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# MORPHOLOGICAL AND FUNCTIONAL ANALYSIS OF CRYOPRESERVED HUMAN SPERM: COMPARISON OF DIFFERENT FREEZING PROTOCOLS

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**Abstract**

**BACKGROUND:** Human semen and epididymal spermatozoa cryopreservation are crucial for men's fertility preservation, particularly for those patients facing neoplastic, autoimmune, urological, and neurological conditions where medical or surgical treatments may pose a risk to fertility or where obstructive or secretory azoospermia is documented. However, there are currently no standardized methods to assure optimal cryosurvival rates. **OBJECTIVE:** To determine the best freezing protocol out of five selected methods based on routine sperm analysis and additional assays including cytofluorimetric analysis, comet assay, and transmission electron microscopy. **MATERIALS AND METHODS:** The study is a cross-sectional analysis of 26 fresh semen samples frozen using five different freezing protocols (or methods, M), varying in cooling phase time and temperatures, and utilizing TEST-Yolk Buffer (TYB) as a cryoprotectant. Data on sperm motility, viability, membrane integrity, DNA fragmentation, and ultrastructural shape post-thawing were collected. **RESULTS:** Our findings showed that the method 1 (M1) and method 3 (M3) (involving a three-phase cooling process with a phase at +4°C, followed by 10 min of exposure to the gas phase of liquid nitrogen before immersion in liquid nitrogen) yielded the best protocols, resulting in minimal deterioration of semen quality. **CONCLUSION:** These results highlight the importance of a pre-freezing phase at +4°C when using TYB cryoprotectant on untreated semen, regardless of the duration, despite the less-than-optimal survival rate achieved. It is crucial to use a range of assays to study the effects of cryopreservation procedures, not only assessing sperm motility and viability, but also evaluating membrane integrity, DNA fragmentation, and ultrastructural shape.

**Keywords:** DNA integrity; fertility preservation; freezing protocol; human sperm cryopreservation; methods; semen analysis; sperm motility; sperm viability.

## INTRODUCTION

Gamete or embryo preservation is crucial for ensuring the possibility of future pregnancy in both men and women under certain circumstances. Many techniques can be applied to gametes and embryos during assisted reproductive procedures, including cryopreservation, which is an essential tool for patients undergoing medical treatments that could potentially affect fertility (1). The improvement in assisted reproductive technologies (ART) and the increase in survival rates of cancer patients have led to significant progress in the field of oncofertility, which brings together oncology and reproductive endocrinology to maximize reproductive potential (1, 2) in affected patients. Furthermore, there is an increasing need to protect fertility over time because of the trend of postponing parenthood for non-medical and sociocultural reasons. Indeed, reproductive success is related mainly to an age-dependent decrease in fertility. That being so, requests for social freezing are increasing rapidly for women (3) and men (4, 5).

Although semen cryopreservation techniques have been investigated for decades, the clinical outcomes of assisted reproduction with frozen semen are lower compared to the fresh samples (6). Since 1997, different protocols have been developed to enable the cryopreservation of small quantities of spermatozoa using the single-sperm cryopreservation technique in clinical practice (7). However, it is important to explore new strategies to potentially improve freezing/thawing protocols, especially when dealing with severely compromised semen.

The yield of the semen cryopreservation procedure is influenced by several factors, including the rate of cooling/thawing of samples, characteristics of cryoprotective agents (8), and the quality of basal semen parameters. While there has been research to improve media for oocytes and embryo culture (9), the composition of routinely used sperm cryoprotective media has remained largely unchanged for many years. Apoptosis of spermatozoa is the prevalent damage induced by cryopreservation (10). Previous studies have shown that sperm DNA fragmentation may occur during cryopreservation due to an increase in oxidative stress rather than caspase activation and apoptosis (11, 12).

Further cryo-damage including the swelling of plasma membranes, acrosomal leakage, and breakdown or ultrastructural injuries to spermatozoa tails, may reduce semen viability and impair the motility of the surviving spermatozoa (13). Previous studies revealed that markers of apoptosis, for instance caspase activation, externalization of phosphatidylserine, alteration of mitochondrial membrane potential, and DNA fragmentation tend to increase in human spermatozoa following freezing/thawing protocols (14).

In a previous study, we compared three different rapid methods of human sperm cryopreservation, showing the impact of the cooling phase at +4°C followed by exposure to liquid nitrogen vapors on sperm motility and viability (15). In the work reported here, we investigated the effect of freezing protocols by changing the cooling phase and analyzing not only basal semen parameters but also DNA and morphology integrity using cytofluorimetric analysis, comet assay, and transmission electron microscopy.

## MATERIALS AND METHODS

### *Study design and samples*

Cross-sectional laboratory studies were perfomed on portions of fresh semen samples not required after routine clinical use, obtained from 26 men (age: 37.7±4.92 years) undergoing first basal semen screening for couple's infertility management or men collected semen samples for ART procedures at the Center for Reproductive Medicine, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. Patients were recruited according to the guidelines of our Ethical Committee and signed informed consent for the use of personal data and for the processing of residual semen for research purpose (Prot. 0020247/22). Semen samples were obtained by masturbation after 2-7 days of sexual abstinence and collected in a sterile container. Routine semen analysis was performed within 1 h of collection, according to the methods described by the 6$^{th}$ edition of the World Health Organization (WHO) manual for semen analysis (16). The system for grading motility was based on the distinction of spermatozoa with progressive (PR) or non-progressive (NP) motility and those that are immotile (IM), as reported by the WHO manual (16). In the present study, we selected only

semen samples from normozoospermic subjects based on parameters detailed in (16): PR + NP ≥ 42%; PR ≥ 30%; the number of spermatozoa ≥ $16\times10^6$/mL; physiological morphology (PHM) ≥ 4%. Then we performed cytofluorimetric analysis, comet assay, and transmission electron microscopy on five of the collected samples, selecting them randomly.

