Novel Sperm Tests and Their Importance

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Introduction

Within living memory, human fertility has always been associated with special fertility symbols such as the prehistoric Venus of Willendorf symbolizing female fertility, which dates back to between 24,000 and 22,000 years BC, or phalli as a male fertility symbol. Such symbols and rituals were thought to have magic effects and thus used by all cultures around the world to assure fecundity in groups or individuals. In this context, infertility is being perceived as a stigma and leads, although mostly not painful, to psychological disorders [1]. Even though women carry the reproductive burden in most societies, men also experience psychological trauma, which leads to damaged self-esteem, inadequacy in the relation, and ridicule [2–4].

Worldwide, an estimated 80 million people are affected by infertility, thus resulting in a prevalence of infertility of 9 % [5]. Initially, the male contribution to infertility was largely ignored because the focus was rather on female infertility. In addition, the male ego and self-image, which, particularly in African and Asian societies, attribute women a low status and regard reproduction related issues as a female duty, whereas the male contribution to human reproduction is either totally underestimated or barely acknowledged. Yet about 50 % of the causes for couple infertility is attributed or partly attributed to male infertility [6]. However, since the advent of assisted reproduction and the improvement of its techniques, scientists increasingly realized that a basic semen analysis, which is still regarded a cornerstone of andrological diagnosis, is sufficient to predict neither the fertilizing potential of a single ejaculate nor the fertility of an individual man. However, although parameters like sperm count or motility or normal sperm morphology are related to fertilization success, results of a standard semen analysis have to be used with caution as they do not necessarily predict the outcome of the assisted reproduction treatment [7, 8].

The reasons for this are manifold and include the fact that the fertilization process in itself is multifactorial and can therefore be limited by numerous sperm parameters [9, 10]. In addition, the quality of ejaculates and the functional parameters of the male germ cell vary on a daily basis and do not necessarily reflect the situation on the day of insemination in an assisted reproduction program [10]. Furthermore, although the number of treatment procedures in assisted reproduction has increased over the past 30 years, pregnancy rates for both in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) remain within a range of 29–33 %, relatively low, [11] and has not significantly increased during that time [12].

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A. Agarwal et al. (eds.), *Non-Invasive Sperm Selection for In Vitro Fertilization: Novel Concepts and Methods,*
DOI 10.1007/978-1-4939-1411-1_3, © Springer Science+Business Media New York 2015

Since standard semen analysis is incomplete and does neither provide information about the functional capacity of the male germ cell, nor shows low variability of the individual parameters such as sperm count or motility, scientists were urged to find other solutions to the problem of accurately predicting male fertility. Yet even parameters with a low biological variability like normal sperm morphology [10] or sperm DNA fragmentation [13] do not detect sperm abnormalities in about 20 % of infertile men, high prevalence of idiopathic infertility is observed [14]. Therefore, some laboratories incorporated advanced sperm tests to determine the functionality of the acrosome, chromatin condensation, or DNA fragmentation into andrological diagnostics. Particularly, the latter one together with high resolution morphological analysis (motile sperm organelle morphology examination; MSOME) has been identified as a valuable parameter [15–17]. In addition, except for MSOME all other methods used to diagnose the male fertility capacity are consumptive, i.e., spermatozoa are used and by the very nature of the procedures involved are devitalized and therefore not suitable for fertilization anymore. Nevertheless, the progress made in improving, standardizing, and validating the methodologies for various male fertility parameters including sperm DNA damage [18, 19], the prediction of male fertility remains controversial [20, 21], and the emphasis for new techniques to predict the male fertility potential is not only on the identification of parameters with low biological variation and the standardization, reliability, repeatability, and validation of the relevant techniques, but also on cost-effectiveness, time consumption as well as the application of non-consumptive tests where the sperm cells can then still be used for insemination purposes.

Techniques that have been shown to have significant importance in the diagnosis of sperm fertilizing potential include sperm DNA fragmentation, mitochondrial membrane potential, sperm binding to hyaluronic acid, MSOME, the determination of reactive oxygen species (ROS), and the total antioxidant capacity (TAC) in the seminal plasma. Furthermore, newly developed techniques that might become important to test male fertility potential are sperm birefringence, proteomics, and DNA microarrays.

