Effect of Swim-up Sperm Washing and Subsequent Capacitation on Acrosome Status and Functional Membrane Integrity of Normal Sperm

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ABSTRACT: Objective-Sperm preparation techniques select sperm population with improved sperm motion characteristics. We sought to determine whether the swim-up technique selects spermatozoa with the ability to undergo hypoosmotic swelling, and how swim-up and subsequent capacitation affect the acrosome reaction rate.

Methods-Semen specimens from 15 normal donors were divided into unprocessed, swim-up, and capacitated groups, and sperm motion characteristics, ability to undergo hypoosmotic swelling, and acrosome reaction rate were measured.

Results-Sperm motility, viability, and all motion characteristics (except linearity) were significantly increased in both swim-up and capacitated specimens. The ability to respond to hypoosmotic swelling was significantly higher in the spermatozoa isolated by swim-up. The percentage of acrosome-reacted spermatozoa remained unchanged in both unprocessed and swim-up groups, but was significantly higher in the capacitated group.

Conclusions-Swim-up isolates sperm with greater ability to undergo hypoosmotic swelling, but does not change the acrosome reaction rate. In vitro capacitation of spermatozoa selected by swim-up enhances the acrosome reaction rate. Int J Fertil 45(5):335-341, 2000

KEY WORDS: spermatozoa, swim-up, functional membrane integrity, hypoosmotic swelling, acrosome

INTRODUCTION

SPERMATOZOA must be washed free from seminal plasma before they are capable of undergoing capacitation, acrosome reaction, and penetration of the zona pellucida [1,2]. Under normal physiological conditions, seminal plasma is removed as the spermatozoa traverse the cervical mucus, and capacitation occurs as they are transported across the cervix, uterus, and fallopian tubes [3].

Prolonged exposure of sperm to seminal plasma results in a marked decline of both motility and viability [4]. Sperm incubated in synthetic culture medium free of seminal plasma contamination show no such declines [5]. It is clearly essential, therefore, that spermatozoa for clinical procedures such as intrauterine insemination or in vitro fertilization be separated from the seminal environment as soon as possible after ejaculation.

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The swim-up procedure remains the simplest means of obtaining a highly motile population of human spermatozoa for assisted reproduction [6,7]. However, high sperm motility is not the only requirement for successful fertilization. Large numbers of spermatozoa with intact acrosome that can undergo normal acrosome reactions are also critical for successful fertilization [8]. The ability of the sperm to undergo the acrosome reaction is dependent on capacitation and correlates with the fertilization rate in vitro and pregnancy outcome [2,9-12]. Although the swim-up procedure involves short-term incubation, it does not seem to induce the biochemical and structural changes that characterize the capacitation phenomenon [9].

A third variable that affects sperm function is membrane integrity. The hypoosmotic swelling (HOS) test assesses the osmoregulatory ability of the sperm membrane in fresh ejaculates, and it can be used to assess the functional membrane integrity [13,14]. Sperm with damaged plasma membranes show no hypoosmotic swelling under hypotonic conditions, and abnormal spermatozoa with poor osmoregulatory capacity swell uncontrollably and rupture [13]. Sperm selected by swim-up may also be evaluated for their ability to undergo hypoosmotic swelling.

The goals of this study were to determine whether sperm washing by swim-up can select not only highly motile sperm but also those with intact acrosomes plus the ability to undergo hypoosmotic swelling, and to study the influence of in vitro capacitation on the acrosome status of spermatozoa selected by swim-up.

MATERIALS AND METHODS

Chemicals

Sperm-washing media (modified Biggers-Whitten-Whittingham [BWW] and Dulbecco’s phosphate-buffered saline [PBS]) were purchased from Irvine Scientific (Santa Ana, CA). Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and bis-benzimide (Hoechst-33258) were obtained from Sigma (St. Louis, MO). A stock solution of FITC-PNA was prepared by dissolving 2 mg FITC-PNA in 1 mL PBS and stored in 600 μL aliquots at -20°C. Hoechst-33258 solution was prepared by dissolving 1 mg Hoechst-33258 in 1 mL PBS; it was then stored in 10-μL aliquots at -20°C. The hypoosmotic swelling (HOS) test solution was prepared by dissolving 7.35 g sodium citrate and 13.51 g fructose in 1 L distilled water [14].

Semen Collection and Assessment of Quality

Semen samples were obtained from 15 normal, healthy volunteers of proven fertility. All subjects were asked to abstain from ejaculation for 48 to 72 hours before their appointments. Semen was collected by masturbation into sterile specimen cups. The ejaculates were allowed to liquefy for 30 minutes at 37°C. A small aliquot was loaded on a counting chamber (Microcell, Conception Technologies, CA) and analyzed on a computer-assisted semen analyzer (CASA, Motion Analysis, Cell-Trak, model VP 110, Santa Rosa, CA) to assess baseline sperm concentration, motility, and motion characteristics. All subjects included in the study had normal semen volume, sperm count, and motility as defined by the World Health Organization criteria [15].

