

Expression of α_6 integrin subunit in bovine oocyte and its potential role during fertilization

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ABSTRACT

Fertilization in mammals requires the successful completion of a sequence of steps, starting with the transport of gametes in the reproductive tract and ending with sperm-egg membrane fusion to produce a zygote. Although some integrin subunits are known to be associated with the plasma membrane of some mammalian oocytes and spermatozoa, the presence of α_6 integrin on bovine oocytes with intact zona pellucida has not been reported. The present study was undertaken to evaluate the expression of α_6 integrin subunit in bovine oocyte and to determine if *in vitro* binding to the zona pellucida and fertilization were affected by treating oocytes with α_6 integrin subunit antibody. The α_6 integrin subunit was identified on the bovine oocyte by immunocytochemistry. *In vitro* fertilization was significantly decreased when *in vitro* matured bovine oocytes were pre-incubated with α_6 integrin subunit antibody at concentration 5 and 20 $\mu\text{g/mL}$, and sperm-oocyte binding increased. These studies demonstrated the presence of α_6 integrin subunit on bovine oocyte, and its importance in fertilization and polyspermy.

Keywords: Integrins; Bovine Oocytes; Fertilization; Sperm-Oocyte Binding

1. INTRODUCTION

Fertilization process is a highly complex and orchestrated 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 by gametes surface proteins [1].

Understanding the role in fertilization of gamete specific surface proteins and other surface proteins expressed, which both contribute to sperm-egg interactions is, at present, a pivotal area of research. Among the best characterized biomarkers for assessment of sperm-egg interactions are the integrins. Integrins are a family of heterodimeric cell adhesion molecules that mediate cell-cell and cell-extracellular matrix interactions. Up to now, 18 α subunits (120 - 180 kDa) and 8 β subunits (90 - 110 kDa) were identified; these combine to make 24 different integrins [2,3]. Many integrins were able to recognize the tripeptide sequence arginine-glycine-aspartic acid (RGD). Integrins further attach to the cell, specifically to the extracellular matrix and participate in cell migration [1].

Integrins may be involved in the process of fertilization [1,4,5]. Coincubation of RGD-containing peptides with human sperm and zona-free hamster eggs or with hamster sperm and zona-free hamster eggs resulted in decreasing number of adherent sperm, egg penetration, and fertilization [4]. Also treatment of bovine sperm and oocytes with RGD peptide or with α_5 or α_V prior to fertilization significantly decreased *in vitro* sperm-egg binding and fertilization [1]. Oocytes and spermatozoa express a number of integrins and molecules that contain integrin recognition sites. Numerous integrin subunits, including α_2 , α_3 , α_4 , α_5 , α_6 , α_V , α_M , β_1 , β_2 , β_3 , and β_5 , have been detected in mammalian oocytes at the protein or RNA level [1-3,6-13]. Similarly, ejaculated spermatozoa and spermatogonial cells may express α_4 , α_5 , α_6 , α_V , β_1 , β_2 , β_3 , and β_5 [12-14]. Confocal microscopy and RT-PCR analysis indicated the presence of α_6 and β_3 integrin subunit at the plasma membrane of bovine oocytes [15].

Although some integrin subunits are known to be associated with the plasma membrane of some mammalian oocytes and spermatozoa, the presence of α_6 integrin on

bovine oocytes with intact zona pellucida has not been reported. The present study was undertaken to evaluate the expression of α_6 integrin subunit in bovine oocyte and to determine if *in vitro* binding to the zona pellucida and fertilization were affected by treating oocytes with α_6 integrin subunit antibody.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tissue culture media 199 (TCM 199 Hepes and Bicarbonate-buffered), PBS, and fetal calf serum (FCS) were obtained from Gibco™, Invitrogen Corporation (Grand Island, NY, USA).

2.2. Oocyte Collection and Maturation

Bovine ovaries were harvested at a local abattoir and transported to the laboratory in PBS at 25°C - 30°C. Once in the laboratory, ovaries were rinsed with warmed PBS. Follicles from 2 to 6 mm in diameter were aspirated using an 18-gauge needle attached to 5 mL syringe. The ovarian follicular fluid was pooled in 15 mL conical tubes and allowed to settle to the bottom of the tube for 10 - 15 min. 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 confocal microscopy and *in vitro* fertilization.

