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Effects of Seminal Plasma on Cooling-Induced Capacitative Changes in Boar Sperm

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ABSTRACT: Porcine seminal plasma (SP) has been shown to contain factors that have a decapacitative or capacitation-inhibiting effect on sperm. The objectives of the present study were to compare the capacitative changes observed in cooled sperm with those seen in sperm after in vitro capacitation and to determine whether SP could prevent these changes. Sperm were subjected to incubation or to slow cooling under noncapacitating or capacitating conditions. The effect of SP on protein tyrosine phosphorylation and the ability of the sperm to undergo an acrosome reaction (AR) were determined. Cooled sperm displayed an increased level of tyrosine phosphorylation and a higher percentage of induced AR sperm compared to incubated sperm. The addition of SP inhibited the number of ARs that occurred during incubation and cooling. These results suggest that cooling of sperm augments the capacitative changes in sperm, and that SP contains a factor(s) that effectively prevents these changes.

Key words: Cryopreservation, semen, capacitation.
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Assisted reproduction is an integral part of pork production. More than 70% of sows and gilts are bred using artificial insemination (AI) in the swine industry (USDA, 2000). As of the year 2000, less than 1% of all AI in the swine industry was performed using frozen-thawed (FT) semen (Johnson et al, 2000). Using standard AI breeding techniques, farrowing rates and the total number of piglets born using FT sperm are generally decreased by 20% to 30% compared to insemination using fresh extended/chilled sperm (Johnson et al, 2000). Although these differences in farrowing rates and litter size can be decreased or eliminated using deep intrauterine insemination or specialized sperm freezing methods (Eriksson et al, 2002; Roca et al, 2003), it is still generally accepted that cryopreserved sperm have a shorter window of optimum fertility relative to fresh extended/chilled sperm (Waberski et al, 1994). This reduction in fertility of FT sperm is believed to be the result of cryoinjury to the sperm acquired during the freezing and thawing process (Bailey et al, 2000). This injury includes capacitation-like changes, which are referred to as cryocapacitation.

Capacitation has been described as a reversible biochemical process that enables the sperm to undergo an acrosome reaction (AR) and penetrate the zona pellucida of the ovum (Austin, 1952; Chang, 1952). Bicarbonate and calcium appear to be major players in the capacitation process of boar sperm by setting in motion an interconnected signaling pathway of adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) (Gadella et al, 2000; Harrison et al, 2000). In the boar, a downstream event from the AC/cAMP/PKA pathway is phospholipid scrambling and cholesterol ejection from the membrane (Flesch et al, 2001). A late event in capacitation is the phosphorylation of tyrosine residues on membrane proteins (Visconti et al, 1995; Flesch et al, 1999).

Tyrosine phosphorylation patterns and chlortetracycline staining patterns that are consistent with capacitation have been observed in capacitated and cooled sperm (Watson, 1996; Bravo et al, 2005; Vadnais et al, 2005a). In boar sperm, changes in temperature induce a lipid phase change from the liquid-crystalline to gel phase during cooling, which results in increased membrane fluidity (Holt et al, 1984; De Leeuw et al, 1990). The major phase change occurs in the vicinity of 15-5°C (Drobnis et al, 1993). Thus, the process of slowly cooling sperm to 5°C results in increased membrane fluidity, which may be responsible for the capacitation-like changes in the sperm.

The addition of seminal plasma (SP) to boar sperm has been shown to reduce the percentages of capacitated and cryocapacitated sperm, as measured by CTC staining, tyrosine phosphorylation, and in vitro fertilization (Zhu et al, 2000; Kaneto et al, 2002; Suzuki et al, 2002; Vadnais et al, 2005b). Similarly, the inclusion of SP at insemination has been demonstrated to increase fertility (Rozeboom et al, 2000; Alghamdi et al, 2004). Since the sperm membrane is coated with epididymal

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proteins during epididymal maturation and with seminal plasma proteins at ejaculation, these proteins have been postulated to act as decapacitation factors that prevent a premature AR (Dukelow et al, 1967; Oliphant et al, 1985; Fraser et al, 1990; Roberts et al, 2003). These specific proteins are lost from the surface of the sperm concomitant with lipid reorganization and cholesterol loss from the sperm membrane during capacitation. Potentially, the loss of these proteins prior to the lipid phase change in the sperm membrane associated with cryopreservation may explain the initiation of cryocapacitation.

