Expression of epithelial cadherin in the human male reproductive tract and gametes and evidence of its participation in fertilization

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Epithelial cadherin (E-cadherin) has been involved in several calcium-dependent cell–cell adhesion events; however, its participation in gamete interaction has not been fully investigated. Our results have demonstrated expression of E-cadherin mRNA in the human male reproductive tract showing higher levels in the caput, corpus and cauda epididymis than in the testis. The mature 122 kDa E-cadherin was detected in epididymal protein extracts and was localized in the epithelial cells from the three epididymal regions. Moreover, the 86 kDa E-cadherin ectodomain was found in cauda epididymal and seminal plasma. Western immunoblotting of human sperm protein extracts allowed the identification of four E-cadherin forms (122, 105, 97 and 86 kDa). The protein was localized in the acrosomal region of intact spermatozoa, remained associated with the head of acrosome-reacted cells and was also detected on the oocyte surface. A similar localization was determined for other proteins of the adhesion complex (β-catenin and actin). Spermatozoa incubated with anti-E-cadherin antibodies showed impaired binding to homologous zona pellucida (ZP); in addition, presence of these antibodies inhibited the penetration of human spermatozoa to ZP-free hamster oocytes. The results presented here describe the expression of E-cadherin in the male reproductive tract and gametes and strongly suggest its involvement in adhesion events during human fertilization. The identification of proteins involved in gamete interaction will contribute to the understanding of the molecular basis of fertilization and help in the diagnosis and treatment of infertility.

Keywords: epididymis; epithelial cadherin; fertilization; gamete interaction; sperm

Introduction

Fertilization results from successful interaction between the female and male gametes. Spermatozoa are produced in the testis, undergo maturation during their transit through the epididymis and, once deposited in the female tract, they suffer a complex series of changes collectively known as sperm capacitation, by which they completely acquire their fertilizing ability. After capacitated spermatozoa traverse the cumulus oophorus cell layer that surrounds the oocyte, they bind to the zona pellucida (ZP) in a species-specific manner. Then, sperm cells undergo exocytosis of the acrosomal granule (acrosomal exocytosis, AE) and penetrate the ZP. Acrosome-reacted spermatozoa bind and fuse with the oocyte plasma membrane (oolemma) and the sperm nucleus decondenses in the ooplasm (Yanagimachi, 1994; Wassarman *et al.*, 2001).

Some members from the superfamilies of adhesion molecules initially described in somatic cells (i.e. selectins, immunoglobulins, integrins and cadherins) are expressed in spermatozoa and oocytes and participate in gamete interaction (Bronson and Fusi, 1996; Geng *et al.*, 1997; Purohit *et al.*, 2004; Inoue *et al.*, 2005; Barraud-Lange *et al.*, 2007; Shao *et al.*, 2008). The cadherin

superfamily is composed of cell surface molecules involved in calcium (Ca^{2+})-dependent adhesion events that include embryonic development and tissue organization (Takeichi, 1995; Gumbiner, 1996; Angst *et al.*, 2001). Epithelial cadherin (E-cadherin) is a member of the classical cadherin family originally found in epithelial adherent junctions and is composed of a highly conserved carboxy-terminal cytoplasmic domain, a single pass-transmembrane domain and five extracellular domains of around 110 amino acids (Blaschuk and Rowlands, 2002). E-cadherin extracellular domains mediate homophilic and heterophilic interactions between adjacent cells (Blaschuk *et al.*, 1990; Nose *et al.*, 1990; Ozawa and Kemler, 1998); the cytoplasmic domain binds to β-catenin that links the adhesion protein to the actin cytoskeleton (Nagafuchi *et al.*, 1993). Studies done in tissues from the male reproductive tract have described the expression of E-cadherin in the human epididymis using immunohistochemistry (Andersson *et al.*, 1994) and molecular biology strategies (Dube *et al.*, 2007). In addition, the detection of E-cadherin in both human (Rufas *et al.*, 2000; Purohit *et al.*, 2004) and rat (Ziv *et al.*, 2002) gametes has been reported. Considering that gamete interaction requires the presence of Ca^{2+} ions (Fraser, 1987; Marín-Briggiler *et al.*, 2003) and that E-cadherin participates in Ca^{2+}-dependent cell–cell adhesion (Takeichi, 1995),

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the involvement of this protein in adhesion events during fertilization could be anticipated.

The present study was undertaken to evaluate the expression of E-cadherin in the human testis and in segments of the epididymis. In addition, identification of E-cadherin protein forms in male tract fluids and sperm extracts, and partial characterization of their association to the sperm membrane, was done. A complete analysis was carried out to assess E-cadherin localization in human spermatozoa incubated under *in vitro* conditions that resemble physiological sperm selection, capacitation and AE events. Immunodetection studies included members of the adhesion complex, such as β-catenin and actin. Finally, assays were performed to investigate the participation of E-cadherin in sperm–oocyte interaction, studies that included immunolocalization of E-cadherin in human and hamster oocytes.

Materials and Methods

All human samples (tissues, cells and fluids) used in the study were obtained under the donor's written consent, and protocols were approved by the Argentine Society of Clinical Investigation Review Board.

Chemicals

Unless specified, chemicals were of analytical grade and purchased from Sigma Chemical Co. (St Louis, MO, USA). Electrophoresis reagents were products of BioRad (Richmond, CA, USA), or as specifically indicated throughout the article. Molecular biology reagents were of the highest quality and purchased from Qiagen (Hilden, Germany) and Invitrogen (Carlsbad, CA, USA), unless otherwise specified.

Antibodies

The following antibodies toward human E-cadherin (anti-E-cadherin) were used throughout the study: a polyclonal antibody, H-108, developed toward amino acids 600–707 (cadherin 5 domain) (Santa Cruz Biotech., Santa Cruz, CA, USA) and the following monoclonal antibodies (i) HECD-1 (cadherin 2 domain) (Becker *et al.*, 2002) (Zymed; South San Francisco, CA, USA), (ii) clone DECMA-1 (cadherin 4–5 domains) (Ozawa *et al.*, 1990) (Sigma), (iii) clone SHE78-7 (cadherin 1 domain) (Laur *et al.*, 2002) (Zymed) and (iv) 610181 (cytoplasmic domain) (Chitaev and Troyanovsky, 1998) (BD Biosciences, San Diego, CA, USA). In addition, antibodies toward β-catenin, 610153 (BD Biosciences) and AB19022 (Millipore, Billerica, MA, USA), and actin, A2668 (Sigma) and Clone ACTN05 (Neomarkers, Basel, Switzerland), were used in the study. Secondary antibodies CY3-labeled anti-rabbit IgG (Chemicon-Millipore) and CY3-labeled anti-mouse IgG (Sigma) were used for immunocytochemistry protocols, as indicated; horse-radish peroxidase-conjugated goat anti-rabbit or anti-mouse IgGs were used in immunohistochemistry and western immunoblotting assays.

Culture media

The standard media used throughout the study were human sperm medium (HSM) (Suarez *et al.*, 1986), human tubal fluid (HTF) medium (Irvine Scientific, Santa Ana, CA, USA) and Biggers–Whitten–Whittingham (BWW) medium (World Health Organization, 1999).

