SEMEN CHARACTERISTICS: ADVANCEMENT IN ANDROLOGICAL ASSESSMENT

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ABSTRACT

Progress in diagnosis of infertility has been dramatically increased during the past decades with changes occurring in virtually all aspects of infertility research, thus providing innovative diagnostic testing and sophisticated instrumentation for improved management and treatment of infertility. There are about 50 % of infertile couples who are suffering because of male infertility. Semen examination is a basic investigation for these infertile couples. It not only reveals the quantity and quality of sperm but also the quality of the seminal plasma, which is essential for normal sperm function. In this review, the recent advancement in investigation procedures has been analyzed which are very important in clinical practice to (a) evaluate the sperm fertilizing ability (Acrosin, aniline blue, HOS), (b) characterization of male accessory sex glands secretions (Fructose, alpha-glucosidase, PSA) and (c) the management of azoospermic patients. It is believed that use of such diagnostic procedures will facilitate wide selection of patients for whom an effective therapy might be then possible.

KEYWORDS

Semen, Sperm, Infertility, Seminal plasma and Epididymis

INTRODUCTION

Infertility is a tragic condition. It causes intense mental agony and trauma that can only be best described by the infertile couples themselves. The prevalence rate of infertility in India is not known. However a WHO¹ study that included India places the incidence of infertility between 10 to 15%. Based on the WHO estimate, approximately 13 to 19 million couples are likely to be infertile in the country at any given time. According to one estimate² about 30% of these infertile couples, infertility is due to male factors alone and in another 20% both partners have a detectable abnormalities. Thus male factors play a significant role in about 50% of infertile couple³.

The causes of male infertility may be due to various reasons and their management and treatment depend on the proper diagnosis of male infertility. Generally the clinicians depend on semen analysis for the assessment of male infertility and also for evaluating/

testing efficacy of male fertility regulating agents.

Apart from the routine semen analysis, specific investigations are required to assess the functional status of sperm. An acrosomal enzyme reflects the sperm fertilizing capacity⁽⁴⁾ Chromatin condensation shows the morphological as well as functional status of the sperms, vital staining is essential to differentiate between necrospemia and immotile syndrome etc. Similarly, Hypo-osmotic swelling (HOS test) is another sperm integrity test which is also equally useful not only to know the physical integrity of the sperm but also the chemical changes in the sperm plasma membrane. Recently, hemi-zona assay is considered to be a reliable method which provides exact information regarding the fertilizing capacity of the sperm which can be used as a marker for sperm fertilizing capacity. Semen CPK, ATP and mitochondrial index activities, which are considered to be energy substrate, have also been shown to correlate with sperm concentration, motility and fertilizing capacity. Reactive oxygen species (ROS) can also be used as diagnostic tools to determine the fertilizing capacities.

Accessory sex glands functions and obstruction can be assessed by fructose, alpha-glucosidase, acid phosphatase and prostate specific antigen (PSA) assays. Some of these investigations are part of the routine semen analysis while the remaining are sophisticated methods, which can only be performed, in se-

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lected patients as and when required. The mixed anti-globulin reaction (MAR) or immunobead binding test detects sperm surface-bound antibodies while genetic evaluation shows the chromosomal status of the sperm. In case of azoospermic patients, the role of testicular examination and serum FSH level has also been discussed for the management of infertility.

In the present review efforts have been made to describe significances of recent diagnostic advancements necessary for the evaluation and management of male infertility.

1. Assessment of Sperm

(a) Sperm Function tests

Test For Acrosomal Enzymes

Acrosin and Hyaluronidase are the two main acrosomal enzymes, which play an important role in fertilization. Assessment of acrosin gives ample indications for fertilizing potential of sperm. Lack of acrosome is a kind of major deformity in sperm and such type of spermatozoa are called as globozoospermia.