The objectives of the experiments described in the present study were to compare capacitation and cooling-induced capacitation in boar sperm by examining protein tyrosine phosphorylation, which is a step in the capacitation signal transduction pathway, and the AR, which is the endpoint of capacitation. The second objective was to determine the effects of SP on these two events.

Materials and Methods

Reagents and Chemicals

Antiphosphotyrosine (4G10) monoclonal IgG conjugated to horseradish peroxidase (HRP) was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-β-tubulin (E7) monoclonal IgG1 was purchased from the Developmental Hybridoma Bank at the University of Iowa (Iowa City, Iowa). Calcium ionophore A23187 and propidium iodide (PI) were purchased from Molecular Probes (Eugene, Ore). Cold-water fish skin gelatin (40% solution) was purchased from Electron Microscopy Sciences (Washington, Pa). Restore Western Blot Stripping Buffer and Super Signal West Pico Chemiluminescent Substrates were purchased from Pierce Chemical Co (Rockford, Ill). All other chemicals and reagents were purchased from Sigma-Aldrich (St Louis, Mo).

Media

The capacitating medium (CM) was composed of 4.8 mM KCl, 1.2 mM KH_2PO_4, 95 mM NaCl, 5.55 mM glucose, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 0.4% BSA, and 2 mM pyruvate (pH 7.4). The noncapacitating medium (NCM) contained 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 5.55 mM glucose, and 2 mM pyruvate (pH 7.4). Both media have been described previously (Tardif, 2001).

Collection of Sperm and Seminal Plasma

Semen was collected using the gloved-hand technique into a gauze-covered container from 3 boars (2 Yorkshires and 1 Duroc) of proven fertility, as defined by the siring of a litter within 6 months prior to collection. The boars were housed at the University of Minnesota Research Farm (St Paul, Minn). Immediately after collection, 2 mL of ejaculate was diluted into 8 mL of NCM, maintained at 39°C, and transported to the laboratory, where a subjective assessment of motility was performed. Samples with fewer than 60% motile sperm were not used. The sperm concentration was determined in the extended semen sample using a hemocytometer (Douglas-Hamilton et al, 2005). The seminal plasma used in the experiments was separated from sperm cells by centrifugation at 1000 × *g* for 30 minutes. Pooled aliquots were stored at -80°C until use.

Incubation and Cooling Treatments

Sperm were suspended in NCM, CM, CM with 10% or 20% (v/v) SP. Incubation was at 39°C in 5% CO_2 in air for 3 hours prior to examination of tyrosine phosphorylation or AR. All of the incubations occurred under these conditions unless otherwise stated. Sperm were cooled from the ambient temperature to 5°C in an Equitainer (Hamilton-Thorne, South Hamilton, Mass) at a rate of 0.3°C per minute (Devireddy, 2002). Immediately after cooling, the sperm were placed in the incubator at 39°C in 5% CO_2 in air for 10 minutes prior to examination of tyrosine phosphorylation or AR.

Acrosome Reaction

The AR was induced in aliquots of sperm (4×10^7 sperm/mL) by adding the calcium ionophore A23187 in dimethylsulfoxide to the sperm suspension to a final concentration of 2 μM and incubating for 30 minutes.