The cumulus-oocyte complexes (25/well) were matured in four-well culture plates (Nunc™, Nunc, Roskilde, Denmark) in 500 μ L TCM-199, 10% fetal calf serum, LH (6 μ g/mL), FSH (8 μ g/mL) (Sioux Biochemical®, Sioux Center, IA, USA), and penicillin (100 units/mL)—streptomycin (100 μ g/mL) for 24 h at 39°C, 5% CO₂ in air (v/v). After maturation, oocytes were prepared for confocal microscopy, sperm binding and fertilization experiments as described below.

2.3. Localization of α_6 Integrin Subunit by Confocal Microscopy

Cumulus cells were removed mechanically by vortexing, 10 min, in 1 mL of maturation medium. All oocytes were fixed with 3.5% (w/v) paraformaldehyde in PBS for 1 h at room temperature (RT). They were washed 3 times, 5 min each, with PBS/PVP, and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 1 h at RT. Subsequently, they were incubated with 10% (v/v) goat serum (Invitrogen) in polyvinyl pyrrolidone (PVP, 0.1% (w/v) in PBS) solution for 30 min at 37°C, with mouse MAB to α_6 integrin subunit (Chemicon International®, Temecula, CA, USA) for 1 h. The oocytes were washed 4 times, 10 min

each, in PBS with PVP. After that they were incubated with Alexa Fluor 488 goat-anti-mouse secondary antibody (Invitrogen®, Eugene, OR, USA) for 1 h at 37°C. They were mounted in a droplet of ProLong® Gold Anti-fade Reagent with DAPI (Invitrogen®) and evaluated for the presence of α_6 integrin subunit using an Olympus BX51 equipped with epifluorescence and a DP71 camera with a 12.5 MPixel CCD microscope.

2.4. Treatments

This study was conducted to determine whether *in vitro* sperm binding to the ZP of intact bovine oocytes and fertilization were affected by treating oocytes with α_6 integrin subunit antibody. *In vitro* matured oocytes were incubated (39°C, 5% CO₂, in air) for 2 h in fertilization medium with: 1) no antibody; 2) 5 μ g/mL α_6 integrin subunit antibody; 3) 20 μ g/mL α_6 integrin subunit antibody; 4) a rabbit IgG against a non-bovine antigen, bacterial histidase (1:2000) [21]. Frozen-thawed bull semen from the same ejaculate was washed using Percoll gradient centrifugation. Briefly, 2 mL of a 90% Percoll solution in modified Tyrode's medium (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. The straw content was layered onto the 45% solution and centrifuge for 30 min at 700 \times g. The pelleted spermatozoa was recovered, assessed for motility and suspended in fertilization medium. Oocytes were inseminated with 10 \times 10⁶ washed spermatozoa in the fertilization medium supplemented with 2 μ g/mL heparin and 20 μ g/mL of PHE solution (20 μ M penicillamine, 10 μ M hypotaurine, 1 μ M adrenaline). Oocytes and spermatozoa were co-incubated for 18 h at 39°C in 5% CO₂ in air (v/v).

2.5. Sperm-Oocyte Binding

After co-incubation oocytes were washed five times in TL-HEPES and placed, 10 per slide, under a coverslip mounted with paraffin wax and petroleum jelly at each corner. The coverslip was gently lowered over the oocytes until they burst, and the cytoplasm was rinsed away leaving the ZP behind. The ZP and spermatozoa attached to it were stained with Hoechst fluorescent dye 33,342 to determine the number of spermatozoa bound to each ZP using fluorescence microscope Olympus BX51 [1].

2.6. In Vitro Fertilization

In vitro matured oocytes were washed twice in HEPES medium, placed in fertilization medium with 2 μ g/mL 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. 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 acetate-orcein [1] the presence of two pronuclei in the cytoplasm of the oocyte indicated normal fertilization.

2.7. Statistical Analysis

Each experiment was repeated four times and data from each experiment were pooled. Approximately 40 - 50 oocytes per treatment for sperm binding, and 80 - 90 oocytes per treatment for fertilization were evaluated in each replicate. ANOVA 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 experiments. Least square means comparisons were used to assess sperm binding and weighted least square means were used to analyze fertilization data. The significance level for these tests was $p < 0.05$.

3. RESULTS

3.1. Localization of α_6 Integrin Subunit by Confocal Microscopy

Expression of α_6 integrin subunit could clearly be detected on the zona pellucida of *in vitro* matured bovine oocytes (Figure 1).