Various methods have been described to assess the acrosin activity in human spermatozoa⁴. The simplest and less time consuming method is determination of proteolytic potential of spermatozoa on gelatin coated plates⁵. Acrosin is released by the rupture of acrosome and leads to halo formation during incubation in a humid chamber at 37°C. The method of gelatinolysis is advantageous, equipments required are simple and acrosin activity can be determined even in single spermatozoa. It shows a good correlation with the biochemical assay also⁶. Normal acrosin activity is observed in man with high fertilization rate, whereas the halo diameters and halo formation rates are smaller in most cases of poor fertilization⁵. Therefore, this method may give the information about the fertilizing potential of sperm population, especially in case of teratozoospermia.

The other method of determination of acrosin is a biochemical assay. The enzyme extraction is performed by acid treatment of spermatozoa. The enzyme activity can be measure by spectrophotometer after colour development. Though it is a time consuming method and not suitable for routine use however it shows a good correlation with gelatin method⁶.

Apart from these methods, some methods based on fluorescence are also in use. A wide variety of lectins that are labelled with fluorescence are used extensively as it provides better result in acrosomal assessment⁷. Antibodies have also been developed for the assessment of the acrosomal status, which detect the intact sperm cells. In other methods monoclonal antibodies like GB24 and MH61 are also being used⁸. Compounds like chlortetracycline (CTC) and calcium

ions bind to membrane and show highly enhanced fluorescence with different patterns^{9, 10, 11, 12}. It can be noted that among all the fluorescent methods, CTC method is easy to perform but it is not suitable for routine use.

Estimation of acrosin should be considered in selected cases of teratozoospermia particularly in polyzoospermic patients to diagnose severe acrosomal dysfunction. In addition, these tests are useful in demonstrating insufficient amount of acrosin in men with idiopathic infertility and before in vitro fertilization.

Determination of Chromatin Condensation

A high proportion of morphologically abnormal spermatozoa were shown to be associated with increased heterogeneity of chromatin structures^{13, 14}. Nuclear shape, which is species specific, depends on chromatin condensation during spermatogenesis as well as on a precise organisation of DNA within the nucleus^{15, 16}. Chromatin condensation is achieved by gradual substitution of lysine rich somatic histones by testis specific histones. It has been suggested that a low DNA content in spermatozoa of subfertile man may be responsible for inducing alternations in sperm shape^{17, 18}. Round-headed spermatozoa shows variable chromatin condensation during morphogenesis and do not attain the same degree of maturity as do normal ones¹⁹.

The degree of biochemical sperm nuclear maturity can be assessed by aniline blue staining²⁰. This technique gives a specific positive reaction of lysine and can reveal differences in basic nuclear protein composition of ejaculated spermatozoa. Histone rich nuclei of immature spermatozoa are rich in lysine; therefore they will be stained blue. Protamine-rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively low lysine^{21, 22} and therefore they will not be stained by acidic aniline blue. The technique is not only less time consuming but also less expensive and therefore may be useful for routine andrological investigations.

Acridine orange method is more sophisticated method²³ and shows green fluorescence when it interacts with double strand DNA and red fluorescence with single stranded (denatured) nucleic acid (DNA or RNA). The spermatozoa are treated under boiling or acidic conditions that decondense the sperm nucleus and destabilize the double strand DNA. If decondensation and DNA denaturation occurs readily, the nucleus will stain red and the spermatozoon may be considered as infertile. Therefore, acridine orange can also be used to determine the stability of the double strand DNA in the sperm nucleus as well as the degree of chromatin condensation. Determination of red/green fluorescence staining patterns can also be performed by light microscopy or more accurately

by flow cytometry. Flowcytometry procedure is more reliable but the instrumentation required is not readily available in routine clinical conditions. This test is meaningful for the patients having abnormal sperm morphology but cannot be useful in routine clinical investigations.

Viability Staining

The viability (live/dead) stain has been used for decades to measure the ability of dyes such as eosin (1% Eosin and 10% Nigrosin) to pass through the sperm membrane. If the membrane is intact, as in the case of viable spermatozoa, dye passage is prevented. If the membrane is broken as often the case with dead or dying spermatozoa, the dye will pass into the sperm's cytoplasm. This test evaluates the integrity of the sperm membrane and can be used as routine clinical investigations.

