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Semen cryopreservation and storage in liquid nitrogen: Impact on chromatin compaction

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Abstract

Background: Sperm cryopreservation is a consolidate option for long-term male fertility preservation. The freezing/thawing procedure causes detrimental effects to spermatozoa, including damage to viability, motility, membrane composition, and DNA, whereas the effect on sperm chromatin compaction is less studied.

Objectives: The primary aim of this study was to investigate the impact of cryopreservation on sperm chromatin compaction. Furthermore, the effect of cryopreservation on sperm parameters (motility, viability, chromatin compaction, and DNA fragmentation) was also assessed in relation to the storage time in liquid nitrogen.

Materials and Methods: Semen samples, collected from 126 (92 normozoospermic and 34 oligozoospermic) patients undergoing routine semen analysis in the Andrology Laboratory of Careggi University Hospital of Florence, were frozen by conventional fast vapor freezing method. Sperm motility, viability, kinematic parameters (by computer-aided sperm analysis [CASA]), chromatin compaction (by staining with both aniline blue [AB] and Chromomycin A3 [CMA3]), and sperm DNA fragmentation (sDF, by TUNEL/Propidium Iodide [PI]) were evaluated before freezing and after thawing at different timepoints.

Results: After 7 days of storage, a significant decline in sperm motility, viability, and kinematics parameters, as well as a significant increase in the percentage of sperm positivity to CMA3, AB, and sDF, were observed. It is noteworthy that while motility and viability decreased in almost all subjects, the increase in CMA3 and AB positivity was observed in 68.0% and 79.2% of samples, respectively. A progressive deterioration of sperm motility and viability, less evident for chromatin structure, was observed at longer times of storage (28 and 180 days).

Discussion: Our results indicate that freezing/thawing procedures can alter chromatin structure. A reduction in protamine content and/or a modification in chromatin assembly can be hypothesized. Furthermore, the length of storage in liquid nitrogen appears

to progressively affect sperm parameters, although it should be confirmed in larger cohort of subjects.

Conclusion: Current sperm cryopreservation protocols need to be improved with new strategies and personalized procedures aimed to minimize the damage.

KEYWORDS
assisted reproduction, chromatin, fertility preservation, human spermatozoa, semen cryopreservation, sperm motility

1 | INTRODUCTION

Semen cryopreservation in liquid nitrogen, which allows long-term storage, is the most consolidate and non-invasive option for male fertility preservation. This possibility is offered to oncological patients undergoing gonadotoxic treatments, but also to men undergoing vasectomy or other urological interventions that may compromise their fertility. Moreover, this option can be offered to male-to-female transgender adults and adolescents before starting hormonal therapy and to male partners of couples undergoing Assisted Reproduction Techniques (ART) when affected by severe oligozoospermia, or unable to ejaculate (patients with spinal cord injuries or those whose spermatozoa are collected by retrograde ejaculation in urine or by surgery from the genital tract), or are unable to provide a fresh sample the day of oocytes pick-up.[1,2] Cryopreservation is also used to store and then distribute semen samples from healthy donors for heterologous artificial insemination.[3] All in vitro sperm manipulation procedures, including those involving semen cryopreservation, can expose male gametes to chemical and physical stress.[4] Our laboratory, which has a long experience in semen cryopreservation, demonstrated that the extent of the damage depends on the initial quality of the semen, varies between individuals and is influenced by the presence of an oncological pathology.[5,6] The worst recovery rate was observed in cases of severe oligozoospermia (sperm concentration below 2 million/mL) in which the average percentage of sperm motility after thawing is 0%.[6]

During freezing and thawing procedures, sperm membranes can undergo phospholipid modifications and peroxidation leading to reduced motility, impaired mitochondrial function, modifications of the acrosome structure and decreased acrosin activity.[7] Furthermore, chromatin compaction and DNA integrity, both critical for successful fertilization, may also be compromised.[8]

The induction of sperm DNA fragmentation (sDF) after cryopreservation is well documented,[9–15] whereas the effect of cryopreservation on sperm chromatin compaction is less studied.

Replacement of histones with protamines during spermatogenesis makes sperm chromatin highly compacted, thereby protecting the integrity of the paternal genome during passage through the male and female reproductive tracts.[16] Several studies reported that abnormalities in sperm chromatin packaging are associated with male infertility.[17–19] Furthermore, protamine-deficient human spermatozoa show a significantly reduced fertilizing ability, worse embryo quality and lower pregnancy rate in ART procedures.[20–24]

The primary aim of this study was to investigate the impact of cryopreservation on sperm chromatin compaction evaluated by staining with Aniline Blue (AB, an index of histone persistence) and Chromomycin A3 (CMA3, an index of poor compaction). In particular, sperm chromatin integrity, motility, and viability were evaluated before and after freezing/thawing in semen samples from both normozoospermic and oligozoospermic men. Furthermore, in normozoospermic samples, the above-mentioned parameters were also assessed in relation to the storage time in liquid nitrogen after 7 (t7), 28 (t28), and 180 (t180) days from freezing.

2 | MATERIALS AND METHODS

2.1 | Reagents

Paraformaldehyde (PFA), CMA3, and AB were obtained from Merck Life Sciences S.r.l. Phosphate-buffered saline (PBS) was obtained from Biosigma S.p.A. Freezing medium test yolk buffer (TYB) containing egg yolk (20%), glycerol (12% v/v), and gentamycin sulfate (10 µg/mL), and human tubal fluid (HTF) were purchased from Fujifilm Italia S.p.A. In Situ Cell Death Detection Kit was purchased from Roche Molecular Biochemicals.

2.2 | Semen sample collection and processing

This study was approved by the local ethical committee (Ref: 23266_bio) and was conducted on male patients undergoing routine semen analysis at the Andrology Laboratory of the Careggi University Hospital of Florence. The only inclusion criterion was obtaining signed informed consent to use the semen remaining after completion of the analysis. Semen samples were obtained by masturbation after a minimum of two and a maximum of 7 days of sexual abstinence. Thirty minutes after semen collection, the volume, viscosity, and pH of the samples were evaluated. Complete semen analysis (sperm concentration, progressive and total motility, viability, and morphology) was performed on fresh semen samples using the Nikon ECLIPSE Ci optical microscope according to the World Health Organization manual.[25]

Sperm concentration was evaluated using an improved Neubauer chamber after dilution of semen samples in formalin-containing buffer. The percentages of progressive, non-progressive, and immotile spermatozoa were assessed on at least 200 spermatozoa using an optical microscope with a 40× objective and a heated plate at 37°C. Sperm viability was evaluated on at least 200 spermatozoa using an eosin test. Sperm morphology was assessed on at least 200 spermatozoa after Diff-Quik staining using a 100× oil immersion objective. Spermatozoa were classified as normal or abnormal.

Based on semen analysis, we selected 92 normozoospermic and 34 oligozoospermic (sperm concentration $\leq 10 \times 10^6$/mL) samples. In order to evaluate the immediate effect of cryopreservation procedure on sperm parameters, 10 samples were thawed immediately after freezing (ti) and after 7 days (t7). As there were no differences in sperm parameters between ti and t7 (data not shown), we decided to eliminate ti in the subsequent experiments. Of the 92 normozoospermic samples, 45 were cryopreserved and stored in liquid nitrogen for 7 (t7) and 28 (t28) days, and 79 for t7 and 180 (t180) days. Only 32 among 92 normozoospermic samples were stored at all timepoints (t7, t28, and t180). Oligozoospermic samples were stored for only 7 days (t7). Sperm motility, viability, kinematics parameters, chromatin compaction, and sDF were evaluated both before cryopreservation (t0) and after different thawing times.

