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Epithelial cadherin is present in bovine oviduct epithelial cells and gametes, and is involved in fertilization-related events

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ABSTRACT

Fertilization is a calcium-dependent process that involves sequential cell–cell adhesion events of spermatozoa with oviduct epithelial cells (OECs) and with cumulus-oocyte complexes (COCs). Epithelial cadherin (E-cadherin) participates in calcium-dependent somatic cell adhesion; the adaptor protein β-catenin binds to the E-cadherin cytoplasmic domain and links the adhesion protein to the cytoskeleton. The study was conducted to immunodetect E-cadherin and β-catenin in bovine gametes and oviduct (tissue sections and OEC monolayers), and to assess E-cadherin participation in fertilization-related events. Epithelial cadherin was found in spermatozoa, oocytes, cumulus cells, and OEC. In acrosome-intact noncapacitated spermatozoa, E-cadherin was mainly localized in the apical ridge and acrosomal cap (E1-pattern; $84 \pm 9\%$; mean $\pm$ standard deviation of the mean). After sperm treatment with heparin to promote capacitation, the percentage of cells with E1-pattern ($56 \pm 12\%$) significantly decreased; concomitantly, the percentage of spermatozoa depicting an E-cadherin staining pattern similar to E1-pattern but showing a signal loss in the acrosomal cap (E2-pattern: $40 \pm 11\%$) increased. After L-α-lysophosphatidylcholine–induced acrosome reaction, E-cadherin signal was mainly localized in the inner acrosomal membrane (E3-pattern: $67 \pm 22\%$). In IVM COC, E-cadherin was immunodetected in the plasma membrane of cumulus cells and oocytes, but was absent in the polar body. The 120 KDa mature protein form was found in protein extracts from spermatozoa, oocytes, cumulus cells, and OEC. β-Catenin distribution followed E-cadherin's in all cells evaluated. Epithelial cadherin participation in cell–cell interaction was evaluated using specific blocking monoclonal antibody DECMA-1. Sperm incubation with DECMA-1 impaired sperm–OEC binding (the number of sperm bound to OEC: DECMA-1 $= 6.7 \pm 6.1$ vs. control $= 29.6 \pm 20.1$; $P < 0.001$), fertilization with COC (% fertilized COC: DECMA-1 $= 68.8 \pm 10.4$ vs. control $= 90.7 \pm 3.1$; $P < 0.05$) or denuded oocytes (% fertilized oocytes: DECMA-1 $= 57.0 \pm 15.2$ vs. control $= 89.2 \pm 9.8$; $P < 0.05$) and binding to the oolemma (the number of sperm bound to oolemma: DECMA-1 $= 2.2 \pm 1.1$ vs. control $= 11.1 \pm 4.8$; $P < 0.05$). This study describes, for the first time, the presence

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of E-cadherin in bovine spermatozoa, COC, and OEC, and shows evidence of its participation in sperm interaction with the oviduct and the oocyte during fertilization.

1. Introduction

Fertilization involves a set of coordinated cell–cell interactions between spermatozoa and the oocyte and between spermatozoa and the oviduct. Spermatozoa that arrive to the oviduct isthmus form a functional sperm reservoir; this cell–cell interaction involves the oviduct epithelial cells (OECs) [1,2] and the sperm head plasma membrane of the acrosomal cap [3,4]. Changes associated with ovulation induce the release of spermatozoa bound to OEC, allowing them to continue their transit to the fertilization site [1,4].

Sperm dissociation from the epithelium of the oviduct has been related to capacitation [5,6], in particular, it has been associated with changes in the sperm membrane composition and fluidity [7] and in protein tyrosine phosphorylation [8], and to the increase in intracellular calcium ion levels [9]. In the bovine, heparin is a glycosaminoglycan highly secreted during the preovulatory period [10]; it is one of the best characterized compounds responsible for the induction of sperm capacitation-related events [11,12], and has a key role in the sperm release from oviduct cells in culture [13]. The addition of glucose to the culture medium containing heparin to promote sperm capacitation (heparin + glucose) is used in the experimental designs as control, because it has been shown to delay some of the heparin effect [14].

In vivo, fertilization takes place in the isthmic–ampullary junction of the oviduct. Spermatozoa make contact with cells from the cumulus mass that surrounds the oocyte of the cumulus oocyte complexes (COCs) and with glycoproteins of the zona pellucida (ZP), the extracellular matrix that surrounds the oocyte. As a result of these interactions, spermatozoa undergo acrosomal exocytosis (AE), a process that involves fusion of the sperm plasma and the outer acrosomal membranes, release of the acrosomal contents, and exposure of the inner acrosomal membrane. The AE can also be induced *in vitro* by sperm incubation with several components, among them L-α-lysophosphatidylcholine (LPC) and calcium ionophore A23187 to heparin-capacitated bovine spermatozoa [11]. Cells that have completed AE and ZP penetration, reach the perivitelline space, bind and fuse to the oolemma. This interaction would initially involve binding of sperm receptors located in the inner acrosomal membrane from the apical region of the head with proteins located in the oolemma microvilli [15,16].

The identification of proteins of both gametes and cells of the female tract involved in fertilization has been the subject of numerous investigations in various experimental models. In the bovine, several proteins that participate in gamete interaction have been reported; examples are the proacrosin/acrosin system [17], ADAM1/ADAM2 [18], P25b [19], PH-20 [20], and more recently IAM38 [21], osteopontin [22], fibronectin, and several integrins [23,24], a lipocalin type prostaglandin-D synthase [25], tetraspanin CD9 [26], and vitronectin [27]. Notwithstanding the extense work done, the molecular mechanisms involved in sperm–oocyte interaction and the entities involved in adhesion events of both gametes in the bovine model have not been completely elucidated.

Among members of cell–cell adhesion proteins, our group has reported a detailed analysis on the presence of epithelial cadherin (E-cadherin) and neural cadherin in human gametes, and evidence supporting their role in gamete interaction [28,29]. Epithelial cadherin is the founder member of the cadherin superfamily, a vast group of membrane glycoproteins that mediate calcium (Ca^{2+})-dependent intercellular adhesion [30]. It is composed of five extracellular domains, a transmembrane domain and a highly conserved cytoplasmic domain. The E-cadherin extracellular domains participate in the formation of dimers in cis (same membrane side) that interact in a homophilic fashion (i.e., same cadherin) with dimers located in trans in a neighboring cell membrane; the intracellular domain anchors the adhesion protein to the actin cytoskeleton through adaptor proteins, among them β-catenin [31,32].

The present study was designed to evaluate the presence and localization of E-cadherin and β-catenin in bovine spermatozoa, OEC and COC cell components, and to assess participation of the adhesion molecule in fertilization-related events.

2. Materials and methods

2.1. Chemicals

All chemicals were of highest analytical and tissue culture grade, and were purchased mainly from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Electrophoresis reagents were products of Bio-Rad (Richmond, CA, USA). Vectashield antifade solution was purchased from Vector Labs (Burlingame, CA, USA).

Unless specifically indicated, sperm handling was done in Sp-TALP medium [11]. IVF procedures and sperm–oolemma assays were carried out in modified synthetic oviduct fluid ([33] supplemented with 3 mg/mL BSA and 60 μg/mL sodium heparin IVF medium). For OEC culture protocols, M199 culture medium (Life, Carlsbad, CA, USA) was supplemented with 10% fetal calf serum (Life), 50 μg/mL gentamicin (Sigma), and 1 μg/mL fungizone (Sigma), as previously described [34].

2.2. Antibodies

The following antibodies were utilized throughout the study, toward (1) E-cadherin (H-108, polyclonal antibody; Santa Cruz Biotech., Santa Cruz, CA, USA; monoclonal antibodies clone DECMA-1 [decompacting monoclonal

antibody; SIGMA]) and 610181 (BD Biosciences, San Diego, CA, USA); (2) β-catenin (610153; BD Biosciences) and AB19022 (Upstate/Chemicon-Millipore, Billerica, MA, USA); (3) cytokeratins (clone AE1/AE3; Dako Cytomation, Carpinteria, CA, USA). Immunoglobulin G (IgG) from normal rabbit, rat, and mouse sera was from SIGMA. Secondary antibodies: Cy3-labeled antirabbit IgG and Cy3-labeled antimouse IgG (Chemicon), fluorescein isothiocyanate (FITC)-labeled antirabbit and FITC-labeled antimouse IgGs (Sigma) were used for immunohistochemistry and immunocytochemistry protocols. Horseradish peroxidase (HRP)-conjugated goat antirabbit and antimouse IgGs (Sigma) was used in Western immunoblotting assays.

In-silico analysis done using the Clustalw2 program (http://www.ebi.ac.uk/Tools/clustalw2/) confirmed 100% conservation of the epitope recognized by the monoclonal antibody 61018 in the bovine E-cadherin amino acid sequence (NM_001002763) compared with the human sequence (NM_004360). For the epitope recognized by the H-108 polyclonal antibody, similarity and identity between human and bovine amino acid sequences was estimated in 89% (786/882) and 82% (725/882), respectively.

DECMA-1 anti–E-cadherin IgGs were purified from the ascitic fluid using protein G agarose affinity chromatography, and used in biological assays (binding to oolemma, IVF, sperm–OEC interaction). Rat IgGs, purified from whole serum using the same procedure, served as control.

To carry out control assays with depleted anti–E-cadherin antibodies, an aliquot (0.5–2 µg/mL) of the H-108 antibody was incubated with $100\times$ recombinant human E-cadherin(rE-cadherin) immobilized onto nitrocellulose pieces. The clone expressing rE-cadherin was kindly provided by Dr. B. Gumbiner to Dr. M. Vazquez-Levin. Protein expression in Chinese Hamster Ovary cells, and purification by protein A agarose affinity chromatography had been previously done by collaborators of the group as described [35].

2.3. Semen samples

Frozen semen doses (0.5-mL straws) from nine fertile bulls were kindly provided by artificial insemination centers CIAVT (Venado Tuerto, Santa Fe, Argentina) and CIALE (Capitan Sarmiento, Buenos Aires, Argentina). Fresh ejaculates were collected with artificial vagina from three bulls of known fertility housed at the School of Veterinary Sciences, University of Buenos Aires (Buenos Aires, Argentina) and the artificial insemination center CRB (Buenos Aires, Argentina). In all cases, semen samples presented more than 40% live spermatozoa, 25% progressively motile sperm cells with vigor 3, and 70% normal sperm morphology (minimum standard parameters established by the Department of Herd Medicine and Theriogenology of the University of Saskatchewan, Canada).

