Sperm functional tests

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Several semen parameters are used to discriminate the fertile male from the subfertile male. The most widely used parameters are sperm concentration, motility, progressive motility, and sperm morphology. Semen analysis is usually applied as described in the World Health Organization manual for semen analysis. In addition to a routine semen analysis, sperm functional tests have been described for many years, which in most cases are regarded as research tools and not part of the routine semen testing in an infertility clinic. In this review we report on the value of four sperm function tests: the sperm penetration assay, the sperm–zona pellucida binding tests, the acrosome reaction, and the hyaluronan binding assay. For each test we describe the current value, the indication for performing the test, how to interpret the results, and its therapeutic implications. Our data show that sperm functional assays are highly predictive of IVF outcome results and have the potential to assist in clinical decision making, especially to avoid the current long-standing treatment with IUI and to direct the patients to intracytoplasmic sperm injection without delay when sperm functional testing fails. We believe that advances in molecular biology techniques will allow us to develop simpler sperm function assays in the near future. This will undoubtedly help clinicians in optimizing male factor infertility diagnosis and treatment. (Fertil Steril® 2014;102: 1528–33. ©2014 by American Society for Reproductive Medicine.)

Key Words: Acrosome reaction, male infertility, sperm functional test, sperm penetration assay, sperm–zona pellucida binding tests

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The basic semen analysis has limited predictive value for pregnancy in couples trying to achieve natural conception and in couples undergoing advanced assisted reproductive technologies (ART) (1). This highlights the need for more extended sperm functional testing. Ideally, the sequential analysis of sperm functions could assist clinicians in planning the therapeutic approach and predicting the outcomes of such treatments (2–8).

In the last two decades the intracytoplasmic sperm injection (ICSI) setting has provided a new and unique arena to evaluate sperm dysfunction. After the first successful ICSI deliveries (9, 10), the clinical focus immediately shifted to gamete manipulation. ICSI quickly became the selected technique for cases of male factor infertility and for couples with previously failed fertilization with conventional IVF. 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 (11, 12). This was followed by achievement of high levels of fertilization with ICSI in the presence of multiple morphological and dysfunctional sperm defects, as well as after the use of ejaculated testicular or epididymal sperm or cryopreserved-thawed sperm and, in cases of obstructive and nonobstructive azoospermia, after sperm extraction from testis or epididymis (13).

In spite of the fact that ICSI has remarkably improved male factor infertility results in ART, we continue to face daily clinical dilemmas. The answer to the many current challenging questions relies on the unveiling of spermatogenesis pathologies and the resulting sperm dysfunctions at the cellular and molecular levels. The role of the various spermatozoal components suspected of actively participating in early human development has being reevaluated (14, 15). The contributions of the fertilizing spermatozoon to the oocyte include, as a minimum, the delivery of the DNA, a putative oocyte-activating factor, most likely phospholipase C zeta (16), and a centriole. Although irrefutable evidence is needed, phospholipase C zeta is now widely considered to be the physiological agent responsible for activating mammalian oocytes (17, 18). It has been also established that the fertilizing spermatozoon may also provide the zygote with a unique suite of paternal mRNAs (19) and that some transcripts might be crucial for early and late embryonic development (20). Clinicians are still looking for the elusive functional test that could be applied universally at the laboratory.

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Work derived from the early IVF days demonstrated that defective acrosome reaction and/or abnormal sperm–zona pellucida (ZP) interaction was frequently observed in the ejaculated sperm of infertile men. Such findings were observed in the presence of normal or abnormal "basic" sperm parameters. Both types of dysfunctions were shown to result in fertilization failure or low fertilization rates. Consequently, acrosome reaction tests and sperm-ZP binding assays were developed to address a real need to assess sperm functional competence in the "extended" evaluation of the infertility workup before conventional IVF was performed. In the current ICSI era, the results of such functional assays can still provide valuable information to the clinician so that he or she may recommend against low complexity alternatives such as IUI therapy and direct couples to ICSI (6, 8, 21–25).

