REPRODUCTIVE BIOLOGY

Human tubal secretion can modify the affinity of human spermatozoa for the zona pellucida

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Objective: To study the effect of the human tubal tissue conditioned medium (CM) on sperm parameters related to sperm–zona pellucida interaction.
Design: Controlled experimental laboratory study.
Setting: Research laboratory.
Subject(s): Semen samples from donors with normozoospermia. Human tubal tissue obtained from women undergoing hysterectomies. Human follicular fluids (hFF) and oocytes collected from patients undergoing IVF-ET.
Intervention(s): Incubation of spermatozoa with CM proteins obtained from human tubal tissue culture; sperm binding to the zona pellucida assessment.
Main Outcome Measure(s): Explants' viability was assessed by tissue DNA analysis. Sperm ability to interact with zona was tested with use of the whole oocyte test. Expression of D-mannose binding sites was assessed with use of a fluorescent probe on mannose coupled to bovine serum albumin. Human FF–induced acrosome reaction was assessed by the *Pisum sativum* technique.
Result(s): Although treatment with 0.8 μg/μL of CM allowed sperm binding to the zona and the expression of D-mannose binding sites comparable with sperm in control medium, with 3.2 μg/mL of CM resulted in a significant decrease of both parameters. No effect of CM on spontaneous or hFF-induced acrosome reaction or in sperm viability was observed.
Conclusion(s): The results indicate that the incubation of spermatozoa in the presence of CM reduces sperm affinity for the zona pellucida. This effect can be partly explained by the decreased expression of D-mannose binding sites on the sperm surface. (Fertil Steril® 2008;■:■–■. ©2008 by American Society for Reproductive Medicine.)

Key Words: Human spermatozoa, zona pellucida binding, D-mannose binding site, human oviduct, acrosome reaction, proteins

The mammalian oviduct provides the microenvironment for gamete transport and storage, fertilization, and embryo development. After ejaculation human mammalian sperm cannot fertilize the oocyte immediately; this ability is acquired after a series of physiologic changes, collectively known as *capacitation,* which is completed during sperm transit through the oviduct (1–3). In humans there are no barriers separating the fallopian tubes and the peritoneal cavity; thus the oviductal environment is composed of transuded serum, follicular fluid, peritoneal fluid, and oviductal tissue secretions (4).

Two hypotheses concerning the role of the oviduct have been suggested (5). The first one considers the oviduct as a passive environment that supplies nutrients and other necessary requirements that allow gamete survival and support the initial embryo development. The second one suggests that the oviduct contains factors involved in the modulation of gamete function and interaction and can also affect the embryo growth and differentiation (6, 7).

Supporting the second hypothesis, our laboratory has described recently that both human follicular and peritoneal fluids regulate sperm binding to the zona pellucida by decreasing the number of available zona receptors on the sperm head (8, 9). For ethical reasons and because of methodologic

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difficulties related to retrieving the fluids of the female genital tract, obtaining human tubal fluid is not recommended. To evaluate the interaction between the human oviductal fluid and spermatozoa, previous studies have reported that coculture of human spermatozoa with oviductal cells or their conditioned medium (CM) improved or maintained sperm motility and sperm survival (10–14). However, different experimental conditions (such as coculture with thawed or fresh cells, coculture with explants, or incubation with conditioned culture medium) were used in those studies. Our laboratory has set up an in vitro system to obtain CM from human oviductal explant culture (15).

Considering that spermatozoa may stay in the oviduct for several hours or even a few days awaiting the arrival of the oocyte (16), it has been suggested that the interaction with fluids present at the female genital tract could modulate the acquisition of sperm fertilizing ability (17, 18). Taking into account that sperm recognition and interaction with the zona pellucida is a critical step in fertilization (2), the aim of the present study was to investigate whether the CM obtained from human oviductal explants affects sperm function by modifying sperm-egg interaction.

MATERIALS AND METHODS

The study protocols were approved by the Institutional Bioethical Board of the School of Biochemical and Pharmaceutical Sciences (National University of Rosario), and written consent was obtained from all donors.

