CHAPTER 7

A BRIEF SUMMARY OF SPERM SELECTION TECHNIQUES AND CURRENT APPROACHES

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INTRODUCTION

Since 15-20% of couples of reproductive age face infertility problems, practical use and development of assisted reproductive techniques (ART) become increasingly necessary with each passing day. Many methods have been developed to isolate sperm capable of fertilizing oocytes for this purpose. In today's world, intracytoplasmic sperm injection (ICSI) is the most widely used method of fertilization. With ICSI applications, the ability to perform fertilization and successful pregnancies even with few and low-quality sperm cells has been a significant development in ART. However, while ICSI practices focus on isolating live and motile sperm in the early period, it has become clear that only these parameters will not be sufficient to select quality sperm for fertilization over time.

Under natural conditions, tens of millions of sperm are released into the vagina, at the cervical os during mating. Sperm cells begin a long and difficult race along the female genital tract to reach the oocyte in the ampulla of the fallopian tube. In humans, only 10% of the total sperm in the ejaculate passes through the cervix, while 1% reaches the uterus and 0.1% reaches the fallopian tube. Finally, between 100-1000 sperm can achieve to reach the oocyte and fertilize it (1). However, in ICSI applications, a random selection takes place among the sperms categorized as high quality, taking into account some criteria of sperm parameters. It's been reported that about half of the sperm injected during ICSI have damaged DNA (2). In vitro injection of a single sperm cell into an oocyte may also bypass many natural elimination factors for fertilization and embryonic development.

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For this reason, some methods are tried to be developed by imitating some natural selection processes in the female reproductive system, and sperms are isolated in a way suitable for natural selection. Increasing success in selecting motile sperm with normal morphology, and maintaining DNA integrity will succeed in ART applications. In this context, by referring to traditional sperm selection methods, approaches to current sperm selection techniques and current selection methods will be evaluated by using sperm membrane properties and electrical charge.

NATURAL SPERM SELECTION

The natural process of fertilizing the oocyte by the sperm includes extraordinary conditions and time-indexed selection processes. The female genital system has a selective and dynamic structure that allows the selection of the best sperm cells that are anatomically, histologically, and physiologically capable of fertilizing the oocyte. This process begins with the release of seminal plasma into the vagina. The sperm cells pass through the cervical canal and travel through the uterus and reach the ampulla of the fallopian tube, where fertilization will take place. Less than 500 sperm meet with the oocyte (1). Although human sperm can survive in the human female reproductive system for up to six days before fertilization (3), oocytes are generally fertilized within hours after ovulation, as the cellular structure of the oocyte is disrupted in less than 24 hours (4). During the sperm's journey in the uterus, interactions with proteins and different type of hormones secreted from the female reproductive system enable the sperm to reach full capacity and move faster with the increase of ions such as intracellular Ca^{2+}, modulating swimming in the direction of the oocyte with a chemotactic and thermotactic effect (5). Ensuring efficient sperm selection by performing the selection process exactly as in the female reproductive system will increase the success of ART. At this point, it is essential to develop models similar to the female reproductive system that distinguish motile and morphologically normal sperm from leukocytes, bacteria, other cell types, and toxic substances. These approaches' simplicity, cheapness, and speed will directly affect reproductive success rates such as fertilization, embryo development, attachment, and successful pregnancy in ART.

SWIM-UP TECHNIQUE

The swim-up method was first described in 1984 with the recovery of motile sperm that migrated through a culture medium placed on seminal plasma (6). This method is considered useful in the World Health Organization laboratory

guideline for examining and processing human sperm (7). Motile sperm selection is based on the sperm's ability to swim into the culture medium. It is performed in both in vitro fertilization (IVF) and ICSI procedures. It is also recommended in cases where the percentage of motile spermatozoa in the sample is low (7). It is known as one of the most basic methods for preparing sperm cells. This application can be applied with a combination of different techniques. By avoiding centrifugation and adding the culture medium directly to the semen, sperm cells can be easily transferred from the seminal fluid to the culture medium. To achieve this, semen is placed in an incubator at 37 °C for 60 minutes, overlapping the culture medium in a 45° inclined tube (7). A second alternative is to add a culture medium to the semen pellet obtained after centrifugation of the sample (8). The swim-up technique allows only mature and motile sperm to be collected. However, multiple tube preparations are required when processing highly concentrated and high-volume samples. This process also increases the workload.

