

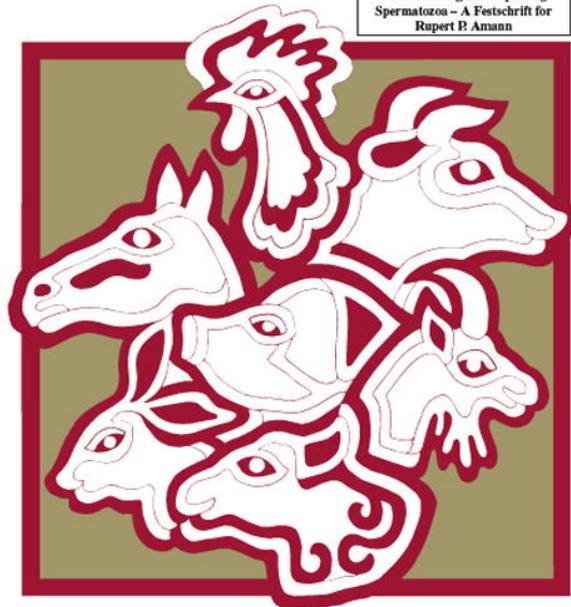
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# ANIMAL REPRODUCTION SCIENCE

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Rupert P. Amann



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# Binding patterns of bovine seminal plasma proteins A1/A2, 30 kDa and osteopontin on ejaculated sperm before and after incubation with isthmic and ampullary oviductal fluid<sup>☆,☆☆</sup>

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## Abstract

Previous studies from our laboratory have reported empirical associations between bovine seminal plasma protein(s) (BSP) A1/A2 and 30 kDa and osteopontin (OPN) in accessory sex gland fluid and bull fertility. These BSP and OPN are believed to bind to sperm at ejaculation and to remain bound until sperm reach the oviduct. The objective of the present study was to evaluate the topographical distribution of BSP A1/A2, 30 kDa and OPN binding on: (1) bovine ejaculated sperm; (2) ejaculated sperm incubated with isthmic oviductal fluid (ODF); (3) ejaculated sperm + isthmic ODF incubated in ampullary ODF. From each of these media, aliquots of sperm for BSP and OPN were processed for immunocytochemistry and analysis by laser scanning confocal microscopy. Isthmic and ampullary ODF was collected from indwelling catheters and used as pools from three cows in the non-luteal phase of the estrous cycle. Anti-BSP A1/A2 was detected bound to the midpiece, post-equatorial and equatorial segments and acrosome of sperm after ejaculation and after incubation with isthmic and ampullary ODF. The BSP A1/A2 fluorescence was more concentrated on the

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midpiece and increased as acrosome-intact sperm came in contact with ODF. As compared with acrosome-intact sperm, non-intact acrosome intact sperm had 39 and 68% reductions of acrosome fluorescence and 36% and 90% increases of post-equatorial fluorescence after contact with isthmic and ampullary ODF ( $P < 0.05$ ). Anti-BSP 30 kDa was more intense on the midpiece than on post-equatorial, equatorial and acrosome regions of sperm after ejaculation and contact with ODF. However, equatorial fluorescence was 141% and 89% more intense and acrosome staining was 80% and 76% less ( $P < 0.05$ ) in non-intact acrosome sperm than in acrosome intact cells, during all ODF incubations. Anti-OPN was identified on the acrosome of ejaculated sperm, but with less fluorescence ( $P < 0.05$ ) on the post-equatorial segment and midpiece. Incubation of sperm with isthmic ODF increased fluorescence on post-equatorial segment ( $P < 0.05$ ). There were 72% and 78% reductions ( $P < 0.05$ ) of acrosome fluorescence and intensification ( $P < 0.05$ ) in equatorial fluorescence in non-intact acrosome sperm as compared with acrosome intact cells incubated with isthmic and ampullary ODF. In summary, interactions of BSP A1/A2 and 30 kDa and osteopontin with the sperm membrane undergo modifications dictated by the oviductal fluid. The BSP are thought to modulate cholesterol and phospholipid movement from the sperm membrane and help sperm binding to the oviductal epithelium. Furthermore, our model suggests that OPN participates in sperm–oocyte interaction, affecting fertilization and early embryonic development.

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**Keywords:** BSP proteins; Fertility; Oocyte; Osteopontin; Oviductal fluid; Sperm

## 1. Introduction

Previous reports from our laboratory indicate empirical associations between proteins in male reproductive fluids and fertility of genetically proven dairy bulls from which semen is frequently inseminated. These associations were derived from the phenotype of reproductively normal bulls with fertility scores calculated from thousands of inseminations. Fertility scores of bulls were positively correlated with amounts osteopontin and phospholipase A<sub>2</sub> in seminal plasma, negatively correlated with spermadhesin Z13 and showed a quadratic association with bovine seminal plasma protein (BSP) 30 kDa (Killian et al., 1993; Moura et al., 2006a). Moreover, accessory sex gland fluid containing more albumin, BSP A1/A2, BSP 30 kDa, clusterin, osteopontin and phospholipase A<sub>2</sub>, and less nucleobindin, was more likely to enhance oocyte-penetrating capacity of cauda epididymal sperm than fluid with opposite characteristics (Moura et al., 2006b). From these studies it was clear that seminal plasma and accessory sex gland fluid contain protein markers of male fertility.

Both *in vivo* and *in vitro* studies have indicated that bovine seminal plasma (BSP) proteins and osteopontin (OPN) were among accessory sex gland proteins related to bull fertility. The BSP comprise approximately 86% of all proteins detected in the bovine accessory sex gland fluid (Moura et al., 2007) and are known to induce phospholipid and cholesterol efflux from the sperm membrane (Manjunath and Thérien, 2002). *In vitro* studies indicate that BSP mediate sperm binding to the oviduct epithelium and maintain sperm motility in that region of the female reproductive tract (Gwathmey et al., 2003, 2006). Osteopontin (OPN) is the most significant marker of fertility in dairy bulls, being 4.5-fold more abundant in the accessory sex gland fluid of sires with the greatest fertility scores (Killian et al., 1993; Moura et al., 2006a). OPN has been detected in several types of tissues, including extracellular matrix of bones (Franzen and Heinegard, 1985) mammary gland, endothelial cells, macrophages, tumor cells, follicles, corpus luteum and trophoblasts (for review: Mazzali et al., 2002; Johnson et al., 2003; Denhardt, 2004; Rangaswami et al., 2006). OPN has been reported to function in cell adhesion and cell-extracellular

matrix communication, stimulation of immune cells, tissue mineralization, cell migration and prevention of apoptosis (see reviews above). Because OPN is a cell adhesion molecule, it was hypothesized that OPN functions in sperm–oocyte interaction and fertilization (Moura, 2005), which has recently been supported by *in vitro* fertilization studies with bovine (Gonçalves et al., 2006a) and porcine gametes (Hao et al., 2006). Accessory sex gland proteins such as osteopontin and BSP are believed to bind to sperm at ejaculation and are assumed to remain bound until sperm reach the site of fertilization.

Information on the localization of fertility-related proteins of accessory sex gland origin on ejaculated sperm and how they may be modified following exposure to oviductal secretions may provide insights into their role in male fertility. The objective of the present immunocytochemical study was to evaluate the topographical distribution and protein intensity of BSP A1/A2, BSP 30 kDa and osteopontin on ejaculated sperm before and after *in vitro* incubation with isthmic and ampullary oviductal fluids.