Tissues, cells and fluids

Human testicular and epididymal tissues were obtained from adult patients undergoing orchiectomy as treatment for prostatic carcinoma and not receiving any hormonal treatment prior to surgery. Human semen samples were provided by normozoospermic donors according to World Health Organization standards (World Health Organization, 1999). Only samples with more than 90% live spermatozoa, 75% progressive motile cells and over 14% normal sperm forms by Kruger (World Health Organization, 1999) were included in the study.

Human oocytes were obtained from women undergoing assisted-fertilization procedures. For immunocytochemical analysis, oocytes were incubated for 6–8 h in HTF medium for maturation. Cumulus oophorus cells were removed by treatment with 80 IU hyaluronidase for 20 s. For hemizona analysis, oocytes were placed at 4°C in 0.1 M Tris buffer (pH 7.0) with 1.5 M

$(NH_4)_2SO_4$ and 0.5% dextran, until used. Hamster oocytes were collected and processed following a protocol for the zona-free hamster oocyte test described by the World Health Organization (World Health Organization, 1999).

Cauda epididymal plasma (CEP) was obtained by retrograde perfusion of the human vas deferens followed by centrifugation at 10 000g for 10 min. Total seminal plasma (SP) was obtained from liquefied semen samples by two sequential centrifugations: one at 600g for 15 min followed by another centrifugation at 10 000g for 10 min at 4°C. Proteins from human urine samples were prepared as previously described (Banks *et al.*, 1995). Human serum was obtained from healthy donors and centrifuged at 15 000g for 20 min. All samples were stored at −20°C until used.

Expression libraries and tissue RNA analyses

An expression cDNA library from human epididymis was constructed using the ZAP Express® vector (Stratagene, San Diego, CA, USA). Aliquots of the epididymal library as well as from a commercial human testis 5′-STRETCH PLUS cDNA expression library (Clontech, Palo Alto, CA, USA) were used in the standard PCR procedures. To characterize the sequence encoding the epididymal E-cadherin mRNA, screening of the epididymal library with the anti-E-cadherin antibody H-108 was done using the *pico*Blue™ Immunoscreening kit (Stratagene). A set of positives were selected and cloned. Purified phagemid DNAs were subjected to PCR with T3 and T7 primers using a standard procedure. Nucleotide sequence analysis of clones was performed at the Core Research Center from the University of Chicago (Chicago, IL, USA) with primers T3 and T7, as well as with internal primers designed from the partial sequencing results. To search for nucleotide sequence similarity between the identified clones and the reported sequence for E-cadherin (NM_004360), the BLAST program was run (Altschul *et al.*, 1990).

Total RNA from human testis and from caput, corpus and cauda epididymal tissue was isolated using the RNeasy kit (Qiagen) according to the manufacture's protocol. Complementary DNA (cDNA) was synthesized using a reaction mixture containing total RNA, oligodT and the SuperScript II reverse transcriptase (Invitrogen), following the procedure suggested by the manufacturer. Negative controls omitting the RNA or the reverse transcriptase were included and tested in the PCR procedure.

Expression libraries and tissue cDNAs were subjected to end-point standard PCR procedures and analysis by electrophoresis in agarose gels. A quantitative analysis was done by real-time PCR with the Applied Biosystems 7500 Real-Time PCR unit, using the SYBR Green® PCR Master Mix (Applied Biosystems, Foster City, CA, USA). In both protocols, E-cadherin primers used were: forward 5′-GACCAAGTGACCACCTTAGA-3′ and reverse 5′-CTCCGAAGAAACAGCAAGAGC-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the housekeeper gene (primers GAPDH forward: 5′-TGCACCACCAACTGCTTAGC-3′, GAPDH reverse: 5′-GGCATGGACTGTGGTCATGAG-3′). The expected sizes for E-cadherin and GAPDH fragments were 172 and 87 bp, respectively. All samples were run in triplicates; there were negative controls (two controls of the reverse transcription assay described in the previous paragraph and the PCR control omitting the DNA template) in all cases. In real-time protocols, melting curves were run to confirm specificity of the signal. Relative E-cadherin expression was carried out using GAPDH as the housekeeper gene, and the results were compared with a sample selected as reference (human testis); the calculation describing these relations is $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [\Delta Ct$ test sample $- \Delta Ct$ reference sample] and $\Delta Ct = [Ct$ gene under study $- Ct$ housekeeper gene].

Immunohistochemistry

Small portions from the caput, corpus and cauda sections of the human epididymis were fixed and processed as previously described (Lasserre *et al.*, 2003). The anti-E-cadherin antibody (H-108) was applied at a concentration of 2 µg/ml. Harris hematoxylin was used for counter staining. Sections were evaluated at ×400 and ×1000 magnifications using an Alphaphot-2 YS2 microscope (Nikon, Tokyo, Japan).

Analysis of membranous vesicles from human SP

Aliquots of SP were supplemented with a cocktail of protease inhibitors and diluted with TBS-Ca^{2+} (30 mM Tris–HCl, pH 7.6, supplemented with 130 mM NaCl and 2 mM $CaCl_2$). This sample was ultracentrifuged at

100 000*g* for 2 h at 4°C. The supernatant, named ultracentrifuged SP (USP) was recovered and stored at −20°C. The pellet was washed with TBS-Ca^{2+} and centrifuged at 100 000*g* for 1 h at 4°C; the resulting pellet was suspended in the same buffer and subjected to filtration on a Sephadex G-200 column (Amersham Pharmacia Biotech, GE Healthcare, Buckinghamshire, UK) as previously described (Stegmayr and Ronquist, 1982). Fractions were collected and monitored at 280 nm, selecting those with high absorbance, which were pooled and centrifuged again (2 h at 100 000*g*). The pellet containing the purified membranous vesicles (pMV) was stored at −20°C until analysis. Evaluation of the aminopeptidase activity in pMV was done as reported (Laurell *et al.*, 1982). In addition, transmission electron microscopy of this fraction was carried out using standard procedures (Fornés *et al.*, 1991).

Tissue and sperm protein extracts

Protein extracts from human epididymides were obtained as part of the procedure designed to isolate total RNA with Trizol reagent (Invitrogen); pellets were sonicated three times at maximal power (Sonifier Cell Disruptor, model W 140, Heat Systems-Ultrasonics, Inc., Plainview, LI, NY, USA) in a period of 30 s, and proteins were stored at −70°C until used.

Total sperm protein extracts were prepared by diluting the sperm suspensions with PBS containing protease inhibitors, followed by centrifugation at 400*g* for 10 min and pellet incubation with Laemmli sample buffer. In some cases, spermatozoa were resuspended with 10 mM Tris–ClH (pH 7.5), 0.25 M sucrose and 50 mM benzamidine (buffer A), supplemented with 1 M NaCl and incubated for 20 min at room temperature. At the end of this incubation, samples were centrifuged at 400*g* for 10 min and the supernatant (high salt extract; HSE) was recovered and analyzed. The procedure was repeated using buffer A containing 2 and 4 M NaCl. The HSEs were concentrated using a Centricon YM-10 (Amicon-Millipore, Bedford, MA, USA).

