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Influence of arginine-glycine-aspartic acid (RGD), integrins (α_V and α_5) and osteopontin on bovine sperm–egg binding, and fertilization in vitro

R.F. Gonçalves [a,b], C.D. Wolinetz [a,c], G.J. Killian [a,*]

[a] *The John O. Almquist Research Center, Department of Dairy and Animal Science, The Pennsylvania State University, University Park, PA, USA*
[b] *Department of Animal Reproduction, College of Veterinary Medicine and Animal Science, São Paulo University, Cidade Universitária, SP, Brazil*
[c] *Federation of American Societies for Experimental Biology, Bethesda, MD, USA*

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Abstract

Osteopontin (OPN), a phosphoprotein containing an arginine-glycine-aspartic acid (RGD) sequence, has been identified in cow oviduct epithelium and fluid. To investigate the potential role OPN in fertilization, we evaluated the ability of RGD peptide (arginine-glycine-aspartic), RGE peptide (arginine-glycine-glutamic acid), integrins α_V and α_5 antibodies and OPN antibody to influence bovine in vitro sperm–egg binding and fertilization. Treatment of sperm or oocytes with the RGD peptide prior fertilization significantly decreased in vitro sperm–egg binding and fertilization compared to the non-treated controls or those treated with RGE peptide. Binding and fertilization were also significantly decreased when in vitro matured bovine oocytes or sperm were pre-incubated with integrins α_V and α_5 antibodies at concentration ranging from 5 to 20 μg/mL. Addition of a rabbit polyclonal IgG antibody against purified bovine milk OPN with sperm or/and oocytes decreased ($P < 0.05$) fertilization compared to the in vitro-fertilized control. These data provided evidence that integrin ligands existed on bovine oocytes and spermatozoa that contained RGD recognition sequences, and that antibody to OPN, a protein that contains that RGD sequence, was capable of reducing sperm–egg binding and fertilization in vitro.

Keywords: Integrin; Osteopontin; Secreted phosphatase 1; Sperm–egg binding; Fertilization; Bovine

1. Introduction

Fertilization is the ultimate event in the series of cellular mechanisms that pass the genome from one generation to the next and initiate development of a new organism. In mammals, fertilization is initiated by the direct interaction of sperm and egg membranes, a process mediated primarily by gamete surface proteins. Therefore, an important task in the study of sperm–egg interaction is to evaluate the role in fertilization of gamete specific surface proteins and other surface proteins more widely expressed. On gametes, these proteins act in a sequential pattern to orchestrate the initial contact and ultimate fusion of the two cells.

Fertilization in mammals is mediated by a series of molecular interactions in which members of the

* Corresponding author. Tel.: +1 814 3290009; fax: +1 814 8630833.
E-mail address: gkillian@psu.edu (G.J. Killian).

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tetraspanin, integrin and ADAM (a disintegrin and metalloprotease) families have been suggested to play a role [1–5]. The importance of CD9 tetraspanin is now clearly established since homozygous null females exhibit severely reduced fertility [6,7]. Secreted phosphatase 1 (SPP1), commonly know as osteopontin (OPN), is a protein that has been identified in cow oviductal epithelium, and fluid [8], bull reproductive tract [9,10], and seminal plasma, where its presence has been correlated with bull fertility [11]. Notably, OPN contains the RGD (arginine-glycine-aspartic acid) peptide sequence. Studies have shown that RGD peptide is capable of blocking fertilization, inducing intracellular Ca^{2+} transients, and initiating parthenogenic development when present during bovine in vitro fertilization [12].

Integrins are a family of heterodimeric cell adhesion molecules that mediate cell–cell and cell–extracellular matrix interactions. To date, 18α subunits (120–180 kDa) and 8β subunits (90–110 kDa) have been identified; these combine to make 24 different integrins [13,14]. Both spermatozoa and oocytes express a number of integrins and molecules that contain integrin recognition sites. The mouse oocyte contains integrin subunits α_2, α_3, α_5, α_6, α_V, β_1, β_3 and β_5 as detected by mRNA, protein analysis, or both [13–16]. Similarly, human oocytes express integrin subunits α_2, α_3, α_5, α_6, α_V, α_M, β_1, β_2, β_3, β_4, β_5 and β_6 [17–19], and ejaculated spermatozoa express β_1 integrins [20].

