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Sperm attraction to a follicular factor(s) correlates with human egg fertilizability

(sperm–egg communication/follicular fluid/chemotaxis/sperm accumulation/fertilization)

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ABSTRACT **Spermatozoa normally encounter the egg at the fertilization site (in the Fallopian tube) within 24 hr after ovulation. A considerable fraction of the spermatozoa ejaculated into the female reproductive tract of mammals remains motionless in storage sites until ovulation, when the spermatozoa resume maximal motility and reach the fertilization site within minutes. The nature of the signal for sperm movement is not known, but one possible mechanism is attraction of spermatozoa to a factor(s) released from the egg. We have obtained evidence in favor of such a possibility by showing that human spermatozoa accumulate in follicular fluid *in vitro*. This accumulation into follicular fluid was higher by 30–260% than that observed with buffer alone and was highly significant ($P < 10^{-8}$). Not all of the follicular fluids caused sperm accumulation; however, there was a remarkably strong correlation ($P < 0.0001$) between the ability of follicular fluid from a particular follicle to cause sperm accumulation and the ability of the egg, obtained from the same follicle, to be fertilized. These findings suggest that attraction may be a key event in the fertilization process and may give an insight into the mechanism underlying early egg–sperm communication.**

Spermatozoa normally meet the egg at the fertilization site (isthmic–ampullary junction of the Fallopian tube) within 24 hr after ovulation (1–3). In women, cervical spermatozoa have been reported to possess prolonged acrosomal integrity (*in vivo*), and this has led to the suggestion that the human cervix may serve as a site of sperm storage (4). In cattle, pigs, and rabbits it has been shown that a considerable fraction of the spermatozoa ejaculated into the female reproductive tract remain in the isthmus of the Fallopian tube until ovulation occurs; they then resume their motility and reach the fertilization site of the ampulla within minutes (2). The mechanisms for sperm selection and the synchronization between ovulation and sperm movement in the female reproductive tract are not known. One plausible mechanism for directed sperm movement would be the attraction of mammalian spermatozoa to factor(s) released from the egg. *In vivo* studies are currently difficult or impossible, and the many techniques available for measuring leukocyte chemotaxis *in vitro* (5) appear to be inadequate for studies on mammalian sperm chemotaxis, primarily because of the complexity and rapidity of sperm motion, which subsequently results in poor signal-to-noise ratios. On the other hand, microscopic methods have been useful for demonstrating chemoattraction of invertebrate spermatozoa to the egg (6, 7). Such studies in the mammal have not been reported to our knowledge. Here we examine the possibility of sperm attraction to follicular factor(s) by measuring sperm accumulation in follicular fluid *in vitro*. We show that human spermatozoa indeed accumulate in follicular fluid and that this accumulation correlates with the ability of the egg to be fertilized.

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MATERIALS AND METHODS

Preparations of Sperm Cells. Human ejaculates were collected by masturbation from normal healthy donors. Each ejaculate was allowed to liquefy at room temperature, and was centrifuged; the pellet was washed twice by suspending in Biggers, Whitten, and Whittingham (BWW) buffer (8) followed by centrifugation. The final pellet was resuspended in BWW buffer in 1/10th of its original volume (which varied from sample to sample). A portion (0.2 ml of the concentrated spermatozoa) was overlaid carefully with 1.8 ml of BWW buffer. The sperm preparations were incubated for 2 hr at 37°C in 5% CO_2/95% air. The upper 1 ml of the buffer was gently aspirated after this time, and the volume was adjusted to give a cellular density of 1–2 million cells per ml ("swim-up" spermatozoa).

Follicular Fluid Preparation. Human follicular fluids were obtained from women undergoing transvaginal aspiration for *in vitro* fertilization, who had been pretreated with human menopausal gonadotropins for ovarian stimulation (9). Follicular fluids were filtered through a 0.45-μm Acrodisc filter to remove cells and cell debris. The filtrates were divided into 200-μl aliquots and stored at −20°C. Follicular fluids were tested fresh or within 2 weeks of storage. After this period of time their attractive activity for sperm gradually decreased. The procedure for fertilization of the egg under *in vitro* conditions was as described (10).

