Review

Genome and Epigenome Disorders and Male Infertility: Feedback from 15 Years of Clinical and Research Experience

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Article

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Abstract: Infertility is affecting around 20 % of couples in the age of procreation but however in some societies as many as one-third of all couples are unable to conceive. Different factors are contributing to male fertility declining such us endocrine disruptors environmental and professional exposures, oxidative stress and life style changes with risks of de novo epigenetics dysregulation. Since the fantastic development of new technologies of Omes and Omics , the contribution of inherited or de novo genomes and epigenome disorders contributions in male infertility are more elucidated and relevant. More than somatic genome investigation of infertile men (from chromosome to single or multiple gene point mutations) many others techniques become available in molecular andrology laboratory to investigate the genome and epigenome integrity, the maturation and the competency of the spermatozoa and its physiological environment. All those new methods of assessment are demonstrating the role of genetics and epigenetics disorders contribution on reproductive pathology and are helping the professional of assisted reproductive technology to propose different management strategy of male infertility to improve the clinical outcomes and minimize the risk of genetics or metabolic disorders at birth.

Keywords: male infertility; genome and epigenome disorders

1. Introduction

Fertility potential declining is a worldwide problem and infertility is affecting an average of 19-20 % of couples. In some societies however as many as one-third of all couples are unable to conceive. In Reproductive pathology investigation practice nearly 20% of cases of genetic counselling is related to fertility problems with at least 15% of men and 10% of women is genetic in origin starting from basic Karyotype abnormalities to specific or multiples genes disorders with adverse effect consequences at epigenetic and proteomic levels.

Standard semen analysis according to the World Health Organization (WHO, 2021) does not allow assessing in a relevant way male fertility [1]. This is due to various factors being not reflected by the examinations recommended.

Basic classical semen analysis is still largely used as the only assessment of male fertility, although it does not reflect either sperm maturity or sperm developmental competency. For instance, conventional sperm analyses not assess factors involved in egg activation, successful fertilization and embryo development.

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Therefore, there is a growing need for the inclusion of new sperm examination methods for evaluating genome and epigenome integrity as well as the type and extent of eventual key proteins. On parallel to somatic genome investigation of infertile men, there is a tremendous amount of evidence that coupling conventional semen analysis with spermatozoa genomic and epigenetic assays would improve the current state of patient counselling and eventual treatment before an assisted reproductive technology cycle.

2. Genetics and male infertility

Genetic causes of male infertility are known in up to 10% of cases mainly in cases of severe quantitative infertility defects, whereas 40–60% of cases with spermatogenic impairment remain unexplained and among moderate oligozoospermia cases, this fraction is close to 80% [2]. The extreme clinical and genetic heterogeneity of male infertility and the reduced reproductive fitness of affected males are two major challenges for the identification of new causative genetic factors.

The karyotype remains the gold standard for genetic evaluation. Although of low resolution, it identifies numerical or structural chromosome abnormalities in nearly 12% of infertile couples, with about 6% of all male infertility associated anomalies such as reciprocal and robertsonian translocations. Klinefelter's syndrome (XXY), a numerical chromosome defect, is common and accounts for nearly 14% of all non-obstructive azoospermia. However, additional molecular analyses are required, even in routine, to identify genetic variants in both Y chromosome and autosomes of infertile men.

The Y chromosome carries multiple genes, essential for testis determination and spermatogenesis. For instance, SRY gene, located on the short arm of the Y chromosome encodes the major regulator of testis formation during early embryonic development. Other Y chromosome genes are involved in germ cell migration, differentiation or function. In 1976 a deletion of the distal portion in the long arm (Yq11) of the Y chromosome was observed in 6 azoospermic men and this suggested the existence of a factor, controlling spermatogenesis in this segment of the Y chromosome [3]. Y chromosome microdeletions are now well characterized as containing genes required for spermatogenesis; they are subdivided into three groups caused by non-overlapping regions: AZFa, AZFb and AZFc. In infertility clinic, the Y chromosome is routinely screened using pairs of Sequence-Tagged-Site (STS) markers for AZF deletions. This screening allows the detection of 95% of the interstitial submicroscopic deletions in azoospermia and oligozoospermic men (<5 million spermatozoa/ml). It is well known that complete AZFa deletion (1% of the AZF deletions) is associated with Sertoli Cell Only Syndrome (SCOS). Nevertheless, deletions within AZFa region were described in cases with variable phenotypes ranging from azoospermia to normospermia.

