1

Sperm Selection Techniques and their Relevance to ART

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1.1 Introduction

Fertilization is now possible using any available sperm through intra-cytoplasmic sperm injection (ICSI) treatment (Palermo *et al*., 1992). As a result, andrological research has raised questions regarding the selection of suboptimal sperm used for assisted reproductive technology (ART) (Avendano and Oehninger, 2011). In recent years, the role of sperm in ART has been highlighted as the sperm provides one half of the genome to the developing embryo. The use of healthier sperm has showed to improved ART outcomes and subsequently, sperm selection has become an integral part of ART procedure (Said and Land, 2011). Since the birth of first *in vitro* fertilization conceived baby in 1978, sperm selection for ART has been focused on selecting physiologically motile and morphologically normal sperm (Bartoov *et al*., 2002). Despite success, it has become evident that physical appearances of the sperm are inefficient to identify the most suitable sperm for ART success (Yetunde and Vasiliki, 2013). Hence, recent research is focused on developing novel sperm biomarkers to identify non-apoptotic sperm with high DNA integrity for successful use in ART.

Our understanding of sperm physiology, as well as the technology to select healthier sperm has progressively been improved. Initially, sperm selection was based on simple semen washing procedures and

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now more sophisticated sperm separation measures have evolved (Simon *et al*., 2015). The sperm is regarded unusable for the use in ART, after being analyzed for its molecular parameters such as DNA integrity, histone retention, protamine content, or ratio, and so on. Therefore, preserving the structural and functional integrity of the sperm is been the goal for recently introduced novel sperm selection approaches (Berkovitz *et al*., 2006a, 2006b). Some novel sperm selection approaches aim to mimic the natural selection process, where the female reproductive tract is known to eliminate poor quality sperm to enhance the chances of a successful fertilization (Holt and Fazeli, 2015). Other methods have focused on sperm physiological changes in the female reproductive tract, like capacitation, which are functionally important for acrosome reaction (Bedford, 1963). Inclusion of such novel biomarkers along with standard sperm preparation procedures has shown promises to enhanced fertilizing ability and improves ART success (Nasr-Esfahani *et al*., 2008a; Kheirollahi-Kouhestani *et al*., 2009; Polak de Fried and Denaday, 2010; Wilding *et al*., 2011).

1.2 Need of Sperm Selection in ART

Human semen is comprised of heterogeneous sperm population with varying degrees of structural differentiation, maturity, fertilizing ability, and functional quality (Huszar *et al*., 1993, 1998). During natural conception the sperm from these subpopulations compete to traverse through several anatomical and physiological barriers in the female tract. The most competent and reproductively efficient sperm are able to migrate through the cervical mucosa, uterus, uterine tube, cumulus cells, zona pellucida, and finally oolemma to participate in the fertilization (Suarez and Pacey, 2006). Further, selection takes place at the level of sperm-oocyte interaction and out of a population of millions, a single sperm is able to fertilize the oocyte and develop into an offspring. These barriers for natural selection exclude the sperm with structural abnormalities as acrosomal absence, flagellar deformity, immature sperm, and sperm with aneuploidy or other chromatin abnormalities from participating in a successful fertilization (Suarez and Pacey, 2006). On the contrary, during ART, sperm are brought in proximity to oocyte, outside the female body, where no such anatomical and physiological barriers exist. Depending upon the technique of ART, either the sperm fertilize the oocyte on their own as in IVF or

the sperm are injected into oocyte for fertilization as in ICSI. During ART, sperm does not have to overcome any anatomical and physiological barriers present in the female reproductive tract, natural sperm selection are bypassed. Therefore, it is imperative to have an efficient artificial selection process that maximizes the probability of successful pregnancy and birth of a healthy offspring. Further, the sperm selection procedures also help to enrich the concentration of good quality sperm that increases the chance of ART success.

Sperm contribute half of the genome to the offspring. Therefore, selection of sperm with intact chromatin and free of chromosomal abnormalities is important for ART success. Studies indicate that even if the best quality sperm are used for ICSI, approximately, 55% of the selected sperm have normal DNA (Ramos *et al*., 2004). The primary objective of sperm selection approaches is to select good quality or healthier sperm. In addition, sperm selection approaches are designed to reduce the physiological and oxidative damage induced to the sperm during the selection process. With these perspectives in sight, recent developments in sperm selection approaches are focused on physiological properties or morphological characteristics or behavior in the electric field or basis on their fluid kinetic properties. This chapter discusses some of the novel sperm selection techniques that have been the focus of recent research and may have the ability to revolutionize ART by improving the success rate, even in patients with severely compromised sperm parameters.

1.3 Methodology of Sperm Selection

1.3.1 Intracytoplasmic Sperm Injection

Intracytoplasmic sperm injection (ICSI) is a very useful gamete micromanipulation technique for treating couples with severely compromised sperm parameters. Since its introduction in 1992, ICSI has revolutionized ART by providing hope to couples to achieve a pregnancy, who had few chances of a natural conception or by *in vitro* fertilization (IVF). The basic principle of ICSI is to manually select the best sperm on the basis of motility and/or morphology and to inject it into an oocyte. The premise for such gamete micromanipulation is that it enables a successful fertilization, when a sperm is unable to fertilize on its own. During this procedure, initial events of fertilization like

acrosome reaction are bypassed and now fertilization is possible with any available sperm.

1.3.1.1 Methodology

The oocytes retrieved after ovarian hyper-stimulation is placed in a petri dish (specific for ART) in which they are fertilized with a sperm. The whole process is done with the help of a CCD attached microscope using a micromanipulator. The basic steps for ICSI manipulation are as follows: the oocytes retrieved after hyper-stimulation are held by a specialized holding pipette in a micromanipulator. The most visually normal sperm by virtue of its motility and morphology are picked by ICSI pipette. During this step, sperm are usually visualized at 400× magnification to increase the chances of detecting and eliminating any sperm with morphological abnormalities. The pipette containing sperm is then carefully inserted through the membrane of the oocyte, into the cytoplasm. A sperm is injected into the cytoplasm and the pipette is carefully removed. The oocytes are then incubated and checked for pronuclear appearance to confirm fertilization after 24 hours. After a successful fertilization, the embryos are cultured until cleavage stage (Day 3 embryo transfer) or until blastocyst stage (Day 5 embryo transfer) into the uterus.

1.3.1.2 Advantages and Limitations

ICSI is the most widely used ART, accounting to 70–80% of the cycles performed (Palermo *et al*., 2009). ICSI has assisted millions of infertile couples to conceive, even with severely compromised sperm parameters, as severe oligozoospermia, asthenozoospermia, or both in the male partner. This technique has dropped down the number of sperm required for fertilization from several thousand to a single viable sperm. In men with obstructive or non-obstructive azoospermia, where there are no sperm in an ejaculate, testicular-epididymal sperm extraction (TESE) combined with ICSI has made it possible to sire a child (Vloeberghs *et al*., 2015).

Although ICSI has fulfilled the dreams of parenthood for millions of infertile couples, but there are risks and concerns for the health of mother and the child associated with this technique. ICSI bypasses numerous physiological events of fertilization, which has always been a safety concern related to this technique. Many hazards are not specific to ICSI they are common to most of the ART. Two specific demerits of ICSI are: the injection of media into an oocyte along with the

sperm and the bypass of natural selection process (Sánchez-Calabuig *et al.*, 2014).

During natural conception, the sperm pass through different barriers with in the female reproductive tract, so that the most capable sperm, with normal morphology and vigorous motility can fertilize the oocyte (Barratt and Kirkman-Brown, 2006; Suarez and Pacey, 2006). Three levels of barriers effectively hinder the reach of an abnormal sperm to an oocyte; (1) the microenvironment of the female reproductive tract, (2) the sperm-oviduct interactions at the caudal isthmus, and (3) the sperm-zona pellucida interaction (Suarez and Pacey, 2006). ICSI bypasses these steps of natural selection to select the best sperm (observed by an embryologist), since it does not involve the sperm–oviduct interaction and other processes as zona pellucida binding-penetration. Further, the presence of acrosomal enzymes from the unreacted acrosome is introduced into the oocyte during ICSI, which may lead to an increased risk of vacuole formation (Morozumi and Yanagimachi, 2005; Morozumi *et al.*, 2006).

