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A REVIEW DIAGNOSING MALE INFERTILITY: BEYOND CONVENTIONAL SEMEN ANALYSIS

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ABSTRACT: *Infertility, defined as the inability of couples to conceive after one year of consistent, unprotected sexual contact, is a widespread concern affecting a significant portion of the population. Notably, between 14 to 17% of couples may encounter infertility at some point during their reproductive journey. This review delves into the realm of diagnosing male infertility beyond conventional semen analysis. Conventional semen analysis, which is a globally accepted tool for male infertility, primarily focuses on assessing sperm count, motility, viability, and morphology, leaving certain aspects of sperm functionality unexplored. These limitations necessitate the integration of advanced semen analysis techniques to provide a more comprehensive evaluation.*

Advanced semen analysis encompasses a range of sophisticated tests that probe deeper into the intricacies of male fertility. These tests include assessing sperm penetration capabilities, acrosomal discharge, and spermatozoa Reactive Oxygen Species (ROS) levels. Moreover, advanced semen analysis encompasses tests like sperm chromatin structure assay, DNA damage assessment, sperm proteomics, sperm metabolomics, and high sperm magnification microscopy, all of which shed light on various facets of sperm function and quality. While advanced semen analysis techniques offer a more comprehensive understanding of male infertility, their uptake and utilization in clinical practice have been limited. This review aims to elucidate the roles, merits, and drawbacks of both conventional and advanced semen analysis in diagnosing male infertility. This review sheds light on the strengths and weaknesses of each approach, it promotes a more nuanced approach to male infertility diagnosis, enhancing the prospects of successful conception for infertility challenged couples.

KEYWORDS: Infertility, Oxidative stress, Spermatogenesis, Azoospermia, Oligozoospermia.

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INTRODUCTION

Infertility is defined as the inability of couples to conceive after one year of consistent, unprotected sexual contact (Ajayi & Akhigbe, 2020). In 20% of couples who have failure in conception, male factor has been the only cause, whereas in 30% to 40% cases, it is a contributing factor (Guzick et al., 2001). Report alluded that about 14 to 17% of couples may at some point in their reproductive lives be affected (Wischmann & Thorn, 2013). Seminal fluid analysis, also known as semen analysis, is a useful and conventional tool in diagnosing male infertility. It assesses sperm count, motility, viability, and morphology. The use of the conventional semen analysis in the evaluation of male infertility is of global acceptance, possibly because it is economical and noninvasive (Agarwal et al., 2008). Although, there are differences in the feature of semen due to the types of human semen used and the procedure of the analysis, these differences may also be due to the procedures used in collecting the semen such as the edition of the guideline of the World Health Organization (WHO) laboratory (Esteves, 2014).

In addition, conventional semen analysis does not reveal the inability of sperm cells to perform certain functions, such as the movement of the sperm to the female gametocyte (Esteves, 2014). Advanced semen analysis includes assessment of sperm penetration (to assess sperm competence in fusion and penetration of the vitelline membrane of the female gametocyte, acrosomal discharge and also the release in the oocyte) (Sharma et al., 2013), and spermatozoa Reactive Oxygen Species, ROS (to assess the level of ROS in the semen) (Sharma et al., 2013), since it has been reported that in about 25 to 40% of infecund men, there is an abnormal increase of the ROS level (Sharma et al., 2013). Other assays include sperm chromatin structure assay, DNA damage test, sperm proteomics, sperm metabolomics, and high sperm magnification microscopy. Despite the shortcomings of the conventional type of semen analysis, it remains the bedrock of accessing male infecundity and the use of advanced semen analysis is yet to gain popularity. Therefore, this study aims to review the roles of conventional and advanced semen analysis in the diagnosis of male infertility, stating their merits and drawbacks.

DEFINITION AND PREVALENCE OF MALE FERTILITY

Infertility is defined as the inability of couples to conceive and produce offspring after a year of constant and unprotected copulation, influencing up to ten to fifteen percent of couples (Turchi, 2015; Zegers-Hochschild et al., 2017; Vander Borght & Wyns, 2018).

CAUSES OF MALE INFERTILITY

Lifestyle factors play a significant role in male infertility. Poor lifestyle choices can negatively impact sperm production, motility, morphology, and overall reproductive health (Durairajanayagam, 2018; Hafedh, 2023) Understanding and addressing these lifestyle causes is crucial for men who are trying to conceive or are concerned about their fertility (Aitken & Baker, 2008).

Lifestyle Factors

Recent reports have highlighted the rising incidence of male infertility due to various factors, including environmental pollution, stress, and lifestyle choices (Huang et al., 2018). This

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review will focus on specific lifestyle elements contributing to the decline in male reproductive health, namely emotional stress, genital heat stress, tobacco use, and alcohol consumption.

Emotional Stress

Psychological stress, in its various forms, can have a substantial impact on male fertility. The reproductive system is closely linked to the autonomic nervous system, adrenal hormones, and the stress response. Social stress, elevated temperatures, surgical procedures, and anxiety can influence body weight, testosterone levels, and copulatory behavior, leading to effects on testicular shape. Research has revealed that emotional stress, ranging from mild to severe, can decrease testosterone levels and disrupt spermatogenesis in human males (Tian et al., 2021). Studies have shown that students experiencing examination stress exhibited reduced levels of seminal antioxidant contents, motility, and morphologically normal spermatozoa (Feng et al., 2022). Work-related stress has also been associated with disturbances in LH pulse, contributing to erectile dysfunction and poor semen quality (Zou et al., 2019). Notably, the quality of semen samples collected from male IVF patients on the day of egg retrieval was lower than that of the initial sample due to the psychological stress associated with the clinical process (Tian et al., 2021).

Genital Heat Stress

Optimal sperm production relies on maintaining testicular temperature slightly below body temperature, typically around 34-35°C (Tian et al., 2021). The temperature range within which spermatogenesis occurs is critical, as lower temperatures reduce metabolic rate and enable longer sperm storage (Gao et al., 2022). Elevated scrotal temperature has been linked to impaired spermatogenesis, with evidence pointing to a connection between fever and reduced semen quality. Prolonged sitting, as seen in professions like driving, is associated with increased scrotal temperatures during the day, which in turn correlates negatively with semen quality. Additionally, wearing tightly fitted underwear elevates testicular temperatures compared to looser clothing. Oligozoospermic men with varicocele exhibit higher scrotal temperatures than normozoospermic men, and corrective varicocelectomy has been found to regulate scrotal temperatures (Gao et al., 2022).