***Sperm cryopreservation***

We compared five different methods of rapid freezing (Fig. 1). The procedure for storing semen in paillettes for cryopreservation was similar but we changed the time course of sperm cooling. An equal volume of sperm cryopreservation TYB medium (Freezing Medium, Irvine Scientific, USA) was added dropwise to an aliquot of the semen sample. The mixture was drawn into 0.3-mL paillettes (CBS, IMV Technologies, France).

We compared five different freezing methods (M) differing for the phase of cooling as described below and incubated at least three paillettes for each one. In the M1, M2, M3, and M4, samples were previously incubated at +4°C for 30 min (M1, M2 or M4) or 2 h (M3). M2 differs because cryoprotectant and semen were pre-cooled before being mixed. M1, M2, and M3 followed the phase at +4°C for 10 min on vapours of liquid nitrogen and finally cooled by plunging them into liquid nitrogen for storage. The exposure to nitrogen vapours was obtained by suspending the samples approximately 10 cm above liquid nitrogen at a controlled temperature of -180°C in a cryogenic dewar (HC-35, Taylor Wharton, USA). M4 introduces 30 min at -20°C and 30 min at -80°C after 30 min at +4°C, before plunging into liquid nitrogen. M5 has the same phases as M4 without pre-cooling at +4°C. M1 is the method used routinely in our lab and is used as a reference method in this work (15).

The samples were thawed at room temperature for 5 min, after at least 1 month of storage in liquid nitrogen. Then, they were washed with 2 mL of Sperm Washing Medium (Modified HTF Medium with Human Serum Albumin, 5.0 mg/mL, Irvine Scientific, USA) and centrifuged at 1800 rpm for 10 min to remove the cryoprotectant.

***Semen analysis***

Semen samples were incubated at +37°C until the analysis was performed. The analysis to assess volume, pH, fluidification, and viscosity

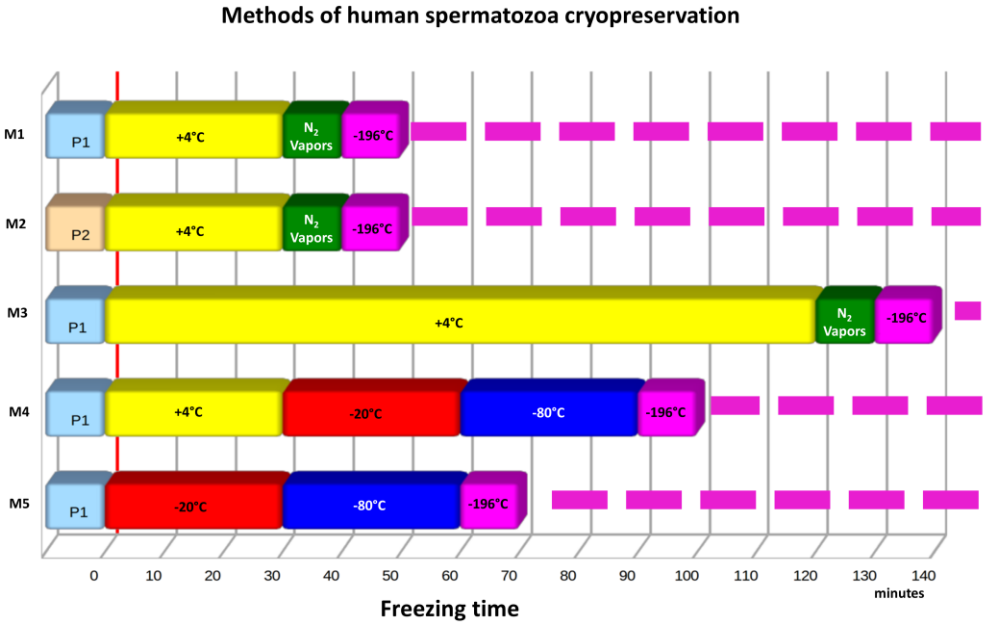


**Figure 1**. Graphic scheme of freezing sperm protocols tested. P1 indicates the traditional method of processing the sample at room temperature by adding equal semen volume to the cryoprotectant; P2 indicates pre-cooling of the cryoprotectant and semen at +4°C separately for 30 min and producing a mixture of them when both sample and cryoprotectant reached +4°C. The different times of cooling are shown by the X-axis. The different temperature of cooling is represented with colors: +4°C (yellow), -20°C (red), -80°C (blue), nitrogen vapors (green), and plunging in liquid nitrogen (pink).

was started within 1 h of the collection to prevent decrease in motility and viability (16).

For sperm morphology, only fresh samples were assessed. We used Diff-Quik-stained slides (Test Simplets, Origio, Denmark). As indicated by the WHO manual (16), restricted criteria by Kruger to analyze at least 200 spermatozoa per sample were used.

Pre-freeze and post-thaw samples were assessed for sperm viability and membrane function by using the Eosin test (Sigma-Aldrich, USA). Briefly, sperm viability was assessed by staining with two drops of 0.5% eosin and 10 µL of the sample. After 1–2 min, at least 200 spermatozoa were counted as stained (dead) or unstained (viable).