Carbohydrate–carbohydrate or carbohydrate–protein interactions are also prevalent in events of fertilization. Sperm membrane proteins recognize and adhere to the sugars of the zona pellucida, essentially acting as a lectin-like molecule [21]. A seminal plasma protein bound to the sperm interacts with fucosyl residues of the oviductal epithelium [22]. The fusion of oolemma and sperm is thought to involve a protein–carbohydrate interface [23]. However, the carbohydrate–sperm protein interactions in the mouse as well as bovine systems appear to be complex, perhaps involving multiple and potentially redundant sugar receptors.

Although integrins are known to be associated with the plasma membrane of some mammalian oocytes and spermatozoa, the presence of these molecules on the surface of bovine oocytes and spermatozoa from bovine has not been reported. The present study was undertaken to determine if in vitro binding to the zona pellucida and fertilization were affected by treating sperm or oocytes with RGD peptide, integrins α_V and α_5 antibodies, and OPN antibody.

2. Materials and methods

2.1. Oocyte collection and maturation

Bovine ovaries were harvested at a local abbatoir and transported to the laboratory in Dulbecco's PBS (pH 7.4) without $CaCl_2$ (Gibco™, Invitrogen Corporation, Grand Island, NY, USA) at 25–30 °C. Once in the laboratory, ovaries were rinsed with 35–37 °C tap water and soaked for 15 min in a solution of 1% Chlorhexiderm (DVM Pharmaceuticals, Miami, FL, USA) and 0.625% 7× Cleaning Solution (ICN Biomedicals, Aurora, OH, USA) in 35–37 °C tap water [24]. After a final rinse in 35–37 °C tap water, ovaries were rinsed with Dulbecco's PBS. Follicles from 1 to 6 mm in diameter were aspirated using an 18 g needle (B-D®, Rutherford, NJ, USA) attached to a 10 cm^3 syringe (B-D®). The aspirate was poured into an Em-Con filter (Immuno Systems®, Spring Valley, WI) and rinsed with low bicarbonate HEPES [25]. The content of the filter was distributed among sterile petri dishes. Oocytes were selected based on the presence of at least one layer of intact cumulus cells for the sperm–oocyte binding experiments, and two or more intact cumulus cell layers for in vitro fertilization.

The cumulus–oocyte complexes (25/well) were matured in four-well culture plates (Nunclon™, Nunc, Roskilde, Denmark) in 500 μL TCM-199 (Gibco™), 10% fetal calf serum (Gibco™), LH (6 μg/mL), FSH (8 μg/mL) (Sioux Biochemical®, Sioux Center, IA, USA), and penicillin (100 units/mL)–streptomycin (100 μg/mL) (Gibco™) for 22 h at 39 °C, 5% CO_2 in air (v/v) [24]. After maturation, oocytes were prepared for sperm binding and fertilization experiments as described below.

2.2. Sperm preparation

Semen was collected from two Holstein bulls (*Bos taurus*) by artificial vagina, pooled and washed twice by centrifugation in 10 mL modified Tyrode's medium at $500 \times g$ for 10 min (MTM) [26]. Spermatozoa (5×10^7 mL) were incubated (39 °C, 5% CO_2 in air) for 2 h with the different treatments. After incubation, spermatozoa were separated from their incubation medium using Percoll (Sigma®, St. Louis, MO, USA) gradient centrifugation. Briefly, 2 mL of a 90% Percoll solution in MTM (v/v) were placed in the bottom of a 15 mL polypropylene tube and 2 mL of a 45% Percoll solution in MTM (v/v) were gently overlaid on top of it. For each treatment, 1 mL of sperm suspension was layered onto the 45% solution and centrifuged for

30 min at 700 × *g*. The pellet spermatozoa were recovered, assessed for motility, and suspended in fertilization medium [25].

2.3. Sperm–oocyte binding

In vitro matured oocytes were vortexed for 2 min to remove cumulus cells, washed twice in low bicarbonate HEPES medium and incubated (25/well) for 2 h in four-well culture plates containing different treatments. Oocytes were denuded before fertilization in order to be able to quantitate sperm bound to the zona pellucidae (ZP) [27]. Oocytes were incubated with 6.25×10^4 spermatozoa from the different treatments described below. Oocytes and spermatozoa were co-incubated for 18 h at 39 °C, 5% CO_2 in air (v/v). After co-incubation oocytes were washed once in HEPES and placed, 10 per slide, under a coverslip mounted with paraffin wax petroleum jelly at each corner. The coverslip was lowered over the oocytes until they burst, and the cytoplasm was rinsed away with HEPES. The ZP and any spermatozoa bound to them were stained with Hoechst fluorescent dye 33342 (Sigma®). The number of spermatozoa bound to each ZP was determined using fluorescence microscopy [27]. Each experiment involving binding studies was repeated four times with 40–50 oocytes per treatment.

