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Impaired E-cadherin expression in human spermatozoa in a male factor infertility subset signifies E-cadherin-mediated adhesion mechanisms operative in sperm–oolemma interactions☆

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Abstract

Cadherins comprise a family of calcium-dependent glycoproteins that function in mediating cell–cell adhesion in virtually all solid tissues of multicellular organisms. We have examined the presence of a cadherin on spermatozoon and its possible involvement in sperm–oocyte interaction. Spermatozoa from fertile human subjects showed the presence of E-cadherin on its head domains, detectable only after permeabilization of the surface membranes. On the contrary, spermatozoa from oligozoospermic subjects did not possess E-cadherin on their principal acrosomal and equatorial domains. Immunoprecipitation and Western blot studies also showed the presence of E-cadherin in spermatozoa from fertile males and its absence in oligozoospermic males. Using RT-PCR, we detected E-cadherin message in the round cells of fertile males, which was absent in the cells from oligozoospermic males. The presence of anti-E-cadherin antibody brought about quantitative reduction in the sperm–oocyte binding in vitro. These observations indicate the possibility of the interplay of a cadherin-dependent homophilic and/or heterophilic adhesion interaction between spermatozoa and oocyte during fertilization. The absence of a key adhesion molecule in a human male infertility disorder points towards genetic defects causing failure in gamete interactions.

The process of mammalian fertilization involves a cascade of cell–cell and cell–matrix interactions. First, sperm bind to the zona pellucida, a large extracellular matrix surrounding the egg. This interaction triggers the acrosome reaction that, in turn, allows sperm to penetrate the zona. Finally, sperm approach, bind to, and fuse with the egg plasma membrane. While details of the molecular interactions between sperm and the egg zona pellucida are beginning to emerge, little is known about the molecular basis for the essential plasma membrane binding and fusion reactions that initiate embryonic development [1–3].

Once in the confines of the perivitelline space the sperm head need traverse but a small distance to bind to the plasma membrane of the egg. In eutherian mammals, binding and subsequent membrane fusion occur at the equatorial region of the sperm head, while the acrosomal region becomes encapsulated in a vacuole [4]. The mechanism of fusion between the mammalian sperm and egg membranes is still largely unknown, but some recent studies have identified a few fusion peptide domains that might play a key role in this process [5]. Out of the long list of molecules that are proposed to mediate sperm–egg interactions, at least three sperm disintegrins (fertilin α, fertilin β, and cyritestin) are shown to mediate sperm–egg binding communicating with multiple integrins on eggs. An α-6/β-1 integrin appears to be able to function as a receptor for fertilin β, but blocking of this integrin does not block sperm–oocyte fusion. Thus, it is possible that other sperm

☆ *Abbreviations:* RT-PCR, reverse transcription polymerase chain reaction; HRP, horseradish peroxidase; PEG, polyethylene glycol; PBS, phosphate-buffered saline.

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disintegrins and perhaps other integrin ligands on sperm could bind to this receptor.

Spermatozoa, like other highly differentiated cells, have large numbers of cell type-specific antigens. Sperm-specific antigens appearing early or late may be stage specific [6–8] in that they appear and disappear during very discrete periods of time. Out of the temporally expressed sperm surface antigens, some are of special importance in that they could act as communication molecules coming into play during gamete recognition and fusion stages. It is well known that Ca^{2+} was required for fusion [3]. If Ca^{2+} was omitted from medium, the sperm bound to eggs, but did not fuse. The role of Ca^{2+} in promoting fusion in phospholipid model system is well worked out. But the precise mechanism through which calcium-dependent gamete recognition and fusion are brought about is not known. In this context, we suspected that a cadherin–cadherin and/or cadherin–integrin interaction might be involved in mediating these physiological interactions. In this study, we report the presence of E-cadherin on the head of normal human spermatozoa and its absence in the spermatozoa from oligozoospermic individuals. Experiments were also designed to evaluate the involvement of cadherin in mediating sperm–oocyte interaction in vitro.

Materials and methods

Reagents. Anti-E-cadherin and biotinylated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, CA. Streptavidin-HRP was from Calbiochem. Ready-To-Go T-Prime kit for reverse transcription was purchased from Amersham Biosciences, Hong Kong. All other reagents used were purchased from Sigma Chemical, WI.

