Human Reproduction, Vol.32, No.10 pp. 2007–2015, 2017
Advanced Access publication on August 31, 2017 doi:10.1093/humrep/dex281

human reproduction

ORIGINAL ARTICLE *Andrology*

New permeable cryoprotectant-free vitrification method for native human sperm

J. Aizpurua[1,2], L. Medrano[1,2,*], M. Enciso[3], J. Sarasa[3], A. Romero[4], M.A. Fernández[1], and M.J. Gómez-Torres[2,4]

[1]IVF-Spain, Av. Ansaldo 13, 03540, Alicante, Spain [2]Human Fertility Chair, University of Alicante, Carretera de San Vicente del Raspeig s/n, 03690, San Vicente del Raspeig, Alicante, Spain [3]iGLS, Carrer Britania 7, 03540, Alicante, Spain [4]Biotechnology Department, University of Alicante, Carretera de San Vicente del Raspeig s/n, 03690, San Vicente del Raspeig, Alicante, Spain

*Correspondence address. E-mail: mll.lopez@ivf-spain.com

Submitted on November 1, 2016; resubmitted on July 20, 2017; accepted on August 6, 2017

STUDY QUESTION: Is permeable cryoprotectant-free vitrification of native sperm samples a good alternative to conventional slow freezing?

SUMMARY ANSWER: The permeable cryoprotectant-free sperm vitrification protocol tested in this study renders considerably better recovery rates of good quality sperm compared to slow freezing.

WHAT IS KNOWN ALREADY: Slow freezing is currently the most commonly used technique for sperm cryopreservation, though this method has been repeatedly shown to have negative effects on both structural and functional sperm features. New alternative methods such as vitrification have been established as a successful alternative in other reproductive cell types, but vitrification of spermatozoa is still a rather unexplored methodology, with limited studies showing its efficacy in male gametes.

STUDY DESIGN SIZE, DURATION: This study included 18 normozoospermic sperm samples from patients seeking ART treatment between 2014 and 2015. The effects of a new vitrification protocol on functional and structural sperm quality parameters in comparison to fresh and slow-frozen samples were assessed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All samples were divided into three aliquots: fresh (F), slow freezing–thawing (S) and vitrification-warming (V). Sperm concentration, motility, morphology, vitality, DNA fragmentation, cytoskeleton integrity and spontaneous acrosome reaction were assessed and compared between the groups.

MAIN RESULTS AND THE ROLE OF CHANCE: Results showed improved preservation of sperm features after vitrification compared to conventional freezing. Permeable cryoprotectant-free vitrification presented a significantly higher percentage of live spermatozoa, than slow freezing, better preservation of acrosomes was achieved in vitrified samples and DNA fragmentation was reduced approximately one-third on average compared to slow freezing. Regarding tubulin assay, three different labelling patterns were observed. The frequency of these labelling patterns was similar in F and V groups but this was not the case of the S group. The multivariate analysis of all sperm quality parameters studied revealed that the V group presented features that are closer to the F group than the S group, indicating that samples are better preserved through vitrification than slow freezing.

LIMITATIONS REASONS FOR CAUTION: This validation has been undertaken only on normozoospermic sperm samples. It would be necessary to compare these results in pathological samples and also to evaluate the influence of the application of this methodology on clinical outcomes.

WIDER IMPLICATIONS OF THE FINDINGS: The sperm vitrification protocol here described warrants better maintenance of sperm quality parameters than traditional freezing methods and may be a good alternative to preserve sperm samples from patients seeking IVF treatment.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by IVF-Spain Foundation. The authors have no conflicts of interest to declare.

Key words: cryopreservation / vitrification / slow freezing / sperm quality / tubulin / spermatozoa / male fertility

Introduction

Cryopreservation of human gametes and embryos is an essential procedure of Assisted Reproduction Technologies (ART). Sperm freezing is particularly important to preserve male fertility and guarantee the availability of sperm at the time of oocyte fertilization (Sanger et al., 1992; Fabbri et al., 2004). Since cryopreservation of human spermatozoa was first introduced in the late 1950s (Polge, 1957), several cryopreservation methods including rapid and slow-freezing techniques have been developed (Di Santo et al., 2012).