2.4. Bovine oviduct tissue handling and oviduct OEC cultures

Bovine oviducts with uterine horns were collected at a slaughterhouse, and transported to the laboratory at 4 °C.

Upon arrival, oviducts were dissected and freed from the surrounding connective tissue, and carefully washed with PBS at 4 °C. The infundibulum and uterine horns were discarded, and only the ampulla and isthmus sections were used. The cycle phase of each tissue was classified using standard parameters (follicular: presence of a mature follicle greater than 12 mm in one ovary and a regressing corpus luteum in the contralateral; luteal: presence of corpus luteum in one or both ovaries, accompanied or not by a white body or follicles of small diameter).

Protocols to perform OEC cultures were essentially as previously reported [34]. To minimize differences between donor cows reported in previous studies [36], tissues from several animals were processed and pooled as part of the cell culture procedure. Epithelial cells from the isthmus and ampulla were recovered after flushing and squeezing of each duct, and kept at 4 °C in PBS. At the end of the isolation procedure, cells were washed two times with PBS, and concentrated by centrifugation for 5 minutes at $150\times$ g each time. The cell pellet was resuspended in M199 culture medium supplemented with 10% fetal calf serum, 50 µg/mL gentamicin, and 1 µg/mL fungizone, and OEC laminae with ciliary beating were placed in 4-cm Petri dishes (Nunc, Rochester, NY, USA) containing four 13-mm round coverslips at the bottom of each well, and kept at 39 °C with 5% CO_2 in humidified air. At 24 to 48 hours of cell culture, follicle-shaped structures were observed; changes with fresh medium were performed every 48 hours until cell confluence was achieved. Cytokeratin immunostaining was performed to confirm 90% epithelial cell content in the pools. On the day of the coculture procedure, coverslips with confluent cell monolayers were transferred to 24-well dishes, washed three times with Sp-TALP medium, and left in the same medium to stabilization during 1 hour before sperm addition.

2.5. Oocyte collection and IVM

Ovaries were collected from a local slaughterhouse and transported to the laboratory at 30 °C. Cumulus-oocyte complexes were retrieved by aspiration of antral follicles. Groups of 50 COCs were matured in a four-well multidish (Nunc) containing 500 µL M199 (Earle's salts, L-glutamine, and 2.2 µg/mL sodium bicarbonate; GIBCO-Life) supplemented with 5% (vol/vol) fetal bovine serum (GIBCO-Life), 0.2 µg/mL porcine FSH (Folltropin-V; Bioniche, Belleville, ON, Canada), 2 µg/mL porcine LH (Lutropin-V; Bioniche), and 50 µg/mL gentamicin sulfate under mineral oil at 39 °C for 22 hours in an atmosphere of humidified CO_2 (5%) in air [37].

In all experiments, oocyte meiotic maturation was assessed on a 10% of the cohort of oocytes by analyzing the chromosomal configuration in metaphase II by means of the Tarkowski technique [38], Giemsa staining and assessment at the optical microscope [39]. In experiments done with denuded oocytes, only those that had completed extrusion of the first polar body were included in the analysis. In biological assays in which oocytes were preincubated with anti–E-cadherin antibody and control
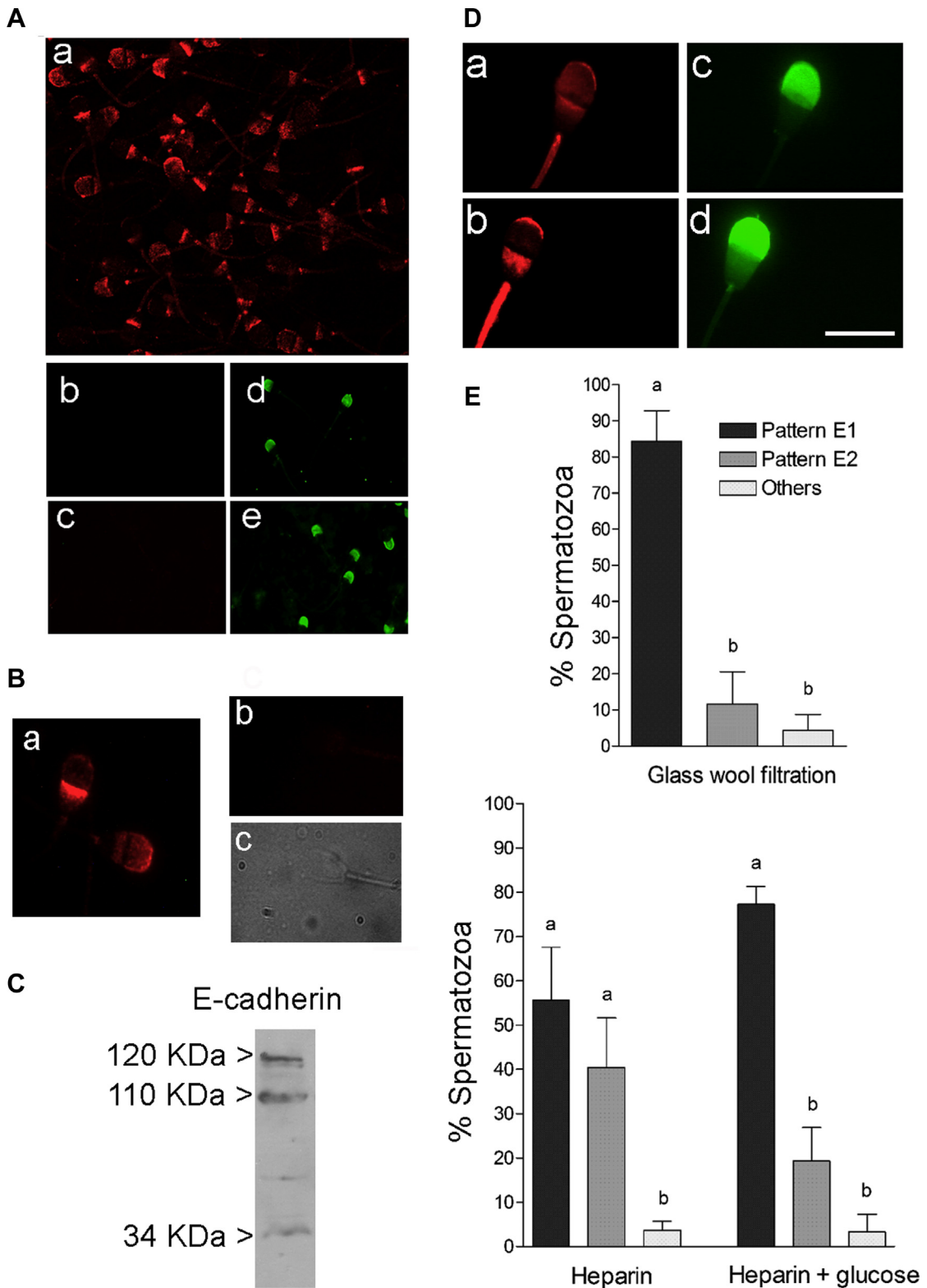
Fig. 1. Immunolocalization of E-cadherin in acrosome-intact bull spermatozoa. (A) Indirect immunofluorescence analysis of frozen-thawed motile bull spermatozoa selected by glass wool filtration, fixed and incubated with anti–E-cadherin antibody (panel a); controls done with rabbit IgG (panel b) and with the anti–E-cadherin antibody preadsorbed with recombinant E-cadherin (panel c). Colocalization with FITC-Pisum Sativum Agglutinin in control samples (panels d and e, respectively). (B) Indirect immunofluorescence analysis of frozen-thawed motile bull spermatozoa selected by glass wool filtration, and incubated with anti–E-cadherin antibody before fixation (panel a); control condition where the first antibody was omitted (panel b) and corresponding brightfield image for the

IgG, cell viability was monitored by oocyte staining with 0.25% (wt/vol) Trypan blue in PBS supplemented with 1% BSA at 37 °C for 10 minutes.

2.6. Assessment of sperm viability, motility, and morphology

The percentage of live spermatozoa was determined using 0.5% (wt/vol) Eosin Y in Sp-TALP medium. The percentage of progressive motile sperm cells was determined in an aliquot of the cell suspension placed on a glass slide, and covered with a prewarmed coverslip. Evaluations were done under direct light microscopy at ×400 magnification in sperm aliquots recovered from all experimental conditions.

Sperm morphology was assessed in spermatozoa from fresh and frozen-thawed semen. Briefly, 10 μL aliquots of the sperm suspensions were smeared onto a slide and air dried. Sperm staining was done for 1 hour with 5% (vol/vol) Giemsa in Sorensen buffer, pH 7.2; excess of dye was removed by washing the cell with distilled water. Sperm cell scoring was done at ×1000 magnification using light microscopy.

2.7. Sperm processing, capacitation, and acrosomal exocytosis

To select a highly motile sperm suspension, fresh and frozen-thawed semen samples were subjected to glass wool filtration following a procedure recently described [40]. Aliquots of selected motile sperm suspensions recovered in the filtrate were subjected to *in vitro* sperm capacitation and induction of AE.

To achieve sperm capacitation, spermatozoa were incubated in Sp-TALP supplemented with 60 μg/mL heparin during 45 minutes for frozen-thawed semen samples [41] or with 10 μg/mL heparin for 4 hours for fresh semen samples [11], at 39 °C with 5% CO_2 in humidified air. In control aliquots, 5 mM glucose was added to the incubation medium [14] or, alternatively, heparin was omitted. At the end of the incubation, cell suspensions incubated with heparin were divided into two fractions: one was supplemented with 100 μg/mL of LPC to promote sperm AE; the other, in which the LPC was omitted, served as control. Samples were incubated for 15 minutes at 39 °C in an atmosphere of 5% CO_2 in air and high humidity [11]. Alternatively to using LPC, 3 μM of calcium ionophore A23187 in DMSO was added to induce AE [42]; aliquots supplemented with DMSO served as control.

In some cases, spermatozoa from frozen-thawed samples were subjected to a procedure reported to remove the acrosomal contents, and to expose the inner acrosomal membrane [21]. Briefly, selected highly motile spermatozoa were sonicated at least three times for 30 seconds at maximal power (Sonifier Cell Disruptor, model W140; Heat Systems-Ultrasonics, Inc., Plainview, NY, USA) until more than 90% of the sperm heads had been dissociated from the flagellum, a procedure monitored using optical microscopy. Sperm heads were isolated by filtration in a column of 80% Percoll (Amersham/GE) in Sp-TALP, and concentrated by centrifugation at 700× *g* for 15 minutes. The excess of Percoll was eliminated by dilution of the cell suspension in Sp-TALP, and centrifugation at 350× *g* for 5 minutes. Removal of the acrosomal content was confirmed by staining the fraction containing the sperm heads with FITC- Pisum sativum agglutinin (FITC-PSA) [43]. Sperm heads were subjected to immunodetection of E-cadherin using the same procedure described for whole cells.

