Chapter

Methods of Sperm Selection for In-Vitro Fertilization

Abimibola Nanna

Abstract

50–60% of infertility cases are as a result of male infertility and infertile men semen sample is characterize with poor motility, abnormal morphology, low sperm concentration, azoospermic and increased levels of sperm DNA damage. As a result of this heterogeneity of the ejaculate, sperm selection has become a necessary step to carry out prior to in vitro fertilization. Furthermore, the choice of sperm cell selection techniques depend on sperm concentration and sperm biology and the recovery of highly functional sperm cell population depend on the combination of more than one technique in some cases. The regular sperm cell selection methods in ART laboratory are swim up, density gradient, simple wash and other advanced and emerging sperm selection techniques which include hyaluronic acid mediated sperm binding, Zeta potential, hypoosmotic swelling test, magnetic activated cell sorting and microfluidic separation of sperm cells. The various methods have its own advantages and disadvantages which may be applicable to the individual need of infertile men and its effect on ART outcome.

Keywords: male infertility, sperm selection techniques, semen

1. Introduction

Male infertility accounts for 50–60% of infertility cases and abnormal semen qualities like low motility, low sperm concentration, abnormal morphology and increased levels of sperm DNA damage are characteristic of infertile men sample [1]. Furthermore high level of reactive oxygen species (ROS) are found in 40–88% of sperm samples of infertile men [2] and physiological sperm functions such as capacitation, acrosome reaction and hyperactivation requires low ROS concentration while ROS overproduction is usually due to the inability of antioxidant to neutralize ROS [3]. Also, decrease sperm motility, DNA integrity and viability, increase midpiece defects are cause by oxidative stress from high level of ROS and decreased levels of antioxidant [3]. In addition, lower in vitro fertilization pregnancy rate, irregular preimplantation development, early loss of pregnancy and decreased rate in ART conceived offsprings are correlated with poor DNA integrity [4].

As a result of the above heterogeneity of ejaculate (understanding of sperm physiology) and male gamete integrity rule in both fertilization and embryogenesis, has led to an increased demand on sperm selection techniques. Sperm biology, sperm concentration, volume and life time invitro are the fundamental challenge of sperm selection and sperm selection process ideal time is about 10 minutes for 1 ml of sperm sample containing 100 million/ml. This shows a very high

biological sorting rate of −100 KHZ and the current cell sorting technologies has lower value of this [5].

There are various sperm selection techniques in Assisted Reproductive Technology (ART) and these techniques try to replicate vivo, the natural process in which quality sperms are selected from other constituents of the ejaculate as they actively move through the cervical mucus [6]. The simple wash, swim up and density gradient are the three most common sperm selective methods for sperm preparation in ART and other additional methods that will be discussed in this chapter.

2. Sperm production

At ejaculation, semen consists of a suspension of spermatozoa which is stored in the epididymis and is mixed with the secretion of the accessory glands. The prostate and the seminal vesicles are the main glands while the bulbourethral glands and the epididymis are responsible for the minor contribution of the ejaculate. The seminal fluid consists of prostatic fraction which is rich in sperm cells and the vesicular fraction which is less in spermatozoa. Furthermore, it is essential to have a complete sample volume collection and not to lose the first rich sperm fraction during masturbation which might make semen analysis difficult. Thus complete semen sample production is the first step throughout sperm preparation [7].

In additional, sample production is carry out through masturbation with sterile specimen container. This is done after abstinences of 2–3 days which maximize conception [8]. Furthermore, patient should be encourage onsite production of semen sample which avoids extreme temperature exposure while offsite semen production should avoid spermicidal effect from lubricant and samples should get to the andrology/in vitro fertilization (IVF) laboratory within 30–40 minutes without extreme environmental temperature exposure [9].

3. Choice of sperm selection techniques

The selection or preparation techniques use in sperm separation in IVF laboratory depends on the characteristic of the semen sample when subjected to semen analysis [10]. Furthermore, sperm cells should be separated from seminal plasma as early as possible and in vitro fertilization capacity diminishes permanently when sperm cells are not separated from seminal plasma within 30 minutes of ejaculation [11]. Furthermore, World Health Organization recommend sperm cell separation from seminal plasma within one hour of ejaculation and this will limit damage from leucocytes and other cells present in the semen [12]. Semen samples characterize with severe oligo and athenozoospermia are separated using simple wash and normazoospermia sample are separated with swim up or density gradient. Also, suboptimal quality semen sample are separated by density gradient [13].