Chemicals and Reagents

Unless mentioned in the text, all the chemicals and reagents were purchased from Sigma-Aldrich Inc. (St. Louis, MO) or from MP Biomedicals Inc. (Burlingame, CA) and were of the highest purity available.

Culture of Human Oviductal Explants

Human oviductal tissues were obtained from premenopausal patients (n = 17, age range 37 to 50 years) with normal menstrual cycles and no clinical history of infection or neoplastic disease, scheduled for hysterectomies as a result of uterine fibromyomas or hypermenorrhea. Thirteen patients were at the periovulatory phase of the menstrual cycle, and the rest were at the mid-follicular phase. No patient received any hormonal treatment before hysterectomy. Patients gave written consent for removal of their oviducts. Tubal tissues were processed within 1 hour after surgery and cultured as described elsewhere (15). In brief, tissue explants were cultured 24 hours in Dulbecco's modified Eagle's medium–Ham's F-12 medium (1:1) (GIBCO BRL, Paisley, Scotland) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) (MP Biomedicals Inc.), fetal bovine serum (FBS, 10% vol/vol; Bioser, Buenos Aires, Argentina), L-glutamine (2 mmol/L; Seromed, Biochrom KG, Berlin, Germany), and HEPES (15 mmol/L, Imperial, United Kingdom). Tissues were then further cultured 24 hours in serum-free medium. After incubation, the CM was collected and centrifuged 5 minutes at 500 × g to remove debris. The clarified media were dialyzed (10 kDa membrane; Sigma-Aldrich, Steinheim, Germany) against distilled water for 24 hours at 4°C, lyophilized (Labconco, Lyph-Lock 12, Kansas City, MO), redissolved in 200 μL of Ham's F-10 medium (Hyclone, Logan, UT) and stored at −20°C until use. Each CM collected was used in independent experiments. Total protein concentration in CM was assessed with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). All cultures were performed at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Collection of Human Follicular Fluid

Human follicular fluids (hFF) were collected during oocyte retrieval from women (n = 6) participating in an IVF program, as described in a previous report (9). Only hFF with no blood contamination and from follicles containing a mature oocyte (metaphase II) were collected. Follicular fluids from each sample were centrifuged (10 minutes, 600 × g) to remove cellular debris, filtered through a 0.22-μm membrane (Millipore Corp., Bedford, MA), and mixed into a pool, before being stored at −20°C.

Tissue DNA Integrity

To evaluate tissue viability after culture, we analyzed DNA integrity of oviductal explants at the end of incubations. In brief, explants were manually disaggregated with buffer B (0.0875 mmol/L NaCl, 9 mmol/L EDTA, 10 mmol/L tris[hydroxymethyl]aminomethane [Tris]). Supernatants were treated for 20 hours at 37°C with sodium dodecyl sulfate (SDS; 20% vol/vol) and proteinase K (10 mg/mL; Promega, MA), after which suspensions were sequentially extracted with phenol, phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform–isoamyl alcohol (24:1) (19). Deoxyribonucleic acid was precipitated by adding NaCl (5 mol/L) and absolute ethanol and incubating for 2 hours at −20°C. After centrifugation at 7,000 × g, DNA was washed with 70% vol/vol ethanol, air dried, resuspended in sterile water, and stored at −20°C until use. Electrophoresis of DNA samples was carried out on 1% vol/vol agarose gels (Bio-Rad) in TBE buffer (0.089 mol/L Tris base, 0.089 mol/L boric acid, 0.02 mol/L EDTA, pH 8.3) for 2 hours at 75 V. A commercial ΦX174 DNA/HaeIII marker (72–1,353 bp; Promega) was used as molecular weight standard. Conditioned media obtained from tissues with altered DNA integrity were excluded from the study.

