Sperm Binding to the Zona Pellucida, Hyaluronic Acid Binding Assay, and PICSI

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Introduction

Current it is estimated that male contributions area causative factors in as many as half of infertile couples. Unequivocal evidence from the IVF setting has shown that sperm quality influences fertilization and cleavage rates, embryo morphology, blastocyst formation, and implantation rates. Currently, the routine semen analysis remains the most common way to evaluate for male factor infertility. Such evaluation typically includes: seminal volume and other semen physical-chemical characteristics, sperm concentration, progressive motility, and strict morphology. However, up to 15 % of patients with male factor infertility have a "normal" semen analysis and a definitive diagnosis of male infertility cannot be made purely based on the results of a routine semen analysis [1]. Furthermore, it was demonstrated that the basic semen parameters of the unprocessed ejaculate or even after separation of the fraction with highest motility had no impact on the outcome of ICSI [2, 3].

Over the last decade a whole body of circumstantial evidence has linked nuclear/DNA damage in human spermatozoa with adverse reproductive outcomes during *IVF* augmentation with *ICSI*. Sperm nuclear factors that may have implications on reproductive outcome include chromatin anomalies, different forms of DNA damage including strand breaks (evidence have been presented that spermatozoa with damaged DNA are more prevalent in infertile versus fertile men), numerical and structural chromosomal abnormalities, Y chromosome micro-deletions, and alterations in the epigenetic regulation of the paternal genome as reviewed in [1, 4]. Currently, there has been no consensus reached as to which test better identifies ejaculated sperm of poor quality. Although nuclear damage in sperm is poorly characterized, it is believed to involve multiple potential pathophysiological mechanisms including: (1) chromatin abnormalities associated with alteration of protamine/histone ratios, (2) hypomethylation of certain genes and DNA, (3) oxidative base damage, (4) endonuclease-mediated cleavage, and (5) the formation of adducts as a results of xenobiotics and the products of lipid peroxidation [4].

The number of de novo structural chromosome aberrations of male descent appears to be increased among children born after ICSI. Although the exact etiology of structural chromosomal aberrations is unknown, miss-repair of double-strand DNA breaks appears to be a prerequisite. Structural chromosomal aberrations such as dysenteric chromosomes, reciprocal translocations, and eccentric fragments represent failure of the oocyte's repair mechanisms that may be overwhelmed by the

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degree of nuclear/DNA damage carried by the fertilizing spermatozoon [1, 4].

The risk of transmitting a genetic or epigenetic lesion to the offspring as a consequence of a combination of such factors is significant. It is believed that nuclear/DNA damage in the male germ-line can be associated with defective preimplantation embryonic development, high rates of miscarriage, and increased rates of morbidity in the offspring, including childhood cancer [4]. The chance of generating a visibly abnormal phenotype is low, but this does not mean that the lesions are not there and will not emerge in future generations. In light of such considerations; it would seem rational (1) to determine the causes of nuclear/DNA damage in the male germ line, (2) to develop efficient systemic preventive and/or therapeutic measures, and (3) to use sperm isolation techniques that will select for gametes possessing minimal-to-none levels of nuclear/DNA damage in assisted conception.

The ICSI technique bypasses multiple steps of the natural fertilization process by introducing a selected and apparently intact spermatozoon into the ooplasm. The utilization of ICSI has become the most common oocyte fertilization method (as compared with standard IVF insemination), being performed in 64 % of IVF cases in the USA, and with an increased worldwide application including Europe that reported 63 % ICSI usage [5, 6]. This strongly suggests that ICSI is being performed for other indications in addition to male factors, for which there is questionable support for their use based on available evidence. It has been reported that the increase in the proportion of ICSI cycles observed in the last decade seems primarily due to an increased use in couples classified as having mixed causes of infertility, unexplained infertility, and advanced age. As a more rare indication, the use of ICSI may represent the solution for oocyte pathology in cases of zona pellucida anomalies, deficiency of the oolemma fusion ability or absence of cortical reaction [1]. These facts stress even further the need for a prioritized examination of sperm-selection techniques for ICSI, and performing long-term follow-up studies on the children born [7].