4. Steps to maximize quality of sperm during sperm selection techniques

A. Bring gradient and sperm wash media to room temperature before use and this will protect sperm cells from cold shock. Also visual detection of contamination on the media will be seen when condensation on the media bottle disappear.

B. Media content should not be use when it is cloudy or hazy.

C. Use individual pipette for each bottle of media.

D. The PH of media is alter with prolong exposure to a 5% co2 environment which will affect their nature and performance.

E. For highly viscous semen, dissolve 5 mg of trypsin in 1 ml of sperm washing media and add it to the highly viscous semen for about 5 minutes before loading the upper gradient. This will allow increase yield of motile sperm without causing any great damage to the motile sperm.

F. Avoid excessive loading of semen on the gradient as this will cause a rafting phenomena. Rafting is define as the present of aggregate of desirable as well as undesirable components of the semen in post centrifuged pellet. For example, a gradient of 1-2 ml of upper and lower gradient, semen volume of 1 ml should be added accordingly.

G. Gradient should be use within one hour of creation. Delay usage of the gradient could lead to the two phase mixing with each other [14].

5. Simple wash method

It involved one or two centrifugation of semen sample in order to separate the sperm pellet from the seminal plasma. This process does not significantly decrease sperm count, normal morphology, and motility remain unchanged but there is increase in rapid forward progression and hypermotility of sperm cells in the post washed sample. Furthermore, this process is use for cases of severe oligospermia, asthenozoospermic semen sample and it is recommended method for insemination-ICSI and not for standard insemination procedure in IVF. Also, centrifugation process in this method causes additional harm to sperm cells by the production of reactive oxygen species (ROS) by leucocytes and abnormal sperm cells. ROS production causes DNA damage in spermatozoa, decreased sperm motility, increase number of apoptotic sperm cells and decrease sperm plasma membrane integrity (**Figure 1**).