During ICSI, the selection of sperm is based on the embryologist experience, which usually rely on the motility and morphology of the sperm. Since, these sperm parameters are not always reflective of sperm DNA integrity, chances of selecting a poor DNA quality sperm for fertilization in ICSI is not ruled out (Celik-Ozenci *et al.*, 2004). Therefore, in ICSI there is a realistic possibility that a sperm with high DNA fragmentation or a aneuploid sperm can be selected for fertilization, which may ultimately lead to adverse consequences from failed fertilization and retarded embryo development to increased rates of miscarriage and diseases in the offspring.

In the post-natal life, children born after ART have been observed to have lower birth weights and higher peripheral fat, blood pressure, and fasting glucose concentrations than controls (Fauser *et al.*, 2014). A meta-analysis of 19 publications selected by a quality score based on sample size and appropriateness of control group observed that major malformation rates ranged from 0 to 9.5% in IVF, 1.1 to 9.7% in ICSI, while 0 to 6.9% after natural conception, leading to a statistically significant overall odd ratio of 1.29 (Rimm *et al.*, 2004). Further, it has been reported that 90–100% of the ART children with Beckwith–Wiedemann had imprinting defects, as compared to 40–50% of the spontaneously conceived children with Beckwith–Wiedemann (Manipalviratn *et al.*, 2009). Similarly, 71% of the Angelman Syndrome cases in ART children were attributed

to epigenetic defects as compared to 5% of the naturally conceived children with Angelman Syndrome (Manipalviratn *et al.*, 2009).

1.3.1.3 Conclusion

ICSI sperm selection is an option for couples who have failed the standard IVF treatment and benefits men with severe male infertility. ICSI selected sperm is directly injected into the oocyte, which provides the best chance of fertilization in couples with fewer available oocytes for treatment. Despite advantages, the absence of natural sperm selection process may lead to an increased risk of miscarriage due to injection of any available sub optimal sperm, which subsequently increases the risk of health issues in ICSI born children. Research into the effects of ICSI sperm selection method is still on going, as this technique is extensive in use for less than two decades. However, ICSI sperm selection method does improve the odds of treating an infertile man, but it does by remove the key elements that often lead to male infertility.

1.3.2 Intracytoplasmic Morphologically Selected Sperm Injection

The introduction of ICSI as a method of insemination revolutionized the treatment of male infertility. With the widespread use of ICSI, contradictory findings were reported in many studies with regard to sperm selection based on morphology. Some studies demonstrate that sperm morphology according to strict criteria (Kruger *et al.*, 1986, 1988) has controversial prognostic value in ICSI outcomes (Svalander *et al.*, 1996; De Vos *et al.*, 2003; French *et al.*, 2010) and does not seem to influence embryo quality or development (De Vos *et al.*, 2003; French *et al.*, 2010). Therefore, need for more stringent sperm selection procedures were recommended to effectively improve ART outcome. As a major development in this direction introduced by Bartoov *et al*. (1994, 2001, 2002), who devised a method of unstained, real-time, high magnification (6600×) examination of sperm called "motile sperm organelle morphology examination" (MSOME). The integration of MSOME with ICSI sperm selection was defined as intracytoplasmic morphologically selected sperm (IMSI) (Bartoov *et al.*, 2003).

During IMSI, the motile sperm morphology that includes normalcy of the sperm nucleus (shape and chromatin content), acrosome size, presence and absence of vacuoles, are observed at high (6600×) magnification instead of around 400× used during conventional ICSI. The

introduction of IMSI facilitated the observation of ultra-structural morphological details of live sperm, thereby assisting in selection of healthier sperm, to be used for ART.

1.3.2.1 Methodology

IMSI is a modification of ICSI, in which the sperm selection is done at magnification many fold higher than ICSI. Its introduction in the field of ART facilitated the observation of live human sperm, particularly by showing sperm vacuoles not necessarily seen at lower magnification. The sperm selection for IMSI relies on the evaluation criterion of MSOME, which evaluates the presence, size, number, and location of vacuoles. According to the MSOME criterion, if the sperm head contains one or more vacuoles (diameter of 0.78 ± 0.18 μm) occupying more than 4% of the normal nuclear area, it is considered abnormal for use in ART (Bartoov *et al*. 1994, 2001, 2002). The MSOME criterion has been modified to a scoring system, to simplify the sperm classification into different grades. Briefly, grade I sperm have normal sperm head and absence of vacuoles and they represent the optimal type. Grade II sperm are characterized by maximum two small vacuoles. Grade III sperm have either more than two small vacuoles or one large vacuole. The grade IV represents the poorest quality sperm, which show large vacuoles fully occupying the head, along with other morphological defects (Vanderzwalmen *et al*., 2008; Greco *et al*., 2013). Cassuto *et al*. (2009) introduced a similar protocol of sperm classification based on the detailed analysis of head, acrosome, vacuoles, base of sperm head, and the presence of cytoplasmic droplet.

1.3.2.2 Advantages and Limitations

The use of IMSI over ICSI or other sperm selection techniques has significantly improved ART success rate, since it involves the selection of sperm with a strictly defined, morphologically normal nucleus. It has been particularly useful for couples with repeated ICSI failure (Bartoov *et al*., 2003; Berkovitz *et al*., 2005; Hazout *et al*., 2006; Antinori *et al*., 2008; Franco *et al*., 2008; Setti *et al*., 2010). It has been reported that IMSI is associated with significantly higher implantation and clinical pregnancy rates and a reduction in the abortion rates (Setti *et al*. 2010, 2011), where the pregnancy rate in IMSI has been observed to be 66% as compared to 30% in ICSI. The reported implantation rate in IMSI is 27.9% while it is 9.5% in ICSI (Bartoov *et al*., 2003; Berkovitz *et al*., 2005). In cases, where no sperm could qualify

for selection in the IMSI procedure, an increase in abortion rate from 10 to 57% has been reported (Berkovitz *et al*., 2005). Further, Cassuto *et al*. (2014) reported a lowering of congenital malformation in IMSI born children to 1.3% as compared to 3.8% born after ICSI. In addition, IMSI improved ART outcome in patients with severe degrees of sperm DNA damage. It has also provided evidence for the association of presence, size, and number of sperm nuclear vacuoles with embryo quality and development, and suggested that high number of vacuoles may account for increased abortions (Figueira *et al*., 2011).

Though, IMSI has been documented to significantly improve the ART outcomes, but the technique has its drawbacks. Undoubtedly, it is a time-consuming technique and selecting a normal sperm in accordance with MSOME criterion may take 60 – 120 min (Antinori *et al*., 2008). Further, the prolonged exposure to the microscope's heated stage may itself cause damage to the sperm, as demonstrated by Peer *et al*. (2007) after 2 h on the microscope's heated stage, sperm nucleus vacuolization significantly increases. Despite these observations, IMSI has proved itself as a valid tool for safe and a non-invasive method of sperm selection.

1.3.2.3 Conclusion

The IMSI sperm selection approach changed the perception of how a sperm suitable for insemination should appear. Sperm, which was considered as normal when observed under a low magnification microscope, is showed to contain ultra-structural defects that may impair ART outcomes. Recent studies have reported that IMSI is associated with improved ART outcomes; specifically, implantation and pregnancy rates, while a reduction in miscarriage rates was observed when compared to conventional ICSI insemination. Despite its advantages, clinical indications for IMSI procedure are still lacking and further prospective randomized clinical trials are required to identify patient groups that are benefited by IMSI sperm selection approach.

