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Review Article

Methods For Assessment Of Testicular Sperm Viability: A Mini-Review

Mohamad Alaa Eldein Hasan, Marwa Mohamed Abdel Meged, Hanan Abd El-Radi Metwally, Ramadan Saleh Abdou

*Department of Dermatology, Venereology and Andrology, Faculty of Medicine, Sohag University

Abstract:

Assessment of testicular sperm viability is crucial for successful intracytoplasmic sperm injection (ICSI) in cases of male infertility involving azoospermia. Traditional methods like staining cannot be used as they compromise sperm viability. This article aimed to provide a comprehensive mini-review of different methods for the assessment of testicular sperm vitality during ICSI. The hypo-osmotic swelling test (HOST) is considered the gold standard, identifying viable sperm based on membrane integrity. However, HOST has limitations with thawed samples and is technically demanding. Chemical agents like pentoxifylline can induce tail movements in immotile sperm to facilitate selection, but with potential toxicity concerns. The sperm tail flexibility test (STFT) relies on mechanically manipulating the tail to detect viability. Laser-assisted immotile sperm selection (LAISS) is an emerging technique using laser pulses to induce tail movements in viable sperm without chemicals. Birefringence polarization microscopy distinguishes viable sperm based on their birefringent properties under polarized light. Raman micro-spectroscopy is a label-free method using molecular fingerprinting to identify biochemical markers of viability. Each technique has advantages and limitations in terms of efficacy, safety, cost and technical requirements. Combining multiple complementary approaches may optimize viable sperm selection for ICSI. Further research is needed to establish the relative performance and refine protocols, with the ultimate goal of improving fertilization and pregnancy outcomes for azoospermic men seeking fertility treatme.

Keywords: Azoospermia, testicular sperm, viability, laser, hypo-osmotic swelling test

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Corresponding Author: **Mohamad Alaa Eldein Hasan** E.mail: mam243@student.bham.ac.uk

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Introduction

Among every seven couples who get married, one couple will suffer from infertility. Males account directly for 20-30% of infertility cases and contribute to around 50% of cases overall. [1] 1% of all men and 15-20% of infertile men suffer from Azoospermia. [2,3]For azoospermic males, the only hope to father a child is to undergo a testicular biopsy. Any sperm found within the testicular tissue shall be utilized to fertilize the partner's oocytes via Intracytoplasmic Sperm Injection (ICSI).

Indeed, a crucial step for a successful ICSI is the selection of viable sperm. Assessment of testicular sperm viability is challenging, as the majority of spermatozoa only attain their motility within the epididymis. [4] Many methods have been used over the past few years to evoke and test the viability of immotile spermatozoa. While it is technically possible to utilize randomly selected immotile spermatozoa, employing only viable immotile spermatozoa is a more favorable strategy that enhances success rates in severe cases.

Staining method is probably the first evolved sperm vitality test. This method depends upon the use of certain dyes to differentiate between viable and non-viable spermatozoa e.g. the Eosin-Nigrosin staining method. [5] While this method holds average sensitivity and specificity ,[6] it obviously compromises sperm viability in the first place and cannot be used prior to ICSI. Hence, the development of efficient laboratory techniques that can differentiate between non-living and viable but immotile spermatozoa is imperative.[7]

This article aimed to provide a comprehensive review of the standard, evolving, as well as state-of-the-art laboratory methods used during ICSI procedures to assess the vitality of testicular sperm.

Methods

A comprehensive literature search was conducted using PubMed to capture studies investigating different methods for the assessment of testicular sperm viability. A combination of two or more of the following search terms was used: “sperm”, “testicular”, “TESE”, “micro-TESE”, “microTESE, “TESA”, “vitality”, “viability”, “ICSI”, “Intracytop-lasmic sperm injection”, “Azoospermia”, “stain*”, “hypo-osmotic”, and “laser”.

