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## Cryobiology

journal homepage: [www.elsevier.com/locate/ycryo](http://www.elsevier.com/locate/ycryo)

## 2 Q1 Assessment of two thawing processes of cryopreserved human sperm in pellets

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## ARTICLE INFO

7  
8  
9  
10  
11 Article history:  
12 Received 16 November 2010  
13 Accepted 9 August 2011  
14 Available online xxx

## Keywords:

15 Cryopreservation  
16 Thawing  
17 Sperm motility  
18 Sperm membrane  
19 Spermatozoa  
20 Acrosome reaction  
21 Oxidative stress  
22  
23

## ABSTRACT

24  
25 In this study, we evaluated the effects of the thawing methodology on sperm function after cryopreser-  
26 vation in pellets. We compared the use of two thawing procedures: method (1) maintaining pellet for  
27 10 min in air at room temperature, then another 10-min period in air at 37 °C followed by dilution in  
28 a thawing medium; and method (2) immersing the pellets directly in thawing medium at 37 °C for  
29 20 min. This procedure leads to a higher rate of temperature increase and a dilution of the glycerol present  
30 in the freezing medium. We analyzed the effect of the thawing procedure on sperm motility, viability,  
31 membrane lipid packing disorder, acrosome status, reactive oxygen species (ROS) level and sperm chromatin  
32 condensation. This study revealed a positive effect of the M2 thawing methodology on sperm  
33 parameters. The percentage of spermatozoa with fast-linear movement is increased (M1: 17.26% vs.  
34 M2: 28.05%,  $p < 0.01$ ), with higher viability (M1: 37.81% vs. M2: 40.15%,  $p < 0.01$ ) and less acrosome damage  
35 (M1: 40.44% vs. M2: 35.45%,  $p = 0.02$ ). We also detected an increase in the percentage of viable spermatozoa  
36 with low membrane lipid disorder (M1: 31.36% vs. M2: 33.17%,  $p = 0.03$ ) and a reduction in  
37 chromatin condensation (44.62 vs. 46.62 arbitrary units,  $p = 0.02$ ). Further studies will be necessary to  
38 evaluate the possible clinical applications.  
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41  
42 Introduction43 Cryopreservation of human semen represents a useful  
44 therapeutic option in the management of infertility with several  
45 possible applications [1–4]. However, during cryopreservation  
46 spermatozoa are exposed to physical and chemical stress, which  
47 result in adverse changes in membrane lipid composition, sperm  
48 motility, viability and acrosome status [5–8]. All these changes  
49 reduce the fertilizing ability of human spermatozoa after cryopres-  
50 ervation [1,2,9].51 Mechanisms of cryodamage to human spermatozoa are thought  
52 to be multifactorial [4,6,10]. Several damaging processes can occur  
53 during the freezing and thawing of human spermatozoa: thermal  
54 shock, formation of intracellular ice crystals, cellular dehydration,  
55 increased concentration of salts, oxidative stress and osmotic  
56 shock [6,11–16].57 Since Bunge et al. [17] reported the first four human pregnan-  
58 cies and births resulting from AI using semen samples that had  
59 been frozen and stored in dry ice at  $-70^{\circ}\text{C}$  for up to 6 weeks,  
60 different methods of cryopreservation have been used [1,18].  
61 However, there is currently no standard freezing and thawing pro-cedure, and methods that optimize motility recovery have not  
been firmly established [19]. Success in the freezing process is re-  
lated to multiple factors including the cryo-protective additives  
used, rate of freezing, freezing medium, volume of sample and  
thawing temperature [4,19,20].The thawing procedure and rate of thawing are important fac-  
tors, as has been previously described [21–23]. However, there  
has been considerable controversy surrounding the optimum rate  
of temperature increase during thawing and efforts must be made  
to improve the basic knowledge of this issue and its clinical appli-  
cation. This is particularly important for frozen sperm in pellets, as  
there is a lack of information about the use of different thawing  
protocols.Another key consideration in sperm freezing is the use of cryo-  
protective agents. Glycerol is a useful cryoprotective agent that has  
been used for sperm preservation for almost 60 years [24]. The  
characteristics of glycerol for freezing are well established, as is  
the damage produced in the spermatozoa, which is mainly related  
to osmotic injury [6,25,26]. This toxic effect could be minimized by  
lowering the glycerol concentration, by lowering the temperature  
at which glycerol is added to the freezing medium, or with a quick  
dilution after thawing [27,28].Spermatozoa in pellets are usually thawed by a simple  
procedure in which they are placed in empty tubes immersed in  
water at room temperature for 10 min, and then maintained in