2.3 | Conventional semen cryopreservation

The conventional cryopreservation protocol involves manual fast vapor freezing. Briefly, samples were diluted (1:1; vol:vol) by dropwise addition of TYB and the mixture was aspirated into 500 µL of high security sperm straws (Cryo Bio System, Groupe I.M.V. Technologies). The straws were exposed to liquid nitrogen vapors for 8 min by placing them in a box with a floating rack maintaining a fixed distance (10 cm) above the liquid nitrogen level at a temperature of −80°[1] with a cooling rate of approximately 12.5°C/min. After exposure to vapors, the straws were plunged into liquid nitrogen (−196°C) and then stored in dedicated tanks.[5]

Thawing was performed by transferring the straws at 37°C for 15 min and then used for the different assessments. In particular, the straws from normozoospermic samples were thawed after 7 (t7), 28 (t28), and 180(t180) days of storage, while those from oligozoospermic samples were thawed after 7 days of storage (t7).

2.4 | Assessment of kinetic parameters

Kinetic parameters were analyzed pre- and post-cryopreservation by computer-aided sperm analysis (CASA, Hamilton Thorne Research) equipped with a Olympus CX41 optical microscope with a 37°C heated plate and 10× objective. Leja slides with two chambers with depth of 20 µm were used for the analysis (Cryo Bio System, Groupe I.M.V. Technologies). The settings used during CASA procedures were those set by the system for human spermatozoa. In particular, analysis duration of 1 s (30 frames); maximum and minimum head size, 50 and 5 μm^2; minimum head brightness, 170; minimum tail brightness, 70.[26] The mean path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), and linearity of progression (LIN, %) were recorded. Two aliquots were analyzed for each sample with a minimum of 200 motile cells, and five fields were analyzed for each aliquot.

2.5 | Sperm chromatin compaction

Sperm chromatin compaction was evaluated before cryopreservation and after thawing by using two different stains: CMA3 and AB. AB is an acidic cytochemical dye, which binds to alkaline amino acids of histones, whereas CMA3 is a fluorochrome which competes with protamines for binding to the minor groove of guanine-cytosine (GC)-rich DNA.[27] For both staining procedures, 1×10^6 washed spermatozoa (with PBS) were fixed in PFA (4% in PBS pH 7.4) for 30 min at room temperature. For CMA3 staining, spermatozoa were incubated with 100 µL of CMA3 solution [0.25 mg/mL in McIlvaine's buffer (0.2 M Na_2HPO_4, 0.1 M citric acid), pH 7.0, containing 10 mM $MgCl_2$], for 20 min at room temperature in the dark. After washing, spermatozoa were resuspended in 10 µL of McIlvaine's buffer, pH 7.0, containing 10 mM $MgCl_2$, smeared on slide, air-dried, and mounted with PBS:glycerol (1:1). Two hundred spermatozoa were analyzed on each slide by fluorescence microscope (Axiolab A1 FL; Carl Zeiss), using an oil immersion 100× objective.[28] Two types of staining patterns were identified: bright green fluorescence of the sperm head (indicating low protamine content and abnormal chromatin packaging) and weak green staining (indicating high protamine content and normal chromatin packaging).[28] For AB staining, after fixation in 4% PFA, spermatozoa were smeared on slide, air-dried, and then incubated with 5% aqueous AB, mixed with 4% acetic acid (pH 3.5) for 7 min at room temperature.[28] Two hundred spermatozoa were analyzed on each slide under a light microscope (Leica DM LS; Leica). Spermatozoa showing dark-blue staining were considered as AB positive (high histone content[29]). All assessments were performed by two blinded observers. The mean inter-observer difference was −0.154 (95% confidence interval [CI]: −1.281 to 0.973) for CMA3 and 0.920 (95% CI: −0.757 to 2.597) for AB, according to Bland and Altman analysis. The differences did not vary significantly between the two different readers.

2.6 | Sperm DNA fragmentation

A total of 10×10^6 spermatozoa were washed twice with HTF medium (from Fujifilm Italia S.p.A.) and fixed with 500 µL PFA 4% for 30 min at room temperature. After two washes with 200 µL of PBS with 1% bovine serum albumin (BSA), the samples were permeabilized with 0.1% Triton X-100 in 100 µL 0.1% sodium citrate for 4 min in ice. Each sample was divided into two aliquots (test sample and negative control) and the labeling reaction was performed using the In Situ Cell Death Detection Kit. Spermatozoa were incubated for 1 h at 37°C in the dark

TABLE 1 Mean ± SD of age, sexual abstinence, and basal semen characteristics of normozoospermic and oligozoospermic subjects included in the study.

	Age (years)	Sexual abstinence (days)	Semen volume (mL)	pH	Sperm total motility (%)	Sperm concentration ($\times 10^6$/mL)	Sperm total count ($\times 10^6$/ejaculate)	Sperm normal morphology (%)
Normozoospermic subjects ($n = 92$)	35.8 ± 7.9	4.2 ± 1.7	3.8 ± 1.2	7.7 ± 0.2	62.1 ± 10.1	88.7 ± 70.2	324.0 ± 247.0	4.2 ± 2.6
Oligozoospermic subjects ($n = 34$)	35.7 ± 8.1	4.1 ± 1.6	4.7 ± 2.3	7.7 ± 0.2	45.3 ± 18.6	5.8 ± 2.8	28.5 ± 21.2	1.4 ± 1.3

TABLE 2 Mean ± SD of sperm progressive and total motility and viability, mean path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), and linearity of progression (LIN) at t0 and after 7 days from cryopreservation (t7) in 92 normozoospermic subjects.

	Sperm progressive motility (%)	Sperm total motility (%)	Sperm viability (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	LIN (%)
t0 ($n = 92$)	55.0 ± 11.8	62.1 ± 10.1	76.2 ± 9.6	29.9 ± 8.3	49.6 ± 13.5	22.2 ± 6.8	42.6 ± 5.7
t7 ($n = 92$)	30.8 ± 14.1*	37.3 ± 14.7*	47.6 ± 13.9*	17.8 ± 7.4*	34.9 ± 13.8*	12.5 ± 5.6*	35.9 ± 8.8*
Cohen's *d* (95% confidence interval [CI])	1.7 (1.4–2.0)	1.8 (1.5–2.1)	1.8 (1.5–2.1)	1.3 (0.9–1.6)	0.8 (0.5–1.1)	1.4 (1.1–1.7)	0.7 (0.4–0.9)

Bottom line: Cohen's *d* (95% CI) for the effect size between t7 and t0.
*$p < 0.001$ versus t0.

with 50 μL of labeling solution (supplied by the kit) containing Terminal Deoxynucleotidyl Transferase (TdT) enzyme (1:10) for the test sample and the labeling solution alone for the negative control. After two washes, samples were resuspended in 300 μL of PBS, stained with PI (30 μg/mL) and incubated in the dark for 5 min at room temperature.