Protein Analysis

Total protein concentration was measured in each CM by Bradford's method (Bio-Rad). Proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with use of 7.5% wt/vol polyacrylamide gels (20), at 100 V in a Mini-protean II system (Bio-Rad). In brief, 10 μg of total protein from each CM in sample buffer (0.2% wt/vol bromophenol blue, 20% vol/vol glycerol, 10% vol/vol

β-mercaptoethanol, 0.125 mol/L Tris-HCl; 4% wt/vol SDS) was loaded per lane. After electrophoresis, proteins were detected by staining the gels with Coomassie blue. A commercial protein marker was used as molecular weight standard (Kaleidoskope; Bio-Rad).

Semen Samples and Sperm Processing

Semen samples were obtained from donors with normozoospermia (n = 26), collected by masturbation after 3 to 5 days of sexual abstinence. After complete liquefaction, semen analysis was performed according to the World Health Organization (WHO) guidelines for the examination of human semen (21). Morphology was analyzed by strict criteria (22), and viability assessed with use of 0.5% wt/vol eosin Y as described in the WHO guidelines (21).

Motile spermatozoa were selected by swim up. In brief, 1 mL of Ham's F-10 medium was layered directly onto 1 mL of liquefied semen and incubated for 1 hour at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The uppermost aliquot (1 mL) containing the motile fraction was aspirated, and sperm concentration assessed. Sperm concentration was adjusted to 4 to 5 × 10^6 spermatozoa/mL and capacitated 22 hours in Ham's F-10 medium supplemented with bovine serum albumin (BSA; 35 mg/mL), streptomycin (100 μg/mL), and penicillin (100 U/mL), at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Effect of CM on Sperm Viability

After isolation, motile sperm were incubated 22 hours under capacitating conditions followed by 2 hours in the presence of CM (0.8 or 3.2 μg protein/μL); viability was assessed with use of 0.5% wt/vol eosin Y as described in the WHO guidelines (21).

Sperm–Zona Pellucida Binding Assay

Unfertilized human oocytes, donated by women undergoing IVF procedures, were stored in a salt solution containing 1.5 mol/L $MgCl_2$, 0.1% wt/vol polyvinylpyrrolidone, and 40 mmol/L HEPES in phosphate-buffered saline solution (pH 7.2) at 4°C, until use. Before oocytes were used for assays, excess salt was removed by washing them in Ham's F-10 and incubating for 20 hours at 37°C in an atmosphere of 5% CO_2/95% air, after which three to six oocytes were placed in a 100 μL oil-overlaid droplet containing Ham's F-10 supplemented with BSA (35 mg/mL, control) in the presence or the absence of 0.8 or 3.2 μg/μL of CM. Drops were inseminated with 1.0 × 10^5 progressively motile sperm/mL and incubated 4 hours in 5% CO_2 in air, after which oocytes were washed by repeated vigorous pipetting and placed on a drop containing 0.5% wt/vol eosin Y solution to exclude dead spermatozoa. The number of tightly bound unstained spermatozoa was registered by a video camera coupled to a phase-contrast inverted microscope (Olympus Optical Co. Ltd., Tokyo, Japan) at ×300 magnification. Results were expressed as the mean percentage of bound unstained spermatozoa (viable) in each droplet.

D-Mannose-binding Sites

Capacitated spermatozoa were exposed 2 hours to 0.8 or 3.2 μg/μL of CM proteins, and the presence of mannose-binding sites was analyzed according to the protocol of Benoff et al. (23). In brief, samples were washed three times with core buffer (30 mmol/L HEPES, 0.5 mol/L $MgCl_2$, 150 mmol/L NaCl, 10 mg/ml BSA, pH 7.0) containing 20 mmol/L [Ca^{2+}] and incubated 30 minutes with 100 μg/mL fluorescein isothiocyanate (FITC) conjugated with mannosylated BSA (Man-FITC-BSA) in calcium-supplemented core buffer at 37°C in an atmosphere of 5% CO_2/95% air. After labeling, motile sperm were pelleted by centrifugation, washed three times with core buffer (without calcium), air dried onto glass slides, and mounted in a glycerol-based medium. Specimens were evaluated at ×1,000 with use of an epifluorescence microscope (Leica Microsystems, Wetzlar, Germany). At least 200 spermatozoa were evaluated per slide. Each spermatozoon was classified according to the pattern of the fluorescent signal as unspecific pattern I (stain on tail and midpiece, observed for all cells), specific pattern II (stain on entire acrosomal cap), or specific pattern III (stain on equatorial/postacrosomal regions). The results were reported as the percentage of cells showing specific patterns II and III in each treatment group.