In other cases, spermatozoa were incubated for 20 min at room temperature in buffer A with 0.25 M NaCl. Cells were centrifuged for 10 min at 600*g*, resuspended in 10 mM Tris–HCl buffer (pH 7.5) containing protease inhibitors and 144 mM NaCl (buffer B) and centrifuged again. Spermatozoa were resuspended in buffer B with or without (control) 5 U of phosphatidyl inositol specific phospholipase C (PI-PLC) and incubated for 2 h at 30°C. Samples were centrifuged at 600*g* for 10 min, and extracted proteins were concentrated by precipitation with trichloroacetic acid.

SDS–PAGE and western immunoblotting

Proteins from tissues, cell extracts and fluids were analyzed by SDS–PAGE followed by western immunoblotting using standard procedures (Lasserre *et al.*, 2003). The type and concentration of the specific primary antibody used is indicated when appropriate.

Sperm handling

Liquified semen samples were subjected to the swim-up procedure, and selected spermatozoa were incubated for 18 h under conditions that promote capacitation. In some cases, capacitated sperm cells were exposed to 10 μM calcium ionophore A23187 for 45 min to induce AE. At the end of the incubations, spermatozoa were washed, fixed and processed for cell staining. A detailed description of these procedures has been previously reported (Marín-Briggiler *et al.*, 2003).

Immunocytochemical analysis

Spermatozoa

Sperm cells were subjected to immunocytochemistry following a procedure previously described (Lasserre *et al.*, 2003). First antibodies were used at the following concentrations: anti-E-cadherin H-108: 2 μg/ml, HECD-1: 20 μg/ml, DECMA-1: 58 μg/ml; anti-β-catenin: 10 μg/ml; anti-actin: 134 μg/ml. For controls, the same concentration of purified IgG from the same specie as the first specific antibody was used. The immunostaining procedure was performed on selected post-swim-up (non-capacitated), 18 h-capacitated (capacitated) and acrosome-reacted human spermatozoa. When indicated, non-capacitated sperm cells were incubated for 1 h with HECD-1 antibody prior to fixation; the procedure was followed as described for fixed cells. E-cadherin immunolocalization analysis was combined with sperm staining with the lectin *Pisum sativum* agglutinin labeled with FITC (FITC-PSA) to assign the acrosomal status of each cell.

Sperm cells were evaluated in a Nikon fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA) coupled to an image analyzer (IPLab Scientific Imaging Software for Windows; BD). When specified, spermatozoa were observed with a Nikon laser confocal microscope C1; images were acquired using an objective PlanApo 60x/1.40 oil, excitation/emission: 488 nm/515–530 nm and 544 nm/570 LP, and analyzed using standard protocols for fluorescent imaging.

Oocytes

Human oocytes in Metaphase II and ZP-free hamster oocytes were incubated for 1 h at 37°C with 20 μg/ml H-108 antibody in HTF medium supplemented with serum substitute (Irvine Sci.). To remove the unbound antibody, female gametes were washed in PBS with 1% BSA, fixed and incubated with the secondary antibody (1 h, room temperature). Oocytes were washed, mounted and analyzed by fluorescence microscopy. Controls omitting the first antibody were included.

Hemizona assay

To perform the hemizona assay (HZA) (Burkman *et al.*, 1988), spermatozoa were pre-incubated in capacitating medium for 4 h followed by 1 h incubation with the anti-E-cadherin antibody (treated condition), or with the antibody's buffer (control condition). At the end of the incubation, the unbound antibody was removed by sperm centrifugation. Each hemi-ZP was placed in a 100 μl drop of a suspension containing 6×10^4 motile spermatozoa. After a 4 h incubation period, each hemi-ZP was washed, and the number of spermatozoa tightly bound to the outer ZP surface was counted under a ×400 magnification using Hoffman interference optics (Modulation Optics Inc., Greenvale, NY, USA). In some cases, the acrosomal status of the sperm cells recovered from the gamete incubation drop was assessed by the FITC-PSA staining. Gamete incubations were carried out in HSM supplemented with 2.6% BSA. Results from the HZA were reported as the number of spermatozoa bound per HZ.

Hamster oocyte sperm penetration assay

The ZP-free hamster oocyte sperm penetration assay (SPA) was done essentially as described (World Health Organization, 1999). To assess the participation of E-cadherin in sperm−oolemma interaction, oocytes were placed for 30 min in medium containing 20 μg/ml of anti-E-cadherin (H-108) or rabbit IgG (control), transferred to a drop containing capacitated spermatozoa and incubated for additional 2.5 h. The presence of at least one swollen head associated with a tail was indicative of successful penetration. Oocytes viability was confirmed at the end of the assay by standard staining with 0.25% Trypan Blue in PBS. The results on the SPA assay were expressed as the percentage of penetrated oocytes, and calculated as [number penetrated oocytes/number inseminated oocytes] ×100.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). The results on the HZA and SPA under different experimental conditions were compared using the Wilcoxon signed-rank test. All statistical analyses were done with an IBM compatible computer using the GraphPad InStat program (GraphPad Software, San Diego, CA, USA).

Results

Expression analysis of E-cadherin in the human male reproductive tract

Assessment of the E-cadherin mRNA expression was done by standard PCR analysis using human epididymal and testicular cDNA libraries, as well as total RNA isolated from testis and from the three epididymal segments (caput, corpus and cauda). Presence of the E-cadherin transcript was evidenced in both tissues (Fig. 1A.1 and A.2). A quantitative analysis by real-time PCR revealed that E-cadherin mRNA levels in the epididymis were 100 to 1000 times
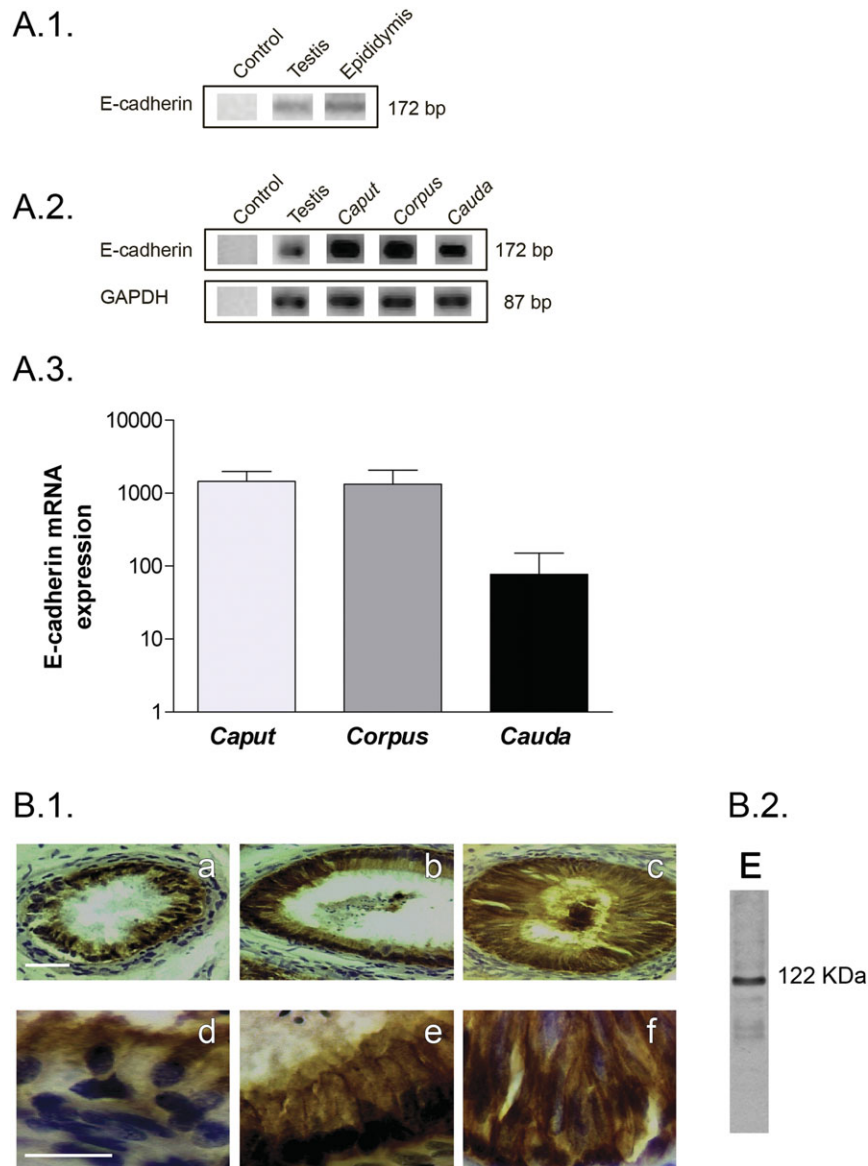
A.1.