Each sample was analyzed to determine sperm count, sperm motility, and kinematics of movement by a trained operator, using a disposable counting chamber (Counting Chamber Makler, Sefi Medical Instruments, Israel) in pre-freeze and post-thaw specimens. We determined the spermatozoa concentration by using the chamber's grid. The number of spermatozoa counted in any strip of 10 squares of the grid indicated their concentration in millions/mL. We counted at least three strips, and the mean value was considered. The chamber has a depth of 10 µm that eliminates blurring and allows sperm to move freely. The motility of each spermatozoon is graded as follows: progressive motility (PR) — spermatozoa moving actively; non-progressive motility (NP) — all other patterns of motility with an absence of progression; immobility (IM) — no movement (16).

***Analysis by flow cytometry***

To establish the best method to maintain nuclear integrity and cell membrane integrity, we studied light scatter patterns by flow cytometry analysis. We applied an unconventional protocol to analyze these parameters using fluorescent dyes for living cells. For these experiments, we have considered only the cells obtained with M1 and M3 because the other tested protocols were discarded due to the poor yield, and all five samples were evaluated in two different aliquots each of which was frozen with M1 or M3.

Following at least 1 month of storage in liquid nitrogen, samples were thawed at room temperature for 5 min and prepared to be analyzed as previously reported. Then, samples were suspended in propidium iodide (PI)/Hoechst 33342 solution (Sigma-Aldrich, USA) in the ratio 1:5 (to identify spermatozoa integrity or alterations, following the kinetic uptake of Hoechst 33342 living stain) and analyzed at different times after staining (1, 10, 30 min).

***Transmission electron microscopy (TEM)***

We applied electron microscopy analysis to investigate sperm ultrastructural details. Only M1 and M3 were considered. Briefly, the pellet of every sample was fixed with a Karnowsky' Diluted solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h and then immersed in cacodylate buffer for 6 h. Spermatozoa pellets were postfixed for 90 min in 1.33% osmium tetroxide in 0.1 M collidine buffer, dehydrated through graded alcohols, and embedded in Epon 812. Semithin sections were prepared and stained with blue toluidine. Thin sections were stained with uranyl acetate and lead citrate and observed by using a Zeiss EM10 electron microscope (10000×).

***Comet assay to measure the sperm DNA fragmentation (SDF)***

To assess the degree of sperm DNA fragmentation (SDF) post-thawing M1 and M3 samples were analyzed by the alkaline comet assay. Spermatozoa, without washing to reduce the DNA damage, were embedded in 1% (w/v) Low Melting Point agarose (Gibco, Sigma-Aldrich, USA) and immediately transferred into the glass microscope slides precoated with 1% (w/v) standard agarose (Gibco, Sigma-Aldrich, USA). The cells were then lysed in a solution containing 2.5 mM NaCl, 0.1 M EDTA, 10 mM Tris-base, and 1% Triton X-100 (pH10), for 1 h at +4˚C. To promote the chromatin decondensation, the lysis buffer was added with 10 mM DTT for 30 min at +4°C and then with 4 mM lithium diiodosalicylate (LIS) for another 90 min at room temperature. Thereafter, slides were equilibrated with electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) for 20 min to unwind the DNA and then electrophoresed for 10 min at 25V. The sperm nucleoids were subsequently washed in neutralizing buffer (0.4 M Tris-HCl, pH 7.5), and stained with Hoechst 33258 (5µg/mL) (17, 18). Hoechst-stained sperm nucleoids were visualized using a fluorescence microscope (Nikon Eclipse E400) with a 40× magnification. Samples were analyzed blindly. For each slide, 100 nucleoids

were scored, classifying them into arbitrary units based on the grade of the damage according to Collins (19).

***Statistical analysis***

Student t-tests were used to analyze differences among protocols. A p-value <0.05 was considered statistically significant. All tests were two-sided. Data analysis was performed with GraphPad Prism 9.0 (San Diego, CA, USA). The percentages of sperm motility and sperm viability are presented as mean and standard deviation (SD) as they were all normally distributed (Shapiro-Wilk test).

## RESULTS

***Effect of cryopreservation on sperm viability***

Reduction of sperm viability, assessed by the eosin test, is evident with all protocols performed after the freezing/thawing phases. Data are reported in detail (Table 1 and Table 2).

Samples thawed with the M3 freezing method showed a similar reduction of sperm viability as M1 without significant differences among the protocols and also between pre-freeze and post-thawed values (Table 1, 2 and Fig. 2C). However, when protocol M2 and M5 were applied, we observed a significant statistical difference ($p<0.05$) between pre-freeze (83.00±6.97%) and post-thaw (75.55±5.41% and 76.10±8.62% respectively) values (Fig. 2C) that suggested these two protocols could be discarded. We observed also a statistically significant reduction of sperm viability, after freezing/thawing procedures, when we used the M4 protocol instead of M1 ($p<0.001$). For these reasons, it is possible to discard M2, M5, and M4 protocols for their low return to viability after viability evaluation.

***Effect of cryopreservation on sperm motility***

A strong significant reduction of sperm motility was observed after freezing/thawing phases, regardless of the method applied.

Comparing the performance of methods tested with each other, a similar reduction of sperm parameters was observed: sperm progress and total motility were significantly different from pre-freeze values ($p<0.001$) but no significantly statistical differences ($p>0.05$) among M1, M2, and M3 were observed (Fig. 2A and 2B). Analysis of data obtained from the comparison among M1, M4, and M5 showed higher recovery of total and progressive motility with M1 (PR+NP: 20.86±11.00%; PR: 14.90±7.65%) in comparison to M4 (PR+NP: 15.90±12.70%; PR: 10.30±8.33%) or M5 (PR+NP: 12.50±8.29%; PR: 8.50±6.62%). Statistical analysis showed a significant difference between M1 and both M4 and M5 for total and progressive motility ($p<0.05$) (Fig. 2A and 2B). After motility evaluation, it was possible to confirm that M4 and M5 should be discarded.

