Spontaneous and Follicular Fluid-Induced Acrosome Reaction in Sperm Samples from In Vitro Fertilizing and Nonfertilizing Normozoospermic Patients

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Submitted: June 18, 1997

Accepted: September 23, 1997

Purpose: One of the most challenging and intriguing groups of infertile patients is that of normozoospermic men who have repeatedly not achieved fertilization in vitro. These cases probably present a wide range of gamete disorders manifested at different stages of the fertilization process. The occurrence of spontaneous and follicular fluid (FF)-induced acrosome reactions (ARs) in in vitro fertilizing and nonfertilizing specimens from normozoospermic IVF patients was assessed, and the effect of Percoll and cryopreservation on the incidence of ARs was evaluated.

Methods: Semen samples from 62 normozoospermic (15 in vitro nonfertilizing and 47 fertilizing) patients were analyzed. Spermatozoa were double-stained with FITC-conjugated Pisum sativum lectin, to assess their acrosomal status, and propidium iodide, to evaluate their vitality, using flow cytometry analysis.

Results: A lower average of AR incidence was observed in the nonfertilizing than in the fertilizing group with all treatments. Both groups exhibited an increase in the proportion of ARs following incubation with FF. This rise was most prominent when Percoll-separated fractions were used (15.8 and 25.6% AR in the nonfertilizing and fertilizing groups, respectively). Thawed cryopreserved and fresh fertilizing samples exhibited similar AR rates.

Conclusions: That normozoospermic recurrently nonfertilizing compared to fertilizing semen samples have a lower capacity to undergo ARs is suggested.

KEY WORDS: acrosome reaction; follicular fluid; human sperm; in vitro fertilization.

INTRODUCTION

One of the most challenging and intriguing groups of infertile patients are normozoospermic men who have repeatedly not achieved fertilization in standard in vitro fertilization (IVF) treatments. The introduction of intracytoplasmic sperm injection (ICSI) has benefited these patients and yielded high fertilization rates using their sperm (1). However, the pathology underlying fertilization failure by standard IVF in these cases (which is probably also the cause of their "unexplained" infertility) remains undefined. These nonfertilizing normozoospermic cases probably represent a wide range of gamete disorders manifested at different stages of the fertilization process. Currently, they are grouped together (and classified as unexplained infertility) due to the lack of practical techniques for clinical evaluation of specific sperm functions. The rationale behind applying ICSI in these cases is that, in this treatment modality, all stages of gamete interaction are bypassed and even sperm maturation and capacitation are not imperative for its success (2,3).

Gamete interaction commences upon binding of the spermatozoon to the oocyte's extracellular glycoprotein vestment, the zona pellucida (ZP). This induces the acrosome reaction (AR), the fusion and vesiculation of sperm plasma and outer acrosomal membranes resulting in the loss of both membranes and the acrosome structure, release of acrosomal contents, and exposure of the inner acrosomal membrane. In many species an intact acrosome is believed to be essential both in vivo and in vitro for ZP binding, while completion of the AR, a prerequisite for ZP traverse, preceding sperm binding and fusion with the oolemma (4).

The ability of spermatozoa to undergo a spontaneous or an induced AR has been reported to correlate with male fertility (5,6). Several assays have been devised to monitor the occurrence of this process, aiming

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mainly at prediction of IVF outcome. These tests usually involve a challenge with different inducers (7,8). A most effective inducer is calcium ionophore, but a variety of physiological factors has also been shown to elicit the AR, including human follicular fluid (FF), cumulus and granulosa cells, whole oocytes, and solubilized ZP (9–11).

It is possible that the unexplained repeated absence of fertilization in IVF cycles of patients with a normal semen analysis may derive in some cases from an inherent inability to undergo the AR or from a defective, premature, or insufficient incidence of AR following exposure to physiological AR inducers. The present study was undertaken to assess the occurrence of spontaneous and FF-induced AR in fertilizing and nonfertilizing specimens of normozoospermic patients.