A.2.

A.3.

B.1.

B.2.

Figure 1: Expression of E-cadherin in the human male reproductive tract.
(**A.1**) Standard PCR analysis of E-cadherin mRNA expression in human testis and epididymis using cDNA expression libraries. A negative control without template was included. The experiment was done three times, obtaining similar results. A negative image of the agarose gel from a typical experiment is shown. (**A.2**) RT–PCR analysis using testis, caput, corpus and cauda RNA. The negative control without reverse transcriptase is shown. GAPDH expression as positive control is shown below each lane. (**A.3**) Quantitative analysis of epididymal E-cadherin mRNA expression by real-time PCR. Relative E-cadherin expression in the caput, corpus and cauda was analyzed using GAPDH as the housekeeper gene, and human testis as the reference sample. Data were processed as described in 'Materials and Methods'. Results are expressed as mean $\pm$ SEM ($n = 3$), and represented in log scale. (**B.1**) Immunohistochemical localization of E-cadherin in different regions of the human epididymis, using an anti-E-cadherin antibody (H-108, Santa Cruz). Images are shown at $\times 400$ and $\times 1000$ magnification: caput (a and d), corpus (b and e) and cauda (c and f). Bar: 50 μm in a–c, and 25 μm in d–f. (**B.2**) Western immunoblot analysis on human epididymal protein extracts (E) using an anti-E-cadherin antibody (H-108). The experiments were performed at least three times obtaining similar results, a typical result is shown.

higher than those found in the testis (Fig. 1A.3). Differences were also observed within the epididymal regions: while comparable expression levels were found in the caput and corpus, lower levels (10 times) were detected in the cauda section (Fig. 1A.3). Expression of E-cadherin mRNA in the epididymis was confirmed by screening of the expression library with an anti-E-cadherin antibody. The analysis of a set of selected clones revealed over 95% sequence identity between the nucleotide sequence cloned from epididymal tissue and that previously reported in other cells (NM_004360) (data not shown).

Immunohistochemical studies showed the expression of the adhesion protein in epithelial cells from all three epididymal segments (caput, corpus and cauda) (Fig. 1B.1, a–c). E-cadherin was immunolocalized to the apical and adjacent cell borders and in the cell cytoplasm (Fig. 1B.1, d–f). A positive immunoreactivity was also observed in the lumen, which may result from protein detection in detached epithelial cells, spermatozoa and other membranous structures.

Western immunoblot analysis of total protein extracts from human epididymal tissue using the anti-E-cadherin antibody H-108 revealed the presence of a 122 kDa E-cadherin form (E-cadherin122) (Figs 1B.2 and 2A). This Mr was reported in other cells and tissues for the mature protein, spanning the five extracellular, the trans-membrane and the cytoplasmic domains (Shore and Nelson, 1991). In some protein extracts, a higher Mr form of around 140 kDa was
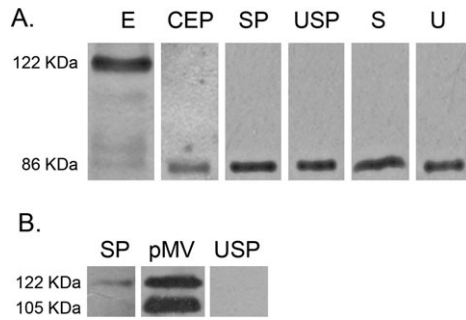
Figure 2: Detection of E-cadherin protein in human epididymis and fluids of the male reproductive tract.
(**A**) Western immunoblot analysis of proteins from human epididymis (E), cauda epididymal plasma (CEP), SP, ultracentrifuged SP (USP), human serum (S) and concentrated human urine (U) using an anti-E-cadherin antibody (H-108). (**B**) Western immunoblot analysis of total proteins from SP, purified membranous vesicles (pMV) and USP using the monoclonal anti-E-cadherin antibody (610181, BD Biosciences).

detected (data not shown), which has been described as the E-cadherin precursor (Ozawa and Kemler, 1990).

Presence of E-cadherin in human fluids from the male reproductive tract

In protein extracts from cauda epididymal plasma (CEP) and SP, an E-cadherin form of 86 kDa (E-cadherin86) was detected (Fig. 2A). This moiety, which is found in human serum and urine (Fig. 2A) and has previously been identified as the E-cadherin ectodomain, comprises the five extracellular domains and is released after protein shedding mediated by metalloproteinases (Wheelock *et al.*, 1987).

The SP contains small membranous vesicles, called prostasomes (prostate-derived vesicles) and epididymosomes (epididymis-derived vesicles), that transfer proteins to the sperm plasma membrane (Saez *et al.*, 2003). To determine whether the signal of E-cadherin protein forms observed in the SP was associated with the soluble or the membranous fractions, pMV were analyzed for the presence of E-cadherin. The efficacy of the purification procedure was verified by the assessment of the aminopeptidase enzymatic activity and transmission electron microscopy analysis in fractions containing the maximum protein contents (data not shown).

Evaluation of the E-cadherin forms present in SP was done using the 610181 antibody; this strategy also allowed the detection of the 122 kDa form (Fig. 2B). The mature E-cadherin and a truncated form of 105 kDa (E-cadherin105) were found in the pMV protein extracts (Fig. 2B). In contrast, these forms were not present in USP protein profiles (Fig. 2B) in which only the 86 kDa ectodomain was identified (Fig. 2A).