For flow cytometry (Ashworth et al, 1995), sperm were resuspended to a concentration of 4×10^6 sperm in 500 μL of NCM at 37°C. Twenty μL of FITC-PSA (0.5 μg per million cells) and 10 μL of PI (4 μg per million cells) were added to the resuspended sperm and incubated for 10 minutes at 37°C. After staining, the cells were immediately analyzed in the FACScan flow cytometer (BD Biosciences, San Diego, Calif) equipped with an air-cooled, 488-nm Argon laser, to determine the proportions of FITC-PSA-bound and PI-stained cells. The FACScan flow cytometer contains 3-color fluorescence detection in addition to the forward and side scatter parameters. The forward (linear) and side (log) light scatter parameters were gated to include only those cells that possessed the light scatter characteristics of sperm for fluorescence analysis. A total of 50 000 events were collected and then gated into a sperm population and analyzed for the log of their fluorescence for each treatment. The green fluorescence (FL1: FITC) was collected through a 525-nm bandpass filter, and the red fluorescence (FL3: PI) was collected through a 620-nm bandpass filter. Single-parameter histograms for FL1 and FL3 were acquired along with 2-parameter cytograms of FL1 and FL3. LN_2-killed sperm stained with PI alone and unstained sperm were used as positive and negative controls, respectively. Figure 1 illustrates the sorting and quantitation of live and dead sperm, and of acrosome-intact and acrosome-reacted sperm.

SDS-Page and Western Blotting

Tyrosine phosphorylated proteins were detected by SDS-PAGE and Western blotting, as described previously (Roberts

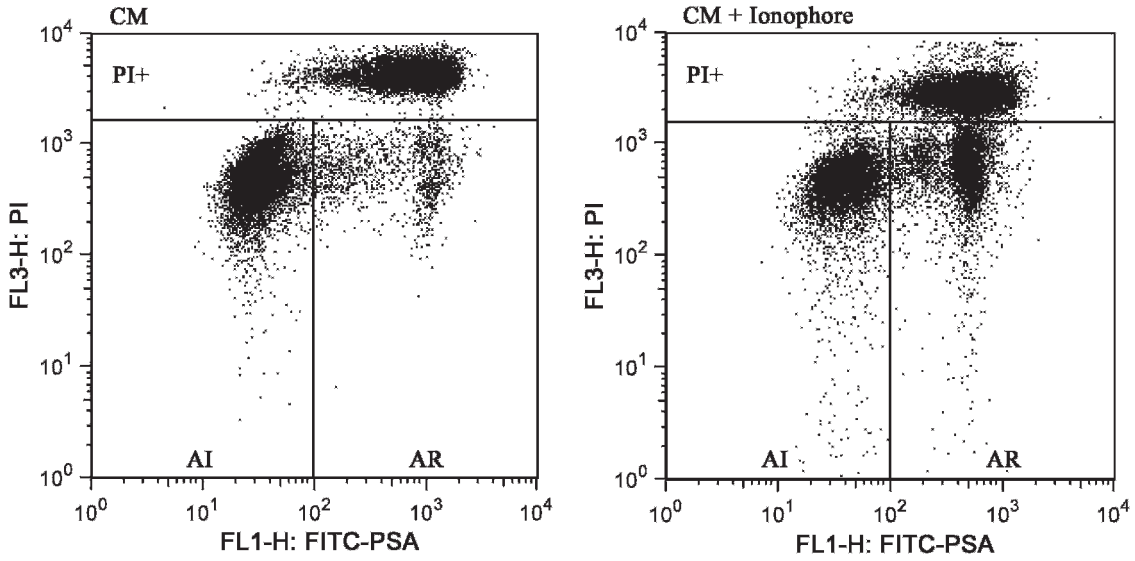

Figure 1. Representative quantitation of live and dead sperm, as well as acrosome-intact and acrosome-reacted sperm after a capacitation incubation in the absence (CM) or presence (CM+I) of ionophore. The fluorescence intensities for FITC-PSA are plotted on the X-axes, and the fluorescence intensities for propidium iodide, a vital dye, are plotted on the Y-axes of the scattergrams. In the absence of ionophore (CM panel, left), most of the PI-negative cells have low FITC-PSA staining and represent acrosome-intact (AI) sperm. With the addition of ionophore (CM+I panel, right), the population of cells that is shifted to the right on the FITC-PSA axis represents sperm that have undergone an AR in response to the ionophore. The sperm that stain positive for PI (PI+) are assumed to be dead. The acrosome-reacted populations of cells, as percentages of the viable cells (PI-negative), are plotted in Figures 2 and 3.