No language restriction was applied. All returned articles had titles and abstracts screened for possible relevance. A full-text review was then conducted for pertinent papers to confirm inclusion. Data related to the assessment of testicular sperm viab-ility during ICSI was extracted from each study. We also included the latest edition of “WHO Labor-atory Manual for the Examination and Processing of Human Semen”.[8]

Results

We found a total of 43 articles addressing methods for the assessment of testicular sperm vitality, in addition to the World Health Organization (WHO) laboratory manual.[8] The time range of those articles ranged from 1950, when the staining method was first reported, to 2023. Seven different categories were identified for the assessment of testicular sperm vitality: 1) Staining methods; 2) Hypo-osmotic swelling test (HOST); 3) Chemical agents; 4) Sperm tail flexibility test (STFT); 5) Laser-assisted immotile sperm selection (LAISS); 6) Birefringence-polarization microscopy; and 7) Raman micro-spectroscopy.

Discussion

1. Staining methods

Staining method is the first invented and most commonly used sperm vitality test, [5] When sperm samples are mixed with the designated stain, viable sperms resist staining, while dead sperms absorb it due to lack of membrane integrity. Eosin-nigrosin is the standard staining method recommended by WHO for assessment of sperm vitality.[8] While eosin is the primary stain for spermatozoa, nigrosin serves to enhance the differentiation between the background and the sperm heads, hence facilitating their visual distinction. Additionally, it allows for the storage of slides for the purposes of re-evaluation and quality control.[9]

When seen under microscopy, healthy spermatozoa exhibit white or mild pink heads (**Figure 1.A**), whereas dead spermatozoa have heads that are stained red or dark pink **(Figure 1.C**). If the stain is

confined only to a specific portion of the neck region while the whole area of the head remains unstained, this phenomenon is referred to as a "leaky neck membrane", and the sperm is still considered as viable **(Figure 1.B**).[10]

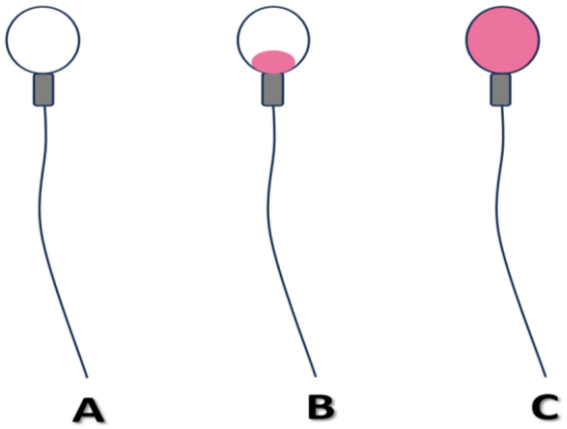

Figure 1. Schematic representation of alive (A, B) and dead sperm (C) after eosin-nigrosin staining.

***Advantages**

Easy, affordable, and standardized method for assessment of sperm vitality.

***Disadvantages**

While staining is a standard method for assessment of sperm vitality in semen analysis samples, this method cannot be used for ART samples, since the stain itself compromises sperm vitality. This is considered the major limitation for this method. Consequently, this method is not advised for testicular samples, which are precious samples that should be either cryopreserved or used directly for ICSI. [11]

2. Hypo-osmotic swelling test (HOST):

HOST measures the sperm's plasma membrane's functional integrity and also acts as a helpful predictor of the sperm's reproductive potential. [12] Sperm reaction in a hypoosmotic media can be used to verify functional integrity. Living cells' semi-permeable membrane causes them to swell in hypotonic solutions because they have intact membranes. The spermatozoa's capacity for in vitro fertilization is highly connected with the great repeatability and accuracy of HOST.

For HOST, a hypo osmotic solution is made by mixing 100 mL of distilled water with 0.735 g of sodium citrate dehydrate and 1.351 g of D-fructose. One milliliter of HOS solution is added to 0.1 milliliters of thoroughly blended semen. The sample is gently mixed using continuous pipette suction and release. The mixture is incubated for 30 minutes for diagnostic purposes, or for 5 minutes for therapeutic purposes as in ICSI. One drop of the semen mixture is then put on a glass slide with a coverslip after incubation. The examination should be carried out in duplicate under a 40-phase contrast lens. Viable spermatozoa exhibit various degrees of tail swelling (**Figure 2**). To obtain a statistically robust result, at least 200 spermatozoa should be evaluated per aliquot for tail swelling/coiling. The number of sperms with tail swelling is divided by the total number of evaluated sperms to obtain the proportion of viable sperms. [8]