Accumulation Assay. The lower wells of the 48-well microchemotaxis-chamber (Neuroprobe AP48, Neuroprobe, Cabin John, MD) were filled with 27 μl of swim-up spermatozoa suspended in BWW medium at a density of 1–2 × 10^6 cells per ml. The upper wells were separated from the lower ones by a polycarbonate Nucleopore filter (8-μm pore diameter) and a gasket. The upper wells were filled with various dilutions of follicular fluid as indicated for the particular experiment. A control with suspending BWW medium only was carried out for each assay. The follicular fluid was diluted in BWW medium [20 mM sodium lactate, 5 mM glucose, 0.25 mM sodium pyruvate, bovine serum albumin (fraction 96–99%), at 3 g/liter, penicillin G at 0.08 g/liter, streptomycin sulfate at 0.05 g/liter, 95 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4$ in 25 mM $NaHCO_3$ buffer (pH 7.4)] (8). The chamber was then incubated for 15 min at room temperature or 10 min at 37°C. The

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accumulation of spermatozoa in the upper wells was measured in one of the following manners.

Endopeptidase activity. We found that endopeptidase activity of spermatozoa is proportional to the number of cells per unit volume and, as such, can serve as a measure of accumulation (unpublished data). An aliquot of 20 μl from each well was transferred into a microtiter plate and assayed for endopeptidase activity as described (11).

Direct counting. After the upper wells were filled and before the 10- to 15-min incubation, the upper wells were covered by another gasket and a microscope slide as described by Gnessi *et al.* (12). The microscope slide had been precoated with polylysine and spermidine to assure good adsorption of the sperm cells to the glass at the subsequent centrifugation step. The coating was performed by incubating the slides in a solution if 0.01% polylysine and 0.5% spermidine overnight (4°C). The slides were air-dried in a 37°C incubator. There was no direct contact between the glass slide and the liquid during the assay. After the incubation step, the chamber was centrifuged upside down at 100 × *g* for 20 min at room temperature. The upper part of the chamber including the glass slide was removed, the excess of liquid in the upper wells was blotted, and the slide-containing part was dried in a 37°C incubator. The dried slide was then separated from the chamber, and the total number of sperm cells in the area above each well was determined by manual counting using a Zeiss phase-contrast microscope (×256).

The absorbance values of the endopeptidase assay or the counted number of cells shown on the ordinate were typically averages of 6–12 determinations of the same follicular fluid and the same sperm (± SEM). The low signal-to-noise ratio of the assay makes the multiple determinations necessary. This assay requires spermatozoa with good motility. Nonmotile or poorly motile sperm samples do not reach the upper wells.

Hormone Level Assay. Progesterone and estrogen were determined by radioimmunoassay with direct solid-phase kits provided by Diagnostic Products (Los Angeles). The sensitivity of the progesterone assay was 0.1 ng/ml, and the intraassay and interassay coefficients of variation were 6% and 10%, respectively. The sensitivity of the estrogen assay was 20 pg/ml, and the intraassay and interassay coefficients of variation were 7% and 10%, respectively.

Analysis of Motility Tracks. The follicular fluid was collected and treated as described above except that, instead of being filtered, the fluid was centrifuged at 200,000 × *g* for 15 min at 4°C in a Beckman TL-100 centrifuge, and only the supernatant fluid was assayed. The swimming of spermatozoa was recorded on a National video recorder and subsequently analyzed by an ExpertVision computerized motion analysis system. Microinjection into 100-μl samples of swim-up spermatozoa (10^5 cells per ml) was carried out as described (7).

Statistical Analysis. All the analyses were made according to unpaired, one-tailed Student's *t* test by using StatWorks software.