The analysis of routine parameters after thawing showed the lower performance of M2, M4, and M5 protocols but did not highlight differences between M1 and M3. For these reasons, we carried out an analysis on M1 and M3, applying assays that investigate membrane integrity, ultrastructural shape, and DNA fragmentation of the spermatozoa.

***Analysis by flow cytometry***

Flow cytometric analysis was preceded by a step to optimize parameters, by performing fluorescence microscopy observations of both fresh and frozen samples, to evaluate the kinetics of uptake of the dye's mixture.

Cytofluorimetric analysis conducted on spermatozoa using a mixture of Hoechst 33342 (HO33342, blue) and propidium iodide (PI, red), allowed the highlighting of macromolecular and cellular damages following the uptake kinetics of the vital dye. The method of the double staining PI/HO is based on different dimensions of these two molecules. The Hoechst 33342 dye is a small molecule (~ 200 kDa) that is able to enter cells with minimal variations of the membrane structure and the efflux pump system, a step having the potential to maintain cell viability. The size of the propidium iodide molecule (~ 668 kDa) is > three-times higher than the Hoechst 33342 and, therefore, it can cross the plasma membrane only if strongly altered, a sign of serious damage leading to cell death.

**Table 1.** Total spermatozoa showing motility (PR+NP) and spermatozoa showing progressive (PR) motility after different freezing/thawing protocols. Data were reported as mean ± standard deviation. Statistical analysis applied to compare motility and vitality from pre-freeze to post-thaw samples was also reported. A p-value <0.05 was considered statistically significant.

| Protocols | Total Motility % (PR+NP) | Progressive motility % (PR) | Vitality % |
|---|---|---|---|
| **Pre-freeze** | 71.05±12.27 | 60.24±13.14 | 83.80±6.97 |
| **M1** | 20.86***±11.00 | 14.90***±7.65 | 80.90±6.05 |
| **M2** | 18.91***±8.54 | 13.09***±6.55 | 75.55**±5.41 |
| **M3** | 18.55***±10.60 | 13.55***±8.07 | 78.91±4.61 |
| **M4** | 15.90***±12.70 | 10.30***±8.33 | 80.10±4.72 |
| **M5** | 12.50***±8.29 | 8.50***±6.62 | 76.10*±8.62 |

Values are mean ± SD

*, P<0.05 vs. Pre-freeze values (Student *t*-test)
**, P<0.01 vs. Pre-freeze values (Student *t*-test)
***, P<0.001 vs. Pre-freeze values (Student *t*-test)

**Table 2.** Statistical analysis was applied to compare motility and viability between different tested protocols. A p-value <0.05 was considered statistically significant.

| Comparison between protocols | Total Motility % (PR+NP) | Progressive motility % (PR) | Viability % |
|---|---|---|---|
| **M1 vs M2** | p=0.3597 | p=0.9609 | p=0.3740 |
| **M1 vs M3** | p=0.4099 | p=0.7200 | p=0.5374 |
| **M1 vs M4** | p=0.0331* | p=0.0129* | p=0.0001*** |
| **M1 vs M5** | p=0.0031** | p=0.0035** | p=0.0133* |

*, P<0.05 vs. M1 (Student *t*-test)
**, P<0.01vs. M1 (Student *t*-test)
***, P<0.001 vs M1 (Student *t*-test)

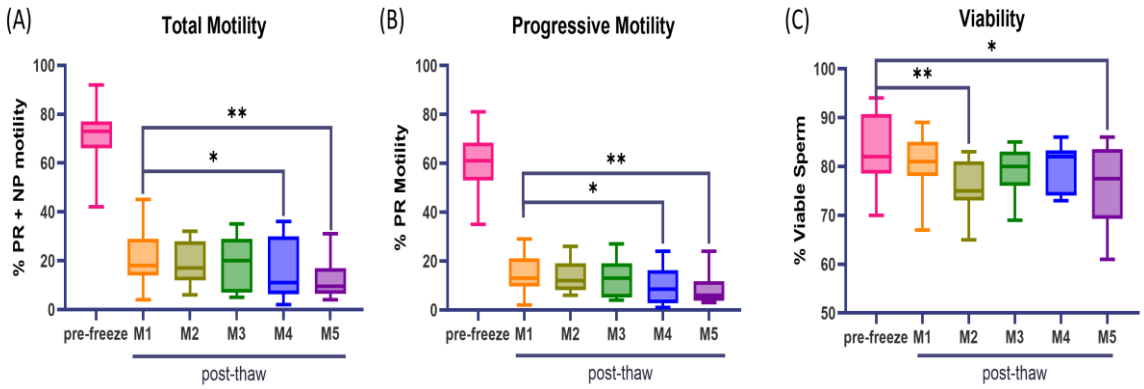


**Figure 2.** Different times of cooling protocols compared. (A) Total motility (PR+NP) of pre-freezing and post-thawing sperm frozen with M1, M2, M3, M4, and M5; and (B) progressive motility (PR) under the same conditions. (C) Sperm viability was observed in pre-freeze and different post-thaw conditions. Results are expressed as a percentage of motility or viability maintained after thawing, compared with the pre-freeze value. Values are shown with Box-and-Whisker plots in which the central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. A line extends from the minimum to the maximum value, excluding outside values which are displayed as separate points. A p-value <0.05 was considered statistically significant (*, p-value < 0.05; **, p-value < 0.01).

