



Sperm function tests in clinical practice

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ABSTRACT

Conventional semen analysis solely is not completely adequate to predict pregnancy outcomes. Therefore, advanced sperm function tests have been developed and introduced to clinical practice. These tests use different methods and techniques to evaluate different stages of fertilization steps. In this review, we reported some commonly used sperm function tests: sperm penetration assay, sperm-zona pellucida binding test (hemizona assay), acrosomal reaction test, hyaluronan binding test, hypo-osmotic swelling test, magnetic-activated cell sorting and zeta sperm selection. We discussed the literature concerning these tests, the utilization techniques and also purpose and mechanism of each test. We emphasized the importance of sperm function tests in predicting in vitro fertilization and pregnancy outcomes and in the management of infertile couples and also the limitations of these tests. Along with improvements in molecular biology techniques, we believe that more applicative and beneficial tests will be developed in the near future.

Keywords: In vitro fertilization; male infertility; sperm functional test.

Introduction

Conventional semen analysis (SA) can not exactly reveal a man's fertility potential. Conventional SA, based on sperm motility and macroscopic evaluation, may not accurately reflect the fertilization ability of the spermatozoa. SA may classify an infertile man according to the type and degree of his spermatogenic defect, but provides limited information about how well a sperm will function in *in vitro* or *in vivo* settings. The basic SA has limited predictive value for pregnancy outcome in couples both trying to achieve conception with or without using assisted reproductive technology (ART).^[1] More importantly, men with normal SA may be infertile or fertile.^[2] This indicates that more comprehensive sperm function tests should be performed.

During the last two decades, intracytoplasmic sperm injection (ICSI) has become the preferred technique in patients with a history of

previously unsuccessful conventional *in vitro* fertilization (IVF) and male factor infertility. It has been shown that the semen parameters of unprocessed ejaculate do not affect ICSI results. Therefore, it is important to identify the sperm dysfunction at the cellular and molecular level.

Ideally, the sequential analysis of sperm functions can help the clinicians in planning therapeutic approach.^[3,4] However, up to date, assessment of sperm function tests has failed to make such a significant impact on the clinical management of infertile couples. Lack of standardized protocols has been the main reason.^[5]

An ideal sperm function test should diagnose a specific sperm dysfunction, predict fertilization and pregnancy rates and indicate an appropriate therapy to alleviate the identified sperm dysfunction. The World Health Organization (WHO) qualified sperm function tests as

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research tests.^[6] These tests were originally designed to evaluate the fertility potential of spermatozoa *in vitro*. But, over time, these tests have become more predictive assays for pregnancy. IVF results have shown that defective acrosomal reaction and/or abnormal sperm-zona pellucida relation are frequently found in sperm of infertile males. Ejaculated sperm must undergo capacitation, recognize, bind to the zona pellucida (ZP) and finally undergo acrosome reaction to fertilize the ovum. Therefore, sperm function tests aim to evaluate the steps of fertilization: (i) sperm binding to ZP, (ii) acrosomal exocytosis, (iii) and fusion with the vitelline membrane of the oocyte (Figure 1).

The acrosome reaction is induced by spermatozoa-ZP binding, right after the lytic acrosomal enzymes are released, and the spermatozoa proceed to the zonal matrix with increased flagellar motility. Sperm-ZP binding tests including sperm-zona binding test and hemizona test (HZA) and acrosome reaction tests have been shown to provide clinically significant information to predict IVF results. The highest specificity and sensitivity associated with sperm-ovum interaction are also provided by the sperm-ZP binding tests. Although there is a significant relation between the *in vitro* results of these tests and fertilization, there are problems about their utilization in routine clinical practice such as difficulties in terms of requirement for human material requirement, challenging occasionally time-consuming and expensive techniques.

The aim of this review is to evaluate and review the clinical utilization of sperm function tests, especially gamete interaction tests.

