to a short exposure of Ca^{2+}-ionophore A23187 (10 µM, 10 min) was explored. The proportion of live, unreacted spermatozoa did not change for P1 but decreased significantly ($P<0.05$) for SRF-P1 (56.22±1.74 *vs* 50.57±1.75), while the proportion of live, acrosome exocytosed spermatozoa increased significantly ($P<0.01$) for either portion, particularly for SRF-P1 (P1, 1.94±0.47 *vs* SRF-P1, 8.47±1.48; $P<0.001$). The total viability (including live acrosome exocytosed and live spermatozoa with intact acrosome) was almost the same in control and ionophore-challenged spermatozoa in each sperm portion. The proportion of live spermatozoa showing acrosome exocytosis was higher in SRF-P1 only when spermatozoa were challenged with ionophore. A representative chart of events is presented in Figure 1.

Figure 2 is a representative time-course cytosolic Ca^{2+} influx measurement by flow cytometry in spermatozoa incubated under capacitating (mBO+) and non-capacitating (mBO-, control) conditions. An

increased population of high Ca^{2+} spermatozoa was observed in the lower right side of each cytogramme. When spermatozoa were incubated in mBO+ medium, the Ca^{2+} content increased (**D** and **E**) up to 30 min while it became inverse (**F**) by 60 min of incubation. A similar pattern of fluorescence was observed in all analyzed samples.

Discussion

The present study revealed that spermatozoa in the SRF-P1 have a tendency to increase membrane instability compared to those in the P1. In both sperm portions, the proportions of live spermatozoa depicting high Ca^{2+} increased over incubation time, particularly in SRF-P1, while

proportions of live spermatozoa with low Ca^{2+}-levels were virtually constant over time. Incubation in capacitation medium dramatically increased the proportions of high- Ca^{2+} spermatozoa particularly among SRF-P1 spermatozoa. There was no difference between alive spermatozoa of P1 or SRF-P1 regarding proportions of cells with intact or burst acrosomes. The ionophore challenge dramatically increased the proportion of acrosome exocytosis in the SRF-P1 portion.

The sperm membrane is directly or indirectly related with many sperm functions, warranting the capability of the cell to maintain homeostasis and depict motility and the capacity to interact with the environment, including the lining epithelium of the female genital tract or the oocyte-cumulus cell-complex (Rodriguez-Martinez, 2003). The aim of the present study was to monitor subtle changes of stability in the lipid plasma membrane between the two portions of SRF. Although they showed a similar fashion of kinematic characteristics and survivability in a previous study (Siqueira et al., 2011), it is worth mentioning that a tendency of instability was occurring in the rest of the SRF (SRF-P1). It is generally known that most alterations during sperm freezing affect the plasma membrane. A significant membrane destabilization was previously observed in frozen-thawed spermatozoa collected in the rest of the ejaculate (P2, i.e. after separating the P1) when compared with the P1 portion (Peña et al., 2003). It might thus be assumed that spermatozoa in the P1 portion would perhaps be more resilient than those in the SRF-P1, particularly during cryopreservation. In both P1 and SRF-P1 portions, less that 25% of the spermatozoa appeared dead while among the surviving spermatozoa a non-significant destabilization occurred among those of the SRF-P1 portion. This destabilization might lead to capacitation-like changes in the spermatozoa. Spermatozoa from P1 were less prompt to depict capacitation-like changes, in contrast to spermatozoa in the bulk ejaculate (Sellés et al., 2001). In contrast, the rate of destabilizing spermatozoa was reported to be similar in frozen-thawed sperm of P1 and P2 (Saravia et al., 2007). One possible explanation may be that in the present study, spermatozoa were incubated in BTS medium while in the study of Saravia et al. (2007), the spermatozoa were incubated in a capacitating medium.