1.3.3 Annexin V Labeling

Annexin V labeling is a well-recognized method to detect bio-molecules at the sperm membrane to identify apoptotic sperm. This method has been widely used to separate the apoptotic sperm from non-apoptotic (healthier) sperm population. This method is based on the affinity of protein coagulant, Annexin V, with a

phospholipid, phosphatidylserine of sperm plasma membrane. In a normal sperm, phosphatidylcholine and phosphatidylserine are asymmetrically distributed, with the former exposed to external leaflet of membrane while the later located at the inner surface of lipid bilayer. However, this asymmetry is disrupted during apoptosis, when the phosphatidylserine is externalized to the outer side of membrane, which facilitates an apoptotic sperm to be recognized by the macrophages and eliminated. A magnetic bead-conjugated annexin V helps in the identification of an apoptotic sperm, in an external magnetic field, annexin-V conjugated to dead and apoptotic sperm by magnetic activated cell sorting (MACS; Figure 1.1).

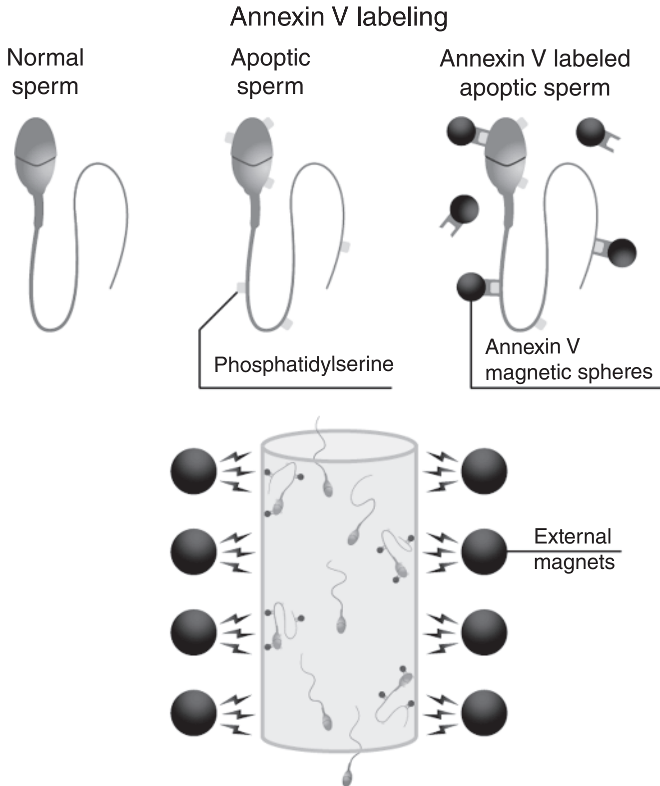

Figure 1.1 Apoptotic sperm are labeled by annexin V magnetic beads. A magnetic field separates the apoptotic sperm. (*See color plate section for the color representation of this figure.*)

1.3.3.1 Methodology

Annexin V is a phospholipid binding protein that has high affinity for phosphatidylserine but lacks the ability to pass through an intact sperm membrane (van Heerde *et al*, 1995). Therefore, in sperm with compromised membrane integrity, the annexin V binding takes place at the phosphatidylserine exposed on the outer layer of membrane (Glander and Schaller, 1999). To separate the apoptotic sperm from non-apoptotic sperm, super-paramagnetic microbeads conjugated with annexin V are used to label sperm with externalized phosphatidylserine. During this procedure of MACS, a mixture of sample and conjugated annexin V is incubated, and loaded on a separation column placed in the magnetic field. The attractive force between the magnetic field around the column attracts the magnetic beads conjugated to annexin V-sperm complex, and hence the annexin V-positive fraction comprising of apoptotic sperm binds to the column, while the annexin V-negative fraction of non-apoptotic sperm elutes through the column. The column is removed from the magnetic field, and annexin V-positive fraction is eluted using the annexin V-binding buffer (Chan *et al*., 2006). Thus, this method yields two fractions: annexin-negative (intact membranes, non-apoptotic sperm) and annexin-positive (externalized phosphatidylserine, apoptotic sperm) (Grunewald *et al*., 2001; Glander *et al*, 2002).

1.3.3.2 Advantages and Limitations

Annexin V labeling is a simple, convenient method for detection and separation of apoptotic sperm. It provides optimal purity with reliable and consistent results. As opposed to other routine methods of sperm separation, which rely on motility and sperm density, this technique acts at the molecular level. Combining this method, with other techniques such as density gradient centrifugation may yield a sperm population with higher motility, viability, and lower number of apoptotic sperm, though it makes the procedure for sperm isolation more time and energy consuming (Said *et al*., 2006a). This technique has been reported to improve acrosome reaction and is associated with higher cleavage and pregnancy rates than spermatozoa selected by density gradient centrifugation in oligoasthenozoospermic men (Dirician *et al*., 2008; Lee *et al*., 2010). Annexin V negative fraction has low amounts of DNA damage, and higher oocyte penetration capacity than annexin V-positive sperm (Said *et al*., 2006). Although, sperm sorting with annexin V method was effective in the treatment

couples with previously failed ICSI outcome (Polak de Fried and Denaday, 2010; Rawe *et al.*, 2010), Romany *et al.* (2014) reported no improvement in ART outcomes when comparing MACS technology to remove apoptotic sperm with swim-up method.

An important limitation of this method is that annexin V may bind with non-apoptotic cells having damaged plasma membrane with the exposed phosphatidylserine and may exaggerate the percentage of apoptotic cells. Secondly, it has been reported that live and healthy macrophages or monocytes, after ingestion of apoptotic bodies or fragments of apoptotic cells become annexin V positive and thus may be misidentified as apoptotic cells (van Engeland *et al.*, 1998). The effect of using magnetic beads in ART has raised concerns that these foreign particles may be accidentally injected to the oocyte along with normal sperm, however this method has shown promise in some trials (Polak de Fried and Denaday, 2010; Rawe *et al.*, 2010), but this technology is yet to be tested in larger randomized trials.

1.3.3.3 Conclusion

Annexin V-conjugated magnetic beads can separate sperm with externalized phosphatidylserine, which is considered one of the early features of late apoptosis. Removal of sperm, which failed to be excluded by the apoptotic machinery or with abnormal membrane protein, should theoretically benefit sperm selection. The separation of non-apoptotic sperm with intact membranes may enhance cryosurvival rates following cryopreservation (Said *et al.*, 2005). Although, this method can effectively remove apoptotic sperm, however there are other components in semen such as leukocytes, debris, and so on that should be removed. Therefore, integration of MACS with density gradient centrifugation can be considered as an effective approach to select non-apoptotic perm (Said *et al.*, 2006b).

1.3.4 Microfluidics

Microfluidics is defined as “precise movement of micro-particles in a controlled microenvironment.” It has recently gained application as an efficient technique for sperm separation. Microfluidics is based on fluid kinetic properties of semen/sperm in a microenvironment. The separation relies on the difference in the physical aspects as density, size, shape, motility, of a good quality sperm and other contaminants, when they are subjected to flow in a network of micro capillaries.

1.3.4.1 Methodology

Typically, a microfluidics based sperm sorting device consists of inlet/outlet ports, sample reservoir, micro-capillaries/micro-channels and a power source. The power source generates fluid flow from sample reservoirs through micro-channel. Various designs for microfluidic sperm sorting devices have been proposed in different studies. Sperm separation through microfluidic based devices are highly dependent upon factors like channel width, height, depth, as well as sperm velocity, viscosity, and contaminant density. In one of the earlier studies using microfluidic technology for separation of sperm, a uniquely configured glass tube was used, which allowed only the motile sperm to progress to an upper arm, which was connected to a reservoir to recover sperm for IVF or IUI (Wang *et al.*, 1992). The author in his later studies reported a higher motility and normal sperm morphology in the isolated sperm from this microfluidic device as compared to swim-up and density gradient separation (Wang, 1995; Figure 1.2).

