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THE CLINICAL SIGNIFICANCE OF SPERM-ZONA PELLUCIDA BINDING

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1. ABSTRACT

The development of homologous functional bio-assay for sperm quality assessment has been a focal point of reproductive biologists; in order to provide a scientific based diagnosis in cases of fertilization failure. The availability of oocytes still remains an important limiting factor for laboratories to embark on the methodology of the assay. The use of zonae pellucidae, derived from post mortem and different *in vitro* fertilization oocytes, enhanced to availability of zonae. Sperm-zona binding has been illustrated to be an essential requisite during human fertilization and can be measured under hemizona assay as well intact zona pellucida conditions. The sensitivity and specificity of sperm-zona binding results indicated the assay to be positively and significantly correlated with *in vitro* fertilization outcome. Furthermore, a highly significant correlation were illustrated to exist between the normal sperm morphology, hyperactivation, sperm creatine kinase activity and the zona binding capacity of a given sperm sample. It was concluded that andrology testing remains an ever-growing component in the work-up 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 intra cellular sperm injection.

2. INTRODUCTION

Perhaps the most remarkable journey made by any cell, is that of the mammalian spermatozoon as it passes from the vas deferens of the male, to the fertilization site. At this site the sperm will subsequently go across the cumulus oophorus and corona radiata cells and bind to the zona pellucida, from where it will start its second intriguing journey progressing through the zona pellucida to the oolemma. The correct sequence of these events leading to fertilization, are imperative and critical analyses of individual processes (sperm binding, penetration and fusion), forms the cornerstone of modern assisted reproductive technology.

Although the establishment of a sperm-zona binding assay initially received prominency, logistical difficulties were encountered during the developmental stages of such an assay, namely; (i) human sperm does not bind adequately to any other zona pellucida due the species-specificity of the sperm-zona receptors (ii) availability of human oocytes (iii) inter oocyte variability and (iv) ethical problems in using fresh viable human oocytes.

Apart from a number of important steps in the fertilization process, the sperm-zona pellucida binding capacity of a given sperm population has been shown to be a crucial event during mammalian fertilization. The results of sperm-zona binding assays provide important evidence on the recognition event leading to fertilization (1, 2, 3, and 4). During a recent consensus workshop on advanced diagnostic andrology techniques, participants proposed that the laboratory evaluation of sperm quality/quantity should be approached using a sequential multi-step diagnostic analysis (5, 6). Significant factors affecting the validity of such an assay include, oocyte sources and maturation, inter-assay and intra-assay variability, sperm motility, morphology and acrosomal status (7).

3. OOCYTE AVAILABILITY

Apart from the use in homologous bio-assays, zonae pellucidae are often used in studies focusing on the glycoprotein components of the zona pellucida, especially the sperm binding proteins (8, 9). The scarcity of human oocytes

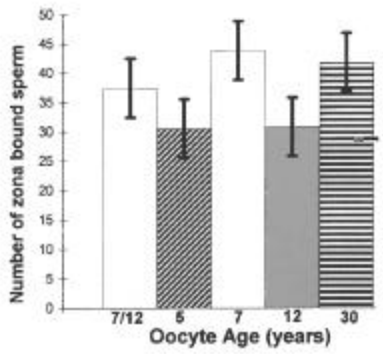

Figure 1. Sperm-zona binding to zonae pellucidae retrieved from human oocytes of different age groups.

stimulated the development of the HZA concept of using identical, matching halves from the same zona pellucida. The availability of oocytes still remains an important limiting factor for laboratories to embark on the methodology of the assay. It was therefore imperative to explore alternative oocyte sources in order to maintain the availability of the assay as a routine laboratory procedure (3, 10, and 11).

During a comparative prospective study on 3639 human prophase oocytes, retrieved from 134 post-mortem derived ovaries (11), optimum sperm-zona binding were recorded using zonae retrieved from oocytes that were aged between 10 to 20 years. Secondly, the sperm binding capacity of zonae pellucidae derived from ovarian tissue of which the ages of the donors ranged between 7 months to 30 years showed no difference in the capacity to bind spermatozoa. The functional integrity of the human zona pellucida, seems therefore to be operational at an early age (figure 1).