Semen collection from humans. Volunteering semen donors who have fathered at least one child and are in the age group 30–40 were short-listed as prospective fertile donors. All the donor semen were analyzed for urogenital tract infections by a collaborating local clinic and the individuals who tested negative and had normal semen parameters (classical parameters including volume, pH, viscosity, sperm number, sperm viability, percentage motility, and fructose content, etc.) were tested for sperm competence by a zona-free oocyte penetration assay in our laboratory. We recruited 20 volunteers who passed all these tests in this study program. They are designated as healthy and fertile males.

Infertile males visiting local infertility clinics for treatment for male factor infertility were invited to volunteer in this study program. Individuals having normal values for testicular volume, serum testosterone levels, serum FSH levels, and patent ducts (as revealed by sonography) were initially short-listed as infertile donors. These donors also were subjected to all the routine pathological examinations and the healthy individuals with sperm count falling between 20 and 60 million/cc with normal motility, viability, and semen volume were designated as prospective infertile donors. The sperm were tested for competence using zona-free oocyte penetration assay. We included 32 patients out of 36 infertile volunteers who failed to pass zona-free oocyte penetration assay in this study program. These participants were designated as infertile males.

Subjects were included in this study program following the guidelines of the institutional ethical committee, and after obtaining the written consent of the donors. Semen samples from donors were collected by masturbation and were evaluated immediately after liquefaction. Sperm concentration, motility, and forward progression were microscopically assessed following World Health Organization standards [9]. Sperm morphology was assessed using strict criteria [10].

Separation of the motile sperm fraction was accomplished after double wash and centrifugation with Ham's F10 medium (Gibco, Grand Island, NY, USA) supplemented with 0.3% BSA. The final sperm pellet was overlaid with 1.0 ml medium and incubated for 1 h at 37 °C in 5% CO_2 in air to allow the motile sperm to swim up. The supernatant was aspirated and evaluated for concentration using a Neubauer hemocytometer.

In vitro capacitation. In vitro capacitation was accomplished following a standard protocol [11]. Briefly, sperm pellets were thawed and washed three times in 155 mM NaCl, 10 mM histidine, pH 7.4, and finally suspended in glucose-free TALP (100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.29 mM KH_2PO_4, 21.6 mM lactic acid, 1.5 mM $MgCl_2$, 1 mM pyruvate, 3 mg/ml bovine serum albumin, and 10 mM Hepes, pH 7.4), containing 10 mg/ml heparin. The cells were incubated in this medium for 4 h at 37 °C with shaking. In control samples (uncapacitated sperm), heparin was omitted from the incubation medium.

Preparation of sperm proteins. Sperm proteins were prepared as previously described [12]. In short cells were suspended in 10 mM histidine, 0.5 mM EDTA, 1 mM PMSF, and 1 mM benzamidine, pH 7.4, brought to 4 °C, and sonicated at 28 kHz for 30 s. The membranes were collected by differential centrifugation and suspended in extraction buffer (110 mM KCl, 5 mM NaCl, and 10 mM Mops, pH 6.8), and the proteins were extracted with 0.5% sodium cholate for 15 min. The proteins were then precipitated by the addition of 30% polyethylene glycol 4000 (PEG). After 1 h the protein was collected by centrifugation at 10,000 rpm for 10 min. The precipitates were redissolved in PBS and the protein concentrations were adjusted to 20 μg/10 μl of the solution. The samples were subjected to SDS–PAGE, followed by electroblotting as described below.

Western blot analysis. The separated proteins were transferred onto PVDF membranes (0.2 μM, Bio-Rad) in the presence of 20% v/v methanol, 25 mM Tris, and 190 mM glycine, pH 8.2 overnight at 30 mA constant current. For development of the blots, the membranes were pre-wet in methanol and then incubated in 5% non fat skimmed milk in TBS containing 0.1% Tween 20 (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) overnight. This was followed by three 10-min washings in TBST. The membranes were incubated with 1:100 dilution of respective primary antibody in TBST. This was followed by 5 washes in TBST, after which the membranes were incubated for 1 h with 1:5000 dilution of appropriate secondary antibody conjugated with HRP. The membranes were washed three times in TBST. The blots were developed by incubating the membranes in 0.05% DAB and 0.1% H_2O_2 and the color was enhanced with 0.04% nickel chloride in 50 mM Tris–HCl, pH 7.5.