Additionally, information gathered from in vitro fertilization (IVF) and embryo transfer data has demonstrated that an abnormal sperm–zona pellucida interaction is frequently observed in infertile men. Impaired sperm–zona pellucida interaction can result in failure of fertilization and can be observed in the presence of normal or abnormal "basic" sperm parameters, resulting in decreasing chances of pregnancy when couples are being subjected to intrauterine insemination (IUI) or IVF therapies when conventional in vitro insemination is performed. Sperm–zona pellucida binding assays were consequently developed to assess sperm functionality and competence in the "extended" evaluation of the infertility work-up [8–10]. The latest World Health Organization (WHO) manual depicts sperm–zona pellucida binding assays as research tests [11]. Furthermore, these bio-assays provide valuable information to the clinician in order to direct clinical management to low complexity alternatives such as IUI or to proceed directly to IVF augmented with assisted microfertilization applying ICSI [1, 10, 12, 13].

Sperm morphology grading is more universally based on WHO or Kruger on stained slides to determine the percentage of 'normal" sperm present. This criterion is also applied by the embryologist when selecting the "best sperm" to inject during ICSI. Semen preparation for IUI and for /IVF/ICSI can be anywhere from a simple "wash" or a more complex series of gradient layers—followed or not by a "swim-up" procedure. Zona-binding assays can be prospectively performed which will provide a binding score for each sample aiding the clinical decision of IUI, IVF, or ICSI [1, 10, 12, 13]. Others have introduced the hyaluronic acid binding assay (HBA) in a commercially available hyaluronic acid-coated plate (PICSI dish) [14, 15]. The objective of this chapter is to critically describe the application of the hemizona assay (HZA), a well characterized sperm–zona pellucida binding assay, and the HBA-PICSI assays, as noninvasive sperm tests used in the ART clinical scenario.

The Hemizona Assay

The HZA has been extensively validated as a diagnostic test for the binding of human spermatozoa to human zona pellucida to predict fertilization

potential [8, 10]. In the HZA, each of the two matching zona hemispheres created by microbisection of a human oocyte provide three main advantages: (1) the two halves (hemizonae) have 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. A highly specific type of binding is essential for fertilization to proceed and therefore, the HZA provides a unique homologous (human) bioassay to assess sperm functionality at the fertilization level [8, 10].

Oocytes recovered from surgically removed ovaries or post-mortem ovarian tissue, and/or surplus oocytes from an IVF program, can be used for this assay. This need for scarce human material makes the test less available and more cumbersome. Since fresh oocytes are not always available to perform this assay, different alternatives for preservation have been implemented. The storage methods of human oocytes include using ultra low temperatures with dimethylsulfoxide (DMSO) as cryoprotectant [16]. Additionally, Yanagimachi et al., showed that high concentrations of salt solutions provided effective storage of hamster and human oocytes and the sperm-binding characteristics of the zona pellucida were preserved [17]. During the developing of the HZA, the binding ability of fresh versus DMSO and salt-stored (under controlled pH conditions) human oocytes were examined and it was concluded that the sperm binding ability of the zona remains intact under all these conditions. Subsequently, the kinetics of sperm binding to the zona was assessed and showed that the maximum binding was at 4–5 h post gamete co-incubation. Interestingly, the binding curves were similar for both fertile and infertile semen samples [18].

This assay has been validated by specifically defining the factors affecting data interpretation; such as, kinetics of binding, egg sources, variability and maturation status, intra-assay variation, and influence of sperm concentration morphology, motility, and acrosome reaction status. Over a period of 90 days' evaluation, spermatozoa from fertile men do not exhibit a time-dependent change in zona binding potential, therefore, reassuring their utilization as controls in this bioassay. Within each pool of donors utilized in the assay, a cut-off value or minimal threshold of binding has to be established in order to validate each assay. The purpose is to identify a poor semen specimen and/or a poor zona control. In the control population, this cut-off value should be approximately 20 sperm tightly bound to the control hemizona (fertile donor). Therefore, it is important that each laboratory statistically assess its own control data in order to establish a reasonable lower limit for assay acceptance. If the control hemizona (matching hemizona exposed to fertile sperm) has a good binding capability, that is, tightly binding of at least 20 spermatozoa after a 4 h incubation period is confirmed (information derived from a statistical evaluation of a pool of fertile donors), then a single oocyte will give reliable information about the fertilizing ability of the tested spermatozoa specimen [18–20].