We also extensively studied the use zonae pellucidae from different types of oocytes for sperm binding capacity. These included oocytes with varying nuclear maturational stages namely; mature (metaphase II) and immature (prophase I). The mean number (±SD) of sperm bound to mature metaphase II oocytes were 115.0±13 compared to 36.0±12 recorded for immature prophase I oocytes. Results from comparative studies showed zonae pellucidae binding to zonae derived from both mature and immature oocytes were able to discriminate between sperm samples with varying zona-binding potentials. This is particularly true for results obtained under hemizona assay conditions. A second group of oocytes that were used during sperm zona binding studies included zonae from inseminated *in vitro* fertilization oocytes that failed to fertilize. Fertilization failure was judged by the absence of pronuclear formation and extrusion of polar body. After removing bound sperm from this zonae, hemizona assays were conducted to evaluate sperm binding capacity. The mean number of sperm reported bound were 114.7±37. However, during parallel studies, HZA results of fertilized, uncleaved oocytes with no further development potential; showed significant less zona bound sperm (6.0±4), compared to the control (116.0±12), using the same fertile sperm in both cases. Fertilized uncleaved oocytes therefore can not be utilized during zona binding experiments.

The sperm binding capacity of inseminated (non-fertilized) metaphase I and II IVF oocytes were recorded during two successive HZA. We therefore addressed the possibility of using recycled hemizonae. The oocytes were used during two successive HZA's, using the same hemizonae during separate assays. After assessing the number of bound sperm to the hemizonae during the first assay, all bound sperm were removed using a hand drawn glass micropipette, slightly smaller than the size of the hemizona with a diameter of 90 micrometers. This procedure sheared sperm off the hemizonae surface, leaving only 2 or 3 sperm with part of the head or entire head embedded in the zona pellucida (11, 12). Sperm-stripped hemizonae were then re-inseminated during a second HZA with another sperm population. Once again after a second 4 hours co-incubation, the number of hemizona bound sperm were reassessed. No differences could be detected between the results obtained for HZA 1 and 2. The exposure to and initial binding of spermatozoa to the hemizonae does not seem to influence the moieties on the zona of a mature oocyte that is responsible for sperm binding; at least under HZA conditions (table 1).

4. SPERM-ZONA BINDING ASSAYS

Originally, human sperm-oocyte interaction was defined in an assay developed to evaluate zona penetration; the methodology of this assay formed the cornerstone of future sperm-oocyte interaction tests (13). This assay outlined bio-assay conditions and oocyte retrieval procedures, used for current sperm zona binding tests, namely, the hemizona assay (HZA) (14). and a competitive intact zona pellucida-binding test (15). Both bioassays have the advantage of providing a functional homologous test for sperm binding to the zona pellucida, comparing populations of fertile and infertile spermatozoa in the same assay.

4.1. The hemizona assay

We have developed a sperm function assay based on the relative binding of patient versus control spermatozoa to the matching halves of a bisected human oocyte (14, 3, 4). The HZA assessed tight binding of sperm to the outer surface of the zona pellucida hemisphere. Clear advantages of the HZA include: (a) the two halves (hemizonae) are functionally (qualitatively) equal zona surfaces, allowing a controlled comparison of sperm-zona binding, (b) the very limited number of recovered human oocytes is amplified, since an internally controlled test can be carried out on a single oocyte; and (c) ethical objections to possible inadvertent fertilization of a viable oocyte are eliminated by first cutting the egg into halves.

4.2. Competitive intact zona pellucida-binding test

A second sperm-zona test is one using oocytes that failed to fertilize *in vitro* to determine a sperm-zona pellucida binding ratio, between control and test spermatozoa (15). Oocytes that showed no evidence of either pronuclei or cleavage 48 to 60 hours after insemination were placed in 1M ammonium sulphate solution and stored at 4^0C. The test is based on competitive binding of two sperm populations (test patient and fertile control sperm donor) to several oocytes. Test and control

Table 1. Mean (±SD) number of zona bound spermatozoa recorded for human zona pellucida derived from various sources

MATURATIONAL STAGE/SOURCE			STORAGE METHOD	
Post mortem PI	IVF donated MII	Reclycled failed IVF MII	Salt stored PI	DMSO
54.0±12[a]	114.7±28[b]	117.8±37[ce]	54.0±12 [d]	49.1±14 [e]

a & b: Franken *et al*., 1994, c,: Franken *et al*., 1989b; d: Franken *et al.*, 1991a; e: Franken *et al*., 1994;

Table 2. Predictive power of HZA to discriminate between successful and failed for fertilization outcome in the IVF setting

	NORFOLK[A]	TYGERBERG[B]
Positive predictive value	82%	81%
Negative predictive value	73%	68%
Sensitivity	83%	75%
Specificity	95%	68%

a: Oehninger *et al.*, 1992; Oehninger *et al.*, 1989, b: Franken *et al.*, 1993; Franken *et al*., 1996a

sperm are labeled with different fluorochrome suspensions namely, FITC; green; tetramethyl rhodamine isothiocyanate; red). Following labeling, sperm populations i.e. control and tests are simultaneously co-incubated with several oocytes, after which zona binding is assessed by counting zona bound sperm under fluorescent microscopy.

