6. Sperm Preparation Methods

Semen is a mixture of motile, non-motile and dead spermatozoa with cells, cellular debris and sometimes micro-organisms.

There are various sperm separation and isolation methods. These methods try to replicate in vitro the natural process in which viable sperm are separated from other constituents of the ejaculate as they actively migrate through the cervical mucus.

The World Health Organization recomandation is to process sperm sample within one hour after ejaculation in order to prevent damage from leukocytes and other cells present in the semen.

The most common methods to separate the motile sperms from the semen semple are washing and centrifugation.

The sperm suspension obtained using these methods is used for IUI, IVF, ICSI and different sperm tests.

The most commonly used sperm preparation methods in ART are :

1. **Simple wash**
   This method is used if the semen sample is very poor and it mainly removes seminal plasma from the sperms.
   This procedure provides the highest yield of spermatozoa. Therefore it is often used for ICSI.
   In this procedure the culture medium is added to the ejaculate after liquefaction and the mixture is centrifuged twice using lower centrifugation forces and fewer centrifugation steps in order to minimize the damage caused by ROS.

The common steps of this method are:

1. Mix the semen sample well
2. Dilute the entire semen sample 1 + 1 (1:2) with supplemented medium to promote removal of seminal plasma.
3. Transfer the diluted suspension into multiple centrifuge tubes, with preferably not more than 3 ml per tube.
4. Centrifuge at 300–500g for 5–10 minutes.
5. Carefully aspirate and discard the supernatants.
6. Resuspend the combined sperm pellets in 1 ml of supplemented medium by gentle pipetting.
7. Centrifuge again at 300–500g for 3–5 minutes.
8. Carefully aspirate and discard the supernatant.
9. Resuspend the sperm pellet, by gentle pipetting, in a volume of supplemented medium appropriate for final disposition, e.g. insemination, so that concentration and motility can be determined (Who laboratory manual for the examination and processing of human semen 2010)

2. **Swim up method**

This technique relies on the ability of spermatozoa to swim out of seminal plasma into culture medium and is suitable for semen with high to moderate motility.

This procedure selects spermatozoa for their motility.

The common steps of this method are:

1. evaluate the precentage of motile and total number of sperm on a Makler Chamber
2. fill the round-bottom test-tubes with 2 ml Sperm Preparation Medium (medium)
3. layer 1ml of the liquefied semen underneath the medium
4. incubate the sample in the incubator for 60 min. Placement of tube at 45° angle creates a larger surface area for sperms to swim-up.
5. aspirate the medium of the upper part of the test-tubes into new prewarmed round-bottom test-tubes without disturbing the lower layer
6. add 5 ml of the medium to the sample and centrifuge 5 min/2000 rpm
7. aspirate the supernatant but save 0,5 ml of the medium to resuspend the pellet
8. evaluate the precentage of motile and total number of sperm on a Makler Chamber

Slika1. Swim up (adopted from http://www.clevelandclinic.org/ReproductiveResearchCenter/info/2010/Beydola-T_Sharma-RK-2013.pdf)

3. **Density-gradient systems**

Density gradient centrifugation separates cells based on their density.

Morphologically normal and abnormal spermatozoa have different densities and at the end of centrifugation they are located accordingly.

The solutions like colloidal silica, poly-sucrose and others have higher density than semen. Because of that this system can separate the debris, cells, micro-organisms and non-motile sperms from the motile ones. Centrifugal force enables the motile sperms to swim from a less dense seminal fluid into a denser solution. Cellular debris and non-motile microorganisms are trapped at the interphase between the two solutions.

