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Andrology

Zona Pellucida Induced Acrosome Reaction, Sperm Morphology, and Sperm–Zona Binding Assessments Among Subfertile Men

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***Purpose*:** The study aimed to evaluate the relationship between the zona pellucida induced acrosome reaction (ZIAR) and (i) percentage normal spermatozoa as well as (ii) sperm–zona pellucida binding potential among men referred for a routine semen analysis.
***Methods*:** Semen samples of 164 consecutive men referred to the andrology laboratory for routine semen analysis were studied. Semen samples were analyzed using the new WHO standards (strict criteria). ZIAR was recorded with a lectin conjugated *Pisum sativum* agglutinin microassay, while sperm–zona binding was evaluated with a standard hemizona assay (HZA).
***Results*:** Andrology patients were divided according to the percentage normal spermatozoa in the ejaculate, namely <4% normal forms ($n = 71$), 5–14% normal forms ($n = 73$), and >14% normal forms ($n = 20$). ZIAR data of the <4%, 5–14%, and >14% groups was $(9.6 \pm 0.6)\%$, $(13.9 \pm 0.5)\%$, and $(15.0 \pm 1.1)\%$, respectively. The ZIAR data of fertile control men was $(26.6 \pm 1.4)\%$ which differed significantly from the three andrology referrals groups. Likewise significant differences were recorded during the hemizona assay namely, 38.0% (<4% normal forms), 54.5% (5–1% normal forms), and 62.6% (>14% normal forms). Among the group with >14% normal forms, five cases had impaired ZIAR outcome (<15%). Three of these men had normal morphology and HZAs.
***Conclusions*:** ZIAR testing should become part of the second level of male fertility investigations, i.e., sperm functional testing, since 15% of andrology referrals revealed an impaired acrosome reaction response to solubilized zona pellucida.

KEY WORDS: acrosome reaction; zona pellucida.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) has provided an unique technique to allow couples, diagnosed with severe male infertility (1), to achieve their reproductive goals (2). However, several questions obviously arise including (i) what are the diagnostic steps that we should use to direct infertile men to a specific therapeutic modality? and (ii) what are the current indications for ICSI? (2).

Despite the questions surrounding the clinical importance of the semen analyses (3), the andrologic investigation still relies on a thorough history and physical examination of the male partner (2). Additionally, an urological and endocrinological workup should be implemented as needed. The semen analysis therefore still remains the cornerstone of the diagnostic management (2,4). A multistep diagnostic approach for the evaluation of the various structural, dynamic, and functional sperm characteristics have

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been advocated by scientists and clinicians (4). This approach has been the result of combined information derived from the basic and clinical areas of the andrology and reproductive endocrinology disciplines. It is our opinion that this diagnostic scheme should include as a first-level assessment the "basic" semen analysis as outlined by the (WHO) (2,5,6). The second level should include functional testing of spermatozoa, i.e., sperm–zona binding (6–8), acrosome reaction (9,10), and chromatin packaging (11).

Sperm morphology is regarded as possibly the most consistent variable that appears to be related to in vitro fertilization success. (12–14). This observation has therefore not only a very important clinical and diagnostic role to play in the structured management of infertile couples, but also serves as a reference point in many research projects that aim to establish the importance of a new diagnostic test (7,9).

This study aimed to evaluate the relationship between the zona pellucida ZP induced acrosome reaction (ZIAR) and (i) percentage normal spermatozoa and (ii) sperm–zona binding potential among men referred for a routine semen analysis.

MATERIAL AND METHODS

Spermatozoa

The semen samples of 164 consecutive men referred to the andrology laboratory at Tygerberg Hospital for routine semen analysis were additionally tested for sperm function. This included sperm–zona binding (hemizona assay, HZA) and acrosome reaction induced by solubilized human ZP (ZIAR). Samples were analyzed based on strict criteria suggested by the WHO's criteria (5,15–18). Results of the semen analyses were kept blind to the diagnostic sperm laboratory personnel until acrosome reaction and sperm–zona binding results were completed.

Microassay for the Evaluation of the Acrosome Reaction

During the initial stages of the work semen samples form 11 fertile sperm donors were used to establish (i) the dose–response curve for acrosome reactions after stimulation with solubilized human ZP and (ii) the inter- and intratechnician and sample variation for sperm morphology and acrosome staining with PSA-FITC. Coefficient of variations for both intra- and interassay and technician values were calculated by dividing the mean with standard deviation $\times$ 100% for each observation. The inter- and intraassay as well as inter- and intratechnician coefficient of variation was <15% among the slides (9,19).

Capacitated motile sperm fractions from the 164 men were incubated for 60 min with (i) HTF medium (spontaneous AR, control) and (ii) 0.6 ZP/μL (ZIAR, test). Results were recorded as the difference between zona induced acrosome reacted and spontaneous acrosome reacted sperm and expressed as percentage ZIAR.