The World Health Organization (26) qualifies sperm functional assays as research tests. These tests were originally conceived as tests to predict the fertilization potential of the male gamete in vitro. Nonetheless, their power to predict pregnancy, a multifactorial outcome, has also become more evident. These bioassays include the examination of sperm binding to the ZP, acrosomal exocytosis, and fusion with the vitelline membrane of the oocyte. The binding of spermatozoa to the ZP initiates the acrosome reaction, releases free and exposes bound lytic acrosomal components, and allows the spermatozoa to penetrate through the zona matrix, driven by the increased flagellar thrusting of hyperactivated motility (27). Although the results of some of these assays correlate with fertilization in vitro with high statistical significance, there are definite drawbacks to their performance, including the need for human material (i.e., ZPs to be solubilized and/or intact eggs), and they are technically and time demanding, making them awkward in the routine clinical laboratory (21).

One of the few published meta-analyses on sperm-oocyte interaction assays revealed that the sperm–ZP binding assays, that is, a sperm-zona binding assay and the hemizona assay (HZA) (28, 29), and acrosome reaction tests, including the examination of ZP-induced acrosome reaction (30, 31), provided clinically useful and prognostic information related to sperm competence to fertilize mature eggs in the IVF setting (22, 24, 32). The HZA also proved to be a predictor of IUI outcome in couples with male factor infertility (33). Within the IVF setting, it was established that the extremely high and frequently observed morphological abnormalities of the male gamete (teratozoospermia) could be used as a biomarker of several gamete dysfunctions, including dyskinetic disorders, and altered capacity to interact with the egg and its vestments (5, 6, 22, 23, 34, 35).

To fertilize the egg, ejaculated spermatozoa must undergo capacitation, recognize and bind to the ZP, and undergo the acrosome reaction. The most significant changes experienced by sperm during capacitation are plasma membrane changes, an increase in certain intracellular messengers, and increased phosphorylation of a set of proteins by different kinases (36–38). Capacitation was first observed in the rat when sperm injected into the periovarian sac of the rat after ovulation did not begin to enter the eggs until 4 or 5 hours later (39, 40). Similar findings were reported in the rabbit when sperm were able to fertilize more eggs if they had first spent about 5 hours in the uterus of another rabbit (41). Sperm capacitation studies require the use of an in vitro fertilizing system. This phenomenon was initially accomplished using cauda epididymal sperm and/or ejaculated sperm incubated under a variety of conditions in defined media mimicking the electrolyte composition of the oviduct fluid.

The molecular and physiological events that enable sperm to fertilize in the female tract are collectively known as capacitation (38, 42–44). The actual capacitation process can be monitored using an antibiotic chlortetracycline that yields different patterns of molecules distribution on the sperm surface that can be visualized as distinct fluorescence patterns depending on the capacitation and the acrosomal status of the sperm (45).

Assessing the ability of human spermatozoa to acquire fertilizing potential (capacitation) by stimulating exocytosis of the contents of the acrosome (acrosome reaction) is thought to have diagnostic potential (44). Calcium-mobilizing agents, such as calcium ionophores (A23187) and P, stimulate the acrosome reaction in vitro (46). Acrosomal status is easily detected using the lectin Pisum Sativum Agglutinin labeled with fluorescein isothiocyanate (47). Defective calcium influx and acrosome reaction (spontaneous and P induced) were found to be compromised in the spermatozoa of infertile men with severe teratozoospermia (48), providing further evidence for the use of capacitation endpoints (acrosome reaction and sperm–ZP binding) as male diagnostic tools.

The aim of this review was to review and highlight the clinical value of sperm functional assays.

SPERM PENETRATION ASSAY (SPA)

This test was one of the first bioassays of sperm function developed (49–51). In this heterologous system, human sperm were subjected to capacitating conditions and incubated with hamster oocytes devoid (enzymatically) of the ZP. The sperm penetration assay with zona-free hamster ova was widely used in the pre-ICSI days. The SPA measures the spermatozoa's ability to undergo capacitation, acrosome reaction, fusion and penetration through the oolemma, and decondensation within the cytoplasm of hamster oocytes and was used to evaluate male fertility potential. However, the results have remained difficult to interpret.

Mao et al. (52) evaluated the clinical relevance of the SPA and reported sensitivity ranges from 0.00 to 1.00 and specificity ranges from 0.95 to 1.00 for diagnosing male factor infertility. As a prognosticator of IVF failure, the sensitivity varied from 0.00 to 0.78 and specificity ranged from 0.51 to 1.00. Similar reports indicated by Vogiatzi et al. (53) reported considerable variation in the diagnostic accuracy values of SPA with wide sensitivity (52%–100%), specificity (0–100%), and positive predictive value (PPV; 18%–100%) and negative predictive value (NPV; 0–100%) together with fluctuation and notable differentiation in the methodology and cutoff values employed by each group. The reproducibility of this assay and standardization of methods between laboratories was low.

