Chemotactic Responsiveness of Human Spermatozoa to Follicular Fluid Is Enhanced by Capacitation but Is Impaired in Dyspermic Semen

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Purpose: Our purpose was to study the chemotactic responsiveness of human spermatozoa from normal and pathological semen samples to follicular fluid (FF), as well as the effect exerted by capacitation on sperm chemotaxis.

Methods: The chemotactic responsiveness of human spermatozoa to FF was tested by an accumulation assay chamber in which they were allowed to migrate through a microporous membrane and accumulate in a compartment filled with FF or control medium. The percentage of cells with hyperactivated motility among migrated sperm was objectively assessed by CASA and its relationship to the accumulation rate was studied. Single FFs were tested with single normospermic or dyspermic semen samples; the same FF was tested with different semen specimens. The influence of capacitation onto the chemotactic responsiveness to FF was investigated by comparing the accumulation rate of spermatozoa from normal and pathological samples after incubation under capacitating conditions for 1 or 6 hr.

Results: Some FFs ("active" FFs) effectively attracted human spermatozoa from normospermic samples up to a dilution factor of 1:500 (v:v) with control medium. A wide range of responses was observed when the same FF was tested with different normal semen samples. A longer time under capacitating conditions increased both the chemotactic responsiveness of normal semen and its ability to undergo the acrosome reaction (AR) in response to A23187. Pathological semen had an impaired chemotactic responsiveness to "ac-

tive" FF that was not enhanced by increasing the time spent under capacitating conditions. Dyspermic semen was also less responsive to A23187, a finding suggesting incomplete capacitation.

Conclusions: Chemotactic responsiveness to FF is acquired in parallel to or as part of the capacitation process. Dyspermic semen samples have an impaired capacity to achieve both capacitation and chemotactic responsiveness to follicular factors.

KEY WORDS: Chemotaxis; dyspermia; follicular fluid; spermatozoa.

INTRODUCTION

Egg-derived, species-specific peptides able to attract spermatozoa have been identified in some invertebrates, whose spermatozoa are spawn into water and must be guided toward eggs to achieve fertilization. Surface receptors for chemoactive peptides have been identified in spermatozoa from echinoderms (1): they are coupled to a guanylate cyclase/cGMP transduction pathway (2) whose activation regulates ion fluxes across the sperm membrane (3), affects the frequency of flagellar beating, and, finally, leads to an oriented movement (4).

In species with internal fertilization, such as mammals, several millions spermatozoa are ejaculated directly into the female genital tract, rapidly transported to the salpinx (5), and stored in the cervix and/or in the oviduct (6), not far from the fertilization site. Although spermatozoa can probably meet the oocyte by pure coincidence, an increasing amount of experimental data suggests the existence of a chemical attraction at distance between human gametes,

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positively affecting their chance to meet and, finally, the probability of fertilization (reviewed in Refs. 7 and 8). Human spermatozoa have been shown to be attracted to follicular fluid (FF) factors in vitro (9–12), and a correlation between the ability of a single FF to attract sperm and the fertilizability of the oocyte contained in it has been described (13). The chemotactic effect of FF, reported to be related to the FF levels of progesterone (14) or atrial natriuretic peptide-like peptides (15,16), has been observed to involve only a small fraction of the sperm population (11,12) and to be a transient characteristic, acquired or lost in a short time (11). Human spermatozoa have been hypothesized to acquire their chemotactic responsiveness as part of the capacitation process, which is transient (from 50 min to 4 hr) and involves only a limited proportion of spermatozoa in a sperm sample (17). Since under physiological conditions only capacitated spermatozoa may undergo the acrosome reaction (AR) and then fertilize, the role of sperm chemotaxis could be the selection of capacitated, highly fertile spermatozoa. Until now, to the best of our knowledge, it is unclear whether mild to moderately dyspermic semen specimens might be poorly fertile or either infertile because of a failure in acquiring a normal chemotactic attitude. The aim of the present study is to investigate in vitro the competence of moderately dyspermic human semen to achieve normal chemotactic responsiveness and a fully capacitated state in response to preovulatory FF.