Acrosome Reaction Assessment

Overnight-capacitated spermatozoa were divided into several aliquots that were incubated 2 hours in the presence or absence of two different protein concentrations of CM (0.8 or 3.2 μg/μL), at 37°C and 95% air and 5% CO_2. Sperm samples were divided into two aliquots and exposed 30 minutes to either control medium (spontaneous acrosome reaction [AR]) or 20% vol/vol hFF (induced AR) at 37°C in an atmosphere of 5% CO_2/95% air, after which sperm viability was assessed. According to the protocol of Cross et al. (24), sperm were treated with FITC-conjugated *Pisum sativum* agglutinin (50 μg/mL) and examined at ×1,000 with use of an epifluorescence microscope (Leica Microsystems). Spermatozoa were classified as [1] nonreacted, if the acrosomal cap was uniformly labeled; [2] reacting, if the acrosomal cap was labeled in a patchy pattern; or [3] reacted, if only the equatorial segment was labeled. At least 200 spermatozoa were evaluated for each slide. The percentage of reacted cells was reported as [2] +[3]. The inducible population was calculated as the difference between the percentage of reacted cells in the presence of hFF (induced AR) and the percentage of reacted cells in the absence of this fluid (spontaneous AR).

Data Analysis

Statistical analysis was performed with use of the GraphPad INSTAT program (GraphPad Software, San Diego, CA). The Kruskal-Wallis nonparametric test and Dunn's test for

multiple comparisons were used to compare the percentage of viable cells and the number of sperm bound to oocytes. Analysis of variance (ANOVA) and the Tukey-Kramer test for multiple comparisons were used to compare the percentages of AR, the percentages of α-D-mannose–binding sites, and the motility of spermatozoa. Data were expressed as the mean ± SEM. A $P<.05$ was considered statistically significant.

RESULTS

Tissue DNA Integrity

To determine tissue viability after culture, we evaluated DNA integrity after incubation. Deoxyribonucleic acid was extracted as described in Materials and Methods and analyzed by agarose gel electrophoresis. Only three of 17 tissue samples (18%) presented an altered DNA pattern determined by the presence of low molecular weight fragments, and these were discarded. It is important to remark that only CM coming from tissues with intact DNA were included in the present study (n = 14).

Protein Analysis

To perform comparable experiments we measured total protein concentrations in each CM. Mean protein concentration in CM was 8.5 ± 1.2 μg/μL (range 2.8 to 15.7 μg/μL). There were no differences in protein concentration based on menstrual cycle phase or patient age. To assess the similarity among the individual CM used, we compared the electrophoresis protein pattern of each sample. Proteins from each CM were analyzed by SDS-PAGE and stained with Coomassie blue G-250. The results showed that the electrophoretic patterns of each CM were comparable.

Effect of CM on Sperm Viability

To exclude any deleterious effect of CM on sperm survival, capacitated spermatozoa were exposed for 2 hours to 0.8 or 3.2 μg/μL of CM protein, and cell viability was assessed with use of 0.5% wt/vol eosin Y. Results showed that, at the doses tested, sperm viability remained >75%, indicating that the presence of CM did not affect sperm viability (Table 1).