Altogether, these evaluations showed the presence of E-cadherin in the human cauda epididymal plasma and SP. In addition to the soluble E-cadherin ectodomain found in the fluid, the mature form was detected in the particulate fraction, suggesting the localization of the adhesion protein in secretory vesicles from the male reproductive tract.

Expression of E-cadherin in human spermatozoa

Western immunoblot analysis was done of E-cadherin in protein extracts from ejaculated spermatozoa freed of SP. Using the anti-E-cadherin antibody H-108, E-cadherin of four high molecular weight forms were identified: E-cadherin122, E-cadherin105, E-cadherin97 and E-cadherin86 (Fig. 3A). Sperm E-cadherin86 and E-cadherin97 were also immunodetected with an anti-E-cadherin antibody toward the N-terminal end (HECD-1) (Fig. 3A). However, these
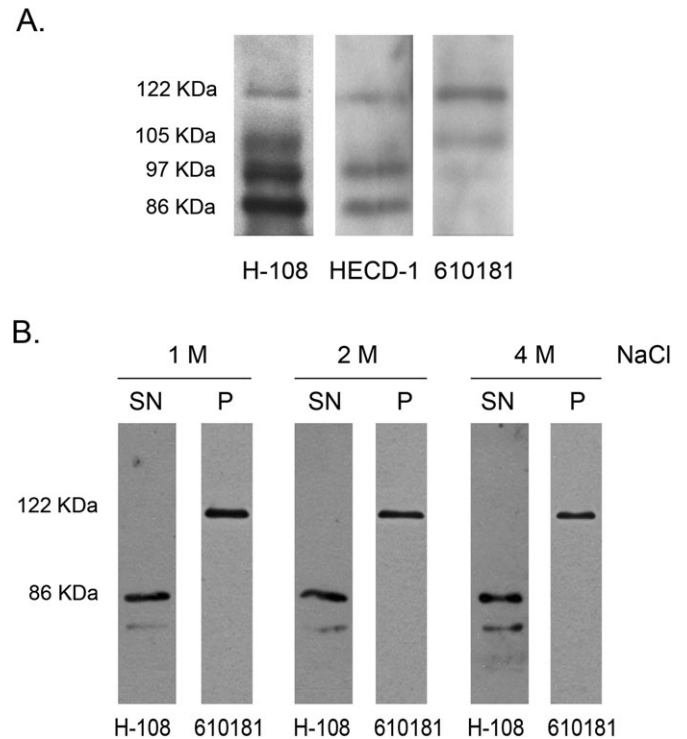

Figure 3: Identification of E-cadherin protein forms in human ejaculated spermatozoa.
(**A**) Western immunoblotting of total human sperm protein extracts using anti-E-cadherin antibodies H-108, HECD-1 (Zymed) and 610181, as indicated. (**B**) Western immunoblot analysis of spermatozoa incubated with NaCl (1, 2 or 4 M). Extracted proteins (SN) were resolved in 7% polyacrylamide gels, and membranes were developed with anti-E-cadherin antibody H-108. Non-extracted proteins (P) were subjected to 10% SDS–PAGE, and membranes were exposed to the monoclonal anti-E-cadherin antibody 610181.

protein forms were not recognized by the antibody directed toward the cytoplasmic domain (610181) (Fig. 3A), suggesting a truncation on their C-terminal end. The sperm E-cadherin105 was detected by the 610181 antibody, but not by the HECD-1 antibody, indicating its processing in the N-terminal end. Immunodetection of E-cadherin forms was specific, since no signal was found when the first antibody was omitted (data not shown).

Next, a series of experiments were carried out to analyze the association of E-cadherin97 and 86 kDa-truncated forms to the sperm plasma membrane. When cells were incubated in the presence of high salt concentrations, only E-cadherin86 was found in the supernatants (Fig. 3B). In somatic cells, the 86 kDa ectodomain results from processing of the mature 122 kDa form, generating a 35 kDa fragment that remains associated with the plasma membrane (Maretzky *et al.*, 2005) and is recognized by the 610181 anti-E-cadherin antibody. The analysis of the cellular pellets after NaCl treatment using this antibody did not reveal the presence of the 35 kDa polypeptide; these findings suggested that the salt extracted 86 kDa form is not originated by proteolysis of E-cadherin122 (Fig. 3B). To evaluate whether E-cadherin97 is associated with the plasma membrane by glyceryl phosphatidil inositol, live spermatozoa were incubated with PI-PLC. After this treatment, E-cadherin97 was not removed; instead, E-cadherin86 was immunodetected. The analysis of these sperm pellets revealed the presence of the 35 kDa fragment, indicating that, in this case, E-cadherin86 resulted from cleavage of the mature protein but not from the PI-PLC enzymatic activity (data not shown).

Taken together, the results from these studies indicate that human spermatozoa express the mature E-cadherin form (E-cadherin122)

already reported in epithelial cells; in addition, other forms truncated at the N- (E-cadherin105) and C- (E-cadherin86 and E-cadherin97) terminal ends were identified. The observation that the 86 kDa ecto-domain can be removed from the cell after high salt treatment suggests a loose association of this protein to the sperm plasma membrane.

Immunolocalization of E-cadherin in spermatozoa

Immunolocalization of E-cadherin in non-capacitated, capacitated and acrosome-reacted spermatozoa was done with polyclonal H-108 and monoclonal DECMA-1 anti-E-cadherin antibodies. The adhesion protein was mainly localized in the acrosomal region of non-capacitated spermatozoa (Fig. 4A.1 and B); this pattern of staining was found in over 75% of the cells (Fig. 4B). In addition, a diffuse and weak signal over the entire head was detected in a small pro-portion of the sperm cells (Fig. 4B). After capacitation, the same E-cadherin distribution was observed in the majority of the sperm cells, although a decrease in the percentage of cells immunoreactive to the acrosomal cap was seen (Fig. 4B). Results were similar with both antibodies. Spermatozoa incubated with an anti-E-cadherin anti-body prior to fixation depicted staining over the acrosome, as found in fixed cells (data not shown). Additionally, co-localization studies with anti-E-cadherin and FITC-PSA indicated that all spermatozoa stained for the adhesion protein in the acrosomal region had an intact acro-some (data not shown). Altogether, these findings favor the notion of E-cadherin localization on the acrosomal surface of intact sperm cells.

To evaluate the fate of E-cadherin after AE, immunocytochemical analyses were performed in human spermatozoa that had been exposed to calcium ionophore A23187. In acrosome-reacted cells, three patterns of protein localization were identified: a signal in the equatorial ring, a staining in the post-acrosomal region and a label over the entire sperm head (Fig. 4C). This latter pattern of E-cadherin staining was predominant in spermatozoa showing an FITC-PSA signal in the equatorial segment (Fig. 4C).

Presence and localization of β-catenin and actin in spermatozoa

In epithelial cells, the E-cadherin cytoplasmic domain binds β-catenin; this molecule links the adhesion protein to the actin cyto-skeleton, contributing to the strength of cell–cell adhesion. The expression and localization of β-catenin and actin in human sperma-tozoa was assessed by immunocytochemistry (Fig. 4A.1). More than 80% of the sperm cells depicted β-catenin and actin immunoreactivity in the acrosomal region. Additionally, western immunoblotting of sperm protein extracts confirmed the presence of a specific signal with the expected Mr for these proteins: 91 kDa for β-catenin and 42 kDa for actin (Fig. 4A.2).

