

Sperm Preparation Techniques for Artificial Insemination - Comparison of Sperm Washing, Swim Up, and Density Gradient Centrifugation Methods

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1. Introduction

The Artificial Insemination (AI) is the first option treatment for infertile couples with cervical factor subfertility, mild-moderate male subfertility and unexplained infertility. With the exception of cases in which the use of in vitro fertilization (IVF or ICSI) is strictly due as a consequence of a severe male or female factor, the artificial insemination must be part of a gradual approach to the techniques of artificial insemination. This is particularly the case since the AI is a valid low-cost method, minimally invasive and easily acceptable for the female's hormone treatment (Aribarg & Sukcharoen, 1995).

The AI, as other assisted reproductive techniques, needs a selection of the ejaculated spermatozoa before the performance of the treatment. In fact, some components of the seminal fluid may become an obstacle to the fertilization when the in vitro fertilization or the intrauterine insemination are performed (Bjorndahl et al., 2005). Spermatozoa and leukocytes produce many oxygen radicals that alter the possibility of the sperm-oocyte fusion after repeated centrifugations. So, the selection of the sperms from the other components with methods like the swim up technique or the gradient density centrifugation must be preferred (Aitken & Clarkson, 1988).

Some different techniques are used to prepare the spermatozoa for the AI, but the choice strongly depend on the quality of the semen, that is on the concentration, motility and morphology, in order to obtain the higher number of good spermatozoa, even from the poorest semens.

The principle techniques of sperm preparation consist of migration, density gradient centrifugation and filtration techniques. While for the migration the method is based on movement of the spermatozoa, for density gradient centrifugation and filtration techniques the method is based on a combination of the motility and the retention at phase borders and adherence to filtration matrices, respectively (Henkel & Schill, 2003).

The main techniques used for the AI are the sperm washing, the swim-up technique, and the density gradient centrifugation and they will be described as follow. The aim of the present chapter is to shed light on the key principles and the best method for sperm selection in order to obtain higher pregnancy rate.

2. Sperm preparation techniques

2.1 Semen collection

The semen consists of a suspension of spermatozoa stored in the epididymes that, at the moment of the ejaculation, is mixed with the secretions of the accessory glands. These glands are mainly the prostate and the seminal vesicles, while the bulbourethral glands and the epididymes represent only the minor contribution of the ejaculate.

Two main fractions are present in the seminal fluid; the first one is prostatic, rich in spermatozoa. The last fraction of the semen consists of vesicular fraction, less rich in spermatozoa (Bjorndahl & Kvist, 2003).

During ejaculation, it is very important to collect the entire volume of the sample: if the first fraction (rich in spermatozoa) is lost, the assessment of the semen features will be more difficult. In case of the AI, the semen sample will not contain the best portion of the spermatozoa.

For these reasons, the first step throughout the sperm preparation, is the correct sperm collection.

The semen collection is strongly recommended after an abstinence period of 2-3 days (Jurema et al., 2005; Marshburn et al., 2010) to maximize the conception rate. A sterile container (non-toxic for the spermatozoa) will be used and the collection of the semen will occur in a private room very close to the laboratory. All of these elements are mandatory for the therapeutic use. After the collection, the name of the couple should be clearly written on the container.

2.2 Choice of the technique

The techniques for the selection of the most efficient spermatozoa are very important for clinical practice. The choice of the best technique for semen preparation, before the AI, strictly depends on the quality of the sample (Canale et al., 1994). So, if we have a sample with normal count, motility and morphology of sperms we choose a sperm washing or a swim up method. By contrast, with a suboptimal quality sample we usually prefer a density gradient centrifugation. With the first methods, we obtain good quality sperms; while the density gradient centrifugation is usually preferred for the greater number of mobile spermatozoa selected from poor characteristics samples (low number, motility and morphology samples). Each technique can be changed or improved with simple changes, in order to optimize the recovery of the sperms.

The efficiency of the sperm selection is expressed as the concentration of spermatozoa with normal motility (that is progressively motile spermatozoa, according to the definition of the World Health Organization Manual of 2010) (WHO Manual, 2010).