During capacitation and acrosome reaction, there is an elevation of sperm intracellular calcium. However, a prompt rise of calcium in cryopreserved spermatozoa is considered as a cause of poor fertility if inseminated (Bailey and Buhr, 1993; McLaughlin and Ford, 1994). Boar spermatozoa are more susceptible to cold temperature damage and they display a large accumulation of Ca^{2+} from internal source immediately after chilling (Bailey and Buhr, 1995). It was evident that chilled boar spermatozoa contain more intracellular calcium than the fresh (Bailey and Buhr, 1995). Thus, the Ca^{2+}

mobility in cryopreserved spermatozoa is somehow different from that of fresh spermatozoa. Alongside with a tendency of membrane instability in SRF-P1, in the present study, a sharp increase of intracellular calcium in SRF-P1spermatozoa was also observed. It might be due to membrane alteration during the cooling/thawing procedure, the membranes became more open and permeable to external Ca^{2+} and disable or alter the Ca^{2+} pumping channels. Alteration of membrane in SRF-P1, which might had opened several Ca^{2+} channels, would have resulted in a rapid Ca^{2+} influx. Connected to this, premature capacitated bull spermatozoa underwent a spontaneous acrosome reaction due to an uncontrolled influx of Ca^{2+} which subsequently reduced oocyte penetration (Kuroda et al., 2007). Ca^{2+} enters into the spermatozoa in two steps, firstly during the capacitation process there is a small intracellular Ca^{2+} surge followed by a mass elevation during the acrosome reaction (DasGupta et al., 1993; Florman, 1994). However, in the present study, a high rate of Ca^{2+} in the early incubation hour and a gradual decline later indicate that membrane instability persists, Ca^{2+} channels are open and an increased cytosolic Ca^{2+} level was present. Irrespective of sperm portions, the influxed Ca^{2+} must be needed for capacitation or the acrosome exocytosis. During the initiation of capacitation, Ca^{2+} that enters the sperm head is either expelled via the plasma membrane or taken up into the acrosome via the acrosomal Ca^{2+}-ATPase. It is possible that cAMP and perhaps increasing pHi open the acrosomal Ca^{2+} channels. The regulation of sperm Ca^{2+} then becomes unstable and as more Ca^{2+} flows into the spermatozoa, the level of Ca^{2+} begins to increase. Such phenomenon seemed true when non-capacitating medium (mBO-) in both the P1 and SRF-P1 was explored with proportions of high Ca^{2+} spermatozoa increased over incubation time. Interestingly enough, a reverse and gradual diminishing scenario was found when spermatozoa were incubated in a capacitating medium (mBO+). It seems that spermatozoa in the non-capacitating medium were unable to utilize its Ca^{2+}, while in a capacitating medium the Ca^{2+} influx occurred immediately after suspension into the medium and was utilized by the spermatozoa in a time-dependent manner. Whatever the mechanism might be, the present study indicates that Ca^{2+} influx is associated with the capacitation process. The proportion of dead spermatozoa in the capacitating medium incubation was much higher than the control. This situation might be explained by the findings of Birck et al. (2009) observing higher proportions of dead spermatozoa in the capacitating medium than that in non-capacitating medium after a 60 min exposure. Thus, capacitating- and membrane-altered spermatozoa that exhibit Ca^{2+} efflux are less stable and prone to die. After incubation, amongst the viable sperm population the proportion of cells with high intracellular Ca^{2+} concentration increased suggesting that cells with low Ca^{2+} lose their membrane integrity. Although speculative, it might be that the

spermatozoa with low Ca^{2+} are those destined to die while those which sustain high Ca^{2+} post-thawing, are the surviving population. Thus cryopreserved spermatozoa are permeable to cellular Ca^{2+} more than the fresh and this quick Ca^{2+} influx might be due to an increase in plasma membrane leakage or depressed Ca^{2+} thrusting out mechanism resulting from cooling, freezing and thawing procedure. It might also be speculated that cryopreservation process depolarizes the plasma membrane which could prime voltage-sensitive Ca^{2+} T-channels and lead to premature Ca^{2+} influx (Hammerstedt et al., 1990).