Hypo-Osmotic Swelling (HOS) Test

This is based on the semi permeability of the intact cell membrane, which causes spermatozoa to swell under hypo osmotic conditions when an influx of water results in an expansion of cell volume²⁴. The plasma membrane may be physically intact but chemically non-functional. Lack of chemically non-functional membrane may impair the ability of spermatozoa to undergo capacitation, acrosome reaction and to bind to the zona pellucida. Through HOS test the ability of the plasma membrane to transport water by subjecting the spermatozoa to hypo-osmotic conditions can be measured. If water transport has occurred, the cytoplasmic volume of the sperm tail will greatly increase, causing the tail fibers to curl within the expanded plasma membrane. Tail curling can be seen under phase contrast microscope. The HOS test is not only an indicator of the chemical integrity of the plasma membrane, but also of its physical integrity (a broken membrane will not allow swelling to occur) therefore, it is not necessary to perform the viability stain if the HOS test is performed. This test can easily be used in andrology laboratory for routine clinical evaluation. The test is also useful for detection of damage during cryopreservation, toxic effect of drugs and chemicals and to assess quality of sperm.

Hemizone Assay

The hemizona assay involves micro dissection of the zona pellucida into two equal halves and testing the binding on each matching half with the same number of test and control spermatozoa. Another assay differentially labels the test sample spermatozoa with one fluorescent dye (e.g. fluorescein) and the control sample with another dye (e.g. rhodamine). The number of spermatozoa from both the test and control are counted and compared. Both zona binding test have been shown to correlate with the success of in vitro fertiliza-

tion. This test is applicable to those couples who undergo in vitro fertilization.

(b) Sperm Energy Substrate Tests

It is arguable whether biochemical tests for mid piece integrity is necessary, once a detailed analysis of sperm motility has been performed through computerized analysis. If the mid piece is not functional, energy will not be generated for sperm movement, particularly under aerobic conditions. However, it is possible that a mid piece component can be used as an indicator of other functional processes besides sperm motion, in which case a test for this indicator may be useful. Mid piece defect are considered due to defective morphology or internal loss of energy therefore, these kinds of investigations are required in the cases of idiopathic asthnoteratozoospermia.

Sperm Mitochondrial Index Activity

This test estimates the incidence of spermatozoa having a full complement of mitochondria containing respiratory enzymes, which are essential for providing energy for sperm motility. Lack of mitochondrial enzymes impair sperm motility and may contribute to subfertility. This test is mandatory in idiopathic asthenozoospermic subjects.

Creatine Phosphokinase (CPK)

Creatine phosphokinase (creatine-N-phosphotransferase) is a very important enzyme⁽²⁶⁾ in the synthesis and transport of energy. Creatine is phosphorylated in the mid piece and transferred to the contractile elements of the tail where creatine phosphokinase uses it for the phosphorylation of ADP to ATP. ATP is important for tubulin-dynein interaction and tail motion. However no correlation has been reported between sperm motility and morphology and the creatine phosphokinase levels²⁷. The outcome of uterine insemination is negatively correlated with sperm creatine phosphokinase activity²⁸. Tentatively, values of lower than 0.250 units/10⁸ sperm are taken as normal. The M-isoform of the enzyme may also further aid in differentiating normal spermatozoa from abnormal spermatozoa²⁹. However, electrophoretic analysis is required for isoform detection, which is somewhat difficult to perform in most clinical settings.

Adenosine Triphosphate (ATP)

The most important property of spermatozoa is adenosine triphosphate (ATP) fuelled motility. The possible value of semen and ATP concentration for predicting male fertility has been studied extensively³⁰. ATP is generated in the sperm mitochondria and the energy is delivered to the sperm tail, which supports the flagellar dynein for the movement of spermatozoa.