Glass-wool columns are reported to be as effective as density gradient for the separation of spermatozoa also with intact acrosome from semen with suboptimal characteristics (Rhemrev et al., 1989; Sterzik et al., 1998), but this technique is less used.

The swim up method and the density gradient centrifugation produce different levels of contamination in the sample in the final preparation. In fact, the swim-up technique produces an higher level of non-sperm components (e.g. debris, bacteria) and the diffusion of other substances (e.g. the prostatic zinc) from the semen into the overlaying medium respect of the density-gradient centrifugation (Bjorndahl et al., 2005). Some differences also exist in the presence and the production of the Reactive Oxygen Species (ROS) and the

sperm DNA damage, associated with high levels of ROS, after the application of the two main techniques (Irvine et al, 2000; Zini et al, 1993, 2009).

The final volume of the preparation depends on the technique performed. If the IntraUterine Insemination (IUI) is performed, 0,3-0,4 milliliters (ml) of spermatozoa resuspended in sterile medium is required. If the case of the Fallopian Tube Sperm Perfusion (FSP), the volume of the suspension must be 4 ml, because it must perfuse the uterus and the both tubes. Because of its simplicity the first technique is the most used, even if some authors, comparing the IUI versus the FSP, demonstrate the superiority of the FSP technique about the pregnancy rate in stimulated cycles (Fanchin, 1995).

3. The sperm count

Before and after the treatment of the seminal fluid, the following parameters must be evaluated in line with the WHO Manual 2010.

- Volume (ml)
- Concentration (millions/ml)
- Motility (Progressive motility)
- Morphology (%normal sperms)

In addition, it is very important to establish the concentration of spermatozoa with progressive motility in the final preparation. The concentration of the progressive spermatozoa is calculated by multiplying the percentage (%) of the progressive sperms for the concentration of the sperms in the final preparation.

$$[PS] = \%PS \times [S]_f \quad (1)$$

The total number of the progressive spermatozoa is calculated by multiplying the concentration of the progressive sperms for the final volume of the suspension.

$$TPS = [PS] \times V_f \quad (2)$$

The total number of the progressive sperms in the preparation before the AI may be defined as a threshold value in predicting outcome in AI. This threshold is not absolute and may vary from study to study, even if some authors have identified this value in 10 million sperms (Miller et al., 2002; Van Voorhis et al., 2001).

4. Sperm washing

For the best quality samples (number and motility of sperms) the sperm washing is often performed (Boomsma et al., 2004) for the AI. The procedure simply consists in the washing of the semen with a sterile medium added with human albumin. After the fluidification of the sample, the entire volume is divided in fractions of not more than 2 ml into centrifuge tubes. The sterile medium of the equal volume (e.g. for the volume of the sample of 2 ml the medium added is 2 ml) is added in each tube and gently mix with a sterile pipette. After

¹ P=Progressive; S=Spermatozoa; [PS]=Concentration of Progressive Spermatozoa; [S]_f=Concentration of the sperms of the final preparation.

² TPS=Total Number of the Progressive Spermatozoa; [PS]=Concentration of Progressive Spermatozoa; V_f =Final volume of the preparation.

that, the samples are centrifuged at 300g (the rpm must be calculated for the centrifuge in each laboratory) for 10 min and then the supernatant is very carefully removed with a sterile pipette. The pellet is resuspended in 1 ml of the medium, gently mixed and centrifuged again for 5 min at 300g. The supernatant is removed again and the final pellet is resuspended in sterile medium for the AI.

It is very important to determine the count and the motility of the final preparation before the insemination.

In spite of the simplicity and velocity of the method, it must be reminded that the repeated centrifugations without the separation of the good sperms from leukocytes and dead sperms can produce many oxidative species and the damage of the sperms function (Aitken & Clarkson, 1988).

5. Swim up method

The swim up is the most common technique used in IVF laboratories and is preferred if the semen sample has a normal number of good sperms (normozoospermia). By this technique, the sperms are selected on their motility and the capability to swim out of the seminal plasma.

