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Microbiological Diagnosis of Male Fertility beyond Semen Analysis

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ABSTRACT

Delivery of genetic materials to the female ovum is the singular purpose of sperm cells production and existence. They are unencumbered by some normal cellular organelles, such as the ribosome, nucleoli, rough endoplasmic reticulum, and Golgi apparatus. The sperm cell structure is divided into the headpiece, middle piece, principal and the tail or flagellum. The genetic materials are located in the headpiece(X and Y chromosomes). Semen analysis is cardinal for the diagnosis and treatment of some infertility cases but in some complicit cases where the causes of the infertility are not established with the traditional method, it becomes paramount to go beyond the conventional semen analysis. Some bacteriological investigations such as semen culture, urethral swab culture, and urine culture are also employed to rule out infection which could be a source of a tubal blockage and anti-sperm antibodies. DNA fragmentation test, flow-cytometer, sperm quality analyzer (SQA-V), intact cell MALDI-TOF MS (ICM-MS) are methods utilized to study the genetic details of the sperm cell. With the advancement in technology, it becomes clearer that there are many other factors apart from the sperm density, motility, morphology and vitality that may impact a deleterious effect on male fertility. Therefore, it becomes imperative that some of these neglected biochemical markers are critically assessed and some sperm functional tests employed to further evaluate "unexplained infertility" among men.

Keywords: *Automation, DNA fragmentation, Male fertility, Microbiology, Molecular, Sperm*

INTRODUCTION

Background

Infertility is prevalent in 15% of couples of reproductive age and is described as the inability to cause pregnancy within 1 year of regular unprotected intercourse. Amongst infertile couples, about 30 - 50% is contributed by male factors alone (Manesh*et al*., 2018; Ashley, 2016).

Semen analysis is routinely performed by macroscopic /microscopic evaluation of the sperm cell using manual techniques by a Medical laboratory scientist/Technician; the result is affected by a wide imprecision due to errors arising from pre-analytical and analytical processes leading to variability of results among the microscopists, affecting its clinical validity (Moazzam*et al*., 2015). Semen microscopic examination lacks information on subcellular/ molecular changes in spermatozoa. The traditional semen analysis has a limitation of poor prediction of fertility since 50% of infertile men have normal semen parameters (Kishore *et al*., 2011; Hazir*et al*., 2015). It is estimated that about 40–50 percent of men presenting for fertility testing may have a deficiency in one of the genes required for spermatozoa to fertilize an ovum, which is not detected by the traditional method of a semen analysis (Jungwirth *et al*., 2012). Therefore, to know the specific functional impairment contained in a sperm cell, specialized semen tests to evaluate specific aspects of spermatozoa function is necessary.

This review aims to discuss the microbiological diagnosis of male fertility beyond the conventional semen analysis.

Materials and Methods

A comprehensive literature search of studies published until June 2018 was performed using

the Pub Med, Medline, and Science Direct databases. The search was strictly limited to full articles in English and studies related to humans. The following keywords were used to extract the articles: men fertility testing beyond semen analysis' 'sperm DNA fragmentation', 'micro-biological diagnosis of male fertility', 'emerging technologies for male fertility testing' and 'male infertility'. Combination of the following words was also used to retrieve articles: sperm DNA methylation, 'sperm DNA damage', 'TUNEL assay', 'MALDI-TOF/MS', 'flow – cytometer laboratory test', 'specific sperm dysfunction test' 'male infertility' magnating resonance and 'advanced sperm test'. Search terms such as CASA, 'SCSA test', 'SCD assay' 'proteomic assay', 'genetics and epigenetics' and 'Comet assay' were also used. Cross-referencing was also looked for and duly cited in the review process.

Literature review through the electronic search of databases produces a total of 701 articles, including both review and original research articles. Thorough screening resulted in the selection of, 271, which are mostly related to different studies on human sperm beyond semen analysis. Further screening leads to the rejected of 222 studies, 49 articles were finally adopted which were strictly related to microbiology diagnosis of male fertility beyond semen analysis.

DISCUSSION

Semen Analysis

Traditional semen analysis has attracted serious criticism in recent time, partly because they are subjective-based on microcopies assessment through the microscope and is subject to variation from time to time and from one diagnostic service delivery point to another, the believed that some sperm cells with certain qualities are necessary to cause fertilization has been proofed wrong some years ago because normal semen analysis does not equal fertility potency simply because the test does not check sperm integrity (Michos *et al.*, 2017; WHO, 2010).Although sperm concentration and shape have shown good correlation with conception, however, a reasonable number of clients with normal semen analysis still lingers with undefined infertility (Mary *et al.*, 2014). Edmund *et al.*, (2016) reviewed the semen parameters of 473 infertile men, and found there was significant overlap between fertile and infertile men with respect to sperm count, motility and morphology, similar results has been reported by many other authors (Kumar et al., 2013, These finding indicate the shortcoming of semen analysis and therefore, cannot be used alone to conclude male fertility. Given this, advanced semen tests have been developed to evaluate genetic components and some other functional aspects of the sperm cell.