Then, samples were acquired by FACScan flow cytometer (Becton Dickinson Biosciences) equipped with a 15 mW argon-ion laser for excitation. Three sperm suspensions were prepared for instrumental setting and data analysis[1]: omitting both PI staining and TdT,[2] omitting only TdT (negative control), and[3] omitting only PI staining (for fluorescence compensation). Green fluorescence of TUNEL was detected by FL-1 detector (515–555 nm wavelength band, voltage set 590) and red fluorescence of PI was detected by FL-2 detector (563–607 nm wavelength band and voltage set 477). A total of 8000 events were recorded within the flame-shaped region characteristic of spermatozoa in the forward-light scatter/side-light scatter dot plot, and sDF was determined by gating the nucleated events (i.e., the events labeled with PI) within this region.[30] A marker, including 99% of total events, was set in the negative control-related dot plot ~~to~~ and translated in the corresponding test sample dot plot, and all the events beyond the marker were considered TUNEL positive. sDF was calculated in both the total and PI Brighter populations, as previously described.[30]

2.7 | Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences version 29.0.2.0 (SPSS) for Windows. Data distribution was verified by Q-QP plot. All variables resulted normally distributed and data are expressed as mean ± SD values. The percentage of decrease or increase for each analyzed parameter was calculated as: {[(Post-thawing value – t0 value)/t0 value] × 100}. Paired *t*-test was used for comparisons among groups. The effect size for a paired samples *t*-test was expressed as Cohen's *d*. A *p* value of 0.05 was considered significant.

3 | RESULTS

Table 1 reports baseline characteristics of the 92 normozoospermic and 34 oligozoospermic men included in the study.

In 92 normozoospermic samples, sperm motility, kinematics parameters, viability, and chromatin compaction were assessed at t0 (before cryopreservation) and t7 (after thawing). As expected, a statistically significant worsening of all analyzed parameters was observed in thawed samples respect to the fresh ones (Table 2). Similar results were observed in oligozoospermic samples (Table 3). Noteworthy, a statistically significant increase in the percentage of spermatozoa positive to both CMA3 and AB staining was observed after 7 days of storage in both normo- and oligozoospermic samples (Figure 1 A,B).

In particular, although the mean percentage decreases of sperm total motility and viability were greater in oligozoospermic than in normozoospermic samples (total motility: −87.3% vs. −40.1%; viability: −60.6% vs. −37.0%, respectively), the percentages of increase in AB and CMA3 positivity were similar (AB positivity: 44.5% vs. 41.7%; CMA3 positivity: 36.7% vs. 35.5%, respectively). Interestingly, when

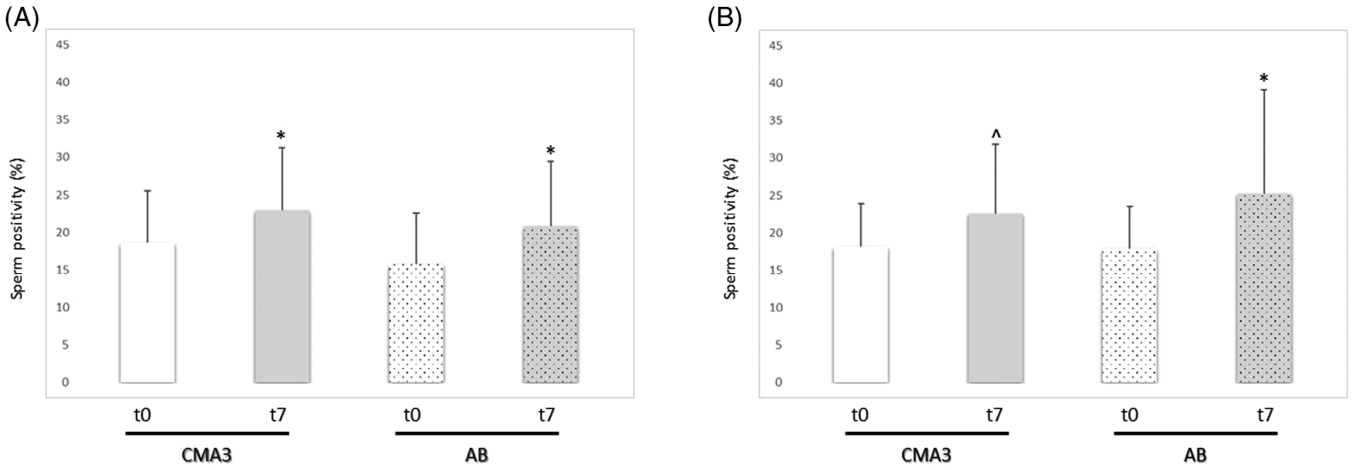

FIGURE 1 Effect of cryopreservation on sperm chromatin compaction. Histograms representing the percentages of sperm positivity to Chromomycin A3 (CMA3) and Aniline Blue (AB) at t0 and after 7 days from cryopreservation (t7) in 92 normozoospermic (A) and 34 oligozoospermic subjects (B). *$p < 0.001$ versus t0; ^$p < 0.05$ versus t0.

TABLE 3 Mean ± SD of sperm progressive and total motility and viability at t0 and after 7 days from cryopreservation (t7) in 34 oligozoospermic subjects.

	Sperm progressive motility (%)	Sperm total motility (%)	Sperm viability (%)
t0 ($n = 34$)	36.2 ± 18.9	45.3 ± 18.6	65.1 ± 17.0
t7 ($n = 34$)	4.2 ± 4.5*	6.1 ± 5.0*	25.2 ± 8.8*
Cohen's *d* (95% confidence interval [CI])	1.9 (1.4–2.5)	2.4 (1.7–3.1)	3.3 (2.4–4.1)

Bottom line: Cohen's *d* (95% CI) for the effect size between t7 and t0.
*$p < 0.001$ versus t0.

individual values of the entire cohort ($n = 126$) were considered, we found that the increase of CMA3 (mean values ± SD: 18.5% ± 6.6 vs. 22.9% ± 8.5) and AB (mean values ± SD 16.0% ± 6.6 vs. 22.1% ± 10.4) positivity at t7 respect to t0 showed a variable trend, with some samples increasing and others decreasing or not changing (Figure 2C,D), despite the decrease in sperm motility and viability observed in almost all samples (Figure 2A,B). We performed a Receiver Operating Characteristic (ROC) analysis to identify the accuracy of pre-cryopreservation semen parameters and patient age in predicting post-thawing CMA3 and AB values, but none resulted predictive (results not shown). On the contrary, basal motility and viability were highly predictive of post-thawing values (not shown), confirming previous results.[5,6] In 45 normozoospermic semen samples, the effect on sperm parameters was evaluated at both 7 and 28 days of storage. A statistically significant reduction of sperm progressive and total motility, viability, and kinematic parameters was observed after 28 days of storage respect to t0 (Table 4). Sperm progressive and total motility were significantly reduced after thawing at t28 respect to t7 (Table 4) suggesting a further detrimental effect with storage time. Conversely, CMA3 and AB positivity, although increased after 7 days of storage, was not significantly modified after 28 days (Table 4).