Tobacco Use

Research suggests a link between smoking and erectile dysfunction, with smoking leading to raised cadmium and lead concentrations in the blood. Infertile smokers have been found to possess higher concentrations of these metals in their semen, along with worse reproductive characteristics compared to non-smokers. Smoking negatively impacts sperm quantity, motility, and morphology. Seminal leukocyte concentrations increase by 48%, reactive oxygen species (ROS) levels increase by 107%, and ROS-TAC scores decrease by 10 points in smokers (Saleh et al., 2003). Moreover, smokers face an elevated risk of sperm aneuploidy, altered sperm plasma membrane phospholipid asymmetry, and sperm DNA fragmentation (Agarwal et al., 2023).

Alcohol Consumption

Alcohol consumption has historically been associated with conditions affecting the reproductive system, including sterility and decreased penile size. Alcohol intake leads to a decrease in male hormone levels, with a study revealing testosterone levels dropping within

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just five days of alcohol consumption among healthy men. This decline continues throughout a four-week study period (Agarwal et al., 2023). Reduced sexual arousal, inadequate-quality sperm production, and decreased sperm quantity are effects of lowered testosterone levels. Some studies even suggest direct toxic effects of alcohol on the testes (Rahul et al., 2022). Additionally, alcohol hampers central nervous system activity, leading to reduced sexual activity. "Brewer's droop," or difficulty in achieving and maintaining an erection, as well as impaired ejaculation control, are common outcomes of alcohol consumption (Rahul et al., 2022). Alcohol may also interfere with sperm structure and movement by disrupting proper vitamin A metabolism in the liver, which is vital for sperm development (Maheshwari et al., 2021). Furthermore, alcohol's impact on zinc absorption is notable; zinc deficiency affects sperm structure, as zinc is crucial for the formation of the sperm cell's outermost covering and tip (Agarwal et al., 2023).

Environmental toxicants

Throughout the world, there is a significant increase in the record of infertility due to decline in semen quality and fecundity in male (Pizzorno, 2018). Pizzorno (2018) revealed that environmental toxicants and occupational exposures to toxicants contribute to the cause of male infertility. Several researchers have grouped environmental toxicants as industrial chemicals, agrochemicals, heavy metals and petroleum products (Osadchuk & Osadchuk, 2023). Heavy metals such as lead (Pb) and cadmium (Cd) are most prominent in the environment and are significantly responsible for defects in semen quality (Ige, 2019). Other heavy metals that have been reported to impair male fertility include aluminum, cobalt, and nickel (Ige, 2019). Reports have it that increased usage of gasoline and other petroleum products, smoking and rapid industrialization are responsible for the increase in the level of Pb and Cd (Ige, 2019). Humans are exposed to these heavy metals via adulterated or contaminated food and water and inhalation of polluted air (Osadchuk & Osadchuk, 2023). Studies have revealed that heavy metals impair spermatogenesis, spermiogenesis and steroidogenesis in the testis by destroying the testicular tissue and sometimes by suppressing the hypothalamic-pituitary-gonadal axis (Roychoudhury et al., 2019). In addition, heavy metal promotes reactive oxygen species (ROS) generation, which out-turn in oxidative stress with testicular and sperm oxidative damage.

Endocrine Factors in Male Infertility

The endocrine system plays a paramount role in regulating reproductive functions (Haywood et al., 2020; Roychoudhury et al., 2021). The intricate interplay of hormones is essential for the orchestration of male reproductive processes. The hypothalamus synthesizes gonadotropin-releasing hormone (GnRH), which stimulates the anterior pituitary to produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones, in turn, influence Sertoli cells for spermatogonia maturation (via FSH) and Leydig cells for testosterone production (via LH) (Roychoudhury et al., 2021). Notably, a delicate balance is required for optimal spermatogenesis; the concentration of sperm cells in the testes must exceed that in the serum. Although this balance is pivotal for spermatogenesis, it indirectly fosters bacterial growth within sperm cells due to testosterone's impact on Sertoli cells (Haywood et al., 2020; Roychoudhury et al., 2021).

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Genetic Cause of Male Infertility

While extensive research into human reproductive physiology persists, the underlying cause of male infertility remains elusive in approximately 50% of cases, categorized as idiopathic infertility (Mazouni et al., 2022). Genetic factors are believed to contribute significantly to this category, particularly considering that the number of genes implicated in human spermatogenesis might surpass 1000. Notably, the cystic fibrosis transmembrane conductance regulator (CFTR) gene and the androgen receptor (AR) gene stand out as genes of clinical relevance. CFTR mutations are tied to cystic fibrosis and absent vas deferens, while AR gene alterations lead to androgen insensitivity syndrome and spermatogenic damage. These genes influence testis determination, descent, and spermatogenesis processes. Chromosomal aberrations and deletions within the azoospermia factor (AZF) regions of the Y chromosome are also recognized biological triggers of spermatogenic dysfunction (Marzouni et al., 2022).

Metabolic Influence on Male Reproductive Health

Androgens, notably testosterone, wield substantial influence over male reproductive function. Leydig cells in the testes are responsible for testosterone synthesis, a pivotal male reproductive hormone and growth steroid. Diminished testosterone levels can disrupt male development and fertility (Kumar et al., 2022). Several factors contribute to low testosterone, encompassing injuries, metabolic disorders, obesity, uncontrolled type 2 diabetes, chemotherapy, radiation exposure, elevated prolactin levels, pituitary dysfunction, medications, alcohol misuse, and estrogen excess (Aitken & Baker, 2008; Durairajanayagam, 2018). Obesity assumes significance in this context due to its multifaceted impact on male fertility. Obesity correlates with elevated fat mass, reduced lean mass, impaired glucose control, diminished insulin sensitivity, and disrupted lipid balance (Heryanto et al., 2022). The consequences of obesity extend to various facets of male reproductive health, including disrupted sperm production, decreased testosterone levels, erectile dysfunction, and diminished sexual desire (Heryanto et al., 2022).