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the incubator at 37 °C for 10 min [29,30]. In this procedure the rate of temperature change is low and glycerol is present in the medium during the whole thawing procedure (20 min). One alternative would be thawing the sperm samples directly in culture medium at 37 °C. This procedure leads to a higher rate of temperature increase and a dilution of the glycerol present in the freezing medium.

The objective of this study was to evaluate the effect of two different procedures for thawing frozen human semen on the functionality of the thawed spermatozoa in terms of motility, viability, membrane lipid disorder, spontaneous acrosome reaction, ROS generation and chromatin condensation.

## Materials and methods

### Ethics

This study was conducted with institutional approval from Instituto Valenciano Infertilidad (IVI-Murcia) and University of Murcia and informed consent from men who visited our centre for infertility screening.

### Sample collection

Semen samples were obtained from 20 normozoospermic patients attending our clinic for infertility screening. Semen samples were obtained by masturbation and collected into sterile containers, following 3–5 days' abstinence from sexual activity. After liquefaction, semen samples were examined for volume, sperm concentration, morphology and motility according to World Health Organization guidelines [31].

### Cryopreservation of semen samples

Semen samples were frozen in pellets on the surface of dry ice using a glycerol-based cryoprotectant with egg yolk (Freezing Medium-Test yolk Buffer, Irvine Scientific, Santa Ana, CA, USA) as previously described [29,30,32]. All the pellets formed were transferred into a cryotube immersed into liquid nitrogen and then immediately moved to semen banks for long-term storage.

The protocol for freezing sperm in pellets has been employed since 1996, when the IVI clinic group reported the first full-term pregnancy achieved with frozen sperm obtained by testicular sperm extraction (TESE) [33]. To date, thousands of live births have been achieved in IVI facilities. Cryopreservation in pellets offers some advantages over other packaging systems. These pellets have a higher surface-to-volume ratio and higher heat exchange through the sample with important implications for the rates of cooling, freezing and thawing of the semen [1,33]. Pellet-freezing of domestic animal semen generally produces the best results [34].

### Thawing process

Frozen semen samples from each patient were divided into two parts, each of which was subjected to one of the different thawing methods being investigated.

#### Method 1 (M1)

Three pellets per sample (total 150 µl) were transferred to a Falcon tube and maintained for 10 min at room temperature, followed by another 10 min period at 37 °C without medium [29,30]. Finally 300 µL of the medium (human tubal fluid, HTF, Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% human serum albumin (HSA, SAGE Coopersurgical, Trumbull, USA) at 37 °C was added before analysis.

#### Method 2 (M2)

Three pellets per sample (total 150 µl) were transferred to a Falcon tube containing 300 µL of HTF + HSA medium at 37 °C and maintained at this temperature for 20 min before analysis.

#### Microscopic evaluation of sperm motility and viability

Motility was evaluated according to World Health Organization guidelines [31]. Sperm vitality in frozen thawed samples was analyzed using the dye exclusion method with eosin–negrosin staining [31] or by applying a combination of the fluorophores carboxyfluorescein diacetate (DCFA) and propidium iodide (PI) [35] on at least 200 cells per sample. Motility was expressed as forward motion percentage (type a + b motility) and viability as percentage of viable cells.

#### Motion parameters measured by CASA

Motion parameters were determined using a computer-assisted sperm analysis (CASA) system (ISAS, Valencia, Spain). The CASA-derived motility characteristics studied were percentage of motility and progressive motility, curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), amplitude of lateral head displacement (ALH, µm), and beat cross-frequency (BCF, Hz) [16,32].

