Calcium influx into equine and bovine spermatozoa during *in vitro* capacitation[1]

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Abstract

Flow cytometry was utilized to measure the influx of calcium into fresh and frozen-thawed equine and bovine spermatozoa that had been treated with either dilauroyl-phosphatidylcholine (PC-12) or the calcium ionophore A23187 (A23187) to initiate sperm capacitation. Sperm suspensions were stained with the fluorescent calcium indicator, Fluo-3, to measure intracellular calcium concentrations and with propidium iodide (PI) to evaluate sperm viability. After staining, sperm were incubated at 37°C in a Hepes buffered salt medium (BGM3,control) with either PC-12 or A23187 and analyzed just prior to (time 0) and 1, 5, 15 and 30 min after addition of capacitating agents. When fresh stallion sperm were treated with either BGM3 (control) or PC-12 neither the percentages of viable sperm or the percentages of sperm with increased levels of intracellular calcium, changed during the incubation period. However, when fresh stallion sperm were treated with A23187, within 15 min of incubation, the percentages of sperm exhibiting high levels of intracellular calcium increased. Treatment of frozen-thawed stallion sperm with A23187 induced an immediate increase in intracellular calcium, while the percentages of control and PC-12 treated sperm having elevated intracellular calcium levels increased after 5 min of incubation. A23187 induced an immediate calcium uptake into both fresh and frozen-thawed bull sperm, while control sperm and sperm treated with PC-12 increased intracellular Ca^{++} levels only after 15 min of incubation. These results indicate that cryopreservation alters the plasma membranes sperm permitting a rapid influx of calcium into the sperm, which was not observed in fresh sperm. In addition, calcium ionophore A23187 induces a rapid influx of calcium into both stallion and bull spermatozoa.

Keywords: sperm capacitation, intracellular calcium, fluo-3, ionophore A23187, dilauroylphosphtidylcholine.

Introduction

Spermatozoal capacitation includes a diverse series of physical and biochemical changes in the spermatozoa that occur within the female reproductive tract. Part of capacitation includes the removal or the alteration of surface-associated components originating from epididymal fluid or seminal plasma. Changes in sperm metabolism and biochemistry also occur during the capacitation process (Zao *et al.*, 1985; Yanagimachi *et al.*, 1988). One result of capacitation is an increase in membrane permeability to calcium, which permits rapid movement of calcium into the spermatozoa. This calcium influx is required for the fusion of the plasma membrane and the outer acrosomal membrane to occur, initiating the acrosome reaction of mammalian sperm (Yanagimachi and Usui, 1974; Talbot *et al.*, 1976; Singh *et al.*, 1978; Triana *et al.*, 1980).

Calcium enters the spermatozoa in a biphasic fashion, with an initial small elevation in intracellular calcium during capacitation followed by a much larger elevation in intracellular calcium that occurs at the time of the acrosome reaction (DasGupta *et al.*, 1993; Adeoya-Osiguwa and Fraser, 1993; Florman, 1994). Although sperm capacitation usually occurs in the female

tract, the development of *in vitro* systems that promote capacitation and fertilization has made it possible to study more carefully the specific requirements for sperm capacitation and fertilizing ability.

In vitro capacitation and induction of acrosome reaction can be accomplished using several treatments (Graham *et al.*, 1986; Suarez *et al.*, 1987; Bird *et al.*, 1988; Tanphaichitr and Hansen, 1994). After capacitation, one primary signal that initiates the acrosome reaction is a change in sperm plasma membrane permeability to calcium. Therefore, divalent cationic ionophores, such as A23187 or ionomycin, artificially induce calcium entry into sperm, resulting in the acrosome reaction in bull (Triana *et al.*, 1980), and stallion (Masgistrine and Palmer,1991; Farlin *et al.*, 1992) spermatozoa. Alternatively fusogenic lipids, such as dilauroylphosphatidylcholine (PC-12) also induce the acrosome reaction in bull, ram and stallion sperm (Graham *et al.*, 1987), possibly by making sperm membranes permeable to calcium.

