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Influence of osteopontin, casein and oviductal fluid on bovine sperm capacitation

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Abstract

Sperm must undergo a process termed capacitation in the female reproductive tract before they are capable of fertilization. Previous studies strongly suggest that capacitation of sperm occurs in the oviduct. The secreted extracellular matrix phosphoprotein osteopontin (OPN) has been positively correlated to fertility in Holstein bull seminal plasma and identified in the bovine oviduct, where we hypothesized it plays a role in sperm capacitation. To determine the effect of OPN on sperm capacitation, sperm were incubated in OPN purified from bovine milk in concentrations ranging from 1 to 20 µg/ml and subjected to dual fluorescence acrosomal and viability staining. Sperm were also incubated in casein (CSN; 1 to 20 µg/ml) and isthmic non-luteal oviductal fluid (INLODF), ampullary non-luteal oviductal fluid (ANLODF) and skim milk. Flow cytometry analysis showed that OPN induced capacitation at low concentration; the largest percentage of capacitated sperm followed after incubation in INLODF. Sperm intracellular calcium levels, an indicator of sperm capacitation, were increased by OPN and INLODF, and OPN had an overall positive effect on sperm viability. There was, however, no effect of any treatment on sperm mitochondrial activity. Experiments utilizing biotinylated OPN and CSN demonstrated that CSN, but not OPN, bound to sperm. The results presented here offer the first direct evidence of the capacitating effect of OPN on bovine sperm and provide confirmation of the increasing number of roles this fascinating protein plays in reproductive biology.

Keywords: osteopontin, capacitation, flow cytometry.

Introduction

Before they are capable of fertilization, sperm must undergo a period of preparation in the female reproductive tract known as capacitation (Austin, 1951; Chang, 1951). While many of the specific changes that sperm experience during this process are not completely understood, they include a number of cellular and molecular changes, including removal and alteration of sperm plasma membrane surface components and changes in intracellular ion concentrations (Yanagimachi, 1994). The exact site of sperm capacitation *in vivo* is still undetermined, although it can occur in the uterus (Austin, 1951; Bedford, 1969, 1970; Barros, 1974) and occurs more rapidly when sperm are exposed to first the uterine and then oviductal environments (Adams and Chang, 1962; Bedford, 1969; Hunter, 1969; Hunter and Hall, 1974). Studies with oviductal fluid and conditioned medium from oviductal epithelium explants suggest that capacitation occurs in the isthmus of the oviduct (Grippo *et al.*, 1995; Lefebvre and Suarez, 1996; Fazeli *et al.*, 1999).

Capacitation has been induced *in vitro* using heparin-like glycosaminoglycans (Parrish *et al.*, 1988, 1994) and oviduct-specific glycoprotein (OSG; McNutt and Killian, 1991; Anderson and Killian, 1994; King *et al.*, 1994), both of which can be found in the bovine oviduct. In addition, sperm can be artificially capacitated using heparin (Parrish *et al.*, 1988). Oviductal fluid (ODF) can capacitate sperm (Parrish *et al.*, 1989; McNutt and Killian, 1991), with more sperm capacitated in non-luteal ODF than in luteal ODF (McNutt and Killian, 1991). Binding of oviductal fluid proteins to sperm has also been demonstrated (McNutt *et al.*, 1992; King and Killian, 1994; Lapointe and Sirard, 1996; Rodriguez and Killian, 1998).

Osteopontin (OPN) is a secreted extracellular matrix phosphoprotein positively correlated to Holstein bull fertility in seminal plasma (Killian *et al.*, 1993; Cancel *et al.*, 1997) and the fluid of the male accessory sex glands (Moura *et al.*, 2006, 2007b). Recent work has indicated that OPN in AGF from Holstein bulls of high fertility is associated with increased sperm penetration of oocytes (Moura *et al.*, 2007a) and OPN may be involved in preventing porcine (Hao *et al.*, 2006) and bovine polyspermy (Erikson *et al.*, 2007) and is a potential ligand for oocyte integrins on bovine sperm during fertilization (Erikson *et al.*, 2007). In the bovine oviduct, only one OPN mRNA transcript was found and amounts did not vary during the estrous cycle, but three different protein isoforms were detected in ODF (Gabler *et al.*, 2003). These isoforms (55, 48 and 25 kDa) were present in ampullary and isthmic ODF during the luteal and non-luteal stages. The 25 kDa isoform was the most prevalent isoform except in the isthmus during the non-luteal phase of the estrous cycle, the time during which sperm enter the oviduct

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and prepare for interaction with the oocyte. This difference in isoform prevalence suggests a role for OPN in the events surrounding capacitation of sperm in the oviduct.

The purpose of this study was to evaluate the effect of purified OPN on sperm capacitation and sperm quality as indicated by mitochondrial activity, viability and intracellular calcium levels. The ability of OPN to bind to sperm was investigated as well, as this could be important to reproductive events.

Materials and Methods

Preparation of incubation treatments

Semen from three Holstein bulls housed at the Almquist Research Center was collected by artificial vagina, pooled, and washed twice in Modified Tyrode's medium (MTM; 700 *x g*; Parrish *et al.*, 1988). Sperm (5 x 10^7/ml) were incubated (39°C, 5% CO_2/air (v/v), 4 h) in the following treatments in MTM: 1) no treatment; 2) 1 µg/ml OPN; 3) 5 µg/ml OPN; 4) 10 µg/ml OPN; 5) 20 µg/ml OPN; 6) 1 µg/ml casein (CSN; Sigma, St. Louis, MO); 7) 5 µg/ml CSN; 8) 10 µg/ml CSN; 9) 20 µg/ml CSN; 10) 10 µg/ml heparin (Sigma, St. Louis, MO); 11) 60% ampullary non-luteal ODF (ANLODF); 12) 60% isthmic non-luteal ODF (INLODF); 13) 60% bovine skim milk (SKM). Oviductal fluid previously collected from cows equipped with indwelling catheters in the ampullary and isthmic oviducts (Kavanaugh *et al.*, 1992) and stored in liquid nitrogen was thawed and pooled from three cows over three non-consecutive estrous cycles. Both ODF and SKM were adjusted to 10 mg total protein/ml using 50 mM sodium bicarbonate, pH 8.5 (Rodriguez and Killian, 1998). Casein was used as a milk protein control for the purified OPN.