The sperm acrosome is one of the structures modulating intracellular Ca^{2+} concentration, since internal Ca^{2+} is stored in the acrosomal proximity where exocytosis occurs (Breitbart, 2002) and is being used as a predictor of sperm fertilizing ability (Makkar et al., 2003). It was evident from the present study that spermatozoa in the SRF-P1 portion undergoing acrosomal exocytosis after Ca^{2+} ionophore (A23187) exposure increased dramatically. However, observed acrosomal exocytosis in SRF-P1 indicates that acrosomal exocytosis could be induced by ionophore in a non-capacitating medium as long as Ca^{2+} was present (Brucker et al., 1994). Data in the present study indicate that ionophore A23187 induced acrosomal exocytosis in SRF-P1 is in line with data obtained from membrane instability followed by a calcium influx. Sperm exposed to control without ionophore did not show acrosomal exocytosis, this finding is in line with Ca^{2+} dependency of acrosome reaction (Bielfeld et al., 1994). Ca^{2+} ionophore A23187 can regulate PLCx activation and PKC activity followed by increasing intracellular Ca^{2+} ion concentration. The PKC opens a voltage-dependent Ca^{2+} channel in the sperm plasma membrane, further increasing Ca^{2+} (Bonaccrsi et al., 1998). Our yet to publish data also indicate that cryopreserved boar spermatozoa follow a PKC mediated Ca^{2+} dependent pathway for protein tyrosine phosphorylation. It might be suggested that spermatozoa in the mBO+ medium compensates for their initial high cytosolic Ca^{2+} by restricting Ca^{2+} uptake and storage during the incubation period. Some lines of evidence suggest that both of cytosolic Ca^{2+} stored in acrosome (Herrick et al., 2005) and protein tyrosine phosphorylation are required for the completion of acrosome reaction process. Thus it is conceivable from this observation that spermatozoa showing higher acrosomal exocytosis in SRF-P1 are a result of membrane instability.

Although right now it is difficult to ascertain the underlying mechanism of this phenomenon, spermatozoa in the P1 or corresponding SP where spermatozoa bathe in might be the mediator of this process. The SP is a heterogeneous fluid containing secretion from the epididymal ducts and the accessory glands and varies in its composition of electrolytes and proteins among ejaculated fractions (Zhu et al., 2000). Compared to P1, the SP of the so called P2 lacks some essential component (Saravia

et al., 2009). Determination of composition and Ca^{2+} in the SP of P1 and SRF-P1 might be effective for a complete understanding of the mechanism. In conclusion, spermatozoa in the P1-portion seem more resilient to express acrosome exocytosis post-thaw compared to those bathing in the rest of the SRF.

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Table 1. Changes in membrane stability and integrity in cryopreserved boar spermatozoa from two different portion of sperm-rich fraction of the ejaculate

Semen fraction	PI+ (%)	An+PI- (%)	An-PI- (%)
P1 (n= 19)	47.08±1.63	2.12±0.52	50.80±1.91
SRF-P1 (19)	45.09±1.54	3.24±0.88	51.67±1.63

Data are presented as means ± standard error of the mean (SEM). PI+ = Sperm with a damaged plasma membrane, An+PI- = Spermatozoa with instable plasma membrane, An-PI- = Viable spermatozoa with stable plasma membrane. P1= the first 10 mL of sperm-rich fraction (SRF), SRF-P1= the rest of the ejaculate from the sperm-rich fraction. All the measurements were performed 15 min after thawing. Nineteen (19) observations were performed for each group.

Table 2. Changes in calcium content in cryopreserved boar spermatozoa from two different portion of sperm-rich fraction of the ejaculate