A partial deletion of AZFa resulting in the absence of the USP9Y gene was reported in a normospermic man [4]. This observation suggests that USP9Y, once considered as a candidate gene for infertility and azoospermia, is not essential for male reproduction and that the AZFa phenotype is likely to be a consequence of the absence of DBY (DEAD-box RNA helicase Y) [5].

In perspective to bridge the gap between AZF microdeletions and karyotypes, Kalantati et al performed a retrospective cohort analysis of 10,388 patients with non-obstructive azoospermia (NOA) and severe oligospermia. They concluded that all chromosomally abnormal NOA cases, except males with a 46, XY/45, X karyotype, were not indicated for AZF screening. On the other hand, the case with Inv(Y) (p11.2q12); isodicentric idic(Y) (q11.2) or idic(y) (p12.2); ring chromosome r(Y); and derivatives as der (y) should also refer to AZF deletion screening. The authors showed that only 1% of cases with sperm count > 1 × 106/mL had Y-chromosomal microdeletions (YCMs) [6]

In the era of assisted reproduction, finding cost-minimizing strategies for infertility clinics without compromising diagnostic quality becomes one of the most important topics for future research. From a diagnostic standpoint, the results reflect the need to reconsider the different karyotype appearance and sperm count thresholds in male infertility guidelines as indicators for YCM screening during infertility assessment [6].

Current gene targeting technologies performed in animal models have identified several hundred of candidate genes involved in spermatogenesis [7]. Nevertheless mutations in only a

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limited number of these genes have been conclusively demonstrated to be associated with human male infertility, altering sperm parameters.

Advances in NGS technologies have significantly contributed to the identification of novel genes responsible for a wide variety of human conditions. In the same way, application of NGS to male infertility allowed to identify several new genetic factors [2]. The last standardized clinical validity assessment of monogenic causes of male infertility published by the International Male Infertility Genomics Consortium (IMIGC) reported 120 genes that are moderately, strongly or definitively linked to 104 infertility phenotypes [8]. This valuable report is a basis for an update of standardized international guidelines for clinical genetic testing in male infertility in the era of NGS by developing well-standardized targeted gene panels.

Identification of the specific genetic cause of male infertility is relevant to optimize the clinical treatment and management of correctable conditions in infertile men and to avoid them unnecessary interventions. It is also helpful for the selection of the best option of assisted reproductive technologies for the couple [8]. An appropriate genetic counseling about the risk assessment for the transmission of infertility to offspring and potential comorbidities can be formulated to patients and couples [9].

3. Sperm genome decays

3.1. DNA fragmentation

A low physiological level of reactive oxygen species (ROS) is necessary for normal sperm function, but if ROS levels exceed standards, they lead to the deterioration of the function of spermatozoa [10]. Unlike somatic cells, spermatozoa are very vulnerable to ROS because their membrane structure has a limited amount of oxidative stress protective enzymes. Sperm DNA breaks (single-stranded or double-stranded) are essentially due to oxidative stress (post-testicular), but may also be caused by the apoptotic intra testicular activity that can be provoked by hyperthermia (varicocele), infection (Chronic prostatitis), age or chronic use of toxic substances (e.g. tobacco, cannabis) [10,11].

It has been shown that sperm with high DNA fragmentation is able to fertilize oocytes with the same efficiency as the sperm without fragmentation. Furthermore, even an apparently normal sperm may have nuclear DNA damage [12,13]. However, if sperm nuclear DNA is damaged, once it is incorporated into the embryonic genome it can lead to errors in DNA replication, transcription and translation during embryogenesis [14] . Nowadays there are sufficient data confirming a negative effect of the use of sperm cells with fragmented DNA including animal models and elevated DNA fragmentation are considered pathological [15].

This was supported over the past years by the increasing number of studies evaluating the input of sperm DNA fragmentation analysis during male fertility work-up and the clinical utility of this analysis. Indeed, elevated sperm DNA fragmentation was shown to impact spontaneous fertility with longer time to achieve pregnancy and increased risk of pregnancy loss [16,17]. Besides, assisted reproductive technology outcomes were also reported to be influenced by sperm DNA fragmentation level. In fact, elevated sperm DNA fragmentation level was associated with lower chances of success after IUI, lower fertilization rate, lower embryo cleavage rate, lower implantation rate and in turn decreased live birth rate[10,18–23]. Based on these evidence sperm DNA fragmentation assessment has a significant value in male fertility evaluation.

