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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Submitted: July 7, 1999
Accepted: September 10, 1999

Purpose: *To develop a method that could accommodate microvolumes of solubilized human zona pellucida (ZP) and sperm for assessing the induction of the acrosome reaction.*
Methods: *A microassay using 1 μl of 2.5, 1.25, 0.6, 0.3, and 0.125 ZP/μl incubated with 1 μl 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/μl, 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. An approximately 30% level of acrosomal exocytosis was induced with 2.5 ZP/μl 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 workup as indicated. Still, the semen analysis remains the cornerstone of diagnostic management. We and others have been promoters of a sequential, multistep 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 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 induc-

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1058-0468/00/0800-0374$18.00/0

ibility (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 volume 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 ± standard deviation): concentration, $117.4 \pm 16 \times 10^6$/ml; sperm motility, 60 ± 5%, and normal morphology (strict criteria), 17 ± 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% CO_2 in air for 3 hr in synthetic human tubal fluid medium (HTF) supplemented with 3% bovine serum albumin (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% 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 USA 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 μm) to separate the ZP from the ooplasm. The separated ZP were then placed in a plastic Eppendorf tube containing HTF medium supplemented with 3% 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 μl 10 mM HCl was added to the zonae pellucidae. Solubilization of the ZP was microscopically observed and controlled after which 5 μl of 10 mM NaOH was added to the ZP to render a final zona volume of 10 μl, containing 5 ZP/μl. 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 ZP 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 ZP recorded over an extended time period.

For the microassay, 1 μl of ZP solution (concentration, 5 ZP/μl stock solution) was aspirated into a Teflon pipette tip (Hamilton Pipette-tip, Cat 84254, Separations, Cape Town, South Africa) fitted to a microsyringe (Hamilton 702, Separations, Cape Town, South Africa) with 1 μl of sperm (10×10^6 sperm/ml, <90% motility), to render a final ZP concentration of 2.5 ZP/μl. Serial dilutions were made from the 5 ZP/μl solutions (1:1, vol:vol) using HTF to equal final ZP concentrations of 2.5, 1.25, 0.6, and 0.3 ZP/μl. One microliter of each dilution was separately added to 1 μl of sperm to equal a final ZP concentration of 1.25, 0.6, 0.3, and 0.15 ZP/μl. 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 of live acrosome-reacted cells were recorded by aspirating/adding 1 μl (1 μg/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 ZP. 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 μl of sperm suspension plus 1 μl HTF or 1μl 10μM Ca^{2+} ionophore (Sigma, Chem. MO, USA, Cat 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 μl of the sperm suspension plus 5 μl of the above-mentioned ZP solutions (i.e., 2.5, 1.2, 0.6, 0.3, and 0.15 ZP/μl), 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) also were 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 isothiocianate (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 micro- and standard acrosome assays were performed where the acrosomal status of ZP-bound sperm was examined after coincubation 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 μl 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 and after pipetting using a fine glass pipette to remove loosely attached sperm and 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 μm inner diameter) pipette. Individual sperm were then placed on a spot glass slide and 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 concentrations on acrosomal exocytosis was assessed by analysis of variance (ANOVA).

RESULTS

The percentage acrosome reaction recorded for spontaneous (in HTF medium) and Ca^{2+} 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

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
Spontaneous AR	Ca^{2+}ionophore-induced AR	Spontaneous AR	Ca^{2+}-ionophore induced AR	% AR of zona-bound sperm
10 ± 2[b]	51 ± 2[c]	10 ± 3[d]	47 ± 14[e]	84 ± 9%

[a] Mean % ± SD; AR, Percentage acrosome-reacted spermatozoa.
[b] vs. [d] Not significant, Fisher exact paired *t*-test.
[c] vs. [e] Not significant, Fisher exact paired *t* test.

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 maximun 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 percentage acrosome-reacted zona-bound sperm was significantly higher than the levels obtained with solubilized ZP or the Ca^{2+} ionophore agent (84 ± 9 % vs. 32 ± 2 and 28 ± 3, respectively, $P < 0.001$).

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.

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

	Standard Assay		Microassay	
Zona pellucida concentration (ZP/μl)	Percentage AR (mean ± SD)	*n*	Percentage AR mean ± SD	*n*
2.5	32 ± 2%[b]	4	28 ± 3%	6
1.25	26 ± 2%[b]	6	23 ± 2%	5
0.6	18 ± 3%[b]	5	17 ± 3%	6
0.3	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.

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 and 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 ZP as detected during sperm-zona binding control assays was, as expected, higher than with the use of solubilized ZP. The incidence of zona-bound acrosome-reacted spermatozoa 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 by the hand of efficacious screening techniques and a new formidable therapy in intracytoplasmic sperm injection (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 ZP protein 3 (rhuZP3) will be the ultimate agonist or trigger substance for human sperm. 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.