Under these conditions, the propidium reaches the nucleus, emitting a red fluorescence, excited by the ray instrument laser. Figure 3 shows an example of cytograms of two aliquots stained with IP/HO33342 solution, reported, and respectively analyzed after 10 min (Fig. 3A C), and 30 min (Fig.3 B, D). At 10 min, the DNA profile of the M1 sample taking PI is like the profile obtained with M3 at the same time. There were also no significant differences between Hoechst 33342 and the cytogram corresponding in both aliquots. Cell membrane alterations were present at all sample aliquots, corresponding to the Gaussian tail of RN2. Similarly, after 30 min of staining with a dye mixture, the percentage of labelled cells was not significantly different in both sample rates (Fig. 3C, D). These data were confirmed for all samples analyzed.

The number of damaged cells for each sample, as shown in the cytograms, was similar using M1 or M3 as the freezing method. The percentage of damaged cells was slightly higher after M1 or M3 as appropriate, with no significant trend in favour of either method.

We also performed some tests to evaluate the uptake kinetics of the Hoechst vital dye 33342 in aliquots of two samples, making the readings during the first 40 s. The recorded

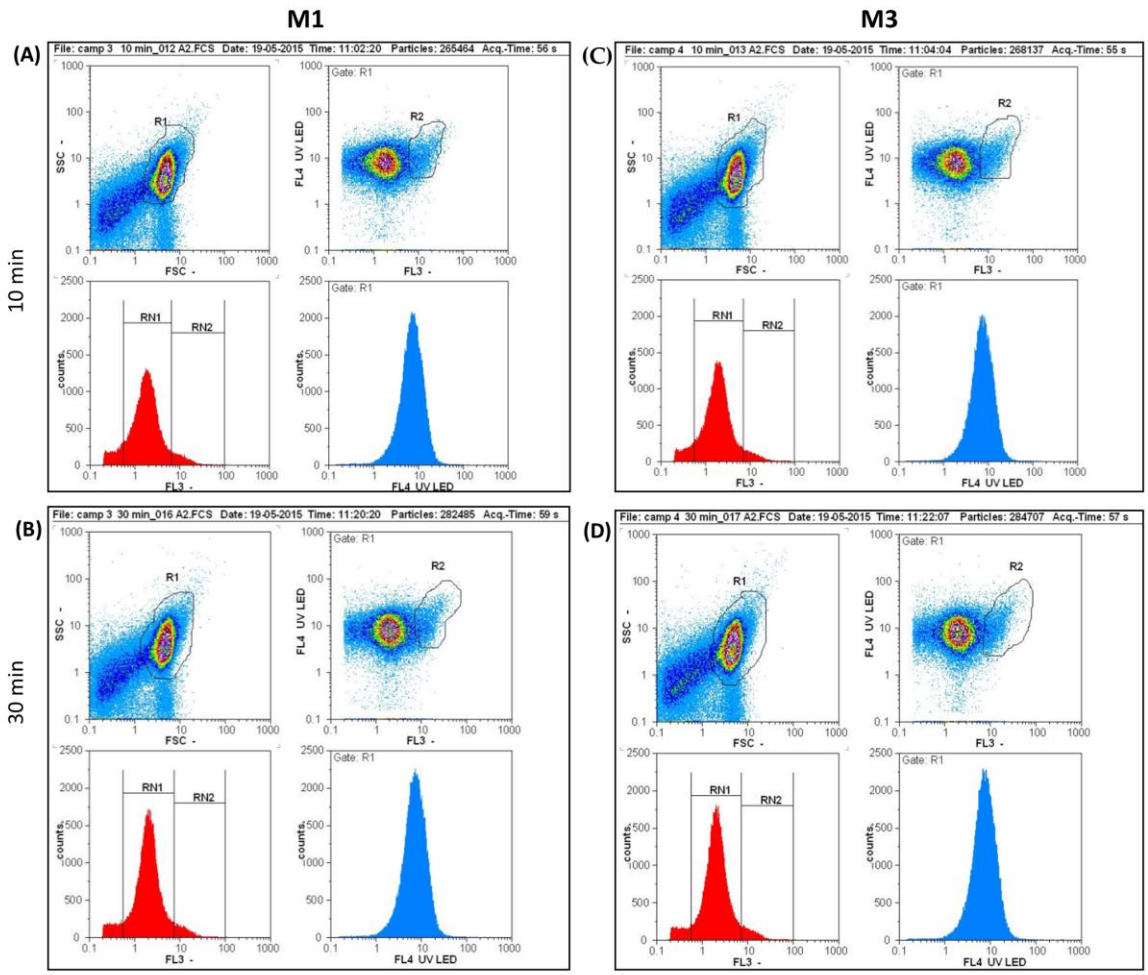


**Figure 3.** Cytograms of two examples of samples (called 3 and 4) stained with PI/HO33342 solution and analyzed after 10 min and 30 min with M1 and M3 (3 and 4 samples, respectively). In a short time (10 min), the DNA profile relating to the uptake of PI of aliquot 3 (A) can be overlaid on the profile of aliquot 4 (B) at the same time. There are also no significant differences in the intake of the vital dye Hoechst since the cytogram is corresponding in both aliquots. It is possible to observe an alteration of the cell membrane in all aliquots corresponding to the tail of the RN2 Gaussian. (C),(D) Similarly, even after 30 min from the intake of the mixture of dyes the percentage of labeled cells is not significantly different in both aliquots of the same sample. These data have also been confirmed for the two aliquots frozen according to the two different freezing methods of the other samples analyzed.

value is an average of a phenomenon dynamically evolving; thus, the data obtained is not at snapshot of the sample situation at a given time, in particular, because they are unfixed. In comparison to the obtained results after 10 and 30 min, the cytogram profiles of the PI uptake were different, which indicated a phase of staining still uncompleted. As far as the profile of Hoechst 33342 was concerned, it was possible to observe a growing curve, not appreciable at 10 and 30 min.