Sperm penetration assay

Sperm penetration assay (SPA) is one of the first sperm function tests developed.^[7] In this heterologous bioassay, human spermatozoa are subjected to the hamster oocytes which are devoid of ZP

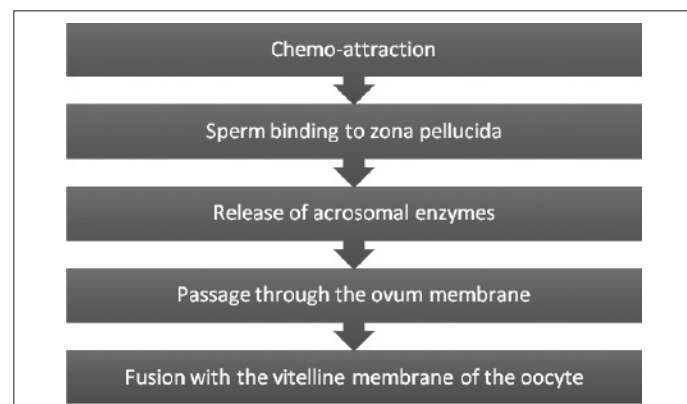


Figure 1. Steps of fertilization

(Figure 2). SPA measures the capacitation ability of spermatozoa, acrosome reaction, fusion and penetration through the oolemma and decondensation within the cytoplasm of hamster oocytes.^[8] Vogiatzi et al.^[9] reported higher sensitivity (52%–100%), specificity (0–100%) and positive predictive value (PPV; 18%–100%) and negative predictive value (NPV; 0–100%) in terms of the diagnostic accuracy of SPA. The standardization and reproducibility of this assay is low. SPA differs from the physiological situation where ZP is absent during the procedure. Oehninger et al.^[8] carried out a meta-analysis of sperm function tests to evaluate their predictive value for (IVF) outcomes. They revealed that SPA had a poor clinical value as a indicative of fertilization after analysis of 2.906 cycles, with high sensitivity but high false-positive rates.^[10] Another meta-analysis collected data on 647 patients in 24 studies. A summary receiver operating characteristics (ROC) curve demonstrated that the sensitivity of SPA was only 37%, with a specificity of 95% and the authors concluded that performing SPA is insufficient for selecting patients for treatment with IVF-embryo transfer.^[11] False-negative results (the spermatozoa fails to respond to SPA but fertilizes the egg successfully) have been also frequently reported.

The anticipation that the strength of the SPA test can be improved with some modifications is not exactly verified.^[12] This expensive and time-consuming test should conceivably not be routinely used to determine fertility potential until the reproducibility and reliability of the test is improved.

Sperm-zona pellucida binding tests

The relation between spermatozoa and ZP is highly critical and indicates many features of sperm function. Some of these functions are achievement of the capacitation and the getting ready for the beginning of the acrosome reaction.^[13,14] HZA^[15] and a competitive intact zona sperm binding assay^[16] are the two most commonly used sperm-ZP binding tests (Figure 3). Although

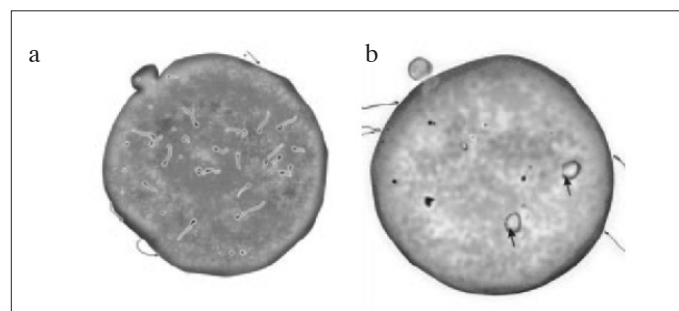


Figure 2. a, b. Illustration of sperm penetration assay. (a) Human spermatozoa are seen in the hamster oocyte cytoplasm. (b) Arrows show sperm heads swelling in the oocyte cytoplasm, indicating sperm decondensation

the methodologies are different, they evaluate the tight binding of sperm to ZP as primary outcome. They revealed a high predictive value for successful IVF outcomes.^[13,17] Impaired sperm-zona binding and zona penetration in conventional IVF are the most common causes of fertilization failure. In oligozoospermic patients, 80% of sperm can not normally bind to ZP.^[18]

Hemizona test is an internally controlled assay and uses matching halves of a human ZP.^[19] HZA should be used in cases with severe oligoasthenozoospermia where recurrent lower or failed fertilization rates were obtained with IVF treatment. In addition, oligozoospermic men were shown to be quite risky of having defective sperm-ZP associations.^[20]

In the HZA test, sperm of fertile men is used as a control because it shows higher binding capacity than infertile male sperm. HZA results are interpreted by an index called hemizona index (HZI). The HZI is the ratio between bound sperm of subfertile men and bound sperm of fertile men ($\text{HZI} = \text{bound sperm from subfertile male} / \text{bound sperm from fertile male} \times 100$).^[15,21] Prospective studies reported a HZI of 35% as a predictive value of ICSI outcome.^[22,23] It has been shown that a HZI of <30 leads to significantly lower pregnancy rates after intrauterine insemination (IUI) treatment, compared to HZI of >30.^[24] Oehninger et al.^[25] concluded in their review that HZA has an excellent predictive power for the outcomes of IUI and IVF, and therefore the assay has a relevance in the clinical diagnostic setting in infertility.