Current Techniques

DNA Fragmentation

Sperm nuclear DNA damage has repeatedly been shown to be associated with male infertility and recurrent pregnancy failure [22, 23] and poor seminal parameters such as motility, abnormal sperm morphology or sperm-head morphology [24–26]. On the other hand, sperm nuclear DNA damage is not only limited to infertile or subfertile patients, but incidences of up to 43 % of the ejaculates showing spermatozoa with DNA damage where the seminal parameters were normal [27]. Nevertheless, concerns were raised about the impact and validity of this parameter on fertilization and pregnancy as conflicting studies from different groups have been reported for IVF and ICSI. While researchers like Sun et al. [28], Benchaib et al. [29], or Huang et al. [30] found a relationship between sperm DNA fragmentation and fertilization rates after IVF, others [31–33] could not find an association with fertilization but with embryo formation and pregnancy rates. This finding was confirmed in a meta-analysis by Li et al. [34] is most probably due to the fact that the male genome with its subsequent gene expression is only switched on as from the four- to eight-cell stage [31, 35] and highlights early and late paternal effects on the embryo.

For ICSI, some studies [32, 36, 37] indicate a predictive value of sperm nuclear DNA fragmentation for pregnancy rates. However, in a subsequent meta-analysis based on 14 studies [38], this could not be confirmed. Instead, sperm DNA fragmentation was rather associated with increased pregnancy loss. This discrepancy might be related to the fact that for ICSI a careful selection of morphologically normal spermatozoa is performed, which might reduce the probability of injecting DNA-damaged sperm into the oocyte [39], seeing normal sperm morphology, particularly in p-pattern sperm morphology patients and

as evaluated under high magnification using MSOME, is negatively related to DNA damage [[26, 40–42], Henkel and Menkveld, unpublished]. It further underlines the possibility that male germ cells with abnormal genetic material are able to fertilize oocytes, thereby posing the risk that such damaged genomes can be manifested in the germ line and contribute to aneuploidy, malformations, miscarriages, and development of early childhood cancer [43–49], particularly after ICSI. Whereas cytoplasmic sperm defects can be repaired by the oocyte immediately after gamete fusion, this appears not to be possible for sperm nuclear damages as they will only be detected once the paternal genome is switched on [50].

Despite the criticism of sperm DNA damage as a prognostic parameter to predict fertilization outcome in assisted reproduction in terms of standardization, reliability, repeatability, and validation of the methods that can be used as "gold-standard" for clinical practice [20, 21], the currently most commonly used techniques, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay, sperm chromatin structure assay (SCSA), COMET assay, and the sperm chromatin dispersion (SCD) test, have been shown to be sensitive and produced clinical thresholds for diagnosis and prediction of success [31, 51–55]. With regard to the mentioned methods, however, one must also keep in mind that they determine different aspects of sperm DNA fragmentation [56], namely "real" DNA damage for the TUNEL assay and "potential" DNA damage in terms of susceptibility to DNA denaturation for the SCSA. Thus, one should clearly distinguish between the different assays, not only practically and methodologically but also linguistically. Therefore, further refinement is necessary. The first steps in this regard have been done for the TUNEL and COMET assay [55, 57].

On the other hand, 8-hydroxy-2-deoxyguanosine (8-OHdG) as one of the major ROS-induced DNA damage products [58], which is mutagenic and cancerogenic [59, 60], has also been shown to be closely linked with oxidative stress (OS) [61], poor sperm quality [62, 63] and function [64]. Several methodologies to detect 8-OHdG including high-performance liquid chromatography (HPLC) and immunofluorescence using microscopic or flow-cytometric analysis are available. While the measurement with HPLC is a rather large-scaled procedure, the determination of the percentage of 8-OHdG-positive cells employing fluorescence methods is easier and has been shown to be effective in predicting clinical pregnancy after intrauterine insemination, but not after ICSI [65]. The reason for this discrepancy might lie in the selection process of spermatozoa for ICSI as indicated above. Nevertheless, the possibility for flow-cytometric analysis is also available and has been shown to be rapid, reproducible, and highly accurate [66]. Yet the latter still needs to be evaluated in an assisted reproduction program for IVF and ICSI.