RESULTS

Accumulation of Spermatozoa in Follicular Fluid. To determine whether spermatozoa are attracted to factor(s) released by the egg or its surrounding cells, we initially used follicular fluids. Since follicular fluid contains secretions of the egg and its surrounding cells, we considered it as a potential as well as convenient source of soluble factors that might be secreted from the egg or its surrounding cells. For the study we used a macroscopic assay that is based on an assay that was initially developed for studying neutrophil chemotaxis (13) and later modified by Gnessi *et al.* (12) for studying sperm chemotaxis. The lower wells of a 48-well chamber were filled with a constant number of spermatozoa as described, and the upper wells, separated from the lower ones by a cell-permeable membrane, were filled with various dilutions of follicular fluid. An accumulation of cells into follicular fluid was clearly observed (Fig. 1). When a similar assay was carried out with the sera of two women from whom at least one follicular fluid caused sperm accumulation, no detectable accumulation of spermatozoa in the sera was obtained (not shown in the figure). Also, when the motility of the spermatozoa was very high and follicular fluid was put in both the upper and the lower wells, no accumulation was observed. However, with spermatozoa of average motility, some accumulation was observed when the follicular fluid was in both the upper and the lower wells, but this accumulation did not equal the accumulation seen when the follicular fluid was only in the top wells.

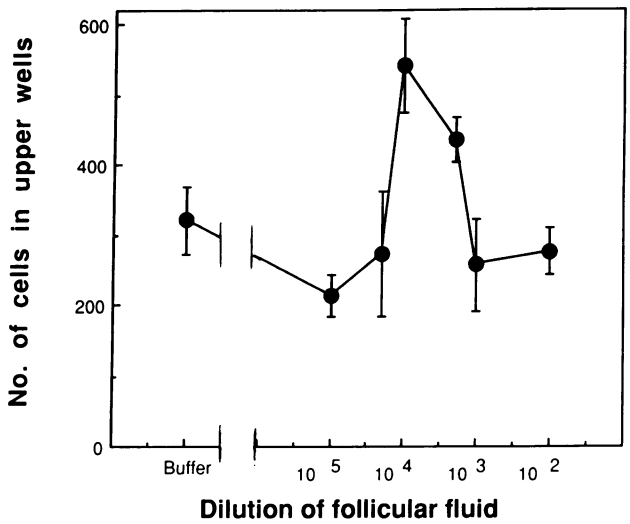

FIG. 1. Accumulation of sperm cells in the upper wells of a chemotaxis chamber that initially had sperm cells in the bottom wells and various concentrations of follicular fluid in the upper wells.

As is evident from Fig. 1, the signal-to-noise ratio in this assay was relatively low–i.e., the peak accumulation in active follicular fluids (see below for definition of active follicular fluid) was usually larger than the control accumulation in the suspending buffer by no more than 2- to 3-fold and often even less. However, such an accumulation was reproducible in >100 assays with active follicular fluids, yielding highly significant results ($P < 10^{-8}$). Possible causes of the relatively low accumulation are, for example, responsiveness of only a fraction of the sperm population to the follicular fluid (see below) or a limiting number of pores in the filter that separates the upper and lower wells. The reasons for the reduction in sperm accumulation when follicular fluid is diluted less than 1:10^4 are considered in *Discussion*.

To assay for changes in swimming pattern by follicular fluid and to determine whether indeed only a fraction of the sperm population responds to follicular fluid, we microinjected follicular fluid into a suspension of spermatozoa as described. Indeed, a fraction of cells changed their swimming patterns from rather straight lines to more circular patterns when they entered the injection area. This resulted in an apparent, transient accumulation of spermatozoa at the site of injection. A similar phenomenon was observed when an egg-conditioned medium was microinjected instead of a follicular fluid. (The conditioned medium is the medium used during an *in vitro* fertilization procedure for incubation of the retrieved egg. This medium contains postovulatory egg secretions and about 5% follicular fluid.) One such experiment is shown in Fig. 2, which includes the traces of spermatozoa immediately after microinjection of a conditioned medium at

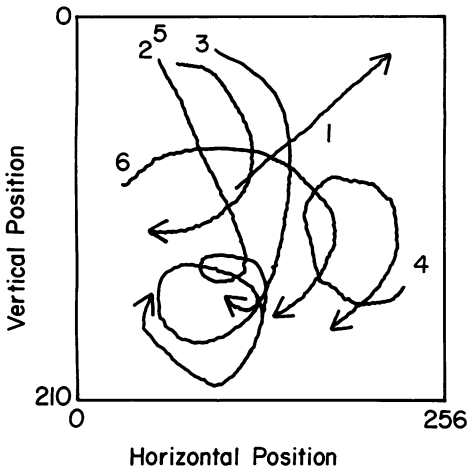

FIG. 2. The response of spermatozoa to microinjection of a conditioned medium. The tracks were drawn by an ExpertVision computerized motion analysis system as described. The microinjection area was the lower left quarter of the field.