Zona pellucida glycoproteins

During sperm-oocyte interaction, acrosome-intact sperm bind to the ZP and ZP induces the acrosome reaction (AR).^[26] There

are four glycoproteins in the human ZP: hZP1, hZP2, hZP3 and hZP4.^[27] Although ZP3 and ZP4 are proposed to act as primer ZP receptors necessary for the initiation of AR, experimental studies have shown that other ZP proteins are also required for human spermatozoa-oocyte relation.^[28]

Occasionally, normozoospermic infertile men have normal sperm-ZP binding but, failed sperm-ZP penetration. This is mostly secondary to impaired ZP-induced AR (DZPIAR) that causes sperms to bind to the ZP but fails to induce AR on the ZP.^[29] Liu et al.^[30] enrolled 51 DZPIAR infertile men in their study and reported that defective protein kinase C (PKC) and protein kinase A (PKA) pathways are highly associated with disordered ZPIAR in normozoospermic infertile men with normal sperm-ZP binding. They also found that phorbol myristate acetate (PMA, PKC activator) and dibutyryl cyclic AMP (AMP, PKA activator) enhanced acrosome reaction among DZPIAR infertile men.^[30] Gupta et al.^[31] underlined the understanding the role of ZP glycoproteins during human fertilization in facilitating the development of new contraceptives and strategies to overcome the problem of infertility.

Role of Zonadhesin during sperm-ovum binding

Zonadhesin is an intra-acrosomal protein. The binding capacity of zonadhesin to ZP was firstly shown in pigs, then in many other animals and most recently in humans.^[32] Zonadhesin is the only sperm protein that shows species specificity in terms of binding to ZP and it is produced during spermatogenesis in the early spermatid period.^[33] It is located at the apical head of spermatozoa and released into the seminiferous tubule. Sperm-egg interaction is assessed by analyzing this protein during fertilization.

Tardif et al.^[34] proposed a model in which sperm could be transiently exposed to acrosomal molecules that adhere to the zona independent from the AR in a 'kiss and run' mechanism. They concluded that this could be important for further investigations and a detailed understanding of the molecular events during sperm-ovum binding is likely to provide new approaches for the design of more effective male contraceptives and better diagnostic methods for sperm dysfunction.^[34]

Acrosome reaction

Sperm binding to the ZP triggers the hydrolyzing enzymes known as AR (Figure 4) and only acrosome-reacted spermatozoa can penetrate ZP, bind the oocyte membrane and fuse with oocyte.^[35] The release of lytic enzymes and the presence of membrane receptors are required for the acrosome reaction, resulting in penetration of sperm through ZP and integration with the oolema.^[36] The AR begins at the ZP after sperm bind-

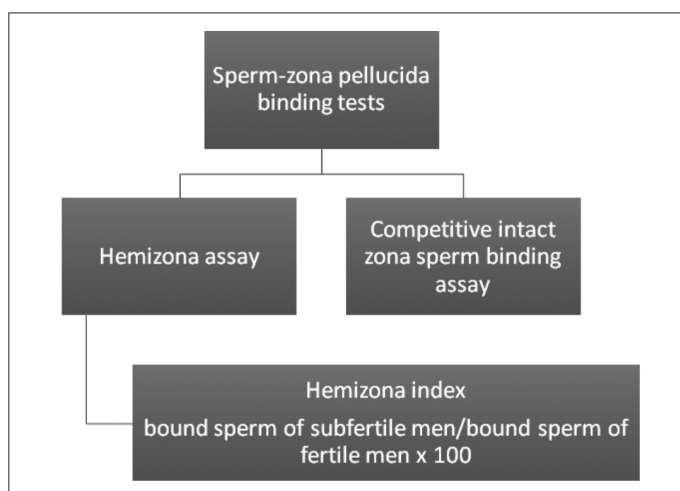


Figure 3. Most commonly used sperm-zona pellucida binding tests

ing. Acrosomal status can be assessed by microscopy, flow cytometry and fluorescently labeled lectins. Calculating the percentage of damaged acrosome containing cells in fresh and stored semen is one of the most important parts of semen assessment.