5. MALE FERTILITY MANAGEMENT

With the onset of assisted reproductive technologies, sperm functional evaluation in the nineties has become a mandatory part of the andrological work-up schedule. The astounding success rates achieve by ICSI (16), in cases of profound male factors, now have enhanced the need to fully understand human fertilization and all subsequent related processes, leading the formation of the normal embryo.

The identification of specific gamete dysfunctions remains one of andrology's most formidable task's (5). Fertilization disorders, due to a defective sperm-zona pellucida interaction, are relatively common in clinical practice, thereby underscoring the importance of sperm-zona binding tests as diagnostic/predictive tests. Independent publications from Norfolk (USA), Melbourne (Australia), Tygerberg (South Africa) and Israel of highly comparable results, confirmed that sperm-zona binding tests are good predictors of fertilization (5, 17, 12, 18) (table 2). The use and development of the indirect zona binding test is advised in cases of total IVF failure and all oocytes should be examined for the presence of spermatozoa bound to be the zona pellucida. While this not as reliable as a direct sperm-zona binding test, being a qualitative rather than a quantitative evaluation, it may be the only source of such information if the direct method is not available. (1).

Contemporary andrology laboratories should be able select the most appropriate form of treatment for each couple, especially those male factor infertility (19). It remains imperative to understand the role and diagnostic power of a sequential analytical approach to properly interpret the existing laboratory methods for evaluation of the male gamete fertilizing ability. For a better understanding of specific defects involved in fertilization failure, the HZA can be extended testing the sperm via sperm-zona pellucida penetration assay (20) and a sperm-oolemma fusion test using either hamster or human oocytes (21).

It is now well established that ICSI allows for successful fertilization and achievement of live births in cases with previously failed fertilization and/or unsuitable sperm parameters for conventional or modified IVF. Because of its success, ICSI is now being performed also in conjunction with urologic interventions including sperm aspiration from the epididymis, sperm retrieved from testicular biopsies and electroejaculation.

5.1. Semen parameters and zona binding

It is interesting to note that sperm parameters in general, are not predictive of fertilization or pregnancy outcome following ICSI. Female factors (age and basal serum levels of FSH, LH and estradiol) are highly predictive of ICSI results, but none of the original or processed sperm parameters showed significant effect (18). This is clearly different from the IVF situation where, normal sperm morphology (as recorded by strict criteria) and sperm-zona pellucida binding capacity (as diagnosed by the HZA or other tests) are highly predictive of fertilization outcome (19, 22). The lack of correlation of any sperm parameter studied so far and ICSI results demonstrates that ICSI is an efficient "bypass" of abnormal sperm-zona pellucida and sperm-oolemma interactions.

The prediction of the capacity of human sperm to achieve fertilization under *in vitro* conditions has been a major objective within the assisted reproductive technology setting. However, because fertilization entails a complex series of events leading to early embryo development, it is unlikely that the evaluation of a single sperm characteristic or function can be predictive in an absolute manner of the fertilization potential of the male gamete. The Tygerberg and Norfolk laboratories, jointly evaluated (i) the relationship between different sperm characteristics (from original semen samples as well from retrieved motile fractions) and sperm-zona binding potential; and (ii) the role of the HZA as a diagnostic tool for predicting fertilization *in vitro*. We prospectively studied a large number of infertile patients before IVF therapy. The main outcome measured included

computerized sperm motion analysis, sperm morphology and HZA data correlated with fertilization rates achieved of the patients under investigation. Multiple regression analyses have demonstrated that sperm morphology is the most significant predictor of sperm-zona binding in the HZA, when compared to other sperm variables from the original semen sample ($r=0.83$, $p=0.0001$). On the other hand, curvilinear velocity (VCL) and hyperactivated motility (HA) were the most significant predictors of successful zona binding, after separation of the motile sperm fraction ($r=0.47$ and $r=0.46$, respectively $p=0.001$) (23).