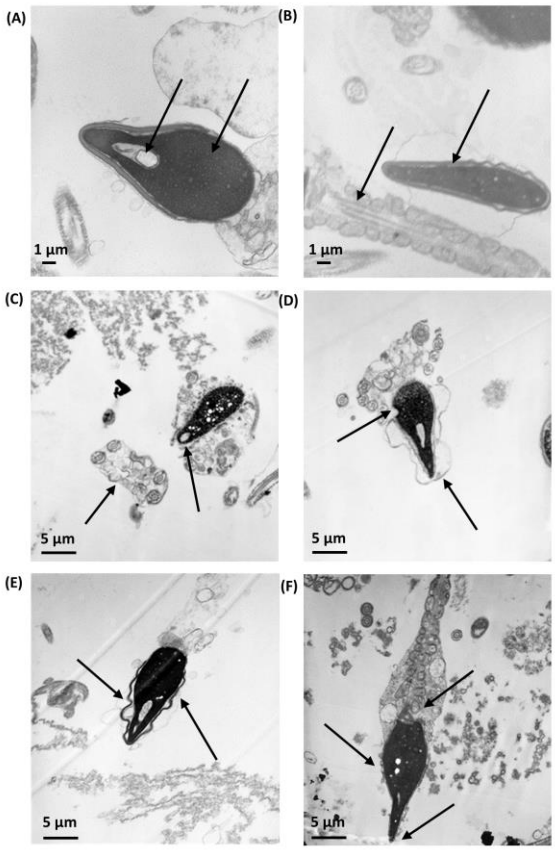


**Figure 4.** TEM micrograph. Ultrastructure of sperm from fresh and frozen seminal fluid (10000×). (A) Head sperm containing the nucleus with condensed chromatin and a vacuole inside and (B) longitudinal section of the sperm head and flagellum, with sections of mitochondria forming the mitochondrial sheath. (C, D) Frozen seminal fluid with M1. (C) A vacuole at the apical end of a nucleus, and a cytoplasmic profile enclosing four axonemes transversally sectioned. (D) One indentation in the nucleus and dilated cell membrane. (E, F) Frozen seminal fluid with M3. (E) The dilated plasma membrane and nuclear envelope and electron-dense nucleus. (F) Some internal nuclear vacuoles are highlighted along with the preserved acrosome and mitochondria at the neck level.

***Ultrastructural morphological analysis by TEM***

The morphological characteristics of the sperm samples were analyzed by two semi-thin sections stained with toluidine blue under light microscopy. These sections were obtained from the sperm pellet after centrifugation of both fresh (controls) and thawed seminal liquid samples.

Preliminary observations revealed differences in sperm morphology, between the fresh samples and the two thawed samples obtained by the M1 and M3 freezing methods.

At the transmission electron microscope, fresh seminal fluid (Fig. 4A, B) contained spermatozoa with well-condensed chromatin, vacuoles scattered in the core level, several limited cellular debris, the acrosome intact as a cap of the head, numerous mitochondria concentrated in the middle part and some transverse sections of the tail that reflect the typical axoneme structure (9 + 2 arrangement of microtubules). The nucleus appeared as homogeneous and compact chromatin condensed in the head.

There was a greater number of tails and cytoplasmic sperm debris detached in frozen samples with the first method (M1) (Fig. 4C, D) compared to those frozen with the other procedure (M3) (Fig. 4E, F). Ultrastructure at the subcellular level of morphology was analyzed using TEM.

A comparison between fresh and frozen samples in all aliquots showed most of the sperm with a "drop" head almost surrounded by acrosome forming a kind of cap. All samples had one large nuclear vacuole, or sometimes very small vacuoles scattered throughout the nucleus.

In some spermatozoa the plasma membrane, not close to the head, seemed broken up, and, in others, it perfectly surrounded the nucleus and the acrosome being in continuity with the plasma membrane surrounding the neck and the tail. In some cells, it was possible to distinguish the plasma membrane from the outer acrosome membrane, as well as the nuclear envelope from the inner acrosome membrane. In the intermediate portion of the sperm it was possible to distinguish the proximal centriole and the spiral of mitochondria (at a higher magnification, the outer and inner sheaths and mitochondrial crests were observed). In all rates, it was also observed the presence of non-germ cells and progenitor cells of the same sperm

(spermatocytes, spermatids) displaying still visible cytoplasmic organelles, such as the Golgi apparatus, the smooth and rough endoplasmic reticulum, vesicles, and granules. Analysing the two frozen rates, some peculiarities were highlighted as compared to fresh semen liquid.

In both frozen samples, some vacuoles appeared as large nuclear recesses with different sizes and in different positions, containing membranes concentrically organized like arranged lamellas as membrane spirals (Fig. 4C, D, E, and F).

Comparing the two rates of frozen samples, it was possible to recognize some of the most seemingly diffuse abnormalities in those that had undergone the M1 treatment; for instance, much-dispersed chromatin, many vacuoles inside the nucleus, dispersions not surrounded by the cytoplasm of the plasma membrane, dispersions of presumed protein clusters arising from the flagella and axonemes with an altered internal structure (Fig. 4C, D).