Effect of CM on Sperm–Zona Pellucida Binding Capacity

To determine whether the presence of CM affects sperm binding to the homologous zona pellucida, we incubated sperm up to 4 hours with oocytes in a medium containing CM (0.8 or 3.2 μg/μL of protein concentration) or in control medium. Results showed that 0.8 μg/μL of CM protein did not affect the number of tightly bound spermatozoa compared with those incubated in control medium (Table 2). However, a concentration of 3.2 μg/μL of CM proteins resulted in a significant ($P<.05$) reduction in the number of spermatozoa bound to the zona. Viability in the treated and control groups was comparable at the end of the assay (data not shown).

TABLE 1

Effect of CM on sperm viability (N = 5).

Treatment	Viable sperm (%)
Post swim up	90.1 ± 1.6
22 h Capacitation	86.0 ± 2.7
CM (μg/μL)	
0.8	76.6 ± 2.8
3.2	75.0 ± 6.0

Note: After isolation, motile sperm were incubated 22 hours under capacitating conditions followed by 2 hours in the presence of CM (0.8 or 3.2 μg/μL) of protein concentration. Viability was assessed after each treatment. Results are expressed as mean ± SEM.

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Effect of CM on the Expression of D-Mannose–Binding Sites

To evaluate whether the reduction in sperm binding to the zona pellucida is related to a lower availability of D-mannose–binding sites on the sperm surface, we exposed overnight capacitated spermatozoa for 2 hours to 0.8 or 3.2 μg/μL of CM protein and evaluated the presence of D-mannose–binding sites. Results indicated that although CM proteins at a concentration of 0.8 μg/μL had no effect on mannose-binding sites, concentrations of 3.2 μg/μL resulted in a significant decrease ($P<.05$) in the expression of specific patterns (II and III) compared with controls (Fig. 1).

Effect of CM on AR

To investigate whether the presence of CM affects the occurrence of the AR, we exposed capacitated spermatozoa to CM or control medium. No statistically significant differences were observed between AR in spermatozoa treated with

TABLE 2

Effect of CM on sperm–zona pellucida–binding capacity.

Treatment	No. of sperm bound
Control (n = 22 oocytes)	16.7 ± 2.3
CM (0.8 μg/μL, n = 23 oocytes)	12.6 ± 2.5
Control (n = 6 oocytes)	18.6 ± 4.2
CM (3.2 μg/μL, n = 7 oocytes)	2.2 ± 0.4[a]

Note: Spermatozoa and oocytes were coincubated 4 hours in the presence of CM (0.8 μg/μL–3.2 μg/μL) or in control medium, after which the number of spermatozoa tightly bound to the zona was determined. Results are expressed as mean ± SEM.

[a] $P<.05$ compared with control.

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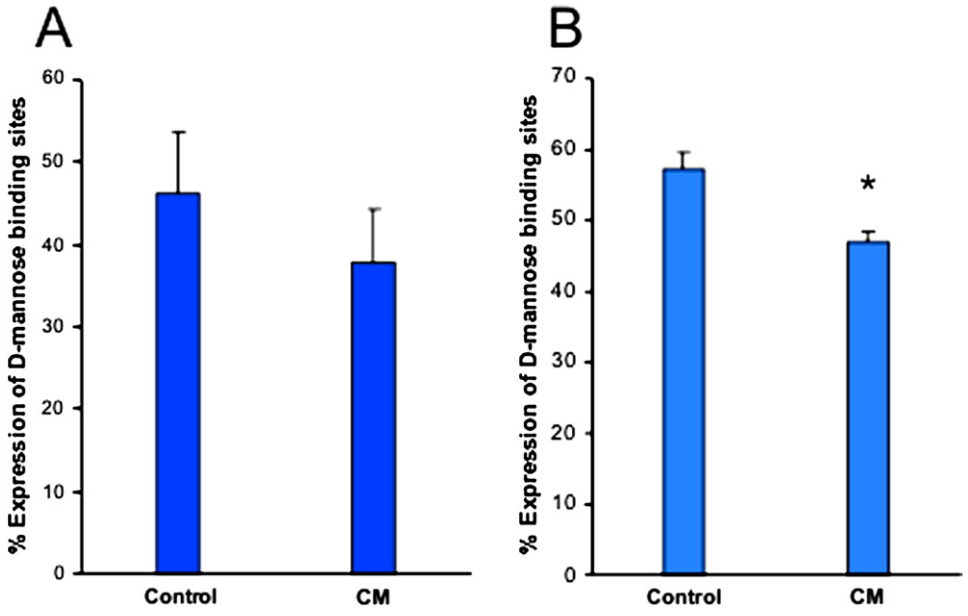
FIGURE 1