Slow freezing is currently the most commonly used technique for sperm cryopreservation (Mocé et al., 2016). This method is based on progressive sperm cooling, either manually or automatically, involving the use of cryoprotectants. Slow-freezing protocols have been shown to produce dramatic changes in sperm quality at both structural and functional levels (O'Connell et al., 2002; Isachenko et al., 2004a; Desrosiers et al., 2006). These changes have been related to the addition and removal of cryoprotective agents and the formation of ice-crystals, causing toxic and osmotic damages respectively (Morris, 2006; Ozkavukcu et al., 2008). Moreover, the method is labour and time-consuming as it requires cooling at very slow rates and the use of permeable cryoprotectants during the process.

Due to these disadvantages, new alternative methods to improve sperm cryopreservation have been developed (Kuznyetsov et al., 2015; Merino et al., 2015; Slabbert et al., 2015). One of the most innovative of these methods is vitrification; this technique is based on the ultra-rapid increase and decrease of temperatures with or without the use of non-permeable cryoprotectants. During the procedure, water is cooled to a glassy state through extreme elevation of viscosity without intracellular ice crystallization making this procedure less labour intensive, quicker and presumably safer compared to traditional slow-freezing protocols (Katkov et al., 2006). The improvement of vitrification has already been demonstrated in other reproductive cell types. In oocytes and embryos, this technique has been shown to be a successful alternative to slow freezing, as it has been confirmed by the birth of numerous healthy babies after its use (Rienzi et al., 2016). Nonetheless, vitrification of spermatozoa is still a rather unexplored methodology, with limited studies showing its efficacy in male gametes. During the last years, different sperm vitrification protocols have been published, most of them developed by Isachenko et al. In 2002, they described their first vitrification protocol without the need to use cryoprotectants (Nawroth et al., 2002). A few years later, the same group presented other vitrification protocols on washed sperm -without seminal plasma-using human tubal fluid (HTF) and sucrose (0.5 M) as cryoprotectants, or using HTF, sucrose and human serum albumin (Isachenko et al., 2008, 2012a,b). Merino et al. (2015) in a recent study described a new method which included butyrate in combination with HTF and sucrose. Not only different combinations of cryoprotectors, or the absence of these, have been proposed but also different devices have been tested: straws (Isachenko et al., 2012a), spheres (Isachenko et al., 2008), chips (Zou et al., 2013) or cooper loops (Isachenko et al., 2004b) with variable volumes cryo-stored per device but always in a range lower than 0.5 ml. These interesting and promising protocols however, have not been implemented in the IVF labs routine yet and slow freezing remains the standard cryopreservation method in most laboratories worldwide (Mocé et al., 2016).

Improved methods that allow for a better maintenance of the functionality and quality of the spermatozoa cryopreserved as compared to slow freezing are still needed. In the present study, we evaluate the performance of a new permeable cryoprotectant-free sperm vitrification protocol that uses native sperm sample and a cryostorage device that allows for the preservation of larger volumes of samples. The effect of this newly described vitrification method on sperm structure and function is evaluated in detail and compared to conventional slow freezing.

Materials and Methods

Sample selection and preparation of spermatozoa

Semen samples from 18 healthy patients (age range 31–40 years old and negative for HepB, C, HIV and syphilis) seeking treatment at our centre (IVF-Spain Clinic, Alicante, Spain) were studied. Samples were obtained after 1–3 days of sexual abstinence. Basic semen analysis including concentration, motility and morphology was performed according to WHO guidelines (WHO, 2010). Only normozoospermic samples were included in this study. The average count (mean ± SEM) and volume (mean ± SEM) was 83.44 ± 13.73 × 10^6 ml^{-1} and 3.79 ± 2.41 ml, respectively.

Ethical approval

Ethical approval for this study was obtained from Alicante General Hospital (Ref. CEIC PI2013/47.1).Signed patient consent for research had been obtained for all patients.

Experimental design

Samples were divided into three aliquots: fresh (F), slow freezing–thawing (S) and vitrification-warming (V). Comprehensive sperm structure and function evaluation was performed in all three groups as depicted in Fig. 1.

