Long-term cryostorage of sperm in a human sperm bank does not damage progressive motility concentration

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**BACKGROUND:** The use of quarantined cryopreserved semen is mandatory in donor insemination programs. Whether sperm cells can survive and retain their ability to fertilize after long-term storage remains a controversial issue. The objective of this study was to determine the effect of the duration of cryostorage in liquid nitrogen on the sperm cells’ progressive motility concentration (PMC) in a large study group.

**METHODS:** A total of 2525 thawed sperm specimens, packed in straws and donated by 72 sperm bank donors for intrauterine insemination (IUI), were evaluated in an assisted reproduction institute. PMC was recorded after 0.5–14.4 years of cryostorage.

**RESULTS:** The mean (± SD) value of PMC of all study samples was 10.8 ± 3.3 × 10⁶/ml after freezing/thawing and before cryostorage (T₀), and 12.3 ± 2.9 × 10⁶/ml after storage and before using the specimen for IUI (T₁, \( P < 0.0001 \)). Specimen storage for different lengths of time revealed that storage duration had no significant influence on the PMC of the specimens (\( r = −0.03, P = 0.08 \)). The PMC of partially filled straws was lower than in full straws. Cryostorage duration made no difference in the PMC of raw and washed sperm specimens.

**CONCLUSION:** Prolonged storage of donated sperm in liquid nitrogen had no influence on the PMC of the specimens and therefore should not alter the fertilization potency of donated sperm. The high post-storage values of the PMC compared with the pre-storage PMC values was probably an artifact of the small volume of the pre-storage sample.

**Key words:** cryostorage / donor sperm / long-term storage / progressive motility concentration

**Introduction**

Successful sperm cryopreservation requires maintaining post-thaw structural and functional integrity. As cryopreservation of human semen results in a significant loss of spermatozoa motility and viability (McLaughlin et al., 1992), only semen derived from a highly selected population of males is suitable for the purposes of insemination (Yavetz et al., 1991).

The use of quarantined cryopreserved semen became mandatory in donor insemination programs following the infection of four recipients with human immunodeficiency virus after insemination with semen from a seropositive donor (Stewart et al., 1985). A safer approach to cryostorage involves the use of small secondary tanks for 6 months before placing the samples into a main storage system (Mazzilli et al., 2006).

Recent guidelines for sperm donation (Practice Committee of the American Society for Reproductive Medicine, 2008) do not include any recommendations or instructions concerning the duration of donor sperm storage. From a biological perspective, sperm could be banked indefinitely or, at least, for a very long period of time. Functional tests revealed that the sperm acrosome reaction and zona pellucida binding were retained after 28 years in liquid nitrogen (Clarke et al., 2006). Two case reports demonstrated that banked sperm cells can survive and retain their ability to fertilize after many years of storage: one live birth followed intracytoplasmic sperm injection using sperm that had been banked for 21 years (Horne et al., 2004) and another live birth followed intrauterine insemination (IUI) treatment using sperm that had been banked for 28 years (Feldschuh et al., 2005).

Proteomic analyses of membranes and cytoskeleton competency of sperm cells have raised the possibility that fertility loss is associated
with time in storage (Desrosier et al., 2006). In another study, long-term (9–13 years) cryostorage of donor samples did not affect DNA integrity any more than those in the short-term (1–5 years) group, but the percentage of motility was significantly lower in the former than in the latter. Only small groups had been compared (16 and 14 specimens) in that earlier study (Edelstein et al., 2008). Therefore, the objective of the current investigation was to determine the effect of time in liquid nitrogen on the progressive motility concentration (PMC) of a large study group of semen samples that underwent cryostorage.