the lower left quarter of the field. Some cells clearly changed their swimming pattern (e.g., cells 2 and 3, the starting points of which were at the upper left corner of the field), and others clearly ignored the injected substance (e.g., cell 1, seen swimming from the center of the field toward the upper right corner). The magnitude of the response–i.e., the fraction of the responding cells–varied from sample to sample. Injection of the suspending medium had no effect (not shown).

In another approach to determine whether or not only a fraction of the spermatozoa respond to follicular fluid, we compared, in a chemotaxis chamber, the activity of the swim-up spermatozoa with that of an equally treated, whole population of spermatozoa of the same sample. Accumulation in follicular fluid was observed only with the swim-up spermatozoa, suggesting that only a fraction of the spermatozoa respond to the follicular fluid, and that this fraction is substantially larger within the group of cells that swim up. The heterogeneity of spermatozoa with respect to their behavior is well-documented (14, 15).

Variations in the Activity of Different Follicular Fluids. To evaluate the variabilities among follicular fluids, we kept the sperm parameters constant as much as possible: the semen was collected from two constant donors, the spermatozoa were subjected to a standard swim-up procedure, and the capacitation time was 2–3 hr.

Only about half of the follicular fluids tested caused sperm accumulation. These follicular fluids have been denoted as "active follicular fluids." The active follicular fluids maintained their activity (as measured by both accumulation assay and microinjection) for about 2 weeks at −20°C or −70°C. After this period of time, the activity was lost. This suggests that the factor(s) responsible for the observed activity of the follicular fluid is labile, or that other processes masking the factor's activity occur in the follicular fluid during storage. Some of the follicular fluids also appeared to have a negative effect on sperm accumulation: the number of spermatozoa accumulated in these follicular fluids was substantially lower than the number accumulated in the control wells containing the suspending BWW medium.

Previous reports on the effects of follicular fluid on the motility of spermatozoa have been conflicting or inconclusive (16–19). On the basis of the data described above, it can be proposed that this may have been due to (*i*) the use of undiluted follicular fluids and (*ii*) the fact that only about half of the follicular fluids appear to be active, as observed here. The loss of activity during storage also could be an important reason for failure. We did not detect any apparent effect of diluted follicular fluids on the percentage motility of spermatozoa.

Correlation with Egg Fertilizability. The accumulation induced by only about half of the follicular fluids raised the question of whether or not fertilization rates would be correlated with this response. Since the eggs from the women had been collected for the purpose of *in vitro* fertilization, they could be divided into two groups: those successfully fertilized and those not. These data could then be compared with the ability of the corresponding follicular fluids to cause sperm accumulation. We assayed in a blind test 62 individual follicular fluids from 40 women with spermatozoa from two constant donors. Sperm accumulation was assayed as in Fig. 1. We defined the ratio between the peak of sperm accumulation in the test follicular fluid and the control accumulation in the suspending medium as "relative accumulation," and we considered it as the parameter of activity of that specific follicular fluid. The activities of follicular fluids varied not only from woman to woman but also from follicle to follicle within the same woman (data not shown). The latter observation indicates that the measured activity cannot be attributed to serum transudate in the follicular fluid; otherwise, the activities of follicular fluids from the same woman should have been similar.

The correlation between the activity of the follicular fluids and the fertilizability of the corresponding eggs was striking. As shown in Fig. 3, which includes the normalized results of all the determinations for the 62 follicular fluids, accumulation was observed in the group of follicular fluids whose corresponding eggs became fertilized. The difference between the two groups was significantly high ($P < 0.0001$). It is interesting to note that within the group of 62 follicular fluids, 5 had negative activity; their corresponding eggs were not fertilized. This explains why the average relative accumulation of the nonfertilized group is slightly lower than the expected value of 1 (Fig. 3).

