Reproductive Sciences (2024) 31:863–882
https://doi.org/10.1007/s43032-023-01405-7

MALE REPRODUCTION: REVIEW

Clinical Utility of Sperm Function Tests in Predicting Male Fertility: A Systematic Review

Debarati Sanyal[1] · Deepshikha Arya[1] · Kumari Nishi[1] · Nafisa Balasinor[1] · Dipty Singh[1]

Received: 11 September 2023 / Accepted: 6 November 2023 / Published online: 27 November 2023

Abstract

Routine semen analysis provides considerable information regarding sperm parameters; however, it is not solely adequate to predict male fertility potential. In the past two decades, several advance sperm function tests have been developed. The present systematic review intends to assess the clinical utility of available advance sperm function tests in predicting the male fertility potential. A systematic literature search was conducted as per PRISMA guidelines using PubMed, MEDLINE, Google Scholar, and Cochrane Library. Different keywords either singly or in combination were used to retrieve the relevant articles related to sperm function tests, male fertility, and pregnancy outcomes. A total of 5169 articles were obtained, out of which 110 meeting the selection criteria were included in this review. The majorly investigated sperm function tests are hypo-osmotic swelling test, acrosome reaction test, sperm capacitation test, hemizona binding assay, sperm DNA fragmentation test, seminal reactive oxygen species test, mitochondrial dysfunction tests, antisperm antibody test, nuclear chromatin de-condensation (NCD) test, etc. The different advance sperm function tests analyse different aspects of sperm function. Hence, any one test may not be helpful to appropriately predict the male fertility potential. Currently, the unavailability of high-quality clinical data, robust thresholds, complex protocols, high cost, etc., are the limiting factors and prohibiting current sperm function tests to reach the clinics. Further multi-centric research efforts are required to fulfil the existing lacunas and pave the way for these tests to be introduced into the clinics.

Keywords Advance sperm function tests · Sperm parameters · Male infertility

Abbreviations

NCD	Nuclear chromatin de-condensation
ART	Assisted reproductive techniques
PCBs	Polychlorinated biphenyls
ROS	Reactive oxygen species
CASA	Computer assisted semen analysis
IVF/IUI/ICSI	In vitro fertilisation/intrauterine insemination/intracytoplasmic sperm injection
ZIAR	Zona induced acrosome reaction
TUNEL	Terminal deoxytransferase mediated deoxyuridine triphosphate (dUTP) nick end labelling
TdT	Terminal deoxyribonucleotide triphosphates
SCSA	Sperm chromatin structure assay
SCD	Sperm chromatin dispersion
TB	Toluidine blue
HOS	Hypoosmotic swelling test
MAR	Mixed antiglobulin reaction
IBT	Immunobead test
DCFDA	2',7'–dichlorofluorescin diacetate
ORP	Oxidation-reduction potential
GM1	Monosialotetrahexosylganglioside
ZP	Zona pellucida
HZA	Hemizona assay
HZI	Hemizona index
AO	Acridine orange
DFI	DNA fragmentation index
HDS	High DNA stainable cells
ASA	Antisperm antibodies
RCR	Respiratory control ratio
MMP	Mitochondrial membrane potential

✉ Nafisa Balasinor
nhbalasinor@gmail.com

✉ Dipty Singh
singhd@nirrch.res.in

[1] Department of Neuroendocrinology, ICMR-National Institute for Research in Reproductive and Child Health, Parel, Mumbai 400012, India

JC-1	Tetraethylbenzimidazolylcarbocyanine iodide
Mt-DNAcn	Sperm mitochondrial DNA copy number
NBT	Nitro blue tetrazolium
8-OHdG	8-Hydroxydeoxyguanosine
SDF	Sperm DNA fragmentation
RPL	Recurrent pregnancy loss
ETs	Embryo transfers
AR	Acrosome reaction
COH	Controlled ovarian hyper stimulation
LBR	Live birth rate
STF	Sperm function test

Introduction

Over the years, there has been a worldwide decline in human semen parameters and overall decline in fertility outcomes due to which infertile men resort to assisted reproductive techniques (ART) [1]. Male factor infertility is the cause of almost 50% of couples wanting to have a child. Male fertility is affected by many conditions like genetic disorders, varicocele, infections, endocrine disturbances, lifestyle choices, and idiopathic factors [2–4]. The continuum of environmental exposures to which a person is exposed at a time includes air pollutants (NO_2, Ozone, etc.), water pollutants like PCBs, and industrial chemicals like phthalates, which may also affect fertility [5]. Other known causes of male infertility include DNA fragmentation, reactive oxygen species (ROS) production, disruption in mitochondrial potential, abnormal epigenetic modifications, protamine depletion, and anti-sperm antibodies against sperm antigens. These factors, solely or in combination, may decrease sperm quality and also have an impact on blastocyst formation and embryo quality, and thereby affecting successful pregnancy [6, 7].

Semen contains secretion from the testis, epididymis, and various other accessory glands, which can convey the physiological/pathological information. Hence, conventional semen analysis is usually the first line of diagnostic tests used to evaluate the male fertility potential [8, 9]. The conventional semen analysis basically relies upon microscopic evaluation of parameters like sperm concentration, motility, and morphology in the ejaculate. Also, physical characteristics of semen like liquefaction time, volume, pH, odour, colour, and viscosity are evaluated. In order to maintain a uniform semen analysis procedure, World Health Organization (WHO) instituted a guideline and published first laboratory manual for semen analysis in 1980. The manual has been updated time to time with the latest version out in 2021 (Table 1) [10].

The conventional manual semen analysis is subjective and susceptible to various errors, which include intra-individual variability, lack of standardization of protocols among laboratories, and absence of training. Additionally, the latest

Table 1 Semen characteristics cut-off reference values as classified by world health organisation (WHO) and their method of detection

Semen characteristics	WHO (1980)	WHO (1987)	WHO (1992)	WHO (1999)	WHO (2010)	WHO (2021)	Method used
Volume (mL)	ND	≥ 2	≥ 2	≥ 2	1.5	1.4	Volume measurement by micropipette
Sperm count (10^6/mL)	20–200	≥ 20	≥ 20	≥ 20	15	16	Microscopic evaluation by phase contrast microscope/using computer assisted semen analysis (CASA)
Total sperm count (10^6)	-	≥ 40	≥ 40	≥ 40	39	39	Microscopic evaluation by phase contrast microscope/CASA
Total motility (% motile)	≥ 60	≥ 50	≥ 50	≥ 50	40	42	Microscopic evaluation by phase contrast microscope/CASA
Progressive motility	≥ 2	≥ 25%	≥ 25% (grade a)	≥ 25% (grade a)	32% (a+b)	30	CASA
Vitality (% alive)	-	≥ 50	≥ 75	≥ 75	58	54	Use of dyes that stain live and dead cells differentially
Morphology (% normal forms)	80.5	≥ 50	≥ 30	14	4	4	Microscopic evaluation by phase contrast microscope. Quantitative evaluation by dyes (Papinicolaou Giemsa, Shorr, and Diff-quik stain)

WHO guidelines are not uniformly adapted in different clinical/research settings, which make it even more difficult to analyse the result obtained from different settings [11]. More importantly, men with normal semen parameters can be both fertile and infertile, and thus advanced sperm function tests in adjunction with routine semen analysis is the need today.

In this systematic review, we have attempted to put together different sperm function tests available in the literature and assessed the clinical utility of these tests in predicting the male fertility potential.

Methods

Literature Search

This systematic literature search was conducted according to the PRISMA guidelines (Fig. 1) using PubMed, MEDLINE, Google Scholar, and Cochrane Library. Different keywords such as sperm DNA fragmentation tests, antisperm antibody test, mitochondrial dysfunction test, advanced sperm function test, hypo-osmotic swelling test, acrosome reaction test, capacitation, hemizona assay, seminal reactive oxygen species, sperm mitochondrial dysfunction, sperm agglutination, sperm nuclear chromatin decondensation, DNA damage and pregnancy, mitochondrial DNA copy number, sperm mitochondrial activity index, sperm intracellular ROS, antisperm antibody, mitochondrial membrane potential, sperm total antioxidant capacity, live birth rate, pregnancy outcome, spontaneous pregnancy, and assisted reproduction technology (IVF/IUI/ICSI) outcomes were used singly or in combination to retrieve the relevant articles.

Inclusion and Exclusion Criteria

Full-text articles having adequate data on human subjects in English language were included in this systematic review. The non-related and non-human studies were excluded. Posters, abstracts, letters to the editor, and editorials were also not considered to compile this systematic review.

Data Extraction

The articles following inclusion criteria were further reviewed independently by two authors and information available on the following sperm function tests was

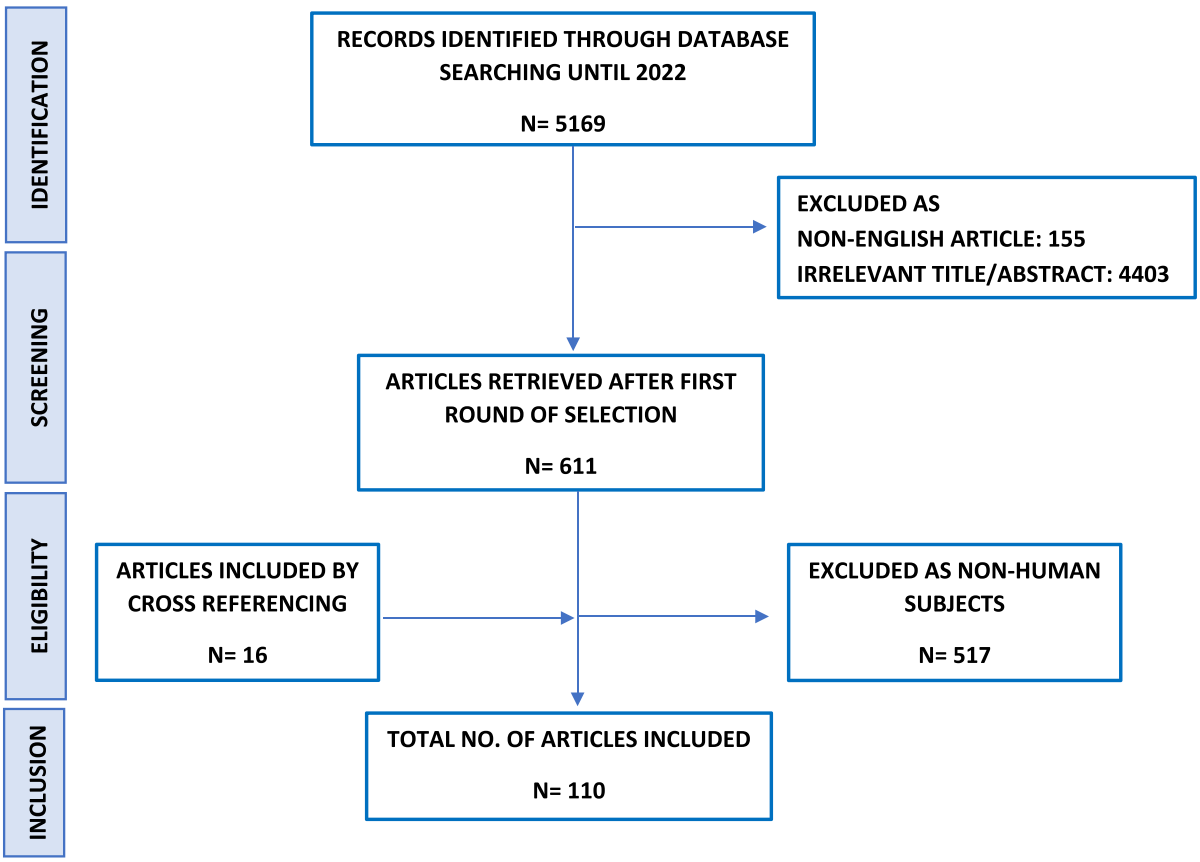

Fig. 1 Study selection criteria according to the PRISMA guidelines

extracted: "Sperm capacitation test, acrosome reaction test, hemizona binding assay, sperm DNA fragmentation test, seminal reactive oxygen species test, mitochondrial dysfunction tests, antisperm antibody test, hypo-osmotic swelling test, nuclear chromatin de-condensation (NCD) test." Also, data on assisted reproduction outcomes was extracted and presented in the review.

