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

Carlos Eduardo A. Souza[1], Arlindo A. Moura, Elisa Monacao, Gary J. Killian

(accepted – September 2007 – Animal Reproduction Science)

Almquist Research Center, Department of Dairy and Animal Science, Pennsylvania State University, USA

This research was funded by USDA grants 2003-34437-13460 and 2004-34437-15106.

[1] Supported by a graduate student fellowship awarded by the Brazilian Research Council (CAPES).

Key words: BSP proteins, fertility, oocyte, osteopontin, oviductal fluid, sperm.

Corresponding authors:
Arlindo A. Moura
Department of Animal Science
Federal University of Ceara
Av. Mister Hull, s/n Campus do Pici
Fortaleza CE Brazil 60021
Phone: 85-3366-9697
Email: arlindo.moura@gmail.com

Gary J. Killian
Almquist Research Center
Penn State University
University Park, PA 16802
Phone: 814-329-0009
Email: garykillian@gmail.com

Abstract

Previous studies from our laboratory have reported empirical associations between bovine seminal plasma (BSP) proteins A1/A2 and 30 kDa and osteopontin (OPN) in accessory sex gland fluid and bull fertility. These BSPs and OPN are believed to bind to sperm at ejaculation and to remain bound until sperm reach the oviduct. The objective of this 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 those media, we processed aliquots of sperm for BSPs and OPN 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 estrus 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. BSP A1/A2 fluorescence was significantly more concentrated on the midpiece and increased as acrosome-intact sperm became 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 stainning was 80 and 76 % lower ($p < 0.05$) 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

54 on post-equatorial segment ($p < 0.05$). There were 72 and 78 % reductions ($p < 0.05$) of
55 acrosome fluorescence and intensification ($p < 0.05$) in equatorial fluorescence in non-intact
56 acrosome sperm as compared with acrosome intact cells incubated with isthmic and ampullary
57 ODF. In summary, interactions of BSP A1/A2 and 30 kDa and osteopontin with the sperm
58 membrane undergo modifications dictated by the oviductal fluid. BSPs are thought to modulate
59 cholesterol and phospholipid movement from the sperm membrane and help sperm binding to
60 the oviductal epithelium. Furthermore, our model suggests that OPN participates in sperm-
61 oocyte interaction, affecting fertilization and early embryonic development.

Introduction

Previous studies from our laboratory have reported empirical associations between proteins in male reproductive fluids and fertility of high use, proven dairy bulls. 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_2 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_2, 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. BSP proteins comprise approximately 86 % of all proteins detected in the bovine accessory sex gland fluid (Moura et al., 2006c) and are known to induce phospholipid and cholesterol efflux from the sperm membrane (Manjunath and Thérien, 2002). In vitro studies indicate that BSP proteins 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) expression 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 highest 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 (Goncalves 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 this 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.

Material and Methods

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 sequencially 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.

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 g for 1 hour at 4 ^{o}C and stored in liquid nitrogen until use. Oviductal fluid and blood samples were obtained during at least two consecutive full 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.

Sperm preparation and incubation with oviductal fluid

After collection by artificial vagina, semen was kept in a water bath at 37 ^{o}C for 10 minutes, and then centrifuged (700g 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 x 10^6 cells were processed for immunocytochemistry using polyclonal antibodies against BSP A1/A2, BSP 30 kDa and osteopontin. From the remaining sperm, 50 x 10^6 cells were taken and incubated for 4 hours (39 ^{o}C, 5 % CO_2) in a media containing isthmic oviductal fluid (50 %, v/v),

MTM containing pyruvate (1 mM) penicillin (100 UI/ml) and streptomycin (100 µg/ml) (Sigma Inc., St. Louis, MO, USA). Following oviduct fluid incubation, aliquots of 5 x 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 hour (39 ^oC, 5 % CO_2). After incubation, aliquots of 5 x 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 1,000 x and digital zoom of 5x.

After each incubation, an aliquot of 100 µl was exposed for 10 minutes (39°C) to 60 µg/mL of lysophosphotidylcholine (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.

Immunocytochemistry

Sperm samples collected before and after incubation with isthmic and ampullary oviductal fluid were fixed in paraformaldehyde (2%) for 10 minutes and washed twice thereafter in PBS using gentle centrifugation (500 xg, 5 min., 4 ^oC). Blocking of nonspecific binding followed with a 2-hour 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 hours under gentle agitation (4 ^oC) and washed three times thereafter with PBS-Tween 20 (500 xg; 5 min.). Sperm were then incubated with anti-IgG conjugated with FITC (1:300; Sigma Inc., St

154 Louis, MI, USA) for 1 hour in a solution of PBS-Tween 20 and BSA (1 %), followed by three
155 washes. Slides were mounted in the dark using antifade reagent (Invitrogen Corp., Carlsbad, CA,
156 USA).

157 Antibodies against BSP A1/A2 and 30 kDa were purified from respective rabbit antisera
158 kindly provided by Dr. Puttaswamy Manjunath (Department of Medicine, University of
159 Montreal, Canada). As described before (Moura et al., 2007), a column with protein-A Sepharose
160 matrice (Sepharose CL-4B; Sigma Co., St. Loius, MO, USA) was washed initially with 50 nM
161 PBS containing 0.15 M NaCl (pH 7.4) and adsorbed proteins were eluted with Glycine-HCl (pH
162 2.5). Fractions with absorbance at 280 nm were pooled, which contained the anti-BSP
163 antibodies, and immediately adjusted to pH 7.4 with 0.1 N NaOH. The antibody solution was
164 then aliquoted and stored in ? 20 ?C until use. For detection of osteopontin in sperm we used a
165 rabbit polyclonal antibody generated against OPN purified from bovine milk (Gabler et al.,
166 2003). For all studies with immunocytochemistry, controls were conducted using incubations of
167 sperm cells with the first antibody only and with the second antibody only. None of theses
168 approaches generated any form of detectable fluorescence.
169
170 **Capture of images and statistical analysis**
171 Images of sperm treated with the first and FITC-conjugated antibodies were captured by
172 confocal microscopy and FluoView^{TM} software (Olympus Inc., Center Valley, PA, USA) as a
173 series of sequential planes taken at every 0.125µm, with a total depth of 5µm. Such images or
174 "stacks" were deconvoluted using AutoDeblur software (Media Cybernetics Inc., Silver Springs,
175 MD, USA) and intensity of fluorescence quantified (in pixels) where staining was detected.
176 Average pixel intensity of all sequential planes taken from each cell were compared by Duncan

177 statistical test (SAS, 2003) among different regions of sperm, within and across three treatments
178 (1. ejaculated sperm; 2. ejaculated sperm plus isthmic oviductal fluid; 3. ejaculated sperm plus
179 isthmic oviductal fluid plus ampullary oviductal fluid). Based on preliminary analysis of images
180 representing binding of all antibodies (against BSP proteins and OPN), pixel intensities were
181 quantified in the following regions of sperm: midpiece, pots-equatorial, equatorial and acrosome
182 (Fig. 1). We separately analyzed cells with intact acrosome and non-intact acrosome.

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 isthmic and ampullary oviductal fluid (Fig. 2). Analysis of different planes from several cells shows fluorescence significantly more concentrated on the entire midpiece than on the other regions (Fig. 3), in all conditions sperm cells were subjected to. 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 contac with isthmic and ampullary oviductal fluid, respectively ($p < 0.05$; Fig. 3).

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 isthmic 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 isthmic 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).

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 (Fig. 6 and 7).