(c) Tests for Sperm Morphology

These tests are routinely used in andrology laboratory, however in cases, particularly in idiopathic teratozoospermia, it is quite necessary to find out the causes of infertility. Different staining procedures are being used accordingly for the assessment of sperm morphology.

Papanicolaou Staining

The papanicolaou staining is widely used in andrology laboratories³¹. It permits staining of the acrosomal and post-acrosomal regions of the head, the mid-piece and the tail. It can be used to differentiate between basophilic and acidophilic cell component and allows a detailed examination of the nuclear pattern and used for routine diagnostic cytology.

Rose Bengal Staining (Triple Staining)

Rose Bengal³¹ staining is used for the morphological assessment of different part of the sperm e.g. sperm head, mid piece and tail. It gives ample information about the abnormalities of sperm head. Though Rose Bengal staining gives consistent results but it is a time consuming technique, therefore papanicolaou staining is used routinely in most andrology laboratory.

(d) Other Cellular Components

Immature Germ Cell

Immature germ cells in the semen can be used to study meiotic chromosomes. Immature germ cells in the semen are generally indication of the disorder of spermatogenesis. These can easily be identified by the use of Bryan Leishman staining³¹ and provides the information for the diagnosis of chromosomal disorders.

Leukocytes (White Blood Cells; WBC)

The role of leukocytes in semen of infertile male is controversial. Some authors reported that leukospermia might indicate genital infection while others reported that the presence of enzymes of leukocytes in semen might impair semen quality^{32, 33}. The differentiation of round cells in semen composed of leukocytes and immature germ cells and can be identified by using peroxidase stains, which allow to identify the polymorphonuclear leukocyte, that constitute 50-80% of all leukocytes. Other leukocytes such as lymphocytes and macrophages are peroxidase negative and therefore they are not stained. However, peroxidase negative leukocytes have cellular features, which are clearly differentiated from those of immature cells. All leukocytes can be identified by using monoclonal antibodies prepared against common leukocytes antigens (CD 45)³⁴.

It is believed that leukospermia reflects sub clinical genital infection. But microbiological investigations fre-

quently fail to detect pathogens, which are responsible for these infections. On the other hand there is no convincing evidence to confirm that the major cause of leukospermia is viral infection of the genital tract³⁵.

Apart from infection, the increased number of leukocytes in semen are reported to be due to abnormal spermatogenesis, cigarette smoking, alcohol intake along with some environmental factors such as irritants and toxins, physical factors such as frequent hot baths, varicocele, abstinence and certain clinical situations like vasovasostomy or homosexuality etc³⁶. Among these possible causes the increased numbers of leukocytes in relation to phagocytes defective sperm is of particularly significance in clinical investigation³⁷. This test is commonly used in andrology laboratory for all types of infertile patients and is a part of routine semen analysis.

2. Assessment of Seminal Plasma

(a) Secretions of Accessory Sex Glands

Seminal plasma contains secretions from various accessory sex glands e.g. seminal vesicles, prostate epididymis etc. By estimating concentrations of various biochemical markers it is possible to evaluate the secretory & functional status of these accessory sex glands e.g. fructose, prostate specific antigens (PSA), citric acid, alpha-glucosidase etc³⁸.

Fructose

The presence of fructose in semen is determined according to the WHO Laboratory Method Manual³¹ by means of a colorimetric reaction. Also an enzymatic assay using spectrophotometry may be performed. In case of azoospermia (caused by congenital absence of the vas deferentia), low fructose levels indicate an associated dysgenesis of the seminal vesicles. Fructose levels are also reduced in case of post-inflammatory atrophy of the seminal vesicle epithelium or relative androgen deficiency. Oral androgen medication (e.g. 120 mg testosterone undecanoate or 75 mg mesterolene) allows differentiation between androgen sensitive and androgen resistant forms of seminal vesicle insufficiency. In case of ejaculatory duct obstruction or agenesis of the vas deferentia and seminal vesicles, semen samples are characterized by low volume, low pH and absence of fructose, which indicates an exclusive presence of prostatic fluid in the ejaculate.