## 2. Materials and methods

### 2.1. Experimental design

Binding patterns of fertility-associated proteins BSP A1/A2, BSP 30 kDa and osteopontin were evaluated in sperm collected from fresh semen and after sperm became sequentially exposed to fluid of isthmus and ampulla oviduct. The experiment used sperm from five Holstein bulls and conducted analysis with sperm in the following conditions: (1) ejaculated sperm; (2) ejaculated sperm incubated with isthmic oviductal fluid; (3) ejaculated sperm + isthmic oviductal fluid (as in 2) incubated in ampullary oviductal fluid. From each of those media (1, 2 and 3), we processed aliquots of sperm for immunocytochemistry and analysis by laser scanning confocal microscopy.

### 2.2. Collection of oviductal fluid

Fluid from isthmic and ampullary oviducts was collected from indwelling catheters inserted in the same cow, as previously described (Kavanaugh et al., 1992). After collection, fluid was centrifuged at  $10,000 \times g$  for 1 h at  $4^\circ\text{C}$  and stored in liquid nitrogen until use. Oviductal fluid and blood samples were obtained during at least two consecutive estrous cycles and peripheral concentrations of progesterone determined by radioimmunoassay. Non-luteal oviductal fluid from days of the estrous cycle when serum progesterone was less than 1 ng/ml were pooled for each cow. For all sperm incubations non-luteal pooled samples of fluid from each oviduct region were combined from three different cows.

### 2.3. Sperm preparation and incubation with oviductal fluid

After collection by artificial vagina, semen was kept in a water bath at  $37^\circ\text{C}$  for 10 min, and then centrifuged ( $700 \times g$  for 15 min) to recover sperm from seminal plasma. Sperm were then washed by two additional centrifugations with modified Tyrode's medium (MTM). Aliquots of  $5 \times 10^6$  cells were processed for immunocytochemistry using polyclonal antibodies against BSP A1/A2, BSP 30 kDa and osteopontin. From the remaining sperm,  $50 \times 10^6$  cells were taken and incubated for 4 h ( $39^\circ\text{C}$ , 5%  $\text{CO}_2$ ) in a media containing isthmic oviductal fluid (50%, v/v), MTM containing pyruvate (1 mM) penicillin (100 UI/ml) and streptomycin (100  $\mu\text{g}/\text{ml}$ ) (Sigma Inc., St. Louis, MO, USA). Following oviduct fluid incubation, aliquots of  $5 \times 10^6$  cells were

removed for immunocytochemistry as indicated above and the remaining pool of sperm incubated in ampullary oviductal fluid/MTM (50%, v/v) for 1 h (39 °C, 5% CO<sub>2</sub>). After incubation, aliquots of  $5 \times 10^6$  cells were taken again for immunocytochemistry. Using a laser scanning confocal microscopy (Olympus Inc., Center Valley, PA, USA), we examined ten cells per animal, per treatment at a magnification of 1000× and digital zoom of 5×.

After each incubation, an aliquot of 100 µl was exposed for 10 min (39 °C) to 60 µg/mL of lysophosphatidylcholine (LPC) containing 50 µg/µl of bovine serum albumin (BSA) to induce acrosome reaction in capacitated sperm (Parrish et al., 1988). After treatment with LPC, an aliquot of 10 µl was then mixed with equal volume of staining solution (Fast Green FCF-Eosin B) and 100 cells per treatment per animal were evaluated by light microscopy. We consider capacitated those live sperm and with reacted acrosome, in response to LPC.

#### 2.4. Immunocytochemistry

Sperm samples collected before and after incubation with isthmic and ampullary oviductal fluid were fixed in paraformaldehyde (2%) for 10 min and washed twice thereafter in PBS using gentle centrifugation (500 × g, 5 min, 4 °C). Blocking of non-specific binding followed with a 2 h incubation in PBS-Tween 20 containing 5% of bovine serum albumin (BSA), under mild agitation at 4 °C. Sperm were again incubated in blocking solution with the primary antibodies (1:200 for anti-BSP A1/A2 or BSP 30 kDa and 1:100 for anti-osteopontin) for 2 h under gentle agitation (4 °C) and washed three times thereafter with PBS-Tween 20 (500 × g; 5 min). Sperm were then incubated with anti-IgG conjugated with FITC (1:300; Sigma Inc., St Louis, MI, USA) for 1 h in a solution of PBS-Tween 20 and BSA (1%), followed by three washes. Slides were mounted in the dark using antifade reagent (Invitrogen Corp., Carlsbad, CA, USA).

Antibodies against BSP A1/A2 and 30 kDa were purified from respective rabbit antisera kindly provided by Dr. Puttaswamy Manjunath (Department of Medicine, University of Montreal, Canada). As described before (Moura et al., 2007), a column with protein-A Sepharose matrice (Sepharose CL-4B; Sigma Co., St. Louis, MO, USA) was washed initially with 50 nM PBS containing 0.15 M NaCl (pH 7.4) and adsorbed proteins were eluted with Glycine-HCl (pH 2.5). Fractions with absorbance at 280 nm were pooled, which contained the anti-BSP antibodies, and immediately adjusted to pH 7.4 with 0.1 N NaOH. The antibody solution was then aliquoted and stored in –20 °C until use. For detection of osteopontin in sperm we used a rabbit polyclonal antibody generated against OPN purified from bovine milk (Gabler et al., 2003). For all studies with immunocytochemistry, controls were conducted using incubations of sperm cells with the first antibody only and with the second antibody only. None of these approaches generated any form of detectable fluorescence.

#### 2.5. Capture of images and statistical analysis

Images of sperm treated with the first and FITC-conjugated antibodies were captured by confocal microscopy and FluoView™ software (Olympus Inc., Center Valley, PA, USA) as a series of sequential planes taken at every 0.125 µm, with a total depth of 5 µm. Such images or “stacks” were deconvoluted using AutoDeblur software (Media Cybernetics Inc., Silver Springs, MD, USA) and intensity of fluorescence quantified (in pixels) where staining was detected. Average pixel intensity of all sequential planes taken from each cell were compared by Duncan statistical test (SAS, 2003) among different regions of sperm, within and across three treatments ((1) ejaculated sperm; (2) ejaculated sperm plus isthmic oviductal fluid; (3) ejaculated sperm plus