Collection and culture of mouse oocytes. Female mice were superovulated by intraperitoneal injection of pregnant mare serum gonadotropin (30 IU/female) in the morning of day 1 and injection of hCG (30 IU/female) in the afternoon or evening of day 3. Mature oocytes were collected from the oviducts of superovulated females between 16 and 18 h after injection of hCG [13]. Oocytes were released into PBS containing 1 mg hyaluronidase/ml. After being rinsed with BSA-free M-2, oocytes were treated for 1 min with 1 mg trypsin/ml in BSA-free M2 to remove the zonae pellucidae. Zona free oocytes were washed in M2 containing 4 mg BSA/ml and cultured in 100 μl drops of TALP-8 medium under mineral oil (37.5 °C under 5% CO_2 in air).

Preparation of human spermatozoa and sperm–oolemma interaction in vitro. Human spermatozoa were collected after swim-up as mentioned earlier in this section. The sperm suspension was centrifuged at 650 g for 5 min. The pellet of spermatozoa was resuspended in TALP medium such that the sperm concentration was approximately 10^7 spermatozoa/ml. An aliquot (200 μl) was placed in a petri dish, covered with mineral oil, and incubated at 37.5 °C under 5% CO_2 in air. After incubation for 18–24 h, 2 μl of progesterone stock solution (100 μg/ml in 10% DMSO in

9% saline w/v) was added to a 200 μl drop of sperm suspension to induce acrosome reaction [14]. The incidence of acrosome reaction under these conditions was approximately 50–70% [14]. Ten minutes later, zona free mouse oocytes were introduced into the sperm suspension containing acrosome reacted spermatozoa. Between 10 and 30 min after insemination, when many spermatozoa were bound to each oocyte, the oocytes were washed gently in TALP and observed.

Immunofluorescence assays. The spermatozoa were suspended in capacitation medium smeared onto a clean glass slide coated with 0.05% poly-L-lysine and the smear was allowed to dry at room temperature. The slides were dipped in 10% neutral formalin for 10 min, followed by neutralization in 0.5 M ammonium chloride for another 10 min. The cells were permeabilized in 0.25% Triton X-100 for 20 min and chilled ethanol for 10 s. Cadherin epitopes were then visualized by indirect immunofluorescence microscopy [15]. Briefly, the samples were incubated with capacitation buffer containing 5% bovine serum albumin, followed by incubation with either 5% pre-immune rabbit serum or with 1:500 (by vol.) dilution of a rabbit anti-cadherin polyclonal antibody [15]. After washing, control and samples were incubated with a 1:300 (by vol.) dilution of a biotinylated rabbit anti chicken IgG antibody for 1 h at 38 °C with fluorescein isothiocyanate-labeled streptavidin (Calbiochem) at the manufacturers recommended dilution, and washed exhaustively. Finally, 10 ml of 10% (by vol.) glycerol was added to each sample and observed under fluorescence microscope (Nikon, 100 × 10 magnification).

RT-PCR of E-cadherin from cDNA prepared from the round cells of the semen. mRNA from the round cells in the semen was extracted using QuickPrep mRNA purification kit (Amersham Biotech). The mRNA concentration was adjusted to 50 μg/ml and the preparations were immediately processed for first strand synthesis. The first strand synthesis was performed with 2 μg mRNA using Ready-To-Go T-Primed first strand synthesis kit (Amersham Biotech) which utilized Moloney murine leukemia virus (M-MuLV) reverse transcriptase and *Not*1-d$(T)_{18}$ primer (5′-d[ACTGGAAGAATTCGCGGCCGCAGG AAT_{18}]-3′). PCR of the first strand was performed using E-cad specific primer pair (CTTGGAGCCGCAGCCTCT as the sense primer and CCTCCGTACATGTCAGCC as the antisense primer). β-Actin was amplified using GGACTTCGAGCAAGAGATGG (forward) and CACCTTCACCGTTCCAGTTT (reverse) primer set. The cycling conditions were: 1 min melting at 94 °C, 45 s annealing at 68 °C, and 1 min primer extension at 72 °C. The cycle was repeated 35 times, with 10 s increments in extension time per cycle. After the completion of the cycles, the reaction was held at 72 °C for 30 min, after which the samples were recovered. Five microliters of the reaction from each of the samples was mixed with 1 μl of 6× gel loading buffer (Sigma) and separated on 4% NuSieve 3:1 agarose (FMN) gel and 1× TAE buffer. Electrophoresis was performed at 5 V/cm^2 for 3 h. The gels were visualized and photographed on an Alpha Innotech imager station.

