Sequential Acquisition of Chemotactic Responsiveness by Human Spermatozoa

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ABSTRACT

Recent studies have indicated that human spermatozoa respond to follicular fluid by attraction to chemotactic factor(s) in the fluid, accompanied by enhancement of motility and ultimately hyperactivation. In this study, we quantified the sperm response. We exposed spermatozoa to a gradient of a chemotactically active fraction of follicular fluid (denoted as "the attractant") and separated the spermatozoa that accumulated in the attractant and those that did not. We thus obtained two subpopulations: one enriched with chemotactically responsive spermatozoa, and one deficient in such spermatozoa. The fraction of the responsive spermatozoa out of the total sperm population was 2–12% at any measured time point. With time, the responsive spermatozoa lost their ability to be attracted, while such activity was gradually acquired by the subpopulation originally deficient in responsive spermatozoa. These results indicate that the identity of responsive spermatozoa is continuously changing. If the in vitro results are representative of the physiological conditions in vivo, they imply that the role of sperm chemotaxis combined with enhanced motility may be to select capacitated spermatozoa and bring them to the egg. Such a mechanism may, over an extended period of time, increase the prospect that an egg will meet capacitated spermatozoa as soon as it ovulates.

INTRODUCTION

Recent findings have revealed the existence of sperm attraction to human follicular factor(s) (in the form of follicular fluid) in vitro, correlated with human egg fertilizability [1, 2]. The attraction is the result of chemotaxis accompanied by enhancement of motility and ultimately hyperactivation [3]. In all the experiments, the extent of the response to follicular fluid was relatively small, probably as a consequence of the responsiveness of only a small fraction of the sperm population. This interpretation was based on analysis of tracks made in vitro by spermatozoa near a well containing an active fraction of follicular fluid, demonstrating that not all of the spermatozoa changed the direction of their swimming towards the well [3]. If this interpretation is correct, it should be possible to separate a sperm population into responsive and nonresponsive subpopulations and demonstrate that only the former population is attracted to follicular fluid. Furthermore, the physiological significance of the sperm fraction's responsiveness is not known. It is also not obvious why there is a need for sperm attraction in humans, where ~2.8 × 10^8 spermatozoa are ejaculated directly into the female genital tract [4] and a sufficient number of spermatozoa may reach the fertilization site by coincidence. On the basis of both the lack of an obvious function and the responsiveness of only a fraction of the sperm population, we have proposed that the physiological role of the attraction in vivo is to select spermatozoa able to fertilize the egg [5], i.e., to select capacitated spermatozoa (or a subfraction thereof) [6, 7]. This requires that only capacitated spermatozoa will have the ability to be attracted to substances secreted from the egg or its surrounding cells [5]. Moreover, we proposed that in order to ensure continuous availability of capacitated spermatozoa for an extended period of time (rather than availability of all at once but for a short period of time), there should be a mechanism of turnover, i.e., a mechanism by which the identity of the capacitated (and therefore responsive) spermatozoa continuously changes. Here we report on the successful separation of a sperm population into two subpopulations, one enriched in responsive spermatozoa and the other deficient in responsiveness, thus demonstrating that indeed only some of the spermatozoa are responsive. We further demonstrate that the responsive spermatozoa do not represent an exclusive subpopulation with properties different from the rest of the population but rather a subpopulation with changing identity, in line with the proposed mechanism of turnover.

MATERIALS AND METHODS

Spermatozoa, Sperm Attractant, and Responsiveness Assays

The methods used were described by Ralt et al. [3]. Briefly, human spermatozoa were collected, washed, and resus-
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Pended in Biggers, Whitten, and Whittingham medium (BWW) [8]. Human follicular fluids were obtained from women undergoing transvaginal aspiration for in vitro fertilization. The attractive fraction of follicular fluid (denoted hereafter as "the attractant") was isolated from follicular fluid by 90% acetone precipitation. The responsiveness of spermatozoa to the attractant was determined in a sealed chamber in which the spermatozoa choose between a well containing the attractant and a well containing BWW [9]. The sperm concentration used in the responsiveness assays was $5 \times 10^8$ cells/ml.