Cryopreservation can damage sub-cellular compartments of spermatozoa resulting in decreased fertilizing potential (Hammerstedt *et al.*, 1990). Perturbations occur in the membranes during cooling and freezing (Parks and Lynch, 1992) which alter the ability of spermatozoa to maintain ion concentration gradients (Plummer and Watson, 1985; Watson, 1981), and lead to potentially lethal increases in intracellular calcium (Watson, 1981). These changes may also adversely affect sperm capacitation, the acrosome reaction and the survival of spermatozoa in the female reproductive tract (Valcarcel *et al.*, 1994).

A true acrosome reaction occurs only in live, membrane intact sperm. Thus, it is important not only to analyze semen for sperm viability but also to simultaneously determine the normalcy of the acrosome (Saacke *et al.*, 1980). The percentage of spermatozoa with morphologically normal acrosomes can be determined in fixed specimens using differential interference contrast microscopy or fluorescent microscopy. Although useful, these classical techniques are time consuming and are inherently variable.

Flow cytometry has been used to analyze the viability (Garner *et al.*, 1986; 1988) and the acrosomal integrity (Hammerstedt, 1975; Graham *et al.*, 1990) of spermatozoa. This technique enables multiple spermatozoal parameters to be measured simultaneously on thousands of individual cells in only a few minutes (Carter and Meyer, 1990). Plasma membrane and acrosomal integrity of bull spermatozoa can be measured simultaneously, using propidium iodide (PI), to evaluate plasma membrane integrity, in conjunction with a fluorescently labeled lectin, *Pisum sativum* agglutinin (PSA), to measure acrosomal membrane integrity

(Graham *et al.*, 1990; Nolan *et al.*, 1992). In addition, the intracellular calcium levels of spermatozoa can be measured using the fluorescent calcium indicator Fluo-3 (Minta *et al.*, 1989). Storey *et al.* (1992) and Bailey and Storey (1994) reported that mouse sperm, preloaded with the Fluo-3, showed an increase in intracellular calcium upon binding to intact mouse zona pellucida.

In the present study, flow cytometry was used to measure the influx of calcium (measured by changes in fluo-3 fluorescence) in viable (measured by PI exclusion) fresh and frozen/thawed equine and bovine spermatozoa, when spermatozoa were treated with either PC-12 or A23187 to induce sperm capacitation.

Material and Methods

Preparation of sperm

Semen from 6 fertile stallions and 6 fertile bulls was obtained using an artificial vagina. Equine semen was diluted 1:1 with Ringer's lactate solution and centrifuged at 300 x g for 7 minutes. The supernatant was discarded and the sperm pellet re-suspended to a final concentration of 200 x 10^{6}cells/mL with a skim-milk egg-yolk freezing extender containing 4% glycerol (Wilhelm *et al.*, 1996) and frozen as described by Wilhelm *et al.* (1996). Briefly, the extended semen was cooled to 5° C over a 2 h period, after which sperm were packaged into 0.5 mL French straws and placed in liquid nitrogen vapor, 7 cm above the nitrogen, for 15 min and then plunged into liquid nitrogen (Wilhelm *et al.*, 1996).

Bovine sperm were diluted in an egg yolk/sodium citrate medium containing 6.4% glycerol (Graham *et al.* 1987), cooled to 5° C over 2 h, packaged into 0.5 mL and frozen in liquid nitrogen vapor, 7 cm above the liquid nitrogen, as described by Graham *et al.* (1987). Both stallion and bull sperm were stored at -196° C until being thawed in a 37° C water bath for 30 seconds just prior to washing, treatment and analysis.

Fresh and frozen/thawed semen samples were washed though a 45/90% discontinuous Percoll (Sigma, St Louis, MO) gradient. The Percoll gradient was prepared by diluting 4.5 mL of 100% Percoll with 0.5-mL of a modified Tyrode's medium (BGM3; Parrish *et al.*, 1989a) containing salts at 10 times normal concentration, and then adding 150μL of a solution containing CaCl$_2$ (30.8 mg/mL) and MgCl$_2$ (8.1 mg/mL) in water and 150μL of NaHCO$_3$ (250mM) in water. This solution, equivalent to a 90% stock isotonic Percoll, was then filtered through a sterile 0.22 μm pore filter. A 45% Percoll working solution was prepared by diluting the Percoll stock with an equal volume of BGM3. The discontinuous Percoll gradient was made by putting 1 mL of 90% Percoll into a sterile 15

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mL conical plastic tube and carefully layering 1 mL of the 45% Percoll solution and 0.5 mL of diluted sperm on top. Fresh sperm were centrifuged for 15 min at 400 x g, the supernatant removed, the sperm resuspended to 3 mL with BGM3 medium and centrifuged a second time for 5 minutes at 400 x g to remove residual Percoll. Frozen-thawed sperm were centrifuged for 10 min at 300 x g, the supernatant removed, the pellet resuspended in 3 mL of BGM3 and washed a second time for 3 min at 300 x g.