et al, 2003). After experimental incubation, 4×10^6 sperm in 500 µL of treatment-specific media were centrifuged at 12 000 $\times$ *g* for 10 minutes. The sperm pellet was washed once with 500 µL of PBS and resuspended in 50 µL of 1$\times$ Laemmli sample buffer. The samples were heated to 96°C for 5 minutes and centrifuged at 12 000 $\times$ *g*. Supernatants were transferred to new tubes and stored at 4°C until use. PAGE with 10 µL of each sample, equivalent to 4×10^6 sperm, was performed with 12% Tris-glycine gels. Proteins were transferred to Immobilon P membranes (Millipore, Bedford, Mass) at 100 V for 1 hour at 4°C. Blots were blocked with 6.5% fish skin gelatin in TTBS (20 mM Tris-HCl, 4 mM Tris base, 150 mM NaCl, 0.1% Tween) for 30 minutes, followed by incubation at room temperature with HRP-conjugated anti-phosphotyrosine antibody (1:15 000) for 1 hour. The blots were then washed in TTBS twice for 10 seconds each, followed by two 5-minute washes, incubation with Super Signal for 5 minutes, and exposure to x-ray film.

Beta-tubulin was used as a loading control for the Western blots. Blots previously probed with the anti-phosphotyrosine antibody were stripped by incubation at room temperature under constant agitation for 1 hour in 15 mL of Restore Stripping Buffer. The blots were blocked in 3% nonfat dry milk in PBS for 30 minutes, reprobed with the anti-beta-tubulin antibody E7 (1:500) in 1% BSA and PBS for 1 hour, and visualized with horseradish peroxidase-conjugated secondary antibody (1:5000) in 1% BSA and PBS-T (0.1% Tween in PBS) for 1 hour. Finally, the blots were washed with 1% BSA and PBS-T twice for 10 seconds each, followed by two 5-minute washes, incubation in Super Signal for 5 minutes, and exposure to x-ray film.

Statistical Analysis

The protein tyrosine phosphorylation gels were analyzed by scanning with Un-Scan-It software version 6.1 (Silk Scientific Corp, Orem, Utah). The flow cytometry data were first analyzed using the FlowJo version 7.1 software (Tree Star, Ashland, Ore), to report the percentages of AR and acrosome-intact viable sperm. The percentages for the AR obtained by flow cytometry were analyzed using the Statistical Analysis Systems software version 9.1 (SAS Institute Inc, Cary, NC). The percentages ($P > .01$) for each response category (AR and acrosome-intact) were analyzed by 1-way repeated-measures ANOVA. The least-squares means for each treatment were back-transformed for the purpose of reporting point estimates and preparing the figures.

Results

Acrosome Reaction

As expected, incubation in NCM resulted in no significant increase in AR-inducible sperm either before or after ionophore induction, while sperm incubated in CM had an AR-inducible fraction of 35% (Figure 2), which confirms the appropriateness of the NCM and CM conditions. In contrast, cooling sperm in NCM resulted in a substantial proportion of the sperm (26% over background) that could be induced to undergo an AR in the presence of ionophore (Figure 2). In addition, cooling the sperm in CM resulted in a further increase in