The common steps of this method are:

1. evaluate the precentage of motile and total number of sperm on a Makler Chamber
2. layer 2 ml of the lower density medium (55% SupraSperm) into conical test-tube
3. put the layer of the high density medium (80% Suprasperm) underneath the upper layer (lower layer will raise and a sharp interface is formed)
4. layer 2 ml of the liquefied semen on the top of the gradient
5. centrifuge the sample 20 min/1200 rpm
6. aspirate the semen sample, upper density layer and the half of the high density layer
7. add 3-5 ml of the sperm preparation medium, centrifuge the sample 5 min/1200 rpm
8. aspirate but save 0,5 ml of the medium to resuspend the pellet
9. evaluate the precentage of motile and total number of sperm on a Makler Chamber

Slika 2. Density gradient centrifugation (adopted from http://www.clevelandclinic.org/ReproductiveResearchCenter/info/2010/Beydola-T_Sharma-RK-2013.pdf)

4. Retrograde ejaculation

Retrograde ejaculation occurs when semen passes into the bladder at ejaculation.
This causes aspermia.
The acidity of the urine kill sperms very quickly. An alkalination of the urine by intake of sodium bicarbonate dissolved in a glass of water is very important in order to recover live motile sperms.
In these cases it is necessary to examine post-ejaculatory urine for the presence of spermatozoa.

Methods of surgical sperm retrieval

In cases of azoospermia sperm can be retrieved from testicular tissue or the epididymis by methods of surgical sperm retrieval.

The main methods of surgical sperm retrieval available include:

- PESA: percutaneous epididymal sperm aspiration
- MESA: microsurgical epididymal sperm aspiration
- TESA: testicular sperm aspiration. This includes testicular fine needle aspiration (TFNA)
- TESE: testicular sperm extraction (spermatozoa retrieved by open biopsy)
- Microdissection TESE
- Perc biopsy: percutaneous biopsy of the testis.

Epididymal aspirates are often minimaly contaminated from red blood cells and non-germ cells which makes isolation and selection of epididymal spermatozoa relatively streightforeward.
Testicular spermatozoa specimens retrieved by open biopsy are contaminated with non-germ cells and large number of red blood cells so additional steps are needed to isolate a clean preparation. In these cases enzymatic or mechanical method is used to free spermatozoa from testicular tissue after which the sample is further procesed for ICSI.
If epididymal and testicular spermatozoa are immotile pentoxifylline can be used to increase their motility.

Which method is to be used depends on the nature of the problem in the male partner, which needs to be explored carefully first.

SPERM SELECTION TECHNIQUES

After the semen sample is processed for IVF further sperm selection techniques can be used to improve results.

Some of the most commonly used are:

Polyvinylpyrrolidone medium (PVP) - ICSI involves the injection of a single sperm directly into a mature egg and is used to treat severe cases of male factor infertility. PVP is used for slowing down the movement and easy immobilization of the sperm for ICSI. It reduces the motility of the sperm and facilitate the capture and loading of a single sperm in the injection pipette without impairing the acrosome reaction.

Physiological Intracytoplasmic Sperm (PICSI) – PICSI is a scientific technique used in a specialised form of IVF to assist the embryologist in the selection of sperm. The PICSI dish provides a functional test based on the ability of sperm to bind to Hyaluronan (HA). The presence of HA binding sites on sperm outer membrane is regarded as a sign of sperm maturity. By selecting the sperm that are bound to HA and using them for ICSI, the embryologists are using the better quality, more mature sperm.

Intracytoplasmic morphologically-selected sperm injection (IMSI) – IMSI is a real-time laboratory technique that involves injection of morphologically selected sperm into the oocite. Using an inverted microscope with much greater magnifying power the embiologists can see the internal morphology of sperm and discard those with abnormalities.

Magnetic-activated cell sorting (MACS) – MACS is magnetic based selection system for sperm cells that can separate early apoptotic from non apoptotic spermatozoa. Apoptotic cells have externalized phospolipid phosphatidylserine (PS) on the sperm plasma membrane. Since PS has high affinity to Annexin V, apoptotic sperm cells bind to Annexin V conjugated paramagnetic microbeads.The magnetically labeled sample is passed through a magnetic column where magnetically labeled apoptotic or dead spermatozoa are retained in the column.
This technique has to be combined with density gradient centrifugation because it is not able to eliminate leukocytes, immature germ cells, seminal plasma and other contaminants from the semen sample.

Slika 3. MACS (adopted from http://www.clevelandclinic.org/ReproductiveResearchCenter/info/2010/Beydola-T_Sharma-RK-2013.pdf

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