The conventional SPA depends on the occurrence of spontaneous acrosome reactions in populations of spermatozoa incubated for prolonged periods in vitro. The fusion of

human spermatozoa to the hamster oocyte is functionally the same as that with the human vitelline membrane, since it is initiated by the plasma membrane overlying the equatorial segment of acrosome-reacted human spermatozoa. The SPA, however, differs from the physiological situation in that the ZP is absent. Since this procedure is less efficient than the biological process and may involve different mechanisms, false-negative results (men whose spermatozoa fail the SPA but successfully fertilize human oocytes in vitro or in vivo) have frequently been recorded. Despite this potentially confounding limitation, the test has provided information on the fusinogenic nature of capacitated sperm. Two of the key intracellular signals that initiate the acrosome reaction after sperm–ZP interaction are an influx of calcium and cytoplasmic alkalinization. As both can be generated artificially with a divalent cation ionophore, an alternative method using ionophore-stimulated spermatozoa was described (54).

A meta-analysis of sperm function assays carried out to determine their predictive value for fertilization outcome in IVF therapy was published by Oehninger et al. (32). 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. The summary receiver operating characteristic (ROC) curve area under the curve was 56%, with a PPV >70% but with a high NPV averaging 50% (low specificity) in studies that were limited by the fact that a fertilization rate cutoff of >0% was established (very low discriminatory range). Another published meta-analysis reached a similar conclusion (55).

Claims that the SPA predictability can be improved by modified versions of the assay have not been corroborated (56, 57). Until the validity and reproducibility of the SPA has been established, this heterologous, time-consuming, and relatively expensive test should probably not be used to evaluate fertility potential.

SPERM–ZP BINDING TESTS

The interaction between spermatozoa and the ZP is a critical event leading to fertilization and reflects multiple sperm functions (i.e., completion of capacitation as manifested by the ability to bind to the ZP and to undergo ligand-induced acrosome reaction) (2, 3, 6, 25, 58). The two most commonly used sperm–ZP binding tests are the HZA (29) and a competitive intact zona sperm binding test (28). Although different in their methodologies, they both use assessment of tight binding of sperm to the ZP as the primary endpoint in an independent comparison in an internally controlled assay and have demonstrated high predictive value for the outcome of fertilization in vitro (6, 21, 24).

The HZA is an internally controlled bioassay that evaluates the ZP binding potential of a sperm population. The HZA provides a functional homologous test for sperm binding to the ZP during which populations of fertile and infertile spermatozoa are compared within the same assay. The assay uses matching halves of a human ZP from an oocyte with no developmental potential (salt-stored or cadaveric) (59). The HZA is indicated in cases where repeated poor or no fertilization is recorded during IVF therapy or in the presence of moderate-severe oligoasthenoteratozoospermia to determine clinical management. Furthermore, it has been demonstrated that oligozoospermic men have a very high frequency of defective sperm-ZP interaction (as examined by sperm-zona binding assays and the ZP-induced acrosome reaction) consistent with their low natural fertility or low fertilization rate in conventional IVF (60).

In the HZA, sperm from fertile men are used as a control and typically exhibit significantly higher binding capacity to hemizonae compared with sperm from infertile patients. The HZA results are expressed as an index known as the hemizona index (HZI), calculated as follows: HZI = bound sperm from subfertile male divided by bound sperm from fertile male × 100 (29, 61). Prospective clinical studies reported a cutoff HZI value of 35% as predictive of IVF outcome (62–64). Of importance, reported clinical results for IUI therapy showed that an HZI <30 was associated with a significantly lower pregnancy rate compared with patients with HZI >30 (11.1% vs. 40.6%, respectively; $P<.05$; relative risk for failure to conceive: 1.5 [confidence interval, 1.2–1.9]) (33).

Consequently, results of this sperm function test are useful in counseling couples before allocating them into alternative therapeutic methods, that is, IUI versus ICSI. Patients with poor sperm-zona binding results should be referred to the ICSI laboratory (22, 23, 53). This is especially true in developing countries where medical insurance and the financial restrictions of the patients are important. Results of the meta-analysis cited above (32) in 978 couples revealed a high predictive power of the sperm-ZP binding assays (HZA and a competitive intact-zona sperm binding test) for fertilization outcome. A summary ROC curve area under the curve showed a PPV >80% and an NPV generally >70% (with low false-negative rates).