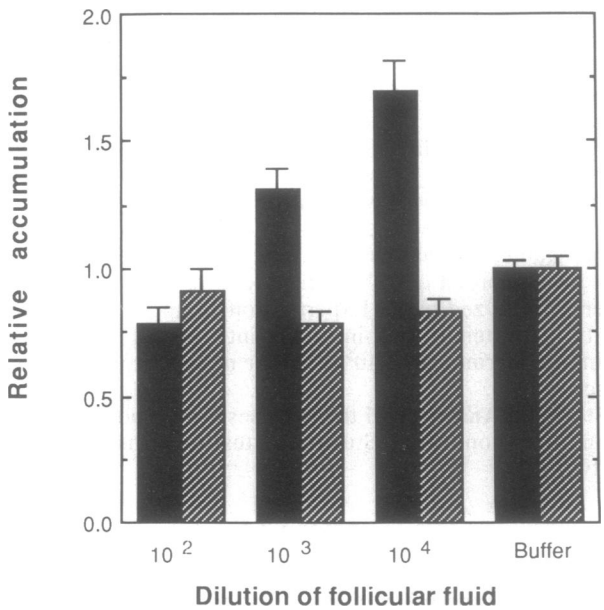

FIG. 3. Correlation between fertility and sperm accumulation in follicular fluid. Sixty-two follicular fluids from 40 women were assayed as in Fig. 1 and grouped according to whether their corresponding egg was fertilized (black columns) or not (grey columns). The bars represent the SEM. The number of determinations with follicular fluids corresponding to fertilized and nonfertilized eggs, respectively, was 76 and 62 at $1:10^2$ dilution of follicular fluid, 195 and 150 at $1:10^3$ dilution, 198 and 152 at $1:10^4$ dilution, and 208 and 159 in buffer only.

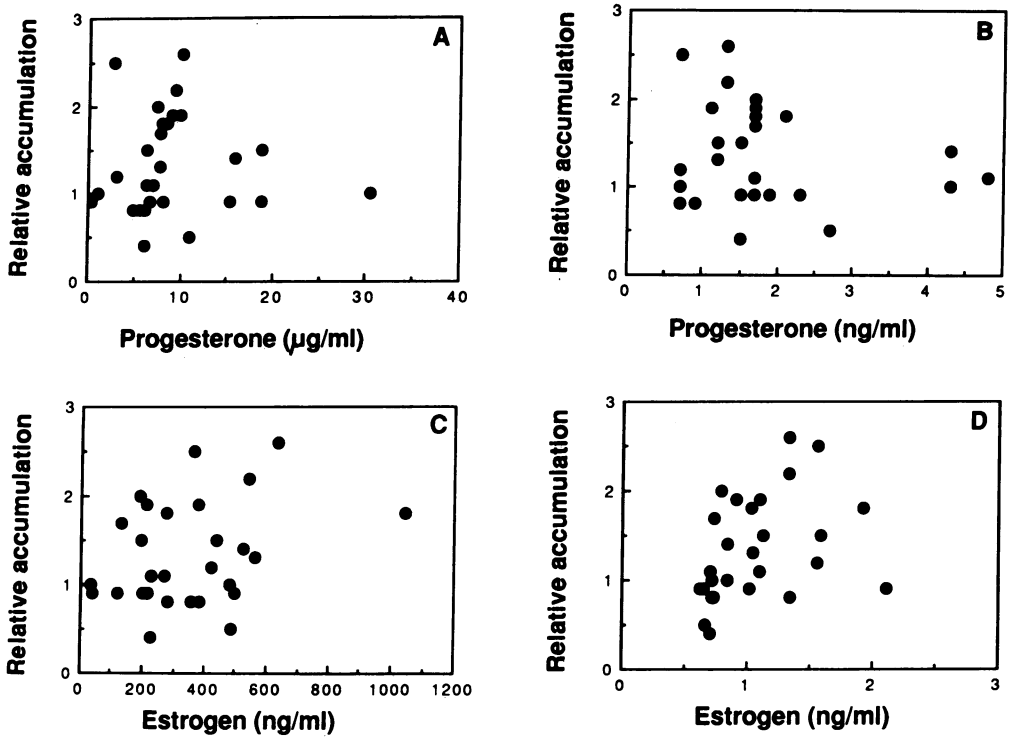

FIG. 4. Lack of correlation between sperm accumulation and the hormonal level in the follicular fluid and serum. (*A*) Progesterone levels in follicular fluid. (*B*) Progesterone levels in serum. (*C*) Estrogen (17β-estradiol) levels in follicular fluid. (*D*) Estrogen levels in serum.