A 7 µl drop of the sperm sample was placed on a warmed (37 °C) slide and covered with a 24 × 24 mm cover slip. The setting parameters were: camera velocity 50 frames/s, evaluation of 100 frames in which spermatozoa had to be present in at least 25 in order to be counted, images obtained at 200× magnification on a contrast phase microscope (Nikon, Tokyo, Japan). Spermatozoa with a VAP < 10 µm/s were considered immotile. A minimum of five fields per sample were evaluated, counting a minimum of 200 spermatozoa per sub-sample.

#### Analysis of seminal parameters by flow cytometry

The flow cytometer was used to evaluate sperm lipid membrane disorder, plasma membrane integrity, acrosome reaction, reactive oxygen species generation and chromatin condensation [16,32,36,37]. Flow cytometric analyses were performed on a Coulter Epics XL cytometer (Beckman Coulter Inc., Miami, FL, USA). The fluorophores were excited by a 15 mW argon ion laser operating at 488 nm. Data from 10,000 events per sample were collected in list mode, and four measures per sample were recorded. Flow cytometric data were analyzed using the program Expo32ADC (Beckman Coulter Inc.) using a gate in forward and side scatter to exclude eventual remaining debris and aggregates from the analysis.

#### Assessment of lipid packing disorder and viability

To detect any increase in plasma membrane lipid packing disorder, sperm samples were stained with merocyanine 540 (M540) and Yo-Pro 1 [35]. Stock solutions of M-540 (1 mM, Sigma–Aldrich Química, S.A, Madrid, Spain) and Yo-Pro 1 (25 µmol/l, Invitrogen S.A, Barcelona, Spain) in DMSO (Sigma–Aldrich Química, S.A, Madrid, Spain), were prepared. For each 1 ml diluted semen sample, 2.7 µl M540 stock solution was added (final concentration 2.7 µmol/l), along with 1 µl of Yo-Pro (25 nmol/l final concentration). M540 fluorescence was collected with a FL2 sensor using a 575 nm band-pass filter and Yo-Pro 1 with a FL-1 sensor using a 525 nm band-pass filter. Cells were classified in three categories: low merocyanine and low Yo-Pro 1 fluorescence (viable, low

disorder, un-capacitated sperm), high merocyanine fluorescence and low Yo-Pro 1 signal (viable, high disorder, capacitated sperm) or Yo-Pro-1 positive (dead).

#### Assessment of acrosome status

We used the fluorescein isothiocyanate-conjugated monoclonal antibody CD46 (FITC-CD46) targeting the inner acrosomal membrane to detect completed acrosome reaction (AR) [38].

Seminal samples (100 µL of diluted semen sample with 5–10 × 10<sup>5</sup> spermatozoa in HTF-BSA medium) were incubated with 3 µL of the antibody CD46 labeled by FITC (dilution 1:10) (Serotec, Oxford, UK) at 37 °C for 1 h in the dark and then fixed with 10 µL of formaldehyde saline solution (1%). FITC-CD46 green fluorescence was collected with a FL1 sensor using a 525 nm band-pass filter. Cells were classified in two categories: low FITC fluorescence (intact, no altered acrosome) and high FITC fluorescence (acrosome damaged).

#### Production of reactive oxygen species (ROS)

Production of ROS was measured by spermatozoa incubation in PBS in the presence of 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) (0.5 µmol/l) for 90 min at 37 °C [32,39]. This dye is a fluorogenic probe commonly used to detect cellular ROS production. H<sub>2</sub>DCFDA is a stable cell-permeable non-fluorescent probe. It is de-esterified intracellularly and turns to highly fluorescent 2',7'-dichlorofluorescein upon oxidation. Green fluorescence was collected with a FL1 sensor using a 525 nm band-pass filter. Measurements were expressed as the mean green intensity fluorescence units (mean channel in the FL1) and this was used as index of ROS generation. The ROS generation was measured at different incubation times (0, 15, 30, 45, 60, 75, 90 min).