2.4. In vitro fertilization

In vitro matured oocytes were washed twice in HEPES medium, placed in fertilization medium with 2 μg heparin and incubated as described above. After 18 h of co-incubation oocytes were vortexed to remove cumulus cells and accessory spermatozoa, and washed twice in HEPES medium [24]. The oocytes were placed 10 per slide under a coverslip mounted at the corners with paraffin wax and petroleum jelly. The coverslip was gently lowered over the oocytes and adhered to the slide with rubber cement. Oocytes were fixed in acid alcohol for 24 h and stained with acetato-orcein [26]. The presence of two pronuclei in the cytoplasm of the oocyte indicated normal fertilization [26]. Each experiment involving in vitro fertilization was repeated four times with 80–90 oocytes per treatment.

2.5. Treatments

2.5.1. RGD and RGE-containing peptides

This study was conducted to determine whether in vitro sperm binding to the ZP and fertilization of bovine oocytes were affected by treating the sperm or oocytes with RGD peptide (arginine-glycine-aspartic acid, a sequence recognized by integrins) or RGE peptide (arginine-glycine-glutamic acid) containing peptides.

In vitro matured oocytes were incubated for 2 h in fertilization medium [18] containing: (1) no peptides (control); (2) 50 μg/mL RGD (GRGDSP sequence; Sigma Genosys®, The Woodlands, TX, USA); (3) 1000 μg/mL RGD; (4) 50 μg/mL RGE (GRGESP sequence; Sigma Genosys®); (5) 1000 μg/mL RGE.

The bovine sperm were incubated for 2 h (concentration, 5×10^7) in MTM with: (6) no peptides (control); (7) 50 μg/mL RGD; (8) 1000 μg/mL RGD; (9) 50 μg/mL RGE; or (10) 1000 μg/mL RGE. Following incubation, treated and untreated oocytes were washed twice in HEPES medium and incubated with 1×10^5 treated or untreated fresh spermatozoa per 10 oocytes. Treated and untreated fresh sperm were recovered from a Percoll gradient (45%/90%) and incubated with treated or untreated oocytes.

2.5.2. Integrins α_V and α_5 antibodies

This study was conduct to determine whether in vitro sperm binding to the ZP of intact oocytes and fertilization were affected by treating sperm or oocytes with integrins α_V and α_5 antibodies. In vitro matured oocytes were incubated (39 °C, 5% CO_2, in air) for 2 h in fertilization medium with: (1) no antibodies; (2) 5 μg/mL α_V antibody (Calbiochem®, San Diego, CA, USA); (3) 20 μg/mL α_V antibody; (4) 5 μg/mL α_5 antibody (Calbiochem®); or (5) 20 μg/mL α_5 antibody. Semen from two different bulls was collected by artificial vagina, pooled, centrifuged at 500 × *g*, 10 min and the pellet washed twice with MTM. Washed sperm were incubated for 2 h in MTM (concentration, 5×10^7) with: (6) no antibodies; (7) 5 μg/mL α_V antibody; (8) 20 μg/mL α_V antibody; (9) 5 μg/mL α_5 antibody; (10) 20 μg/mL α_5 antibody. Following incubation, treated and untreated oocytes were washed twice in HEPES medium and incubated with 1×10^5 treated or untreated fresh spermatozoa per 10 oocytes.

2.5.3. Osteopontin (OPN) antibody

This study was conduct to determine whether in vitro fertilization was affected by pretreating either the sperm or/and oocytes with OPN antibody (the IgG fraction of the serum). A study using rabbit IgG antibody prepared against a non-bovine antigen was performed to assess the effect of non-specific IgG. The IgG used in this experiment was prepared as stated by [28], and was specific for bacterial histidase (anti-hist). In vitro matured oocytes were incubated (39 °C, 5% CO_2, in air) for 2 h in fertilization medium with: (a) no antibody; (b) a rabbit

polyclonal antibody IgG against purified bovine milk OPN (from our laboratory; α-OPN); or (c) anti-hist. The bovine sperm from two different bulls was incubated for 2 h at 5×10^7 concentration in MTM with: (d) no antibody; (e) a rabbit polyclonal antibody IgG against purified bovine milk OPN (α-OPN; 1:200); or (f) anti-hist (1:2000). For this experiment, we had six discrete treatments: (1) no antibody (control); (2) α-OPN at fertilization time; (3) α-OPN treated oocytes; (4) α-OPN treated sperm; (5) anti-hist treated oocytes; or (6) anti-hist treated sperm. Following incubation, treated and untreated oocytes were washed twice in HEPES medium and incubated with 1×10^5 treated or untreated fresh spermatozoa per 10 oocytes.