Participation of E-cadherin in gamete interaction

Immunocytochemical studies showed localization of E-cadherin and components of the adhesion complex in human sperm regions that par-ticipate in sperm–oocyte interaction. To assess the involvement of E-cadherin in adhesion events during fertilization, the effect of anti-E-cadherin antibodies upon sperm interaction with the ZP and with the oocyte plasma membrane was evaluated.

Participation of E-cadherin in sperm–ZP interaction

To determine the participation of E-cadherin on human sperm inter-action with homologous ZP, the HZA was done with spermatozoa incubated for 1 h with monoclonal (10 and 50 μg/ml of SHE78-7) or polyclonal (10 and 100 μg/ml of H-108) anti-E-cadherin antibodies

prior to their incubation with the ZP. Cellular exposure to both con-centrations of the SHE78-7 and to 100 μg/ml of H-108 antibodies resulted in a significant ($P < 0.05$) decrease in the number of sperma-tozoa bound to the ZP (Table I). The inhibitory effect could not be attributed to a decrease in either sperm viability or motility. Moreover, inhibition could not be explained due to a premature AE induced by the antibodies, since the percentages of acrosome-reacted cells at the end of the assay were similar in treated and control conditions (data not shown). The ability of the anti-E-cadherin antibody H-108 to recognize cadherin-like epitopes in the human ZP was demonstrated by immunocytochemical analysis done on *in vitro* matured human oocytes (Fig. 5).

Participation of E-cadherin in sperm–oolemma interaction

The interaction between the acrosome-reacted spermatozoa and the oocyte plasma membrane is a cell–cell adhesion event. E-cadherin localization in sperm cells that have undergone AE (Fig. 4C), as well as in the oolemma of human oocytes (Rufas *et al.*, 2000; Fig. 5), prompted us to test its participation in this step of the fertiliza-tion process. To performing the study, the SPA was carried out. Prior to perform the assay, localization of E-cadherin on the oolemma of the ZP-free hamster oocytes was confirmed by immunocytochemical analysis (Fig. 6A.1); also, the expression of proteins from the adhesion complex (E-cadherin, β-catenin and actin) in hamster oocytes was determined by western immunoblotting (Fig. 6A.2). Pre-incubation of the female gametes with the anti-E-cadherin antibody (H-108; 20 μg/ml) resulted in a significant ($P < 0.05$) decrease in the percen-tage of penetrated oocytes (Fig. 6B).

In conjunction, these results suggest the participation of E-cadherin in sperm interaction with the ZP and the oocyte plasma membrane during fertilization.

Discussion

The participation of cadherins in homotypic and heterotypic contact between somatic cells has been extensively documented (reviewed by Angst *et al.*, 2001). However, the role of these proteins in gamete interaction remained elusive. The present study was aimed at characterizing the expression of E-cadherin in tissues and fluids of the human male reproductive tract, evaluating its localization in both gametes and assessing its participation in fertilization.

Expression of E-cadherin in human tissues and fluids of the male reproductive tract

Our results have shown the expression of E-cadherin mRNA in the human testis and epididymis. The caput and corpus epididymal seg-ments displayed a higher mRNA expression than the cauda, in agree-ment with a recent report showing the lowest transcriptional activity in the distal epididymal region (Thimon *et al.*, 2007). The results of western immunoblot studies of protein extracts, as well as sequence analysis of clones, from an expression library would indicate that mature epididymal E-cadherin is identical to that described in other somatic cells (Mansouri *et al.*, 1988). The protein was localized to the apical surface of epididymal epithelial cells, leading us to suggest its involvement in the formation and maintenance of the blood-epididymal barrier, as reported in rodent models (Levy and Robaire, 1999). Epididymal E-cadherin could also be related with the sperm maturation process that takes place in this organ.

In cauda epididymal plasma and SP, an 86 kDa E-cadherin form was detected. This moiety is similar to the soluble E-cadherin ectodomain found in other biological fluids (Banks *et al.*, 1995), which is the result of the shedding mediated by metalloproteinases,
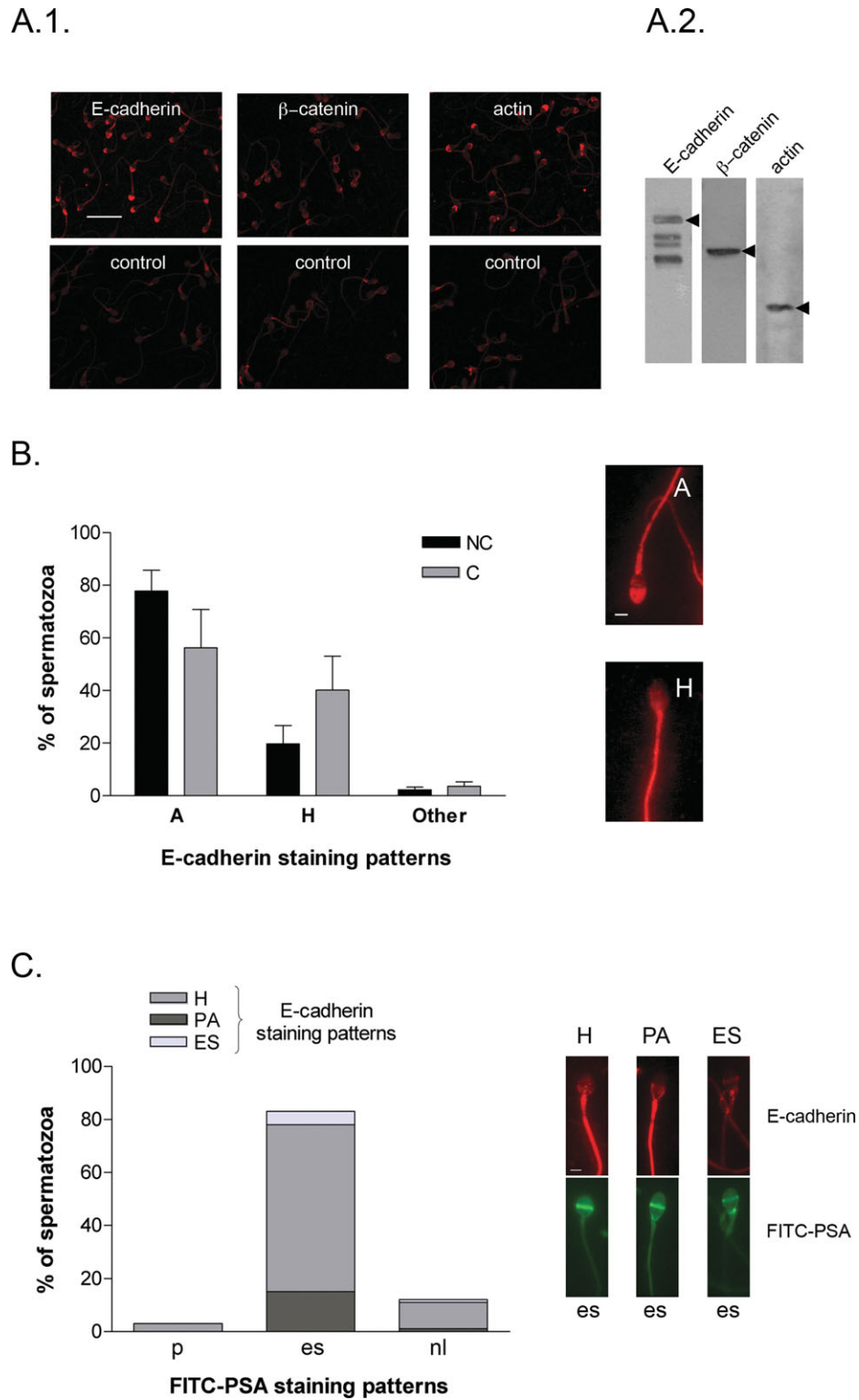
A.1.