Capacitation and acrosome reaction of sperm

Following the incubations described above, samples were divided in half and treated either with the fusogenic lipid lysophosphatidyl choline (LPC; Sigma, St. Louis, MO) to induce the acrosome reaction or an MTM negative control (Parrish *et al.*, 1988; McNutt and Killian, 1991). The ability of LPC to induce the acrosome reaction in live sperm was used as a bioassay to measure rates of sperm capacitation. Lipid lysophosphatidyl choline was prepared as previously described (McNutt and Killian, 1991). A 500-µl volume of sperm suspension was incubated with 60 µg/ml LPC or in MTM alone. After LPC was added, bovine serum albumin (BSA; Sigma, St. Louis, MO) was added to each tube at a final concentration of 5 mg/ml, and the tubes were incubated for 10 min at 39°C. Sperm were then washed twice and stained for viability and the acrosome reaction.

Detection of the acrosome reaction and sperm viability

Ethidium monoazide (EMA; Molecular Probes, Carlsbad, CA) was used to detect cell viability following the treatments described above. Sperm (5 x 10^7/ml) were incubated in 10 µg/ml EMA in PBS for 10 min on a white metal pan 20 cm below a fluorescent light to induce photochemical covalent binding of EMA to nucleic acids in cells with compromised membranes (Riedy *et al.*, 1991). After two washes in PBS, cells were fixed in 2% paraformaldehyde (PFA) and washed twice more in PBS. Sperm were then incubated in 1 µg/ml fluorescein-labeled *Pisum sativum* agglutinin (FITC-PSA; Sigma, St. Louis, MO) in PBS for 30 min to detect intact sperm (Cross, 1986), washed in PBS and analyzed on a Beckman-Coulter EPICS XL-MCL flow cytometer using System II software.

Detection of sperm intracellular calcium and cell viability

Semen was collected and pooled as described. After two washes in MTM, 5 x 10^7 sperm/ml were incubated (39°C, 5% CO_2/air (v/v), 4 h) in the treatments listed above. Sperm were washed twice in MTM and incubated (39°C, 5% CO_2/air (v/v), 30 min) in 5 µM fluo-3 AM (Molecular Probes, Carlsbad, CA) in MTM. The cell permeant fluo-3 AM is not fluorescent unless bound to Ca2+. The fluorescent emission of Ca2+-bound fluo-3 is detected using the same methods as FITC. Following two washes with MTM, sperm were suspended in PBS and stained with propidium iodide (10 µg/ml, PI; Molecular Probes, Carlsbad, CA) to measure sample viability. Propidium iodide is a cell permeant used routinely to detect dead or dying cells; it only enters compromised membranes and binds to nucleic acids. The samples were then analyzed by flow cytometry. Only viable cells that did not stain with PI were selected and analyzed for calcium content.

Detection of mitochondrial activity in sperm

Semen was collected, pooled and washed in MTM as described above. Sperm (5 x 10^7/ml) were incubated (39°C, 5% CO_2/air (v/v), 4 h) in the treatments listed. Sperm were washed twice in MTM and incubated (39°C, 5% CO_2/air (v/v), 15 min) in 5 µM MitoTracker Red CMXRos (Molecular Probes, Carlsbad, CA) in MTM. Following two washes with MTM, sperm were suspended in PBS and stained with PI (10 µg/ml) and analyzed by flow cytometry. Only viable cells that did not stain with PI were selected and analyzed for mitochondrial activity.

Binding of biotinylated osteopontin and casein to sperm

Osteopontin was purified from commercially-available skim milk as previously described (Bayless

et al., 1997). Osteopontin and CSN were biotinylated using a method previously used to label oviductal fluid proteins (Rodriguez and Killian, 1998). Sperm (5 x 10^7/ml) were incubated (39°C, 5% CO_2/air (v/v), 4 h) in the following treatments in MTM using biotinylated OPN and CSN: 1) no treatment; 2) 1 µg/ml OPN; 3) 5 µg/ml OPN; 4) 10 µg/ml OPN; 5) 25 µg/ml OPN; 6) 50 µg/ml OPN; 7) 100 µg/ml OPN; 8) 1 µg/ml CSN; 9) 5 µg/ml CSN; 10) 10 µg/ml CSN; 11) 25 µg/ml CSN; 12) 50 µg/ml CSN; or 13) 100 µg/ml CSN. After two washes in MTM, sperm were fixed in 2% PFA, washed twice in PBS and incubated for 30 min in 100 µg/ml FITC-labeled streptavidin (Sigma, St. Louis, MO). Following a wash in PBS, protein binding to sperm was analyzed by flow cytometry.

Statistical analysis

In each experiment, 10^4 sperm from each sample were analyzed by flow cytometry. Three replicates of each experiment were performed and data were pooled. Data were analyzed using the Kolmogorov-Smirnov test to compare means between different treatments. All treatments were compared to the MTM negative control in each experiment. The significance level for all tests was $P < 0.05$.