5.1 Method

1. Semen sample is mix well.

2. Add supplement media to semen sample in a ratio of 1:1 in 15 ml conical tube.

3. Centrifugation is done at 1800 g for 5–10 minutes.

4. Aspirate the supernatant.

5. Add 0.1–0.5 ml of the culture media to the pellet for swim up.

6. Insemination can be done and sperm concentration and motility can be determined using WHO 2010 protocol [15, 16].

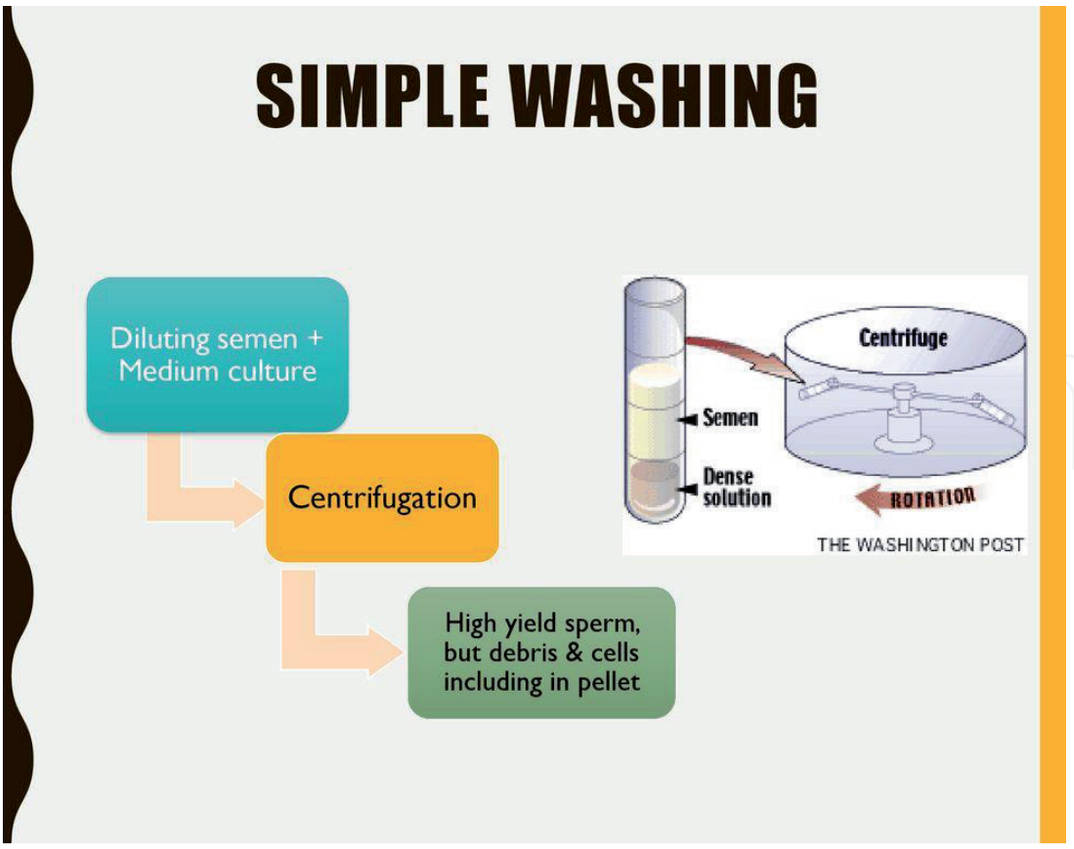

Figure 1.
Above indicate a typical simple wash procedure.

6. Swim up from semen

It is recommended for normazoospermic semen quality with high sperm count and good motility. Furthermore, it is carry out in round bottom tube place at an angle of 45 degree and this method favors the selection of motile sperm with intact membrane which result in higher clinical pregnancy rate in the IVF laboratory (**Figure 2**).

6.1 Method

1. Incubate 4–5 falcon sterile round bottom tube at 37 degree Celsius for few hours and add 1–2 ml of sperm wash medium.

2. Allow semen to liquefy and carry out semen analysis using WHO protocol.

3. Gently underlay liquefied semen (1–2 ml) in the bottom of the sterile tube.

4. Place the round bottom tube at 45 degree position in a humidified incubator at 37 degree or at room temperature for 30–60 minutes with tubes tightly capped. The motile sperm cells migrate upward into the sperm wash medium.

5. Aspirate the upper and middle section of the medium in the tubes and combined it in a conical centrifuge tube.

6. Centrifuge the pooled swim up fractions at 300 g for 10 minutes and remove supernatant and repeat centrifugation with additional 2 ml sperm wash.

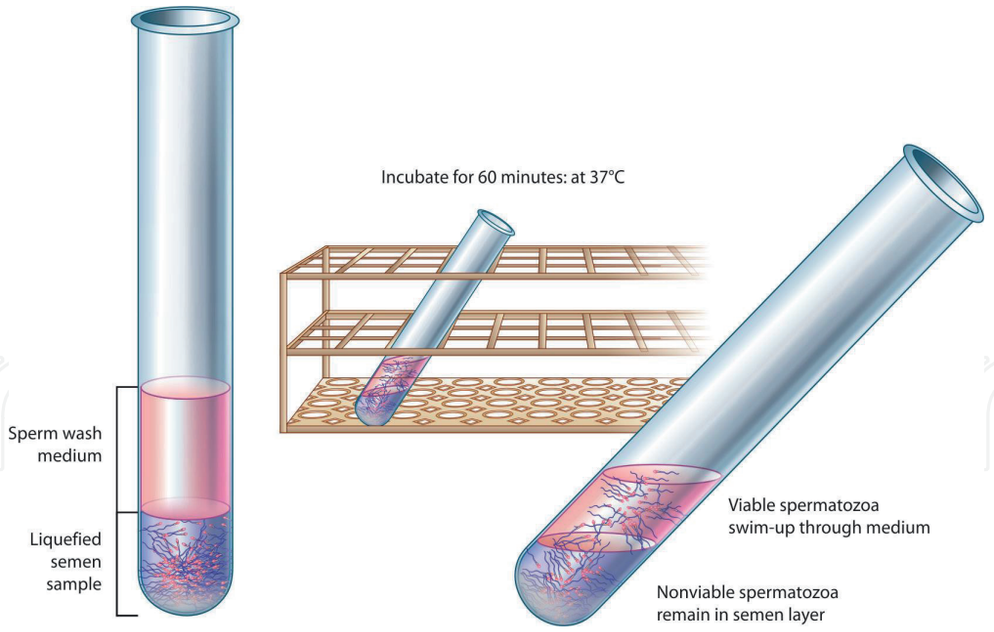

Figure 2.
Swim up from semen where the round bottom tube is place at an angle of 45 degree for 30–60 minutes. Active sperm cells swim to the top and are aspirated.