Effect of CM on the expression of D-mannose–binding sites. Overnight-capacitated spermatozoa were exposed 2 hours to CM (0.8 or 3.2 μg/μL) or control medium, after which the presence of sugar binding sites was determined by labeling sperm with Man-FITC-BSA. (**A**): 0.8 μg/μL (n = 4). (**B**): 3.2 μg/μL (n = 4). Results are expressed as mean ± SEM. *$P<.05$ versus control.



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CM with respect to controls (Fig. 2). Whereas hFF significantly induced the AR in all assays, the presence of CM did not increase the rate of AR at any of the concentrations tested, because values remained similar to those of controls (data not shown).

DISCUSSION

Although millions of spermatozoa are deposited in the vagina, the number of sperm that reach the site of fertilization is very low (16, 25). In humans, intercourse may occur at any time during the menstrual cycle, and spermatozoa can retain their physiologic function within the female tract for several days after copulation (16, 25, 26). This "surviving ability" may be due in part to the assistance of specific factors and proteins secreted by the female genital tract, which can modulate sperm function (17, 27–31). The aim of the present report was to investigate the effect of the CM obtained from oviductal tissue culture on parameters related to sperm-egg interaction.

We recently have detected at least 16 de novo synthesized proteins in the CM from human oviductal tissue culture (15). In the present study, explant quality was assessed at the end of the culture period by evaluating tissue DNA integrity. Results indicated that 82% of the collected CM were obtained from viable tissues. This inclusion criterion was considered critical for the study, because the composition of the CM could be affected by the functional integrity of the oviductal tissue. As far as we know, this is the first report where the tubal tissue integrity was assessed after culture and previous to CM-spermatozoa incubation.

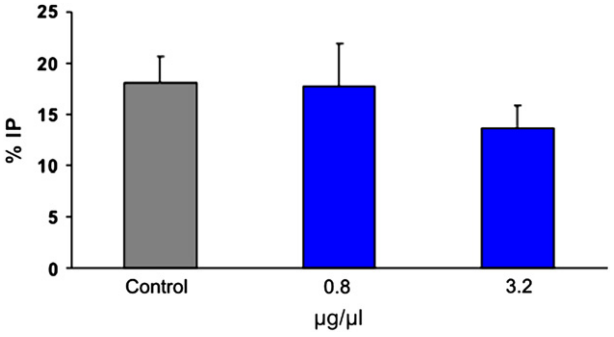
Because we found a variable range in the total protein concentration of CM from different tissues (2.8 to 15.7 μg/μL) and assuming that protein factors could modulate sperm function, to perform comparable assays, we exposed spermatozoa to CM by setting the protein concentration to known values (0.8 and 3.2 μg/μL). In contrast, in previous studies spermatozoa were incubated with the pure CM or at different vol/vol ratios (10–14).

In the presence of CM no alteration in sperm viability was observed, because values were similar to those in control medium. In agreement with this result, a previous report by Yeung et al. (10) showed that the exposure of spermatozoa to CM obtained from cultures of cryopreserved oviductal cells also maintained sperm viability.

Tight binding of human spermatozoa to the human zona pellucida is an early and critical step in gamete interaction leading to fertilization (2). Our data show that in the presence of 3.2 μg/μL of CM protein, there is a significant reduction in the number of viable bound spermatozoa to the homologous oocytes with respect to those incubated in control medium.