MATERIALS AND METHODS

Sperm Preparation

Human semen samples were obtained by masturbation from 71 normospermic and 38 dyspermic men participating in our artificial insemination (AIH) or in vitro fertilization–embryo transfer (IVF-ET) programs. After liquefaction (30 min at 37°C), samples were analyzed according to the current WHO criteria and subsequently classified as “normospermic” or “dyspermic.” The latter group included semen samples with mild to moderate oligoasthenoteratozoospermia (OAT; $n = 19$ samples), oligoasthenozoospermia (OA; $n = 13$), and asthenoteratozoospermia (AT; $n = 6$). All samples were washed twice in BWW (2:1, v:v, with semen) in order to eliminate seminal plasma, and motile spermatozoa

were subsequently harvested by the swim-up technique (37°C at 5% CO₂ atmosphere for 1 hr) using previously equilibrated IVF medium (Medicult, Denmark). The motile-rich sperm fraction was centrifuged at 600g for 10 min at room temperature, and the sperm concentration was adjusted to $10 \times 10^6/\text{ml}$ by removing or adding IVF medium.

Follicular Fluid Preparation

Follicular fluids from $n = 52$ patients were used. Donors were women participating in the IVF-ET program because of male-related infertility. Superovulation was induced by desensitizing the pituitary gland with a GnRH analogue, and then administering 150–225 IU/day of HP-FSH (Metrodin HP, Serono, Italy) starting from the day in which serum E₂ levels fell below 20 pg/ml (conversion factor to SI unit, 3.671). From day 8 of ovarian stimulation, follicular growth was monitored daily by ultrasound measurements and serum E₂ determinations. On the day the leading follicle reached 18 mm, with appropriate E₂ levels, 10,000 IU of hCG (Profasi HP, Serono, Italy) was administered. Ultrasound-guided oocyte retrieval was scheduled 36 hr after hCG injection, and FFs were aspirated under sterile conditions during this procedure. Only blood-free FFs containing an oocyte–cumulus complex were further processed. They were centrifuged (1500g at room temperature for 10 min), and the supernatant was aspirated and filtered into plastic sterile tubes (0.22- μm Swinnex Millipore filters; Millipore, Milford, MA). Various 1-ml aliquots of FF from each woman were stored at –20°C for 1 to 10 days until analysis.

Chemotaxis Assay

Sterile, 24-well chemotaxis chambers (Transwell, Costar, Cambridge, MA) were used. This kind of chamber is similar to those employed in studies about sperm chemotaxis (13,18) and drug-conditioned sperm motility (19). One-half milliliter of the sperm suspension obtained from each sperm sample after the swim-up procedure was placed in the lower compartment of each well, and 60 μl of FF or IVF medium (control) was delicately placed in the upper compartment. These volumes were chosen in order to minimize the mixing of the fluids by means of an optimal relative meniscus level in the two compartments. Preliminary tests in which Trypan blue-colored FF was used showed a progressive FF concentration difference gradually developing from the upper to the