206 Incubation of sperm with isthmic ODF for 4 hours increased the fluorescence on post-equatorial
207 segment ($p < 0.05$), but without alterations on the acrosomal cap or midpiece, in comparison with
208 the same regions on ejaculated sperm. Subsequent contact with ampullary fluid did not change
209 fluorescence on either midpiece, post-equatorial segment or acrosome (Fig. 7). Cells with non-
210 intact acrosome showed pronounced reductions ($p < 0.05$) of the fluorescence on the acrosome,
211 when compared with acrosome intact cells incubated in isthmic and ampullary oviductal fluid
212 (72 and 78 % reductions, respectively). Also, there was intensification ($p < 0.05$) in equatorial
213 fluorescence in non-intact acrosome sperm as compared with acrosome intact cells, in both
214 conditions. Antibody binding to the midpiece remained without significant changes (Fig. 7).
215 The highest percentage of spermatozoa undergoing capacitation and capable of acrosome
216 reaction when treated with LPC occurred after incubation with isthmic (39.8 and 79 %) and
217 ampullary oviductal fluid (20.5 and 69.3 %, respectively), in comparison with ejaculated
218 spermatozoa mixed with MTM only (12.3 and 49.3 %) or with heparin (23.7 and 38.9 %; Fig. 8).

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 exposed to 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 proteins 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 proteins even when antibody concentrations were 50 % greater than those used in the present study. Association of BSP proteins with membranes of the ejaculated sperm head is in accord with experimental evidence that BSP proteins participate in cholesterol and phospholipid removal from the membrane (Manjunath and Thérien, 2002). In this model, BSP proteins remain bound to sperm as they traverse the female reproductive tract. After reaching the oviduct, interaction of sperm-bound BSP proteins occur with HDL triggering a second cholesterol efflux from the membrane which initiates capacitation. Studies also suggest that BSP proteins 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 proteins in sperm capacitation assumes that they remain attached to ejaculated sperm in the female reproductive tract. Our results monitoring BSP proteins 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

242 contact with both isthmic and ampullary oviductal fluids. Such modifications may be the result
243 of fusion of the acrosome membrane caused by the acrosome reaction and/or rearrangements of
244 the sperm membrane triggered by movement of cholesterol and phospholipids, some of which
245 are modulated by BSP proteins. Oviductal fluids have the ability to induce acrosome reaction of
246 bovine sperm in vitro (Killian, 2004). ODF also contains apolipoprotein A-I and high density
247 lipoproteins, known to interact with both phospholipids and BSP proteins (Manjunath and
248 Thérien, 2002; Thérien et al., 1997). We have also determined that albumin comprises more than
249 85 % of all proteins detected in both isthmic and ampullary oviductal fluid (unpublished results).
250 Albumin is known to facilitate cholesterol efflux from membranes and may promote sperm
251 capacitation as well (Singleton and Killian, 1983; Go and Wolf, 1985; Visconti and Kopf, 1998).
252 Taken together with the current findings it appears that oviductal fluid creates an environment in
253 which direct or indirect modifications of the sperm membrane occur and result in altered staining
254 patterns of BSP proteins bound to the head and acrosome of ejaculated sperm.
255 The pronounced BSP protein immunofluorescence at the sperm midpiece, which was much
256 greater than in the other regions of the sperm at all treatments, is intriguing. Intense
257 immunoreaction of anti-BSP A1/A2 (Pdc 109) at the midpiece has been reported previously for
258 ejaculated bovine sperm (Aumuller et al., 1988). They suggested that localization of BSP
259 proteins in the midpiece close to the mitochondria may indicate that BSP proteins affect sperm
260 motility. Although protein receptors for BSP proteins have not been identified, it has been shown
261 that BSP A1/A2 stimulates sperm motility and membrane-bound calcium ATPase activity in
262 bovine sperm (Sanchez-Luengo et al., 2004). The functional connection between binding of BSP
263 proteins to the midpiece and stimulation of mitochondrial activity and sperm motility suggests

multifaceted role for BSP proteins as sperm are prepared for fertilization. Although it has not been tested, BSP proteins may influence hyperactivation of sperm as well as sperm capacitation.

In the present study we observed an unexplained increase in fluorescence associated with BSP A1/A2 binding to the midpiece of sperm 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 proteins are expressed in either isthmic and ampullary ODF. On the other hand, there were reductions in BSP A1/A2 fluorescence of the acrosome and increases in the post-equatorial segment of non-acrosome intact sperm 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 protein binding to sperm exposed to ODF is that components of oviduct fluid potentiate membrane remodeling and clustering of BSP proteins 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 poteins.

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 obviouslyy 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 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 equaorial/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 Figure 9. In addition to sperm binding, osteopontin also has the ability to interact with the zona pellucida and oolema of bovine oocytes (Figure 10). In light of these findings, we propose that OPN adheres to sperm and such sperm-

310 OPN complex binds to the zona pellucida. Another possibility is that sperm-OPN connects to the
311 OPN already bound to the zona because osteopontin is capable of forming bonds with other OPN
312 molecules with high affinity (Kaartinen et al., 1999; Goldsmith et al., 2002). The oviductal fluid
313 has osteopontin (Gabler et al., 2003), which may attach to the zona before spermatozoa even
314 reach the site of fertilization. After entering the periviteline space, OPN attached to the post-
315 equatorial segment would mediate the interaction of sperm with the oolema, also through
316 integrins and/or CD44. Our hypothesis that osteopontin binds to sperm and oocytes through
317 integrins and CD44 is supported by observations that a_v and a_5 integrins have been identified in
318 the bovine (Erikson et al., 2003) and human spermatozoa (Fusi et al., 1996; Reddy et al., 2003),
319 as well as on the oolema (D'Cruz, 1996). CD44 transmembrane glycoproteins are also present in
320 bovine sperm (Bains et al., 2002) and oocyte (Schoenfelder and Einspanier, 2003) and
321 osteopontin has been reproted to interact with integrins and CD44 in several other cell types
322 (Mazalli et al., 2002; Rangaswami et al., 2006).
323 The importance of OPN in reproduction is also demonstrated by experiments using in vitro
324 fertilization. Addition of OPN antibodies to fertilization media reduced both the number of
325 sperm bond to oocytes and the percentage of fertilized bovine oocytes (88.64 ± 3.0 % vs 28.7 ±
326 3.2 %; Goncalves et al., 2006a). Treatment of eggs with non-luteal oviductal fluid and anti-OPN
327 also diminished the percentage of blastocysts on day 8 (from 22 ± 1 % to 10.5 ± 0.5 %) and
328 hatched blastocysts on day 11 (from 8 ± 1 to 3 ± 0.5 %; Goncalves and Killian, 2004). The RGD
329 amino acid sequence in the OPN molecule mediates its link with a5 and av integrins (Denhardt
330 et al., 2001; Wai and Kuo, 2004) and the ability of osteopontin to support cell adhesion is
331 prevented when the RGD sequence is mutated (Liaw et al., 1995; Xuan et al., 1995). Treatment
332 of sperm or oocytes with an RGD peptide, but not with an RGE sequence, or with a5 and av

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 (Goncalves 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 ± 1.3 to 85.8 ± 1.4 %), blastocyst development at day 8 (from 24.2 ± 1.2 to 33.8 ± 1.4 %) and hatched blastocysts at day 11 (from 10.6 ± 1.6 to 18.5 ± 1.4 %; Goncalves 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 caused greater in vitro fertilization rates (85 to 78 ± 4 % versus 75 to 69 ± 4 %) and blastocyst development at day 8 (45 ± 2.9 to 37 ± 1.6 % versus 33 ± 2.3 to 29 ± 2.8 %) in comparison with untreated semen (Goncalves 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, incluing activation of Map kinases, phosphoinositide (PI) 3-kinase/Akt-dependent NFkB, IKK/ERK-mediated pathways, which stimulates uPA-dependent MMP-9 activation, PLC-g/ PKC/PI 3-kinase pathways (Wai and Kuo, 1998; Rasgawami et al., 2006; Chakraborty et al., 2006; Khodavirdi et al., 2006; Lee et al., 2007). It has been well documented that numerous intracellular signals are activated in the egg (such as those involving Src kinases and PKC) once 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; 2002a,b). Our hypothesis, as summarized in Figure 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-fertilizatin 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., 200a,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 proteins to sperm modulate 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.