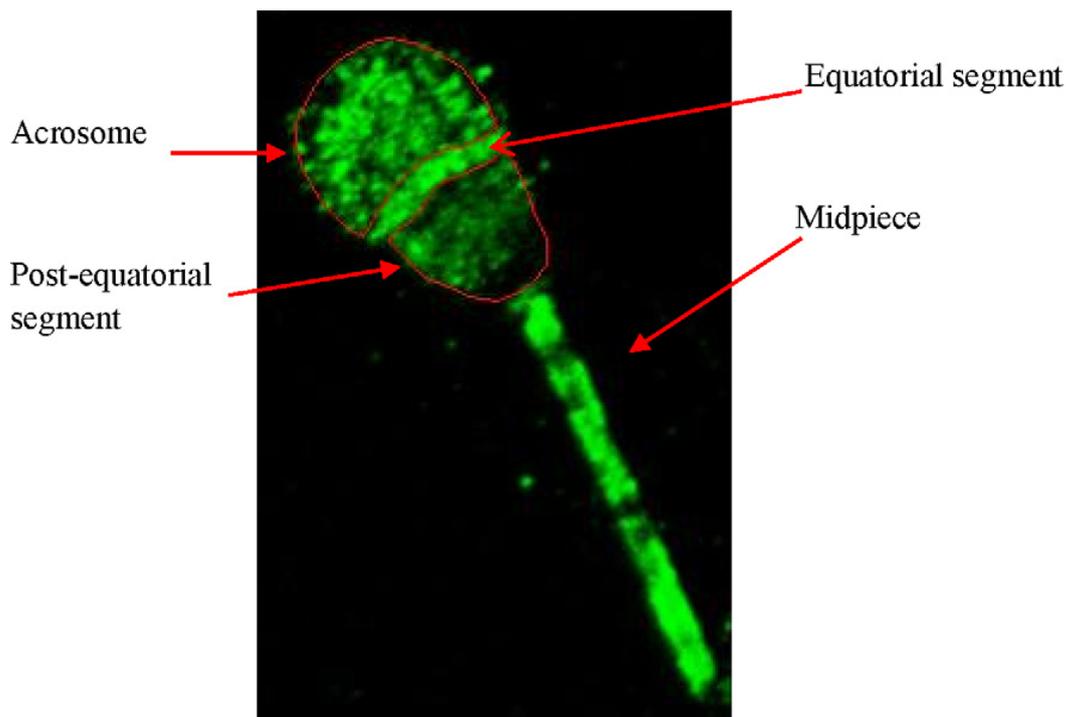


Fig. 1. Diagram showing the regions of sperm where pixel intensity was quantified. The image represents indirect immunofluorescence specific for BSP 30 kDa on ejaculated sperm.

isthmus oviductal fluid plus ampullary oviductal fluid). Based on preliminary analysis of images representing binding of all antibodies (against BSP and OPN), pixel intensities were quantified in the following regions of sperm: midpiece, post-equatorial, equatorial and acrosome (Fig. 1). Cells were analyzed separately with intact acrosome and non-intact acrosome.

### 3. Results

The antibody against BSP A1/A2 was detected bound to the midpiece, post-equatorial and equatorial segments and acrosome of sperm after ejaculation and after incubation with non-luteal isthmus and ampullary oviductal fluid (Fig. 2). Analysis of different planes from several cells shows fluorescence was more concentrated on the entire midpiece than on the other regions (Fig. 3), in all conditions to which the sperm cells were subjected. However, binding intensity of anti-BSP A1/A2 on the midpiece of acrosome-intact sperm increased as cells became in contact with oviductal fluids, while immunoreactivity on the other regions remained unchanged (Fig. 3). Cells with non-intact acrosome showed the same topographical distribution of anti-BSP A1/A2 binding on midpiece, post-equatorial and equatorial regions. As compared with acrosome-intact sperm, non-intact acrosome intact sperm had 39% and 68% reductions of acrosome fluorescence and 36% and 90% increases of post-equatorial fluorescence after contact with isthmus and ampullary oviductal fluid, respectively ( $P < 0.05$ ; Fig. 3).

The anti-BSP 30 kDa reaction appeared more intense on the midpiece than on post-equatorial, equatorial and acrosome regions of sperm evaluated after ejaculation and contact with isthmus and ampullary oviductal fluid (Figs. 4 and 5). However, equatorial fluorescence was 141% and 89% more intense ( $P < 0.05$ ) in non-intact acrosome sperm than in acrosome intact cells, during both isthmus and ampullary ODF incubations, respectively. Acrosome fluorescence, in turn, was 80% and 76% less prominent ( $P < 0.05$ ) in non-intact acrosome sperm than in acrosome intact cells, at the same conditions (Fig. 5).

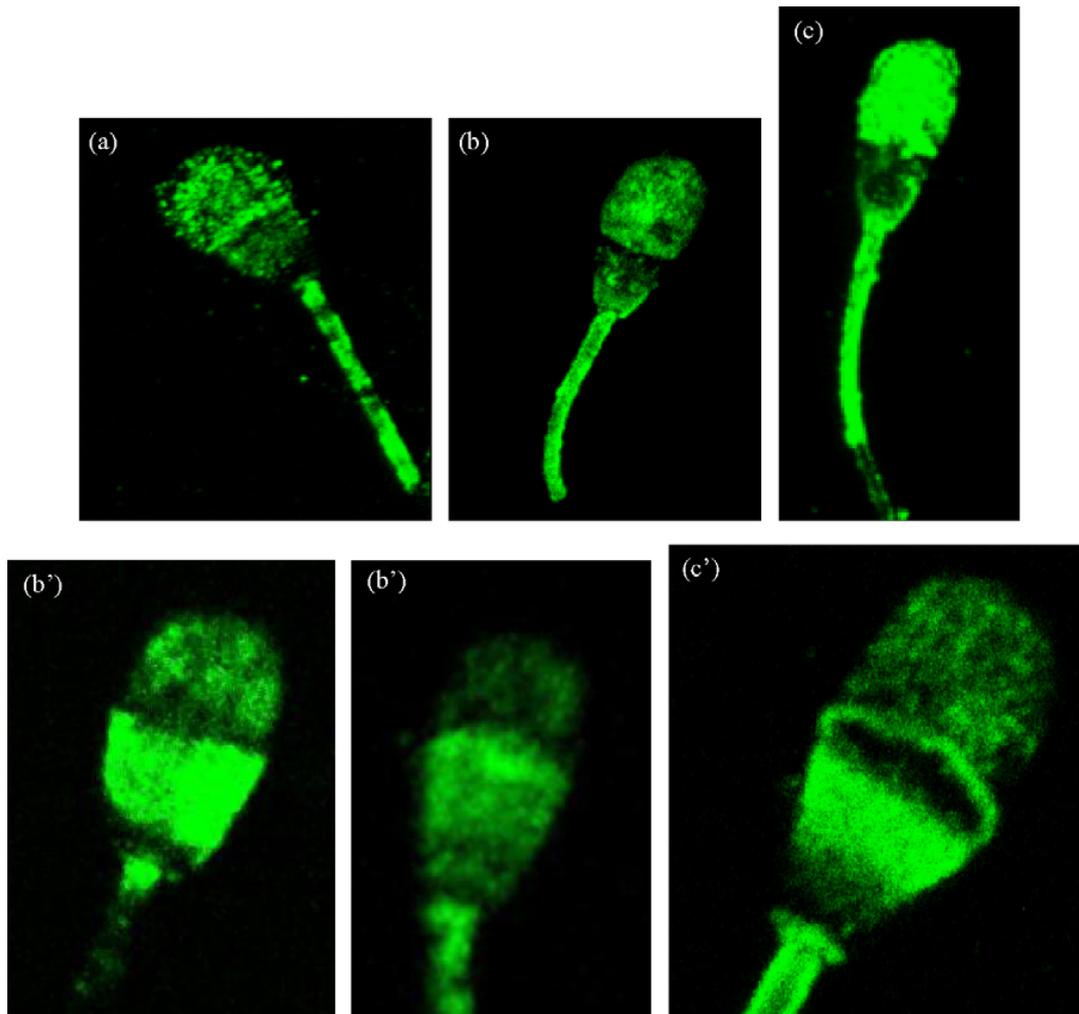
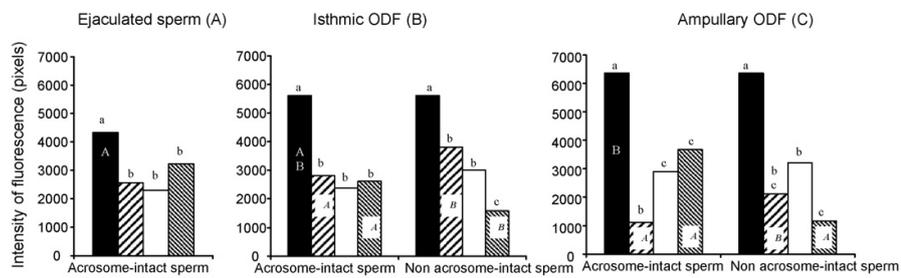


Fig. 2. Patterns of BSP A1/A2 binding to ejaculated sperm (a) and to sperm with intact acrosomes after incubation with non-luteal isthmic (b) and ampullary (c) oviductal fluid. BSP A1/A2 staining in sperm with acrosome that was not intact is shown, respectively, in (b') and (c'). Information was generated by indirect immunofluorescence, laser scanning confocal microscopy and FluoView<sup>TM</sup> and AutoDeblur softwares.