Conventional slow freezing and thawing

Sperm Cryoprotect™ II with glycerol (Nidacon, Mölndal, Sweden) freezing medium was slowly added to semen samples to achieve 1:1 dilution. This mix was packaged into 1.8 ml NuncCryotubes® (Brand Products, Roskilde, Denmark), initially kept at 4°C for 30 min, then exposed to liquid nitrogen (LN_2) vapour for 30 min and finally, plunged and stored for at least 10 days in LN_2 (−196°C).

After storage, samples were thawed at room temperature for 30 min, washed using wash medium (PureSperm®wash, Nidacon, Mölndal, Sweden) centrifugation at 300 g, 10 min and then resuspended in 300 µl of wash medium.

Sperm vitrification and warming

For sperm vitrification the recently developed Easy-Sperm® kit (iGLS, Alicante, Spain) was used following manufacturer's instructions. In brief, vitrification medium (V1) was added to semen samples to achieve 1:1 dilution. The mix was incubated 5 min at 37°C and then plunged drop by drop (20 µl) into medical LN_2 free from contaminants so that small solid spheres are formed. A disposable container for LN_2 is used and changed between patients. Around fifty spheres were then packaged into 1.8 ml cryotubes and these plunged and stored for at least 10 days in LN_2 (−196°C). After storage, sample warming was performed by adding the small spheres contained in a 1.8 ml cryotubes into 4 ml of previously warmed solution (V2).

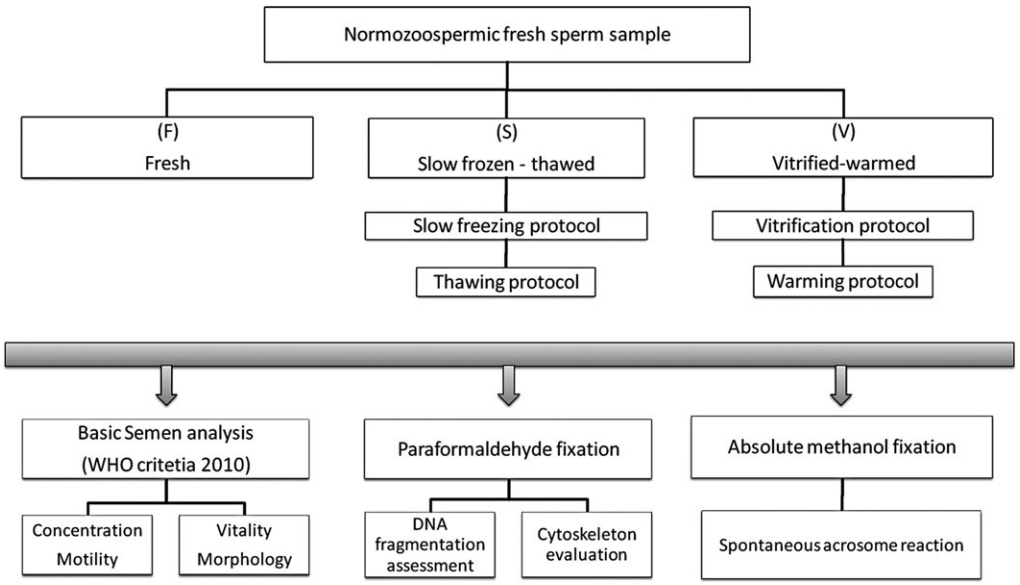

Figure 1 Experimental design. Sperm samples were divided into three groups: fresh (F), slow freezing–thawing (S) and vitrification-warming (V). Comprehensive sperm analysis was performed in all three study groups.

After 5 min at 37°C, the sample was centrifuged at 300 × *g* for 10 min and then resuspended in 300 µl of wash medium. Before using this kit, a toxicity test of both V1 and V2 solutions was performed. For this, five sperm samples were incubated in V1 and V2 for up to 10 min at 37°C. No negative effect on motility, vitality or acrosome integrity was observed (Supplementary Figure S1). The vitrification protocol was also tested for reproducibility; sperm motility, morphology and vitality were analysed in three replicates from five different normozoospermic donors. No significant differences were found in the parameters analysed (Supplementary Table S1).

