Detection of osteopontin on Holstein bull spermatozoa, in cauda epididymal fluid and testis homogenates, and its potential role in bovine fertilization

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Abstract

Osteopontin (OPN) is a secreted extracellular matrix phosphoprotein identified in various tissues and fluids including those of the male and female reproductive tracts. OPN was previously identified as a 55 kDa high fertility marker in Holstein bull seminal plasma, produced by the ampulla and the vesicular gland. The objectives of this study were to characterize OPN on ejaculated and cauda epididymal sperm using immunofluorescence and western blot analysis, and to assess the role of sperm OPN in fertilization. Solubilized sperm membrane proteins from ejaculated and cauda epididymal sperm were separated by 1D SDS-PAGE, transferred to nitrocellulose, and probed with an antibody to bovine milk OPN. A 35 kDa protein was detected by this antibody in both ejaculated and cauda epididymal sperm membranes. Analyses also recognized OPN at 55 and 25 kDa in cauda epididymal fluid and testicular parenchyma homogenates respectively. Immunofluorescent analysis of ejaculated and cauda epididymal sperm showed OPN localization in a well-defined band in the postacrosomal region of the sperm head and also on the midpiece. Results of in vitro fertilization experiments showed that sperm treated with an antibody to OPN fertilized fewer oocytes than sperm treated with control medium while increasing incidence of polyspermy, suggesting a role of sperm-associated OPN in fertilization and a block to polyspermy. These studies demonstrate that OPN exists at multiple molecular weight forms in the bull reproductive tract and its presence on ejaculated sperm may signal its importance in fertilization by interacting with integrins or other proteins on the oocyte plasma membrane.

Reproduction (2007) 133 909–917

Introduction

Osteopontin (OPN) is a secreted phosphoprotein first identified in the mineralized matrix of bovine bone (Franzen & Heinegard 1985). More recently it has been detected in many tissues and fluids, including urine (Sorenson et al. 1995), milk (Sorensen & Petersen 1993, Sorensen et al. 2003), kidney (Xie et al. 2001), uterine endometrium of rabbits (Apparao et al. 2003), sheep (Johnson et al. 2003, Kimmins et al. 2004), cows (Kimmins et al. 2004) and pigs (Garlow et al. 2002), the bovine oviduct (Gabler et al. 2003), and on the luminal surfaces of epithelial cells of human gastrointestinal and reproductive tracts, gall bladder, pancreas, lung, breast, urinary tract, salivary glands, and sweat glands (Brown et al. 1992). OPN may be glycosylated, phosphorylated and sulfated, and its expression and post-translational modifications are tissue-specific and regulated by many hormones and growth factors (Denhardt & Guo 1993). Bovine OPN contains a thrombin cleavage site, a calcium-binding site and binds to various integrins through an RGD amino acid sequence which allows OPN to participate in cell adhesion and intracellular communication (Denhardt & Guo 1993). Integrin binding may also be RGD-independent through the SVVYGLR motif, and OPN binds to different isoforms of the hyaluronic acid receptor CD44 (Mazzali et al. 2002).

OPN has been detected in the seminal plasma of Holstein bulls, where a 55 kDa isoform was positively correlated to fertility (Cancel et al. 1999). OPN has been detected in bull accessory sex gland fluid (AGF), seminal vesicle fluid and ampullary fluid (Cancel et al. 1999), the epithelium of the male reproductive tract in humans (Brown et al. 1992), and rat testis, epididymis (Sitteri et al. 1995, Luedtke et al. 2002) and sperm (Sitteri et al. 1995, Luedtke et al. 2002).
Although previous studies failed to detect OPN in bovine sperm by immunofluorescence (Cancel et al. 1999), its presence in the male reproductive tract and seminal plasma and correlation with male fertility suggests that OPN has some association and function in ejaculated bovine spermatozoa. In support of this notion, OPN has been described as a sperm surface molecule in rats (Siiteri et al. 1995), as associated with sperm during development in the testis (Luedtke et al. 2002), and while sperm are transported and stored in the epididymis (Siiteri et al. 1995, Luedtke et al. 2002), and is present in AGF (Cancel et al. 1995) at ejaculation. In addition, proteins from AGF are known to bind to sperm during ejaculation (Manjunath et al. 1994).

The goals of this study were to characterize OPN on the plasma membrane of bovine spermatozoa and to assess the functional role of OPN as a sperm ligand during sperm–egg binding and fertilization using in vitro fertilization (IVF).

