



Brief Communication

# Inter-sample variability in post-thaw human spermatozoa<sup>☆</sup>

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## Abstract

Semen cryopreservation is a useful tool for preserving fertility in men who have been diagnosed with cancer and will undergo chemotherapy, radiotherapy or testicular surgery. Semen is also commonly cryopreserved prior to its use in assisted reproductive techniques such as in vitro fertilization and intracytoplasmic sperm injection. The post-thaw quality of banked sperm can vary, which may negatively affect fertilization rates. The objective of our study was to assess the pre-freeze and post-thaw variability of sperm parameters in patients who used our sperm banking services. Multiple samples obtained after a short period of sexual abstinence were examined for variation in sperm characteristics. Semen samples showed a high degree of post-thaw inter-sample variability in sperm motility, motion characteristics, and percentage cryosurvival rate compared with the pre-freeze inter-sample variability. Further research is necessary to understand the mechanism(s) responsible for this variability. This may also assist clinicians utilize semen samples with optimum semen quality in ART procedures.

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Cryopreservation of human semen is the most commonly accepted method of preserving reproductive capacity. Semen can be collected and banked for use in assisted reproductive techniques (ART) [3,5,8,10]; especially in cases where the patient elects to undergo vasectomy for contraception [5] or, most importantly, when a patient is

diagnosed with cancer and the treatment may render him infertile [2]. The latter is often a major issue for young men who desire to father a healthy biological child sometime in the future. Banking semen specimens prior to treatment enables these patients to fulfill their wish.

Semen samples that are collected at different times may show a high degree of sample-to-sample (inter-sample) variability in sperm parameters. This may subsequently affect their fertilizing potential [13,14,17]. According to current semen

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banking guidelines, two to six ejaculates should be collected from a patient after a sexual abstinence of 2–3 days [1]. Several factors affecting the cryo-survival of spermatozoa have been described [11,12,15]. Although, a direct relationship has been reported between pre-freeze and post-thaw motility [9], studies comparing inter-sample variability in pre-freeze and post-thaw parameters of cryopreserved sperm are lacking. Information on inter-sample variability and its causes could potentially help fertility specialists select ideal samples for cryopreservation for future use in assisted reproduction. Therefore, the objective of our study was to examine the pre-freeze and post-thaw variability of sperm parameters in semen samples that were collected within a short abstinence period from a cohort of patients undergoing sperm banking and from fertile donors.

Patient history as well as pre-freeze and post-thaw information on manual semen analyses was obtained from 109 men who were referred to the Sperm Bank at the Cleveland Clinic Foundation. The study population consisted of 16 fertile men who were scheduled to undergo vasectomy (these men were considered fertile donors), patients with male factor infertility (MFI,  $n = 19$ ), patients with testicular cancer ( $n = 22$ ), patients with Hodgkin's lymphoma ( $n = 15$ ), and non-Hodgkin's lymphoma ( $n = 18$ ), and 19 patients with other types of cancers (leukemia = 8; soft tissue sarcoma = 2; cancer in the chest = 3; bladder cancer = 3; and brain cancer = 3).

At least two samples were collected from each study participant after a period of sexual abstinence. The period of abstinence lasted no more than 10 days for the first sample and no more than 3 days for all subsequent samples. All sperm evaluations were performed using a 20 $\mu$  Microcell chamber (Conception Technologies, San Diego, CA).

Sperm motion characteristics were assessed using computer-assisted semen analysis (CASA, Cell-Trak, version 4.24 Motion Analysis Corporation, Palo Alto, CA). The CASA setting was as follows: frame rate (frames/second): 30; duration of data capture (frames): 15; minimum motile speed ( $\mu\text{m/s}$ ): 600; distance scale factor ( $\mu\text{m/pixel}$ ): 0.9457; centroid cell size minimum (pixels): 2; cen-

teroid cell size maximum (pixels): 8; number of cells to find per well: 200; and the minimum number of fields per sample: 3.