Basic sperm analysis: sperm count, motility, morphology and vitality

Sperm count, motility and morphology were assessed immediately after liquefaction in fresh samples and after thawing and warming in frozen and vitrified samples. Makler® counting chamber (Sefi Medical Instruments, Haifa, Israel) was used for sperm count and motility scoring according to WHO guidelines (2010). Sperm morphology was assessed using Kruger strict criteria (Kruger *et al.*, 1987). Two hundred spermatozoa per sample were analysed. Defects were subdivided into: head damage, mid-piece damage, cytoplasmic droplet or tail damage according to WHO guidelines (2010). Teratozoospermia index (TZI), i.e. the number of abnormalities present per abnormal spermatozoa, was calculated for each of the study groups (F, S, V).

For sperm vitality evaluation, Sperm VitalStain™ (Nidacon, Mölndal, Sweden) following manufacturer's instructions was used. A total of 200 spermatozoa per sample were assessed.

Spontaneous acrosome reaction measurement

Spontaneous acrosome reaction assessment was performed using the acrosomal marker Lectin from *Pisum sativum* (pea)-FITC conjugate (FITC-PSA) (Sigma-Aldrich, Steinheim, Germany) following the protocol described elsewhere (Lybaert *et al.*, 2009). A minimum of 200 spermatozoa per sample were evaluated using LEICA DMRB fluorescence microscope under 100× magnification.

Sperm DNA fragmentation assessment

Sperm DNA fragmentation (SDF) assessment was performed using TUNEL (In Situ Cell Death Detection Kit, Roche, Mannheim, Germany) according to the manufacturer's instructions. A minimum of 200 spermatozoa per sample were evaluated using LEICA DMRB fluorescence microscope under a 100× magnification.

Immunocytochemistry of α-tubulin

Sperm cytoskeleton was studied by fluorescence immunocytochemistry of α-tubulin performed using monoclonal anti-α-tubulin antibody produced in mouse (Sigma-Aldrich, Steinheim, Germany).

A sperm sample previously fixed in paraformaldehyde (1%, Sigma-Aldrich, Steinheim, Germany) was permeabilized with 1% Triton X-100 in phosphate-buffered saline (PBS) 1× for 10 min. Slides were then blocked with 10% bovine serum albumin for 1 h, washed in PBS 1× and incubated with anti-α-tubulin antibody (1:600 dilution, 1 h). Slides were rinsed in PBS 1× during 15 min and incubated with Dylight™488 Donkey anti-mouse IgG (1:300 dilution, 1 h) (Jackson Inmuno Laboratories, West Grove, USA). Finally samples were washed in PBS 1× and mounted with complete mounting medium (100 nM DAPI, Sigma-Aldrich, Steinheim, Germany). A minimum of 200 spermatozoa per sample were assessed under LEICA DMRB fluorescence microscope at 100× magnification.

Statistical analyses

Descriptive and statistical analyses were performed using SPSS version 22.0 (IBM, New York, USA) and the SYSTAT® 11 software. A *P*-value <0.05 was considered to be statistically significant. ANOVA or Friedman test, respectively, was used as needed to assess significant differences. *Post hoc* analysis and Wilcoxon signed-rank tests were conducted with a

Bonferroni correction applied. In addition to univariate analysis, a principal component analysis (PCA) model was employed to calculate individual-specific patterns that account for most of the variability observed among sample types.

Results

The evaluation of the influence of slow freezing and vitrification on functional and structural sperm features showed differential effects of each one of the procedures tested on sperm quality and function.

With regard to conventional sperm parameters, a significant decline in progressive sperm motility was observed after both cryopreservation methods as compared to fresh samples (ANOVA, $P < 0.001$) (Table I). However, progressive sperm motility after vitrification displayed significantly higher levels of motility compared with slow-frozen samples ($P < 0.05$) (Table I).

In terms of sperm morphology, the percentage of normal spermatozoa was also significantly reduced after cryopreservation (Friedman's test, $P < 0.05$) (Table I). Better preservation of sperm morphology was achieved through vitrification compared to slow freezing ($P < 0.05$). Tail morphology was particularly affected by cryopreservation; a significant increase in the frequency of tail abnormalities was detected in slow-frozen samples compared to vitrified sperm ($P < 0.05$). No significant differences between fresh and cryopreserved groups were found with regards to other morphological abnormalities evaluated (Table I).

