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REVIEW

New insights in sperm biology: How benchside results in the search for molecular markers may help understand male infertility

Sara Marchiani, Lara Tamburrino, Monica Muratori, Elisabetta Baldi

Sara Marchiani, Lara Tamburrino, Monica Muratori, Elisabetta Baldi, Department of Experimental and Clinical Biomedical Sciences, University of Florence, 50139 Florence, Italy

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Correspondence to: Sara Marchiani, PhD, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy. sara.marchiani@unifi.it
Telephone: +39-055-2758235

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Abstract

The male factor is responsible for about 40% of couple infertility cases and such percentage is expected to increase in the future because of several likely factors including the presence of endocrine disruptors in the environment, changes in lifestyle habits and advanced couple aging. How such factors affect male fertility status, however, should be clarified. Most studies on male fertility status have focused on parameters analyzed using a spermiogram test, the primary diagnostic tool in the routine assessment of male infertility, which is, however, poorly predictive of both natural and medically assisted conception. For these reasons it is mandatory for the scientific community to identify new molecular markers to incorporate into the existing diagnostic tests of male fertility. Ideally, such markers would be detected in mature spermatozoa to avoid invasive procedures for the patient. This review summarizes the recent advancements in benchside approaches that appear most promising for the development of new diagnostic sperm fertility tests, or identification of therapeutic targets, and, illustrates their advantages and limits.

Key words: Sperm markers; Male infertility; Genetic and epigenetic approaches; Proteomic approach; Ion channels

Core tip: This review focuses on genetic, epigenetic, proteomic, and post-translational protein modification and ion channel studies present thus far in the literature to identify possible sperm markers that could be helpful for new diagnostic tests or represent possible therapeutic targets for male infertility.

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INTRODUCTION

Infertility is a worldwide health problem affecting about 15% of couples[1]. Although the World Health Organization (WHO, 5th edition[2]) defines it as a disease of the reproductive system, infertility also influences emotional, social and psychological spheres. The male factor is involved in about 40% of couple infertility cases, with the highest incidence rates in Eastern Europe and Africa[1]. Male infertility, affecting presently 7% of the worldwide population, is expected to double over the coming years. Some possible explanations reside in the rise in hectic lifestyles, in the increase of pollution and in socio-economic changes that delay couples in starting a new family. Currently, how all these factors affect male fertility status is not clear.

The increase in reproductive age is becoming an important social problem, which can be particularly noted in industrialized countries. The role of advanced maternal age in the lower success of natural and medically assisted reproduction has been well established[3]. A recent trend among young women is to freeze their oocytes for social reasons, such as desire to have a career, delaying the age of the first conception. Not surprisingly, some multinational American corporations offer to pay for an oocyte preservation procedure for their female employees to allow for career advancement. In contrast with the maternal age, whether paternal age affects fertility is still highly debated. Despite some authors not finding correlations between paternal age and infertility[4,5], others have shown that a forward shift in male age represents a further risk factor for the failure to conceive[6,7], for the success of assisted reproductive techniques (ART) and for the health of offspring[8]. Advanced age may lead to changes in hormonal profile[9] and germinal epithelium disorders with the consequent alterations in seminal parameters[10,11]. Decreased sperm quality may be due to alterations in the expression of some proteins[11,12], as well as an increase in sperm DNA fragmentation (sDF)[13,14] or of other types of DNA damage[11]. In addition, it has been demonstrated that the higher number of *de novo* mutations found in offspring of increasingly older fathers can mostly be attributed to paternal transmission[15-17].

As mentioned above, besides male aging, there are several other factors contributing to the decrease in male fertility potential with similar pathogenic mechanisms, such as the ever increasing presence of endocrine disrupting chemicals in the environment[18] and the changes in lifestyle with an increased prevalence of obesity and metabolic syndrome[19].