2.6. Statistical analysis

Each experiment was repeated four times and data from each experiment were polled. Approximately 40–50 oocytes per treatment for sperm binding, and 80–90 oocytes per treatment for fertilization were evaluated in each replicate. Analysis of variance using a general linear model was performed using mean number of spermatozoa bound per ZP for each treatment in the sperm oocyte binding experiments, and a weighted mean based on the number of oocytes per treatment in the fertilization. Least square means and Bonferroni means comparisons were used to assess sperm binding. Weighted least square means were used to analyze fertilization data (SAS) [29]. The significance level for all tests was $P < 0.05$.

3. Results

3.1. RGD and RGE containing peptides

More sperm bound to the ZP of untreated or RGE treated oocytes than those incubated with the RGD peptide (mean ± S.E.M., Fig. 1a): 71.2 ± 4.1 and 71.9 ± 4.2 (control with oocytes or sperm); 33.2 ± 4.2 and 29.8 ± 4.2 (50 μg/mL RGD); 24.2 ± 4.1 and 19.8 ± 4.2 (1000 μg/mL RGD); 69.5 ± 4.1 and 68.9 ± 4.2 (50 μg/mL RGE); 70.2 ± 4.2 and 70.6 ± 4.2 (1000 μg/mL RGE). Treatment of oocytes or sperm with the RGD peptide decreased ($P < 0.05$) fertilization compared to the untreated controls or those treated with RGE peptide (Fig. 1b): 80 ± 3.0% and 78.9 ± 3.0% (control with oocytes or sperm); 42 ± 3.0% and 41.3 ± 3.0% (50 μg/mL RGD); 30.2 ± 3.0% and 29.1 ± 3.0% (1000 μg/mL RGD); 78.5 ± 3.0% and 79.2 ± 3.0% (50 μg/mL RGE); 79.1 ± 3.0% and 80.2 ± 3.0% (1000 μg/mL RGE).

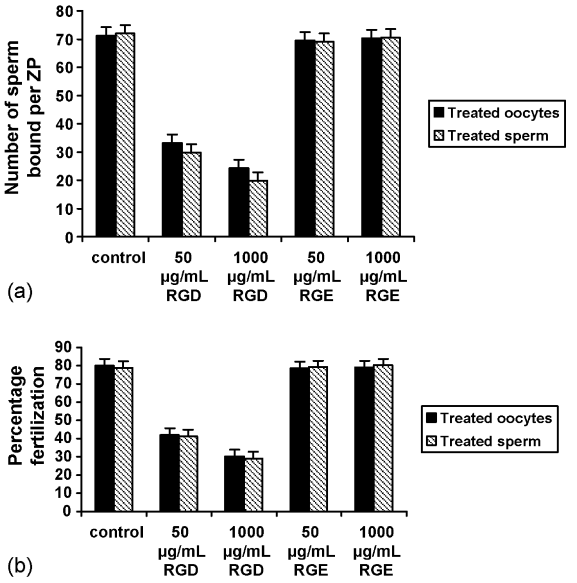

Fig. 1. (a) Mean (±S.E.M.) number of sperm bound per zona pellucida (ZP); (b) mean percentage of fertilized eggs following exposure of oocytes or sperm to the treatments: no peptide (control), 50 μg/mL RGD, 1000 μg/mL RGD, 50 μg/mL RGE, 1000 μg/mL RGE. Untreated control and RGE peptide oocytes and sperm were greater than the other treatments ($P < 0.005$). There were no difference among the 50 μg/mL RGD, 1000 μg/mL RGD, 50 μg/mL RGE, and 1000 μg/mL RGE with oocytes or sperm. The study was repeated four times using 40–50 (sperm–egg binding) and 80–90 (fertilization) oocytes per treatment.

