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The clinical significance of sperm-zona pellucida binding: 17 years later

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1. ABSTRACT

The development of homologous functional bio-assays for sperm quality assessment has been a focal point of reproductive biologists in order to provide a scientifically- based diagnosis in cases of fertilization failure. The availability of viable oocytes still remains an important limiting factor for laboratories to embark on the methodology of assays that examine sperm-oocyte interaction. The use of zonae pellucidae obtained from oocytes derived from post mortem tissue and failed *in vitro* fertilization cycles, enhanced the availability of zona material. Sperm-zona pellucida binding has been illustrated to be an essential requisite during human fertilization. This fundamental biological step can be measured under hemizona assay as well intact-oocyte test 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, highly significant correlations were demonstrated between normal sperm morphology, hyperactivated motility, 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 intracytoplasmicc sperm injection.

2. INTRODUCTION

Infertility affects about 15% of couples of reproductive age and has a major impact on public health; its treatment still remains a burden on health systems. Since the birth of the first ICSI baby in 1992 (1) the clinical importance of semen analysis and sperm preparation techniques has become under scrutiny (2). The question has often been asked “is sperm quality still important for the outcome of ART?” Eventually it was thought that all infertile couples could be helped with intracytoplasmic sperm injection (ICSI), since sperm concentration, motility, morphology and sperm antibodies did not seem to play a role with the achievement of fertilization using microfertilization (3). It was believed that only a few spermatozoa are necessary to obtain fertilization, and that good embryo development would therefore lead to a healthy pregnancy.

This approach has been abandoned, since today we know that in the clinical management of infertility, allocation of patients between standard *in vitro* fertilization and ICSI is mainly decided on the basis of assessment of sperm characteristics (4). Although ICSI could be used for all classes of male infertility and irrespective of sperm quality, the cost of the procedures involved, compels most clinics to reserve ICSI treatment for patients with moderate to severe sperm anomalies (5). Furthermore, the

indiscriminate use of ICSI appears to deviate from the principles of evidence-based medicine (6). On the other hand, we know now that patients with subtle sperm defects that often influence sperm-oocyte interaction are more efficiently treated with ICSI (4, 7). Reliable sperm functional tests that assist in the diagnosis of infertility and that can be used to reasonably select treatment are valuable tools in an infertility programme.

In 1976, Overstreet *et al.*, first described sperm-zona pellucida binding and penetration using nonviable human oocytes (8). During that study it was suggested that testing the ability of sperm to interact with the zona pellucida might provide information on specific sperm defects responsible for the couple's infertility problem. Due to the scarcity of human oocytes, the work received little attention until an additional source became available with the onset of IVF programmes (9).

3. SPERM-ZONA PELLUCIDA BINDING ASSAYS

In the late 80's two bioassays were developed in parallel using oocytes retrieved either from failed *in vitro* fertilization cycles or post mortem ovarian tissue (10, 11, 12). Both bioassays, i.e., the hemizona assay (HZA) and a sperm-zona binding ratio test (sperm-ZP binding ratio test) 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. In the last years Liu and Baker repeatedly proposed the assessment of the sperm-ZP binding using 4 intact zonae for each sample, considering < 40 bound spermatozoa as a low binding. The internal control offered by the microbisection of a single oocyte in the HZA represents an advantage above the sperm-ZP binding ratio assay by decreasing the number of oocytes needed during the assay and diminishing the intra-assay variation (13, 14, 15). Since then several additional tests have been developed for assessing various aspects of sperm-oocyte interaction, including zona induced acrosome reaction assays (ZIAR) (7, 16, 17). sperm zona pellucida penetration tests (18, 19) and a sperm-oolemma binding assay (16).

3.1. The hemizona assay (HZA)

In the HZA, two matched zona hemispheres created by microbisection of the human oocyte provide three main advantages: 1) the two halves (hemizonae) are functionally equal surfaces allowing controlled comparison of binding and reproducible measurements of sperm binding from a single egg, 2) the limited number of available human oocytes is amplified because an internally controlled test can be performed on a single oocyte, and 3) because the oocyte is split microsurgically, even fresh oocytes cannot lead to inadvertent fertilization and pre-embryo formation (20, 21).