To further investigate the effect of the storage time in liquid nitrogen, we compared sperm parameters after thawing at t7 and at t180 in a group of 79 normozoospermic samples. The data, reported in Table 5, show a statistically significant progressive reduction of sperm motility and viability, and of some kinematic parameters at 180 days of storage. A statistically significant increase in CMA3 and AB positivity was observed at both thawing times respect to t0. A slight but significant increase in the percentage of AB-positive spermatozoa was observed at t180 respect to t7, whereas CMA3 positivity remained similar (Table 5).

To better evaluate the effect of storage time, 32 semen samples were thawed at both t7, t28, and t180. Figure 3 shows a gradual deterioration of average values of sperm motility and viability. On the contrary, sperm positivity to CMA3 and AB confirms the results reported in Table 5 also in this caseload (Table 6). The impact of cryopreservation on sDF was evaluated in 10 samples at t0, t7, and t28. Our results (Table 7) demonstrated that total and PI Brighter sDF increased immediately after freezing and no changes were observed at t7 and t28.

4 | DISCUSSION

The advent of cryopreservation has revolutionized the field of assisted reproduction by giving many men the opportunity to become father. However, this procedure, which involves sperm freezing/thawing, inevitably has deleterious effects on seminal quality. The present study confirms that cryopreservation causes a decline in sperm total and progressive motility, viability,[6,31,32] and sperm motion characteristics measured by computer-aided analysis, corroborating previous studies.[33,34] As a novel finding, we show here that sperm motility progressively deteriorates with storage time and that sperm chromatin compaction is altered by cryopreservation procedure.

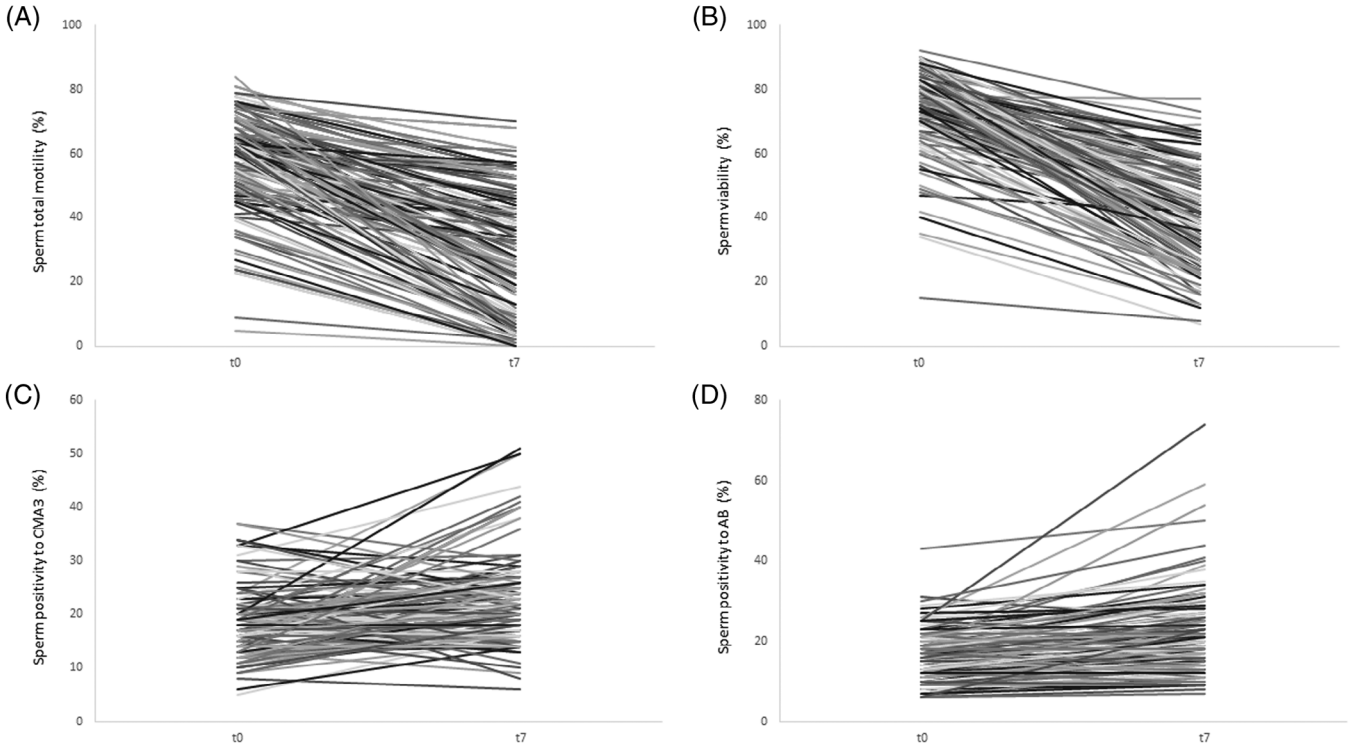

FIGURE 2 Individual effect of cryopreservation on sperm parameters. Line graphs showing individual values of sperm total motility (A), viability (B), positivity to Chromomycin A3 (CMA3) (C), and Aniline Blue (AB), (D) at t0 and after 7 days from cryopreservation (t7) in the whole cohort of 126 semen samples.

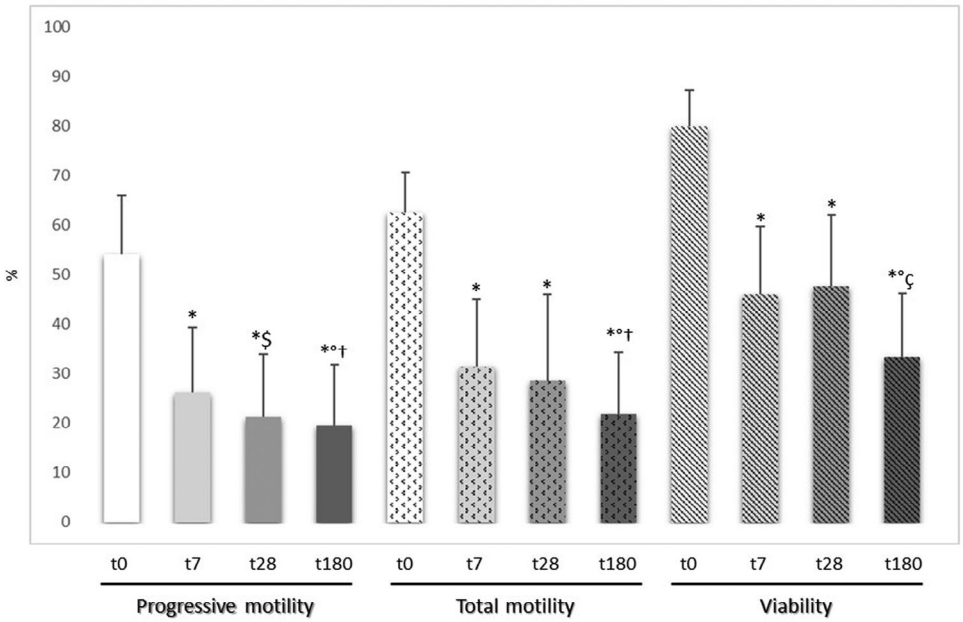

FIGURE 3 Effect of storage time on sperm parameters. Histograms representing the percentages of sperm progressive and total motility and viability at t0 and after 7 (t7), 28 (t28), and 180 (t180) days from cryopreservation in 32 normozoospermic subjects. *$p < 0.001$ versus t0; °$p < 0.001$ versus t7; $^{\$}p < 0.05$ versus t7; $^{ç}p < 0.001$ versus t28; $^{\dagger}p < 0.05$ versus t28.