lower compartment in about 1 min and persisting over 30 min; FF became moderately diluted and its approximate final concentration in the upper compartment was estimated to be 85% by evaluating the change of intensity of Trypan blue. The lower and upper compartments were separated by a 8.0- μ m-pore size, tissue culture-treated polycarbonate membrane filter, 10 μ m thick, having a total area of 6.5 mm². On top of the chemotaxis chamber rested a cover slide, which entirely covered the apparatus without sealing it, but was proven to be effective in minimizing evaporation during the test time. The chamber was incubated at 37°C in 5% CO₂, humidified atmosphere. At the end of incubation the cover slide was taken off, and the upper compartment of each well suddenly removed. Preliminary tests made with dead spermatozoa showed that the probability of aspirating immotile spermatozoa into the upper compartment as a result of surface tension during this procedure was negligible. Even the flushing of the microporous membrane with IVF medium allowed us to retrieve a negligible number of spermatozoa, indicating that only a few sperm could remain trapped in the membrane. After removal of the upper compartment, the test medium, or the control medium contained in it, where motile spermatozoa had migrated and accumulated, was completely aspirated. After thorough mixing, 10 μ l of either suspension was placed in a Makler chamber (Sefi Instruments, Israel) and the number of motile spermatozoa was immediately assessed. When motility was measured, 20 μ l of either suspension was immediately analyzed by means of Hamilton Thorn algorithm, which objectively measured sperm concentration and motility parameters, automatically calculating the rate of spermatozoa showing hyperactivated motility. Hyperactivated motility was defined as a curvilinear velocity >100, a linearity <65, and a lateral head displacement >7.5. The total motile sperm count in the lower compartment being known, the chemotactic activity of FF or of the control medium toward a sperm sample was expressed as the percentage of motile spermatozoa accumulating in the upper compartment of the well.

Normospermic ($n = 49$) and dyspermic ($n = 38$) semen samples were treated as described, and the ability of motile spermatozoa to accumulate in FF or IVF medium was tested during 30 min of incubation in the chemotaxis chamber. Each semen specimen was tested with a single FF. The motility of spermatozoa from normospermic ($n = 16$) and dyspermic ($n = 8$) specimens was objectively assessed before

and after migration in the upper compartment of the well. The tests with normal semen allowed us to identify (a) a group of chemotaxis-responsive semen samples and (b) "active" FFs ($n = 18$), when the difference (Δ) between the percentage of sperm accumulated in them and the percentage of sperm accumulated in the control medium was greater than 25. Only these "active" FFs were used in the case of dyspermic semen specimens. To test the intraassay variability of the method, accumulation assessment was made in triplicate in some cases. Moreover, three "active" FFs were individually tested with normospermic samples ($n = 7$) to obtain information about the different responsiveness among sperm samples. Further, normospermic samples ($n = 10$) were also tested with pentoxifyllin-supplemented IVF medium (1 mg/ml) vs IVF medium alone, with the purpose of verifying if a known motility-enhancing substance (20) could affect the rate of accumulation in the upper compartment of the chemotactic chamber.

In another experiment, two aliquots of the same sperm sample ($n = 11$ chemotaxis-responsive, normospermic and $n = 7$ dyspermic semen specimens) were incubated in IVF medium for 1 or 6 hr, respectively, to achieve different proportions of capacitated spermatozoa. They were subsequently tested with the same FF or IVF medium during 30 min of incubation in the chemotaxis chamber. To estimate capacitation, the percentage of sperm undergoing AR after exposure to Ca²⁺-ionophore A23187 (10 μ M for 30 min) was assessed using a FITC-labeled PSA fluorescent probe according to Cross *et al.* (21).

Statistical Analysis

Since the distribution of data proved to be skewed in the Kolmogorov–Smirnov (Lilliefors) test, the percentages of motile spermatozoa migrated into FF or in the control medium were compared by the nonparametric Kruskal–Wallis test for unpaired data. The correlation between the percentage of migrated sperm and that of hyperactivated spermatozoa was calculated by Spearman's correlation test. The STATGRAPHICS 6.0 software was used for statistical analysis.