Leptin, secreted by adipocytes, plays a pivotal role in regulating body weight and energy balance. A direct association between BMI and leptin levels is established (Fariello et al., 2021), and excessive leptin production appears to contribute to androgen impairment and reduced reproductive function in obese men (Fariello et al., 2021). Importantly, obesity's impact on fertility is not confined to men but extends to women as well (Heryanto et al., 2022). In males, this entails disruptions in sex hormone levels and diminished sperm parameters, potentially influencing embryo development, live birth rates, and miscarriage rates in humans (Fariello et al., 2021).

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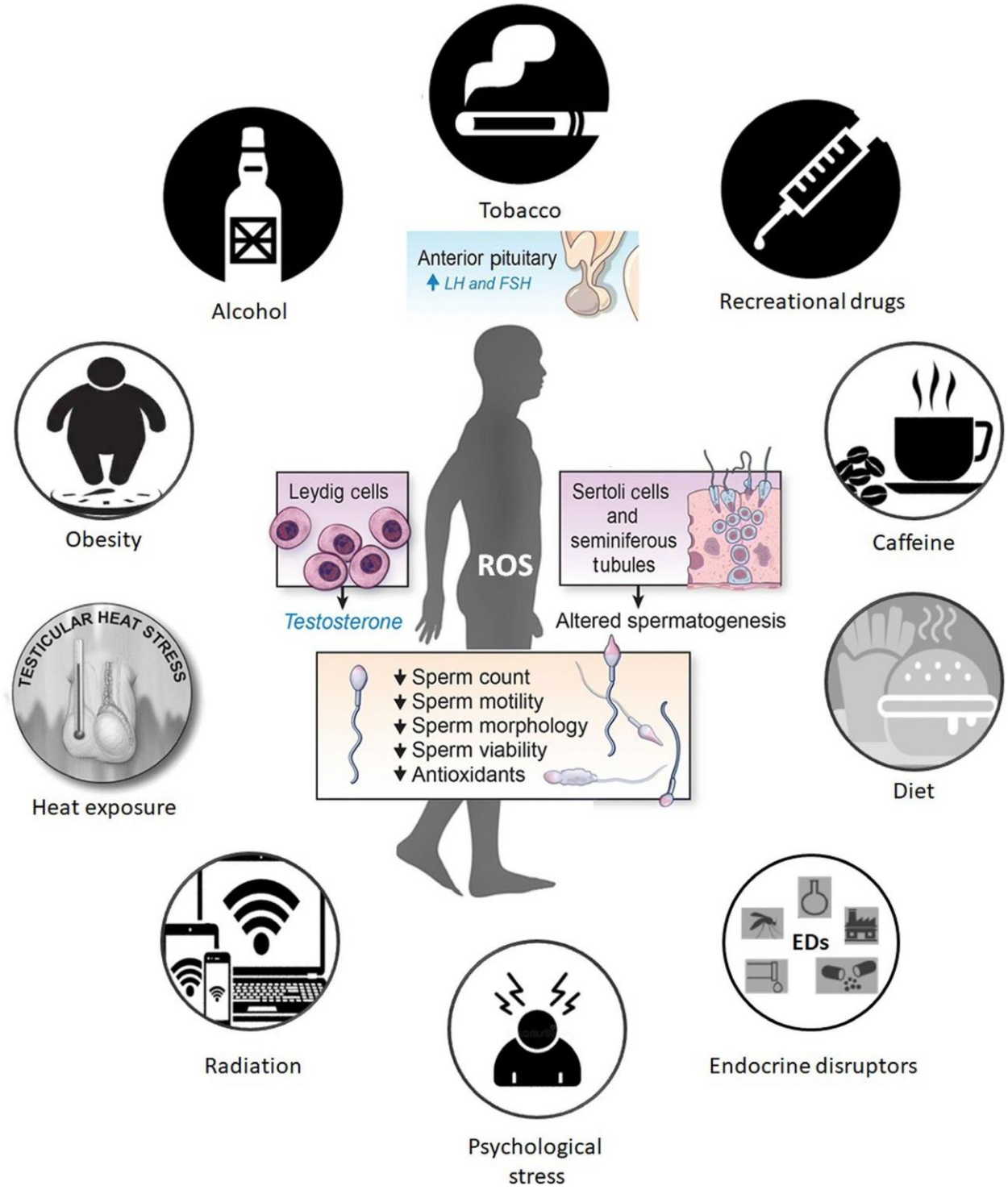

FIGURE 1 Lifestyle factors and their effects on male fertility (Huang *et al*., 2018)

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CONVENTIONAL SEMEN ANALYSIS

The conventional semen analysis is an insufficient means of examining male fertility, although it is of high importance in testing male fertility (Koju et al., 2021). However, standard semen analysis provides important information about sperm generation, movement of sperm and survival, male genital tract patency, discharges of the related organs, as well as ejaculation and expulsion. These tests however show that the primary examination of infertile male is not a fertility test (Koju et al., 2021) but does not show any possible function of the spermatozoon to fertilize an ovum or to undergo the constant maturation processes required to achieve fertilization. It is essential to know that the results may seem to correlate with "fertility," the assessment is not a direct measure of fertility (Guzick et al., 2001).

The traditional semen analysis and the sperm functional assay, which indirectly assesses a spermatozoon's capacity to deliver the correct combination of chromosomes to an ovum, must work in harmony. Sperm must be created in adequate quantities, have normal motility and morphology, pass through the cervical mucus, uterus, and oviduct ampulla after experiencing capacitation, acrosomal response, the binding of the zona pellucida and nuclear recondensation, in order to do this. If any of these complex events is impaired, it can result in male infertility and it is of importance to understand these tests and their basic explanation. (Eliasson, 2010; Koju et al., 2021).

TYPES OF CONVENTIONAL SEMEN ANALYSIS

1. Volume and pH

Conventional semen analysis begins with measuring the semen volume and pH level. Normal semen volume typically ranges from 1.5 to 5 milliliters. A low volume might indicate a problem with seminal vesicle function, while a high volume could suggest accessory gland abnormalities. The pH level, which should be slightly alkaline (7.2 to 8.0), is essential for the sperm's survival in the female reproductive tract.