Pharmacological treatment of the male partner can only be successfully applied to non-idiopathic causes (such as hypogonadropic hypogonadism), whereas for idiopathic infertility, despite many attempts, virtually no effective treatment is currently available[1]. A recent meta-analysis has concluded that gonadotropin therapy is a possible choice to improve fertility, especially in case of post-pubertal onset hypogonadotropic hypogonadism[20]. Efforts to treat idiopathic male infertility, for instance using gonadotropins, or anti-aromatase, anti-estrogen and anti-oxidant drugs, have not demonstrated a conclusive, beneficial effect of said therapies[21]. Until robust results are obtained, ARTs remain, for idiopathic male infertility, the option with the highest chance of achieving pregnancy.

Although ARTs have expanded globally over the last few decades, these procedures remain inaccessible in many parts of the world and are quite expensive. Moreover, despite ARTs' success rate having approved greatly over the past few years, the current live birth outcome remains low, averaging just 34%[22], with important economic and psychological consequences for couples. For these reasons it is mandatory, for the scientific community, to identify the causes of infertility in order to find effective treatments and new sperm markers to improve the accuracy of diagnosis.

The primary diagnostic tool in the routine assessment of male infertility is semen analysis (spermiogram), which consists in the evaluation of the macroscopic (volume, pH, liquefaction) and microscopic (number, motility and morphology) characteristics of seminal fluid. Despite the fact that WHO issued detailed laboratory guidelines to standardize the methods and has established normal reference values[2], spermiogram has a high operator variability, high intra-individual variation[23] and is not highly predictive of the fertility status[24,25]. The diagnosis of infertility results as being accurate only in the case of azoospermia and severe oligozoospermia. Semen analysis does not provide information about the molecular status of spermatozoon and the functions necessary for oocyte fertilization. For this reason, identification of new semen or sperm molecular markers able to discriminate between fertile and infertile men is one of the main goals of current research. Markers that single out spermatozoa with a higher fertilizing ability could lead also, in the future, to a better sperm selection for ARTs. Indeed, although new advanced tools for sperm selection have been developed based on sperm surface charge, apoptotic or maturity sperm markers and sperm ultramorphology, more studies are needed before introducing advanced sperm selection methods in ART[26]. Based on current published data, sperm selection using real-time motile sperm organelle morphology examination at high magnification coupled with intracytoplasmic morphologically selected sperm injection seems to be a promising method with benefits for late ART outcomes (pregnancy, live birth and abortion rates)[27].

This review will focus on the recent advancements of benchside approaches that appear most promising for the identification of new sperm/germ cells as molecular markers of infertility.

GENETIC AND EPIGENETIC STUDIES ON TESTICULAR GERM CELLS AND MATURE SPERMATOZOA

At least 15% of male infertility cases are due to genetic alterations[28], including Y chromosome microdeletions, present in about 20% of cases of azoospermia or severe oligozoospermia[29]. Innovative approaches implying whole-genome analysis, such as the evaluation of single nucleotide polymorphisms and copy number variations, could be helpful in the search for new gene candidates having a role in male infertility[30-32]. For instance, a recent study by Yatsenko *et al*[33] identified hemizygous mutations in the *TEX11* gene as one of the causes of meiotic arrest and azoospermia in infertile men. A microarray study found a different expression of genes linked to spermatogenesis in testis RNA from non-obstructive azoospermic (NOA) men when compared to commercial RNA from normal testicular tissue[34,35]. We expect that other genes responsible for azoospermic/severe oligozoospermic phenotypes will be discovered in the future.

Whereas genetic studies are of great help in identifying the genes involved in testicular disorders that lead to severe alterations in sperm number, the search for genetic modifications leading to sperm dysfunctions in idiopathic infertility appears to be a sort of "fishing expedition". Conversely, the use of genetic, epigenetic and proteomic approaches on ejaculated spermatozoa could allow researchers to characterize the complete spectrum of sperm phenotypes present in infertile subjects better and, accordingly, to understand the leading causes of infertility in depth.