DENSITY GRADIENT CENTRIFUGE

Density gradient centrifuge is among the frequently preferred methods to select motile sperm in cases of asthenozoospermia, severe oligozoospermia, and teratozoospermia. Sperms are forced to cross a colloidal silical gradient in this procedure. Motile sperms are separated from dead sperms, leukocytes, and other seminal plasma components by showing separation according to their density. The continuous or discontinuous density gradients are used for the centrifuge. Sperm cells are placed on these gradients and centrifuged. Separation occurs when the most motile, mature and best morphological sperm accumulate at the lower region of the tube (9). The density and motility are the key factors for the separation. Sperm samples are left in a higher density solution in a tube, and the tube is centrifuged for about half an hour. Instead of being exposed to centrifugation kinetics, motile sperm swim vigorously to form a soft pellet in the lower region of the tube. In contrast, dead sperm cells, leukocytes, bacteria, and residues pass through the gradients and remain in the intermediate areas (10). Different density gradient media are commercially available or prepared to form density layers in stable solutions that are non-toxic and will not cause any pH or osmolarity changes.

Different parameters such as centrifugation time, sample concentration, number of layers, sample volume, and g-force may affect morphology and motility ratios in each application. The disadvantages of this method are that the application time is long, low efficiency is obtained in samples with high concentrations, the

mechanical stress that occurs during centrifugation increases ROS production, and it costs more than the swim-up technique (11). Recent research suggests that this approach may increase sperm DNA fragmentation, a factor that has been linked to poor reproductive results following ARTs (12).

These techniques may cause DNA breaks and damage. Low IVF fertilization and pregnancy rates, abnormal preimplantation development, early pregnancy loss, and neonatal morbidity have been linked to decreased or inadequate DNA integrity. In conclusion, these widely used methods are not a best selection methods based on DNA integrity, apoptosis, ROS production, membrane maturation, and sperm structure.

MICROFLUIDIC SYSTEMS

One of the fastest-growing areas for sperm selection in ART applications has been adapting microfluidic-based technologies to sperm selection and preparation. In recent years, microfluidics has come to the forefront as a successful method applied in various fields, such as cryopreservation, single-cell analysis, biological and chemical analyses, forensic analysis, and medical diagnosis. It provides high efficiency, high accuracy, and sensitive results (13, 14). Microfluidics, which were developed based on the forward-looking characteristic of sperm, has been increasingly used in ART. Smith and Takayama conducted one of the earliest research projects in this field and they published a series of publications demonstrating that microfluidic applications may increase not just sperm quality but also laboratory productivity (15, 16). During this time, positive results have been tried to be obtained by integrating different approaches such as flow, electrophoresis, and chemical gradients into microfluidic systems (17-19). The hydrostatic pressure model method to help select the most motile and robust sperms helped achieve the most significant results. Two parallel capillary channels are used in this method proposed by Smith and Takayama. While motile sperm can be separated by moving along the capillary channels and traveling in separate channels, cell debris and non-motile cells are passively transported from the capillary canal's entry to its exit in the same plane. Compared with classical swim-up and density gradient centrifuge techniques, it was determined that the selected sperms improved significantly in terms of both motility (98%) and morphology (22%) parameters (20). With these and similar studies, it has been suggested that using microfluidic may eliminate the mechanical stress induced on sperm during preparation, such as the centrifugation step, create a suitable environment similar to the female genital system with stable osmolality, temperature, and pH. Oxidative

stress and DNA damage may be prevented as a result (21, 22). In addition, it may be associated with an improvement in the pregnancy rate of selected sperm with the use of microfluidic in couples undergoing ICSI (23).

Advances in microfluidic systems may support the development of novel strategies for sperm selection that cause the least amount of damage and have the most excellent chance of success. Parallel development of a microenvironment that closely resembles natural conditions within the female reproductive system, including geometry, temperature, and fluid composition, enables biosimilar-based sperm selection approaches that are more similar to the in vivo environment. This selection may also offer advantages in fertilization and the embryo's development (24). Using the microfluidic sperm selection method has potential benefits and some disadvantages. The complexity of the use of microfluidic systems and the time-indexed variability of sperm selection stands out as obstacles to routine clinical practice.