Materials and Methods

Isolation of sperm membranes from ejaculated sperm

Semen was collected through an artificial vagina from eight Holstein bulls housed at the Almquist Research Center. Following evaluation of sperm concentration and motility, each sample was centrifuged (600 g) for 10 min at room temperature (RT). Seminal plasma was removed by aspiration and the sperm pellet was washed twice by centrifugation in warm (37 °C), sterile phosphate buffered saline (PBS). Sperm membrane solubilization was performed as previously described (Jones 1989, McNutt et al. 1992). Sperm were incubated in sperm membrane solubilization buffer (0.4% sodium deoxycholate, 0.26 M sucrose, 10 mM Tris (pH 8.5)) with 200 μM phenylmethylsulfonyl fluoride (PMSF) at a concentration of 2.5 × 10⁸ sperm/ml for 1 h at 4 °C. Samples were then centrifuged (10 000 g) for 30 min at 4 °C and the supernatant containing sperm membrane proteins was dialyzed overnight at 4 °C against 50 mM ammonium bicarbonate and vacuum-concentrated. Protein concentration was determined (Lowry et al. 1951) and sperm membrane proteins were stored at −80 °C until use in SDS-PAGE and western blot analysis.

Isolation of cauda epididymal sperm membranes and preparation of cauda epididymal fluid (CEF)

Testes and epididymides from five Holstein bulls were collected at slaughter and transported on ice to the laboratory. Sperm and fluid from the cauda region of each epididymis were recovered by back-flushing the epididymis through the vas deferens with sterile PBS (Killian & Amann 1972). Flushes from each epididymis were centrifuged (600 g) for 10 min at RT. CEF was aspirated from the sperm pellet and centrifuged (10 000 g) for 60 min at 4 °C to remove remaining sperm. The supernatant containing the CEF was aspirated, the protein concentration was determined (Lowry et al. 1951), and samples were stored at −80 °C until use. Cauda epididymal sperm were washed twice in sterile PBS and membranes were solubilized and stored as previously described.

Collection and preparation of testis tissue samples

Whole testes and epididymides from five Holstein bulls were obtained at slaughter and transported on ice to the laboratory. Sections of testicular parenchyma (TP) were excised from the testes of all five bulls, snap frozen in liquid nitrogen, and stored at −80 °C. Testis samples were later homogenized in protein extraction buffer (10 mM Tris (pH 8.0), 0.2 M sucrose, 0.2 mM EDTA (pH 8.0), 50 mM NaCl, 1% Triton X-100, 200 μM PMSF) for 30 s, using 10 ml buffer per 1 g tissue. The homogenates were then centrifuged (10 000 g) for 30 min at 4 °C and the supernatant containing testis proteins was dialyzed overnight against 50 mM ammonium bicarbonate at 4 °C. Following determination of protein concentration (Lowry et al. 1951), the samples were frozen at −80 °C until use in SDS-PAGE and western blot analysis.

1D SDS-PAGE and western blot analysis

Ejaculated sperm membrane (100 μg), cauda sperm membrane (100 μg), epididymal fluid proteins (100 μg), and TP homogenates (100 μg) were separated by 1D SDS-PAGE (10–17.5% gradient gels) under denaturing conditions as previously described (Cancel et al. 1999) and transferred to nitrocellulose (Schleicher and Schuell Bioscience, Keene, NH, USA) at 208 mA for 1 h using a Multiphor II NovaBlot (Amersham Pharmacia Biotech). Blots were incubated overnight at 4 °C in PBS containing 0.5% Tween 20 (v/v), 5% heat-inactivated normal goat serum (v/v), and 3% BSA (w/v; blocking buffer 1) with gentle rocking to reduce nonspecific antibody binding. Blots were incubated with an affinity-purified polyclonal rabbit antibody to bovine milk OPN (anti-OPN; 1:2000, w/v in blocking buffer 1; Gabler et al. 2003) or normal rabbit serum (1:2000, v/v in blocking buffer 1) for 2 h at RT and then washed in PBS/Tween (PBST) (3 × 20 min). After washing, blots were incubated in anti-rabbit IgG peroxidase conjugate (Sigma; 1:7500, w/v in blocking buffer 1) for 1 h. Following washes in PBST (3 × 20 min), blots were visualized using ECL (Amersham Biosciences) and developed onto radiography film (Kodak). The developed film was subsequently scanned using an imaging densitometer (Bio-Rad).
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Immunofluorescence of ejaculated and cauda epididymal spermatozoa