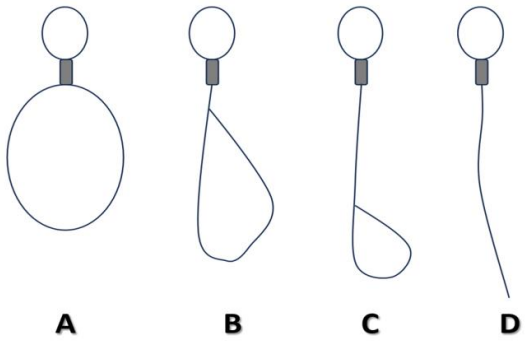

Figure 2. Schematic representation of alive (A, B, C) and dead sperm (D) using HOST.

***Advantages**

Due to its efficacy, feasibility, affordability, and safety, HOST is considered the gold standard test for assessment of sperm viability during ICSI. [7,13]

***Disadvantages**

The main limitation of HOST is its use on thawed samples. Up to 10% of thawed sperms after cryopreservation could exhibit spontaneously developed tail swellings (SDTS), jeopardizing the accuracy of HOST if used on those samples. [14] A recent study with sibling oocyte split design reported lower fertilization and blastulation rates with HOST-immotile sperm selection compared to motile sperm selections for ICSI using thawed ejaculated or testicular sperm samples. [15] On the contrary, HOST-immotile sperm selection showed comparable results to motile sperm selection for ICSI using fresh ejaculated and testicular sperm samples. Nevertheless, clinical, obstetric, and neonatal outcomes were comparable across all fresh and thawed samples.

HOST is also a technically demanding procedure. Swollen sperms should be detected and utilized rapidly with minimal exposure time to the hypo-osmotic medium before possible steric problems occur. In addition, HOST is more time consuming when compared to the readily motile spermatozoa provided by motility provoking substances such as pentoxifylline. [7]

***Modified hypo-osmotic swelling test:**

Modified HOST was developed to simplify the test and facilitate its use in ICSI procedures. The solution is prepared using 50% sterile deionized water and 50% sperm washing medium. Water creates hypo-osmotic pressure upon the sperm, while washing medium provides the essential nutrients and delays the potential harmful effects of hypo-osmosis, increasing HOST safety [16]. Salam and his colleagues investigated the outcome of ICSI using modified HOST (50% culture medium + 50% Milli-Q grade water) versus sperm selection based on morphology in both ejaculated and testicular sperm samples, and reported a comparable or superior fertilization, pregnancy, and ongoing pregnancy rates. [17,18]

3. Chemical substances for induction of tail movements

Chemical substances such as pentoxifylline and theophylline, which are methylxanthine derivatives, have been used to induce tail movements in immotile sperm for selection in assisted reproductive techniques. These drugs work by inhibiting phosphodiesterases (PDEs), which increases intracellular cyclic AMP levels and improves sperm motility. [7] Several studies have shown benefits of using these drugs to select sperm from immotile testicular or ejaculated samples for intracytoplasmic sperm injection (ICSI). For example, pentoxifylline reduced the ICSI procedure time from 120 minutes down to 30 minutes by provoking motility to facilitate viable sperm selection. [19] Compared to no chemical activation, pentoxifylline improved the number of accessible embryos. However, pregnancy rates were unchanged. Another study found higher fertilization and pregnancy rates with pentoxifylline versus

selection based on HOST, likely by reducing false positives.[20] Remarkably, pentoxifylline enabled pregnancy using immotile ejaculated sperm from a patient with Kartagener's syndrome that initially seemed impossible to use.[21]

Similarly, theophylline transiently improves sperm motility, allowing quick selection and immobilizeation for later ICSI use. This increased fertilization rates, blastulation, implantation and pregnancy rates with no observed birth defects.[7,22,23] Comparisons of pentoxifylline and theophylline found both effective in ~90% of cases, but lower miscarriage rates with theophylline (1.2% vs. 9%). [24]

*Advantages

The main advantages of these drugs seem to be enabling rapid, reliable identification of motile sperm.