In another study, a chamber consisting of central loading well surrounded by slightly depressed side wells was devised as the microfluidic cell sorter (Lih *et al.*, 1996). The motile sperm would migrate and would concentrate up to 13-fold in these side wells, yield sufficient number of sperm that can be used for ICSI. A modification in this device was reported in a later study, in which hamster oocyte was placed within the side wells. The oocyte served as repositories and

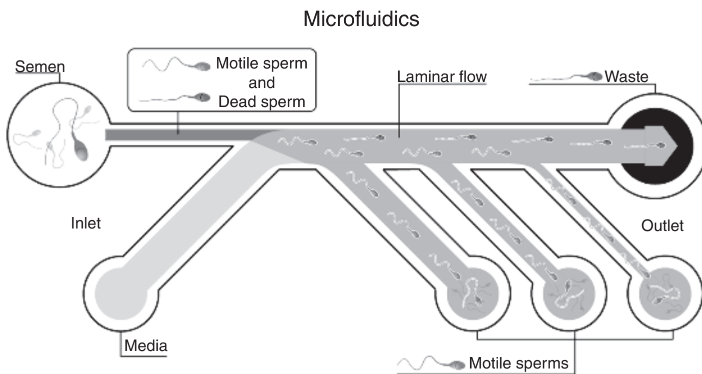

Figure 1.2 Separation of motile sperm using microfluidics. (*See color plate section for the color representation of this figure.*)

resulted in hamster oocyte penetration in 64% of the cases (Gordon and Chen, 1995).

Another approach employing microfluidics for separation of motile sperm used a micro-device made of poly dimethyl siloxane. The semen sample and media from separate inlet ports, joined a convergent micro-channel, and only the motile sperm could traverse the border that separates the parallel stream of diluted semen and fresh medium. Thus, the laminar flow properties exhibited by media in micro-channels allowed motile sperm to swim away from non-motile sperm, debris, and seminal plasma and collect in a separate outlet reservoir (Cho *et al.*, 2003). The novel approach appeared to offer a feasible alternative to isolate sperm from oligozoospermic patients for use in ICSI.

1.3.4.2 Advantages and Limitations

A potential benefit of microfluidics for sperm separation over traditional methods as density gradient, swim up, or simple dilution and washing is that sperm isolated using microfluid device have been reported to have significantly lower levels of DNA damage and improved motility (Schulte *et al.*, 2007). During the semen preparation for these traditional methods, the sperm is subjected to physical stresses as centrifugal force, which may induce reactive oxygen species production, ultimately leading to sperm DNA damage. Increased sperm DNA damage during ART correlates with reduction in embryo morphology at early cleavage stages (Virant-Klun *et al.*, 2002), failure to advance to the blastocyst stage *in vitro* (Benchaib *et al.*, 2003; Seli *et al.*, 2004), decreased pregnancy rates (Bungum *et al.*, 2004; Henkel *et al.*, 2004; Tesarik *et al.*, 2004), and increased spontaneous abortions (Carrell *et al.*, 2003). Thus, microfluidic sperm sorting may allow for selection of higher quality sperm, without causing oxidative stress induced DNA damage, potentially leading to improved ART outcome. Furthermore, the microfluidic sperm sorting has a higher sperm recovery rate particularly in patients with severe oligozoospermia. Such samples have large amount of debris and recovery rates from oligozoospermic sperm samples have been reported to be as low as 0.8% for swim-up method (Englert *et al.*, 1992; Smith *et al.*, 1995). One limitation of sperm separation using microfluidics is that the application is unable to identify non-motile but viable sperm for ICSI, which is considerably relevant in severe or complete asthenozoospermic patients.

1.3.4.3 Conclusion

An ideal sperm isolation technique should be simple, rapid, and should not cause any damage to the sperm genome. It should also be able to isolate a sufficient number of good quality sperm, which is potentially used for ART. Microfluidics based sperm sorting offers better potential for this compared to the conventional methods of sperm separation. Sperm sorting through microfluidics is high-speed and high-throughput compared to other available options. Use of the microfluidics technique is less labor intensive and less time consuming.

1.4 Electrophoretic Sperm Separation

John Aitken at the University of Newcastle, Australia first proposed electrophoretic sperm selection approach where sperm is selected based on its negative charge potential (Ainsworth *et al*., 2005). Mature sperm acquires a negative charge as it passes through the epididymis, where a number of negatively charged glycoproteins are bound to the sperm membrane. Here, sperm membrane charge is used as a biomarker to select mature sperm. Two models of electrophoretic system have been built to separate negatively charged sperm from semen based on the size and charge. A four-chambered device consisting of two inner and two outer compartments, where the inner chambers are used for inoculation and collect sperm. Approximately 2 mL of semen is added to the inoculation chamber and 400 µL of sperm is collected from the collection chamber. A polycarbonate separation membrane with pore size 5 µm and membrane area of 30 × 15 mm separates these chambers. The two-chambered system consists of an inoculation and collection chambers separated by a polycarbon membrane (Figure 1.3). The device hosts two platinum-coated titanium mesh electrodes and two 12 V buffer pumps to circulate buffer through the chambers at a flow rate of 1.6 L/min. Raw semen and buffer (10 mm HEPES, 30 mm NaCl and 0.2 m sucrose; pH 7.4 and 310 mOsm/L) were loaded into the inoculation and separation chambers, respectively and allowed to equilibrate for 5 min prior to application of electric current. The samples were run at 23°C with a constant applied current of 75 mA and a variable voltage of between 18 and 21 V (Ainsworth *et al*., 2011). During electrophoresis, the sperm with negative charge move from the inoculation chamber to the

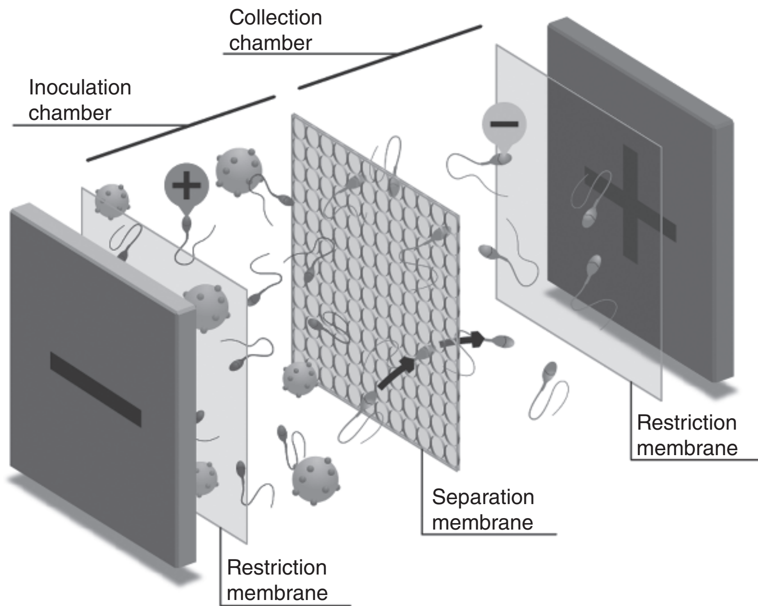

Figure 1.3 Schematic diagram showing the apparatus for the electrophoretic sperm separation. (*See color plate section for the color representation of this figure.*)

collection chamber through the polycarbon separation membrane. The 5-μm pores size of the membrane allows the passage of morphologically normal sperm while larger cells such as immature germ cells, leukocytes, any contaminant and large debris are left behind.