Results

Effect of treatments on capacitation and acrosome reaction

Sperm that had undergone the acrosome reaction and were viable based on lack of EMA staining were considered functionally capacitated, as dead sperm often suffer membrane damage leading to removal of the acrosome. Therefore, the percent of acrosome-reacted live (ARL) sperm were measured and compared in each treatment (Fig. 1). In all treatments, the percent of ARL sperm was greater than sperm incubated in MTM (negative control; $P < 0.05$). Osteopontin capacitated significantly more sperm than MTM and casein at incubation concentrations of 1 µg/ml and 10 µg/ml, but was not different than casein at other concentrations. Surprisingly, 1 µg/ml OPN capacitated significantly more sperm than other concentrations and treatments, suggesting that small amounts of OPN can have positive effects on sperm capacitation. The largest percentage of ARL sperm occurred in INLODF, with skim milk containing the second largest population of functionally capacitated sperm (Fig. 2).

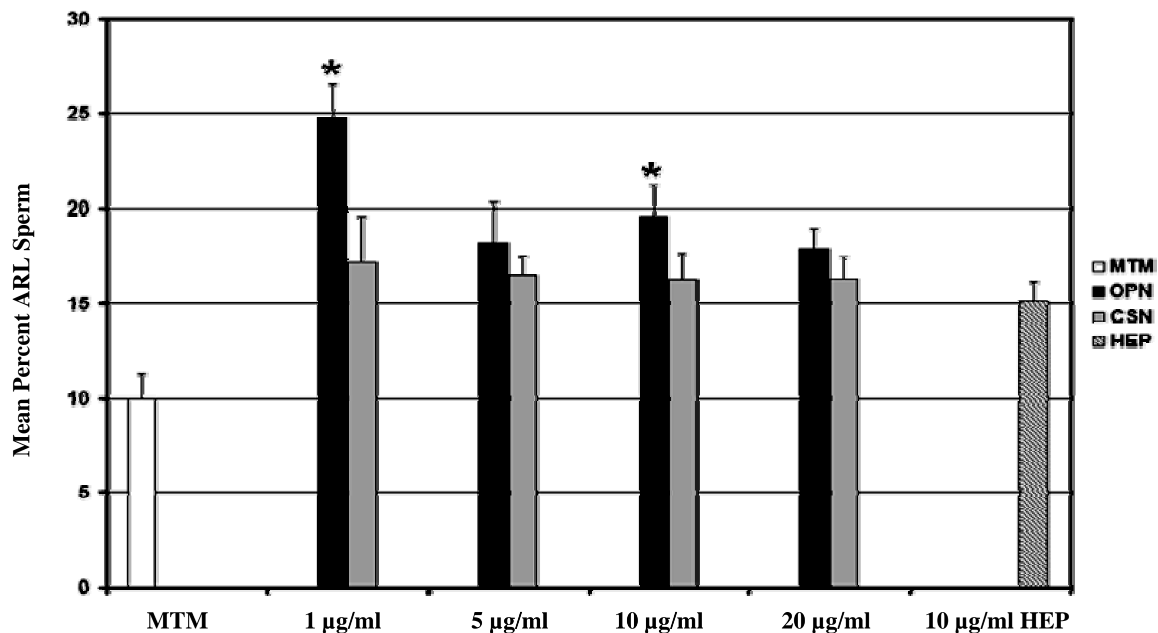

Figure 1. Effect of osteopontin treatment on sperm capacitation, represented as the mean (± SEM) percentage of acrosome-reacted live (ARL) sperm in each treatment. Sperm were incubated (39°C, 5% CO2/air, 4 h) in 1, 5, 10, or 20 µg/ml osteopontin (OPN) or casein (CSN), 10 µg/ml HEP in Modified Tyrode's medium (MTM), or MTM alone, and the acrosome reaction was induced in capacitated sperm using lysophosphatidylcholine (LPC). Sperm were then stained with ethidium monoazide (EMA) and fluorescein-labeled Pisum sativum agglutinin (FITC-PSA) to evaluate viability and acrosome status of sperm. Cells (10^4 per sample) were analyzed by flow cytometry. Three replicates were performed and data were pooled. The percentage of functionally capacitated sperm (ARL) from each treatment is represented. OPN = osteopontin; CSN = casein; HEP = heparin; MTM = Modified Tyrode's medium. Bars marked with asterisks are significantly different than MTM and corresponding CSN control ($P < 0.05$).