References

Aumuller, G., Vesper, M., Seitz, J., Kemme, M., Scheit, K.H. 1988. Binding of a major secretory protein from bull seminal vesicles to bovine spermatozoa. Cell Tissue Res. 252, 377-84.

Bains, R., Adeghe, J., Carson, J. 2002. Human sperm cells express CD44. Fert. Steri. 78, 307-312.

Bavister, B.D., Leibfried, M.L., Lieberman, G. 1993. Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol. Reprod. 28, 235-247.

Cancel, A.M., Chapman, D.A., Killian, G.J. 1997. Osteopontin is the 55-kilodalton fertility-associated protein in Holstein bull seminal plasma. Biol. Reprod. 57, 1293-1301.

Chakraborty, G., Jain, S., Behera, R., Ahmed, M., Sharma, P., Kumar, V., Kundu, G.C. 2006. The multifaceted roles of osteopontin in cell signaling, tumor progression and angiogenesis. Curr Mol Med. 6, 819-30.

D'Cruz, O.J. 1996. Adhesion molecules in human sperm-oocyte interaction: relevance to infertility. Front. Biosc, 1:161-176

Denhardt, D.T. 2004. The third international conference on osteopontin and related proteins, San Antonio, Texas, May 10-12, 2002. Calcif. Tiss. Int. 74, 213-219.

Denhardt, D.T., Giachelli, C.M., Rittling, S. 2001. Role of osteopontin in cellular signaling and toxicant injury. Annu. Rev. Pharmacol. Toxicol. 41, 723-749.

Eliyahu, E., Kaplan-Kraicer, R., Shalgi, R. 2001. PKC in eggs and embryos. Front. Biosci. 6, 785-791.

Eliyahu, E., Shalgi, R. 2002a. A Role for Protein Kinase C During Rat Egg Activation. Biol. Reprod. 67, 189–195.

394 Eliyahu, E., Talmor-Cohen, A., Ruth Shalgi, R. 2002b. Signaling through protein kinases during
395 egg activation. J. Reprod. Immunol. 53, 161–169.
396 Erikson, D., Chapman, D., Ealy, A., Killian, G. J. 2003. Immunodetection of osteopontin on
397 Holstein bull sperm and av and a5 integrins on bovine oocytes. Biol. Reprod. 68(Suppl.
398 1):575 (abstract).
399 Franzen, A., Heinegard, D. 1985. Isolation and characterization of two sialoproteins present only
400 in bovine calcified matrix. Biochem. J. 232, 715-724.
401 Fusi, F.M., Tamburini, C., Mangili, F., Montesano, M., Ferrari, A., Bronson, R. 1996. The
402 expression of alpha v, alpha 5, beta 1 and beta 3 integrin chains on ejaculated human
403 spermatozoa varies with their functional state. Mol. Hum. Reprod. 2, 169-175.
404 Gabler, C., Chapman, D.A., Killian, G.J. 2003. Expression and presence of osteopontin and
405 integrins in the bovine oviduct during the estrous cycle. Reproduction 126, 721-729.
406 Gaddum-Rosse, P. 1985. Mammalian gamete interactions: what can be gained from observations
407 on living eggs? Am. J. Anat. 174, 347–356.
408 Go, K.J., Wolf, D.P. 1985. Albumin-mediated changes in sperm sterol content during
409 capacitation. Biol. Reprod. 32, 145–153.
410 Goldsmith, H.L., Labrosse, J.M., McIntosh, F.A., Maenpaa, P.H., Kaartinen, M.X., McKee,
411 M.D. 2002. Homotypic interactions of soluble and immobilized osteopontin. Ann. Biomed.
412 Eng. 30, 840-850.
413 Goncalves, R., Chapman, D.A., Killian, G.J. 2003. Effect of osteopontin on in vitro bovine
414 embryo development. Biol. Reprod. 68, Suppl. 1 (abstract 545).

Gonçalves, R.F., Bertolla, R.P., Eder, I., Chapman, D.A., Killian, G.J. 2006b. Effect of frozen semen with osteopontin on in vitro bovine fertilization and embryo development. Reprod. Fert. Dev. 19, 263 – 264.

Goncalves, R.F., Wolinetz, C.D., Killian G.J. 2006a. Influence of arginine-glycine-aspartic acid (rgd), integrins (a_V and a_5) and osteopontin on bovine sperm-egg binding, and fertilization in vitro. Theriogenology 2006 Oct 6; [Epub ahead of print]

Guillaume Halet, G. 2004. PKC signaling at fertilization in mammalian eggs. Biochimica et Biophysica Acta 1742, 185– 189.

Gwathmey, T.M., Ignotz, G.G., Mueller, J.L., Manjunath, P., Suarez, S. 2006. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. Biol. Reprod. 75, 501-507.

Gwathmey, T.M., Ignotz, G.G., Suarez, S. 2003. PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium in vitro and may be involved in forming the oviductal sperm reservoir. Biol. Reprod. 69, 809–815.

Hao, Y., Mathialagan, N., Walters, E., Mao, J., Lai, L., Becker, D., Li, D., Critser, J., Prather, R.S. 2006. Osteopontin reduces polyspermy during in vitro fertilization of porcine oocytes. Biol. Reprod. 75, 726–733.

Hasler, J.F., Henderson, W.B., Hurtgen, P.J., Jin, Z.Q., McCauley, A.D., Mower, S.A., Neely, B., Shuey, L.S., Stokes, J.E., Trimmer, S.A. 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 43, 141-152.

Hong Wu, H., Colin Pritchard, C., Peter S. Nelson, P.S., and Pradip Roy-Burman, P. 2006. Increased Expression of Osteopontin Contributes to the Progression of Prostate Cancer. Cancer Res. 66, 883-888.