ACROSOME REACTION

Sperm binding to the ZP triggers the release of hydrolyzing enzymes known as the acrosome reaction. The acrosome reaction is an exocytotic process of spermatozoa and a prerequisite for fertilization. Only acrosome-reacted spermatozoa are able to pass through the ZP, bind the oocyte plasma membrane, and fuse with the oocyte (27). Previous studies showed that a defective ZP-induced acrosomal reaction was recorded in 25% of normozoospermic subfertile men (65).

The physiological acrosome reaction occurs at the ZP after sperm binding. The ZP-induced acrosome reaction can be assessed on spermatozoa removed from the surface of the ZP or exposed to disaggregated human ZP proteins (30, 31, 66). These tests are limited by the restricted availability of human ZPs. Zonae from other primates cannot be used as surrogates because of their restricted binding specificity (67, 68). Other stimuli, such as calcium ionophores, will induce the acrosome reaction, but the results are not related to those obtained from the ZP-induced acrosome reaction (69). Acrosomal status after induction of the acrosome reaction can be assessed by microscopy, flow cytometry, or fluorescently labeled lectins, such as Pisum Sativum Agglutinin (47, 70).

In advanced studies, the induced acrosome reaction assay is performed using ZPs of a single human oocyte that were aggregated in acid Tyrode's solution at a concentration of 0.5 zonae/mL. After an incubation period with a highly motile sperm population, the acrosomal status of the sperm is calculated using a fluorescent stain. The zona-induced acrosome reaction (ZIAR) is calculated as the difference between the ZIAR (stimulated) and the spontaneous (unstimulated) acrosome reaction results. A clinical cutoff value of 15% was reported, that is, the difference between ZIAR and spontaneous acrosome reaction (71, 72). The mean percentage of acrosome-reacted sperm recorded after exposure to solubilized human ZP also showed statistically significant differences: 13.4%, 16.1%, and 22.8%, and for sperm morphology groups (strict criteria) <4%, 5%–14%, and >14%, respectively (35). Similar to the failure to pass zona-binding assays, cases with poor acrosome reactivity to solubilized ZP should be referred to ICSI. Results of the meta-analysis cited above also revealed a high predictive power of the induced acrosome reaction tests for the prediction of fertilization (32). In 797 subjects, a summary ROC curve area under the curve showed PPV >75%, NPV >65%, 80% sensitivity, and a 20% false-positive rate.

HYALURONAN BINDING ASSAY

Hyaluronic acid binding by human spermatozoa indicates cellular maturity, viability, and spermatozoa with intact acrosomes (73, 74). Only mature, motile spermatozoa bind to hyaluronan through specific receptors. This ability to bind to hyaluronan is not present in immature spermatozoa. Furthermore, hyaluronan binding assay (HBA) represents a more convenient and reproducible laboratory test for identifying candidates for ICSI. HBA results may assist clinicians in the therapeutic approach, that it, in assigning patients for either IVF or ICSI treatment (75).

The use of hyaluronan-bound spermatozoa for an ICSI procedure has the advantage that mature spermatozoa, with high DNA integrity and low frequency of chromosomal aneuploidies, will be selected for injection (76). The HBA scores are also significantly associated with the fertilization rates and biochemical pregnancies (77). No correlation was recorded between HBA and the standard semen parameters (77, 78).

Huszar et al. (75) described a second sperm maturation marker, identified as a testis-expressed protein HspA2. The HspA2 acts as a hyaluronic acid (HA) receptor during normal fertilization (75, 79). It has been shown that HA-bound spermatozoa have increased developmental maturity, including enhanced chromatin integrity, normal morphology, and increased functional potential (80). Furthermore HA-bound sperm showed decreased aneuploidy and decreased active caspase-3 (73, 77).

CONCLUDING REMARKS

This analysis underscores the clinical need for sperm functional testing in the advanced workup of the infertile couple. It also points out to limitations of the currently available tests, manifested by the need for standardization of methodologies, the cumbersome nature of some of the assays, and the need for human material, and also provides objective evidence on which clinical management and future research can be based (7, 81).

The prediction of male fertility potential is probably an elusive goal owing to the multifactorial nature of conception. Here we presented objective data that demonstrated that sperm functional assays are highly predictive of IVF results. However, the implementation of ICSI has basically eliminated the need for such tests. It is our opinion that this does not represent a right approach. On the contrary, sperm functional tests have the potential to assist the clinician in the decision-making process. If patients fail sperm functional testing, then that would eliminate the time, effort, and expense of couples undergoing lower complexity therapies such as IUI and direct them to ICSI without delay.