Anti-osteopontin binding was primarily identified on the acrosomal cap of ejaculated sperm with less fluorescence ( $P < 0.05$ ) on the post-equatorial segment and midpiece (Figs. 6 and 7). Incubation of sperm with isthmic ODF for 4 h increased the fluorescence on post-equatorial segment ( $P < 0.05$ ), but without alterations on the acrosomal cap or midpiece, in comparison with the same regions on ejaculated sperm. Subsequent contact with ampullary fluid did not change fluorescence on either midpiece, post-equatorial segment or acrosome (Fig. 7). Cells with non-intact acrosome showed pronounced reductions ( $P < 0.05$ ) of the fluorescence on the acrosome, when compared with acrosome intact cells incubated in isthmic and ampullary oviductal fluid (72% and 78% reductions, respectively). Also, there was intensification ( $P < 0.05$ ) in equatorial fluorescence in non-intact acrosome sperm as compared with acrosome-intact cells, in both conditions. Antibody binding to the midpiece remained without significant changes (Fig. 7).

The greatest percentage of spermatozoa undergoing capacitation and capable of acrosome reaction when treated with LPC occurred after incubation with isthmic (39.8% and 79%) and ampullary oviductal fluid (20.5% and 69.3%, respectively), in comparison with ejaculated spermatozoa mixed with MTM only (12.3% and 49.3%) or with heparin (23.7% and 38.9%; Fig. 8).



Lower cases represent significant differences ( $P < 0.05$ ) among regions of acrosome intact or non acrosome intact sperm, within each treatment (A, B and C).

Capital letters show significant ( $P < 0.05$ ) variations across treatments (A, B and C), for each specific region of sperm.

Fig. 3. Quantitative BSP A1/A2 binding to midpiece:

- post-equatorial
- ▨ equatorial
- ▧ acrosomal

regions of ejaculated sperm (A) and of sperm incubated with non-luteal isthmic (B) and ampullary (C) oviductal fluid (ODF). In panel A, fluorescence is shown for sperm with intact acrosome only. Within panels B and C, anti-BSP A1/A2 staining is shown for acrosome intact and non acrosome-intact sperm. Information was generated by indirect immunofluorescence, laser scanning confocal microscopy and FluoView™ and AutoDeblur softwares.

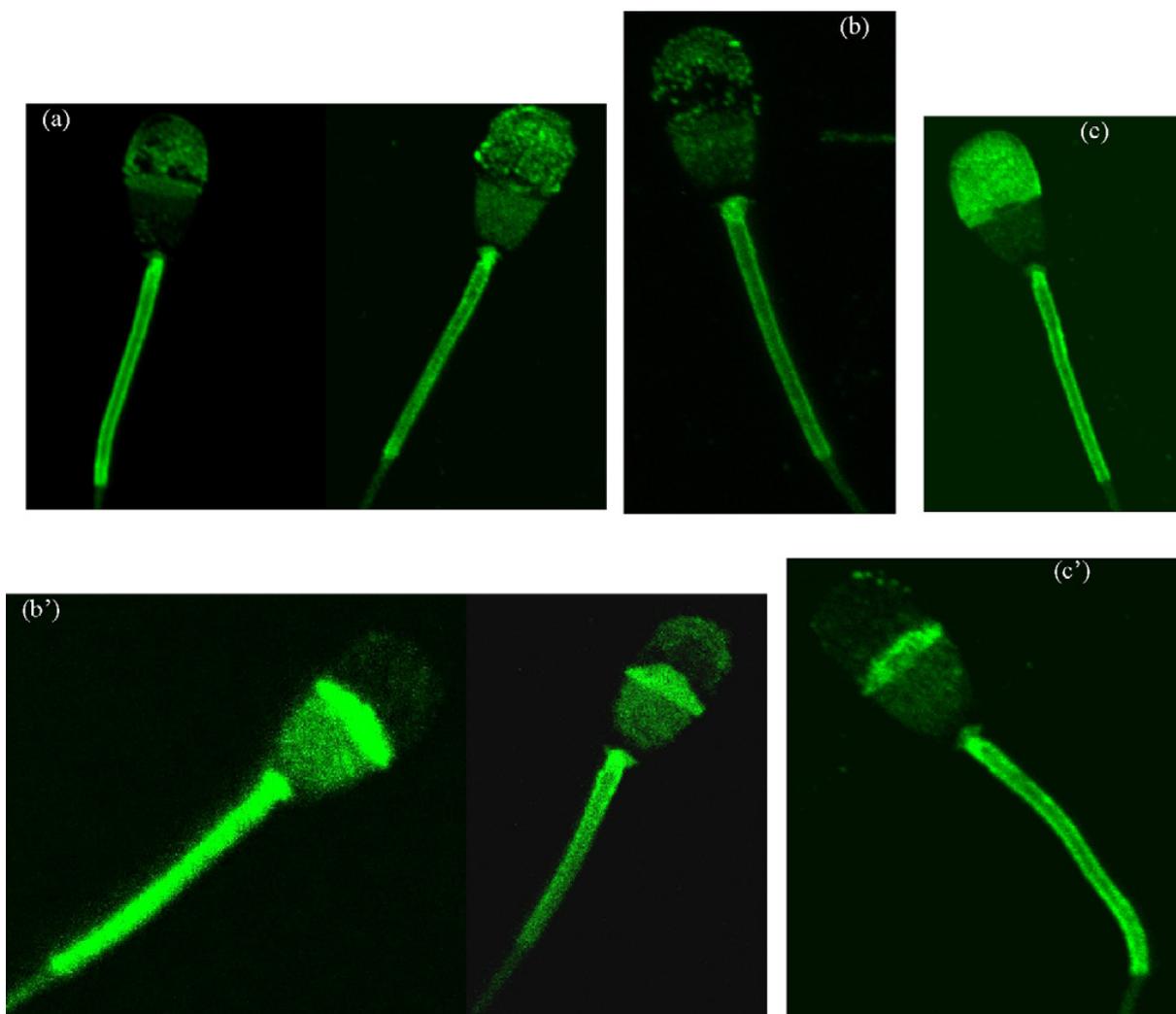
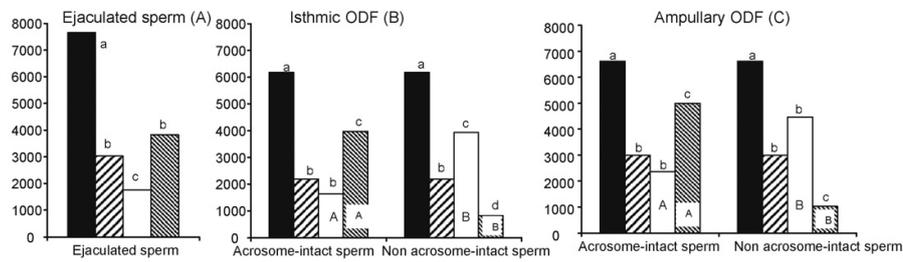


Fig. 4. Patterns of BSP 30 kDa binding to ejaculated sperm (a) and to sperm with intact acrosome after incubation with non-luteal isthmic (b) and ampullary (c) oviductal fluid. BSP 30 kDa staining in sperm with acrosome that was not intact is shown, respectively, in (b') and (c'). Information was generated by indirect immunofluorescence, laser scanning confocal microscopy and FluoView<sup>TM</sup> and AutoDeblur softwares.