2. Sperm Concentration

Sperm concentration, also known as sperm count, measures the number of sperm cells per milliliter of semen. A normal sperm concentration is typically greater than 15 million sperm/mL. Semen counts are given as millions/mL. Azoospermia which is a condition referred to as the lack of semen in the sperm sac can be noticed and/or oligozoospermia (also often called oligospermia) which refers to seminal plasma concentration less than 20 million per milliliter can also be evident.

3. Sperm Motility

Sperm motility assesses the percentage of sperm that exhibit progressive forward movement. Mobility is crucial as it determines the sperm's ability to reach and fertilize the egg. Normal motility should be over 40% to 50%. Reduced motility, known as asthenospermia, can impair fertility (Zhong et al., 2021). The movement of spermatozoa through the cervical mucus relies on steady progressive motility (Zhong et al., 2021) that is, spermatozoa with a forward progression of at least 25 μm/s. reduced sperm motility may be a sign of conditions affecting the production of hormones and progressive draining of male peripheral reproductive organs. The percentage (range 0%–100%) of the rate at which 200 sperm cells migrate with flagellar

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motion in a specific amount is used to determine rapid and slow sequential locomotion. Fast gradual movement is defined as speeds above 25 m/s at 37°C and greater than 20 m/s at 20°C. 25 m is about equivalent to 5 head lengths or half of a tail length. Nonprogressive motility is noticeable when sperm movement is <5 μm/s.

4. Sperm Morphology

Sperm morphology evaluates the percentage of sperm with normal shape and size. Head defects include large, tiny, tapering, pyriform, spherical, amorphous, and vacuolated patches (>20% of the head area is taken up by these areas, which are unstained). Acrosomal areas that make up less than 40% of the head area, twin heads, or any combination of these. Deformities of the neck and mid-piece include bent necks, asymmetrical mid-piece insertions into the skull, thick, crooked mid-pieces, abnormally thin mid-pieces, and any combination of these. Tail flaws include short, numerous, hairpin, broken, bent, kinked, coiling, and coiled tails.

5. Viability and Vitality

Sperm viability tests, which are recommended when sperm movement is less than five percent to ten percent, are used to determine if nonmotile sperm are viable or not. They are useful in primary ciliary dyskinesia where ultrastructural defects in sperm flagella result in absent or very low motility but with high viability (Finelli et al., 2021). Sperm from surgically removed testicular tissue is also used to select sperm for intracytoplasmic sperm injection (ICSI). Sperm are viable but typically non motile due to absence of the transport of the epididymis (Agarwal et al., 2008).

Viability testing also uses dye exclusion assays and the hypothesis osmotic sperm swelling test. It depends on live sperm's capacity to withstand particular dyes' absorption, whereas these dyes infiltrate and taint nonliving sperm cells. The commonly used stains are Trypan blue and Eosin Y stains because they do not taint viable semen. However, as the technique requires air drying after staining, sperms are killed and not practically useful. (Finelli et al., 2021).

6. White Blood Cells (WBCs)

The presence of white blood cells in semen suggests inflammation or infection in the male reproductive tract. Elevated levels of WBCs (pyospermia) may indicate an underlying condition that affects fertility.

7. Coloration

Seminal coloring can be clinically caused by new blood, medications (pyridium), jaundice, or infection of the sperm from urination (example: malfunction of the bladder neck). Long-term abstinence samples have a biological yellowish tint that is caused by the carotenoid’s pigmentation, and sperm oxidation creates odor.

8. Semen Viscosity

Sperm viscosity gauges the flow barrier of the seminal fluid. The measurement of the movement of sperm, level, and antibody coating may be influenced by high viscosity. Semen often begins to agglomerate after ejaculation and liquefies after fifteen to twenty minutes. Semen that remains a coagulum is termed non liquefied, whereas that which pours in

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thick strands instead of drops is termed hyperviscous (Schallmoser et al., 2021) Precise time of liquefaction is of no clinical significance except it is greater than two hours with no significant difference. The inability to liquefy is usually a sign that there is inadequate secretion by the prostate of the proteolytic enzymes fibrinolysin, fibrinogenase, and aminopeptidase (Schallmoser et al., 2021) conversely, a lack of agglomeration can be linked to a blocked ejaculatory pathway or a genetic lack of seminal blood vessels.

9. Fructose Measurement

Fructose, a sugar produced by the seminal vesicles, is an essential energy source for sperm. Low levels of fructose in the semen may indicate a blockage or absence of the seminal vesicles.

ADVANTAGES OF CONVENTIONAL SEMEN ASSAY

Sperm cell quality can be evaluated through various approaches, including conventional semen analysis, a technique employing light microscopy to assess sperm quantity, motility, viability, and morphology. This method not only sheds light on potential infertility causes but also lays the groundwork for subsequent investigative processes. One notable benefit of conventional semen analysis is its speed, affordability, and relative simplicity, as it does not demand extensive expertise.

Recently, an intriguing advancement involves the creation of smartphone-based devices for semen analysis. These devices, such as those compatible with iOS 8, iOS 9, and Android 4.4, linked to a single-ball lens microscope, have been introduced to gauge sperm concentration and motility in comparison to existing CASA systems (Lammers et al., 2021). Additionally, other smartphone setups, like the combination of Galaxy S7 or iPhone 7 with a YO device, have emerged to assess motile sperm motility (Agarwal et al., 2014). Using magnified smartphone screens, these gadgets facilitate manual tracking of sperm movement and density.

A more recent development is the smartphone-based CASA system, which offers real-time video display of sperm motility, automated counting, and the ability to detect sperm concentration and motility. This innovation underscores the potential of smartphone-based diagnostic CASA systems for automating semen analysis in screening for male infertility among couples seeking medical intervention or for individuals interested in assessing their fertility prior to marriage. Notably, smartphone-based CASA offers rapid, automated, cost-effective, and user-friendly features, making it a promising addition to the global healthcare landscape (Park et al., 2021).