Results

A total of 5169 articles were obtained, out of which 155 were excluded as non-English articles, 4403 articles were excluded as they were not directly related to the addressed topic and 517 articles were excluded as they dealt with non-human subjects.16 cross-references were included and finally 110 articles were included in writing the review (Fig. 1). The majorly investigated sperm function tests are hypo-osmotic swelling test, acrosome reaction test, sperm capacitation test, hemizona binding assay, sperm DNA fragmentation test, seminal ROS test, mitochondrial dysfunction tests, antisperm antibody test, and nuclear chromatin de-condensation (NCD) test. Table 2 represents the data extracted from these eligible articles, which enlist the details of various sperm function tests reported in the literature.

Hypo-osmotic Swelling Test

The hypo-osmotic swelling test (HOS) is a simple, easy to perform, and accurate test that determines the sperm plasma membrane integrity. The test evaluates the ability of the spermatozoa to respond to changes in the osmolarity of the medium and its response to a hypo-osmotic environment. When the viable sperms are exposed to a hypo-osmotic medium, there is an influx of fluid, which causes the tail to swell and coil and this change in the tail morphology can be visualized under a phase contrast microscope. Higher the percentage of sperms with a swollen tail more likely is the fact that they have an intact plasma membrane. Sperm membrane integrity and its susceptibility to changes is very crucial for various events associated with fertilization like sperm capacitation, acrosome reaction, and spermatozoa binding to the zona pellucida layer of the ovum. Thus, an assay designed to evaluate the status of the spermatozoa membrane is of utmost importance in predicting fertility outcomes [40]. The spermatozoa are exposed to 50–100 mOsm lactose or sucrose solution and the fluid from the hypo-osmotic solution is exported across the plasma membrane and the sperm membrane integrity can be measured. The percentage of sperm with swollen or coiled tail (HOS+) are then evaluated [41]. If 60% of the spermatozoa are coiled, then it is considered as normal, and if 40% of the coiled spermatozoa are present in the semen sample, then it is considered as abnormal for fertility [42]. The HOS test induces several distinct categories of swelling, which are present in the sperm tail region. Tip swelling is when the rest of the tail is normal, but the tip of the tail is swollen; hairpin swelling is when the tail swells at the mid-piece and main piece junction and the tip may or may not show a swelling, shortened and thickened tail is when the tail swells that leads to the constriction of the surface causing shortening and partly or completely enveloped sperm tail where the tail balloons from swelling [40]. The HOS test is valuable in predicting the sperm variability, helps in infertility diagnosis, and widely used in sperm selection in assisted reproduction settings [40].

Sperm Capacitation Test

Capacitation is a biologically controlled phenomenon which is highly selective and is associated with a cascade of changes that occur once the sperm enters the female reproductive tract [43, 44]. The events involved in the capacitation of sperm renders it responsive to signals that originate from the cumulus-oocyte complex [43, 45]. The events involved in sperm capacitation are interconversion of various reactive nitrogen and oxygen species [43], loss of cholesterols from the plasma membrane involving a reversible lowering of the cholesterol/phospholipid ratio in the membranes leading to changes in membrane fluidity [43, 46], increase in tyrosine phosphorylation involving Src and cABL family kinases in the presence of ATP and appropriate pH [43, 47, 48] and calcium mediated hyper activated motility which is important for the penetration of the zona pellucida layer of the oocyte [49–51].

Moody et al. (2017) [12] established a laboratory-based protocol involving monosialotetrahexosylganglioside (GM1) localization measured by cap score sperm function test as a reliable, accurate and highly precise test that can be used to differentiate between sperms that respond to stimuli for capacitation and undergo various physiological changes required to fertilize the ovum from those that did not respond to cues of capacitation. Matamoros-Volante et al. (2018) [13] described another semi-automatized analysis using image-based flow cytometry as a powerful tool to study the intracellular events in a sperm to enable the study of various sperm factors associated with signalling with emphasis on tyrosine phosphorylation which is a hallmark in sperm capacitation. This study confirmed the role of PYK2 kinase and FYK2 kinase in tyrosine phosphorylation and provided evidence of the FYK2 expression and its subcellular localization in mammalian spermatozoa. Immunocytochemistry can also detect the subcellular localization of the tyrosine phosphorylation with the limitation that only a small number of cells can be analysed at a given time [52]. In conclusion, these techniques are useful in understanding the differential

Table 2 Details of sperm function tests reported in literature

Sperm attributes/functions	Tests available	Test details/method used	References
Sperm capacitation	CAP score sperm function test	GM1 localization measured by cap score; fluorescence microscopy	[12]
	Tyrosine phosphorylation	Subcellular localization of tyrosine phosphorylation; flow cytometry	[13]
Acrosome reaction	Zona induced acrosome reaction (ZIAR), induction by calcium ionophore	Microscopy, flow cytometry and fluorescently labeled agglutinin	[14] [15]
	Bismarck brown and rose bengal stain	Light microscope	[16]
	CD46 (present in the inner acrosome membrane)	Flow cytometry	[17]
Sperm zona pellucida interaction	Hemizona assay and competitive intact zona sperm binding test	Micromanipulation	[18] [15]
Sperm DNA damage	TUNEL assay	TdT and dUTP; flow cytometry/fluorescence microscopy	[19] [20]
	COMET assay	Spermatozoa are lysed with detergent and stained with SYBR Green I; gel electrophoresis and fluorescence microscopy	[21]
	SCSA assay	(Acridine orange) fluorescence microscopy	[22]
	SCD assay	(Acid denaturation) florescence microscopy	[23]
	TB test	(Toluidine blue staining) light microscope	[24] [25]
Sperm chromatin integrity	NCD test	(SDS and EDTA) light microscope	[26]
Sperm plasma membrane integrity	Hypoosmotic swelling test (HOS).	(Lactose or sucrose) light microscope	[27]
Antisperm antibody	Mixed antiglobulin reaction (MAR)	Light microscope	[28]
	Gel agglutination test	Light microscope	[29]
	Immunobead test (IBT)	Light microscope	[30]
Mitochondrial function	Mitochondrial reactive oxygen species (ROS) test	MitoSOX Red; DCFDA flow cytometry/fluorescence microscopy	[31–33]
	Mitochondrial membrane potential test (MMP)	JC-1 fluorescence; flow cytometry/fluorescence microscopy	[34]
	Mitochondrial copy number	Real-time PCR	[35–37]
Seminal reactive oxygen species	Direct tests: -chemiluminescence using luminol, nitro-blue tetrazolium, cytochrome C reduction test, fluorescein probe, electron spin resonance, and oxidation-reduction potential (ORP)	-	[38]
	Indirect tests: measurement of Endtz test, lipid peroxidation, chemokines, antioxidants/micronutrients/vitamins, ascorbate, total antioxidant capacity	-	[39]

phosphorylation status of the sperm during various stages of capacitation and hence can help in predicting male fertility status.

Acrosome Reaction Test

Through the process of fertilization, the sperm must first undergo the acrosome reaction before it can penetrate the zona pellucida (ZP) layer and fuse with the oolemma. Progesterone secreted by the cumulus cells is an important factor influencing capacitation as well as the acrosome reaction [53]. Acrosome reacted sperm can only penetrate the ZP and premature occurrence of the acrosome reaction can lead to an inability to penetrate the ZP layer. Defects in ZP induced acrosome reaction have been a rising cause of male infertility [54, 55].

Acrosome reaction can be evaluated using flow cytometry, immunofluorescent assays using fluorescently labelled lectins and chlortetracycline assays. Several factors have been known to induce the acrosome reaction, which includes cumulus-corona complex, follicular fluid, progesterone and intact or acid solubilised ZP [14, 56–58]. A study by Liu and Baker (1996) [14] compared the chemically induced acrosome reaction by the calcium ionophore A23187 and

by ZP. Sperm samples were collected from men with normal semen parameters according to the WHO criteria and subjected to acrosome reaction by A23187, intact ZP, and solubilised ZP. The acrosome reacted spermatozoa were distinguished by labelling with fluorescein labelled PSA and if more than half of the head showed a fluorescence, then the acrosome was considered to be intact and spermatozoa without any fluorescence was considered to be acrosome reacted. Sperm samples bound to intact ZP had a higher acrosome reaction than those subjected to solubilised ZP. So, the assessment of acrosome reaction should be done with sperms bound to intact ZP. A modification of the acrosome test, which does not require special reagents and equipment, has been described by Glazier et al. (2000) [16] which uses a combined approach of using HOS test and a double stain (Bismarck brown and rose bengal) and requires the usage of basic reagents and a light microscope. HOS test was used to detect viable sperms and the Bismarck brown and rose bengal was used to stain the post acrosome and acrosome region, respectively. A clear colour on the sperm head depicted a complete acrosome reaction. The cut-off set by this protocol was similar to that set by Carver-Ward et al. (1996) [17], in which CD46 present in the inner acrosome membrane was used as a marker to evaluate a viable acrosome reaction and analysed by flow cytometry. Zona induced acrosome reaction (ZIAR) is another method for induction of acrosome reaction by calcium ionophore. Here, the acrosomal status can be evaluated using microscopy, flow cytometry, and fluorescently labelled agglutinin.

Hemizona Binding Assay

Sperm zona pellucida binding tests most commonly includes the hemizona assay (HZA) and competitive intact zona sperm binding assay. HZA is an internally controlled assay and has been recommended as a diagnostic test to assess the binding of the sperm to zona pellucida to predict the fertilization outcome [15, 18]. In the HZA assay, each zona pellucida is cut into equal two halves or hemispheres by micromanipulation and one half is exposed to abnormal spermatozoa and the other half to spermatozoa from a normal sample. The results of the HZA are evaluated using the hemizona index [HZI = (bound sperm from sub-fertile men/bound sperm from fertile men) × 100]. The use of the HZA confers several advantages, which include the two halves of the zona pellucida, created by microdissection are equivalent a controlled comparison can be made, an internally controlled test can be carried out in the same oocyte, which circumvents the problem of using multiple oocytes and also ethical objections that might arise due to inadvertent fertilization of live viable oocyte is also eliminated [18]. Defective zona pellucida interactions have been seen in men with oligozoospermia and hence HZA assay should be performed in their case to predict their success rate in ART procedures.