3.2. Binding of Sperm Incubated with α_6 Integrin Subunit Antibody to Oocytes

More sperm bound to oocytes treated with a 5 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$ α_6 integrin subunit antibody than oocytes incubated in control medium and a rabbit IgG against a non-bovine antigen, bacterial histidase (Figure 2(a)): control (18.3 ± 1.7) 5 $\mu\text{g}/\text{mL}$ of α_6 integrin subunit antibody (80.7 ± 1.9); 20 $\mu\text{g}/\text{mL}$ of α_6 integrin subunit antibody (82.5 ± 2.5); non-bovine antigen (20.2 ± 1.4).

3.3. *In Vitro* Fertilization

Treatment of oocytes with α_6 integrin subunit antibody decreased fertilization compared to the untreated control (Figure 2(b)): $84\% \pm 1.5\%$ (control); $40.2\% \pm 1.7\%$ (5 $\mu\text{g}/\text{mL}$ of α_6 integrin subunit antibody); $38.2\% \pm 1.5\%$ (20 $\mu\text{g}/\text{mL}$ of α_6 integrin subunit antibody); $82.8\% \pm 1.6\%$ (non-bovine antigen).

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 and

fertilization remains incomplete. Integrins are cell surface receptors that mediate cell-cell and cell-extracellular matrix interactions in many cell types. Many molecules have been characterized and suggested to participate in the fertilization process, having integrin as receptors such as CD46, members of the A Desintegrin and A Metalloprotease domains (ADAM) family, secreted proosphoprotein one (SSP1 or OPN) and molecules with RGD peptides sequence [1-6,9,11,18-22]. Inhibition of sperm-oocyte adhesion/fusion can be performed by monoclonal antibodies, RGD or desintegrin-like peptides and RNAi-mediated knockdown [16-19].

Integrins are expressed in an inactivated or activated state [23]. Monoclonal antibodies or ligands bind only the activated state. Integrin affinities advance is normally consistent with a conformational change in extracellular domain induced from inside the cell through cytoskeletal modifications or from outside the cell activation through cooperative ligand/receptor systems [24,25]. Several integrin subunits have been detected in mammalian oocytes at the mRNA or at the protein level, including sea urchin, mouse, hamster, human, pig, bovine [1-3,11,15, 26-33].

Bronson and Fusi (1990) were the first to show either with peptides containing the RGD sequence or with anti-integrin monoclonal antibodies that some integrin subunits were present on Syrian hamster unfertilized oocytes (α_2 , α_5 , $\alpha_6\beta_1$, $\alpha_v\beta_3$) and on human oocytes (α_2 , α_5 , $\alpha_6\beta_1$) [4,5,28]. Expression of α_3 , α_5 and $\alpha_6\beta_1$ were identified both at the protein and mRNA levels in unfertilized mouse oocytes [2,27]. De Nadia *et al.* (1996) have studied membrane receptor conservation among species, and through immunofluorescence labeling and Western blot analysis using a variety of mouse anti-integrin antibodies [26]. The same authors have demonstrated the presence of α_2 , α_5 , and β_1 integrin subunits on sea urchin, hamster

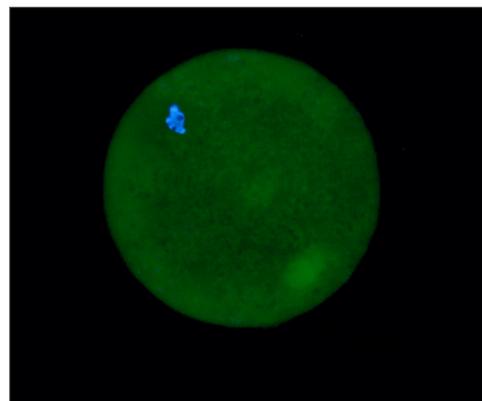


Figure 1. Cumulus denuded ZP intact bovine matured oocyte labeled with mouse MAB to α_6 integrin subunit and Alexa Fluor 488 goat-anti-mouse combined with ProLong[®] Gold Antifade Reagent with DAPI. Bar = 25 μm .