Materials and Methods

Specimens

A possible effect of long-term cryostorage was assessed on a total of 2525 thawed sperm specimens donated by 72 sperm bank donors. Additional 12 donors were recruited into the study to evaluate the influence of the frozen sample volume on the PMC of the thawed samples. The participants were composed of young (mean ± SE, 25 ± 0.4 years of age, median of 24 years) students, most of whom were born in Israel. They all fulfilled the criteria for suitable sperm donors as previously described (Yavetz et al., 1991; Botchan et al., 2001): sperm concentration >40 × 10^6/ml, sperm motility at first hour >50% motile (most in progressive motility), normal morphology >14% (strict criteria) and post-thaw motility >40% motile. All had proven fertility as judged by pregnancies that had been achieved with their sperm donations. The donors gave informed consent to use their samples and provided permission to use their specimens for future studies. The current study was approved by the local Institutional Review Board Committee in accordance with the Helsinki Declaration of 1975.

The ‘long-term storage’ group included a total of 1239 samples (72 sperm donors, 17 ± 12.9 samples each), which had been cryostored for women who already had one child and had requested the preservation of specimens from the same donor for future use. The only determinant for inclusion of donor specimens in this group was the date a new chamber for sperm concentration evaluation had been installed in the laboratory: the Makler chamber had replaced the Horwell chamber in July, 1992. For purposes of homogeneity, only samples that had been frozen after that date were included in the current study. They were thawed for IUI after 1.08–14.42 years of storage.

A second group was composed of 1286 specimens that were donated by 19 sperm donors out of the 72 that were included in the long-term storage group (68 ± 14.4 samples each). These specimens were kept in the bank for routine inseminations and were cryostored for 0.5–2.75 years: they served as a control ‘short-term storage’ group.

Semen analysis

All participants were instructed to observe 2–3 days of abstinence from sexual activity before each donation. Sperm quality was assessed prior to and immediately after undergoing the freezing procedure by thawing a small sample, to ensure that it conformed to the required quality. Each ejaculation was allowed to liquefy for at least 30 min at 34°C. The volume was determined by drawing up the entire sample into a disposable syringe. A sample was taken to evaluate sperm concentration (by the Makler chamber) and the percentage of motile spermatozoa (by microscopic assessment, ×300 magnification). At least five microscopic fields were assessed for motility evaluation. The percentage of morphologically normal sperm was assessed according to strict criteria (×1500 magnification, Menkveld and Kruger, 1995; WHO, 1999) on a Papanicolaou-stained smear. Straight-forward rapid PMC (type ‘A’ according to WHO, 1999) in the thawed sample of each cryopreserved ejaculate was also evaluated. PMC evaluation after storage (T1) was performed by a laboratory technician unaware of the values immediately after freezing (at T0). The whole process for each specimen, including semen analyses before and after freezing, was conducted in the same laboratory. Most of the laboratory technicians (four of five) were employed over the entire 14.4-year study period.

The objective of the freezing procedure was to obtain a PMC of 8–12 × 10^6/ml in each thawed straw, a value that was found to be adequate for cervical and IUI in our hands (Yogev et al., 2004). To achieve this goal, a total count was calculated for each ejaculate by multiplying the volume and concentration. In the previous freezing procedure, the number that was found appropriate to achieve one thawed straw with 8–12 × 10^6/ml PMC was used to divide the total count result. The quotient determined the number of straws to be prepared. The specimen was washed with human tubal fluid medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 1% human serum albumin (Bio Products Laboratory Dagger Lane, Elstree, Herts, W5D 3BX, UK), and an aliquot of the medium was added to the pellet in a volume sufficient for the previously calculated number of straws plus one specimen to be thawed immediately after the freezing process. A total of 60 × 10^6/ml were used for the first freezing of a sample from a new donor. The results of the tested freezing–thawing process facilitated calibration of the ensuing freezing procedure. When the concentration of the raw specimen was high enough to achieve the minimum of 8 × 10^6/ml PMC without any need for further concentration, the freezing process was performed without pre-freezing sperm preparation.

Our laboratory has been under the external quality assessment scheme for Andrology by United Kingdom National External Quality Assessment Scheme, Manchester, UK (UKNEQAS) since 1997.

Freezing and thawing

Sperm specimens were cryostored either in a raw form or after washing, and freezing of both types was carried out as previously described (Yogev et al., 2004). Each specimen was diluted by adding an equal volume of freezing medium test yolk buffer (Irvine Scientific, Santa Ana, CA, USA). After equilibration for about 15 min at room temperature, the specimen was sealed in 0.5-ml straws (IMV, Paris, France) and cooled in a semi-programmable freezer (Nicool LM-10; Air Liquid, Paris, France). The straws were cooled gradually and then transferred to liquid nitrogen for storage.