Nuclear Chromatin De-condensation (NCD) Test

During the process of spermatogenesis there are several changes that occur in the haploid spermatid which includes elongation phase, cytoplasmic droplet ejection, and finally differentiation into mature spermatozoa [59]. During the process of mature sperm formation, diploid spermatogonia divide and differentiates into haploid round spermatids, which undergo cyto-differentiation including changes in chromatin. Testes specific histone variants, transition proteins and protamines replace the classical DNA bound histones and lead to a highly condensed paternal genome, which helps in the protection of the genome from various exogenic insults [60, 61]. The highly condensed nature of the nuclear chromatin in spermatozoa is due to the presence of S-S cross-links between the histone units [26]. In the NCD assay, the cleavage of the S-S links is induced by incubating the sperm, sodium dodecyl sulphate and ethylene diamine tetra-acetic acid together and the sample is visualized under a phase contrast microscope. Under the phase contrast microscope, the spermatozoa that do not decondense appear bright. Sperm nuclear chromatin condensation or chromatin integrity is also measured by analysing the levels of histone and protamine protein or transcript in sperm. A study done by Hamad 2019 [62] demonstrated the ratio of sperm's nuclear histones/protamine transcripts (H2A+H2B)/(PRM1+PRM2) were higher in infertile patient samples as compared to the normal samples and there was a negative correlation between the histones/protamine transcript ratio and other sperm parameters like sperm count, motility, progressive motility, membrane integrity, normal morphology, and was positively correlated with chromatin decondensation [62]. The data suggests that histones/protamine ratios are important measures for sperm quality and could be used to predict male infertility.

Sperm DNA Fragmentation Test

Damaged DNA in human spermatozoa is the rising cause of male infertility according to recent clinical evidences. It has been observed that the amount of damaged DNA is much greater in infertile males than among fertile males [63]. The main cause of sperm DNA damage could be: Intrinsic factors such as defects during the spermatozoa maturation process, impaired protamination and defects in chromatin remodeling; Extrinsic factors include both dietary and various environmental effects and finally a defect in the apoptotic pathway, which increases the cellular stress and hence leads to increased sperm DNA damage [64]. Different assays

are described for comprehensive analysis of sperm DNA fragmentation [65].

a) ***Terminal deoxytransferase mediated deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL):*** The TUNEL assay first described by Gorczyca et al. (1992) [19] utilizes the property of the terminal deoxyribonucleotide triphosphates (TdT) to non-preferentially add deoxyribonucleotide residues (dUTP) to the 3'OH groups of single and double stranded DNA. These cells are then subjected to flow cytometry and the spermatozoa positive for the TUNEL assay are shown to have more DNA damage. Sharma et al. (2016) [20] established a standard protocol, reference values, sensitivity, and specificity of the TUNEL assay using a new bench top flow cytometer which could make a prediction that there is an increased DNA damage associated with infertile men.

b) ***Alkaline comet assay:*** First introduced by Ostling and Johanson (1984) [21], the alkaline comet assay is also known as single cell gel electrophoresis. In this assay, spermatozoa are lysed with detergent after they are embedded in the agarose gel and the migration of the fragments is noted with gel electrophoresis. After the electrophoresis is complete, the slides are stained with SYBR Green I and visualized with the help of a fluorescence microscope. Intact DNA without any fragmentation remains in the head and the fragmented DNA moves farther than the head in the gel forming a tail [25]. Comet assay is a sensitive, cheap, and reliable method that can be used as an advanced sperm function test in routine semen analysis. To make the comet assay more accurate and informative, Albert et al. (2016) [66] developed a standardized automated high throughput comet (HT-COMET) assay that could be used in fertility clinics as a diagnostic test to assay the degree of sperm DNA damage. The comet assay is a low throughput technique and cannot be used, when there is a large cohort of sample are to be analysed because of its manual selection procedure, focusing, imaging, and semi-automatized screening. The automated HT-COMET assay is at advantage because of its high accuracy, evenness, and ability to analyse a large cohort of samples in a much reduced time. HT-COMET assay uses the Komet ^{KM} software, which allows the automatic scoring and screening of comets. HT-COMET assay as established in this study could also be used in the assessment of DNA damage profiles which is a reflection of the extent of chromatin damage.

c) ***Sperm chromatin structure assay (SCSA):*** SCSA is the most widely used technique that is routinely used in laboratories as a suitable test to detect the extent of sperm DNA damage. SCSA evaluates the ratio of single and double stranded DNA after the sperm nuclei is stained with Acridine Orange (AO) following denaturation by acid exposure. This is an indirect assay where; upon acid denaturation the DNA breaks are exposed which is then detected by AO. AO when bound to dsDNA (intact DNA) emits green fluorescence and when bound to ssDNA (denatured DNA) it emits red fluorescence. The SCSA evaluates the DNA fragmentation index (DFI), which is a ratio of the red to the total fluorescence intensity indicating the amount of denatured DNA over the total DNA present. DFI is a suitable indicator of the rate of fragmentation and is the most important parameter of SCSA. DFI value of 30% is considered to be clinically useful [22]. The SCSA is also used in the evaluation of high DNA stainable cells (HDS). HDS represents a distinct set of immature spermatozoa with incomplete chromatin condensation [22]. Another study by Enciso et al. (2013) [67] investigated the possibility of correlation between numerous chromosomal abnormalities and DNA damage in infertile men. Samples were evaluated for DNA damage using the SCSA assay and fluorescence in situ hybridization (FISH) with probes specific to chromosome 13 was used for detection of chromosomal abnormalities. The data obtained showed a significant correlation between the extent of DNA damage and the proportion of sperm with a numerical chromosome abnormality ($p < 0.05$) and concluded that the spermatozoa with chromosome abnormalities are more prone to DNA damage.

d) ***Sperm Chromatin Dispersion Test (SCD) Test/Halo:*** SCD test is an indirect technique developed by Fernandez et al. (2003) [23]. In this technique, when relaxed DNA after acid denaturation is loaded into agarose, it produces halos/chromatin dispersion, which can be visualized by fluorescence microscopy. Since sperms with more damage have more ssDNA and fragmented DNA have a characteristic small halo. A study by Greze et al. (2019) [68] compared the efficacy of TUNEL and SCD assay, which are two sperm DNA damage tests to determine whether they can be used interchangeably and concluded that despite of a good correlation value the SCD assay gives slightly underestimated measurements as compared to the TUNEL assay and requires further validation from each laboratory because these results cannot be considered absolutely conclusive because of the small sample size used for the study.

e) ***Toluidine blue staining test:*** This assay is a rapid, simple, and effective method that helps in the assessment of the integrity of the sperm chromosomal DNA. A thin smear of the semen sample after fixation and treatment with acid is stained with toluidine blue stain. The heads of the spermatozoa which have high chromatin DNA integrity are stained blue and the damaged DNA stains

purple [25]. The toluidine blue (TB) test when compared to SCSA and TUNEL assay, showed a strong correlation between the proportion of damaged cells detected by TUNEL and SCSA assay and that detected by the TB test. The proportion of cells with defective DNA integrity as detected by TB test was almost equal to the sum of two SCSA parameters (DNA fragmentation index and the proportion of high DNA stainable cells) [24]. TB positive cells indicate that the DNA is both fragmented with an abnormal chromatin structure and hence being an inexpensive method can be used in andrology laboratories worldwide to test for DNA integrity along with other routine semen analysis parameters.

Antisperm Antibody Test

Antisperm antibodies, which were first described by Rumke et al. (1954) [28], are present in both the semen and the cervical mucus and have an inhibitory effect on the sperm function. Antisperm antibodies negatively impact the motility of the sperm through the cervical mucus, uterine cavities, and fallopian tubes and also block the events of fertilization like sperm capacitation, acrosome reaction, and sperm–egg interaction [69].

To detect the presence of antibodies on the surface of the sperm membrane mixed antiglobulin reaction (MAR) and direct immunobead test (D-IBT) are routinely used in andrology laboratories worldwide according to the standards set by WHO. Gel agglutination test (GAT) is also one of the most significant diagnostic tests to determine the presence or absence of antisperm antibodies in the semen of infertile men [29]. Immunobead test (IBT) allows for the rapid detection of IgA and IgG which are bound to washed spermatozoa and also detects the location of binding and provides an estimate regarding the proportion of spermatozoa which are bound to the antibodies [70]. Almeida et al. (1986) [30], in their study, evaluated whether IBT could be used as a screening test for antisperm antibodies in the men with accuracy and reliability. The majority of men who tested positive for IBT had both IgA and IgG on their surface and the immunobeads were predominantly localized over the head and the end piece of the tail but were also localized over the whole spermatozoa with identical binding pattern of IgA and IgG antibodies. IBT positive samples had impaired sperm penetration of the cervical mucus evaluated by cervical mucus *in vitro* penetration test (CMPT) and there was also an increased incidence of circulating antibodies in the serum. Thus, IBT can be used as a routine screening assay which would help in better selection of sperms for ART.

While evaluating male infertility, one of the parameters that should be evaluated before screening for ASA is sperm agglutination, which is due to binding of antibodies on the surface of the spermatozoa. Study by Gatimel et al. (2018) [71] suggests that the tests for the presence of antisperm antibodies should only be done when there is a presence of sperm agglutination during routine analysis.

Mitochondrial Dysfunction Tests

Mitochondrial dysfunctions in human spermatozoa are increasingly recognized as a cause of male infertility. The mitochondria present in the somatic cells are different from that present in the spermatozoa as during germ cell differentiation, there is a rapid loss of the sperm's antioxidant defences. ROS such as hydroxyl radicals, superoxide anions, and hydrogen peroxide are generated in terms of oxidative stress in the mitochondria during cellular respiration. The antioxidant defences in the cell prevent the cell from succumbing to damages caused by ROS but when this balance is dismantled in the spermatozoa, there is an increased progression toward decreased motility. Mature spermatozoa contain 50–75 mitochondria, which are helically arranged around the mid-piece axoneme and are responsible for the generation of ATP by oxidative phosphorylation (OXPHOS) system, a key event required for sperm motility in the female reproductive tract [6, 72].

a) ***Mitochondrial reactive oxygen species (ROS) tests:*** Numerous assays designed to measure the ROS levels in spermatozoa have gained significance over the years. Cassina et al. (2015) [73] have shown that sperm mitochondrial dysfunction evaluated using respiratory control ratio (RCR) measured by high resolution respirometer is correlated to sperm motility. Sperm mitochondrial oxygen consumption is dependent on various sperm parameters and this study showed that there is a positive correlation of RCR with sperm morphology, sperm viability, and sperm concentration. The study showed a positive correlation of both progressive and total motility with oxygen consumption measured as RCR with mean RCR values were lower in groups with <32% motility than those with motility greater than this value. MitoSOX; a fluorochrome has been routinely used in flow cytometric analysis for the detection of mitochondrial ROS in cells including sperm and semen [31, 74]. The study by Marques et al. (2014) [31] showed that the semen sample is actually heterogeneous with different categories of sperm population and can be divided into MItoSOX- (one that included better quality sperm and hence was not stained by the probe), MitoSOX+ and MitoSOX++ which had worsened sperm characters. Thus, MitoSOX can be used to predict sperm mitochondrial ROS levels and help in better selection of spermatozoa for ART.
b) ***Mitochondrial membrane potential test:*** The reduction in mitochondrial membrane potential (MMP) has been

considered a suitable prognostic indicator of abnormal sperm associated with reduced male fertility. A study by Espinoza et al. (2009) [75] recommended that the measurement of MMP as a routine test to evaluate semen quality. The study analysed three interrelated events namely ROS levels, phosphatidylserine (PS) externalization and sperm mitochondrial potential in fertile individuals and those undergoing fertility treatments using the fluorescent compounds dihydroethidium, JC-1 and annexin V-FITC, respectively. The results indicated that men with normal semen parameters had a higher MMP than men with abnormal semen parameters. MMP positively correlates with sperm motility. In addition, MMP showed a significant correlation with all sperm parameters analysed, namely, motility, viability, morphology, sperm count, and volume. The correlation between prohibitin (a major mitochondrial inner membrane protein) and mitochondrial membrane potential (MMP) has been described by Chai et al. (2017) [34] using JC-1 assay.

c) ***Mitochondrial DNA copy Number:*** Sperm mitochondrial DNA copy number (mtDNAcn) accounts for mitochondrial DNA copies per nuclear DNA copy [76]. Several human studies have shown the association of high mtDNAcn with poor semen parameters. Few studies have also reported the association of mtDNAcn with sperm motility and lower pregnancy rate [77–80]. These findings indicate that sperm mtDNAcn may be a novel biomarker of semen quality and pregnancy outcome. To estimate the mtDNAcn, quantitative real-time PCR (qPCR) has been used most frequently and current gold standard method due to its low cost and quick turnaround time [35]. Few studies have reported digital PCR (dPCR) as the new gold standard for mtDNAcn estimation technique due to its sensitivity and absolute copy number quantify ability [36, 37].