TABLE 4 Mean ± SD of sperm progressive and total motility and viability, mean path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), linearity of progression (LIN), Chromomycin A3 (CMA3), and Aniline Blue (AB) positivity at t0 and after 7 (t7) and 28 (t28) days from cryopreservation in 45 normozoospermic subjects.

	Sperm progressive motility (%)	Sperm total motility (%)	Sperm viability (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	LIN (%)	CMA3 (%)	AB (%)
t0 ($n = 45$)	52.4 ± 12.8	61.0 ± 9.6	77.9 ± 9.0	30.9 ± 6.5	51.2 ± 10.4	23.1 ± 5.8	42.7 ± 6.2	16.3 ± 5.6	12.6 ± 5.0
t7 ($n = 45$)	28.4 ± 13.6*	33.9 ± 13.8*	46.8 ± 13.0*	17.1 ± 6.8*	33.7 ± 13.1*	12.3 ± 5.8*	36.6 ± 10.1^	21.3 ± 7.9*	16.9 ± 6.3*
t28 ($n = 45$)	23.1 ± 12.3*,°	29.7 ± 15.6*,$	46.4 ± 13.2*	20.0 ± 7.8*	41.1 ± 17.2*	13.6 ± 6.4*	33.1 ± 10.5	22.6 ± 10.4*	17.8 ± 8.2*
Cohen's *d* (95% confidence interval [CI]) t7 versus t0	1.6 (1.2–2.1)	1.9 (1.4–2.4)	2.3 (1.7–2.8)	1.6 (0.9–2.2)	1.1 (0.6–1.6)	1.6 (0.9–2.2)	0.5 (0.1–0.9)	−0.6 (−0.9 to −0.3)	−0.8 (−1.1 to −0.4)
Cohen's *d* (95% CI) t28 versus t0	2.2 (1.7–2.8)	1.9 (1.5–2.5)	2.6 (1.9–3.2)	1.9 (0.9–2.8)	1.6 (0.7–2.4)	1.3 (0.5–2.1)	0.3 (−0.3 to 0.8)	−0.6 (−0.9 to −0.3)	−0.8 (−1.1 to −0.4)
Cohen's *d* (95% CI) t28 versus t7	−0.6 (−0.9 to −0.3)	−0.3 (−0.6 to −0.03)	−0.03 (−0.3 to 0.3)	−0.2 (−0.8 to 0.4)	0.1 (−0.4 to 0.7)	−0.6 (−1.2 to 0.1)	−0.4 (−1.1 to 0.2)	0.1 (−0.2 to 0.4)	0.1 (−0.2 to 0.4)

Bottom lines: Cohen's *d* (95% CI) for the effect size between t7 and t0, t28 and t0, t28 and t7.

*$p < 0.001$ versus t0.

^$p < 0.05$ versus t0.

°$p < 0.001$ versus t7.

$$p < 0.05$ versus t7.

TABLE 5 Mean ± SD of percentages of sperm progressive and total motility and viability, mean path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), linearity of progression (LIN), Chromomycin A3 (CMA3), and Aniline Blue (AB) positivity at t0 and after 7 (t7) and 180 (t180) days from cryopreservation in 79 normozoospermic subjects.

	Sperm progressive motility (%)	Sperm total motility (%)	Sperm viability (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	LIN (%)	CMA3 (%)	AB (%)
t0 ($n = 79$)	58.0 ± 11.4	62.8 ± 9.7	76.7 ± 9.3	29.8 ± 8.5	49.2 ± 13.9	22.2 ± 6.8	42.9 ± 5.6	19.1 ± 7.1	16.5 ± 6.8
t7 ($n = 79$)	30.1 ± 14.2*	36.6 ± 14.9*	47.4 ± 14.3*	17.8 ± 7.8*	34.4 ± 14.1*	12.7 ± 5.9*	36.6 ± 8.3*	22.5 ± 8.1*	21.4 ± 8.8*
t180 ($n = 79$)	24.3 ± 14.4*,°	27.6 ± 15.3*,°	36.2 ± 13.4*,°	18.9 ± 7.3*	40.2 ± 16.8^,$	11.9 ± 4.9*	30.4 ± 8.3*,°	22.3 ± 6.6*	22.8 ± 7.8*
Cohen's *d* (95% confidence interval [CI]) t7 versus t0	1.8 (1.5–2.2)	1.9 (1.5–2.1)	1.8 (1.4–2.1)	1.2 (0.9–1.6)	0.8 (0.5–1.1)	1.3 (0.9–1.7)	0.7 (0.4–0.9)	−0.4 (−0.6 to −0.2)	−0.7 (−0.9 to −0.5)
Cohen's *d* (95% CI) t180 versus t0	2.4 (1.9–2.8)	2.5 (2.0–2.9)	2.8 (2.3–3.2)	1.0 (0.7–1.3)	0.4 (0.1–0.6)	1.5 (1.1–1.8)	1.3 (0.9–1.7)	−0.5 (−0.7 to −0.2)	−0.8 (−1.1 to −0.6)
Cohen's *d* (95% CI) t180 versus t7	−0.7 (−0.9 to −0.4)	−0.9 (−1.2 to −0.7)	−0.9 (−1.2 to −0.6)	−0.1 (−0.4 to 0.1)	−0.3 (−0.5 to 0.003)	0.1 (−0.2 to 0.3)	0.5 (0.3–0.8)	−0.03 (−0.3 to 0.2)	0.2 (−0.02 to 0.4)

Bottom lines: Cohen's *d* (95% CI) for the effect size between t7 and t0, t180 and t0, t180 and t7.

*$p < 0.001$ versus t0.

^$p < 0.05$ versus t0.

°$p < 0.001$ versus t7.

$$p < 0.05$ versus t7.

TABLE 6 Mean ± SD of percentages of sperm progressive and total motility and viability, mean path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), linearity of progression (LIN), positivity to Chromomycin A3 (CMA3), and Aniline Blue (AB) at t0 and after 7 (t7), 28 (t28), and 180 (t180) days from cryopreservation in 32 normozoospermic subjects.

