Submitted: 18 October, 2024 Accepted: 25 November, 2024 Published: 28 February, 2025 DOI:10.22514/jomh.2025.016

REVIEW

JOMH Journal of Men's Health

In vivo versus *in vitro* sperm selection

Michal Ješeta[1,*], Jana Antalíková[2], Adéla Doubravská[1], Lenka Mekiňová[1], Bartosz Kempisty[3,4,5], Pavel Ventruba[1], Igor Crha[1,6]

[1]Department of Gynecology and Obstetrics, University Hospital Brno and Faculty of Medicine, Masaryk University Brno, 62500 Brno, Czech Republic
[2]Laboratory of Reproductive Physiology, Institute of Animal Biochemistry and Genetics, Centre of Biosciences, Slovak Academy of Sciences, 84005 Bratislava, Slovak Republic
[3]Department of Veterinary Medicine, Nicolaus Copernicus University, 87-100 Torun, Poland
[4]Department of Human Morphology and Embryology, Division of Anatomy, Faculty of Medicine, Wroclaw Medical University, 50-368 Wroclaw, Poland
[5]Physiology Graduate Faculty, North Carolina State University, Raleigh, NC 27606, USA
[6]Department of Health Sciences, Faculty of Medicine, Masaryk University, 62500 Brno, Czech Republic

***Correspondence**
jeseta.michal@fnbrno.cz
(Michal Ješeta)

Abstract

The separation of human spermatozoa is an important step in therapy of human infertility. Given that male fertility is decreasing and, as a result, fertilization techniques based on microinjection of sperm into the cytoplasm are being used more intensively, this question is becoming increasingly relevant. In recent years, microfluidic sperm processing techniques have been increasingly used. These methods are simple and easy to use, however, the question is to what extent they select the correct sperm. They are essentially based mainly on motility and do not reflect other navigational approaches such as chemotaxis, thermotaxis or rheotaxis. This review compares traditional, advances and novel *in vitro* methods of sperm separation which are commonly used during human infertility therapy in context of *in vivo* sperm separation in female reproductive system.

Keywords

Spermatozoa; IVF; Sperm separation; ICSI; Microfluid separation; Thermotaxis; Chemotaxis

1. Introduction

In the ejaculate of a healthy man, there are 40–500 million sperm, but only a few dozen to a maximum of hundreds reach the ovulated oocyte. This means that there is usually a huge surplus of sperm and there is a lot of room for selection. Sperm separation *in vivo* is, therefore, very intense and is a multistep process that starts immediately after ejaculation at the level of cervix uteri. Once the sperm penetrates the uterus, it is phagocytosed by leukocytes that penetrate the endometrial wall. It is only after the sperm has overcome this barrier that they enter the isthmus of the oviduct, where they are in an environment of relative safety, and from where they move in waves into the oviduct in search of the ovulated oocyte. This is the next selection step, and only spermatozoa with the highest fertilization capability and the best features for supporting embryo development have the opportunity to fertilize. Spermatozoa populations exhibit significant heterogeneity, with only a small subset within the ejaculate retaining the capacity for fertilization.

In modern assisted reproduction techniques, there is an increasing utilization of *in vitro* fertilization (IVF) employing intracytoplasmic sperm injection (ICSI). Multiple approaches exist for sperm separation under *in vitro* conditions. Typically, there is an abundance of spermatozoa available, allowing for rigorous selection of spermatozoa. Unfortunately, current methods for sperm selection are not perfect and do not allow to make an excellent selection for specific subpopulation best for IVF [1]. This phenomenon is attributable to several factors. Certain methodologies were initially developed for conventional IVF techniques and subsequently adapted for ICSI. In some instances, limitations are imposed by the operational environment and specimen handling procedures. Frequently, this aspect of IVF is underestimated in clinical settings, and due to the efficiency of the process and the ease of specimen handling, the most straightforward method is often selected. While this approach yields satisfactory results, it is often used and predominantly based on the principle of sperm selection according to motility. Especially in ICSI, sperm separation is crucial due to the absence of a natural barrier. Selecting defective sperm or those with DNA fragmentation can harm fertilization, embryonic genome activation and overall embryo development.

2. Sperm selection *in vivo*

2.1 Sperm selection at the level of cervical mucus

After coitus, sperm migrates from the seminal plasma after liquefaction, from the acidic environment of the vagina into the cervical mucus. The structure of cervical mucus helps

remove adsorbed molecules and sterols from the sperm plasma membrane. A large number of sperm cells do not pass through cervical mucus. No dead, immobile, or morphologically abnormal sperm cells pass through this barrier. Leukocytes infiltrating the cervical mucus, together with sperm, produce reactive oxygen species (ROS) and trigger sperm capacitation. Cervical mucus contains prostaglandins, which stimulate sperm motility and penetration [2]. Only hyperactivated sperm cells can swim through this environment. The movement of spermatozoa in the cervical mucus occurs mainly at the level of the interstitial spaces in mucin micelles. A reduction in these spaces leads to reduced motility and fertility. During ovulation, the cervical mucus is highly watery, and is the most permeable to sperm. In the post-ovulatory period, thicker mucus is secreted, which forms a barrier and prevents sperm from penetrating the endometrial space [3].

2.2 Sperm selection in the oviduct/fallopian tube

Sperm reaches the fallopian tube roughly 5–10 minutes after ejaculation. After transport via utero-tubal junction, sperm interacts with the epithelium and forms a reservoir in isthmus. Sperm function is further regulated in oviduct by different ways, including proteins, mRNA and extracellular vesicles [4]. Oviductal fluid is hyperosmotic (350–355 mOsm/kg) in comparison to seminal plasma and has a higher pH (7.4) compared to the uterus (6.8) [5]. This environment modulates sperm motility, viability and the process of capacitation consisting of several events such as an increase in sperm plasma membrane fluidity with cholesterol efflux, phospholipid scrambling, an increase of intracellular HCO_3^-, and activation of Cyclic Adenosine Monophosphate/Protein Kinase A (cAMP/PKA) pathway. Exposure of sperm to large changes in HCO_3^-, Ca^{2+} as well as H^+, Na^+ and K^+ affects membrane potential (Em) and the intracellular pH [6]. In human sperm, the regulation of Em has been linked to male fertility through modulation of ion channels and transporters such as CatSper (sperm-specific Ca^{2+} channel) and voltage-gated proton channel (Hv1) [7, 8]. The idiopathic and asthenozoospermic infertile men had more depolarized Em than fertile men [9].

Sperm cells do not capacitate synchronously and the effect of oviductal fluid is depending on estrous cycle. The entire process of sperm navigation from the ejaculation site to the ovulated oocyte is controlled by three systems: chemotaxis, thermotaxis and rheotaxis.

