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Repeated slow programmable freezing and thawing

An equal volume of warm (37 °C) sperm cryopreservation medium (SpermFreeze; FertiPro NV, Beernem, Belgium) was added dropwise to an aliquot of processed sperm sample. The mixture was loaded into a 0.25-ml straw and left at room temperature for 10 min. The straw was inserted into a programmable freezer (Planer Kryo 10 series III) and cooled at a rate of −1 °C per min from 20 °C to −5 °C. The straw was then cooled at a rate of −10 °C per min to −80 °C per min. After a holding for 5 min at this temperature, the straw was plunged into liquid nitrogen.

After greater than or equal to one week of storage in liquid nitrogen, the samples were thawed by plunging the straw into water bath at 25–28 °C. The thawed sample was first diluted with 0.5 ml of EBSS supplemented with human serum albumin, pyruvate and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and the remaining 3.5 ml of supplemented EBSS was added gradually over a period of 3–4 min. The suspension was then centrifuged at 250g for 5 min to remove the cryoprotectant. Post-thaw pellet was resuspended in 0.4 ml of EBSS with supplements, and 0.1 ml was removed and immediately assessed for sperm kinematics and viability. The remaining 0.3 ml was cryopreserved by adding sperm cryopreservation medium a second time, as previously described.

After storage for 2–5 days, the sample was thawed and the cryoprotectant was removed. A small aliquot (0.1 ml) from the second thaw was assessed for sperm kinematics and viability. The remaining sample (0.3 ml) was then cryopreserved for a third time and thawed few days later. An aliquot from the third thaw was similarly studied for kinematics and viability.

Repeated rapid freezing (RF)

Our method of sperm freezing was previously described.8 In brief, the aliquot was mixed dropwise with an equal volume of cold cryopreservation medium (4 °C). The mixture was loaded into a pre-cooled 0.25-ml straw and left to incubate at 4 °C for 10 min in a refrigerator. The cryopreservation medium for rapid freezing was a modified human sperm preservation medium,10 in which 50 mmol l−1 of sucrose was replaced by 100 mmol l−1 of trehalose. The concentration of glycerol was decreased to 10%, whereas that of human serum albumin was increased 20-fold. After 10 min, the straw was inserted into a hole in a pre-cooled homemade aluminium block, previously immersed in liquid nitrogen for 20 min before use. The aluminium block remained immersed in liquid nitrogen during the whole process of freezing. The straw was held in the aluminium block for 3–5 min before transfer into a liquid nitrogen storage dewar.

Thawing was done in the same way as that described for the slow freezing technique. The thawed sample was washed with EBSS to remove the cryoprotectant. A small aliquot was removed for kinematic and survival study and the remaining sample was again re-frozen as previously described. After 3–7 days, the same procedure of thawing and freezing was done, until three cycles of freezing/thawing were completed.

Determination of sperm motility parameters

A 5-μl volume of sample was loaded into a disposable counting chamber (20-μm depth; Leja, Niew-Vennep, The Netherlands) for the evaluation of sperm motility and kinematics of movement, using a computer-aided semen analyzer (CASA; HTM-IVOS, Hamilton Thorne Biosciences, Beverly, MA, USA), equipped with a clinical human motility program version 12. The following parameter settings were used: frame rate, 60 Hz; 30 acquisition frames; straightness threshold, 80%; minimum contrast, 80; minimum cell size, 3 pixels; non-motile head size, 6 pixels; non-motile head intensity, 160; illumination intensity, 2128; magnification, ×1.9; temperature, 37 °C; and chamber depth, 20 μm. At least 400 sperm trajectories were analyzed.

Determination of sperm morphology

A CASA (HTM-IVOS) was used to evaluate Diff-Quick stained slides for sperm morphology. Strict criteria were employed to analyze at least 200 sperm per sample, using Dimension Strict Morphology software on HTM-IVOS CASA. This program has been shown to give excellent repeatability of normal and abnormal cells, and to agree well with the manual strict criteria.11

Determination of sperm viability

Sperm viability was assessed by staining with 0.5% eosin-Y.9 After 1–2 min, 100 sperm or more were counted as stained (dead) or unstained (viable).

Determination of sperm DNA integrity by modified alkaline single-cell gel electrophoresis (comet) assay

The status of sperm DNA was evaluated using a modified alkaline single-cell gel electrophoresis (comet) assay, as described by Hughes et al.12 and Donnelly et al.13 In brief, the method involves embedding the sperm in agarose, lysing the membranes and breaking down the protein matrices. Fragmented strands of DNA are drawn out by electrophoresis to form a comet ‘tail’, leaving a ‘head’ of intact DNA. The amount of intact and damaged DNA is quantified using an epifluorescence microscope, equipped with an excitation filter (515–560 nm), 100 W mercury lamp and barrier filter (590 nm). At least 100 sperm were captured and analyzed by image analysis software (LUCIA Comet Assay; Laboratory Imaging, Prague, Czech Republic). Our intra-assay coefficient variation for this assay was 7%.

