Semen collection, analysis and preparation: a mini review

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Summary

Assisted reproductive techniques (ARTs) begin with spermatozoa processing, with the purpose to select the best spermatozoa for insemination. An appropriate semen analysis involves the evaluation of sperm concentration, motility and morphology. These parameters are still the mainstay in the assessment of male reproductive potentiality. The quality of semen sample influence directly the preparation method to perform. Different sperm separation approaches exist to maximize the yield of motile sperm. Sperm processing techniques should be gentle to avoid free radical formation. Moreover these procedures should prevent sperm damage, avoid non-physiological alterations, eliminate dead sperm and other cells or debris. The most common techniques used in andrology laboratories and ART programs are swim-up, density gradient and simple washing. Generally, simple washing and swim-up procedures are favourite with good quality semen samples, whereas density gradient centrifugation is ideal with lower concentrated samples. Several efforts have been made to improve selection techniques. However methods recently proposed to further investigate the competence of spermatozoa are still not validated. To date, further randomized studies are needed before recommending these advanced sperm selection techniques in the clinical practice.

KEY WORDS: sperm analysis, sperm preparation, swim-up, density gradient, simple washing.

Introduction

At ejaculation millions of spermatozoa are released by male partner, but only very few of them are able to reach the fertilization site. A progressive selection mechanism occurs in the female tract; in fact, it has been estimated that only about 0,1% of the total starting sperm population reaches the fallopian tube in human species (1), however it is still unclear if this final sperm population is really composed of the most fecund cells from the ejaculate (2). Assisted reproductive techniques (ARTs) also begin with spermatozoa processing, with aim to select the best available spermatozoa for insemination. Thus, different sperm separation approaches have been developed. Despite controversy about the clinical value of semen analysis, the assessment of sperm concentration, motility and morphology is still essential. Therefore, when accomplished according to WHO recommendations, it still has a significant role in the evaluation of male fertility potential (3).

Sample collection and analysis

In order to not alter sperm quality and integrity, patients should have clear instructions for sam-
ple collection. They are asked to abstain from sexual intercourse for at least 2-7 days, as recommended by WHO guidelines (4). For sample collection, semen is produced by masturbation and only sterile plastic-containers must be used to avoid microbiological contamination. Before use, containers must be labelled with patient’s name, identification number, date and time of production. During sample collection lubricants must be avoided since they can impact semen quality. One recommendation is to keep the sample at room temperature (from 20° to 37°C) and to perform semen analysis within one hour from collection (4).

**Semen analysis: macroscopic examination**

According to WHO guidelines (4), after semen collection, several parameters need to be evaluated to perform initial macroscopic investigation. The overall appearance of the sample in terms of colour, pH, volume, viscosity and liquefaction is estimated. A normal sample has a homogeneous appearance with a white to opalescent grey colour. Volume can be measured by using graduated pipettes, or as an alternative, graduated containers to collect the sample. The lower reference limit is 1.5ml (5th centile, 95% confidence interval 1.4-1.7) (4). Viscosity can be valued by aspirating the sample into a plastic pipette. By flowing drop by drop, it is possible to evaluate if the semen forms a thread rather than a drop. If the sample has increased viscosity, it can be reduced by adding washing medium and repeated pipetting. The use of a narrow-gauge needle to aspirate semen with high viscosity must be avoided since this procedure can severely damage sperm motility (5). PH should be measured after liquefaction using a ph paper with a range from 6.0 to 10.0. Normal sample has a value of ≥ 7.2 (4). Finally, sample generally liquefies within 15 minutes, then it is possible to proceed with microscopic investigation of the semen sample.