After the second centrifugation the supernatant was removed and the sperm resuspended in 1 mL BGM3 medium containing 0.98 mM calcium and 6% BSA. The sperm concentration was then determined using an hemacytometer and the final sperm concentration adjusted to 50×10^6 cell/mL with BGM3 medium containing 0.98 mM calcium and 6% BSA. The percentages of motile sperm in each sample was assessed visually, by a single observer using a phase contrast microscope equipped with a heated stage (37° C) with an attached television monitor, final magnification 600X, prior to and after washing.

Fluo-3 AM and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Fluo-3 AM was prepared as a 1mM stock solution in DMSO plus 2.5% Pluronic F-127; the Pluronic F-127, a dispersing agent that facilitates loading of the tetra-AM ester of Fluo-3 into cells, was stored at room temperature as a 25% (w/w) solution in DMSO.

A volume of 10μL of the Fluo-3 AM solution was added to sperm creating a final loading concentration of 10 μM indicator and 0.025% Pluronic F-127. The sperm/Fluo-3AM suspension was sealed with a snap cap and incubated for 20 min at 25^oC in the dark to permit loading of Fluo-3 AM into the sperm. Samples consisting of 500μL of diluted Fluo 3-loaded sperm suspension were then stained with 15μL PI (125mg/mL dissolved in Dulbecco's phosphate buffer saline).

Dilauroylphosphatidylcholine liposomes (PC-12) were prepared at a concentration of 800 μM/mL, as described by Graham *et al.* (1986) and stored in 0.5 mL aliquots at B80^{o}C until use. A23187 was diluted in DMSO at a concentration of 5μM/mL and stored at room temperature.

After staining, sperm were incubated at 37° C in each of the following treatments: 1) BGM3 (control); 2) PC-12 (100 μM final concentration); or 3) A23187 (100 nM final concentration) for up to 30 min prior to analysis.

Just prior to (time 0) and 1, 5, 15 and 30 min after addition of capacitating agents, sperm samples were prepared for analysis by diluting 50 μL of sperm into 450 μL of BGM3, filtering the sperm suspension through a 40 μm nylon mesh to remove debris and immediately analyzing the sample using a flow cytometer.

Flow Cytometric analysis

The percentages of viable cells and the percentages of viable cells possessing either low or high levels of intracellular calcium were determined using an Epics V flow cytometer (Coulter Electronics, Miami, FL) fitted with a beveled tip to orient the sperm (Johnson and Pinkel, 1986). The flow cytometer was equipped with an argon laser tuned to 488 nm with the voltage set to 100 mW of power to excite both Fluo-3 and PI. The filter setup to detect sperm fluorescence included a 515 nm long pass filter with a 457-505 nm laser blocker; a 550 nm dichroic beam splitter and a combination of a 525 nm band pass with a 560 nm short pass filter to detect Fluo-3 and a 610 nm long pass filter to detect PI.

The sperm samples were separated into live (PI negative) and dead (PI positive) populations as previously described (Graham *et al.*, 1990). Within the live population, subpopulations of sperm possessing low levels of intracellular calcium and high levels of intracellular calcium were detected (Fig. 1) and the percentages of the live cells in each category (low or high intracellular calcium) were determined by the software analyzing the cells (Cyclops, Cytomation, Ft. Collins, CO).
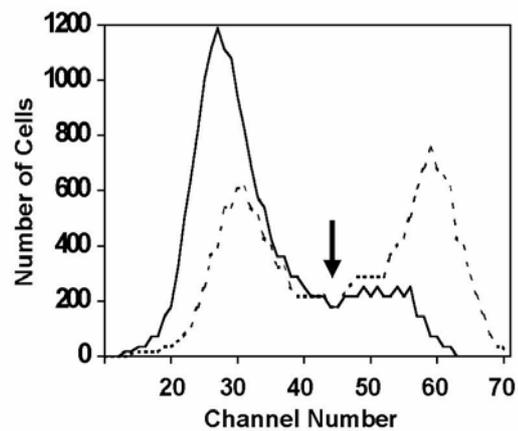
Figure 1. Frequency histograms of live sperm from one stallion evaluated by flow cytometry after addition of fluo-3 AM and incubation in either BGM-3 medium alone for 5 min (———) or in BGM-3 with 100 nM ionophore A23187 for 5 min (- - - - - -). The arrow indicates the channel corresponding to the minimum fluorescence required for high calcium classification (high calcium cells to right of the arrow).

