

PHYSIOLOGY

Physiological Induction of the Acrosome Reaction in Human Sperm: Validation of a Microassay Using Minimal Volumes of Solubilized, Homologous Zona Pellucida

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Purpose: The objective was to develop a method that could accommodate microvolumes of solubilized human (ZP) and sperm for assessing the induction of the acrosome reaction.

Methods: A microassay using 1 μ l of 2.5, 1.25, 0.6, 0.3, and 0.125 ZP/ μ l incubated with 1 μ l of a highly motile sperm suspension for 60 min. As a control and parallel to the microassay a standard acrosome reaction technique was performed.

Results: No significant differences were observed between the percentage acrosome-reacted sperm reported by the two assays under basal conditions (spontaneous) or after induction with a Ca^{2+} ionophore or solubilized ZP. At a ZP concentration of 0.6 ZP/ μ l, the percentages of acrosome-reacted spermatozoa in both techniques were significantly higher compared to the spontaneous acrosome reaction results, namely 18% and 17%, compared to 10% and 10%, respectively. Approximately a 30% level of acrosomal exocytosis was induced with 2.5 ZP/ μ l in both methods.

Conclusions: This newly devised microtechnique is easy and rapid to perform, is repeatable, and facilitates the use of minimal volumes of solubilized human ZP (even a single ZP) for assessment of the inducibility of the acrosome reaction of a homologous sperm population.

KEY WORDS: Human sperm; acrosome reaction; zona pellucida; microassay.

INTRODUCTION

The andrologic evaluation of the male partner relies on a thorough history and physical examination, followed by a urologic and endocrinologic work-up as indicated. Still, the semen analysis remains the cornerstone of diagnostic management. We and others, have been promoters of a sequential, multi-step diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics (1–3). The proposed diagnostic scheme should include (i) assessment of the “basic” semen analysis and (ii) functional testing of spermatozoa (4,5).

Different laboratories have highlighted the diagnostic power of a variety of tests that examine the functional competence of the male gamete. The World Health Organization has incorporated some of them under the category of functional tests (4). At a recent Consensus Workshop in Advanced Andrology (6) it was concluded that because of their validation and unquestioned clinical value, the homologous sperm–zona pellucida (ZP) binding tests should be incorporated in the advanced stages of the workup. However, it also was agreed that better standardization of the currently used acrosome reaction techniques should be implemented prior to their introduction as a routine clinical tool. At the present time, there seems to be general agreement that more clinical information can be gained by the analysis of a stimulant-induced acrosomal exocytosis compared to the assessment of the

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spontaneous frequency of acrosome reactions (basal rate). The most widely utilized method is the challenge with a calcium ionophore agent where the acrosome reaction is identified with defined lectins in combination with indirect immunofluorescence (6).

The acrosomal response of a given sperm sample has been illustrated to be a crucial event leading to fertilization and many reports have aimed to correlate acrosome reaction response with *in vitro* fertilization rates. Moreover, the precise timing of the acrosome reaction formed the rationale for the development of the ARIC test (7,8). The concept of acrosomal inducibility (9,10) and the ARIC test have gained more recognition, and there seems to be agreement that this method of evaluation is a more reliable predictor of sperm-fertilizing ability than those tests that simply measure the frequency of spontaneous acrosome reactions. The inducibility of the acrosome reaction, i.e., the difference between spontaneous and percentage acrosome-reacted sperm after induction, correlates significantly with *in vitro* fertilization outcome (9).

The ZP in both the intact and solubilized state has been demonstrated to be a powerful and physiological inducer of the acrosome reaction (11–16). During fertilization, acrosome reaction failure can be caused by multiple factors, such as (i) inadequate sperm capacitation; (ii) an inability of the sperm membrane to undergo specific structural–functional changes after binding to the ZP; or (iii) an impaired capacity of the ZP of a specific oocyte to induce the acrosomal cascade.

In this investigation, we aimed to develop and validate a simple and rapid microassay for the accurate determination of the human sperm acrosome reaction mediated by minimal volumes of solubilized (or even a single ZP) homologous ZP.

MATERIALS AND METHODS

Preparation of Sperm Samples

Ejaculates from fertile men (donors) were used in these studies after approval by the local ethics committee. The sperm parameters of samples used were as follows (mean \pm standard deviation): concentration, $117.4 \pm 16 \times 10^6$ /ml; sperm motility, $60 \pm 5\%$, and normal morphology (strict criteria), $17 \pm 2\%$. Motile sperm fractions (10×10^6 cells/ml, $>90\%$ motility) were retrieved using a double-wash swim-up technique (15,16). Before the onset of acrosome reaction studies, sperm were allowed to capacitate at 37°C in $5\% \text{CO}_2$

in air for 3 hr in synthetic human tubal fluid (HTF) medium supplemented with $3\% \text{BSA}$ (17).

