Effect of Different Thawing Temperatures on the Motility Recovery of Cryopreserved Human Sperm

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Abstract

Introduction: Standard protocol designed to optimize sperm motility recovery after thawing has not yet been established.

Purpose: We aimed to study the effect of different thawing temperatures on the motility recovery of cryopreserved human sperm.

Patients and Methods: Fifty semen samples of males complaining of primary infertility and oligozoospermia. A semen sample was obtained and evaluated. Every sample was divided into three straws that underwent cryopreservation, then thawing at three different temperatures; 25°C, 37°C and 40°C. Pre-cryopreservation sperm motility was compared to the post-thawing motility at each of the three thawing temperatures.

Results: Total motility percentage was better at thawing temperatures of 37°C and 40°C compared to 25°C. Rapidly progressive motility was the highest at thawing temperature 40°C compared to the other thawing temperatures; 25°C and 37°C as p-value was 0.08, <0.001 and <0.001 respectively denoting no significant difference in change of the rapidly progressive motility before cryopreservation and at thawing temperature 40°C.

Conclusion: Thawing cryo-preserved semen samples with severe oligozoospermia at 40°C provides highest potential for restoring sperm motility.

Key Words: Thawing temperatures – Sperm motility recovery – Cryopreservation.

Introduction

CRYOPRESERVATION is an essential tool routinely implemented in Assisted Reproduction Technologies and semen banking [1]. It is widely settled in IVF centers for the different Assisted Reproduction Technologies like insemination, in vitro fertilization and intracytoplasmic sperm injection. The assessment of spermatozoa viability, motility and sometimes DNA integrity after freezing/thawing has been considered enough to guarantee the safety and effectiveness of the technique [2].

Over the last few decades, sperm freezing has been technically improved. However, there is no standard protocol settled to optimize sperm motility recovery after thawing [3].

There are different systems which designed the optimal rate of cooling to freeze and the optimal rate of warming to thaw with regard to the cryo damage [4]; these protocols require further resolution for mammalian sperm [5].

During cryopreservation, there are physical and chemical stresses that affect the spermatozoa [6], changes in lipid composition of the sperm plasma membrane [7], decrease in the head size [8], and externalization of phosphatidylserine [9]. Other factors that contribute to these changes are the degree of sperm maturation and the type of cryoprotectant used [10]. In a given system, the warming rate depends on the cooling rate; the faster the cooling rate, the faster the warming rate should be [5]. It was found that to get a higher post-thaw sperm viability, it is preferably to use rapid warming during thawing [11]. Mostly all cryopreservation protocols use 37°C as an optimal temperature of thawing and avoid higher thawing temperatures because of the possible risk of cell damage. The recently introduced sperm stress tests require incubation of spermatozoa at 40°C for 4 hours and it was found that it is a useful predictor of pregnancy rate in ARTs [12,13]. However, the effect on sperm viability and actual time of exposure to 40°C has not yet investigated. Therefore, the main objective of this study was to assess the effects of thawing at temperatures at 25°C, 37°C and 40°C on sperm post-thaw motility.
The plasma membrane of mammalian spermatozoa has a high polyunsaturated fatty acid content, which makes it highly susceptible to lipid peroxidation. This could contribute to sperm cryodamage. Human spermatozoa are particularly susceptible to oxygen radical-induced lipid peroxidation [14,15]. Lipid peroxidation reactions have been shown to have a steep temperature coefficient and, therefore, there may be a greater risk of peroxidative damage at 40°C compared with at lower temperatures. Human spermatozoa have enzymatic antioxidant protection, which has wide variation between individual sperm samples [15]. Peroxidative reactions cause phospholipid damage that results in increase the permeability of the sperm membrane and loss of ATP with negative impact to the sperm motility in return [16,17]. Intracellular ATP is of paramount importance that maintain sperm motility [18]. Loss of ATP is a major indicator of membrane damage leading to loss of viability and sperm fertilizing ability.

Patients and Methods

This is a prospective study involving fifty males consulting a private ART center for reason of primary infertility for more than one year on account of oligo-astheno-teratozoospermia in the period between May 2012 and July 2013. Each couple was counseled for ICSI procedure after evaluation of the female partner and ensuring that they were fit for starting the protocols established for ovarian induction by the gynecologist. All couples were informed about the aim of the present study and that it is totally irrelevant to the ICSI procedure they are going to do. They were also informed that they will only participate in the study through providing a semen sample after that one which will be used for the ICSI procedure.