Semen from four Holstein bulls housed at the Almquist Research Center was collected and evaluated as previously described. A volume of semen containing 5×10^7 sperm was washed twice by centrifugation (1000 g, 5 min) with warm (37 °C), sterile PBS. Sperm were fixed in warm (37 °C) 2% paraformaldehyde for 10 min at 4 °C, washed twice in PBS (10 000 g, 5 min), and incubated at RT in 1 ml PBS containing 5% BSA (w/v) and 0.1% Tween 20 (v/v; blocking buffer 1) for 2 h with gentle rocking. After blocking, sperm were incubated in 1 ml anti-OPN (10 μg/ml in blocking buffer 2), 1 ml normal rabbit serum (1:100, w/v in blocking buffer 2), or blocking buffer 2 alone overnight at 4 °C with gentle rocking. Following two washes in PBS, sperm were incubated in 1 ml PBS containing 1% BSA (w/v) and 0.1% Tween 20 (v/v; blocking buffer 3) with FITC-labeled anti-rabbit IgG (Sigma; 1:300, w/v) for 1 h with gentle rocking. Sperm were then washed twice with PBS, smeared onto slides, mounted with AntiFade (Molecular Probes, Eugene, OR, USA), and analyzed using fluorescence microscopy. Alternatively, cauda epididymal sperm from five bulls were obtained as previously described and subjected to the same immunocytochemical staining procedure as ejaculated sperm. In order to confirm the specificity of anti-OPN for OPN on sperm, anti-OPN was adsorbed with OPN purified from bovine skim milk (Bayless et al. 1997). This adsorbed antibody was then used for immunofluorescent detection of sperm as previously described.

Oocyte collection and maturation

Bovine ovaries were harvested at an abattoir and placed in Dulbecco’s PBS (Invitrogen; 35 °C) prior to an ~2 h transport to the laboratory. At the laboratory, ovaries were rinsed with Dulbecco’s PBS (39 °C) and oocytes were aspirated from visible ovarian follicles and washed in low bicarbonate Hapes medium (Bayister et al. 1983). Those with at least one intact cumulus cell layer were matured in vitro in medium M199 containing 10% fetal bovine serum (v/v), luteinizing hormone (6 μg/ml), follicle-stimulating hormone (8 μg/ml), and penicillin (100 units ml^-1)/ streptomycin (100 μg/ml) for 22–24 h at 39 °C in 5% CO₂/air (Hasler et al. 1995). After maturation oocytes were prepared for sperm binding and fertilization experiments.

Sperm preparation

Semen from three fertile Holstein bulls was collected through artificial vagina, pooled and the sperm washed twice (700 g) in modified tyrode’s medium (MTM;

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Sperm–oocyte binding

In vitro matured oocytes were vortexed for 2 min to remove cumulus cells, washed twice in low bicarbonate Hapes medium and placed in Nunclon 4-well culture dishes (Fisher Scientific, Pittsburgh, PA, USA) containing 0.5 ml fertilization medium. Twenty to twenty-five oocytes were inseminated with 125 000 spermatozoa from (a) no anti-OPN; (b) 2.5 μg/ml anti-OPN; (c) 5 μg/ml anti-OPN; or (d) 10 μg/ml anti-OPN for 2 h at 39 °C in 5% CO₂/air (v/v).

IVF of oocytes with sperm incubated in OPN antibody

In vitro matured oocytes were washed twice in low bicarbonate Hapes medium, placed in fertilization medium with 2 μg heparin, and inseminated as previously described. After 20-h co-incubation, oocytes were vortexed to remove cumulus cells and accessory spermatozoa and washed in low bicarbonate Hapes medium. Oocytes were fixed in acid alcohol for 24 h and stained with aceto-orcein (Sirard et al. 1988). The presence of two pronuclei in the cytoplasm of the oocyte indicated normal fertilization.

Statistical analysis

Densitometry data comparing OPN on ejaculated and epididymal sperm was analyzed using Student’s t-test. The significance level for this test was P<0.05.