TZI, i.e. the number of abnormalities present per abnormal spermatozoon was also calculated and compared. No significant differences were observed between F and V groups (Table I). However, a significant difference in this index was observed when comparing F and S groups (1.24 ± 0.04 vs. 1.46 ± 0.05; $P < 0.05$).

Sperm vitality was also evaluated. The percentage of viable sperm decreased significantly after cryopreservation (ANOVA, $P < 0.001$). This decrease was significantly higher after slow freezing compared to vitrification ($P < 0.05$), with a 32.6% reduction in vitality observed in the S group and an 11.37% reduction observed in the V group compared to the F group (Table I).

The number of sperm with intact acrosome also decreased significantly after both cryopreservation techniques (Friedman's test, $P < 0.05$) (Fig. 2a). However the vitrification protocol provides significantly stronger prevention against 'cryo-capacitation' compared to conventional freezing (with the percentage of acrosome-reacted sperm cells being $44.27\% \pm 3.42$ in the V group vs $58.19\% \pm 3.43$ in the S group; $P < 0.05$).

Another sperm parameter affected by cryopreservation is DNA integrity. Significantly higher SDF indexes were observed in both S and V groups as compared to fresh ejaculates (ANOVA $P < 0.05$) (Fig. 2b). Nevertheless these levels of DNA-damaged spermatozoa were significantly higher in groups cryopreserved by slow freezing compared to vitrified groups ($P < 0.001$).

Advanced analysis of the sperm cytoskeleton was also performed. Differences on the impact of slow-freezing and vitrification protocols on this essential cellular structure were observed. Three different α-tubulin labelling patterns were detected in F, S and V groups: (i) total labelling (T-tub; the whole sperm tail is labelled), (ii) discontinuous labelling (D-tub; sperm tail presents a discontinuous labelling pattern) and (iii) final labelling (F-tub; only the end of the sperm tail is labelled) (Fig. 3a).

The differences in the frequencies of these patterns between the study groups were observed (Fig. 3b). Results showed that the α-tubulin distribution seen in fresh spermatozoa was not affected by the vitrification-warming method used. No significant differences were detected between F and V groups in any of the α-tubulin distribution patterns observed. But this was not the case in the slow-freezing group, frequencies of D-Tub and F-Tub patterns in S group were significantly different to those found in F and V groups (ANOVA, $P < 0.05$).

Finally, to examine the effects of vitrification on sperm quality, we performed a PCA based on 16 sperm biomarkers (Table II and Fig. 4). The PCA provided strong evidence that sperm biomarkers vary in freezing and vitrification methods. The first two components of the PCA (PC1-2) with eigenvalues >2 account for more than 50% of the variance. Likewise, significant differences (ANOVA; $P < 0.01$) were observed only for PC1 among F, S and V groups. The PC1 (44.063% of the variance) separates clearly (Bonferroni *post hoc*; $P < 0.01$) the slow frozen, vitrified and fresh semen samples mainly clustered with

Table I Conventional sperm quality parameters (mean ± SEM) for fresh (F), slow frozen–thawed (S) and vitrified-warmed (V) groups.

Parameter	Fresh (F)	Slow frozen–thawed (S)	Vitrified-warmed (V)
Progressive motility (%)	47.67 ± 4.08	11.33 ± 2.70*¥	18.17 ± 2.70*¥
Immotility (%)	34.39 ± 4.06	72.89 ± 4.59*¥	65.28 ± 4.53*¥
Normal morphology (%)	28.10 ± 1.48	16.35 ± 1.77*¥	22.24 ± 1.14*¥
Head damage (%)	44.83 ± 3.05	53.03 ± 3.77	47.88 ± 2.87
Mid-piece damage (%)	15.82 ± 1.28	16.02 ± 2.00	17.92 ± 4.13
Cytoplasmic droplet (%)	3.12 ± 0.44	1.76 ± 0.38	3.49 ± 1.71
Tail damage (%)	25.98 ± 2.94	51.62 ± 3.38*¥	36.88 ± 3.11*¥
Vitality (%)	87.75 ± 1.66	55.13 ± 4.79*¥	76.38 ± 1.53*¥
TZI	1.24 ± 0.04	1.46 ± 0.05*¥	1.33 ± 0.05¥

*Groups are significantly different to control (F) ($P < 0.05$).
¥Study groups (S and V) are significantly different between them ($P < 0.05$). TZI = Teratozoospermia index, i.e. the number of abnormalities present per abnormal spermatozoon.