Similar to zona binding tests, patients with poor AR test should be referred to ICSI. Meta-analysis revealed a high predictive power for AR tests for the prediction of fertilization.^[8] In a total of 797 subjects, ROC curve showed higher PPV (>75%), NPV (>65%), 80% sensitivity and 20% false-positive rates. Nevertheless, AR is currently used for research purposes rather than clinical purposes.

Hyaluronan binding assay (HBA)

This test is based on the binding of hyaluronic acid to sperm with better morphological features and intact acrosome.^[37] Hyaluronic acid (HA) surrounds the human oocyte and acts as a natural spermatozoa selector. Human spermatozoa express HA receptors and only spermatozoa with normal shape, minimal DNA fragmentation and lesser chromosomal aneuploidies can bind to HA. Spermatozoa, expressing the HA receptor and having hyaluronidase activity at high levels, are more likely to pass the extracellular matrix, bind to the ZP and fertilize the ovum.^[38]

In hyaluronan binding assay (HBA), HA is binded by human spermatozoa and indicates the presence of cellular maturity, viability and spermatozoa with intact acrosomes because HA is the main component of extracellular matrix of cumulus oophorus. Intact HA binding sites on the sperm plasma membrane indicate sperm maturity.^[37] Therefore, HBA is a reliable and reproducible test to identify IVF or ICSI candidates.^[37]

Two methods have been examined for the selection of ideal sperm with high expression of HA activity: a HA culture dish

(PICSI [physiological intracytoplasmic sperm injection] Sperm Selection Device) and viscous medium containing HA (Sperm Slow). HA sperm are bound by the head to the bottom, however, in Sperm-Slow HA, spermatozoa has a very slow motility. However, in clinical practice the authors concluded that the rates of achieving embryos of improved quality were similar between two groups.^[39]

Hyaluronic acid is also found in cervical mucus and oviducal fluid. It has been demonstrated that human spermatozoa expressing HA receptor (CD44) display better plasma membrane structure, mitochondrial membrane potential, fertilization potential and maturation characteristics compared to non-CD44.^[40] Therefore, CD44 present in the spermatozoa is a good indicator of sperm quality, which is also recognized by the female genital tract.

Evidence is insufficient to show whether sperm selection by HA binding improves live birth or pregnancy rates in ART. There is also no sufficient evidence to show difference in efficiency between the HA binding methods SpermSlow and PICSI. No randomised evidence describes the evaluation of sperm selection by sperm apoptosis, sperm birefringence or surface charge.^[41]

Hypo-osmotic Swelling Test

Hypo-osmotic swelling test (HOST) is based on the intact membranes of the viable spermatozoa. An intact sperm membrane plays an important role during fertilization. HOST is indicated in cases with very few or no motile sperm in the ejaculate. In hypo-osmotic conditions, intact sperm cell's cytoplasmic space swells and its tail curls. Dead sperm with non-intact membrane can not swell in hypotonic media and exhibits these features (Figure 5). The results of this test correlate with other semen assessments such as morphology and motility, but the data on its effects on fertility are not satisfactory.^[42]

Magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) depends on selecting preferred cells based on membrane surface markers. In this assay, healthy spermatozoa are separated magnetically by maintaining the structure, viability and functioning of the spermatozoa (Figure 6).

In somatic cells, normally, phosphatidylserine (PS) is located in the internal layer of plasma membrane. Externalization of PS from inner to outer layer of plasma membrane can be assessed by Annexin V and is considered as one of the early signs of apoptosis before capacitation.^[43] However, this process is a nor-

