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Authors
Dantas, ZN
Araujo, E
Tadir, Y
et al.

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Effect of freezing on the relative escape force of sperm as measured by a laser optical trap*†

Zoetania Nery Dantas, M.D.‡  Michael W. Berns, Ph.D.§
Edilberto Araujo, Jr., M.D.‡  Michael J. Schell, Ph.D.‖
Yona Tadir, M.D.§§  Sergio C. Stone, M.D.¶¶

University of California, and Beckman Laser Institute and Medical Clinic, Irvine, California

Objective: To determine the possible effect of freezing on sperm escape force as measured by laser trap.

Design: Controlled clinical study.

Setting: Normal volunteers, academic setting.

Patients: Normal, healthy volunteers.

Intervention: Sperm selection, freezing and thawing. Measurement of relative (sperm) escape force before and after freezing.

Main Outcome Measure: Comparison of escape force as a measurement of freezing effects.

Results: Wide individual variations noted, with some individuals showing significant increases or decreases. The relative escape force in fresh (76.1 ± 31.1 mW) and in frozen-thawed samples (75.6 ± 40.0 mW) were similar.

Conclusion: Freezing does not affect the relative escape force of normal sperm. Any possible detrimental effect of freezing on sperm may be related to other sperm functions.

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Key Words: Normal sperm, freezing, thawing, escape force

Recent advances in laser technology have allowed the application of laser microbeams to human gametes (1-4). Human sperm motility was studied by Tadir et al. (3) using the force generated by radiation pressure of an infrared laser beam. A second study (4) applying micromanipulation of human spermatozoa with a laser-generated optical trap assessed the relative force generated by each single spermatozoan. The swimming force generated by sperm is a function of flagellar movement. It is not only a fundamental expression of the sperm viability but probably also it is essential for reproductive capabilities (3). In the Tadir study (3), the measurements were performed by gradually decreasing the laser power until the sperm were able to swim out of the trap. These values were defined as the relative escape force. Therefore, the force generated by each sperm, defined as relative escape force, is proportional to the threshold trapping power required for immobilization.

This novel technology was applied in the present study to assess the relative escape force of human gametes.
ejaculated sperm in fresh and frozen-thawed samples from the same subject. In addition, we hoped to determine possible adverse effects of the freezing process on sperm swimming force as measured with an optical trap.

MATERIALS AND METHODS

Ten normal sperm samples were selected for this study based on the selection criteria of the World Health Organization (WHO) (5). The semen analysis protocol consisted of a 48-hour abstinence period, collection by masturbation, and examination within 1 hour of collection, using WHO standard protocol for the evaluation of seminal specimens. Motility was estimated as the proportion of sperm showing progression on a glass slide under ×400 magnification, counting 100 sperm per field in at least 10 fields. Morphology was evaluated by preparing a stained specimen with methylene blue and counting 100 sperm under ×400 magnification.

After semen analysis, each sample was placed in a sterile container and diluted in HEPES-buffered human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA) supplemented with 0.5% human serum albumin. The samples were centrifuged once at 200 × g for 10 minutes, and the resulting pellet was resuspended in HTF. At this point, each individual sample was split into two aliquots; one was taken for laser analysis within an interval of 2 to 3 hours, and the other half was frozen in vials in a yolk buffer freezing media (Irvine Scientific). As per our routine, they were frozen by the manual method of hanging the semen and yolk buffer mixture in liquid nitrogen vapor. Two to 10 days later, the frozen sample was thawed at room temperature, washed, resuspended in HTF, and taken for laser analysis. The same technique for freezing-thawing was applied to all samples, performed by the same biologist.

A total of 2,130 morphologically normal spermatozoa with normal motility were trapped at room temperature: 1,160 sperm from fresh samples and 970 from matched frozen-thawed samples. An average of 100 sperm were trapped in each aliquot; the number of sperm trapped ranged from 57 to 135 per aliquot. The sperm randomly selected for analysis were the ones that present visible swimming movement around the laser beam trap.

The laser optical trap employed in the study was a titanium-sapphire laser (model 899-01; Coherent Innova, Palo Alto, CA), operating continuous wave, at an infrared wavelength of 800 nM and with a power at the focal point ranging from 0 to 300 mW. The beam was directed into a Zeiss photo microscope (Zeiss, Thornwood, NY) and focused using a 100× Neofluar objective (1.3 numeric aperture). A variable attenuator (model 930; Newport Corp., Fountain Valley, CA) was used to control the laser power. A beam polarizer (Melles Griot; Glen Thompson, Irvine, CA) was used also to obtain a linear calibration curve. The spot diameter at the focal plane was in the range of 2 to 3 μm. The sperm were confined in the optical trap while the trapping power was attenuated gradually until the sperm could swim away. Remote real-time viewing of the sperm was performed using a video camera (Series 68; Dage-MTI Inc., Michigan City, IN) par focal with the trapping beam. A photodiode and calorimeter were used to calibrate the optical trap.