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Statistical Analysis

The percentages of live sperm and the percentages of sperm having high levels of intracellular calcium were analyzed using multivariate analysis for repeated measures using a general-linear-models procedure (SAS, 1986). Sires were considered as a random variable and treatments as fixed. Treatment means, for both the percentages of live sperm and the percentages of live sperm possessing high intracellular calcium, at each time period were separated using Student-Newman-Kuels multiple range test (SAS, 1986).

Results

The mean percentage of motile sperm cells immediately before and after washing through the Percoll gradient for fresh and frozen equine and bovine sperm are showed in Table 1.

Table 1. The percentages of motile cells in fresh and frozen-thawed sperm samples from six stallions and six bulls prior to and after centrifugation through a 45/90% discontinous Percoll gradient.

| | Fresh Sperm | | Frozen-thawed sperm | | |
Species	Pre-Centrifuge	Post-Centrifuge	Pre-Centrifuge	Post-Centrifuge	SEM
Stallion	73[a]	87[a]	44[b]	86[a]	5
Bull	73[a]	82[a]	37[b]	64[a]	6

[a, b] Means with different superscripts within a row (species) are different at $P < 0.05$.

The relative changes in intracellular calcium levels in fresh and frozen-thawed sperm from bulls and stallions, after treatment with either BGM3, PC-12 or A23187 for 30 min to induce sperm capacitation in vitro, are presented (Fig. 2 and 3). The percentages of viable cells in the samples of fresh stallion sperm did not change over the 30 min incubation period when sperm was treated with either BGM3 or with PC-12 (means of 64 8% and 63 19%, respectively). However, when sperm were treated with A23187, there was a significant decrease in the percentage of live sperm by 5 min (from 72 16% to 57 18%; $P<0.05$), but there was no further decrease in sperm viability with longer incubation. Fresh stallion sperm incubated with BGM3 (Control) or PC-12 showed no increase in the percentages of sperm having high levels of intracellular calcium over the 30 min incubation time (Fig. 2a). However, when stallion sperm were incubated with A23187, the percentages of sperm exhibiting high levels of intracellular calcium increased from about 20%, prior to treatment, to over 60% after A23187 treatment by 15 min and remained at this level through 30 min.
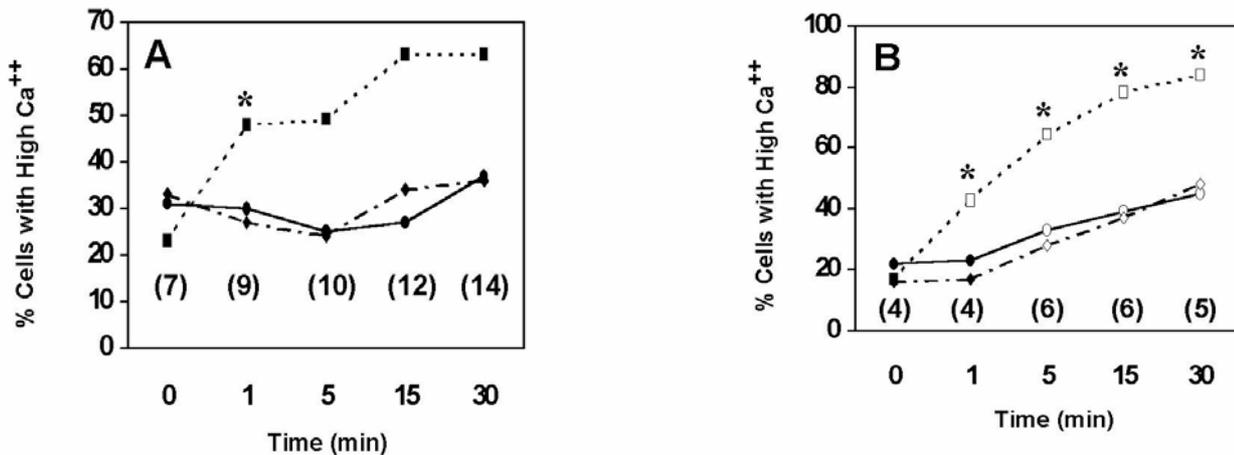
Figure 2. The percentages of viable fresh (A) or frozen-thawed (B) stallion sperm possessing high intracellular calcium concentration when sperm were incubated in BGM-3 medium (control; ————), BGM-3 with 100 µM dilaurylphosphatidylcholine (— —— —— —) or BGM-3 with 100 nM ionophore A23187 (- - - - - - - -) for up to 30 min. Numbers in parentheses are the standard error of the means associated with means within each time period. An asterisk (*) indicates the mean value, within a time period, is different from incubation in BGM-3 alone ($P<0.05$). Open symbols ("G) indicate the mean value, within a specific treatment, is different from the mean of that treatment at time zero ($P<0.05$).