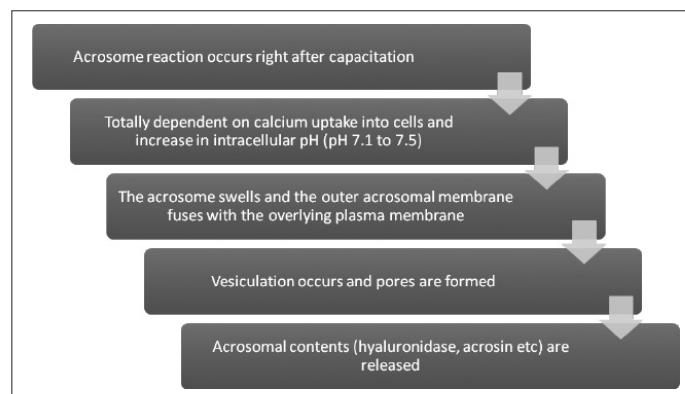


Figure 4. Steps of acrosome reaction

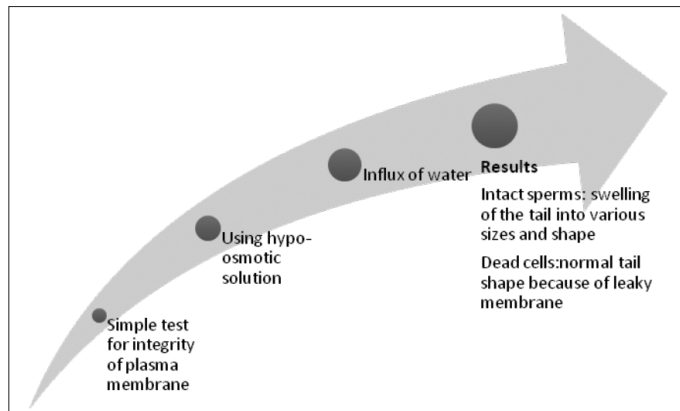


Figure 5. The flow chart of hypo-osmotic swelling test

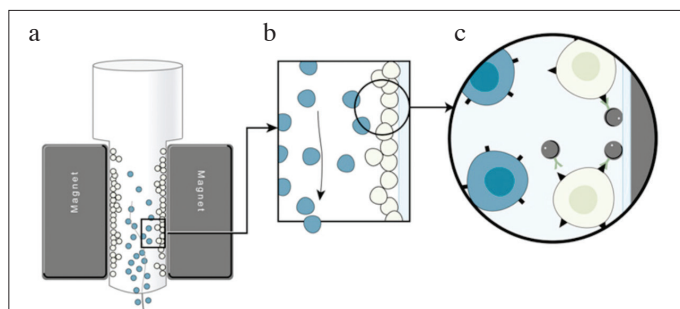


Figure 6. Magnetic-Activated Cell Sorting. (a) Annexin V molecule is attached to magnetic microspheres (b) When sperm go through a magnetic field, those who attach to the microspheres are retained and discarded (c) Annexin V binds to a marker (phosphatidylserine) present only in the membrane of sperm with fragmented DNA

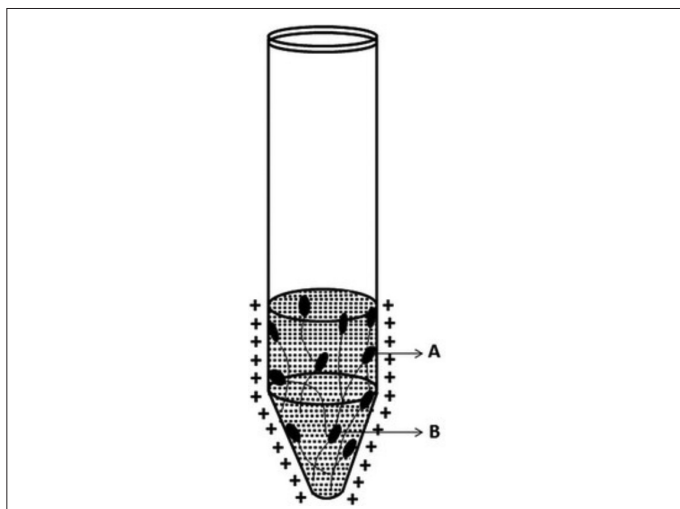


Figure 7. Sperm selection using zeta test. (A) Negatively charged mature sperm will move to the positively charged tube surface. (B) Immature spermatozoa will be discarded

mal phenomenon observed during capacitation. This dual functioning role of PS in human spermatozoa makes interpretation and test design very difficult. At this point, MACS can isolate sperm at the molecular level using Annexin V.^[44]

Said et al.^[45] reported higher embryo cleavage and pregnancy, but lower fertilization or implantation rates with MACS technique. It has been emphasized that the MACS assay has the potential to isolate spermatozoa with normal acrosomes and protamine content and to have a higher significance in severe teratozoospermic patients.^[46]

Zeta sperm selection

A mature sperm possess a surface electric charge ranging from -16 to -20 mV, termed as the zeta potential which decreases with capacitation.^[47] Normally differentiated sperms are charged electronegatively (Figure 7). Many studies have shown that sperms selected according to the membrane zeta potential levels exhibit lower DNA fragmentation.^[48] From this point of view, sperm selection according to electric charge could improve the ICSI results.

