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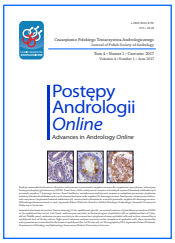

NOT JUST THE MARRIAGE OF FIGARO: BUT THE MARRIAGE OF WHO/ESHRE SEMEN ANALYSIS CRITERIA WITH SPERM FUNCTIONALITY

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Received: 23.04.2017 r. Accepted: 21.06.2017 r.

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Abstract

The authors present a critical review of the WHO5 (2010) manual of semen analysis and what it should be used for: The analysis of sperm quality and not analysis to predict fertility outcome per se. We show the strengths and shortcoming of WHO5 and then ask for a better "marriage" among these parameters and the outcome of sperm functionality and fertilization/live birth outcome. For many decades the basis of the WHO manual for semen analysis has not changed and we emphasize that sperm functionality testing has not really been considered/performed in the routine andrology laboratory. There is a need to first develop more objective and quantitative

methodology such as computer-aided sperm analysis, to analyse sperm quality and sperm functionality that relates in many instances to fertilization/live birth outcome: 1) sperm cervical mucous penetration using computer aided sperm analysis (CASA), 2) endpoint of capacitation, hyperactivation as measured accurately by CASA, 3) acrosome reaction quantitatively, 4) chromatin maturity and DNA fragmentation quantitatively, 4) where possible oocyte binding tests (hemizona), 5) relationships of vitality and hypo-osmotic swelling test using modern technology 6) measurement of oxidative stress, 7) analysis of semen using proteomics (proteins that are importantly functionally expressed in seminal plasma) as well as 8) metabolomics representing a systematic study of the unique metabolic fingerprints (chemical) that specific cellular processes leave behind and inform us about function/dysfunction, 9) patient profile (obesity, smoking, age, stress, female cryptic choice, environment and many other patient characteristics) as important determinants in fertility outcome. We believe we can intelligently in the end construct a matrix which combine all these factors and others in the future that inform us about potential fertility outcome. But then realize WHO5/ESHRE current guidelines are not particularly informative in the above context.

Key words: semen, sperm, WHO5, sperm functionality

Abbreviations

AB – aniline blue staining, ALH – amplitude of lateral head displacement, AO – acridine orange test, AR – acrosome reaction, ART – assisted reproductive techniques, CASA – computer-aided sperm analysis, CE-MS – capillary electrophoresis-mass spectrometry, CMA3 – chromomycin A3, DAF-2DA – 4,5-diaminofluorescein diacetate, DCF – 2,7-dichlorofluorescein, DHE – dihydroethidium, ESHRE – European Society of Human Reproduction and Embryology, FISH – fluorescent in situ hybridization, GC-MS – gas chromatography coupled to mass spectrometry, HA –hyperactivation, Ha – hyaluronic acid, HBA – hyaluronan binding assay, HOS test – hypo-osmotic swelling test, HPLC – high-performance liquid chromatography, HspA2 – heat shock protein A2, HZA – hemizona assay, HZI – hemizona index, ICSI – intracytoplasmic sperm injection, IUI – intrauterine insemination, IVF – *in vitro* fertilization, LIN – linearity, MAI – multiple anamolies index, MS – mass spectrometry, NMR – nuclear magnetic resonance, ORP – oxidation reduction potential, ROS – reactive oxygen species, SCD/Halo – sperm chromatin dispersion test, SCA – Sperm Class Analyzer, SCMPT – sperm cervical mucous penetration test, SCSA – sperm chromatin structure assay, SDF – sperm DNA fragmentation, STR – straightness, PCR – polymerase chain reaction, PNA – *Arachis hypogaea* agglutinin, PSA – *Pisum sativum* agglutinin, QPCR – quantitative PCR, TAC – total antioxidant capacity, TB – toluidine blue, TUNEL – terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling, TZI – teratozoospermic index, WHO – World Health Organization, WHO4 – WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction 4th ed. (1999), WHO5 – WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. (2010), VAP – average path velocity, VCL – curvilinear velocity, ZP – zona pellucida

Introduction

Just like in *The Marriage of Figaro*, the second play in a trilogy of operas (following *The Barber of Seville*), Sperm Functionality Testing should become an integral part of testing potential male fertility and as part of manuals of guidance bodies such as the World Health Organization /European Society of Human Reproduction and Embryology (WHO/ESHRE).

In essence there should be a matrimonial bond between Sperm Functionality and the existing Basic Semen Analysis. However, such a bond will come with various challenges similar to those faced in *The Marriage of Figaro*. Our title suggests that a great deal more is needed than just standardized semen analysis and our basic aim is to suggest a more holistic approach to better understand male fertility as related to semen analysis, sperm functional testing and associated molecular biology. This should include the marriage of many complimentary analyses and approaches as well as proper investigation of the patient and the couple; but not exclude any new developments as we envision that emerging technologies will continue to be included as "new plays" in this evolving field of male fertility diagnostics.

Historical background on standardization of semen analysis

The first WHO semen analysis manual was published in 1980, and it was updated in 1987, 1992, 1999 (so called WHO4) and then lastly in 2010 (WHO5). In all these editions there were concerted efforts to standardize and improve methodology and technology to evaluate semen quality. It was particularly the first three editions that came under scrutiny for the lack of detailed descriptions, procedures and standardization for the various semen analyses procedures. Improved methodology and standardization required internal and external quality control and resulted in cut-off values for semen quality to be modified (table 1 after *Esteves*, 2014). It was only in the 1999 (WHO4) and 2010 (WHO5) editions that more progress was made; emphasis in the WHO4 was based on new scientific information, while with WHO5 was more based on clinical outcomes, but certainly not without some criticisms.

Basic semen analysis (WHO5)

Is basic semen evaluation useful in predicting fertility? It is believed that many users of the WHO5 manual are

Table 1. Cut-off reference values for semen characteristics as published in consecutive WHO (World Health Organization) manuals (modified from *Esteves*, 2014)

Semen/sperm characteristics	WHO 1980	WHO 1987	WHO 1992	WHO 1999	WHO 2010
Semen volume (mL)	ND	>2	>2	>2	1.5
Sperm concentration (10^6/mL)	20–200	>20	>20	>20	15
Total sperm in ejaculate (10^6)	ND	>40	>40	>40	39
Total percentage motile sperm	60	>50	>50	>50	40
Progressive motility (5%)	>2	25	>25 (grade a)	>25% (grade a)	32% (a + b)
Vitality (% alive)	ND	>50	>75	>75	>58
Normal morphology (%)	80.5	>50	>30	14	4

ND – non detected

Table 2. Distribution of values, lower reference limits and their 95% CI for semen and sperm parameters from fertile men whose partners had a time-to-pregnancy of 12 months or less. Light grey shaded area currently accepted (WHO5) (modified from WHO5 and *Cooper et al.* (2010)

Semen/sperm characteristics	N	Centiles			
		2.5	95% CI	5	95% CI
Semen volume (mL)	1941	1.2	1.0–1.3	1.5	1.4–1.7
Sperm concentration (10^6/mL)	1859	9	8–11	15	12–16
Total sperm in ejaculate (10^6)	1859	23	18–29	39	33–46
Total percentage motile sperm (%)	1781	24	33–37	40	38–42
Progressive motility (%)	1780	28	25–29	32	31–34
Vitality (% alive)	428	53	48–56	58	55–63
Normal morphology (%)	1851	3	2–3	4	3–4

CI – confidence interval; N – number of subjects; WHO5 – World Health Organization Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. (2010)

under the wrong impression that it predicts human fertility outcome. This is incorrect for many reasons, but at the same time it does not imply that it is of limited or no use (*Jequier*, 2010). It certainly supports defining the general quality of a semen sample as poor, medium or good, but cannot predict fertility *per se*. It certainly points in the direction of potential fertility particularly in view of the ranges of cut-off values as proposed by *Cooper et al.* (2010) (table 2) and provide a much better basis for measuring sperm quality rather than a single cut-off value for each parameter. This has fortunately been incorporated in WHO5 as an Appendix. Apart from predicting the quality of a semen sample it may also indicate the general reproductive health of the donor and it is important from a general Andrology point of view of understanding the patient.