Epigenetic alterations derived from environmental pollution, toxicants and nutritional habits could impair both sperm quality and embryo development[36,37], increasing the risk in offspring of developing chronic diseases, such as type 2 diabetes, obesity, cardiovascular disease and cancer[38,39]. Evidence in animal models suggests that some epigenetic markers can be inherited by the offspring through parents' gametes[39]. Rodent studies have demonstrated that paternal diet affects pregnancy achievement and offspring metabolism[40,41]. In two recent studies evaluating genome wide sperm DNA methylation, such an epigenetic pattern was found to differ significantly between *in-vitro* fertilization (IVF) patients and normozoospermic fertile men[42] and between men achieving pregnancy within two months and men who did not obtain pregnancy within twelve months, despite similar semen quality[43]. These studies identified candidate methylation loci to be explored in future studies in order to consolidate the results. Epigenetic inheritance related to spermatozoa includes not only DNA methylation but also other epigenetic factors such as histone retention or non-coding RNA (ncRNA). In view of the recent observation that histone retention in specific loci is important for subsequent embryo development[44,45], new sperm diagnostic tests based on histone enrichment in specific genes could be developed in the future. Alterations in ncRNAs may also impair embryo development and transgenerational inheritance. Among ncRNA, the occurrence of miRNA in sperm, seminal fluid and testicular tissue has been reported recently[46]. The fundamental role of miRNA during spermatogenesis is demonstrated by the fact that the knockout of the Dicer enzyme, which is responsible for the cleavage from immature to mature forms of miRNA, leads to infertility[47]. What remains to be determined is whether miRNAs are required also for human spermatogenesis. Recently, an alteration of five miRNAs in subfertile and NOA subjects has been shown[48]. Similarly, employing next generation sequencing, Jodar *et al*[49] found a set of sperm RNA elements required to achieve live births in couples with idiopathic infertility undergoing non-invasive fertility treatments, such as timed intercourse or intrauterine insemination (IUI). However, the absence of such RNA elements does not appear to be critical when ARTs are employed.

Whereas the above described potentially new tools for male infertility diagnosis are still a long way off from use in clinical practice, sDF tests are utilized at present in many ART laboratories in support of traditional semen analysis. Many studies, summarized in the meta-analysis by Zini[13], have evaluated the effect of high sDF levels on the outcomes of both natural conception and ART. The meta-analysis concluded that pregnancy rate is negatively associated with sDF in natural insemination, IUI and IVF but not in intra-cytoplasmatic sperm injection (ICSI). These results were confirmed in a later meta-analysis[50]. Even more disturbing, the risk of miscarriage resulted as being strongly related to sDF levels in couples undergoing both IVF and ICSI[13]. Also these results were confirmed in recent meta-analyses[51,52]. Interestingly, the review by Robinson *et al*[51], pointed out the importance of the methodology used to evaluate sDF, as a subgroup analysis demonstrated that the association with miscarriage is strongest for studies employing the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

The methodology used in sDF studies represents an important issue. Among the various techniques employed to detect sDF[53], Sperm Chromatin Dispersion assay is the only standardized one and the only one for which there is enough agreement on the reference values across studies. Conversely, for the other methods, such as the widely employed TUNEL or COMET assays, standardization is lacking and established cut-off levels for fertility differ in the various studies. Recently, our group has set up a new refined flow cytometric method, TUNEL/propidium iodide, which allows a more accurate measure of sDF[54,55], eliminating all semen confounders[56]. Employing such a method, we have established a cut-off level for fertile subjects and demonstrated that sDF is able to discriminate between fertile men and patients regardless of age and semen quality[57]. sDF analysis in live sperm[58,59]

is an advancement of the TUNEL technique allowing clinicians to detect the damage in the sperm population which participates in the fertilization process. Another advancement is the possibility of assessing, in the same COMET slides, sDF and the presence of oxidative damage[60].

Despite the presence, in the literature, of many studies evaluating the impact of sDF on reproduction, a position report from the European Society of Human Reproduction and Embryology[61] and the guidelines for male infertility drafted by the American Society for Reproductive Medicine Practice Committee[62] claim that evaluation of sDF cannot be considered as a diagnostic test until "randomized, well-designed, adequately powered studies comparing infertile couples to a population of men with demonstrated recent fertility, and excluding cases with female infertility" are conducted in great number. However, as has recently been, introducing sDF among the diagnostic tests of male infertility could improve IVF success rate[63].