Sperm Washing and Capacitation Procedures

After the initial semen analysis, each semen specimen was divided into three equal aliquots. The first aliquot (the unprocessed sample) received no subsequent treatment. The second and third aliquots were prepared by the swim-up method [12]. Briefly, the liquefied semen sample was diluted with an equal volume of BWW. After centrifugation at 300 g for 10 minutes, the supernatant was removed and the pellet resuspended in 2 mL of BWW. A second centrifugation was followed by resuspension to a final volume of 600 μL of BWW supplemented with 0.3% bovine serum albumin factor V (BSA) (Irvine Scientific, Santa Ana, CA). This small volume, containing a large number of spermatozoa, was divided into three aliquots of 200 μL. Each of them was underlayered beneath 800 μL of BWW supplemented with 0.3% BSA. The tubes were loosely capped and placed in a 37°C incubator, under 5% carbon dioxide in air, at a 45° angle for one hour. During this period, motile spermatozoa migrated from the underlayered sperm suspension to the upper layer. Subsequently, the top 600-700 μL was removed with extreme care to avoid disturbing the interface of the two layers. The supernatant thus contained actively motile spermatozoa.
After swim-up, the second aliquot (swim-up) received no further treatment, and the third aliquot (capacitated) was capacitated for three hours by incubation in a BWW medium with 3% BSA at 37°C, under 5% carbon dioxide in air [2,16]. Unprocessed, swim-up, and capacitated specimens were analyzed by CASA for percent motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), and amplitude of lateral head displacement (ALH).

Calibration Setup of the Motion Analyzer

The CASA calibration setup was as follows: 2-well, 20 μm, duration of data capture (frames): 15 (unprocessed) and 30 (washed); maximum motile speed (μm/sec): 600 (unprocessed) and 800 (washed); distance scale factor (μm/pixel): 0.9457; centroid cell size minimum (pixels): 2; centroid cell size maximum (pixels): 8; number of cells to find per well: 200; minimum number of fields per sample: 3. High correlation between the CASA and manual sperm counts (r² = 1, slope 1) and motility (r² = 0.97, slope 0.97) established the accuracy of CASA measurements [17].

Assessment of Sperm Functional Membrane Integrity

A 100-μL aliquot of the liquefied ejaculate (unprocessed) or resuspended sperm specimens (swim-up and capacitated) was added to 1 mL of the HOS test solution and then incubated for one hour at 37°C. After the incubation period, a 20-μL drop was loaded on a microscope slide and covered with a cover slip. Slides were examined under phase-contrast illumination at 400x magnification to assess sperm tail swelling; swelling in the tail indicated that the spermatozoon was viable with functionally intact plasma membrane. Absence of swelling indicated that the spermatozoon was nonviable and had a functionally damaged membrane.

Assessment of Acrosome Status

The acrosome status in unprocessed, swim-up, and capacitated specimens was evaluated by FITC-PNA in conjunction with Hoechst-33258 as a viability test [18,19]. Simultaneous assessment of viability by Hoechst-33258 and acrosome status by FITC-PNA was done by adding 100 μL of semen specimen to 100 μL of 2 μg/mL Hoechst-33258 solution and incubating for 10 minutes in the dark. The spermatozoa were then washed in PBS solution by centrifugation at 300 g for five minutes to remove excess stain, and the pellet was resuspended in 100 μL of BWW. Twenty microliters of this solution was subsequently smeared on a microscope slide and allowed to dry. The slides were then immersed in ice-cold methanol for 30 seconds to render the sperm membranes permeable and allowed to air dry. The fixed smears were immersed in 40 μg/mL FITC-PNA solution, incubated at room temperature for 20 minutes in a foil covered Coplin jar, and washed gently in PBS to remove the excess label. Scoring was completed within 48 hours of staining.

A Leitz Orthoplan fluorescence microscope equipped with an epi-illumination module and a mercury ultraviolet source (Leitz, Germany) was used to examine the smears. Filter cube 1.2 was used for FITC-PNA, which fluoresces apple green, and cube A.2 for Hoechst-33258, which fluoresces a bright medium blue. The same smear was examined for FITC-PNA labeling and for Hoechst-33258 staining by interchanging the two filters.

Staining Patterns

Viable spermatozoa fluoresce pale blue, and the nonviable spermatozoa fluoresce bright blue-white with Hoechst-33258. A total of 200 spermatozoa per sample were scored. With FITC-PNA labeling, the uniform apple-green fluorescence in the acrosomal region of the sperm head indicated an intact acrosome. In a reacted acrosome, only the equatorial segment of the acrosome was stained. The percentages of acrosome-intact and acrosome-reacted spermatozoa were calculated only for the viable spermatozoa.