2.2.1 Chemotaxis

Chemoattractants produced by the female reproductive tract stimulate the movement of sperm towards the oocyte. However, only hyperactivated spermatozoa can respond to secretions from the cumulus cells of the oophore and oocyte [2]. A subpopulation of sperm cells exhibits a chemotactic response, so they have a greater probability of locating and fertilizing the egg [10]. Moreover, less than 30% of human sperm cells express C-X-C chemokine receptor type 4 (CXCR4) [11], suggesting that only a portion of sperm cells would be responsive to the stromal cell-derived factor 1 (SDF1) chemokine. If sperm chemotaxis is crucial for fertilization, it is expected that the chemoattractant gradient would be maintained throughout the period of the oocyte's viability and fertilization potential (in humans, about 24 hours after ovulation) [12]. This would require a constant supply of chemoattractant, rather than a single event like the limited release of follicular fluid during ovulation. Thus, it is more likely that the cumular cells produce sperm chemoattractant(s) after ovulation, when it is located at the fertilization site within the oviduct (Fig. 1). These factors are similar in mammals and human sperm can even respond to follicular factors from other mammalian species [13]. The primary chemoattractants found in follicular fluid include progesterone, atrial natriuretic peptides (ANPs), RANTES (regulated on activation, normal T cell expressed and secreted) chemokines and other substances. While progesterone effectively attracts sperm cells in laboratory settings, it lacks a rational basis for the mechanism of sperm selection *in vivo*. Chemokine ligand 20 (CCL20), which is present in follicular fluid and is produced by oocytes and surrounding follicular cells, attracts sperm via the C-C chemokine receptor type 6 (CCR6) receptor (expressed on human sperm cells), and neutralizing of CC chemokine CCL20 in follicular fluid inhibits chemotaxis [14]. Specific odorant receptors corresponding to chemoattractants (*e.g.*, human olfactory receptor 17-4 (hOR17-4)) have been detected on the surface of sperm plasma membranes. This type of receptor is mainly localized in olfactory cells, but more than 20 species of this receptor family are also present in the midpiece and flagellum of the sperm [15]. The signal is transmitted via G-proteins (GPCRs), which activate adenylate cyclase and guanylate cyclase. Increased synthesis of cAMP and cGMP (cyclic guanosine monophosphate) opens the CatSper channel and allows Ca^{2+} to enter cells. This increases sperm motility towards the concentration gradient of chemoattractants [16].

2.2.2 Thermotaxis

However, chemotaxis acts only at a short distance from the oocyte because of the peristaltic activity of fallopian tubes. In contrast, thermotaxis is resistant to fallopian tube movements, and thus acts over longer distances (Fig. 1). Similarly to chemotaxis, only capacitated spermatozoa present thermotaxis [17]. Sperm cells can move along a temperature gradient from cooler areas to areas of higher temperatures. The human sperm cells are sensitive to a temperature difference [10]. During ovulation, the temperature is lower (34.7 °C) at the site of the oviductal reservoir in the isthmic part of the fallopian tube and, conversely, the temperature is elevated by almost two degrees (36.4 °C) at the oocyte fertilization site [18]. Thus, capacitated spermatozoa respond more strongly to thermotaxis and later to chemotaxis [19]. Opsins function as thermosensors in mammalian spermatozoa and act as photosensors in the visual pathway. The most common in sperm cells are rhodopsins and melanopsins, which are mainly located in the equatorial plane of the head, the postnuclear cap and the medial region. As with vision, opsins trigger different signaling pathways in the sperm. Rhodopsins stimulate the transducin/cyclic nucleotide pathway and melanopsins stimulate the phospholipase C (PLC) pathway [20]. The PLC signaling pathway is activated through GPCRs and leads to the opening of TRPV1 (transient receptor potential cation channel subfamily V). In humans, TRPV1 is

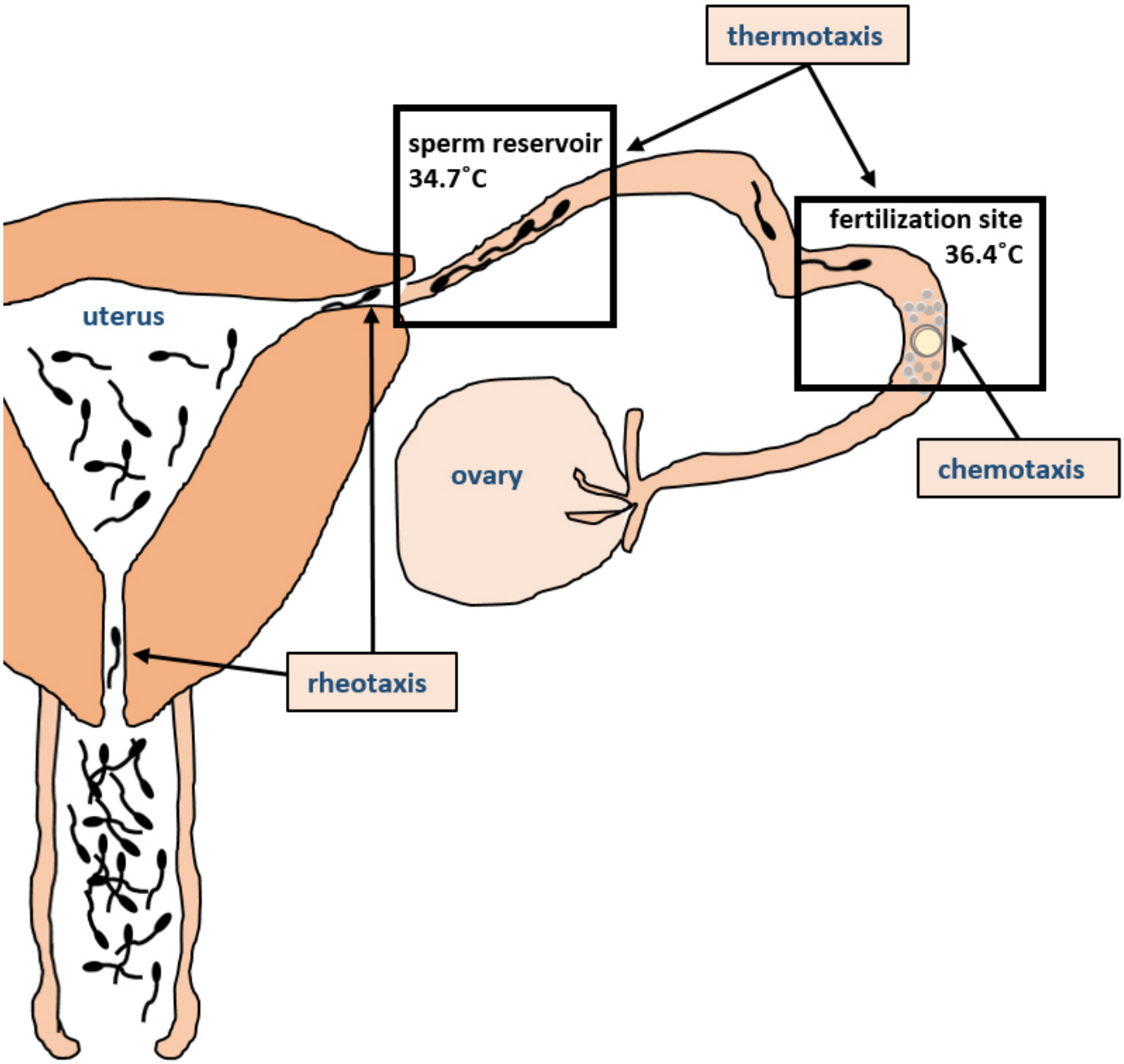

FIGURE 1. Sperm selection *in vivo*. Rheotaxis works at the level of the cervix uteri and uterotubal junction, thermotaxis navigate the sperm from the isthmus of the oviduct to the site of fertilization, and chemotaxis ensures the navigation of the sperm directly to the ovulated oocyte.

found in both sperm and testes. It serves as one of the receptors that respond to temperature fluctuations and increase motility in the direction of the temperature gradient [21].