RESULTS

When normospermic specimens were tested with single FFs ($n = 49$ tests), the median percentage of

motile sperm accumulated in the 85% FF-containing compartment after 30 min was significantly higher than that observed in the IVF medium-containing compartment (59 vs. 34; $P < 0.003$) (Fig. 1A). This experiment allowed us to identify 18 “active” FFs and, on the other side, chemotactic-responsive semen specimens. The existence of semen samples with a good, or, respectively, poor, response to FF was confirmed when seven semen samples were tested with the same “active” FF; in this experiment, which was repeated three times with three different FFs, a wide range of accumulation rates was observed (from 21 to 66%; not shown). This variability was due to intrinsic properties of the semen and not to the assay, as when the accumulation rate was measured in triplicates, the intraassay coefficient of variation did not exceed 12%. The chemotactic effect of FF was maintained up to a dilution factor of 1:500 (v:v) with IVF medium

(Fig. 2), it still appeared after 5 min of incubation, when accumulation in FF was already significantly higher than in the control medium, and reached a “plateau” approximately after 20 min of incubation, then remaining constant (not shown). The overall proportion of sperm with hyperactivated motility in the upper compartment (calculated in $n = 16$ tests) was low, reaching a maximal value of 13%; however, it was significantly higher in FF than in the control medium (median, 10 vs 0.8; $P < 0.001$) (Fig. 1C). No correlation was observed between the proportions of migrated and hyperactivated sperm in FF (Spearman’s coefficient, 0.1979; $P = \text{ns}$), whereas the two variables were directly correlated in the control medium (Spearman’s coefficient, 0.6334, $P < 0.02$). The lack of a direct relationship between accumulation and hyperactivated motility was confirmed when pen-toxyfillin-supplemented IVF medium aliquots ($n =$

Fig. 1. Chemotactic and chemokinetic effects of single FFs toward normospermic (A, C) and dyspermic (B, D) semen specimens. (A) Median and range of the percentage of motile spermatozoa from normospermic samples accumulated in the upper compartment of a well containing either FF (white column) or control medium (gray column) ($n = 49$; ** $P < 0.003$). (B) Median and range of the percentage of motile spermatozoa from dyspermic samples accumulated in the upper compartment of a well containing either FF (white column) or control medium (gray column) ($n = 38$; $P = \text{ns}$). (C) Median and range of the percentage of spermatozoa from normospermic samples showing hyperactivated motility among those accumulated in the upper compartment of a well containing either FF (white column) or control medium (gray column) ($n = 16$; *** $P < 0.001$). (D) Median and range of the percentage of spermatozoa from dyspermic samples showing hyperactivated motility among those accumulated in the upper compartment of a well containing either FF (white column) or control medium (gray column) ($n = 8$; * $P < 0.05$).

10) were tested in comparison with IVF medium alone. In these experiments, no significant difference in the median accumulation rate was observed (23 vs 20, respectively; $P = \text{ns}$), despite the relevant increase in sperm with hyperactivated motility in the pentoxifyllin-containing compartments (median, 8 vs 0.4; $P < 0.02$) (Fig. 3).

In tests employing dyspermic samples ($n = 38$ tests), only "highly chemotactic" FFs were used, but in spite of this, motile sperm accumulated more in IVF medium than in FF, although the difference was not significant (median, 33 vs. 28; $P = \text{ns}$) (Fig. 1B). The overall proportion of hyperactivated spermatozoa in the upper compartment (calculated in $n = 8$ tests) was very low; however, it was significantly higher in FF (median, 2.6 vs 0.1; $P < 0.05$) (Fig. 1D). No relevant differences were observed in the median accumulation rates when the three types of dyspermia (OTA vs OA vs AT) were compared.

Time spent under capacitating conditions appeared to affect positively the responsiveness of sperm to "active" FFs (Fig. 4). A higher proportion of motile

spermatozoa was observed to accumulate in FF when chemotaxis-responsive, normospermic sperm samples were incubated under capacitating conditions for 6 hr (median, 62 with 27% induced ARs) vs 1 hr (median, 44 with 13% induced ARs), although this difference was not statistically significant (Fig. 4A). In both cases, however, the percentage of sperm accumulated in FF was significantly higher ($P < 0.02$) in comparison to the control medium (median, 30 with 9% induced ARs) (Fig. 4A). On the contrary, both the chemotactic responsiveness and the ability to respond to A23187 of dyspermic semen specimens were not increased after 1 or 6 hr of incubation under capacitating conditions (median, 14 with 7% induced ARs and 16 with 8% induced ARs, respectively) with respect to what was observed after 6 hr in IVF medium (median, 15 with 6% ARs) (Fig. 4B).