2.8. Assessment of sperm acrosomal status

The proportion of acrosome-intact and acrosome-reacted (AR) spermatozoa was assessed after cell labeling with FITC-PSA [44]; parallel evaluations with Nomarski differential interference contrast microscopy were done, validating its use for the assessment of acrosomal integrity, as previously reported [45]. Aliquots of sperm cells recovered after each treatment were fixed with 2% (vol/vol) formaldehyde in Dulbecco-PBS (D-PBS), washed, smeared onto slides and air dried. Spermatozoa were incubated with 50 μg/mL FITC-PSA in PBS during 1 hour, washed, mounted, and analyzed at ×400 magnification using a Nikon epifluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) (excitation at 510 nm) coupled to an image analyzer (IPLab Scientific Imaging Software for Windows; BD). Cells were scored as follows: (1) "acrosome-intact" spermatozoa, showing a homogenous signal over the entire acrosomal region; (2) "acrosome-reacting (patchy)" spermatozoa, showing a wrinkled acrosomal border or presence of disruptions in the acrosome; and (3) "acrosome-reacted" spermatozoa, showing a signal over the equatorial segment or lack of fluorescence over the acrosomal region. Epithelial cadherin immunolocalization results in "acrosome-intact" and "acrosome-reacted" spermatozoa will only be shown in this report. All incubations were performed in darkness.

2.9. Assessment of sperm capacitation status

Aliquots (200 μL) of the sperm suspension were mixed with an equal volume of chlorotetracycline-HCl (CTC) solution (750 μM CTC in 20 mM Tris buffer, pH 7.8, supplemented with 130 mM NaCl and 5 mM ʟ-cysteine) and fixed with glutaraldehyde [46]. Samples were mounted with coverslips, protected from the light, and evaluated using an epifluorescence microscope at ×400 magnification (excitation at 400–450 nm). Spermatozoa were classified according to the three staining patterns, as follows:

control condition (panel c). (C) Western immunoblot analysis of protein extracts from 30 million spermatozoa and anti–E-cadherin (610181, BD) monoclonal antibody. (D) Detailed indirect immunofluorescence analysis of frozen-thawed motile bull spermatozoa selected by glass wool filtration, fixed and incubated with anti–E-cadherin antibody. Predominant E-cadherin staining patterns: E1, staining in the apical ridge, acrosomal cap, and proximal postacrosomal region (panel a); E2, strong signal in the apical ridge and postacrosomal region (panel b). Colocalization analysis with FITC-PSA (panels c and d, respectively). Bar = 10 μm. (E) Distribution analysis of E-cadherin immunostaining patterns (E1, E2, and others) in frozen-thawed glass wool selected spermatozoa (top panel), and in sperm cells incubated under capacitating (heparin) and control (heparin + glucose) conditions (bottom panel). [a,b] Different superscripts indicate significant differences between E-cadherin patterns (P < 0.05). E-cadherin, epithelial cadherin; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G.

a homogenous fluorescence signal over the entire head (noncapacitated cells, pattern F), a fluorescence staining over the entire acrosome (capacitated cells, pattern B), and a bright fluorescent band along the equatorial segment accompanied with a low fluorescence signal over the entire head (AR cells, pattern AR).

2.10. Immunodetection of E-cadherin and β-catenin using immunocytochemistry

Immunolocalization studies of E-cadherin were done in fresh and frozen-thawed spermatozoa from all experimental conditions (selected motile, in vitro capacitated and AR and controls), and in immature and IVM COCs, cumulus mass cells and denuded oocytes, and in OEC cultures.

Cells obtained as indicated above were fixed with 2% (vol/vol) formaldehyde (spermatozoa in D-PBS; COCs and OECs in M199 medium). Protocols for immunolocalization of β-catenin included an additional step of cell permeabilization with 0.1% (vol/vol) Triton X-100 in PBS during 15 minutes at 4 °C. Nonspecific binding sites were blocked by cell incubation with a solution of PBS containing 1% (vol/vol) normal goat serum, 0.5% (wt/vol) BSA, 0.01% (vol/vol) Triton X-100, and 100 mM glycine at room temperature for 30 minutes. Immediately after, cells were placed for 16 to 18 hours with anti–E-cadherin or anti–β-catenin overnight at 4 °C. Unbound antibodies were washed with PBS, and Cy3-conjugated rabbit or mouse IgG or FITC in PBS with 4% BSA (wt/vol) was added as secondary antibody for 1 hour at room temperature in darkness. Samples were mounted with Vectashield antifade solution, and observed in an epifluorescence microscopy at ×400 magnification. In some cases, cells were analyzed in a Nikon laser confocal microscope C1; images were acquired using an objective of 60×/1.40 oil, excitation and/or emission: 488 nm/515 to 530 nm and 544 nm/570 LP.

Colocalization studies of sperm acrosomal contents with FITC-PSA and of cell DNA content with Hoechst 33342 were performed when indicated. In some cases, gamete incubation with the anti–E-cadherin antibody H-108 was carried out before cell fixation.

2.11. Immunodetection of E-cadherin and β-catenin by immunohistochemistry

Tissue sections from the oviduct ampulla and isthmus regions were fixed with 10% formaldehyde in PBS during 6 hours, dehydrated and paraffin embedded; ultrathin sections were mounted for further analyses. Hematoxylin–eosin counter staining was done using standard procedures in all immunohistochemical evaluations. Staining protocols for control samples included incubation with Hoechst 33342 (10 μg/mL in PBS for 5 minutes at room temperature) for nuclear DNA staining.

2.12. Western immunoblotting

Protein extracts from selected motile spermatozoa recovered in the glass wool filtrate, and from isolated cumulus cells and denuded oocytes from IVM COCs were prepared in Laemmli sample buffer containing 2% SDS (wt/vol) and 5% β-mercaptoethanol (vol/vol), run on 10% SDS-PAGE and electrotransfered onto nitrocellulose membrane (Hybond; Amersham/GE) as previously described [28]. The amount loaded for each cell type is indicated in the figure legend. Detection of E-cadherin and β-catenin protein forms was achieved after membrane incubation with specific antibodies (610181: 0.25 μg/mL; 610153: 1.25 μg/mL), followed by incubation with HRP-conjugated horse antimouse or HRP-conjugated goat antirabbit IgG secondary antibodies and development with enhanced chemiluminescence (ECL kit; Amersham/GE) and membrane exposure to X-ray films.

2.13. Sperm interaction assays with OEC, COCs, and denuded oocytes. Effect of anti–E-cadherin blocking antibodies upon cell–cell interaction

2.13.1. OEC and spermatozoa cocultures

Motile spermatozoa ($0.5–1 \times 10^6$ sperm per mL of Sp-TALP per well) were added to the OEC monolayers in a total volume of 500 μL in each well. After 1 hour of cell coincubation, unbound spermatozoa were removed by extensive washing. This sperm population was recovered and concentrated by centrifugation and subjected to evaluation (sperm capacitation and acrosomal status; E-cadherin immunolocalization). Quantification of spermatozoa bound in the cocultures was done after addition of a solution of Hoechst 33342 in Sp-TALP medium to the cocultures previously fixed during 2 minutes with 2% (wt/vol) paraformaldehyde in PBS. The amount of spermatozoa bound to the OECs, and their acrosomal and capacitation status, was scored after counting at least 10 fields at ×400 magnification using a Nikon Optiphot microscope equipped with epifluorescence optics (Nikon, Japan).

Spermatozoa bound to OEC monolayer were released after adding 100 μg/mL heparin in Sp-TALP medium during a 10-minute incubation at 39 °C in 5% CO_2 in humidified air, as previously described [34]. At least 200 spermatozoa were counted and evaluated (sperm acrosomal and capacitation status; E-cadherin immunolocalization).

To test the effect of anti–E-cadherin antibodies upon sperm–OEC interaction, 15 minutes before addition to the OEC monolayers, glass wool selected spermatozoa at a 1×10^6 sperm per mL concentration were preincubated with 70 μg/mL of purified DECMA-1 anti–E-cadherin antibody or purified IgG from normal rat serum (control). Sperm cells were then transferred to allow interaction with the confluent OEC monolayers, and incubated for 1 hour (final concentration: 5×10^5 spermatozoa per mL). At the end of cell coincubation protocol, unbound spermatozoa were removed, and cocultures were subjected to DNA-staining with Hoechst 33342 as described previously. The number of spermatozoa associated with the OEC under each experimental condition was scored using fluorescent microscopy, as previously indicated.

2.13.2. IVF using COC and denuded oocytes

Highly motile sperm cells were selected from frozen-thawed samples by glass wool filtration, and placed for 45 minutes under capacitating conditions in IVF medium
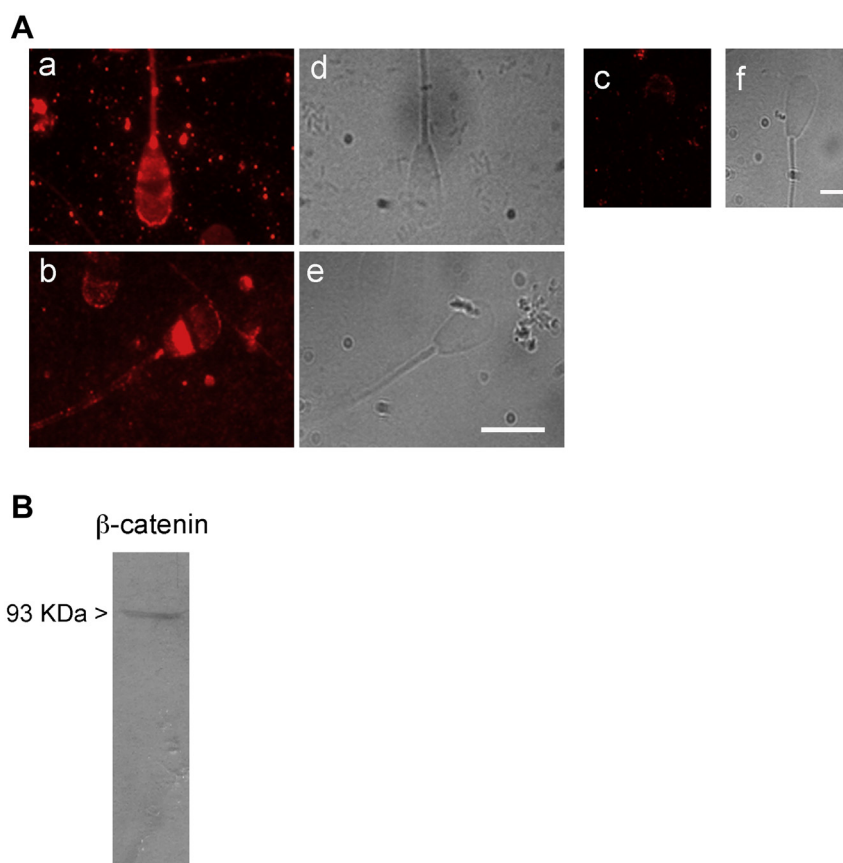
Fig. 2. Immunolocalization of β-catenin in acrosome-intact bull spermatozoa. (A) Indirect immunofluorescence analysis of frozen-thawed motile bull spermatozoa selected by glass wool filtration, fixed and incubated with anti–β-catenin antibody (panels a and b). Control done with rabbit IgG (panel c). β-catenin pattern B1: apical ridge, acrosomal cap, and postacrosomal region (panel a), and B2: apical ridge and postacrosomal region (panel b). Corresponding brightfield images (panels d, e, and f). Bar = 10 μm. (B) Western immunoblot analysis with anti–β-catenin (610153, BD) on protein extracts from 30 million spermatozoa.