**Sperm Separation Procedure**

Separation of spermatozoa according to their responsiveness was carried out in the apparatus, schematically shown in Figure 1. With the valve open, the connecting tube (200 μl in volume) was filled with BWW. The valve was then closed, and chamber no. 1 was filled with 1 ml spermatozoa (unless mentioned otherwise, $10^8$ cells/ml, suspended in BWW) and chamber no. 2 with 1 ml attractant diluted in BWW. On each day of the experiment, the dilution of the attractant was the dilution in which optimal accumulation was observed in the responsiveness assays of that day (diluted $10^{-3}$-$10^{-4}$-fold). The separation was initiated upon opening the valve. The apparatus was incubated at room temperature (21–24°C) for 60 min (unless mentioned otherwise), during which time a concentration gradient of the attractant was established by diffusion (and verified, in a parallel experiment, by monitoring the time-dependent color change of phenol red dye in the apparatus after the addition of 0.1 N HCl to one of the wells). The sperm suspensions from both chambers were collected and washed twice by centrifugation ($120 \times g$, 15 min). The original population was similarly washed as a control.

**RESULTS**

**Sperm Separation into Subpopulations**

To examine the possibility that not all spermatozoa can simultaneously respond to follicular factors, we first attempted to separate the responsive and nonresponsive spermatozoa. This was accomplished with the use of an apparatus consisting of two chambers connected by a tube (Fig. 1). One chamber (no. 1) contained spermatozoa and the other (no. 2) contained the attractant. As before [1], follicular fluid served here as a source of soluble factors that might be secreted from the egg or its surrounding cells. As a control and as a means of distinguishing between attraction and coincidental arrival, the separation procedure was repeated, with BWW replacing the attractant in chamber no. 2. We tested various separation periods and, as shown in Figure 2, at any given time period more spermatozoa reached chamber no. 2 when it contained the attractant than when it contained BWW. From this ratio the extent of enrichment with responsive spermatozoa was calculated, indicating that the shorter the separation time, the higher was the enrichment (e.g., at 15 min the number of spermatozoa that passed to chamber no. 2 when it contained the attractant was twice the number that passed when it contained BWW; at 60 min the ratio was 1.3 only). Nevertheless, in order to optimize between reasonable enrichment on the one hand, and a number of spermatozoa in chamber no. 2 that would be sufficiently large for testing their responsiveness on the other hand, we chose 60 min as the separation time in our next experiments. During this separation period, an average of $28 \pm 3\%$ (mean ± SD for all the experiments included in Table 1) of the spermatozoa in chamber no. 1 moved to chamber no. 2 when the latter contained the attractant. In all the experiments, the number of spermatozoa that passed to chamber no. 2 when it contained the attractant was larger than the number that passed when the chamber contained BWW. The difference at 60 min was $4 \pm 3\%$ (mean ± SD) of the original sperm population. The range of the difference at any time point was 2–12%. This indicates that about 2–12% of the spermatozoa in the original sperm population were responsive.

**Responsiveness of the Separated Subpopulations**

To examine whether the separation indeed resulted in two different subpopulations, one enriched and one deficient in responsive spermatozoa, we determined the re-
sponsiveness of each subpopulation and compared it to that of the original population (Table 1). The sperm suspension collected from chamber no. 2 (attractant-containing) displayed higher responsiveness than that of the original sperm population. On the other hand, the remaining sperm suspension collected from chamber no. 1 was significantly less responsive. Such a statistically significant difference in responsiveness, either between the separated subpopulations or between them and the original population, was not observed when the separation had been carried out, as a control, with BWW substituted for the attractant (Table 1). To rule out the possibility that the observed increase in the sperm responsiveness was the consequence of the sperm incubation with the attractant, we incubated the original spermatozoa with the attractant (diluted as in the separation procedure) and then washed them and reexamined their responsiveness to it. As shown in Figure 3, the incubation with the attractant (unlike incubation in the absence of the attractant), not only did not increase the sperm responsiveness but had a negative effect on it. (Such a decrease in responsiveness is known in other sensory systems, e.g., the olfactory system [10] or leukocyte chemotaxis [11, 12], and is possibly indicative of desensitization of the spermatozoa to the attractant.) The above results indicate that the spermatozoa collected from chamber no. 2 were indeed enriched with responsive spermatozoa whereas those