DISADVANTAGES OF CONVENTIONAL SEMEN ANALYSIS

Conventional analysis gives considerable information; it does not assess the presence of deregulated programmed cell death (apoptosis) in spermatozoa, which may be partially responsible for the low fertilization and implantation rates seen with assisted reproduction. Also, since it is more like a manual means of semen investigation and it is been carried out by a laboratory scientist there is possibility of having error in the test result leading to a idiopathic male fertility, it is usually not precise when number is involved e.g. sperm count (Koju et al., 2021). Hence, the conventional semen analysis is an insufficient and unreliable means of examining male fertility (Li et al., 2019).

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Despite the fact that standard semen analysis is still the gold standard for evaluating male patients with infertility, it does not accurately identify the origins of infertility or foresee reproductive fitness (Li et al., 2019). Routine semen analysis produces inconsistent results due to inter- and intra-observer variability, and it provides no information on sperm dysfunctions at the cellular and molecular levels (Esteves and Gupta, 2014; Henkel et al., 2003). Furthermore, the WHO's lower reference limits do not apply to all men because the values of semen parameters overlap in fertile and infertile men. As a result, the actual cause of undiagnosed arthritis is unknown. Even after routine semen analysis, the cause of unexplained male infertility remains unknown, even when routine semen analysis is performed (Hamada et al., 2011). Normal spermatozoa fertilization potential does not equate to normal sperm parameters (Hamada et al., 2011). As a result, more advanced tests are needed to correctly diagnose male infertility and foresee pregnancy in couples simply trying natural conception or couples using artificial reproductive technologies ART (Henkel et al., 2003).

Despite the fact that developed smartphone-based CASA systems can measure sperm concentration and motility, a particularly notable limitation of the technology is that the system does not measure sperm morphology, oxidation reduction potential, or DNA integrity for successful reproductive outcome. Another limitation is that these smartphone-based CASA systems need a high-resolution camera at the periphery of the field of view of the captured image (Henkel et al., 2003). The limitations of this study had to do with the small number of participants and that the smartphone-based CASA system is currently available only for iOS. An additional limitation of our study was that consumers did not examine the smartphone-based CASA system (Lammers et al., 2021; Park et al., 2021).

ADVANCED SEMEN ANALYSIS

The standard of care for determining a male patient's fertility status is a conventional semen analysis. It however has some flaws, which include the inability to correctly identify the etiology of fertility problems, intra- and inter observer variability, and incomplete information on sperm function (Wang et al., 2021). Advanced sperm tests, such as sperm function tests, oxidative stress (OS), and sperm DNA fragmentation (SDF) tests have been developed to investigate male infertility in light of these drawbacks. This study explains the most common sperm function test as well as the assays used to assess SDF and OS and their diagnostic value (Gill et al., 2019; Rahman et al., 2019).

TYPES OF ADVANCED SEMEN ANALYSIS

Semen analysis has long been a cornerstone of male infertility assessment, providing valuable insights into sperm quantity, motility, and morphology. However, the limitations of conventional semen analysis in capturing the multifaceted nature of male fertility have led to the development of advanced techniques that offer a more comprehensive and nuanced evaluation. These advanced semen analysis methods delve deeper into various aspects of sperm health, DNA integrity, and functional attributes, contributing to a more accurate diagnosis and tailored treatment strategies for couples struggling with infertility.

1. Acrosome reaction (AR)

After sperm capacitation and sperm–zona pellucida binding, the acrosomal reaction (AR) occurs naturally. Proteolytic enzymes stored in the acrosome are released during AR to allow

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sperm–zona pellucida penetration and sperm cells to fertilize the oocyte as in figure 2 (Agarwal et al., 2008). Flow cytometry and fluorescence microscopy using lectins or antibodies, electron microscopy, bright-field light microscopy, and chlortetracycline fluorescence are some of the laboratory tests conducted to assess spermatozoa's ability to undergo AR. A baseline and an induced AR are determined in order to investigate acrosomal functionality (Cissen et al., 2016). The proportion of spermatozoa that randomly release their acrosomal content, as well as the percentage of sperm cells that are acrosome-reacted after an in vitro induction of AR, are assessed in AR testing (Cissen et al., 2016; Zheng et al., 2023).

The distinction between induced and spontaneous AR is regarded as the inducibility of AR which is the ability of sperm cells to undergo AR (Zheng et al., 2023). The effective methods for AR testing are electron microscopy and flow cytometry. Nevertheless, their effectiveness is hampered by the fact that both techniques are costly. Other techniques are simpler to use, but they have drawbacks such as being labor-intensive and making it difficult to correctly identify the AR. Essential information is obtained by differentiating between spontaneous and AR after calcium ionophore or low temperature induction (Ghajeri et al., 2022). Ultimately, assessing AR can provide useful information about the ability of sperm cells to fertilize (Ghajeri et al., 2022). It was stated that in all cases studied, an acrosomal response of 31.3 percent is a marker of fertilization failure (Shan et al., 2022). A meta-analysis deduced that AR is a good predictor of the outcome of in vitro fertilization (IVF) (Sharma et al., 2013). As a result, patients who have failed ART and have a poor acrosomal reaction should be referred to ICSI (Cissen et al., 2016; Zheng et al., 2023). The zona pellucida-induced acrosome reaction (ZIAR), which is influenced by dissolved human zona pellucida (ZP), is a more complex test for AR that can distinguish between fertile and sub-fertile males (Ghajeri et al., 2022). Overall, the information obtained from AR testing aids in the better management of male infertility cases.

2. Sperm capacitation test

Capacitation is a series of biochemical and structural changes that spermatozoa go through to be able to fertilize. The process takes place in the female genital tract but can be induced in vitro by incubating spermatozoa with capacitation-inducing media. Sperm capacitation test also plays an important role in preventing the release of lytic enzymes until spermatozoa reaches the oocyte. One of the basic signs of capacitation is the display of hyper activation by spermatozoa (Sáez-Espinosa et al., 2020).

In the female genital tract, spermatozoa go through a capacitation phase. This biological process encompasses all of the modifications that allow spermatozoa to undergo acrosome reaction and thus become fertilization competent. A test to determine the spermatozoa's ability intends to stimulate sperm capacitation under laboratory conditions by placing the sperm cells in a capacitating medium such as human tubal fluid (HTF) medium enriched with 3% albumin (Henkel et al., 2003). Cap-ScoreTM Sperm Function Test (Cap-ScoreTM) is a new test that assesses the spermatozoa's capacitation possibility.