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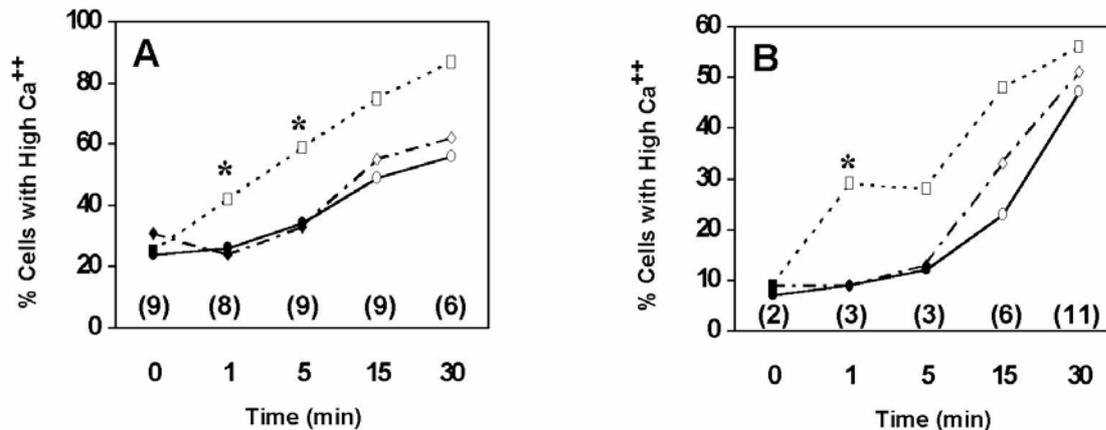
Figure 3. The percentages of viable fresh (A) or frozen-thawed (B) bull sperm possessing high intracellular calcium concentrations when sperm were incubated in BGM-3 medium (control; ———), BGM-3 with µ100 M dilaurylphosphatidylcholine (− —— − — −) or BGM-3 with 100 nM ionophore A23187 (- - - - - - - -) for up to 30 min. Numbers in parentheses are the standard error of the means associated with means within each time period. An asterisk (*) indicates the mean value, within a time period, is different from incubation in BGM-3 alone (P<0.05). Open symbols (" G) indicate the mean value, within a specific treatment, is different from the mean of that treatment at time zero (P<0.05).

The percentages of viable frozen-thawed stallion sperm decreased from initial values by 5 min, even in the control samples (73 to 62%, 76 to 61% and 72 to 57% respectively for control, PC-12 and A23187 treatment; P>0.05), however, there was no further decrease in sperm viability after 30 min incubation (60%, 60% and 53%, respectively). The percentages of sperm having high levels of intracellular calcium also increased for control samples and samples treated with PC-12 by 5 min and continued to increase throughout the 30 min incubation time period (Fig. 2b). Treatment with A23187, induced an immediate increase in intracellular calcium, with the mean percentage of sperm having high levels of intracellular calcium increasing from 17% prior the addition of A23187 to 43%, after addition of A23187 for 1 min. (P<0.05).