Semen fraction	Time	Control (mBO-)			mBO+		
		High calcium (%)	Low calcium (%)	Dead (%)	High calcium (%)	Low calcium (%)	Dead (%)
P1 (n=19)	15 min	0.78 ± 0.10^{a}	11.5 ± 0.48^{a}	87.4 ± 0.56^{c}	16.3 ± 3.04^{b}	10.6 ± 0.35^{a}	72.7 ± 2.96^{a}
	30 min	5.75 ± 1.08^{b}	11.6 ± 0.44^{a}	82.3 ± 1.37^{bc}	12.8 ± 2.60^{ab}	10.4 ± 0.38^{a}	76.5 ± 2.54^{ab}
	60 min	14.5 ± 2.33^{c}	12.7 ± 0.50^{a}	72.6 ± 2.48^{ab}	10.8 ± 2.18^{a}	10.5 ± 0.35^{a}	78.4 ± 2.09^{b}
SRF-P1 (n=19)	15 min	1.28 ± 0.17^{a}	13.2 ± 0.94^{ab}	85.0 ± 1.08^{c}	19.1 ± 2.83^{c}	10.7 ± 0.35^{a}	69.8 ± 2.63^{a}
	30 min	7.35 ± 0.94^{bc}	13.1 ± 0.54^{ab}	79.0 ± 1.03^{b}	15.3 ± 2.29^{b}	10.5 ± 0.34^{a}	73.7 ± 2.23^{a}
	60 min	22.1 ± 2.03^{d}	14.2 ± 0.53^{b}	64.2 ± 1.95^{a}	12.5 ± 1.92^{ab}	10.8 ± 0.36^{a}	76.5 ± 1.87^{ab}

Data are presented as means ± standard error of the mean (SEM). ^{a,d}Values with different superscripts within a column indicate significant differences ($P<0.05$). P1= the first 10 mL of sperm-rich fraction (SRF), SRF-P1= the rest of the ejaculate from sperm-rich fraction. mBO-, (control medium without bicarbonate, Ca^{2+}, caffeine or BSA, 300 mOsm hereafter named as mBO- medium). mBO+, modified Brackett-Oliphant medium (capacitating medium).

Table 3. Ionophore (A23187) challenged acrosomal integrity in cryopreserved boar spermatozoa from two different portion of sperm-rich fraction of the ejaculate

Semen fraction	Control				Ionophore challenged			
	Live intact (%)	Dead intact (%)	Dead exocytosed (%)	Live exocytosed (%)	Live intact (%)	Dead intact (%)	Dead exocytosed (%)	Live exocytosed (%)
P1 (n=19)	58.52±2.38[a]	29.62±1.73[a]	10.91±1.65[a]	0.72±0.13[a]	56.22±1.74[a]	29.61±1.20[a]	11.89±1.46[a]	1.94±0.47[a]
SRF-P1 (n=19)	58.23±2.14[a]	29.09±1.46[a]	11.24±1.22[a]	1.13±0.16[a]	50.57±1.75[b]	28.48±1.36[a]	12.19±1.01[a]	8.47±1.48[b]

Data are presented as means ± standard error of the mean (SEM). [a,b]Values with different superscripts within a column indicate significant differences ($P<0.05$). P1= the first 10 mL of sperm-rich fraction (SRF), SRF-P1= the rest of the ejaculate from sperm-rich fraction. All the measurements were performed 10 min after thawing.