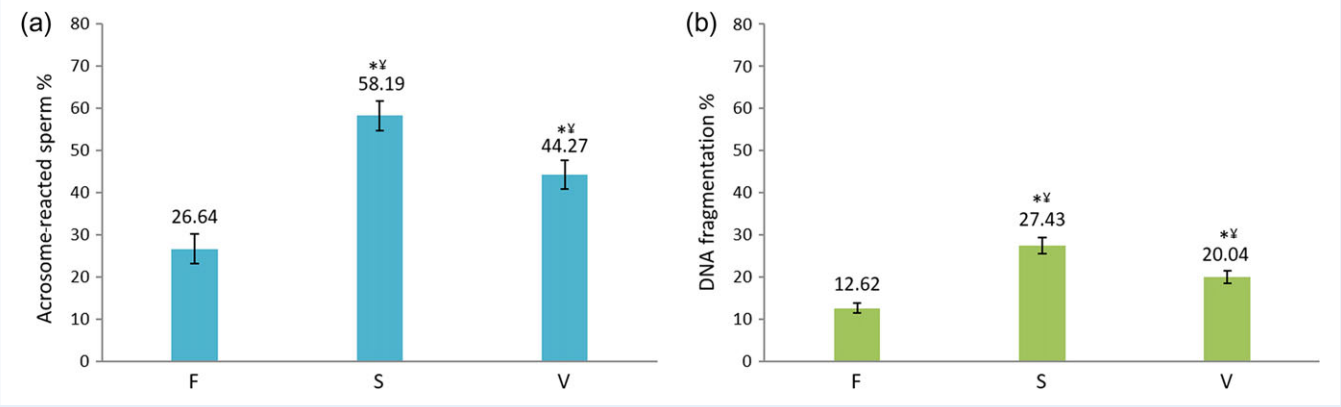

Figure 2 Impact on sperm characteristics of slow-freezing and vitrification protocols. (**a**) Frequency of spontaneous acrosome-reacted sperm in fresh, slow-freezing and vitrification groups. (**b**) Frequency of DNA-damaged spermatozoa in fresh, slow-freezing and vitrification groups. F = fresh ejaculate; S = slow frozen/thawed; V = vitrified/warmed. *Groups are significantly different to control (F) ($P < 0.05$). ¥Study groups (S and V) are significantly different between them ($P < 0.05$).

negative values. Several biomarkers such as motility, DNA fragmentation, acrosome reaction, tubulin labelling and tail damage load most heavily ($r > 0.5$; $P < 0.01$) on the PC1 axis, since the fresh semen samples display lower values than the slow-frozen and vitrified groups, with significantly greater values for slow-frozen samples. Instead, other biomarkers including the progressive motility, discontinuous labelling, vitality and normal morphology show significant negative correlations ($r > -0.5$; $P < 0.01$), because of decreasing values in both the S and V groups compared with those reported from the F group. Therefore, the sperm biomarkers with the greatest correlation at multiparametric scale report the significant changes during slow-freezing and vitrifying processes (Table II). The PC2 (14.054% of the variance) discriminates within individual semen samples mostly affecting the S group in sperm head ($r = 0.563$; $P < 0.01$) and tail-damages ($r = 0.480$; $P < 0.01$) and, marked lower values in D-Tub labelling ($r = 0.422$; $P < 0.01$). Otherwise, negative scores along the PC2 associate semen samples from F and V groups with similar values in T-Tub labelling ($r = -0.796$; $P < 0.01$) and morphologically normal sperm ($r = -0.582$; $P < 0.01$).