**Semen analysis: microscopic examination**

A microscopic examination involves the evaluation of sperm concentration, motility and morphology. These parameters are still the mainstay in the assessment of male reproductive potentiality (4). To perform these evaluations a wet preparation is needed. A drop of undiluted semen, previously mixed very well to provide the most representative data, is placed on prewarmed slide and covered with a cover slip. To allow free movement of the sperm during examination, a 20µm-deep chamber is recommended, for example taking 10µl of native semen and using a 22mm x 22mm coverslip (4). In presence of viscous semen, the use of a positive displacement pipette results helpful. It is important to begin the evaluation only when the “flow” has stopped, to avoid bias during evaluation. The wet preparation allows insight also for other elements present in the seminal fluid, such as round cells, debris, epithelial cells, aggregation and agglutination. Immotile spermatozoa adhering each other or motile spermatozoa adhering to mucus strand, cells or debris should be recorded as nonspecific aggregations. Whereas, when motile spermatozoa aggregate tail to tail, head to head or both, should be recorded as specific agglutinations and could be suggestive of the presence of anti-sperm antibodies (4). Furthermore when round cells are found in semen it is important to differentiate the leukocytes from immature cells, thus a special method for detection of inflammatory cells should be used.

**Sperm concentration**

The evaluation of sperm concentration could give indications about the most appropriate insemination technique to be used (standard IVF vs ICSI). For evaluation a phase contrast microscope optics (200-400x) is used. Different types of counting devices exist, with at least two counting chambers, different design and grid placement and may require different calculation to obtain the final sperm concentration. However, the use of 100-µm-deep haemocytometer chamber (i.e., improved Neubauer) is suggested as the most accurate by WHO guidelines (4). To assess semen concentration, usually 1:1 dilution is performed; however the dilution can be varied according to initial semen concentration (i.e., 1:5, 1:20, 1:50) (4). Duplicate wet preparations ensure accurate assessments with at least 200 spermatozoa counted per replicate (4). The total
Semen number of the entire ejaculate is calculated multiplying the sperm concentration by the semen volume. According to WHO the lower reference limit for sperm concentration is $15 \times 10^6$ spermatozoa per ml ($5^{th}$ centile, 95% CI 12-16 $\times 10^6$), whereas for total sperm number is $39 \times 10^6$ ($5^{th}$ centile, 95% CI 33-46$\times 10^6$) (4).

When no spermatozoa are visualized at wet preparation, a centrifugation at high speed of the sample (3000g for 15 minutes) is necessary prior to confirm the total absence of spermatozoa. After removal of the supernatant, the pellet needs to be resuspended and carefully scanned under a microscope. If no or very few spermatozoa are found in the pellet, a second ejaculation or a testicular biopsy can be considered.

**Sperm motility**

Motility should be assessed as soon as possible to avoid deleterious effects such as dehydration, changes in pH and temperature, that might cause erroneous interpretations. As mentioned above, to perform motility assessment a wet preparation needs to be prepared (4). As for concentration, the evaluation of motility is performed with a phase contrast optics at 200x or 400x, assessing at least 200 spermatozoa per replicate, and looking for sperm in a central area of the slide (≥5mm from the edge of the coverslip) to avoid air-drying artefacts, at room temperature or at 37°C. The fifth edition of WHO recommend to evaluate only three different types of sperm motility (6):

- **progressive motility (PR):** sperm that is able to move actively, linearly or in a large circle;
- **non progressive motility (NP):** sperm that shows movement without progression i.e. swimming in small circles;
- **immotile (IM):** all sperm without movement.

According to WHO, the lower reference limit for total motility (PR+NP) is 40% ($5^{th}$ centile, 95% CI 38-42), whereas for progressive motility (PR) is 32% ($5^{th}$ centile, 95% CI 31-34).