2.2.3 Rheotaxis

One of the major orientation mechanisms in the female reproductive system is positive rheotaxis for sperm (Fig. 1). This involves movement against fluid flow. After coitus, under the influence of prolactin, secretion increases in the epithelium of the fallopian tube, and oviductal secretion is driven by cilia and muscle contractions toward the uterus [22]. There is a smooth flow of secretions from the oviduct into the uterus [23, 24]. The rotation of spermatozoa about their longitudinal axis, called "rolling", stimulates the movement of the sperm against the flow of the fluid.

After reaching the oocyte, sperm cells are further selected as they penetrate the cumulus oophorous, a structure surrounding the oocyte, consisting of granulosa cells embedded within the extracellular matrix, in which hyaluronan is one of the main components. Only spermatozoa with a high fertilization capacity are able to penetrate, bind to the zona pellucida of the oocyte and undergo acrosomal exocytosis. During this process, the fusion of the plasma and the outer acrosomal membranes is followed by the release of the acrosomal content, leaving the surface of the sperm head covered only by the inner acrosomal membrane and exposing new proteins required for binding and fusion with the oolemma [25].

Following insemination, the female reproductive system

eliminates hundreds of millions of sperm cells within hours through phagocytosis. This process occurs without triggering inflammation due to apoptosis, which ensures that the sperm cells undergo controlled death. The apoptotic cascade generates markers such as phosphatidylserine that enable the recognition and subsequent removal of dying sperm cells. This mechanism facilitates quiet phagocytosis of aging sperm without provoking an inflammatory response [26]. Human sperm cells exhibiting externalized phosphatidylserine show reduced oocyte penetration capacity [27].

3. Sperm selection *in vitro*

Since the ICSI technique is used extensively for IVF of human oocytes, and sperm is usually in great surplus, it is very important to carefully select a sperm suitable for fertilization. In the case of the ICSI technique, the above-mentioned *in vivo* mechanisms are not used and the embryologist selects the sperm they consider the best *in vitro* and then injects it into the cytoplasm of the oocyte. Several separation systems have been developed based on various principles. It should be noted that these systems have a number of positive outcomes, but sometimes these methods can have a negative impact on the sperm. Sperm separation methods are inspired by the naturally occurring processes in the female reproductive tract. The traditional sperm selection methods are based on sperm motility. More advanced methods use a variety of approaches, including the assessment of morphology, sperm membrane surface charge, sperm membrane integrity, and sperm binding properties. In general, the most commonly used sperm separation techniques in IVF laboratories are swim-up and density-gradient sperm centrifugation.

3.1 Traditional separation methods

In the group of traditional methods are the oldest methods which are mainly based on sperm motility and morphology. However, these methods have been thoroughly tested and are still among the most commonly used until today.

3.1.1 Swim-up

Swim-up is a simple method based on sperm motility that separates motile from non-motile sperm. Ejaculate and sterile medium are introduced into a tube and gently homogenized using a sterile pipette. Subsequently, the samples are centrifuged at 300g for 10 minutes, after which the supernatant is carefully removed. The resulting pellet is resuspended in 1 mL of medium, gently homogenized, and centrifuged again for 5 minutes at 300g. The supernatant is once more removed, and the final pellet is resuspended in sterile medium. After centrifugation, the sperms are in a medium containing glucose and calcium ions to promote sperm movement. To increase the surface area, the tube is placed at an angle of 45° (Fig. 2). This step increases the surface between the medium and the semen and improves the capability of the sperm cells to swim out of the semen and to reach the medium. Incubation is carried out for up to one hour at 37 °C, it is important to monitor the sample and possibly terminate the swim-up earlier if the concentration of motile sperm is too high, and there is a risk that non-mobile/non-progressive sperm will also be recovered. Sperm cells prepared using the swim-up method are referred to as having longer telomeres and higher DNA integrity [28–30]. Samples selected by the swim-up technique contain spermatozoa with higher motility and fewer apoptotic and necrotic spermatozoa [31] compared with density gradient centrifugation [32]. However, a disadvantage of this method, may be the initial centrifugation, which leads to an unnatural accumulation of all cellular structures of the ejaculate into a single pellet. In particular, in patients with higher round cell counts, this may result in a negative effect of dead or damaged sperm cells or leukocytes on live motile sperm. Moreover, repeated centrifugation can increase the levels of oxygen radicals that damage the integrity of sperm DNA [33]. This method is based only on motility, and in the case of dead spermatozoa and leukocytes, there are risks related to oxidative stress and iatrogenic damage after centrifugation. For this reason, a direct swim-up is sometimes used, where sperm cells are only allowed to travel from the ejaculate into the medium without centrifugation.

3.1.2 Density gradient centrifugation

This method is based on the centrifugation of semen through a gradient of dense solutions (usually two, *e.g.*, 40% and 80%). During this process, sperm is separated from bacteria, fungi, and, to some extent, viruses. At the end of the centrifugation process, each sperm is at a different depth in the column according to its own density. Morphologically normal, viable and motile sperm cells reached the bottom of the tube. They have a density higher than 1.12 g/mL. Morphologically immature and immobile spermatozoa were observed in the upper layers of the medium. The density of these spermatozoa is low, in the range of 1.06–1.09 g/mL. In the layer above immature spermatozoa, other particles of the ejaculate, such as leukocytes, debris or dead spermatozoa are found. At the top of the medium, the seminal plasma remains [34] (Fig. 3). It has also been shown to produce better results in sperm capacitation, acrosomal reaction, and faster sperm hyperactivation than swim-up techniques. On the other hand, more oxygen radicals were produced in sperm using this method than when the direct swim-up method is used [35]. Occasionally, the swim-up method was combined with a density gradient. This step increases the proportion of motile sperm and decreases the proportion of pathological sperm compared with these separation methods used alone. The combination of density gradient centrifugation followed by the swim-up method is particularly suitable for ICSI because of the low number of spermatozoa that are separated [36]. However, this method requires a sufficient number of motile spermatozoa to begin; therefore, this combination of methods is not suitable for severe oligoasthenozoospermia.

3.2 Advanced separation methods

In the group of advanced methods are methods aimed at selecting sperm on the basis of various physiological parameters. These include methods that remove apoptotic sperm from sample or methods that select more matured sperm. These methods often experienced a time when they were popular, but