Results

Expression of E-cadherin in human spermatozoa

On Western blots, protein extracts from the spermatozoa of all fertile males showed a prominent band at 120 kDa that was recognized by anti-E-cadherin antibody. Fig. 1 shows six representative samples from our study group of fertile males (Fig. 1, lanes 2–7). This band was not detectable in the protein preparations from oligozoospermic individuals (lanes 8–15). Lane 1 show the molecular weight markers.

Immunolocalization of E-cadherin on sperm surfaces

Initial studies with spermatozoa that were not permeabilized did not exhibit detectable levels of fluorescence (data not presented). Since permeabilization of sperm membranes as detailed in the methods improved the localization substantially, we followed this protocol for performing the experiments included in this section. The spermatozoa from normal and oligozoospermic individuals were permeabilized were probed with anti-E-cadherin followed by incubation with a biotinylated secondary antibody and streptavidin conjugated with FITC. In case of normal spermatozoa, the whole sperm head showed heavy localization of E-cadherin. While the localization on equatorial domain and the post-acrosomal domain were very intense, the pattern of cadherin localization over the principal acrosomal domain was moderate (Fig. 2A). However, in the case of spermatozoa from infertile males, the spermatozoa did not show the presence of cadherin in majority of the sperm

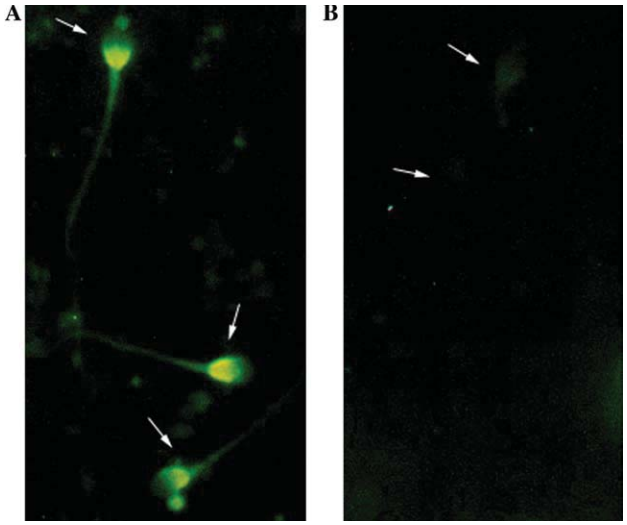

Fig. 2. Immunofluorescence analysis of spermatozoa from fertile (A) and infertile (B) human males. Arrows indicate the positions of sperm head.

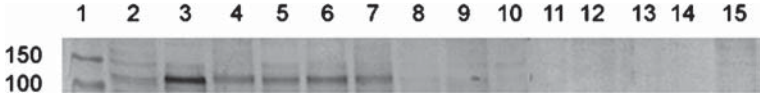

Fig. 1. Western blot analysis of E-cadherin on spermatozoa, showing representative samples from fertile and infertile human males. Sperm proteins immunoprecipitated with anti-E-cadherin antibody were analyzed on Western blots. A prominent 120 kDa band was present in samples from fertile males (lanes 2–7), which was absent in infertile semen samples (lanes 8–15). Lane 1 represents molecular weight markers.

cells. Very rarely, we saw a few spermatozoa that showed very weak staining over the post-acrosomal domain (Fig. 2B). The mid-piece and tail stained negative with anti-E-cadherin antibody.