at a concentration of 10×10^6 spermatozoa per mL. Sperm cells were incubated for additional 15 minutes with 35 or 70 μg/mL of purified anti–E-cadherin antibody DECMA-1 or control rat IgG. *In vitro*–matured COC or denuded oocytes (identified by presence of the first polar body) were inseminated with control (IgG) or treated (anti–E-cadherin) spermatozoa to a final concentration of 5×10^6 spermatozoa per mL. After a 5-hour incubation at 39 °C in 5% CO_2 in humidified air, oocytes were washed by several passages in IVF medium to remove loosely bound spermatozoa, cumulus cells, and excess of antibody, and left for additional 13 to 14 hours. Oocytes were labeled with 10 μg/mL of Hoechst 33342 in IVF medium during 5 minutes, mounted, and scored. Fertilization was confirmed by the presence of a decondensed sperm head or pronuclear formation; contrasting, the presence of the oocyte metaphase II was indicative of lack of fertilization. Oocyte viability was evaluated by means of Trypan blue staining, as indicated previously.

2.13.3. Sperm–oolemma binding assay

The sperm–oolemma binding assay was carried out essentially as previously described [47]. Briefly, highly motile spermatozoa were incubated under capacitating conditions for 45 minutes at 39 °C in 5% CO_2 in humidified air; the sperm AE was stimulated by incubation with 100 μg/mL LPC for 15 minutes. This treatment was followed by a 15-minute incubation with 70 μg/mL purified DECMA-1 anti–E-cadherin antibody or control rat IgG. Mechanically denuded IVM oocytes were treated with 0.5% (wt/vol) pronase in IVF medium to remove the ZP. Then, 5×10^3 sperm cells were added to 50 μL drops of IVF medium containing 10 oocytes, and incubated at 39 °C in 5% CO_2 in humidified air for 1 hour. After removal of nonspecifically bound spermatozoa by extensive washing, oocytes were fixed with 1% (wt/vol) paraformaldehyde in PBS for 5 minutes, stained in suspension with 1 μg/mL Hoechst 33342 in IVF medium, placed onto a microscope slide, and stained for 1 hour with FITC-PSA. At the end of the incubation, oocytes were mounted and analyzed under fluorescent microscopy at ×400 magnification to assess the number of spermatozoa bound (Hoechst) and their acrosomal status (FITC-PSA). In experiments run during optimization of the procedure, it was established that under the conditions assayed, only AR spermatozoa were bound to the oolemma.
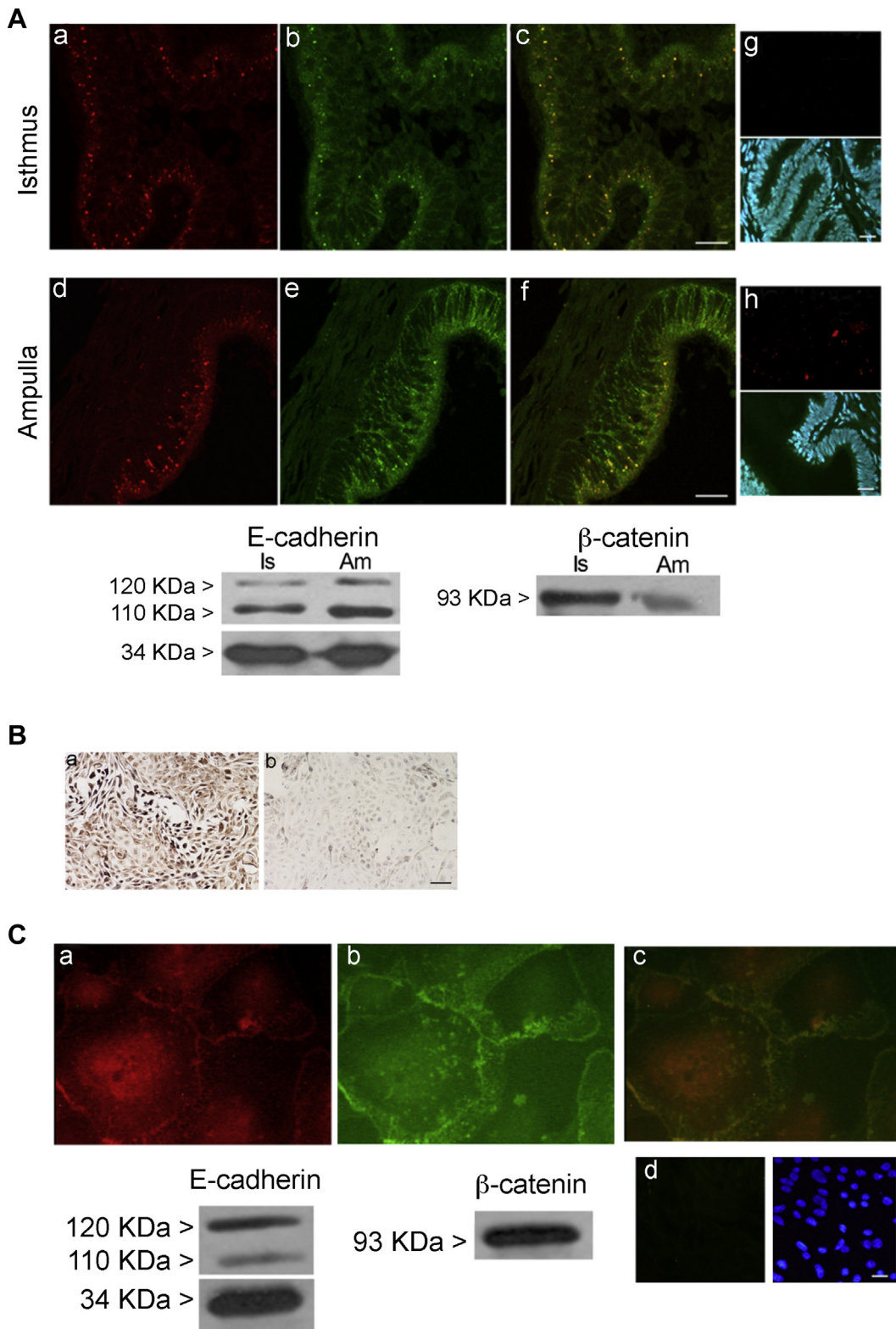
Fig. 3. Immunodetection of E-cadherin and β-catenin in the cow oviduct. (A) Immunohistochemical localization of E-cadherin (H-108, Santa Cruz Biotech, and antirabbit-Cy3) (panels a and d), β-catenin (610153, BD Bioscience, and antimouse-FITC) (panels b and e) and merged (panels c and f) images on oviduct tissue sections: isthmus (panels a–c) and ampulla (panels d–f). Confocal microscopy analysis. Bar = 20 μm. Control: panels g and h = IgG from normal mouse and rabbit sera (top); cell nuclei are stained with Hoechst 33342 (bottom). Confocal microscopy analysis. Bar = 50 μm. Western immunoblotting of total protein extracts (50 μg) from isthmus (Is) and ampulla (Am) oviduct tissues. Evaluations were done with anti–E-cadherin (610181, BD) and anti–β-catenin (610153, BD)

2.14. Statistical analysis

Results were expressed as mean ± standard deviation of the mean (SDM). Percentages of different sperm parameters and scores registered after assessment of immunocytochemical staining patterns were transformed by arcsine, and analyzed with ANOVA and post-ANOVA for comparisons of means. The Wilcoxon rank-sum test was applied to evaluate fertilization rate using COCs and denuded oocytes, the number of sperm bound to the oolemma and to OEC, and sperm release by heparin. In all cases, a P < 0.05 was considered of statistical value. The Statistix 8 program (Analytical Software, Tallahassee, FL, USA) was used for data analysis.

3. Results

3.1. Immunolocalization of E-cadherin in noncapacitated and capacitated spermatozoa

Frozen-thawed cell suspensions recovered after glass wool filtration was enriched in highly motile acrosome-intact and noncapacitated sperm cells [40]. These sperm suspensions were subjected to fluorescence immunocytochemistry of E-cadherin. A strong signal for the adhesion protein was found in a high proportion of the sperm cells in all samples analyzed (Fig. 1A, panel a). Staining was specific, because no signal was obtained in protocols replacing the anti–E-cadherin antibody with the same amount of purified control IgG or with the primary specific antibody preadsorbed with rE-cadherin (Fig. 1A, panel b and c, respectively). A specific signal was also obtained when spermatozoa were incubated with the first antibody before cell fixation, suggesting E-cadherin localization in the sperm plasma membrane (Fig. 1B, panel a). Western immunoblot analysis of protein extracts from spermatozoa freed of seminal plasma revealed a specific signal for the 120 KDa full-length E-cadherin (Fig. 1C); additional forms of lower molecular weight (110 and 34 KDa). These truncated protein forms most likely resulted from proteolytic processing by specific proteinases, as previously reported in the human model [28].