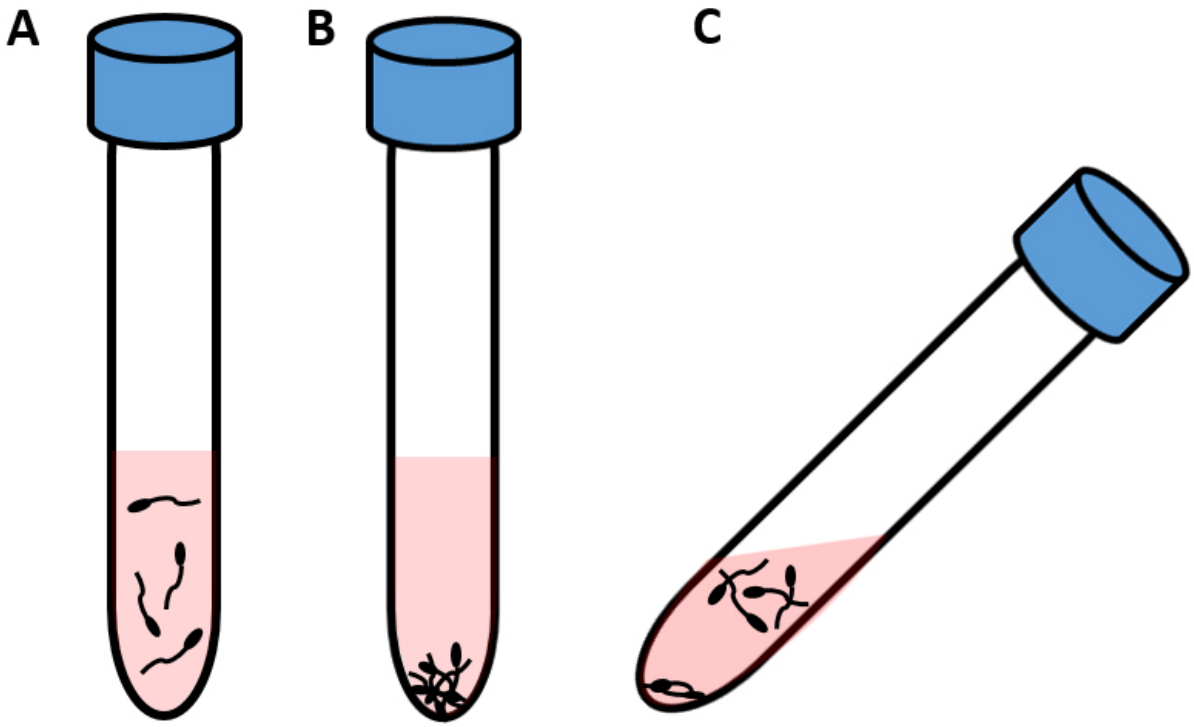

FIGURE 2. Swim-up method. (A) semen sample before centrifugation; (B) sample after centrifugation, all cells are in the pellet; (C) sample after 45–60 minutes of incubation at a 45° angle, motile spermatozoa are at the surface, while non-motile spermatozoa remain in the pellet.

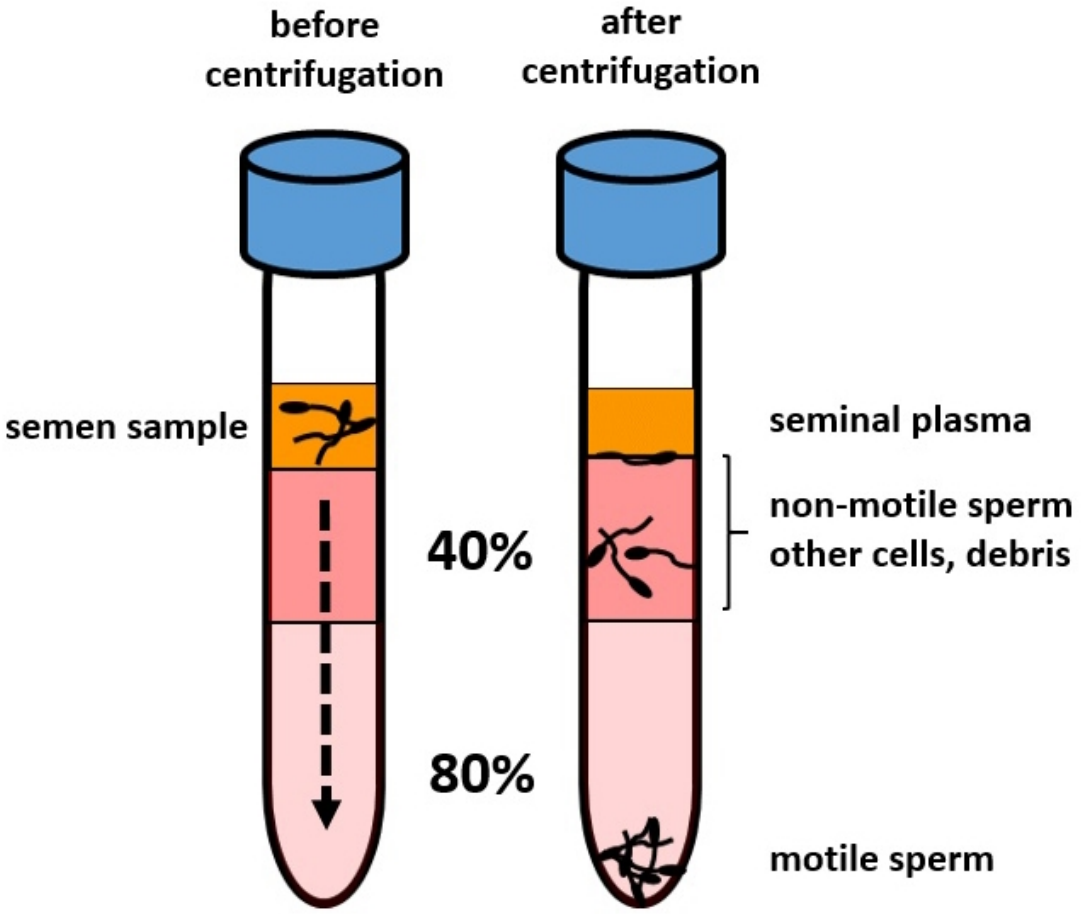

FIGURE 3. Sperm separation by density gradient centrifugation.

most of them are not widely used today.

3.2.1 Magnet activated sperm sorting

Magnetic-activated cell sorting (MACS) is based on the separation of apoptotic sperm cells using a magnet. Apoptotic sperm have phosphatidylserine on their membrane surfaces [37]. Increased amounts of externalized phosphatidylserine are associated with poorer semen parameters such as reduced motility, abnormal sperm morphology and lower ejaculate concentration [38]. The MACS method uses Annexin V protein, which has a high affinity to phosphatidylserine (typical for apoptotic cells). For this method, Annexin V is conjugated to magnetic nanoparticles that, after a short incubation, bind to the phosphatidylserine residues of apoptotic sperm to form magnetic objects. In the column, labeled apoptotic sperm cells were collected using a magnet (Fig. 4). Non-apoptotic sperm pass through the column and are used for further processing [39]. In MACS, it is better to use a basic separation technique (density gradient or swim-up) as the first step and after that MACS. MACS has limitations in terms of sperm concentration and loading volume because of its use of a column. Indeed, loading of raw semen onto the MACS column may reduce the filtering function, and a meta-analysis reported a positive effect of MACS on IVF outcomes in terms of pregnancy and miscarriage rates [40]. Studies focusing on the proportion of spermatozoa with fragmented DNA after MACS are more relevant for adequate evaluation of MACS. From this perspective, it was reported that the MACS method yields very good results (a strong reduction in the proportion of spermatozoa with fragmented DNA) in some patients [41]. This method is unsuitable for intrauterine insemination (IUI) because of the limited number of spermatozoa obtained after MACS. The preparation of spermatozoa for IUI can reduce the total number of spermatozoa suitable for IUI, which can be especially limiting in this procedure.