**Sperm morphology**

Evaluation of sperm morphology is a further tool to investigate male fertility, reproductive health and testicular stress. Nevertheless, it is still a controversial aspect of semen analysis because of the subjectivity of the human eye and the different staining techniques involved. Moreover, the definition of “normal sperm morphology” still lacks of a scientific evidence (7). The first studies investigating features of sperm able to fertilize an egg were conducted on spermatozoa recovered from the endocervical mucus (8). This selected sperm population has been considered as reference of potentially fertilizing spermatozoa. As proposed by Mekveld et al., in 1990 (9), a sperm should be considered morphologically normal, if it has the following characteristics (strict criteria):

- **head** with oval profile, a well-defined acrosome occupying about 40-70 % of the sperm head;
- **acrosomal region** should contain no large vacuoles (and no more than two small vacuoles occupying more than 20% of the sperm head);
- **head length** should be between 3-5µm and width between 2-3 µm;
- **neck, mid-piece and tail** should be without defects and aligned with major axis of the sperm head;
- **residual cytoplasm** is considered normal only if it doesn’t exceed 1/3 of the sperm’s head size.

This strict criteria for the evaluation of sperm morphology became the standard method of evaluation adopted by WHO manual, despite several criticisms. After mixing, a smear of the sample is prepared applying 5-10µl of semen on a slide (depending on sperm concentration) and pulling the drop of the semen over the whole surface of slide using a second slide at an angle of about 45° (4). Then the smear is air dried, fixed and finally stained. Evaluation of the slide is conducted by bright field optics at 1000x magnification with oil. It is recommended to use Papanicolaou, Shorr or Diff Quick to stain the smear and highlight details of spermatozoa, in particular:

- the **acrosomal region** will result stained pale blue, whereas dark blue will be stained in the **post acrosomal region**;
- **midpiece** may show red staining as well as excess residual cytoplasm.

During sperm morphology assessment, also the type of defects should be analysed:

- **head defects:** tapered, pyriform, round with...
small or no acrosome, amorphous, vacuolated;
- **neck and midpiece defects**: bent neck, asymmetrical, thick or thin insertion;
- **tail defects**: short, bent or coiled;
- **excess residual cytoplasm**: ≥1/3 head.

According to WHO, the lower reference limit for the normal form is 4% (5th centile, 95% CI 3.0-4.0).

**Sperm preparation techniques**

Different methods for sperm preparation have the purpose to select sperm potentially able to fertilize an egg. Selection techniques should reproduce as much as possible the natural selection that occurs during the journey across the female genital tract (1). An ideal sperm preparation procedure should minimize sperm damage, avoid non-physiological alterations, eliminate dead sperm and other cells or debris (5). The preparation should be gentle in order to minimize the production of reactive oxygen species (ROS) (5). The choice of sperm separation method depends mainly on the quality of semen sample. The most common techniques used in andrology laboratories and ART programs are swim-up, density gradient and simple washing (Figure 1).

**Swim-up**

First described by Mahadevan and Baker in 1984 (10) it is still mainly adopted by IVF laboratories worldwide (5). Normal sperm parameters and high sperm motility are prerequisite to adopt this method, based on the ability of sperm to swim actively from the pre-washed pellet into a placed over medium (11). This very easy and cheap procedure allows for the recovery of a clean fraction of highly motile sperm (5). On the other hands, during the centrifugation steps, leukocytes and other pellet components may induce oxidative stress. To overcome this disadvantage, it is possible to proceed directly from liquefied semen avoiding the washing step procedure (4,5). In both cases, culture medium supplemented with nutrient is gently stratified over the sample; the use of conical tubes incubated with an inclination of about 45° enhances the interface between the semen and culture medium thus maximizing the yield of sperm (4,5). After an incubation time of about 30-60 minutes at 37°C, the cul-
ture medium is recovered with a sterile pipette, beginning from the meniscus downwards, taking care to avoid the interface region (5). The portion of culture medium recovered, enriched with sperm, can be analysed to estimate final motility and concentration of the processed sample.