In whole spermatozoa, two predominant patterns of E-cadherin localization were identified: E1-pattern, depicting a signal for the adhesion protein in the apical ridge and proximal postacrosomal region and a less intense label in the acrosomal cap; E2-pattern, with a strong label in the apical ridge and whole postacrosomal region, and a faint or absent signal over the acrosomal region (Fig. 1D, panels a and b, respectively). Spermatozoa were classified as intact with the PSA lectin in both E1 and E2 patterns (Fig. 1D, panels c and d, respectively). In addition to the sperm head, E-cadherin was immunodetected in the flagellum of all spermatozoa, although the signal intensity varied with the antibody lot. In the glass wool filtrate, a high percentage of sperm cells depicted the E1-pattern (E1 = 84 ± 9%; E2 = 12 ± 9%; mean ± SDM; n = 16; Fig. 1E, top panel). A similar high proportion of sperm depicting the E1-pattern was also observed in filtrates from fresh semen samples (E1 = 79 ± 28%, E2 = 19 ± 30%; n = 3).

In spermatozoa incubated under capacitating conditions with heparin, the same E-cadherin localization patterns were identified, although changes in their abundance were detected. Although a decrease in the percentage of sperm depicting the E1-pattern was observed after incubation with heparin (+heparin = 56 ± 12%, heparin + glucose = 77 ± 4%; n = 4; P < 0.05), a concomitant increase in the percentage of spermatozoa depicting the E2-pattern was observed (+heparin = 40 ± 11%, heparin + glucose = 19 ± 8%; P < 0.05; Fig. 1E, bottom panel). An additional control done in parallel in which heparin was not added to the sperm capacitation medium gave similar results to the (heparin + glucose) control condition (−heparin: E1 = 79 ± 5%, E2 = 14 ± 1%; n = 4).

A similar trend was found when spermatozoa from fresh semen were evaluated (E1-pattern: heparin = 64 ± 3% vs. heparin + glucose = 89 ± 8%; E2-pattern: heparin = 30 ± 2%, heparin + glucose = 11 ± 8%; n = 3; P < 0.05).

3.2. Immunolocalization of β-catenin in noncapacitated and capacitated spermatozoa

Immunolocalization analysis of β-catenin was done in noncapacitated and capacitated frozen-thawed spermatozoa using fluorescence immunocytochemistry. Two patterns were distinctive and predominant: B1, with a signal of similar intensity in the acrosomal and postacrosomal regions and in the apical ridge, and B2, with the same protein distribution as B1 but signal of higher intensity in the postacrosomal region (Fig. 2A, panels a and b). The B1-pattern was predominant in spermatozoa recovered immediately after glass wool filtration (B1 = 78 ± 8; B2 = 20 ± 4). After incubation under capacitating conditions, a specific decrease in the percentage of spermatozoa depicting the B1-pattern was observed, with a concomitant increase in the percentage of sperm cells stained with the B2-pattern (+heparin: B1 = 60 ± 1; B2 = 35 ± 9; heparin + glucose: B1 = 74 ± 6; B2 = 16 ± 7). In total protein sperm extracts, β-catenin was immunodetected as a 93 KDa protein form (Fig. 2B).

3.3. E-cadherin localization and sperm interaction with OEC

Based on the changes observed in E-cadherin localization on spermatozoa incubated for in vitro capacitation under defined conditions, in addition to the fact that

antibodies. The estimated molecular mass of each protein form is indicated. (B) In vitro cultured bovine oviduct epithelial cells (OECs). Immunocytochemical localization of cytokeratin (clone AE1/AE3 and DAKO LSAB + System-HRP detection system) (panel a); control: IgG from normal mouse serum (b). Cell nuclei staining with hematoxylin-eosin. Bar = 50 μm. (C) In vitro cultured bovine OECs. Immunocytochemical localization of E-cadherin (610181, BD and antimouse-FITC) (panel a) and β-catenin (AB19022, Upstate/Chemicon-Millipore and antirabbit-Cy3) (panel b) in oviduct epithelial cell monolayers; merged image (panel c). Control: IgG from normal rabbit and mouse sera (panel d). Cell nuclei stained with Hoechst 33342 (right). Confocal microscopy analysis. Bar = 20 μm. Western immunoblotting of total protein extracts (50 μg) from OEC monolayers, using anti–E-cadherin (610181, BD) and anti–β-catenin (610153, BD) antibodies. E-cadherin, epithelial cadherin; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G.
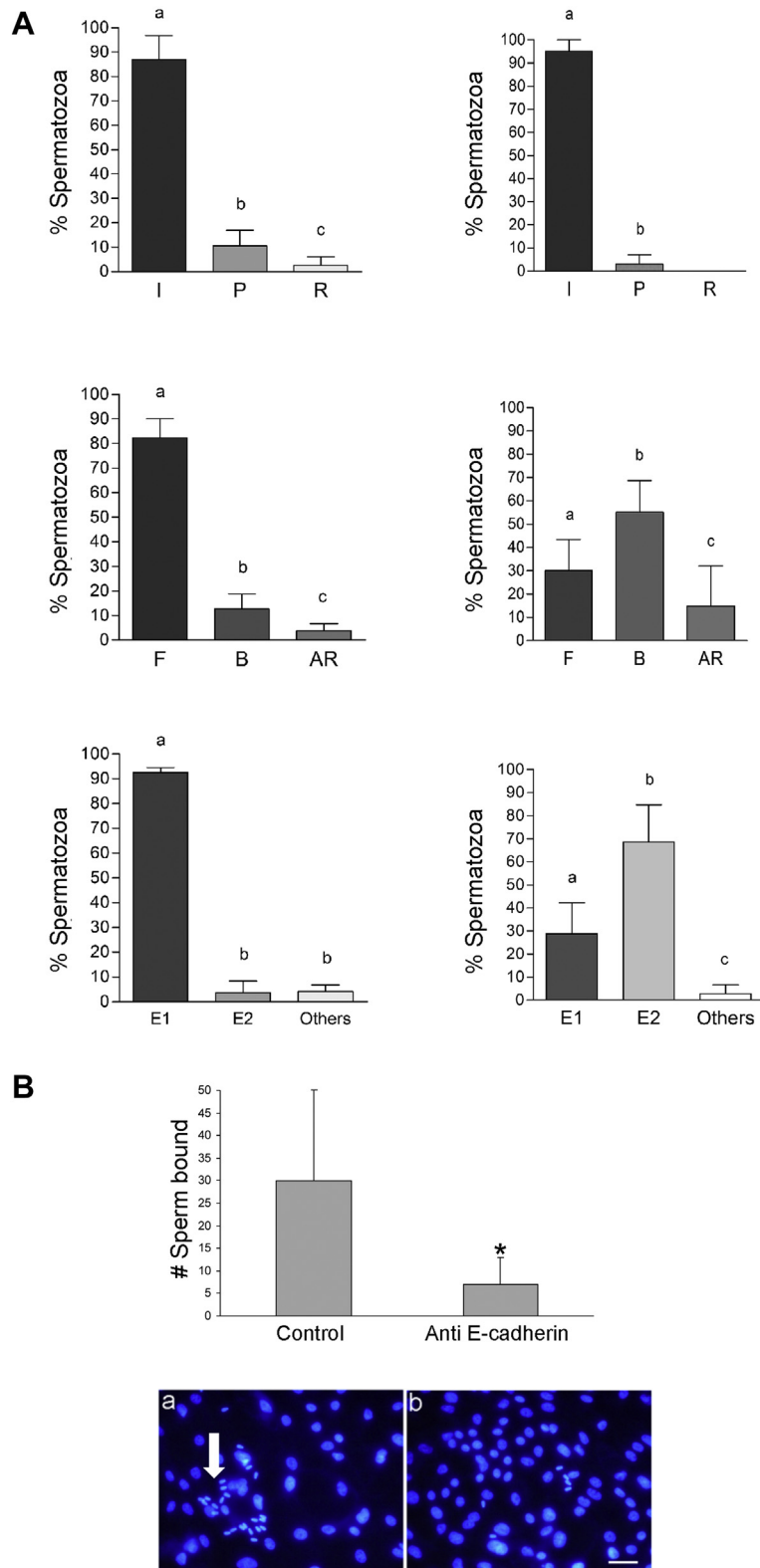
Fig. 4. Sperm interaction with OEC. Acrosomal and capacitation status and E-cadherin immunolocalization. Effect of DECMA-1 antibody upon sperm–OEC interaction. (A) Top panel: assessment of the sperm acrosome status by FITC-Pisum Sativum Aggutinin staining in spermatozoa recovered after glass wool filtration (left top panel) and in spermatozoa released from the OEC after addition of heparin (right top panel). Distribution of different patterns: acrosome-intact (I), acrosome-reacting (P; patchy), and acrosome-reacted (R) spermatozoa. [a,b,c] Different superscripts indicate significant (P < 0.05) differences in acrosome status

E-cadherin participates in cell–cell adhesion events, and also that there is a relationship between sperm interaction with the OEC and capacitation-related events, we hypothesized that E-cadherin localized to the plasma membrane overlying the acrosomal region participates in sperm–OEC interaction and formation of the sperm reservoir, and changes in its localization are related to sperm dissociation from OEC.

To test this hypothesis, the expression of E-cadherin and β-catenin in the cow oviduct was first evaluated. Histologic sections of isthmus and ampulla regions of the oviduct were recovered from cows in the follicular and luteal phases of the reproductive cycle. As expected, differences were observed between both phases of the cycle in hematoxylin–eosin stained sections, with a predominant ciliated epithelium observed in the follicular phase and luteal phase characterized by the presence of a secretory epithelium (data not shown). Sections subjected to immunolocalization studies of E-cadherin and β-catenin revealed a specific signal in epithelial cells in both regions of the oviduct, and in both phases of the reproductive cycle (not shown). Moreover, the signal was observed in both, basolateral and apical, regions of the epithelial cells (Fig. 3A). Western immunoblot analysis of protein extracts from ampulla and isthmus regions of the oviduct specifically revealed the 120 KDa E-cadherin full-length protein and truncated forms of 110 and 34 KDa. Immunostaining protocols applied to detect β-catenin resulted in the detection of a protein form of the expected molecular weight (93 KDa; Fig. 3A).

Bovine OEC were isolated and cultured following the procedure described in Section 2. Cells in culture displayed a characteristic epithelial polygonal shape and rarely overlapped; confluence was achieved around 7 days after starting the culture in the plastic surface. Epithelial cell purity of the culture was confirmed by immunocytochemical analysis using anti-cytokeratin antibodies (Fig. 3C). Epithelial cadherin and β-catenin localized on cells from cultured OEC monolayers, depicting a signal in the cell membrane and the cytoplasm. Western immunoblot analysis of OEC protein extracts showed a specific signal for E-cadherin and β-catenin protein forms of the expected molecular weight (120 and 93 KDa, respectively) and some additional degradation products of E-cadherin (110 and 34 KDa) (Fig. 3C).