Preparation of Solubilized ZP

Human oocytes were retrieved from postmortem-derived ovarian tissue following approval by the local ethics committee. Oocytes were stored in DMSO/sucrose at -196°C in liquid nitrogen (18). Twelve hours prior to each experiment, oocytes were removed from storage and thawed at 37°C . Oocytes were placed in 0.25 M sucrose and $3\% \text{BSA}$ in HTF medium for 20 min at room temperature, after which the oocytes were placed under mineral oil (Sigma Chem. Co., St Louis, MO; Cat. No. M-3516) until used.

On the day of each experiment, 50 oocytes were vigorously pipetted with a small-bore glass pipette (inner diameter $80 \mu\text{m}$) to separate the ZP from the ooplasm. The separated ZP were then placed in a plastic Eppendorf tube containing HTF medium supplemented with $3\% \text{BSA}$. The tubes were centrifuged for 15 min at $1800 \times g$, after which the HTF medium was carefully removed under microscopic vision (Olympus SZ40, Wirsam Scientific, Cape Town, South Africa), leaving only 50 ZP at the bottom of the tube. A total volume of $5 \mu\text{l}$ 10 mM HCl was added to the zona pellucidae. Solubilization of the ZP was microscopically observed and controlled after which $5 \mu\text{l}$ of 10 mM NaOH was added to the ZP to render a final zona volume of $10 \mu\text{l}$, containing $5\text{ZP}/\mu\text{l}$. The final pH of the zona solution was 7.4.

Acrosome Reaction

Two sets of experiments, each using different volumes of solubilized ZP, were performed in a parallel fashion, namely, a microassay and a standard acrosome reaction assay. Following solubilization zona pellucidae were kept at 4°C for 7 days during which all experiments were performed. Ongoing studies in our laboratory currently evaluate the acrosome reaction inducibility of solubilized zona pellucidae recorded over an extended time period.

For the microassay, $1 \mu\text{l}$ of ZP solution (concentration, $5 \text{ZP}/\mu\text{l}$ stock solution) was aspirated into a Teflon pipette tip (Hamilton Pipette-tip, Cat. No. 84254, Separations, Cape Town, South Africa) fitted to a microsyringe (Hamilton 702, Separations, Cape Town, South Africa) with $1 \mu\text{l}$ of sperm (10×10^6 sperm/ml, $>90\%$ motility), to render a final ZP concentration of $2.5 \text{ZP}/\mu\text{l}$. Serial dilutions were made from the $5 \text{ZP}/\mu\text{l}$ solutions (1:1, vol:vol) using HTF to equal final zona

concentrations of 2.5, 1.25, 0.6, and 0.3 ZP/ μ l. One microliter of each dilution was separately added to 1 μ l of sperm to equal a final ZP concentration of 1.25, 0.6, 0.3, and 0.15 ZP/ μ l. Prior to aspiration into Teflon tips, all sperm/ZP suspensions were gently mixed in a well of a microtiter plate (Microtest plate Cat. No. P43 Laboratory and Scientific, Cape Town, South Africa). To prevent evaporation from the Teflon tips, aspirating HTF droplets into both sides of the Teflon tip sealed off sperm–ZP suspensions. Each sperm–ZP suspension was separated from the HTF droplets by air bubbles on both sides. Due to the small volumes involved, progressive motility and percentage live cells (4) for both acrosome reaction techniques were manually performed on spotted slides (MAGV, Germany, XER 201B). Control and treated sperm samples were carefully removed from the teflon tips and placed on separate spots on the spotted slide and immediately evaluated for percentage live sperm under inverted phase contrast microscope (Nikon TMS-F, Research Instruments, Johannesburg, South Africa). During both techniques the percentage live acrosome reacted cells were recorded by aspirating/adding 1 μ l (1 μ g/ml) Hoescht dye (supravital stain Hoechst 33258, B-2883; Sigma Chemical Co, St Louis, MO) 5 min before termination of incubation of sperm and solubilized zona. During the evaluation of the acrosome status of each experiment, only live acrosome-reacted spermatozoa were recorded. In each study, negative and positive control experiments consisted of 1 μ l of sperm suspension plus 1 μ l HTF or 1 μ l 10 μ M Ca^{+2} ionophore (Sigma, Chem. MO, Cat. No. C7522) incubated as the test conditions at 37°C, 5% CO_2 , 95% humidity for 1 hr. For the standard acrosome reaction assay, larger volumes [i.e., 5 μ l of the sperm suspension plus 5 μ L of the above-mentioned zona solutions (i.e. 2.5, 1.2, 0.6, 0.3, and 0.15 ZP/ μ l)] were incubated in 0.4-ml Eppendorf tubes under similar laboratory conditions for 1 hr. Prior to the onset of the study, control experiments (i.e., exposure of sperm to HTF and Ca^{2+} ionophore) were also included in the standard acrosome assay as described above.