#### Determination of chromatin condensation

Sperm chromatin was stained with propidium iodide for the determination of sperm chromatin condensation [16,32,39]. Samples were centrifuged (1200g × 3 min) and the pellet resuspended in a solution of ethanol and phosphate buffered saline (PBS) (70/30 v/v) to permeabilise the sperm membrane, followed by storage overnight at -20 °C until evaluation. The samples were then centrifuged, the supernatant discarded and the pellet resuspended in a PI solution (10 mg/ml) in PBS. Samples were maintained in the dark for 1 h before flow cytometric analysis. Red PI fluorescence was collected with a FL3 sensor using a 650 nm band-pass filter. Measurements were expressed as the mean red intensity fluorescence units (mean channel in the FL3) and this was used as an index of the state of the chromatin condensation, which is inversely related to the PI uptake by DNA.

#### Experimental design

Frozen samples in pellets from 20 donors were thawed using two methods. Semen samples were divided into two parts, each of which was subjected to one of the thawing methods being investigated. After a thawing process of 20 min, motility, viability, mem-

brane lipid disorder, acrosome status, ROS generation and chromatin condensation were evaluated.

#### Statistical analysis

Data are expressed as the mean ± SEM and analyzed by ANOVA, considering the specific sperm treatment and donor as the main variables. When ANOVA revealed a significant effect, values were compared by the least significant difference pair wise multiple comparison post hoc test (Tukey). Differences were considered statistically significant at *p* < 0.05.

Cluster analysis was applied to analyze and compare the motility parameter values associated with each spermatozoon so as to identify sub-groups within the sperm population [40,41]. Data sets for analysis were prepared by merging raw data files from every measured sperm sample (values for VAP, VSL, BCF and ALH). Upon completion of the cluster analysis, each individual spermatozoon was categorized as belonging to one of the three groups, or sub-populations, described above. Once the sub-populations had been identified, the relative frequencies of spermatozoa within each experimental sample, and belonging to each group, were compared by ANOVA.

#### Results

The sperm characteristics for the fresh semen were: sperm concentration 70.95 ± 15.29 × 10<sup>6</sup> cells/ml and progressive motility 57.55 ± 3.23%.

#### Microscopic evaluation of sperm motility and viability

When microscopic evaluation of spermatozoa was carried out by an experienced observer no differences were found between thawing groups in terms of progressive motility (grades a + b) (M1: 16.87 ± 0.97% vs. M2: 16.06 ± 0.99%, *p* = 0.56), or viability as measured by EN staining (M1: 30.85 ± 1.91% vs. M2: 30.03 ± 1.97, *p* = 0.77) and carboxifluorescein diacetate/propidium iodide staining (M1: 29.72 ± 1.92% vs. M2: 28.22 ± 1.96, *p* = 0.59).

#### Motion parameters measured by CASA

According to CASA evaluation, no differences were found in the percentage of motile and progressively motile sperm between the two thawing methods (Table 1, *p* > 0.05). However, the use of the M2 thawing method modified the pattern of movement compared to the M1 group and most of the motion parameters were increased. The velocities of the spermatozoa (VCL, VSL and VAP) were higher in the M2 group than in the M1 group (Table 1, *p* < 0.01), and the linearity, straightness, wobble and beat cross-frequency were also increased in M2 (Table 1, *p* < 0.01). The amplitude of lateral head displacement also tended to be higher in M2 than in M1 (Table 1, *p* = 0.08).

To investigate the data in more detail we therefore used a cluster analysis, which aims to classify the entire set of individual