The HZA has been validated by a clear-cut definition of the factors effecting data interpretation, i.e., kinetics of binding, egg variability and maturation status, intra-assay variation and influence of sperm concentration morphology, motility and acrosome reaction status (13, 22, 23, 24, 25, 26). Because of the definition of the assay's limitations and its small intra-assay variation (less than 10%) the power of discrimination of the HZA has been maximized. Hemizona assay results are expressed as hemizona index (HZI), a value that is calculated by the number of sperm tightly bound to the hemizona for the patient/control x 100.

3.2. Sperm-zona pellucida binding ratio test

A second sperm-zona binding assay uses oocytes that failed to fertilize *in vitro* to determine a sperm-zona pellucida binding ratio, between control and test spermatozoa (27). Oocytes that showed no evidence of either pronuclei or cleavage 48 to 60 hours after insemination were placed in a concentrated salt solution (1M ammonium sulphate) and stored at 4°C. The test is based on competitive binding of two sperm populations (test patient and fertile control sperm donor) to a single oocyte. Several oocytes have to be used because of the high inter-egg variation and in fact a high intra-assay coefficient of variation has been reported. Test and control sperm are labeled with different fluorochrome suspensions namely, fluorescein isothiocyanate (green fluorescence) and tetramethylrhodamine isothiocyanate (red fluorescence). A mixture of equal numbers of motile test and control sperm is incubated for 2 hours with at least four oocytes. After incubation, oocytes are evaluated for numbers of tightly bound sperm. The ratio between test and control sperm is calculated. Liu and Baker have proposed the assessment of the sperm-ZP binding using 4 intact zonae for each sample, considering < 40 bound spermatozoa as a low binding (28, 29, 30).

3.3. Oocyte sources

Different sources of human oocytes can be used for sperm-zona binding assays. For example, oocytes recovered from surgically removed ovaries or post-mortem ovarian tissue, and surplus oocytes from IVF treatment (following patients' consent in approved protocols). Since fresh oocytes are not always available for the test, different storage alternatives have been described. One of the more popular storage methods is a short-term storage technique (7-days) for human oocytes. A highly concentrated salt solution provided an effective storage method ensuring maintenance of the sperm-binding characteristics of the zona pellucida (26, 31). In cases where multiple oocytes are available, a long-term storage method is advised. In the latter case a dimethylsulfoxide (DMSO) solution at ultra-low temperatures in liquid nitrogen is recommended (32). The long-term storage method can be used to store oocytes up to 12 months without influencing the sperm binding capacity.

During the development stages of the HZA, we have evaluated the sperm binding ability of oocytes retrieved from different sources namely, fresh, long term DMSO stored and short term salt-stored human oocyte. We concluded that sperm binding ability of the zona is maintained under all these storage conditions (9, 26). In most of our studies we have used salt-stored oocytes (short term storage) and recorded the kinetics of sperm binding to the zona, showing maximum binding at 4 hour of gamete co incubation. Similar binding curves were described for

semen samples obtained from both fertile and infertile men (24, 26).

We also extensively studied the use of 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 were able to discriminate between sperm samples with varying zona-binding potentials. This is particularly true for results obtained under HZA 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 these hemizonae, the hemizonae were used in assays to evaluate sperm binding capacity; the mean number of sperm bound to these hemizonae was 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 controls (116.0±12), using the same fertile sperm in both cases. Fertilized, uncleaved oocytes therefore cannot be utilized during zona binding experiments (9, 10).

The sperm binding capacity of inseminated (non-fertilized) metaphase I and II IVF oocytes were recorded during two successive HZA experiments. We therefore addressed the possibility of using recycled hemizonae. The oocytes were used during two successive HZAs, 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, 33). 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 was 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 do not seem to influence the moieties on the zona of a mature oocyte that is responsible for sperm binding; at least under HZA conditions.

4, CLINICAL RELEVANCE

In prospective and blinded studies, Oehninger and co-workers investigated the relationship between sperm binding and hemizona assays results and IVF outcome (15, 33, 34, 35, 36). Results highlighted the prognostic value of the HZA and concluded that sperm-zona pellucida binding can successfully distinguish the population of male-factor patients at risk for failed or poor fertilization. Using either a cut-off value of fertilization rate of 65% (mean minus 2 standard deviations of the overall fertilization rate in the Norfolk Program for non-male-factor patients), or distinguishing between failed vs. successful fertilization (0% vs. 1-100%), the hemizona assay results expressed as HZI provide a valuable means to separate these categories of patients (17, 34, 36).