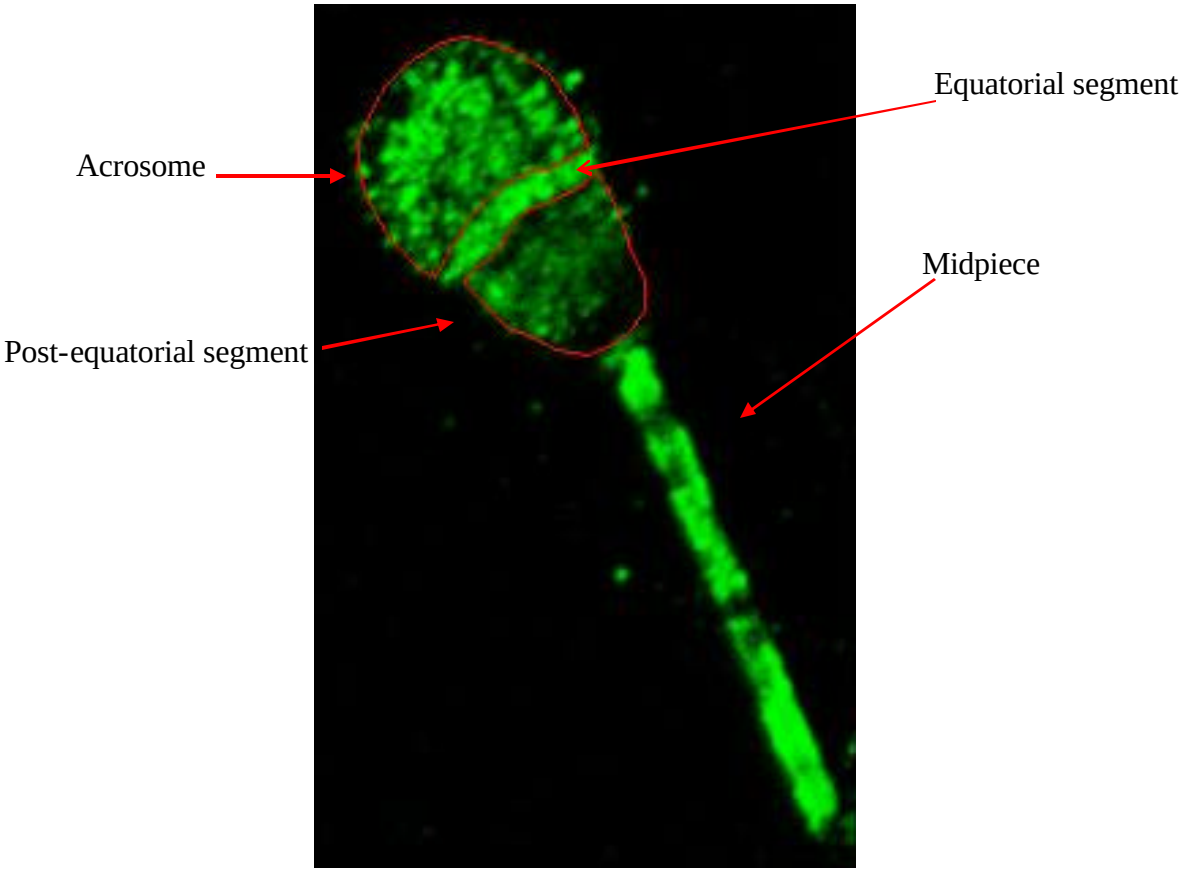
438 Johnson, G.A., Burghardt, R.C., Bazer, F.B., Spencer, T.E. 2003. Osteopontin: roles in
439 implantation and placentation. Biol. Reprod. 69, 1458-1471.
440 Kaartinen, M.T., Pirhonen, A., Linnala-Kankkunen, A., Mäenpää, P.H. 1999. Cross-linking of
441 osteopontin by tissue transglutaminase increases its collagen binding properties. J. Biol.
442 Chem. 274, 1729-1735.
443 Kavanaugh, J.F., Grippo, A.A., Killian, G.J. 1992. Cannulation of the bovine ampullary and
444 isthmic oviduct J. Invest. Surg. 5, 11–17.
445 Khodavirdi, A.C., Song, Z., Yang, S., Zhong, C., Wang, S., Wu, H., Pritchard, C., Nelson, P.S.,
446 Roy-Burman, P. 2006. Increased expression of osteopontin contributes to the progression of
447 prostate cancer. Cancer Res. 66, 883-888.
448 Killian, G.J. 2004. Evidence for the role of oviduct secretions in sperm function, fertilization and
449 embryo development. Anim. Reprod. Sci. 82-83, 141-53.
450 Killian, G.J., Chapman, D.A., Rogowski, L.A. 1993. Fertility-associated proteins in bull seminal
451 plasma. Biol. Reprod. 49, 1202-1207.
452 Lee, J-L., Wang, M-J., Sudhir, P-R., Chen, G-D., Chi, C-W., Chen, J-Y.1. 2007. Osteopontin
453 Promotes Integrin Activation through Outside-In and Inside-Out Mechanisms: OPN-CD44V
454 Interaction Enhances Survival in Gastrointestinal Cancer Cells. Cancer Res. 67, 2089-2097.
455 Liaw, L., Birk, D.E., Ballas, C.B., Whitsitt, J.S., Davidson, J.M., Hogan, B.L.M. 1998. Altered
456 wound healing in mice lacking a functional osteopontin gene (spp1). J. Clin. Invest. 101,
457 1468-1478.
458 Liaw, L., Lindner, V., Schwartz, S.M., Chambers, A.F., Giachelli, C.M. 1995. Osteopontin and ß3
459 integrin are coordinately expressed in regenerating endothelium in vivo and stimulate arg-gly-
460 asp–dependent endothelial migration in vitro. Circulation Research 77, 665-672.

461 Lynda K. McGinnis, L.K., David F. Albertini, D.F., William H. Kinsey, W.H. 2007. Localized
462 activation of Src-family protein kinases in the mouse egg. Dev. Biol. 306, 241–254.
463 Manjunath, P., Chandonnet, L., Leblond, E., Desnoyers, L. 1993. Major proteins of bovine
464 seminal vesicles bind to spermatozoa. Biol. Reprod. 49, 27-37.
465 Manjunath, P., Thérien, I. 2002. Role of seminal plasma phospholipid-binding proteins in sperm
466 membrane lipid modification that occurs during capacitation. J. Reprod. Immunol. 53, 109-
467 119.
468 Mazzali, M., Kipari, T., Ophascharoensuk, V., Wesson, J.A., Johnson, R., Hughes, J. 2002.
469 Osteopontin: a molecule for all seasons. Q. J. Med. 95, 3-13.
470 Moura, A.A. 2005. Seminal plasma proteins and fertility indexes in the bull: the case for
471 osteopontin. Anim. Reprod. 2, 3-10.
472 Moura, A.A., Chapman, D.A., Killian, G.J. 2006b. Proteins of the accessory sex glands
473 associated with the oocyte-penetrating capacity of cauda epididymal sperm from Holstein
474 bulls of documented fertility. Mol. Reprod. Dev. 74(2): 214 - 222.
475 Moura, A.A., Koc, H., Chapman, D.A., Killian, G.J. 2006a. Identification of accessory sex gland
476 fluid proteins as related to fertility indexes of dairy bulls: a proteomic approach. J. Androl.
477 27, 201-211.
478 Moura, A.A., Koc, H., Chapman, D.A., Killian, G.J. 2006a. Identification of accessory sex gland
479 fluid proteins as related to fertility indexes of dairy bulls: a proteomic approach. J. Androl.
480 27, 201-211.
481 Moura, A.A., Koc, H., Chapman, D.A., Killian, G.J. 20076c. A comprehensive proteomic
482 analysis of the accessory sex gland fluid of mature Holstein bulls. Anim. Reprod. Sci. 98(3-
483 4): 169-188, 2007.

484 Parrish, J.J., Susko-Parrish, J., Winer, M.A., First, N.L. 1988. Capacitation of bovine sperm by
485 heparin. Biol. Reprod. 38, 1171-80.
486 Rangaswami, H., Bulbule, A., Kundu, G.C. 2006. Osteopontin: role in cell signalling and cancer
487 progression. Trends Cell Biol. 16, 79-87.
488 Reddy, V.R.K., Rajeev, S., Gupta, V. 2003. $a_6ß_1$ integrin is a potential clinical marker for
489 evaluating sperm quality in men. Fert. Steril. 79, 1590-1596.
490 Sanchez-Luengo, S., Aumuller, G., Albrecht, M., Sen, P.C., Rohm, K., Wilhelm, B. 2004.
491 Interaction of PDC-109, the major secretory protein from bull seminal vesicles, with bovine
492 sperm membrane Ca2+-ATPase. J. Androl. 25, 234-44.
493 SAS Institute Inc., SAS/STAT® User's Guide, Version 6, Fourth Edition, Volume 2, Cary, NC:
494 SAS Institute Inc., 2003.
495 Schoenfelder, M., Einspanier, R. 2003. Expression of hyaluronan synthases and corresponding
496 hyaluronan receptors is differentially regulated during oocyte maturation in cattle. Biol.
497 Reprod. 69, 269–277.
498 Singleton, C.L., Killian, G.J. 1983. A study of phospholipase in albumin and its role in inducing
499 the acrosome reaction of guinea pig spermatozoa in vitro. J Androl. 4, 150-156.
500 Talmor-Cohen, A., Tomashov-Matar, R., Eliyahu, E., Shapiro, R., Shalgi, R. 2004. Are Src
501 family kinases involved in cell cycle resumption in rat eggs? Reproducion 127, 455-463.
502 Therien, I., Soubeyrand, S., Manjunath, P. 1997. Major proteins of bovine seminal plasma
503 modulate sperm capacitation by high-density lipoprotein. Biol. Reprod. 57, 1080-1088.
504 Visconti, P.E., Kopf, G.S. 1998. Regulation of protein phosphorylation during sperm
505 capacitation. Biol. Reprod. 59, 1–6.
506 Wai, P., Kuo, P.C. 2004. The role of osteopontin in tumor metastasis. J. Surg. Res. 121, 228-241.