For the microassay, 1 μL of ZP solution (concentration 5 ZP/μL) 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 (stock solution). The stock solution was stored at 4°C for a maximum period of 7 days. On each day of the experiment 1 μL volumes were removed from this solution to perform serial dilutions using HTF to equal a final zona concentration (after adding 1 μL sperm) of 0.6 ZP/μL.

Prior to aspiration into Teflon tips, all sperm/ZP suspensions were gently mixed in a well of a microtitre plate (Microtest plate cat No. P43, Laboratory and Scientific, Cape Town, South Africa). To prevent evaporation from the Teflon tips, sperm/ZP suspensions were sealed off by aspirating HTF droplets into both sides of the Teflon tip. Each sperm/ZP suspension was separated from the HTF droplets by air bubbles on both sides. Monitoring of progressive motility for both acrosome reaction techniques was manually performed on spotted slides (MAGV, Germany, XER 201B). Sperm droplets were carefully placed on separate spots and immediately evaluated for percentage live sperm under inverted phase contrast microscope (Nikon TMS-F, Research Inst., Johannesburg, South Africa) (20).

Sperm–Zona Binding

For the HZA, oocytes were microbisected into two identical hemizonae using previously reported micromanipulation techniques (7). In each assay, matching hemizonae were separately incubated to a sperm concentration of 5×10^5/mL from a fertile donor (control) and patient (test). After 4 h of coincubation (at 37°C, in 5% CO_2 in air), hemizonae were rinsed in medium (HTF10) by pipetting 5$\times$ with a finely drawn micropipette (100 μm inner diameter) to dislodge

Table I. Dose–Response Results of Percentage Acrosome Reacted Sperm Mediated by Varying Concentrations of Solubilized Human Zona Pellucida

Concentration (ZP/μL)	Acrosome reacted sperm (%)
Control	10 ± 0.2a
0.3	14.8 ± 0.3b
0.6	19.29 ± 0.4c
1.25	25.1 ± 0.4d
2.5	32.5 ± 0.7e

Note. Fisher's exact tailed test: a vs. b, $p > 0.5$; a vs. c, $p = 0.001$; a vs. d, $p = 0.001$, a vs. e, $p = 0.001$.

loosely attached sperm. HZA results were calculated as the absolute number of tightly bound sperm per hemizona, and results were expressed as hemizonae indices (HZI).

Statistical Analysis

Comparisons between normal sperm morphology, percentage acrosome reacted sperm and sperm–zona binding data were done with Fisher's exact *t* test and the Wilcoxon *t* test. The association between percentage normal spermatozoa and percentage acrosome reacted sperm were reported by using a correlation analysis. The discriminating power of sperm morphology and sperm–zona binding as a screening test for the identification of acrosome reaction responsiveness was illustrated with the Receiver Operating Characteristics (ROC) curve analysis.

RESULTS

The dose–response results recorded with varying dosages of solubilized human ZP and the acrosome reaction data are represented in Table I. Because of the scarcity of human material, a ZP concentration of 0.6 ZP/μL was chosen for acrosome induction studies.

Sperm parameters and results from the functional assays, i.e., acrosome reaction data as well as the hemizona assay data of the fertile control group and 164 andrology patients, are depicted in Table II. A significant difference was recorded between the percentage of morphological normal spermatozoa among the fertile controls compared to that of the andrology patients ($p = 0.0001$, Fisher's exact *t* test). Furthermore, significant differences existed between ZIARs ($p = 0.001$) and sperm–zona binding data ($p = 0.001$) of the fertile and subfertile groups.

The andrology patients were further subdivided into three groups according to the percentage normal morphology present in the semen, i.e., $\leq$4% (P-pattern, $n = 71$), 5–14% (G-pattern, $n = 73$), >14% (normal, $n = 20$) (Table III). Since we did not have fertilization rates to calculate cutoff values for the ZIAR results, the data was analyzed according to the distribution plots for the percentage ZIAR recorded among each morphological group (see Fig. 1). Impairment ZIAR results were identified in cases where the values fell outside the lower 95% confidence interval (95% CI) of the group. For the morphological normal group (>14% normal forms) the lower 95% CI for the ZIAR was 12.7%, for G-patterns (5–14% normal forms) lower 95% CI was 10%, and for the P-patterns (<4% normal forms) the ZIAR was 6%. In the normal, G-pattern, and P-pattern groups, 75% (15 out of 20), 76% (55 out of 73), and 59% (49 out of 71) men, respectively, had ZIAR results above the lower 95% CI of that group. Five cases (25%) among the normal group reported in Table III had impaired ZIAR results, i.e., <12.7% ZIAR. The HZA results of three of the five cases were normal HZA (hemizona index >40%), while two men also had impaired sperm–zona binding (HZI < 40%). The HZI is defined as a ratio between the number of patient sperm bound to the ZP divided by the number of control sperm bound to the zona. All five cases had >14% normal spermatozoa (Table IV).