Limitations of conventional semen analysis and factors that can affect the evaluation

Semen quality is commonly taken as a surrogate indicator of male fertility. However, there is little consensus as to the power of conventional semen analysis in predicting future fertility. Nonetheless, a multitude of studies proclaim significant correlations between individual parameters and fertilization, pregnancy and birth rate after both natural conception or assisted reproductive techniques (ART) interventions.

For the first time the reference values for semen analysis as included in WHO5 are based on controlled studies comprising fathers with a known time to pregnancy. The goal of WHO5 was therefore to provide evidence-based thresholds through the lower reference limits to help clinicians approximate a patient's fertility. What needs to be kept in mind is that meeting the lower reference values does not ensure fertility or *vice versa*. Basic semen analysis merely acts as a tool to quantify semen quality and we should not place excessive expectations on it.

Conventional semen analysis including the use of the WHO5 manual provide very good guidelines for standardization in determining important semen characteristics such as semen volume, agglutination, viscosity, sperm concentration, sperm motility, sperm morphology, vitality and many other facets such as immunological tests and biochemistry of seminal plasma such as fructose, citric acid and zinc (figure 1). It is sometimes ignored that the WHO5 manual has a subtitle, *"examination and processing of human semen"*, and should be used in this context and not as an absolute reference to what is fertile or sub-fertile or infertile.

As with any predictive formulae, the WHO5 guidelines have some limitations and caveats (*Bonde*, 2010; *Boyd*, 2010, *Esteves et al.*, 2012). We would like to highlight a few limitations of the original design and study that led to establishing the new WHO5 lower reference values for semen analysis:

- the studies and data used were not sufficiently representative. Only 10% of the study population came

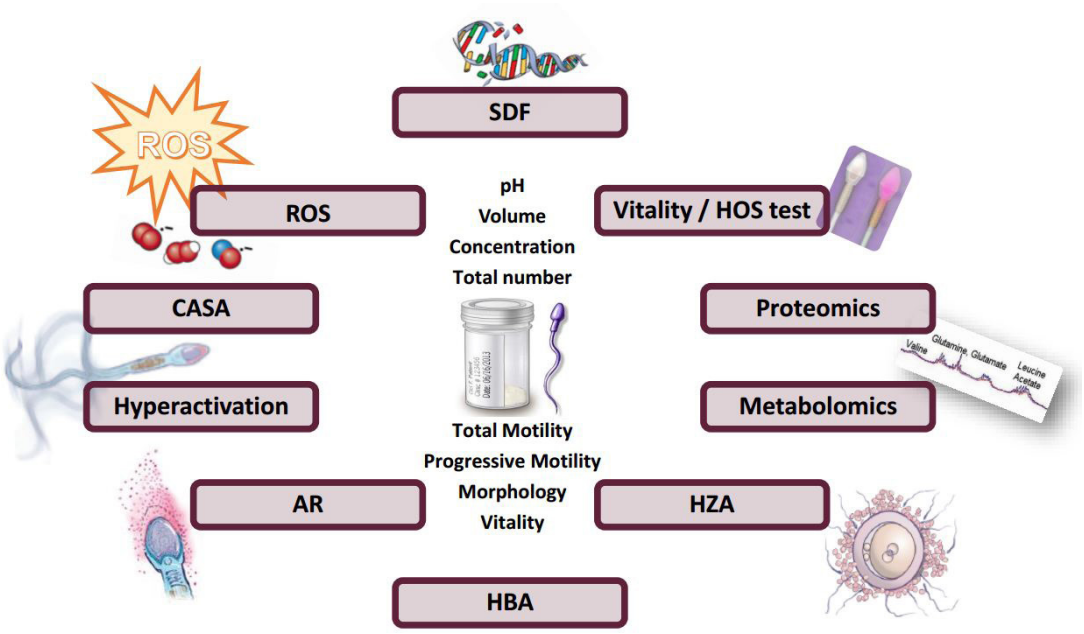

Fig. 1. Basic and functional semen tests. AR – acrosome reaction; CASA – computer-aided sperm analysis; HBA – hyaluronan binding assay; HOS test – hypo-osmotic swelling test; HZA – hemizona assay; ROS – reactive oxygen species; SDF – sperm DNA fragmentation

from the Southern hemisphere (Australia), while 55% of data originated from four European cities and 30% from the USA. A large proportion of studies have overlapping authorship or collaboration among authors,

- only a single semen sample was collected to represent each man, which is problematic due to the heterogeneity of semen,
- different evaluation criteria were used for morphology analysis (Tygerberg "strict" vs. David's).

A further limitation of basic semen analysis is that it cannot predict and diagnose idiopathic or unexplained infertility as ± 30% of men with difficulty of fathering a child display no demonstrable abnormalities; yet again pointing towards inclusion of functional assays to test the impact of sperm dysfunction.

While the manual has been put together by experts with very good consequence (*Jequier*, 2010) there are shortcomings relating to both the manual itself, but more so to the use and application of the manual globally (*Handelsman* and *Cooper*, 2010a, 2010b; *Lu* and *Gu*, 2010). The main shortcoming relates to the fact that many of the central tests such as sperm motility, sperm morphology and vitality rely on subjective manual observations and according to Eliasson are not outcome-based as the WHO5 claims (*Eliasson*, 2010).

Handelsman and *Cooper* (2010b) provide an excellent summary on the major objections to WHO5. Both *Eliasson* (2010) and *Björndahl* (2010) raise valid objections to WHO5 combining "a" and "b" motility ratings for fast progressive sperm. *Handelsman* and *Cooper* (2010b) furthermore quote *Eliasson* stating his serious objections to strict criteria for many reasons. *Auger* (2010) states: "… whether the actual sperm morphology of 4% *per se* is useful is questionable: the likely more relevant parameter is the total number of morphologically normal spermatozoa in the ejaculate and men with even lower percentages of normal forms than fathers may well have far fewer total spermatozoa as well" (*Handelsman* and *Cooper* (2010b). *Sadeghi* (2010) in an editorial raises similar objections.

Moreover, a diagnosis for fertility cannot be based on one semen parameter alone. Various factors can affect basic semen analysis, as described in WHO5, and thus impact on the clinical utility thereof. These issues can render the results obsolete or difficult to interpret if not placed in perspective; therefore, physicians should exercise caution when making inferences. This can include factors such as, but not limited to: intra-patient variability of semen parameters with repeat testing, ethnic and geographical variations in semen parameters, declining sperm quality, incorrect laboratory handling of sperm, inter-technician variability, lack of standardization and consensus on appropriate techniques.

It is well known that there is inherent variability from one ejaculate to the next due to pre-analytic influences (e.g. environmental exposures, abstinence duration) as well as intrinsic biological variation. Within-patient coefficient of variation for all semen parameters between two routinely performed semen analyses were reported to be between 28–34% (*Leushuis et al.*, 2010). Similarly, other studies demonstrated significant changes in volume (decrease), concentration (decrease) as well as motility (increase) with shorter abstinence periods (*Mayorga-Torres et al.*, 2015). Similarly, variations in semen parameters have also been shown based on ethnicity and geographical location, e.g. a significant proportion of Asian men had values below that of the WHO reference values or their European counterparts (*Barazani et al.*, 2014). It is

tempting to suggest that semen quality (specifically sperm count) is on the decline (*Sengupta et al.*, 2017), however, there are many critics negating this hypothesis. In contrast, it is fair to say that male reproductive health does appear to be under threat (testicular dysgenesis syndrome) due to environmental exposure, which could ultimately impact sperm quality (*Bay et al.*, 2006; *Lewis*, 2007).

