Sperm Selection Using Three Semen Processing Techniques

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ABSTRACT

Objective: This study aimed to assess the efficiency, in terms of recovered motile spermatozoa with normal morphology, of three sperm selection techniques: migration-sedimentation (SS), swim-up from fresh semen (SF), and swim-up from washed (SL) sperm.

Methods: Samples from 20 normozoospermic men were divided into three equal aliquots and processed in parallel. SS was performed in a Jondet tube, using 1 ml of semen and 2.5 ml of Human Tubal Fluid medium (HTF+10% Synthetic Serum Supplement, Irvine, USA). For SF, 1 ml of HTF was layered over 1 ml of fresh semen (SF). For SL, 1 ml of sperm was first centrifuged (300 g, 10 min) and the pellet resuspended in 1 ml of HTF; a second layer of HTF was placed on top. Migration time was 1h (SF and SL) and 1h30' for SS at 37°C. After migration, 200 µl were removed from the top layer (SF, SL) and from the central cone (SS). Concentration, morphology and motility were determined.

Results: Recovery rates were 25% for SS, 10.1% for SF and 4.5% for SL. SS recovery rate was significantly higher ($P<0.01$) than the two swim-up techniques. Total motility was statistically different ($P<0.001$), with 93.6% for SS, 91.2% for SF, and 77% for SL. Sperm morphology was similar between the three techniques ($P=0.12$).

Conclusions: SS is an efficient technique for the recovery of motile spermatozoa from native semen preparations and yielded better results than SF and SL. Routine use for assisted reproduction remains to be evaluated.

Keywords: Assisted reproduction, Sperm preparation, Sperm selection, Migration-sedimentation

INTRODUCTION

With the advancement of human assisted reproduction technologies, there is a need for reliable, simple, secure methods for the selection of fertile spermatozoa (Mortimer, 2000). The increasing demand for sperm preparations in the context of intrauterine insemination (IUI) or in vitro fertilization (IVF) has provided better knowledge on how sperm contributes to the success of such techniques.

Theoretically, the ideal sperm processing technique should allow the selection of many motile spermatozoa of good quality, maintain physiological conditions, allow the removal of dead cells, microorganisms, and reactive oxygen species (ROS), avoid irreversible injuries to the sperm membrane, enable the processing of large semen volumes, and be quick, affordable and safe (Henkel & Schill, 2003). The most used modern sperm selection methods include swim-up and discontinuous density gradient filtration (Henkel, 2012). The swim-up technique consists of carefully layering a culture medium over fresh or washed semen. With time, the motile spermatozoa that have progressively colonized the upper medium layer can be recovered by gentle aspiration. In the density gradient

technique, motile spermatozoa aided by the centrifugation force pass through several layers of increasing density and are recovered in the densest bottom layer, while dead cells, debris, and microorganisms are withheld in the upper layers. The recovered motile fraction needs then to be washed twice with culture medium to remove the gradient compounds. Washing procedures are considered critical for the maintenance of sperm fertilizing ability. For instance, pelleting native semen by centrifugation may enhance ROS production from leucocytes, leading to peroxidative damage of spermatozoa and to their obliteration or reduction of their fertilizing capacity (Mortimer, 2000). In addition to the two techniques described above, the migration-sedimentation method, although described in 1985 (Tea *et al.*, 1984), has never been widely applied. It is based on the use of a device in which motile spermatozoa that have undergone a swim-up are progressively collected in a central cone as spermatozoa are submitted to spontaneous gravitational sedimentation (Henkel & Schill, 2003).

The purpose of our study was to compare the efficiency of recovering motile spermatozoa with normal morphology from three sperm selection techniques: migration-sedimentation (SS), swim-up from fresh semen (SF), and swim-up from washed (SL) sperm.