As shown here, a competent interaction of spermatozoa with the ZP is critical, both in terms of binding and induction of the acrosome reaction, for successful fertilization. Notwithstanding the fact that other processes can be impaired in some patients (i.e., sperm abnormalities that are unveiled after ZP interaction steps, such as oolemma fusion, pronuclear formation, and others), the evidence presented herein underscores the fact that a high number of males with subfertility can be identified by these tests. It is our expectation that advances in molecular biology techniques may allow for the development of simpler assays, maybe based on recombinant ZP proteins or analogs, both in the form of solid- and/or soluble-phase assays, leading to simpler, improved, and physiologically oriented sperm–ZP binding and acrosome reaction assays (30, 31, 82–84), These newly developed assays may indeed have a true impact on the implementation of novel diagnostic and therapeutic changes for optimized management of male factor infertility.

REFERENCES

1. Weber RF, Dohle GR, Romijn JC. Clinical laboratory evaluation of male subfertility. Adv Clin Chem 2005;40:317–64.
2. Oehninger S, Toner J, Muasher SJ, Coddington C, Acosta AA, Hodgen GD. Prediction of fertilization in vitro with human gametes: is there a litmus test? Am J Obstet Gynecol 1992;167:1760–7.
3. Oehninger S, Franken D, Alexander N, Hodgen GD. Hemizona assay and its impact on the identification and treatment of human sperm dysfunctions. Andrologia 1992;24:307–21.
4. Amann RP, Hammerstedt RH. In vitro evaluation of sperm quality: an opinion. J Androl 1993;14:397–406.
5. Oehninger S. An update on the laboratory assessment of male fertility. Hum Reprod 1995;10(Suppl 1):38–45.
6. Oehninger S. Clinical and laboratory management of male infertility: an opinion on its current status. J Androl 2000;21:814–21.
7. Oehninger S. Clinical management of male infertility in assisted reproduction: ICSI and beyond. Int J Androl 2011;34:e319–29.
8. Barratt CL, Mansell S, Beaton C, Tardif S, Oxenham SK. Diagnostic tools in male infertility-the question of sperm dysfunction. Asian J Androl 2011; 13:53–8.
9. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 1992; 340:17–8.
10. Van Steirteghem AC, Liu J, Joris H, Nagy Z, Janssenswillen C, Tournaye H, et al. Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. Hum Reprod 1993;8:1055–60.