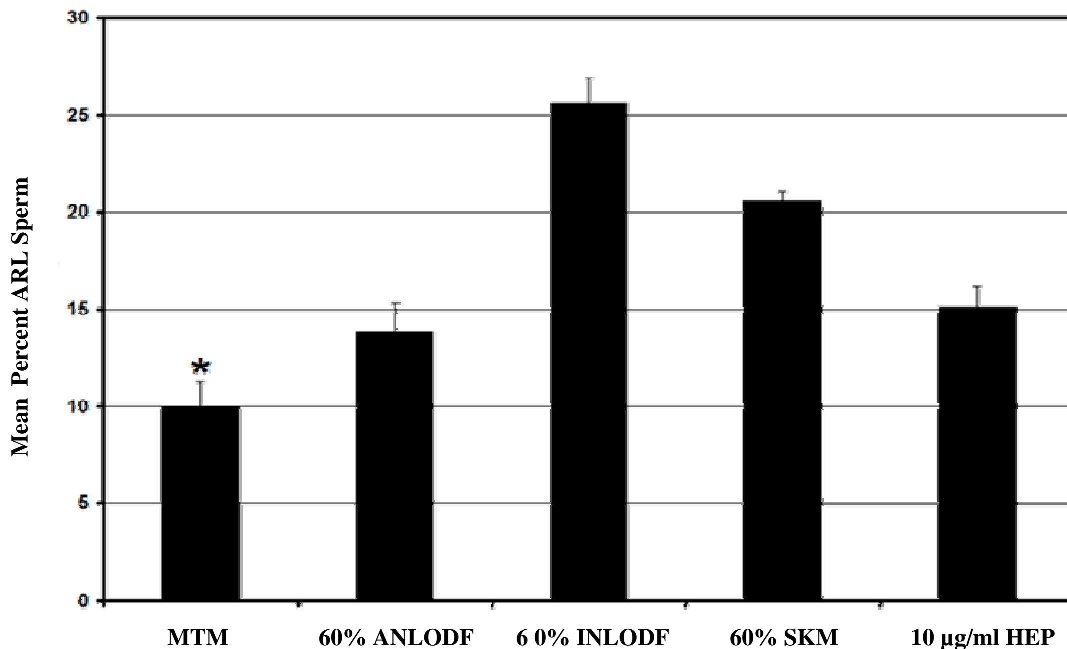

Figure 2. Effect of oviductal fluid treatment on sperm capacitation, represented as the mean (± SEM) percentage of acrosome-reacted live (ARL) sperm in each treatment. Sperm were incubated (39°C, 5% CO2/air, 4 h) in 60% ANLODF, 60% INLODF, 60% SKM, or 10 µg/ml HEP in Modified Tyrode's medium (MTM) or MTM alone. The acrosome reaction was induced in capacitated sperm using lysophosphatidylcholine (LPC). Sperm were then stained with ethidium monoazide (EMA) and fluorescein-labeled Pisum sativum agglutinin (FITC-PSA) to evaluate viability and acrosome status of sperm. Cells (10^4 per sample) were analyzed by flow cytometry. Three replicates were performed and data were pooled. The percentage of functionally capacitated sperm (ARL) from each treatment is represented. MTM = Modified Tyrode's medium; ANLODF = ampullary non-luteal oviductal fluid; INLODF = isthmic non-luteal oviductal fluid; SKM = skim milk; HEP = heparin. ANLODF, INLODF and SKM were adjusted to 10 mg total protein/ml with 50 mM sodium bicarbonate, pH 8.5 prior to dilution in MTM. All treatments resulted in more ARL sperm than the MTM control ($P < 0.05$).

Effect of treatments on mitochondrial activity

Addition of OPN to sperm had no significant effect on MitoTracker Red CMXRos uptake versus the MTM and CSN controls at all incubation concentrations (Fig. 3; $P > 0.05$). Sperm treated with heparin, ANLODF, INLODF, and skim milk were also not different from the negative control (Fig. 4).

Effect of treatments on intracellular calcium

Osteopontin had no effect on intracellular content of calcium in sperm versus the MTM control (Fig. 5; $P > 0.05$). However, OPN caused a significant increase in intracellular calcium versus CSN in the 20 µg/ml treatment. Incubation of sperm in heparin, ANLODF and INLODF all increased intracellular calcium levels over the MTM control, with INLODF showing the greatest effect (Fig. 6; $P < 0.05$). Skim milk caused a decrease in intracellular calcium in sperm following incubation.

Effect of treatments on sperm viability

In all treatment groups, OPN significantly increased the percentage of viable sperm versus the MTM control as measured by exclusion of PI (Fig. 7; $P < 0.05$). This was different than the casein control in only the 1 µg/ml treatment group. Sperm samples treated with heparin, ANLODF, INLODF, and skim milk all contained fewer viable sperm than the MTM control (Fig. 7; $P < 0.05$).

Osteopontin and casein binding to sperm

More CSN associated with sperm as the incubation concentration of casein increased (Fig. 8; $P < 0.05$). All concentrations of biotinylated casein 10 µg/ml and greater showed significantly more fluorescence on sperm than sperm incubated in MTM alone. In addition, all concentrations of casein 10 µg/ml and greater associated with sperm in higher amounts compared to OPN at the same incubation concentration. At no concentration did OPN bind to sperm in greater amounts than the negative control.

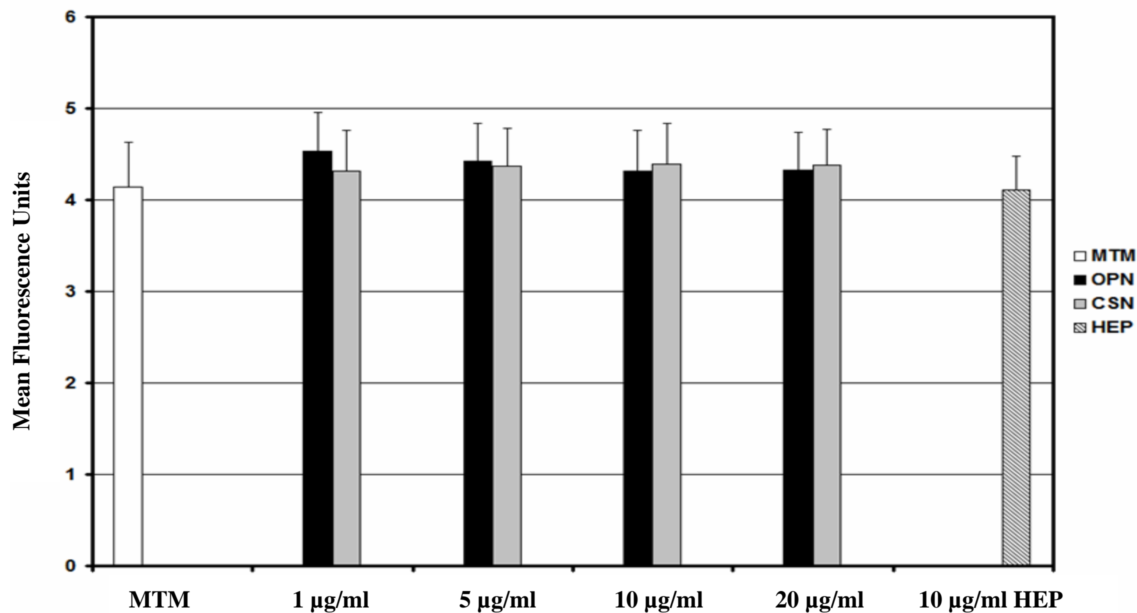

Figure 3. Effect of osteopontin treatment on mitochondrial activity in sperm, represented as mean (± SEM) fluorescence. Sperm were incubated (39°C, 5% CO2/air, 4 h) in 1, 5, 10, or 20 µg/ml osteopontin (OPN) or casein (CSN), 10 µg/ml HEP in Modified Tyrode's medium (MTM), or MTM alone and stained with MitoTracker Red CMXRos to evaluate mitochondrial status and propidium iodide (PI) to ensure that only live cells were evaluated. Cells (10^4 per sample) were analyzed by flow cytometry. Three replicates were performed and data were pooled MTM = Modified Tyrode's medium, OPN = osteopontin; CSN = casein; HEP = heparin. No significant differences were detected ($P < 0.05$).