507 Xuan, J., Hota, C., Shigeyama, Y., D'Errico, J.A., Somerman, M.J., Chambers, A.F. 1995. Site-
508 directed mutagenesis of the arginine-glycine-aspartic acid sequence in osteopontin destroys
509 cell adhesion and migration functions. J. Cell Biochem. 57, 680-690.

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



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

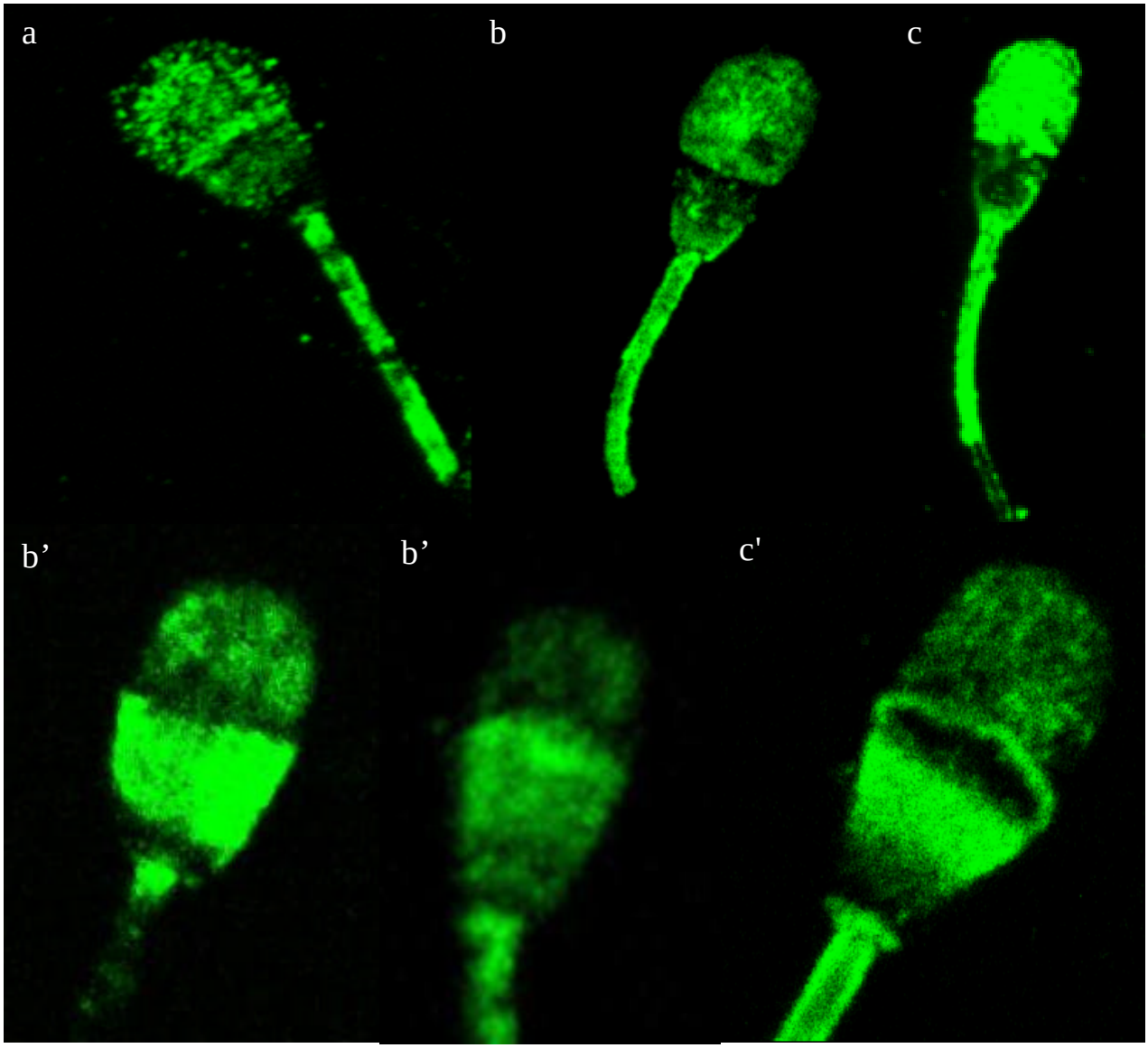
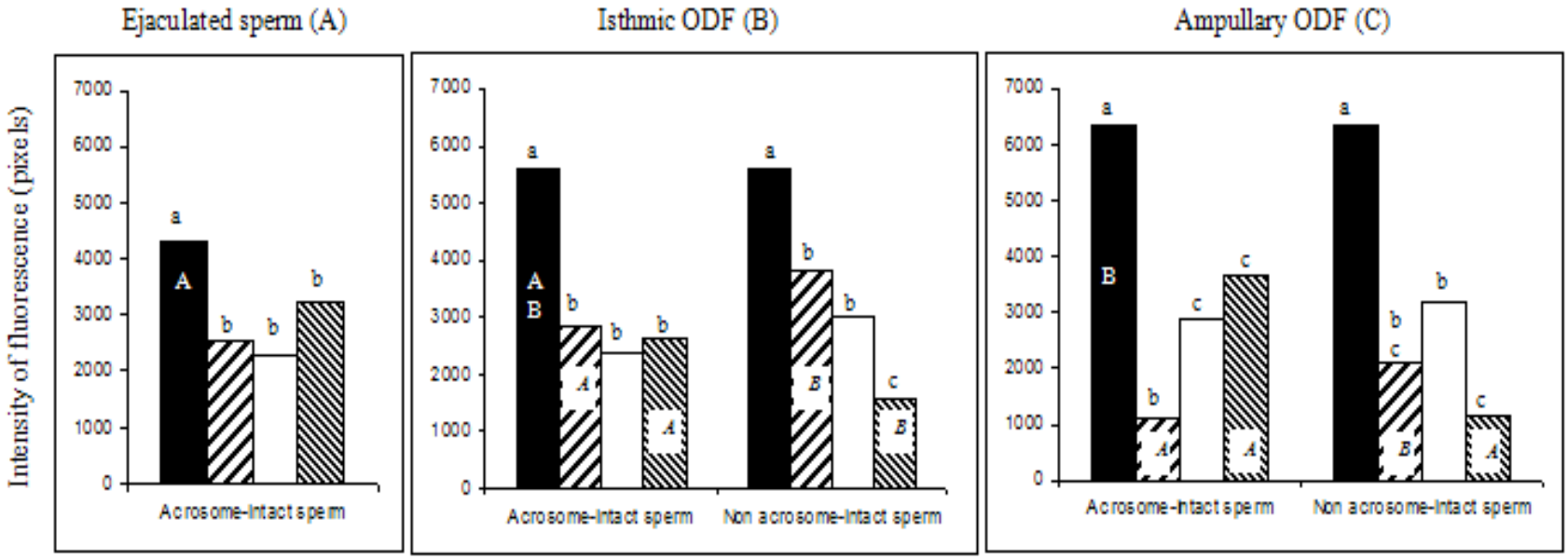


Figure 3. Quantitative BSP A1/A2 binding to midpiece (■), post-equatorial (◪), equatorial (□) and acrosomal (▩) regions of ejaculated sperm (A) and of sperm incubated with non-luteal isthmic (B) and ampullary (C) oviductal fluid (ODF). In panel A, fluorescence is shown for sperm with intact acrosome 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.