A concern on the actual clinical relevance of the sperm-ZP binding tests is the high correlation of its results with sperm morphology (17). Relevant information would be the predictive value of the test in non-teratozoospermic patients. The clinical value of the HZA is the very high negative predictive value for IVF outcome (17), whereas the positive predictive value is not so high, as sperm dysfunction may occur in the steps following the ZP-binding. Accordingly our results and especially those of Liu and Baker (37), ZIAR (17), or ZPIAR (37) , performed in cases with positive sperm-ZP binding, could reduce false positive (good) results.

A meta-analysis examined the validity of the currently available sperm functional bio-assays based upon the results obtained from 2,906 subjects evaluated in 34 published and prospectively designed, controlled studies. That study aimed to examine through evidence-based objective approach the predictive value of four categories of sperm functional assays (computer-aided sperm motion analysis or CASA, induced-acrosome reaction testing, sperm penetration assay or SPA, and sperm-zona pellucida binding assays) for *in vitro* fertilization (IVF) outcome (15). Results of this meta-analysis demonstrated a high predictive power of the sperm-zona pellucida binding and the induced-acrosome reaction assays for fertilization outcome under *in vitro* conditions (15). On the other hand, the findings of that study indicated a poor clinical value of the SPA as predictor of fertilization and a real need for standardization and further investigation of the potential clinical utility of CASA systems. On the other hand, at least in 2 prospective studies on the occurrence of natural pregnancies a predictive power significantly higher than that of conventional semen parameters has been demonstrated for enhanced versions of SPA with A23187 (38) and with TEST-yolk buffer (39). Accordingly with the ESHRE Andrology Special Interest Group (40), the enhanced versions of the SPA are more promising that the conventional version of the test.

Although this study provided objective evidence in which clinical management and future research may be directed, the analysis also pointed out to limitations of the current tests and the need for standardization of present methodologies and development of novel technologies. It is important to note that there are no studies addressing the validity and predictive power of these assays for natural conception. Furthermore, in a study during which the statistical weights of several semen parameters (sperm concentration, morphology and motion characteristics), were also calculated, the HZA results proved to have the highest predictive power with progressive motility as the second best predictor of fertilization outcome (34). It would appear that although important in achieving binding, motility may be more important for cumulus penetration and zona pellucida penetration, factors not directly evaluated in the HZA. Logistic regression analysis

provided a robust HZI range predictive of the oocytes potential to be fertilized. This HZI cut-off value is approximately 35%. Overall, for failed vs. successful and poor vs. good fertilization rate, the correct predictive ability (discriminative power) of the HZA was 80 and 85%, respectively. The information gained may be extremely valuable for counseling patients in the IVF setting (i.e., considering a HZI below 35% the chances of poor fertilization are 90-100%, whereas for the HZI over 35%, the chances of good fertilization are 80-85%) (10, 20, 33, 34, 35).

In general, the HZA results from the Tygerberg and Norfolk assisted reproductive programmes have illustrated an excellent sensitivity and specificity with a low incidence of false positive results. For an HZI of 35%, the positive predictive value of the HZA is 79% and its negative predictive value is 100% (considering good vs. poor fertilization rates). In the HZA, false positive results can be expected, since other functional steps follow the tight binding of sperm to the zona pellucida and are essential for fertilization and pre-embryo development (20, 35) (23, 36).

5. NEW DEVELOPMENTS IN SPERM FUNCTIONAL TESTING

We have advocated a sequential, multistep diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics. 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 such a diagnostic scheme should include (i) assessment of the "basic" semen analysis and (ii) functional testing of spermatozoa (14, 36).

Following the sperm-zona binding studies the role of the zona pellucida as physiological inducer of the acrosome reaction was addressed by different diagnostic laboratories (7, 16, 17). The development of a micro-assay for the assessment of acrosomal status of a sperm population circumvented the oocyte scarcity problem (4, 7, 16, 41). The micro acrosome assay facilitates the use of a single oocyte to serve as inducer of the acrosome reaction. By using micro-volumes of solubilized human zonae pellucidae, we examined 35 couples attending an *in vitro* fertilization (IVF) programme (7). Sperm morphology of the husbands was classified as g-pattern (5-14% normal forms) and/or normal patterns (>14% normal forms). All the couples had a history of repeated poor or failed *in vitro* fertilization rates from previous IVF attempts.