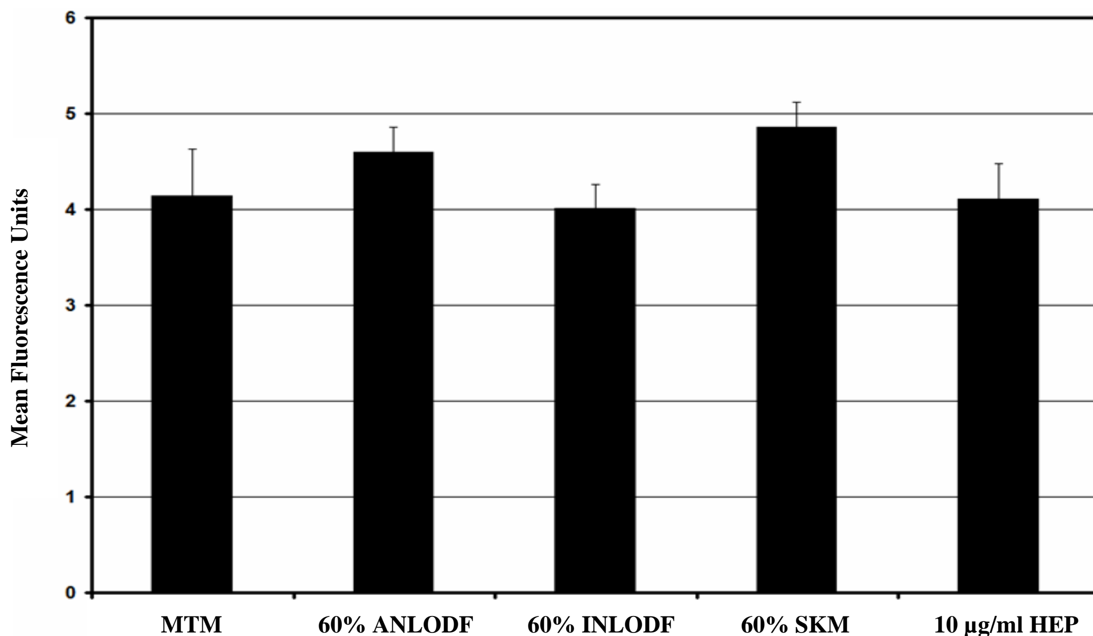

Figure 4. Effect of oviductal fluid treatment on mitochondrial activity in sperm, represented as mean (± SEM) fluorescence. Sperm were incubated (39°C, 5% CO2/air, 4 h) in 60% ANLODF, 60% INLODF, 60% SKM or 10 µg/ml HEP in Modified Tyrode's medium (MTM) or MTM alone. Cells were stained with MitoTracker Red CMXRos to evaluate mitochondrial status and propidium iodide (PI) to ensure that only live cells were evaluated and analyzed (10^4 per sample) by flow cytometry. Three replicates were performed and data were pooled. MTM = Modified Tyrode's medium; ANLODF = ampullary non-luteal oviductal fluid; INLODF = isthmic non-luteal oviductal fluid; SKM = skim milk; HEP = heparin. ANLODF, INLODF and SKM were adjusted to 10 mg total protein/ml with 50 mM sodium bicarbonate, pH 8.5 prior to dilution in MTM. No significant differences were detected ($P < 0.05$).