In a search for other parameters of follicular fluid with which the fertilizability of the eggs could be correlated, we analyzed the levels of progesterone and 17β-estradiol in both the follicular fluids and sera of the women. No correlation between the accumulation (Fig. 4) or between the fertilizability of the eggs (not shown) and the level of any of these hormones in the follicular fluid could be found. Similarly, there was no apparent difference between the fertilized and nonfertilized eggs with respect to morphology and size of the egg–corona complex. Thus, of the parameters tested, the fertilizability of the egg is correlated only with accumulation of spermatozoa into follicular fluid. It should be noted that only female factors were investigated, as the fertilization of the egg was with the husband's sperm, whereas the accumulation assays were carried out with spermatozoa from two constant donors. The infertility of all the couples used in this study appeared related to female problems, most of which were attributed to Fallopian tube mechanical problems. None appeared due to a male factor.

DISCUSSION

Here, we report that human spermatozoa accumulate in follicular fluid and that the accumulation is strongly correlated with fertilizability of the egg from the same follicle.

A few questions that may result from the above observations merit further consideration:

What is the cause of the sperm accumulation in follicular fluid? In other systems, accumulation of cells in response to stimuli has been shown to be a consequence of modulation of the direction of travel and/or speed of travel (e.g., ref. 5), phenomenologically defined as chemotaxis and chemokinesis, respectively. Accumulation of spermatozoa in the chemotaxis chamber could thus result from chemotaxis; chemokinesis; a negative effect of follicular fluid on motility; a change in swimming behavior from, for example, more linear paths to circular paths; mechanical trapping (20); or any combination thereof. Preliminary data suggest that neither trapping nor chemokinesis are the cause of the phenomenon (D.R. and M.E., unpublished data).

What is the cause of the lower sperm accumulation in those wells that contained relatively high concentrations of follicular fluid (Fig. 1)? This could result from a number of reasons. (*i*) It is possible that the concentrations of the active factor established at the lower wells by diffusion were already at saturation, thus preventing attraction to the upper wells. A similar decreased accumulation at high attractant concentrations has been observed in chemotaxis and chemokinesis assays for leukocytes (5) and in capillary assays of bacterial chemotaxis (21). (*ii*) The lower accumulation could be the result of repulsion from the same or other factors in the follicular fluid; this possibility has not been eliminated. (*iii*) Reduced adsorption of the spermatozoa to the polylysine-coated glass could occur at high follicular fluid concentrations; this artifact has been eliminated as an explanation (not shown).

In this study we found that only a fraction of the sperm population responds to the active factor(s). It is reasonable to propose that attraction by an active factor(s) may be a means for selecting spermatozoa more competent for fertilization or a way to coordinate sperm behavior and ovulation, or both. The observation that in humans only about 200 spermatozoa are found at the region of the fertilization site in the Fallopian tube [out of about 280×10^6 spermatozoa ejaculated into the vagina (22)] supports our finding that only a fraction of the sperm population is responsive to the factor(s). There may be an additional negative mechanism that prevents spermatozoa from reaching the fertilization site. In other words, those spermatozoa that are capable of fertilizing the egg (e.g., capacitated spermatozoa) may be attracted to the egg, whereas the rest (too mature or premature spermatozoa) may be repelled or deactivated by the same or other factor(s) released from the egg and its surrounding cells. Further studies are required for determining whether or not this is indeed the case and for determining the identity of the factor(s) and its cellular origin(s). (The egg, cumulus oophorus, and/or granulosa cells could be sources.)

Whatever the physiological significance of these findings, the data presented suggest that immediately after ovulation there is sperm–egg communication (or communication between nongerm ovarian cells and spermatozoa). Data in favor of such an interaction prior to actual sperm–egg contact have not been shown in the mammal before, to our knowledge. If indeed this interaction enables successful fertilization, new approaches for studying cases of sterility of unknown origin may develop.

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