Chapter 2
Sperm Processing for IVF

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Introduction

The birth of Louise Brown, the first human who was born on 25 July 1978 after the application of assisted reproduction techniques hallmarks the advent of the era of assisted reproductive technology (ART) in the human [1]. The first in vitro fertilization (IVF) cases, including that of Louise Brown, were performed to treat female tubal infertility. Subsequently, the demand for assisted reproduction techniques grew dramatically with the increasing number of men showing poor semen quality. Hence, scientists and clinicians were prompted to develop a wide array of different laboratory techniques focusing on the selection and enrichment of motile and functionally competent spermatozoa from the ejaculate. Since our understanding of the fertilization process and various sperm functions also extended significantly and the percentage of andrological cases with even poorer semen quality increased rapidly, the initial simple sperm separation techniques were not sufficient enough anymore, and new improved sperm separation techniques had to be developed.

Spermatozoa are not only the smallest but also the most polarized cell in the body (sperm head and a flagellum) that even fulfill their physiological functions, fertilization of oocytes, outside the body in a different individual, the female genital tract. Thus, the male germ cell is a very specialized and in certain aspects very sensitive cell. The latter is based on the sperm cell's special composition of its plasma membrane with an extraordinary high amount of poly-unsaturated fatty acids, particularly docosahexanoic acid with six double bonds in the molecule [2]. This feature is the foundation of the high membrane fluidity which is essential in maintaining the extreme polarization of the sperm and is required for normal sperm function [3]. Together with the sperm cell's lack of intrinsic reactive oxygen

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species (ROS)-scavenging systems due to a lack of cytoplasm, which is harboring repair mechanisms in any other cell, this renders the male germ cell extremely susceptible to oxidative stress by ROS [4] impairing the membrane function and resulting in loss of fertilizing potential [5, 6], serious damage of the DNA [7, 8] or even cell death.

In vivo, after normal ejaculation for sexual intercourse, spermatozoa depend on scavenging systems provided by the seminal plasma, which is the biological fluid that contains more antioxidant substances than any other physiological fluid does. The most important natural antioxidants in seminal plasma seem to be vitamin C and E [9, 10], superoxide dismutase [11], uric acid [12], glutathione [13], or the polyamine spermine that acts directly as a free radical scavenger [14]. If these protective substances are removed by means of any sperm separation technique, this can cause severe damage to the sperm cell and its membranes since sperm functions like motility or acrosome reaction are basically membrane functions. This damage is set by ROS either by the sperm cells themselves or leukocytes [15–17], which produce about 1,000 times more ROS than spermatozoa [18, 19].

On the other hand, even the extreme polarization of the male germ cell can be a direct target of damage through excessive centrifugation, resuspension, and vortexing of semen samples [15 16 20] as the mechanical stress imposed on the sperm can be harmful to the cell and its functions leading not only to decreased percentages of motile and vital sperm but also to a reduced mitochondrial membrane potential, which is essential for normal sperm function [21–23].

Sperm Processing Methods

In vivo, separation of motile sperm most capable of fertilizing oocytes from immotile sperm, debris, seminal plasma, and leukocytes is taking place in the female genital tract by active migration through the cervical mucus [24]. Yet, this process does not only separate motile and potentially fertile sperm, but it also prepares and enables male germ cells for the fertilization process by means of fundamental physiological changes called capacitation. In turn, the capacitation process involves, among others, changes in the motility pattern, metabolism, and the removal of cholesterol from the plasma membrane leading changed fluidity of the sperm plasma membrane, eventually enabling the sperm to undergo acrosome reaction and thus penetrate and fertilize oocytes [25–29].

The first sperm separation methods that were developed only comprised of one or two washing procedures to eliminate seminal plasma with subsequent resuspension of the male germ cells [30]. Following these first reports on human sperm processing, more sophisticated methods were developed in order to obtain sufficient amounts of motile, functionally competent spermatozoa for IVF, one of them being a swim-up procedure from a washed cell pellet.

Except for the first washing procedures, modern sperm processing techniques can be differentiated in migration, filtration, and density-gradient centrifugation. While

for all migration techniques (conventional swim-up and migration-sedimentation), the sperm cells' most obvious feature, self-propelled movement, is an essential prerequisite; the separation principle in the filtration and density-gradient centrifugation techniques is based on a combination of the sperms' own motility and their adherence to filtration matrices and the retention at phase borders, respectively.

Apart from possible financial considerations, sperm physiology and the physiology of the fertilization process have to be taken into account for any method of assisted reproduction in order to better select functional sperm or better protect and "preserve" sperm functions from damages caused by the separation process.