**Table 1**  
Motility parameters measured by CASA. Comparison of thawing frozen human sperm using two methods.

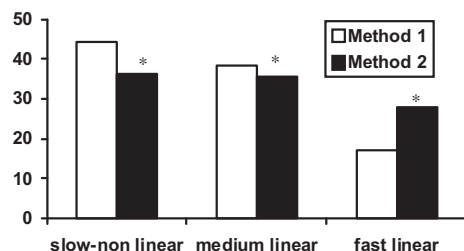
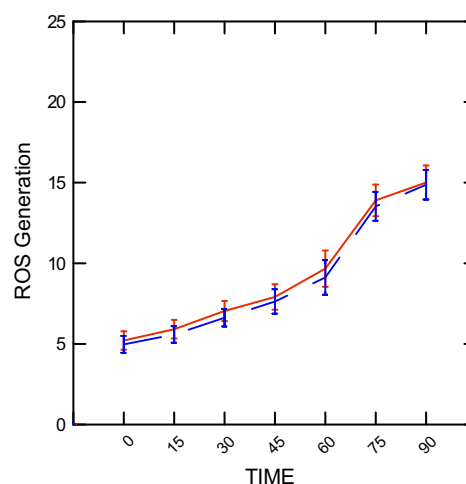
	Motility (%)	Progressive motility (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm/s)	BCF (Hz)
Method 1	28.44 ± 0.64	17.49 ± 0.37	49.40 ± 1.24 <sup>a</sup>	26.40 ± 0.98 <sup>a</sup>	33.62 ± 0.98 <sup>a</sup>	52.18 ± 1.02 <sup>a</sup>	75.49 ± 0.90 <sup>a</sup>	67.39 ± 0.79 <sup>a</sup>	1.78 ± 0.05	7.04 ± 0.23 <sup>a</sup>
Method 2	26.70 ± 0.60	16.53 ± 0.39	53.39 ± 1.25 <sup>b</sup>	30.96 ± 0.99 <sup>b</sup>	38.01 ± 1.01 <sup>b</sup>	55.36 ± 1.03 <sup>b</sup>	78.01 ± 0.91 <sup>b</sup>	69.00 ± 0.80 <sup>b</sup>	1.84 ± 0.05	7.47 ± 0.23 <sup>b</sup>
<i>p</i> -value	0.42	0.31	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.08	<0.01

VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, LIN: linearity of the curvilinear trajectory, STR: straightness, WOB: Wobble (VAP/VCL), ALH: amplitude of lateral head displacement; BCF: beat cross-frequency.

<sup>a,b</sup>: numbers within columns with different superscripts differ (*p* < 0.01).

**Table 2**  
Summary of group mean ( $\pm$ sem) sperm motion parameters derived from a cluster analysis of motility data.

Cluster group	(%)	VSL ( $\mu$ m/s)	VAP ( $\mu$ m/s)	ALH ( $\mu$ m/s)	BCF (Hz)
1. Fast-linear spermatozoa	20.21	52.14 $\pm$ 1.40	58.91 $\pm$ 1.34	2.06 $\pm$ 0.09	9.98 $\pm$ 0.39
2. Medium-linear spermatozoa	37.69	30.46 $\pm$ 0.62	38.00 $\pm$ 0.64	1.99 $\pm$ 0.08	8.36 $\pm$ 0.27
3. Slow-non-linear spermatozoa	42.10	15.35 $\pm$ 0.47	22.32 $\pm$ 0.53	1.54 $\pm$ 0.07	4.94 $\pm$ 0.28

**Fig. 1.** Summary of group mean (%) sperm motion parameters derived from a cluster analysis of motility data. Comparison of thawing frozen human sperm using two methods ( $p < 0.01$ ).**Fig. 2.** ROS generation (arbitrary fluorescent units). Comparison of thawing frozen human sperm using two methods (Method 1, blue line, Method 2, red line) ( $p = 0.42$ ).

M1:  $8.90 \pm 0.51$  vs. M2:  $9.24 \pm 0.52$   $p = 0.42$ ; Arbitrary Fluorescent Units, Fig. 2).

#### Determination of chromatin condensation

The grade of chromatin condensation, measured by propidium iodide staining (condensation is inversely related to PI uptake by DNA), decreased in the M2 group compared to M1, (M1:  $44.62 \pm 0.48$  vs. M2:  $46.27 \pm 0.52$  arbitrary fluorescent units;  $p = 0.02$ ).

#### Discussion

Sperm cryopreservation represents a common and important process in assisted reproductive treatments [4]. However, standard protocols that minimize the cryodamage produced during the process of freezing and thawing are not currently well established. Freeze–thawing causes physical and chemical stress to the sperm membrane, leading to loss of sperm viability and fertilizing ability [4,42–44]. The survival of functional spermatozoa during freezing and thawing is affected by many factors. These include cryopreservation methods and cryoprotectants, composition of the preserving medium, freezing rate and thawing temperature [4,42,43].