In contrast to many animal species, human semen is virtually unique because of its heterogeneity in sperm morphology (pleomorphism); both fertile and infertile men produce high numbers of morphologically abnormal spermatozoa (24). This phenomenon is of paramount clinical importance as sperm morphology is regarded as a significant prognostic factor for fertilization and pregnancy outcome in the IVF/GIFT/IUI settings (24, 25, 26). The percentage of normal spermatozoa bound to the zona pellucida under HZA conditions reported for both normo-and teratozoospermic men showed significant improvement when compared to the percentage of normal spermatozoa found after the swim-up procedure. The mean (±SEM) percentages of normal forms for normozoospermic men in the fresh semen specimen, after swim-up and after zona binding, were 21.5±1.6, 27.5±2.9 and 44.8±3.4, respectively. A significantly higher percentage of normal forms were found among zona-bound sperm, compared to swim-up forms ($p=0.02$) and seminal spermatozoa ($p=0.02$). Significant differences existed between the percentage of normal forms found in the swim-up and zona-bound spermatozoa ($p<0.01$ and $p<0.0003$, respectively), compared to the original ejaculates (27). Abnormal spermatozoa are therefore regarded as dysfunctional and unable to bind to the zona pellucida.

Sperm morphology remains, not only one of the best predictors of the ability of sperm to bind to the zona pellucida, but also it correlates well with fertilization outcome under IVF conditions. This might due to the presence of a specific membrane/receptor deficiency among teratozoospermic men. When we removed the HZA results from a logistic regression analysis in order to identify the predictive value of other sperm parameters (sperm concentration, morphology and motion characteristics), the percentage progressive motile cells was the best predictor of fertilization outcome (19, 28). Logistic regression analysis provided a robust HZI range predictive of the oocyte's potential to be fertilized. This cut-off value was determined to be 35%. The discriminating power of the hemizona assay is constant for different sperm samples from the same patient in different (consecutive) IVF cycles (5).

Furthermore, a highly significant inverse correlation exists between sperm creatine kinase activities and sperm concentrations among fertile and infertile men. In the sperm fraction selected by swim-up, the creatine kinase activities were lower that the initial semen specimen. This indicates biochemical subpopulations of sperm within the same ejaculate. A preferential zona binding by biochemical mature spermatozoa as detected by creatine kinase activity and zona binding was later reported by the same group (29,30).

6. SPERM-ZONA BINDING AS A DIAGNOSTIC TOOL

The fact that sperm-zona binding follows a dose-response curve can be employed in a clinical setting as a diagnostic tool to establish the sperm concentration, which will ensure optimal zona binding under IVF conditions. Sperm binding kinetic studies indicated that 250 000 motile spermatozoa/ml was the minimum number of sperm required to sustain a valid hemizona assay (31). The number of sperm bound to the zona decreased significantly with sperm concentrations <250 000 sperm/ml. At a standard sperm insemination concentration of 500 000 sperm/ml, fertile controls had a significantly higher number of tightly bound sperm when compared with teratozoospermic patients (133.5±6 versus 32.1±2, $p<0.00001$). By increasing the number of spermatozoa/ml, teratozoospermic men revealed individualized sperm concentrations necessary to achieve zona binding parity, compared to the number of bound sperm representing the lower 95% confidence interval for the control sample with the matching hemizona. The sperm concentrations needed to reach parity among the patient population varies from 500 000 sperm/ml to $>4x10^6$ sperm/ml (22). These results emphasized functional individuality of sperm samples as defined by the zona pellucida binding capacity.

Quality control is a crucial aspect of sperm-zona binding testing and it is important that each laboratory establishes an adequate pool of fertile men to be used reliably as internal controls. If the sperm zona binding capacity of the fresh samples is known, comparative studies can be performed after cryopreservation/thawing. Some groups use pooled cryopreserved sperm from fertile controls (6, 17). We have compared various sperm features of fresh versus cryopreserved/thawed sperm donors. Parameters assessed included motion characteristics, ATP levels, intra-cellular changes of calcium and sperm zona binding. Results showed that in this fertile population the cryopreservation stress does not alter sperm zona binding capacity in a clinical significant fashion (32).

7. THE ROLE OF THE ZONA PELLUCIDA AND OOPLASMA

The basic underlying gamete defects responsible for failed fertilization is poorly understood in the majority of cases. The development of zona binding technology enhanced the awareness of sperm dysfunction so far as zona binding is concerned. On the other hand it is accepted that oocyte abnormalities may also be responsible for fertilization failure, excluding "descriptive" oocyte morphology abnormalities. The role of the zona pellucida, the primary structure responsible for gamete recognition, has been evaluated in the context of the IVF setting using oocytes from failed fertilization attempts (33, 34). These immunocytochemical studies showed a statistical significant incidence of zona pellucida abnormalities in patients with both morphologically normal and abnormal oocytes and in the absence of sperm defects (18, 35).