DISCUSSION

In the present study, motile spermatozoa from several normospermic semen specimens were able to

Fig. 2. Chemotactic effect of FF at various dilutions or a control medium toward normospermic semen specimens. Median and range of the percentage of motile spermatozoa accumulated in the upper compartment of a well containing either FF at various (v:v) dilutions with IVF medium (white columns) or control medium (gray column). $N = 9$; * $P < 0.05$ vs all other columns.

Fig. 3. Effect of pentoxifyllin-added IVF medium on sperm accumulation and hyperactivation. (A) Median and range of the percentage of motile sperm accumulated in the upper compartment of a well containing pentoxifyllin-supplement IVF medium (white column) or IVF medium alone (gray column). (B) Median and range of the percentage of spermatozoa showing hyperactivated motility among those accumulated in the upper compartment of a well containing pentoxifyllin-supplement IVF medium (white column) or IVF medium alone (gray column). $n = 10$; * $P < 0.02$.

accumulate in the FF-containing compartments of a chemotactic chamber, apparently responding to the chemical attraction provided by “active,” chemotactic FFs. The chemotactic responsiveness of normal spermatozoa to FF was greatly variable from one sperm sample to another and involved only part of the cells in a responsive sample; furthermore, it was acquired rapidly (5 min), reached a “plateau” after 20 min of exposure to FF, and was enhanced by prolonging preincubation under capacitating conditions. Both highly diluted (1:500, v:v, with culture medium) and highly concentrated (85% FF) “active” FFs were proven effectively to attract normal spermatozoa. On the other side, the responsiveness of moderately dyspermic semen samples to FF was poor, despite that only “active” FFs, with proven effectiveness toward normal semen, were employed. Spermatozoa from pathological semen specimens showed a limited ability to respond to FF even after 6 hr of incubation under capacitating conditions, suggesting an impaired capacity of acquiring normal chemotactic responsiveness, hyperactivated motility, and responsiveness to AR inducers.

The observation that only some FFs actively attract spermatozoa (“active” FFs), whereas others have a very limited, if any, effectiveness, agrees previous reports by others (11,13). The analysis of some clinical features of our patients (age, duration of infertility, indication to treatment) and of some oocyte-related parameters (total dose of gonadotropins employed, follicle size at pickup, total number of follicles, oocyte morphological score) did not allow us to determine the reason(s) for such a big difference in chemotactic activity among different FFs and why some of them completely lack chemotactic properties. Unfortunately the concentration of progesterone, which was claimed to be the effector (or one of the effectors) of FF-derived chemoattraction on human spermatozoa (14), was not measured in the FFs used herein. Since the chemotactic ability of FF toward sperm was previously correlated with the capacity of the oocyte to allow fertilization (13), it could be speculated that the lack of chemotactic properties of some FFs could represent a novel factor underlying some cases of unexplained infertility.

In the present study, “active” FFs caused the accu-

mulation of responsive spermatozoa independently of the dilution at which they were tested. The possibility that chemokinesis (chemical enhancement of sperm motility) or sperm trapping could interfere with the observed results has already been signaled when highly concentrated FF is employed in an accumulation assay (11). Indeed, FF is a well-known activator of sperm motility and is able to increase significantly the proportion of hyperactivated spermatozoa in a sample (22–24). Since hyperactivated motility is known to cause a loss of linearity and the onset of a more confused movement path, hyperactivated spermatozoa could have accumulated in the upper compartment of our test chamber because of their inability to come back (sperm trapping). Previous studies on sperm chemotaxis in which an accumulation assay was employed already addressed the problem of trapping, demonstrating that the trapping effect is negligible under experimental conditions very similar to ours (13). On the other hand, chemokinesis might