3.2.2 Selection by sperm binding to hyaluronic acid

Hyaluronic acid (HA) is a glycosaminoglycan found in the expanded cumulus complex of oocytes and cumulus cells. This is the first barrier on the oocyte side encountered by the sperm. Sperm have different affinities for hyaluronic acid, which is used for sperm separation. In general, mature sperm cells preferentially bind to the hyaluronic acid envelope. Immature sperm cells have low levels of Heat Shock Protein Family A (HspA2) protein, which is responsible for binding to hyaluronic acid. The HspA2 protein also regulates chromatin remodeling, and sperm cells deficient in this protein are often

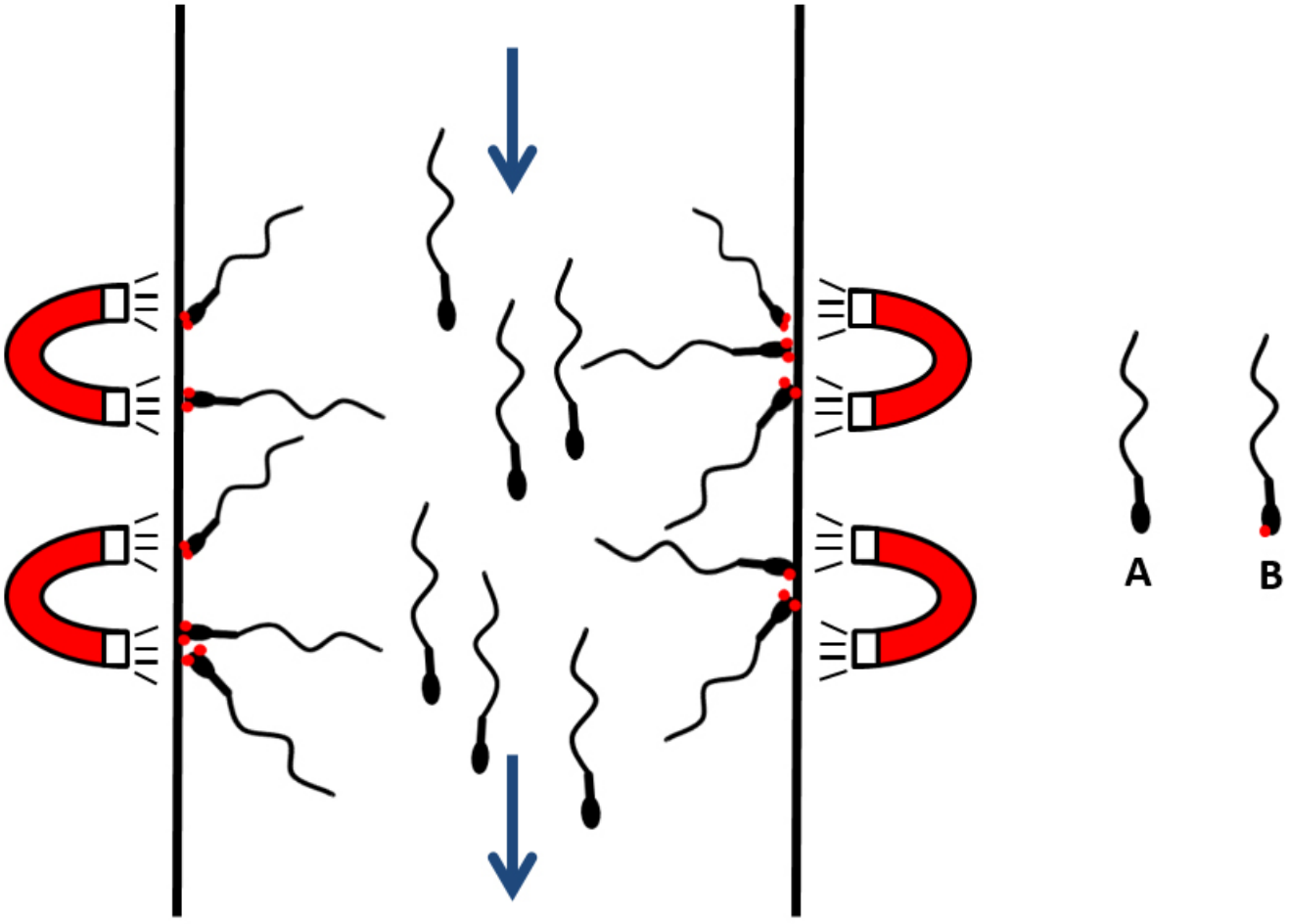

FIGURE 4. MACS selection system. (A) nonapoptotic spermatozoa, (B) apoptotic spermatozoa with connected Annexin V with magnetic particle.

aneuploid [42]. This property is used to positively select sperm prior to ICSI, a method known as PICSI (Physiology election spermatozoa for ICSI). In this method, the heads of matured sperm cells were bound to a hyaluronic acid hydrogel at the bottom of a PICSI dish. The unmated sperm floats freely and does not bind [43]. The selected sperms were then used for ICSI. A methodologically easier alternative to PICSI is the SpermSlow method, which uses viscous medium containing hyaluronic acid. The matured sperm in this medium slow down their movement. Hyaluronic acid-binding methods reduce the frequency of chromosomal disomy and diploidy and there have been fewer early miscarriages [44]. Comparison of PICSI and MACS, when the quality of embryo blastulation, number of implanted blastocysts and number of clinical pregnancies were evaluated, showed no significant difference between the methods. Both methods were found to be similarly effective in sperm selection [45].

3.2.3 Sperm selection using Zeta potential

This method is based on sperm maturation and currently is not frequently used. This is based on the fact that mature sperm have an electrical charge on their surface, known as zeta potential, ranging from −16 to −20 mV, which decreases during capacitation. The matured, negatively charged sperm adheres to the positively charged tube wall, and during centrifugation, the adherent sperm cells are separated from the other sperm. Mature sperm cells are then obtained by washing the tube with medium [46]. This method is fast, simple and inexpensive. Selected sperm have higher DNA integrity and better morphology. The method is suitable for ICSI as well as for selection of thawed sperm after cryopreservation [47]. Compared to the MACS method, the Zeta method yielded a higher percentage of sperm with a normal acrosome, but at the same time, more sperm with fragmented DNA were separated than the Zeta potential selection method. When compared to the sperm-hyaluronic acid binding method, Zeta potential selection appears to be a better separation technique, particularly as the percentage of sperm with fragmented DNA was significantly reduced [48].

3.2.4 IMSI

The IMSI (Intracytoplasmic Morphologically Selected sperm Injection) method involves the selection of sperms according to their morphology. A microscope system acquired with camera and specific software with total magnification (6000×) [49, 50] was used to select spermatozoa on the basis of the morphology of the acrosome, post-acrosomal lamina, neck, flagellum, mitochondria and nucleus. Presence of vacuoles was assessed and they did not occupy more than 4% of the head surface area. Vacuoles may adversely affect capacitation and acrosomal response. Sperms with large vacuoles are also more likely to have fragmented DNA [51]. The disadvantage of this method is the risk of reduced viability during microscopic evaluation [52]. This method has now been replaced by more modern methods.

3.3 Novel approaches in sperm separation

New approaches in sperm separation are still being intensively developed. All three navigational processes (thermotaxis, chemotaxis and rheotaxis) are often used in this research. Microfluidic separation chip methods are simple, reliable and are becoming more widely used.

3.3.1 Microfluidic separation chips

Microfluidic sperm separation chip (MFSS) techniques mimic the natural selection of sperm in the female reproductive tract better than the previous methods. These methods do not centrifuge the semen. This eliminates the risk of secondary damage due to centrifugation. This method works with a small sperm sample volume, short processing time, and separation is a one-step process without centrifugation and other manipulation with samples in the laboratory. Microfluidic chip separation techniques can be divided into active and passive sperm separation methods. Active microfluidic devices use external forces, such as electrophoresis or hydrostatic pressure [53, 54]. Sometimes a continuous flow of medium is used and motile sperm can swim in different directions from the media flow and can be collected in a separate chamber. Passive sperm separation is the most commonly used technique for sperm separation which use passive barriers, labyrinths or semipermeable membranes. These systems can separate sperm without damaging their morphology or DNA integrity by mimicking the *in vivo* environment, allowing selection of the best and most competent sperm. A partial limitation of this sorting method is its capacity to accommodate only a small sample volume. It can make problems in patients with very low concentration in normal or higher volume of ejaculate, when classical swim-up can be more effective. This constraint arises from the inherent design and mechanism of the microfluidic system, which relies on lamellar flow and controlled diffusion, achievable only in micrometer-scale channels [44]. On the other hand, the ability to process even very small volumes of ejaculate with brief separation times can be very suitable in patients with normal concentration in very small volume of ejaculate. This procedure is a single-step process that eliminates the need for centrifugation or further manipulation of the sperm. As a result, the negative effects of centrifugation and the risk of exposure to high concentrations of oxygen radicals are eliminated, thus the DNA is better protected from fragmentation in contrary to classical methods [55]. These techniques work directly with liquefied ejaculate, without further dilution or pipetting. This minimizes the risk of confusion or damage due to carelessness in the laboratory. Owing to limitations in sample size, it is considered a method for characterizing spermatozoa rather than sperm cell processing. However, these methods are currently used more often for their advantages.