None of the capacitation treatments affected the viability of fresh bull sperm (85 6%, 83 8% and 86 5% for control, PC-12 and A23187 treatment respectively; P>0.05) during the incubation time. The calcium ionophore A23187 induced an immediate uptake of calcium in fresh bull sperm (Figure 3a). However, unlike stallion sperm, fresh bull sperm treated with BGM3 (control) or PC-12 also showed an increase in intracellular calcium, to the level by 15 min.

The percentages of viable cells in frozen-thawed bull sperm samples decreased within 5 min, similar to that seen for stallion sperm (80 to 74%, 80 to 75% and 81 to 74% respectively for control, PC-12 and A23187; P<0 05). Treatment with A23187 caused an immediate increase in the percentages of viable sperm having high levels of intracellular calcium (the percentage increased from 9% to 29% in 1 min) and the percentages of viable sperm with high levels of intracellular calcium continued to increase throughout the 30 min incubation period (Fig. 3b). Frozen-thawed control bull sperm and bull sperm treated with PC-12 did not show an immediate increase in the calcium influx into the cells, however, the percentages of sperm with high intracellular calcium increased by 15 min of incubation for these treatments and by 30 min of incubation were similar to samples treated with A23187.

Discussion

The results of this study confirm the findings of previous reports, that separation of spermatozoa by Percoll gradient selects spermatozoa with higher motility (Arcidiacomo *et al*., 1983; Lessley and Garner, 1983; Kaneko *et al*., 1986; Le Lannou and Blanchard, 1988; Moohan and Lindsay, 1995). Although there was no statistical difference between the percentages of motile cells just after collection and after washing through Percoll gradient for fresh bovine and equine semen, the percentages of motile frozen-thawed sperm was significantly higher after washing sperm through

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the Percoll gradient.

Cryopreservation and subsequent thawing resulted in a significant decline in the number of motile and viable spermatozoa. Furthermore, evidence from human studies suggests that the presence of non-functional gametes in sperm suspensions impairs the fertilizing ability of normal spermatozoa, and influences sperm movement characteristics (Aitken *et al*., 1988). Aitken and Clarkson (1988) found that a sub-population of spermatozoa having poor motility could be isolated in the low density portion of Percoll gradient, and that removing that population selects a sperm population with high progressive motility.

Cytoplasmic membranes of ejaculated spermatozoa contain high concentrations of cholesterol and are covered by proteins and glycoproteins originating from epididymal fluid or seminal plasma, all of which stabilize the sperm membrane and prevent a premature acrosome reaction (Triana *et al*., 1980). Capacitation induces morphological changes in sperm and a decrease in the concentrations of cholesterol in the external surface of the plasma membrane overlying the sperm head (Lin and Kan, 1996). Cholesterol restricts lateral mobility of membrane components, thereby, stabilizing the membranes. During capacitation, cholesterol is lost from sperm plasma membrane and this destabilizes the plasma membrane making it more permeable to calcium ions (Langlais and Roberts, 1985) and capable of fusing with the underlying outer acrosomal membrane (Lin and Kan, 1996).

It is generally accepted that capacitation involves many biochemical changes in the membrane of the sperm head, which alters the sperm membrane permeability to calcium (Yanagimachi and Usui, 1974; Zao *et al*., 1985; Frazer and McDermott, 1992). The specific actions of calcium, however, are not well understood. It has been proposed (see Meizel, 1978 for review) that calcium is involved in the activation of proacrosin to acrosin prior to the acrosome reaction. Calcium also activates the adenylate cyclase-cyclic AMP system, thereby increasing intracellular concentrations of cAMP (Morton *et al*.,1974; Hyne and Garbers, 1979a, b). Increased intracellular calcium levels are also required for membrane fusion events associated with acrosomal reaction (Green, 1978; Singh *et al*., 1978). However, high intracellular calcium levels for prolonged periods also leads to cell death if fertilization does not occur (Hunter, 1987).