The purpose of this test is to detect and analyze the localization patterns of the ganglioside GM1 (a lipid raft marker in the sperm membrane), which is critical for determining the spermatozoa's ability to fertilize the oocyte. This test could be used as a screening tool because it has the ability to foresee high versus low pregnancy rates and is highly associated with the possibility of pregnancy (Cissen et al., 2016; Nakidkina and Kuzmina, 2019).

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3. Sperm–oocyte penetration assay

The sperm–oocyte penetration assay, also known as the zona-free hamster oocyte penetration assay, was one of the first methods for assessing sperm function. It assesses spermatozoa's ability to undergo capacitation, acrosome reaction, fusion, and penetration through the oolemma. It also examines sperm heads' capacity to decondense within the cytoplasm of hamster oocytes (Oehninger & Kruger, 2021). It also removes the zona pellucida, of human spermatozoa undergoing capacitation and attaches to the oolemma of trypsinized hamster oocytes (Cissen et al., 2016). This test does not precisely predict the outcome of fertilization (Sharma et al., 2013). This test is inadequate for IVF patient selection because it cannot foresee successful IVF (Sharma et al., 2013). Finally, the sperm–oocyte test is not recommended for regular use because it is an expensive and time-consuming test with low clinical significance (Cissen et al., 2016; Zheng et al., 2023).

4. Sperm-zona pellucida binding test

Sperm cells must pass through the zona pellucida to reach the oolemma and, ultimately, the nucleus of the oocyte. Sperm–zona pellucida binding abnormalities are the most frequent cause of IVF (Shan et al., 2022) and IUI (Arslan et al., 2006) failure. The hemizona assay and the competitive zona binding assay are the two most frequently used tests to assess sperm–zona pellucida binding capacity (Shan et al., 2022). Sperm–zona pellucida binding assays have a higher prediction accuracy for the outcome of fertilization (Sharma et al., 2013). These tests may be suggested for patients who have failed standard IVF and have unidentified primary fertility problems (Samplaski et al., 2010). As a result, patients who have sperm–zona pellucida binding defects are encouraged to consider ICSI (Arslan et al., 2006).

5. Hemizona assay

Pre-ovulatory, unfertilized, or recycled failed-fertilized human oocytes can be used in the hemizona assay (HZA) (Lewis et al., 2013). Because oocytes are separated into two equal equal parts under microscopic regulation and the ooplasm is removed, there is no fully functioning, live oocyte for fertilization, only a vacant hemizona with no ability. One hemizona is sub-cultured with fertile donor spermatozoa (as a positive control), while the other half is incubated with the patient's spermatozoa (sub-fertile) (Agarwal et al., 2014). The hemizona index (HZI) is calculated as the ratio of patient to control. A HZI value of 30 percent is considered abnormal (Arslan et al., 2006). The hemizona assay can tell the difference in both fertile and infertile male patients (Bastiaan et al., 2002) and may be suggested to patients with oligoasthenoteratozoospermia (OAT) and recurrent IVF failures (Arslan et al., 2006; Sharma et al., 2013). Patients with a HZI of 30 percent had lesser fertility rates than patients with a HZI of more than 30 percent, 11.1 percent and 40.6 percent, respectively (Arslan et al., 2006). Moreover, patients with oligozoospermia have reduced or normal ZP binding but low ZIAR, which is coherent with their reduced likelihood of natural or conventional IVF fertility (Wang et al., 2021). This test has an elevated clinical significance and provides valuable information about the physiology of sperm cells. However, due to its labor-intensive nature, the need for specialized costly equipment, and the scarcity of human zonae pellucidae, the hemizona assay is rarely done.

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6. Hypo osmotic swelling test

The hypo-osmotic swelling test can be used to determine sperm vitality and plasma membrane integrity (Agarwal et al., 2016). Under hypo-osmotic stress (150 mOsmol/L), viable spermatozoa with intact membrane swell and curl their tail, which is a characteristic of viable spermatozoa with intact membrane. When fluid reaches the cell's intact membrane, this occurs as a result of the membrane's semipermeability. The plasma membrane of dead spermatozoa is not intact, resulting in a leaky membrane. While live cells keep ions and other osmotically active molecules outside and only allow water to penetrate into the cell, resulting in cellular swelling, the plasma membrane of dead spermatozoa is not intact, resulting in cellular swelling. As a result, dead spermatozoa do not swell and their tail shape does not change (Agarwal et al., 2014). Patients who have few or no motile sperm cells in their seminal fluid are regarded infertile.

Fluorescence microscopy and flow cytometry coupled with fluorescent dyes can be used to evaluate mitochondrial function, especially the mitochondrial membrane potential (MMP) (Moraes & Meyers). The assay's principle is that MMP is commensurate with the fluorescence color and intensity (Moraes & Meyers). The most commonly used fluorescent dyes for analyzing MMP in human spermatozoa are JC-1 and TMRM (tetramethylrhodamine methyl ester perchlorate) (Moraes and Meyers). TMRM is preferred for human spermatozoa analysis (Kumar, 2023). TMRM is a simple, time-saving method that can accurately identify changes in laboratories equipped with flow cytometry technology.

This test can differentiate between normal and poor sperm samples (Kumar, 2023), and it is connected to semen parameters like progressive motility, viability, normal morphology, sperm count, and seminal volume for all parameters (Kumar, 2023). This test also differentiates between astheno- and oligoasthenozoospermia patients, disclosing critical information about mitochondrial function in sperm samples (Paoli et al., 2011; Park & Pang, 2021). As a result, this test can be used to augment basic sperm analysis (Kumar, 2023).

7. Comet assay

The Comet assay, also known as single-cell gel electrophoresis, is a single-cell test that shows sperm ssDNA and dsDNA breaks (Enciso et al., 2009). This method is premised on the assumption that DNA fragments move in an electric field from the anode to the cathode depending on their weight, resulting in the generation of a comet tail emanating from the nucleoid (Enciso et al., 2009). High-molecular-weight DNA fragments that are intact move very slowly or not at all during agarose gel electrophoresis, remaining at the 'head of comet.' Low-molecular-weight DNA fragments that are broken, on the other hand, move in the shape of a comet's tail (Simon et al., 2013). The decondensed sperm DNA is stained with a fluorescent DNA-binding dye under neutral or alkaline denaturing conditions (Lewis et al., 2013). An electron microscope is used to view the level of DNA fragmentation, and the comet tail dimensions and fluorescence intensity are assessed (Panner Selvam et al., 2021). Recognition of dsDNA breaks is feasible using the Comet assay under neutral pH conditions for lysis and electrophoresis. The DNA is decondensed in the alkaline Comet assay, which allows for the recognition of both ssDNA and dsDNA breaks without difference (Enciso et al., 2009).