Discussion

Results from the present study show that the new vitrification protocol evaluated here adequately preserves sperm quality and renders significantly better results in terms of structural and functional features compared to slow freezing. Regarding sperm motility, vitrification results in higher rates compared to slow freezing. Several studies have described a correlation between poor sperm motility and mitochondrial damage (O'Connell *et al.*, 2002; Piomboni *et al.*, 2012). Compromised ATP production derived from mitochondrial cryo-damage may be transferred to the microtubules participating in motility and hence reduce their activity (O'Connell *et al.*, 2002). Improved preservation of mitochondrial structure and function may be achieved through vitrification compared to traditional freezing.

The assessment of the impact of cryopreservation on sperm morphology showed better results after vitrification in comparison to slow freezing. Ozkavukcu *et al.* (2008) state that extracellular ice crystal formation is the main factor physically affecting cell morphology. They also note that rapid osmolality changes during the thawing-freezing process caused by the addition and removal of cryoprotectants along with the water-entry and exit, may be responsible for the abnormalities observed in the tail. These results are in agreement with our findings revealing a higher number of damaged tails with tail winding in the S group compared to the V group. A reduction of nearly 20% of this type of damage on vitrified spermatozoa may be the result of not applying a permeable cryoprotectant, thus avoiding the negative effect of osmotic changes resulting from ice crystal formation (Isachenko *et al.*, 2004a; Morris, 2006).

Regarding sperm vitality, we observed a decrease of this parameter after both cryopreservation protocols, the decrease being significantly higher on frozen spermatozoa compared to vitrified sperm. The reduction of live spermatozoa observed in both groups, may be due to membrane alterations caused by temperature variations or breakages and micro-breaks that can produce sub-lethal damage (Alvarez *et al.*, 1993; Ozkavukcu *et al.*, 2008). A number of authors have proposed that the decrease in the rate of live spermatozoa after cryopreservation may be due to the osmotic changes, together with the dehydration and rehydration resultant from the water-cryoprotective agent (CPA) exchanges and also to the intracellular crystal formation or ice recrystallization (Morris, 2006; Chaytor *et al.*, 2012). This may explain why the impact on sperm vitality is reduced in the vitrified group since only dehydration by means of the use of non-permeable cryoprotectant is performed and no water-CPA exchanges are produced (Isachenko *et al.*, 2008). Membrane alterations resulting from intracellular crystal formation or ice recrystallization and osmotic and temperature changes may also explain the higher percentage of spontaneous acrosome reaction detected after traditional freezing and the better prevention against cryo-capacitation observed in the vitrification protocol. The temperature drop associated with slow freezing may increase the levels of cytoplasmatic calcium, induce capacitation-related changes and acrosomal-content exocytosis (Silva and Gadella, 2006). In the V group the temperature drop is faster and hence there may be no time for acrosome content release.