Several MFSS methods use a semi-permeable membrane separating the ejaculate and a clean medium into which the sperm swims through the pores in the membrane, which are 8 μm in size. This technique is simple, user-friendly and yields good results when used for sperm separation before ICSI. After transferring 850 μL of ejaculate, approximately 500 μL of sperm-containing medium was obtained. The disadvantage of this method is that it does not work well in patients with

concentrations below 10 mil/mL. Since the maximum capacity of this method is 800 μL of ejaculate, it is not suitable for preparing sperm for IUI.

3.3.2 Methods based on positive rheotaxis

The most basic method for separating sperm via rheotaxis involves a narrow channel connecting the two droplets. The smaller droplet contains the ejaculate, while the larger droplet has a greater volume of the medium. Owing to the volume difference, fluid flows from the larger droplet to the smaller droplet, and sperm swims upstream into the larger droplet through positive rheotaxis. However, more sophisticated devices are now available [56]. For instance, a K-shaped microfluidic chip features two inputs and outputs linked by channels. In this system, non-motile sperm follow the laminar flow across the channel, whereas motile sperm with sufficient velocity can move out of the laminar flow into the multilayer flow and reach the exit [57]. This category also includes methods using specialized petri dishes designed for ICSI, covered with a semicircular double-layered film. One layer of the film contains 60 patterned microchannels and is partially covered by a film that divides the entry and exit points. The medium fills the exit site and microchannels, while sperm is placed at the entry site. Only motile sperm cells that move along the channel edges reach the central exit site in the petri dish [58].

3.3.3 Separation techniques on the base of chemotaxis and thermotaxis

Acetylcholine, progesterone (P4) and atrial natriuretic peptides function as chemoattractants [59].

A concentration gradient of these substances was established. This methodology effectively separates less motile spermatozoa while concurrently isolating a higher percentage of spermatozoa with normal morphology, intact acrosome and a reduced proportion of spermatozoa with fragmented DNA compared to the swim-up method. It is noteworthy that only capacitated spermatozoa exhibit a chemotactic response, resulting in a mere 2–12% of sperm reacting to chemoattractants in laboratory conditions. However, the efficacy of this method is significantly enhanced when combined with thermotaxis. The temperature at the outlet side is maintained at human body temperature, while the inlet side is set 2 °C lower. Consequently, a greater number of spermatozoa reach the exit side compared to the utilization of the chemotaxis method in isolation [56]. Chemotaxis is the most specific sperm navigation system based on the guidance of sperm over short distances. Only a small part of the sperm is sensitive to chemotaxis, which makes this system an interesting model for efficient sperm separation before micromanipulation techniques, when we only need a small amount of the best spermatozoa anyway. In evaluation of microfluidic system employing chemotaxis and thermotaxis, sperm population was selected with better morphology, motility, acrosome reactivity and DNA integrity in comparison with the swim-up method [60].

There are a number of methods that can be used for separation of human sperm before they are used to therapy of human infertility. When choosing which separation methods to use, it is very important to first know the spermiogram and the method which will be used for fertilization. It is also advisable to know the level of DNA integrity and the level of reactive oxygen radicals in the seminal plasma. If the sperm are intended for intra uterine insemination, it is not advisable to use methods that significantly select the sperm so that only a very small amount of sperm remains in the sample after separation (MACS and MFSS methods working with a small volume of ejaculate).

For *in vitro* fertilization methods, this does not apply and all the mentioned techniques can be used. The most frequently used are traditional methods swim-up and density gradient centrifugation (DGC). These methods are more and more replaced by the MFSS method, which probably replace them in the future [61]. If the man has a high proportion of sperm with fragmented DNA, it is advisable to supplement the traditional separation in combination with the MACS method or to use one of the MFSS methods which significantly reduce the proportion of sperm with fragmented DNA [62] and had a higher production of top quality blastocyst after IVF in compare to DGC [63]. If is the man with a leucocytospermia it is not advisable to use methods where the sample is centrifuged (swim-up) to avoid iatrogenic damage to the sperm by leucocytes [64]. In men with very low sperm concentrations, traditional methods of separation (DGC) should be used with caution and, if necessary, the volume of the wash media should be modified to avoid sperm loss during the washing and centrifugation process. In the case of patients with high levels of oxygen radicals in seminal plasma, is not recommended to use swim-up and it is advisable to remove sperm from the seminal plasma immediately after liquefaction by DGS or some MFSS methods can be used successfully [65].

4. Conclusion

Historically, sperm separation techniques relied on large ejaculate volumes and centrifugation. Currently, efficient microfluidic chip systems which are used in clinical embryology can select the most viable sperm based on their motility. While this method is highly effective, it only considers a single sperm characteristic. There are a several separation approaches and it is important to choose the appropriate method based on the parameters of the spermiogram as well as the planned therapeutic methods. Natural sperm selection involves multiple stages, suggesting that *in vitro* selection should also incorporate various criteria. Consequently, an ideal approach may combine a passive separation method based on sperm movement with an active method utilizing positive thermotaxis or chemotaxis.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

AUTHOR CONTRIBUTIONS

MJ, JA, PV and IC—Conception and supervision. MJ, JA, BK and AD—Manuscript draft. MJ and LM—Preparation of table and figure. All authors have read and agreed to the published

version of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

FUNDING

This work was supported by Czech Ministry of Education, Youth and Sports (MSMT INTER-COST LUC 23009), Czech ministry of Health (MH CZ-DRO FNBr 65269705), project Andronet-COST CA20119 and Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences (VEGA-2/0074/24).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1] Pérez-Cerezales S, Ramos-Ibeas P, Acuña OS, Avilés M, Coy P, Rizos D, *et al*. The oviduct: from sperm selection to the epigenetic landscape of the embryo. Biology of Reproduction. 2018; 98: 262–276.

[2] Fernandes CB, Loux SC, Scoggin KE, Squires EL, Troedsson MH, Esteller-Vico A, *et al*. Sex-steroid receptors, prostaglandin E2 receptors, and cyclooxygenase in the equine cervix during estrus, diestrus and pregnancy: gene expression and cellular localization. Animal Reproduction Science. 2017; 187: 141–151.

[3] Olaniyan OT, Dare A, Okotie GE, Adetunji CO, Ibitoye BO, Eweoya O, *et al*. Ovarian odorant-like biomolecules in promoting chemotaxis behavior of spermatozoa olfactory receptors during migration, maturation, and fertilization. Middle East Fertility Society Journal. 2021; 26: 3.