Seminal Reactive Oxygen Species Test/Oxidative Stress Test

Oxidative stress is one of the most well-established factors associated with male infertility due to its effect on both DNA integrity and impairment of sperm motility [81]. Various tests for detection of oxidative stress include reactive oxygen species (ROS) assays, total antioxidant capacity (TAC) assay, and malondialdehyde (MDA) assay [38].

Nitro blue tetrazolium (NBT) assay is a photometric method of detection of coloured formation that is an indicator of seminal ROS production [82]. NBT assay is a simple and inexpensive test which can be used routinely but suffers from the lack of standardization protocol. Tunc et al. (2010) [82] developed a standardized protocol for the NBT assay and correlated the results obtained with those obtained from assessing sperm DNA integrity, apoptosis, and sperm motility. In their study, semen samples from both fertile and etiology infertile men were assessed for seminal ROS (NBT assay), sperm DNA integrity (TUNEL assay), apoptosis (Annexin V), and sperm motility. The production of seminal ROS was higher in infertile men than in fertile men. A negative correlation existed between formazan production and sperm motility, which suggested that with an increase in ROS production there is a decrease in the sperm motility. 8-Hydroxydeoxyguanosine(8-OHdG) is a DNA adduct that can act as a biomarker and can be commonly used for detection of DNA damage due to oxidative stress. Another study by Kao et al. (2008) [83] demonstrated that sperm which has higher oxidative damage (measured by levels of 8-OHdG and lipid peroxides) have a lower antioxidant capacity and negatively correlated with sperm motility. The levels of various antioxidants like retinol, α-tocopherol, ascorbate, and protein thiols were measured, and it was seen that the antioxidant capacities were lower in men diagnosed with infertility.

A study by Shen et al. (2000) [84] established 8-OHdG as a suitable and quantitative biomarker for the oxidative DNA damage induced by ROS in human sperm along with TUNEL, single cell gel electrophoresis (SCGE) and Comet assay. Majzoub et al. (2018) [85] assessed the relationship between seminal oxidation-reduction potential (ORP) and sperm DNA fragmentation (SDF) for the first time and also evaluated its effect on different sperm parameters. ORP was measured by the male infertility oxidative system (MiOXSYS) that measures the net balance between the oxidants and reductants in any given media. In the control patients, there was no correlation between SDF and ORP, but there was a significant correlation between ORP and SDF levels in infertile group.

Homa et al. (2019) [86] evaluated the efficacy of two different assays for measuring oxidative stress in seminal plasma, namely; chemiluminescence assay and oxidation-reduction potential (sORP). Semen samples collected were measured for seminal ROS using either a chemiluminescence assay or an electrochemical assay to measure the oxidation-reduction potential. In samples with abnormal semen parameters, ROS, sORP, DFI, and HDS were elevated as compared to the control samples but in samples which had polymorphonuclear leucocytes (PMN); there was an increased ROS level but there was no change in the sORP and DFI. This assay validated the need of using both tests in conjunction for the evaluation of oxidative stress in different samples with abnormal semen parameters with special emphasis on those samples where leucocytes are detected.

The Suitability of Sperm Function Tests in Predicting Male Fertility and Pregnancy Outcomes

Routine semen analysis is widely used in andrology laboratories for the male infertility diagnosis but its limited predictive value emphasizes on the need to include more

advanced sperm function tests. Number of sperm function tests has been reported in the literature; however, very few studies report their predictive value for fertilization and pregnancy rates (Table 3).

A randomized control study ($n = 79$) by Sallam et al. (2005) [98] has concluded that sperm selected via modified HOS test is better suited for ICSI as compared to selection based on morphology alone. Low HOS test scores can act as a predictor of the formation of defective embryos which translates to a lower fertilization rate [101]. A study by Tartagni et al. (2002) [102] reported prediction of a successful pregnancy with the HOS test > 50%, had a sensitivity of 64%, a specificity of 75%, respectively.

The HZA can evaluate the relationship between the sperm-zona binding and sperm fertilizing ability in IVF settings [103]. Oehninger et al. (1989) [18] indicated that patients with poor fertilization rates in IVF showed a lower binding than successful fertilization cases which makes HZA is a useful tool for the evaluation of dysfunctional sperm-zona pellucida binding. Further, HZA along with other factors like sperm motility, sperm morphology and sperm viability can predict IVF outcome. Franken et al. (1989) [97] demonstrated that binding to the hemizona was significantly higher for the group with reported IVF success as compared to the failure group. Another study by Gamzu et al. (1994) [92] aimed to assess the prognostic value of HZA in determining the success of IVF with a large sample of patients to establish its validity. The threshold value for HZI that gave the best predictive value in this analysis was 23%, and thus this could be used as a pre selection test before IVF. HZA can also be used as a predictor of pregnancy outcome in patients undergoing controlled ovarian hyper stimulation (COH) and intrauterine insemination (IUI) and thus help in the counselling of couples before any treatment is advised. Patients having HZI < 30 had a lower pregnancy rate as compared to patients with HZI ≥ 30. HZA can act as a predictor of fertilization in IVF but it can also help in the prediction of pregnancy in IUI. The threshold value HZI < 30 for poor fertilization established previously for IVF was also statistically valid in the IUI setting [87].

The assessment of sperm chromatin is essentially important and helps in the prediction of fertility. SCSA assesses the sperm nuclear chromatin integrity. Studies on the assessment of SCSA as a predictor of fertility/pregnancy outcome have shown varied results. Bungum et al. 2004 [22] correlated the results of SCSA with the outcome of IVF, IUI, and ICSI in 306 couples undergoing ART. The assay showed that pregnancy is possible despite of high DFI because oocyte and the embryo can repair the DNA damage to a certain extent. The chances of pregnancy/delivery were higher in those groups were the DFI ≤ 27% and HDS ≤ 10% in all the 3 ART groups. Interestingly, the pregnancy outcomes in the group with DFI >27% were better in ICSI cases than IVF group [22]. Study by Lin et al. (2008) [95] has shown that men with high sperm DFI (> 27%) and HDS >15% could achieve successful pregnancy and delivery after either IVF or ICSI; however, the abortion rates were increased in IVF cases suggesting that ICSI could be recommended in such cases. On the other hand, Virro et al. (2004) [104] have shown that men with high levels of DNA fragmentation (> or =30% DFI) had low blastocyst rates and pregnancy rate but the fertilization rate was not affected [95]. Nijs et al. [105] also confirmed the threshold of ≤ 15% HDS for obtaining successful fertilization and pregnancy in IVF; however, ICSI outcome was not affected by any conventional or functional sperm parameters as it avoids the biological anomalies in sperm [105]. Meseguer et al. (2011) [96] later showed that the effect of SDF on pregnancy was independent of the sperm origin (fresh or thawed) and the fertilization procedure. For every 10% increase in DFI there was a decrease in the chances of achieving pregnancy by 1.31, if oocytes from fertile women were used. The investigators attributed the heterogeneity in the results of DNA fragmentation in predicting pregnancy outcomes to the DNA damage repair capacity of the oocyte. A systematic review and meta-analysis suggested that the recent SDF tests are not sufficient to discriminate couples who have a low chance to conceive from those who have a greater chance of conceiving after medically assisted reproduction (MAR) [106]. HOST being an easy to conduct test can be used as an alternative. Stanger et al. (2010) [107] have established that low HOST values in neat semen samples were significantly associated with increased DNA damage ($p < 0.01$) evaluated by both the TUNEL and SCSA assay. Esteves et al. (2022) [91] assessed the reliability of the SCD assay to evaluate sperm DNA damage in infertile men ($n = 219$). They classified the infertile men in normal, intermediate, and high SDF categories and evaluated the reliability of the assay by re-testing SDF within a 3-month interval under similar conditions. Their observations overall support SCD test for patient classification using predefined SDF thresholds.

Recurrent pregnancy loss is a devastating problem which affects couple worldwide and standard semen parameters are not sufficient enough to predict this recurrent pregnancy loss (RPL). An assessment of the sperm DNA integrity may act as a prognostic factor for the determination of idiopathic recurrent pregnancy loss (iRPL) following spontaneous conception. A systematic review and meta-analysis by Mcqueen et al. (2019) [108] suggested that there is an association between RPL and DNA fragmentation and there is a requirement for further studies to correlate the DNA fragmentation rate with miscarriage frequency, live birth rates, etc.

Table 3 Studies reporting suitability of different sperm function tests in predicting male fertility and pregnancy outcomes

Author, year	Purpose of sperm function test	Study type	Study group	Method used	Outcomes	Conclusion
Arslan et al. (2006) [87]	HZA value is used as a predictor of pregnancy in patients undergoing controlled ovarian hyperstimulation (COH) and intrauterine insemination (IUI)	Prospective clinical study	$n = 82$ couples with unexplained or male factor infertility that underwent 313 IUI cycles	Hemizona assay (HZA)	Patients with a Hemizona index (HZI) of <30 had a significantly lower pregnancy rate compared to patients with an HZI of ≥30	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
Bielsa et al. (1994) [88]	The presence of abnormal acrosomes results in poor fertilization rates and infertility	Prospective study	$n = 156$ couples attending an infertility clinic Three groups of men were studied: Group I ($n = 27$), the control group, consisted of healthy fertile men with normal semen parameters Group II ($n = 16$) consisted of infertile men, whose partner became pregnant by intra-uterine insemination (IUI) and a female factor could therefore be ruled out Group III ($n = 140$), men with primary infertility > 12 months	Acrosome reaction tests	Acrosome alterations were significantly more frequent in group III compared with group II: 21.4 ± 0.7 and 5.9 ± 1.7, respectively	High numbers of acrosome-reacted spermatozoa are required for good fertilization rates
Bungum et al. (2004) [22]	Identifies the spermatozoa with abnormal chromatin packaging defined as susceptibility to acid-induced DNA denaturation in situ	Cohort study	$n = 306$ consecutive couples undergoing assisted reproduction were included. IUI was performed in 131, IVF in 109, and ICSI in 66	Sperm chromatin structure assay (SCSA)	For IUI, the chance of pregnancy/delivery was significantly higher in the group with DFI 27% or HDS >10%	No statistical difference between the outcomes of IVF versus ICSI was observed in the group with DFI 27% group, however, the results of ICSI were significantly better than those of IVF

Table 3 (continued)