The value of post-thaw PMC before commencement of storage was evaluated by thawing one straw (filled partially with only about 0.2 ml of sperm suspension for the sake of saving cells for fertilization) from each frozen specimen after about 30 min in liquid nitrogen. Thawing was carried out at 34°C for 5 min. The criteria for the recruitment of sperm bank donors, freezing procedure, equipment and medium composition remained unchanged throughout the entire study period.

Influence of frozen specimen volume

To eliminate the factor of the length of time in cryostorage and to find a possible contribution of the volume of a specimen frozen in 0.5-ml straws, donor specimen (n = 12) were frozen, each of them in two straws: one to full capacity (0.5 ml) and the other with only 0.2 ml. Both straws were thawed following 30 min of storage in liquid nitrogen, and the PMC was assessed.

Sperm washing procedure

Specimens were washed with human tubal fluid medium (Irvine Scientific) supplemented with 1% human serum albumin. After centrifuging and removal of the supernatant, an aliquot of the medium was added to the
pellet in a volume sufficient for the previously calculated number of straws plus one additional specimen to be thawed immediately after the freezing process (Yoge et al., 2004).

Statistical analysis

Results are presented as means ± SD, except for few occasions when ±SE and median were used. PMC levels before and after cryostorage were compared using the paired sample t-test and the Wilcoxon signed-rank non-parametric paired test. The association between storage time and PMC was assessed by the Pearson correlation coefficient. This analysis was performed in each group and for each donor separately for all specimens. The PMCs of the subgroups of specimens that were formed according to the length of time in cryostorage were compared using a one-way analysis of variance. Pairwise comparisons between groups were carried out using the Ryan–Einot–Gabriel–Welch method using a one-way analysis of variance. Pairwise comparisons between groups were carried out using the Ryan–Einot–Gabriel–Welch method for multiple comparisons. An additional analysis using the mixed model was performed with the ejaculated sample as the unit of analysis. This model addresses the hierarchical structure of the data, where different numbers of straws were analyzed for each ejaculated sample. The mixed model was used to study the effect of storage time on the level of change in PMC. Only specimens stored for specific women (long-term storage subgroup) were included in this analysis. All analyses were performed using the SAS system for Windows (Cary, NC, USA).

Results

The mean ejaculate volume for all donors was 3.1 ± 1.15 ml (mean ± SD), and the mean sperm concentration, motility and normal morphology percentages were 110 ± 36.9 x 10^6/ml, 56 ± 4.4 and 18 ± 3.6%, respectively. Sperm specimens, which were cryopreserved for women who wished an additional pregnancy with the same sperm donor from whom they already had a child, had been stored for a mean length of about 6 years (5.9 ± 0.08 years, n = 1286). Specimens in the control group that were taken from the 'general' sperm bank had been cryopreserved for 1.5 ± 0.1 years (n = 1286). All 2525 samples that were included in the present study had a mean PMC of 10.8 ± 3.30 x 10^6/ml with a median of 10 x 10^6/ml after thawing and before cryostorage (T0) and a mean PMC of 12.3 ± 2.93 x 10^6/ml, with a median of 11 x 10^6/ml, after cryostorage and before using the specimen for IUI (T1, P < 0.0001).

Influence of specimen volume on freezing results

The mean difference between the PMC at T0 and the PMC at T1 was 1.34 ± 4.66 (10^6/ml). To eliminate the storage length factor and to find the possible contribution of the specimen volume frozen in the 0.5-ml straws, each of fresh 12 donor specimens was divided into two straws: one full (0.5 ml) and the other with 0.2 ml only. Both straws were frozen and thawed following 30 min of liquid nitrogen storage. The mean (±SE) PMC of the partially filled straws (specimen volume of 0.2 ml) was 9.5 ± 0.82 (10^6/ml). The mean (±SE) PMC of the full straws was significantly higher, i.e. 13.4 ± 1.4 (10^6/ml) (P < 0.001, paired t-test).