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.

Figure 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™ and AutoDeblur softwares.

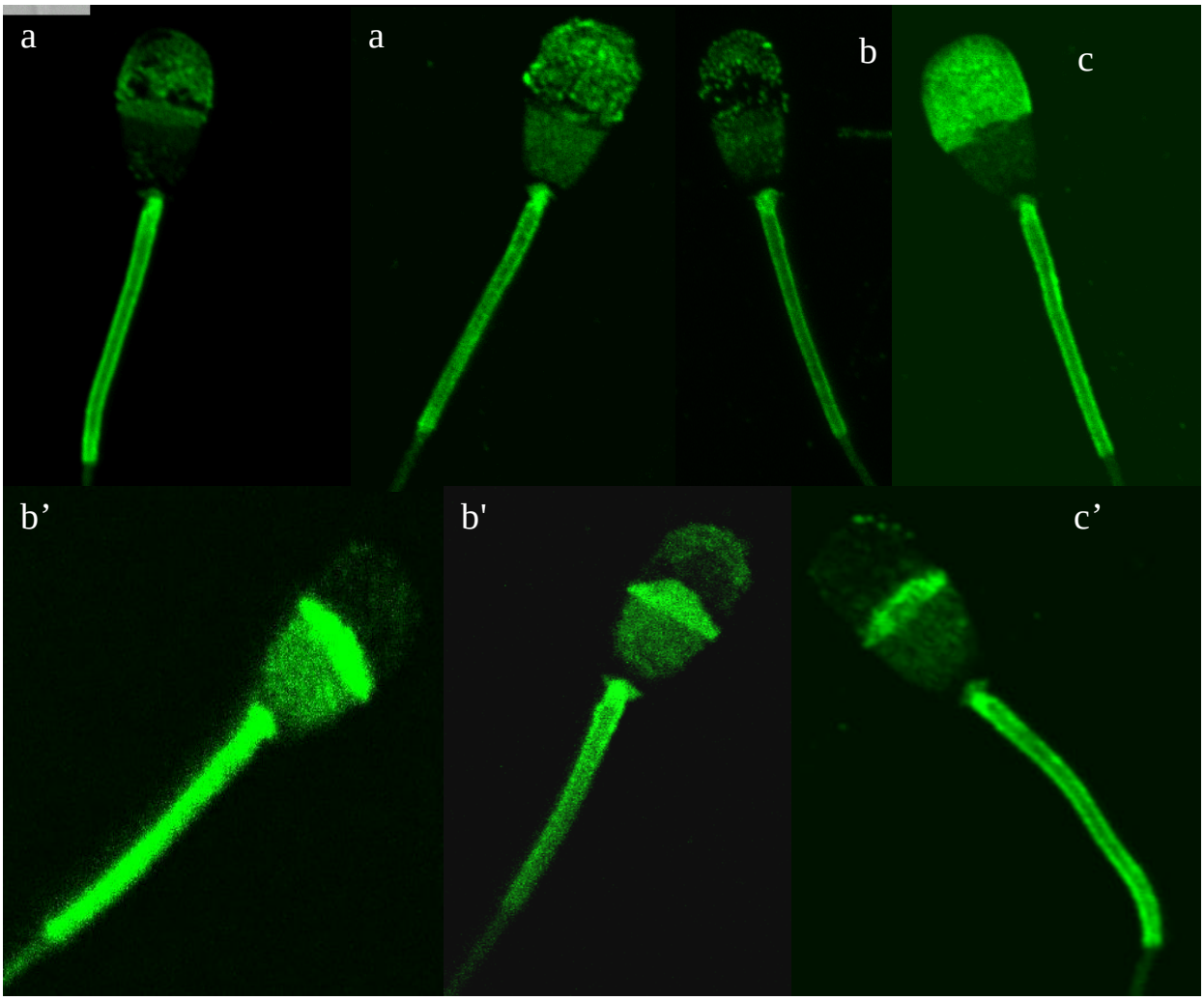
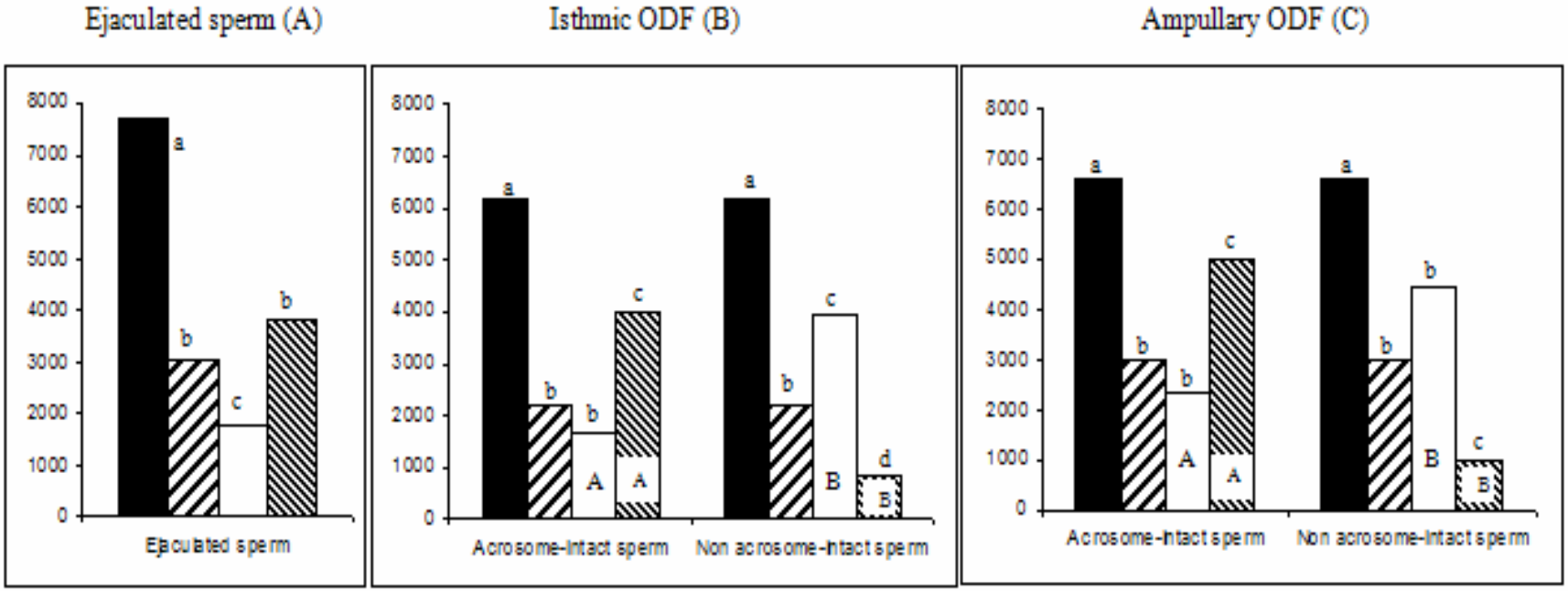


Figure 5. Quantitative BSP 30 kDa binding to midpiece (■), post-equatorial (◪), equatorial (□) and acrosomal (▨) regions of ejaculated sperm (A) and of sperm incubated with non-luteal isthmic (B) and ampullary (C) oviductal fluid. In panel A, fluorescence is shown for sperm with intact acrosome only. Within panels B and C, anti-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.