As mentioned previously incorrect handling of the semen sample and spermatozoa in the laboratory can also affect the outcome of semen analysis. Excessive centrifugation can lead to reactive oxygen species (ROS) generation, whilst removal of the seminal plasma also leads to removal of important antioxidants, rendering the spermatozoa even more vulnerable to oxidative stress in the short term. Exposing the gametes to, type/intensity of light sources, temperature fluctuations and inconsistencies with regards to the time before analysis occurs are additional factors that can cause variation in results (*Lewis*, 2007).

Despite the improvement in training there still remains paucity in continuous proficiency testing of technicians (*Franken* and *Oehninger*, 2012). Due to human subjectivity inter- and intra-technician inconsistency regularly leads to discrepancies when evaluating the same specimen (*Alvarez et al.*, 2005; *Riddel et al.*, 2005). Many studies have shown that the coefficient of variation for technicians in evaluating sperm morphology range from 10 to 80%, thus questioning the usefulness of these parameters for measuring sperm quality and leave alone fertility (*Handelsman* and *Cooper*, 2010b). Other studies also proved manual analysis to be subjective and accordingly variable (*Esteves*, 2014; *Pacey*, 2006) and that as much as 12% of errors surround the diagnostic process and actually impact on patient care (*Goldschmidt* and *Lent*, 1995).

The lack of standardization within and between laboratories as well as the dearth in consensus on specific tests and analysis methods are also causes of concern. In a study conducted on more than 500 laboratories in the USA it became evident that nearly 40% did not report abstinence length or the specific criteria used for morphology analysis, while more than half of them did not perform quality control for any of the commonly assessed parameters (*Keel et al.*, 2002). These findings were corroborated by surveys on laboratory practices conducted in the UK (*Riddel et al.*, 2005) as well as shortcomings related to standardized methodology and problems associated with subjective sperm evaluations in many laboratories globally (*Esteves et al.*, 2012; *Lu et al.*, 2010; *Pacey*, 2010; *Walczak-Jedrzejowska et al.*, 2013). It is therefore vital that quality assurance must form an integral part of any laboratory, whilst appropriate record keeping will also help with proper clinical diagnosis and management of the infertile male. The importance of specialised Andrology laboratories with expertize from embryologists to clinicians (Urologists) can therefore not be underestimated.

One of the authors (*Gerhard van der Horst*) visited 40 "sperm analysis laboratories" in 30 countries (mainly Europe and Russia) during the past five years and observed the following typical deviations from WHO5/ESHRE guidelines that may affect the outcome of basic semen analysis:

- non-standardization of temperature control of semen sample, consumables and temperature stage,
- inconsistent timing related to determination of sperm motility and sperm vitality after collection,
- inaccurate semen volume determination through measuring in a graduated tube instead of weighing the semen sample on a sensitive balance,
- not using positive displacement pipettes when determining sperm concentration,
- sub-optimal microscope settings, because of incorrect setting of Köhler illumination and critical illumination, essential for meeting optical resolution requirements,
- incorrect methods for making sperm morphology and vitality smears,
- not adhering to WHO5 guidelines for re-assessing concentration, motility and morphology when differences between replicate counts are not acceptable.

The informative paper of *Walczak-Jedrzejowska et al.* (2013) reported similar and additional shortcomings in a recent survey of Polish laboratories evaluating semen quality. In ESHRE accredited facilities well defined internal and external quality assurance is performed by qualified embryologists, spermatologists and andrologists. This is also supported through guidelines and subsequent training courses (*Björndahl et al.*, 2002; *Pacey*, 2010). On the other hand, *Jequier* (2005) is of strong opinion that quality assurance in semen analysis is not essential because the results generated do not adequately predict fertility. *Pacey* (2006) contests both aspects and makes a case based on the paper by *Bonde et al.* (1998) that some aspects of semen analysis do relate to fertilization outcome. In contrast, *Jequier* (2005) makes such an important statement and point about semen analysis and infertility: "What the clinician needs for the correct management of infertility in the male is a diagnosis. Infertility is not a diagnosis: it is only a symptom. The analysis of semen only occasionally gives the clinician a diagnosis, as for the most part, the changes that take place in semen are largely non-specific". It brings us back to the point that we need different strategies for evaluating semen quality and what it means *versus* what is regarded as a fertile male *versus* what is needed in assisted reproductive strategies.

As long as the three critical aspects of basic semen analysis as well as other facets such as semen viscosity are not analysed objectively and/or with automated proven technology, there will always be discrepancies that are simply unscientific, leave alone outcome-based and may be to the disadvantage of the patient. There is a paucity by WHO5 to update the importance of newer

computer-aided sperm analysis (CASA) technology in measuring most of the semen/sperm parameters objectively and reliably (despite shortcomings; see section on CASA). Also the use of CASA in the objective quantification of sperm functionality is very much under played (See section under sperm functionality relating to CASA) (*Mortimer et al.*, 2015). At least *Handelsman* and *Cooper* (2010b) make some positive comments as to how CASA could be used to predict progressive motility more accurately and objectively for a potential WHO6 manual.

Very few semen analysis laboratories make use of any sperm functional tests as suggested in the WHO5 manual and this is surprising as most of these tests are listed under the heading Research and thus not considered as a core part of the analysis. Many important and existing sperm functional tests actually relate to the challenges that sperm experience in the female reproductive tract and are merely mentioned or not even included in WHO5 despite their ability to shed light on fertilization outcome (see later). There is accordingly a great need to make these shortcomings more widely known and develop measures that will decrease the misuse of clearly prescribed and standardized conditions and analysis of semen parameters.

Sperm functional testing and beyond: the role of computer-aided sperm analysis (CASA)

CASA has developed remarkably fast during the past 30 years. Initially it was predominantly used for measuring sperm concentration and sperm motility and later also used for sperm morphology assessment (*van der Horst* and *Maree*, 2009; *Maree et al.*, 2010, *Maree* and *van der Horst*, 2013; *Mortimer et al.*, 2015). While the early developmental stages of CASA introduced a new era of objective analysis, it was not commonly used in semen evaluation in the clinical setting and even currently there is some scepticism about its role in clinical spermatology (*Talarczyk-Desole et al.*, 2017; *WHO*, 2010). In contrast many clinics across the globe use CASA systems because of more objective analysis and despite the shortcomings in clinical practise support its use as a consistent clinical tool (*Mortimer* and *Mortimer*, 2013; *Mortimer et al.*, 2015) and in a Urology setting it has proved to be invaluable in varicocelectomy (*Ariagno et al.*, 2017) showing decreased sperm motility.

Mortimer et al. (2015) alluded to the problems of CASA in routine semen analysis and indicated that ideally it should be used for sperm functional studies. Earlier in this review it was indicated that more attention should be devoted to sperm functionality and its relationship to fertility. Particularly in the last decade much attention has been devoted to relate sperm functional techniques to fertility outcome but also develop CASA techniques/modules that can measure some of these functional aspects automatically, objectively and quantitatively (see aspects below).

Sperm cervical mucous penetration test

The sperm cervical mucous penetration test (SCMPT) has its origins in 1866 when Sims (quoted by *van der Horst*, 2016) showed that fertile males had many sperm passing through the cervical mucous. More recently, two versions of the test namely vanguard distance and actual swim-up sperm count or decrease of sperm count along a capillary tube has been used and it was established that the sperm count provided a better outcome in relation to sperm motility (*Ola et al.*, 2003). However, this test was adopted only much later, but in a more detailed form. The WHO5 provided detailed instructions to the sampling, storage (freezing) of cervical mucous and subjective evaluation of this test. Apart from the fact that it is difficult for most andrology/fertility centres to routinely sample/obtain cervical mucous of similar or a particular quality (close to ovulation), it can be conceived that the characteristics of the cervical mucous vary from female to female. It is accordingly most difficult to standardize SCMPT in any setting including establishing accurately on a subjective basis which sperm pass the SCMPT assay.