RT-PCR analysis of E-cadherin

RT-PCR analysis showed transcripts of E-cadherin in the round cells of normal volunteers. Normal individuals presented the expression of two variants of E-cadherin transcripts, with a larger one at around 3.0 kb and the smaller one at around 2.2 kb positions (Fig. 3A). However, the mRNA preparation from the round cells of oligozoospermic individuals did not show any amplification with this primer set under identical experimental set-up (Fig. 3B). Internal control amplification with β-actin primer set was also performed using the cDNA prepared from fertile (Fig. 3C) and oligozoospermic (Fig. 3D) semen.

Sperm agglutination assay

Spermatozoa from fertile donors were washed and resuspended in Ham's F-10. These spermatozoa were mixed with a series of dilutions of anti-E-cad antisera (ranging from 1:100,000 to 1:50). The preparations were observed on slides to examine the occurrence of agglutination. Even at the highest concentration of anti-E-cadherin antibody used in this study, we failed to observe agglutination in washed and capacitated spermatozoa (Fig. 4B), indicating that the E-cadherin is not accessible on the surfaces of intact spermatozoa. A

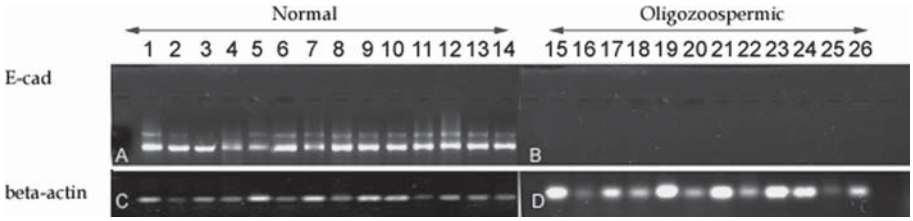

Fig. 3. RT-PCR analysis of E-cadherin message in the round cells of human semen. Representative amplifications of E-cadherin from cDNA prepared from the semenial cells of 14 fertile males are shown (A). Corresponding β-actin levels are represented in (C). RT-PCR performed for detecting E-cadherin in cDNA from the seminal cells of oligozoospermic males are shown in (B). Corresponding β-actin amplification is shown in (D).

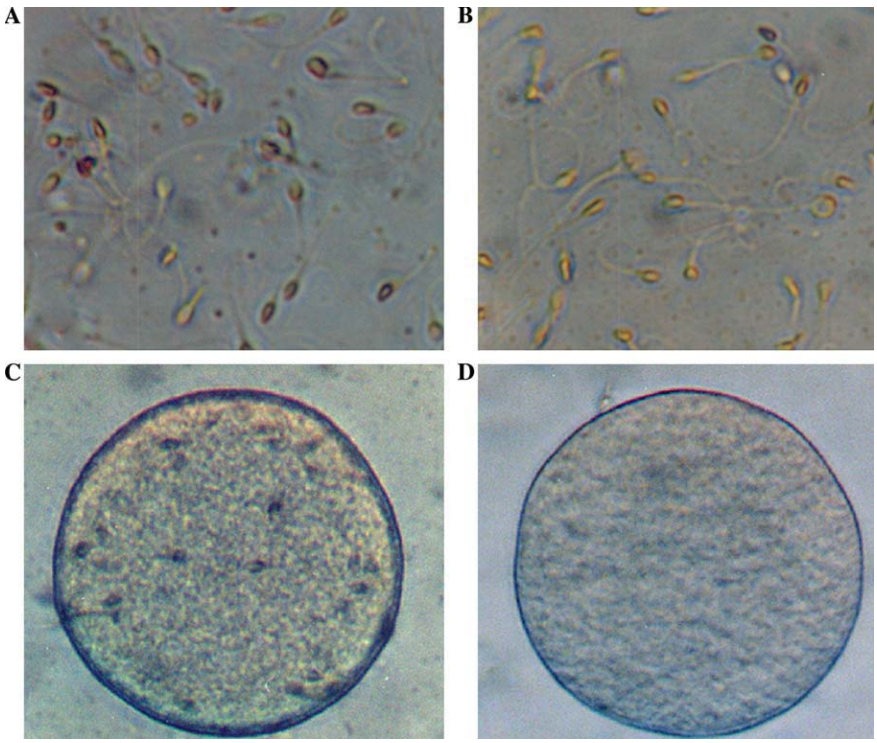

Fig. 4. Agglutination assay of capacitated human spermatozoa in presence of 1:50 dilution of anti-E-cadherin antibody (B). A control sperm preparation is shown (A) for comparison. Human sperm–mouse oolemma interactions in the absence (C) and presence (D) of E-cadherin antibody are shown.

control sperm preparation that did not receive any anti-E-cadherin antibody in the incubation is shown in Fig. 4A.