Alpha-glucosidase

The main source of alpha-glucosidase is the epididymis, particularly from the corpus and cauda epididymis. Distal ductal obstruction shows significantly decreased alpha-glucosidase values. In case of an occlusion at the level of rete-testis, alpha-glucosidase

activity in seminal plasma will be in normal range. Though this test is simple, economic and less time-consuming³¹ but functional disturbances at the level of the epididymis cannot be characterised by the alpha-glucosidase assay. Future research will hopefully provide more insight and sensitive markers of epididymal function.

Prostate Gland Secretion

Citric acid, zinc and prostatic acid phosphatase³¹ are the marker for prostate gland secretion. However, in clinical use, determination of prostate gland secretion is of not great diagnostic value to localise obstructive disease. Apart from that assessment of the prostatic specific antigen (PSA; Kallikrein-like protease) may give information about inflammatory reactions and tumor cells in the prostate.^{38, 39} PSA can be determined in semen and serum by means of a commercially available radioimmunoassay kit. This determination is used as a screening test to exclude prostatic cancer or chronic inflammatory process within the prostatic tissue. Absence of fructose with a low pH and a less ejaculatory volume indicate men with obstruction in the periphery. Therefore, estimation of biochemical markers can play very important role in assessing the functional status of accessory sex glands and can provide easy access to find out the obstructive azoospermia also.

Zinc

Zinc is the secretion of the prostate gland and contributes significantly in seminal plasma. The determination of zinc in the laboratory can be performed by a simple calorimetric assay⁴⁰. Zinc values in human seminal plasma obtained by this assay were found to correlate well with those obtained by flame atomic absorption spectroscopy. This shows that calorimetric assay can be performed for the determination of zinc in seminal plasma as routine andrological investigations.

Acid Phosphatase

Acid phosphatase is one of the important secretions of the prostate. The determination of acid phosphatase in laboratory can be performed by taking the fresh seminal plasma and processed for stabilisation of the acid phosphatase for few hours at room temperature or at 4°C for few days or -20°C for several months. By diluting the seminal plasma the sample is reacted with the substrate at 37°C. The enzymatic reaction can be stopped by adding NaOH; absorbance can be read at 405 nm against the assay blanks.⁴¹

Transferrin

Transferrin, a glycoprotein involved in iron transport has been detected in human sertoli cell in large amount. The role of transferrin in human seminal

plasma is considered to be an important constituent for sperm membrane integrity and functional competence⁴². Studies have shown that the transferrin concentration in obstructive azoospermia is always lower than that of normal range therefore; it is considered to be a reliable indicator of seminal tubule function⁴³. A sensitive enzyme immunoassay has been developed for transferrin assay to use as an effective marker of sertoli cell function. Although it gives information about the function of the seminal tubule and especially the Sertoli cell in obstructive azoospermic patients, however it cannot be used as routine method for andrology laboratory.

(b) Secretions of Genital Tract

The role of genital tract markers is also equally important to identify men with silent genital tract inflammation. Semen pattern with $>1 \times 10^6/\text{ml}$ specific neutrophil granulocytes (peroxidase-positive round cells) also known as leukocytospermia shows a genital tract infection. Therefore, biochemical markers have been suggested to be used as sensitive indicators of an inflammatory reaction⁴⁴ particularly elastase, C'3 complement component, ceruloplasmin and ROS are the factors which indicate the genital tract related inflammation. An enzyme immunoassay for the determination of elastase in seminal plasma, as a specific inflammatory parameter of polymorphonuclear (PMN) granulocytes, enables the diagnosis of a silent genital tract inflammation⁴⁵.

(c) Casual Factors

In cases of idiopathic infertility where sperm as well as seminal plasma parameters are normal, there are some other factors, which are associated with infertility. These factors are commonly known as casual factors. To evaluate these factors some specialised investigations need to be carried out which are not routinely used for the diagnosis of male infertility.