Mortimer and *Mortimer* (2013) have come forward with a technique to measure a subpopulation of sperm with specific kinematics to penetrate through seminal plasma (surrogate to cervical mucous). Instead of exposing sperm in semen to cervical mucous or similar medium, it is exposed to its own seminal plasma. Two main advantages of this approach is that seminal plasma share some viscosity characteristics of cervical mucous, and, secondly, sperm is challenged by the viscosity of its own seminal plasma and makes more physiological sense since the seminal plasma actually represents the first barrier for sperm to cross. Kinematic cut-off points (average path velocity – VAP>25um/s; straightness – STR>80%; amplitude of lateral head displacement – ALH>2.5) are then used to determine the number of sperm in the ejaculate that pass these criteria (ideally >5 million sperm/ejaculate). In view of the common current use of CASA in the clinical setting this surrogate of SCMPT could be valuable as a further adjunct to sperm functionality. Many studies, even in the past, have in principle shown that ability for sperm to pass through cervical mucous are of prognostic value (*Aitken et al.*, 1985; *Eggert-Kruse et al.*, 1989).

Vitality and hypo-osmotic swelling

The eosin-nigrosin test or dye exclusion test is universally accepted for determining the percentage live sperm in a sperm sample. The WHO5 manual suggests an eosin-nigrosin combination dye made up in 0.9% saline and evaluation should include the use of an × 100 objective lens. The basic rationale and validity of the eosin-nigrosin test is undisputable. However, there are three major disadvantages to this technique as described in WHO5; in low concentration samples it may take very

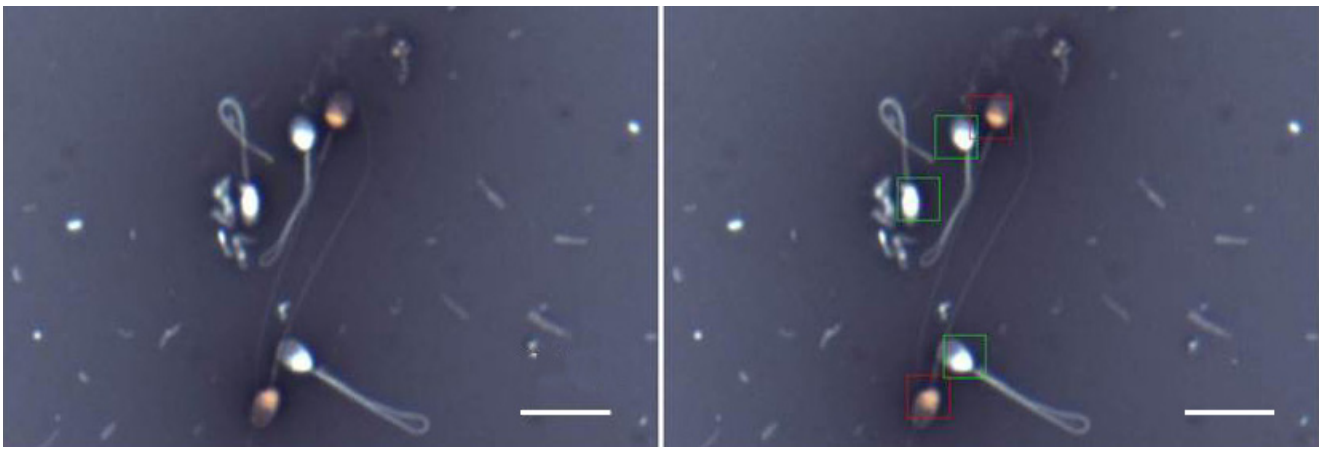

Fig. 2. Measuring vitality of spermatozoa. White sperm cells are alive and underwent hypo-osmotic swelling (note sperm tail swelling after exposure to the hypo-osmotic solution), while pink sperm cells are dead and show no swelling. The panel on the right shows the correct classification with the BrightVit module of the SCA CASA (Sperm Class Analyser computer-aided sperm analysis system) SCA 6.2 (Miroptic SL, Barcelona, Spain[1]): viable cells framed by green squares and non-viable cells framed by red squares. Scale bar = 10 µm

1 Specialized functional test allows simultaneous assessment of the sperm head (eosine staining) and tail (hypo-osmotic swelling) cellular membrane integrity (editor footnote).

long to count 100 to 200 cells; it is in several instances difficult on a subjective basis to distinguish between live and dead; because of the background noise it cannot be used in CASA analysis.

A modified eosin-nigrosin stain, BrightVit, has since been developed by Microptic SL (2016) that is also hypo-osmotic and amenable to CASA analysis using the 20× objective. Accordingly, at low magnification it is faster since more cells are captured per field and as many as 500 cells can be captured in less than 5 minutes and it is objective using the relevant SCA (Sperm Class Analyser) CASA module. The same slides can also be used for the determination of hypo-osmotic swelling (HOS test) and this is almost equivalent to the percentage live cells. In both instances vitality should not be less than the percentage sperm motility and be higher than 58% to qualify for a good semen sample (figure 2).

A more accurate method to determine vitality is by using a fluorescent technique involving Sybr-14 or Hoechst in combination with propidium iodide and a fluorescent microscope (*Garner* and *Johnson*, 1995). CASA systems such as the SCA (Microptic SL, Barcelona) has automated this and vitality measurement is objective and very fast.

The vitality tests discussed above are particularly useful in cases of asthenozoospermia (very low sperm motility), and when to decide in the *in vitro* fertilization (IVF) laboratory on the best strategy when there is only 10% motility but 60% vitality for example. The cut-off point for good quality sperm for the HOS test is similar than for vitality (>58%).

Hyperactivation

Hyperactivation (HA) was first described independently by *Yanagimachi* (1969) as well as *Gwatkin* and *Andersen* (1969) in the hamster. Hyperactivation of sperm is associated with vigorous motility (*Yanagimachi*, 1969), including rapid speed and large high amplitude flagellar waves (whiplash tail movements) with a large space gain compared to non HA sperm (*Mortimer et al.*, 2015). It was soon established that HA is an important endpoint of capacitation required for the acrosome reaction and eventual fertilization. HA is typically associated with various Ca^{2+} signals (*Alasmari et al.*, 2013) and particularly Ca^{2+} moving into sperm and involving CatSper 1[1] directly or indirectly (*Tamburrino et al.*, 2015). Progesterone is among several chemicals that seem to play an important role in priming sperm to become hyperactivated.

It was only in 1984 that *Burkman et al.* showed by means of CASA the relationship of HA with fertile and oligozoospermic groups in humans, while *Mortimer* (1997), and *Mortimer et al.* (2015) confirmed the importance of HA in sperm functional testing and its relationship with fertility. It is currently accepted that HA >20% relates to a high quality sperm and fertilization success in humans and animals (*Burkman et al.*, 1984; *McPartlin et al.*, 2009; *Mortimer et al.*, 2015).

Different modern CASA systems such as the HT[2]-IVOS II and the SCA versions 5 and 6 are programmed to measure sperm hyperactivation routinely in capacitation medium using Boolean arguments for different kinematic parameters such as curvilinear velocity (VCL), linearity (LIN) and ALH and or the D fractal (*Mortimer et al.*, 2015). If performed under standardized conditions this provides an objective assessment to potential fertility outcome (figure 3).