Each male has been subjected to history taking including personal, sexual, medical, and surgical history and history of medical treatment for infertility. They underwent physical examination; general examinations was performed with special emphasis on secondary sexual characters, distribution of facial and body hair, presence of gynecomastia, trunkal obesity, and any syndromal features such as Klinefelter syndrome. Genital examination was conducted with emphasis on testicular volume and consistency, assessing presence of varicocele, penile size and hair distribution. Then the subjects were given clear written and spoken instructions concerning the collection of the semen sample. They were informed that the semen sample needs to be complete and that they should report any loss of any fraction of the sample. Samples were obtained by masturbation after 2-7 days of abstinence and evaluated according to the laboratory manual for the Examination and processing of human semen [19]. Sperm motility was assessed as soon as possible after liquefaction of the sample, at around 30 minutes, but in any case within 1 hour following ejaculation, to limit the deleterious effects of dehydration, pH or temperature changes on motility. A wet preparation 20μm deep was prepared in a pre-warmed Makler chamber, and motility was assessed at 37°C. Motility was classified into progressive, non-progressive and immotile, then further classified as per progression into rapidly progressive motility (type A), slowly progressive motility (type B), non-progressive motility (type C) and immotile (type D). If the sperm was swimming forward at ≥5 times the length of its own head in one second it was classified as (rapidly progressive) and if the sperm was moving forward at an otherwise velocity (i.e. moving between 1 and 5 head lengths per second) it was classified as (slowly progressive). Sperm morphology was assessed using strict criteria established by [20]. Every semen sample underwent the same processing steps and cryopreserved according to the standard vapor freezing procedure.

The whole volume of the sample was poured in a tube of 5ml capacity, to which was added HEPES buffered Ham F-10 as washing medium, and centrifuged for 15 minutes with a speed 2000 R.P.M (round per minute). The supernatant was then discarded and the sediment dissolved in 1ml Ham's F-10. Cryoprotectant (SpermFreezeTM Fertipro) was equilibrated at room temperature to avoid cold shock to spermatozoa, then 1ml was added to the sample. The medium was added in drops while gently swirling. After that the mixture was left for 10 minutes at room temperature for equilibration. The sample/medium mixture was then divided and aspirated into three freezing straws of 0.5ml capacity. Sealing of the straws was done using heat sealer. The three straws were placed in a metal can which was placed into liquid nitrogen vapor for 25 minutes (manual freezing). Then it was inserted into the respective sperm freezing canister, plunged into liquid nitrogen, and the storage details were recorded. After one month from cryopreservation, the three straws were thawed by rapid thawing technique. Every straw was rapidly dipped into temperature-controlled glass water bath for 3 minutes at three different temperatures; 25°C (referred to the room temperature), 37°C and 40°C respectively. The temperature was monitored using calibrated digital thermometer for standardization. The ends of the straws were
cut off with sterile scissor and the contents were expelled into labeled tubes.

After centrifugation and washing, the supernatant was discarded and the pellet was dissolved equal volume of Ham's F-10 medium. A 20µm deep wet preparation on pre-warmed slides and cover-slips was evaluated for motility as aforementioned. The pre-cryopreservation sperm motility was compared to post-thawing motility at the three different temperatures tested. Date were statistically described in terms of range, mean ± Standard Deviation (±SD), frequencies (number of cases) and percentage when appropriate.

Different statistical tests (Mann-Whitney and \( t \)-test) were used in order to compare the pre-thawing sperm motility and that of the three different temperatures tested. Correlations were made to other findings from the history and examination.

**Statistical analysis:**

All statistical calculations were done using computer programs Microsoft Excel 2010 (Microsoft Corporation, NY, USA) and SPSS (statistical package for social science; SPSS Inc; Chicago, IL, USA) version 19 for Microsoft Windows. Results were expressed in mean ± Standard Deviation (SD), frequencies (number of cases) and percentages when appropriate. Comparison of means was performed using paired-samples \( t \)-test. A probability value (\( p \)-value) less than 0.05 was considered statistically significant.

**Results**

**Table (1):** Total motility percent before cryopreservation, and after thawing at temperatures 25ºC, 37ºC and 40ºC.

<table>
<thead>
<tr>
<th>Total motility %</th>
<th>Mean ± SD</th>
<th>Med</th>
<th>Min</th>
<th>Max</th>
<th>% decrease of the med</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before cryo</td>
<td>28.1 ±14.1</td>
<td>27.5</td>
<td>5</td>
<td>65</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>At 25ºC</td>
<td>10.1 ±6.7</td>
<td>10</td>
<td>0</td>
<td>25</td>
<td>63.6%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>At 37ºC</td>
<td>14 ±8.1</td>
<td>10</td>
<td>2</td>
<td>30</td>
<td>63.6%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>At 40ºC</td>
<td>13.3 ±7.7</td>
<td>13</td>
<td>1</td>
<td>35</td>
<td>52.7%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Comparison of means was performed using paired-samples \( t \)-test. A probability value (\( p \)-value) less than 0.05 was considered statistically significant.