We used the pellet methodology for cryopreservation of human samples. This could be considered a low-tech approach when compared to the automated freezing systems now available. However, cryopreservation in pellets offers some advantages over other packaging systems. These pellets have a higher surface-to-volume ratio with important implications for the rates of cooling, freezing and thawing of the semen [1]. Pellet-freezing of domestic animal semen generally produces the best results after thawing [34]. Pellets also offer advantages in the management of the frozen samples, because it is possible to easily select the number of pellets required for an assisted reproduction treatment and store the remaining pellets for future treatments [45]. Some authors have

spermatozoa into functionally meaningful subsets, so that the data can be interpreted more easily and more accurately [40,41]. The initial data set ( $N = 20$  donors) consisted of 23,460 cases that contained data values for VAP, VSL, BCF and ALH. After undertaking a cluster analysis, the data were separated into three new groups, summarized in Table 2. Group 1 represented the fast-linear spermatozoa, group 2 represented medium velocity linear spermatozoa and group 3 represented slow non-linear sperm population. The use of M2 as the thawing process increased the percentage of fast-linear spermatozoa compared to control (28.05% vs. 17.26%, Fig. 1,  $p < 0.01$ ), and reduced the percentage of slow non-linear spermatozoa (36.42% vs. 44.24%, Fig. 1,  $p < 0.01$ ) and medium linear spermatozoa (35.54% vs. 38.50%, Fig. 1,  $p < 0.01$ ).

#### Assessment of lipid packing disorder and viability

The use of flow cytometry allowed detection of a small increase in the percentage of viable spermatozoa with low membrane lipid disorder in the M2 group (Table 3,  $p = 0.03$ ) and a reduction in the total percentage of dead spermatozoa when compared with the M1 group (Table 3,  $p < 0.01$ ). The percentage of viable spermatozoa with high lipid disorder was similar in both experimental groups (Table 3,  $p = 0.36$ ).

#### Assessment of acrosome status

When the sperm acrosome status was evaluated a reduction in the percentage of sperm with acrosome damage was observed in the M2 group (M1:  $40.44 \pm 1.50$  vs. M2:  $35.45 \pm 1.49$ ,  $p = 0.02$ ).

#### Production of reactive oxygen species (ROS)

No significant differences were found between treatments for the level of ROS during 90 min incubation at 37 °C (Mean value

**Table 3**  
Membrane lipid packing disorder. Sub-populations of spermatozoa after staining with merocyanine 540 and Yo-Pro 1 and examination by flow cytometry. Comparison of thawing frozen human sperm using two methods.

	Viable low lipid disorder (%)	Viable high lipid disorder (%)	Dead (%)
Method 1	31.36 $\pm$ 0.46 <sup>a</sup>	6.46 $\pm$ 0.32	62.19 $\pm$ 0.42 <sup>a</sup>
Method 2	33.17 $\pm$ 0.52 <sup>b</sup>	6.98 $\pm$ 0.41	59.85 $\pm$ 0.34 <sup>b</sup>
<i>p</i> -value	0.03	0.36	<0.01

<sup>a,b</sup>: numbers within columns with different superscripts differ ( $p < 0.05$ ).



suggested that direct application of sperm to the surface of dry ice potentially increases the risk of cross contamination [46], although the pellets are prepared in the flow chamber on new dry ice and later stored in cryotubes in the cryobank in similar biosecurity conditions used with other freezing procedures. Another possible drawback of the method is that variability between samples could be detected, which could be related to the lack of control of the freezing process compared to automated freezing systems [45].

As Nijs et al. suggested [43], specific freezing and thawing rates have to be determined for each specific cryoprotectant mixture and for each type of vial used, as each system has a different conductivity. In the present study, we compared the effect of two thawing methodologies on sperm motility, kinematics and sperm functionality in a population of healthy donors. This study revealed a significant effect of the thawing methodology on motion parameters, viability, sperm membrane lipid disorder, acrosome status and sperm nuclear chromatin condensation when CASA and flow cytometry were used for the assessment. However, when microscopic observation was used no differences were found. It is well established that flow cytometry offers a higher degree of accuracy and repeatability of sperm assessment [47–50].