The variability between eggs is high for oocytes representing different stages of maturation (immature versus mature eggs), as well as within a certain population of eggs at the same maturational stage as well as cohort variations. However, this factor is internally controlled (eliminated as a variable) in the assay by the use of matching hemizonae from the same egg. This allows a comparison of fertile versus an infertile semen sample binding in the same assay under the same oocyte quality conditions. Incubating matching hemizona from eggs at the same maturational stage with homologous spermatozoa from the same fertile ejaculate, established a low (<10 %) intra-egg (intra-assay) viability both for human and monkey (cynomolgus) oocytes [21–23].

Importantly, it has been shown that sperm with full meiotic competence were associated with an increased zona pellucida binding potential to human and monkey oocytes. Furthermore, the specificity of the interaction between human spermatozoa and the human zona pellucida under HZA conditions is strengthened by the fact that

the sperm tightly bound to the zona are acrosome reacted. Moreover, results of interspecies experimented performed with human, cynomolgus monkey, and hamster gametes have demonstrated a high species specificity of human sperm/zona pellucida functions under HZA conditions, thus providing further support for the use of this bioassay for infertility testing [24, 25].

Prospective blinded studies have reported a robust statistical association between sperm binding to the hemizona and conventional IVF. These studies suggest that the HZA can successfully be used to differentiate between the populations of male-factor patients that are at risk for failed or poor fertilization with high predictive value. Using a cut-off value of fertilization rate of 65 ± 2 % of the overall fertilization rates for non-male-factor patients, or distinguishing between failed (0 %) versus successful fertilization (1–100 %) the hemizona assay results expressed as a Hemi-zona-Index (HZI) can provide a valuable tool to distinguish between different categories of patients. The HZI is calculated as the number of bound sperm from the test sample/number of bound sperm from the control sample × 100. Interpretation: a HZI >30–35 is associated with successful fertilization in IVF and with pregnancy in IUI and IVF. A powerful statistical analysis (logistical regression), provides strong support to the clinical the application of the HZA in the prediction of fertilization and provides a robust HZI range predictive of an oocyte's potential to be fertilized [24–27].

It has been reported by Liu [28] that embryo quality and implantation rates were significantly improved and resulted into more pregnancies when zona pellucida-bound sperm ICSI were used as compared to a conventional ICSI; however the difference in fetal heart pregnancy rate was not significant. In another study by Casciani et al. [29] also evaluated whether zona binding sperm selection could be utilized to select superior spermatozoa for ICSI. Spermatozoa that were tightly bound to the zona pellucida were used for micro-injection (ZP-ICSI) versus the conventional method of sperm selection for ICSI. Results showed no significant difference in fertilization, pregnancy, implantation, and take-home-baby-rates. Interestingly, the authors confirmed previous reports by Oehninger et al. [12] that higher sperm concentration and morphology correlated with higher zona pellucida-sperm binding. Additionally, patients with higher zona binding seemed to have improved pregnancy and take-home-baby rates. It was concluded that ZP-ICSI is not superior compared to conventional ICSI, but that some clinical ICSI outcomes were improved in the presence of adequate sperm–zona pellucida binding.

A meta-analytical approach to examine the predictive value of four categories of sperm functional assays and for predicting fertilization outcome have been reported by Oehninger et al. [30]: computer-aided sperm motion analysis (CASA); induced-acrosome reaction testing; heterologous hamster oocyte-sperm penetration assay (SPA); and sperm–zona pellucida binding assays (including the HZA). Subsequent studies have been reported by Arslan et al. [13] that investigated the predictive value of the HZA assay for pregnancy outcome in patients undergoing intrauterine insemination (IUI) with controlled ovarian hyperstimulation (COH). The European Society of Human Reproduction and Embryology (ESHRE) and the World Health Organization (WHO) have recognized the value of sperm binding assays as research tests [10, 11]. In addition, results of the HZA function test can be effectively applied to counseling couples before allocating them into COH/IUI, IVF, or ICSI therapies.

The Hyaluronic Acid Binding Assay

The sperm Hyaluronic Binding Acid (HBA) assay can be applied to select sperm for ICSI. It has been proposed that the results of the test indicate that the selected spermatozoa have undergone normal spermatogenesis [14, 15]. The rationale is that during the events of human spermiogenesis, spermatids undergo alterations in their plasma membranes that involve formation of HA-binding sites. Original studies by Huszar et al. [14] reported on the effect that HA had on the stimulation of sperm motility and by later Slotte et al. [31] on the acrosome reaction.

