INFLUENCE OF ARTIFICIAL STIMULATION ON UNPROCESSED AND PERCOLL-WASHED CRYOPRESERVED SPERM

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The ability of pentoxifylline and 2-deoxyadenosine to stimulate sperm motility and motion characteristics was assessed in unprocessed and processed (Percoll-separated) cryopreserved specimens. Specimens from 12 healthy volunteers were obtained, and the motion characteristics were analyzed; half the sample was immediately cryopreserved and the other was washed using the Percoll gradient technique. To study stimulation, samples were thawed and divided into four aliquots: One was used as a control, and the others were incubated with 2.5 mM 2-deoxyadenosine, 2.5 mM pentoxifylline, or 5.0 mM pentoxifylline for 60 min. Sperm characteristics were analyzed on a sperm motion analyzer at 0 and 60 min incubation. In both unprocessed and processed samples, percentage motility improved significantly after stimulation with 2-deoxyadenosine or pentoxifylline \( p = .003 \) or \( p = .0002 \), respectively; other characteristics improved to varying extent after 2-deoxyadenosine or pentoxifylline stimulation. Comparison after stimulation revealed that sperm motion characteristics in the unprocessed specimen were similar to sperm motion characteristics in the processed group.

Keywords cryopreservation, 2-deoxyadenosine, pentoxifylline, Percoll wash, spermatozoa

Cryopreservation has become a useful tool for preserving semen from men who are expected to experience subfertility after events such as chemotherapy, spinal cord injury, or because sperm aspirated from the epididymis remain after intracytoplasmic sperm injection. However, cryopreservation and thawing result in a significant reduction not only in the sperm motion characteristics but also in the fecundity and pregnancy rates [9, 24].

Several laboratory techniques are used to select motile sperm in fresh specimens [1, 12, 13]. The discontinuous Percoll gradient technique is universally accepted because it separates a highly motile subpopulation of spermatozoa that has a high fertilizing capacity and an increased longevity [5]. However, the quality of cryopreserved specimens separated on a Percoll gradient is controversial [2, 9]. Sperm motility can also be increased with artificial stimulants

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173
such as pentoxifylline, a methylxanthine derivative, and 2-deoxyadenosine, an analogue of adenosine that inhibits cyclic adenosine 3',5'-monophosphate (cAMP) phosphodiesterase [7, 10, 11].

This study evaluated whether incubation of cryopreserved unprocessed and processed (Percoll-separated) specimens with 2-deoxyadenosine or varying concentration of pentoxifylline can improve sperm motion characteristics.

MATERIALS AND METHODS

_Sperm Preparation._ Semen samples from healthy donors (_n_ = 12) were evaluated as per World Health Organization criteria [25]. All subjects were asked to abstain from sexual activity for 48 h. The samples were obtained by masturbation into a sterile specimen cup. The ejaculate was allowed to liquefy at 37°C, and the semen characteristics were analyzed. After analysis, the samples were divided into two aliquots; one aliquot was frozen without any processing (unprocessed). The second aliquot was separated on a discontinuous density gradient (Percoll, Perception Fertility Technologies, Natick, MA, USA) (processed). For processing on Percoll density gradient the pellet was prepared as described earlier [15]. In brief, Percoll gradient consisted of the lower phase (90%) and the upper phase (47%) reconstituted from 100% Percoll with Heps-buffered human tubal fluid (HTF). Two milliliters of the lower phase was transferred into a 15-mL centrifuge tube and 2 mL of the upper phase was gently layered onto the lower phase to create a clear interface. Once the gradient was prepared, 0.5 to 1.0 mL of the ejaculate was layered on the top and centrifuged at 300g for 20 min. The supernatant was removed and resuspended in HTF and centrifuged at 300g for 7 min. The final pellet was resuspended in HTF.

_Cryopreservation._ Semen samples (unprocessed and processed) were diluted by dropwise addition of Test Yolk Buffer (TYB)-freezing medium (Irvine Scientific, Santa Ana, CA, USA) [15]. Four additions were done to provide a final semen to TYB ratio of 1:1 (vol/vol). After the aliquot was placed in 1.2-mL screw-top disposable, sterile cryogenic vials, the vials were attached to the canes and cryopreserved by slow freezing at −20°C for 8 min, followed by immersion in liquid nitrogen vapor at −79°C for 2 h and finally submersion in liquid nitrogen at −196°C.