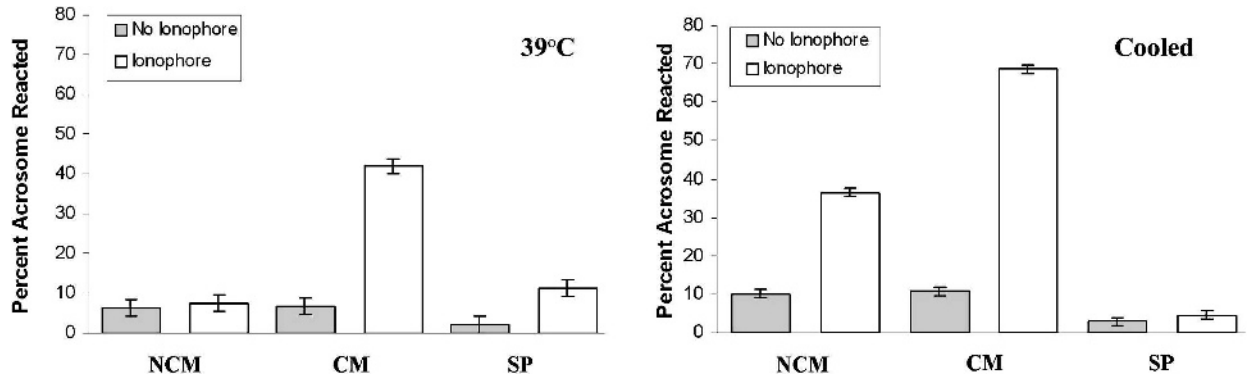

Figure 2. The percentages of acrosome-reacted (AR) boar sperm (± SE) after incubation for 3 hours at 39°C (left panel) or cooled (right panel) in noncapacitating media (NCM) or capacitating media (CM) without and with 20% (v/v) seminal plasma (SP). Spontaneous AR is indicated by gray bars (no ionophore), and induced AR is indicated by white bars (ionophore). Sperm were counted by flow cytometry, as illustrated in Figure 1.

the AR-inducible fraction (58% over background) compared to sperm incubated in CM. These results indicate that cooling has a capacitating effect on sperm that is exacerbated in a medium that supports capacitation, as measured by the percentage of cells that are able to undergo an ionophore-induced AR.

The inclusion of 20% SP during incubation or cooling in CM significantly reduced the percentage of sperm that were able to undergo the AR when challenged with ionophore (Figure 2). There was no significant difference between the ionophore-inducible fractions of the incubated or cooled sperm with SP inclusion. These results demonstrate a capacitation-inhibiting effect of SP, as indicated by sperm that were unable to undergo an ionophore-induced AR when SP was included with the sperm.

Protein Tyrosine Phosphorylation

To determine if the effects of SP on sperm include suppression of the signal transduction cascade established for capacitation, we assessed the levels of tyrosine phosphorylation induced by capacitation and cooling. As shown in Figure 3, protein tyrosine phosphorylation increased in sperm incubated in CM as compared to NCM. Attenuation of protein tyrosine phosphorylation occurred with the inclusion of 10% and 20% (v/v) SP. Although these Western blots were only semiquantitative, densitometric scans of these blots confirmed a dose-dependent trend in tyrosine phosphorylation inhibition by SP. The suppression of tyrosine phosphorylation appeared to affect all phosphorylated proteins, although the suppression of a 32-kd phosphorylated protein was particularly pronounced and consistent. The addition of 20% SP (v/v) decreased the tyrosine phosphorylation band intensity by 250% compared to CM tyrosine phosphorylation, as measured by densitometry.

Sperm cooled to 5°C exhibited increased protein tyrosine phosphorylation, including that of the 32-kd protein, when suspended in either NCM or CM (Figure 3). There was also a relative decrease in the intensity of tyrosine phosphorylation of the band that migrated above 25 kd relative to the band immediately below it in the sperm cooled in either NCM or CM. As with the incubated sperm, SP conferred a dose-dependent suppression of phosphorylation. The inclusion of 20% (v/v) SP resulted in the most significant stabilization of cooling-induced capacitation, decreasing the 32-kd band intensity by 175% compared to sperm cooled in CM.