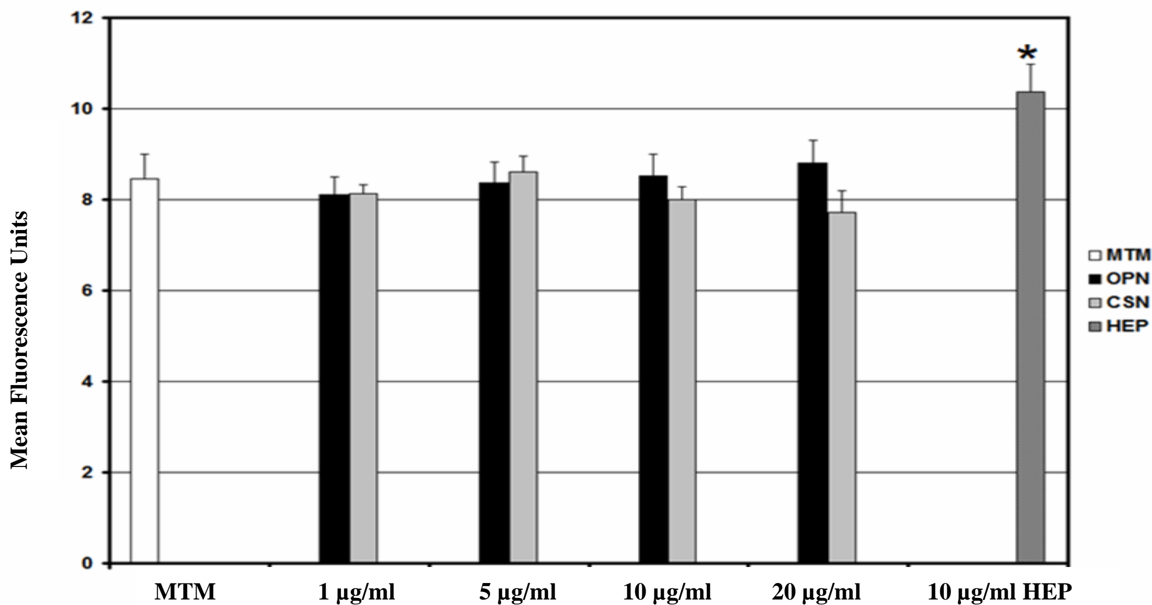

Figure 5. Effect of osteopontin treatment on intracellular calcium content in sperm, represented as mean (± SEM) fluorescence. Sperm were incubated (39°C, 5% CO2/air, 4 h) in 1, 5, 10, or 20 µg/ml osteopontin (OPN) or casein (CSN), 10 µg/ml heparin (HEP) in Modified Tyrode's medium (MTM) or MTM alone and stained with fluo-3 AM to evaluate calcium content. Sperm were also stained with propidium iodide (PI) to ensure that only live cells were evaluated. Cells (10^4 per sample) were analyzed by flow cytometry. Three replicates were performed and data were pooled. OPN = osteopontin; CSN = casein; MTM = Modified Tyrode's medium. Only HEP treatment resulted in increased levels of intracellular calcium versus MTM control ($P < 0.05$).

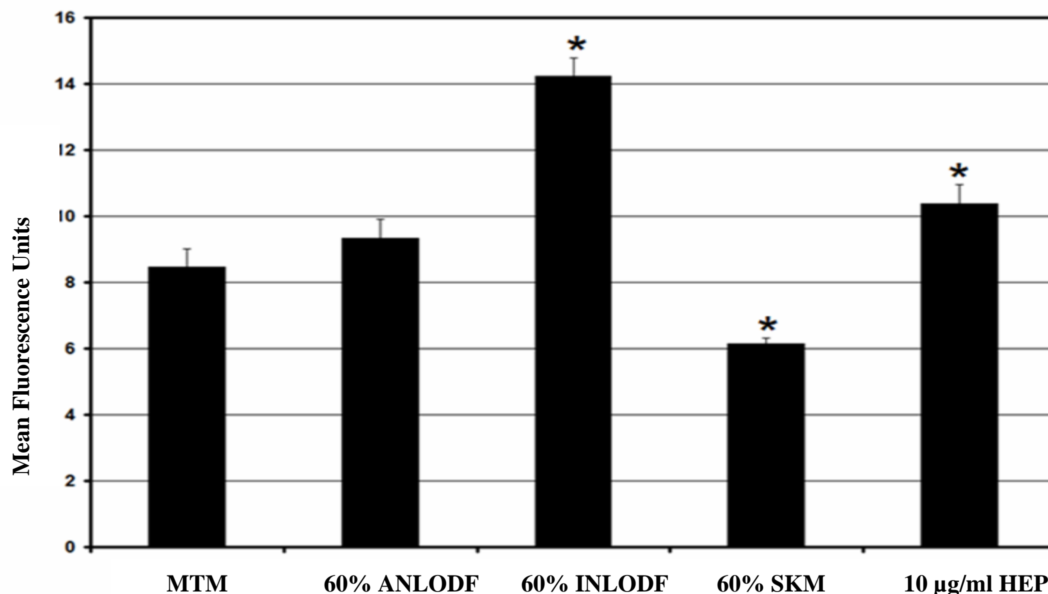

Figure 6. Effect of oviductal fluid treatment on intracellular calcium content in sperm, represented as mean (± SEM) fluorescence. Sperm were incubated (39°C, 5% CO2/air, 4 h) in 60% ANLODF, 60% INLODF, 60% SKM or 10 µg/ml HEP in Modified Tyrode's medium (MTM) or MTM alone and stained with fluo-3 AM to evaluate calcium content. Sperm were also stained with propidium iodide (PI) to ensure that only live cells were evaluated. Cells (10^4 per sample) were analyzed by flow cytometry. Three replicates were performed and data were pooled. ANLODF = ampullary non-luteal oviductal fluid; INLODF = isthmic non-luteal oviductal fluid; SKM = skim milk; HEP = heparin. ANLODF, INLODF and SKM were adjusted to 10 mg total protein/ml with 50 mM sodium bicarbonate, pH 8.5 prior to dilution in MTM. Bars marked with asterisks are significantly different than the MTM control ($P < 0.05$).