Table II. Results of Semen Parameters from 164 Andrology Patients to Determine Acrosome Reactions Mediated by Human Zona Pellucida and Sperm–Zona Binding Capacity

	Acrosome reacted sperm (mean $\pm$ SEM)		
	Fertile controls ($n = 11$)	Andrology patients ($n = 164$)	p values Fisher's exact t test
Sperm concentration (10^6/mL)	199.4 ± 7.8	74.1 ± 6.6	
Motile cells (%)	56.5 ± 0.8	52.1 ± 1.5	
Normal cells (%)	15.8 ± 0.5a	5.5 ± 0.1e	a vs. e, $p = 0.0001$
Spontaneous acrosome reaction (%)	10.33 ± 0.55b	12.1f ± 1f	b vs. f, $p = 0.0001$
ZIAR (0.6 ZP/μL) (%)	26.56 ± 1.4c	11.9 ± 0.5g	c vs. g, $p = 0.0001$
Hemizona assay (%)	84.8 ± 2.9d	48.3 ± 1.9h	d vs. h, $p = 0.001$

Table III. Results (Mean ± SEM) of Acrosome Reactions Mediated by Human Zona Pellucida, Sperm–Zona Binding Capacity According to Percentage Normal Spermatozoa

	P-pattern ($n = 71$)	G-pattern ($n = 73$)	Normal ($n = 20$)	p value (unpaired t test)
Sperm concentration (10^6/mL)	42.4 ± 6.6	89.5 ± 6.3	121.7 ± 16.0	
Normal cells (%)	2.2 ± 0.1a	6.9 ± 0.2b	14.5 ± 0.1c	a vs. b, $p \leq 0.0001$; a vs. c, $p \leq 0.0001$; b vs. c, $p < 0.0001$
Motile cells (%)	46.0 ± 1.9	55 ± 1.2	58.5 ± 2.2	p values > 0.05
Spontaneous acrosome (%)	12.2 ± 0.3d	11.9 ± 0.3e	11.9 ± 0.6f	d vs. e, $p \leq 0.003$; d vs. f, $p \leq 0.003$; e vs. f, $p \leq 0.002$
ZIAR (Mean ± SEM)(%)	9.6 ± 0.6g	13.9 ± 0.5h	15.0 ± 1.1i	g vs. h, $p \geq 0.05$; g vs. i, $p \geq 0.05$; h vs. i, $p \geq 0.05$
ZIAR median (range) (%)	9.0 (2 – 23)j	14.0 (4 – 26)k	15.0 (6 – 26)l	j vs. k, $p < 0.0001$; j vs. l, $p \leq 0.001$; k vs. l, $p \geq 0.05$
Hemizona index (%)	38.0 ± 1.6m	54.5 ± 2.2n	62.6 ± 4.2o	m vs. n, $p \leq 0.01$; m vs. o, $p \leq 0.001$; n vs. o, $p \geq 0.05$

Receiver Operator Characteristics

To evaluate the relationship of the ZIAR results and percentage normal spermatozoa the data were analyzed with the ROC curve analyses. ZIAR data were able to discriminate (sensitivity 60% and specificity 82%) between sperm populations with sperm morphology of >4% and <4% normal forms at a cutoff value for percentage ZIAR of 13%. The areas under the curve for ZIAR and HZI were 0.76 (95% CI, 0.67–0.82) and 0.80 (95% CI, 0.72–0.86), respectively (see Fig. 2). This implies that a randomly selected individual from the >4% normal spermatozoa group has a ZIAR value larger than that for a randomly chosen individual from a <4% morphology group in 76% of cases. Likewise, a randomly selected individual from the >4% normal spermatozoa group has an HZI value larger than that for a randomly chosen individual from a <4% morphology group in 80% of cases. The calculated cutoff values for ZIAR and HZI were 13 and 46%, respectively.

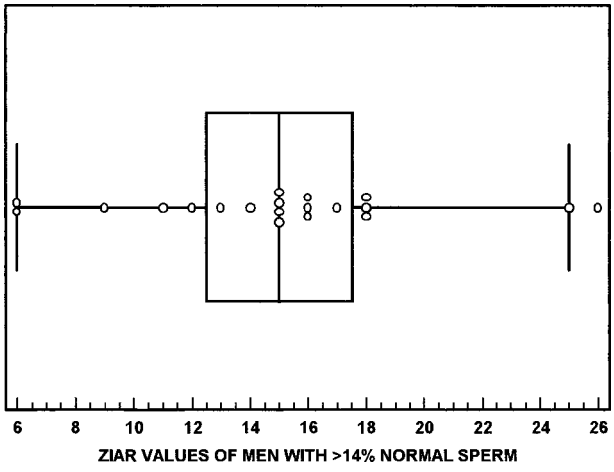

Fig. 1. Distribution of ZIAR data recorded for normozoospermic men.