All semen specimens were cryopreserved using Test-yolk buffer (TYB, Irvine Scientific, Santa Ana, CA), a glycerol-based cryoprotectant [7]. An aliquot of the freezing medium equal to 25% of the semen sample volume was added to the specimen and gently mixed for 5 min using a Hema-Tek aliquot mixer (Miles Scientific, Elkhart, IN). This was repeated to give a final 1:1 (v/v) ratio of freezing medium and the sperm samples. Specimens were placed in cryogenic vials (1.5 mL, Corning, Fisher Scientific, Pittsburg, PA) and loaded into cryocanes and placed in at  $-20^\circ\text{C}$  for 8 min and thereafter in liquid nitrogen vapor at  $-100^\circ\text{C}$  for 2 h. The cryovials were finally transferred to liquid nitrogen tanks at  $-196^\circ\text{C}$ . Twenty-four hours after the semen was frozen, a vial was removed and thawed by incubating it at  $37^\circ\text{C}$  for 20 min. An aliquot of well-mixed sample was analyzed immediately without removal of cryopreservative to assess for manual sperm motility and sperm motion characteristics by CASA.

The percentage of sperm that survived the cryopreservation process (percentage cryosurvival) was calculated using the following formula:  $100 \times \text{post-thaw total motile sperm} / \text{pre-freeze total motile sperm}$ . Variability was calculated from 334 samples obtained from the 109 study participants. Among the 16 fertile donors, 95% provided two to three samples each. Among the 93 patients, 70% provided three to seven samples each and 30% provided two to three samples each. The variability was calculated between individual parameters of each sample and expressed as the coefficient of variation:  $\text{CV} (\%) = 100 \times \text{standard deviation} / \text{mean value}$ . All statistical analysis was performed with GraphPad Software Version 3.20 (1998, GraphPad Software, San Diego, CA). The Wilcoxon rank-sum test was used to compare the pre-free and post-thaw semen characteristics of the fertile donors with those of the patients and to compare the semen characteristics among the five patient groups.

Our results revealed that the overall semen quality (concentration, motility, and morphology) was similar between the fertile donors and cancer

patients before and after freezing. However, in patients with MFI, poor semen quality (lower sperm concentration, percentage motility, and percentage normal morphology) was seen compared with the other patient groups.

Irrespective of the sperm motion characteristics or the study population, a large variability was seen both in the pre-freeze and post-thaw sperm motion characteristics from the multiple samples frozen from the same individual or from the individuals in any given group. The mean and the range (minimum and maximum) of the variability in semen characteristics from fertile donors and men with male factor infertility patients as well as various cancers are shown in Table 1. A large variation was seen in percent motility both for pre-freeze as well as post-thaw samples. In the pre-freeze group, the maximum variability was observed in samples obtained from donors, male factor patients, and men with Hodgkin's lymphoma. In the post-thaw samples, a large but similar variability was seen in all patient samples.

Both the mean and the range of pre-freeze curvilinear velocity curvilinear velocity (VCL) values were comparable all across the groups. The post-thaw values displayed a large range all across the study groups although this was smaller for donors and men with other cancers. The mean pre-freeze straight-line velocity (VSL) values as well as the range of variability were comparable all across the groups. Men with male factor infertility showed a larger variability when compared with other groups. In the post-thaw samples, although VSL increased, the extent of variability was similar.

The mean average path velocity (VAP) was comparable and the variability was similar in pre-freeze samples all across the groups. An increase in mean post-thaw values was seen in men with male factor infertility and testicular cancer when compared both with donors and other groups, however, this increase was also evident by the increase in the range of values for VAP.