Storage length

Calculations of the differences between the PMCs of thawed samples before and after cryostorage [Δ(T1 – T0)] revealed that the length of time in storage had no significant influence on the ΔPMC of any of the specimens (n = 2525, r = −0.03, P = 0.08). Specimens that were cryopreserved in the 'general' sperm bank (short-term storage group, n = 1286) were used for insemination within 3 years. Changes that took place in the PMC with this length of time in storage are presented in Table I. The mean Δ(T1 – T0) was 1.6 ± 4.32. There was no significant difference between the three subgroups (0.5–1, >1–2 and >2–2.75 years) for T0 (P = 0.116), T1 (P = 0.72) or Δ(T1–T0) (P = 0.62).

The mean Δ(T1 – T0) was 1.34 ± 4.66 for the specimens that were kept in storage for women for future inseminations (long-term storage group). A comparison between the five subgroups which were formed according to length of time in cryostorage (0.5–3, >3–5, >5–7, >7–10 and >10 years, Table II) revealed a significant difference between the subgroups before cryostorage (T0, P = 0.009) and for the ΔPMC obtained immediately after freezing and before preservation (T0) to that at the time of thawing for use (T1) (P = 0.041). There was no difference, however, between the subgroups that could be related to the length of time in cryostorage, nor was there any difference between the short- and long-term cryostorage subgroups in terms of the ΔPMC (P = 0.15). To address the hierarchical structure of the data, where for each sample different number of straws were analyzed following different length of storage, the mixed model was used. No effect of storage time on the level of changes in PMC was found (P = 0.89).

PMC in the same 19 donors after short-term and long-term storages are presented in Table III. A difference in the PMC was found in 6 of 19 donors, but there was no tendency towards either an increase or a decrease in PMC after long-term storage. As such, no difference was found between T1 of the short-term storage group and the same 19 donors in the long-term storage group (P = 0.544).

Table I

<table>
<thead>
<tr>
<th>Storage (years)</th>
<th>Subgroup size (% of total group)</th>
<th>T0 PMC (mean and median)</th>
<th>T1 PMC (mean and median)</th>
<th>Δ(T1–T0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5–1</td>
<td>148 (12)</td>
<td>10.8 ± 4.93 (9.0)</td>
<td>12.1 ± 5.60 (10.0)</td>
<td>1.3 ± 4.61</td>
</tr>
<tr>
<td>&gt;1 to &lt;2</td>
<td>1056 (82)</td>
<td>10.1 ± 3.86 (9.0)</td>
<td>11.7 ± 4.90 (11.0)</td>
<td>1.6 ± 4.28</td>
</tr>
<tr>
<td>&gt;2</td>
<td>82 (6)</td>
<td>9.9 ± 2.63 (10.0)</td>
<td>11.6 ± 3.99 (11.0)</td>
<td>1.8 ± 4.27</td>
</tr>
</tbody>
</table>

Three subgroups were formed according to the time of storage length (n = 1286). There are no significant differences between the three subgroups for T0 (P = 0.116), T1 (P = 0.72) and Δ(T1–T0) (P = 0.62). PMC, progressive motility concentration (10^6/ml), T0, value of sample after freezing/thawing and before storage, T1, value after storage and thawing before insemination.
Effect of storage length on individual donors

Testing the possible influence of length in cryostorage on individual donors revealed a significant influence in 11 of 72 donors, but the findings were not uniform; four sperm donors had a negative Pearson correlation (a damage effect), whereas the remaining seven showed a positive one. The influence of length of time in cryostorage on the PMC in 20 individual sperm donors who contributed a relatively large number of specimens (more than 50 each) is shown in Table IV. Only two of them showed a significant storage influence (Nos. 8 and 13), and both showed a positive correlation, i.e. the length of time in cryostorage ‘increased’ the specimen quality.

Similar results were also shown when several aliquots (at least three) from the same ejaculate were thawed after different lengths of time in storage (n = 483 aliquots, r = −0.04, P = 0.27, Fig. 1).