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 acrsome intact and non-acrsosome intact

Figure 6. Patterns of osteopontin binding to ejaculated sperm (a) and to sperm with intact acrosome 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^{TM} and AutoDeblur softwares.

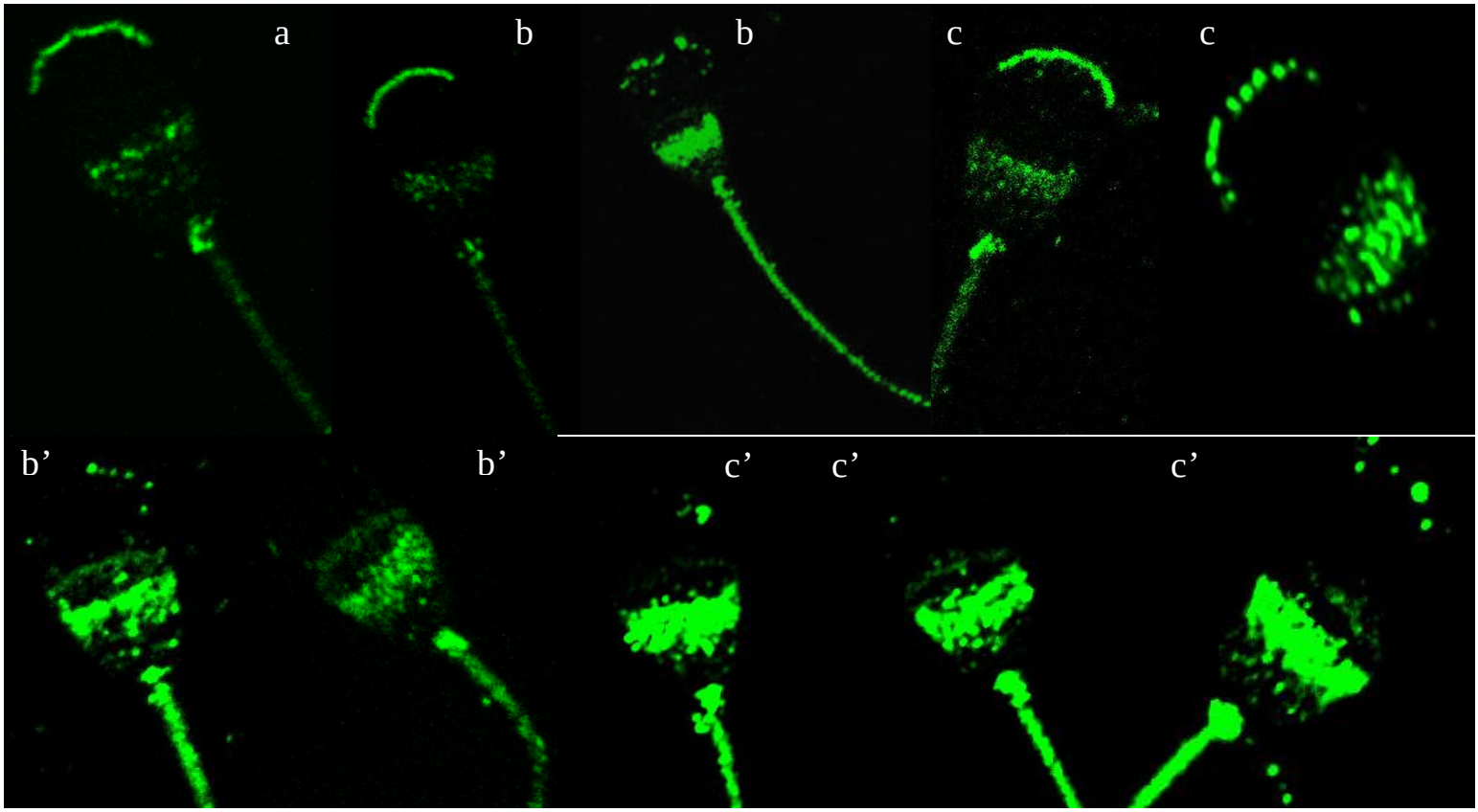
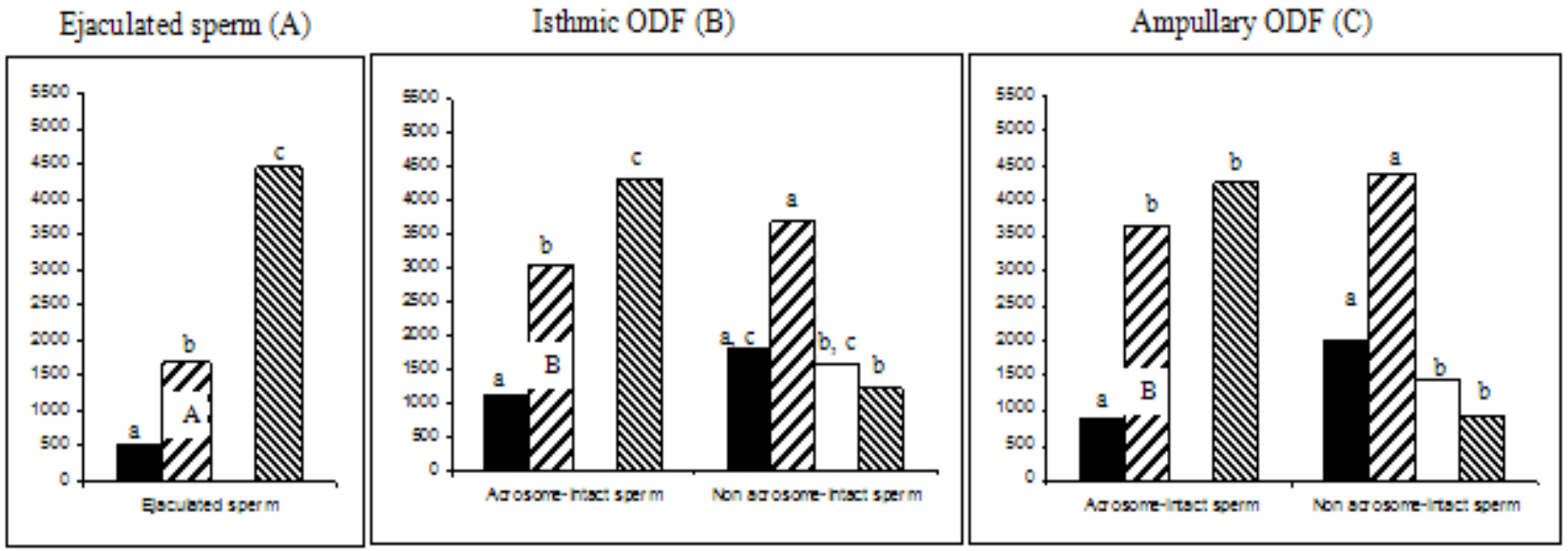


Figure 7. Quantitative osteopontin binding to midpiece (■), post-equatorial (◪), equatorial (□) and acrosomal (▩) regions of ejaculated sperm (A) and of sperm incubated with non-luteal isthmic (B) and ampullary (C) oviductal fluid. In panel A, fluorescence is shown for sperm with intact acrosome only. 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.