DISCUSSION

Standard IVF requires good sperm function, particular sperm–zona binding, and penetration that are essential for fertilization. With ICSI, several sperm functions are not required for fertilization, especially those associated with sperm–ZP interaction. Couples with severe spermatozoal defects such as teratozoospermia can usually be identified by routine semen analysis (5), and ICSI is recommended for the first treatment. However, couples with unexplained infertility with normal semen analysis are usually treated with standard IVF. Studies have shown that between 10% (9) and 25% (21) of these couples may have a low ZIAR result and are at risk of zero or very low fertilization rates in standard IVF (9). Although these couples can be treated with ICSI in the second cycle, there is a high cost to the patients both financially and emotionally. Failed attempts can also decrease the confidence of the patient in the therapy and therefore reduce the chance of success during future attempts.

Table IV. Results of Hemizona Assay and Sperm Morphology of Five Cases with Impaired ZIAR

Case	Hemizona index (%)	ZIAR (%)	Morphology (% normal)
1	77	11	16
2	92	6	16
3	63	9	15
4	26	6	14
5	37	12	14

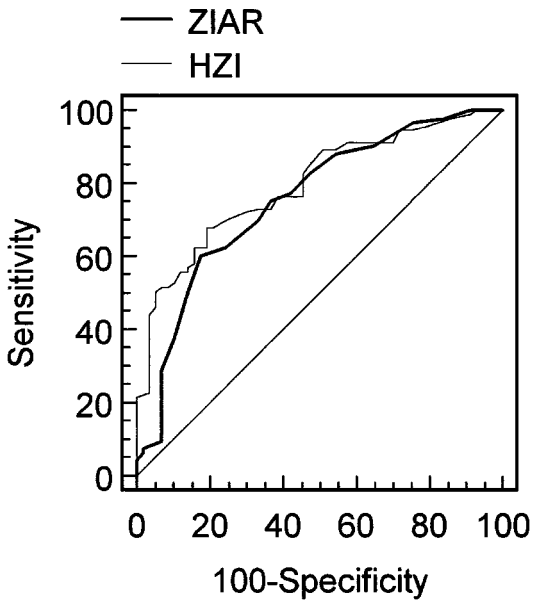

Fig. 2. ROC analyses percentage ZIAR and HZI versus sperm morphology.

Sperm morphology has been recognized as a clinical discriminator of male fertility potential (12). Likewise in close correlation with the percentage of normal spermatozoa sperm–ZP binding was described as an additional clinical important characteristic of spermatozoa (4,7).

Sperm functional information is important since it could assist in therapeutic choices, such IVF or ICSI. The role of impaired ZIAR in a clinical setup is crucial when reported among cases with apparently normal sperm characteristics. Five of the 20 cases (25%) with normozoospermic semen had a ZIAR value of <12% which is an indication of slight impairment of acrosomal response to the zona pellucida. Esterhuizen *et al.* (9) described among IVF couples two groups of patients, i.e., ZIAR < 15% and ZIAR > 15% with mean fertilization rates of 49 and 79%, respectively. Although the present report does not include IVF results, we believe that the reported decrease in the percentage ZIAR, especially among the three men (Cases 1, 2, and 3) with normal semen parameters and HZAs, should be regarded as a clinical warning.

In a selected patients population, ZIAR results can be used to indicate in vitro fertilization failure in >90% of cases and patient could accordingly be referred to an ICSI program (9,22,23). It is known that about 10% of patients repeatedly have zero or low fertilization with standard IVF. Although oocyte immaturity or abnormalities can contribute to fertilization failure, sperm defects are regarded as the most frequent contributors in cases where complete fertilization failure is reported (21,22).

Present results and those reported by others (9,21) underline the importance of a multistep diagnostic approach to identify the specific cause of male factor infertility. When sperm morphology is used as a first-step clinical guideline of male factor infertility, investigations such as sperm–zona binding and acrosomal response to homologous zona pellucida play an important role during the diagnostic procedure (24). We suggest that ZIAR evaluation should not be part of the first level of clinical approach, but instead form part of the second level of the diagnostic scheme that includes testing of the functional capacity of spermatozoa. In conclusion, the implementation of acrosome assays using small volumes of human solubilized zonae pellucidae (22), biologically active recombinant human ZP3 (25), or active, synthetic ZP3 peptides (or analogs) (26) will probably allow for the design of improved, physiologically oriented assays (27).

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