[4] Soto-Heras S, Sakkas D, Miller DJ. Sperm selection by the oviduct: perspectives for male fertility and assisted reproductive technologies. Biology of Reproduction. 2023; 108: 538–552.

[5] Lavanya M, Archana SS, Swathi D, Ramya L, Arangasamy A, Binsila B, *et al*. Sperm preparedness and adaptation to osmotic and pH stressors relate to functional competence of sperm in Bos taurus. Scientific Reports. 2021; 11: 22563.

[6] Molina LCP, Luque GM, Balestrini PA, Marín-Briggiler CI, Romarowski A, Buffone MG. Molecular basis of human sperm capacitation. Frontiers in Cell and Developmental Biology. 2018; 6: 72.

[7] Darszon A, Labarca P, Nishigaki T, Espinosa F. Ion channels in sperm physiology. Physiology Review. 1999; 79: 481–510.

[8] Lishko PV, Kirichok Y, Ren D, Navarro B, Chung JJ, Clapham DE, *et al*. The control of male fertility by spermatozoan ion channels. Annual Review of Physiology. 2012; 74: 453–457.

[9] Calzada L, Tellez J. Defective function of membrane potential (psi) on sperm of infertile men. Archives of Andrology. 1997; 38: 151–155.

[10] Teves ME, Guidobaldi HA, Uñates DR, Sanchez R, Miska W, Publicover SJ, *et al*. Molecular mechanism for human sperm chemotaxis mediated by progesterone. PLOS ONE. 2009; 4: e8211.

[11] Zuccarello D, Ferlin A, Garolla A, Menegazzo M, Perilli L, Ambrosini G, *et al*. How the human spermatozoa sense the oocyte: a new role of SDF1-CXCR4 signalling. International Journal of Andrology. 2011; 34: e554–e565.

[12] Harper MJK. Sperm and egg transport. In Austin CR, Short RV (eds.) Reproduction in Mammals. I. Germ cells and fertilization (pp. 102–107). 2nd edn. Cambridge University Press: Cambridge, UK. 1982.

[13] Sun F, Giojalas L, Rovasio RA, Tur-Kaspa I, Sanchez R, Eisenbach M. Lack of species-specificity in mammalian sperm chemotaxis. Developmental Biology. 2003; 255: 423–427.

[14] Duan YG, Wehry UP, Buhren BA, Schrumpf H, Oláh P, Bünemann E, *et al*. CCL20-CCR6 axis directs sperm-oocyte interaction and its dysregulation correlates/associates with male infertility. Biology of Reproduction. 2020; 103: 630–642.

[15] Sun F, Bahat A, Gakamsky A, Girsh E, Katz N, Giojalas L, *et al*. Human sperm chemotaxis: both the oocyte and its surrounding cumulus cells secrete sperm chemoattractants. Human Reproduction. 2005; 20: 761–767.

[16] Ali MA, Wang Y, Qin Z, Yuan X, Zhang Y, Zeng C. Odorant and taste receptors in sperm chemotaxis and cryopreservation: roles and implications in sperm capacitation, motility and fertility. Genes. 2021; 12: 488.

[17] Bahat A, Tur-Kaspa I, Gakamsky A, Giojalas L, Breitbart H, Eisenbach M. Thermotaxis of mammalian sperm cells: a potential navigation mechanism in the female genital tract. Nature Medicine. 2003; 9: 149–150.

[18] Bahat A, Caplan SR, Eisenbach M. Thermotaxis of human sperm cells in extraordinarily shallow temperature gradients over a wide range. PLOS ONE. 2012; 7: e41915.

[19] Bahat A, Eisenbach M, Tur-Kaspa I. Periovulatory increase in temperature difference within the rabbit oviduct. Human Reproduction. 2005; 20: 2118–2121.

[20] Pérez-Cerezales S, Boryshpolets S, Afanzar O, Brandis A, Nevo R, Kiss V, *et al*. Involvement of opsins in mammalian sperm thermotaxis. Scientific Reports. 2015; 5: 16146.

[21] Roy D, Levi K, Kiss V, Nevo R, Eisenbach M. Rhodopsin and melanopsin coexist in mammalian sperm cells and activate different signaling pathways for thermotaxis. Scientific Reports. 2020; 10: 112.

[22] Ramal-Sanchez M, Bernabò N, Valbonetti L, Cimini C, Taraschi A, Capacchietti G, *et al*. Role and modulation of TRPV1 in mammalian spermatozoa: an updated review. International Journal of Molecular Sciences. 2021; 22: 4306.

[23] Ishimoto K, Gaffney EA. Fluid flow and sperm guidance: a simulation study of hydrodynamic sperm rheotaxis. Journal of The Royal Society Interface. 2015; 12: 20150172.

[24] Zhang Z, Liu J, Meriano J, Ru C, Xie S, Luo J, *et al*. Human sperm rheotaxis: a passive physical process. Scientific Reports. 2016; 6: 23553.

[25] Florman H, Ducibella T. Fertilization in mammals. In Neil JD, Knobil E, Neil JD (eds.) Physiology of reproduction (pp. 1–3296). 3rd edn. Elsevier: Amsterdam. 2006.

[26] Aitkem RJ, Baker MA. Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. International Journal of Developmental Biology. 2013; 57: 265–272.

[27] Grunewald S, Said TM, Paasch U, Glander HJ, Agarwal A. Relationship between sperm apoptosis signalling and oocyte penetration capacity. International Journal of Andrology. 2008; 31: 325–330.

[28] Santiso R, Tamayo M, Gosálvez J, Meseguer M, Garrido N, Fernández JL. Swim-up procedure selects spermatozoa with longer telomere length. Mutation Research. 2010; 688: 88–90.

[29] Marchesi DE, Biederman H, Ferrara S, Hershlag A, Feng HL. The effect of semen processing on sperm DNA integrity: comparison of two techniques using the novel Toluidine Blue Assay. European Journal of Obstetrics, Gynecology, and Reproductive Biology. 2010; 151: 176–180.

[30] Zhao F, Yang Q, Shi S, Luo X, Sun Y. Semen preparation methods and sperm telomere length: density gradient centrifugation versus the swim up procedure. Scientific Reports. 2016; 6: 39051.

[31] Ricci G, Perticarari S, Boscolo R, Montico M, Guaschino S, Presani G. Semen preparation methods and sperm apoptosis: swim-up versus gradient-density centrifugation technique. Fertility and Sterility. 2009; 91: 632–638.

[32] Rao M, Tang L, Wang L, Chen M, Yan G, Zhao S. Cumulative live birth rates after IVF/ICSI cycles with sperm prepared by density gradient centrifugation *vs*. swim-up: a retrospective study using a propensity score-matching analysis. Reproductive Biology and Endocrinology. 2022; 20: 60.

[33] Lee M, Park JW, Kim D, Kwon H, Cho MJ, Lee EJ, *et al*. Viscous cervical environment-on-a-chip for selecting high-quality sperm from human semen. Biomedicines. 2021; 9: 1439.

[34] Yamanaka M, Tomita K, Hashimoto S, Matsumoto H, Satoh M, Kato H, *et al*. Combination of density gradient centrifugation and swim-up methods effectively decreases morphologically abnormal sperms. Journal of Reproduction and Development. 2016; 62: 599–606.

[35] Ali AH, Ajina T, Ali MB, Mehdi M. Efficacy of density gradient centrifugation technique (DGC) in enhancing sperm cell DNA quality for assisted reproductive technique. Middle East Fertility Society Journal. 2022; 27: 22.