have contributed to the accumulation. The following considerations, however, suggest that chemotaxis is the major cause of sperm accumulation in our experiments. (a) A 30-min exposure to FF (even if highly concentrated) is too short to allow massive hyperactivation, which requires a longer period of incubation (24). In fact, the average hyperactivation rate of spermatozoa in the FF-containing compartments was low (maximal value, 13%). (b) For both normal and dyspermic semen specimens, no correlation between the rate of accumulation and that of hyperactivation in FF was observed, suggesting a limited importance of chemokinesis in causing the described results. (c) The addition of pentoxifyllin to the culture medium, although effective in increasing sperm motility, did not cause accumulation in our assay.

Moderately dyspermic semen samples were shown to be unresponsive to FF, as neither hyperactivation nor chemotactic accumulation was observed during the incubation time. The lack of chemotactic respon-

Fig. 4. Effect of two capacitation times on the chemotactic responsiveness of 11 normospermic and 7 dyspermic semen specimens toward FF or a control medium. Median of the percentage of motile spermatozoa accumulated in the upper compartment of a well containing either FF (white columns) or control medium (gray column). Lane a: Normospermic semen, 1-hr capacitation time and then exposure to FF. Lane b: Normospermic semen, 6 hr of capacitation and then exposure to FF. Lane d: Dyspermic semen, 1-hr capacitation time and then exposure to FF. Lane e: Dyspermic semen, 6 hr of capacitation and then exposure to FF. Lanes c and f: Six-hour capacitation time and then exposure to a control medium. Narrow, dotted gray columns: median of the percentage of acrosome reactions in response to Ca^{2+} -ionophore A23187. * $P < 0.02$.

siveness to FF seemed to be unrelated to the kind of seminal pathology, as no significant differences were noticed comparing oligoasthenoteratozoospermic, oligoasthenozoospermic, and asthenoteratozoospermic samples. However, the low number of samples in the three subgroups does not allow us to draw conclusions in this regard, which eventually will be achieved by more targeted, further studies. Since both the onset of hyperactivated motility and the ability to sense a chemical stimulus involve the sperm membrane as the first effector of response, membrane alterations could be basis of the impaired chemotactic responsiveness of dyspermic sperm samples. Interestingly, a prolonged preincubation under capacitating conditions enhanced the chemotactic responsiveness of normal semen to FF as well as its ability to undergo Ca^{2+} -induced AR but had no effect on dyspermic semen, which remained poorly responsive to both chemotactic and AR-inducing stimuli.

Although a cause–effect, direct relationship between chemotaxis and capacitation was not demonstrated in the present study, data presented herein reinforce the concept that chemotactic responsiveness is acquired in parallel with capacitation (expressed as hyperactivated motility and AR responsiveness to inducers). Capacitation and chemotactic responsiveness could be synergic in vivo, the former activating sperm motility and responsiveness to AR inducers, the latter orienting the movement of sperm toward the egg. Since in this study, as in previous ones (11–13), it has been shown that only part of the sperm population possesses the ability to respond to chemotactic factors, chemoattraction could even be regarded either as a mechanism to select spermatozoa able to undergo complete capacitation and finally fertilize or, eventually, as a useful resource to avoid excessive sperm concentration around the egg, thus preventing polyspermia. On the other hand, incompetence to respond to chemical attraction could represent a further factor leading to a low probability of spontaneous conception for subjects with mild-to-moderate dyspermia. Better knowledge about the intimate mechanisms of chemotaxis in humans and its alterations in infertile subjects will probably help to identify, and possibly treat, previously undiagnosed defects responsible for reproductive failure. An intriguing hypothesis for further work is also the use of chemotactic in vitro assays to select the most fertile sperm subpopulation when IVF for tubal or unexplained infertility is required.

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