3.2. Integrins α_V and α_5 antibodies

More sperm bound to the ZP when the oocytes or sperm were untreated than those incubated with α_V and α_5 (Fig. 2a): control (oocytes or sperm; 73.2 ± 2.5 and 72 ± 2.7); 5 μg/mL α_V antibody (36 ± 2.7 and 35 ± 2.1); 20 μg/mL α_V antibody (33 ± 2.4 and 33 ± 2); 5 μg/mL α_5 antibody (43 ± 2.4 and 42 ± 1.4); 20 μg/mL α_5 antibody (40 ± 2.2 and 39 ± 1.8). Treatment of sperm or oocytes with either the α_V and α_5 antibodies decreased fertilization compared to the untreated control (Fig. 2b): 82 ± 2% and 81 ± 1.5% (control; oocytes and sperm); 41 ± 2.1% and 43 ± 2.2% (5 μg/mL α_V antibody); 37 ± 2.3% and 39 ± 2.3% (20 μg/mL α_V antibody); 48 ± 2.2% and 49 ± 2.8% (5 μg/mL α_5 antibody); 44 ± 2% and 46 ± 2.1% (20 μg/mL α_5 antibody).

3.3. Osteopontin (OPN) antibody

Addition of a rabbit polyclonal IgG antibody against purified bovine milk OPN with sperm or/and oocytes decreased fertilization compared to the in vitro-fertilized control (Fig. 3) as follows: 88.64 ± 3.0% (no antibody); 28.7 ± 3.2% (α-OPN at fertilization

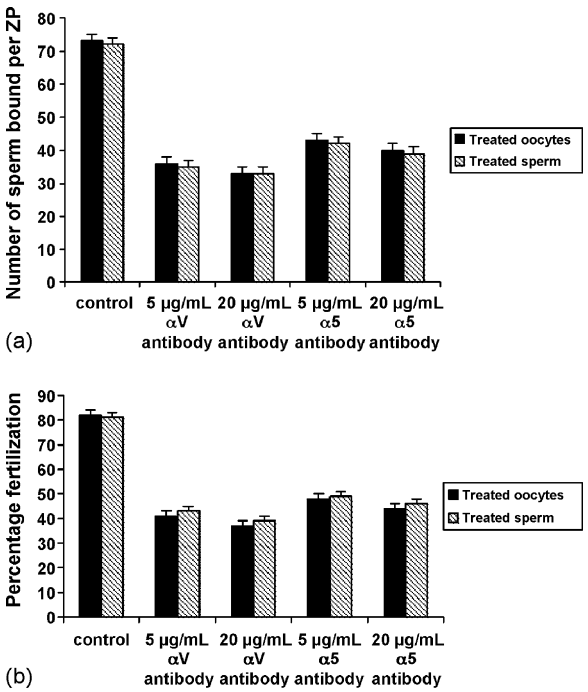

Fig. 2. (a) Mean (±S.E.M.) number of sperm bound per zona pellucida (ZP); (b) mean percentage of fertilized eggs following exposure of oocytes or sperm to the treatments: no antibody (control), 5 μg/mL α_V antibody, 20 μg/mL α_V antibody, 5 μg/mL α_5 antibody, 20 μg/mL α_5 antibody. Binding of spermatozoa, and also fertilization to the untreated control oocytes or sperm were greater than the others treatments ($P < 0.005$). The study was repeated four times using 40–50 (sperm–egg binding) and 80–90 (fertilization) oocytes per treatment.

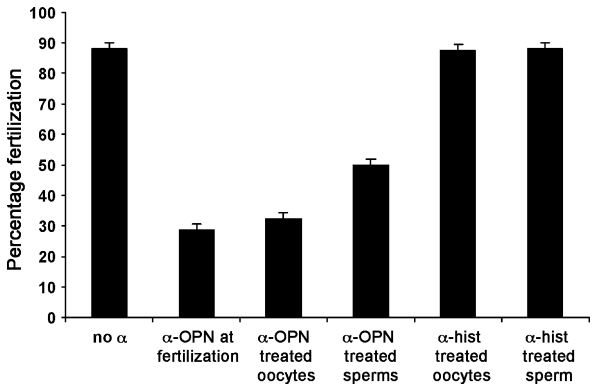

Fig. 3. Mean (±S.E.M.) percentage of fertilized eggs following exposure of oocytes and sperm to the: no antibody (control); α-OPN at fertilization time; α-OPN treated oocytes; α-OPN treated sperm; anti-hist treated oocytes; and anti-hist treated sperm. Addition of a rabbit polyclonal antibody against purified bovine milk OPN with sperm or/and oocytes decreased ($P < 0.05$) fertilization compared to the in vitro-fertilized control and the anti-hist treated oocytes and sperm. There were no differences among the other treatments. The study was repeated four times using 80–90 oocytes per treatment.

time); 32.42 ± 3.5% (α-OPN treated oocytes); 49.87 ± 3.0% (α-OPN treated sperm); 87.56 ± 3.5% (anti-hist treated oocytes); 88.08 ± 3.0% (anti-hist treated sperm). The anti-hist did not decrease fertilization.