7. Remove the supernatant.

8. Suspend the pellet in equilibrated bicarbonate based IVF medium (0.1–0.5 ml) and incubate at 37 degree in 5–6% co2 environment for 30 minutes.

9. Post wash analysis is carry out for sperm concentration and motility.

10. Use for IVF or ICSI insemination [15, 17].

7. Density gradient centrifugation

It separates spermatozoa based on their density and at the end of each centrifugation, each spermatozoon is found at the gradient level that corresponds to its density. Furthermore, normal morphological spermatozoon has at least 1.10 g/ml density while abnormal one has density between 1.06–1.09 g/ml [18].

In addition, following centrifugation, the leucocytes, cell debris and sperm cell with abnormal morphology with poor motility are found at the interphase between seminal plasma and 45%, 45% and 90%. Also, the pellet at the bottom of the tube is characterized with highly motile, morphologically normal, viable spermatozoa [19]. Furthermore, if the volume of each gradient is lower than 1 ml, greater number of motile spermatozoa can be recovered, as a result of the spermatozoa have to migrate for a less distance between layers [20]. This method is used for normazoospermic semen and suboptimal semen qualities and it is recommended method for insemination IVF or ICSI [15].

7.1 Gradient preparation

1. Add 1-2 ml of lower phase (90%) at the bottom of the 15 ml conical tube.

2. Add 1–2 ml of upper phase (45%) on top without mixing the two gradients.

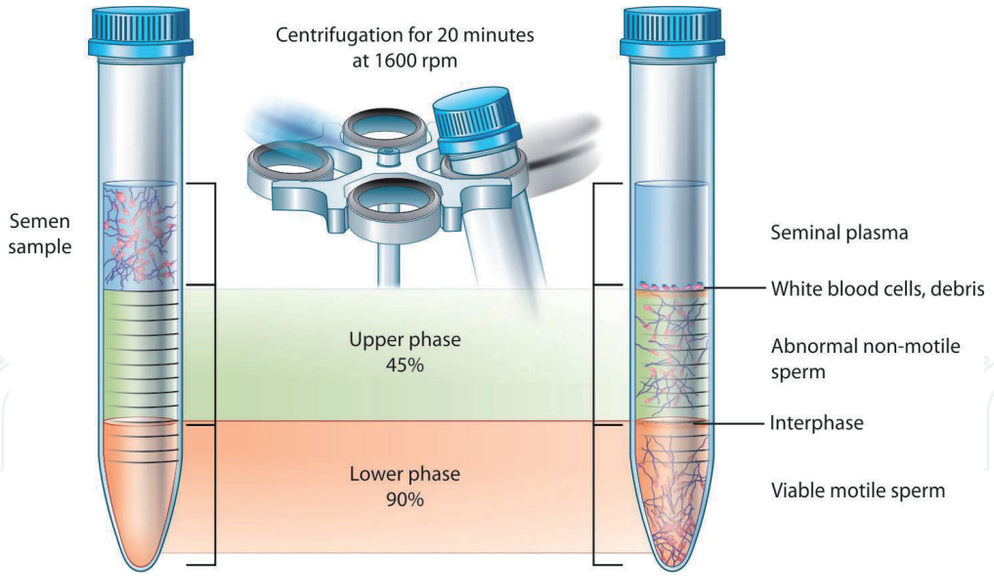

Figure 3.
Indicate sperm cell separation of density gradient.

3. Add 1-2 ml of liquefied semen on top and centrifuged for 15–20 minutes at 1600–3000 rpm.

4. Remove supernatant without disturbing the pellet at the bottom of the tube.

5. Aspirate the pellet by adding 0.1mlof sperm wash and transfer it to fresh conical tube containing 2–5 ml of sperm wash media.

6. Centrifuge at 1600–3000 rpm for 10 minutes, discard the supernatant and repeat this step.

7. Re-suspend the pellet in 0.1–0.5 ml of culture media (Fertilization media) and to allow swim up for 15–20 minutes in the incubator.