Otherwise, sperm after the freezing with the second method (M3) had vacuoles smaller (Fig. 4E, F) than those observed in the M1 aliquot and, in many cases, the plasma membrane was raised to the level of the head. More flagella were surrounded by a unique plasma membrane,

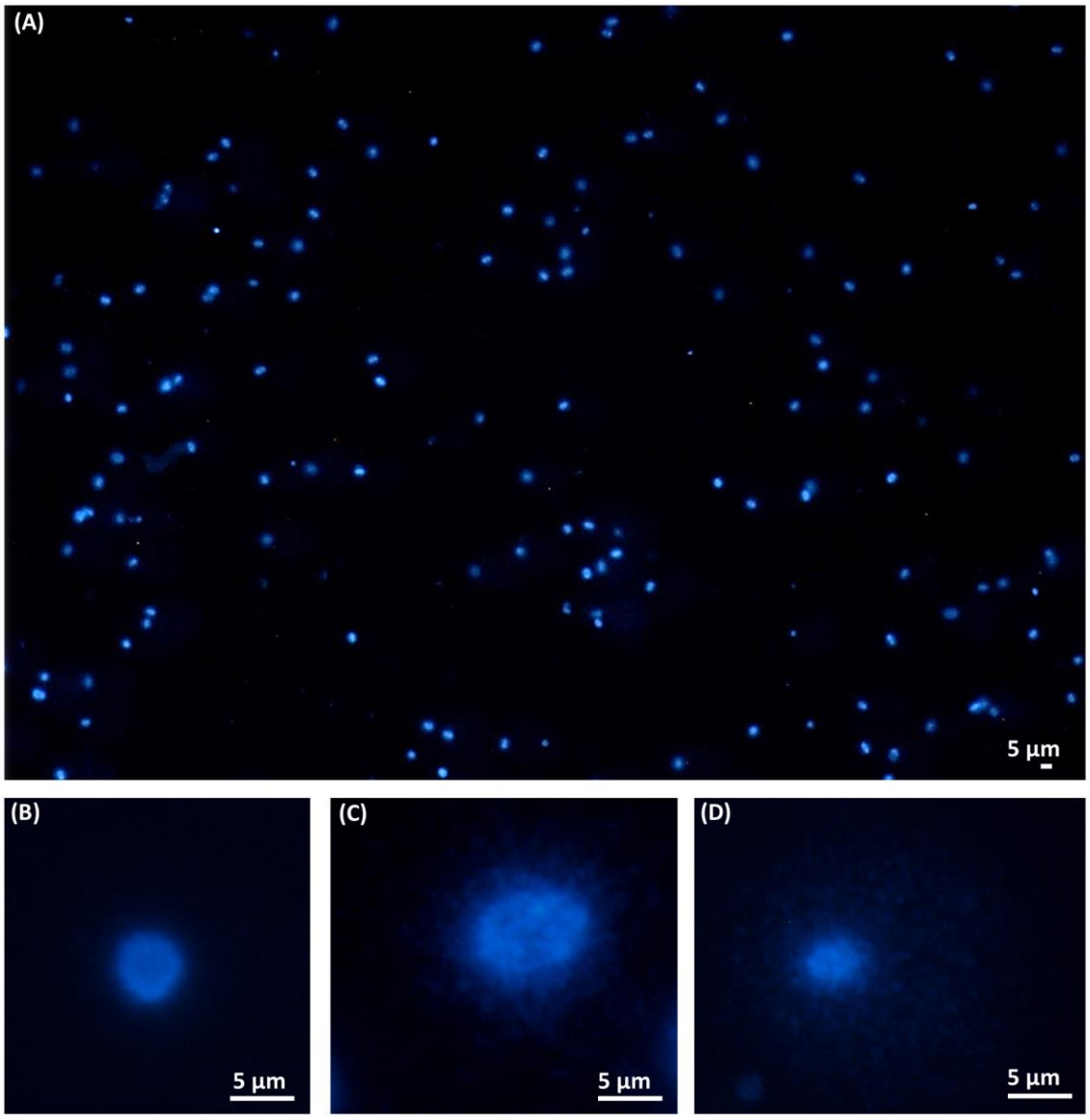


**Figure 5.** Comet assay showed DNA damage by immunofluorescence microscopy after being stained with Hoechst 33258. Example of a sample showing each sperm head stained at 10× of magnification (A). At 100× it is possible to appreciate the comet on sperm with a different number of labile sites: (B) sperm without DNA fragmentation; (C) sperm with DNA fragmentation; (D) sperm with high DNA fragmentation.

as an artefact due to the sample preparation procedure. The sperm head was surrounded by a dilated plasma membrane; also, the nuclear envelope was dilated and in continuity with the high electron-dense acrosome (Fig. 4E, F).

***Comet assay***

Figure 5 shows data about sperm DNA fragmentation (SDF) induction in samples analysed post-thawing after freezing procedures with two methods (M1 and M3). Different percentages of SDF were observed among patients; one case reported low values of SDF (under 100 A.U.), while another showed, higher levels. Samples frozen with M1 and M3 were combined and the analysis of SDF did not reveal statistically significant differences (Fig. 6). Between the two methods (M1 and M3) in the same patient, the SDF was very similar, without statistically significant differences. In addition, the total amount of SDF for each method was almost the same, demonstrating that the two methods applied did not induce differences in sperm DNA damage.

## DISCUSSION

Fertility preservation counselling is an essential component of education and informed consent prior to cancer treatment for young individuals at high risk of compromising their reproductive ability (20, 21). The increased survival rate of cancer patients has oriented healthcare providers to find strategies to improve the quality of life over time. Indeed, the ability to become parents in the future is an important part of well-being in both sexes (22).

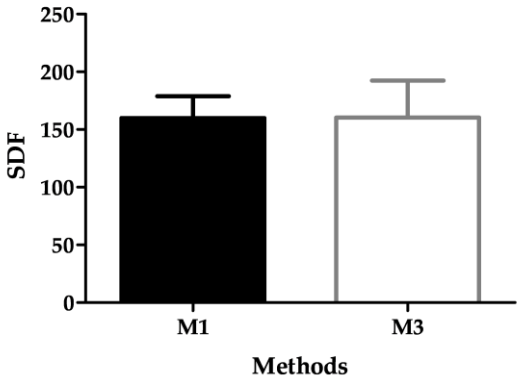


**Figure 6.** The number of labile sites counted in the samples frozen with two different methods, M1 and M3. No statistical differences were observed.