MATERIALS AND METHODS

Study Group

Sixty-two normozoospermic [according to World Health Organization criteria (12)] patients undergoing IVF treatment cycles during the study period were included; 15 of them had not obtained fertilization in standard IVF cycles and were presently offered micromanipulation assisted fertilization. The other 47 patients attained high fertilization rates in the present as in previous trials. In addition, 7 cryopreserved donor semen samples used in standard IVF cycles were examined, to assess the effect of the cryopreservation-thawing process on the incidence of AR.

Preparation and Incubation of Samples

Fresh Samples. Leftover portions of semen samples used for in vitro insemination were used in this study. A routine semen analysis was carried out before and after sperm preparation using the Mackler counting chamber (Sefi Medical Instruments, Haifa Israel). Half of each sample was washed twice by centrifugation at 300g for 10 min in culture medium [Ham's F-10 (GIBCO BRL, Life Technologies, Paisley, Scotland) supplemented with 25 mM sodium bicarbonate, 1.33 mM lactic acid (Sigma Chemical Co., St. Louis, MO), and penicillin-streptomycin (GIBCO)], while the other half was separated on a triple layer of discontinuous Percoll gradients (90, 70, and 40%; isotonic Percoll; Sigma). Pellets were resuspended with culture medium enriched with 10% synthetic serum substitute (Irvine Scientific, Santa Ana, CA) and incubated at 37°C and

5% CO₂ for 6 hr, until an aliquot was removed for insemination. The remainder of each sample was further incubated overnight at room temperature and subjected to AR induction the following day.

Cryopreserved Samples. Seventeen cryopreserved donor sperm samples thawed for in vitro insemination were examined. Raw semen samples obtained from normozoospermic healthy donors were mixed with an equal volume of test-yolk buffer (Irvine Scientific), cooled in liquid nitrogen vapor for 10 min, and stored in liquid nitrogen until required. Semen was thawed at room temperature washed in Ham's F-10 culture medium and processed as described for fresh samples.

Spontaneous and Induced AR

Each of the overnight-incubated samples was divided again: half served to assess the incidence of spontaneous AR after a further 5 hr of incubation at 37°C in 5% CO₂. The other half was incubated for 5 hr in human FF (1:1, v/v) under conditions similar to these for AR induction. FF aspirated during several oocyte retrievals was collected. The same pool of frozen FF was used throughout the study.

Double-Staining for Assessment of Acrosomal Status and Viability

Samples were incubated in 10 µg/ml propidium iodide (PI) to enable differentiation between physiological and degenerative AR. They were then washed twice in DPBS (Beit Haemek, Israel), fixed in 95% ethanol for 30 min, and washed again in DPBS prior to acrosome labeling by exposure to 10 µg/ml fluorescein isothiocyanate-conjugated lectin, *Pisum sativum* agglutinin (PSA; Sigma), for 60 min.

Fluorescent Microscopy Scoring

In 10 samples manual assessment of AR was scored using a Leitz epifluorescent microscope in parallel to computerized flow cytometry scanning. Dead spermatozoa, as evidenced by PI staining, were excluded. The acrosomal status was determined by scoring the fluorescence emitted by the PSA-labeling on 100 consecutive cells. Spermatozoa exhibiting green fluorescence of the acrosomal region were considered nonreacted.

Flow Cytometry Scoring

All samples were subjected to flow cytometry analysis using a FACS IV flow cytometer (Beckton Dick-

son Corp., Mountain View, CA). Fluorescence was recorded for both markers simultaneously on individual cells. Four types of cells were thus defined: live acrosome-reacted, live nonreacted, dead reacted, and dead nonreacted spermatozoa. The proportion of these was calculated by computed analysis of 10,000 spermatozoa in each sample. Dead spermatozoa were then excluded and AR rates recalculated as the proportion of reacted cells within the live population alone.

Statistical Analysis

Results are expressed as means $\pm$ SE. Differences between groups were analyzed using either paired or unpaired Student's *t* test. *P* values of <0.05 were considered significant.