Following the motility assessments, sperm droplets were allowed to air dry, after which the sperm were fixed in 70% ethanol for 20 min and evaluated for percentage live cells. Acrosomal status for both assays (and respective control conditions) were evaluated using fluorescein isothiocyanate (FITC)–*Pisum sativum agglutinin* (PSA) staining (Sigma Chemicals, MO, L0770), with epifluorescence microscopy (Olympus BX40, Wirsam Scientific, Cape Town, South Africa) (11,12,19,20). Two hundred cells were counted in a

blinded fashion in each well of the spotted slide and results were expressed as percent acrosome-reacted sperm. The following staining patterns were evaluated as acrosome-reacted spermatozoa: (i) patchy staining on acrosomal region, (ii) distinct staining in the equatorial region occurring as a equatorial bar, and (iii) and no staining observed over entire sperm surface.

Intact ZP-Induced Acrosome Reaction

Additional acrosome reaction studies were performed on intact ZP. Using the same sperm samples, parallel experiments to the microassay and standard acrosome assay were performed where the acrosomal status of ZP-bound sperm was examined after co-incubation of the male gametes with intact, previously salt-stored human oocytes. It has been demonstrated that oocytes stored under these conditions retain biophysical, biochemical, and functional properties (21–23). At the time of the experiments, the oocytes were desalted, washed in culture medium, and microbisected into matching hemizonae as previously described (22). A total of 60 hemizonae (matching pairs from 30 oocytes) were individually incubated under oil in 50 μ L droplets containing 25×10^6 /ml motile sperm (post-swim-up) for 60 min at 37°C in 5% CO_2 in air. The hemizonae were then removed from the suspension; after pipetting using a fine glass pipette to remove loosely attached sperm, the number of sperm tightly bound to each hemizonae were counted under phase-contrast microscopy (22,23). Thereafter, the sperm tightly bound to the zona were removed (stripped) by a shearing action, using a small-bore glass (60 μ m inner diameter) pipette. Individual sperm were then placed on a spot glass slide, allowed to air dry, after which the acrosome reaction was determined as described above. The number of sperm tightly bound to each hemizona under these conditions was always >300 cells. Most of the zona-bound sperm could be stripped during the experiments for all hemizonae evaluated and at least 200 spermatozoa (per hemizona) could be assessed for acrosomal status.

Statistical Analyses

Comparisons of the percentage acrosome reaction for both methods (micro- and standard assays) under different experimental conditions (i.e., spontaneous, Ca^{2+} ionophore, and ZP-induced conditions) were performed with Fisher's exact paired *t* test. The overall dose-dependency effect of varying solubilized ZP con-

Table I. Acrosome Reaction Results Recorded with a Microassay and a Standard Technique Using Calcium Ionophore-Induced and Spontaneous Reactions

Standard assay ^a		Microassay ^a		Intact zona pellucida (% AR of zona bound sperm)
Spontaneous AR	Ca ²⁺ ionophore induced AR	Spontaneous AR	Ca ²⁺ ionophore induced AR	
10 ± 2 ^b	51 ± 2 ^c	10 ± 3 ^d	47 ± 14 ^e	84 ± 9%

^a Mean % ± SD.

^b b versus d: not significant, Fisher exact paired *t* test.

^c c versus e: not significant, Fisher exact paired *t* test. AR; percentage acrosome reacted spermatozoa.

centrations on acrosomal exocytosis was assessed by analysis of variance (ANOVA).

Ca²⁺ ionophore agent (84 ± 9 % vs. 32 ± 2 and 28 ± 3, respectively, *P* < 0.001).