Finding the causes responsible for the generation of sperm DNA breaks could be the basis for the development of new therapeutic strategies to prevent the onset of sDF in infertile men. As oxidative stress is considered the main insult generating DNA damage in spermatozoa[64] and infertile men have lower levels of antioxidants and higher reacting oxygen species (ROS) amount in their semen compared to fertile men[65-67], many studies have investigated the effect of antioxidant administration on sDF. A recent Cochrane review[68] concluded that the current body of evidence does not allow for the deducing of clear conclusions regarding the role of antioxidants in the treatment of idiopathic infertility. Further well-designed randomized controlled trials are necessary in order, on one hand, to demonstrate the real efficacy of antioxidants and, on the other hand, to evaluate any eventual adverse events and their side effects[69]. Interestingly, we have recently demonstrated that sDF is mostly established in the testis as a result of an apoptotic process, whereas oxidative DNA damage occurs mostly during transit in the male genital tracts[70]. Accordingly, testis apoptosis should be the primarily target of therapies aimed to reduce sDF. Among these, treatment with follicle-stimulating hormone appears promising[71-73]. However, the complex role of apoptosis in human health makes it difficult to develop anti-apoptotic treatments for male infertility, whereas antioxidants remain an interesting object of study.

PROTEOMIC STUDIES ON MATURE SPERMATOZOA

In recent years, proteomic studies have been conducted in order to define sperm protein profiles and to characterize the role of different proteins in sperm functions. Over the years multiple strategies have been set up to study sperm proteome. In general, the first step is the isolation of spermatozoa from the complex semen matrix, then proteins are separated by various methods (Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis, two dimensional-gel electrophoresis, two dimensional fluorescence difference gel electrophoresis), analyzed by liquid chromatography-mass spectroscopy and identified by a database. Isolation of spermatozoa from semen matrix is a tricky step, representing a major limitation of these studies, as density gradient centrifugation or swim up (*i.e.*, the collection of a fraction of motile spermatozoa moving from semen to an upper medium) procedures, although they eliminate most immature germ cells and leukocytes, may lead to selection of a sperm population which is not representative of the entire sperm population present in the ejaculate. Another stumbling block in performing proteomic analysis is the poor amount of available sperm material in cases of oligozoospermia, thus leaving out a considerable portion of infertile subjects, as in many cases oligozoospermia is accompanied by other sperm defects, such as low motility and abnormal morphology.

In initial studies, few sperm proteins were detected, but the optimization of proteomic technologies has allowed, in recent years, to characterize more than 6000 proteins[74], even though proteins whose concentration is under the dynamic range of instruments remain undetected.

To investigate the roles of sperm proteins in male infertility, studies comparing proteomic profiles of different sperm samples have been performed. They compared infertile *vs* fertile subjects[75-78], asthenozoospermic *vs* normozoospermic men[79-83], male partners of couples undergoing successful ART *vs* those who failed[84-86], subjects with high sDF *vs* low sDF[87,88], men displaying elevated *vs* low ROS levels[89,90], and patients with metabolic disorders *vs* healthy men[91-93]. Overall, these studies led to the identification of a variable number of proteins, likely implicated in male infertility, that are down- or up-regulated in specific sperm defects. Results are, however, often inconsistent among the various studies, probably because of a high intra- and inter-variability of proteomic sperm profiles[94,95], the frequent use of pooled samples and problems related to sperm isolation (see above).

A further progression of proteomic studies is the isolation of proteins from specific sperm compartments leading to the association of the identified protein with its cellular localization and thus with its specific function. Using these approaches, several proteins have been assigned to the main compartments, including histone variants, transcription factors and zinc finger proteins in the nucleus[96,97], several receptors (progesterone receptor, metabotropic glutamate receptor, transforming beta growth factor receptor, Neurotensin receptor 3) to the sperm head[98] and proteins related to energetic metabolism, structure, and motility to the tail[82]. In the latter compartment, also proteins involved in lipid metabolism, mitochondrial oxidation and ADP/ATP carriers[99,100] have been found. Further studies are

needed to understand if these proteins are differentially expressed or mislocalized in spermatozoa from men with defects in motility or morphology.