Statistical analysis was performed by using the paired Student's t-test to compare the two groups considered (fresh versus frozen) in the study and using Student's t-test to compare the two groups for individual patients. The minimum sample size in any group was 57. Correlation of the sperm count, sperm motility, sperm normal forms, and logarithm of the total motile count with relative escape force was examined for fresh and frozen sperm using Pearson's correlation coefficient.

RESULTS

The mean relative escape force of the ejaculated sperm in fresh samples was 76.1 ± 31.1 mW (mean

<table>
<thead>
<tr>
<th>Patient</th>
<th>Fresh</th>
<th>Frozen</th>
<th>Probability values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>127.0 ± 37.1</td>
<td>108.7 ± 42.1</td>
<td>0.001</td>
</tr>
<tr>
<td>2</td>
<td>63.9 ± 41.1</td>
<td>73.9 ± 41.6</td>
<td>NS†</td>
</tr>
<tr>
<td>3</td>
<td>116.4 ± 55.0</td>
<td>84.5 ± 47.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>86.8 ± 34.3</td>
<td>91.4 ± 32.4</td>
<td>NS†</td>
</tr>
<tr>
<td>5</td>
<td>45.0 ± 19.0</td>
<td>77.5 ± 17.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6</td>
<td>40.5 ± 10.5</td>
<td>118.9 ± 10.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>7</td>
<td>104.5 ± 18.6</td>
<td>129.9 ± 27.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>8</td>
<td>60.6 ± 18.7</td>
<td>19.5 ± 10.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>9</td>
<td>45.4 ± 11.3</td>
<td>19.6 ± 12.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10</td>
<td>70.9 ± 15.4</td>
<td>32.6 ± 15.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>All</td>
<td>76.1 ± 31.1</td>
<td>75.6 ± 40.0</td>
<td>NS*</td>
</tr>
</tbody>
</table>

* Values are means ± SD.
† NS, P value not significant.
The results of this preliminary study show that the overall mean relative escape force of the ejaculated sperm in fresh samples was not significantly different from the mean relative escape force in the frozen-thawed samples ($P > 0.05$). Given the sample size used ($n = 10$), we would have been able to detect a 50% difference in the mean relative escape force between fresh and frozen-thawed sperm with 80% power. However, wide individual variations were observed. In five subjects, the fresh samples had higher relative escape force than the frozen-thawed samples, whereas in three other subjects the ordering was reversed ($P < 0.001$). Yet, the same technique for freezing-thawing was performed by the same biologist, thus minimizing experimental condition variations that could account for the observed intersample differences. The variability of intrapatient differences was surprisingly high (SD = 38.2). These intrapatient comparisons were made on sperm taken from a single split sample. We suspect that the sample-to-sample variability is much larger than the intrasample variability, contributing to the nonsignificant effect seen overall.

Detrimental effects of the freezing-thawing process were noticed in the conventional sperm parameters measured, except for morphology (Table 2). Despite the reduction in the number of motile sperm in the cryopreserved sample compared with the fresh counterparts, the relative escape force of sperm was not altered significantly. It appears that there is a population of sperm that survive the freezing-thawing in five subjects (nos. 1, 3, 8, 9, and 10) and increases in three others (nos. 5, 6, and 7). In two subjects the increases were not significantly different (Table 1).

Table 2 depicts the sperm conventional parameters in the fresh and frozen-thawed groups before trapping. As expected, there was a significant decrease in percentage of sperm motility as well as total motile count for all subjects in the frozen-thawed group. Also, progression was noticed to be adversely affected. Yet, no difference was noted in the number of normal sperm forms.

Significant correlations were seen with the logarithm of the total motile count with relative escape force for fresh and frozen sperm ($r = 0.73$, $P = 0.02$ and $r = 0.68$, $P = 0.03$, respectively) and between sperm motility and relative escape force in frozen sperm. Positive but not significant correlations were observed between sperm normal forms with relative escape force for either fresh or frozen sperm.

**DISCUSSION**

The results of this preliminary study show that the overall mean relative escape force of the ejaculated sperm in fresh samples was not significantly different from the mean relative escape force in the frozen-thawed samples ($P > 0.05$). Given the samples...
studies have pioneered standardization of the methodology to some degree; however, we agree that future research is needed to replicate the methods and produce a body of solid conclusions.

Westphal et al. (6) have demonstrated that sperm exposed to cumulus oophorus exhibited a significant increase in relative escape force. In a different study, Araujo et al. (7) found that the average relative escape force of epididymal sperm was 60% weaker than that of ejaculated sperm. However, general consensus is that it is still too early to assess the direct relevance of this parameter as a predictive marker of fertilization capacity in vitro or the implication on therapeutic donor insemination with frozen sperm. Different techniques of freezing and thawing may have different effects on sperm function. Absolute velocity and relative escape force are different physiological parameters that may not correlate, as was demonstrated in a previous study (4). Thus, the assessment of relative escape force and fertilization in vitro may add valuable information on sperm performance and possibly offer a new tool for choosing a preferred cryopreservation method for different patients. Studies evaluating these parameters may provide the most useful information about male gamete function. This research is now in progress in our laboratory.

REFERENCES