REFERENCES

1. Oehninger S, Acosta A, Veeck L, Brzyski R, Kruger TF, Muasher SJ, Hodgen GD: Recurrent failure of in vitro fertilization: Role of the hemizona assay in the sequential diagnosis of specific sperm-oocyte defects. Am J Obstet Gynecol 1991;164:1210–1215
2. Amman RP, Hammerstedt RH: In vitro evaluation of sperm quality: An opinion. J Androl 1993;14:397–406
3. Oehninger SC, Franken DR, Kruger TF: Approaching the next millenium: How should we manage andrology diagnosis in the intracytoplasmic sperm injection era? Fertil Steril 1997;67:434–436
4. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction, 3rd ed. Cambridge, England, Cambridge University Press, 1992
5. Oehninger SC: An update on the laboratory assessment of male fertility. Hum Reprod 1995;10(Suppl 1):38–45
6. Consensus Workshop on Advanced Diagnostic Andrology Techniques. ESHRE Andrology Special Interest Group. Hum Reprod 1996;11:1463–1479
7. Cummins JM, Pember SM, Jequier AM, Yovich JL, Hartman PE: A test of the human sperm acrosome reaction following ionophore challenge: Relationship to fertility and other seminal parameters. J Androl 1991;12:98–103
8. Tesarik J. Acrosome reaction testing. *In* Consensus Workshop on Advanced Diagnostic Andrology Techniques. ESHRE Andrology Special Interest Group. Hum Reprod 1996;11:5–8
9. Henkel R, Müller C, Miska W, Gips H, Schill W-B: Determination of the acrosome reaction in human spermatozoa is predictive of fertilization in vitro. Hum Reprod 1993;12:2128–2132
10. Henkel R, Franken D.R. Habenicht UF: Zona pellucida as physiological trigger for the induction of the acrosome reaction. Andrologia 1998;30:275–280
11. Cross NL, Morales P, Overstreet JP, Hanson FW: Induction of the acrosome reaction by the human zona pellucida. Biol Reprod 1988;38:235–244
12. Mahony M, Oehninger S, Clark G, Acosta A, Hodgen GD: Inhibition of zona pellucida-induction of the acrosome reaction in human spermatozoa by the complex polysaccharide, fucoidin. Contraception 1991;44:657–665
13. Bielfeld PP, Faridi A, Zaneveld LJD, de Jonge CJ: The zona pellucida induced acrosome reaction of human spermatozoa is mediated by protein kinases. Fertil Steril 1994;61:536–541
14. Liu DY, Baker HWG: Disordered acrosome reaction of spermatozoa bound to the zona pellucida: A newly discovered sperm defect causing infertility with reduced sperm-zona penetration and reduced fertilization in vitro. Hum Reprod 1994;9:1694–1700
15. Franken DR, Morales P, Habenicht UF: Inhibition of G protein in human sperm and its influence on acrosome reaction and zona pellucida binding. Fertil Steril 1996;66:1009–1011
16. Franken DR, Bastiaan HS, Kidson A, Wranz P, Habenicht U-F: Zona pellucida mediated acrosome reaction and sperm morphology. Andrologia 1997;29:311–317
17. Quinn P, Kerin JF, Warnes GM: Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 1985;44:493–498
18. Hammit DG, Syrop CH, Walker DL, Bennet MR: Conditions of oocyte storage and use of non-inseminated as compared with inseminated, nonfertilized oocytes for the hemizona assay. Fertil Steril 1993;60:131–136
19. Morales P, Cross NL, Overstreet JW, Hanson FW: Acrosome-intact and acrosome-reacted human sperm can initiate binding to the zona pellucida. Dev Biol 1989;133:385–392
20. Morales PJ, Cross NL: A new procedure for determining acrosomal status of very small numbers of human sperm. J Histochem Cytochem 1989;37;1291–1292
21. Yanagimachi R, Lopata A, Odom CB, Bronson RA, Mahi CA, Nicolson GL: Retention of biologic characteristics of zona pellucida in highly concentrated salt solution: The use of salt-stored eggs for assessing the fertilizing capacity of spermatozoa. Fertil Steril 1979;31:562–567
22. Burkman LJ, Coddington CC, Franken DR, Kruger TF, Rosenwaks Z, Hodgen GD: The hemizona assay: Development of a diagnostic test for the binding of human spermatozoa to the human zona pellucida to predict fertilization potential. Fertil Steril 1988;49:688–693
23. Franken DR, Oosthuizen WT, Cooper S, Kruger TF, Burkman LJ, Coddington CC, Hodgen GD: Electron microscopic evidence on the acrosomal status of bound sperm and their penetration into human zonae pellucidae after storage in a buffered salt solution. Andrologia 1991;23:205–208
24. Brewis IA, Clayton R, Barratt CLR, Hornby DPJ, Moore HDM: Recombinant human zona pellucida glycoprotein 3 induces calcium influx and acrosome reaction in human spermatozoa. Hum Reprod. 1996;2:583–589
25. Chapman NR, Barrattt LR: The role of carbohydrate in sperm-ZP3 adhesion. Mol Hum Reprod 1996;2:767–774
26. Coddington CC, Fulgham DC, Alexander NJ, Johnson D, Herr JC, Hodgen GD: Sperm bound to zona pellucida in hemizona assay demonstrate acrosome reaction when stained with T-6 antibody. Fertil Steril 1990;54:504–508
27. Mortimer D: The essential partnership between diagnostic andrology and modern assisted reproductive technologies. Hum Reprod 1994;9:1209–1213