Sperm–oocyte interaction assay

The binding of spermatozoa to the oocyte in control preparations could be represented by Fig. 4C, where a heavy attachment of spermatozoa to the oocyte could be visualized. The binding of human spermatozoa to mouse oolemma was significantly inhibited by the presence of anti-E-cadherin antibody (Fig. 4D).

Discussion

Cellular interactions in the testis and epididymis are an important prerequisite for spermatogenesis and sperm maturation, and involve a well-developed complex of intercellular junctions. Of special interest are the essential mechanisms involved in sperm–oolemma fusion. Recognition and binding is initiated by specific cell surface receptor engagement between gametes. Fusion between oolemma and spermatozoa is prevented in the presence of trypsin in Ca^{2+}-free media, as is oocyte activation, implicating a cadherin-like adhesion [16]. Cadherins are cell surface proteins which mediate intercellular Ca^{2+}-dependent adhesion and are believed to be fundamentally important for cell–cell interactions and for maintaining multicellular structures [17]. Using primer sets specific for mouse N-cadherin and rat testicular RNA for RT-PCR, a full-length complementary DNA (cDNA) coding for rat testicular N-cadherin was isolated. The deduced amino acid sequence of rat N-cadherin yielded an 883-amino acid polypeptide that displayed a 98.6% identity with the mouse homolog. N-Cadherin was found to be expressed by Sertoli and germ cells in the rat testis by RT-PCR. Since N-cadherin plays a crucial role in facilitating invasive capacity of metastatic tumor cells, the observation of germ cell-released factor(s) in affecting Sertoli cell N-cadherin expression may suggest its possible role in facilitating germ cell migration during spermatogenesis [18]. Available literature presents some inconsistency in the expression of various cadherin derivatives in testis and on spermatozoa. Thus, Northern blot analysis of rat testicular RNA indicates the presence of N-Cad and P-Cad transcripts, with no detectable E-Cad message [19]. Andersson et al. [20] supported this observation by showing no expression of E-cadherin in the human testis [20]. In rat sperm lysate, both E- and N-cadherin were demonstrated as major protein bands but a series of lower molecular weight proteins (that may represent protein degradation) were also detected [21], raising a possibility that 7E-cadherin could be incorporated onto spermatozoa outside the testis. In the human epididymis E-cadherin, but not N-cadherin, was expressed and localized to the surface of the principal epithelial cells as shown by immunohistochemistry [20], supporting the extratesticular origin of E-cadherin on the spermatozoa. On the contrary, immunostaining of human spermatozoa with pan-cadherin, N-cadherin and E-cadherin antibodies by another group confirmed the presence of E-cadherin on human spermatozoa, mostly confined to different head regions [22]. The presence of N-cadherin on the spermatozoa has been confirmed by the identification of antigenic epitopes recognized by N-cadherin monoclonal antibodies diffusely distributed over the entire sperm head, and by presence of anti-N-cadherin reactive protein on Western blots [16]. However, human sperm lysates showed the presence of E-cadherin, but not N-cadherin [22]. Notwithstanding the inconsistencies, it is clear that various members of cadherin family of proteins are present on plasma membranes of spermatozoa from human and other animal origin, which may play a role in the intricate recognition process preceding gamete fusion. These observations also suggested the need to analyze cadherin transcripts from small numbers of spermatozoa from a variety of donors to determine if defects in cadherin distribution or structure may predict reduced male fertility.

We observed that spermatozoa from all the fertile human subjects possessed E-cadherin on its head domains, covering the principal acrosomal, equatorial, and the post-acrosomal domains (Fig. 2A). On the contrary, spermatozoa from oligozoospermic subjects did not possess E-cadherin on their principal acrosomal, and equatorial domains (Fig. 2B). Using immunoprecipitation followed by Western blot analysis, we observed a band of 120 kDa staining immunopositive in sperm protein extracts from fertile males, which was absent in samples from oligozoospermic males (Fig. 1). Using RT-PCR analysis, we could detect E-cadherin message in the testicular mRNA preparations from fertile males, but was not detectable in the case of the oligozoospermic individuals included in this study (Fig. 3). The spatio-temporal location of E-cadherin on the sperm head domains and the inhibition in sperm–oolemma interaction in anti-E-cadherin antibodies indicate the possibility of the interplay of a cadherin-dependent homophilic and/or heterophilic adhesion interaction between spermatozoa and oocyte during fertilization.