1.4.1 Methodology

This method of sperm separated is rapid, free from contaminant cells and debris. In addition, the sperm population obtained is showed to have high percentage of morphologically normal and motile sperm with intact DNA (Ainsworth *et al.*, 2005). Such rapid isolation of viable sperm without any centrifugation procedure prevents the sperm from oxidative mediated DNA damage (Aitken *et al.*, 2011). The 5-μm polycarbonate membrane separating the inoculation and collection chambers allows the passage of sperm, but not contaminant

cells including precursor germ cells, leukocyte subtypes, viable, and non-viable sperm. Heat generated during electrophoresis is prevented by maintaining the buffer at 25°C and circulating the excess buffer stored in the reservoir around the instrument using a pump. The sperm obtained from the collection chamber could be directly used for ART. The main drawback of the electrophoretic system is the laborious procedure for cleaning the instrument following sperm separation. The separation cartridges have to be autoclaved after each separation and the electrophoresis buffer is removed and rinsed by sterile distilled water and replaced with cleaning buffer (0.1 M NaOH) overnight. The next day, the cleaning buffer is removed and the system is washed three times with distilled water (Fleming and Aitken, 2011).

1.4.2 Advantages and Limitations

The sperm population obtained after electrophoretic sperm separation is extremely pure with no contaminant cells detected (Ainsworth *et al*., 2005). In addition, 43% of the sperm is recovered from the collection chamber following 15 min electrophoresis (Ainsworth *et al*., 2005). This method can also separate slightly motile and viable testicular sperm from testicular biopsy materials, leaving the contaminant cells behind (Ainsworth *et al*., 2007). When cryopreserved semen was used in the inoculation chamber, the sperm population isolated after 5 min of electrophoresis showed significantly improved motile and viable sperm compared to the inoculant (Ainsworth *et al*., 2007). The percentage of morphologically normal sperm obtained after electrophoresis sperm separation was higher at all electrophoretic current settings and duration of electrophoresis (Ainsworth *et al*., 2005). While the sperm deformity index in the selected sperm population was significantly lower than the inoculant. A reduction in sperm DNA damage was observed for all time-points up until 10 min of electrophoresis, while prolonged electrophoresis did not result in a significant reduction in sperm DNA damaged (Ainsworth *et al*., 2005). In addition, a reduction is sperm DNA damage was observed after the use of cryopreserved and testicular biopsy samples (Ainsworth *et al*., 2007). The use of sperm selected from the electrophoretic systems in ART should have no impact on the gender of the resultant offspring (Ainsworth, *et al*., 2011).

During the electrophoretic selection method, sperm is not only selected based on its charge but sperm motility plays an important

role in the selection process. When semen is loaded in the inoculation chamber and prior to the start of electrophoresis, 3.2% of sperm is showed to pass through the polycarbon membrane to the collection chamber irrespective to the charge of the sperm (Ainsworth *et al.*, 2005). The percentage of sperm motility obtained from the collection chamber is comparable to the original raw semen and the recovery of motile sperm did not change with different electrophoretic duration (Ainsworth *et al.*, 2011). However, a progressive loss of total sperm motility was observed at high electrophoretic current settings (Aitken *et al.*, 2011). The viability of the sperm population did not increase following electrophoretic sperm separation when compared to the raw semen at all electrophoretic power settings (Ainsworth *et al.*, 2005).

1.4.3 Clinical Importance of Sperm Preparation by Electrophoresis

A successful pregnancy following electrophoretically selected sperm was reported by Ainsworth *et al.* (2007). Later, a prospective controlled clinical trial was performed comparing electrophoretically separated sperm with DGC selected sperm (Fleming *et al.*, 2008). In this split-cohort study, no statistical difference in fertilization rate, embryo cleavage rate, top quality embryo, or clinical pregnancy was observed between the two insemination groups. The lack of statistical significance in fertilization rate and embryo quality was observed in both IVF and ICSI patient groups (Fleming *et al.*, 2008). Although no statistical significant between the two insemination groups, this study provides the proof-of-principle, that electrophoretically separated sperm could be used for ART.

1.4.4 Conclusion

Electrophoretic sperm separation procedure is an extremely versatile and cost-effective method of preparing sperm based on their negative membrane charge. The sperm selected using this approach have shown adequate recovery rate and a significant improvement in sperm morphology and vitality. DNA damage is reduced in the selected sperm population as this procedure excludes the centrifugation step, which is known to induce oxidative stress on sperm. The sperm obtained from this method could directly be used for ART. However, to date there is no conclusive evidence to confirm the effectiveness of this

approach in the management of male infertility and the selected sperm could effectively improve ART success. Further evidence research is required to prove the effective use of this sperm selection method in ART success.

1.5 Zeta Test

All biological and non-biological particles in nature are known to have an electrostatic potential. During sperm maturation in the epididymis, negative charged glycoproteins are added to the sperm membrane (Veres, 1968), which provides the sperm its characteristic negative membrane potential (Bedford, 1983). The sperm negative membrane charge was termed the zeta potential or electro-kinetic potential by Ishijima *et al*. (1991). In the Zeta sperm selection method, this negative membrane charge is utilized as a biomarker to select mature sperm. Later, Chan *et al* (2006) developed the Zeta test to select sperm according to its electrostatic potential.

1.5.1 Methodology

The zeta sperm selection method is performed on density gradient centrifugation selected sperm. A new centrifuge 15 mL tube is used as a platform to isolate highly negatively charged sperm. The electrostatic charge of an untouched centrifuge tube is positively and will attract negatively charged sperm. Touching the tube without the use of glove will ground the tube, resulting in the loss of electrostatic potential. Extreme care should be taken to place the tube inside a latex glove up to the cap and hold the cap of the tube at all time. DGC washed sperm (0.1 mL) is diluted with 5 mL of serum-free HEPES – HTF medium and gently pipetted in the tube. The tube with the sperm sample should be rotated two or three turns clockwise and incubated at room temperature (23°C) for 1 min to allow adherence of the charged sperm to the wall of the centrifuge tube. Following incubation, the tube is centrifuged at 200 × *g* for 5 min and the tube is slowly inverted to drain out all non-adhering sperm and other contaminant cells. The excess liquid at the mouth of the tube is removed by placing the tube upside down on a tissue paper. Three percent serum supplemented with HEPES – HTF medium (0.2 mL) is pipetted into the tube, by allowing the medium to trickle down the side of the tube wall. This process helps to neutralize

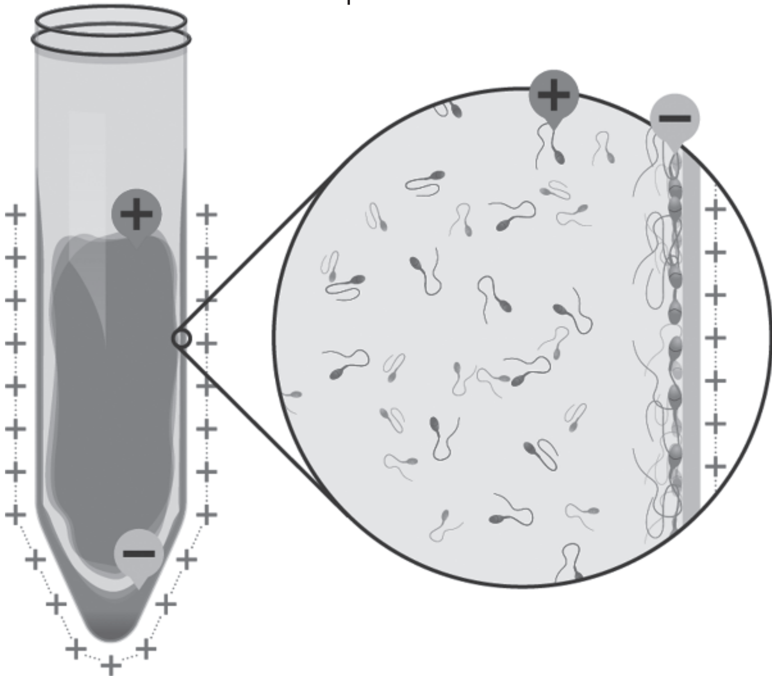

Figure 1.4 Diagram of sperm selection using the Zeta test. Negatively charged mature sperm is adhered to the positively charged tube surface, while immature sperm remain suspended in the media. (*See color plate section for the color representation of this figure.*)

the positive charge of the tube and detach the adhering sperm from the wall (Figure 1.4). The collected medium at the bottom of the tube is re-pipetted and used to rinse the wall of the same tube several times to increase the concentration of recovered sperm (Chan *et al.*, 2006; Kam *et al.*, 2007; Khajavi *et al.*, 2009).

