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

David W Erikson, Amy L Way¹, David A Chapman and Gary J Killian

Department of Dairy & Animal Science, John O Almquist Research Center, The Pennsylvania State University, University Park, Pennsylvania 16802, USA and ¹Department of Health Science, Lock Haven University, Clearfield Campus, Clearfield, Pennsylvania 16830, USA

Correspondence should be addressed to G J Killian; Email: gkillian@psu.edu

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.

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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 (Siiteri *et al.* 1995, Luedtke *et al.* 2002) and sperm (Siiteri *et al.*

1995). 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.* 1999) 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 2.5×10^8 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, w/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).

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 2) 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 on 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 Hepes medium (Bavister *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;

Parrish *et al.* 1988). Sperm (5×10^7 ml⁻¹) were incubated in MTM containing: (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).

Sperm–oocyte binding

In vitro matured oocytes were vortexed for 2 min to remove cumulus cells, washed twice in low bicarbonate Hepes 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 sperm treatments. Heparin (2 µg) was added to each well at the time of insemination (Hasler *et al.* 1995). Oocytes and spermatozoa were co-incubated for 20 h at 39 °C in 5% CO₂/air (v/v). After co-incubation, oocytes were washed once in low bicarbonate Hepes and stained with Hoechst fluorescent dye 33342 (Sigma #B-2261; Way *et al.* 1997). The number of sperms bound to each zona pellucida was evaluated by fluorescence microscopy.

IVF of oocytes with sperm incubated in OPN antibody

In vitro matured oocytes were washed twice in low bicarbonate Hepes 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 Hepes 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: $93\% \pm 0.82\%$ of ejaculated sperm exhibited staining on the head and midpiece, $3\% \pm 1.63\%$ exhibited staining on the head alone, and $4\% \pm 2.16\%$ 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

Figure 1 Representative western blot of osteopontin (OPN) in ejaculated and cauda epididymal solubilized sperm plasma membranes from two Holstein bulls. Ejaculated and epididymal sperm were washed, solubilized, and 100 µg each sample were separated by 1D SDS-PAGE under denaturing conditions and transferred to nitrocellulose. Resultant blots were probed with an affinity purified polyclonal rabbit antibody to bovine milk OPN (1:2000, w/v) and visualized using ECL and developed onto radiography film. ES, ejaculated sperm; CS, cauda epididymal sperm; $M_r \times 10^{-3}$, molecular mass markers.

Figure 2 Representative western blot of osteopontin in cauda epididymal fluid and homogenates of testicular parenchyma from Holstein bulls. Cauda epididymal fluid and sections of testicular parenchyma were obtained from bulls at slaughter. Sections of testicular parenchyma were homogenized in protein solubilization buffer. Testis homogenates and cauda epididymal fluid were separated by 1D SDS-PAGE (100 µg each sample) under denaturing conditions and transferred to nitrocellulose. Resultant blots were probed with an affinity purified polyclonal rabbit antibody to bovine milk OPN (1:2000, w/v) and visualized using ECL and developed onto radiography film. CEF, cauda epididymal fluid; TP, testicular parenchyma; $M_r \times 10^{-3}$, molecular weight markers.

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

Figure 3 Densitometric analysis of osteopontin in ejaculated Holstein bull sperm membranes and cauda epididymal Holstein bull sperm membranes. Solubilized ejaculated and cauda epididymal sperm plasma membrane proteins from five bulls were separated by 1D SDS-PAGE (100 µg each sample) and transferred to nitrocellulose. Resultant blots were probed with an affinity purified polyclonal rabbit antibody to bovine milk OPN (1:2000, w/v), visualized using ECL and developed onto radiography film. Film was then analyzed on an imaging densitometer. The absorbance for each is shown (mean ± S.E.M.). There was no significant difference between the two values ($P=0.06$).

rates were evaluated, excluding polyspermic fertilization ($P<0.05$; Fig. 6B). The highest percentage of polyspermic fertilizations occurred when sperm were incubated in 10 µg/ml anti-OPN ($P<0.05$), with polyspermy occurring in nearly 22% of all fertilizations by sperm treated with this antibody concentration (Fig. 6C).

Binding of sperm incubated with OPN antibody to oocytes

Fewer sperm bound to oocytes treated with a 2.5 µg/ml anti-OPN than sperm incubated in control medium (Fig. 6D, $P<0.05$). However, sperm treated with 5 or 10 µg/ml antibody actually bound more sperm per oocyte than sperm incubated with 2.5 µg/ml anti-OPN or control medium.

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

Figure 4 Immunofluorescent localization of osteopontin (OPN) on ejaculated and cauda epididymal Holstein bull sperm. Ejaculated sperm were washed to remove seminal plasma, 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) brightfield image of same sperm. Cauda epididymal sperm were collected from dissected epididymides from bulls at slaughter and washed to remove epididymal fluid. Cells were 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. (C) FITC-labeled epididymal sperm and (D) brightfield image of same sperm.

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.

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 IP_3 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 6 (A) Mean percentage $\pm$ S.E.M. of oocytes fertilized, including normal and polyspermic fertilizations. Oocytes were inseminated with spermatozoa incubated in MTM, 2.5 μ g/ml affinity purified polyclonal rabbit antibody to bovine milk osteopontin (anti-OPN), 5 μ g/ml anti-OPN, or 10 μ g/ml anti-OPN prior to insemination. (B) Mean percentage $\pm$ S.E.M. of oocytes fertilized normally, excluding polyspermic fertilizations. Oocytes were inseminated with spermatozoa incubated in MTM, 2.5 μ g/ml anti-OPN, 5 μ g/ml anti-OPN, or 10 μ g/ml anti-OPN prior to insemination. (C) Mean percentage $\pm$ S.E.M. of polyspermic fertilizations, expressed as a percentage of total fertilizations. Oocytes were inseminated with spermatozoa incubated in MTM, 2.5 μ g/ml anti-OPN, 5 μ g/ml anti-OPN, or 10 μ g/ml anti-OPN prior to insemination. (D) Mean number $\pm$ S.E.M. of sperm bound per zona pellucida (ZP). Oocytes were inseminated with spermatozoa incubated in MTM, 2.5 μ g/ml anti-OPN, 5 μ g/ml anti-OPN, or 10 μ g/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 IP_3 (Fenichel & Durand-Clement 1998). Integrins, such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$, 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. $\alpha_v\beta_3$, β_1 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.

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