8. Carry out post wash analysis for sperm concentration and motility and inseminate for IVF (100,000 progressive motile sperm/ml) and ICSI [15].

One of the advantage of this method is that, it eliminates the majority of leucocytes in the ejaculate, easy to carry under sterile condition, it consume less time and major disadvantage is that spermatozoa recovered from this method has lower DNA integrity as compare to spermatozoa from swim up method (**Figure 3**) [21].

8. Sperm preparation for testicular sperm, epididymal sperm and retrograde ejaculation

Sperm Cells can be gotten from cases of epididymal obstruction or complete azoospermia through their epididymis or the testicular tissue. These sperm cells gotten from the process can be separated through Simple wash technique if the number of sperm cells isolated is low while density gradient is used when the number of sperm cells collected is enough.

8.1 Epididymal sperm preparation

1. Examine the epididymal aspirate when pour into the culture dish through the dissected microscope.

2. From your view, determine if simple wash method or density gradient will be use.

3. Pour the aspirate into the conical tube and add 1–2 ml of sperm wash media.

4. Centrifuge the conical tube at 400 g for 10 minutes and discard the supernatant or use density gradient base on the number of sperm cells.

5. Wash with RBC lysis buffer if the pellet is mix with blood cells.

6. Add 2 ml of RBC lysis buffer and gently mix, centrifuge at 400 g for 5 minutes.

7. Aspirate the supernatant without affecting the pellet.

8. Add 1–2 ml of sperm wash to the pellet and centrifuge at 400 g for 10 minutes.

9. Pellet is suspended in 0.5 ml of sperm wash, for ICSI purpose.

8.2 Testicular sperm preparation

1. Add 1-2 ml of culture medium to the testicular tissue to wash away the red blood cells.

2. Transfer the tissue to Petri dish and add another few drops of culture medium and this is to keep it moist.

3. Add additional 1 ml of culture medium and minced with sterile scissors.

4. Aspirate the suspension and pass it through 21 gauze needle which is attached to a 3 ml syringe. The passage is done 2–3 times and the process, sperm cells are released from the seminiferous tubules in the small pieces and also dislodges the sperm from the lumen.

5. Transfer the suspension into a conical tube and allow standing at room temperature for 5 minutes. Tissue clumps and seminiferous tubules will settle at the bottom of the tube.

6. Supernatant is transfer to another conical tube and centrifuged at 300 g for 10 minutes.

7. Supernatant is discarded.

8. If there are red blood cells with the pellet, wash with 2 ml of RBC lysis buffer by centrifuging at 300 g for 5 minutes.

9. Remove supernatant and add 0.5-1 ml of culture media to the pellet and it ready for ICSI [22, 23].

8.3 Retrograde ejaculation sample preparation

1. Patients abstain for 3 days.

2. The evening before the day of sample production, patient drink 250 ml of alkaline drink (Bicarbonate of soda).

3. The morning of production, patient empty his bladder.

4. Patient takes another 250 ml of alkaline drink.

5. One hour later, when patient has the urge to pass urine, 3–4 sterile container containing 20 ml of sperm medium each is given to the patient to pass urine and bring to the IVF laboratory.

6. All tubes are centrifuge together at 400 g for 15 minutes.

7. Supernatant is discarded and pellet is re-washing again with 5 ml of sperm medium at 400 g for 10 minutes.

8. Supernatant is discarded and 0.5–1 ml of sperm medium is added to the pellet for swim up.

9. Wash sperm ready for post wash sperm concentration and motility assessment, ICSI procedure [15, 23].

9. Emerging sperm selection techniques before ICSI procedure

There are various advanced techniques of sperm preparation which is based on spermatozoa surface charge and morphology and this has overcome the limitation of classical sperm selection procedures. This techniques include Zeta Potential, Hypoosmotic Swelling Test (HOST), Hyaluronic acid-mediated sperm selection, magnetic-activated cell sorting (MACS) and the microfluidics which is the latest.

10. Zeta potential

It is the electrical charge potentials that exist between the sperm surrounding and negatively charge sperm membrane. The epididymal protein present in the surface of sperm membrane is responsible for the negatively charge on this sperm membrane [24] and this is lower in sperm cells with damage DNA [25]. Furthermore, the mature sperm has zeta potentials between −16 to −120 mV [26].