POST-TRANSLATIONAL PROTEIN MODIFICATIONS IN MATURE SPERMATOZOA

Another point that increases the complexity of proteomic analysis is post-translational protein modifications (PTMs) that carry out an important role in the regulation of functions of mature spermatozoa which, being transcriptionally and translationally silent, mostly rely on PTMs to accomplish important and complex processes necessary for oocyte fertilization, such as capacitation, development of hyperactivated motility and acrosome reaction[101]. For this reason, expression levels *per se* could not have biological relevance for those proteins undergoing PTMs for their functionality. Phosphorylation is a well described PTM in spermatozoa and human phosphoproteomic studies found numerous differently regulated phosphoproteins involved in sperm capacitation[102] and motility[80]. Early studies by Buffone *et al*[103] demonstrated that spermatozoa from asthenozoospermic men showed a reduced protein tyrosine phosphorylation during capacitation *in vitro*, which may be related to a decrease in membrane fluidity leading to the inability to achieve a hyperactivated motility[104]. Among the proteins that are highly phosphorylated in tyrosine during the process of capacitation, A-kinase-anchoring proteins (for review see[105]), structural proteins of the sperm tail, represent an interesting target of these studies, in light of their involvement in motility.

Although ubiquitination is another important PTM, which most likely acts as a sperm quality control system during epididymal transit[106,107] and is related positively to normal sperm morphology[108], most ubiquitin-modified proteins in spermatozoa are still unknown. A similar PTM to ubiquitination is sumoylation, which is associated with poor motility, occurrence of DNA damage and recognition of morphologically defective spermatozoa[109,110]. Recently, Vigodner *et al*[109] identified by mass spectrometry several sumoylated proteins, whose role in sperm functions remains undefined.

Clearly, proteomic studies on spermatozoa are still in their infancy and need to be further validated in field trials before drafting a complete list of sperm proteins that may differentiate fertile and infertile subjects.

SPERM ION CHANNELS

In the attempt to find new male infertility markers, researchers have focused their attention on sperm ion channels having a central role in sperm physiology and in the fertilization process[111]. In particular, proton voltage-gated ion channels (Hv1) induce intracellular pH (pHi) modification involved in the capacitation process[112]. pHi regulation and the role of Hv1 channels has assumed importance with the discovery of two pHi- and voltage-sensitive ion channels, namely Slo3 and Cation channel of sperm (CatSper), that may be connected functionally to the regulation of important sperm activities. Slo3 is a sperm-specific potassium channel involved in mouse sperm capacitation[113], whose role in human sperm functions has yet to be defined. Recent studies have shown that Slo3 channel activity may be regulated also by intracellular calcium increase[114]. Calcium is a well-studied sperm second messenger, whose role in the fertilization process has been widely demonstrated over the last 15 years. Many different types of calcium channels have been described in spermatozoa. Among them, the CatSper calcium channel[115] appears to play a key role in intracellular calcium regulation. CatSper knock-out mice are unable to develop hyperactivated motility, and, for this reason, to reach and fertilize the oocyte[115-117]. Similarly, men with *CatSper* gene mutations leading to a lack of expression of the protein are infertile[118,119]. CatSper gained further importance when, in 2011, two independent groups of research[120,121] demonstrated that it is activated, in human spermatozoa, by progesterone which is considered the main candidate for stimulating the acrosome reaction process in the fertilizing spermatozoon[122,123]. We have demonstrated recently that sperm CatSper expression is lower in asthenozoospermic men and correlates positively with progressive and hyperactivated motility[124,125]. In addition, we found that CatSper (but none of the parameters evaluated by routine semen analysis) accurately predicts the ability of the sample to hyperactivate[125]. Conversely, the involvement of CatSper in the acrosome reaction process, although expected, is debated in the literature[124,126,127]. CatSper and Slo3 expression and activity may be related to the fertility status of the patient and may be involved in the pathogenesis of asthenozoospermia. However, introduction of CatSper or Slo3 evaluation in the diagnosis of male infertility is presently unlikely. Indeed, the techniques to evaluate their function or expression (patch clamping, flow cytometry and Western blot) are costly and/or need skilled personnel, becoming unsuitable for routine clinical practice. Studies on *CatSper* gene mutations or polymorphisms[118,128], if conducted in a large cohort of infertile men, could help to identify novel gene candidates for male infertility. In addition, both channels represent an attractive target for development of a male contraceptive[129,130], being expressed only in germ cells[114,115].