Immunological Considerations

The patient suffering from the antisperm antibodies in their semen is quite significant in Indian populations. There are several factors, which contribute to this kind of deformity. Prolonged use of alcohol and smoking and environmental pollution may be a cause for the same. This can be a genetic cause in a number of sub-populations. The correlations of antisperm antibodies (ASA) with some cases of unexplained infertility suggest a role of these antibodies in inhibiting fertilization.⁴⁶ The incidence of immunity to sperm in infertile couples is estimated to be 9-36%⁴⁷. In contrast, the prevalence of ASA in the general population is approximately 0-2%⁽⁴⁸⁾. ASA is thought to impair fertility by inhibiting sperm motility⁴⁹, sperm cervical mucus penetration⁵⁰, capacitation⁵¹, acrosome reaction⁵² or they may involve the complete cascade resulting in sperm

lysis.^{52, 53} A complete understanding of the mechanism behind immunologic infertility as well as improved diagnosis and treatment, depends on knowledge of specific sperm antigens capable of eliciting the production of functionally relevant sperm antibodies.

The Mixed anti-globulin test (MAR test) is routinely used in andrology laboratory to diagnose the sperm antibody. The kits are commercially available in the market. This is also helpful for the patients going for Assisted Reproductive Technologies (ART) and for patients of idiopathic infertility.

Genetic Evaluation

Patients of idiopathic infertility or suffering from oligozoospermia and azoospermia with family history of genetic disorder need to undergo genetic evaluation. Among couples with fertility problems, oligozoospermia and azoospermia are frequently observed. Genetic macroscopic defects (detected by cytogenetic method) and genetic microscopic defects (detected by molecular biology method) have been identified in such infertile patients⁵⁴. The genetic information for the control of spermatogenesis carried out on the Y chromosomes cytogenetic analysis.

0.5% infertile men had microscopic deletion of the distal long arm of the Y chromosome. The presence of an azoospermic factor in the long arm of Y chromosome has been studied⁵⁴. With the polymerase chain reaction (PCR) and construction of a Y chromosome sequence tagged site map⁵⁵ micro deletions were detected at a frequency of 5-18 % in the azoospermic factor (AZF) region of oligozoospermic and azoospermic men^{56, 57}. The micro deletions were mapped to three close sub-region labelled AZFa, AZFb and AZFc^{58, 59}. So far two candidate genes have been identified; the RNA-binding motif (RBM) gene was isolated from the AZFb region and deleted in azoospermia (DAZ) gene was identified in several copies in the AZFc region. The RBM gene is expressed specifically in the testis⁵⁷ and belongs to a large family of genes spread over the Y chromosome⁶⁰. The DAZ gene is also expressed specifically in the testis and bears an RNA recognition motif⁶¹. An autosomal homologue to DAZ (DAZH-DAZLA) was identified and mapped in human chromosome⁶². This gene is expressed in both gonads but at a lower extent in the ovary. The definite proof of the involvement of these genes in the spermatogenesis would be the identification of a point mutation in one of them, leading to oligozoospermia or azoospermia.

Sperm Chromosome Analysis

Chromosomal analysis by means of Fluorescence in situ hybridization (FISH) is becoming increasingly important. This method is based on marking single chromosome with specific DNA sequences to which fluo-

rescent stains are bound covalently. The DNA probes attach to the complementary sequences of the chromosomal DNA sequence and on exposure to UV light shows a characteristic emission pattern. The light signal within a cell shows the number of copies of the chromosome in question. Because of the small size of a sperm, the number of the chromosomes analysed simultaneously is generally limited to three. Many studies initially concentrated on identifying numeric chromosome aberrations in fertile volunteers^{63, 64} agreed upon a rate of 0.1 to 0.4 % per chromosome. This technique is not for routine laboratory use but in experimental or specific cases, it can be a useful tool.