4. Discussion

The molecular events of sperm–oocyte binding and fusion have been studied extensively, but identification of the molecules involved in sperm–oocyte interaction remains incomplete.

Carbohydrate–protein interactions also are prevalent in the process of fertilization. Zona pellucida sugars have also been shown to play a role in the zona reaction [30], as well as having been implicated in the fertility of individual oocytes [31]. A great deal of effort has been exerted to determine which terminal sugar(s) on the ZP are involved in sperm binding, resulting in a striking lack of consensus within any given species. In pigs and cows, evidence has long suggested that simple, terminal monosaccharides are less important than complex carbohydrates. In the bovine, high-mannose-type chains containing *N*-acetyllactosamine repeats in the nonreducing portion had been hypothesized as a sperm ligand [32], but more recent studies support the idea that nonreducing, terminal α-mannosyl residues may be responsible for gamete interaction in this species [33]. However, as in the mouse, the carbohydrate–protein interactions in the bovine system appear to be complex, perhaps involving multiple and potentially redundant sugar receptors.

In this study, we demonstrated a potential role for RGD-containing peptide on in vitro bovine egg binding and fertilization. Previous experiments demonstrated that the coincubation of RGD-containing peptides in a heterologous system (human sperm and zona-free hamster eggs) or a homologous system (hamster sperm and zona-free hamster eggs) resulted in a significant decrease in the number of adherent sperm, egg penetration, and fertilization [34,35]. RGD peptide is capable of blocking fertilization, inducing intracellular Ca^{2+} transients, and initiating parthenogenic development when present during bovine in vitro fertilization [12]. In this study the incubation of bovine oocytes and/or spermatozoa with RGD peptide significantly inhibited in vitro sperm–egg binding and fertilization, suggesting that the RGD peptide is involved in the interaction between spermatozoa and oocyte during fertilization.

Integrins have been shown to be involved in the process of fertilization [34–36]. Both spermatozoa and

oocytes express a number of integrins and molecules that contain integrin recognition sites. Among the many different integrin subunits expressed on oocytes, available results suggest a role of $\alpha_6\beta_1$ integrin in the process of gamete binding and/or fusion and as a potential marker for evaluating sperm quality in men [20,36,37]. Conversely, members of the RGD-binding integrin subfamily (including $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$) are expressed by oocytes and are implicated in fertilization by studies using RGD peptides in IVF assays [18]. Biochemical analyses have implicated the α_v integrin subunit on the pig oocyte in the recognition of isolated pig sperm membrane proteins [38], but direct involvement of RGD-sensitive integrins should be confirmed. We have demonstrated that treatment of bovine or spermatozoa with integrins α_V and α_5 antibodies significantly decreased in vitro sperm–egg binding and fertilization, suggesting that both integrins may be involved in bovine fertilization.

Osteopontin (OPN) was originally identified in the mineralized matrix of bovine bone [39], but has subsequently been reported in a variety of organs, including female reproductive tissues as the human and bovine ovary [40,41], the human and ovine endometrium [40,42,43], and the human and bovine oviduct [8,33]. Its presence has been also shown in several organs of the male reproductive tract including the bovine seminal vesicle and ampulla [9], the epithelium of the human testis and prostate gland [40], and the rat testis and epididymis [44]. In situ hybridization revealed that bovine spermatozoa located within the epididymis and ampulla contained OPN mRNA [10]. The relative amount of OPN in bovine seminal plasma was correlated positively with the fertility of bulls [11]. In the present study, we demonstrated that antibody to OPN reacts with both oocytes and spermatozoa resulting in inhibition of fertilization in vitro.