Criteria for a "good" sperm selection are as follows:

- Elimination of seminal plasma, decapacitation factors, and debris
- Elimination/reduction of dysfunctional and ROS-producing sperm
- Elimination/reduction of leukocytes
- Elimination/reduction of bacteria
- Enrichment of functional sperm in terms of motility, DNA integrity, acrosome reaction, and normal sperm morphology
- Cost-effectiveness
- Easy and quick to perform
- Allow processing of larger volumes of ejaculates

However, since none of the different methods available meets all the criteria, it is mandatory for every Andrology laboratory/IVF unit to be able to perform a variety of different sperm separation methods having the individual circumstances of the patients as first priority in mind.

Liquefaction of Ejaculates and Viscous Ejaculates

An essential requirement for any sperm preparation method to be performed is that the semen is liquid. Normally, human semen liquefies after 15–30 min, and these ejaculates can directly be subjected to a sperm processing technique. In the laboratory, semen liquefaction is normally performed by placing the semen sample for 30 min into an incubator at 37°C. However, if the ejaculate does not liquefy after 60 min, which can be the case in about 10% of infertile patients [31], seminal viscosity remains high and can severely interfere with sperm motility resulting in poor yield of motile sperm after sperm separation.

Semen viscosity, which is sometimes referred to as "consistency," can be tested by means of gently aspirating a semen sample into a Pasteur pipette and then allowing the ejaculate dropping out. Normally, the seminal fluid is running out in discrete drops. Threads longer than about 2 cm are indicative of high viscosity and poor liquefaction, i.e., viscosipathy [32]. Since such ejaculates cannot well be processed, liquefaction can be achieved by enzymatic digestion using bromelain (1 mg/mL) [32] or a small spatula tip of approx. 2–5 mg crystalline α(alpha)-chymotrypsin to be mixed into the semen and incubated for 15–30 min at room temperature [33].

However, one still has to consider that these enzymes might be harmful to the sperm or oocyte and have to be removed by washing procedures as soon as possible. Alternatively, viscous semen can also be mixed with IVF medium in order to decrease its viscosity.

NB: Recommendations to force viscous seminal fluid through 18 or 23 gauge needles should be refrained from since such procedure can severely damage sperm.

Swim-Up

Since the introduction of the so-called conventional swim-up procedure by Mahadevan and Baker [34], this method has been successfully used in many IVF units around the world with excellent fertilization rates and is still in use for all techniques in assisted reproduction including intrauterine insemination (IUI) and IVF. This method is simple and cheap and does not require sophisticated equipment or highly specialized skills.

Procedure

Typically, 1 mL of the liquefied semen is mixed with 4 mL of IVF medium containing 10 mg/mL serum albumin or 10% inactivated serum and centrifuged for 10 min at $300 \times g$. Then, the supernatant is carefully taken off with a pipette and discarded. For this step, it is essential that the surface of the pellet is not disturbed or that parts of the pellet are sucked into the pipette as this would result in a decreased yield and motility in the sperm suspension. Subsequently, fresh medium is layered very carefully by letting 0.5–1 mL slowly rinse down the inner surface of the test tube tilted at an angle of about 45° on top of the pellet and incubated for 30–60 min at 37°C in an incubator. After this incubation period, the test tube is taken out of the incubator, and the medium is carefully aspirated into a pipette again without disturbing the pellet which can become quite soft during the incubation period. Ideally, sperm fractions with >90% motility can be obtained using this technique.

NB: Considering that the success of the swim-up technique strongly depends on the initial motility in the ejaculate and the size and quality of the surface of the pellet obtained after the centrifugation step, the yield of this technique is rather limited. Therefore, it is advisable to run two or three or more tubes from one semen sample at the same time in order to increase the yield. Since the use of medium that does not contain macromolecules like albumin is detrimental to spermatozoa, it is not advisable to use unsupplemented medium as it might result in sperm sticking to the surface of the test tube leading to a lower yield and motility.

Advantages/Disadvantages of the Technique

Normally, this sperm selection method recovers a very clean fraction of highly motile sperm that can directly be used for insemination in IVF or for IUI. For the latter, it is important that not more than 0.5 mL of the isolated sperm fraction after swim-up is inseminated into the uterus as higher volumes might leak out.

With regard to the quality of the ejaculates, the conventional swim-up technique is rather restricted to good quality ejaculates with high sperm concentration and good motility. With regard to the application of the swim-up for artificial insemination, it is not only important to know that the yield is rather low but also that sperm can be massively damaged by ROS because of the close cell-to-cell contact during the centrifugation step. This is particularly the case in patients with male genital tract infections. If, despite good motility and sperm count in the ejaculate, the expected sperm concentration is less than 10^6/mL after the swim-up, one should consider the effect of ROS.