Mitochondrial Membrane Potential

Spermatozoa and essentially their functions depend on the functionality of the mitochondria, which can be measured by determining the inner mitochondrial membrane potential ($\Delta\psi_m$). The $\Delta\psi_m$ has been described as a sensitive indicator of mitochondrial function in terms of the functionality of the mitochondrial electron transfer chain [67]. Therefore, $\Delta\psi_m$ has been widely used in cell biology to investigate metabolism, viability and cell functionality including apoptosis. Several cationic lipophilic dyes have been used to determine the $\Delta\psi_m$. One of those dyes that were originally used is rhodamine 123 (Rh123). However, mitochondria have been found to have several energy-dependent Rh123-binding sites [68], which render this probe not very useful for the determination of $\Delta\psi_m$. In contrast, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimiddazolyl-carbocyanine iodide (JC-1) was found to evaluate changes in $\Delta\psi_m$ accurately [69] and specifically [70].

In spermatozoa, $\Delta\psi_m$ has repeatedly been associated with poor sperm motility, elevated levels of sperm ROS production, and parameters of apoptosis such as annexin V-binding, DNA fragmentation or caspase activity [71–75]. In two separate studies including 28 and 91 patients,

respectively, Marchetti and coworkers [70] revealed a positive and significant relationship between sperm $\Delta\psi_m$ and the fertilization rate in vitro after IVF. Although the correlation coefficients in both studies were relatively low ($r=0.36$ and $r=0.24$, respectively), the test was suggested to as one of the most sensitive parameters of functional quality of spermatozoa and therefore useful in diagnosis of male factor infertility and the prediction of fertilization in IVF [74, 76–78]. On the other hand, a recent study by Zorn et al. [79] comparing various clinical and sperm parameters including sperm DNA damage and $\Delta\psi_m$ revealed that the DNA damage predicted the occurrence of natural pregnancy better than all other parameters investigated. Thus, more work has to be carried out in order to evaluate and most importantly standardize and validate this certainly important functional parameter of spermatozoa.

Hyaluronic Acid (Hyaluronan) Binding

Hyaluronan is the main glycosaminoglycan secreted by the cumulus mass [80] which spermatozoa have to penetrate before reaching the oocyte. During this process, interaction between the male germ cell and the female organism takes place and spermatozoa are thought to bind to hyaluronan via a receptor on their membranes [81]. Considering that this appears as an essential step in the fertilization process, sperm able to bind to hyaluronan are regarded as mature [82] and have been shown to have normal general and nuclear morphology and functions. Moreover, they exhibit lower rates of aneuploidies and DNA damage [83–85]. Interestingly, the physiologic response of human spermatozoa in terms of tyrosine phosphorylation patterns does not differ after sperm binding to zona pellucida or hyaluronan. In turn, immature sperm fail to execute this important physiologic process [86].

ICSI performed with hyaluronan-selected sperm resulted in high quality embryos and improved life birth rates [87, 88] and Worrilow et al. [89] showed in a multicenter, double-blinded, randomized controlled study that ICSI with spermatozoa from men who were prescreened with less than 65 % hyaluronan-bound spermatozoa had a significantly higher chance of an ongoing pregnancy after ICSI if spermatozoa were selected by means of hyaluronan-binding. Nevertheless, the test is not without any criticism by well-known scientists. Van den Bergh et al. [90] found no significant differences in fertilization rates and zygote scores by hyaluronan-bound and non-hyaluronan-bound spermatozoa in their controversially received study [91]. On the other hand, other recent studies revealed that hyaluronan-binding was not able to predict the results of the sperm penetration assay [92], pregnancy rates in intrauterine insemination cycles [93], and IVF [94].

The failure of the hyaluronan binding test to predict fertility indicates only a limited role of isolated hyaluronan in sperm selection [95] because both components of the cumulus, the extracellular matrix with its hyaluronan content, and the cumulus cells with their conversion of glycodelin-A and -F into glycodelin-C contribute to the male germ cells' ability to penetrate the cumulus and modulate sperm functions [96, 97]. The reason for this failure of hyaluronan-separated spermatozoa to achieve higher implantation rates might reside in the nature of the method because other factors such as glycodelin-C are missing.