Taking into account that spermatozoa in the oviduct reservoir have been reported to have intact acrosomes, and classified mainly as noncapacitated [5,6,48], frozen-thawed spermatozoa were processed using glass wool filtration to obtain sperm suspensions that resemble cell populations arriving to the oviduct isthmus. The sperm suspensions recovered in the filtrate had a high proportion of motile cells with intact acrosomes (% sperm depicting I pattern (FITC-PSA staining = 87 ± 10; Figure 4A, left upper panel), and were mainly noncapacitated (% sperm depicting F pattern [CTC-staining]: 83 ± 13; Fig. 4A, left middle panel). Moreover, over 90% spermatozoa from this suspension displayed the characteristic E1-pattern of E-cadherin immunolocalization (93 ± 2%; Fig. 4A, left lower panel).

Spermatozoa from these suspensions were placed in contact with the OEC monolayers. The interacting spermatozoa were found to have a strong flagellar beating; moreover, OEC–sperm cell contact appeared to involve the sperm acrosomal region, as suggested by a preliminary study done using transmission electron microscopy (data not shown). The sperm acrosomal and capacitation status of cells bound to the OEC monolayer were analyzed in fixed preparations by means of the FITC-PSA and CTC staining, respectively. Of all, 96 ± 4% sperm cells were found to have intact acrosomes, and 88 ± 10% spermatozoa were scored as noncapacitated (I and F pattern, respectively). Sperm bound to the OEC were also immunoreactive to E-cadherin, with a signal in the apical and acrosomal region of the sperm head (E1-pattern), although quantification could not be achieved, because OECs in coculture were also immunoreactive to E-cadherin, and made difficult the assessment. Contrasting with the characteristics of bound sperm cells, 30 ± 9% of unbound spermatozoa recovered in the medium at the end of 1 hour of cell coculture and stained with CTC depicted the F pattern of noncapacitated cells, and the other spermatozoa were classified either as capacitated (B pattern: 32 ± 3%) or acrosome-reacted (AR pattern: 37 ± 7%; n = 3) cells. Epithelial cadherin signal was absent or restricted to the postacrosomal region in this sperm subpopulation (data not shown).

Sperm–OEC cocultures were incubated for 15 minutes in the presence of heparin to induce the release of bound spermatozoa. This procedure resulted in a significant decrease in the number of the sperm bound to the OEC compared with the cocultures control incubated under the same conditions but omitting heparin (% released spermatozoa after addition of heparin; bull 1: 79 ± 8%; bull 2: 67 ± 4%; bull 3: 58 ± 22%; and bull 4: 68 ± 1%; n = 14). Heparin-released spermatozoa were classified as acrosome-intact in most of the cases (97 ± 3%, n = 4; FITC-PSA staining Fig. 4A, right upper panel). Contrasting with spermatozoa bound to the OEC, the released sperm cell suspensions were enriched in spermatozoa classified as

patterns scored. Results expressed as mean ± SDM; n = 5 assays. Middle panel: assessment of the sperm capacitation status by CTC staining in spermatozoa recovered after glass wool filtration (left panel) and in spermatozoa released from the OEC after addition of heparin (right panel). Distribution of different patterns: noncapacitated (F), capacitated (B), and acrosome-reacted (AR) patterns in spermatozoa from the glass wool selected cell population. [a,b,c] Different superscripts indicate significant (P < 0.05) differences in CTC patterns scored. Results are expressed as mean ± SDM; n = 5 assays. Bottom panel: distribution of E-cadherin patterns E1, E2, and others in spermatozoa recovered after glass wool filtration (left), and in spermatozoa released from the OEC after addition of heparin (right) by glass wool filtration. [a,b] Different superscripts indicate significant (P < 0.05) differences in E-cadherin patterns scored. Results are expressed as mean ± SDM; n = 5 assays. (B) Effect of anti–E-cadherin antibody upon sperm–OEC interaction. The number of bound spermatozoa to OEC in cocultures incubated in the presence of anti–E-cadherin antibody (clone DECMA-1, SIGMA; purified) or IgG form normal rat serum (control). A total of 10 fields (with up to 75 spermatozoa per field) were scored in each case. Results are expressed as mean ± SDM. ∗ P-value less than 0.05 between experimental conditions. Representative images from incubations done with IgG control (a) or anti–E-cadherin antibodies (b). Cell nuclei are stained with Hoechst 33342. Bar = 20 μm. CTC, chlorotetracycline; E-cadherin, epithelial cadherin; FITC, fluorescein isothiocyanate; OEC, oviduct epithelial cell; SDM, standard deviation of the mean.
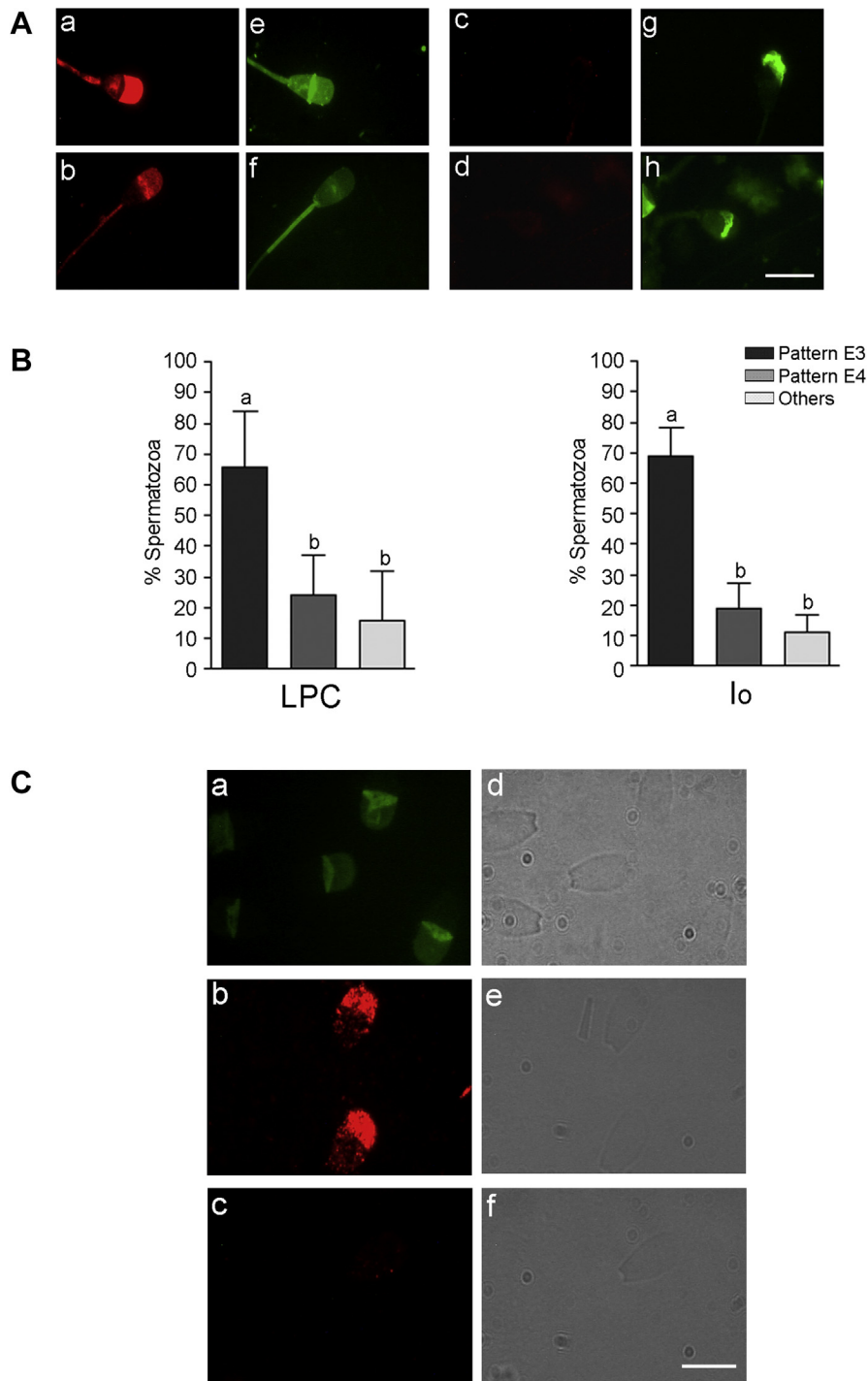
Fig. 5. Immunolocalization of E-cadherin in AR bull spermatozoa. (A) Indirect immunofluorescence analysis of frozen-thawed motile bull spermatozoa selected by glass wool filtration incubated under capacitating conditions and exposed to ʟ-α-lysophosphatidylcholine (LPC) to induce the AE. Anti–E-cadherin (panels a and b). Controls done with rabbit IgG (panel c) and with the anti–E-cadherin antibody preadsorbed with recombinant E-cadherin (panel d). Predominant E-cadherin staining patterns E3: staining in the inner acrosomal membrane (panel a), E4: staining in the equatorial segment (panel b). Colocalization with FITC-PSA (panels e–h, respectively). Bar = 10 μm. (B) Distribution analysis of E-cadherin immunostaining patterns (E3, E4, and others) in spermatozoa incubated under capacitating conditions followed by exposure to LPC or Ionophore A23187 (Io), and classified as "acrosome-reacted" spermatozoa after FITC-PSA staining. (C) Immunolocalization of E-cadherin in sonicated bull sperm heads. Indirect immunofluorescence analysis of frozen-thawed motile spermatozoa selected by glass wool filtration, sonicated, and subjected to immunodetection of E-cadherin (panel b) and control done with normal rabbit IgG (panel c). Staining of bull sperm heads with FITC-PSA (panel a). Corresponding brightfield images (panels d–f). Bar = 10 μm. AE, acrosomal exocytosis; E-cadherin, epithelial cadherin; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G.

capacitated (55 ± 14%, n = 8) at the CTC assay (Fig. 4A, right middle panel). With regard to E-cadherin localization, spermatozoa in these suspensions depicted the E2-pattern in 69 ± 16% of the cells (Fig. 4A, right lower panel). Changes in E-cadherin localization found in spermatozoa after interacting with OEC were similar to those observed in cells incubated *in vitro* under capacitating conditions in a defined medium.