11. Nagy ZP, Liu J, Joris H, Verheyen G, Tournaye H, Camus M, et al. The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. Hum Reprod 1995;10:1123–9.
12. Oehninger S, Veeck L, Lanzendorf S, Maloney M, Toner J, Muasher S. Intracytoplasmic sperm injection: achievement of high pregnancy rates in couples with severe male factor infertility is dependent primarily upon female and not male factors. Fertil Steril 1995;64:977–81.
13. Devroey P, Van Steirteghem A. A review of ten years experience of ICSI. Hum Reprod Update 2004;10:19–28.
14. Sutovsky P, Schatten G. Paternal contributions to the mammalian zygote: fertilization after sperm-egg fusion. Int Rev Cytol 2000;195:1–65.
15. Barroso G, Valdespin C, Vega E, Kershenovich R, Avila R, Avendaño C, et al. Developmental sperm contributions: fertilization and beyond. Fertil Steril 2009;92:835–48.
16. Swann K, Larman MG, Saunders CM, Lai FA. The cytosolic sperm factor that triggers Ca2+ oscillations and egg activation in mammals is a novel phospholipase C: PLCzeta. Reproduction 2004;127:431–9.
17. Taylor SL, Yoon SY, Morshedi MS, Lacey DR, Jellerette T, Fissore RA, et al. Complete globozoospermia associated with PLCζ deficiency treated with calcium ionophore and ICSI results in pregnancy. Reprod Biomed Online 2010;20:559–64.
18. Nomikos M, Kashir J, Swann K, Lai FA. Sperm PLCζ: from structure to Ca2+ oscillations, egg activation and therapeutic potential. FEBS Lett 2013;587:3609–16.
19. Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. Lancet 2002;360:772–7.
20. Avendaño C, Franchi A, Jones E, Oehninger S. Pregnancy-specific {beta}-1-glycoprotein 1 and human leukocyte antigen-E mRNA in human sperm: differential expression in fertile and infertile men and evidence of a possible functional role during early development. Hum Reprod 2009;24:270–7.
21. ESHRE Andrology Special Interest Group. Consensus Workshop on Advanced Diagnostic Andrology Techniques. Hum Reprod 1996;11:1463–79.
22. Oehninger S, Franken D, Kruger T. Approaching the next millennium: how should we manage andrology diagnosis in the intracytoplasmic sperm injection era? Fertil Steril 1997;67:434–6.
23. Oehninger S, Mahony M, Ozg€ur K, Kolm P, Kruger T, Franken D. Clinical significance of human sperm–zona pellucida binding. Fertil Steril 1997;67:1121–7.
24. Liu DY, Garrett C, Baker HW. Clinical application of sperm-oocyte interaction tests in in vitro fertilization–embryo transfer and intracytoplasmic sperm injection programs. Fertil Steril 2004;82:1252–63.
25. Oehninger S. Molecular basis of human sperm–zona pellucida interaction. Cells Tissues Organs 2001;168:58–64.
26. World Health Organization (WHO). WHO laboratory manual for the examination and processing of human semen. 5th ed. Switzerland: WHO Press; 2010:56–8.
27. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, editors. The physiology of reproduction. 2d ed. New York: Raven Press; 1994:189–317.
28. Liu DY, Lopata A, Johnston WI, Baker HW. A human sperm–zona pellucida binding test using oocytes that failed to fertilize in vitro. Fertil Steril 1988;50:782–8.
29. Burkman LJ, Coddington CC, Franken DR, Krugen TF, Rosenwaks Z, Hogen GD. The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. Fertil Steril 1988;49:688–97.
30. Franken DR, Bastiaan HS, Oehninger SC. A micro-assay for sperm acrosome reaction assessment. J Assist Reprod Genet 2000;17:156–61.
31. Franken DR, Bastiaan HS, Oehninger SC. Physiological induction of the acrosome reaction in human sperm: validation of a microassay using minimal volumes of solubilized, homologous zona pellucida. J Assist Reprod Genet 2000;17:374–8.
32. Oehninger S, Franken DR, Sayed EM, Barroso G, Kohm P. Sperm function assays and their predictive value for fertilization outcome in IVF therapy: a meta analysis. Hum Reprod Update 2000;6:1160–8.
33. Arslan M, Morshedi M, Arslan EO, Taylor S, Kanik A, Duran HE, et al. Predictive value of the hemizona assay for pregnancy outcome in patients undergoing controlled ovarian hyperstimulation with intrauterine insemination. Fertil Steril 2006;85:1697–707.