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The Comet assay is a responsive and low-cost technique that allows for the evaluation of DNA damage in only one spermatozoon rather than a general percentage of DNA fragmentation in the whole semen sample (Lewis et al., 2013). Since this assay requires only a small number of cells, DNA fragmentation can even be deduced in samples with low sperm concentration. The rate of sperm cells containing fragmented DNA is used to assess DNA authenticity (Enciso et al., 2009). Evaluating about 50 to 500 spermatozoa is sufficient to obtain a clear picture of the DNA damage status of the entire sperm specimen with a covariance less than 4% (Lewis et al., 2013). This method detects both protamine- and histone-bound chromatin breaks (Lewis et al., 2013). A frail link was observed between DNA fragmentation as assessed by the neutral Comet assay and sperm parameters (Lewis et al., 2013). Furthermore, the neutral Comet assay was unable to differentiate between fertile and infertile men, resulting in no diagnostic value (Simon et al., 2011). The alkaline Comet assay was suggested by (Simon et al., 2013) as a screening aid for male infertility and IVF outcomes. Low fertility and pregnancy rates, as well as poor embryo quality, were linked to higher levels of DNA fragmentation in spermatozoa (Simon et al., 2011). TUNEL, SCD, and SCSA were the best predictors of male infertility, followed by the alkaline Comet assay (Simon et al., 2011). The alkaline Comet assay has the limitation of being a laborious assay with multi laboratory variation, making it a less preferable diagnostic test (Simon et al., 2013).

8. TUNEL assay

The TUNEL assay is a straightforward test that measures actual sperm DNA damage (Hassanen et al., 2019) (Atala, 2020). This approach uses the enzyme TdT (DNA polymerase), which non preferentially ads fluorescein-labeled deoxyribonucleotides (dUTP) to free single- and double-stranded 3′-hydroxyl (OH) break ends (Sharma et al., 2016). The unification of dUTP into DNA breaks is measured as a percentage of fluorescent spermatozoa using this method (DFI) as seen in figure 3 (Hassanen et al., 2019). Flow cytometry as well as fluorescence microscopy can be used (Atala, 2020). However, the DFI derived through cytometry and fluorescence microscopy cannot be directly compared to the TUNEL assay results because the two assays assess various aspects of sperm DNA damage (Hassanen et al., 2019). Sharma et al. (2016) normalized the TUNEL assay, and at a trimmed value of 16.8 percent, the test demonstrated high specificity (91.6 percent). SDF was markedly larger in infertile men compared to control men. While the controls had an upper limit of SDF of 19.6 percent, infertile patients had a maximum limit of SDF of 68.9 percent (Sharma et al., 2016). Sperm parameters like morphology, motility, and progressive motility were found to be related to the DFI (Henkel et al., 2003). In addition, a significant correlation was found between DFI and sperm parameters such as total sperm count, concentration, motility, and normal sperm morphology in ICSI patients (Borini et al., 2006). DNA fragmentation as measured by the TUNEL assay is an efficiency indicator of pregnancy (Borini et al., 2006), fertilization and pregnancy loss while others claim there is no link (Henkel et al., 2003). A meta-analysis written by (Cui et al., 2015), concluded that the TUNEL assay obtained greater results.

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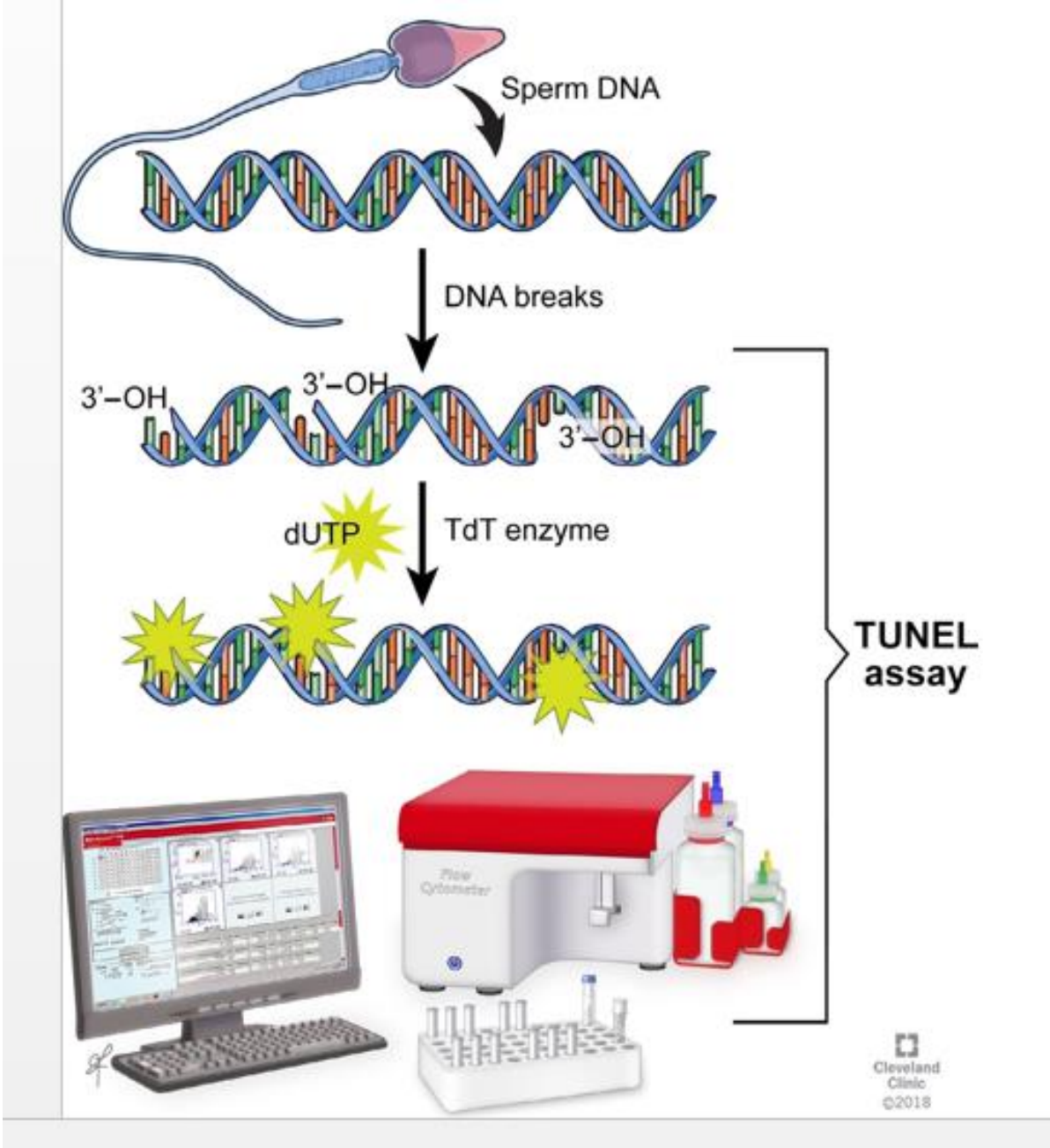