If the "direct swim up" is performed, after the fluidification of the sample, the entire volume (well mixed) is divided in fractions of 1 ml into centrifuge tubes (round bottom is preferred). 1,3 ml of culture medium is placed over the semen with extreme attention in each tube. The tubes must be put in the incubator, inclined at an angle around 45° and incubated at 37°C for 30-60 min. By inclining the tubes at 45°, we increase the surface between the medium and the semen and we improve the capability of the sperms to swim out of the semen and to reach the medium. After that, the tube must be returned in the vertical position and 1 ml of the supernatant of each tube can be gently removed, aspirating the sperms from the upper meniscus downwards with a sterile pipette (Henkel et al., 2003).

In alternative, the culture medium can be placed in each tubes and the semen can be stratified under the medium, in order to obtain a much cleaner surface between the semen and the medium. In addition, the recovery of the sperms can be optimized by increasing the number of the tubes and decreasing the volume of the semen in each tube. 2 ml of medium are added to the supernatant of each tubes and then centrifugated at 300g for 10 minutes. The supernatant is removed again and the pellet is resuspended in the sterile medium for the AI.

The "not direct" swim up from pellet is performed with the centrifugation of the semen followed by the stratification of the medium over the resuspended pellet. The liquefied semen is divided in fractions of 1 ml into each tubes, the medium is added (1:1) and after the centrifugation the supernatant is gently removed. Over the resuspended pellet, 1,3 ml of medium is replaced with caution and the tubes is put into the incubator from 30 to 60 min at 37°C (inclined at 45°); after the migration of the sperms, the volume of the semen for the AI is removed and the sperm count and motility are assessed.

The centrifugation for the direct swim up occurs after the migration of the sperms, that is, after the separation of the good sperms from the leukocytes and dead sperms. These species, usually produce the reactive oxygen species after the centrifugation (Irvine et al, 2000; Zini et al, 1993, 2009) so the direct swim up is the preferred method respect to the "not direct" swim up to select sperms for the AI.

6. Density gradient centrifugation

This is the preferred technique to select the greater number of motile spermatozoa in cases of severe oligozoospermia, teratozoospermia or asthenozoospermia. In this method, good quality sperms can be separated from dead sperms, leukocytes and the other components of the seminal plasma by a density discontinuous gradient. Cells with different density and motility can be selected during the centrifugation by the colloidal silica coated with silane of the gradient; the sperms with high motility and good morphology are at the bottom of the tube, finally free from dead spermatozoa, leukocytes, bacteria and debris.

The most applied discontinuous density-gradient is a two layers density-gradient, formed by a top layer of 40% (v/v) and a lower layer of 80% (v/v). Density gradient media are available in commerce ready to use or ready to make the different density layers; the top layer phase (40%) is prepared by adding 4 ml of density gradient medium to 6 ml isotonic sterile medium (BWW, Earle, Ham F-10 or HTF) supplemented with HAS (Human Serum Albumin); the lower layer phase (80%) is prepared by adding 8 ml of density gradient medium to 2 ml of isotonic sterile medium. The density gradient is prepared by layering 1 ml of 40% medium over the 80% medium, or by layering the 80% medium under the 40% medium in a conical centrifuge tube (not the round bottom tube!). The number of the tubes depends on the volume of the semen sample, but the total volume could be divided in not more of 1 ml of semen per tube.

After the fluidification, 1 ml of the semen is layered over the upper layer (40%) and centrifuged at 300g for 15 minutes. If the volume of each layer is reduced (<1ml) the spermatozoa have to migrate for a less distance between the layers and so the greater number of motile spermatozoa can be recovered. The centrifugation time and force can be varied depending on the quality of the sample: for example, the centrifugation time can be increased for specimens with high viscosity. After the centrifugation, most of the supernatant must be gently removed and the pellet is placed into a new, clean tube; here, the pellet is well resuspended in 5 ml of medium to remove the density gradient medium. It is centrifuged at 200g for 10 minutes. At the end of the centrifugation, the supernatant is removed and 5 ml of new medium are added. The centrifugation is repeated again and the final pellet is resuspended in the sterile medium for the AI.