34. Schuffner AA, Bastiaan HS, Duran HE, Lin ZY, Morshedi M, Franken DR, et al. Zona pellucida–induced acrosome reaction in human sperm: dependency on activation of pertussis toxin-sensitive G(i) protein and extracellular calcium, and priming effect of progesterone and follicular fluid. Mol Hum Reprod 2002;8:722–7.
35. Bastiaan HS, Windt ML, Menkveld R, Kruger TF, Oehninger SC, Franken DR. Relationship between zona pellucida–induced acrosome reaction, sperm morphology, sperm–zona pellucida binding, and in vitro fertilization. Fertil Steril 2003;79:49–55.
36. Storey BT. Interactions between gametes leading to fertilization: the sperm's eye view. Reprod Fertil Dev 1995;7:927–42.
37. Baldi E, Luconi M, Bonaccorsi L, Krausz C, Forti G. Human sperm activation during capacitation and acrosome reaction: role of calcium, protein phosphorylation and lipid remodeling pathways. Front Biosci 1996;1:d189–205.
38. Visconti PE, Kopf GS. Regulation of protein phosphorylation during sperm capacitation. Biol Reprod 1998;59:1–6.
39. Austin CR. Observations on the penetration of the sperm in the mammalian egg. Aust J Sci Res B 1951;4:581–96.
40. Austin CR. The capacitation of the mammalian sperm. Nature 1952;170:326.
41. Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 1951;168:697–8.
42. Zaneveld LJ, De Jonge CJ, Anderson RA, Mack SR. Human sperm capacitation and the acrosome reaction. Hum Reprod 1991;6:1265–74.
43. Zaneveld LJ, Anderson RA, Mack SR, De Jonge CJ. Mechanism and control of the human sperm acrosome reaction. Hum Reprod 1993;8:2006–8.
44. De Jonge CJ, Barratt CL, Radwanska E, Cooke ID. The acrosome reaction–inducing effect of human follicular and oviductal fluid. J Androl 1993;14:359–65.
45. Saling PM, Storey BT. Mouse gamete interactions during fertilization in vitro: chlortetracycline as fluorescent probe for the mouse sperm acrosome reaction. J Cell Biol 1979;83:544–55.
46. Brucker C, Lipford GB. The human sperm acrosome reaction: physiology and regulatory mechanisms. An update. Hum Reprod Update 1995;1:51–62.
47. Cross NL, Meizel S. Methods for evaluating the acrosomal status of mammalian sperm. Biol Reprod 1989;41:635–41.
48. Oehninger S, Blackmore P, Morshedi M, Sueldo C, Acosta AA, Alexander NJ. Defective calcium influx and acrosome reaction (spontaneous and progesterone-induced) in spermatozoa of infertile men with severe teratozoospermia. Fertil Steril 1994;61:349–54.
49. Barros C, Gonzalez J, Herrera E, Bustos-Obregon E. Human sperm penetration into zona-free hamster oocytes as a test to evaluate the sperm fertilizing ability. Andrologia 1979;11:197–210.
50. Binor Z, Sokoloski JE, Wolf DP. Penetration of the zona-free hamster egg by human sperm. Fertil Steril 1979;32:664–70.
51. Rogers BJ, Van Campen H, Ueno M, Lambert H, Bronson R, Hale R. Analysis of human spermatozoal fertilizing ability using zona-free ova. Fertil Steril 1980;33:321–7.
52. Mao C, Grimes DA. The sperm penetration assay: can it discriminate between fertile and infertile men? Am J Obstet Gynecol 1988;159:279–86.
53. Vogiatzi P, Chrelias C, Cahill DJ, Creatsa M, Vrachnis N, Iliodromiti Z, et al. Hemizona assay and sperm penetration assay in the prediction of IVF outcome: a systematic review. Biomed Res Int 2013;2013:945825.
54. Aitken RJ, Buckingham DW, Fang HG. Analysis of the responses of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. J Androl 1993;14:132–41.
55. Mol BW, Meijer S, Yuppa S, Tan E, de Vries J, Bossuyt PM, et al. Sperm penetration assay in predicting successful in vitro fertilization. A meta-analysis. J Reprod Med 1998;43:503–8.
56. Aitken RJ, Thatcher S, Glasier AF, Clarkson JS, Wu FC, Baird DT. Relative ability of modified versions of the hamster oocyte penetration test, incorporating hyperosmotic medium or the ionophore A23187, to predict IVF outcome. Hum Reprod 1987;2:227–31.