3.2. Sperm chromatin decondensation

A mature sperm has a tightly compacted chromatin, because more than 80% of the histones have been replaced by protamine's, during spermatogenesis. Two types of protamine's were investigated, Protamine 1 and Protamine 2, and a ratio close to 1 reflects a good quality of chromatin compaction [24]. Any disjunction of the chromatin condensation can potentially result in defects in fertilization and early embryonic development [24]. These may materialize, in IVF/ICSI embryonic cell stage blocking before or after genome activation, or result in spontaneous miscarriages. The mechanism of

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the origin of sperm DNA decondensation is still poorly known. Failure of sperm chromatin condensation or premature chromatin decondensation exposes sperm DNA to an increased risk of DNA fragmentation [24].

Truthfully, the paternal genome in sperm is condensed in a specific way, certainly to protect DNA during the transit of the sperm to the oocyte, before fertilization. The existence of this unique packaging of chromatin has a significant impact on fertility and embryonic development. A sperm DNA decondensation rate higher than 30% is held for abnormal by some researchers while others consider that the cut-off is 20% [12,13]. No consensus was found on a cut-off value and standardize protocols are still required. However, sperm chromatin dispersion provides informative insight to predict assisted reproduction outcomes.

3.3. Sperm parameters declining and specific genes defects

3.3.1. Reduced sperm counts

Sperm protamine, **PRM1** (MIM 182880) is the basic protein that replaces histones during spermiogenesis. Considerable interest was given to the impact of mutations or variants in the protamine genes on male fertility [25]. Variants were found to be associated with oligozoospermia [26]. Nuclear Subfamily, group A, member 1: **NR5A1** (MIM 184757) is a 461 amino acid protein belonging to the nuclear receptor superfamily. NR5A1 is a key transcriptional regulator of genes involved in hypothalamic-pituitary-steroidogenic axis and is expressed in developing and adult gonad. Mutations in NR5A1 cause a large spectrum of phenotypes, including 46, XY partial and complete gonadal dysgenesis with or without adrenal failure, penoscrotal hypospadias, micropenis with anorchidia, 46, XX primary ovarian insufficiency [27]. NR5A1 mutations are also found in 4% of men with severe spermatogenic failure [28].

Methylentetrahydrofolate reductase, **MTHFR** (MIM 607093) is a key enzyme in the folate metabolism pathway. MTHFR also plays critical a role in DNA, protein and phospholipid methylation and is a nucleotide supplier for DNA synthesis and repair [29]. Methionine synthase reductase, **MTSR** (MIM 602568), encoded by MTRR gene, activates methionine synthase in a cobalamin dependent manner. Human spermatogenesis can be affected by changes in folate status through the impact of folate metabolism on DNA methylation and gene expression or by inducing uracil disincorporation during DNA synthesis leading to errors in DNA repair, strand breakage and possibly chromosomal anomalies Although MTHFR, MTRR and Hcy are involved in spermatogenesis [30], the relationship between the variants in these genes and spermatogenesis is still not conclusive [31].

3.3.2. Asthenozoospermia

Cation channel of sperm 1 and 2, **CatSper1** (MIM 606389) and **CatSper2** (MIM 607249), regulate intracellular calcium channels and potassium currents in sperm. Reduction of CatSper protein and expression levels was reported to reduce sperm motility [32].

Mutations in genes encoding proteins of axonemal dynein's clusters, dynein, axonemal, heavy chain 1, 5, 11 (**DNAH1** [MIM 603332], **DNAH5** [MIM 603335], **DNAH11** [MIM 603339]) and the gene encoding Tektin-1 (**TEKT1**, an alpha helical protein required for flagella assembly) are associated with asthenozoospermia [33].

3.3.3. Teratozoospermia

Aurora Kinase C (**AURKC**, MIM603495), is highly expressed in the testis and is involved in cytokinesis, mitosis and meiosis. The deletion of a cytosine in exon3 (c.144delC) is associated with large-headed, multiflagellar polyploidy spermatozoa (MIM: 243060) mainly in North African populations [34]. Spermatogenesis-associated 16, **SPATA16** (MIM 609856), specifically expressed in human testis, may play a role in acrosome formation during spermiogenesis. The mutation in exon4, c.848G>A, is predicted to result in the p.R283Q amino acid change located at the C-terminal end and it is associated with globozoospermia (MIM 102530) [35]. **DPY19L2** (MIM 613893) is an

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uncharacterized protein whose deletion causes male infertility; as a result, globozoospermia is due to sperm head elongation and acrosome formation blockage [36].