In this study we observed that the percentages of live cells in the samples of fresh stallion semen did not change over the 30 minutes incubation with either BGM-3 or PC-12. However after 5 min incubation of sperm with A23187 more dead cells were found. These results agree with others studies, where the treatment of sperm with A23187 reduced sperm motility or reduced cell viability (Schams-Borhan and Harrison, 1981; Far-

lin *et al*., 1992). Calcium ionophore forms a lipophilic complex with calcium and facilitates its transport across the sperm plasma membrane, resulting in the generation of the acrosome reaction (Talbot *et al*., 1976). The drop in motility reported in earlier papers and the reduced viability seen in samples treated with A23187, in this and previous studies, may be related and are likely associated with calcium's effect on cellular ATPases (Fraser and McDemott, 1992). In somatic cells and in spermatozoa, a calcium-ATPase located in the plasma membrane is important in maintaining low intracellular calcium levels by pumping calcium out of the cell (Schatzmann, 1982). The high influx of calcium into sperm treated with A23187 may be too great for the calcium-ATPase to maintain anormal intracellular calcium concentrations. This may result in high intracellular calcium concentrations not only in the sperm head, but may also induce high calcium concentrations in the sperm midpiece, where it may affect mitochondrial function.

Calcium levels of fresh stallion sperm incubated with BGM-3 (control) or PC-12 did not increase during the 30 min incubation period, whereas intracellular calcium levels did increase in similarly treated bull sperm. This may be due to the fact that stallion sperm are more difficult to capacitate *in vitro* than sperm from other domestic species (Didion *et al*., 1989) and only a very small proportion of stallion sperm undergo a spontaneous acrosome reaction *in vitro* (Casey *et al*., 1993).

PC-12 liposomes have previously been used to induce stallion spermatozoa to undergo an acrosome reaction enabling them to penetrate into zona-free hamster oocytes (Graham *et al*., 1987). We did not observe any significant changes in intracellular calcium levels when fresh stallion sperm were treated with PC-12. This difference may be due to the difference in the concentration of PC-12 used in this and the previous experiment. Graham *et al*. (1987) achieved the highest rate of sperm penetration into zona-free hamster oocytes when stallion sperm were treated with 51 μM PC-12. However, fewer sperm were motile after 30 minutes of incubation with this concentration of PC-12 (25%) than in control samples (61%; Graham *et al*., 1987). We utilized a much lower PC-12 concentration to treat sperm in this study (100 nM) in an effort to maintain sperm viability and motility throughout the incubation period. It appears that this concentration was too low to initiate a change in sperm intracellular calcium levels.

When fresh stallion spermatozoa were incubated with A23187, the percentage of sperm that exhibited high levels of intracellular calcium increased after 5 min of incubation reached a maximum by 15 min and remained high through 30 min of incubation. However, there were large variations in the way sperm from individual stallions responded to A23187, resulting in high

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STD errors for the means. Zhang *et al*. (1991) and Baranska and Tischner (1995) also reported large individual stallion differences in the morphologic and functional changes exhibited by spermatozoa in response to treatment with A23187. One of the reasons for the differences in capacitation capacity of spermatozoa from different stallions may be due to differences in the individual biochemical makeup or molecular structure of the plasma membranes of spermatozoa from the individual sires. Similarly, Davis (1981) suggested that variations in the capacitation rate of human sperm may be related to differences in the cholesterol/phospholipid ratio of sperm from individual men.

None of the capacitation treatments affected sperm viability in fresh bull sperm samples. Bull spermatozoa undergo an acrosome reaction in presence of PC-12 (Graham *et al*., 1986; 1987), likely due to its ability to intercalate into the sperm plasma membrane lipid bilayer and creating pores that increase membrane permeability to ions (Nolan *et al*., 1992). Once incorporated into the sperm plasma membrane outer leaflet, PC-12 alters the properties of the plasma membrane permitting fusion of the plasma membrane and outer acrosomal membrane, in presence of calcium. Accumulation of PC-12 in the outer leafl*et al*so causes a shift in the trans-bilayer distribution of cholesterol between the inner and the outer monolayer of the plasma membrane, resulting in a cholesterol-poor, and less stable inner leaflet. It is this inner leaflet, which is ultimately involved in fusion with the outer acrosomal membrane (Nolan *et al*., 1992). Similar to our results with stallion spermatozoa, the concentration of PC-12 that we treated bull spermatozoa with in this experiment did not reduce the percentages of viable cells after 30 min of incubation.