SPERM SELECTION ACCORDING TO ELECTRICAL CHARGE

The sperm membrane is necessary for cellular interactions such as sperm-oocyte contact and plays an important role in sperm maturation, capacitation, and fertilization. The membrane is negatively charged between −16 to −20 mV and covered with a 20-60 nm thick coating (25). Thanks to the negatively charged glycocalyx in the membrane, nonspecific attachment of sperm to the genital system epithelium are prevented (26). Mature sperm cells, according to research, have a higher net negative charge than immature sperm cells (27, 28). High levels of sialic acid residues on the sperm membrane are assumed to indicate normal spermatogenesis and sperm maturation. Morphologically normal and motile sperm cells have a higher net negative charge than abnormal and immature sperm cells (29). This difference in electrical charge observed in the sperm membrane has opened the door to investigate different methods in sperm selection, mainly Zeta test and electrophoresis methods.

SPERM SELECTION WITH THE ZETA TEST

In a protein-free environment, the sperm membrane's negative electrical charge, known as the zeta potential or electrokinetic potential, causes the sperm to adhere to surfaces. In the culture medium without serum or protein supplement, the sperm adhere to the glass slides. The sperm demonstrate increasing motility when the supplement is added to the culture media. The additional proteins are considered to neutralize the sperm's net negative zeta potential charge (30). Sperm

cells do not require expensive electrophoresis equipment, extreme pH conditions, UV irradiation, or buffers, because they are not exposed to high voltage electricity (30, 31). Zeta potential is higher in sperms with preserved chromatin integrity and lower DNA damage rate (32). One of the advantages of the Zeta method is that it may be applied effectively on sperm that has been frozen and stored for a long time (33). However, it is not preferred for oligozoospermic patients due to its low sperm recovery rate. Compared to classical methods, it has been shown to isolate mature sperm with better morphology, higher motility, and preserved DNA integrity (34, 35).

SPERM SELECTION BY ELECTROPHORESIS

Classical electrophoresis principles are used in this method, and negatively charged sperms are drawn towards the positive electrode (36). The primary rationale for this application is based on the idea that sperm cells with negatively charged membranes are motile and have normal morphology and DNA structure. This method allows sperms to be protected from oxidative stress damage and rapidly differentiate morphologically normal and motile sperm from other cells. It has been suggested that sperm obtained using this technique are not contaminated with different cell types and show progressive motility, normal morphology, and lower DNA damage levels (19).

Recently, it has been demonstrated that sperm selection by micro-electrophoresis results in relatively more minor DNA damage. As a result of negatively charged sperm selection using micro electrophoresis, increased fertilization rate and blastocyst development were observed (37). Micro-electrophoresis, an exceedingly fast and simple procedure that does not require complex instruments, may be used to select strongly negatively charged sperm. The healthier sperm cells may be selected using micro-electrophoresis, considering the inverse ratio between the high negative charge and apoptotic findings (38).

SPERM SELECTION WITH ANNEXIN V

Annexin V is a 35 kDa phospholipid-binding protein with a high affinity for the negatively charged phospholipid phosphatidylserine (PS) in the presence of physiological Ca^{2+}. It cannot pass through healthy sperm membranes (39). The binding of Annexin V to a sperm indicates that the sperm enters the apoptotic

process, membrane integrity is impaired, and oocyte fertilization capacity is lost (40). As an early indicator of apoptosis, the PS moves from the inner to the outer region of the plasma membrane. Annexin V may be used to detect this, and spermatozoa that are undergoing apoptosis may be eliminated (41).

MAGNETIC ACTIVATED CELL SORTING

In the light of Annexin V approach, magnetically activated cell sorting (MACS) is another technique that uses sperm membrane properties to isolate sperm that do not show the phospholipid phosphatidylserine in the outer membrane (Annexin V-negative). This application was first applied by the Glander group in Leipzig, Germany (42). In the affinity column with the sperm suspension, annexin V-conjugated paramagnetic microspheres are exposed to a magnetic field. When annexin V-positive apoptotic sperm cells are exposed to a strong magnetic field, they bind to these microspheres and remain in the separation column. Annexin V-negative sperm cells with normal membrane and not undergoing the apoptotic process are not affected and pass freely through the colon. Magnetic field-activated cell separation efficiently removes apoptotic sperm cells from sperm suspensions and selects sperm with normal head, neck and tail regions and significantly less DNA damage (43, 44).