Figure 3: TUNEL assay for the assessment of sperm DNA fragmentation (Hassanen *et al*., 2019).

ADVANTAGES OF ADVANCED SEMEN ANALYSIS

Infertile couples undergo a series of tests to learn more about the cause(s) of their problems, to help them choose the best course of action, and to predict the probable outcome of their treatment. In males, this assessment almost entirely consists of routine semen analysis, which has been done in the same way for decades. The first WHO manual introduced standardized processes, which was a significant advancement.

In terms of diagnostics, these lengthy microscopic examinations do provide some insight into testicular and genital tract function. If an absolute abnormality is not found (e.g., azoospermia,

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necrospermia, asthenospermia, and globozoospermia), they provide little information on which a clinician can advise and act.

Routine semen analysis, in terms of prognosis, is unable to accurately assess fertility potential or predict reproductive outcome. It is unsurprising that only a small percentage of laboratories actually follow the WHO guidelines (Sharma et al., 2016). However, recent approaches to studying male reproductive function have been accompanied by the development of tools that can reveal previously unknown features and have the ability to reveal the true state of sperm motility and sperm quality. Some have been included as optional or research procedures in subsequent versions of the WHO manual.

Old parameters are being assessed in new ways in the sense that skills and knowledge of the scientists performing the laboratory tests evaluate the accuracy and reliability of semen analysis in most laboratories. Several semi-automated and fully automated computer-aided sperm analysis (CASA) methodologies have been introduced to improve precision, accuracy, and repeatability while eradicating human subjective nature. Quality and timeliness have improved because of modifications and the integration of new or different sample preparation procedures (Finelli et al., 2021). Few laboratories use CASA routinely, and then only as an effective alternative to established procedures, as it requires similar standardization and quality control as manual analysis.

DISADVANTAGES OF ADVANCED SEMEN ANALYSIS

The hemizona assay is of high clinical importance and gives critical information concerning the normal function of the sperm cells. However, due to its labor-intensiveness, the need for specialized and costly facilities and the fact that human zonae pellucidae are not easily obtainable, the hemizona assay is rarely done. The best techniques for acrosomal reaction testing are electron microscopy and flow cytometry. However, their effectiveness is hindered by the fact that both techniques are costly and labor-intensive, and it can be difficult to accurately identify the acrosomal reaction (Agarwal et al., 2021). The sperm-oocyte assay test does not accurately predict the outcome of fertilization (Sharma et al., 2013). The accuracy and sensitivity, positive and negative predictive values (Cissen et al., 2016). This test is also inadequate for IVF patient selection because it cannot foresee productive IVF (Bastiaan et al., 2002). Conclusively, sperm–oocyte assay is not suggested for regular use because it is a costly and time-consuming test with low clinical significance (Cissen et al., 2016). The hyaluronan binding assay is a test which measures how well hyaluronan binds. However, a study concluded that this test is unable to differentiate between patients with elevated, minimal, and failed fertilization rates (Borini et al., 2006). Furthermore, there was no link between this and other reproductive results like fertilization rate, implantation, or fetal death (Borini et al., 2006). The ASA IBT (immunobead binding test) is a sensitive and specific test. It is, however, a costly option to use because it is time-consuming and requires a skilled and experienced operator, and the results are difficult to comprehend (Mazumdar & Levine, 1999). Correspondingly, the MAR test is a quick specific assay that necessarily involves the use of a highly trained and professionally experienced operator. Furthermore, the test's sensitivity is unknown, and the costs are outrageously high (Mazumdar & Levine, 1999).

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CONCLUSION AND RECOMMENDATION

Conclusion

Since conventional semen analysis has a low predictive value for pregnancy outcomes, sperm functional tests are required in the infertile couple's enhanced evaluation and treatment. Sperm function tests can help physicians decide which care method is best for infertile couples. A few advanced semen analysis tests are now readily accessible, and their clinical significance has been thoroughly investigated. Specific sperm function tests, such as sperm DNA fragmentation (SDF), as well as tests that measure Reactive oxygen species (ROS), antioxidants, and ORP, are among the tests available. Sperm function tests can assess important sperm cells features and foresee spermatogenesis and perinatal mortality in infertile couples. Oxidation-reduction potential (ORP) has been ascertained to be the most effective test for assessing oxidative stress (OS) state.

Majority of studies reveals a correlation between SDF and assisted reproduction success; other studies have been unable to demonstrate this correlation. As a result, assays aimed at testing spermatozoa function and measuring DNA damage and OS should be better regulated so that they can be incorporated into WHO guidelines.

Recommendation

Advanced sperm testing can help couples who are trying to conceive naturally or who are undergoing ART better predict pregnancy.

The advanced semen analysis comprises improved standardized methodologies useful in the assessment of male infertility which improve clinical significance.

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