57. Johnson A, Smith RG, Bassham B, Lipshultz LI, Lamb DJ. The microsperm penetration assay: development of a sperm penetration assay suitable for oligospermic males. Fertil Steril 1991;56:528–34.
58. Liu DY, Baker HW. Tests of human sperm function and fertilization in vitro. Fertil Steril 1992;58:465–83.
59. Franken DR, Oosthuizen WT, Cooper S, Kruger TF, Burkman LJ, Coddington CC, et al. Electron microscopic evidence on the acrosomal status of bound sperm and their penetration into human hemizonae pellucida after storage in a buffered salt solution. Andrologia 1991;23:205–8.
60. Liu DY, Baker HW. High frequency of defective sperm–zona pellucida interaction in oligozoospermic infertile men. Hum Reprod 2004;19:228–33.
61. Oehninger S, Morshedi M, Franken D. The hemizona assay for assessment of sperm function. Methods Mol Biol 2013;927:91–102.
62. Oehninger S, Coddington CC, Scott R, Franken DA, Burkman LJ, Acosta AA, et al. Hemizona assay: assessment of sperm dysfunction and prediction of in vitro fertilization outcome. Fertil Steril 1989;51:665–70.
63. Franken D, Kruger T, Oehninger S, Coddington CC, Lombard C, Smith K, et al. The ability of the hemizona assay to predict human fertilization in vitro in different and consecutive IVF/GIFT cycles. Hum Reprod 1993;8:1240–4.
64. Franken DR, Oehninger SC. The clinical significance of sperm–zona pellucida binding: 17 years later. Front Biosci 2006;11:1227–33.
65. Liu DY, Clarke GN, Martic M, Garrett C, Baker HW. Frequency of disordered zona pellucida (ZP)-induced acrosome reaction in infertile men with normal semen analysis and normal spermatozoa-ZP binding. Hum Reprod 2001;16:1185–90.
66. Liu DY, Baker HW. Disordered acrosome reaction of spermatozoa bound to the zona pellucida: a newly discovered sperm defect causing infertility with reduced sperm–zona pellucida penetration and reduced fertilization in vitro. Hum Reprod 1994;9:1694–700.
67. Bedford JM. Sperm/egg interaction: the specificity of human spermatozoa. Anat Rec 1977;188:477–87.
68. Oehninger S, Mahony MC, Swanson JR, Hodgen GD. The specificity of human spermatozoa/zona pellucida interaction under hemizona assay conditions. Mol Reprod Dev 1993;35:57–61.
69. Liu DY, Baker HW. Relationship between the zona pellucida (ZP) and ionophore A23187-induced acrosome reaction and the ability of sperm to penetrate the ZP in men with normal sperm-ZP binding. Fertil Steril 1996;66:312–5.
70. De Jonge CJ, Barratt CL. Methods for the assessment of sperm capacitation and acrosome reaction excluding the sperm penetration assay. Methods Mol Biol 2013;927:113–8.
71. Esterhuizen AD, Franken DR, Lourens JG, van Rooyen LH. Clinical importance of a micro-assay for the evaluation of sperm acrosome reaction using homologous zona pellucida. Andrologia 2000;33:87–93.
72. Bastiaan HS, Menkveld R, Oehninger S, Franken DR. Zona pellucida induced acrosome reaction (ZIAR), sperm morphology and sperm-zona binding assessments among subfertile men. J Assist Reprod Genet 2002;19:329–34.
73. Cayli S, Jakab A, Ovari L, Delpiano E, Celik-Ozenci C, Sakkas D, et al. Biochemical markers of spermatozoa function: male fertility and spermatozoa selection for ICSI. Reprod Biomed Online 2003;7:462–8.
74. Cayli S, Sakkas D, Vigue L, Demir R, Huszar G. Cellular maturity and apoptosis in human spermatozoa: creatine kinase, caspase-3 and Bclx expression in mature and diminished maturity sperm. Mol Hum Reprod 2004;10:365–72.
75. Huszar G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E, Vigue L. Hyaluronic acid binding by human spermatozoa indicates cellular maturity, viability, and unreacted acrosomal status. Fertil Steril 2003;79(Suppl 3):1616–24.
76. Nasr-Esfahani MH, Razavi S, Tavalaee M. Failed fertilization after ICSI and spermatozoaiogenic defects. Fertil Steril 2008;89:892–8.
77. Worrilow KC, Eid S, Matthews JM, Pelts EJ, Khoury C, Lieberman J. A multisite clinical trial evaluating PICSI® versus intracytoplasmic spermatozoa injection (ICSI): positive clinical outcomes observed in a prospective, randomized and double-blinded study. Fertil Steril 2009;92:S36–7.
78. Nijs M, Creemers E, Cox A, Janssen M, Vanheusden E, Van der Elst J, et al. Relationship between hyaluronic acid binding assay and outcome in ART: a pilot study. Andrologia 2010;42:291–6.
79. Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Clampaglia W, Filicori M. "Physiologic ICSI:" hyaluronic acid (HA) favours selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. Fertil Steril 2010;93:598–604.
80. Keel BA, Kelly RW, Webster BW, Zumbach KL, Roberts DK. Application of a bovine cervical mucus penetration test. Syst Biol Reprod Med 1987;19:33–41.
81. Franken DR, Oehninger S. Semen analysis and sperm function testing. Asian J Androl 2012;14:6–13.
82. Dong KW, Chi TF, Juan YW, Chen CW, Lin Z, Xiang XQ, et al. Characterization of the biologic activities of a recombinant human zona pellucida protein 3 expressed in human ovarian teratocarcinoma (PA-1) cells. Am J Obstet Gynecol 2001;184:835–43.
83. Chapman NR, Barratt CL. Sperm-zona interaction and recombinant DNA technology. Mol Hum Reprod 1997;3:646–50.
84. Hinsch E, Oehninger S, Schill WB, Hinsch KD. Species specificity of human and murine anti-ZP3 synthetic peptide antisera and use of the antibodies for localization and identification of ZP3 or ZPC domains of functional significance. Hum Reprod 1999;14:419–28.