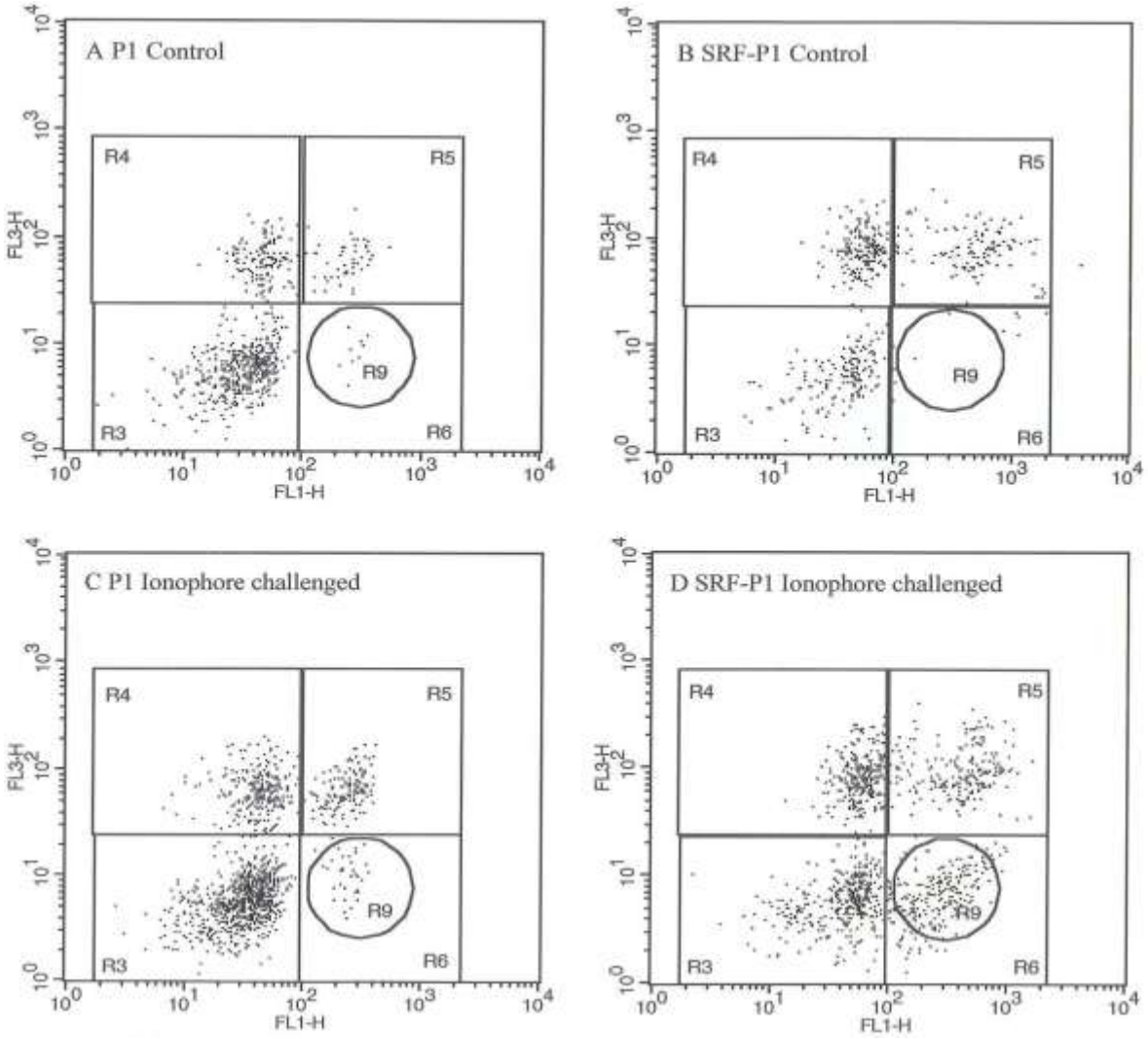

Figure 1. Representative dot plot displaying ionophore (A23187)-challenged acrosome exocytosis in cryopreserved boar spermatozoa from one of two different portions (P1 and SRF-P1) of the sperm-rich fraction (SRF) of the boar ejaculate. In each cytogram, **R3** represent live acrosome-intact (PI-negative & FITC-PNA-positive), **R4** dead acrosome-intact (PI-positive & FITC-PNA-negative), **R5** dead acrosome-exocytosed (PI-positive & FITC-PNA-positive), **R6** live acrosome-exocytosed (PI-negative & FITC-PNA-positive) and **R9** is more restricted live reacted (PI-negative & FITC-PNA-positive) spermatozoa. Dot plot **A** and **B** represent, respectively control and ionophore (A23187) challenged acrosome exocytosis in **P1** portion of sperm-rich fraction. Dot plot **C** and **D** represent, respectively control and ionophore (A23187) challenged acrosome exocytosis in **SRF-P1** portion of sperm-rich fraction.