1 CatSper1 is a member of the sperm specific cation channels (editor footnote).
2 Hamilton Thorne Inc (editor footnote).

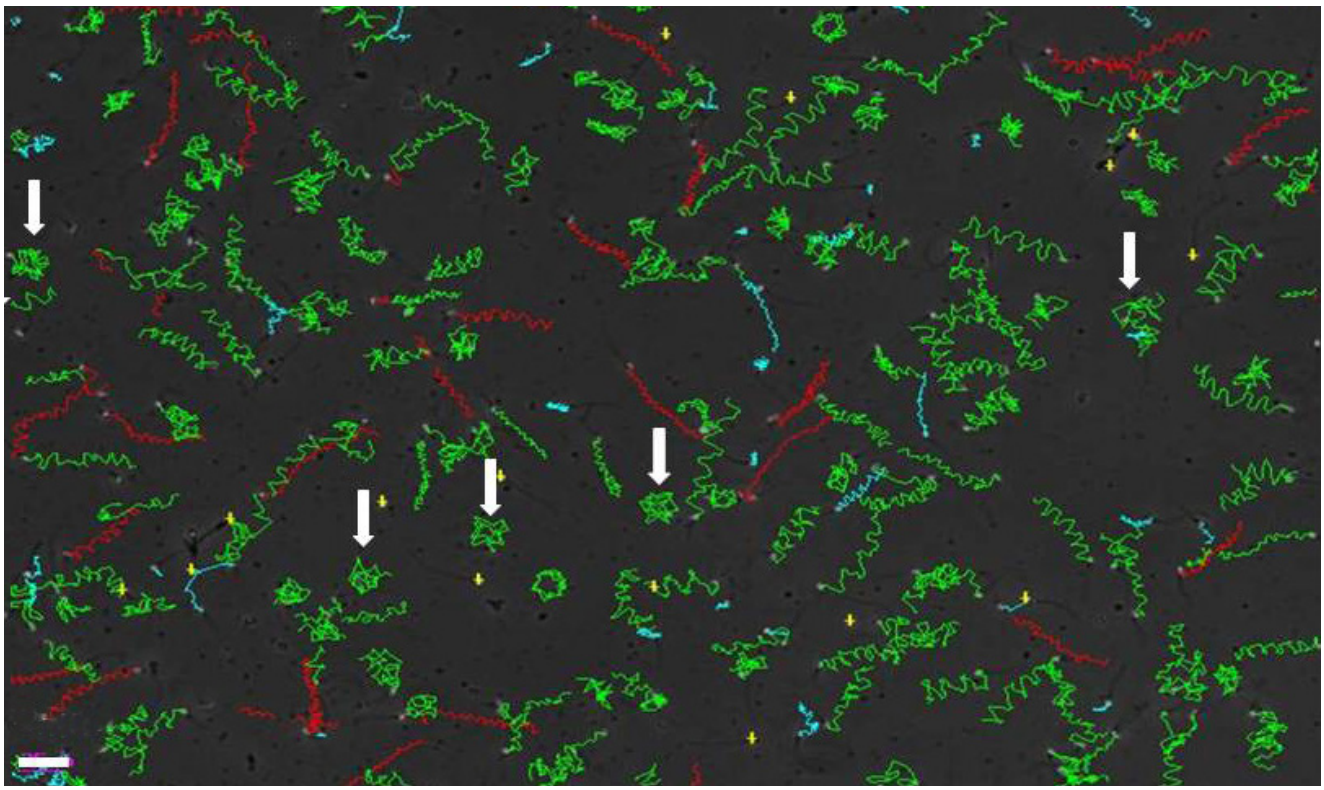

Fig. 3. CASA (computer-aided sperm analysis) tracks of human spermatozoa showing several hyperactivated spermatozoa (see arrows pointing to starspin type hyperactivation tracks). A capacitation medium was used and this example has > 30% hyperactivation. Scale bar = 25 μm

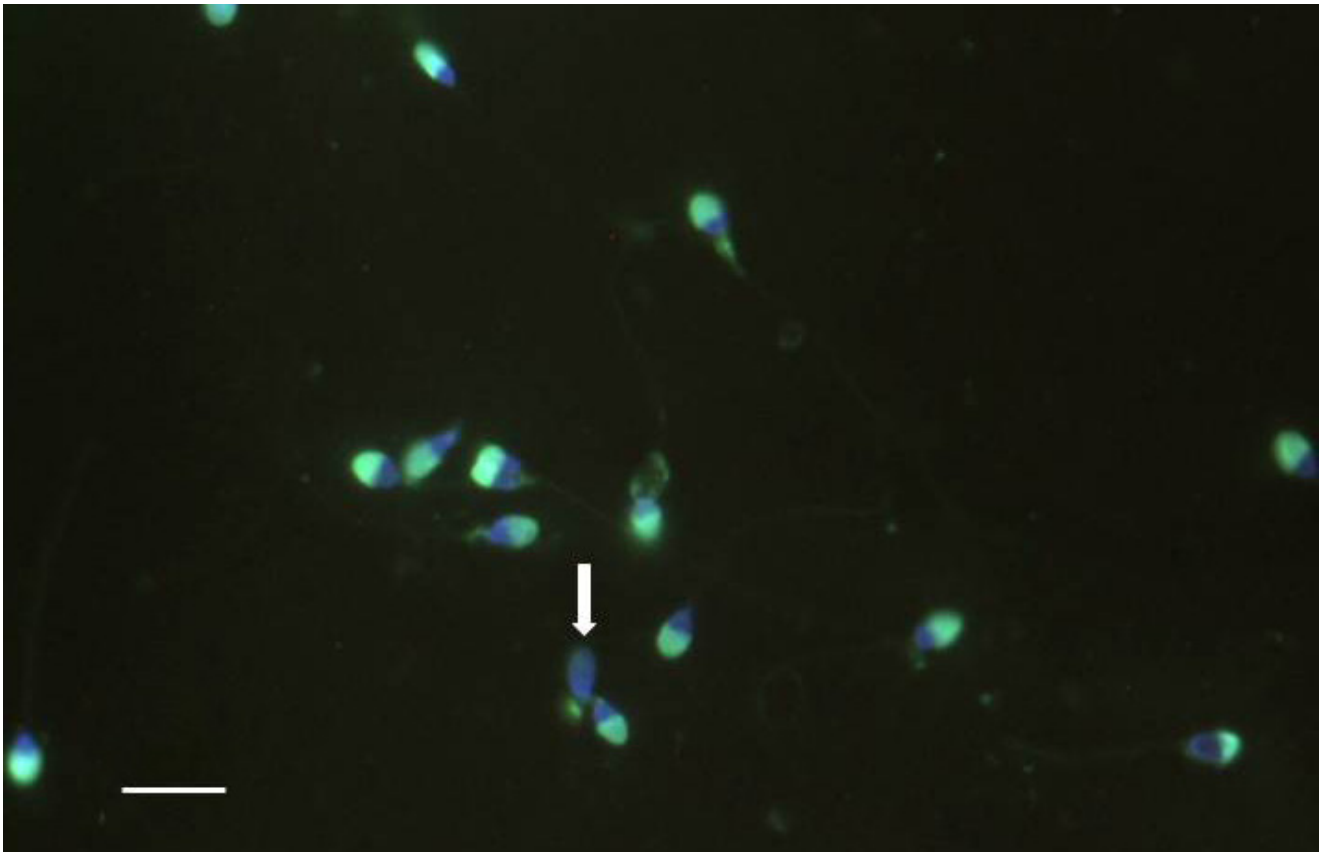

Fig. 4. Measuring acrosomal status of human spermatozoa with the FluoAcro kit (Microptic SL, Barcelona, Spain[1]). Sperm head with intact acrosome fluoresces green (stained with PNA – Peanut agglutinin derived from Arachis hypogaea[2]), while with reacted acrosome (arrow) fluoresces only blue (stained with Hoechst). Scale bar = 10 μm

1 Specialized functional test allows simultaneous assessment of the sperm acrosome status (PNA staining) and sperm viability (Hoechst staining) (editor footnote).
2 Fluorescein isothiocyanate-conjugated peanut agglutinin – FITC-PNA binds to the outer acrosome membrane (editor footnote).