To our knowledge, this is the first report identifying the potential role of OPN and its ligands in the process of bovine fertilization. The results indicated that the sperm–egg interaction may be mediated through the cell surface receptors that contain RGD recognition sequences. OPN may bind primarily to an integrin on tissues via its GRGDS sequence to promote cell–cell attachment, cell spreading and extracellular matrix communication. Our results, combined with those of others, demonstrated a role of carbohydrates in sperm binding and fertilization and suggested that multiple mechanisms operate during sperm–oocyte binding fertilization to ensure a fail-safe system.

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References

[1] Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, editors. The physiology of reproduction. New York: Raven Press; 1994. p. 189–317.
[2] Rajeev SK, Reddy VRK. Integrins and desintegrins; the candidate molecular players in sperm–egg interactions. Indian J Exp Biol 2000;38:1217–21.
[3] Glande HJ, Schaller J. Semen samples with teratozoospermia show a lower percentage of spermatozoa with dectable adhesion molecules. Int Urol Nephrol 1993;25:485–9.
[4] Wassarman PM, Jovine L, Litscher ES. A profile of fertilization in mammals. Nat Cell Biol 2001;3:E59–64.
[5] Primakoff P, Myles DG. Penetration, adhesion, and fusion in mammalian sperm–egg interaction. Science 2002;29:2183–5.
[6] Le Naour F, Rubinstein E, Jasmin C, Prenant M, Boucheix C. Severely reduced female fertility in CD9-deficient mice. Science 2000;287:319–21.
[7] Miyado K, Yamada G, Yamada S, Hasuwa H, Nakamura Y, Ryu F, et al. Requirement of CD9 on the egg plasma membrane for fertilization. Science 2000;287:321–4.
[8] Gabler G, Chapman DA, Killian GJ. Expression and presence of osteopontin and integrins in the bovine oviduct during the oestrous cycle. Reproduction 2003;126:721–9.
[9] Cancel AM, Chapman DA, Killian GJ. Osteopontin localization in the Holstein bull reproductive tract. Biol Reprod 1999;60 :454–60.
[10] Rodriguez CM, Day JR, Killian GJ. Osteopontin gene expression in the Holstein bull reproductive tract. J Androl 2000;21: 414–9.
[11] Cancel AM, Chapman DA, Killian GJ. Osteopontin is the 55-kilodalton fertility-associated protein in Holstein bull seminal plasma. Biol Reprod 1997;57:1293–301.
[12] Campbell KD, Reed WA, White KL. Ability of integrins to mediate fertilization, intracellular calcium release, and parthenogenetic development in bovine oocytes. Biol Reprod 2000;62:1702–9.
[13] Tarone G, Russo MA, Hirsch E, Odorisio T, Altruda F, Silengo L, et al. Expression of beta 1 integrin complexes on the surface of unfertilized mouse oocyte. Development 1993;117:1369–75.
[14] Evans JP, Schultz RM, Knopf GS. Identification and localization of integrin subunits in oocytes and eggs of the mouse. Mol Reprod Dev 1995;40:220–1.
[15] Zuccotti M, Giogi Rossi P, Fiorillo E, Garagna S, Forabosco A, Redi CA. Timing of gene expression and oolemma localization of mouse alpha6 and beta 1 integrin subunits during oogenesis. Dev Biol 1998;200:27–34.
[16] Burns KH, Owens GE, Fernandez JM, Nilson JH, Matzuk MM. Characterization of integrin expression in the mouse ovary. Biol Reprod 2002;67:743–51.
[17] Campbell S, Swann HR, Seif MW, Kimber SJ, Aplin JD. Cell adhesion molecules on the oocyte and preimplantation human embryo. Hum Reprod 1995;10:1572–8.