Experimental design (Figure 1)

Experiment 1: Comparison of repeated freezing/thawing of sperm, using the SPF and RF techniques (11 subjects). In this experiment, we divided processed sperm samples into three aliquots. The first aliquot was not frozen, and was immediately assessed for sperm kinematics, morphology and viability. The second and third aliquots were cryopreserved by the SPF and our in-house method of RF, respectively. Three cycles of repeated freezing/thawing were performed. We assessed sperm viability, motility (World Health Organization grade a and b movement), morphology and kinematics immediately after each freeze/thaw cycle.

Experiment 2: Effect of three repeated freezing/thawing cycles by the RF technique on sperm DNA fragmentation. Sperm samples from 20 men were processed and divided into two aliquots. One aliquot was not frozen and was immediately assessed for sperm kinematics, morphology and DNA fragmentation by the alkaline comet assay. Another aliquot was subjected to three cycles of rapid freezing/thawing. A 0.1 ml aliquot from each thaw was assessed for sperm kinematics, viability, morphology and DNA fragmentation.

Experiment 3: Sperm samples from 10 men were processed and subjected to repeated cycles of RF until no motile sperm were observed.

Statistical analysis

We used STATA program version 8.2 (College Station, TX, USA) to perform all the statistical analyses. Data were expressed as mean±standard deviation (s.d.). A normality test was performed using the Shapiro–Wilk test. Mean percentages of motility, normal morphology, sperm kinematics, viability and DNA fragmentation before and after each freezing/thawing
cycle were compared by repeated measure ANOVA or Friedman nonparametric repeated-measure tests, as appropriate. A comparison was considered to be statistically significant if the $P$ value was $<0.05$.

**RESULTS**

**Experiment 1**
The mean age±s.d. of 11 subjects in this study was 31.2±5.6 years. Sperm parameters were: volume, 2.1±0.9 ml; concentration, $75\times10^6\pm22\times10^6$ per ml; motility, 72%±11%; viability, 82%±8%; and normal morphology, 22%±9%. The average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH) and linearity (LIN) are shown in Table 1. After each freezing/thawing cycle, sperm viability and motility in the SPF group decreased more than those in the RF group, but there was no meaningful difference in sperm morphology and LIN between the two groups (Table 1).

**Experiment 2**
The mean age±s.d. of the 20 subjects in this study was 34.7±7.6 years. Sperm parameters were: volume $2.9\pm0.8$ ml; concentration, $68\times10^6\pm24\times10^6$ per ml; and motility 61%±10%.
Sperm motility decreased by 18.5%, 53.3% and 69.1% after the first, second and third freeze/thaw cycles, respectively (Friedman test; P<0.001). Sperm viability decreased by 20.4%, 38.9% and 54.6% after the first, second and third cycles, respectively (Friedman test; P<0.001). Cryopreservation caused a decrease (Friedman test; P<0.01) in VAP, VSL and VCL, but it had little effect on ALH and LIN (Friedman test; P>0.05) (Table 2). Sperm DNA integrity was affected by repeated freezing/thawing as evidenced by considerable changes in the comet assay of sperm head and tail DNA, and tail moment (Table 2). Moreover, there was high variability in sperm DNA fragmentation between individual cases (P<0.0001).

**Experiment 3**

Ten subjects in this study had a mean±s.d. age of 32.8±6.9 years. Sperm parameters were: volume, 2.6±0.5 ml; concentration, 69×10⁶±23×10⁶ per ml; motility, 84%±4%; and viability 87%±3%. The mean percentages±s.d. of sperm motility and viability after the first to the eighth cycle of freezing/thawing are shown in Table 3. The median number of repeated freezing/thawing cycles that yielded no motile sperm was seven (range: 5–8; mode: 6, 7 and 8; mean: 6.8).

**DISCUSSION**

After three cycles of repeated freezing/thawing, the RF method gave better sperm survival and motility than the SPF. We washed the sperm and added fresh cryoprotectant in between each freeze–thaw cycle. This process could induce repeated cycles of osmotic shock to sperm, in addition to the mechanical damage and oxidative stress from centrifugation.14,15 Previous studies, that employed similar washing steps,4–6 consistently reported sperm motility as low as 2.0%–5.5% after three repeated cycles of freezing/thawing by slow programmable or conventional liquid nitrogen vapor freezing. This was in agreement with our study in the SPF group (motility: 4.1%). In contrast, the study that refreeze the samples in their original cryoprotectant without washing in between showed a better motility, which could be as high as 11.4%.7 Although not directly comparable, the result was still inferior to our repeated freezing/thawing by the RF method (motility: 18%).