Acrosome reaction

HA and acrosome reaction (AR) are intimately related. AR cannot occur if sperm capacitation has not taken place with HA as a central capacitation landmark. Many of the mechanisms associated with HA, such as an increase in intra cellular calcium and Ca^{2+} signalling also relates to the acrosome reaction and progesterone also appears to be important in the AR.

Two important facets are required when considering the acrosome reaction. Firstly, it is important to establish that most sperm are acrosome intact. Secondly, sperm need to be able to undergo the acrosome reaction (*Cummins et al.*, 1991). The most commonly accepted techniques also prescribed by WHO5 is the use of the agglutinins from *Pisum sativum* (PSA) and *Arachis hypogaea* (PNA) among others but a brightfield microscopic technique using a tri-stain combination have been used in the past with success (*Henkel et al.*, 1993; *Talbot* and *Chacon*, 1981). In this latter case solubilized zonae have been used to induce the acrosome reaction and have been found to be of prognostic value.

Jamil and *White* (1981) established the principle of the acrosome induced reaction test using Ca^{2+} ionophore. *Mortimer* (1994) described a very detailed modified technique for AR based on the principles of and a combination of PNA and Hoechst for the evaluation of live and dead acrosome intact/reacted sperm followed by exposure to Ca^{2+} ionophore to induce the acrosome reaction. WHO5 (2010) cautioned that the concentration of Ca^{2+} ionophore may be too high and needs to be reduced to what may be more physiological. It was demonstrated by several researchers that 5 µmol/L Ca^{2+} ionophore can be used to induce the acrosome reaction and that it may have a potential in the clinical laboratory and relate to fertilization outcome (*Pampiglione et al.*, 1993; *Zeginiadou et al.*, 2000). Figure 4 depicts intact acrosomes using PNA and Hoechst staining showing acrosomes in green (intact) and one sperm stained by Hoechst only (acrosome reacted) (*van der Horst*, unpublished using Microptic FluoAcro kit). We have also established that a 1 µmol/L Ca^{2+} ionophore concentration is ideal for inducing the acrosome reaction and is in line with previous suggestions to lower Ca^{2+} ionophore concentrations to acceptable (physiological?) levels.

Oxidative stress

The concept that excessive production of ROS is related to abnormal semen parameters, sperm damage and impaired sperm function has been generally accepted. ROS, such as hydrogen peroxide, superoxide anions, and hydroxyl radicals are formed as by-products of oxygen metabolism and in semen it can originate from either intrinsic production by the spermatozoa themselves or from external sources such as leukocytes that are mostly omnipresent in the ejaculate (*Lackner et al.*, 2010).

Physiological levels of ROS are vital for a number of critical sperm functions such as capacitation and the acrosome reaction (reviewed in *du Plessis et al.*, 2015). However, when excessive amounts of ROS are produced and the enzymatic (e.g. catalase, superoxide dismutase, glutathione peroxidase) and non-enzymatic antioxidants (e.g. Vitamin C, glutathione, albumin) are unable to eradicate it, oxidative stress develops with damaging consequences to the spermatozoa (*Kothari et al.*, 2010). Due to their inability to repair damage and high levels of polyunsaturated fatty acids sperm are particularly susceptible to ROS-mediated damage (e.g. lipid peroxidation of plasma membrane, impaired motility, nuclear and mitochondrial DNA fragmentation) (*du Plessis et al.*, 2010). Several studies have reported that 25–40% of infertile men show elevated ROS levels and reduced total antioxidant capacity (TAC) in their semen compared to fertile counterparts (*Agarwal et al.*, 2014; *Barazani et al.*, 2014; *Mayorga-Torres et al.*, 2017; *du Plessis et al.*, 2008; *Tremellen*, 2008).

Tests to determine potential oxidative injury include assessment of ROS generation as well as antioxidant capacity analysis (*Lewis*, 2007). For various reasons these tests are not routinely included during the screening and evaluation of men with fertility problems, despite their superior diagnostic and prognostic properties. Multiple assays to measure ROS exist and the most commonly performed analysis are chemiluminescent based. This includes the use of probes such as luminol, lucigenin, dihydroethidium (DHE), 2,7-dichlorofluorescein (DCF) and 4,5-diaminofluorescein diacetate (DAF-2DA) of which the illumination can be detected by a chemiluminometer, flow cytometer or fluorescent microscope (*Hamada et al.*, 2013; *Lampiao et al.*, 2006a, 2006b; *Mahfouz et al.*, 2010).

Total antioxidant capacity (TAC) of the seminal plasma can be measured colorometrically and together with the ROS results a ROS-TAC score can be calculated. Unfortunately, these methods have limitations to be used routinely for diagnostic purposes as they require large volumes of semen and are cumbersome, costly and time consuming. Recently it has been shown that oxidation reduction potential (ORP), which is a direct measurement of oxidative stress or redox potential can be determined with great success in small volumes of semen in real time (*Agarwal et al.*, 2016b). This relatively inexpensive test provides boundless research and clinical opportunities.

The extent of ROS induced oxidative stress damage can also be assessed indirectly by measuring the levels of lipid peroxidation and DNA damage sustained by spermatozoa. Despite the use of ROS testing by certain andrology laboratories as an advanced test in the evaluation and diagnosis of unexplained male infertility, universally acceptable and standardized seminal ROS, ROS-TAC and ORP assays as well as cut-off ranges remains to be established in order to successfully predict fertility outcomes and guide treatment options (ART and antioxidant therapy).

Sperm DNA fragmentation

Sperm DNA fragmentation (SDF) has been linked to various pathologies (e.g. varicocele) and abnormal sperm parameters. However, impaired sperm chromatin is also found in men displaying semen parameters within the normal ranges (*Agarwal et al.*, 2016a; *Esteves*, 2016). During spermatogenesis histones are replaced by protamines which help to compact and protect the DNA during transit. Within limits, a certain amount of SDF can be repaired by the oocyte's cytoplasm; if this damage exceeds the repair threshold it can cause infertility issues (*Agarwal et al.*, 2016a).

Various assays have been developed to measure SDF and chromatin abnormalities ranging from rather sophisticated to relatively simple cytochemical assays, with some relating to DNA maturity/compaction, while others measure the levels of SDF directly or indirectly (table 3). Of these assays the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), the sperm chromatin structure assay (SCSA) and the sperm chromatin dispersion test (SCD) are the most routinely used and cut-off levels have been defined in the literature. As these tests measure different expressions of sperm DNA damage the results are not necessarily interchangeable (*Esteves*, 2016).

Fluorescence *in situ* hybridization analysis (FISH) of sperm is another useful test related to DNA and chromosomes. This cytogenetic technique is able to detect sperm aneuploidy and helps to quantify complex chromosomal rearrangements, such as translocations and inversions. FISH analysis of sperm can be successfully used as a screening tool for men with severe male factor infertility, especially in cases of prior repeated IVF/intracytoplasmic sperm injection (ICSI) failure or recurrent pregnancy loss (*Hwang et al.*, 2010).