Motile Sperm Organelle Morphological Examination (MSOME)

Normal sperm morphology has been regarded as a good predictor of male fertility potential, particularly if a strict evaluation approach is followed [98–100]. Nevertheless, this classic methodology of assessing normal sperm morphology is a relatively large-scaled procedure and consumptive, i.e., the spermatozoa that are assessed are no longer available for fertilization. In addition, the evaluation must be carried out in a semen sample different from that used for insemination. These obvious disadvantages can be overcome by a method developed by Bartoov et al. [101], which evaluates sperm morphology

at higher, digital magnification (6,300×) using Nomarski interference contrast. Using this technique, a finer morphological status of acrosome, post-acrosomal lamina, neck, mitochondria, flagellum, and the nucleus can be examined. For the latter, the shape, as well as the presence and size of vacuoles, is observed. Since MSOME identifies objects undetectable by light microscopy, such as nuclear vacuoles, which are indicative of abnormal chromatin packaging [102], this method is regarded more stringent than the evaluation of sperm morphology according to strict criteria [103].

High resolution of specific morphologic features like nuclear vacuolization and sperm head morphometry as evaluated by MSOME has been shown to correlate very well with various other sperm parameters including sperm concentration and motility [26], capacitation and acrosomal status [104], and DNA integrity [42, 105, 106]. Since MSOME is thought to identify good quality spermatozoa, the technique has been included in ICSI protocols in an increasing number of groups (intracytoplasmic morphologically selected sperm injection; IMSI). In turn, using IMSI, not only fertilization rates but also implantation and pregnancy rates could be improved [107, 108]. These results were confirmed in a recent meta-analysis [109]. On the other hand, Balaban et al. [110] restrict the beneficial effects of IMSI to selected male factor patients and only with lower rates of aneuploidy and miscarriage [109, 111].

In contrast to these positive results, other studies indicate that the association of the occurrence of large nuclear vacuoles with sperm DNA damage is only valid if the nuclear vacuoles are taking up more than 50 % of the nuclear volume [112]. This assumption is supported by Watanabe et al. [113] showing that only 7 (=3.1 %) of spermatozoa with large vacuoles out 227 were TUNEL-positive suggesting that ICSI using spermatozoa selected for injection by MSOME from patients with high quality semen is not necessary. This assumption can be supported by the study of Tanaka et al. [114] who showed that sperm head vacuoles do not affect the outcome of ICSI. Although this methodology is appealing because it is non-consumptive, the procedure, for diagnostic (MSOME) and treatment (IMSI), is time consuming and little practical for routine semen testing. In addition, MSOME has not been properly validated yet.

Reactive Oxygen Species (ROS)/Total Antioxidant Capacity (TAC)

Reactive oxygen species (ROS) are highly reactive radical derivatives of oxygen that are produced by any living cell, including spermatozoa, in the mitochondria. These molecules are chemical intermediates that have one or more unpaired electrons, which causes them to be highly labile and results in extreme reactivity. Examples of biologically relevant ROS are hydroxyl radicals (·OH), superoxide anion ($\cdot O_2^-$), or hydrogen peroxide (H_2O_2). ROS have a high oxidative potential and therefore very short half life-times in the nanosecond (10^{-9} s) (·OH; hydroxyl radicals) to millisecond range (10^{-3} s) ($\cdot O_2^-$; superoxide anion) [115]. Consequently, these molecules essentially react at the site of generation.

Considering that male germ cells exhibit a specially composed plasma membrane with an extraordinary high amount of polyunsaturated fatty acids, which is essential for normal sperm functions, spermatozoa are very sensitive to oxidative damage by ROS [for review see: [116]]. Despite the detrimental effect that ROS have on spermatozoa causing lipid peroxidation or DNA fragmentation by means of oxidative stress (OS), ROS also exert important physiologic roles by triggering cellular events such as sperm capacitation, hyperactivation, and the penetration of the zona pellucida [117–119], and thereby modulating acrosome reaction as key event in the fertilization process [120, 121].

Considering the two important features of ROS, namely, causing OS if present in excessive amounts [116, 122–125], thus having detrimental effects, and on the other hand, having beneficial effects by triggering essential cellular functions, the male and female organisms must counteract excessive OS for spermatozoa. For this purpose, seminal plasma contains more antioxidant compounds than any other physiological fluid, including vitamins C and E

[126, 127], superoxide dismutase [128], glutathione [129], glutathione peroxidase [130], or uric acid [131]. Except for the semen-specific polyamines spermine and spermidine [132], the female organism also provides these radical scavengers [133, 134], and a lack thereof will result in disturbed reproductive functions [135, 136]. Thus, finding the correct balance between oxidation and reduction is crucial for normal sperm function and fertilization [137, 138] as reductive stress is as dangerous as OS [116, 139].