#### 4. Discussion

The present study shows detailed analysis of binding patterns of fertility-associated proteins BSP A1/A2, BSP 30 kDa and osteopontin in ejaculated sperm and after sperm were treated with non-luteal isthmic and ampullary oviductal fluid. Because none of the antibodies against these proteins showed detectable immunoreaction with bull sperm collected from the cauda epididymidis (data not shown), it is clear that BSP and osteopontin originate from the accessory sex glands and bind to sperm at ejaculation. Manjunath et al. (1993) described some immunoreactivity of anti-BSP 30 kDa with cauda epididymal sperm, but we were unable to detect BSP even when antibody concentrations were 50% greater than those used in the present study. Association of BSP with membranes of the ejaculated sperm head is in accord with experimental evidence that BSP participate in cholesterol and phospholipid removal from the membrane (Manjunath and Thérien, 2002). In this model, BSP remain bound to sperm as they traverse the female reproductive tract. After reaching the oviduct, interaction of sperm-bound BSP occur with HDL triggering a second cholesterol efflux from the membrane which initiates capacitation. Studies also suggest



Lower cases represent significant differences ( $P < 0.05$ ) among regions of acrosome intact or non acrosome intact sperm, within each treatment (A, B and C).

Capital letters show significant ( $P < 0.05$ ) variations in equatorial fluorescence between acrosome intact and non-acrosome intact sperm.

Fig. 5. Quantitative BSP 30 kDa binding to midpiece:

- post-equatorial
- ▨ equatorial
- ▩ acrosomal

regions of ejaculated sperm (A) and of sperm incubated with non-luteal isthmic (B) and ampullary (C) oviductal fluid. In panel A, fluorescence is shown for sperm with intact acrosomes only. Within panels B and C, anti-BSP 30 kDa staining is shown for acrosome intact sperm and non acrosome-intact sperm. Information was generated by indirect immunofluorescence, laser scanning confocal microscopy and FluoView™ and AutoDeblur softwares.

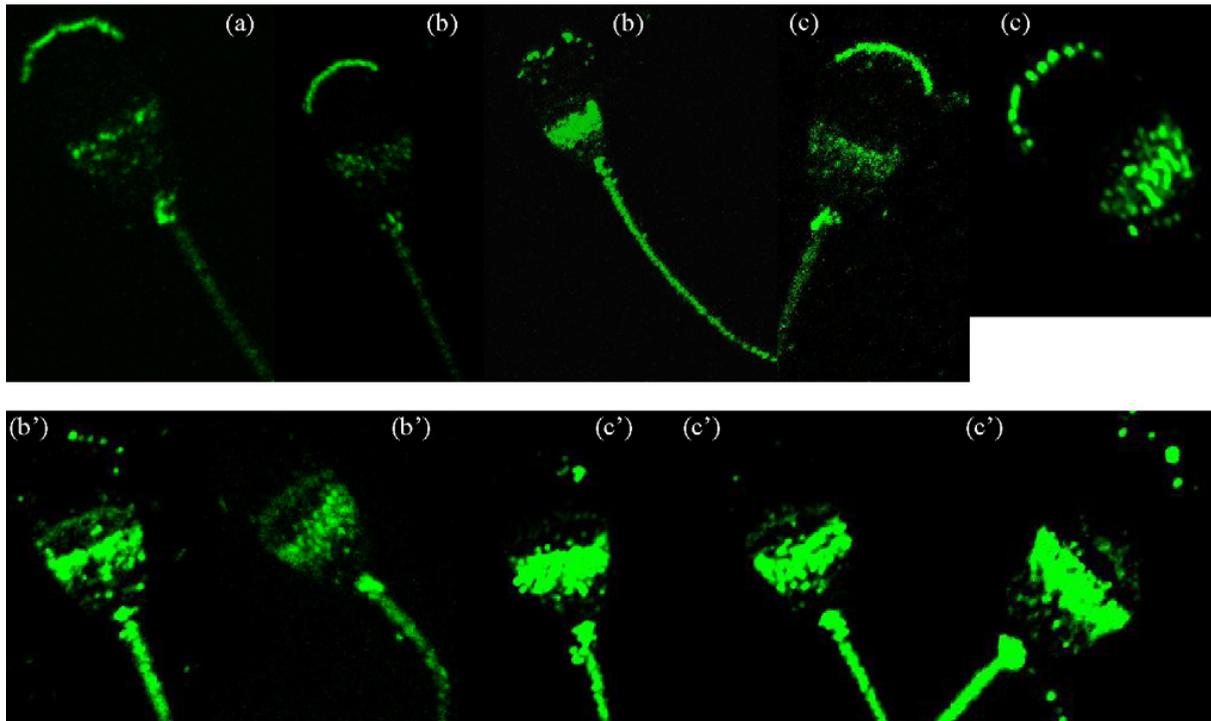
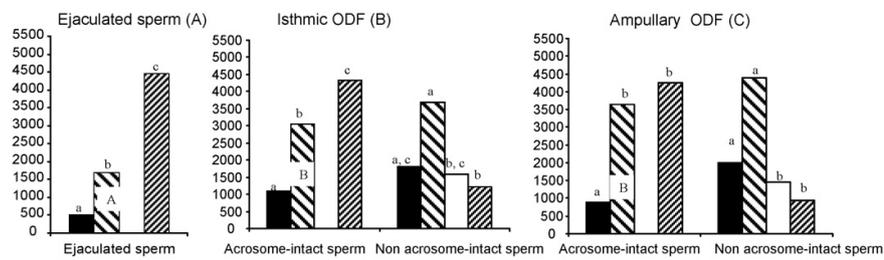


Fig. 6. Patterns of osteopontin binding to ejaculated sperm (a) and to sperm with intact acrosomes after incubation with non-luteal isthmic (b) and ampullary (c) oviductal fluid. Osteopontin staining in sperm with acrosome that was not intact is shown, respectively, in (b') and (c'). Information was generated by indirect immunofluorescence, laser scanning confocal microscopy and FluoView™ and AutoDeblur softwares.

that BSP mediate sperm binding to the oviductal epithelium, helping to preserve sperm viability and motility while in the oviductal reservoir (Gwathmey et al., 2006).