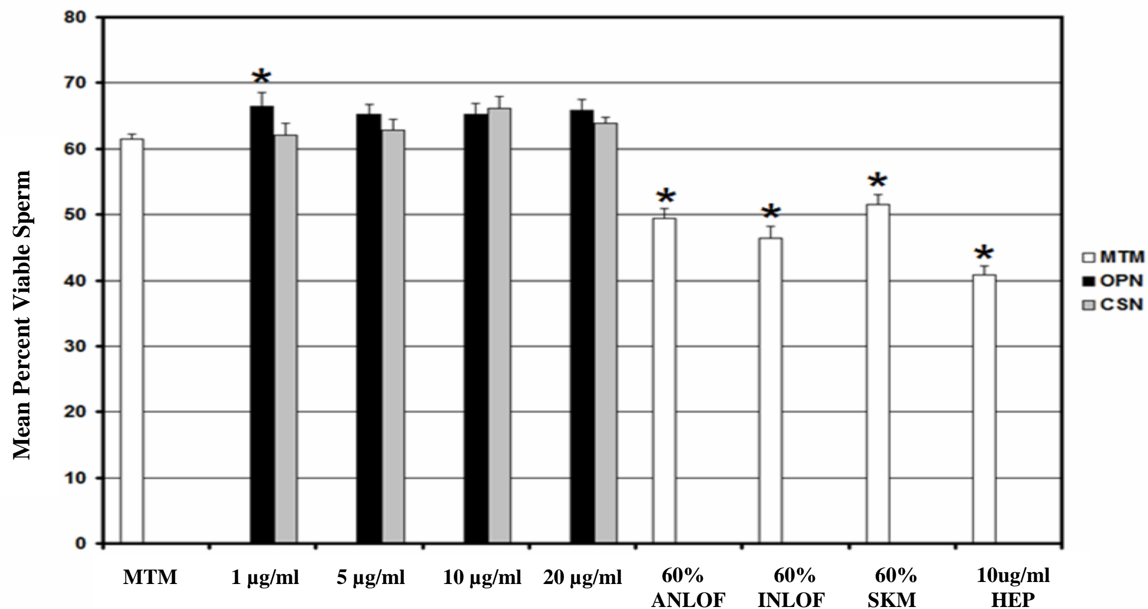

Figure 7. Effect of osteopontin and oviductal fluid treatment on sperm viability, represented (± SEM) as mean percentage of viable sperm. Sperm were incubated (39°C, 5% CO2/air, 4 h) in 0, 1, 5, 10 or 20 µg/ml osteopontin (OPN) or casein (CSN), 60% ANLODF, 60% INLODF, 60% SKM or 10 µg/ml HEP in Modified Tyrode's medium (MTM) and stained with propidium iodide (PI) to evaluate sperm viability. Cells (10^4 per sample) were analyzed by flow cytometry. Three replicates were performed and data were pooled. OPN = osteopontin; CSN = casein; ANLODF = ampullary non-luteal oviductal fluid; INLODF = isthmic non-luteal oviductal fluid; SKM = skim milk; HEP = heparin. ANLODF, INLODF and SKM were adjusted to 10 mg total protein/ml with 50 mM sodium bicarbonate, pH 8.5 prior to dilution in MTM. Bars marked with asterisks are significantly different than MTM control (P < 0.05).

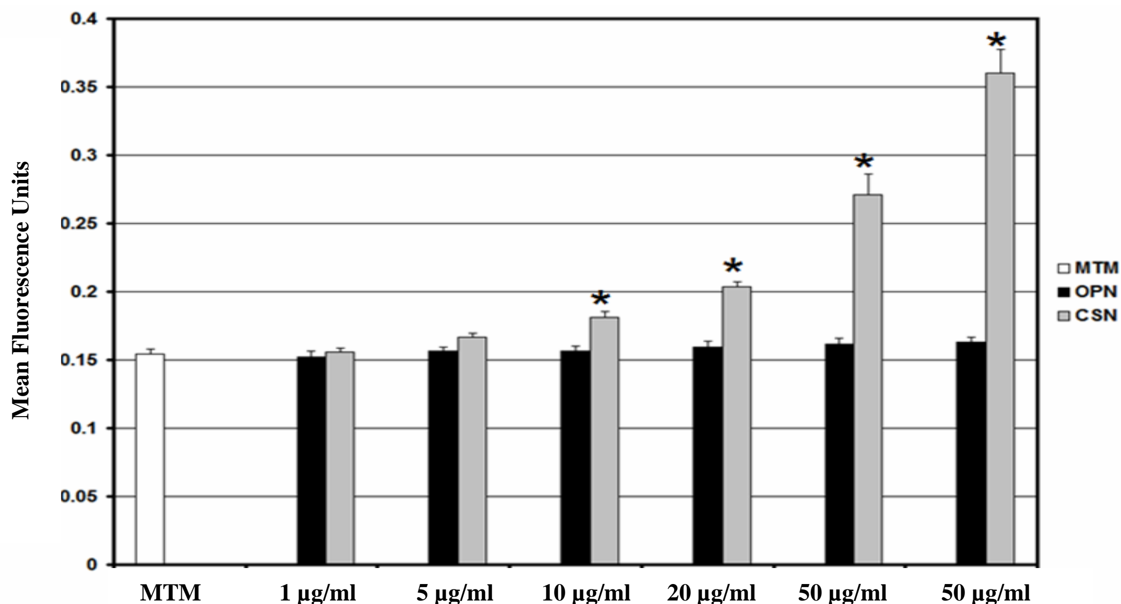

Figure 8. Association of biotinylated OPN and casein with sperm, represented as mean (± SEM) fluorescence. Sperm (5 x 10^7/ml) were incubated (39°C, 5% CO2/air, 4 h) in 1, 5, 10, 20, 50, or 100 µg/ml biotinylated osteopontin (OPN) or biotinylated casein in Modified Tyrode's medium (MTM) or MTM alone and stained with fluorescein-labeled streptavidin (FITC-SA). Cells (10^4 per sample) were analyzed using flow cytometry. Three replicates were performed and data were pooled. Bars marked with asterisks are significantly different than MTM and OPN at same incubation concentration (P < 0.05).

Discussion

These studies were conducted to examine the physiological effects of OPN on sperm and sperm capacitation. Sperm were incubated in concentrations of OPN believed to be similar to those of seminal plasma (Cancel *et al.*, 1997) and ODF. While the milk OPN preparation used in this study was greatly enriched, a small amount of contaminating CSN could not be eliminated. Therefore, CSN was included as a control whenever purified milk OPN was used. Sperm were incubated in the various treatments for 4 h, mimicking the appropriate time for heparin to capacitate sperm (Parrish *et al.*, 1988). Heparin capacitation was used as a control in all experiments to compare the effect of OPN on capacitation and other physiological parameters against a defined capacitating agent.