Median total motility percent before cryopreservation of the spermatozoa was 27.5% and that of thawing temperatures 25ºC and 37ºC was 10%, while that of the thawing temperature 40ºC was 13%.

There was marked negative impact of the cryopreservation on the total motility of the sperm as shown in the 3 different thawing temperatures if compared to the precryopreservation total motility as the \( p \)-value was <0.001 in 25ºC, 37ºC and 40ºC but noticeably the percent decrease in the median of total motility at thawing temperature 25ºC and 37ºC was the same and greater than that at 40ºC.

**Table (2):** Type (A) motility percent before cryopreservation, and after thawing at temperatures 25ºC, 37ºC and 40ºC.

<table>
<thead>
<tr>
<th>Type A motility %</th>
<th>Mean ± SD</th>
<th>Med</th>
<th>Min</th>
<th>Max</th>
<th>% decrease of the med</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before cryo</td>
<td>4 ±4.6</td>
<td>3.5</td>
<td>0</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At 25ºC</td>
<td>1 ±2.4</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>100%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>At 37ºC</td>
<td>1.4 ±2.6</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>100%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>At 40ºC</td>
<td>3 ±3.5</td>
<td>1.5</td>
<td>0</td>
<td>10</td>
<td>57.1%</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Comparison of means was performed using paired-samples \( t \)-test. A probability value (\( p \)-value) less than 0.05 was considered statistically significant.

The previous table shows that the median of the type A motility percent before cryopreservation was 3.5%, and that of both thawing temperatures 25ºC and 37ºC was 0%, even though the median of thawing temperature 40ºC was 1.5%.

No significant difference in the rapidly progressive motility at thawing temperature 40ºC compared to the precryopreservation rapidly progressive motility, on the other hand there was great reduction in the percentage of the rapidly progressive motility of both thawing temperatures 25ºC and 37ºC compared to the rapidly progressive motility of the precryopreservation state.

**Table (3):** Type (B) motility percent before cryopreservation, and after thawing at temperatures 25ºC, 37ºC and 40ºC.

<table>
<thead>
<tr>
<th>Type B motility %</th>
<th>Mean ± SD</th>
<th>Med</th>
<th>Min</th>
<th>Max</th>
<th>% decrease of the med</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before cryo</td>
<td>10.7 ±6.7</td>
<td>10</td>
<td>0</td>
<td>25</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>At 25ºC</td>
<td>3 ±3.3</td>
<td>2</td>
<td>0</td>
<td>15</td>
<td>80%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>At 37ºC</td>
<td>6.3 ±4.6</td>
<td>5</td>
<td>0</td>
<td>20</td>
<td>50%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>At 40ºC</td>
<td>6 ±4.4</td>
<td>5</td>
<td>0</td>
<td>20</td>
<td>50%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Comparison of means was performed using paired-samples \( t \)-test. A probability value (\( p \)-value) less than 0.05 was considered statistically significant.

The previous table shows that the median of type B motility percent before cryopreservation was 10% and the median of the thawing temperatures 25ºC, 37ºC and 40ºC was 2%, 5% and 5% respectively.
Unlike the rapidly progressive motility; the slowly progressive motility showed a statistically significant difference at thawing temperatures 25ºC, 37ºC and 40ºC denoting that the slowly progressive motility percentage markedly decreased in the three thawing temperatures compared to the precryopreservation state taking in consideration that it was almost similar in both thawing temperatures 37ºC and 40ºC.

Comparison of means was performed using paired-samples t-test. A probability value (p-value) less than 0.05 was considered statistically significant.

The previous table shows that the median of type C motility percent before cryopreservation was 10% and the median of the thawing temperatures 25ºC, 37ºC and 40ºC was 5%.

The p-value was <0.001 in the three thawing temperatures compared to the precryopreservation state emphasizes that there is great decrease in the percentage of the non-progressive motile sperms after the thawing.

### Discussion

Our results showed that there was marked negative impact of cryopreservation on the total motility of the spermatozoa in the 3 different thawing temperatures if compared to the pre-cryopreservation total motility. This also matches the results of [21] who referred this reduction in motility to the fact that the cryopreservation increases the rate of lipid peroxidation and that this might be related, at least in part, to the loss of the sperm antioxidant enzymatic activity.

The negative impact of the cryopreservation on the motility recovery of the spermatozoa was less at thawing temperature 40ºC. However, the negative effect of the cryopreservation was almost the same on thawing temperatures 25ºC and 37ºC.