An influence of the storage length on Δ(T1 − T0) could not be demonstrated.

Comparison between raw and washed sperm specimens storage

To evaluate whether raw specimens (cryopreserved with seminal fluid) can survive cryostorage differently to washed sperm samples (i.e. after seminal fluid has been replaced by a human tubal fluid medium), specimens were divided into two groups by type of sample preparation before cryostorage. Raw specimens were mixed with the freezing medium in Group A (n = 1157) and the specimens were washed before cryostorage in Group B (n = 1365). The mean
storage lengths of the two groups were $4.2 \pm 0.09$ and $3.2 \pm 0.05$ years, respectively. The mean change in PMC, between the thawed samples that were used for treatment (T1) and those that were thawed immediately after freezing and before cryopreserving (T0), was similar ($1.56 \pm 0.12$ and $1.38 \pm 0.13 \times 10^6/\text{ml}$, respectively). Observation of the PMC after storage revealed that the length of time in cryostorage did not influence the PMC values in either Group A or Group B ($r = 0.02$, $P = 0.48$ and $r = 0.042$, $P = 0.12$, respectively).

### Discussion

This evaluation of the largest ever-reported number of cryopreserved sperm bank donor specimens revealed that up to ~14 years in storage caused no demonstrable decrease in progressive motility. This finding is very encouraging, because progressive motility percentages and concentrations are reportedly the best prognostic parameters for pregnancy in IUI with donor semen (Larsen et al., 2000; Freour et al., 2009). Moreover, sperm motility after thawing provides the most significant parameter for predicting donor sperm fertility potential (Marshburn et al., 1992).

Whatever procedure of freezing is used, sperm cells are damaged by undergoing either structural or functional changes or both (O’Connell et al., 2002; Thomson et al., 2009). Damage to human spermatozoa can be recognized immediately after freezing—thawing as a loss in motility, but there is a possibility that further harm can be caused to the sperm cells during storage (Desrosier et al., 2006).

Sperm frozen in seminal fluid has appeared to be more resistant to damage associated with freezing than washed sperm (Donnelly et al., 2001; Saritha and Bongso, 2001). When we assessed the influence of the length of time in cryostorage on the PMC of specimens cryopreserved with and without seminal plasma, however, we found no difference between the two groups.

Unexpectedly, there was a significant difference in PMC after freezing process survival of samples between partially filled and totally filled straws. Consequently, instead of showing zero value of $\Delta(T1 - T0)$ because of the absence of storage influence, an increase in PMC that was not influenced by the storage length could be demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated. In order to establish the quality of each freezing (before storage), we therefore recommend using a full straw that demonstrated.
There were varying degrees of influence of length of time in cryostorage on individual donors. There was a decrease of PMC in some and an increase in others. Interestingly, three ejaculates of the same donor (Fig. 1E) showed greater, less and no influence of the length of time they were in cryostorage. These results support the position that there is no remarkable and lasting change in PMC after long-term cryostorage.

In a previous report from our sperm bank, an overall pregnancy rate of 12.1% was achieved with frozen-thawed spermatozoa (Botchan et al., 2001). A comparison between the fertility rate of the cryopreserved semen after short- and long-term storage would obviously contribute to the significance of the present study. However, the older age of the women in the present study taken together with the extended storage time can be expected to dramatically reduce the value of such a comparison.

We concluded that the length of time during which sperm is stored in liquid nitrogen has no injurious effect on PMC and consequently, on the fertilizing potency of donated sperm. When the effect of freezing is examined by the thawing of a small sample volume in partially filled straws, it should be borne in mind that the PMC will be lower in the sample than in the rest of the stored specimen.

Authors’ roles

L.Y.: conception and design, collection, assembly of data and writing the manuscript. S.E.K.: critical revision of the manuscript. E.S.: analyzing the data and performing the statistical evaluation. A.B.: revision of the manuscript. G.P.: contribution to the conception of the model; revision of the manuscript. R.H.: critical revision of the manuscript; approval of the final version. O.L.: design and coordination of the research. H.Y.: conception and design of the research, data interpretation and critical revision of the manuscript.

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