Defective protamination and abortive apoptosis can possibly explain the generation of DNA fragmentation within the testis, while outside of the testis oxidative stress is the major cause of SDF during transit through the epididymis and post-ejaculation (*Esteves*, 2016). It has been well documented that SDF correlates significantly with impaired reproductive outcomes (*in vivo* and *in vitro* conception, pregnancy loss, health of offspring), however, some controversy regarding the validity and clinical significance of these techniques still exist. It is commonly accepted that consensus should be reached regarding standardization, clinical significance and establishing of recognized reference ranges. Despite recently conceding that SDF results might be clinically informative w.r.t. intrauterine insemination (IUI), IVF and ICSI, the *Practice Committee of the American Society for Reproductive Medicine's* (2013; 2015) practice guidelines currently still recommend against the routine use of sperm DNA testing; however, it should be noted that SDF testing is not a replacement for the standard semen

Table 3. Different sperm DNA integrity testing methods

Assay/Test	Principle/Method	Outcome/Product
Acridine orange test (AO)	Fluoresces green when bound to ds (non-denatured) DNA Fluoresces red when bound to ss (denatured) DNA	Provide quantity of sperm with DNA damage (%SDF)
Aniline blue staining (AB)	Stains remnant histones dark blue Protamine-rich nuclei remain unstained	Marker of sperm chromatin maturity and compaction
Toluidine blue (TB)[1]	Normal sperm appear light blue abnormal DNA condensed sperm appear dark blue or violet	Measures damaged chromatin (%SDF)
Chromomycin A3 (CMA3)[2]	Protamine deficient sperm appear bright yellow Sperm with normal protamination appear yellowish-green	Associated with poorly packaged sperm chromatin
Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL)	Quantifies the enzymatic incorporation of dUTP (modified nucelotides) into free ends of DNA (nicks)	Measures "true" ss and ds DNA fragmentation (%SDF)
Sperm chromatin dispersion test (SCD/Halo)	Different patterns of DNA dispersion after denaturation; Big or medium halo – non-fragmented DNA; Small or no halo – fragmented DNA	Measures fragmented DNA (%SDF)
Sperm chromatin structure assay* (SCSA)	Measures DNA stability and susceptibility to denaturation	SDF index (%)
Single cell gel electrophoresis assay (Comet)	Quantify the amount of DNA fragments migrating from the sperm head	Analyse different types of DNA damage (ss/ds breaks, altered bases); Quantifies the degree of SDF
Quantitative PCR (QPCR)	QPCR of nuclear and mitochondrial DNA	Quantitative data on the number of DNA lesions per kb of DNA
Fluorescent in situ hybridization (FISH)	Cytogenetic technique using chromosome specific probes	Detects aneuploidy

* – modification of the acridine orange test; ds – double stranded; SDF – sperm DNA fragmentation; dUTP – deoxyuridine triphosphate; PCR – polymerase chain reaction; ss – single stranded (adapted from *Agarwal et al.*, 2016a; *Aitken*, 2006);

1 TB has affinity to sperm DNA phosphate residues (editor footnote).
2 CMA3 competitively binds to GC-reach sequence of DNA (editor footnote).

analysis, but should rather be seen as an adjunct (*Agarwal et al.*, 2013).

Sperm zona pellucida binding testing

During sperm-egg interaction the zona pellucida (ZP), which consists of various glycoproteins (ZP1, ZP2, ZP3) and surrounds the oocyte, is responsible for species specific sperm recognition. However, it also serves as a binding site for sperm and acts as a natural ligand to induce the acrosome reaction (*Aitken*, 2006; *Oehninger et al.*, 2014). The interaction between spermatozoa and the ZP is a critical event leading to fertilization and reflects multiple sperm functions (*Vasan*, 2011). Quantification of sperm-ZP binding led to the development of the hemizona assay (HZA) (*Burkman et al.*, 1988). The HZA is a highly significant internally controlled functional bioassay and is one of only a few sperm-oocyte interaction tests.

The HZA is performed by incubating matching halves of a ZP with sperm from a patient and fertile donor (control) respectively. Binding capacity is expressed as a hemizona index (HZI) and calculated by expressing the number of tightly bound patient sperm as a percentage of the number of tightly bound control sperm. However, as the binding is species specific it limits the usefulness of this assay as only human zona can be used (*Vasan*, 2011).

It is well documented that the HZI relate to spermatozoal events leading to fertilization as only capacitated and acrosome reacted sperm (thus normal functioning spermatozoa) can bind to the ZP (*Franken* and *Oehninger*, 2012). The HZA is also highly predictive of IVF (*Oehninger et al.*, 2000) and IUI fertilization and pregnancy outcomes (*Arslan et al.*, 2006).

Results from this functional assay help to determine the clinical management of men for whom conventional IUI and IVF therapy is likely to be unsuccessful and whom should rather be referred to ICSI (*Aitken*, 2006; *Oehninger et al.*, 2014).

Hyaluronian binding assay

Sperm plasma membrane remodelling occurs during the maturational steps of spermiogenesis. This promotes the formation of ZP-binding sites and the expression of Hyaluronic acid (Ha) receptors which are localised on the acrosome membrane (*Cayli et al.*, 2003). These membrane changes are further accompanied by cytoplasmic extrusion and synthesis HspA2, another cellular marker of maturity.

Ha-binding has been shown to correlate significantly with viability, acrosome intactness and sperm maturity (*Huszar et al.*, 2003). Not all sperm binds to Ha and various experiments have shown that those able to bind Ha have completed cytoplasmic extrusion and membrane remodelling, as well as the replacement of histones with protamines (*Huszar et al.*, 2006). Sperm motility and viability is a prerequisite for Ha-binding ability, while only sperm with an intact or slightly reacted acrosomal cap are able to bind. Furthermore, enrichment of morphologically normal sperm, as evaluated by Tygerberg Strict Criteria, was also observed in Ha-bound spermatozoa. Interestingly, it is estimated that the selection power of Ha for normal spermatozoa are relatively similar to that of ZP (*Prinosilova et al.*, 2009; *Ye et al.*, 2006).

Positive correlations were found between the hyaluronian binding assay (HBA) test and total motile sperm count, progressive motility and sperm concentration, thereby proving to be a useful tool in verifying sperm quality (*Yildirim et al.*, 2015). The HBA also selects for sperm with less DNA fragmentation and low frequency of chromosomal abnormalities (*Nasr-Esfahani et al.*, 2008).

HBA binding has diagnostic and prognostic utilities. *Huszar et al.* (2006) were able to identify and classify three sperm populations based on Ha-binding, i.e. (i) sperm that bind permanently (mature), sperm that continuously bind and release (intermediate maturity) as well as those that exhibit no binding (immature). These HBA results can assist clinicians in the therapeutic approach to ART as it is a convenient and reproducible laboratory test for identifying and assigning patients for either IVF or ICSI treatment (*Oehninger et al.*, 2014). HBA scores are not only significantly associated with fertilization rates and biochemical pregnancies (*Worrilow et al.*, 2013), but Ha selected sperm will also ameliorate the risks related to ICSI fertilization with sperm of diminished maturity (*Huszar et al.*, 2006).

Proteomics

Proteomics allows for the characterisation of the semen profile at a molecular level as it offers a comprehensive analysis of all the proteins expressed by the spermatozoon or those present in the seminal plasma (*Kashou et al.*, 2011; *du Plessis et al.*, 2011). Sperm proteomics are evolving rapidly and researchers belief that identification of proteins expressed differentially between normal and diseased state holds the key to better diagnosing and understanding of phenotypical and functional aberrations leading to male infertility (*Barazani et al.*, 2014).

Human sperm and seminal plasma are particularly suited for non-invasive proteomic analysis as it is easily obtained, isolated and purified. Several methods have been developed to separate and digest the proteins where after the peptides are subjected to liquid chromatography and mass spectrometry. This helps to identify the proteins by mapping peptide mass as well as by sequencing the peptides by fragmentation characteristics according to mass-to-charge ratio of ions. The data acquired are analysed via bioinformatics through submitting the

amino acid sequences into various data basis (e.g. Mascot, SEQUEST) to search for matching peptide sequences in order to identify the most likely protein(s). Pathway analysis can also be performed (using e.g. Reactome) to reveal the cellular, metabolic and regulatory roles of these proteins.