MATERIALS AND METHODS

Biological material and sperm analysis

Sperm samples were obtained from 20 normozoospermic men on abstinence for 2-5 days seen at an IVF clinic in Itajai, Brazil. After liquefaction, sperm concentration, motility, and morphology were determined following the WHO laboratory guidelines (WHO, 2010). Concentrations were briefly measured after a dilution of 1:10 with a fixative solution (1% formaldehyde) using a Neubauer chamber (WHO, 2010). Motility was graded in three categories: A: linear or largely circular motion, regardless of speed; B: local displacement without forward progression; and C: motionless. A Makler chamber was used to count a minimum of 100 spermatozoa. For morphology, smears were prepared and air-dried, and then submitted to a Diff-Quick staining procedure (Kruger *et al.*, 1987). A single observer examined the prepared slides. Head, midpiece, and tail morphology was described as being normal or having malformations. A total of 100 spermatozoa were counted.

Set-up of the migration systems

Each sample was divided into three equal aliquots, and each was processed in parallel using three different sperm selection procedures. In all cases, HEPES-buffered Human Tubal Fluid medium (HTF, Irvine Scientific, USA) supplemented with 10% Synthetic Serum Supplement (SSS, Irvine Scientific, USA) was used. For the swim-up from fresh semen (SF) procedure, the medium was first placed on a 15-ml conical sterile Falcon tube; semen was then gently placed underneath it using a

Figure 1. Schematic view of migration-sedimentation (A) and a picture of the RI-MSC™ tube (B). Upward arrows indicate regions of migration out of the seminal plasma into the medium (M). Downward arrows indicate direction of sedimentation. OR: outer rim, CC: central cone.

Pasteur pipette at a ratio of 1:1. For the swim-up from washed (SL) procedure, the sperm aliquot was mixed (2:1) with washing medium and centrifuged (8 min, 350 g). The resulting pellet was then resuspended in HTF medium up to a volume equal to that of the sperm aliquot. A migration set-up similar to that of SF was then prepared for SL. Both the SF and SL tubes were then placed in an incubator at 37°C at a 45° angle for one hour.

A Jondet tube (Research Instruments, England) was kindly provided by Vitrolife (São Paulo, Brazil) for the migration-sedimentation (SS) procedure. The semen aliquot was placed in the external rim of the tube, and subsequently 2.5 ml of the HTF medium were slowly poured into the inner cone (Figure 1). The tube was then incubated for 1.5 hours at 37°C.

After the migration period, 200 µl were removed from the top layer (SF, SL) and from the central cone (SS), and then submitted to sperm analysis. Concentrations and motility grades were determined for each sperm suspension immediately after the end of the selection procedure.

Calculation of recovery ratios (R)

$R = c_f / c_i \times 100$ where c_i and c_f are the initial and final concentrations, respectively.

Statistics

Statistical analysis and graphic representations were produced using the Prism 6 software package (GraphPad, La Jolla, CA 92037 USA). Incidences and frequency distributions were compared using ANOVA with Tukey's test.

Review board

The research project was submitted to the Ethics Review Board of UNIVALI University in Itajaí (Comissão de Ética em Pesquisa, CEP No 644.949). Informed consent was obtained from the sperm donors as part of routine sperm analysis procedures.

RESULTS

The mean recovery and motility rates of twenty tests are presented on Table 1. Significantly ($P < 0.01$) higher recovery rates were obtained with the SS method (25.0%) versus swim-up techniques SF (10.1%) and SL (5.4%); SF and SL were not statistically different.

The percentage of motile spermatozoa (A + B) was significantly lower in SL (77%) when compared to SS (93.6%) and SF (91.2%). Progressive forms (A) were significantly more present in SF (80.4%) and SS (85.6%) when compared to SL (57.6%), whereas non progressive forms (B) were more present in SL (19.1%) than in SF (10.7%) or SS (8.0%).

The percentage of normal forms was significantly ($P = 0.03$) lower in SL (3.5%) than in fresh semen (5.8%), SF (5.2%), or SS (5.5%), and in the differentiated sperm morphology there were no significant alterations between the three tested methods (Table 2).

DISCUSSION

The results of the study showed that SF and SS were equally effective at selecting motile spermatozoa, while SS allowed the recovery of a larger amount of spermatozoa. None of the methods allowed the enrichment of normal forms in the case of normozoospermic samples. SL clearly reduced motility rates, probably as a consequence of exposure to ROS.