Computer-Aided Sperm Analysis (CASA)

Computer-assisted sperm analysis (CASA), is software developed and dedicated only to sperm analysis. Computer-aided semen analysis help to study sperm parameters such as concentration, motility, and morphology; this was developed to reduce human error as much as possible and they also help in the precise assessment of the sperm head and the flagella. A field of view is enlarged and digitalized and sperm parameters are analyzed for each sperm cell, which is then computerized. Semen parameters are generated as observed, morphology and active progressive motility concerning their kinematic values, which cannot be determined by standard microscopic examination. Although these characteristics have been successfully correlated with the rates of IVF fertilization, CASA cannot authentically predict spontaneous fertilization outcomes (Mary *et al.*, 2014; Boyers *et al.*, 2018). They are very expensive and not suitable for general laboratories with small batches of samples for semen analysis.

Morphometry

Stained semen smear or unstained semen sample can be examined using software developed for this purpose e.g. AAV Technology(negative phase contrast in 40) is examined under the microscope and image of about 100 – 200 sperm cells are captured by a special camera. The image captured is analyzed concerning standard normal/ abnormal sperm cells template software. The result is usually expressed as a percent of normal /abnormal, this method produces a result equivalent to WHO standard (Marato- Morales *et al.*, 2016 and Maree *et al.*, 2010).

Semen Quality Analyzer (SQA –V)

Sperm concentration, volume, morphology, progressive motility, and viscosity can be determine employing SQA-Version, according to the WHO laboratory manual for examination and processing of human semen, 5th Edition, lay down criteria. Using SQA- Version, complete semen analysis for male fertility can be achieved, it's also recommended for post-vasectomy semen analysis and for a sample to be used for insemination. SQA-V can be used interchangeably with the manual method for the examination of sperm concentration and

motility. Automated SQA-V analyzer is more precise and more accurate in grouping normal and abnormal sperm cell morphology than the manual method (Ashok *et al*., 2007; Boyers *et al.*, 2018).The SQA-V method of semen analysis is more reliable and faster compared to manual.

Flow Cytometry

Flow cytometry is a process in which fluorescently labeled spermatozoa cells travel singly at high speed (hundreds or thousands per second) through a flow cell, where they are reflected by one or more lasers light (Kumar *et al.*, 2012). This causes light scattering and causes excitation of markers located on specific parts of the sperm cells, which is then carried up by photo detectors and sent to a computer program (Kumar *et al.*, 2012). The computer program presents the information in the form of relative fluorescent intensity units, which are typically displayed as either scattered plots or histograms (Martinez-Pastor *et al.*, 2010).To define the region of interest related to the light scattering behavior of sperm, a "sperm window "must be identified by analyzing samples containing the isolated sperms(Farrera *et al.*, 1997). The percentage of sperm population with certain fluorescent characteristics within a total sample, median fluorescence intensity can be measured (Chelsey *et al.*, 2011). There is an advancement in the flow cytometric studies of semen samples which is more sensitive, objective, rapid, and measures multiple parameters of the sperm cell to know the fertility potential of the sperm cell. Fluorescent dyes can be employed in a flow cytometer to study mitochondria function, ATP production and motility of the sperm cell effectively.

Molecular Approach

The discovery of the polymerase chain reaction (PCR) as a method to specifically and selectively identify amplify DNA and RNA sequences has greatly brought a transformation to the practice of molecular medicine. Utilization of these newly developed molecular biology techniques in the area of male infertility has made the "unexplained infertility" known through the identification of genital tract infection, immune system activation within the genital tract, mutations in sperm mitochondrial or chromosomal DNA damage, alterations in sperm components involved in receptor-ligand interactions, and production of sperm auto antibodies which were neglected to play an important role in male infertility.

Polymerase Chain Reaction (PCR)

The conventional semen analysis shows very little about the sperm cell nucleus and the chromatin content, damage in the mitochondrial DNA or messenger RNA (mRNA) cannot be detected and or the presence of abnormal surface molecules activated immune system or inherent bacterial infections. PCR techniques are just enough to be utilized to diagnose some of these factors that exert a great negative influence on male fertility in this new age of technology (Regmi *et al.*, 2015).

Polymerase chain reaction (PCR) is being utilized to identify infectious micro-organisms present in the male reproductive system refers to as sexually transmitted diseases. The technique is based on the amplification of specific microbe DNA one million-fold in few hours, offering high specificity and sensitivity in the diagnosis of infectious diseases (Alkhayal *et al*., 2013).