To evaluate whether E-cadherin may participate in sperm–OEC interaction, selected motile spermatozoa recovered after glass wool filtration were preincubated with purified DECMA-1 anti E-cadherin monoclonal antibody, and later added to OEC monolayers to allow interaction. As shown in Figure 4B, an inhibitory effect of the E-cadherin antibody upon sperm–OEC interaction was achieved in assays using spermatozoa from frozen-thawed samples of four different bulls; the effect was evidenced by the significant (P < 0.001) reduction in the number of spermatozoa bound to the OEC and was specific, because it was not observed in spermatozoa preincubated with control rat IgG added at the same concentration as the DECMA-1 antibody (number of sperm bound to OEC: DECMA-1 = 6.7 ± 6.1 vs. control = 29.6 ± 20.1).

3.4. Fate of E-cadherin during acrosomal exocytosis

Once capacitated spermatozoa interact with the COC cell vestments, a series of changes occur that result in the induction of AE in response to physiological stimuli. To assess localization of E-cadherin in spermatozoa that have undergone AE, *in vitro* capacitated sperm cells were incubated with LPC-treated or calcium ionophore A23187-treated spermatozoa. Because only part of the sperm population undergoes acrosomal loss after exposure to the inducer (% AR spermatozoa, LPC = 48 ± 14%; control = 19 ± 8%; P < 0.05; A23187-calcium ionophore = 51 ± 14%; control = 19 ± 5%; P < 0.05; n = 4), colocalization analyses were done by staining with FITC-PSA (see Section 2) to determine the sperm acrosomal status in each sperm cell; AR spermatozoa were identified by a characteristic signal in the equatorial segment.

All AR spermatozoa lost the signal for E-cadherin in the apical ridge. Moreover, two main E-cadherin localization patterns were identified: E3-pattern, with a strong signal in the apical portion of the sperm head that would correspond to the inner acrosomal membrane; and E4-pattern, with a distinctive signal in the equatorial segment (Fig. 5A, a and b, respectively). A quantitative analysis done on AR frozen-thawed spermatozoa showed E3 as the predominant pattern, whether AE was induced with either LPC or A23187-calcium ionophore (Fig. 5B). The E3-pattern was the only one found in acrosome-reacting and AR spermatozoa recovered from fresh semen (data not shown).

To rule out the possibility that E3-pattern could result from immunodetection of E-cadherin from remnants of the sperm head plasma membrane, a protocol involving sperm sonication before immunodetection was carried out. This procedure has been reported to effectively remove the sperm plasma and outer acrosomal membranes and the acrosomal content of porcine and rat

spermatozoa [21]. Bovine sperm heads detached from the tails showed a strong signal in the equatorial segment in 100% of the cells when stained with FITC-PSA (Fig. 5C, a), confirming the effectiveness of the procedure. In these samples, 61 ± 2% (n = 3) of the sperm heads displayed a strong and specific signal for E-cadherin to the apical portion of the head (Fig. 5C, anti–E-cadherin: b; control IgG: c), reinforcing the idea of E-cadherin localization on the inner acrosomal membrane. The other sperm cells evaluated were negative to the immunostaining.

3.5. Immunolocalization of E-cadherin and β-catenin in COCs

Cumulus-oocyte complexes were subjected to immunocytochemical analyses to evaluate expression and localization of E-cadherin and β-catenin using confocal microscopy. Immature and IVM COC exhibited a specific highly intense E-cadherin signal mainly in the cell periphery, corresponding to the oocyte oolemma (Fig. 6A, left column, panels a and d, respectively). Moreover, β-catenin signal followed E-cadherin distribution (Fig. 6A, middle column, panels b and e). Colocalization studies using confocal microscopy showed overlapping of both signals (Fig. 6A, right column, merged image).

In mature oocytes, the signal for E-cadherin and β-catenin appeared slightly weaker in the membrane region localized near the polar body than in other regions of the cellular perimeter (panels d, e, and f). This distinct distribution contrasted with the continuous signal observed in the immature oocytes (panels a, b, and c). Moreover, the first polar body was not immunoreactive to E-cadherin (panels e and f, arrow). Staining was specific, as indicated by the lack of signal in protocols replacing the primary specific antibody by IgG (Fig. 6A panels g, h, and I, brightfield image).

The cumulus and corona cells recovered by mechanical dissection of IVM COC were also immunoreactive to both proteins (Fig. 6B).

Western immunoblot analysis of E-cadherin and β-catenin was done in protein extracts from cumulus cells and oocytes from immature and IVM COCs. These cells complexes revealed the presence of the 120 KDa E-cadherin full-length form in all samples evaluated with different signal intensity; in addition, the 34 KDa truncated E-cadherin fragment was also observed in some cases. In all cell extracts, β-catenin was immunodetected as a 93 KDa protein form (Fig. 6C).

3.6. Effect of anti–E-cadherin antibodies upon bovine sperm oocyte interaction

To evaluate the participation of E-cadherin in sperm interaction with the COC, a set of biological assays were carried out. The experimental design involved sperm preincubation with the DECMA-1 anti–E-cadherin monoclonal antibody (or an equivalent amount of control IgG in the control condition) followed by evaluation of their performance in IVF and sperm–oolemma assays.

3.6.1. IVF analysis

Highly motile spermatozoa recovered from glass wool filtration of frozen-thawed cells were preincubated under
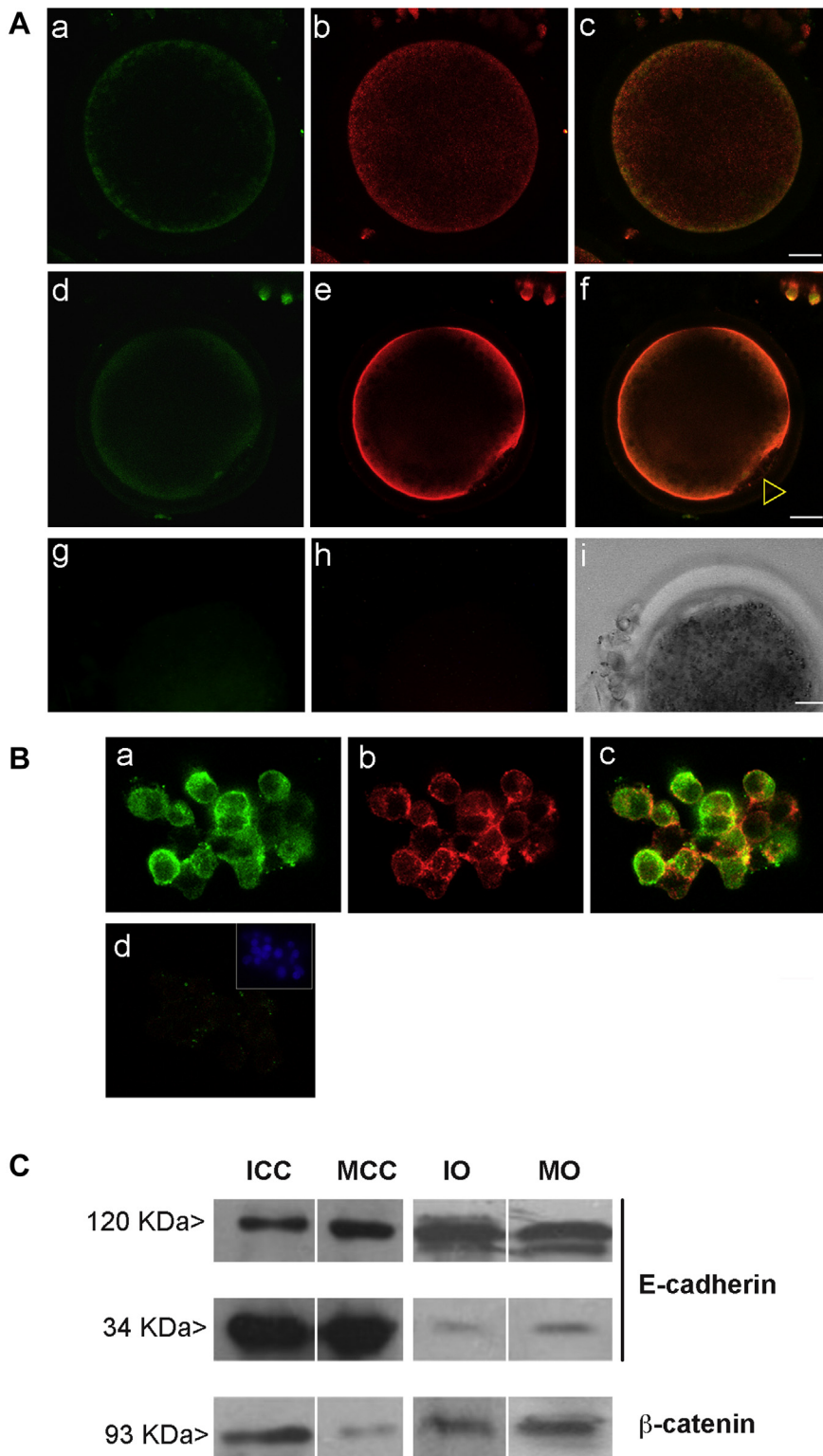
Fig. 6. Immunolocalization of E-cadherin and β-catenin in cumulus-oocyte complexes (COCs). Immunostaining procedures were applied to a total of 139 immature class A and 190 IVM COCs, including those incubated with the first specific antibody or IgG control before or after cell fixation with or without permeabilization. (A) Representative image of class A immature (panel a) and IVM (panel d) COCs fixed and subjected to immunocytochemical localization of E-cadherin (left panels) and β-catenin (middle panels) using confocal microscopy. Colocalization of both proteins in ooplasma and oolemma with absence in the first polar body (panel f, yellow arrow) is shown in the merge images (right panels). Bar = 20 μm. Rabbit and mouse IgG control (panels g and h, respectively) evaluated under fluorescence microscopy and its correspondent brightfield (panel i). Bar = 20 μm. (B) Immunolocalization of E-cadherin and β-catenin in

capacitating conditions with purified IgGs from the DECMA-1 anti–E-cadherin antibody or normal rat serum (control), and challenged with IVM COC or *in vitro* denuded mature oocytes. Incubations done with 35 μg/mL of anti–E-cadherin antibody resulted in a significantly lower fertilization rate of COC (P < 0.05) and denuded oocytes (P < 0.05; Table 1) than controls. IVF experiments done with COC and 70 μg/mL of the DECMA-1 IgGs, showed a higher inhibitory effect of the antibody, reaching levels similar to those obtained with denuded oocytes (57.8 ± 8.1% vs. 57.0 ± 15.2%, respectively). In all cases, gamete viability or sperm motility were not affected by exposure to the antibodies (data not shown).