A.2.

B.

C.

Figure 4: Immunolocalization of E-cadherin and proteins of the adhesion complex in human spermatozoa.
(**A.1**) Indirect immunofluorescence of human spermatozoa using anti-E-cadherin (H-108), anti-β-catenin (AB19022, Millipore) or anti-actin (A2668, Sigma) anti-bodies, or the same concentration of specific IgGs as control (bottom panels). Samples were analyzed using a laser confocal microscope. Bar: 20 μm. (**A.2**) SDS–PAGE and western immunoblotting of sperm protein extracts using anti-E-cadherin (H-108), anti-β-catenin (610153, BD Biosciences) or anti-actin (Clone ACTN05, Neomarkers) antibodies. The arrowheads indicate 122 kDa for E-cadherin, 91 kDa for β-catenin and 42 kDa for actin. (**B**) Distribution of E-cadherin immunostaining patterns in non-capacitated (NC) and capacitated (C) spermatozoa using the H-108 antibody. A, label in the acrosome; H, diffuse and weak label over the entire head. The results are expressed as mean ± SEM, $n = 5$. Representative images of the A and H patterns are shown. Bar: 2.5 μm. (**C**) Distribution of E-cadherin immunostaining patterns in acrosome-reacted spermatozoa. Cells were stained with anti-E-cadherin antibody (H-108) and CY3-labeled secondary antibody followed by incubation with FITC-PSA to assess the acrosomal status. E-cadherin staining patterns: a signal over the entire head (H), in the post-acrosomal region (PA) or in the equatorial segment (ES). FITC-PSA staining patterns: a 'patchy' signal on the acrosomal cap (p), a label in the equatorial segment (es) or no label (nl), $n = 5$. Representative images of the H, PA and ES E-cadherin patterns in 'es' acrosome-reacted cells are shown. Bar: 2.5 μm.

Table I. Effect of sperm pre-incubation with an anti-E-cadherin antibody on sperm binding to the homologous ZP.

Antibody	Number of bound spermatozoa per HZ		*n*
	Treated condition	Control condition	
SHE78-7, 10 µg/ml	10 ± 3**	19 ± 5	8
SHE78-7, 50 µg/ml	20 ± 6*	34 ± 6	9
H-108, 10 µg/ml	20 ± 10	18 ± 6	5
H-108, 100 µg/ml	18 ± 5*	31 ± 9	7

HZ, hemizona; the results are expressed as mean $\pm$ SEM.
**$P < 0.01$, *$P < 0.05$ versus control condition, Wilcoxon signed-rank test.
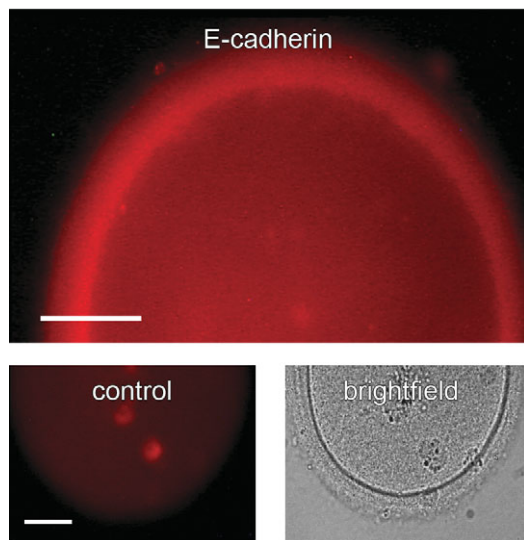
Figure 5: Immunolocalization of E-cadherin in human oocytes. Immunodetection of E-cadherin in human oocytes using the H-108 antibody. Control, in which the first antibody was replaced by rabbit IgG, and the corresponding brightfield photomicrograph. Bar: 25 µm.

such as matrilysin (MAT) and ADAM10 (Noe *et al.*, 2001; Maretzky *et al.*, 2005). MAT and members of the ADAM family have been described in the male reproductive tract (Wilson *et al.*, 1995; Primakoff and Myles, 2000); however, their involvement in the release of E-cadherin86 to these fluids remains to be determined.

E-cadherin protein forms in human spermatozoa

Using a set of antibodies directed toward different E-cadherin domains, several sperm protein forms were detected; in addition to the mature E-cadherin122, truncated forms of 105, 97 and 86 kDa were observed. These truncated forms could result from digestion by acrosomal enzymes released during AE or cell death. A truncated form of 97–100 kDa appears to be generated from proteolysis of the E-cadherin cytoplasmic domain by calpain (Rios-Doria *et al.*, 2003). The calpain/calpastatin enzymatic system has been described in human spermatozoa (Rojas *et al.*, 1999); however, the ability of these proteases to cleave the sperm E-cadherin is still unknown. The sperm E-cadherin86 ectodomain was detected after cellular treatment with NaCl, indicating its loose association to the sperm surface. This form may interact with a sperm transmembrane E-cadherin (Wheelock *et al.*, 1987; Noe *et al.*, 2001) or with other proteins associated with the plasma membrane.

E-cadherin forms identified in human spermatozoa could be of testicular origin, as this protein has been identified in testicular rat

spermatozoa (Ziv *et al.*, 2002); E-cadherin could also be acquired during epididymal sperm maturation and/or during sperm contact with SP components. In support of the last possibility, the E-cadherin122 and 105 forms were identified in membranous vesicles recovered from the SP fluid. The acquisition of sperm E-cadherin during maturation and ejaculation is currently under investigation in our laboratory.