Discussion

We have shown that cooling sperm to 5°C results in capacitation-like changes. When the AR was measured by flow cytometry, there was a significant increase in the inducible AR fraction of viable sperm cooled in NCM or CM. When the AR was examined by fluorescent microscopy using fluorescein-labeled *Pisum sativum* agglutinin, a similar pattern of cooling-induced AR was observed (data not shown).

Although these experiments do not prove that the changes induced by cooling represent true capacitation, the fact that cooling increased both the level of protein tyrosine phosphorylation of specific sperm proteins and the percentage of AR-inducible sperm suggests that cooling stimulates the true capacitation pathway. On the other hand, the fact that cooling also increased the number of sperm undergoing an induced AR in NCM, in the absence of calcium, bicarbonate, and cholesterol-binding proteins, suggests that cooling bypasses certain requirements of normal capacitation. One explanation for the NCM cooling data is that the increased

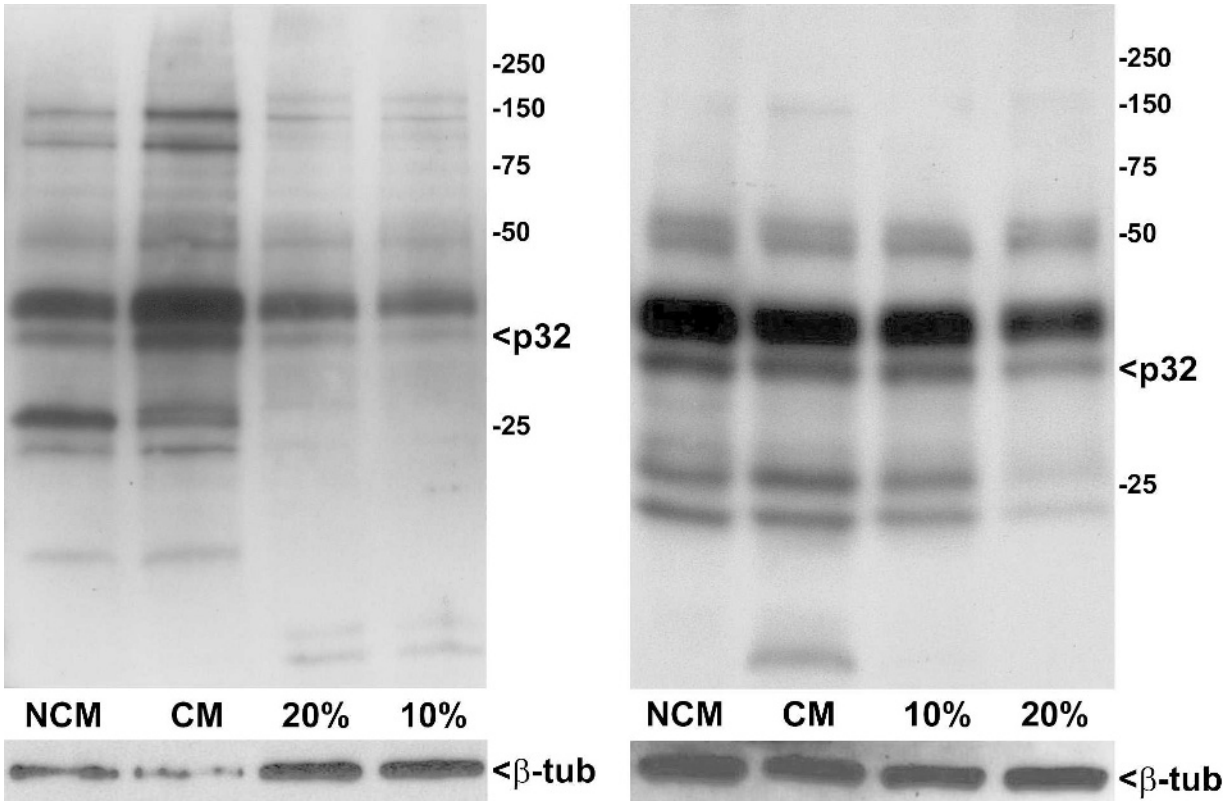

Figure 3. Western blot analysis of protein tyrosine phosphorylation in boar sperm. The upper panels show tyrosine phosphorylation of total proteins from sperm incubated for 3 hours at 39°C (left) or cooled (right) in noncapacitating media (NCM), capacitating media (CM) with and without the inclusion of 10% or 20% (v/v) seminal plasma. The position of p32, a protein shown to be phosphorylated during capacitation, is noted. In the lower panels, the blot was stripped and reprobed for beta-tubulin (β-tub) as a loading control.

percentage of dead and lysing cells may have altered the composition of the medium, thereby supplying factors necessary for capacitation. Similarly, cell death and lysis caused by cooling may release reactive oxygen species, which have been demonstrated to play a role in capacitation, specifically tyrosine phosphorylation (Aitken et al, 1995; Rivlin et al, 2004).

Consistent with other studies, we have demonstrated increases in protein tyrosine phosphorylation in both incubated and cooled sperm, including an increase in the phosphorylation of a 32-kd protein band (Green et al, 2001; Kaneto et al, 2002; Tardif et al, 2003; Bravo et al, 2005). This 32-kd protein has recently been identified as proacrosin-binding protein, and its phosphorylation has been clearly shown to be associated with capacitation (Dube et al, 2005). This study also demonstrated suppression of ionophore-induced AR and protein tyrosine phosphorylation, including the 32-kd protein band, with the addition of SP to both capacitated and cooled sperm. The highest level of suppression occurred in the presence of 20% (v/v) SP. Thus, one or more components of SP appear to have the ability to inhibit capacitation.