RESULTS

The percentage acrosome reaction recorded for spontaneous (in HTF medium) and Ca²⁺ ionophore-induced did not differ between the micro- and standard acrosome reaction assays (Table I). Table II shows acrosome reaction results for the various solubilized ZP concentrations used. Again, there were no differences in the induction of acrosomal exocytosis for the two methods. The percentage of live acrosome reacted sperm in both techniques were >80%. In addition, an obvious dose-dependent effect of solubilized ZP on the acrosome reaction was observed reaching an approximate 30% induction using a maximum dose of 2.5 ZP/μl for both the micro- and standard assays. Following a 1-hr incubation with intact hemizonae (intact ZP), on the other hand, the percent acrosome-reacted zona-bound sperm was significantly higher than the levels obtained with solubilized ZP or the

DISCUSSION

The need for a microvolume assay to assess acrosome reaction has been identified previously (20). This is due to the fact that diagnostic andrology laboratories often lack sufficient biological material (i.e., human ZP) to perform a defined and specific test such as the examination of the physiological acrosome reaction. This is true for the natural ZP protein(s), but also will be relevant when recombinant human ZP proteins are to be tested for corroboration of their biological activity (24,25).

The newly described assay is simple, can be performed quickly, and the results are reliable and repeatable. Therefore, because of the small volumes employed, it is an ideal technique for testing native and recombinant ZP (highly precious or scant material). The results of the present study indicated the use of a single ZP to be adequate for mediating the acrosome reaction of a sperm population in suspension. At a ZP concentration of 0.6 ZP/μL, the percentage acrosome-reacted sperm, as determined by both the standard and microassays, was significantly higher than the spontaneous reaction, i.e., 18% (standard assay) and 17% (microassay) compared to the spontaneous reaction namely, 10% (standard assay) and 10% (microassay). The maximum levels of acrosomal exocytosis (28%) induced with the highest ZP concentration (2.5 ZP/μl final concentration) in the microassay were similar to the ones observed with the standard assay in our laboratory and in those of others (12,14,15). On the other hand, acrosome reaction induction by intact zonae pellucidae as detected during sperm-zona binding control assays as expected was higher than with the use of solubilized ZP. The incidence of zona-bound acrosome reacted spermatozoa

Table II. Mean (SD) Percentage Acrosome-Reacted Spermatozoa Recorded for Varying Solubilized ZP Concentrations^a

Zona pellucida concentration (ZP/μl)	Standard assay		Microassay	
	Percentage AR (mean ± SD)	<i>n</i>	Percentage AR mean ± SD	<i>n</i>
2.50	32 ± 2% ^b	4	28 ± 3%	6
1.25	26 ± 2% ^b	6	23 ± 2%	5
0.60	18 ± 3% ^b	5	17 ± 3%	6
0.30	14 ± 2%	6	16 ± 2%	4
0.15	14 ± 3%	4	14 ± 3%	6
Spontaneous	10 ± 2% ^b	6	10 ± 3%	6

^a Overall dose-dependency effect for both methods, *P* < 0.0001 by analysis of variance.

^b *P* = < 0.05 for 2.5, 1.25, and 0.6 ZP/μl when compared to spontaneous acrosome reaction results.

found here is similar to the one we reported using the hemizona assay model combined with transmission electron microscopy and the monoclonal T-6 antibody (23,26). A more adequate configuration of the ZP proteins in the intact zona matrix and a higher number of zona protein molecules in the hemizona (as compared to lower numbers present in the very small volumes of solubilized ZP used here) are a possible explanation for this finding.

The routine introduction of a simple and reliable assay for the evaluation of the physiologically induced acrosome reaction as a component of the previously proposed sequential diagnostic workup program will assist in the identification of specific sperm defects and may allow the development of more directed therapies. Andrology testing remains, in our opinion as well as those of others (27), an ever-growing component in the workup of the infertile couple. We enter the next millennium with many questions that remain to be answered through efficacious screening techniques and a new formidable therapy in ICSI (3). The analysis of the inducibility of the acrosome reaction, a critical step during fertilization, aids the clinician in the management of male infertility. Finally, it is accepted that, once available, a biologically active recombinant human zona pellucida protein 3 (rhuZP3) will be the ultimate agonist or trigger substance for human sperm acrosome reaction. Such a test will most certainly become the basis of the ideal acrosome reaction test (8). Once available in sufficient amounts, rhuZP3 moieties will have to go through comparative evaluation studies, using natural (solubilized) ZP as controls in subsequent (microvolume) acrosome reaction assays.

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