10.1 Method

1. Pipette 100 ul of wash sperm into 15 ml conical tube which is suspended in serum free HEFES-HIF medium.

2. Rotate or rub the tube on a latex glove a couple of times. This will make a negative charge sperm to stick to the wall of a positively charge plastic tube.

3. Invert the tube and immature abnormal sperm cells are discarded.

4. Tube is place on the rack for 1 minute at room temperature without agitation, then centrifuge at 300 g for 5 minutes.

5. Suspend the pellet in 0.2 ml of serum supplement medium, examine under the microscope after swim up and use for ICSI [25].

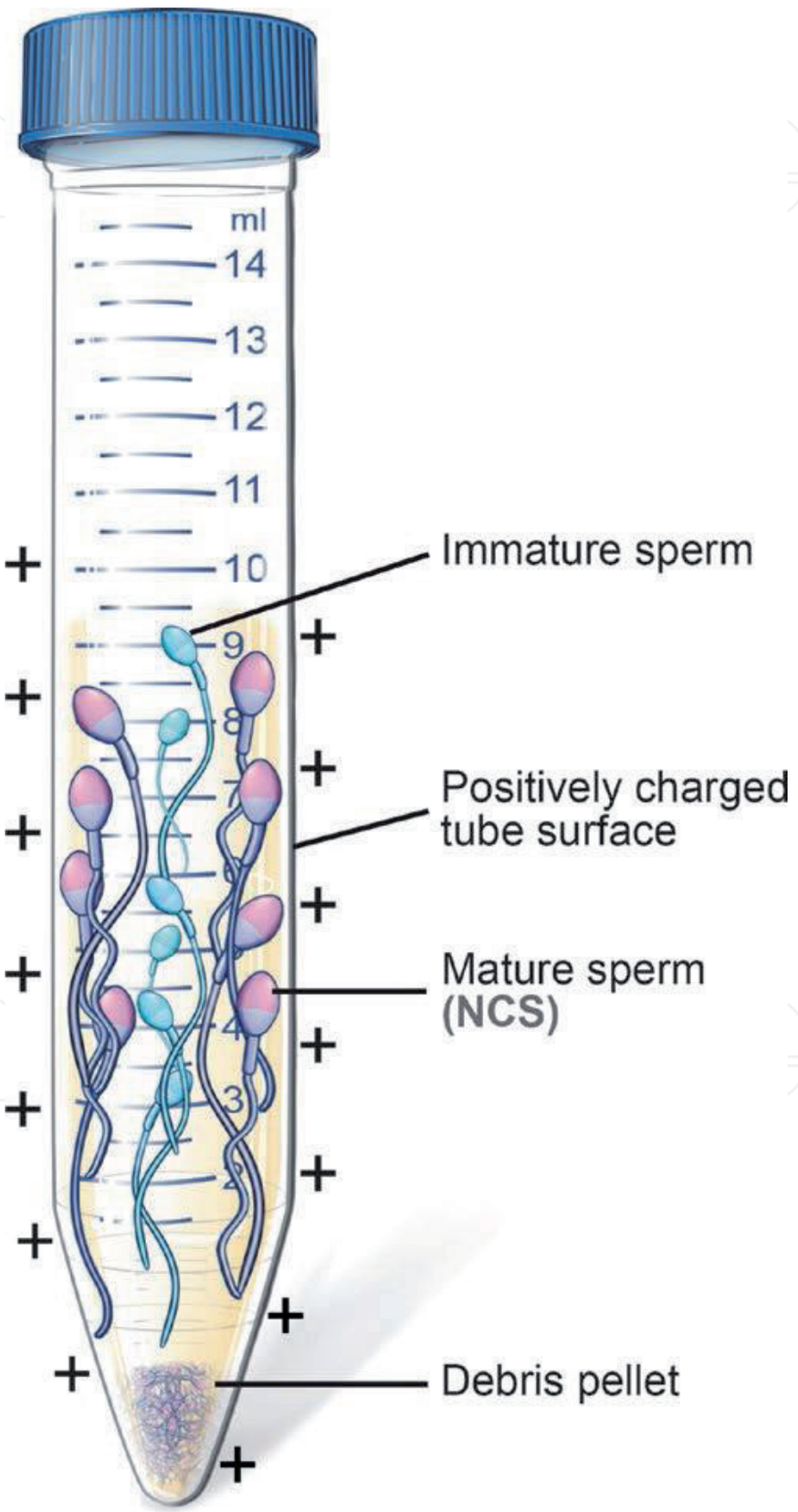

Figure 4.
Above show zeta potential process during the separation of mature and immature spermatozoa.