That being so, it is not only mandatory to propose a freezing program for fertility preservation but also to investigate suitable strategies for better reproductive outcomes after thawing gametes or embryos.

Recent studies showed the effects of cryopreservation on the motility and viability of spermatozoa after thawing in men who have undertaken the procedure because of oligozoospermia, cancer, or other pathologies (23). If basal semen parameters were below the 5$^{th}$ percentile of WHO reference values, the yield after thawing was low. In men with testicular cancer who cryopreserved semen, the motility and viability of spermatozoa before freezing were worse than those of other types of cancer. This translates into a small number of patients using their frozen samples for reproductive purposes, especially if the cryo-preservation process has an impact on sperm motility and viability (24).

This evidence leads us to consider the importance of trying to improve freezing protocols to ensure a better quality of thawing semen to maintain the same chance to achieve a pregnancy (25).

In this perspective, the study here presented had the purpose of comparing different freezing protocols obtained through little variations in the phases of cooling. We have previously shown that implementing a slow cooling phase at +4°C, instead of how it is suggested in the WHO manual (16), before nitrogen vapours and plugging into the nitrogen liquid, positively influenced the yield in the cryopreservation process when TYB cryoprotectant was used (15). In the first part of the present study, we modified times and temperatures during the cooling phase to assess the impact on thawing sperm quality. We first considered the motility and viability of spermatozoa to compare the various protocols tested. The obtained results highlighted the importance of maintaining the passage at +4°C because when this phase is eliminated the seminal quality of thawed samples worsened dramatically. We did not observe any significant difference between M1 and M3 protocols. For this reason, to establish the best method of cryopreservation, we introduced a different approach by studying DNA and morphology integrity using cytofluorimetric analysis, comet assay, and transmission electron microscopy. Flow cytometry compared the DNA profile of each samples obtained by the two selected M1 and

M3 methods to analyse the macromolecular and cellular damages following the uptake kinetics of two vital dyes (Hoechst 33342 and propidium iodide). The number of damaged cells for each sample was similar using both freezing methods, with slight variations in trend which were not significant for both methods. The data were verified through comet assay analysis, which was conducted on samples from the same patient using two methods (M1 and M3). The results indicated that sperm DNA fragmentation was almost identical for both protocols, suggesting that neither method resulted in differences in sperm DNA damage.
The data were also confirmed qualitatively through ultrastructural analyses by TEM. Compared to the fresh sample, both frozen samples showed the presence of large membranous vacuoles of varying sizes. Morphological abnormalities observed at chromatin, vacuoles, nucleus, and plasma membrane levels in the two thawed samples, obtained using the M1 and M3 freezing methods, were largely similar.

Although no significant difference emerged when samples were cooled for 30 min (M1) or 2 h (M3) it is clear when the TYB with glycerol cryoprotectant is used that maintaining the cooling phase at +4° C is useful to prevent damage from freezing. The evidence that the time of this exposure does not influence sperm quality favours a highly versatile and flexible variability of protocol application. It allows the management of cryo-lab procedures by modifying the times of the first cooling phase according to the schedule in routine activities without affecting the performance of the cryopreservation process. The cryo survival factor (CSF) or "percent return of motility", a commonly used index to define the yield of cryopreservation protocol, was often under 50% and this also happened when applying the M1 or M3 freezing protocol despite being the highest values found in the tested protocols (15, 26).

In conclusion, we suggested that when TYB with glycerol was used as a cryoprotectant, the best practice in sperm cryopreservation included previous incubation at +4°C for at least 30 min and then exposure of 10 min on vapors of liquid nitrogen before finally being cooled by plunging paillettes into liquid nitrogen for storage. In fact, TEST-yolk buffer is a semen extender and cryobuffer that can be paired with glycerol to achieve high sperm motility and viability and to recover more motile sperm post-thaw than glycerol-only media (27, 28). We believe further studies are needed to improve sperm cryopreservation protocols by changing not only the time or temperature of cooling but also the type of cryoprotectant solution, testing for example the vitrification method.

The study here presented could show not only the effect induced by a different type of freezing protocol but also propose alternative tools to deepen the investigation of sperm characteristics, beyond the basal screening semen analysis by performing not routine assays, such as cytofluorimetric analysis, comet assay, and transmission electron microscopy.

A fertility preservation program in women is an established reality following in-depth experience with oocytes and embryo vitrification. Among all the fertility preservation strategies currently proposed, cryopreservation of oocytes (or embryos) is the only one recommended as a standard procedure for the preservation of fertility in cancer patients by the American Society of Clinical Oncology (ASCO) and the European Society for Medical Oncology (ESMO). Since January 2013, the ASCO and ASRM have validated the technique of cryopreservation of oocytes which is no longer considered an experimental technique (29). Other techniques currently in use include medical or surgical procedures mainly designed to preserve the recovery of gonadal function (ovarian cycle) from iatrogenic or pharmacological damages. However, their efficiency in preserving fertility in terms of reproductive prognosis remains uncertain and strongly variable (30).

The last guidelines of major cancer societies (31, 32) confirmed the importance of preserving fertility in several oncological and non-oncological diseases. In women wishing to postpone maternity and in transgender individuals the vitrification of metaphase II oocyte is the preferred option. For men, testicular tissue cryopreservation should be recommended only in pre-pubertal boys, whereas semen cryopreservation is the only established method under many circumstances. However, we still lack clear recommendations about the protocol to be used. This evidence pinpoints the importance of investigating sperm cryopreservation protocols.

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