The proposed role of BSP in sperm capacitation assumes that they remain attached to ejaculated sperm in the female reproductive tract. Results of the present study monitoring BSP of sperm before and after incubation in oviductal fluids support this notion although changes in staining intensity were observed. In non-intact acrosome sperm, BSP A1/A2 and BSP 30 kDa fluorescence intensities significantly decreased in the acrosome region after sperm came in contact with both isthmic and ampullary oviductal fluids. Such modifications may be the result of fusion of the acrosome membrane caused by the acrosome reaction and/or rearrangements of the sperm membrane triggered by movement of cholesterol and phospholipids, some of which are modulated by BSP. Oviductal fluids have the ability to induce acrosome reaction of bovine sperm *in vitro* (Killian, 2004). ODF also contains apolipoprotein A-I and high density lipoproteins, known to interact with both phospholipids and BSP (Manjunath and Thérien, 2002; Thérien et al., 1997). Albumin comprises more than 85% of all proteins detected in both isthmic and ampullary oviductal fluid (unpublished results). Albumin is known to facilitate cholesterol efflux from membranes and may promote sperm capacitation as well (Singleton and Killian, 1983; Go and Wolf, 1985; Visconti and Kopf, 1998). Taken together with the current findings it appears that oviductal fluid creates an environment in which direct or indirect modifications of the sperm membrane occur and result in altered staining patterns of BSP bound to the head and acrosome of ejaculated sperm.

The pronounced BSP immunofluorescence at the sperm midpiece, which was much greater than in the other regions of the sperm at all treatments, is intriguing. Intense immunoreaction of anti-BSP A1/A2 (Pdc 109) at the midpiece has been reported previously for ejaculated bovine sperm (Aumuller et al., 1988). They suggested that localization of BSP in the midpiece close



Lower cases represent significant differences ( $P < 0.05$ ) among regions of acrosome intact or non acrosome intact sperm, within each treatment (A, B and C).

Capital letters show significant ( $P < 0.05$ ) variations across treatments (A, B and C), for each specific region of sperm.

Fig. 7. Quantitative osteopontin binding to midpiece:

- post-equatorial
- ▨ equatorial
- ▩ acrosomal

regions of ejaculated sperm (A) and of sperm incubated with non-luteal isthmic (B) and ampullary (C) oviductal fluid. In panel A, fluorescence is shown for sperm with intact acrosome only. Within panels B and C, anti-osteopontin staining is shown for acrosome-intact sperm and non acrosome-intact sperm. Information was generated by indirect immunofluorescence, laser scanning confocal microscopy and FluoView™ and AutoDeblur softwares.

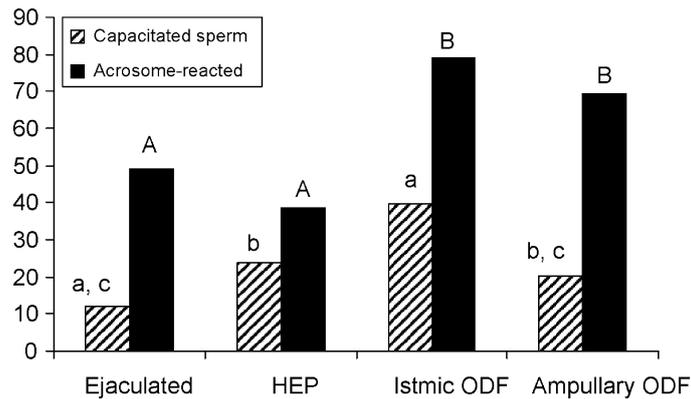


Fig. 8. Percentage of ejaculated spermatozoa undergoing capacitation and the acrosome reaction after incubation with heparin and non-luteal isthmic and ampullary oviductal fluid.

to the mitochondria may indicate that BSP affect sperm motility. Although protein receptors for BSP have not been identified, it has been shown that BSP A1/A2 stimulates sperm motility and membrane-bound calcium ATPase activity in bull sperm (Sanchez-Luengo et al., 2004). The functional connection between binding of BSP to the midpiece and stimulation of mitochondrial activity and sperm motility suggests multifaceted role for BSP as sperm are prepared for fertilization. Although it has not been tested, BSP may influence hyperactivation of sperm as well as sperm capacitation.

In the present study, an unexplained increase in fluorescence associated with BSP A1/A2 binding to the midpiece of sperm was observed after exposure to oviductal fluid. Based on earlier reports (reviewed in Killian, 2004) and an analysis of ODF composition by 2-D electrophoresis and mass spectrometry (Souza et al., 2007), there is no evidence that BSP are expressed in either isthmic and ampullary ODF. There were reductions in BSP A1/A2 fluorescence of the acrosome and increases in the post-equatorial segment of non-acrosome intact sperm, however, after incubations with oviductal fluids. Anti-BSP 30 kDa reaction also changed, as equatorial fluorescence was more intense and acrosome fluorescence, in turn, less prominent in non-acrosome intact sperm than in acrosome intact cells. One plausible explanation for the changes in BSP binding to sperm exposed to ODF is that components of oviduct fluid potentiate membrane remodeling and clustering of BSP with the midpiece membrane. Alternatively, components of ODF may remove or modify other surface proteins in specific regions of sperm membrane and alter a quenching effect they may have had on FITC detection of BSP.

Anti-osteopontin binding was present on the post-equatorial segment and acrosomal cap of ejaculated sperm, with less fluorescence on the midpiece. Incubation of sperm with isthmic ODF increased the fluorescence on post-equatorial segment, but without alterations on the acrosomal cap or midpiece, in comparison with the same regions on ejaculated sperm, but subsequent contact with ampullary fluid did not change fluorescence. These findings are intriguing and have not been previously reported. Oviductal fluid contains OPN (Gabler et al., 2003) and it is possible that such OPN binds to sperm, causing the increase in fluorescence seen in the post-equatorial region. However, reductions on acrosome fluorescence and intensification in equatorial fluorescence in non-intact acrosome sperm in the isthmic and ampullary ODF, as compared with acrosome intact cells, are obviously caused by other mechanisms. These changes may be the consequence of membrane fusion and remodeling which occurs between the plasma and outer acrosomal membranes prior to the acrosome reaction. Although coincidental increases in acrosome reacted sperm (in response to LPC) occurred after incubation with oviductal fluids, the theory that partial loss