**Density gradient centrifugation**

Two types of density gradients, continuous or discontinuous, can be prepared. While in continuous gradients, a gradual increase in density is created in the conical test tube, in a discontinuous gradient a clear boundary between the two phases is present (5). Nowadays, a discontinuous gradient, the most widely used, contains colloidal silica coated with silane having different densities, 40% (v/v) and 80% (v/v) (4). This substances replaced the use of percoll, which is a PVP-coated silica, with known detrimental effects on sperm membranes (12). Semen sample is placed on the top of density media. During centrifugation cells stratify in different layer of the gradient, according to their density. Motile and morphologically competent sperm have higher density (1,10g/ml) compared to immature or abnormal sperm (1.06-1.09g/ml) (13), thus they form a pellet at the bottom of the tube. On the contrary debris, bacteria, leucocytes and abnormal sperm stratify in the upper layer (5). After removal of supernatant, the pellet is resuspended with washing medium and eventually centrifuged again before sample determination (4). This is the ideal procedure when the sample has scarce parameters, thus it is mainly used for oligozoospermic, asthenozoospermic and teratozoospermic patients. Although density gradient recovers a clean fraction of highly motile sperm and reduces reactive oxygen species, this technique is more time consuming and expensive compared to swim-up procedure (5).

**Simple washing**

This technique is the most easy and quick to fulfil; no filter is used to select spermatozoa as it is performed by mixing the semen sample with a washing medium and centrifuging the mixture twice at 300-500g for 5-10 min (4). The supernatant is discarded and the pellet is resuspended. As well as for the above mentioned techniques, these steps remove harmful components present in the ejaculate and concentrate sperm from the total sample. Simple washing procedure normally is used with semen samples with a good concentration of highly motile sperm.

**Sperm retrieval in azoospermic patients**

In case of azoospermia, sperm can be recovered from epididymis and from testicular tissue. Recovering of sperm from epididymis aspiration is indicated in case of obstructive azoospermia, due to bilateral obstruction of the seminal ducts. This procedure recovers sperm with minimal red blood cells and non-germ cells contamination. WHO guidelines recommend to process the sample performing density gradient centrifugation (4).

In patients with non obstructive azoospermia, where spermatogenic failure occurs, sperm retrieval is possible by testicular sperm extraction (TESE) or the most accurate alternative microdissection TESE. The material obtained from TESE is contaminated by high number of erythrocytes. The freshly retrieved tissue is washed with buffered medium, then it is disrupted using two glass slides or fine needles to achieve a small pieces suspension. The suspension is then centrifuged at 300g 8-10 min and the pellet is analysed to assess the presence of sperm. As alternative to the mechanical method, an enzymatic digestion using collagenase (from clostridium histolyticum type 1A, for 1.5- 2 hours at 37°C vortexing every 30 min) can be considered to release tissue bound spermatozoa (4). This procedure is more invasive and generally recovers sperm without motility, fragile and with non-germ cells contaminating elements. Generally after conventional TESE, very low sperm numbers are yielded, in comparison to sperm retrieval rate achieved performing microTESE. This is a complex and time-consuming procedure, especially recommended in case of non-
obstructive azoospermia coexistent with testicular cancer during radical orchiectomy, in order to maximize sperm retrieval rates (16).

Conclusions

Semen analysis is an important step to start a treatment cycle in ARTs. After sample collection and liquefaction it is recommended to perform the evaluation of semen volume, viscosity, appearance and pH measurement. Next step involves motility, concentration and morphology assessments. Some of these parameters are susceptible to change over time, thus, it is imperative to perform them as soon as possible. If an appropriate semen analysis is achieved, then the appropriate preparation technique can be chosen. Generally, simple washing and swim-up procedures can be performed with good quality semen samples, whereas density gradient centrifugation is preferred with lower concentrated samples.

Several efforts have been made to identify novel sperm quality indicators and improve selection techniques. Despite noteworthy progresses, our knowledge about the natural sperm selection that occurs during the journey across the female reproductive tract is still fragmentary (1). Methods recently proposed to further investigate the competence of spermatozoa, such as the examination of motile sperm-organelle-morphology (MSOME) or the ability of sperm to bind hyaluronic acid (17, 18), are still not validated (19, 20). To date, further randomized studies are needed to improve the evidence quality before recommending all these advanced sperm selection techniques in the clinical practice.

References