**TABLE 1. Relative responsiveness of the separated subpopulations.**

<table>
<thead>
<tr>
<th>Separation procedure</th>
<th>Relative responsiveness of the populations ± SEM</th>
<th>n²</th>
<th>p value³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Original Passed Remaining F-test ş</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attractant</td>
<td>1.82 ± 0.13 2.13 ± 0.16 1.28 ± 0.06 14.5 12</td>
<td>0.0001</td>
<td>0.072</td>
</tr>
<tr>
<td>BWW</td>
<td>1.94 ± 0.22 1.40 ± 0.08 1.66 ± 0.14 3.3 7</td>
<td>(insignificant)</td>
<td></td>
</tr>
</tbody>
</table>

*The sperm separation was carried out as described in Materials and Methods.
**The relative responsiveness was calculated as the ratio between the maximal sperm densities in the right half (comprising the attractant-containing well) and the left half (BWW-containing well) of the device, integrated over the whole observation period (10 min).
°The statistical significance was calculated by ANOVA with repeated measures.
²n, the number of experiments. The experiments were carried out with 3 sperm donors and active fractions from 6 follicular fluids.
³The p value refers to the original, passed, and remaining populations together, as calculated by ANOVA.
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To determine whether or not the identity of the responsive spermatozoa was continuously changing, we studied the responsiveness of the original population and its subpopulations as a function of the time after separation. As shown in Figure 4, the passed subpopulation had very little responsiveness left at 100 min after the separation (i.e., 50 min after the first measurement). At the same time, the remaining subpopulation acquired responsiveness that reached a value close to that of the original subpopulation at 50 min and at 100 min was 95%.

collected from chamber no. 1 were deficient in responsive spermatozoa.

DISCUSSION

The results of this study demonstrate that in humans, unlike organisms in which fertilization is external [13–15], only a fraction of the spermatozoa at a given time are responsive to the attractant. They further show that the identity of the responsive spermatozoa changes with time: responsive spermatozoa lose their activity while others acquire it.

These findings, made in vitro, indicate what might occur in vivo. They suggest that spermatozoa in the female genital tract undergo a continuous process of acquiring and losing responsiveness to the attractant, so that throughout the lifetime of spermatozoa in the female genital tract there is always a sufficient number of responsive spermatozoa "on call," ready to be attracted to the egg as soon as it ovulates. Accordingly, if the responsive spermatozoa are the capacitated ones or a subpopulation thereof (yet to be established), it is reasonable that the role of this sperm responsiveness (comprising chemotaxis, enhancement of motility, and ultimately hyperactivation) might be to increase, over an extended period of time, the prospects that an egg will meet capacitated spermatozoa as soon as it ovulates, and that this is done by selecting capacitated spermatozoa and bringing them to the egg [5]. This notion is well in line with a number of in vivo observations. 1) In hamsters, mice, rats, and rabbits, only capacitated or hyperactivated spermatozoa are transported from the oviductal isthmus to the ampulla (the site of fertilization) [16–19]. 2) The fertilizing potential of spermatozoa recovered from the oviduct (as expressed by the number of spermatozoa that should be supplied for fertilizing an egg, or by the time required for penetration of eggs in vitro) is much higher than that of ejaculated spermatozoa or spermatozoa recovered from the uterus [20–22]. 3) In mammals, a considerable fraction of the spermatozoa ejaculated into the female reproductive tract is retained with reduced motility in storage sites; when ovulation occurs, some of them resume high motility and travel the distance between the storage and the fertilization sites within minutes [23–26] (see [5] for review). The observation that a dye injected into the lowermost region of the oviductal isthmus of pigs or hamsters is rapidly transported to the upper ampulla shortly before, during, and within a few hours after ovulation, suggests that the oviduct as a whole exhibits contractile movements [27, 28]. 4) The same holds for humans, it seems that a gradient of an attractant cannot be established over long distances in the fallopian tube, suggesting that the range of sperm attraction to the egg and therefore of sperm selection may be relatively short. It is perhaps also possible that the cumulus oophorus provides a viscoelastic milieu that resists the stirring action of the contractions and of the oviductal cilia.

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REFERENCES