The pre-freeze mean values of linearity (LIN) were similar all across the groups except in patients with male factor infertility. These patients, however, displayed a wide range in linearity. Post-thaw variability both in the mean values as

Table 1  
Pre-freeze and post-thaw variability (%) in donors and patient population

Sperm parameters	Fertile donors (n = 16)		Male factor infertility (n = 19)		Testicular cancer (n = 22)		Hodgkin's lymphoma (n = 15)		Non-Hodgkin's lymphoma (n = 18)		Other cancers <sup>a</sup> (n = 19)	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Motility (%)	24.5 (1.9–102.0)	31.4 (3.3–55.1)	31.7 (2.4–84.6)	32.4 (0–108.6)	22.9 (1.2–58.7)	36.5 (4.4–71.6)	31.0 (4.8–80.3)	45.0 (2.4–141.4)	19.5 (5.2–46.8)	48.1 (14.8–99.0)	17.2 (3.0–45.0)	33.6 (0–86.6)
VCL (µm/s)	19.3 (0.5–63.0)	15.7 (0.2–42.8)	20.3 (3.2–44.1)	44.3 (11.9–61.5)	18.4 (1.3–38.9)	31.9 (1.8–133.7)	20.7 (4.8–44.6)	28.3 (4.3–88.9)	19.6 (0.2–73.3)	31.2 (13.8–110.6)	19.8 (2.2–54.0)	18.2 (1.1–88.6)
VSL (µm/s)	21.4 (3.5–36.2)	18.5 (0.5–53.5)	27.5 (1.3–91.9)	35.9 (6.73–66.5)	23.7 (2.7–42.7)	33.1 (0.8–92.5)	20.8 (4.3–40.4)	29.5 (7.9–91.7)	21.2 (3.9–45.4)	25.5 (1.6–47.8)	20.1 (6.1–37.9)	24.0 (4.9–102.7)
VAP (µm/s)	21.2 (0.5–58.2)	18.6 (1.2–52.7)	26.1 (0.5–62.7)	41.5 (22.3–62.0)	20.7 (4.2–38.6)	31.2 (2.5–102.9)	19.4 (1.9–40.0)	27.2 (8.5–92.0)	20.8 (6.0–42.2)	26.4 (9.1–43.7)	18.9 (4.1–42.9)	20.8 (2.8–95.7)
LIN (%)	9.8 (1.3–59.6)	9.2 (0–37.7)	20.0 (3.1–124.3)	19.7 (1.3–53.3)	11.0 (0–39.0)	20.0 (2.2–131.2)	12.1 (0–26.9)	18.5 (2.8–86.8)	13.58 (3.5–86.9)	18.71 (3.9–103.5)	9.1 (0–39.3)	15.2 (1.2–90.0)
ALH (µm)	12.6 (0.0–42.7)	11.1 (0–42.0)	33.2 (0.8–141.4)	57.6 (2.2–173.2)	20.2 (1.3–64.9)	43.9 (1.8–147.1)	17.6 (1.7–57.5)	27.7 (4.9–68.0)	28.7 (6.2–141.4)	28.0 (2.9–72.9)	11.9 (0–39.3)	37.9 (3.7–173.2)
Cryosurvival (%)		32.0 (1.5–141.4)	54.5 (4.2–141.4)		38.8 (9.0–74.6)		45.9 (5.9–141.5)		49.5 (4.6–102.0)		37.2 (0.3–95.8)	

Values in parentheses represent the range. VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; and ALH, amplitude of lateral head displacement.

well as in the range of variability appeared to be smaller in donors. The post-thaw linearity was higher than donors but was comparable across all groups that also displayed a large range in between the lowest and the highest values.

As regards the amplitude of lateral head displacement (ALH), both the mean and the range of pre-freeze variability were similar in donors and in men with Hodgkin's and other cancers. Also, both the mean and the range of variability were higher in men with male factor infertility, non-Hodgkin's lymphoma, and other cancer. Similarly in post-thaw samples, a significant increase was seen in the mean values all across the groups, as was the increase in the range of variability.

Table 2 shows the percentage variability in pre-freeze and post-thaw sperm motility and motion parameters in the overall study population. According to our results, the percentage of post-thaw variability was significantly higher than the pre-freeze variability for the following parameters: [motility ( $P < 0.0001$ ), VSL ( $P = 0.02$ ), VAP ( $P = 0.006$ ), LIN ( $P = 0.009$ ), and ALH ( $P < 0.0001$ )]. The VCL was also higher but did not reach statistical significance ( $P = 0.07$ ).