Future studies could look into whether the migration-sedimentation technique might be applied successfully to IUI or IVF-ICSI procedures. There are only a few studies in the literature using migration-sedimentation devices, but the ones comparing swim-up and migration-sedimentation have reported similar conclusions, in that the recovery rates of spermatozoa were higher with the migra-

Table 1. Sperm recovery rates (%) and motility grades A and B (%) determined after selection by three semen processing techniques: migration-sedimentation (SS), swim-up from fresh sample (SF), and swim-up from washed sample (SL), expressed as mean $\pm$ SEM.

Parameters	Fresh semen	SF	SL	SS
Concentrations (10⁶/ml)	77.0 $\pm$ 4.3	10.0 $\pm$ 15.2	5.0 $\pm$ 6.7 ^a	22.5 $\pm$ 33.7 ^a
Recovery rates (%)	10.1 $\pm$ 2.4	5.4 $\pm$ 0.9 ^a	25.0 $\pm$ 5.7 ^a	
Motility grades				
A+B (%)	77.0 $\pm$ 11.0	91.2 $\pm$ 2.0 ^a	77.0 $\pm$ 2.8 ^{a,b}	93.6 $\pm$ 1.4 ^b
Progressive, A (%)	80.5 $\pm$ 2.4 ^a	57.6 $\pm$ 4.4 ^{a,b}	85.6 $\pm$ 2.5 ^b	
Non-progressive, B (%)	10.7 $\pm$ 1.4 ^a	19.1 $\pm$ 3.2 ^{a,b}	8.0 $\pm$ 1.5 ^b	

^{a,b}: statistically different ($P < 0.01$)

Numbers without or with different superscripts are not statistically different.

Data were compared using ANOVA and Tukey's test.

Table 2. Sperm morphology determined after selection by three semen processing techniques: migration-sedimentation (SS), swim-up from fresh sample (SF), and swim-up from washed sample (SL), expressed as mean $\pm$ SEM.

Parameters	Fresh semen	SF	SL	SS
Normal (%)	5.8 $\pm$ 0.5	5.2 $\pm$ 0.5	3.5 $\pm$ 0.4 *	5.5 $\pm$ 0.8
Piriform	9.0	10.4	9.2	11.9
Elongated	14.9	14.8	11.4	13.6
Macrocephalic	3.3	1.2	0.8	1.6
Microcephalic	2.0	2.6	4.2	2.5
Amorphous	7.5	7.9	9.4	7.7
Vacuoles	27.4	26.6	26.6	27.9
Midpiece defects	26.7	29.5	28.0	27.4
Tail defects	8.6	7.0	10.3	7.5

*. statistically different ($P < 0.05$).

tion-sedimentation technique than with regular swim-up. Recovery rates ranged from 35% (Cardona Maya *et al.*, 2007) to 58% (Tea *et al.*, 1984), but this variation may have been affected by the way recovery rates were calculated, incubation times, and whether motility was included in the formula (Cardona Maya *et al.*, 2007). In the present study, recovery rates were calculated by taking into account only the number of spermatozoa. The 25.0% recovery rate for SS was significantly higher than that of both swim-up procedures (10.1% and 5.4% for SF and SL, respectively) (Table 1). Migration time is clearly an important factor that plays a greater role in migration-sedimentation than in swim-up, as in the latter case a plateau is reached as soon as an equilibrium between actively upwards moving and downwards sedimenting forms is reached. The amount of medium sampled after migration is another key value that might explain differences between studies; this value was set to 200 μ l for standardization purposes between the three tested techniques in our study.