Sperm with high negative surface electrical charge are mature and more likely to have intact chromatin. In a recent prospective study, a total of 203 ICSI cycles studied, 101 cycles were included to density gradient centrifugation (DGC)/Zeta group and the remaining 102 in the DGC group. The authors observed a significant increase in top quality embryos and pregnancy rates in the DGC/Zeta group compared to DGC group. They concluded that zeta method improves the percentage of embryo quality and pregnancy outcomes.^[49] Some substantial studies evaluating sperm function tests are summarized in Table 1.

In conclusion, sperm function tests have a role in further evaluation of infertile couples. It is not always possible to predict pregnancy outcomes by conventional semen analysis due to multifactorial nature of fertilization. Therefore, functional tests evaluating the male fertility potential at the molecular and cellular level are of importance. On the other hand, these tests have some limitations: standardization is required in methodologies, some tests are useless, expensive and time consuming and human material is required. The development of molecular biology techniques and the optimization of these tests will lead to novel diagnostic and therapeutic improvements in the management of male factor infertility.

With the advances in technology, to take part in routine clinical practice, these tests must be (i) accurate; (ii) cost-effective; (iii) easy to use; and (iv) clinically useful.

Table 1. Studies evaluating sperm function tests

Authors	Year	Objective	Outcome
Vogiatzi et al. ^[9]	2013	To review SPA's predictive power in IVF outcomes, together with statistical significance of diagnostic power of the assay	Considerable variation was noted in the diagnostic accuracy rates of SPA with higher sensitivity (52–100%), specificity (0–100%), and PPV (18–100%) and NPV (0–100%) rates together with fluctuation and notable differentiation in methodology and cut-off values employed by each group
Oehninger et al. ^[8]	2000	An objective, outcome-based examination of the validity of the currently available assays was performed based upon the results obtained from 2906 subjects evaluated in 34 prospectively designed, controlled studies	Results indicated a poor clinical value of the SPA as a predictor of fertilization after assessment of a total of 2,906 cycles, with good sensitivity but very high false-positive rates
Liu et al. ^[20]	2004	To determine frequency of defective sperm–ZP (zona pellucida) interaction in oligozoospermic infertile men.	Oligozoospermic men have a very high rates of defective sperm–ZP interaction, consistent with their low natural fertility or low fertilization rates in conventional IVF
Oehninger et al. ^[22]	1989	To assess the relationship between tight- sperm binding in the HZA and sperm fertilizing ability in IVF	HZA is a valuable tool for evaluating dysfunctional sperm–zona pellucida binding, with good predictive value for <i>in vitro</i> fertilization
Franken et al. ^[23]	2005	To assess the clinical significance of sperm–zona pellucida binding	The sensitivity and specificity of sperm–zona binding results indicated the assay to be positively and significantly correlated with <i>in vitro</i> fertilization outcomes. 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
Arslan et al. ^[24]	2006	To evaluate the value of the HZA as a predictor of pregnancy in patients undergoing COH and IUI	The HZA predicted pregnancy in the IUI setting with high sensitivity and negative predictive value in couples with male infertility. Results of this sperm function test are useful in counseling couples before allocating them into COH/IUI therapy.
Vogiatzi et al. ^[9]	2013	To review predictive power of HZA in IVF outcomes, with statistical significance of diagnostic power of the assay	HZA should be used in a sequential fashion with semen analysis and potentially other bioassays in an IVF setting.
Oehninger et al. ^[8]	2000	To examine the predictive value of sperm–zona pellucida binding assay for IVF outcomes	Results demonstrated a high predictive power of the sperm–zona pellucida binding and the induced-acrosome reaction assays for fertilization outcomes
Said et al. ^[44]	2008	To investigate whether the MACS technology could be used to improve ART outcomes	Integrating MACS as a part of sperm preparation techniques will improve semen quality and cryosurvival rates by eliminating apoptotic sperm
Said et al. ^[45]	2011	To systematically review the literature describing these advanced sperm selection methods focusing on their anticipated benefits on sperm quality and ART outcomes	Fertilization and pregnancy rates showed improvement following some of the advanced sperm selection techniques such as MACS
Chan et al. ^[47]	2006	To develop a simple zeta potential method for sperm isolation, and to analyze the sperm maturity, morphology, kinematic, and DNA parameters.	The zeta method improved sperm parameters associated with increased fertilization and pregnancy rates after assisted reproduction procedures

SPA: sperm penetration assay; IVF: in vitro fertilization; HZA: hemizona assay; COH: controlled ovarian hyperstimulation; IUI: intrauterine insemination; MACS: magnetic activated cell sorter; ART: assisted reproductive technique; PPV: positive predictive value; NPV: negative predictive value

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