For fertilization and sperm binding, each experiment was repeated thrice and data from each experiment were pooled. ANOVA using a general linear model was performed using mean number of spermatozoa bound per zona pellucida for each treatment in the sperm–oocyte-binding experiments, and a weighted mean based on the number of oocytes per treatment in the fertilization experiments. Least square means comparisons were used to assess sperm binding and weighted least square means were used to analyze fertilization data (Way et al. 1997). The significance level for these tests was P<0.05.
Results

**OPN on sperm and in CEF and testis homogenates**

Solubilized sperm membrane proteins subjected to 1D SDS-PAGE and western blot analysis showed that anti-OPN recognized a 35 kDa protein in ejaculated and cauda epididymal sperm (Fig. 1). The 55 and 25 kDa protein in CEF and TP respectively, were also recognized by the antibody to OPN (Fig. 2). While a majority of immunoreactivity occurred at 55 and 25 kDa in CEF and TP respectively, immunoreactive bands also appeared at 60, 40, and 22 kDa in TP and 45 kDa in CEF. A similar pattern of OPN detection at multiple molecular weights was previously reported in rat testis homogenates (Siiteri et al. 1995). Densitometry analysis of OPN in ejaculated and epididymal sperm from eight bulls revealed that, while not significantly different \( (P = 0.06) \), ejaculated sperm contained approximately 50% more OPN than epididymal sperm (Fig. 3).

**Immunofluorescence of ejaculated and epididymal sperm**

Immunofluorescent analysis of ejaculated sperm showed that the same antibody that reacted to a 35 kDa protein on western blots of sperm membrane proteins recognized a protein on intact sperm membranes from four Holstein bulls (Fig. 4). Immunofluorescence occurred in a well-defined band in the postacrosomal region on the sperm head, as well as on the midpiece. Not all sperm in a given sample showed consistent staining for OPN and the possibility of different populations of OPN-positive sperm exists in a given ejaculate: 93% ± 0.82% of ejaculated sperm exhibited staining on the head and midpiece, 3% ± 1.63% exhibited staining on the head alone, and 4% ± 2.16% exhibited staining on the midpiece alone. In addition, fluorescence appeared to be more intense in ejaculated than in epididymal sperm (Fig. 5). Sperm incubated in normal rabbit serum as a control exhibited no fluorescence, indicating that the binding of OPN antibody to sperm was specific. Negative controls using no OPN antibody and no normal rabbit serum exhibited no fluorescence, indicating that no nonspecific binding of the FITC-labeled secondary antibody occurred. In addition, anti-OPN adsorbed with purified OPN did not bind to sperm, confirming the specificity for the antibody of OPN on sperm.

**IVF of oocytes with sperm incubated in OPN antibody**

Due to high levels of polyspermic fertilization in some treatment groups, analysis of fertilization was presented as percentage total fertilization, percentage normal fertilization, and percentage polyspermic fertilization. Fewer oocytes were fertilized by sperm treated with anti-OPN than sperm treated in control medium, with sperm incubated in 5 µg/ml antibody fertilizing the smallest percentage of oocytes \( (P < 0.05; \) Fig. 6A). This was also true when normal fertilization conditions were used.
rates were evaluated, excluding polyspermic fertilization ($P<0.05$; Fig. 6B). The highest percentage of polyspermic fertilizations occurred when sperm were incubated in 10 mg/ml anti-OPN ($P<0.05$), with polyspermy occurring in nearly 22% of all fertilizations by sperm treated with this antibody concentration (Fig. 6C).

**Discussion**

This is the first study to describe OPN on the bovine sperm membrane and relate it to sperm function. Western blot analyses showed that OPN was present in ejaculated and cauda epididymal solubilized sperm plasma membranes, CEF and TP homogenates. Immunofluorescent analyses of ejaculated and cauda epididymal sperm resulted in fluorescence of a distinct band on the postacrosomal region of the sperm head. Although earlier studies from our laboratory were unable to detect OPN on bovine sperm membranes (Cancel et al. 1999), the work used an antibody specific to the 55 kDa isoform of OPN found in Holstein bull seminal plasma. Since the completion of the study, we produced the affinity-purified antibody in rabbits to bovine milk OPN used in the present study, which reacts with multiple isoforms of OPN (Gabler et al. 2003).