1.5.2 Advantages and Limitations

The Zeta sperm selection method is a simple, cost effective, and rapid method of selecting mature sperm (Chan *et al.*, 2006). A recent study proved that sperm selected based on its Zeta test are more mature when assessed for markers such as protamine content, ability to resist DNA fragmentation, and apoptotic markers such as TUNEL,

or acridine orange (Kheirollahi-Kouhestani *et al.*, 2009). In addition, this method is showed to isolate sperm with significantly increased normal morphology, hyperactivation, DNA integrity and maturity. However, the motility of the sperm is reduced due to the process of sperm binding to the surface charge of container (Chan *et al.*, 2006; Kam *et al.*, 2007; Nasr-Esfahani *et al.*, 2008a; Khajavi *et al.*, 2009; Razavi *et al.*, 2010). This method can be effectively used on cryopreserved-thawed semen (Kam *et al.*, 2007). Sperm selected using Zeta method is showed to have low percentage of DNA damage when compared with DGC (Khajavi *et al.*, 2009; Kheirollahi-Kouhestani *et al.*, 2009).

1.5.3 Clinical Importance

Kheirollahi-Kouhestani *et al.* (2009) performed a study where sibling oocytes from patients undergoing ART were split into two groups and inseminated by sperm prepared by DGC and DGC/Zeta. Fertilization rate was significantly higher in sibling oocytes group inseminated by sperm prepared by DGC/Zeta compared to DGC group. However, embryo cleavage rate and embryo quality on Day 2 were not significantly different between the two insemination groups. Embryo quality on Day 3 showed a slight improvement after DGC/Zeta selected sperm, but was not statistically significant. Similarly, an increase in implantation and pregnancy rates were observed after the DGC/Zeta insemination method, but these improvements were not statistically significant. Another study by Deemeh *et al.* (2010) showed that oocytes inseminated by sperm selected from DGC/Zeta method resulted in a high fertilization rate and good quality embryos, leading to a pregnancy following Day 3 embryo transfer. These studies provide proof-of-principle that Zeta method may aid the selection of good quality sperm for ART. However, large randomized controlled clinical trials are required to identify the beneficial effect if this method on ART outcomes.

1.5.4 Conclusion

The Zeta selection method is inexpensive, easy to perform, and does not require any complex equipment to select mature sperm. This method is able to recover highly negatively charged sperm with an increase in normal morphology and DNA integrity; however, the motility of the sperm is severely reduced. Hence the sperm selected

using this method is applicable for the ICSI insemination method. Due to low recovery of sperm, this method is of limited use in patients with oligozoospermia. This method of sperm selection may not be applicable to testicular sperm, as immature sperm lacks membrane potential (Chan *et al*., 2006).

1.6 Microelectrophoresis Sperm Selection

The microelectrophoresis sperm selection method has identical principles to the Zeta test, where sperm surface charge is utilized to select mature sperm. Immature sperm entering the epididymis have a positive membrane charge. During the epididymal maturation process, sperm acquires the characteristic negative charges by the addition of negatively charged glycoproteins to its membrane (Saxena *et al*., 2002). Using the sperm membrane charge as a biomarker, researchers at University of Utah, USA have developed a novel method of sperm selection known as Microelectrophoresis (Simon *et al*., 2015). They also found that the negative membrane charge could be partially removed from the sperm by a simple sperm wash procedure, resulting in sperm with variable level of negative charge to positively charged sperm. The negatively charged glycoproteins are known to accumulate as the sperm transit through the epididymis. Therefore, the ejaculated population of sperm would acquire a variable level of glycoproteins. However, the majority of the sperm is shown to have negative charge (Simon *et al*., 2015). During sperm wash by density gradient centrifugation, immature sperm with low negative charge and sperm undergoing apoptosis or capacitation will completely loose its charge, resulting in positively charged sperm. However, highly negative charged sperm would retain some of its charge. A slight variation in charge difference can be visualized during the Microelectrophoresis sperm selection procedure and sperm having high charge can be identified and isolated for ART (Simon *et al*., 2015).

1.6.1 Methodology

The micro-electrophoresis sperm separation unit consists of three parts as described in Simon *et al*. (2015). The power supply, consists of a basic power-pack unit that can control and supply 0–300 V and 0–300 mA of electricity (Bio-Rad Laboratories, Inc.). The platinum reusable connecting electrodes are used to connect the

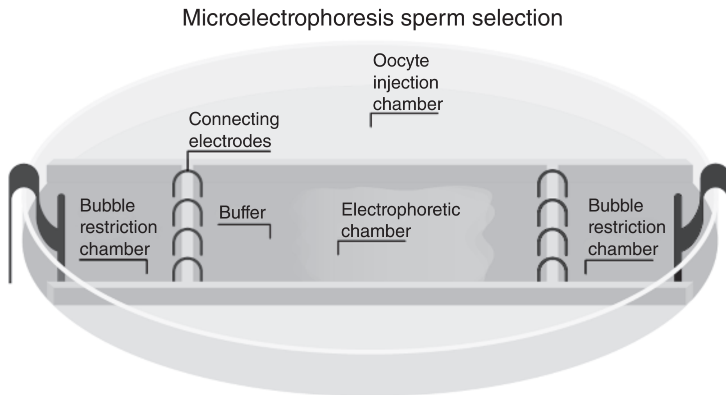

Figure 1.5 Schematic representation of microelectrophoresis unit. (*See color plate section for the color representation of this figure.*)

electrophoresis unit to the power supply. Sperm separation is performed in the disposable sterile electrophoresis unit (Figure 1.5). The basic methodology for micro-electrophoresis is essentially the same as ICSI sperm selection procedure. The micro-electrophoresis apparatus is set-up on the ICSI stage of an inverted microscope and sperm were viewed at 200× magnification and picked up with a beveled, glass ICSI pipette connected to a CellTram Vario manual microinjector (Eppendorf). Two mL of electrophoresis buffer (10 mM Tris, 20 mM NaOH, pH: 7.8) is added to the electrophoresis chamber. Approximately 10–15 μL sperm were added to the electrophoretic buffer and allowed to settle for 2 min. Electrophoresis is performed by applying current between 6–14 mA (increased from low to high) at variable 30–100 V. Sperm is collected during electrophoresis (as soon as the electrophoresis is initiated, but not later the electrophoresis is completed). In patients where the number of charged sperm was low, sperm were identified within the electrophoretic chamber at different microscopic field of view by moving the ICSI stage. During electrophoresis, the sperm were viewed through the ICSI inverted microscope and the charge of the sperm is observed by visualizing the direction of sperm movement under the influence of current (PCS move towards cathode and NCS move towards the anode). During electrophoresis, sperm were assessed for their morphology, and sperm with normal morphology and negative charge were selected using the ICSI pipette.

1.6.2 Advantages and Limitations

Negative charged sperm was shown to have relatively low DNA damage. When sperm were selected based on their charge (positive, negative, and neutrally charged) and compared with control, negatively charged sperm showed significantly lower DNA damage than positive charged sperm and control (Simon *et al*., 2015). While positively charged sperm showed the highest level of DNA damage. Subsequently, when negatively charged sperm were isolated at three different electrophoretic current settings, low level DNA damage was observed at all current settings when compared with positively charged sperm and their corresponding unselected control. The percentage of sperm with normal histone retention was directly proportional to the percentage of negatively charged sperm and inversely proportional to the percentage of positively charged sperm. While the percentage of sperm with abnormally high histone retention was inversely proportional to the percentage of negatively charged sperm and directly proportional to the percentage of positively charged sperm (Simon *et al*., 2015).