The zona induced acrosome reaction (ZIAR) test was performed using homologous 0.25 zona pellucida/ml incubated with sperm to induce the AR. Acrosome reactions were measured with FITC-PSA staining, and expressed as the difference between zona induced and spontaneous AR spermatozoa. Results indicated that microvolumes of solubilized human zona pellucida could successfully be used to determine the acrosomal status of spermatozoa. The results were compared with *in vitro* fertilization rates of metaphase II oocytes and analysed with Receiver Operating characteristics curve (ROC). ROC analyses divided the patients into 2 groups i.e. ZIAR<15% and ZIAR>15%. The sensitivity and specificity for ZIAR results versus fertilization were 93% and 100%, respectively. The correlation coefficient between ZIAR and *in vitro* fertilization was r= 0.94 ($p<0.0001$). ZIAR data can be used as an indictor for fertilization failure, thus assisting clinicians to refine the therapeutic approach of infertile couples prior to the onset of the treatment. A similar method has been introduced by Liu and Baker and denominated as ZPIAR (18,30).

6. PERSPECTIVES

Specific sperm function/biochemical tests may be of highest value in order to direct a couple to ART. Assisted reproduction can be indicated as a result of several factors that play a role in the fertility prognosis of a couple, namely (a) failure of urological/medical treatments, (b) the diagnosis of "unexplained" infertility in the couple; (c) the presence of "basic" sperm abnormalities of moderate-high degree; or (d) abnormalities of sperm function as diagnosed by predictive bio-assays (such as the HZA or ZIAR).

The HZA and the ZIAR are validated and useful functional tests to predict the outcome of fertilization under *in vitro* conditions. Consequently, the HZA and sperm-zona pellucida binding ratio test, and the ZIAR and ZPIAR tests should be added to the diagnostic work-up schedule in the clinical arena to assess male fertility potential. The use of these tests in a sequential manner will enhance sensitivity and specificity. Used as part of a sequential diagnostic scheme, these bioassays offer the clinician useful means to direct therapy in clinical assisted reproduction. Due to the high expertise needed for performing these tests and the low availability of human oocytes, it is difficult to envision their use on a large scale, apart from laboratories part of an IVF setting. It is of paramount significance therefore that continued studies are carried out to develop simpler methodologies. It is also important to emphasize that more prospectively designed studies are needed to establish the value of these tests to predict pregnancy outcome in the IUI and natural reproduction settings. In preliminary studies, we have examined the predictive value of the HZA for pregnancy outcome in couples with unexplained or male factor infertility undergoing controlled ovarian hyperstimulation and IUI therapy (42). The HZA demonstrated high sensitivity and positive predictive power for conception.

The question is often asked whether sperm quality still plays a role in the outcome of ART. In light of the present evidence it is clear that the evaluation and adequate processing human semen samples has an important role in the male fertility workup (6, 33, 36). Analyzing the quality of a sperm sample will define the type of ART proposed to the patient. Clinically relevant cut off values have been validated especially for normal morphology, sperm zona binding and zona induced acrosome reactions. These cut off values can be used to identify those samples to be used in a first line infertility treatment regime such as IUI (intrauterine insemination)

before introducing the more costly and more intense IVF or ICSI procedures.

Furthermore, the study of the individual spermatozoon injected during ICSI procedure should become a priority research effort. This is especially important since we are still not certain that ICSI therapy is without any risk factor. For example, different morphological-ICSI studies revealed a relationship between morphology of the injected spermatozoon and its genetic constitution.

Biochemical markers such acrosin, aniline blue reactive oxygen species, creatine kinase, hypo-osmotic swelling test and others can also be used in clinical practice to further evaluate sperm fertilizing potential. Moreover, sperm nuclear DNA stability and chromatin packaging and apoptotic events are equally important clinical issues that need to be address to fully understand the fertilizing capacity of the ICSI injected spermatozoon and to address the important question of paternal contributions to embryogenesis.

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