The inhibitory effect of SP on capacitation may be the result of SP proteins binding to the surface and preventing the membrane changes required for capacitation and/or inhibiting the signal transduction pathways of capacitation at other points. In boars, more than 90% of the SP proteins are sperm adhesion proteins (Topfer Petersen, 1998). There are 2 main groups of sperm adhesion proteins. The first group (AQN-1, AQN-3, AWN) comprises heparin-binding proteins that stabilize the plasma membrane and are lost during capacitation (Calvete, 1997). The second group includes PSP-I and PSP-II, which are non–heparin-binding proteins that form the major portion of sperm adhesion proteins in SP (Nimtz, 1999). Recently, Garcia et al (2006) have shown that the addition of boar non–heparin-binding SP proteins PSPI/PSPII protects sperm against the damaging effects of dilution, including decreases in viability, motility, and mitochondrial activity (Garcia et al, 2006). The PSPI/PSPII heterodimer protein also suppresses spontaneous ARs in diluted boar sperm incubated at 38°C for several hours, with the beneficial effects being largely conserved in the PSP-II subunit (Centurion, 2003; Garcia, 2006).

There are other possible explanations for the capacitation-inhibitory activity of SP. It could be that the antioxidant effects of the added SP are partially responsible for the prevention of cooling-induced capacitation and the subsequent AR. Alternatively, proteins in the SP may function to stabilize the membrane against cholesterol loss and lipid reorganization, similar to the way that lipoproteins in egg yolk are hypothesized to act (Benson et al, 1967; Bergeron et al, 2004). In any case, the inhibitory effects of SP on capacitation are likely to be reversible, since sperm capacitate in the female tract after dilution of the SP. Time-course studies examining the ability of sperm to capacitate after SP removal are in progress in our laboratory.

In conclusion, the processes of capacitation and AR represent a continuum of membrane alterations and signaling events, all if which end in the ability of the sperm to fertilize an oocyte. Sperm are subjected to protein additions from the epididymis and SP, which is a product of the accessory sex glands. These proteins may function to stabilize the sperm against premature capacitation and spontaneous AR. Significantly, these proteins may also protect the sperm from cooling-induced damage, such as cryocapacitation.

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