Also, the method is inexpensive, easy to carry out. Sperm with intact DNA, superior motility and normal morphology can be separated with this method and negative zeta potentials sperm in IVF had a higher fertilization (65.79%) compared with sperm selected with double density gradient centrifugation. Furthermore, there is increase fertilization rate and possible pregnancy rate in infertile couples with male factor infertility whose semen sample was selected with double density gradient/zeta potential as compared to double density gradient alone during an ICSI procedure in a randomized prospective study (**Figure 4**) [27].

11. Hyaluronic acid-mediated sperm binding (HA)

The plasma membrane of acrosome-intact sperm cell has hyaluronic acid receptors (HA) which are indicative of sperm maturity. Also, the extracellular matrix of the cumulus oophorus contains hyaluronic receptors as its main component. Mature sperm cell bind with hyaluronic acid in the oocytes, therefore having a better chance of oocyte fertilization.

Physiological intracytoplasmic sperm injection (PICSI) plastic dish has spots containing HA which is attached to its base or a viscous medium containing HA (Sperm slow). 2ul of sperm suspension is added to the spots and incubated under oil for 5 minutes. Mature sperm will attached its head to HA and the sperm can easily be selected by ICSI injecting pipette for ICSI procedure [28].

Furthermore, the selection of sperm with reduced oxidation stress gotten with the use of HA binding which excludes immature sperm with cytoplasmic extrusion, presence of sperms with histones and DNA fragmented sperm cells. Also, significant increased fertilization rate of oocyte injected with HA-selected sperm has been reported in one study [29] while better embryos quality has also been reported in another study [30].

12. Magnetic activated cell sorting (MACS)

It uses a strong magnetic field principle where non-apoptotic and apoptotic sperm cells are pass through this field. It is only the non-apoptotic sperm cell that is retain in the field and separated from the apoptotic ones. Furthermore, phosphatidylserine is a phospholipid found on the plasma membrane of spermatozoa and it moves to the outer surface when the plasma membrane is damaged (apoptotic sperm cell) and this aid selection of non-apoptotic spermatozoa in ART [31].

The combination of density gradient and MACS has given higher quality sperm than density gradient alone. MACS removes already damaged spermatozoa with altered membrane, activated apoptosis signaling and DNA fragmentation while density gradient removes immature sperm cells, debris and leucocytes. Furthermore, the use of MACS techniques allows the selection of sperm cells with improved morphology, motility, viability and greatly improved fertilization rates [32]. Thus, increase cleavage rate and clinical pregnancy rate has been reported for sperm cell used in ICSI selected by MACS compared with density gradient in oligo-astheno- and teratozoospermic men [33].

13. Microfluidic separation of sperm cells

This is the latest sperm selection techniques and the device is made up of microchannels made from polydimethylgiloxane (PDMS) silicon polymers which are nontoxic and transparent [34].

The boundary following behavior is the principle use in selection of spermatozoa in the popular passive microfluidic device. It consists of a radial network of channels (52u width) which is involved in the separation of spermatozoa into left, right and straight swimmers. 200 ul of raw semen is loaded into the inner ring with plastic syringe and kept undisturbed for 15 minutes at 37 degree Celsius. Motile sperm cells are collected at the outlet microchannels while dead or immotile sperm cells are retained in the inlet.

Furthermore, small volume of sperm is required for this process and DNA fragmentation is significantly decreased in sperm separated with this method (Sperm damage in swim up is 16.4 and and microfluidics is 8.4%). Furthermore, the use of microfluidics device in sperm selected for ICSI treatment in porcine, yielded short time for ICSI treatment and increased the number of viable embryos without the reduction of the invitro production efficiency [35].