This has serious consequences for andrological diagnostics as both parameters, sperm ROS levels [137, 140] as well as the so-called total antioxidant capacity (TAC) [141, 142], have to be tested in order to obtain a picture of the seminal redox status reflecting the seminal OS. This concept also explains the inconsistency reported in the literature about the impact and importance of ROS as well as that of leukocytes. Therefore, it is not sufficient to measure only one of these parameters, the ROS levels or seminal TAC, because both parameters may vary between different patients. For example, a patient might have high numbers of leukocytes present in the ejaculate, but if the patient also shows high levels of TAC, the seminal redox status and therefore the fertility might not be compromised. On the other hand, a patient might have low numbers of activated seminal leukocytes, but a very low TAC which do not scavenge ROS production sufficiently. In the latter case, the patient might be infertile as the system between oxidation and reduction is not in balance. Thus, for spermatozoa this system is like a "balancing act", they will only have functional competence if the system of seminal oxidants and antioxidants as a whole does not deviate to either side [116, 137, 138].

For ROS, the most commonly used test system is based on chemiluminescence with luminol [140] or lucigenin [143] as probes. The difference between these two chemiluminescent probes is that chemiluminescence of luminol appears to be dependent on the myeloperoxidase-H_2O_2-Cl^- system [144], hydroxyl radicals in vivo [145], or neutrophils in vitro [146], while lucigenin is rather specific for extracellularly released superoxide [147–149]. Furthermore, lucigenin rather measures extracellular ROS production, which is clinically more important as they are capable of damaging surrounding spermatozoa and might therefore be more suitable as a diagnostic tool [147]. Nevertheless, numerous groups are using luminol as chemiluminescent probe as it is cheaper and easy to use. Thus, the determination of ROS in seminal fluid is recommended by a number of groups to improve the management of male infertility [150–152], particularly if measured in neat semen [153]. Higher seminal ROS levels were not only significantly negatively correlated with sperm motility and concentration [154], but also with fertilization and pregnancy rates as well as embryo quality after IVF and ICSI [155].

On the other hand, Yeung et al. [156] concluded that the determination of ROS in a sperm suspension after swim-up has no diagnostic impact. In contrast, it might even play a positive role for fertilization, which then refers to the beneficial aspects of ROS. This is in line with data of Henkel and coworkers (unpublished) who showed that ROS in the medium after sperm separation is weakly, but significantly correlated with fertilization after IVF ($r=0.148$; $P=0.0454$; $n=183$). Furthermore, a positive trend was observed between sperm ROS production after sperm separation and the 4-cell stage formation ($r=0.135$, $P=0.0695$; $n=183$), possibly retrospectively reflecting the sperm cells' ability to undergo capacitation and acrosome reaction. The latter events are triggered by ROS physiologically produced by spermatozoa [121].

For the analysis of the antioxidative protection system for spermatozoa provided by seminal plasma several techniques are available including the oxygen radical absorbance capacity (ORAC) [157], ferric reducing ability (FRAP) [158], phycoerythrin fluorescence-based assay (PEFA) [159], and Trolox-equivalent antioxidant capacity (TEAC) [160]. While the latter test is most frequently used [141, 161, 162], the ORAC is high specificity and responds to numerous antioxidants [157]. On the other hand, the chemiluminescent detection of the antioxidant capacity and subsequent comparison to the water-soluble tocopherol equivalent Trolox is also time-consuming and

requires fresh preparation of chemicals each time the assay is run. Milner and coworkers [163] developed an inexpensive colorimetric alternative using 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and was commercialized. Said et al. [164] compared both assays, the chemiluminescent and colorimetric, and concluded that the colorimetric measurement is reliable and accurate and might therefore be an easy-to-perform, rapid, and cheap alternative. Yet none of these techniques has been evaluated with regard to its predictivity of male fertility.