Electron Microscopy (EM)

The role of electron microscopy (scanning and transmission) in the investigation of sperm abnormality is quite helpful to understand the defects in sperm. A fertile and an infertile spermatozoon show considerable morphological variations when observed under electron microscopy. In infertile patients, specific anomalies of sperm can be observed only under scanning and electron microscope. It is specifically used for idiopathic patients or for research purposes and cannot be used as a routine investigation.

Sperm Nitric Oxide (NO)

Nitric oxide (NO) has been implicated in a number of diverse physiological processes including smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation and penile erection. NO is an important messenger molecule for most of the biological processes. NO act as an atypical neurotransmitter in the central nervous system and as an agent of non-adrenergic non-cholinergic (NANC) autonomic neurotransmitter in the periphery. In the urogenital tract, NO play important functions in the penis, urethra and bladder.^{65, 66} Prime evidence in support of this prospect derives from investigations of penile erection. NO synthase (NOS) the enzyme that synthesises NO is localised at nerves in rat, canine, bovine and human pelvic and genital structures. Additionally, electrically induced relaxation of isolated corporal smooth muscle, tissue from rabbits and men as well as penile nerve stimulated erections in rabbits, rats and dogs are blocked by specific inhibitors of NO and these effects are elicited with agents that facilitates NO pathways.

There is now mounting evidence in support of a role for NO in sperm function and dysfunction. Like other reactive oxygen species (ROS) such as super oxide anion, hydrogen peroxide and hydroxyl anion, NO may be involved in both pathologic and physiologic processes. It has been shown that NO generated by NO donors, can influence sperm motility.

Reactive Oxygen Species (ROS)

The role of reactive oxygen species (ROS) in the pathophysiology of human sperm function has been emphasised in recent years. ROS production in semen has been associated with loss of sperm motility and decreased ability of fertilization. In men from couples consulting for infertility, 72% of washed sperm preparations from infertile men produced detectable amounts of reactive oxygen species (ROS) compared to only 25 % of preparations from a population of fertile donors⁶⁷. High ROS production was associated with oligozoospermia and poor sperm function. Various assays have been developed to monitor ROS generation in semen or sperm. The usefulness of these experiments in clinical analysis is understudy. The question whether administering antioxidants to men with sub-normal semen quality has any beneficial effect, is controversial⁶⁸.

DNA Flow Cytometry

Flow cytometry (FCM) is a technique, which provides a strong advantage over light microscopic techniques in terms of speed, multiple parameters /cell measurement, objectivity and number of cells measured/sample and thus provide a very high degree of statistical power. Apart from that, FCM can potentially sort out unique cells that comprise <1% of the total population for further study or use in assisted reproduction. By this technique, it is very easy to estimate relative proportions of the various cell populations e.g. mature haploid and diploid spermatozoa, cellular fragments, immature germ cells (haploid round spermatids, diploid cells) and leukocytes as indicators of infection. This technique is not meant for routine investigation however the technique is very important for the research purposes.

3. Other Clinical Investigations for Azoospermia

Testicular Examination

In cases of non-obstructive azoospermia, testicular volume is an important parameter to assess the testicular atrophy that may lead to the reduced spermatogenesis or absence of spermatogenesis. Testes are examined for tenderness, size and consistency in both the supine and standing position. A normal testis is over 4 cm in length with a volume >16cc. The seminiferous tubules contain the spermatogenic region of the testis and occupy 80% of its volume. Reduction in the number of tubular cells can manifest itself as testicular atrophy. Abnormally soft testicles and the presence of small, hard testis also suggest impaired spermatogenesis. In case of testicular atrophy, serum FSH levels needs to be measured.