[18] Ji YZ, Wolf JP, Jouannet P, Bomsel M. Human gamete fusion can bypass beta 1 integrin requirement. Hum Reprod 1998;13:682–9.
[19] Sengoku K, Takuma N, Miyamoto T, Horikama M, Ishikawa M. Integrins are not involved in the process of human sperm–oolemmal fusion. Hum Reprod 2004;19:639–44.
[20] Reddy VRK, Rajeev SK, Gupta V. Alpha 6 beta 1 integrin is a potential clinical marker for evaluating sperm quality in men. Fertil Steril 2003;79(Suppl 3):1590–600.
[21] Töpfer-Peterson E. Cabohydrate-based interactions on the route of spermatozoa to fertilization. Hum Reprod Update 1999;5: 314–29.
[22] Suarez S. Carbohydrate-mediated formation of the oviductal sperm reservoir in mammals. Cells Tissues Organs 2001;168: 105–12.
[23] Gougoulidis T, Trounson A, Dowsing A. Inhibition of bovine sperm–oocyte fusion by the carbohydrate GalNAc. Mol Reprod Dev 1999;54:179–85.
[24] Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, et al. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 1995;43:141–52.
[25] Bavister BD, Leibfried ML, Lieberman G. Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol Reprod 1993;28:235–47.
[26] Sirad MA, Parrish JJ, Ware CB, Leibfried-Rutledge ML, First NL. The culture of bovine oocytes to obtain developmentally competent embryos. Biol Reprod 1988;39:546–52.
[27] Way AL, Schuler AM, Killian GJ. Influence of bovine ampullary and isthmic oviductal fluid on sperm–egg binding and fertilization in vitro. J Reprod Fertil 1997;109:95–101.
[28] King RS, Anderson SH, Killian GJ. Effect of bovine oviductal estrus-associated protein on the ability of sperm to capacitate and fertilize oocytes. J Androl 1994;15:468–78.
[29] SAS Institute Inc., 1989–1996. SAS for Windows Version 6.12. Gary, NC.
[30] Dolci S, Bertolani MV, Canipari R, De Felici M. Involvement of carbohydrates in the hardening of the zona pellucida of mouse oocytes. Cell Biol Intern Reprod 1991;15:571–8.
[31] Talevi R, Gualtieri R, Tartaglione G, Fortunato A. Heterogeneity of the zona pellucida carbohydrate distribution in human oocytes failing to fertilize in vitro. Hum Reprod 1997;12:2773–80.
[32] Katsumata T, Noguchi S, Yonezawa N, Tanokura M, Nakano M. Structural characterization of the N-linked carbohydrate chains of the zona pellucida glycoproteins from bovine ovarian and fertilized eggs. Eur J Biochem 1996;240:448–53.
[33] Amari S, Yonezawa N, Mitsui S, Katsumata T, Hamano S, Kuwayama M, et al. Essential role of the nonreducing terminal alpha-mannosyl residues of the N-linked carbohydrate chain of bovine zona pellucida glycoproteins in sperm–egg binding. Mol Reprod Dev 2001;59:221–6.
[34] Bronson RA, Fusi F. Evidence that an arg-gly-asp adhesion sequence plays a role in mammalian fertilization. Bio Reprod 1990;43:1019–25.
[35] Bronson RA, Fusi F. Sperm–oolemmal interaction: role of Arg-Gly-Asp (RGD) adhesion peptide. Fertil Steril 1990;54: 527–9.
[36] Almeida EA, Huovila AP, Sutherland AE, Stephens L, Calarco PG, Shaw LM, et al. Mouse egg integrin α6β1 functions as a sperm receptor. Cell 1995;81:1095–104.
[37] Takahashi Y, Yamakawa N, Matsumoto K, Toyoda Y, Furukawa K, Sato E. Analysis of the role of egg integrins in sperm–egg binding and fusion. Mol Reprod Dev 2000;56:412–23.
[38] Linfor J, Berger T. Potential role of alpha v and beta 1 integrins as oocyte adhesion molecules during fertilization in pigs. J Reprod Fertil 2000;120:65–72.
[39] Frazen A, Heinegard D. Isolation and characterization of two sialoproteins present only in bovine calcified matrix. Biochem J 1985;232:715–24.
[40] Brown LF, Berse B, Van de Walter L, Papadopoulos-Sergiou A, Perruzzi CA, Manseu EJ, et al. Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. Mol Biol Cell 1992;3:1169–80.
[41] Brunswig-Spickenheier B, Mukhopadhyay AK. Expression of osteopontin (OPN) mRNA in bovine ovarian follicles and corpora lutea. Reprod Domest Anim 2003;38(3):175–81.
[42] Nomura S, Wills AJ, Edwards DR, Heath JK, Hogan BL. Developmental expression of 2ar (osteopontin) and SPARC (osteonectin) RNA as revealed by in situ hybridization. J Cell Biol 1988;106:441–50.
[43] Johnson GA, Spencer TE, Burghardt RC, Bazer FW. Ovine osteopontin. I. Cloning and expression of messenger ribonucleic acid in the uterus during the preimplantation period. Biol Reprod 1999;61:884–91.
[44] Siiteri JE, Ensrud KM, Moore A, Hamilton DW. Identification of osteopontin (OPN) mRNA and protein in the rat testis and epididymis, and on sperm. Mol Reprod Dev 1995;40:16–28.