Antisera against synthetic ZP3 peptides were used to identify ZP3 proteins in various mammalian oocytes. An antiserum, called ZP3-6, generated against a conserved epitope, reacted strongly with human ZP3 protein. Using ZP3-6 as a probe, human metaphase II oocytes studied under a variety of conditions (i.e., fresh in culture medium, refrigerated at 4^0C or salt-stored), revealed a strong and similar reactivity with the antiserum (33). The hypothesis that zona pellucida abnormalities could be present in unfertilized oocytes following IVF treatment was investigated. Immunocytochemical studies showed a significant incidence of zona pellucida defects (of the protein backbone) in different cohorts of oocytes, demonstrating that the antiserum can be used as a biomarker of oocyte integrity/function.

In the sequential pathways of fertilization, sperm-zona binding, penetration and subsequent formation of an early embryo, is accompanied by specific post fertilization changes in the composition of the human zona pellucida. Successful ICSI is dependent upon the competence of the oocyte to respond to the injection of a spermatozoon in the presence of calcium. The human zona pellucida protein 2 (hu ZP2) is known to undergo a detectable shift in its biochemical composition, which is related to the cytoplasmic activation. One likely explanation for this discrepancy is specific oocytes that failed to fertilize have completed nuclear in the absence of subsequent cytoplasmic maturation.

8. PERSPECTIVE

The dramatic increase in the knowledge of human gamete biology and reproductive medicine has been the consequence of extensive basic scientific research and the expanding use of assisted reproductive technologies. The successful implementation of intra cellular sperm injection (ICSI) has provided innovative treatment modality to couples suffering from severe male infertility to achieve their reproductive goals. However, despite the great therapeutical advantages, ICSI provides solutions to clinicians often in the absence of an etiological or pathophysiologic diagnosis. Questions that obviously arise are (i) what diagnostic steps should be used to direct infertile men to a specific therapeutical modality? (ii) what are the current indications for ICSI?

The semen analysis still remains the cornerstone of the diagnostic management. We have been promoters of a sequential, multistep diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics (2). It is our opinion that this diagnostic scheme should include (i) a first level of investigation namely, assessment of the semen parameters and (ii) a second diagnostic tier i.e. functional testing of spermatozoa.

The semen analysis performed by the infertility specialist should include the assessment of physical semen characteristics (volume, pH agglutination, viscosity), evaluation of sperm concentration, progressive motility, normal morphology (strict criteria) (24) and viability, presence of leukospermia and immature sperm cells, detection of antisperm antibodies and a bacteriologic investigation (18). If abnormalities are found during the basic investigation, the work-up should progress to the examination of specific sperm functions.

Today the second level of male work up schedule includes four categories of tests have been proposed: (i) computer-assisted evaluation of sperm motion characteristics (CASA), (ii) inducibility of the acrosome reaction, and bio-assays that sequentially assess gamete interaction including (iii) sperm-zona pellucida binding tests and (iv) sperm-hamster egg penetration assay (36,18, 1). Different laboratories have highlighted the diagnostic power of these tests and the World Health Organization (WHO) has incorporated them under the category of functional test (36). However, as discussed at a recent Consensus Workshop in Advanced Andrology (ESHRE Special Interest Group) it was agreed that better standardization of CASA methods and acrosome reaction techniques should be implemented prior to its introduction as a routine clinical tool (1). Importantly, among the bio-assays of sperm-egg interaction, it was concluded that because of the powerful evidence for prediction of both fertilization and its failure in the IVF setting, sperm-zona binding tests should be favored among the functional assays. Notwithstanding some practical limitations of these assays, we have incorporated the hemizona assay (HZA) as part of our routine advanced diagnostic scheme.

ICSI is indicated today after the failure of other therapeutical approaches or as the initial treatment of choice depending upon the degree of sperm dysfunction and numbers. Patients are selected for the ICSI programme following these indications; (i) poor semen parameters - predictive of fertilization failure- (i.e. <1 million total spermatozoa with adequate progressive motility after separation and/or poor zona pellucida binding capacity - hemizona index <30% (1,6,7), (ii) previous failed fertilization and (iii) presence of obstructive or non-obstructive azoospermia where ICSI is combined with sperm extraction from the testes or epididymis (9,10). In these cases, genetic counseling is recommenced to the couples.

Only the identification of specific sperm defects will allow the development of directed therapies. Andrology testing remains, in our opinion as well as those of others (15), an ever-growing component in the work-up of the infertile couple. We enter the next millennium with many questions that remain to be answered by the hand of efficacious (but definitely improvable) screening techniques and a new formidable therapy in ICSI.

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