FSH

Serum FSH is an important tool for the patients of azoospermia. The role of FSH in the spermatogen-

esis is quite evident. Therefore, before going for the Fine Needle Aspiration Cytology (FNAC), the patient is recommended for this investigation. Serum FSH reflects the completeness of spermatogenesis in the seminiferous tubules. In general, a man with normal spermatogenesis in a solitary testis will have an elevated FSH. Where as, a man with non-obstructive azoospermia and maturation arrest in both testes will also have a normal serum FSH levels but may not have sperm in the testis. In addition, a man with diffuse sertoli cell only pattern and isolated packets of spermatogenesis will have an elevated FSH levels. If the FSH levels are more than two to three times of normal values, the patients have some degree of testicular failure and the chances of finding obstruction is very low. Testicular biopsy may be performed for confirmation to evaluate the prognosis for sperm retrieval and to rule out intratubular germ cell neoplasia (Testicular carcinoma in situ). Potential causes of non-obstructive azoospermia includes acquired (e.g. mumps, orchitis) genetic (klinefelter's) and idiopathic patients with a sertoli cell only pattern in testicular biopsy and have an elevated FSH in 90% of cases while leutenizing hormone (LH) & Testosterone (T) remain normal.

A low FSH and LH combined with a reduced serum testosterone level are indicative of hypogonadotropic hypogonadism. The condition may be congenital (e.g. Kallman's syndrome), acquired (e.g. pituitary tumour) or idiopathic. Such conditions require imaging of the pituitary with magnetic resonance imaging (MRI) along with further hormonal testing. Prolactin (PRL) levels should also be measured. In the presence of acquired or congenital hypogonadotropic-hypogonadism, some patients will have panhypopituitarism while other might show significant elevations of PRL levels. High circulating PRL level causes decrease gonadotropins release and may have direct detrimental effects on the testis as well. Tumours usually are managed medically with oral bromocriptine administration. Deficiencies in GnRH or gonadotropins (LH and FSH) are managed with hormone replacement (partially purified or recombinant LH or FSH).

Testicular Biopsy

If the testicular volume and serum FSH levels are normal than to differentiate obstruction from abnormal spermatogenesis testicular biopsy is essential for evaluation. It can be accomplished under general or local anaesthesia through a small "window" scrotal incision.

Microscopic evaluation of biopsy specimens initially should be quantified by evaluating the average number of elongated spermatid per round seminiferous tubule. If >15 to 20 mature spermatid per tubule are present in an azoospermic patient, than a diagnosis of obstruction can be made. Seminiferous tubules fre-

quently appear in a mosaic of different spermatogenic patterns. These tubules generally fall in to the following categories:

Normal

Seminiferous tubules are separated by a thin interstitium containing acidophilic leydig cells, macrophages, blood vessels, lymphatic and connective tissue. Sertoli cell and spermatogonia line the basement membrane of the tubules. Germ cells in all stages of spermatogenesis are visible in seminiferous tubule of normal testis.

Hypospermatogenesis

In this condition, seminiferous tubule contains reduced number of all germinal elements. The germinal epithelium may be disorganised in places with immature germ cells in the lumen. The interstitium and the leydig cells are normal. Diffuse type of spermatogenesis may manifest clinically with oligospermia or azospermia.

Maturation Arrest

In this condition normal spermatogenesis can be seen up to a specific stage, and further development is inhibited. The arrest may be at primary or secondary spermatocyte or late spermatid stage. Mixed pattern occur occasionally and in some cases of late maturation arrest it may be difficult to differentiate from normal spermatogenesis. Most patients with maturation arrest have non-obstructive azospermia.

Sertoli Cell Only Syndrome

In this condition histologically there is complete absence of germ cells in seminiferous tubules, diameter of the tubules is reduced and interstitium may be minimally altered or demonstrate leydig cell hyperplasia. The sertoli cells are identified easily by their basal location in the seminiferous tubules and presence of single prominent nucleolus in the nucleus of each sertoli cell. Typically these patients have bilateral atrophy, soft testicles and elevated levels of FSH.

Sclerosis

During this conditions seminiferous tubules and the surrounding interstitium becomes sclerotic and hyalinisation, germ cells are absent and leydig cells may be present in hypertrophic nodules. On physical examination, these testes are usually small in size with hard tissue. Patients with klinefelter's syndrome may exhibit these symptoms.

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