_Motility Stimulation._ For stimulation study, the cryopreserved specimens were thawed by incubating them at room temperature for 5 min followed by incubation at 37°C for 20 min. They were washed once with HTF, centrifuged at 300g for 7 min, and then resuspended in HTF to a concentration of 25 to 30 × 10⁶ sperm/mL. A 5 mM stock solution of 2-deoxyadenosine and a 10 mM stock solution of pentoxifylline (Sigma Chemical, St. Louis, MO, USA) were prepared in HTF and used as motility stimulants. The sperm suspensions were divided into 4 aliquots, one each for 3 concentrations of the stimulant, and one for a control. The stimulants were dissolved in HTF to give the following final concentrations: 2-deoxyadenosine 2.5 mM (1:1 vol/vol) and pentoxifylline 5 mM (1:1 vol/vol) and 2.5 mM (1:1 vol/vol from a 5 mM solution). The control aliquot was mixed with HTF alone. The samples were then analyzed immediately before stimulants were added (0 min incubation) and after a 60-min incubation at 37°C.
Semen Analysis. Sperm samples were analyzed on a computer-assisted motion analyzer (Cell-Trek, Model VP 110, Santa Rosa, CA, USA). For each measurement, a 5-µL sample aliquot was loaded on a MicroCell slide (Conception Technologies, La Jolla, CA, USA) and analyzed for percent motility, curvilinear velocity (total distance traveled by a given sperm divided by the total time elapsed), straight-line velocity (straight-line distance from the beginning of the sperm track divided by time), average path velocity, linearity (ratio of straight line velocity to curvilinear velocity), and amplitude of lateral head displacement (mean width of sperm head oscillation). To verify the accuracy of CASA results, manual counts and motility readings were taken on each specimen [16].

Statistical Analysis. Repeated measures analysis of variance (ANOVA) was used to assess the effect of stimulants on each sample, and a paired t test for analyzing the differences between the processed and unprocessed specimens using the SAS statistical software package (SAS Institute, 1992). A p value of \leq 0.05 was considered statistically significant. Semen specimens were studied individually to avoid possible interaction from pooling the specimen.

RESULTS

Compared with prefreeze values, sperm motility significantly decreased in both unprocessed and processed specimens after freezing, but the difference was not significant when post-thaw motility was compared in the two groups. Both 2-deoxyadenosine and pentoxifylline significantly improved sperm motion characteristics. Values for all motion characteristics increased significantly in unprocessed and processed specimens except for linearity in the raw specimens (Table 1). Compared to 0 min incubation (control), 2-deoxyadenosine significantly increased values for all motion characteristics. Similarly, pentoxifylline significantly improved sperm motility at 2.5 and 5 mM concentrations (Table 2). Sperm characteristics of artificially stimulated processed specimens showed no improvement when compared with unprocessed samples (Figure 1).

DISCUSSION

Poor sperm motility after cryopreservation is associated with a significant decrease in fecundity, pointing to the importance of preserving motility in cryopreserved sperm [6]. Considerable variation exists in the ability of sperm from individual donors to survive the rigors of cryopreservation, so predicting the outcome of ejaculates subjected to freeze–thaw procedures is difficult [8].

The mechanism by which the Percoll gradient selects and possibly protects sperm is not fully understood. It may reduce sperm membrane damage caused by excessive production of reactive oxygen species or may eliminate factors that inhibit one of the steps in the fertilization process [20]. However, separation of samples on the Percoll gradient after cryopreservation not only does not improve motion parameters but also further decreases motility, velocity, linearity, viability, and the number of intact acrosomes [3]. This decrease indicates that Percoll separation of cryopreserved samples is not a suitable technique for post-thaw separation of motile spermatozoa [3]. Quite possibly, the frozen spermatozoa have already been damaged during freezing and have lost the ability to retain their viability and motion parameters, and further treatment may not be helpful. In an earlier study, we reported that when spermatozoa
Table 1. Influence of time and artificial stimulation on motion characteristics in unprocessed and processed cryopreserved spermatozoa (n = 12)

<table>
<thead>
<tr>
<th>Motion characteristics</th>
<th>Time with no stimulant (0)</th>
<th>Time with stimulants* (0)</th>
<th>Time and stimulant interaction (0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>NS</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>VCL</td>
<td>0.001</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>VSL</td>
<td>NS</td>
<td>0.001</td>
<td>0.02</td>
</tr>
<tr>
<td>VAP</td>
<td>0.001</td>
<td>0.0</td>
<td>NS</td>
</tr>
<tr>
<td>LIN</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ALH</td>
<td>0.001</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Processed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>NS</td>
<td>0.0001</td>
<td>0.04</td>
</tr>
<tr>
<td>VCL</td>
<td>0.01</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td>VSL</td>
<td>NS</td>
<td>0.0002</td>
<td>NS</td>
</tr>
<tr>
<td>VAP</td>
<td>NS</td>
<td>0.002</td>
<td>NS</td>
</tr>
<tr>
<td>LIN</td>
<td>0.004</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>ALH</td>
<td>0.002</td>
<td>0.0004</td>
<td>NS</td>
</tr>
</tbody>
</table>

Note. Repeated measures ANOVA, p < .05 is statistically significant. NS, not significant; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; ALH, amplitude of lateral head displacement.