Some of these micro-organisms may be attached to the sperm cell and reduce their potency to reach the fallopian tubes as well as their ability to cause fertilization. Among these micro-organisms are Mycoplasma genitelium and Ureaplasmaurealyticum which they have strong adhesion and can attach to the sperm head and midpiece and antagonized the performance of the sperm cell. Antibodies produced against these bacteria can equally adhere to sperm cell rendering them vulnerable to phagocytosis causing their destruction (Abbas *et al*., 2014).

C. trachomatis infection has been associated with the development of an autoimmune response to spermatozoa in the male genital tract. Other viral or bacterial infections of the male genital tract may also stimulate local immune activation and lead to the recognition of sperm antigens by auto reactive T cells (Abbas *et al.*, 2014).

Sperm cells require a great deal of energy for mobility to the cervical mucus up to the fallopian tubes and the penetration of the zona pollucida, the energy required for the tactful movement is obtained from the mitochondria. Mitochondria have their genetic material, like cellular DNA, but mitochondria are naked (unprotected by histones and DNA binding proteins) and deficient in an effective efficient DNA repair mechanism, thus exposing the mitochondrial DNA to damage mostly by the free radicals and ROS which are continuously produced by the mitochondria during their normal aerobic metabolism. Specific mitochondrial DNA deletion was shown to be associated with diminished fertility and motility of human

spermatozoa, this phenomenon was discovered among infertile men with low motility compared to those with normal sperm motility.

PCR has employing to detect the present or absent of the specific mitochondrial DNA. Those with DNA deletion were found to have a nucleotide product size of 4977 bp in variation to the normal mitochondrial DNA. Deletion in mitochondrial DNA would usually compromise the ability of the sperm from the synthesis of mitochondrial proteins necessary for mitochondrial function resulting in a subsequent decline in sperm motility (Kao et al, 1995). Thus identify-cation of these deficiencies and the manifestation of the defect to mitochondria in the spermatozoa by using a standardized PCR protocol to detect these deletions in mitochondrial DNA and the application of these techniques to sperm from men with impaired motility will hopefully allow a more definitive diagnosis of this problem.

The absent of DNA repair mechanism in spermatid and spermatozoa is a major drawback in the life of a sperm cell because they are susceptible to both internal and external environmental factors that can cause damage (Alex *et al*., 2017). There are many types of damages or abnormalities that can occur to a sperm DNA structure depending on the rate of individual exposure to these internal and external factors such as antioxidant and ascorbic acid which can result to chromosomal rearrangement leading to chromosomal breaks and fragments compromising quality of spermatozoa and inefficiency in pregnancy sustainability (Jeremias and Witkin1996). There two genes present on the Y chromosome: the sex-determining gene (SRY) and the azoospermia factor gene (AZF), which are involved in male fertility. Deletions or mutations involving the SRY/ AZF genes may result in a variety of clinical states ranging from testicular absence to male sterility. These deficiencies can be comprehensively detected through the use of a standardized PCR method as reported by Jaffe and Oates, 1994

PCR analysis and sequencing of the LH P β subunit gene can also be achieved which often revealed whether there is a missense mutation (Weiss *et al.*, 1992). A thorough study of the regulation of expression of the genes coding for adhesion molecules in spermatozoa using RT-PCR or Western blotting is recommended. Identifying the factors that interfere with fibronectin, vitronectin, laminin and integrin gene activation in testicular cells might provide a mechanism for previously unexplained infertility in some men as well as suggest novel methods of fertility regulation (Khaldoun*et al.*, 2013).

Altered expression or abnormal binding capabilities of CD46 may affect fertilization by as yet unidentified mechanisms. Human lymphocytes contain two major CD46 isoforms whose relative expression has been shown to vary according to an autosomal co-dominant polymorphism (Russell *et al*, 1992). PCR is used to amplify the polymorphic region of the C3 gene which has a deleterious effect on male fertility.

Microarray Technology

Spermatozoa RNA provides a historical record of spermatogenesis, based on a template obtained using transcriptional profiling, and these have been investigated as markers of fertility (Moldenhauer *et al*., 2013). This technology may be used to investigate the response of cells to conditions that alter mRNA expression, allowing insight into the mechanisms and effects of specific diseases. Microarrays from fertile and infertile men are compared for the identification of genes that are important for successful fertilization and pregnancy, or biomarkers for infertility (Garrido *et al*., 2013). Transcriptomes at different stages of sperm-atogenesis are also used for the identification of genetic aberrations which may provide some details for couples with repeated spontaneous abortions. However, this technology has some limitations for ART, because spermatozoa used for ICSI bypass the normal body's natural selection process, which may allow the transmission of junked genes (Zhu *et al*., 2016).