Migration-Sedimentation

The migration-sedimentation technique, which was originally developed by Tea et al. [35], is a more sophisticated method of sperm processing. Like the swim-up technique, it is based on the sperm cells' own motility, yet combined with a sedimentation step. However, special, commercially available "Tea-Jondet tubes" have to be used. Contrary to the conventional swim-up method, sperm swim up directly from the liquefied semen sample into the supernatant medium, deposit, and accumulate into the inner cone of the tube. Since such a migration technique directly from the semen is the gentlest method of sperm processing, several modifications have been developed, which are even used for ICSI [36–38].

Procedure

In order to process semen for IVF using the migration-sedimentation technique, the "Tea-Jondet tubes" are filled up with 1–2 mL IVF medium supplemented with 10 mg/mL serum albumin or 10% inactivated serum. The overlaying medium must not be too high above the edge of the inner cone. Then, approx. 0.5 mL of liquefied semen is placed in the outer ring around the cone carefully paying attention that no ejaculate is running into the inner cone. After incubating the tubes for 1–2 h at 37°C, the supernatant medium, including the medium in the inner cone, is carefully aspirated with a Pasteur pipette without aspirating remnants of the ejaculate. Typically, sperm fractions with motility rates of more than 90% can be obtained and after adjustment of sperm concentration directly used for insemination.

For the use in ICSI, the whole liquefied ejaculate (or part of it) of the respective patients is centrifuged for 10–15 min at 300×*g*, and the supernatant except for about 0.3–0.5 mL which contains the sperm is discarded. This "concentrated" ejaculate is filled into the "Tea-Jondet tubes" as described above and incubated. In order to obtain higher numbers of motile sperm, processing can be carried out in duplicate.

NB: Like in the swim-up method, the success of isolating motile sperm using migration-sedimentation is dependent on initial quality of the ejaculate. For its application in ICSI, it is, however, important that the semen must *not* be diluted with medium for centrifugation. The seminal plasma is then still able to have at least some protection against oxidative stress.

Advantages/Disadvantages of the Technique

Migration-sedimentation is one of the gentlest methods of processing sperm and usually results in a very clean fraction of highly motile, functional sperm.

Considering the very low recovery rate in its original protocol, this method is rather restricted to higher quality ejaculates. The technique is also a bit more expensive than the conventional swim-up and requires advanced skills.

Glass Wool Filtration

Using a different approach Paulson and Polakoski [39] succeeded to separate motile from immotile sperm by means of densely packed glass wool fibers. The principle for the separation of motile spermatozoa from immotile, debris and leukocytes is not only rested in the self-propelled movement of the sperm cells but also on a filtration effect of the glass wool fibers, for which both are responsible, a mechanical retention of the bigger particles in the ejaculate as well as the adhesion of these particles to the surface of the glass fibers [40]. Hence, the kind of glass wool used plays a cardinal role in the success of this technique, and it is not possible to use any kind of glass wool for sperm processing [41]. This adhesion and filtration process is a feature of glass wool filtration that may contribute to the selection of spermatozoa with matured nuclei, i.e., with good chromatin condensation [42].

Recently, Grunewald et al. [43] developed a glass wool, which chemically activated to bind annexin V to the surface of the glass wool fibers. Annexin V binds to externalized phosphatidyl serine and is an indicator of apoptotic cells [44, 45]. While the conventional glass wool filtration is in routine use in many IVF centers, clinical data supporting the efficiency of this new molecularly activated glass wool remains to be seen.

Procedure

Useful glass wool is available from Manville Fiber Glass Corp. (code 112; Denver, CO) or glass wool columns from TransMIT (SpermFertil®; Giessen, Germany). While from the former, 15–30 mg of glass wool has to be loosely packed in a Pasteur pipette or small syringe, the latter are sterile, ready-to-use glass wool columns. In order to eliminate loose glass particles, both columns have to be flushed carefully with at least 2 mL sterile IVF medium. Subsequently, the glass wool column must be put on a new sterile test tube and filled with 1–3 mL *liquefied* fresh semen. Filtration should take place in an incubator at 37°C with occasional visual control in order to avoid the column falling dry. Immediately after the semen has passed through the glass wool, the column is washed with 0.5 mL fresh IVF medium. Finally, the filtrated semen will be diluted in the ratio 1:5 with fresh IVF medium and centrifuged for 10 min at 300×*g*. The supernatant will then have to be carefully discarded and the pellet resuspended in fresh IVF medium. For better washing of sperm, this procedure can be repeated. This sperm suspension can then be used for insemination after adjusting sperm concentration.

NB: As high concentrations of "particles" might block the column and prevent semen from being filtrated properly, the amount of semen used depends on sperm and debris concentration. Therefore, in cases of high "particle" concentrations, the ejaculate can be divided on 2–3 glass wool columns for filtration and the filtrates eventually be combined. For gentle sperm processing using glass wool filtration, it is fundamentally important that the sperm separation takes place first. Only afterward, washing of sperm should take place.