Later, Huszar et al. [15, 32] reported a correlation between the percentage of HA-bound sperm and their maturation and functional status. It was suggested that this observation could be used for fertility diagnosis as well as for the selection of functional spermatozoa for ICSI.

Hyaluronic acid (HA) is an integral component of the extracellular matrix of the cumulus oophorus [33] and is composed of alternating repeats of D-glucuronic and *N*-acetyl-D-glucosamine residues [34]. In humans, oocytes are naturally surrounded by HA during the fertilization process and it is also the environment where natural sperm selection takes place. It has been proposed that such spermatozoa are mature and have the best chance penetrating the oocyte and subsequently fertilizing it, by forming a complex with a glycodelin-interacting protein which retains and concentrates glycodelin-C, a component that is crucial for sperm–zona binding as reported by Chung et al. [35]. In the human, final maturation steps of spermatogenesis involve plasma membrane modifications that prepare the male germ cell for binding to hyaluronan and subsequently to the zona pellucida [36]. Furthermore, HA receptors are present in mature human spermatozoa and at least three hyaluronan-binding proteins are involved in sperm maturation, acrosome reaction, motility, hyaluronidase activity, and sperm-zona binding [37–40].

The application of HA as a "physiologic selector" in vitro has been acknowledged: reports have demonstrated that the spermatozoa that were immobilized and bound to HA in vitro had also completed their plasma membrane remodeling, cytoplasmic extrusion, and nuclear maturation. These spermatozoa are also believed to have a reduced risk of chromosomal imbalance or chromatin anomalies [41, 42]. It could be argued that the above consequences of selection of spermatozoa by HA-binding prior to ICSI, might contribute in optimizing ART outcome.

According to Jakab et al. [41] HA-bound sperm have completed the process of spermiogenesis with cytoplasmic extrusion and demonstrate enhanced levels of the testis-expressed HspA2 chaperone protein. Furthermore, Cayli et al. [43] reported that HA-bound sperm are devoid of DNA fragmentation and the apoptotic marker, caspase-3. Most significantly, sperm bound to hyaluronan display a reduced frequency of chromosomal aneuploidies in comparison to their nonbinding counterparts. Each of these biochemical and molecular parameters of developmental maturity play a critical role in the paternal contribution to successful preimplantation embryogenesis.

Recent studies have provided data that indicated that HA-bound sperm that were selected for ICSI lead to increased implantation rates. In one such study, Parmegiani et al. [44] reported that in 293 couples treated with HA-ICSI compared with 86 couples treated with conventional ICSI, all outcome measures (fertilization, embryo quality, and implantation and pregnancy rates) were at least similar or improved in the HA-bound sperm group. Furthermore, the implantation rate was increased from 10.3 % in conventional ICSI to 17.1 % in the HA-bound group. Studies by Worrilow et al. [45, 46] reported improved clinical pregnancy rates when using HA-selected sperm were compared with conventional sperm selection criteria for ICSI. These authors also showed that in patients with a prescreened binding efficiency of <65 % HA-binding efficiency before ICSI, the rates of pregnancy loss were slightly higher. In patients with a HA-binding score of 65 % or greater, an implantation rate of 37.4 % compared with 30.7 % for control subjects ($P>0.05$) was reported. Additionally, they reported a 50.8 % clinical pregnancy rate in patients randomized to the HA-binding group of compared with 37.9 % for those randomized to the control group ($P>0.05$). Importantly, for patients with HA binding score of higher that 65 %, there was a significant reduction in their pregnancy loss rate from 15.1 % in the control group down to 3.3 % in HA group ($P=0.021$). In contrast, Tarozzi et al. [47] reported that the application of HA was not useful in the context of the limited use of oocytes under Italian law.

ICSI performed with HA-bound spermatozoa has been defined as "physiologic ICSI" and currently, two systems, specially designed for sperm-HA binding selection are available. Due to the different design of these systems, mature HA-bound spermatozoa behave different in each.

Firstly: a special culture dish with microdots circling the area of attached HA hydrogel to the bottom of the dish (PICSI Sperm Selection Device; MidAtlantic Diagnostic–Origio) [48]. In the PICSI-dish sperm are bound by the head to the bottom of the dish, and the tail depicts vigorous spinning (in circles) around their bound head. Secondly: a viscous medium containing HA (Sperm Slow; MediCult–Origio) [49] is available. In the viscous HA containing Sperm Slow medium, HA-bound sperm exhibits very low progression and are therefore easier to be morphologically evaluated. The above described technical differences makes selection and recovery with both these HA systems difficult, therefore the embryologist should be able to choose the system most suitable to their own ability.