Author, year	Purpose of sperm function test	Study type	Study group	Method used	Outcomes	Conclusion
Calvo et al. (1994) [89]	Assessment of acrosome reaction (AR) inducibility is a useful diagnostic tool in the prospective evaluation of a man's fertilizing potential	Prospective study	$n = 232$ infertile patients going for in vitro fertilization (IVF)	Acrosome reaction tests	AR-positive patients are 2.9 times more likely to achieve fertilization than patients with a failed AR	AR has a greater effect on the fertilization rate.AR assessment is a clinically useful diagnostic tool in determining a patient's likelihood of achieving fertilization at IVF
Esteves et al (2007) [90]	Direct immunobeads test is carried out for detecting antisperm antibodies in semen. Formation of antisperm antibodies (ASA) may be a consequence of rupture in the blood testis barrier	Retrospective study	$n = 986$ patients data reviewed submitted to ICSI cycles	Screening for the presence of antisperm antibodies (ASA) in the semen by using the direct immunobeads test (IBT)	Fertilization, cleavage rate, velocity, percentage of good quality embryos, as well as clinical pregnancy and miscarriage rates did not differ among different ASA level groups	ICSI outcomes are not influenced by ASA
Esteves et al. (2022) [91]	Sperm chromatin dispersion (SCD) test, a novel assay for detecting sperm DNA fragmentation (SDF)	Diagnostic test reliability study	$n = 219$ men with infertility SDF assessment in two ejaculates of the same subjects within a 3-month interval, using the SCD assay performed and analyzed by the same observers under similar testing conditions	Sperm chromatin dispersion (SCD) assay	The agreement rate was highest (approximately 80%) in ejaculates initially classified as either normal or high and lowest (approximately 60%) among those with intermediate SDF levels	SDF assessments by the SCD assay are stable enough to be of potential clinical value A substantial intraindividual agreement of paired SCD assay results to classify men with infertility into three SDF categories: normal, intermediate, and high
Gamzu et al. (1994) [92]	Detection for binding of human spermatozoa to human zonae pellucidae to predict fertilization potential	Case-control study	$n = 133$ patients who were referred for semen evaluation Thirty samples were tested twice to assess interassay variation. Seventy couples were also referred for IVF	Hemizona assay (HZA)	The intra-assay and interassay coefficient of variation were 8% and 14%, respectively. Hemizona assay results had the highest correlation with sperm morphology Of all parameters evaluated, fertilization rates were best predicted by hemizona index (HZI)	The HZA is a valuable prognostic test for IVF. With a threshold HZI of 23%, it has a good predictive value for fertilization rates in IVF and may thus be used for patient preselection before IVF

Table 3 (continued)

Author, year	Purpose of sperm function test	Study type	Study group	Method used	Outcomes	Conclusion
Hervás et al. (2022) [93]	Terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) assay for determining sperm DNA fragmentation (SDF)	Multicenter retrospective cohort study	Data from $n = 864$ couples using donated eggs and undergoing ICSI were analyzed	TUNEL assay	A total of 1,903 ICSI cycles were considered, encompassing 6340 donated oocytes, 2543 embryos, and 1145 ETs. Comparing ≤15% SDF (low) with >15% SDF (high) or by 10% SDF ranges, the live birth rate (LBRs) per first embryo transfer (ET) and per all ETs did not significantly differ.	High SDF does not affect the live birth rate (LBR) Also, the cumulative chance to have a child, when calculated per embryo transfer (ET), per embryo replaced (EmbR), and per donated metaphase II oocyte used in couples undergoing ICSI cycles, is not affected
Janssen et al. (1992) [94]	To investigate the influence of antisperm antibodies in the male, the female, or both partners on the outcome of IVF treatment	Prospective study	$n = 207$ patients attending the infertility clinic	Sperm antibodies in the patient serum were detected using an ELISA kit	-	IVF provides an equal chance of conception in couples with antisperm antibodies compared to couples with no antisperm antibodies, provided all the other sperm functions are normal
Lin et al. (2008) [95]	To investigate the relationship between sperm chromatin structure assay (SCSA) parameters, DNA fragmentation index (DFI) and high DNA stainability (HDS), and outcomes of IVF and intracytoplasmic sperm injection (ICSI)	Retrospective review and prospective study	$n = 223$ couples undergoing conventional IVF ($n = 137$) and ICSI ($n = 86$)	Sperm chromatin structure assay (SCSA)	There were no significant differences in IVF and ICSI fertilization rate, good embryo rate, and pregnancy rate (PR) between high, moderate, and low DFI or HDS groups A statistically insignificant trend was toward an increased abortion rate in the high DFI (>27%) group	Sperm DNA fragmentation probably affects sperm motility. The DFI correlated negatively with sperm motility, and HDS correlated negatively with sperm morphology and concentration The potential adverse effect of sperm DNA damage on the quality of post-implantation embryos and spontaneous abortion should be a concern

Table 3 (continued)

Author, year	Purpose of sperm function test	Study type	Study group	Method used	Outcomes	Conclusion
Meseguer et al. (2011) [96]	To quantify the effect of sperm DNA fragmentation (SDF) on reproductive outcomes by evaluating the most statistically significant bias factors using logistic regression	Prospective blind observational cohort study	$n = 210$ male partners of couples undergoing in vitro fertilization (IVF) or first intracytoplasmic sperm injection (ICSI) cycles with fresh or thawed sperm with the women's own or donated oocytes	Sperm DNA fragmentation (SDF) assay	SDF had a statistically significant negative impact on the chance of pregnancy. For every 10% increase in SDF, the probability of not achieving pregnancy increased by 1.31	Oocyte quality conditions the extent of the negative impact of SDF on pregnancy; this can be overcome when good-quality oocytes are employed
Oehninger et al. (1989) [97]	Detection for binding of human spermatozoa to human zonae pellucidae to predict fertilization potential	Case-control study	Case $n = 28$ men participated in the IVF program with two or more years of infertility Control $n = 4$ proven fertile donors who had fathered a child in the last 2 years.	Hemizona assay (HZA)	Patients with poor fertilization rates in IVF had significantly lower binding than those with successful fertilization	The HZA is a valuable tool for evaluating dysfunctional sperm-zona binding, with good predictive value for IVF results
Sallam et al. (2005) [98]	Selection of immotile testicular spermatozoa in patients treated with ICSI	Randomized controlled study	$n = 79$ couples with immotile testicular spermatozoa treated with ICSI were randomly assigned into two groups Group I: spermatozoa used for injection were selected using the modified HOS test Group II: spermatozoa were selected based on their morphology	Modified hypo-osmotic swelling (HOS) test	Fertilization rate in HOS test group: 43.6% Fertilization rate in no-HOS test group: 28.2% The pregnancy and ongoing pregnancy rates were higher in the HOS test group compared with the no-HOS test group	Modified HOS test selected viable sperm from among immotile testicular spermatozoa for ICSI results in higher fertilization, pregnancy, and ongoing pregnancy rates compared to morphological selected
Sukcharoen et al. (1995) [99]	Acrosome reaction test is the predictor for the ability of human spermatozoa to achieve fertilization in vitro	Prospective study	$n = 41$ couples who underwent IVF-ET therapy	Acrosome reaction tests	Spermatozoa that had completely lost their outer acrosomal membranes exhibited a strong negative correlation with fertilization rate	Decrease in the acrosome reacted sperm, there is a decrease in the fertilization rate in IVF and ICSI

Table 3 (continued)

Author, year	Purpose of sperm function test	Study type	Study group	Method used	Outcomes	Conclusion
Zorn et al. (2003) [100]	High seminal reactive oxygen species (ROS) are related to poor semen quality and impaired fertilization.	Prospective study	$n = 147$ male partners of infertile couples	ROS were assessed with luminol chemiluminescence assay	High seminal ROS levels are associated with impaired sperm fertilizing ability and lower pregnancy rates after IVF. In ICSI, a negative association of ROS with embryo development to the blastocyst stage has been observed	Negative correlation between ROS production and embryo development

Hervás et al. (2022) [93] studied the effect of SDF (TUNEL assay) on ICSI outcomes by evaluating the per embryo transfers (ETs) collective live birth rates (CLBRs), embryos replaced (EmbR), and metaphase II (MII) oocytes required in successive treatments to attain the first newborn. They concluded that high SDF does not affect the LBR. Also, the cumulative chance to have a child in couples undergoing ICSI cycles is not affected per ET, per EmbR, and per donated MII oocyte used.

Generation of short-lived reactive oxygen radicals has a detrimental effect on various sperm functions and may cause premature acrosome reaction, lipid peroxidation, which can induce the apoptotic cascade causing DNA fragmentation and can cause a decrease in the mitochondrial membrane potential. Hammedah et al. (2006) [109] showed that there is a negative correlation between the production of ROS and fertilization rate in IVF and ICSI programs. Zorn et al. (2003) [100] also found that ROS level had a negative correlation with pronuclear formation and sperm MMP is a negative correlation between ROS production and embryo development. In ICSI patients, the sperm MMP was affected by both H_2O_2 and O_2^- but H_2O_2 exclusively has a role in the prevention of pronucleus formation [110]. The reason for the poor pronuclear formation in the presence of intracellular ROS is attributed to the destruction of microtubules and cytoskeletal structures by the high ROS levels.

Progesterone-induced acrosome reaction is an extremely important event that dictates the success of fertilization, and hence more research in this area guarantees better treatment for male infertility [111]. Throughout the literature there has been conflicting evidence regarding the use of acrosome reaction tests in the prediction of fertilization rates with some studies claiming that with a decrease in the acrosome reacted sperm, there is a decrease in the fertilization rate in IVF and ICSI [88, 89, 99, 112], and some studies claim no correlation at all between the status of acrosome reaction and the fertilizing ability [113]. Acrosome reaction (AR) and sperm DNA fragmentation are good predictors of fertilization and blastocyst rate, respectively. Recently, an equation combining sperm chromatin integrity and AR developed by investigators can work in the individual level and predict fertilization and blastocyst rates [114].

Antisperm antibodies are known to hamper the process of fertilization by hindering the passage of the sperm through the female genital tract. ASAs interfere by binding to specific antigens present on the sperm membrane, preventing the interaction of the sperm with the oocyte and also preventing the acrosome reaction to occur. A study by Janssen et al. (1992) [94] concluded that IVF provides an equivalent conception chance in couples with antisperm antibodies provided all the other sperm functions are normal. The results obtained by Vujisic et al. (2005) [115] and N. Sukcharoen et al. (1995) [116] further stated that the presence of

antisperm antibodies in the semen or the follicular fluid does not have an impact on the IVF outcome. Esteves et al. (2007) [90] have shown in their study that ICSI outcomes are also not influenced by ASA.

Discussion

Conventional semen analysis has been an integral part of clinical andrology and male fertility evaluation. While the test gives a fair idea of the seminal characteristics, it cannot be used as an accurate predictor of male fertility potential and fecundity [8, 11]. This notion comes from the fact that around 10% of men with normal semen analysis are infertile and similar percentage of men with abnormal semen analysis is fertile. Hence, the low predictive capacity of conventional semen analysis has necessitated the quest to develop more sensitive and specific sperm function tests on which clinicians can base their preliminary diagnosis [11, 117]. However, it may not be necessary to go for specialized sperm function tests for every individual presenting to andrology clinic. Men with fairly good semen parameters may not require to be tested. But for those with borderline semen parameters or with history of infertility, it becomes necessary to perform a battery of tests to evaluate sperm quality [11, 118].