The concentration and the motility after the preparation can be determined. It must be stressed that the sterile conditions and materials are essential when we perform the technique for therapeutic applications.

Nevertheless, the two main techniques produce different levels of contamination in the sample and of the production of ROS (see above). In addition, the swim-up technique produces an higher level of non-sperm components respect of the density-gradient centrifugation. The density gradient centrifugation recovers spermatozoa with improved motility but lower DNA integrity instead of the swim up technique, as the literature suggests (Zini et al., 1999, 2000). Several studies demonstrate that sperm DNA damage is associated with lower natural pregnancy rates (Loft et al., 2003; Spano et al., 2000) and lower IUI pregnancy rates (Evenson et al., 2008).

7. Conclusions

The AI is the still most used reproductive technique as it is relatively simple and low-cost. It is essential to select the most motile and normal morphological spermatozoa from the

ejaculate as soon as possible, first because some components of the ejaculate contrast with the fertilizing capability of the spermatozoa (Bjorndahl et al., 2005; Mortimer et al., 1998). Then, because spermatozoa and leukocytes produce many oxygen radicals that can negatively influence the fertilizing sperm function (Agarwal & Sekhon, 2010; Aitken et al., 1998; De Jonge, 2002; Shamsi et al., 2008; Sharma & Agarwal, 1996; Zini & Sigman, 2009). So, the methods who separates the functional sperms from the other cells must be preferred (Aitken & Clarkson, 1988). The choice of the best method to select the functionally competent sperms depends on the features of the samples.

The swim up technique and the density gradient centrifugation have different efficiency in separating the sperms: the sperms isolated with the swim up are clean and motile, but damaged by the ROS and with higher DNA integrity; the sperms isolated with the density gradient centrifugation are not damaged by the ROS but with low DNA integrity.

When we compare the pregnancy rate after artificial insemination obtained with the sperm washing, the advanced sperm preparation methods (swim up and density gradient centrifugation) offer the higher rate of pregnancies (Carrell et al., 1998). These data indicate that the correct choice of the method of sperm selection can represent a good chance of pregnancy, after ovarian stimulation, in the artificial insemination.

Finally, the threshold value of 10 million sperms in the final preparation for the IUI has a predictive value for the pregnancy rate in IUI. Some authors demonstrate that when the total count of the progressive sperms is less than 10 millions the pregnancy rate decreased, even if, in practice, a pregnancy is also possible with an inferior total sperm count (Miller et al., 2002; van Weert et al., 2004; Van Voorhis et al., 2001).

If the total sperm count is very low, and in presence of a severe male factor, other alternative must be considered, like the In Vitro Fertilization (IVF).

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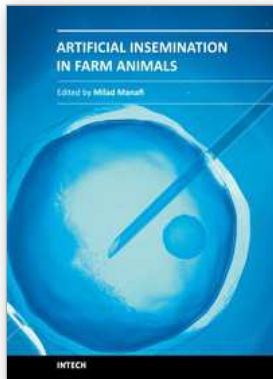
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Artificial insemination is used instead of natural mating for reproduction purposes and its chief priority is that the desirable characteristics of a bull or other male livestock animal can be passed on more quickly and to more progeny than if that animal is mated with females in a natural fashion. This book contains under one cover 16 chapters of concise, up-to-date information on artificial insemination in buffalos, ewes, pigs, swine, sheep, goats, pigs and dogs. Cryopreservation effect on sperm quality and fertility, new method and diagnostic test in semen analysis, management factors affecting fertility after cervical insemination, factors of non-infectious nature affecting the fertility, fatty acids effects on reproductive performance of ruminants, particularities of bovine artificial insemination, sperm preparation techniques and reproductive endocrinology diseases are described. This book will explain the advantages and disadvantages of using AI, the various methodologies used in different species, and how AI can be used to improve reproductive efficiency in farm animals.

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