[36] Saylan A, Erimsah S. High quality human sperm selection for IVF: a study on sperm chromatin condensation. Acta Histochemica. 2019; 121: 798–803.

[37] Hernández-Silva G, López-Torres AS, Maldonado-Rosas I, Mata-Martínez E, Larrea F, Torres-Flores V, *et al*. Effects of semen processing on sperm function: differences between swim-up and density gradient centrifugation. The World Journal of Men's Health. 2021; 39: 740–749.

[38] Berteli TS, Da Broi MG, Martins WP, Ferriani RA, Navarro PA. Magnetic-activated cell sorting before density gradient centrifugation improves recovery of high-quality spermatozoa. Andrology. 2017; 5: 776–782.

[39] Tavalaee M, Deemeh MR, Arbabian M, Nasr-Esfahani MH. Density gradient centrifugation before or after magnetic-activated cell sorting: which technique is more useful for clinical sperm selection? Journal of Assisted Reproduction and Genetics. 2012; 29: 31–38.

[40] Pacheco A, Blanco A, Bronet F, Cruz M, García-Fernández J, García-Velasco JA. Magnetic-activated cell sorting (MACS): a useful sperm-selection technique in cases of high levels of sperm DNA fragmentation. Journal of Clinical Medicine. 2020; 9: 3976.

[41] Ješeta M, Boženková E, Žáková JŽ, Ventruba P, Crha I, Lousová E, *et al*. Magnetic-activated cell sorting in combination with swim-up efficiency improve effectivity of spermatozoa separation. Medical Journal of Cell Biology. 2018; 6: 55–60.

[42] Razavi SH, Nasr-Esfahani MH, Deemeh MR, Shayesteh M, Tavalaee M. Evaluation of zeta and HA-binding methods for selection of spermatozoa with normal morphology, protamine content and DNA integrity. Andrologia. 2010; 42: 13–19.

[43] Witt KD, Beresford L, Bhattacharya S, Brian K, Coomarasamy A, Hooper R, *et al*. Hyaluronic acid binding sperm selection for assisted reproduction treatment (HABSelect): study protocol for a multicentre randomised controlled trial. BMJ Open. 2016; 6: e012609.

[44] Jakab A, Sakkas D, Delpiano E, Cayli S, Kovanci E, Ward D, *et al*. Intracytoplasmic sperm injection: a novel selection method for sperm with normal frequency of chromosomal aneuploidies. Fertility and Sterility. 2005; 84: 1665–1673.

[45] Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Taraborrelli S, Arnone A, *et al*. Comparison of two ready-to-use systems designed for sperm–hyaluronic acid binding selection before intracytoplasmic sperm injection: PICSI *vs.* Sperm Slow: a prospective, randomized trial. Fertility and Sterility. 2012; 98: 632–637.

[46] Chan PJ, Jacobson JD, Corselli JU, Patton WC. A simple zeta method for sperm selection based on membrane charge. Fertility and Sterility. 2006; 85: 481–486.

[47] Said TM, Land JA. Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. Human Reproduction Update. 2011; 17: 719–733.

[48] Kam TL, Jacobson JD, Patton WC, Corselli JU, Chan PJ. Retention of membrane charge attributes by cryopreserved-thawed sperm and zeta selection. Journal of Assisted Reproduction and Genetics. 2007; 24: 429–434.

[49] Lo Monte G, Murisier F, Piva I, Germond M, Marci R. Focus on intracytoplasmic morphologically selected sperm injection (IMSI): a review. Asian Journal of Andrology. 2013; 15: 608–615.

[50] Lukaszuk K, Jakiel G, Wocławek Potocka I, Kiewisz J, Olszewska J, Sieg W, *et al.* IMSI-Guidelines for sperm quality assessment. Diagnostics. 2022; 12: 192.

[51] Teixeira DM, Miyague AH, Barbosa MA, Navarro PA, Raine-Fenning N, Nastri CO, *et al*. Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction. Cochrane Database of Systematic Reviews. 2020; 2: CD010167.

[52] Gat I, Raviv G, Baum M, Orvieto R. MSOME and IMSI: reasonable rationale, selective clinical value. Clinical and Experimental Obstetrics & Gynecology. 2019; 46: 679–689.

[53] Seo DB, Agca Y, Feng ZC, Critser J. Development of sorting, aligning, and orienting motile sperm using microfluidic device operated by hydrostatic pressure. Microfluidics and Nanofluidics. 2007; 3: 561–570.

[54] Ainsworth C, Nixon B, Aitken RJ. Development of a novel electrophoretic system for the isolation of human spermatozoa. Human Reproduction. 2005; 20: 2261–2270.

[55] Ashghar W, Velasco V, Kingsley JL, Shoukat MS, Shafiee H, Anchan RM, *et al*. Selection of functional human sperm with higher DNA integrity and fewer reactive oxygen species. Advanced Healthcare Materials. 2014; 3: 1671–1679.

[56] Huang J, Chen H, Li N, Zhao Y. Emerging microfluidic technologies for sperm sorting. Engineered Regeneration. 2023; 4: 161–169.

[57] Phiphattanaphiphop C, Leksakul K, Phatthanakun R, Khamlor T. A novel microfluidic chip-based sperm-sorting device constructed using design of experiment method. Scientific Reports. 2020; 10: 17143.

[58] Xiao S, Riordon J, Simchi M, Lagunov A, Hannam T, Jarvi K, *et al*. FertDish: microfluidic sperm selection-in-a-dish for intracytoplasmic sperm injection. Lab on a Chip. 2021; 21: 775–783.

[59] Khodamoradi M, Rafizadeh Tafti S, Mousavi Shaegh SA, Aflatoonian B, Azimzadeh M, Khashayar P. Recent microfluidic innovations for sperm sorting. Chemosensors. 2021; 9: 126.

[60] Doostabadi MR, Mangoli E, Marvast LD, Dehghanpour F, Maleki B, Torkashvand H, *et al*. Microfluidic devices employing chemo- and thermotaxis for sperm selection can improve sperm parameters and function in patients with high DNA fragmentation. Andrologia. 2022; 54: e14623.

[61] Jahangiri AR, Ziarati N, Dadkhah E, Bucak MN, Rahimizadeh P, Shahverdi A, *et al*. The future of sperm selection in assisted reproduction. Andrology. 2024; 12: 1236–1252.

[62] Kocur OM, Xie P, Cheung S, Souness S, McKnight M, Rosenwaks Z, *et al*. Can a sperm selection technique improve embryo ploidy? Andrology. 2023; 11: 1605–1612.

[63] Keskin M, Pabuçcu EG, Arslanca T, Demirkıran ÖD, Pabuçcu R. Does microfluidic sperm sorting affect embryo euploidy rates in couples with high sperm DNA fragmentation? Reproductive Sciences. 2022; 29: 1801–1808.

[64] Henkel RR, Schill WB. Sperm preparation for ART. Reproductive Biology and Endocrinology. 2003; 1: 108.

[65] Khodamoradi K, Kuchakulla M, Narasimman M, Khosravizadeh Z, Ali A, Brackett N, *et al*. Laboratory and clinical management of leukocytospermia and hematospermia: a review. Therapeutic Advances in Reproductive Health. 2020; 14: 2633494120922511.

How to cite this article: Michal Ješeta, Jana Antalíková, Adéla Doubravská, Lenka Mekiňová, Bartosz Kempisty, Pavel Ventruba, *et al*. *In vivo* versus *in vitro* sperm selection. Journal of Men's Health. 2025; 21(2): 1-10. doi: 10.22514/jomh.2025.016.