Over the past two decades, a number of sperm function tests have been developed to determine the quality of spermatozoa. These tests basically evaluate the intact sperm plasma membrane and acrosome, acrosome reaction, capacitation, sperm–zona pellucida interactions, chromatin quality, nuclear and mitochondrial sperm DNA integrity, mitochondrial functions, etc [118]. These SFTs can help clinicians to make more precise diagnosis of male factor contributing to infertility. This may also help in treating the underlying conditions and may in turn, help in improving sperm quality/functions and pregnancy outcomes. ART is the ultimate disease management option for infertility [119]. These tests could also help in better patient counselling and more tailored and effective ART.

However, majority of these sperm function tests singly or together do not have proven clinical value in predicting fertility and have not been adopted into routine clinical use as yet. The basic limitation of these sperm function tests to reach the clinics is unavailability of high-quality clinical data correlating the sperm function to fertilization or pregnancy outcome [11]. Any test to be clinically relevant, it necessarily has strong positive predictive value for fertilization capacity and pregnancy outcome as well as have small overlap between fertile and infertile samples. Also, there is a need to undertake multi-centric trials which can establish the robust thresholds for these SFTs. The other inherent technical issues limit these tests to reach to the routine clinical practice are complexities to set up these tests and equipment costs. Requirement of more robust, simplified, reliable, cheaper, and reproducible SFTs in male andrology work flow is evident.

So far, growing literature on sperm OS and SDF measurement has highlighted its clinical utility [100, 109, 120]. The condition of oxidative stress is present in 30% to 80% of cases and sperm DNA damage is observed in approximately 5% to 25% of infertile men with normal and/or abnormal semen analysis [22, 95, 104, 105]. Hence, including both these sperm function tests in to male infertility diagnostic workup could be relevant.

Conclusion

Conventional semen analysis has low prognostic value in predicting pregnancy outcomes. Therefore, advance sperm functional tests are much needed in the workup of the infertility clinics. This could be helpful to guide the clinician to make more precise clinical decisions. Various attributes together contribute to sperm function and fertilization capability. Hence, any single sperm function test may not be able to predict its quality and functions adequately. An array of high-sensitivity tests with high pregnancy predictive value and low false positive rates are required to be introduced to the andrology clinical practice. Currently, unavailability of the high-quality clinical data, robust thresholds, complex protocols, and high cost, etc. are the limiting factors prohibiting current sperm function tests to reach to clinics. Further multi-centric research efforts are required to fulfil the existing lacunas and pave the way for these tests to be introduced into the clinics. Including OS and SDF tests for male fertility evaluation could be clinically useful.

Acknowledgements The authors acknowledge the support from ICMR-National Institute for Research in Reproductive and Child Health. Debarati Sanyal and Deepshikha Arya acknowledge the Indian Academy of Science (Summer Fellowship) and UGC (Junior Research Fellowship), respectively.

Author Contribution DS and NB conceived the idea of the systematic review. DS conducted the systematic review and drafted the manuscript. DS and DA crosschecked the review process and organized the manuscript with necessary additions. NB and KN proofread the draft and gave critical inputs. All authors agreed upon the final draft.

Data Availability Not applicable.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable

Consent to Participate Not applicable

Consent for Publication Not applicable

Conflict of Interest The authors declare no competing interests.

References

1. Mann U, Shiff B, Patel P. Reasons for worldwide decline in male fertility. Curr Opin Urol. 2020;30(3):296–301. https://doi.org/10.1097/MOU.0000000000000745.
2. Sharlip ID, Jarow JP, Belker AM, Lipshultz LI, Sigman M, Thomas AJ, Schlegel PN, Howards SS, Nehra A, Damewood MD, Overstreet JW, Sadovsky R. Best practice policies for male infertility. Fertil Steril. 2002;77(5):873–82. https://doi.org/10.1016/s0015-0282(02)03105-9.
3. Kumar N, Singh AK. Trends of male factor infertility, an important cause of infertility: a review of literature. J Hum Reprod Sci. 2015;8(4):191–6. https://doi.org/10.4103/0974-1208.170370.
4. Esteves SC. Novel concepts in male factor infertility: clinical and laboratory perspectives. J Assist Reprod Genet. 2016;33(10):1319–35. https://doi.org/10.1007/s10815-016-0763-8.
5. Krzastek SC, Farhi J, Gray M, Smith RP. Impact of environmental toxin exposure on male fertility potential. Transl Androl Urol. 2020;9(6):2797–813. https://doi.org/10.21037/tau-20-685.
6. Durairajanayagam D, Singh D, Agarwal A, Henkel R. Causes and consequences of sperm mitochondrial dysfunction. Andrologia. 2021 Feb;53(1):e13666. https://doi.org/10.1111/and.13666.
7. Arya D, Balasinor N, Singh D. Varicocoele-associated male infertility: cellular and molecular perspectives of pathophysiology. Andrology. 2022;10(8):1463–83. https://doi.org/10.1111/andr.13278.
8. Esteves SC. Clinical relevance of routine semen analysis and controversies surrounding the 2010 World Health Organization criteria for semen examination. Int Braz J Urol. 2014;40(4):443–53. https://doi.org/10.1590/S1677-5538.IBJU.2014.04.02.
9. Vasan SS. Semen analysis and sperm function tests: how much to test? Indian J Urol. 2011 Jan;27(1):41–8. https://doi.org/10.4103/0970-1591.78424.
10. Björndahl L. O-285 The 6th edition of the WHO manual for the examination and processing of human semen: how should it affect our practice? Human Reproduction. 2023;38(Supplement_1):dead093-345.
11. Douglas C, Parekh N, Kahn LG, Henkel R, Agarwal A. A novel approach to improving the reliability of manual semen analysis: a paradigm shift in the workup of infertile men. World J Mens Health. 2021;39(2):172–85. https://doi.org/10.5534/wjmh.190088.
12. Moody MA, Cardona C, Simpson AJ, Smith TT, Travis AJ, Ostermeier GC. Validation of a laboratory-developed test of human sperm capacitation. Mol Reprod Dev. 2017;84(5):408–22. https://doi.org/10.1002/mrd.22801.
13. Matamoros-Volante A, Moreno-Irusta A, Torres-Rodriguez P, Giojalas L, Gervasi MG, Visconti PE, Treviño CL. Semi-automatized segmentation method using image-based flow cytometry to study sperm physiology: the case of capacitation-induced tyrosine phosphorylation. Mol Hum Reprod. 2018;24(2):64–73.
14. Liu DY, Baker HW. A simple method for assessment of the human acrosome reaction of spermatozoa bound to the zona pellucida: lack of relationship with ionophore A23187-induced acrosome reaction. Hum Reprod. 1996;11(3):551–7. https://doi.org/10.1093/humrep/11.3.551.
15. Oehninger S, Morshedi M, Franken D. The hemizona assay for assessment of sperm function. Methods Mol Biol. 2013;927:91–102. https://doi.org/10.1007/978-1-62703-038-0_9.
16. Glazier DB, Marmar JL, Diamond SM, Gibbs M, Corson SL. A modified acrosome induction test. Arch Androl. 2000;44(1):59–64. https://doi.org/10.1080/014850100262425.
17. Carver-Ward JA, Jaroudi KA, Hollanders JM, Einspenner M. High fertilization prediction by flow cytometric analysis of the CD46 antigen on the inner acrosomal membrane of spermatozoa. Hum Reprod. 1996;11(9):1923–8. https://doi.org/10.1093/oxfordjournals.humrep.a019518.
18. Oehninger S, Coddington CC, Scott R, Franken DA, Burkman LJ, Acosta AA, Hodgen GD. Hemizona assay: assessment of sperm dysfunction and prediction of in vitro fertilization outcome. Fertil Steril. 1989;51(4):665–70. https://doi.org/10.1016/s0015-0282(16)60618-0.
19. Gorczyca W, Bruno S, Darzynkiewicz R, Gong J, Darzynkiewicz Z. DNA strand breaks occurring during apoptosis—their early insitu detection by the terminal deoxynucleotidyl transferase and nick translation assays and prevention by serine protease inhibitors. Int J Oncol. 1992;1:639–48. https://doi.org/10.3892/ijo.1.6.639.
20. Sharma R, Ahmad G, Esteves SC, Agarwal A. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using bench top flow cytometer for evaluation of sperm DNA fragmentation in fertility laboratories: protocol, reference values, and quality control. J Assist Reprod Genet. 2016 Feb;33(2):291–300. https://doi.org/10.1007/s10815-015-0635-7.
21. Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun. 1984;123(1):291–8. https://doi.org/10.1016/0006-291x(84)90411-x.
22. Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination. IVF and ICSI. Hum Reprod. 2004;19(6):1401–8. https://doi.org/10.1093/humrep/deh280.
23. Fernández JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl. 2003 Jan-Feb;24(1):59–66.
24. Erenpreiss J, Jepson K, Giwercman A, Tsarev I, Erenpreisa J, Spano M. Toluidine blue cytometry test for sperm DNA conformation: comparison with the flow cytometric sperm chromatin structure and TUNEL assays. Hum Reprod. 2004;19(10):2277–82. https://doi.org/10.1093/humrep/deh417.
25. Panner Selvam MK, Agarwal A. A systematic review on sperm DNA fragmentation in male factor infertility: laboratory assessment. Arab J Urol. 2018;16(1):65–76. https://doi.org/10.1016/j.aju.2017.12.001.
26. Gopalkrishnan K, Hinduja IN, Kumar TC. In vitro decondensation of nuclear chromatin of human spermatozoa: assessing fertilizing potential. Arch Androl. 1991;27(1):43–50. https://doi.org/10.3109/01485019108987650.
27. Carreras A, Ramirez JP, Mendoza C. Sperm plasma membrane integrity measurement: a combined method. Andrologia. 1992;24(6):335–40. https://doi.org/10.1111/j.1439-0272.1992.tb02665.x.
28. Rumke PH. The presence of sperm antibodies in the serum of two patients with oligospermia. Vox Sang. 1954;4:135–40.
29. Andolz P, Bielsa MA, Martínez P, García-Framis V, Benet-Rubinat JM, Egozcue J. Detection of anti-sperm antibodies in serum, seminal plasma and cervical mucus by the immunobead test. Hum Reprod. 1990;5(6):685–9. https://doi.org/10.1093/oxfordjournals.humrep.a137168.
30. De Almeida M, Soumah A, Jouannet P. Incidence of sperm-associated immunoglobulins in infertile men with suspected autoimmunity to sperm. Int J Androl. 1986;9(5):321–30. https://doi.org/10.1111/j.1365-2605.1986.tb00894.x.