Statistical Analysis

Repeated-measures analysis of variance (ANOVA) was used to test for statistical differences in sperm motion char-
acteristics and for differences in functional membrane integrity in unprocessed, swim-up, and capacitated specimens. Owing to the paired nature of the study design, each donor served as his own control. Wilcoxon's signed-rank test was used to compare acrosome status between the groups. An alpha level of 0.05 was considered statistically significant for pairwise comparisons. Statistical analysis was performed with the SAS (v 6.0) statistical software package (Cary, NC).

RESULTS

Sperm characteristics in the unprocessed, swim-up, and capacitated specimens are compared in Table I. Swim-up and capacitated specimens had significantly higher percent motility and improved motion characteristics (P <.04), except linearity, compared to the unprocessed specimens. Sperm motion characteristics of the capacitated group were similar to those of the swim-up group, although the VCL, VAP, and ALH values were higher in the former. Viability scores in both processed specimens (swim-up: 84.5% ± 9.2%; capacitated: 85.3 % ± 8.4 %) were significantly higher than in unprocessed ones (74.8 % ± 12.3 %; P = 0.04 and 0.03, respectively). Similarly, the percentage of spermatozoa with functionally intact membranes was significantly higher in both processed specimens (swim-up: 88.2% ± 5.9%; capacitated: 88.7% ± 6.5%) than in unprocessed specimens (80.2% ± 11.0%; P = 0.03 and 0.01, respectively). Capacitation of spermatozoa selected by swim-up had no effect on functional membrane integrity status (P = 0.82).

In unprocessed specimens, the acrosome reaction in viable sperm occurred spontaneously, at a rate of about 13%. After sperm processing by swim-up, there was no further increase in the rate of acrosome reaction (Table II). However, the percentage of viable acrosome-reacted spermatozoa in the capacitated group (median 20.5 %) was significantly higher than in both unprocessed (median 13.1%) and swim-up groups (median 13.0%; P<.001).

DISCUSSION

Sperm processing is an integral step in assisted reproduction technologies. The most common methods are the swim-up technique and separation through a discontinuous gradient. The advantage of these methods is that they select a highly motile sperm population, in contrast to the non selective concentration of spermatozoa obtained through a simple wash procedure [5,7]. Spermatozoa selected by the swim-up method show enhanced sperm penetration results in the zona-free hamster egg sperm penetration assay (SPA) [20,21]. Although motility is an important aspect of sperm fertilizing ability both in vivo and in vitro, it is not the only one. Efforts have been directed recently to understanding sperm function and developing assays that can predict the fertilization potential of a given sperm population [10,21,22].

TABLE I

Characteristics of unprocessed, swim-up and capacitated sperm specimens in 15 normozoospermic men.

<table>
<thead>
<tr>
<th>Sperm Characteristic</th>
<th>Unprocessed</th>
<th>Swim-up</th>
<th>P*</th>
<th>Capacitated</th>
<th>P†</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (%)</td>
<td>75.8 ± 11.6</td>
<td>91.0 ± 5.1</td>
<td>&lt;0.001</td>
<td>90.2 ± 4.0</td>
<td>&lt;0.001</td>
<td>0.49</td>
</tr>
<tr>
<td>VCL (μm/s)</td>
<td>37.2 ± 7.6</td>
<td>97.4 ± 18.9</td>
<td>&lt;0.001</td>
<td>102.9 ± 21.1</td>
<td>&lt;0.001</td>
<td>0.34</td>
</tr>
<tr>
<td>VSL (μm/s)</td>
<td>16.2 ± 4.0</td>
<td>39.3 ± 5.2</td>
<td>&lt;0.001</td>
<td>37.5 ± 8.7</td>
<td>&lt;0.001</td>
<td>0.42</td>
</tr>
<tr>
<td>VAP (μm/s)</td>
<td>24.5 ± 5.4</td>
<td>62.1 ± 10.2</td>
<td>&lt;0.001</td>
<td>66.2 ± 10.3</td>
<td>&lt;0.001</td>
<td>0.20</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>42.2 ± 5.5</td>
<td>42.9 ± 7.2</td>
<td>0.09</td>
<td>38.4 ± 8.8</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>ALH (μm)</td>
<td>2.4 ± 0.4</td>
<td>3.2 ± 0.7</td>
<td>0.02</td>
<td>3.3 ± 0.7</td>
<td>&lt;0.001</td>
<td>0.52</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>74.8 ± 12.3</td>
<td>84.5 ± 9.2</td>
<td>0.04</td>
<td>85.3 ± 8.4</td>
<td>0.03</td>
<td>0.84</td>
</tr>
<tr>
<td>Hypoosmotic swelling (%)</td>
<td>80.23 ± 11.0</td>
<td>88.2 ± 5.9</td>
<td>0.03</td>
<td>88.7 ± 6.5</td>
<td>0.01</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Values are mean ± SD. P <.05 considered significant.
* Comparison between unprocessed and swim-up groups
† Comparison between unprocessed and capacitated groups
‡ Comparison between swim-up and capacitated groups

VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; LIN = linearity; ALH = amplitude of lateral head displacement.
TABLE II

Frequency (%) of acrosome reaction in unprocessed, swim-up and capacitated specimens.

<table>
<thead>
<tr>
<th></th>
<th>Unprocessed</th>
<th>Swim-up</th>
<th>Capacitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.1 (11.0-15.7)</td>
<td>13.0 (8.5-17.0)</td>
<td>20.5 (17.2-37.8)</td>
</tr>
<tr>
<td>Values are median and interquartile range (25%-75%).</td>
<td>P* &lt;0.05 considered significant.</td>
<td>P† &lt;0.001</td>
<td>P‡ &lt;0.001</td>
</tr>
</tbody>
</table>

* Comparison between unprocessed and swim-up groups
† Comparison between unprocessed and capacitated groups
‡ Comparison between swim-up and capacitated groups

The hypoosmotic swelling (HOS) test can assess sperm viability because it assesses the osmoregulatory ability of the spermatozoon, an important aspect of functional membrane integrity [13]. The ability of the HOS test to differentiate the viability of spermatozoa has been demonstrated only in fresh, not cryopreserved, specimens [14]. Normal sperm morphology [5,20], sperm-zona pellucida (ZP) binding [21-24], and normal intact acrosomes have been shown to correlate with the fertilization rate in vitro and with pregnancy outcome [2,9-11] Therefore, the ideal method for processing sperm for assisted reproduction techniques should be able to select spermatozoa with normal morphology, functionally intact plasma membranes, forward progression, high zona-binding capacity, and normal intact acrosomes.

In the present study, the swim-up preparation produced a highly motile, viable sperm population with functionally intact membranes. Additionally, the swim-up technique did not change the percentage of viable acrosome-reacted sperm over the baseline spontaneous results seen in the unprocessed specimens, since we observed similar rates of acrosome-reacted spermatozoa in both unprocessed and swim-up specimens. A plausible explanation for this finding is that the swim-up procedure eliminates immotile and dead spermatozoa along with exfoliated cells, cellular debris, and amorphous material. The major component of the swim-up fluid is live sperm exhibiting good motility and the ability to undergo hypoosmotic swelling, which is correlated with viability [5,14].

During the swim-up procedure, the spermatozoa escape the inhibitory factors in the seminal plasma and can, therefore, undergo capacitation. Ejaculated sperm cannot undergo the acrosome reaction if they do not complete capacitation [25]. Processed specimens that have not undergone capacitation are expected to have low baseline acrosome reaction rates [25]. Abnormally high rates (>20%) of acrosome reaction unrelated to capacitation are seen in males with unexplained infertility [26]. In our study, the viable, spontaneous acrosome reaction rate was about 13% in unprocessed semen. With swim-up, the acrosome reaction rate was also 13%, which suggests that the impact of swim-up may not have negative clinical implications, since this processing step did not increase the percentage of acrosome-reacted spermatozoa. Other investigators have also shown that a small proportion of the spermatozoa from fertile men is already acrosome-reacted after ejaculation [12,27]. On the other hand, we observed that further incubation of spermatozoa selected by swim-up under culture conditions that promote capacitation increased the acrosome reaction rate. From our data, it seems clear that induction of capacitation is not achieved by swim-up alone, even though this procedure involves short-term incubation.

Following ovulation, the oocyte has an average life span of 12 to 24 hours during which fertilization can occur [28]. Capacitation requires from 3 to 12 hours for sperm obtained from male infertility patients [1]. Appropriate timing of insemination is, therefore, clinically important for such men, particularly when IUI is to be performed. Prolonged sperm incubation under culture conditions may actually inhibit the ability to achieve pregnancy by IUI, since the frequency of acrosome reaction increases with the duration of incubation [27].

In conclusion, the swim-up preparation selects sperm with better motility and ability to undergo hypoosmotic swelling. Our study further establishes that sperm processing by swim-up does not have a negative impact on acrosome status, since the frequency of acrosome reaction is unchanged in a comparison with unprocessed semen. Prolonged in vitro incubation of spermatozoa selected by swim-up enhances the acrosome reaction, and this may be detrimental to use for intrauterine insemination.
REFERENCES


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