Our findings showed that the motility percentage and grade was always higher in the pre-freezing compared to the post-thawing samples. This matches the opinion of [23] study who pointed out that generally the better the pre-freeze sperm motility is, the better the post-thaw motility tends to be [22,23]. Also proved that pre-cryopreservation sperm concentration, progressive motility and normal morphology were significantly associated with the post-thawing progressive motility recovery rate of human frozen spermatozoa [23]. However, [3] stated that the cryopreservation has no negative impact on the motility recovery of the spermatozoa. It may be due to that the samples involved in their study were normozoospermic but in our study was oligoasthenoteratozoospermic [3].

Our study also showed that the recovery of sperm total motility after cryopreservation was better at thawing temperature 40ºC than thawing temperatures 25ºC and 37ºC.

The present study showed that there was no marked change in the rapidly progressive motility of thawing temperature 40ºC compared to the precryopreservation rapidly progressive motility. On the other hand, there was great reduction in the percentage of the rapidly progressive motility of both thawing temperatures 25ºC and 37ºC compared to the rapidly progressive motility of the pre-cryopreservation state. These findings come in agreement with [8]. They designed their study on normozoospermic samples. Their study showed that the increase in motility recovery observed after thawing of cryopreserved sperm at 40ºC was much better than the standard thawing temperature of 37ºC. Sperm thawing at 40ºC showed better sperm recovery compared to thawing at 37ºC by 23.1%.

[24] tried to find an explanation for the observed increase in motility recovery after thawing at 40ºC compared to thawing at 37ºC, that it is due to a faster rate of recovery of sperm enzymatic antioxidant activity. They proposed that cell damage during thawing may be affected by either 1). The level of oxygen free radical produced. 2). The recovery rate of antioxidant enzymatic activity. In higher temperature 40ºC the recovery rate of antioxidant enzymatic activity increased and be able to overcome the oxygen free radical production better than that occurred at 37ºC. However, the use of higher temperatures during thawing should be highly controlled in terms of magnitude and duration to avoid cell damage. Thawing at 40ºC during 3 minutes was better because it did not result in cell damage compared to thawing at 37ºC and at the same time, resulted in a better motility recovery [24].
Unlike the rapidly progressive motility; our study reported that the slowly progressive motility showed a great reduction at the three thawing temperatures 25ºC, 37ºC and 40ºC if compared to the pre-cryopreservation state. The difference in reduction of the slowly progressive motility between thawing temperatures 37ºC and 40ºC was almost the same.

Regarding the non-progressive motility; there was also marked decrease in its percentage at the three thawing temperatures if compared to the pre-cryopreservation non-progressive motility. The difference in reduction in the non-progressive motility between the three thawing temperature was almost the same.

Conclusion:

In conclusion, this study showed that motility recovery of the spermatozoa in oligoasthenoteratozoospermic cases after cryopreservation was markedly affected negatively at the three thawing temperatures 25ºC, 37ºC and 40ºC. Total motility percentage was better at thawing temperature 40ºC than 25ºC and 37ºC. On the other hand the rapidly progressive motility percentage was the highest at thawing temperature 40ºC if compared to the other thawing temperatures; 25ºC and 37ºC. Thus it is recommended for oligoasthenoteratozoospermic cases, who will be subjected to cryo-preservation, to undergo thawing of their semen samples at temperature of 40ºC. This is recommended for the aim of getting the best sperm motility quality to be used for ICSI.

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This study was self-funded by the authors.

Conflict of interest:

None.

References


الملخص العربي

استهدف هذا البحث دراسة تأثير درجات الحرارة المختلفة (20° و 25° و 30° من حفرة) المستخدمة في إذابة الحيوانات المنوية بعد تجميدها على حركة تلك الحيوانات المنوية.

وقد تمثل هذه الدراسة على فحص عدد الحيوانات المنوية من الطرف الأولي لأكثر من عام ونتائج فحص الحيوانات المنوية أظهرت أنهم يعانون بنقص في الحركة وانخفاض نسبة تشوهات الحيوانات المنوية.

وقد أظهرت هذه الدراسة أن انتظام حرارة الحيوانات المنوية في حالات نقص عدد ونقص حركة وانخفاض نسبة تشوهات الحيوانات المنوية بعد الحفظ بالتبريد قد تأثر سلبًا بشكل ملحوظ في درجات الحرارة 25° و 30°، التي استخدمت في درجات حرارة 20°، 25° و 30°. من ناحية أخرى، بلغ إجمالاً نسبة الحركة لفترة أعلى في درجة حرارة 20°. 25° و 30° من حيث الحيوانات المنوية إذا ما قورنت بدرجات حرارة النوبان الأخرى 25° و 30°.