1.6.3 Clinical Importance

The charge of the sperm analyzed using microelectrophoresis was associated with ART outcomes. IVF fertilization rate was directly proportional to the percentage of negatively charged sperm, while positively charged sperm was inversely proportional to fertilization rate. No correlation was observed between the sperm charge and ICSI fertilization rate (Simon *et al*., 2015). The percentage of negatively charged sperm following density gradient centrifugation was directly proportional to the percentage of embryos that developed to blastocyst and inversely associated with the percentage of arrested embryos. In contrast, an inverse association was observed between the percentage of positively charged sperm and embryo quality and development (Simon *et al*., 2015). The implantation rate was higher in the patient group containing greater than 15% negatively charged sperm after DGC compared with the patient group containing less than 15% negatively charged sperm. While, couples achieving a successful clinical pregnancy had a higher percentage of negatively charged sperm and lower percentage of positively charged sperm, than couples who did not achieve clinical pregnancy (Simon *et al*., 2015).

1.6.4 Conclusion

The microelectrophoresis sperm selection approach is designed to utilize sperm surface charge as a biomarker to identify mature sperm. Some of the advantages of this method of sperm selection are that it is extremely versatile, easy to use, not time consuming, does not require complex instruments, and does not require additional qualified technicians. The sperm selected by this method could be directly used for ICSI insemination. The negatively charged sperm obtained by this method are relatively free of DNA damage. The preliminary results obtained thus far are promising; however, this proof-of-principle is yet to be confirmed in the context of ART.

1.7 Raman Spectroscopy

When photons from a light source are focused on a particle, the interaction causes a change in the frequency and wavelength of the photons. The particle absorbers energy, resulting in a scattering of diminished energy photons. The change in wavelength of photon may vary depending on the particle's molecular composition such as, atomic mass, compaction of atoms, free electrons, type of atomic bonds, arrangement of atoms, and so on (Ellis *et al.*, 2013). A shift in wavelength and photon frequency when scattered by a molecule is known as the Raman effect. During the Raman sperm selection approach, light wavelength of specific frequency is focused on the sperm head; the scattered light provides a chemical fingerprint of the structure of the sperm nuclear DNA (Sanchez *et al.*, 2012). Using this innovative technique, it is now possible to identify sperm with intact DNA.

1.7.1 Methodology

In the past decade, few research groups have used Raman spectral scattering on sperm to fingerprint the sperm nuclear DNA for damage. The current protocol of Raman spectral analysis is performed on dried sperm (Sanchez *et al.*, 2012). Sperm is washed and smeared on a suprasil slide and air-dried. To acquire each spectrum, the cell was centered, and the laser directed at the postacrosomal region of the sperm head. A confocal Raman spectroscopy microscopy equipped with a 632.8 nm He-Ne laser is used for light source (Figure 1.6). The light

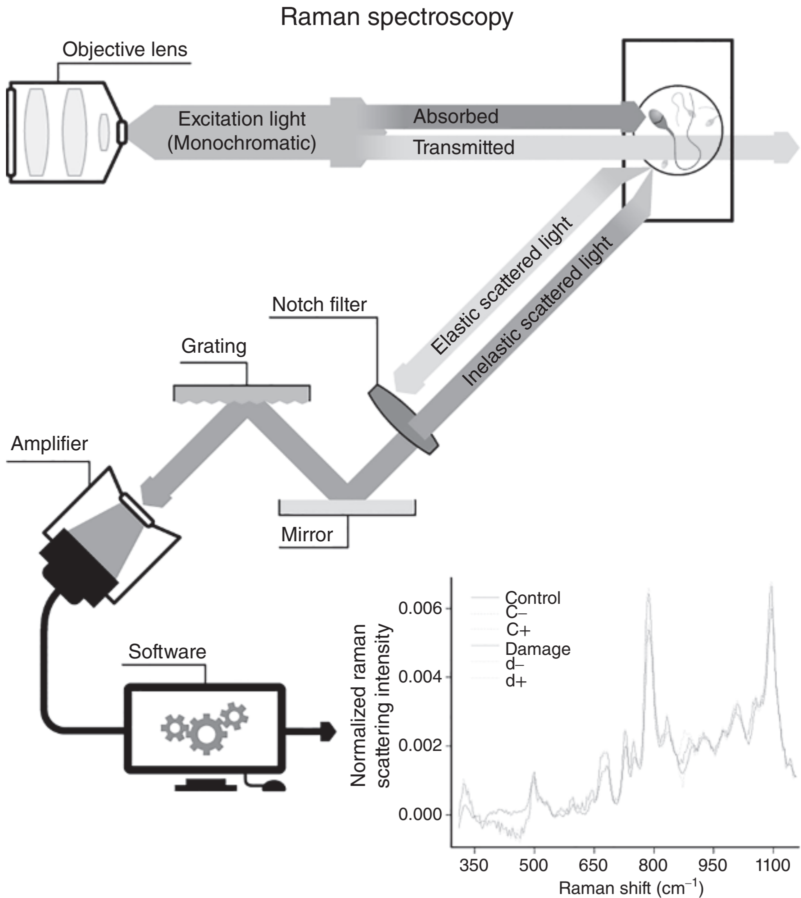

Figure 1.6 A typical micro-Raman spectroscopy setup as utilized in the analysis of sperm. (*See color plate section for the color representation of this figure.*)

source is focused on the sperm head using a confocal pinhole with appropriate notch filter and imaged using a CCD detector. Spectral acquisition is performed for 30 s and the scattered wavelengths ranging between 600 and 1800 cm^{s-1} were collected. The scattering profile is collected on one sperm per acquisition and a total of 200 sperm per sample are randomly analyzed at different microscopic field. Subtracting the spectral background performs a baseline correction. To

enhance resolution without losing spectral information, the spectra are filtered using a noise reduction algorithm and setting the maximum intensity signal normalizes the spectra. In addition, mapping is performed on the *z*-axis to identify the optimal focus point on the sperm head. This step helps to obtain the maximum signal strength, which ensures to obtain a best spectral quality (Sanchez *et al.*, 2012).

1.7.2 Advantages and Limitations

In the past decade, a few research groups have analyzed the Raman spectral analysis on sperm (Huser *et al.*, 2009; Meister *et al.*, 2010; Mallidis *et al.*, 2011). These studies have identified distinctive Raman spectra specific to regions of the sperm. Although these studies have identified a large DNA-rich region of the sperm head, there is a number of disagreements between these studies, owing to variations in peak intensity and ratios (Mallidis *et al.*, 2011). The peaks that were reported to predict normal sperm morphology (Huser *et al.*, 2009) were not observed in later studies (Mallidis *et al.* 2011). Similarly, spectral peaks representing neck and mid-piece region indicative of the presence of mitochondria reported by Meister *et al.* (2010) were not confirmed in the other studies (Huser *et al.*, 2009; Mallidis *et al.*, 2011). However, all the studies confirm the importance of the peak at 1092 cm^{-1} that corresponds to the PO_4 backbone of sperm DNA. Mallidis *et al.* (2011) reported a difference in status of the sperm nuclear DNA based on the signal intensity and associated increase in the peak at 1040–1050 cm^{-1}, which is related to fragmented nuclear DNA. The change associated to the peak intensity caused by the fragmentation of DNA has been confirmed by recent studies (Sanchez *et al.*, 2012; Lang *et al.*, 2013). Accurate and reproducible results identifying nuclear DNA fragmentation in sperm have been reported by Sanchez *et al.* (2012).

1.7.3 Clinical Importance

To date, spectral analysis on sperm has been performed on sperm fixed to slides (dead sperm), therefore such sperm has not been used for ART. However, Raman spectral analysis has gained significant importance in examining spent embryo culture media. Seli *et al.* (2007) were the first to report a significant difference in the day 3 culture media, particularly the increase in the relative amount of –SH and decrease in the relative amounts of –CH and –NH in embryo media that were

implanted compared to the ones that were not. Based on these spectral differences, the authors have also calculated an index that could help to predict the viability of individual embryos (Seli *et al.*, 2007). A strong association between the metabolic profile and clinical outcome was observed on day 3 and day 5 culture media with high sensitivity and specificity (Scott *et al.*, 2008). Recently, Zhao *et al.* (2013) also found that the Raman detection of compounds such as sodium pyruvate and phenylalanine levels in culture medium was indicative of ART success.