By comparing findings from studies on proteins from spermatozoa or seminal plasma, from infertile men with those from normozoospermic fertile men, putative biomarkers have already been identified that will aid in clinical application for functional diagnosis of e.g. idiopathic infertility. A number of studies have characterized irregularities in proteins from asthenozoospermic (*Zhao et al.*, 2007), oligozoospermic (*Hosseinifar et al.*, 2013) and immature samples (*Sharma et al.*, 2013c). Proteomics also exposed reduced protamine content in infertile men, which relates to DNA fragmentation (*De Mateo et al.*, 2007; *Intasqui et al.*, 2013). Pathological conditions such as varicocele (*Hosseinifar et al.*, 2013) and elevated ROS (*Hamada et al.*, 2013; *Sharma et al.*, 2013a, 2013b) levels displayed differentially expressed protein profiles.

The groundwork of ascertaining and cataloguing seminal protein profiles has already been laid. Many of the proteins differentially expressed between control and pathological sperm and seminal plasma samples represents potential novel proteomic biomarkers for diagnosing male infertility with prognostic abilities of identifying the best treatments (e.g. therapeutic, surgical, ART) (*Barazani et al.*, 2014).

Metabolomics

Metabolomics is a systematic approach to study the metabolites within cells or fluids as small-molecule biomarkers which represent chemical phenotyping. Identifying the metabolome and its dynamic changes can subsequently be associated with the physiological or pathological state (*Courant et al.*, 2013; *Deepinder et al.*, 2007; *Egea et al.*, 2014). This can be performed through various techniques including e.g. nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), gas chromatography coupled to mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), capillary electrophoresis-mass spectrometry (CE-MS), and optical spectroscopy.

Recent studies have demonstrated the potential role of this rapid, noninvasive analysis in the investigation of infertile men. To date a total of 69 metabolites have been identified in spermatozoa (*Paiva et al.*, 2015), while metabolomic profiling of seminal plasma is also explored as an approach for acceptable diagnosis in the evaluation and characterization of male fertility/infertility such as idiopathic infertility, testicular failure, azoospermia and ductal obstruction (e.g. differences in citrate, lactate, glutamate, cholesterol glycerylphosphorylcholine and glycerylphosphorylethanolamine) (*Deepinder et al.*, 2007; *Hamamah et al.*, 1998; *Zhang et al.*, 2015; *Zhou et al.*, 2016). *Gupta et al.*, (2011) was able to identify 10 seminal plasma metabolites of which 5 could possibly be used as biomarkers of infertility.

Zhou et al. (2016) also concluded in their novel study that plasma metabolomics has a diagnostic future as they were able to discriminate with very high sensitivity and specificity between controls, men with seminal plasma abnormalities and those with erectile dysfunction. However, more studies are necessary to identify the complete sperm and seminal plasma metabolome in order to recognise infertility biomarkers with certainty (*Egea et al.*, 2014).

Concluding remarks

It is clear that there are many conflicts in this Marriage of Figaro. It is not only a conflict of manual *versus* more objective analysis of basic semen parameters or actually adhering to the WHO5 guidelines or the importance of using sperm functional tests. It is of importance to realize what each of these aspects is intended for, their strengths, and weaknesses and how they can be combined in good matrimony to advise us better about male fertility.

Firstly, the WHO5 manual and similar guidelines for ESHRE are intended to provide basic guidelines and standard methodology for semen and sperm quality determination and in this respect remains a cornerstone. It is not a manual to be used for evaluating fertilization success or live birth outcome. One of the major problems is that in many (most?) andrology, embryology and fertility laboratories or centres the WHO5 is used to provide some kind of fertility outcome. The authors are in agreement that several facets of the WHO5 semen analysis are "outcome-based", but then a single or two or three parameters cannot be used to predict fertilization success or live birth outcome or determine a specific assisted reproductive technique. In this context much work is needed to develop mathematical models that will use various sperm parameters and sperm functional aspects to construct a greater likelihood for fertilization success than before.

Secondly, the unfaithful *Figaro Marriage* continues despite the fact that methodology is abused in many semen analysis laboratories. Thirdly, the very good intention of including sperm functionality in WHO5 is almost never used/clinically applied but reserved for research only. There are many sperm functional techniques that have been simplified and particular in view of the fact that CASA will/should become more common many of these aspects can be incorporated as routine tests.

The question remains "So how will these conflicts be resolved in order to routinely assess male fertility potential better in the laboratory?" The following provide some guidelines and are not rules or absolute endpoints:

- the WHO5 manual for semen analysis should be used for what it is intended for, i.e. to evaluate semen quality according to very specific consensus methodologies. However, this will only be realized if laboratories follow these procedures correctly and this is not currently the case,
- more objective technologies such as CASA should be used to replace manual methods, but only if these new technologies have proven to be more consistent. The CASA technologies should include at least fully automated analysis of sperm concentration, sperm motility, sperm morphology including the multiple anamolies index (MAI) and the teratozoospermic index (TZI), sperm vitality and HOS test and sperm fragmentation,
- sperm functionality as outlined should be seriously addressed and incorporated; especially those functional aspects relating to challenges in the female reproductive tract and actually relate to fertility (SCMPT, capacitation with HA as endpoint, acrosome reaction and sperm zona binding). Most of these aspects can be objectively determined using various CASA systems while several can also be performed manually (acrosome reaction),
- one of the reasons why there may have been "resistance" to apply sperm functional analysis in the routine laboratory is because they are too tedious, too complex and takes too much time. But many of these tests have been simplified and fully automated for CASA such as sperm mucous penetration, HA and some chromatin assays,
- a real problem is what happens from one WHO edition to the next? Several years lapse, despite development of newer and sometimes better technologies which unfortunately do not receive WHO/ESHRE accreditation/approval in the *interim*. Thus are they now by default disqualified despite that they may represent new information for improvement or often new innovations? While WHO/ESHRE guidelines must serve as a "watchdog" for standardization of semen analysis, it must not exclude new/alternative developments and different views based on good scientific/patient outcome basis particularly if they can be defended.,
- the marriage requires that we systematically establish a matrix where sperm functional tests, sperm quality parameters and many other factors such as female cryptic choice and psychological factors including stress, as well as the total patient/couple is considered as well as combined in models that assist us to predict better,
- the challenge then is: "Does all of this strengthen the marriage or might it lead to matrimonial problems and ultimately a divorce?" It is strongly suggested that we accept this challenge and in future provide simple, but more comprehensive semen/sperm analysis, including many complimentary techniques providing us with a better understanding of male fertility/infertility instead of just qualifying the semen by quality mainly as has been done for four decades.

It is perhaps useful to quote *Aitken* (2010) on commenting on WHO5 and pointing to future needs: "Clearly, laboratory seminology is still very much in its infancy. In as much as the creation of a conventional semen profile will always represent the foundations of male fertility evaluation, the 5th edition of the WHO manual is a definitive statement on how such assessments should be carried out and how the quality should be controlled. However, future editions of the WHO manual will inevitably move beyond the provision of consensus protocols for the conventional semen profile and into the assessment of biochemical criteria, which will shed light on the underlying pathophysiology of the infertile condition and suggest strategies for its effective management and prevention".

The average andrology laboratory needs to be accommodated in this respect and also in terms of sperm functionality in the broadest sense with techniques that are simple to perform, provided they are objective and move away from the current subjectivity. In this context the WHO/ESHRE guidelines need to move faster, more boldly, steer clear of subjective methods and adopt fresher ones. Hopefully a new edition will soon adopt more quantitative sperm functional aspects with hopefully better fertility prediction.

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