*Disadvantages

possible disadvantages include added incubation times, cost, and uncertainties about long-term safety. Animal studies using high concentrations of pentoxifylline showed some detrimental effects on embryos, [25] but no defects have been reported in humans after therapeutic use in fertility treatments. [26]

In summary, pentoxifylline and theophylline hold promise for enabling use of immotile sperm samples for ICSI, with rapid selection, good efficacy, and no clear evidence of defects in resulting children. However, some embryo toxicity has been observed at high concentrations in animals. Further optimization and long-term follow-up studies in humans would help realize the full potential of these drugs to improve outcomes for severe male infertility.

4. Sperm tail flexibility test (STFT)

The sperm tail flexibility test (STFT) is an additional technique for determining if immotile spermatozoa are viable. In essence, this test entails agitating the sperm tail mechanically while applying lateral pressure with the microinjection pipette to see if it is moving.[27] The immotile sperm is deemed alive and may be chosen for ICSI if the tail moves independently of the head. In contrast, a spermatozoa is nonviable when its tail remains stiff in reaction to the same stress. The pregnancy and take-home baby percentages employing immotile and motile sperm were discovered to be comparable when sperm were chosen based on this approach, either from frozen or fresh testicular tissue samples. [28]

***Advantages**

A straightforward and affordable technique that avoids the dangers of chemical solutions and preserves the structural integrity of the sperm.

***Disadvantages**

The primary drawback of this mechanical touching approach is the lack of sufficient evidence comparing its outcomes to those of other techniques, particularly with its reliance on the practitioner's individual training and expertise. [29]

5. Laser-assisted immotile sperm selection (LAISS)

The use of lasers in ART has evolved substantially over the past decade. Initial applications focused on assisted hatching and embryo biopsy. [30,31] More recently, novel uses for lasers on sperm have emerged, including laser-induced immobilization and permeabilization of the sperm membrane [32,33] and LAISS. [34,35]

The first described technique involves using a laser to permeabilize and immobilize the sperm membrane, which is often done using an intracytoplasmic sperm injection (ICSI) pipette for high fertilization rates. While manual permeabilization is consistent, it relies heavily on the embryologist’s skill and dexterity. Replacing the needle with a 2-3 millijoule laser pulse can permeabilize more accurately and reliably, reducing operator dependency. This technique's utility was established over a decade ago, with the first successful birth reported. [33]

The invention of a method for the detection of viable but immotile spermatozoa (LAISS) marked a further advancement in the application of lasers for sperm selection.[34] In LAISS, a single 129 μJ laser pulse lasting roughly 1.2 ms is shot at the flagellum's tip. A viable immotile sperm will exhibit tail twisting or coiling. On the other hand, a dead sperm will show no response.

The technique's capacity to detect the presence of viable spermatozoa in a sample was equivalent to that of the HOS test (22.0% vs. 21.5%) when applied to ejaculated asthenozoospermic samples

and immotile spermatozoa from testicular biopsies. [34] The take-home baby rate rose from 5.9% to 19.0% as the fertilization rate climbed noticeably from 20.4% in the randomly chosen TESE spermatozoa group to 45.4% in the laser selection group. [36] LAISS can be also used with cryopreserved sperm samples. [37] Pregnancy and live birth were successfully reported after using LAISS-ICSI for cases with primary ciliary dyskinesia, even resistant to pentoxifylline. [38,39]

***Advantages**

LAISS is a rapid, simple, and safe procedure, which is supported by the reported birth of healthy offspring after its application [40]. The LAISS method is slowly gaining ground as the method of choice for identifying a viable spermatozoon. The key benefit of LAISS, apart from its speed, is that it doesn't require chemical substances to either increase motility or produce spermatozoa flagellum curling; therefore, there won't likely be any concomitant adverse effects.