At all incubation concentrations, OPN produced more acrosome-reacted live (ARL) sperm than heparin as detected by our dual staining method, but only at concentrations of 1 µg/ml and 10 µg/ml did OPN capacitate a higher percentage of sperm than the corresponding CSN control. It is noteworthy that the percent of ARL sperm produced by OPN was greater than that of heparin at the same time point, while CSN did not capacitate more sperm than heparin at any concentration. Choosing a protein control for capacitation is difficult, as even BSA, a protein typically used as a negative control in *in vitro* assays, can also induce sperm capacitation (Davis, 1976, 1981; Davis *et al.*, 1979; Go and Wolf, 1985; Langlais and Roberts, 1985; Suzuki and Yanagimachi, 1989). While there was no dose-dependent effect of CSN in any of the assays, the possible contribution of residual CSN to the capacitating effect of OPN cannot be overlooked.

Previous studies have shown the ability of ODF to capacitate sperm. Estrual ODF capacitated sperm at higher rates than heparin (Parrish *et al.*, 1989) and similar experiments using regional-staged ODF showed that INLODF capacitated more sperm than ANLODF (Grippo *et al.*, 1995; Way *et al.*, 1997; Topper *et al.*, 1999). The effect of INLODF was expected, as sperm become capacitated in the isthmus of the oviduct *in vivo* (Grippo *et al.*, 1995; Lefebvre and Suarez, 1996; Fazeli *et al.*, 1999). While the conditions of these experiments yielded slightly different numbers of capacitated sperm than treatments in the present study, the overall effect was the same with INLODF capacitating more sperm than ANLODF and heparin.

Increased intracellular calcium is a characteristic of capacitated sperm (Singh *et al.*, 1978) and can be used as an indicator of the induction of sperm capacitation (Handrow *et al.*, 1989; Nolan *et al.*, 1992; Parrish *et al.*, 1999). There are two distinct waves of calcium uptake during capacitation (Singh *et al.*, 1978), the first of which is associated with an acrosomal calcium reservoir (Parrish *et al.*, 1999). In the experiments described here, OPN showed no positive effect on intracellular calcium levels in sperm, suggesting its effect on capacitation is through different means.

The effect of OPN on sperm mitochondrial activity was also examined. Mitochondria have a negative interior membrane potential, in which fluorescent cations are able to accumulate (Johnson *et al.*, 1980). Functional mitochondria enable the uptake of MitoTracker Red CMXRos based on membrane potential (Poot *et a*l., 1996). These experiments showed that increasing the concentration of OPN in the sperm environment had no direct effect on MitoTracker Red CMXRos uptake by sperm, indicating that the presence of OPN has no effect on mitochondrial membrane potential.

The possibility that OPN and casein could bind to sperm was also investigated in this study. Previous studies have shown that proteins in ODF can associate with sperm (King and Killian, 1994; Abe *et al.*, 1995; Boatman and Magnoni, 1995; Lapointe and Sirard, 1996; Rodriguez and Killian, 1998), including oviduct-specific glycoprotein (OSG; King and Killian, 1994). Based on data in the present study, OPN did not bind to sperm, but CSN did in a dose-dependent manner. This is the first report showing association of CSN, the major protein in milk and a commonly-used semen extender with bovine sperm. The data presented here appear to contradict previous data from our laboratory which suggested that OPN from accessory sex gland fluid (AGF) appears to bind to sperm during ejaculation (Erikson *et al.*, 2007). It is possible that OPN alone is not able to associate with sperm, but requires an additional protein or enzyme interaction to bind. Another possibility is that ejaculated sperm used in these studies are already saturated with OPN acquired from AGF during ejaculation, occupying available OPN binding sites on the sperm membrane. If ejaculated sperm are saturated with OPN from seminal plasma, they may be in equilibrium with OPN provided in the incubations. This would not preclude an exchange of OPN between the sperm membrane and the incubation medium, although the net amount of OPN measured on the sperm may be unchanged. This exchange process may provide the stimulus needed to facilitate capacitation.

The final parameter studied was sperm viability. Osteopontin has been shown to stimulate cell survival by inhibiting apoptosis in various cell types (Denhardt and Noda, 1998; Giachelli and Steitz, 2000; Mazzali *et al.*, 2002). While there was again no dose-dependent effect of OPN or CSN on cell survival, there was an overall increase in sperm viability in samples incubated with OPN or CSN. This could indicate an important function for OPN in oviductal fluid as sperm can reside in the oviduct for up to 20 h before fertilization (Hunter and Wilmut, 1984). A recent report demonstrated that milk caseins prevent binding of BSP proteins to sperm during sperm storage, thus preventing membrane lipid loss and the detrimental effects of these proteins on the sperm membrane (Bergeron *et al.*, 2007). While that study did not demonstrate binding of milk caseins to the sperm membrane during storage at 4°C, our study was conducted at 39°C and utilized

incubation in isolated caseins which did bind to sperm in our study. Still, the protective effect of CSN on the sperm membrane could explain the stimulation of sperm survival shown here.

An unexpected result from this study occurred when bovine skim milk was included as a treatment in the experiments. Although there were a high number of ARL sperm after treatment in milk, intracellular calcium was decreased. Cell viability was similar to that of sperm incubated in ANLODF, which has been shown to enhance the ability of sperm to fertilize (Grippo *et al.*, 1995; Way *et al.*, 1997; Topper *et al.*, 1999). While sperm samples treated with heparin, ANLODF, INLODF and skim milk all contained fewer viable sperm than the MTM control, this may have been due to an artifact of capacitation, as the sperm membranes may have become permeable to PI and may not be indicative of the true number of viable cells. Milk has long been used as an extending agent for bovine semen cryopreservation, and our data suggest milk may promote sperm capacitation through the presence of OPN and CSN. However, since milk contains a multitude of components which could affect sperm function, the potential for many undetermined mechanisms of sperm capacitation in milk exist.

In summary, OPN promotes capacitation of sperm through undetermined means and enhances sperm viability, possibly by blocking apoptotic pathways. Osteopontin had no effect on sperm mitochondrial membrane potential or intracellular calcium content, and purified OPN did not bind to sperm *in vitro*. These data provide further insight into the various roles OPN seems to play in reproductive events.

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