31. Marques M, Sousa AP, Paiva A, Almeida-Santos T, Ramalho-Santos J. Low amounts of mitochondrial reactive oxygen species define human sperm quality. Reproduction. 2014;147(6):817–24. https://doi.org/10.1530/REP-13-0644.
32. Eruslanov E, Kusmartsev S. Identification of ROS using oxidized DCFDA and flow-cytometry. Methods Mol Biol. 2010;594:57–72. https://doi.org/10.1007/978-1-60761-411-1_4.
33. Royall JA, Ischiropoulos H. Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. Arch Biochem Biophys. 1993;302(2):348–55. https://doi.org/10.1006/abbi.1993.1222.
34. Chai RR, Chen GW, Shi HJ, WS O, Martin-DeLeon PA, Chen H. Prohibitin involvement in the generation of mitochondrial superoxide at complex I in human sperm. J Cell Mol Med. 2017, 21(1):121–9. https://doi.org/10.1111/jcmm.12945.
35. Ashar FN, Zhang Y, Longchamps RJ, Lane J, Moes A, Grove ML, Mychaleckyj JC, Taylor KD, Coresh J, Rotter JI, Boerwinkle E, Pankratz N, Guallar E, Arking DE. Association of mitochondrial DNA copy number with cardiovascular disease. JAMA Cardiol. 2017;2(11):1247–55. https://doi.org/10.1001/jamacardio.2017.3683.
36. Li B, Kaushik S, Kalinowski P, Kim B, Gershome C, Ching J, Poburko D. Droplet digital PCR shows the D-Loop to be an error prone locus for mitochondrial DNA copy number determination. Sci Rep. 2018;8(1):11392. https://doi.org/10.1038/s41598-018-29621-1.
37. Ye W, Tang X, Liu C, Wen C, Li W, Lyu J. Accurate quantitation of circulating cell-free mitochondrial DNA in plasma by droplet digital PCR. Anal Bioanal Chem. 2017;409(10):2727–35. https://doi.org/10.1007/s00216-017-0217-x.
38. Agarwal A, Roychoudhury S, Bjugstad KB, Cho CL. Oxidation-reduction potential of semen: what is its role in the treatment of male infertility? Ther. Adv Urol. 2016;8(5):302–18. https://doi.org/10.1177/1756287216652779.
39. Agarwal A, Qiu E, Sharma R. Laboratory assessment of oxidative stress in semen. Arab J Urol. 2017;16(1):77–86. https://doi.org/10.1016/j.aju.2017.11.008.
40. Ramu S, Jeyendran RS. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. Methods Mol Biol. 2013;927:21–5. https://doi.org/10.1007/978-1-62703-038-0_3.
41. McCue PM. Hypo-osmotic swelling test. In Dascanio JJ, McCue PM (eds.) Equine reproductive procedures. Wiley Online Library, 2014;481-482. https://doi.org/10.1002/9781118904398.ch147
42. Zubair M, Ahmad M, Jamil H. Review on the screening of semen by hypo-osmotic swelling test. Andrologia. 2015 Sep;47(7):744–50. https://doi.org/10.1111/and.12335.
43. Aitken RJ, Nixon B. Sperm capacitation: a distant landscape glimpsed but unexplored. Mol Hum Reprod. 2013;19(12):785–93. https://doi.org/10.1093/molehr/gat067.
44. Umehara T, Kawai T, Goto M, Richards JS, Shimada M. Creatine enhances the duration of sperm capacitation: a novel factor for improving in vitro fertilization with small numbers of sperm. Hum Reprod. 2018;33(6):1117–29. https://doi.org/10.1093/humrep/dey081.
45. Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS. A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J Cell Sci. 1998;111(Pt 5):645–56. https://doi.org/10.1242/jcs.111.5.645.
46. Davis BK. Timing of fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. Proc Natl Acad Sci U S A. 1981;78(12):7560–4.
47. Baker MA, Hetherington L, Ecroyd H, Roman SD, Aitken RJ. Analysis of the mechanism by which calcium negatively regulates the tyrosine phosphorylation cascade associated with sperm capacitation. J Cell Sci. 2004;117(Pt 2):211–22. https://doi.org/10.1242/jcs.00842.
48. Krapf D, Arcelay E, Wertheimer EV, Sanjay A, Pilder SH, Salicioni AM, Visconti PE. Inhibition of Ser/Thr phosphatases induces capacitation-associated signaling in the presence of Src kinase inhibitors. J Biol Chem. 2010;285(11):7977–85. https://doi.org/10.1074/jbc.M109.085845.
49. White DR, Aitken RJ. Relationship between calcium, cyclic AMP, ATP, and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. Gamete Res. 1989;22(2):163–77. https://doi.org/10.1002/mrd.1120220205.
50. Suarez SS, Ho HC. Hyperactivation of mammalian sperm. Cell Mol Biol (Noisy-le-grand). 2003;49(3):351–6.
51. Aitken RJ, McLaughlin EA. Molecular mechanisms of sperm capacitation: progesterone-induced secondary calcium oscillations reflect the attainment of a capacitated state. Soc Reprod Fertil Suppl. 2007;63:273–93.
52. Godbole GB, Modi DN, Puri CP. Regulation of homeobox A10 expression in the primate endometrium by progesterone and embryonic stimuli. Reproduction. 2007;134(3):513–23. https://doi.org/10.1530/REP-07-0234.
53. Patrat C, Serres C, Jouannet P. The acrosome reaction in human spermatozoa. Biol Cell. 2000;92(3-4):255–66. https://doi.org/10.1016/s0248-4900(00)01072-8.
54. Liu DY, Baker HW. Acrosome status and morphology of human spermatozoa bound to the zona pellucida and oolemma determined using oocytes that failed to fertilize in vitro. Hum Reprod. 1994;9(4):673–9. https://doi.org/10.1093/oxfordjournals.humrep.a138570.
55. 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(9):1694–700. https://doi.org/10.1093/oxfordjournals.humrep.a138776.
56. Tesarík J. Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in human sperm populations of proven fertilizing ability in vitro. J Reprod Fertil. 1985;74(2):383–8. https://doi.org/10.1530/jrf.0.0740383.
57. Cross NL, Morales P, Overstreet JW, Hanson FW. Induction of acrosome reactions by the human zona pellucida. Biol Reprod. 1988;38(1):235–44. https://doi.org/10.1095/biolreprod38.1.235.
58. Brucker C, Lipford GB. The human sperm acrosome reaction: physiology and regulatory mechanisms. An update. Hum Reprod Update. 1995;1(1):51–62. https://doi.org/10.1093/humupd/1.1.51.
59. Hecht NB. Regulation of 'haploid expressed genes' in male germ cells. J Reprod Fertil. 1990;88(2):679–93. https://doi.org/10.1530/jrf.0.0880679.
60. Oliva R. Protamines and male infertility. Hum Reprod Update. 2006;12(4):417–35. https://doi.org/10.1093/humupd/dml009.
61. Rogenhofer N, Dansranjavin T, Schorsch M, Spiess A, Wang H, von Schönfeldt V, Cappallo-Obermann H, Baukloh V, Yang H, Paradowska A, Chen B, Thaler CJ, Weidner W, Schuppe HC, Steger K. The sperm protamine mRNA ratio as a clinical parameter to estimate the fertilizing potential of men taking part in an ART programme. Hum Reprod. 2013;28(4):969–78. https://doi.org/10.1093/humrep/des471.
62. Hamad MF. Quantification of histones and protamines mRNA transcripts in sperms of infertile couples and their impact on sperm's quality and chromatin integrity. Reprod Biol. 2019;19(1):6–13. https://doi.org/10.1016/j.repbio.2019.03.001.
63. Durmaz A, Dikmen N, Gündüz C, Göker EN, Tavmergen E. Fluctuation of "sperm DNA integrity" in accordance with semen parameters, and it's relationship with infertility. J Assist Reprod Genet. 2014;31(12):1665–71. https://doi.org/10.1007/s10815-014-0364-3.

64. Zhang Y, Trussell JC, Chohan KR. Detecting and minimizing sperm DNA damage. Semin Reprod Med. 2013;31(4):267–73. https://doi.org/10.1055/s-0033-1345274.
65. Ribas-Maynou J, García-Peiró A, Fernández-Encinas A, Abad C, Amengual MJ, Prada E, Navarro J, Benet J. Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. Andrology. 2013;1(5):715–22. https://doi.org/10.1111/j.2047-2927.2013.00111.x.
66. Albert O, Reintsch WE, Chan P, Robaire B. HT-COMET: a novel automated approach for high throughput assessment of human sperm chromatin quality. Hum Reprod. 2016;31(5):938–46. https://doi.org/10.1093/humrep/dew030.
67. Enciso M, Alfarawati S, Wells D. Increased numbers of DNA-damaged spermatozoa in samples presenting an elevated rate of numerical chromosome abnormalities. Hum Reprod. 2013;28(6):1707–15. https://doi.org/10.1093/humrep/det077.
68. Grèze C, Guttmann A, Pons-Rejraji H, Vasson MP, Lornage J, Ouchchane L, Brugnon F. Can the SCD test and terminal uridine nick-end labeling by flow cytometry technique (TUNEL/FCM) be used interchangeably to measure sperm DNA damage in routine laboratory practice? Basic Clin Androl. 2019;26(29):17. https://doi.org/10.1186/s12610-019-0098-2.
69. Shibahara H, Koriyama J. Methods for direct and indirect antisperm antibody testing. Methods Mol Biol. 2013;927:51–60. https://doi.org/10.1007/978-1-62703-038-0_6.
70. Pretorius E, Franken DR. The immunobead technique: an indicator of disturbed sperm cervical mucus interaction. Andrologia. 1988;20(1):5–9. https://doi.org/10.1111/j.1439-0272.1988.tb02351.x.
71. Gatimel N, Moreau J, Isus F, Moinard N, Parinaud J, Leandri RD. Anti-sperm antibodies detection by a modified MAR test: towards a better definition of its indications. Reprod Biomed Online. 2018;37(6):717–23. https://doi.org/10.1016/j.rbmo.2018.09.011.
72. Harper ME, Bevilacqua L, Hagopian K, Weindruch R, Ramsey JJ. Ageing, oxidative stress, and mitochondrial uncoupling. Acta Physiol Scand. 2004;182(4):321–31. https://doi.org/10.1111/j.1365-201X.2004.01370.x.
73. Cassina A, Silveira P, Cantu L, Montes JM, Radi R, Sapiro R. Defective human sperm cells are associated with mitochondrial dysfunction and oxidant production. Biol Reprod. 2015 Nov;93(5):119. https://doi.org/10.1095/biolreprod.115.130989.
74. Kauffman ME, Kauffman MK, Traore K, Zhu H, Trush MA, Jia Z, Li YR. MitoSOX-based flow cytometry for detecting mitochondrial ROS. React Oxyg Species (Apex). 2016;2(5):361–70. https://doi.org/10.20455/ros.2016.865.
75. Espinoza JA, Schulz MA, Sánchez R, Villegas JV. Integrity of mitochondrial membrane potential reflects human sperm quality. Andrologia. 2009;41(1):51–4. https://doi.org/10.1111/j.1439-0272.2008.00878.x.
76. Malik AN, Czajka A. Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction? Mitochondrion. 2013;13(5):481–92. https://doi.org/10.1016/j.mito.2012.10.011.
77. Song GJ, Lewis V. Mitochondrial DNA integrity and copy number in sperm from infertile men. Fertil Steril. 2008 Dec;90(6):2238–44. https://doi.org/10.1016/j.fertnstert.2007.10.059.
78. May-Panloup P, Chrétien MF, Savagner F, Vasseur C, Jean M, Malthièry Y, Reynier P. Increased sperm mitochondrial DNA content in male infertility. Hum Reprod. 2003;18(3):550–6. https://doi.org/10.1093/humrep/deg096.
79. Rosati AJ, Whitcomb BW, Brandon N, Buck Louis GM, Mumford SL, Schisterman EF, Pilsner JR. Sperm mitochondrial DNA biomarkers and couple fecundity. Hum Reprod. 2020;35(11):2619–25. https://doi.org/10.1093/humrep/deaa191.
80. Tian M, Bao H, Martin FL, Zhang J, Liu L, Huang Q, Shen H. Association of DNA methylation and mitochondrial DNA copy number with human semen quality. Biol Reprod. 2014;91(4):101. https://doi.org/10.1095/biolreprod.114.122465. Epub 2014 Sep 10. Erratum in: Biol Reprod. 2015 Mar;92(3):61
81. Desai N, Sharma R, Makker K, Sabanegh E, Agarwal A. Physiologic and pathologic levels of reactive oxygen species in neat semen of infertile men. Fertil Steril. 2009;92(5):1626–31. https://doi.org/10.1016/j.fertnstert.2008.08.109.
82. Tunc O, Thompson J, Tremellen K. Development of the NBT assay as a marker of sperm oxidative stress. Int J Androl. 2010 Feb;33(1):13–21. https://doi.org/10.1111/j.1365-2605.2008.00941.x.
83. Kao SH, Chao HT, Chen HW, Hwang TIS, Liao TL, Wei YH. Increase of oxidative stress in human sperm with lower motility. Fertil Steril. 2008;89(5):1183–90. https://doi.org/10.1016/j.fertnstert.2007.05.029.
84. Shen H, Ong C. Detection of oxidative DNA damage in human sperm and its association with sperm function and male infertility. Free Radic Biol Med. 2000;28(4):529–36. https://doi.org/10.1016/s0891-5849(99)00234-8.
85. Majzoub A, Arafa M, Mahdi M, Agarwal A, Al Said S, Al-Emadi I, El Ansari W, Alattar A, Al Rumaihi K, Elbardisi H. Oxidation-reduction potential and sperm DNA fragmentation, and their associations with sperm morphological anomalies amongst fertile and infertile men. Arab J Urol. 2018;16(1):87–95. https://doi.org/10.1016/j.aju.2017.11.014.
86. Homa ST, Vassiliou AM, Stone J, Killeen AP, Dawkins A, Xie J, Gould F, Ramsay JWA. A comparison between two assays for measuring seminal oxidative stress and their relationship with sperm DNA fragmentation and semen parameters. Genes (Basel). 2019;10(3):236. https://doi.org/10.3390/genes10030236.
87. Arslan M, Morshedi M, Arslan EO, Taylor S, Kanik A, Duran HE, Oehninger S. Predictive value of the hemizona assay for pregnancy outcome in patients undergoing controlled ovarian hyperstimulation with intrauterine insemination. Fertil Steril. 2006;85(6):1697–707. https://doi.org/10.1016/j.fertnstert.2005.11.054.
88. Bielsa MA, Andolz P, Gris JM, Martínez P, Egozcue J. Which semen parameters have a predictive value for pregnancy in infertile couples? Hum Reprod. 1994;9(10):1887–90. https://doi.org/10.1093/oxfordjournals.humrep.a138353.
89. Calvo L, Dennison-Lagos L, Banks SM, Dorfmann A, Thorsell LP, Bustillo M, Schulman JD, Sherins RJ. Acrosome reaction inducibility predicts fertilization success at in-vitro fertilization. Hum Reprod. 1994;9(10):1880–6. https://doi.org/10.1093/oxfordjournals.humrep.a138352.
90. Esteves SC, Schneider DT, Verza S Jr. Influence of antisperm antibodies in the semen on intracytoplasmic sperm injection outcome. Int Braz J Urol. 2007;33(6):795–802. https://doi.org/10.1590/s1677-55382007000600007.
91. Esteves SC, López-Fernández C, Martínez MG, Silva EA, Gosálvez J. Reliability of the sperm chromatin dispersion assay to evaluate sperm deoxyribonucleic acid damage in men with infertility. Fertil Steril. 2022;117(1):64–73. https://doi.org/10.1016/j.fertnstert.2021.08.045.
92. Gamzu R, Yogev L, Amit A, Lessing J, Homonnai ZT, Yavetz H. The hemizona assay is of good prognostic value for the ability of sperm to fertilize oocytes in vitro. Fertil Steril. 1994;62(5):1056–9. https://doi.org/10.1016/s0015-0282(16)57073-3.
93. Hervás I, Pacheco A, Gil Julia M, Rivera-Egea R, Navarro-Gomezlechon A, Garrido N. Sperm deoxyribonucleic acid fragmentation (by terminal deoxynucleotidyl transferase biotin dUTP nick end labeling assay) does not impair reproductive success measured as cumulative live birth rates per donor metaphase II