1.7.4 Conclusion

Raman spectroscopy was first employed in forensic medicine to identify semen; however, in recent years, the information obtainable through this method in reproductive medicine is constantly evolving. Recent developments in reproductive medicine are due to the advanced resolution of instruments meaning it is now possible to examine microstructures and even single molecules through Raman scattering. Raman scattering may provide a means of accurate and harmlessly assessment to identify healthier sperm and such sperm could be selected and used in ART (Mallidis *et al.*, 2011; Sanchez *et al.*, 2012). Despite advancements in instrumentation and technology, Raman spectral analysis has been performed on air dried and fixed sperm, which are unusable for ART.

1.8 Hyaluronic Acid Binding Assay

Hyaluronic acid was first discovered by Karl Meyer as a polymer of disaccharides composed of d-glucuronic acid and d-N-acetylglucosamine, linked via alternating β-1,4 and β-1,3 glycosidic bonds (Meyer and Palmer, 1934). Many mammalian tissues, including cumulus cells, secrete hyaluronic acid. The hyaluronic acid is recognized by the receptor PH-20 and HspA2 present on the plasma membrane and on the inner acrosomal membrane of mammalian sperm head (Huszar *et al.*, 2003). Several functions of sperm have been attributed to the binding of hyaluronic acid to sperm receptors, such as intracellular signaling, zona pellucida adhesion, and sperm penetration into the oocyte. During the last stages of spermatogenesis following histone-protamine replacement and extrusion of cytoplasm, the zona pellucida binding site and hyaluronic acid binding site are formed.

Therefore, only mature sperm would have the ability to bind to hyaluronic acid (Huszar *et al.*, 2003). Using hyaluronic acid receptors as a biomarker, it is now possible to select and isolate mature sperm for ART.

1.8.1 Methodology

The hyaluronic acid binding assay is performed on DGC separated sperm. After DGC, the sperm concentration was adjusted to 1×10^6 cells in 1 mL. Droplets of diluted hyaluronic acid were placed on a falcon dish and allowed to air dry under the sterile laminar flow hood. The coated hyaluronic acid spot was covered with 15 to 75 µL of DGC washed sperm and incubated at 22°C for 10 min. The sperm droplets were washed twice with media to remove unbound and motile sperm. The hyaluronic acid bound sperm that showed vigorous tail movement without forward motility were selected using an ICSI pipette and transferred to another droplet, preferably with PVP, where morphologically normal sperm were selected for ICSI insemination (Nasr-Esfahani and Marziyeh, 2013).

1.8.2 Advantages and Limitations

Sperm that bind to hyaluronic acid *in vitro* have been shown to reduce chromosomal abnormalities (Jakab *et al.*, 2005; Parmegiani *et al.*, 2010), intact plasma membrane (Cayli *et al.*, 2003), normal morphology (Prinosilova *et al.*, 2009), nuclear condensation (Nijs *et al.*, 2009), and cytoplasmic and nuclear maturity (Huszar *et al.*, 2003, 2007; Cayli *et al.*, 2004). Huszar *et al.* (2003) reported that only mature and motile sperm have the ability to bind to hyaluronic acid through specific receptors and such an ability is not present in immature sperm. In addition, hyaluronic acid bound sperm are shown to have reduced DNA fragmentation (Parmegiani *et al.*, 2010; Catenacci *et al.*, 2012). In contrast, Huang *et al.* (2015) reported no association between hyaluronic acid bound sperm and DNA integrity. Nijs *et al.* (2010) reported no significant correlation between hyaluronic bound sperm and sperm parameters.

1.8.3 Clinical Importance

The use of hyaluronic acid bound sperm in ART has the advantage of selecting mature sperm with relatively low levels of DNA

fragmentation and low frequency of sperm with chromosomal abnormalities (Nasr-Esfahani *et al*., 2008b). An increase in the percentage of sperm with the hyaluronic acid binding ability has been associated with improved fertilization rate and biochemical pregnancies (Worrilow *et al*., 2009). The use of hyaluronic acid bound sperm in ART has showed to improve embryo quality and development (Parmegiani *et al*., 2010; Kim *et al*., 2014). However, Nijs *et al*. (2010) reported that no association between hyaluronic acid bound sperm with fertilization rate, embryo quality or pregnancies. In a retrospective study of patients undergoing ART (Sollmann and Krebs, 2013), no improvement in any of the ART outcomes (fertilization rate, embryo quality, and clinical pregnancy) was observed in patients with oligozoospermic, asthinozoospermic, and normozoospermic profiles. Cortes *et al*. (2012) reported no improvement in fertilization rate and embryo quality but an increase in pregnancy rates was observed in the use of hyaluronic acid bound sperm following ICSI. An increase in implantation and clinical pregnancy and a decrease in miscarriage rates were observed by the use of hyaluronic acid bound sperm with normal MSOME in ICSI than conventional ICSI (Cubillos *et al*., 2010). In a recent multicenter clinical trial, the use of hyaluronic acid bound sperm was shown to improve clinical pregnancy rates when compared with conventional ICSI (Worrilow *et al*., 2013).

1.8.4 Conclusion

It is well known that human oocytes are naturally surrounded by hyaluronic acid, which is involved in the mechanism of sperm selection. Recent studies show that only mature sperm have specific receptors that bind to hyaluronic acid. The quality of hyaluronic acid bound sperm has been accessed in a number of studies, while most studies have suggested an improvement cytoplasmic and nuclear maturity, normal morphology, functional potential, and relatively low frequency of sperm with DNA damage and chromosomal abnormalities. Despite substantial evidence showing an improvement in sperm quality, the use of hyaluronic bound sperm in ART has resulted in mixed outcomes. Additional studies with larger sample sizes are required to determine the beneficial impact of the use of hyaluronic acid bound sperm in ICSI.

1.9 Future Perspective

Although sperm selection techniques have been demonstrated to enhance the ART success rate, the full potential of these techniques is yet to be uncovered. The present techniques for sperm selection typically utilize a single characteristic of sperm such as membrane surface property, chromatin integrity, optical property, motility, morphology, or kinetic behavior in fluid. Development of techniques that could more stringently analyze multiple characteristics in a single platform would help in selection of sperm that is more reproductively efficient and therefore may improve the success rate of ART and reduce ART related complications.

The discussed methods of sperm selection vary in terms of time and instrumentation required. Techniques such as Zeta potential and Annexin V have low input costs and are less time consuming whereas IMSI, HA binding, and electrophoretic separation are more time consuming and need more sophisticated instrumentation. Future research should seek to ensure that the modified sperm separation protocols are simple and do not require complicated and costly equipment that limit widespread use. The development of a more efficient technique for sperm selection should also ensure that, unlike IMSI, it does not require a high level of expertise. Technical modifications in future developments warrant careful assessment of processing time, since prolonged sperm exposure to non-physiologic conditions may induce iatrogenic damage (Agarwal *et al.*, 1994). Prolonged processing times have been reported to cause sperm DNA damage (Twigg *et al.*, 1998), aberrant embryo development, and abnormalities in offspring presenting as birth defects or genetic disorders (Marchetti and Wyrobek, 2005; Verhofstad *et al.*, 2008).

Furthermore, there is a lack of consensus in data establishing a universal technique that can be applied for sperm selection in all categories of infertile men. Further research is needed to identify which infertility cases, if not all, will benefit from the application of these selection methods. The efficacy of the techniques has been shown to vary in different studies. The future research in this area should also focus to develop a protocol that is not only universal for infertile men to which it could be applied but also should have a high throughput with the capability of efficient selection of the best gametes. In addition, large scale randomized controlled clinical

trials are required to illustrate the benefits of these sperm selection methods for ART success.

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