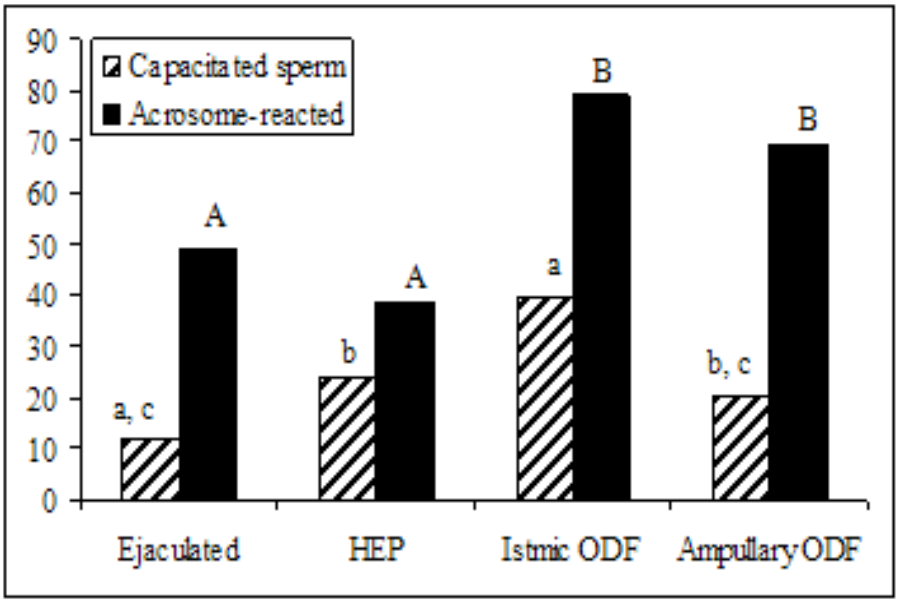


Lower cases represent significant differences ($p < 0.05$) among regions of acrosome intact or non acrosome intact sperm, within each treatment (A, B and C).

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

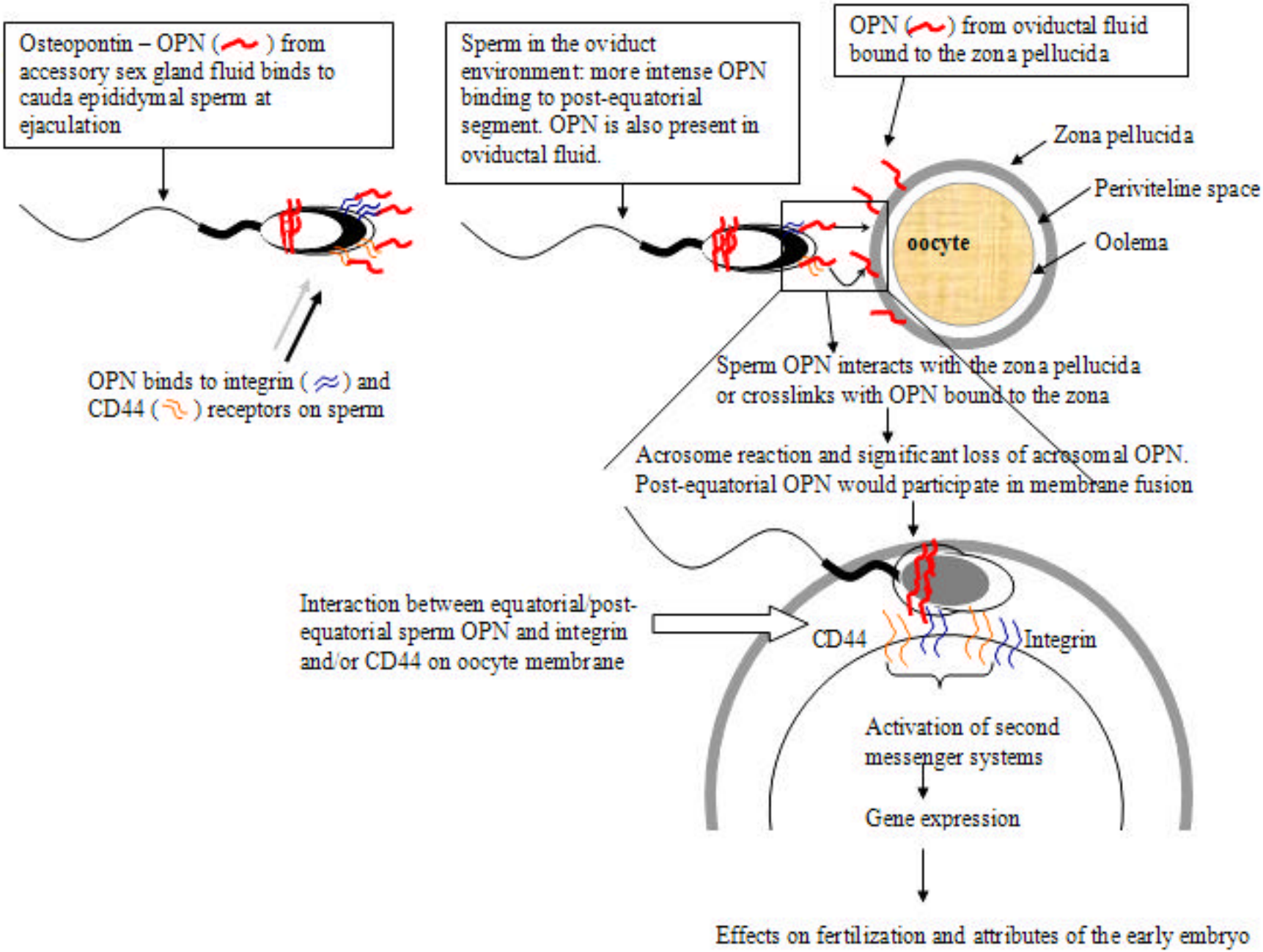
543 Figure 8. Percentage of ejaculated spermatozoa undergoing capacitation and acrosome reaction

544 after incubation with heparin and non-luteal isthmic and ampullary oviductal fluid.



Letters represent significant differences ($p < 0.05$) among the percentages of capacitated (lower cases) and acrosome reacted (capital letters) sperm across treatments.

Figure 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 once in the periviteline space, with the oocyte membrane. This last phase may also involve ONP 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 ONP 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.


Osteopontin – OPN ( ) from accessory sex gland fluid binds to cauda epididymal sperm at ejaculation
OPN binds to integrin ( ) and CD44 ( ) receptors on sperm
Sperm in the oviduct environment: more intense OPN binding to post-equatorial segment. OPN is also present in oviductal fluid.
OPN ( ) from oviductal fluid bound to the zona pellucida
Zona pellucida
Periviteline space
Oolema
oocyte
Sperm OPN interacts with the zona pellucida or crosslinks with OPN bound to the zona
Acrosome reaction and significant loss of acrosomal OPN. Post-equatorial OPN would participate in membrane fusion
Interaction between equatorial/post-equatorial sperm OPN and integrin and/or CD44 on oocyte membrane
CD44
Integrin
Activation of second messenger systems
Gene expression
Effects on fertilization and attributes of the early embryo

573 Figure 10. Immunofluorescence detection of osteopontin on bovine oocytes pre-incubated with
574 bovine oviductal fluid, which contains OPN (Gabler et al., 2003) . Oocytes were matured in
575 vitro, followed by removal of cumulus cells (Hasler et al., 1995; Goncalves et al., 2006a;
576 Bavister et al. 1983). After culture, eggs were washed twice in fresh TL-HEPES and incubated
577 with anti-bovine milk osteopontin (100 µg/ml) for 1 hour, at 39°C (5% CO2 in air), the same
578 antibody used for sperm immunocytochemistry. After washing twice with fresh TL-HEPES,
579 eggs were incubated for 30 minutes with goat F (ab)2 –anti rabbit IgG (H+L)-FITC (1:300;
580 Southern Biotech Associates, Birmingham, AL, USA) and washed twice again. Images were
581 analyzed under laser scanning confocal microscopy and FluoView^{TM} and AutoDeblur softwares,
582 as described for all studies with sperm cells.