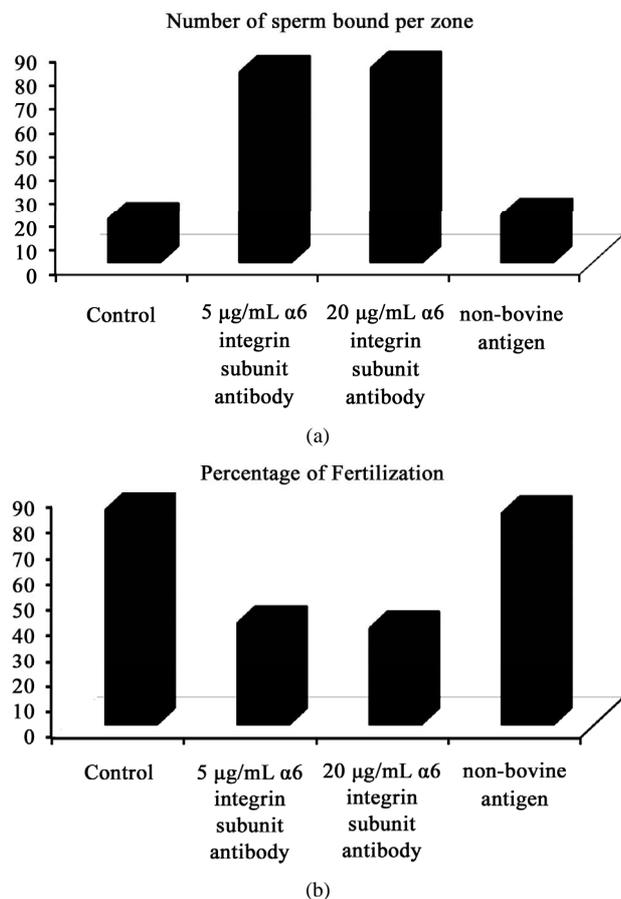


Figure 2. (a) Mean (\pm SEM) number of sperm bound zona pellucida (ZP); (b) mean percentage of fertilized eggs following exposure of oocytes to the treatments: control, 5 μ g/mL α_6 integrin subunit antibody, 20 μ g/mL α_6 integrin subunit antibody, a rabbit IgG against a non-bovine antigen, bacterial histidase (1:2000). The study was repeated four times using 40 - 50 (sperm-oocyte binding) and 80 - 90 (fertilization) oocytes per treatment.

and human unfertilized oocytes. They also have found by immunofluorescence studies that both hamster and human oocytes expressed $\alpha_M\beta_2$, a β_2 class antigen, as already suggested by Anderson *et al.* (1993) [34]. Linfor and Berger (2000) have demonstrated the presence of β_1 , α_1 , α_2 , α_3 , α_4 , α_5 , α_6 , and α_V integrin subunits on the plasma membrane of pig oocytes by immunocytochemistry and confocal microscopy [33].

Evidence for sperm-oocyte interaction through integrins have been established by the presence of α_6 , α_4 , α_2 , α_V , β_3 , and β_3 integrin subunits on the bovine oocyte plasma membrane and also supported by the ability of RGD-containing peptides to induce intracellular calcium transients. Indeed, the parthenogenetic development and the decreased *in vitro* fertilization have both provided a pivotal role for integrins at oocyte surface, influencing sperm-oocyte interaction [1,29,31,32]. This is the first study showing the α_6 integrin on the bovine oocytes with the

zona pellucida intact by confocal microscopy. The binding of monoclonal antibody to α_6 integrin subunit to the ZP of bovine matured oocyte indicates that α_6 integrin is part of the bovine oocyte.

The inhibition of sperm-oocyte plasma membrane binding and antibody recognizing of the α_6 integrin subunit implies involvement of the α_6 in sperm-oocyte interaction. This data also suggest that α_6 integrin subunit may be involved in preventing polyspermy in bovine oocytes. Mammalian oocytes have capability to block polyspermy both at the zona pellucida and at the plasma membrane. While zona polyspermy blocking has been well characterized, little is known regarding molecular events surrounding the plasma membrane capabilities in avoiding polyspermy [19,35].

Bovine sperm bound in higher numbers to oocytes treated with anti- α_6 integrin subunit than to oocytes incubated in control medium, nevertheless incidence of polyspermic fertilization also increased with antibody-treated oocyte. While it is possible that sperm bound in higher numbers to antibody-coated oocytes than control oocytes through IgG-ZP interactions, a decrease in fertilization rates coupled with an increase in polyspermic fertilizations suggests that α_6 integrin subunit on bovine oocyte may participate in the induction of polyspermy blocks in bovine oocytes and in fertilization.

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