RESULTS

Comparison of Fluorescent Microscopy and Flow Cytometry Results

The manual and computerized methods for AR scoring were compared in 10 FF-treated samples. The mean AR rates were 23.4 ± 5.8 and 24.42 ± 6.5 as assessed by fluorescent microscopy and flow cytometry, respectively. No significant intrasubject differences were observed as determined by paired *t*-test analysis ($P > 0.5$). Based on this result the less tedious and more objective computerized method was adopted, and all data presented in this study were obtained by flow cytometry.

Spontaneous AR

Following prolonged (overnight) incubation a marginally significant ($P = 0.08$) lower average incidence of spontaneous AR was observed in the nonfertilizing group (5.6 ± 1.68) compared to the fertilizing group (13.2 ± 2.36) (Fig. 1A). The scatter plots (Figs. 1B and C) presenting the raw data of AR rates in individual samples reveal the wide divergence observed in the fertilizing group.

Effect of Percoll

AR rates did not differ between washed and Percoll-separated aliquots from the same sample in the fertilizing group ($P > 0.5$), denoting that Percoll segregation neither is affected by the cell's acrosomal status nor acts as an AR inducer. Percoll-treated samples of the

Fig. 1. Proportions of acrosome-reacted cells in fertilizing and nonfertilizing samples of normozoospermic patients. Mean $\pm$ SE of the nonfertilizing (white bar) and fertilizing (black bar) groups (A). Distribution of spontaneous AR rates in the nonfertilizing (B) and fertilizing (C) groups, presenting the raw data on AR rates in individual samples.

Fig. 2. Effect of density-gradient separation on AR rates in the fertilizing and nonfertilizing groups: Mean + SE before (lightly shaded bars) and after (darkly shaded bars) exposure to Percoll.

nonfertilizing patients, however, showed a significant rise ($P = 0.03$) in the average percentage of acrosome-reacted cells compared to the nonseparated cell population from the same sample (Fig. 2). The difference observed in the mean AR rates of Percoll-separated samples between the fertilizing and the nonfertilizing groups was not significant.

Effect of FF

In both study groups FF elevated the mean proportion of live acrosome-reacted cells. This rise was only "marginally significant" ($P = 0.09$) in washed aliquots of nonfertilizing samples, while of definite statistical significance ($P = 0.0002$) for the washed samples from the fertilizing group. The mean AR rate following exposure to FF was significantly lower ($P = 0.03$) for the nonfertilizing compared to the fertilizing washed specimens (Fig. 3).

The effect of FF was most prominent in samples separated on Percoll gradients prior to incubation with

Fig. 3. Effect of incubation in FF on AR rates in the fertilizing and nonfertilizing groups: mean + SE before (lightly shaded bars) and after (darkly shaded bars) induction.

the inducer. The mean rate of acrosome-reacted cells was significantly higher in the fertilizing group, reaching 25.6 ± 2.45 , compared to that in the nonfertilizing group, 15.8 ± 3.3 ($P = 0.04$) (Fig. 4A), while the rise in AR rates within the samples was significant in both study groups ($P < 0.002$). The scatter plot depicts the calculated difference in AR incidence in each sample between aliquots incubated with and those without FF for 5 hr (Figs. 4B and C). The induction was more effective in the fertilizing samples than in the nonfertilizing ones, as the mean rise in AR following FF incubation was significantly higher: 9.27 ± 1.04 , compared to 5.06 ± 1.44 in the nonfertilizing group ($P = 0.04$).

The proportion of live cells in the samples did not differ between the two groups, either in whole semen preparations or after Percoll separation (not shown).

AR in Frozen/Thawed Samples

Frozen donor sperm samples thawed for in vitro insemination displayed a similar ($P > 0.5$) average of live acrosome-reacted spermatozoa both with and without FF induction as corresponding preparations of fresh samples from fertile patients (Fig. 5). The intrasample rise following exposure to FF was statistically significant ($P = 0.02$) in the thawed as observed in the nonfrozen samples.

DISCUSSION

The AR is a fundamental step in the sequence of events comprising the fertilization process, essential for sperm crossing of the ZP that leads to gamete membrane fusion. The requirement of an intact acrosome for ZP binding in humans remains controversial (13,14), but it is generally accepted that spermatozoa that do not complete the AR will not fertilize (15).