***Disadvantages**

ICSI microscope has to be equipped with a laser, 409 Laser objective for analysis required and experienced lab personnel. [7] Unfortunately, due to its cost, the laser is not yet a 'must-have' equipment for the standard IVF laboratory. [41]

6. Birefringence-polarization microscopy

This method of operation assumes that a viable sperm has a birefringent head and midpiece (reacted sperm) under polarization microscopy. Dead, necrotic spermatozoa lack birefringence under pathological conditions due to the full absence of the typical conventional sperm surface and texture; whereas live human spermatozoa are naturally and physically birefringent. [42] Because different sperm regions react to polarised light differently, birefringence capacity may be able to distinguish between them. For example, an immotile viable spermatozoon shows a non-luminous or partially luminous acrosome, whereas the completely luminous acrosome can be seen in the anomalous spermatozoa.[43,44] The different types of birefringent spermatozoa increase the likelihood and potential for identifying essential spermatozoa with completely intact DNA, even if they are not motile.[45]

A recent study investigated ICSI using polarization microscope for samples with complete athenozoospermia (83 ejaculated and 109 testicular samples).[44] The authors reported significantly higher rates of fertilization, embryo development and implantation when using birefringent sperm versus absence of birefringence. Cumulative live birth rates were 53.6% and 9.0%, respectively ($P < 0.001$). Furthermore, Spermatozoa with partial head birefringence yielded significantly higher fertilization and embryo utilization rates compared with total birefringence. The cumulative live birth rates showed the same trend (62.7% and 46.7%, respectively, $P = 0.048$). [44]

***Advantages**

A rapid and simple procedure, without need to interfere with the sperm mechanically or chemically.

***Disadvantages**

This process is quite expensive. In addition, more comparative and virtual research investigations must be carried out. [41]

7. Raman micro-spectroscopy

Raman microscopy is a label-free, non-invasive optical technique that probes the molecular composition of cells. It measures the inelastic scattering of monochromatic light as it interacts with molecular vibrations, providing a molecular fingerprint of the sample. This fingerprint contains detailed information about the biochemical makeup, including DNA, proteins, lipids, and metabolites. [46]

Raman spectroscopy has emerged as a powerful tool in reproductive biology research. [46] Studies have demonstrated its ability to detect sperm DNA damage and identify aneuploid sperm based on DNA content differences. [47] Its molecular sensitivity and non-invasive nature make it promising for assessing the viability of immotile sperm samples. By analyzing the Raman spectra of individual immotile sperm, researchers could identify biochemical markers associated with viability. For example, differences in DNA packaging, membrane integrity, or metabolic activity could serve as viability indicators. Machine learning algorithms could be trained on Raman data to

classify sperm as viable or non-viable with high accuracy. [47,48]

Integrating Raman microscopy into ICSI workflows could improve sperm selection and fertilization rates. Immotile sperm identified as viable by Raman analysis could be injected with higher confidence, potentially reducing the need for backup sperm samples or additional cycles. Furthermore, Raman microscopy could be used for quality control, ensuring only viable sperm are used. [47,49]

Except for nuclear DNA, research on sperm assessment has so far produced conflicting results, and it is yet unknown how to identify and assign spectral bands in Raman-profiles to the various sperm areas. [48] Although studies are still in their early stages, Raman micro spectroscopy is a promising method for assessing male fertility. [49]

Conclusion

In conclusion, the assessment of testicular sperm viability remains a critical challenge for achieving successful pregnancies through ICSI for azoospermic men. While the hypo-osmotic swelling test is still considered the gold standard, several emerging techniques show promising potential. Laser-assisted immotile sperm selection provides a rapid, safe and effective approach, though it requires specialized equipment. Birefringence polarization microscopy is another optical method that can identify viable sperm without compromising the sample. Raman micro-spectroscopy is an exciting label-free technique that could revolutionize viable sperm selection by detecting molecular biomarkers of viability. Chemical agents like pentoxifylline induce motility for easier selection, but with potential toxicity tradeoffs. Looking ahead, combining multiple complementary techniques may prove most effective for optimizing viable sperm recovery and improving ICSI outcomes.

Continued research focusing on refining protocols, evaluating efficacy compared to current methods, and long-term safety assessment will be crucial for translating these innovative approaches into widespread clinical practice. Ultimately, advancing techniques for testicular sperm viability assessment holds great promise for helping more azoospermic couples overcome male infertility and achieve their dreams of parenthood.

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