Cadherins comprise a family of calcium-dependent glycoproteins that function in mediating cell–cell adhesion in virtually all solid tissues of multicellular organisms. In epithelial cells, E-cadherin represents a key molecule in the establishment, and stabilization of cellular junctions. On the cellular level, E-cadherin is concentrated at the adherens junction and interacts homophilically with E-cadherin molecules of adjacent cells [23]. Recent success in solving the three-dimensional structure of an extracellular cadherin domain

provides a structural basis for understanding the homophilic interaction mechanism and the calcium requirement of cadherins. According to the crystal structure, individual cadherin molecules cooperate to form a linear cell adhesion zipper [23]. Triton X insoluble E-cadherin, α-catenin, and β-catenin colocalized along cell–cell contacts in spatially discrete micro-domains which we designate "puncta," and the relative amounts of each protein in each punctum increased proportionally. As the length of the contact increases, the number of puncta increases proportionally along the contact and each punctum is associated with a bundle of actin filaments. These results indicate that localized clustering of E-cadherin/catenin complexes into puncta and their association with actin is involved in initiating cell contacts. Subsequently, the spatial ordering of additional puncta along the contact may be involved in zippering membranes together, resulting in rapid strengthening of adhesion [24]. Using time-lapse imaging and photobleach recovery analyses of a fully functional E-cadherin/GFP fusion protein, three sequential stages in cell–cell adhesion were defined which provided evidence for mechanisms involving E-cadherin, and the actin cytoskeleton in transitions between these stages. In the first stage, membrane contacts between two cells initiated coalescence of a highly mobile, diffuse pool of cell surface E-cadherin into immobile punctate aggregates along contacting membranes. These E-cadherin aggregates were spatially coincident with membrane attachment sites for actin filaments branching off from circumferential actin cables that circumscribe each cell. In the second stage, circumferential actin cables near cell–cell contact sites separated, and the resulting two ends of the cable swing outwards to the perimeter of the contact. Concomitantly, subsets of E-cadherin puncta were also swept to the margins of the contact where they coalesced into large E-cadherin plaques. This reorganization resulted in the formation of a circumferential actin cable that circumscribes both cells, and was embedded into each E-cadherin plaque at the contact margin. At this stage, the two cells achieved maximum contact, a process referred to as compaction [25]. Cadherin-mediated cell–cell adhesion is initiated by *cis* dimerization of cadherin ectodomains at the cell surface followed by an antiparallel *trans* interaction of dimers on opposing cells [26]. It is becoming clear that variation in their extracellular region leads to the large potential for recognition properties of cadherin superfamily, as exemplified by the recently discovered FYN-binding CNR-protocadherins; these exhibit alternative expression of the extracellular portion, which could lead to distinct cell recognition in different neuronal populations, whereas their cytoplasmic part, and therefore intracellular interactions, is constant [27]. A recent report has shown that the gene-poor, hominid-specific Yp11.2/Xq21.3 X–Y homology block encodes two members of the protocadherin group of cell surface molecules, PCDHX and PCDHY [28,29]. PCDHX/Y are encoded by at least 11 exons spanning more than 700 kb, with many of the exons located at the 5′ and 3′ ends of PCDHX/Y displaying differential and alternative splicing [29]. Studies on NTERA pluripotential cell line (which differentiates along neuronal and spermatogenic pathways in response to retinoic acid) demonstrated that the X- and Y-linked genes are regulated differently [28]. It is not clear whether the E-cadherin identified on the spermatozoa from fertile human males is a variant of PCDHX/Y. However, the identification of E-cadherin on human sperm heads, and the absence of E-cadherin message and the protein in a subset of male infertility point out a molecular defect in cell-adhesion mechanisms associated with human infertility.

Acknowledgments

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