With CASA, an objective assessment of sperm kinematics can be performed to better determine which parameter may be more indicative of sublethal injury to human sperm. Previous studies concluded that the percentage of progressive motility, VAP, VSL and VCL but not ALH and LIN, were sensitive and useful indicators to determine adverse effects of toxic agents on human sperm19 and rat sperm motility.17 By time-lapse photography, McLaughlin et al.18 also observed the average velocity and the percentage of progressive motility, but not ALH, were indicative of cryodamage to human sperm. In our study (experiments 1 and 3), we found a similar adverse effect of cryoinjury on sperm kinematics, after three cycles of repeated freezing/thawing in the RF group. As LIN is defined as the ratio of VSL to VCL, the small changes observed in our study after repeated freezing/thawing suggested a decrease of both VSL and VCL to nearly the same extent.

In the second experiment, we focused our attention on the extent of DNA damage after three cycles of repeated freezing/thawing by the RF method. We used the alkaline comet assay as it allowed for sensitive detection of both single and double-stranded DNA breaks.19,20 In a recent study,21 the test had been shown to be useful both for the diagnosis of male factor infertility and prediction of in vitro fertilization outcome. The percentage of migrated DNA (7.2% tail DNA) before freezing in our study was compatible with the background damage obtained by others under a similar condition (~10% tail DNA).20,22 It was reassuring that after three cycles of repeated freezing/thawing, the mean level of DNA fragmentation by the alkaline comet assay was still below the diagnostic threshold of 25%, which predicted subfertility and below 42% that predicted failure to achieve a pregnancy after in vitro fertilization.21 In our study, a substantial

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**Table 1** Repeated freezing/thawing cycles in the rapid (RF) and slow programmable freezing (SPF) groups (n=11)

| Freeze/thaw cycle no. | RF | SPF | P* |
|-----------------------|----|-----|----|
| **First cycle**       |    |     |    |
| Viability (%)         | 66.8±7.2 | 55.4±8.6 | 0.003 |
| Morphology (%)        | 15.5±5.4 | 19.8±9.9 | 0.213 |
| Motility (%)          | 66.3±12.4 | 38.2±14.4 | 0.002 |
| VAP                   | 65.7±7.4 | 52.2±8.9 | 0.004 |
| VSL                   | 55.7±7.8 | 43.5±7.9 | 0.006 |
| VCL                   | 112.0±12.6 | 95.6±1.3 | 0.024 |
| ALH                   | 5.4±0.9 | 4.9±0.5 | 0.151 |
| LIN                   | 49.3±5.9 | 46.6±6.1 | 0.197 |
| **Second cycle**      |    |     |    |
| Viability (%)         | 54.1±5.2 | 27.6±7.6 | 0.000 |
| Morphology (%)        | 16.8±4.9 | 15.2±8.0 | 0.572 |
| Motility (%)          | 30.9±10.4 | 14.0±5.9 | 0.001 |
| VAP                   | 51.0±7.7 | 39.2±7.4 | 0.000 |
| VSL                   | 44.3±7.7 | 32.2±6.3 | 0.000 |
| VCL                   | 94.1±11.9 | 78.8±17.7 | 0.005 |
| ALH                   | 4.3±0.7 | 4.5±1.5 | 0.775 |
| LIN                   | 47.2±6.8 | 43.6±9.0 | 0.193 |
| **Third cycle**       |    |     |    |
| Viability (%)         | 40.8±12.9 | 14.2±4.6 | 0.000 |
| Morphology (%)        | 16.3±6.9 | 11.8±6.2 | 0.129 |
| Motility (%)          | 18.4±8.7 | 4.1±2.6 | 0.000 |
| VAP                   | 47.3±10.6 | 20.2±13.7 | 0.001 |
| VSL                   | 39.4±7.9 | 16.9±11.6 | 0.001 |
| VCL                   | 93.7±23.5 | 42.2±29.2 | 0.004 |
| ALH                   | 4.9±1.8 | 1.6±2.3 | 0.004 |
| LIN                   | 43.9±8.8 | 39.3±25.9 | 0.557 |

Abbreviations: ALH, amplitude of lateral head displacement (μm); LIN, linearity (%); VAP, average path velocity (μm s⁻¹); VCL, curvilinear velocity (μm s⁻¹); VSL, straight line velocity (μm s⁻¹).

Values are expressed as means±s.d.

* Friedman non-parametric tests.