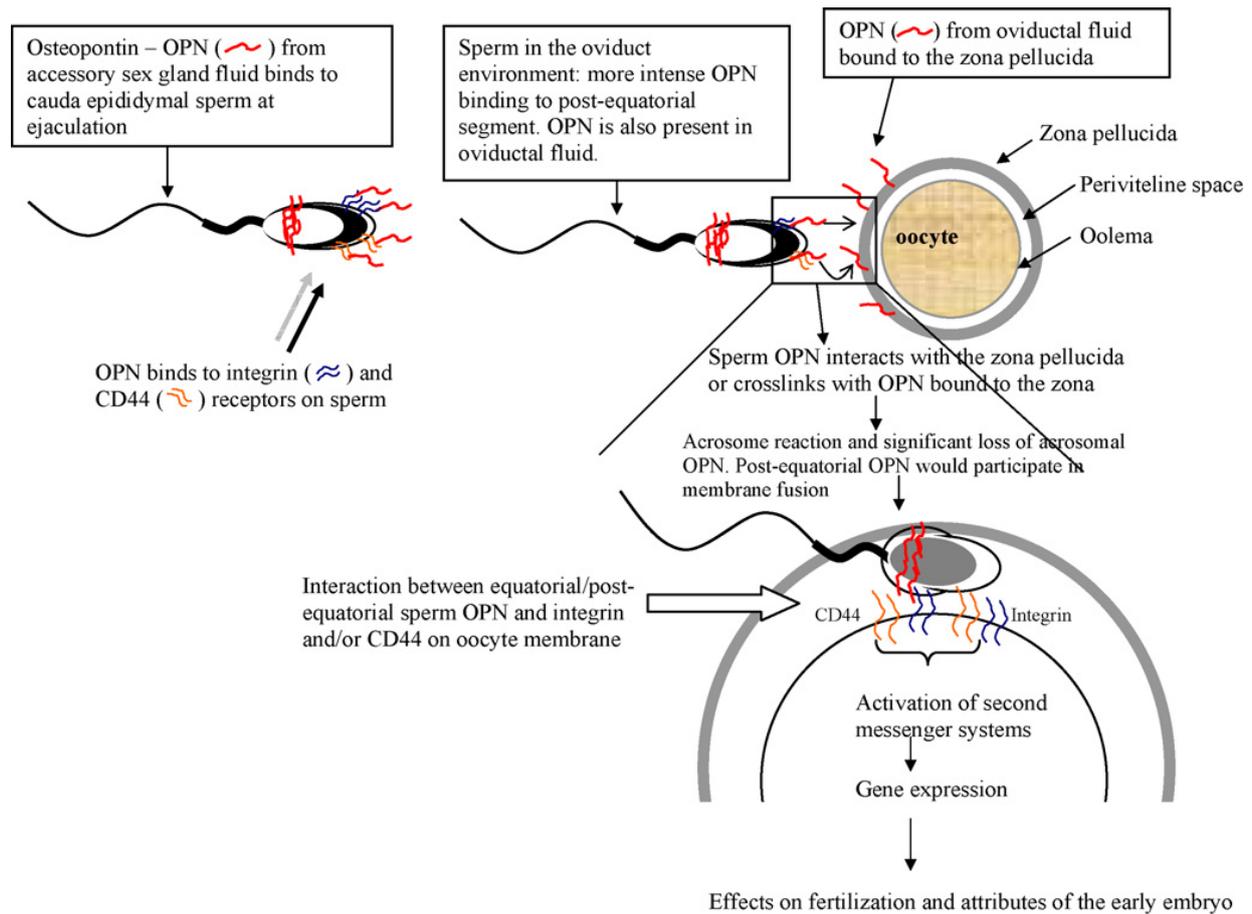


Fig. 9. Proposed mechanisms by which osteopontin interacts with sperm and with the oocyte. Osteopontin will bind to sperm at ejaculation, possibly through integrin and/or CD44 receptors, and will remain bound to it until it reaches the site of fertilization. Contact with the isthmic and ampullary oviduct will decrease binding of OPN to the acrosome region of some sperm. Reaching the oocyte, sperm-bound OPN will interact with the zona pellucida and after in the perivitelline space, with the oocyte membrane. This last phase may also involve OPN bound to the post-equatorial region of the acrosome-reacted sperm, a region that is typically involved in sperm–oolema fusion. Link of sperm-bound OPN with integrin and/or CD44 receptors on oocyte membrane would inevitably trigger intracellular signaling, affecting post-fertilization events. This would explain why exogenous OPN has positive effects on early embryo development.

of acrosome OPN is a consequence of membrane remodeling awaits further confirmation. Nevertheless, it seems that spermatozoa will reach the site of fertilization with OPN steadily bound to the equatorial/post-equatorial region but varying degrees of OPN attachment to the acrosomal cap. However, it remains to be determined if such different populations of cells also have different fertilizing abilities. Moreover, link of OPN to the central region of sperm is of special relevance because membrane fusion is thought to occur using the segments close to the equatorial region of sperm (Gaddum-Rosse, 1985).

Osteopontin is a multifunctional molecule typically involved in cell adhesion and tissue remodeling, but other events also occurs (for reviews see: Liaw et al., 1998; Mazzali et al., 2002; Wai and Kuo, 2004; Denhardt, 2004). Despite substantial information about the actions of osteopontin in several tissues, an understanding of its function in male reproduction is far from complete. Based on general attributes of osteopontin and results from our earlier studies, we hypothesized that OPN mediates sperm–oocyte interaction and fertilization (Moura, 2005; Moura et al., 2006a). Results presented here and elsewhere further support this hypothesis and allow us to refine our model about OPN roles, as summarized in Fig. 9. In addition to sperm binding, osteopontin also has the