In addition to our previous work on OPN protein localization (Cancel et al. 1999) and gene expression in the bull reproductive tract (Rodriguez et al. 2000), OPN has been identified in the reproductive tract of the male rat and human male. Siiteri et al. (1995) identified OPN...
in rat epididymal fluid in a broad band at 27–32 kDa, and on the surface of epididymal sperm and in sperm detergent extracts and testis homogenates. The amount of OPN exhibited on epididymal bovine sperm in the present study was visibly less than that of ejaculated sperm, but was at the limit of significance. This suggests that some OPN from AGF becomes associated with sperm during ejaculation. It is possible that the different isoforms of OPN detected in AGF, CEF, and sperm membranes have different functions in the bull reproductive tract, although determining these roles was beyond the scope of this investigation. While the 55 kDa form of OPN in Holstein bull seminal plasma has been correlated to fertility, no such correlation has been made to the other isoforms in the ejaculate or on sperm.

OPN was previously identified in epididymal tissue and germ cells in the spermatogonial stage of spermatogenesis in rats. The authors suggested that OPN functioned as an adhesion molecule, binding these germ cells to the basement membrane of the seminiferous tubule and to adjacent Sertoli cells (Luedtke et al. 2002). Primordial germ cells utilize integrins and extracellular matrix proteins to maintain contact with the Sertoli cells and other primordial germ cells (Kierszenbaum 1994; Siiteri et al. 1995) suggested that Sertoli cells in the rat testis secrete OPN based on its localization to the basal and adluminal region of the seminiferous tubule. In the bull, OPN was expressed in the seminiferous tubule, but only in tubules that contain elongated spermatids, suggesting a stage-related expression pattern (Rodriguez et al. 2000). Data showing the presence of OPN protein in homogenates of bovine TP along with OPN gene expression in the bovine seminiferous tubule (Rodriguez et al. 2000) suggest that OPN may be expressed by Sertoli cells in the later stages of spermatogenesis in the Holstein bull.

The presence of OPN in the epididymis and on epididymal sperm may be important in regulating calcium content of the sperm and epididymal lumen (Luedtke et al. 2002). OPN contains a calcium-binding site and causes calcium release in osteoclasts through an integrin-stimulated IP₃ pathway (Denhardt & Guo 1993). When secreted into the proximal tubule of the mouse nephron, OPN suppressed the accumulation of calcium oxalate crystals, most likely by interfering with the crystallization process (Shiraga et al. 1992). Calcium crystal deposits have also been shown in human rete testis, efferent ducts, and epididymis (Nistal et al. 1989, 1996). Luedtke et al. (2002) suggested that OPN in epididymal fluid may prevent calcium crystallization.

Figure 5 Side-by-side comparison of osteopontin localization on Holstein bull sperm. Ejaculated and cauda epididymal sperm were washed, fixed, and incubated with an affinity purified polyclonal rabbit antibody to bovine milk OPN (1:100, w/v). Sperm were then stained with FITC-labeled anti-rabbit IgG (1:300, w/v) to visualize OPN on the sperm. (A) FITC-labeled ejaculated sperm and (B) FITC-labeled cauda epididymal sperm.

Figure 6 (A) Mean percentage±S.E.M. of oocytes fertilized, including normal and polyspermic fertilizations. Oocytes were inseminated with spermatozoa incubated in MTM, 2.5 μg/ml affinity purified polyclonal rabbit antibody to bovine milk osteopontin (anti-OPN), 5 μg/ml anti-OPN, or 10 μg/ml anti-OPN prior to insemination. (B) Mean percentage±S.E.M. of oocytes fertilized normally, excluding polyspermic fertilizations. Oocytes were inseminated with spermatozoa incubated in MTM, 2.5 μg/ml anti-OPN, 5 μg/ml anti-OPN, or 10 μg/ml anti-OPN prior to insemination. (C) Mean percentage±S.E.M. of polyspermic fertilizations, expressed as a percentage of total fertilizations. Oocytes were inseminated with spermatozoa incubated in MTM, 2.5 μg/ml anti-OPN, 5 μg/ml anti-OPN, or 10 μg/ml anti-OPN prior to insemination. (D) Mean number±S.E.M. of sperm bound per zona pellucida (ZP). Oocytes were inseminated with spermatozoa incubated in MTM, 2.5 μg/ml anti-OPN, 5 μg/ml anti-OPN, or 10 μg/ml anti-OPN prior to insemination. Bars with the same letter are not significantly different.
that can be detrimental to sperm motility and fertility. Our observations that OPN is present in epididymal fluid and increased fertility of dairy bulls is correlated to greater amounts of OPN in seminal plasma (Cancel et al. 1997) support this claim.