TAC as measured by means of the FRAP method has been shown to correlate significantly with seminal parameters such as sperm concentration ($r=0.533$), motility ($r=0.530$), and normal sperm morphology ($r=0.533$) [165]. In addition, this group confirmed earlier data by Mahfouz et al. [142] using the colorimetric TEAC that TAC levels in abnormal ejaculates or from infertile patients were significantly lower. These authors also calculated a cut-off of 1,420 μM Trolox equivalent with a sensitivity of the assay of 76 % and as specificity of 64 %. Considering that there are significant correlations between TAC and serum prolactin and tetraiodothyronine levels, but not with gonadotropins, testosterone, or estradiol, Manchini et al. [166] suggest that systemic hormones might play a role in the regulation of seminal TAC.

Birefringence

A technique that can evaluate life sperm cells is polarization microscopy. In this approach, which was pioneered by Baccetti [167] to identify functional spermatozoa for ICSI, the birefringence (double refraction) of light caused by the anisotropic properties of the compact textures of the sperm nucleus, acrosome, and flagella permits the evaluation of the organelle structure of the male germ cell. Gianaroli et al. [168, 169] used the technique to distinguish acrosome-reacted from non-reacted spermatozoa. In a more recent report from the same group, Magli et al. [170] showed a strong relationship between partial birefringence and acrosome reaction. Yet the patterns of birefringence, total or partial, depends to some extend on motility and normal sperm morphology.

Collodel et al. [171] tried to evaluate the diagnostic value of the technique and used sperm birefringence to estimate viability and normal morphology. The morphology was compared with the standard technique after Papanicolaou (PAP) staining. Although there was no significant difference ($P=0.308$) between PAP and the evaluation with polarization microscopy, receiver operating characteristics (ROC) curves always showed a greater area under the curve for polarization microscopy than for PAP staining, indicating a better a higher diagnostic value. The authors suggest a cutoff value of 20 % of spermatozoa showing birefringence as indicator for fertility.

Later, Collodel and coworkers [172] confirmed positive relationships between sperm cell birefringence and motility as well as the fertility index calculated by a mathematical formula after transmission electron microscopy [173]. The authors concluded that polarization microscopy offers several advantages and that it should be considered in sperm analysis [172].

Contrary, Petersen et al. [174] challenged the positive reports with regard to sperm DNA fragmentation. These authors showed a significantly higher percentage of sperm with DNA damage in sperm presenting with total head birefringence than in those with partial head birefringence. This was in support of findings by Vagnini et al. [175] that the patterns of birefringence (total or partial) could not discriminate between sperm with normal and abnormal chromatin packaging. Gianaroli et al. [169] report significantly higher implantation, clinical pregnancy and ongoing pregnancy rates in ICSI cycles where spermatozoa selected by means of polarization microscopy were injected. The authors conclude that injection of acrosome-reacted spermatozoa seems to result in more viable embryos. Nevertheless, as reported for other tests systems, a proper clinical evaluation of the technique in terms of the establishment of reliable cutoff values has not been carried out yet.

"Omics" as Molecular Techniques

In the light of the limited predictive value of the currently used parameters, scientists started to look at biomarkers as a novel approach to identify infertile men, in recent years. Biomarkers are "distinctive biological or biologically derived indicators (as a biochemical metabolite in the body) of a process, event, or condition (as aging, disease, or exposure to a toxic substance)" that can be utilized as an objective and quantitative measure to identify infertile patients [176]. In addition, for clinical application, these biomarkers should be able to indentify infertile me easily, accurately, and cost-effectively [177]. Principally, this identification can make use of genomic, proteomic, or metabolomic techniques.

Proteomics

Considering that RNA is translated into proteins and sperm proteins not only come from the testis but are also derived from the epididymis or other accessory sex glands, and are modified and incorporated into sperm surface [178, 179], the actual protein expression in spermatozoa differs from their gene expression [177, 180, 181], this approach is of particular importance. However, scientists are facing grave problems as two compartments of the semen can be analyzed, namely, the seminal fluid and the male germ cell itself. With regard to the seminal plasma, the protein composition has multiple origin as the seminal fluid is composed of secretions from testis (about 5 %), seminal vesicles (about 60 %), prostate (about 30 %), and the bulbo-urethral glands (about 5 %) [182]. Therefore, seminal plasma markers might rather reflect pathologies of the respective glands, which, of course, can also contribute or be a cause of male infertility. In addition, the composition of seminal fluid also depends on other factors such as the general health of a particular man; for example diabetes, flu, alcohol consumption, or smoking can cause variability of the seminal fluid [8, 183]. All this makes the analysis and identification of specific male "infertility markers" in seminal plasma rather difficult [184]. Nevertheless, a number of recent studies report on the proteomic analysis of seminal plasma and found relevant differences between fertile and infertile men.