3.3.4. Proteome dysregulation

Sperm proteins have been largely investigated in the past decade and some are of particular interest in in the context of low fertilization potential of sperm. For instance, Phospholipase C Zeta (PLCζ) that is a protein located in the equatorial and acrosomal regions of spermatozoa. It is a crucial player in the initiation of the signaling cascade marking the first steps of fertilization. Indeed, it induces the intracellular calcium (Ca2+) oscillations necessary for the activation of oocytes after fertilization [37–39]. During fertilization, PLCζ is released into the cytoplasm of the oocyte and induces Ca2+ oscillations via the inositol 1,4,5-triphosphate signaling pathway [38].

In cases of absence (KO) or deficiency of sperm in PLCζ, in animal models, the rates of fertilization and early embryonic development were negatively impacted with an inability to induce Ca2+ oscillations [40]. In addition, microinjection of PLCζ protein or mRNA into mouse eggs corrected the phenotype observed in KO mice, restoring normal fertilization and embryonic development [41–43].

The evaluation of PLCζ protein levels in spermatozoa during a male infertility work-up could then help to detect patients at risk of poor fertilization of total fertilization failure. This will help for patient counselling and management. Indeed, men with low levels of PLCZ detected would be to propose to go for in vitro fertilization rather than IUI. In such cases, artificial oocyte activation could be induced artificially (mechanically or chemically). This method is expected to significantly improve fertilization and oocyte activation rates in patients with PLCζ deficiency [44–46]. There is Work aimed at identifying a pathological threshold value of PLCζ is in progress.

3.3.5. Epigenetic marks and sperm immaturity

The term “epigenetics” comprises all the modifications that regulate gene expression by mechanisms other than changes in the underlying DNA sequence. Epigenetic modifications in mature spermatozoa include histone modification, protamine’s, small non-coding RNA and DNA methylation and possibly architecture of sperm nuclei. Epigenetics modifications work all together to establish and maintain genes expression status [47]. Apart from small non-coding RNA, all these epigenetic marks are represented and explained in the figure 4 extracted from “epigenetics of the male gamete” [48].

3.3.5.1. Histones

Histones are alkaline proteins that package DNA into structural units called nucleosomes. Nucleosomes are octameric complexes composed of two copies, each of the four highly conserved core DNA-binding histones, H2A, H2B, H3 and H4. Histone H3 and H4 have long tails protruding from the nucleosome which can undergo post-translational modifications at several places, altering the interaction of histones with DNA. The most important histone post-translational modifications are ubiquitination, lysine acetylation, lysine and arginine methylation and serine and threonine phosphorylation. Gene expression is determined by methylation, acetylation, ubiquitination and phosphorylation of the histone depending on the nature and position of the modification of the amino acid involved [49]. Furthermore, histone modifications are crucial in spermatogenesis and early embryonic development [50, 50].

3.3.5.2. Protamine’s

During sperm maturation, 90-95% of the core histones surrounding DNA are replaced by small basic arginine-rich nuclear proteins termed protamine’s (protamine 1: P1 and Protamine2: P2). This replacement of histones by protamine’s, called protamination, leads to a highly condensed and transcriptionally silent chromatin. This high level of chromatin condensation is necessary for sperm

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motility and is believed to protect the sperm genome against endogenous and exogenous agent such as nuclease, free radicals and mutagens [49].

3.3.5.3. RNA associated gene silencing

Small non-coding RNAs can regulate gene expression by binding to complementary mRNA and subsequently inducing their degradation. Recently, a number of small noncoding RNAs have been identified in male germ cells [51]. A disruption in noncoding RNA pathway may lead to spermatogenic failure [52]. However the association of non-coding RNAs with male infertility is poorly documented.

3.3.6. Methylome Unbalance

The establishment of DNA methylation in the male germline is not only important for ensuring normal sperm function but it also because it contributes to embryonic development and in turn impacts the health of the children born. A decreased level of methylation, also known as hypo methylation, of sperm DNA is associated with altered testicular histology, reduced sperm production and male infertility [53,54]. The preliminary observations made in rodents were also described in humans. Indeed, the methylation of sperm DNA was also found to be altered in the sperm of men exposed to environmental factors and also in men suffering from infertility [55–64]. Further investigations showed that these alterations in the methylation profile can affect the entire genome [56,63].