	Sperm progressive motility (%)	Sperm total motility (%)	Sperm viability (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	LIN (%)	CMA3 (%)	AB (%)
t0 ($n = 32$)	53.3 ± 12.5	62.1 ± 8.3	79.6 ± 7.5	31.4 ± 5.9	50.7 ± 9.4	23.6 ± 4.8	43.8 ± 6.0	16.5 ± 6.0	13.3 ± 5.3
t7 ($n = 32$)	25.9 ± 13.0*	31.3 ± 13.3*	45.8 ± 13.6*	16.9 ± 8.6*	30.1 ± 13.1*	13.2 ± 7.1*	40.4 ± 7.5	19.5 ± 6.8^	16.8 ± 6.7*
t28 ($n = 32$)	20.9 ± 12.6*,$	28.2 ± 17.3*	47.1 ± 14.4*	21.8 ± 7.8*	49.4 ± 19.9^	13.1 ± 5.2*	27.9 ± 9.1*	21.4 ± 9.5^	17.8 ± 8.4*
t180 ($n = 32$)	19.0 ± 12.4*,°,†	21.2 ± 12.7*,°,†	32.7 ± 13.0*,°,ç	19.7 ± 7.4*,†	41.7 ± 16.0$	12.2 ± 4.9*,†	28.9 ± 7.9*,$	20.2 ± 5.1*	20.7 ± 6.9*,°,†
Cohen's *d* (95% confidence interval [CI]) t7 versus t0	1.8 (1.3–2.4)	2.3 (1.6–2.9)	2.4 (1.7–3.1)	1.6 (0.7–2.4)	1.5 (0.6–2.3)	1.4 (0.5–2.1)	0.3 (−0.2 to 0.9)	−0.4 (−0.7 to −0.02)	−0.6 (−0.9 to −0.2)
Cohen's *d* (95% CI) t28 versus t0	2.6 (1.9–3.3)	1.9 (1.4–2.6)	2.3 (1.7–2.9)	1.7 (0.9–2.4)	1.0 (0.3–1.7)	1.7 (1.1–2.4)	1.7 (0.9–2.4)	−0.5 (−0.9 to −0.2)	−0.7 (−1.1 to −0.3)
Cohen's *d* (95% CI) t180 versus t0	2.8 (2.1–3.6)	3.7 (2.7–4.7)	3.7 (2.7–4.6)	1.1 (0.5–1.8)	0.5 (−0.07 to 0.9)	1.7 (0.9–2.5)	1.2 (0.5–1.8)	−0.6 (−1.0 to −0.3)	−1.6 (−2.1 to −1.1)
Cohen's *d* (95% CI) t28 versus t7	0.5 (0.2–0.9)	0.2 (−0.1 to 0.6)	−0.1 (−0.4 to 0.2)	−0.5 (−1.2 to 0.2)	−0.3 (−1.1 to 2.4)	−0.6 (−1.3 to 0.1)	−0.6 (−1.3 to 0.1)	−0.2 (−0.5 to 0.2)	−0.2 (−0.5 to 0.2)
Cohen's *d* (95% CI) t180 versus t7	0.8 (0.4–1.2)	1.1 (0.7–1.6)	1.0 (0.6–1.4)	−0.5 (−1.1 to 0.1)	−0.8 (−1.5 to −0.2)	−0.1 (−0.7 to 0.5)	0.8 (0.2–1.5)	−0.1 (−0.4 to 0.3)	−0.7 (−1.1 to −0.3)
Cohen's *d* (95% CI) t180 versus t28	0.4 (0.04–0.7)	0.6 (0.2–0.9)	1.1 (0.6–1.5)	0.9 (0.1–1.6)	0.7 (−0.01 to 1.4)	0.8 (0.1 to 1.5)	0.6 (−0.05 to 1.3)	0.1 (−0.3 to 0.4)	−0.5 (−0.9 to −0.1)

Bottom lines: Cohen's *d* (95% CI) for the effect size between t7 and t0, t28 and t0, t180 and t0, t28 and t7, t28 and t7, t180 and t7, t180 and t28.

*$p < 0.001$ versus t0.

^$p < 0.05$ versus t0.

°$p < 0.001$ versus t7.

$$p < 0.05$ versus t7.

ç$p < 0.001$ versus t28.

†$p < 0.05$ versus t28.

TABLE 7 Mean ± SD of percentages of sperm DNA fragmentation (sDF) at t0 and after 7 (t7) and 28 (t28) days from cryopreservation in 10 normozoospermic subjects.

	PI brighter sDF (TUNEL/PI, %)	Total sDF (TUNEL/PI, %)
t0 ($n = 10$)	26.2 ± 5.9	40.6 ± 9.0
t7 ($n = 10$)	47.1 ± 6.9*	57.1 ± 10.0*
t28 ($n = 10$)	45.9 ± 7.5*	57.9 ± 10.6*
Cohen's *d* (95% confidence interval [CI]) t7 versus t0	−2.9 (−4.4 to −1.5)	−2.2 (−3.3 to −0.9)
Cohen's *d* (95% CI) t28 versus t0	−2.6 (−3.9 to −1.2)	−2.3 (−3.5 to −1.1)
Cohen's *d* (95% CI) t28 versus t7	0.2 (−0.4 to 0.8)	−0.1 (−0.7 to 0.5)

Bottom lines: Cohen's *d* (95% CI) for the effect size between t7 and t0, t28 and t0, t180 and t0, t28 and t7, t28 and t7, t180 and t7, t180 and t28.
*$p < 0.001$ versus t0.

As already observed in our previous study including data from 1973 cryopreserved semen samples,[6] we show here that the damage on sperm motility appears to be greater in the cases of oligozoospermic patients in whom the recovery rate after thawing is dramatically worse than that of normozoospermic subjects. Osmotic changes occurring during the process, which lead to morphological alterations of the tail and several changes in mitochondrial structure and function, are most likely responsible for the decrease in sperm motility.[35,36]

In addition to the structural damage that compromises sperm motility and viability, DNA integrity can also be damaged after cryopreservation. In particular, several studies demonstrated an increase of sDF after freezing/thawing,[9,12–15,28] including the present one. Less investigated is the impact of this procedure on sperm chromatin compaction, which is important for protecting the paternal genetic heritage during the journey to the oocyte. Our data show a significant decrease in sperm chromatin compaction evaluated by two different techniques, AB and CMA3 staining, indicating that the freezing/thawing procedure can alter the chromatin structure endangering sperm DNA integrity. Osmotic stress resulting from cryopreservation may lead to protein degradation and membrane damage, favoring the loss of intracellular constituents of spermatozoa, possibly explaining the reduction of protamine content. It can be hypothesized that some subjects are more prone to osmotic stress than others resulting in a greater protein loss. The mechanism(s) underlying the increase of AB positivity, that is, a greater AB binding to histones after freezing/thawing, is(are) more difficult to understand in a transcriptionally and translationally silent cell such as the spermatozoon. Mass spectrometry-based studies evidenced numerous changes in sperm proteomic profile after cryopreservation[37,38] and, in particular, Bogle et al.[39] found an increase of histone H4 expression levels. Furthermore, cryopreservation also leads to increased post-translational protein modifications.[40,41] Among these, acetylation (a modification of the lysine residues that are abundant in histones) weakens electrostatic DNA–histone interactions, modifying chromatin assembly and, possibly, leading to an increase of AB binding. It is also possible that a decrease in protamine content after cryopreservation renders chromatin more accessible to AB.