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**Table 2** Sperm parameters before and after each freezing/thawing cycle (n=20)

| Parameters | Pre-freeze | Cycle 1 | Cycle 2 | Cycle 3 | P* |
|------------|------------|---------|---------|---------|----|
| Viability (%) | 85.6±9.2 | 68.1±6.4 | 52.3±9.9 | 38.9±2.4 | <0.001 |
| Morphology (%) | 20.6±6.1 | 18.6±6.2 | 18.0±4.4 | 17.5±5.8 | 0.018 |
| Motility (%) | 81.3±8.2 | 66.3±9.4 | 37.9±15.3 | 25.1±7.2 | <0.001 |
| VAP | 69.2±13.4 | 61.9±12.7 | 48.3±13.1 | 50.4±16.1 | 0.003 |
| VSL | 56.4±9.2 | 52.0±11.8 | 40.8±13.4 | 43.1±15.9 | 0.003 |
| VCL | 120.2±34.9 | 107.6±20.6 | 87.7±17.9 | 96.7±26.5 | 0.003 |
| ALH | 5.3±1.2 | 5.0±0.8 | 4.2±1.5 | 4.5±1.9 | 0.089 |
| LIN | 49.1±7.9 | 49.0±7.4 | 46.1±9.6 | 45.9±11.9 | 0.764 |

Comet assay

**Head DNA (%)** | 92.9±5.9 | 91.0±6.5 | 84.0±8.5 | 75.5±12.8 | <0.001 |

**Tail DNA (%)** | 7.2±5.9 | 9.0±6.5 | 15.9±8.5 | 24.5±12.8 | <0.001 |

**Tail moment** | 13.6±16.0 | 25.4±20.8 | 32.6±18.9 | 63.0±37.7 | <0.001 |

Abbreviations: ALH, amplitude of lateral head displacement (μm); LIN, linearity (%); VAP, average path velocity (μm s⁻¹); VCL, curvilinear velocity (μm s⁻¹); VSL, straight line velocity (μm s⁻¹).

Values are expressed mean±s.d.

* Friedman nonparametric repeated measure tests.
Recurrent rapid freezing/thawing of human sperm

Table 3 Sperm motility and viability before and after each freezing/thawing cycle

| Subject | Motility (%) | Viability (%) |
|---------|--------------|---------------|
|         | Freezing/thawing cycle no. | Freezing/thawing cycle no. |
| No.     | 0 1 2 3 4 5 6 7 8 | 0 1 2 3 4 5 6 7 8 |
| 1       | 88 75 50 30 20 12 7 3 0 | 90 85 62 41 28 22 14 10 8 |
| 2       | 80 55 50 34 16 5 0 0 0 | 82 63 53 36 21 17 3 0 0 |
| 3       | 84 54 52 15 13 4 2 0 0 | 87 61 54 36 22 12 8 2 0 |
| 4       | 92 55 41 20 10 0 0 0 0 | 92 55 41 20 10 0 0 0 0 |
| 5       | 80 72 56 32 16 1 0 0 0 | 88 80 61 42 25 12 3 0 0 |
| 6       | 85 70 40 20 15 10 6 2 0 | 90 69 51 36 30 18 12 8 4 |
| 7       | 80 68 58 21 11 6 1 0 0 | 85 70 59 34 24 10 9 4 0 |
| 8       | 85 70 35 16 10 3 1 0 0 | 90 72 36 28 22 18 10 3 0 |
| 9       | 80 50 40 36 13 2 0 0 0 | 85 60 44 40 18 3 2 0 0 |
| 10      | 81 70 40 24 14 8 4 2 0 | 85 72 47 32 20 12 9 5 3 |
| Mean±s.d. | 83±4 64±9 46±8 25±8 14±3 5±4 2±3 1±1 0 | 87±3 69±9 51±9 35±7 22±6 12±7 8±4 5±3 5±2 |

In conclusion, we demonstrated that repeated freezing/thawing of processed human sperm by our in-house method gave better results than standard slow programmable freezing. Our in-house method can help maximize the use of precious cryopreserved sperm samples in assisted reproduction technology. The method has been validated only on semen samples of high quality, which is normally the case for samples from sperm banking used for donor insemination. However, the method has not yet been proven valid for samples of patients with moderate or poor semen quality.

AUTHOR CONTRIBUTIONS
TV made substantial contributions to conception, design, analysis and interpretation of the data, and drafted the manuscript. WL participated in its design and coordination, carried out experiments 1 and 2, and helped to draft the manuscript. WP participated in the design, carried out experiments 2 and 3, and helped in data analysis and revising the manuscript critically for important intellectual content. SS participated in the acquisition of data in experiments 1–3, and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS
All authors have no potential conflicts of interest, whether of a financial or other nature, with any pharmaceutical company.

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