Incubating bull spermatozoa with A23187 induces sperm capacitation and acrosomal loss (Fraser *et al*., 1995). Similarly, we observed an immediate increase in intracellular calcium when fresh bull spermatozoa were treated with A23187. Spermatozoa treated with A23187 rapidly become immobile when BSA is omitted from the medium (Suarez *et al*., 1987; Fraser, 1982), probably due to extremely high increases in intracellular calcium concentrations, which affect mitochondrial capacity to produce ATP (Bradley *et al*., 1979). However, sperm motility can be maintained if BSA is added to the medium. Albumin sequesters the calcium ionophore from the plasma membrane, thereby permitting membrane to more effectively regulate the movement of calcium across the membrane and inhibit the accumulation of intracellular calcium to toxic levels (Sarkadi *et al*., 1976). The BGM-3 medium utilized in this experiment contained 6% BSA which prevented the influx of calcium into the bovine sperm to toxic levels, and maintained sperm viability throughout the incubation period.

An understanding of how sperm regulate intracellular calcium levels also has important implications in cryopreserving spermatozoa. The intracellular calcium levels of frozen-thawed spermatozoa increase more rapidly than fresh sperm. Cryopreservation essentially initiates sperm capacitation and this may lead to premature sperm death (Parrish *et al*., 1989b). In the experiments presented here, the percentages of viable frozen-thawed equine and bovine sperm decreased in samples after 5 minutes of incubation, even in the control samples. Cryopreservation alters sperm membrane structure which can ultimately alter sperm membrane permeability to calcium and lead to potentially lethal increases in intracellular calcium concentrations (Parks and Lynch, 1992). Therefore, it was not unreasonable to see high percentages of frozen-thawed equine sperm with high intracellular calcium levels even in untreated sperm samples after as little as 5 min incubation.

Hoshi *et al*. (1990) reported that spermatozoa with lower cholesterol/phospholipid ratios required less time to capacitate than spermatozoa having higher ratios of cholesterol. Although stallion spermatozoa do not undergo a spontaneous acrosome reaction, freezing and thawing may cause sufficient cholesterol loss from the plasma membranes to alter the cholesterol/phospholipid ratio of the membranes significantly (Parks and Lynch, 1992) and alter sperm capacitation (Wheeler and Seidel, 1987; Slonina *et al*., 1995; Lin and Kan, 1996).

In addition, spermatozoa undergo a series of changes in cell volume which result in the aggregation of intramembranous particles of the lipoproteins in cell membrane, during cryopreservation (Holt and North, 1984). Rearranging the protein structure of membranes may also greatly affect membrane function after thawing (Hammerstedt *et al*., 1990). It is likely that the differences in the way fresh and frozen spermatozoa are affected A23187, and PC-12 may be related to differences in the stability of membranes that have never been frozen and those that have been frozen and thawed.

Similar intracellular calcium changes were observed in bull sperm. Fresh and frozen-thaw bull sperm responded similarly to A23187 by showing an immediate rise in the percentage of cells with high intracellular calcium. However, unlike stallion spermatozoa in which the percentages of cells with high concentrations of intracellular calcium leveled of after 15 min, the percentages of bull sperm with high concentrations of intracellular calcium continued to increase during the 30 min incubation period.

Frozen-thawed bull sperm, either untreated or treated with PC12, did not show an immediate increase in the levels of intracellular calcium, but the percentages of spermatozoa with high intracellular calcium levels increased significantly by15 min of incubation

and were similar to those for samples treated with A23187 by 30 min. Again, sperm cryopreservation, likely induced changes in sperm membrane permeability resulting in a more rapid influx of calcium, which was not seen in fresh sperm.

Sperm capacitation is a poorly understood phenomenon, partly due to a dearth of techniques to study it. Although several staining techniques can be used to identify sperm in different stages of capacitation (Farlin *et al.*, 1992; Cheng *et al.*, 1996) these techniques are plagued with inconsistencies and the most commonly used technique to assess sperm capacitation is evaluating the ability of sperm to undergo an acrosome reaction. Using Fluo-3, changes in intracellular calcium levels, one part of the capacitation process, can be effectively monitored and treatments that affect changes in intracellular calcium levels evaluated. These studies indicate that the calcium ionophore, A23187, increases calcium movement into fresh and frozen-thawed spermatozoa from stallions and bulls, however, PC-12, at the concentrations utilized, failed to do so. In addition, cryopreservation altered the influx of calcium into both stallion and bull sperm, likely due to membrane changes induced by the freeze-thaw process making the plasma membranes more permeable to calcium. Perhaps more importantly, this technique provides a useful tool to rapidly evaluate sperm capacitation.

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