CONCLUSION

Follow-up studies reveal that ART children present an increased incidence of birth defects, prematurity and low birth weight[131], congenital malformations[132] and imprinting disorders[133] when compared to naturally conceived children. A large study conducted in Australian

Table 1 Promising sperm markers of male infertility based on so far published literature

Approach type	Main outcomes	Ref.	Advantages (+)/disadvantages (-)
Semen analysis			
	Macroscopic and microscopic evaluation of semen according WHO guidelines	[2]	(+) Established reference values (-) High operator variability (-) Poorly predictive of fertility
Genetic and epigenetic			
	NGS: Found a set of sperm RNA elements required to achieve live births	[47]	(+) Broad-spectrum analysis
	miRNA: Alteration of 5 miRNAs in subfertile and NOA subjects compared to controls	[46]	(-) Lack of validation (-) Not independently predictive of fertility
	DNA methylation: Different methylation pattern between fertile and infertile subjects	[42,43]	(-) Too early for diagnostic purpose
	sDF: Discrimination between fertile and infertile subjects	[55,56,58]	(+) Presently adopted in many ART laboratories (+) Prediction of fertility independent from semen quality (-) Employment of different techniques to detect sDF (-) Lack of agreement on cutoff values
Proteomic			
	> 6000 proteins (histone variants, transcription factors, zinc finger proteins, receptors, proteins related to metabolism, structure and motility, carriers)	[80,95-98]	(+) Broad-spectrum analysis (-) Isolation of spermatozoa (-) Low available sperm material in oligozoospermic subjects (-) Intra- and inter-variability of proteomic profiles
PTMs			
	Phosphorylation: Reduced tyrosine phosphorylation in asthenozoospermic subjects	[101]	(+) Higher biological relevance compared to gene or protein expression *per se*
	Ubiquitination: Sperm quality control system	[104]	(-) No target proteins identified
	Sumoylation: Marker of defective sperm	[107,108]	(-) Too early for diagnostic purpose
Ion channels			
	Slo3: Involved in hyperpolarization during sperm capacitation	[111,112]	(+) Analysis free from confounders
	CatSper: Involved in sperm progressive and hyperactivated motility	[123]	(-) Skilled personnel and advanced instruments are required (-) Too early for diagnostic purpose

PTMs: Post-translational protein modifications; WHO: World Health Organization; NGS: Next-generation sequencing; NOA: Non-obstructive azoospermia; sDF: Sperm DNA fragmentation; ART: Assisted reproduction technique.

ART couples demonstrated that, after multivariate adjustments for male and female factors of infertility, the risk for any birth defect retained statistical significance only for ICSI, hypothesizing that differences in male infertility factors, which lead to the use of ICSI, may underlie the phenomenon. Similarly, a recent large and well-designed retrospective study demonstrated that ICSI children have an increased incidence of neuro-developmental disorders[134]. Identifying the possible causes of male infertility may lead, in the future, to a decrease in ART children's anomalies, not only because of the possible development of new therapeutic strategies for male infertility but also because of the establishment of new technologies for a better sperm selection for ARTs. However, despite the urgency of establishing new diagnostic tests and defining new sperm markers of male infertility to be used in conjunction with semen analysis, new tests based on "omics" studies or in evaluating sDF (Table 1), are not routinely made a part of the diagnosis of infertile men, mainly because of a lack of standardized procedures, the need to validate the results, and the establishment of clinically accepted cut-off values.

Researchers' efforts should be devoted to gradually translating their acquired knowledge to clinical practice. In this respect, a continuous discussion between clinicians and researchers is desirable, so that basic research will be conducted on the real needs of the medical practice. This will allow for research innovations to be transformed into new diagnostic or therapeutic methods in order to achieve a more successful natural or assisted conception and delivery of healthy babies. The inclusion in clinical practice of new markers, employing advanced technologies, could be more expensive and may require skilled personnel compared to semen analysis, however, once such predictive markers are validated and, consequently, widely employed to diagnose male infertility, their costs will likely decrease, allowing a breakthrough in the management of infertile couples.

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