Our results, obtained by evaluating chromatin compaction with two different methods, confirm and extend data of previous studies[42–44] despite having been performed with different cryopreservation protocols. It has been demonstrated that AB and CMA3 are not strictly related[22] and are differently associated with ART outcomes,[22,45,46] likely revealing different aspects of chromatin structure; therefore, it is important to use both probes to study the chromatin compaction. Of note, whereas increased CMA3 and AB positivity was observed in 68.0% and 79.2% of samples, respectively (Figure 2 C,D), motility and viability decreased in almost all subjects (99.2%, Figure 2A,B), indicating that chromatin damage depends more on individual characteristics of the samples. At present, which semen characteristic(s) make(s) chromatin more vulnerable to cryopreservation damage is unclear, as none of the basal semen parameters is predictive of the damage. Spermatozoa with poor DNA chromatin compaction are more susceptible to nuclease cleavage and consequently more prone to damage[47] with consequences on their fertilizing capacity. In agreement, we show here that cryopreservation also induces an increase in sDF, confirming previous studies.[12,14] No changes in average levels of sDF were detected with increased storage time in liquid nitrogen, however, further studies are needed to clarify this point. An altered composition of sperm nuclear proteins was observed in infertile men, underling the importance of histone to protamine ratio in natural fertility.[48] Also in ART cycles, a negative correlation was observed between chromatin immaturity and fertilization rate, embryo quality, and live birth rate.[22,24,49]

Although the assessment of sperm chromatin has been included in the latest edition of WHO laboratory manual for the examination and processing of human semen[1] among advanced examinations and it is currently performed only for research purpose, our findings highlight the importance of considering this parameter especially when cryopreserved spermatozoa are used in ART cycles. The few studies comparing the use of fresh or frozen spermatozoa in ART cycles are reassuring, reporting no statistically significant differences in reproductive outcomes.[50–53] Furthermore, to date, no increase in genetic or phenotypic anomalies has been reported in offspring obtained with ART cycles using frozen spermatozoa versus fresh.[54] However, it cannot be excluded that the observed reduction in chromatin compaction (present study,[55,56]) and the epigenetic modifications resulting from sperm cryopreservation[37,40,57] may have potential consequences on the health of offspring.

Our results, in addition to confirming that cryopreservation procedure causes damage to spermatozoa, evidenced a progressive worsening of sperm motility and viability parameters after 7, 28, and 180 days of storage. In a large study on cryopreserved semen samples for donation,[58] no adverse effects on sperm motility due to long-term cryostorage were observed. It should be noted that, although the data by Yogev et al. are robust, they compared short and long storage in the same samples only in 19 donors. Our work, besides demonstrating an effect of storage time on standard parameters of motility and viability in a larger caseload, also indicates that chromatin compaction is less affected by time spent in liquid nitrogen. In contrast, a previous

study by Fortunato et al.,[55] reported a progressive detrimental effect of chromatin with storage time in liquid nitrogen at 10 and 90 days.

Neither good practice recommendations in reproductive donation by ESHRE[59] nor the guidance regarding gamete and embryo donation by Practice Committee of the American Society for Reproductive Medicine[60] include indications concerning the duration of donor sperm storage. If our data will be confirmed in larger cohort of subjects, the guidelines and the management of donor semen samples should consider also this important aspect.

In conclusion, our data show that cryopreservation and the length of storage in liquid nitrogen may alter several sperm parameters including chromatin compaction suggesting the necessity to improve current protocols with new strategies and personalized procedure aimed to minimize the damage.

AUTHOR CONTRIBUTIONS

Conceptualization: Lara Tamburrino and Sara Marchiani. *Data curation*: Lara Tamburrino and Giulia Traini. *Formal analysis*: Giulia Traini and Maria Emanuela Ragosta. *Investigation*: Giulia Traini, Maria Emanuela Ragosta, Sara Vezzani, and Flavia Scarpa. *Validation*: Lara Tamburrino, Sara Dabizzi, and Sara Marchiani. *Writing—original draft*: Lara Tamburrino. *Writing—review & editing*: Sara Dabizzi, Elisabetta Baldi, and Sara Marchiani. *Supervision*: Linda Vignozzi and Elisabetta Baldi. *Funding acquisition*: Linda Vignozzi. All the authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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APPENDIX: BASIC HUMAN SEMEN EXAMINATION—CHECKLIST FOR AUTHORS

When publishing results of basic human semen examination in Andrology, it is requested that it is transparent which laboratory methods were used and that these methods are adequate for the purpose. Thus, the journal requests that methods are clearly described in the manuscript. Additionally, the authors must also fill in the semen examination methodology checklist. The checklist is for ejaculate examination (modified from [1]) and it is based on the ISO Standard on basic semen examination,[2] the current WHO recommendations ("WHO6"),[3] and on general scientific standards. Essential training for laboratory personnel is described in ref. [4] For data obtained by computer-aided sperm analysis (CASA) requirements for validation, verification, and personnel training are described in ref. [5]

A deviation from this checklist does not necessarily mean that the study cannot be accepted for publication. Still, deviations must be transparently described in the manuscript, including their impact on the accuracy and measurement uncertainty of the data. This is essential to allow the reader to evaluate the quality of the analyses performed. For studies not reporting all characteristics of a basic semen examination, the checklist includes the option "Not applicable to the study."

All manuscripts that report results from basic human semen examination should be accompanied by a signed copy of the checklist at the initial submission. That includes studies that report clinical, experimental, and epidemiological results, as well as manuscripts potentially describing other types of studies.

Any scientific rationale for not complying with the guidelines, which is not included in the Materials and Methods section of the manuscript, must be substantiated to the Editor and Reviewers.

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2. International Organization for Standardization. *ISO 23162:2021 Basic Semen Examination — Specification and Test Methods*. ISO; 2021:32.
3. World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*. 6th ed. World Health Organization; 2021.
4. Mortimer D. *Practical Laboratory Andrology*. Oxford University Press; 1994:393.
5. ESHRE Special Interest Group in Andrology. Guidelines on the application of CASA technology in the analysis of spermatozoa. In: Mortimer S, Mortimer D, Fraser L, eds. *Hum Reprod*. 1998:142–145.

1. PATIENTS

Explanation for non-Compliance

☐ Not applicable to the study

X 1.1 The studied population (e.g., patients or volunteers) has been declared in the manuscript, together with the recruitment method and inclusion and exclusion criteria.

☐ 1.2 In a study concerning couples being investigated for infertility, the following is specified in the manuscript: fertility status of the female partner; and all investigations carried out in the male partner.

The study does not investigate parameters related to couples' infertility.

☐ 1.3 If used in the manuscript, the term "male factor" is completely defined.

The term is not used.

X 1.4 Reference limits provided in WHO5 or fifth percentile of the distribution of semen examination results in WHO6 have not been used to define a man as fertile or infertile.

2. GENERAL ASPECTS

Explanation for non-compliance

☐ NOT APPLICABLE TO THE STUDY

X 2.1 Patients were instructed to maintain 2–7 days of sexual abstinence before collecting an ejaculate for investigation.

X 2.2 Patients were informed about the importance of reporting any missed ejaculate fractions, and their responses were noted on the laboratory record.

X 2.3 Ejaculates were collected at the laboratories.

The samples were collected

☐ At the home of the subject.

☐ At another location:

_____

For specimens not collected at the laboratory:

1. Were patients instructed to avoid cooling (under 20°C) or heating (above 37°C) the semen specimen during transport to the laboratory?

☐ Yes; ☐ No

2. Were patients instructed to deliver the semen specimen to the laboratory within 60 min?

☐ Yes; ☐ No

X 2.4 In the laboratory, specimens were kept at 37°C before initiation of and during the analysis in case of sperm motility assessment.