Sperm DNA Fragmentation

Sperm chromatin tightly packed due to cross-linkages, sperm DNA fragmentation (DFI) was first described in 1993 and it correlates with poor semen parameters, leukocytospermia, elevated oxidative stress which is closely associated with unexplained infertility, spontaneous miscarriages. Sperm DNA integrity and morphology are poorly correlated (Ebner*et al*, 2011). Rather, DNA sperm integrity correlates with reproductive success, while elevated DFI is associated with low spontaneous pregnancy and assisted reprod-uction success rates low (Edmund *et al.*, 2016, Ebner*et al.*, 2011). Several different methods have developed to assess the percentage of sperm with reasonable DNA damage. Some of these methods include; Comet assay, TUNNEL assay, sperm karyotyping, sperm FISH analysis and sperm chromatin structure analysis (SCSA) (Andersen, 2000).

Sperm Chromatin Structure Analysis (SCSA)

Here the sperm cells are treated with a special dye which stains more of the sperm DNA fragmented cells and less of sperm cells with normal DNA, this differential staining is recognized with the aid of a flow – cytometer in conjunction with a computer program that select the sperm DNA fragment which are reported in percentage or in graphical form (Pandiyan*et al.*, 2017). The pregnancy rate in patients with SCSA less than 30% fragmentation (the normal group) was 62%. The pregnancy rate in patients with SCSA greater than 30% (abnormal group) was 81%. SCSA test result has been consistent compared to semen analysis (Morris, 2018).

MALDI-TOF MS

Recent advances in MS instrumentation in combination with the development of bio-informatics tools have allowed the development of untargeted, much simpler and less expensive clinical proteomic approaches based on MALDI-TOF MS profiling. One of these approaches is Intact Cell MALDI-TOF-Mass Spectrometry (ICM-MS), a method that relies on the identification of cell-specific peptide/ protein MS fingerprints. "Intact cell" means that whole cells are subjected directly to MS analysis without any preparatory steps, which is highly convenient in diagnostics.

This approach has been proven useful for routine high-throughput bacterial and yeast biotyping in clinical specimens, but it has also been applied in superior eukaryotic cells such as macrophages or neuroglia cells (Laura *et al.*, 2016).

Spectra were successfully developed to generate fertility-predictive models showing a better diagnostic performance than any traditional in-vitro sperm quality tests. The identification of the peptides/proteins represented in the ICM-MS spectra determined that this method evaluates different sperm functions at once, and helped us identifying particular proteins whose relative abundance might be linked with fertility ((Laura *et al.*, 2016).

Bacteriological Tests

Male urinary tract infection (UTI) is one of the most important causes of male infertility, being associated with 8%-35% of male infertility (Melania *et al.*, 2016). Pathogenic bacteria may interfere with infertility treatment involving the application of in vitro fertilization. Microorganisms might affect the spermatozoa function in different ways: (a) By direct contact on sperm cells; by the help of some organelles such as pili; causing agglutination of motile sperm, reducing the ability of the acrosome reaction, and also causing alterations in cell morphology. (b) Trigger a local inflammatory reaction leading to an increase in reactive oxygen species (ROS). (c) Induction of sperm autoantibodies. (d) Production of cytotoxic factors. (e) Infection treatment with antibiotics for a long time may lead to a defect in the sperm. The most frequently isolated bacteria from semen samples include Staphylococcus aureus, Escherichia coli, Streptococci, Klebsiellasp, Mycoplasma hominis, Chlamydia trachomatis, and Enterococcus faecalis. The infection with these bacteria has a significant negative effect on sperm parameters and DNA integrity (La Vignera*et al.*, 2011; Leterrier*et al.*, 2011; Abbas *et al.*, 2014). DNA fragmentation may cause infertility, miscarriage, and birth defects in offspring (Tiwari *et al*., 2016). Therefore it may be a more objective marker of sperm function. The exact molecular mechanism of how bacteria affect chromatin and sperm nuclear protein still unknown. The bacterial infections lead to premature emergence of histone H3 methylation at lysine 79 (trimethylated H3K79) and hyper-acetylated H4 which simultaneously occurred with transition protein TNP1. In mammals, reduced levels of histone H4 hyperacetylation correlates with impaired fertility (Zeyad*et al.*, 2017; Meares and Stamey, (1968).

Male partners attending fertility clinics should be bacteriologically tested for UTI to rule out the possible implication of microorganism infection through culture and sensitivity test and or used molecular method available which is more specific, sensitive and faster. Semen, urine and urethral swab samples are to be utilized along with appropriate culture media (Tiwari *et al*., 2016).

Conclusion

Based on a modern computer and advanced image processing techniques for a clinical test of sperm quality, it is becoming clearer that there are many other factors apart from the sperm density, motility and morphology that may impact a deleterious effect on male fertility. Thus, it becomes imperative that some of these neglected biochemical markers be critically assessed and more advanced sperm function tests be employed to further evaluate "Unexplained infertility" among men.

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