In the present study, motility rates were equal between SF and SS (Table 2). This was also reported by other authors (Yener *et al.*, 1990), but one study found significantly higher levels of motility using a migration-sedimentation device. Obviously, it should be pointed out that native semen quality affects recovery and motility rates of washed samples (Chan *et al.*, 1991, Hinting *et al.*, 2001); therefore, native semen parameters should be taken into consideration in the selection of the appropriate sperm selection procedure (Cardona Maya *et al.*, 2007, Chan *et al.*, 1991). In the present study, sperm motility in the SL technique was significantly lower than in SF and SS (Table 1). This illustrates the negative effects of centrifugation (Mortimer, 2000), which have been associated with the negative effects of ROS (Henkel *et al.*, 2005). The quantification of sperm motility in this study was based on subjective parameters. Variations of 30% to 60% in the assessment of sperm motility may occur due to limitations of the human eye in quantifying the various sperm subpopulations in a sample (Verstegen *et al.*, 2002). Computer assisted sperm analyzers (CASA) have been shown to identify motility patterns reliably (Matos *et al.*, 2008). Although it is unclear which kinetic characteristics determined by the CASA system may be used to predict fertility or fertilization rates (Ferreira *et al.*, 1997), the use of such a system might shed some light on the particular kinetic behaviors seen in the three analyzed methods.

Enrichment of normal forms using the swim-up procedure has led to contradictory results, with some studies showing higher values than others (Angelopoulos *et al.*, 1998; Hammadeh *et al.*, 2000, 2001). This might be partly due to the subjectivity of morphologi-

cal evaluation, which might vary considerably between laboratories. In this study, a single observer made all determinations using Kruger's strict criteria (Kruger *et al.*, 1986) in order to mitigate uncontrolled subjective appreciation of sperm morphology. However, here again, the quality of the native semen needs to be taken into account, as poor samples may behave differently from normozoospermic ones. Selection of motile spermatozoa is an essential step in assisted reproduction procedures, as spermatozoa need to be freed from decapacitation factors present in the seminal fluid for successful fertilization to take place. In vivo, this occurs spontaneously as the spermatozoon traverses the cervical mucus. In vitro, centrifugation and replacement of the seminal plasma by culture medium is the simplest way to achieve this goal, but it has been recognized that pelleting spermatozoa may cause the release of reactive oxygen species (ROS), which by their turn might induce loss of motility (Henkel *et al.*, 2005), reduction of fertilization capacity, and DNA damage (Henkel *et al.*, 2004). Swim-up from native sperm and use of density gradient have thus become the usual practice over the past decades (Mortimer, 2000). A less common technique, despite its promising features, was developed in the early 1980s by a French group (Tea *et al.*, 1984). It is based on the migration of motile spermatozoa into the medium followed by the recovery of spontaneously sedimenting spermatozoa into a separate receptacle. Various systems have been developed differing in shape and materials (Tea *et al.*, 1984, Ebner *et al.*, 2011), but all maintain a surface of contact between semen and culture medium and a receptacle where spontaneously sedimenting spermatozoa may accumulate. The most recent system reduces the sperm/medium contact to a capillary bridge created by a separate u-ring (Ebner *et al.*, 2011). The system used in the present study is similar to a culture plate, with a central collecting well and an outer rim (Figure 1), and is quite similar to the description of a Jondet (Tea *et al.*, 1984).

Spermatozoa may be exposed to ROS and suffer DNA damage in vivo during sperm migration into the urogenital tract (Pasqualotto *et al.*, 2008), but also in vitro during sperm preparation procedures (Henkel, 2012). Sperm DNA fragmentation has been shown to negatively affect pregnancy rates in IUI treatments, but not in ICSI procedures (Thomson *et al.*, 2011). However, it is generally recognized that increased sperm fragmentation is associated with increased incidence of miscarriage (Pasqualotto *et al.*, 2002, Dar *et al.*, 2013). Techniques that avoid centrifugation and close contact between spermatozoa and ROS producing leucocytes, such as the migration-sedimentation technique, were shown to have the potential to reduce DNA alterations (Ebner *et al.*, 2011).

One might conclude that the migration-sedimentation technique (SS) offers advantages over the traditional methods tested (SF or SL) in terms of sperm recovery and quality. Future studies should confirm that the migration-sedimentation technique can efficiently protect spermatozoa from molecular and structural damage, thus allowing better results in IUI or IVF-ICSI procedures.

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CONFLICT OF INTERESTS

No conflict of interest have been declared.

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