BINDING TO HYALURONIC ACID

Hyaluronic acid is an essential component of the extracellular matrix that surrounds the cumulus-oocyte complex (25). It forms a compound with a glycodelin-interacting protein that captures and concentrates glycodelin-C, which is required for the attachment of sperm and the zona pellucida (45). Polysaccharide-binding receptors can only be found on the outer membrane of sperm cells that have undergone spermatogenesis and maturation (46, 47). According to recent research, the percentage of sperm linked to hyaluronic acid represents sperm maturation and function. By exploiting these characteristics, physiological intracytoplasmic sperm injection (PICSI) by incubating sperm cells in a culture medium containing hyaluronic acid or hyaluronan-coated chambers (48, 49). Although sperm cells with low chromatin damage and better chromatin condensation may be selected with this routinely applied method, findings supporting a clinically positive outcome in studies on fertilization and pregnancy rates are controversial (49, 50).

INTRACYTOPLASMIC MORPHOLOGICALLY SELECTED SPERM INJECTION

Morphological assessment of sperm has been used for a long time to analyze semen quality in classical sperm selection practices. With the introduction of digital microscopes, it has become possible to examine the motile sperm organelle morphology (51). Intracytoplasmic morphologically selected sperm injection (IMSI) is a combination of motile sperm organelle morphological examination and ICSI application. It has been routinely used in many ART centers (52). Microscopes with 6000x magnification, integrated with the micromanipulation system, made it possible to select motile sperm with a small number of vacuoles, nuclei with normal morphology, and normal head, mid-piece, and tail structure (53). IMSI has been shown to increase fertilization and implantation rates when compared to traditional ICSI techniques (54). Some studies, on the other hand, found no significant differences in fertilization and pregnancy rates between these methods (55). Although some studies implied that the existence of big vacuoles in the sperm nucleus is linked to a high amount of nuclear fragmentation, others claim that the presence of vacuoles in the nucleus has no relation to DNA fragmentation (56). IMSI applications require a high level of technical experience. It stands out as a promising method for patients diagnosed with severe oligoasthenoteratozoospermia and patients with only a few oocytes. Although findings in the literature do not support each other, selection based on motile sperm organelle morphology is seen as a potential method for selecting high-quality sperm.

FUTURE APPROACHES IN SPERM SELECTION

There is a need to develop non-invasive, low-cost, easy-to-apply, and safe methods in clinical applications. Although some recently developed techniques have a more sophisticated infrastructure, their use in ART is being investigated with experimental analysis. Among these, the Raman microspectrophotometer is among the well-known methods.

With Raman microspectrophotometer, analysis of sperm intracellular components, analysis of DNA structure, and detection of possible damages may be possible. It is not an invasive method and is far from causing damage to the sperm during analysis. These advantages make this method preferable. However, the changes in ambient temperature during the investigation affect the results and

the preparation of the samples to be analyzed takes time (57). Another method is interferometric phase microscopy. With this application, sperm cells can be viewed quantitatively, and no staining is required. However, in the application of the method, the step of immobilizing the sperms may bring about the possibility of damaging the sperms. Another important technique is confocal light absorption and scattering spectroscopic microscopy. In this application, which emerged as a combination of two different methods, more specific images can be obtained in the depth dimension of the cell at the micrometer level (58). No damage to cellular structures and DNA comes to the forefront as an advantage. However, sample preparation takes time, and standardization processes are very sensitive, which is among the limiting factors for the method.

Artificial intelligence technology has started taking its position among sperm analysis methods with experimental investigations, having evolved as a preferred approach to do visual inspection, categorization, and choosing tasks in various applications in medical and other sectors. In these applications, machine learning has been applied to categorize sperm cells according to manually detected properties or dictionary models loaded with image data (59, 60). Appropriate findings were obtained to select single sperm with high DNA integrity, which were directly consistent with sperm selection using manual microscopy (61).

CONCLUSION

Despite the significant development in sperm preparation methods in recent years, each emerging approach has advantages and disadvantages. Although many new techniques are tried to be put into practice routinely today, no application has emerged that has an extraordinary effect or achieves outstanding success in increasing pregnancy rates. At this point, it is more clearly understood that there is a need to observe natural processes from deeper and different perspectives to increase ART success rates. As our knowledge of sperm selection processes in the female reproductive system improves, scientists may design more advanced methods to mimic these processes. Successful selection of sperm with higher quality and genomic integrity will increase fertilization, embryo development, and pregnancy rates. In the future, the development of indication-specific sperm isolation techniques and their specific application to the individual may be a factor that increases the pregnancy success rate.

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