3.6.2. Sperm binding to oolemma

To further evaluate the participation of E-cadherin in adhesion events during sperm–oocyte interaction, the effect of sperm preincubation with the anti–E-cadherin antibody upon sperm binding to the oocyte plasma membrane was evaluated. Sperm interaction with the oolemma in the presence of the anti–E-cadherin antibody was significantly impaired, as reflected by the lower (P < 0.05) number of spermatozoa bound to ZP-free oocytes (Table 1).

4. Discussion

This report describes, for the first time, a thorough analysis on immunolocalization of E-cadherin and β-catenin in bovine spermatozoa, COC, and OEC. Moreover, it provides evidence of E-cadherin involvement in sperm interaction with OEC and COC, by demonstrating the ability of a well-established blocking monoclonal anti–E-cadherin antibody to specifically impair sperm interaction with both OEC and COC in standard *in vitro* assays.

Immunodetection studies in bull spermatozoa were done with a commercial polyclonal anti–E-cadherin antibody (H-108; Santa Cruz Biotech) developed toward an extracellular domain of human E-cadherin. The high amino acidic sequence identity between human and bovine E-cadherin supported the use of the antibody for immunodetection studies. The strong signal obtained in fresh and frozen-thawed spermatozoa, together with the lack of signal obtained when using either the antibody preadsorbed with the rE-cadherin or rabbit IgG, instead of the primary antibody, confirmed the antibody ability to specifically recognize bovine E-cadherin. The specific signal for the 120 kDa full-length protein in sperm extracts was in line with the immunodetection studies in whole cells.

A similar E-cadherin distribution observed in spermatozoa incubated with the first antibody before or after cell fixation strongly suggests E-cadherin localization in the sperm plasma membrane, as previously reported in the human model [28]. Differences in the proportion of spermatozoa depicting E1 and E2 patterns of E-cadherin localization in association to sperm incubation under capacitating conditions, and the ability to prevent them by either omitting heparin or adding glucose, lead us to propose changes in E-cadherin signal distribution to capacitation-related events. Differences between E1 and E2 patterns may result from E-cadherin relocalization; in this regard, modifications in the plasma membrane of bovine spermatozoa during sperm capacitation have been initially evidenced in studies using lectins [49] and more recently characterized for proteins and lipids [7,50,51]. In somatic cells, a highly mobile diffuse E-cadherin protein pool was found at the plasma membrane in cells not engaged in cell adhesion [52], and caveolin-1 was reported to colocalize with E-cadherin, and to promote its membrane localization [53]. Caveolin-1 has been detected in spermatozoa, and was found to re-colocalize during capacitation from the acrosome to the apical ridge [50]. Other mechanisms could involve masking of E-cadherin epitopes by other proteins and/or E-cadherin processing with a concomitant loss of epitopes recognized by the antibody.

In vitro studies using OEC monolayers have been a useful tool to assess the molecular events of the oviduct cells in sperm selection and maintenance of the adhesion, and in their release [48,34] and have proven the ability of OEC to select morphologically normal noncapacitated acrosome-intact spermatozoa [5,6,48]. In addition, these studies have also shown that the mechanisms involved in the release of spermatozoa from the OEC cocultures are linked to molecular events of sperm capacitation [48,12]. Using this experimental design, the shift in E-cadherin localization from E1 to E2 pattern was also observed in spermatozoa released from cocultures, suggesting the involvement of the adhesion protein in assembly or disassembly of the oviduct–sperm reservoir, as part of the capacitation-related events. Regarding the reservoir assembly, structural studies have pointed at the interaction between the rostral portion of the acrosome of the bull sperm and the OEC microvilli [3,36,54]. Several studies have suggested the participation of species-specific carbohydrate recognition in sperm–oviduct interaction [55–57], and specific proteins have been proposed to play a role in the formation of the oviduct sperm reservoir; among them are seminal plasma protein PCD-109 (BSP-A1/A2) [58,59], BSP-A3, and BSP-30-kDa–related proteins [60] in bovine spermatozoa. These proteins appear to be lost from the plasma membrane overlying the head during sperm capacitation, and this event is associated to a decrease in OEC–sperm binding [59,61,62]. Epithelial cadherin may be part of this complex array of membrane proteins participating in this event to assure sperm association and/or release from the oviduct epithelium during sperm transit through the female reproductive tract.

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cumulus cells (panels a and b, respectively) isolated from IVM bovine oocytes. Colocalization of both proteins is shown in the merge image (panel c). A representative image of confocal microscopy analysis is shown. Rabbit and mouse IgG control (panel d) evaluated under fluorescence microscopy, and its correspondent cell nuclei stained with Hoechst 33342 (inset). (C) Western immunoblot analysis using anti–E-cadherin (610181, BD) and anti–β-catenin (610153, BD) antibodies on protein extracts of mechanically isolated cumulus cells recovered from immature (ICC) and IVM COC (MCC; 50 μg total protein), and of denuded oocytes from immature (IO) and IVM oocytes (MO; 20 COCs or denuded oocytes in each case). E-cadherin protein forms of 120 and 34 kDa are shown. E-cadherin, epithelial cadherin; IgG, immunoglobulin G.

Table 1
Results of IVF and sperm–oolemma binding assay done with DECMA-1 anti–E-cadherin antibody and control rat IgG.

Assay	DECMA-1 (n)	Control IgG	P
IVF			
COC	68.8 ± 10.4 (115)	90.7 ± 3.1 (119)	<0.05
Denuded oocytes	57.0 ± 15.2 (122)	89.2 ± 9.8 (66)	<0.05
Sperm–oolemma binding	2.2 ± 1.1 (35)	11.1 ± 4.8 (35)	<0.05

Abbreviations: COC, cumulus-oocyte complex; E-cadherin, epithelial cadherin; IgG, immunoglobulin G.

Because E-cadherin is mainly involved in events mainly in a homophilic (same cadherin) cell–cell adhesion, its expression in the oviduct was assessed. The comparable expression of E-cadherin observed in the isthmus and ampulla tissue sections would be in agreement with previous reports describing sperm binding to the epithelium of both anatomic regions of the oviduct regardless the time of the cycle [63].

Cell suspensions recovered in the glass wool filtrate resemble those that encounter the oviduct epithelium (i.e., intact acrosomes) [49], and were adequate for the OEC cocultures. Bound spermatozoa had intact acrosomes, and were mainly classified as noncapacitated, in agreement with previous reports [49,64], supporting the use of this experimental model. Participation of E-cadherin in the formation of the reservoir could be proposed from the results of the competition assays, in which the DECMA-1 blocking anti–E-cadherin antibody significantly inhibited sperm binding to the OEC monolayers.

The release of spermatozoa bound to OEC was induced by the addition of heparin [12]. Released spermatozoa were found to be mainly acrosome-intact, as previously reported [49], but changed their functional status, reflected by a significant increase in the percentage of capacitated sperm cells, and in the proportion of spermatozoa depicting the E2-pattern of E-cadherin localization. The shift in E-cadherin localization may be associated with heparin and the induced increase in intracellular calcium [49], causing E-cadherin relocalization and/or proteolysis, as reported in somatic cells [65,66], allowing sperm release from the oviduct, thus contributing to the reservoir disassembly.

After capacitation, a strong signal was still detected for E-cadherin in the acrosomal ridge; and postacrosomal region. Epithelial cadherin could interact with its counterpart on the plasma membrane of cumulus cells, also immunoreactive to the adhesion protein in a classical hemophilic interaction. These somatic cells could act as holder of functional spermatozoa that arrive at the fertilization site, a role previously proposed for these cells [67]. Alternatively, if functional intact sperm cells interact with ZP components, E-cadherin in this structure could participate in this molecular event; in this regard, several ZP-binding proteins have been identified in purified apical plasma membrane preparations [52,68].

In AR spermatozoa, the signal for E-cadherin in the apical ridge was lost in all cells. Nevertheless, a specific and strong signal was observed on the apical sperm head region in a large proportion of cells that had completely lost the plasma and outer acrosomal membranes (monitored by simultaneous analysis with FITC-PSA). The use of the sonication procedure that exposes the inner acrosomal membrane [21] confirmed the results. The inner acrosomal membrane has been proposed to participate in interactions between AR spermatozoa and the ZP and its penetration and in sperm adhesion to the oolemma [15]. Other proteins, among them IAM38 [21], SAMP32 [69], SAMP14 [70], 26S proteasome [71], and complement regulatory proteins CD46, CD55, and CD59 [72] have been localized to this structure, and biochemical and functional data supports their involvement in sperm–oocyte interaction.

To further evaluate the effect of the anti–E-cadherin antibody upon adhesion events during sperm–oocyte interaction, IVF and sperm–oolemma assays were done. Immunolocalization studies in immature and IVM oocytes showed a specific E-cadherin signal in the oolemma. Epithelial cadherin distribution along the plasma membrane except in the region next to the first polar body would be consistent with a role for the adhesion protein in sperm binding before fusion, as shown for other proteins. The significant inhibitory effect of the DECMA-1 anti–E-cadherin antibody upon IVF of COC and denuded oocytes and upon sperm binding to the oolemma favors the notion of E-cadherin participation in sperm–oocyte adhesion.

The antibody may have interfered with the formation of E-cadherin cis-dimers, with their lateral clustering in the plasma membrane and/or with the interaction of E-cadherin with counterparts on the other cell [73]. The DECMA-1 anti–E-cadherin monoclonal antibody was selected for the *in vitro* cell–cell interaction studies, because this antibody has been widely accepted, and used as a tool to block E-cadherin mediated cell–cell adhesion in somatic cells [74–76] and compaction of preimplantation embryos [74]. The antibody was added at a concentration similar to that reported for other cell types [74]. Sperm interactions with the OEC and COC membrane vestments mediated by E-cadherin may be dynamically regulated through mechanisms previously characterized in somatic cells, among them Ser-Thr/Tyr phosphorylation mediated by Src kinases [77,78]; in this regard, expression of members of Src kinases have been reported in bull spermatozoa [79].

4.1. Conclusions

In conclusion, findings presented in this report constitute the first thorough analysis characterizing the immunolocalization of E-cadherin and β-catenin in bovine gametes and OEC, and provides evidence of the involvement of E-cadherin in cell–cell adhesion events during fertilization. The identification of molecules that participate in gamete interaction will greatly contribute to our understanding of this fascinating complex process.

Acknowledgments

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