In relation to motility parameters, we observed an increase in all the motion parameters (except for a tendency for ALH) when M2 was used, along with an increase in the subpopulation of fast-linear spermatozoa. These observations of motility are in concordance with data reported previously [21,23,28], where an increase in motility recovery and motion parameters is seen when a high rate thawing protocol is used. However, other studies have not found any difference in sperm parameters when different thawing rates were used [22,51]. The interaction between cooling and thawing could be the cause of these differences: it is well established that the freeze-thaw process is most successful when sperm are thawed at warm temperatures (37 °C) after rapid freezing, but more successful for thawing at lower temperatures (room temperature) after slower, computer-controlled freezing [28]. The differences in the pattern of movement reported in this study showed that optimization of the thawing process is important to ensure recovery of a higher percentage of spermatozoa with a fast-linear movement. This sperm subpopulation could be related to the fertilizing capacity [52,53].

In addition to the increase in motility seen with M2, this method was also associated with an increase in viability and reduction of acrosome damage, which means a reduction in the membrane cryodamage. Membrane lipid disorder is one of the early signals of the process of sperm capacitation [35] and also an index of stress during cryopreservation [14]. We found a higher proportion of viable spermatozoa with a low lipid disorder and a lower percentage of acrosome damage when warm media was used, a finding which could be related to lower damage induced by ice crystal formation [14,44] and an increase in the rate of recovery of the enzymatic antioxidant activity [23]. The fast thawing rate in this method is likely to be responsible for these improved results, because the rapid thawing process prevents recrystallization and reduces membrane damage [14]. The better result obtained by thawing in warm media is probably based on the rapid progression through the critical phase of the freezing-thawing process (from 5 to –5 °C) [54].

Previous studies have shown that freezing and thawing induce important changes in sperm chromatin resulting in greater compactness, which is related to an alteration in the interactions between DNA and nuclear proteins [8,55,56]. Thus, chromatin condensation and stability may be critical factors to consider when using frozen semen [32]. In this study, we observed a lower condensation of the nucleus of the spermatozoa when M2 was used, a finding that could suggest that this methodology is more appropriate than M1, as it reduces “hyper condensation” induced by

freezing and the alteration of the nucleo-proteins. Similar observations have previously been reported in studies comparing computerized slow-stage and static liquid nitrogen vapor freezing methods [57] and in similar conditions as when we added antioxidant compounds to the thawing extender [16,32].

Regarding the effect of thawing temperature, some authors have hypothesized that the degree of cell damage could be related to an increase in the rate of recovery of the enzymatic antioxidant activity when higher temperatures are used in thawing [23], resulting in more efficient neutralization of oxygen radicals produced during thawing, these being the predominant cause of human sperm DNA fragmentation [15]. However, ROS generation measured in this study was similar for both thawing treatments and viability was increased in the M2 group. This fact suggests that the negative effect of the presence of glycerol may also be important, as previously reported [6,28]. According to some authors, much of the reduction in sperm functionality observed during the freezing and thawing of human spermatozoa is due to exposure to cryoprotectants [25,58,59]. Another factor that could explain the improved results achieved with M2 is the quick dilution of the sperm in the presence of media with human albumin (HAS), which has a protective effect on spermatozoa [60]. We have detected significant differences in sperm parameters using very accurate technology. The use of this simple thawing methodology with warm media could easily be employed for frozen samples under clinical conditions. However, it is necessary to carry out further studies to analyze the differential effect of dilution and temperature of thawing on sperm characteristics and it is necessary to correlate this improvement in sperm parameters with fertilizing potential [61,62].

In conclusion, we have observed that the changes made to the thawing process have improved the subsequent sperm functionality. The percentage of spermatozoa with fast-linear movement is increased, with higher viability and less acrosome damage. We also detected an increase in the percentage of viable spermatozoa with low membrane lipid disorder and a reduction of the chromatin condensation. Further studies will be necessary to confirm our findings and to evaluate the possible clinical applications.

#### Acknowledgment

We acknowledge with gratitude Ruth Tunn (University of Oxford) for help with the language in the manuscript.

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