Identification of OPN on ejaculated spermatozoa may signal its importance in fertilization, as indicated by a decrease in fertilization by treating sperm with anti-OPN. Abundant evidence points to the involvement of an RGD-mediated mechanism in binding and fusion of sperm to oocytes in several mammalian species (Evans 1999, 2001; Wassarman et al. 2001, Evans & Florman 2002). Further, studies have shown that RGD peptides can activate, induce calcium transients into, and induce parthenogenetic development in bovine oocytes (Campbell et al. 2000). We suggest that the OPN localized to the postacrosomal region on sperm membranes may participate in bovine fertilization by interacting with egg integrins. Integrin receptors are found on the oolema of sea urchin, mouse, hamster, human (Fenichel & Durand-Clement 1998), and bovine unfertilized oocytes (Goodison et al. 1999, Campbell et al. 2000). Integrins may act as co-receptors during fertilization by transducing a signal to initiate and propagate calcium release through IP3 (Fenichel & Durand-Clement 1998). Integrins, such as αβ3 and α2β1, which recognize the RGD peptide that is characteristic of OPN binding are among those integrins present on mammalian oocytes (Fusi et al. 1992, 1993, Evans 1999). OPN is known to associate with these integrin subunits and the RGD peptide can competitively inhibit fertilization and induce intracellular calcium transients in oocytes and initiate parthenogenetic development of oocytes (Campbell et al. 2000). It is likely that sperm-associated OPN plays a role in bovine fertilization by promoting sperm–egg binding and oocyte activation.

OPN is also a known ligand for the CD44 family of plasma membrane receptors. CD44 and its splice variants are members of the hyaluronic acid receptor family, ubiquitously expressed and can bind extracellular matrix proteins, such as OPN in addition to its primary ligand hyaluronic acid (Goodison et al. 1999). While Smith et al. (1999) claim that CD44–OPN interactions may not be common in vivo and Katagiri et al. (1999) have shown that OPN binds only to CD44 variants in an RGD-independent manner, multiple studies argue that OPN, CD44, and RGD-binding integrins (e.g. αβ3, β1 subunit) may cooperate in adhesion, signaling pathways, and stimulation of motility in various cell types (Chellaiah et al. 2003, Gao et al. 2003, Marroquin et al. 2004, Zhu et al. 2004). CD44 is expressed on the acrosomal region of human sperm cells (Bains et al. 2002), on porcine oocytes (Kimura et al. 2002) and cumulus cells (Yokoo et al. 2002), and bovine oocytes and early embryos (Furnus et al. 2003). Sperm-associated OPN may interact with CD44 on bovine oocytes during fertilization to facilitate adhesion and signaling.

The IVF data suggest that OPN may be involved in a block to polyspermy. A recent study indicates that OPN may be involved in preventing polyspermy in porcine oocytes in vitro (Hao et al. 2006). Mammalian eggs establish blocks to polyspermy both at the level of the zona pellucida (ZP) and the plasma membrane. While the zona block to polyspermy has been well characterized (Yanagimachi 1994), little is known of the molecular events surrounding the plasma membrane block to polyspermy (Evans 2003). Sperm treated with anti-OPN bound to oocytes in higher numbers than sperm incubated in control medium, and the incidence of polyspermic fertilization also increased with antibody-treated sperm. The localization of OPN on sperm in the postacrosomal region makes it unlikely that it participates in zona interactions, but a likely candidate for interaction with the plasma membrane of oocytes. While it is possible that antibody-coated sperm bound in higher numbers to oocytes than control sperm through IgG–ZP interactions, a decrease in fertilization rates coupled with an increase in polyspermic fertilizations suggests that OPN on sperm may participate in the induction of polyspermy blocks in bovine oocytes at the level of the plasma membrane.

The results of this study show that OPN is present on bovine sperm membranes and confirms previous results showing that multiple isoforms of OPN exist in the Holstein bull reproductive tract. While the exact nature of its association with sperm is not known, it is likely that sperm acquire OPN in the testis and that sperm-associated OPN is involved in the fertilization process and a block to polyspermy. Other isoforms present in the bull reproductive tract may have different functions such as calcium regulation in the epididymis, although those roles were not investigated in this study.

Acknowledgements

We thank the staff and students at the Almquist Research Center for technical assistance. This study was funded in part by USDA grants 2003–3447–3460 and 2004–34437–15106. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 25 September 2006
First decision 30 October 2006
Revised manuscript received 7 December 2006
Accepted 23 January 2007