*Comparison of overall effect of 2-deoxyadenosine and pentoxifylline.

from healthy donors were Percoll-separated before cryopreservation, they remained motile for a longer period (24 h) than did unprocessed samples, suggesting that Percoll treatment lessens the damage associated with time [15]. Therefore, sperm preparation techniques if used before freezing can make samples resistant to structural and biochemical damage induced during freezing. Because sperm motility is critical for all assisted reproductive procedures, methods that help maintain motility for a sufficient time to permit completion of intrauterine insemination and other reproductive procedures are vital. The physiology of cryopreserved sperm suffi-

Table 2. Effect of stimulants on unprocessed and processed cryopreserved spermatozoa (n = 12)

<table>
<thead>
<tr>
<th>Motion characteristics</th>
<th>0 vs. 2-DA (2.5 mM)</th>
<th>0 vs. PTX (2.5 mM)</th>
<th>0 vs. PTX (5 mM)</th>
<th>2-DA (2.5 mM) vs. PTX (2.5 mM)</th>
<th>2-DA vs. PTX (5 mM) vs. PTX (5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>0.003</td>
<td>0.0002</td>
<td>0.0005</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>VCL</td>
<td>0.0003</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>VSL</td>
<td>0.004</td>
<td>NS</td>
<td>NS</td>
<td>0.0007</td>
<td>0.0001</td>
</tr>
<tr>
<td>VAP</td>
<td>0.0002</td>
<td>NS</td>
<td>NS</td>
<td>0.009</td>
<td>0.0001</td>
</tr>
<tr>
<td>LIN</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.0001</td>
</tr>
<tr>
<td>ALH</td>
<td>0.0001</td>
<td>NS</td>
<td>NS</td>
<td>0.008</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Note. p ≤ .008 was considered significant by paired t test. 2-DA, 2-deoxyadenosine; PTX, pentoxifylline; NS, not significant; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; ALH, amplitude of lateral head displacement.
Stimulation of Cryopreserved Sperm

Figure 1. Sperm motion characteristics in (A) unprocessed and (B) processed semen specimen. Values are means ± SD (n = 12).

Spermiolytically differs from fresh sperm to narrow the fertilizing ability of frozen sperm [15]. The Percoll gradient technique selects sperm with better morphological quality, and such selection leads to improved fertilization rates [2, 23].

Sperm motility decreases 25 to 75% after cryopreservation [15]. Previously, we established the optimum concentrations and incubation time at which pentoxifylline and 2-deoxyadenosine maintain improved motion variables in cryopreserved raw sperm samples from normozoospermic volunteers [16, 18]. Mbizvo et al. [11] reported longer-lasting effects with 2-deoxyadenosine than pentoxifylline, and Imoedemhe et al. [7] found 2-deoxyadenosine improved fertilization rates and motility. The increase in motility suggests that pentoxifylline and 2-deoxyadenosine may act on a subpopulation of cryopreserved sperm that have poor motility or are metabolically quiescent and that become active in response to an increase in cAMP levels induced by these stimulants. The increase in cAMP is probably the main mechanism underlying the increased motility observed with pentoxifylline; pentoxifylline also inhibits reactive oxygen
species formation [17], causes hyperactivation of sperm [14, 19], and enhances the acrosome reaction [4].

In the present study, all concentrations of 2-deoxyadenosine and pentoxifylline tested enhanced cryopreserved sperm motility in unprocessed as well as processed specimens at an incubation of 60 min. Earlier, we reported that motion parameters are similar in fresh raw and fresh processed specimens at short incubation intervals of 1 to 3 h; it was only after 6 h of incubation that washed specimens had a smaller loss of motility compared to raw specimens [15]. The effect of these stimulants is maintained for as long as 3 h [18]. Therefore, if Percoll separation is to be used in cryopreserved specimens for maintaining improved motion characteristics, artificial stimulants may not further improve the motion characteristics beyond a few hours. However, the net effect of these stimulants may not be limited to increased motility alone; other sperm functions required for successful fertilization, such as egg penetration, may also be modulated by cAMP [21, 22]. Whether Percoll processing before freezing improves fertilization outcome also warrants further investigation. Randomized well-designed clinical studies are needed to confirm pregnancy rates after using these stimulants in cryopreserved samples.

In conclusion artificial stimulants improve sperm motion characteristics in both unprocessed and processed sperm specimen after freezing. Addition of stimulants does not confer any added advantage in combination with processing of semen specimen by Percoll separation.

REFERENCES