Semen samples from a considerable number of patients exhibited increased post-thaw variability. Overall, 34% of the cryopreserved samples showed increased post-thaw inter-sample variability in motility, and 46–65% of the samples exhibited in-

creased post-thaw variability in other motion parameters such as VCL (46.9%), VSL (53.8%), VAP (52.3%), LIN (63.7%), and ALH (65.5%). These findings are important as sperm motility and motion characteristics measured by CASA are believed to be critical indicators especially of sperm cervical mucus penetration [4]. Cryopreservation adversely affects sperm quality [1,6,13], which in turn affects post-thaw motility and the success of ART.

The median (25th, 75th percentiles) percentage cryosurvival was similar between the patients with MFI [31 (22.5, 38.7)] and those with cancer [39.8 (25.9, 55.6)] but was significantly lower compared with fertile donors [42.8 (27.0, 53.5),  $P = 0.02$ ]. No correlation was seen between the extent of post-thaw variability and patient age, diagnosis and history of smoking or alcohol intake.

We detected a significant increase in inter-sample variability after post-thaw in the percent recovery (motility and motion kinetics) of cryopreserved sperm from semen specimens collected at intervals of 2–3 days compared with the pre-freeze variability. The exact cause(s) behind the increased variation in post-thaw sperm quality is unclear. All cryopreservation protocols were identical for all the samples; both inter- and intra-observer variability was carefully recorded and was within the acceptable range.

The average period of abstinence for 84% of the donors of the initial samples was 3.5 days (range: 1–5 days). Only 9% of the samples were collected after a period of abstinence lasting 5–10 days and 1% of the samples were collected after more than 10 days of abstinence.

It is unlikely that the post-thaw variability was caused solely by the varied abstinence time. Two previous studies from our group reported that semen parameters did not change with the abstinence period. We therefore recommended using an abstinence period of 1–3 days for sperm banking [1,13]. Seasonal and/or diurnal causes for post-thaw variability [16] is also an unlikely cause as all the samples were obtained within a short period of time and during the day. Although the inter-sample variability can be partially attributed to the difference in the abstinence period, it may be mainly idiopathic in nature.

Table 2

Overall variability between pre-freeze and post-thaw sperm parameters in fertile donors and patients with male factor infertility or cancer ( $n = 109$ )

Sperm parameters	Pre-freeze variability (%)	Post-thaw variability (%)	$P$ Value <sup>a</sup>
Motility (%)	24.1	37.7	<0.0001
VCL ( $\mu\text{m/s}$ )	19.6	24.3	0.07
VSL ( $\mu\text{m/s}$ )	22.4	27.5	0.02
VAP ( $\mu\text{m/s}$ )	20.8	26.8	0.006
LIN (%)	12.4	16.7	0.009
ALH ( $\mu\text{m}$ )	20.5	34.8	<0.0001

<sup>a</sup> Wilcoxon rank-sum test was used for comparison, and  $P < 0.05$  was significant. VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; and ALH, amplitude of lateral head displacement.

In conclusion, we report significant increase in inter-sample variability in post-thaw sperm motility, motion kinetics, and cryosurvival after freezing in comparison to pre-freeze variability. This increase in the inter-sample variability in post-thaw sperm parameters occurred in semen samples from healthy men, infertile men, and men with cancer. Further research is necessary to identify the exact mechanism(s) of increased inter-sample variability seen in our study. Identifying factors that influence the recovery of sperm motility and motion characteristics after cryopreservation may help fertility specialists select ideal semen samples for freezing. Our study results suggest that multiple semen samples should be cryopreserved for future use—irrespective of the pathological disorder—to ensure that sperm of the highest quality are available for use in ART. In addition, a test vial should be thawed and assessed following 24 h of cryopreservation to monitor the response of spermatozoa to cryodamage.

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