There are other some reports comparing conventional ICSI with "physiologic"HA-ICSI. HA represents also a more natural alternative for handling spermatozoa before ICSI than the potentially toxic PVP used in conventional ICSI [45, 50–54].

On the other hand, Ye at al. [55] and Nijs et al. [56] reported that even though spermatozoa bound to HA had inferior DNA damage and improved chromatin condensation as compared to the control group, the HB-assay failed to predict fertilization, pregnancy, and baby take-home rate after IVF and ICSI and concluded that it has no predictive value as a clinical test. Similarly, Petersen et al. [57], reported no differences in the percentages of normal spermatozoa in the HA-bound and nonbound fractions. Van den Bergh et al. [50], also found no significant differences in fertilization rates and zygote score. HA-binding did not predict spontaneous fertilization in patients with unexplained infertility undergoing IVF/ICSI treatment. When it was used for "screening" it did not help to select the method of fertilization [58]. Therefore, the true benefit/advantage of HB-bound sperm selection needs to be confirmed in larger-scale studies.

HA-containing products have no known negative effects on post-injection zygote development and can be metabolized by the oocyte [50–52]. The failure of the HBA binding test to predict fertility may indicate only the partial role of isolated hyaluronan in sperm selection. Sperm function and the spermatozoa's ability to penetrate the cumulus depend on a combination of components from the cumulus (extracellular matrix containing hyaluronan) and the cumulus cells (converting glycodelin-A and -F into glycodelin-C) [59, 60].

Summary and Conclusions

There is unequivocal evidence to support the use of sperm–zona binding bioassays, including the HZA, in the clinical setting. Results obtained from these prospectively performed assays assist in the clinician's direct management towards IUI, IVF, or ICSI. Unfortunately, the need for human material (eggs) makes the assay cumbersome and difficult to be performed by most laboratories. Efforts to use recombinant zona pellucida proteins in soluble or solid-phase assays have not been successful [7, 61]. On the other hand, the HBA test is easier to perform, but contradictory clinical results have limited its value.

The use of alternative molecular binding methods for sperm selection for ICSI needs to be further explored to be able to drawn firm conclusions about their clinical value [62]. These methods include the use of annexin V microbeads which are based on the identification of apoptotic markers such as the presence of externalized phosphatidylserine on the surface membrane of spermatozoa [63]. Flow cytometric cell sorting technique—a procedure that utilizes fluorescence labeled Annexin V to mark phosphatidylserine positive spermatozoa, is highly effective in separating a subpopulation of spermatozoa with normal morphology, as developed by Hoogendijk et al. [64]. Other methods of sperm selection for ICSI have been introduced such as the (1) zeta potential based on sperm membrane charge [65] and (2) an electrophoretic technique where functional sperm penetrates through a polycarbonate membrane and separates highly motile sperm with good DNA integrity and morphology [66].

Sperm selection can also be attempted though microscopic methods. It remains to be established whether any of these molecular binding assays is superior, or can be additive to morphological

evaluations using motile sperm organelle morphological examination (MSOME) or ICSI using morphologically selected sperm injection (IMSI) [67–72]. With this technique, selection of sperm cells is performed using an inverted microscope equipped with Nomarski optics coupled with a digital system to reach a final magnification of >×6,000. Other novel methods are also being investigated. Huser et al. [73] reported that Raman spectroscopy of DNA packaging in individual human spermatozoa cells distinguishes normal from abnormal cells. Gianaroli et al. [74] used polarized light that permitted microscopic analysis of the pattern of birefringence in the human sperm head to examine the impact of acrosomal status on ICSI outcome. We estimate that novel and emergent noninvasive technologies should take into consideration the morphological normalcy of the spermatozoa, because such spermatozoa are the ones typically selected for ICSI, and may have "hidden" DNA as described by Avendaño et al. [75, 76].

We conclude that more well-designed studies are needed to confirm the clinical utility, cost-efficiency, and temporal aspects of application (learning curves and real time needed to complete sperm selection in the laboratory) of all these tests, in order to determine accuracy for sperm selection and safe use in the IVF setting.

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