Proteomic Analysis of Seminal Plasma

Seminal plasma is abundantly available in both donors and patients and its protein concentration is with about 58 mg/mL approximately as high as in serum. The concentration of albumin, however, is markedly lower [185] and one of the major components are seminogelin I (MM 49.9 kDa) and II (MM 63.5 kDa), which are involved in the gel formation [186]. Seminal plasma is a rich source of thousands of proteins mainly belonging to three major groups; proteins carrying fibronectin type II modules, spermadhesins, cysteine-rich secretory proteins (CRISPs) [187], and approximately 25 % of the proteins are secretory [188, 189].

In an in-depth analysis of human seminal plasma, Rolland and coworkers [190] initially identified 699 proteins. However, in a subsequent comparison with previous descriptions, 2,545 unique proteins were identified, of which 83 were of testicular origin, 42 derived from the epididymis, 7 from the seminal vesicles, and 17 from the prostate. For the testis-specific proteins, three (TKTLI, LDHC, and PGK2) germ cell expression was confirmed and a difference in their expression between fertile and infertile men was established, thus highlighting these proteins as possible diagnostic biomarkers. Similarly, Milardi et al. [181] identified 83 seminal plasma proteins, including seminogelin I and II, olfactory receptor 5R1, lactoferrin, hCAP18, spindlin, and clusterin as possible target proteins to identify infertile patients. Other proteins were specifically identified in subgroups of patients showing a high percentage of DNA damage [15], an important aspect of sperm function. These proteins were associated with increased immune response, sperm motility, or inhibition of mitochondrial apoptosis.

Although the proteomic analysis of seminal plasma is a good approach for andrological diagnostics as it is non-consumptive of spermatozoa, the methodology is still in its infancy and specific marker proteins still have to be validated for their use. Eventually, normal values have to be established.

Proteomic Analysis of Spermatozoa

On the other hand, the analysis of the sperm cells themselves might give a better idea of the actual fertilizing potential of spermatozoa from a specific man. Considering that the male germ cell is highly specialized and differentiated, and has also to interact not only with the female reproductive tract [for review see [191]], but also with the cumulus oophorus, the zona pellucida, and the oolemma, this approach would make spermatozoa a primary target for a proteomic analysis. In this context, sperm surface proteins are of particular interest as the interaction between spermatozoa and the female genital tract as well as the oocyte must take place at this level for the female to select the most capable spermatozoon to fertilize the oocyte. This natural selection process is most stringent as it selects only one spermatozoon out of about 10^7 spermatozoa that are ejaculated into the upper part of the vagina.

In contrast to the analysis of seminal plasma, proteomic analysis of spermatozoa is more difficult and might therefore be limited for various reasons. In spermatozoa, not only the protein concentration is much less than for seminal plasma, but the number of spermatozoa available for the analysis varies individually and might even reach the detection limit if the seminal sperm count is very low, particularly in patient samples. Moreover, the risk of contamination of the samples by leukocytes or other non-sperm cells is high, and therefore the probability of a detection of non-sperm proteins, if the spermatozoa are not properly separated from the seminal plasma and debris prior to the analysis [192].

For human spermatozoa, the number of identified proteins varies considerably from 1,760 [193] to 4,675 of which 227 were shown to be testis-specific [194]. In a very recent literature review analyzing 30 studies, Amaral et al. [195] even report a total number of identified sperm proteins of 6,198 of which about 30 % are of testicular origin. This high number of proteins indicates the complex composition and function of the male germ cell and the proteins showed to be associated with various essential cellular functions such as sperm motility, capacitation, sperm–oocyte binding, metabolism, apoptosis, cell cycle, or membrane trafficking [195, 196]. It also makes the task of identifying highly specific diagnostic markers difficult. Nevertheless, using MALDI-TOF/TOF analysis of protein spots after 2D-gel electrophoresis, Xu et al. [197] identified 24 differentially expressed proteins in infertile patients, of which 9 (including TGF-β1, MYC, MYCN, TP53) are involved in main physiological pathways. With respect to seminal oxidative stress, Hamada et al. [198] revealed a significantly different expression of proteins related to the protection against oxidants, with 6 proteins decreased and 25 proteins increased in patients exhibiting seminal oxidative stress. Yet the methodology for a diagnostic approach has still to be standardized as the use of different detergents for the solubilization of membrane proteins results in different proteins that can be detected after electrophoresis (Fortuin and Henkel, unpublished). Moreover, none of the currently employed proteomics methodologies is properly evaluated for clinical use.