X 2.5 For specimens collected adjacent to the laboratory, analysis was initiated after completion of liquefaction and within 30 min after ejaculation. If some of the specimens were collected at the laboratory and others collected at home, the influence on the data is declared and discussed in the manuscript.

Samples included in the study were all collected to the laboratory.

X 2.6 Liquefaction was first checked within 30 min after ejaculation.

X 2.7 Volume was assessed by weighing.

X 2.8 Viscosity was measured using a wide-bore pipette or a glass rod.

X 2.9 pH was assessed by spreading a drop of well-mixed semen on a pH test strip (with the range of 6.0–10.0)

X 2.10 All staff members who performed the analyses have been trained in basic semen analysis (ESHRE Basic Semen Examination Course—or equivalent—with further in-house training to establish competency) and regularly participate in internal quality control.

X 2.11 When more than one method exists for a particular assessment, only one was used in the study.

☐ 2.12 For a multicenter study, all laboratories used the same method or variable methods are declared in the manuscript.

This is not a multicenter study.

3. SPERM CONCENTRATION ASSESSMENT

Explanation for non-compliance

☐ Not applicable to the study

X 3.1 Semen aliquot to be diluted for sperm concentration assessment was taken with a positive displacement pipette (i.e., a "PCR pipette") using a recommended diluent (state which diluent:

_____

_____

_____).

X 3.2 Only standard dilutions were used (1:100, 1:50, 1:20, or 1:10, i.e., 1 + 99, 1 + 49, 1 + 19, or 1 + 9).

X 3.3 Sperm concentration was assessed using counting chamber of type hemocytometer:

X 3.3.1 Counting chamber with Improved Neubauer ruling.

☐ 3.3.2.a Other hemocytometer:

_____

☐ 3.3.2.b If other hemocytometers were used, adjusted calculation factors were employed

X 3.4 Hemocytometers were allowed to rest for 10–15 min in a humid chamber to enable sedimentation of the suspended spermatozoa onto the counting grid before counting.

X 3.5 Sperm counting was done using phase contrast microscope optics (200–400×).

X 3.6 Comparisons were made between replicate counts (two dilutions, one count of each), and the two counts were within the acceptable variations

X 3.7 At least 200 spermatozoa were counted in each replicate assessment.

4. SPERM MOTILITY ASSESSMENT

Explanation for non-compliance

☐ Not applicable to the study

X 4.1 Motility assessments were performed by laboratory personnel

In case computer-aided sperm analysis (CASA) was used:

- Product name and version of the equipment:

_____

- validation and verification procedures

_____

- training of personnel:

_____

X 4.2 Motility assessments were performed at 37 °C ± 0.5 °C.

X 4.3 Motility assessments were initiated within 30–60 min after sample collection.

X 4.4 Motility assessments were performed using phase contrast microscope optics (200–400×).

☐ 4.5 Sperm motility was classified using a four-category scheme: rapid progressive, slow progressive, non-progressive, and immotile.

For this study, sperm motility was classified using a three-category scheme according to WHO 5th edition.

X 4.6 Motility assessments were done in replicate (two aliquots), and the two were within the acceptable variations.

☐ 4.7 The wet preparation was made using
a drop of __10____ µL
and a coverslip of ___21__ × __26___ mm
to obtain a preparation depth of _18.3____ µm
(must be at least 10 µm depth, but not too deep to allow spermatozoa to move freely in and out of focus; typically ca. 20 µm).

X 4.7 At least 200 spermatozoa were assessed in each replicate motility count.

X 4.9 At least five microscope fields of view were examined in each replicate count.

5. SPERM VITALITY ASSESSMENT

Explanation for non-Compliance

☐ Not applicable to the study

X 5.1 A validated supravital stain was used to assess sperm vitality, specify: Klicka eller tryck här för att ange text.

X 5.2 At least 200 spermatozoa were evaluated.

X 5.3 Assessments were done under high magnification (1000–1250×) using a 100× high-resolution oil immersion objective and bright field microscope optics.

6. SPERM MORPHOLOGY ASSESSMENT

Explanation for non-compliance

☐ Not applicable to the study

X 6.1 Tygerberg Strict Criteria were used to evaluate human sperm morphology.

Another classification could be used for scientific studies with specific aims if the classification is described or referenced. Depending on the objective of the study, the evaluation of particular abnormal forms might be useful:

☐ 6.2 Abnormalities are recorded for the four defined regions of the spermatozoon (head, neck/midpiece, tail, and cytoplasmic residue).

Sperm morphology was classified as typical and abnormal forms.

X 6.3 The Papanicolaou staining method adapted for assessing human sperm morphology was used. Other staining methods could be used for specific aims but must then be declared and explained.

We used Diff-Quik staining.

X 6.4 At least 200 spermatozoa were assessed in each ejaculate.

X 6.5 Assessments were done under high magnification (1000–1250×) using a 100× high-resolution oil immersion objective and bright field microscope optics.

7. EXTERNAL QUALITY ASSESSMENT (EQA)

Explanation for non-compliance

☐ Not applicable to the study

X 7.1 The laboratory participated in EQA for the semen examination methods used to obtain data for the study. Our laboratory/ies participated in EQA regarding:

☐ Sperm concentration/sperm number
☐ Sperm motility.
☐ Sperm vitality
☐ Sperm morphology

Our laboratory participates in EQA regarding: sperm concentration/number, sperm motility, and sperm morphology.

X 7.2 Name of the EQA scheme:

UK-NEQAS (United Kingdom National External Quality Assessment Service)

8. OTHER FINDINGS

Explanation for non-Compliance

☐ Not applicable to the study

X 8.1 The presence of abnormal clumping (aggregates and agglutinates) was recorded.

X 8.2 Abnormal viscosity was recorded.

9. ANALYZING DATA

Explanation for non-compliance

☐ Not applicable to the study

X 9.1 The actual duration of sexual abstinence (in "hours" or "days") was recorded for each specimen and included in the data reported in the manuscript.

X 9.2 As a minimum in clinical studies, semen volume, sperm concentration, total number of spermatozoa per ejaculate, and abstinence time are given to reflect sperm production and output; only samples identified as having been collected completely were included in the study.

☐ 9.3 Confounding factors have been considered for statistical analysis: for example, abstinence time and age, to consider secular or geographical variations in sperm concentration or sperm count.

In our study, we evaluated semen samples pre- and post-cryopreservation, thus no confounding factors have been considered in statistical analysis.

☐ 9.4 If appropriate, optional biochemical markers for prostatic, seminal vesicular, and epididymal secretions were analyzed and reported, both as concentration and total amount.

For our study these markers were not relevant.

X 9.5 Signs of active infection/inflammation were noted and considered in the analysis of data in the study (e.g., presence of non-germ line round cells, inflammatory cells, impaired sperm motility, possibly also anti-sperm antibodies, or reduction of secretory contributions).

X 9.6 If the manuscript also includes results of other types than those originating from basic semen examination (e.g., sperm DNA damage, acrosome reaction, etc.), the methodology used to obtain the results is clearly reported.

DECLARATION BY THE CORRESPONDING AUTHOR

The information provided in this checklist is solemnly declared to be true.

Date: 23.10.2024

Signature: 

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