FIGURE 2

Effect of CM on sperm AR. Overnight-capacitated sperm were incubated 2 hours in the presence of 0.8 or 3.2 μg/μL of CM proteins and divided into a control aliquot and an aliquot exposed 30 minutes to 20% vol/vol of hFF, after which AR was assessed. The IP was calculated as the difference between the percentage of reacted cells in the presence of hFF and the percentage of reacted cells in the absence of this fluid. Results are expressed as means ± SEM (n = 5).



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The present results are in accordance with a previous report of Yao et al. (32), who, using the hemizona assay, observed that the CM also decreased the ability of spermatozoa to interact with the zona. However, they did not analyze any mechanisms involved in this effect.

As far as we know, this is the first study in which sperm-oocyte interaction was evaluated in a drop containing a supravital staining (eosin Y). Only those unstained spermatozoa (viable), tightly bound to the zona, were recorded, and, thus, sperm bound during the IVF procedure were excluded.

Because sperm binding capacity to human oocytes may provide predictive information of sperm fertilizing potential (33), our data suggest that the presence of the CM reduces this parameter. However, little is known about the possible mechanisms involved in that inhibition.

It is widely accepted that carbohydrates mediate sperm-egg interaction on the basis of the specific binding of sperm-surface carbohydrate-binding proteins to glycoconjugates present in the zona pellucida, which sets off the signal transduction pathways that result in the AR (34). It has been demonstrated that D-mannose residues participate in sperm–zona pellucida binding (35–37). With the aim to analyze whether the reduction in zona binding in the presence of CM was related to a decrease in the number of available zona receptors on the sperm surface, we also assessed the presence of D-mannose binding sites. The results show that in the presence of human oviductal CM (3.2 μg/μL of protein), there is a significant reduction in the number of detectable binding sites on the sperm surface ($P<.05$). This is the first study where the decrease in sperm-binding capacity caused by CM was associated with a decreased expression of D-mannose binding sites on the sperm surface. Although the presence of CM decreased the number of D-mannose binding sites in about 19%, a higher rate of reduction in the number of bound spermatozoa to the zona was observed (approximately 88%). This finding suggests that other carbohydrate-ligand systems responsible for the binding to the zona pellucida (for review see Oehninger [38]) also could have been blocked by the presence of CM. However, further experiments are necessary to elucidate this assumption.

The occurrence of the AR triggered at the zona pellucida is an essential prerequisite for fertilization (2). In this study, capacitated spermatozoa pretreated with CM were induced with hFF as a physiologic stimulus, and the AR analyzed. Results indicated that the inducible population (which represents the fertilizing population) ranged between 13% and 22%, in agreement with the hypothesis that capacitation is an asynchronic and reversible event (39). Because the oviductal tissue is devoid of steroidogenic activity, P is absent in the CM (data not shown) justifying in part the lack of AR-inducing activity of CM itself. Progesterone is the main component of the hFF that induces the AR (40). Because P receptors and D-mannose binding sites seem to be related topographically (41), it would be expected that if some molecules from CM blocked the D-mannose binding sites, the neighboring sites could also be blocked. However, preincubation with CM did not prevent the ability of spermatozoa to further undergo the hFF-induced AR. Although there was a trend indicating that the hFF-induced AR was decreased at the highest dose of protein used, values among treated and untreated groups were not statistically different.

Considering that only intact spermatozoa could interact with the zona (42), it is expected that during oviductal transit they conserve their acrosomes intact (2, 43). Thus, the reduction on sperm binding described in this article was not a consequence of an effect on the occurrence of the AR.

In this sense, in a recent study we observed that CM decreased the ionophore-induced AR (15). Taking into account that ionophore acts as a pharmacologic AR inducer, it is possible that CM interferes with its mechanism of action, which does not involve the P receptor activation as with hFF-induced AR (44, 45).

In conclusion, our model suggests that tubal secretions could regulate the number of viable spermatozoa with the ability to interact with the homologous zona pellucida. These results reinforce the idea that the human oviduct could be considered as a sperm "reservoir," which maintains and prepares the spermatozoa for successful fertilization.

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