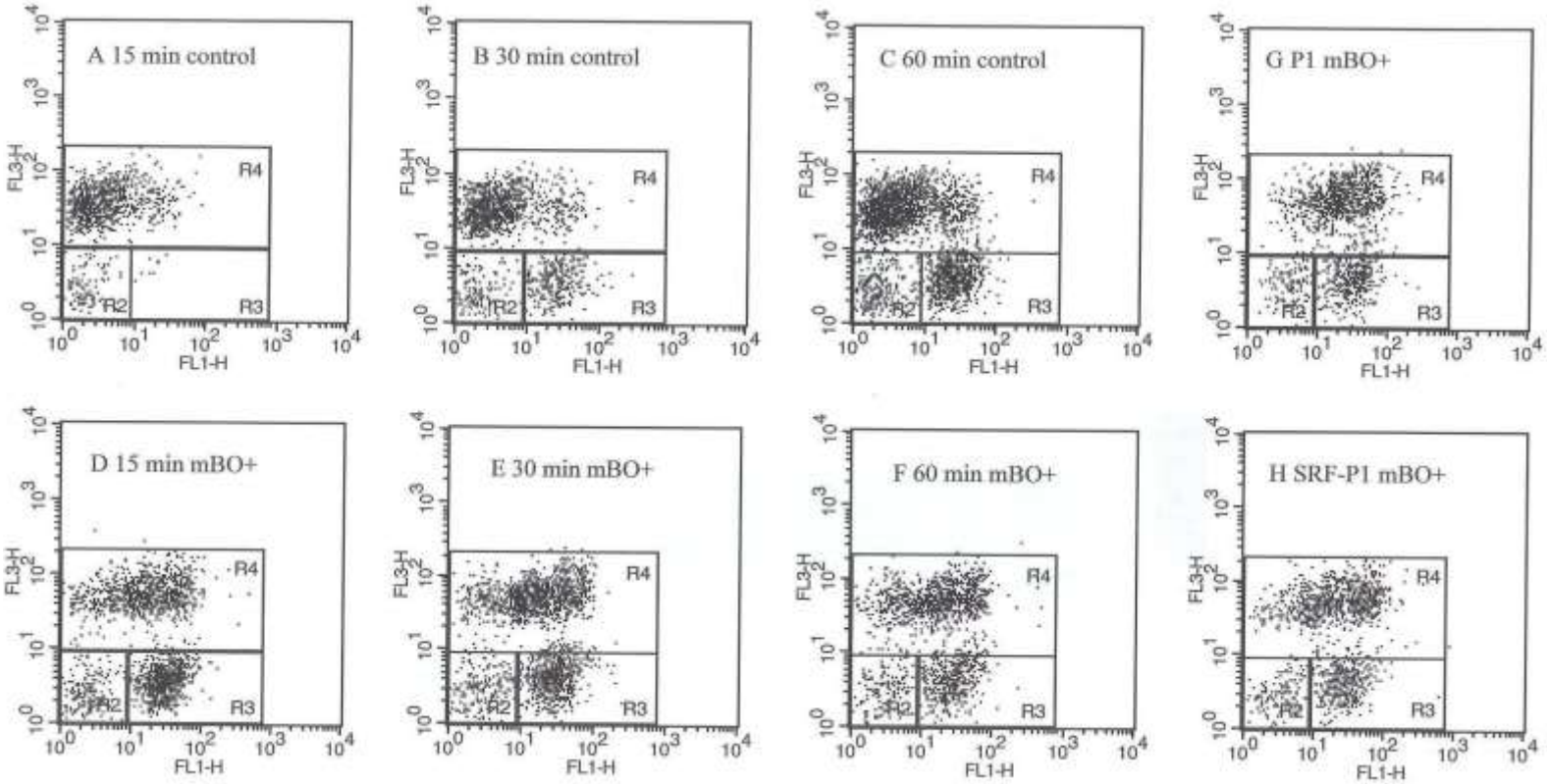

Figure 2. Representative dot plot displaying time-dependent changes of calcium influx in different medium (Control and mBO+) and different portion (P1 and SRF-P1) of sperm-rich fraction. A detailed description of staining is described in the *Materials and Methods*. For all the cytograms, the region **R2** represent live spermatozoa with low calcium, **R3** live sperm with high calcium and **R4** is dead sperm without calcium. Dot plots **A** and **B** represent change of calcium content at 15 minutes, **B** and **E** at 30 minutes, and **C** and **F** at 60 minutes changes. Dot plot **G** and **H** indicate difference in calcium content between P1 and SRF-P1 portion of sperm-rich fraction.