14. Hypoosmotic swelling test (HOS test)

Jeyendran et al. developed hypoosmotic swelling test to evaluate the functional integrity of the sperm membrane [36]. Tail swelling and curving is exhibited by viable sperm with normal membrane function when exposed to hypoosmotic condition [37]. Furthermore, its application seems to be a promising method to identify live spermatozoa for ICSI and study conducted showed that injected spermatozoa selected by HOS test gave higher fertilization rate than randomly selected spermatozoa [38].

In another study, 50% sperm media and 50% of Millipore-grade water was used to prepare HOS test microdroplet. Immotile spermatozoa was introduced into the microdroplet and after 10 seconds, the viable spermatozoa whose tails were curved and swollen, were further selected, transferred to hepes-buffered microdroplet and wash three times is sperm medium for re-equilibration before been transferred to PVP for ICSI purpose. According to the group, improved fertilization and pregnancy rates were recorded as compared with non use of HOS test (**Figure 5**) [39].

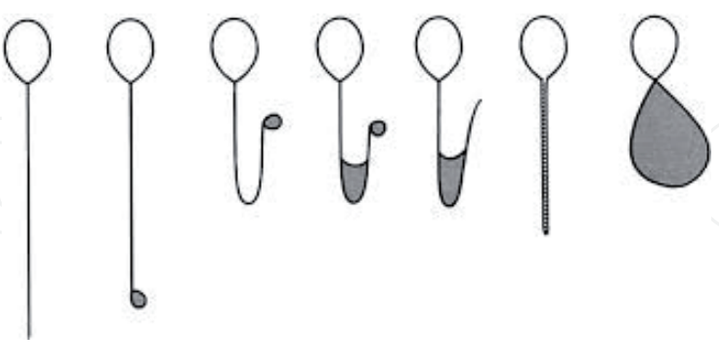

Figure 5.
Indicate viable spermatozoa with curved tails while non-viable spermatozoa is without curved tail when subjected to hypoosmotic condition and under microscopic observation.

15. Conclusion

Sperm selection techniques are very important and the choice of technique depends on semen quality of each male partner. The appropriate selection of sperm cells with lower DNA damage will improve the chance of fertilization rate, cleavage rate, Implantation and pregnancy rate in IVF laboratory.

Summary of the advantages and disadvantages of each method.

Methods	Advantage	Disadvantage
Simple wash	It is simple to perform and produce high yield of spermatozoa.	Increase DNA damage of spermatozoa.
Density gradient	It is easy to perform under sterile condition in a short time.	Contamination with endotoxins a risk factor.
	It eliminates majority of leucocytes in the ejaculate.	Sperm cells recovered with this method has lower DNA integrity.
Swim up	Spermatozoa with high DNA integrity are recovered.	It consumes more time of preparation.
	It is simple and not expensive.	
MACS	Molecular level is involved.	It is use in conjunction with density gradient to remove substance like leucocytes, seminal plasma and apoptotic spermatozoa.
	It is non-invasive, convenient and rapid.	
	It differentiate and separate non-apoptotic spermatozoa from apoptotic spermatozoa.	
HA	It supports sperm cells selection with good DNA integrity in severe oligozoospermic sample.	It is done in conjunction with intracytoplasmic sperm injection (ICSI) and simple wash method.
HOS test	Identification of live spermatozoa in severe asthenozoospermic sample.	Additional time needed to perform this method before ICSI.
	It is use for normal semen sample for identification of intact membrane sperm cell before ICSI.	
	It simple to perform.	
Zeta potential	It is not costly and easy to carry out.	It operates in conjunction with density gradient procedure.
	Recovery of spermatozoa with normal morphology, superior motility and intact DNA.	
Microfluidic device	It uses small volume of semen volume.	Not yet available done in human IVF treatment but available in porcine spermatozoa.
	Spermatozoa with significant decrease of DNA fragmentation are recovered.	

DOI: http://dx.doi.org/10.5772/intechopen.99874

Author details

Abimibola Nanna[1,2,3,4,5]

1 University of Benin, Benin City, Nigeria

2 University of Leeds, Leeds, United Kingdom

3 Delta State University Teaching Hospital, Oghara, Nigeria

4 Shepherd Hospital and Fertility Centre, Warri, Nigeria

5 St. Jude's Fertility Institute, Abuja, Nigeria

*Address all correspondence to: bolanannal@gmail.com

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