However, the debate remains open concerning the trend of global changes in sperm DNA methylation in infertile men compared to normospermic controls. The contradictory nature of the results obtained calls for new investigations to better characterize the DNA methylation errors associated with disturbed spermatogenesis and to elucidate the biological mechanisms and the clinical consequences of these epimutations.

Analysis of the sperm methylation profile will certainly have its place in the diagnosis of male infertility because an alteration can lead to potential sperm defects associated with fertility disorders. In addition, an alteration in the methylation profile can be transmitted trans generationally to the offspring leading to the heredity of various pathologies such as spermatogenesis defects, male infertility, breast cancer, kidney, prostate and immune dysfunctions [65,66].

3.3.7. Nuclear architecture disorganisation

The three dimensional organization of nuclear DNA has been shown to play a role in gene-expression of different cell types [67]. Still, mechanistic insights on how this works in detail are far from being understood. Even though nuclear architecture of normal, human sperm is quite similar to genetic active other cells, like fibroblasts or lymphocytes [68], studies on effects of supernumerary normal or marker chromosomes still are not systematically done [69]. This is unfortunate, as e.g. the presence small supernumerary marker chromosomes (irrespective of its chromosomal origin and genetic content) may lead to male infertility, specifically to oligoastenoterato (zoo) spermia [70].

3.3.8. Peripheral free circulating DNA and sperm characteristics

Data on cf-DNA levels in the seminal plasma of men with sperm alterations have not previously well been documented and published. The presence of free nucleic acids in seminal plasma has not been well documented. Li *et al.* showed that seminal cf-DNA levels in a small cohort of patients with azoospermia were significantly higher than in individuals without sperm abnormalities [71]. Costa et al 2017 suggested that elevated seminal cf-DNA levels are related to defects in sperm motility and morphology. In 2018 Chen et al observed an association between seminal free mitochondrial copy number and sperm parameters. Our Study compared men with abnormal sperm characteristics (n=21) with normospermic controls (n=21). The PCR assay evidenced significantly higher mean Cf-DNA levels in patients with sperm abnormalities than in controls (2.09 vs 1.18 μg/mL, respectively; p=0.0003). The Cf-DNA levels were notably higher in men with azoospermia (3.65 μg/mL, vs 1.34

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μg/mL in matched controls; p=0.03) and men with teratozoospermia (1.80 μg/mL, vs 1.29 μg/mL in matched controls; p=0.008). Our data report a significant association between elevated Cf-DNA levels and sperm abnormalities. These results may open up new diagnostic and prognostic perspectives in male infertility [72].

4. What for the future?

In daily practice of male infertility investigation it is important to change our approach to sperm investigation by adding genome, epigenome and mitochondria dysfunction analysis because paternal genetic and epigenetic disorders are contributing to the current decline of male fertility and abnormal embryo formation.

In a recent study Denomme et al. (2018) provided evidence of the potential for epigenetic sperm alterations to affect embryogenesis and IVF outcome. Comparing IVF/preimplantation genetic screening (PGS) couples with oligoasthenoteratozoospermia (OAT) to female age-matched controls, the study reports elevated rates of miscarriage and altered embryo DNA methylation and gene expression. The authors suggest an association between altered embryo methylomes and gene expression abnormalities, thus adding new data in support of the role of sperm epigenetics in embryo competence. Some studies already demonstrated methylation abnormalities in specific loci of imprinted genes [73].

Moreover, it is well established that, while one part of the embryo genome undergoes early embryonic demethylation, some gamete DNA methylation is retained. Therefore, it is likely that sperm DNA methylation both directly and indirectly affects the re-methylated DNA profile of the embryo.

However, it is difficult to perform the methylation analysis of so limited numbers of cells. Therefore, we have to consider the impact of life lifestyle and habits including diet, obesity, medications, endocrine disorders and smoking.

Inherited or de novo, epigenetic disorders can also produce problems of oocyte activation and embryo development, implantation failures, miscarriages, risks of imprinting diseases at post-natal(pre pubertal and adult stages) as well. It is time to revisit our practice and services to support clinicians and patients for better clinical management and therapy.

Regarding the state of the art, genetic factors of male infertility are well documented but further studies are required to clarify the implication and the role of epigenetic marks in the onset and spermatogenic disruption. It is of particular interest to focus additional studies on the effects of environment and ageing on epigenetic landscape in sperm and how they lead to male infertility. Finally, we believe that epigenetic embryo screening will likely provide the next revolution in assisted reproductive technology.

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