Completion of the postejaculatory sperm membrane modifications, collectively named capacitation, renders the spermatozoa the competence to undergo the AR in response to an effective inducer. A very potent agent commonly used for experimental AR induction in vitro is the calcium ionophore A23187, which originates a massive influx of calcium ions (4). AR rates achieved by this method have been suggested to predict sperm fertilizing potential (8). However, the validity of the ionophore-challenged AR (the ARIC test) has been questioned lately by several investigators. It was shown to have a low reproducibility due to a high degree of intrasubject variability (16), and in some laboratories it did not correlate with IVF results (17)

Fig. 4. AR induction by human FF in Percoll-separated samples. Mean + SE without (white bars) and following (black bars) induction (A). Rise in AR rates following FF incubation presenting the raw data on the difference in AR rates (with and without induction) in individual samples in the nonfertilizing (B) and fertilizing (C) groups.

Fig. 5. Effect of FF on fresh and frozen/thawed fertilizing samples. Mean + SE AR rates before (white bars) and following (shaded bars) induction.

or with AR rates achieved using physiological inducers (18).

Physiological inducers of the human AR include the different egg investments, i.e., the cumulus oophorus (11), granulosa cells (10), ZP (9), and FF (11). The AR-inducing factor in the latter has been identified as progesterone (19), but immunoglobulins isolated from human FF may be involved in eliciting this response (20). The choice of FF for induction of physiological AR in this study was due mainly to its availability. It is an abundant by-product of oocyte retrieval and can be easily stored for successive experiments.

Spontaneous AR occurs to some extent under in vitro conditions and is enhanced by separation procedures and by prolonged incubation. The baseline values following overnight incubation were used in this study to allow maximal capacitation and expression of spontaneous AR potential and measurement of the additive value of AR induction exceeding this rate.

The phenomena of cell death and membrane degeneration falsely enlarge the population of spermatozoa detected as acrosome reacted, affecting results, especially when aged samples are studied. It is therefore important to ascertain the viability of each spermatozoon evaluated for acrosomal status when examining either spontaneous or induced AR. In this study this was achieved by double-staining the samples with PI and PSA and recording both markers for individual cells by computerized flow cytometry.

The use of frozen semen is now compulsory in many countries in donor insemination programs to curb the spreading of sexually transmitted diseases. Cryopreservation is known to cause a decline in the number of motile spermatozoa as well as their velocity and also to induce membrane disruption (21). Here, degenerative acrosome loss was distinguished from genuine AR, as only live cells were included. As "fresh" (as opposed to frozen/thawed) samples were examined after pro-

longed incubation, the difference in AR rates between fresh and cryopreserved samples was not as evident as noted by McLaughlin *et al.* (22).

A lower average AR incidence was observed in the nonfertilizing group compared to the fertilizing group in all treatments. The great interpatient variability observed in this study affected the statistical significance of the differences between the two groups after their initial preparation. Yet a marked difference was observed following exposure to FF induction using either washed or Percoll-separated fractions.

The results presented here suggest a lower capacity to undergo AR in normozoospermic nonfertilizing compared to fertilizing semen samples. Fertilization failure cannot be attributed to a single cause common to all the patients in this group, but it is conceivable that an AR-associated dysfunction is represented. Spermatozoa that bind to the ZP but fail to undergo AR will not fertilize but may impede the binding of other sperm cells. Thus, if a significant proportion of such "malfunctioning" cells is present in the sample, it can hinder fertilization in vitro as well as in vivo.

Currently, although several hypotheses exist [reviewed by Brucker and Lipford (4)], the nature of the molecules participating in the signal transduction pathway responsible for AR and its regulating factors remain unclear. In situ molecular measurements of the AR capacity of spermatozoa therefore cannot be devised. This is essential for advancing a more scholastic approach to the task of explaining "unexplained" recurrent fertilization failure.

ACKNOWLEDGMENTS

This work forms part of the Ph.D. thesis of O. Rufas, Tel-Aviv University.

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