Advantages/Disadvantages of the Technique

Principally, glass wool filtration is an easy to perform technique, which results in the recovery of spermatozoa with good motility. Since the whole ejaculate can be filtrated, ejaculates from patients with oligozoospermia can be processed. In addition, the technique eliminates up to 90% of leukocytes contaminating the semen and therefore reduces ROS significantly [46]. As a disadvantage, it can be brought bear that the filtration does not result in as clean fractions as the swim-up procedure does. There is always some debris or immotile sperm that pass through the mesh.

Density-Gradient Centrifugation with Different Media

Density-gradient centrifugation is another method for gentle sperm separation. Its principle is based on the ability of motile sperm cells to penetrate concentration boundaries in direction of the centrifugation force. This penetration is quicker the

faster sperm swim; thus, highly progressively swimming sperm are reaching the bottom of a centrifuging test tube quicker than immotile or poorly motile sperm, which are retained at the boundaries of interphases.

The first reports employing density-gradient centrifugation to isolate progressively motile human spermatozoa date back to 1981 and 1983 [47, 48]. Initially, Percoll®, a medium containing polyvinylpyrrolidone-coated silica particles, was used to create media with different densities. Since 1996, however, Percoll® has been withdrawn from the market for use in assisted reproduction because of its possible risk of being contaminated with endotoxins [49, 50]. Yet, other density media have emerged from the market with good results in separating highly motile sperm, competent for fertilization. These new density media are based on silane-coated silica particles and have been proven to have very low toxicity. The most commonly used new density media are SilSelect® (FertiPro N.V., Beernem, Belgium), SupraSperm® (MediCult, Jyllinge, Denmark), PureSperm® (NidaCon International AB, Mölndal, Sweden), or ISolate® (Irvine Scientific, Santa Ana, CA, USA).

Like for glass wool filtration, the selection of nuclear matured sperm in terms of chromatin condensation has been described for density-gradient centrifugation [51, 52]. Moreover, while recent studies showed that processing of sperm with PureSperm® resulted in significantly lower percentages of sperm exhibiting DNA damage and extended survival rate [53, 54], older studies using Percoll® rather revealed adverse effects on the sperm DNA [55, 56]. Moreover, for the high motility fraction of density gradient prepared spermatozoa lower percentages of sperm with externalized phosphatidyl serine and disrupted mitochondrial membrane potential have been described [57].

Procedure

There are two different kinds of density gradients that can be performed, the continuous or the discontinuous. Nowadays, most labs are using the discontinuous gradient employing a two-step gradient consisting of 40% (45%) and 80% (90%) density medium. These density gradients can either be bought ready to use or can be made from high-concentration stock solutions. Depending on the product used, the two-step gradient is prepared by *carefully* overlaying 1–2 mL of the high-density solution (80%/90%) with 1–2 mL of the low-density solution (40%/45%). A distinct phase border between the two layers must be visible. After the gradient is equilibrated in 5% CO_2 at 37°C in an incubator, 1 mL of the liquefied and thoroughly mixed semen sample is *carefully* dispensed on top of the prepared gradient. Again, a distinct phase border should be visible. Subsequently, the gradient is centrifuged for 20–30 min at 300–400×g.

After this centrifugation step, the soft pellet can be obtained in two ways. Firstly, by carefully aspirating the pellet with a Pasteur pipette. By applying this procedure, nothing of the overlaying layers should be aspirated. Secondly, the top layers of the gradient can be removed and discarded first, and then the pellet can be aspirated and

placed into a clean test tube. This sample has then to be resuspended in 3–5 mL fresh sperm preparation or IVF medium and centrifuged 10 min at 300–400×*g* again. This procedure has to be repeated twice. After adjusting sperm concentration, the finally resulting sperm suspension can be used for insemination.

NB: Overloading the gradient with either too much volume or a too high number of "particles" (spermatozoa and/or debris) will result in poor sperm separation. On the other hand, in cases of oligozoospermia or asthenozoospermia, more than one test tube can be prepared in order to obtain a sufficient number of competent sperm for fertilization.

Advantages/Disadvantages of the Technique

Normally, density gradient centrifugation results in a good and clean fraction of highly motile spermatozoa with good recovery. Even ejaculates with low sperm counts from patients with oligozoospermia can be processed. The possibility to process the full volume of the ejaculate increases the yield. Considering that the technique eliminates leukocytes to a large extent, ROS are significantly reduced.

The formation of good interphases between the different media gradients is essential for the quality of the sperm separation and needs special attention during the preparation. After the density-gradient centrifugation the "soft pellet" containing the high-quality sperm has to be washed in order to eliminate the density medium. Thus, an additional centrifugation step is required.

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