Genomics

DNA Microarrays

The progress in genomic biotechnology revealed genetic testing to be a viable alternative in andrological diagnostics, particularly as the prevalence of genetic abnormalities causing male infertility was found to between 15 and 30 % [199]. Due to the rapid improvement of technologies, which make it possible that very small genomic regions can now be analyzed and have already been found to be responsible for infertility [200–202], it is likely that this number would increase in near future since even single nucleotide modifications can be detected [201].

Currently, two main genetic tests are carried out, karyotyping and fluorescence in situ hybridization (FISH). While these techniques are limited in their ability to diagnose and specifically identify larger numbers of infertile men, and need a specific sequence of interest before determining this region in specific patients, respectively, microarrays not only allow the examination of a higher number of men but also the detection of copy number variations, gene expression levels, and

single nucleotide polymorphisms [177]. Using the microarray technology, Park et al. [203] and Lee et al. [204] were able to identify copy number variations and Y-chromosomal microdeletions outside the AZF regions.

Spermatozoa do not only store and transport the male genetic material in form of DNA, but RNA obtained from ejaculated spermatozoa also reflects gene expression during spermatogenesis [205, 206]. Although spermatozoa are transcriptionally silent [207], spermatozoa RNAs play a vital role not only in the development of the male germ cells but also in early embryo development [208, 209], which lead to the development of novel approaches in the diagnostics of male infertility using microarrays [210]. In fact, Ostermeier and coworkers [211] were able to distinguish between sperm populations exhibiting rapidly degrading and stable spermatozoa RNAs. Following this initial discovery, Krawetz et al. [212] revealed a complex population of small noncoding RNA (sncRNA) that is available at fertilization. MicroRNA (miRNA), which is a subclass of sncRNA, appears to play a modifying role in early post-fertilization [213–215]. In infertile patients, Montjean and coworkers [216] found a 33-fold lower gene expression of genes involved in spermatogenesis and sperm motility. These authors conclude that the spermatozoal transcription profile in idiopathic infertility differs significantly from that in fertile men. Although these technologies seem to be appealing for diagnostic purposes, they are still in infancy stages as relevant biomarkers have yet to be identified and validated.

Conclusion

Considering that standard semen analysis fails to predict male fertility in up to about 40 % of the cases, scientists searched for novel parameters and methodologies to close this obvious gap in andrological diagnostics. Requirements for such new tests are that they should not only be reproducible, effective, properly validated and cost-effective and time-effective, but also be nonconsumptive and stable. Particularly, the latter represents an essential condition and might even be one of the biggest challenges for novel sperm tests, as the diagnostics are carried out way before assisted reproduction treatment, and standard semen parameters vary considerably, even on a daily basis. Techniques such as determination of sperm DNA fragmentation, mitochondrial membrane potential, and hyaluronan binding refer to essential sperm functions and have been investigated for a number of years already. Attempts have been made to establish clinically significant cutoff values. However, except for the hyaluronan binding test, the consumptive nature of these test parameters still remains unexposed. Novel nonconsumptive parameters such as the high resolution evaluation of sperm morphology by MSOME, the determination of seminal ROS and/or TAC, as well as the evaluation of the birefringence of spermatozoa seem to point to alternative ways. Yet proper determination of clinical significance in terms of the fertilizing capacity of spermatozoa and valuation thereof are also still outstanding. In recent years, new promising molecular approaches to identify biomarkers of male fertility in terms of proteomic or genomic analyses of the male germ cells and seminal plasma, respectively, have been made available. On the other hand, some researchers were able to distinguish between fertile and infertile men using DNA/RNA microarrays. However, although "omics" approaches in the male infertility diagnostics are very appealing, both proteomic and genomic methodologies are still lacking the indubitable identification of markers that meet all the criteria for a good clinical marker as well as the necessary validation. Therefore, the implementation of these novel techniques in clinical routine will still take some time.

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