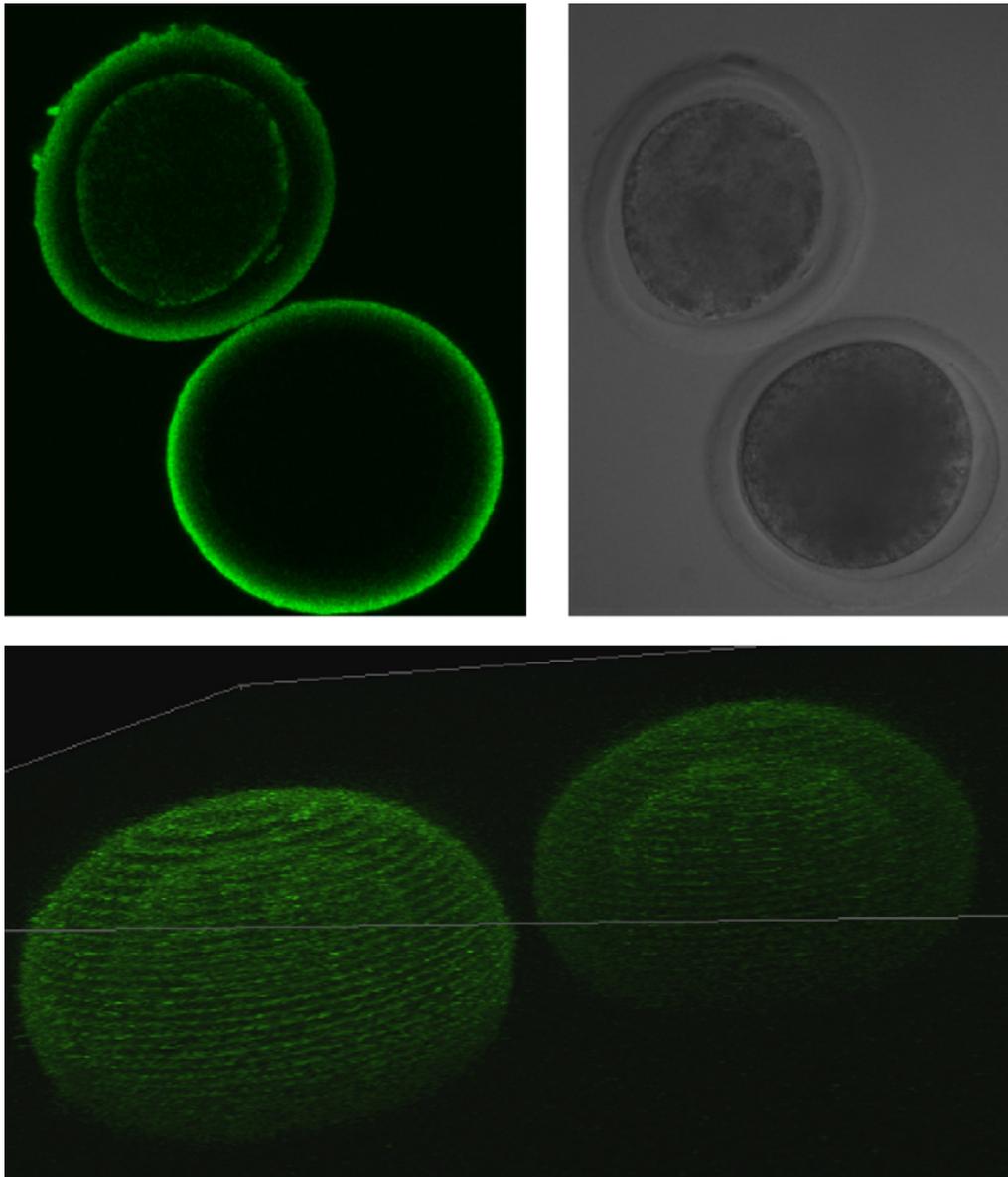


Fig. 10. Immunofluorescence detection of osteopontin on bovine oocytes pre-incubated with bovine oviductal fluid, which contains OPN (Gabler et al., 2003). Oocytes were matured *in vitro*, followed by removal of cumulus cells (Hasler et al., 1995; Gonçalves et al., 2006a; Bavister et al., 1993). After culture, eggs were washed twice in fresh TL-HEPES and incubated with anti-bovine milk osteopontin (100  $\mu\text{g}/\text{ml}$ ) for 1 h at 39 °C (5% CO<sub>2</sub> in air), the same antibody used for sperm immunocytochemistry. After washing twice with fresh TL-HEPES, eggs were incubated for 30 min with goat F (ab)2 –anti rabbit IgG (H+L)-FITC (1:300; Southern Biotech Associates, Birmingham, AL, USA) and washed twice again. Images were analyzed under laser scanning confocal microscopy and FluoView™ and AutoDeblur softwares, as described for all studies with sperm cells.

ability to interact with the zona pellucida and oolema of bovine oocytes (Fig. 10). In light of these findings, we propose that OPN adheres to sperm and such sperm-OPN complex binds to the zona pellucida. Another possibility is that sperm-OPN connects to the OPN already bound to the zona because osteopontin is capable of forming bonds with other OPN molecules with high affinity (Kartinen et al., 1999; Goldsmith et al., 2002). The oviductal fluid has osteopontin (Gabler et al., 2003), which may attach to the zona before spermatozoa even reach the site of fertilization. After entering the perivitelline space, OPN attached to the post-equatorial segment would mediate the interaction of sperm with the oolema, also through integrins and/or CD44. Our hypothesis that

osteopontin binds to sperm and oocytes through integrins and CD44 is supported by observations that  $\alpha_v$  and  $\alpha_5$  integrins have been identified in cattle (Erikson et al., 2003) and human spermatozoa (Fusi et al., 1996; Reddy et al., 2003), as well as on the oolema (D’Cruz, 1996). The CD44 transmembrane glycoproteins are also present in bovine sperm (Bains et al., 2002) and oocyte (Schoenfelder and Einspanier, 2003) and osteopontin has been reported to interact with integrins and CD44 in several other cell types (Mazzali et al., 2002; Rangaswami et al., 2006).

The importance of OPN in reproduction is also demonstrated by experiments using *in vitro* fertilization. Addition of OPN antibodies to fertilization media reduced both the number of sperm bound to oocytes and the percentage of fertilized bovine oocytes ( $88.64 \pm 3.0\%$  compared with  $28.7 \pm 3.2\%$ ; Gonçalves et al., 2006a). Treatment of eggs with non-luteal oviductal fluid and anti-OPN also diminished the percentage of blastocysts on day 8 (from  $22 \pm 1\%$  to  $10.5 \pm 0.5\%$ ) and hatched blastocysts on day 11 (from  $8 \pm 1\%$  to  $3 \pm 0.5\%$ ; Gonçalves et al., 2003). The RGD amino acid sequence in the OPN molecule mediates its link with  $\alpha_5$  and  $\alpha_v$  integrins (Denhardt et al., 2001; Wai and Kuo, 2004) and the ability of osteopontin to support cell adhesion is prevented when the RGD sequence is mutated (Liaw et al., 1995; Xuan et al., 1995). Treatment of sperm or oocytes with an RGD peptide, but not with an RGE sequence, or with  $\alpha_5$  and  $\alpha_v$  integrin antibodies reduced the number of sperm bound to the zona pellucida and fertilization rates, similar to what was found using antibodies against osteopontin. Results obtained with the use of antibodies against osteopontin, the RGD peptide and integrins (Gonçalves et al., 2006a) not only support the notion that OPN participates in sperm–oocyte interaction but also that such event may involve integrins, although further experimental evidence is needed to substantiate such hypothesis. Addition of osteopontin to sperm and oocytes also had positive influence on the outcome of IVF. Incubation of oocytes with OPN purified from bovine skim milk led to increases in cleavage rates at day 4 (from  $78.1 \pm 1.3\%$  to  $85.8 \pm 1.4\%$ ), blastocyst development at day 8 (from  $24.2 \pm 1.2\%$  to  $33.8 \pm 1.4\%$ ) and hatched blastocysts at day 11 (from  $10.6 \pm 1.6\%$  to  $18.5 \pm 1.4\%$ ; Gonçalves et al., 2003). Following this same line of research, Hao et al. (2006) found that treatment of fertilization media with rat recombinant OPN enhanced fertilization rates of swine oocytes by as much as 41%. Moreover, bull semen frozen with different concentrations of OPN induced greater *in vitro* fertilization rates ( $85\text{--}78 \pm 4\%$  compared with  $75\text{--}69 \pm 4\%$ ) and blastocyst development at day 8 ( $45 \pm 2.9\%$ – $37 \pm 1.6\%$  compared with  $33 \pm 2.3\%$ – $29 \pm 2.8\%$ ) in comparison with untreated semen (Gonçalves et al., 2006b). The fact that osteopontin has effects on post-fertilization events is indeed exciting but how it happens remains to be elucidated. OPN has known activities in antiapoptosis and cell survival through activation of integrins and CD44 membrane receptors and signal transduction mechanisms, including activation of Map kinases, phosphoinositide (PI) 3-kinase/Akt-dependent NF $\kappa$ B, IKK/ERK-mediated pathways, which stimulates uPA-dependent MMP-9 activation, PLC-g/PKC/PI 3-kinase pathways (Wai and Kuo, 2004; Rangaswami et al., 2006; Chakraborty et al., 2006; Khodavirdi et al., 2006; Lee et al., 2007). Numerous intracellular signals are activated in the egg (such as those involving Src kinases and PKC) after sperm–egg fusion occurs. Such intracellular messengers are thought to play important roles in the resumption of meiosis after fertilization and consequent development of the zygote (McGinnis et al., 2007; Talmor-Cohen et al., 2004; Halet, 2004; Eliyahu et al., 2001; Eliyahu and Shalgi, 2002; Eliyahu et al., 2002). Our hypothesis, as summarized in Fig. 9, is that OPN binding to the oolema triggers some of those intracellular signals via interaction with integrins and/or CD 44. This would explain the positive effects of osteopontin on post-fertilization events.

In summary, since the empirical associations between osteopontin, BSP proteins and male fertility were first described (Killian et al., 1993; Cancel et al., 1997; Moura et al., 2006a,b), we have gathered additional information about their actions, especially in the case of osteopontin.

Interactions of both BSP proteins and osteopontin with gametes undergo modifications dictated by the oviductal fluid. Binding of BSP to sperm modulates sperm membrane cholesterol and phospholipid content, contact of sperm with the oviduct epithelium and possibly sperm motility. Our model for OPN action suggests that it participates in sperm–oocyte interaction, affecting fertilization and early embryonic development. Studies aimed to understand how fertility-related proteins interact with gametes will support their potential use of fertility markers in the male.

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