oocyte used. Fertil Steril. 2022;118(1):79–89. https://doi.org/10.1016/j.fertnstert.2022.04.002.
94. Janssen HJ, Bastiaans BA, Goverde HJ, Hollanders HM, Wetzels AA, Schellekens LA. Antisperm antibodies and in vitro fertilization. J Assist Reprod Genet. 1992;9(4):345–9. https://doi.org/10.1007/BF01203957.
95. Lin MH, Kuo-Kuang Lee R, Li SH, Lu CH, Sun FJ, Hwu YM. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. Fertil Steril. 2008;90(2):352–9. https://doi.org/10.1016/j.fertnstert.2007.06.018.
96. Meseguer M, Santiso R, Garrido N, García-Herrero S, Remohí J, Fernandez JL. Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. Fertil Steril. 2011;95(1):124–8. https://doi.org/10.1016/j.fertnstert.2010.05.055.
97. Franken DR, Burkman LJ, Oehninger SC, Coddington CC, Veeck LL, Kruger TF, Rosenwaks Z, Hodgen GD. Hemizona assay using salt-stored human oocytes: evaluation of zona pellucida capacity for binding human spermatozoa. Gamete Res. 1989;22(1):15–26. https://doi.org/10.1002/mrd.1120220103.
98. Sallam HN, Farrag A, Agameya AF, El-Garem Y, Ezzeldin F. The use of the modified hypo-osmotic swelling test for the selection of immotile testicular spermatozoa in patients treated with ICSI: a randomized controlled study. Hum Reprod. 2005;20(12):3435–40. https://doi.org/10.1093/humrep/dei249.
99. Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Predicting the fertilizing potential of human sperm suspensions in vitro: importance of sperm morphology and leukocyte contamination. Fertil Steril. 1995;63(6):1293–300. https://doi.org/10.1016/s0015-0282(16)57614-6.
100. Zorn B, Vidmar G, Meden-Vrtovec H. Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional in vitro fertilization and intracytoplasmic sperm injection. Int J Androl. 2003;26(5):279–85. https://doi.org/10.1046/j.1365-2605.2003.00424.x.
101. Check JH, Stumpo L, Lurie D, Benfer K, Callan C. A comparative prospective study using matched samples to determine the influence of subnormal hypo-osmotic test scores of spermatozoa on subsequent fertilization and pregnancy rates following in-vitro fertilization. Hum Reprod. 1995 May;10(5):1197–200. https://doi.org/10.1093/oxfordjournals.humrep.a136118.
102. Tartagni M, Schonauer MM, Cicinelli E, Selman H, De Ziegler D, Petruzzelli F, D'Addario V. Usefulness of the hypo-osmotic swelling test in predicting pregnancy rate and outcome in couples undergoing intrauterine insemination. J Androl. 2002;23(4):498–502.
103. Vogiatzi P, Chrelias C, Cahill DJ, Creatsa M, Vrachnis N, Iliodromiti Z, Kassanos D, Siristatidis C. Hemizona assay and sperm penetration assay in the prediction of IVF outcome: a systematic review. Biomed Res Int. 2013;2013:945825. https://doi.org/10.1155/2013/945825.
104. Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. Fertil Steril. 2004;81(5):1289–95. https://doi.org/10.1016/j.fertnstert.2003.09.063.
105. Nijs M, Creemers E, Cox A, Franssen K, Janssen M, Vanheusden E, De Jonge C, Ombelet W. Chromomycin A3 staining, sperm chromatin structure assay and hyaluronic acid binding assay as predictors for assisted reproductive outcome. Reprod Biomed Online. 2009;19(5):671–84. https://doi.org/10.1016/j.rbmo.2009.07.002.
106. Cissen M, Wely MV, Scholten I, Mansell S, Bruin JP, Mol BW, Braat D, Repping S, Hamer G. Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: a systematic review and meta-analysis. PLoS One. 2016;11(11):e0165125. https://doi.org/10.1371/journal.pone.0165125.
107. Stanger JD, Vo L, Yovich JL, Almahbobi G. Hypo-osmotic swelling test identifies individual spermatozoa with minimal DNA fragmentation. Reprod Biomed Online. 2010;21(4):474–84. https://doi.org/10.1016/j.rbmo.2010.06.026.
108. McQueen DB, Zhang J, Robins JC. Sperm DNA fragmentation and recurrent pregnancy loss: a systematic review and meta-analysis. Fertil Steril. 2019;112(1):54–60.e3. https://doi.org/10.1016/j.fertnstert.2019.03.003.
109. Hammadeh ME, Radwan M, Al-Hasani S, Micu R, Rosenbaum P, Lorenz M, Schmidt W. Comparison of reactive oxygen species concentration in seminal plasma and semen parameters in partners of pregnant and non-pregnant patients after IVF/ICSI. Reprod Biomed Online. 2006;13(5):696–706. https://doi.org/10.1016/s1472-6483(10)60661-x.
110. Ghaleno LR, Valojerdi MR, Hassani F, Chehrazi M, Janzamin E. High level of intracellular sperm oxidative stress negatively influences embryo pronuclear formation after intracytoplasmic sperm injection treatment. Andrologia. 2014;46(10):1118–27. https://doi.org/10.1111/and.12202.
111. Chen X, Zheng Y, Zheng J, Lin J, Zhang L, Jin J. The progesterone-induced sperm acrosome reaction is a good option for the prediction of fertilization in vitro compared with other sperm parameters. Andrologia. 2019;51(6):e13278. https://doi.org/10.1111/and.13278.
112. Schill WB. Some disturbances of acrosomal development and function in human spermatozoa. Hum Reprod. 1991;6(7):969–78. https://doi.org/10.1093/oxfordjournals.humrep.a137471.
113. Plachot M, Mandelbaum J, Junca AM. Acrosome reaction of human sperm used for in vitro fertilization. Fertil Steril. 1984;42(3):418–23. https://doi.org/10.1016/s0015-0282(16)48083-0.
114. Tello-Mora P, Hernández-Cadena L, Pedraza J, López-Bayghen E, Quintanilla-Vega B. Acrosome reaction and chromatin integrity as additional parameters of semen analysis to predict fertilization and blastocyst rates. Reprod Biol Endocrinol. 2018;16(1):102. https://doi.org/10.1186/s12958-018-0408-0.
115. Vujisić S, Lepej SZ, Jerković L, Emedi I, Sokolić B. Antisperm antibodies in semen, sera and follicular fluids of infertile patients: relation to reproductive outcome after in vitro fertilization. Am J Reprod Immunol. 2005;54(1):13–20. https://doi.org/10.1111/j.1600-0897.2005.00274.x.
116. Sukcharoen N, Keith J. The effect of the antisperm auto-antibody-bound sperm on in vitro fertilization outcome. Andrologia. 1995;27(5):281–9. https://doi.org/10.1111/j.1439-0272.1995.tb01106.x.
117. Kızılay F, Altay B. Sperm function tests in clinical practice. Turk J Urol. 2017;43(4):393–400. https://doi.org/10.5152/tud.2017.96646.
118. Talwar P, Hayatnagarkar S. Sperm function test. J Hum Reprod Sci. 2015;8(2):61–9. https://doi.org/10.4103/0974-1208.158588.
119. Tournaye H. Management of male infertility by assisted reproductive technologies. Baillieres Best Pract Res Clin Endocrinol Metab. 2000;14(3):423–35. https://doi.org/10.1053/beem.2000.0089.
120. Agarwal A, Virk G, Ong C, du Plessis SS. Effect of oxidative stress on male reproduction. World J Mens Health. 2014;32(1):1–17. https://doi.org/10.5534/wjmh.2014.32.1.1.