E-cadherin localization in human gametes and participation in fertilization

Results from the present study have shown that a high proportion of human spermatozoa displayed E-cadherin in the acrosomal region. The findings presented here are in agreement with the observations from our group in mouse, rat and bovine models (Veiga and Caballero *et al.*, unpublished results); however, they contrast with those from previous reports in which a weak signal for E-cadherin in other sperm regions was described (Rufas *et al.*, 2000; Purohit *et al.*, 2004). Differences may be attributed to the protocols used for E-cadherin staining (i.e. cell permeabilization and antibodies selected for evaluation), or to other causes not identified by these authors. After sperm incubation in conditions that promote capacitation, a decrease in the percentage of stained cells was observed. This finding could be explained by the removal of loosely bound E-cadherin86 ectodomain, or changes in E-cadherin epitope accessibility due to protein relocalization during capacitation (Yanagimachi, 1994). Alternatively, E-cadherin may be processed by acrosomal enzymes released during *in vitro* incubation. In spermatozoa that have undergone AE, the low immunoreactivity toward E-cadherin on the entire head would indicate protein localization in the inner acrosomal membrane, and in the plasma membrane of the equatorial segment and the post-acrosomal region. Future ultrastructural studies will confirm the precise localization of E-cadherin in acrosome-reacted cells.
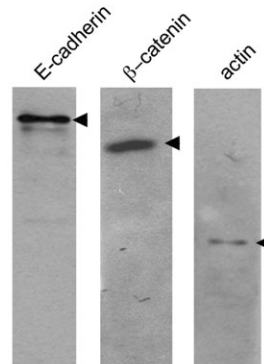
Detection of E-cadherin in sperm subcellular regions involved in adhesion events during fertilization led us to consider its potential role in sperm–oocyte interaction. The inhibitory effect of different anti-E-cadherin antibodies upon sperm–ZP binding favored the notion of its involvement in the early steps of fertilization. The antibody may have interfered with the formation of E-cadherin *cis*-dimers (Brieher *et al.*, 1996), with their lateral clustering in the plasma membrane (Angres *et al.*, 1996; Yap *et al.*, 1997) and/or with the interaction of E-cadherin with counterparts on apposing structures (Shiraishi *et al.*, 2005). In this regard, a specific immunoreactivity toward E-cadherin was detected in the human ZP. Antibody blockage of gamete interaction by steric hindrance cannot be ruled out; however, the same amount of a specific antibody toward neuronal cadherin did not alter sperm binding to the ZP, even though this protein was immunolocalized in the sperm acrosomal cap (Marín-Briggiler *et al.*, unpublished results).

The presence of an anti-E-cadherin antibody during sperm–oolemma interaction also led to a significant decrease in the percentage of penetrated oocytes. The inhibition levels reached may have resulted from the use of a gamete assay optimized to obtain high penetration rates ($>70\%$ penetrated oocytes), as well as to the antibody and experimental protocols used. Moreover, the involvement of other proteins should also be considered, since gamete adhesion/fusion events would be mediated by several proteins (Yanagimachi, 1994; Wassarman *et al.*, 2001). Participation of E-cadherin in this process may imply the formation of E-cadherin *trans*-dimers; in support of this possibility, E-cadherin and other members of the adhesion complex (β-catenin and actin) were detected in human spermatozoa and hamster oocytes. E-cadherin was also immunolocalized in the oolemma of human oocytes (present report and
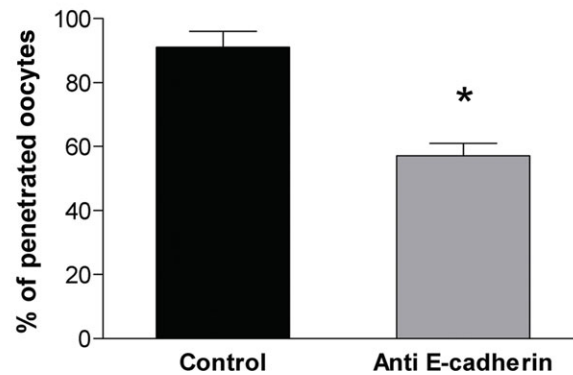
A.1.
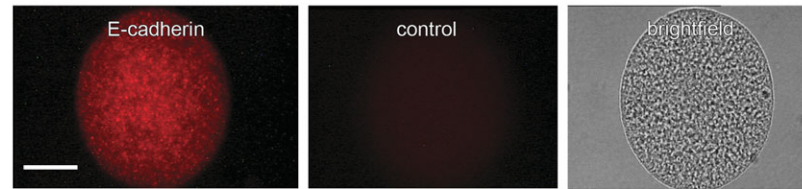
A.2.

B.

Figure 6: Presence of E-cadherin and proteins of the adhesion complex in hamster oocytes, and effect of anti-E-cadherin antibodies in sperm−oolemma interaction. (**A.1**) Immunodetection of E-cadherin in ZP-free hamster oocytes using the H-108 antibody. Control, in which the first antibody was replaced by rabbit IgG, and the corresponding brightfield photomicrograph. Bar: 25 μm. (**A.2**) Western immunoblot analysis of hamster oocyte protein extracts using anti-E-cadherin (H-108), anti-β-catenin (610153) or anti-actin (Clone ACTN05) antibodies. The arrowheads indicate 122 kDa for E-cadherin, 91 kDa for β-catenin and 42 kDa for actin. (**B**) Effect of the anti-E-cadherin antibody upon sperm−oolemma interaction using the ZP-free hamster oocyte sperm penetration assay. Female gametes were pre-incubated with the H-108 anti-E-cadherin antibody or with rabbit IgG (control). The percentage of penetrated oocytes was determined, and the results were expressed as [number penetrated oocytes/number inseminated oocytes] × 100 (mean ± SEM, $n = 6$ assays). *$P < 0.05$.

Rufas *et al*., 2000). Alternatively, E-cadherin could interact with other proteins present in the counterpart gamete. Previous studies have shown the ability of E-cadherin to interact with neuronal cadherin (Volk *et al*., 1987) and with integrins α2β1 (Whittard *et al*., 2002) and αEβ7 (Cepek *et al*., 1994). The expression of neuronal cadherin and several members of the integrin family in mammalian gametes have been documented (Rufas *et al*., 2000; Evans, 2002; Ziv *et al*., 2002; Barraud-Lange *et al*., 2007). The interaction between E-cadherin and integrins during fertilization deserves further investigation.

Cell−cell adhesion mediated by E-cadherin is a highly dynamic event; changes in the adhesive properties of E-cadherin have been attributed to several post-translational modifications in proteins of the adhesion complex, within them Ser-Thr and Tyr phosphorylation mediated by Src and casein kinases (Matsuyoshi *et al*., 1992; Lickert *et al*., 2000; Dupre-Crochet *et al*., 2007); in this regard, evidence on the expression of members of the casein and Src kinases (Ruzzene *et al*., 1992; Mitchell *et al*., 2008) in the male gamete has been reported. In addition, disruption of cell−cell adhesion due to

E-cadherin relocalization/proteolysis by calcium influx has been reported (Pey *et al*., 1998; Ito *et al*., 1999); a massive increase in intracellular calcium concentrations has been associated with the AE (Yanagimachi, 1994). Whether any of these mechanisms regulates E-cadherin-mediated adhesion events during fertilization remains to be investigated.

In conclusion, the present study has characterized the expression of E-cadherin in the human epididymis and has identified several E-cadherin protein forms in male reproductive tract fluids and ejaculated spermatozoa. This study has described E-cadherin localization in spermatozoa under conditions that resemble sperm transit through the female tract and has presented evidence of its participation in fertilization. The identification of proteins involved in these events is of great importance to understand the mechanism of mammalian fertilization; moreover, it may contribute to the development of new diagnostic assays, and eventually may help in the treatment of male infertility as well as in the design of novel contraceptive tools.

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