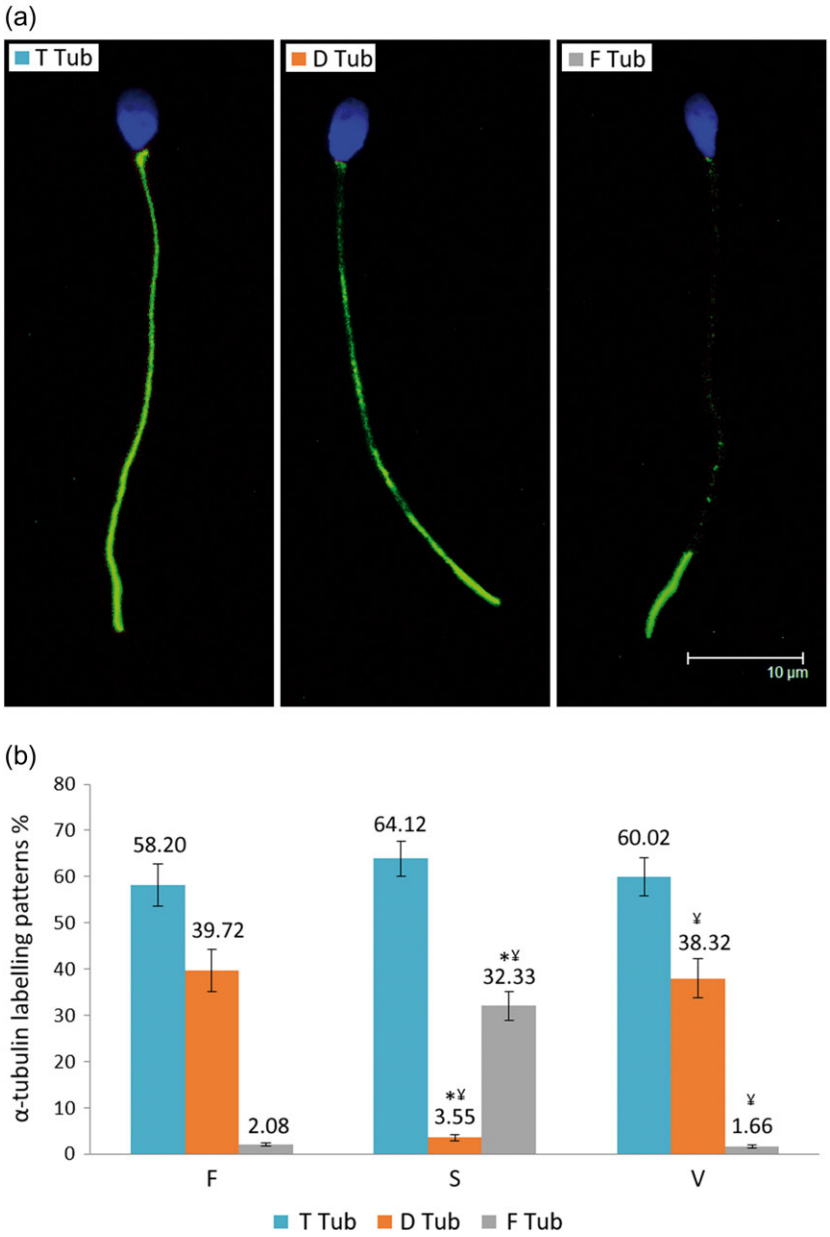

Figure 3 α-Tubulin patterns and their distributions on slow freezing and vitrification. (**a**) α-Tubulin patterns: T-Tub, total labelling; D-Tub, discontinuous labelling; F-Tub, final (tail end) labelling. (**b**) Slow freezing (S) and vitrification (V) impact on α-tubulin distribution patterns. Frequency of spermatozoa with total (T-Tub), discontinuous (D-Tub) and final (F-Tub) labelling. F = fresh ejaculate; S = slow frozen/thawed; V = vitrified/warmed. *Groups are significantly different to control (F) ($P < 0.05$). ¥Study groups (S and V) are significantly different between them ($P < 0.05$).

Cytoskeleton evaluation using α-tubulin detection showed similar distribution patterns and frequencies on F and V samples. A significantly different frequency of α-tubulin patterns was observed however in S samples. These results confirm the low impact of vitrification on the above-mentioned molecule. Previous studies have reported alterations in α-tubulin detection after sperm freezing and have listed several possible causes: (i) weakening of the tail structure caused by the freezing–thawing process; (ii) susceptibility of α-tubulin to posttranslational changes which affects the binding site of tubulin antibody, and hence its binding and signal and (iii) the disassembling of tubulin molecules produced as a result of an income of calcium ions during slow freezing (Doi *et al.*, 2003; Desrosiers *et al.*, 2006; Bhagwat *et al.*, 2014; Gomez-Torres *et al.*, 2017). These three hypotheses, may also explain the changes in distribution observed in the present study between the S group and the F and V groups, indicating that slow freezing leads to α-tubulin localization alterations that are not observed in vitrified or fresh

Table II Variance explained and factor loadings by the first two PCs based on sperm biomarkers within semen samples.

Principal component (PC)	1	2
% variance	44.063	14.054
Eigenvalue	5.261	2.695
Biomarker	***r***	***r***
Immotility	0.827*	−0.086
TUNEL	0.670*	0.191
Acrosome reaction	0.665*	0.355*
Final labelling	0.612*	0.266
Tail damage	0.559*	0.480*
Total labelling	0.407*	−0.796*
Tail damage/total damage	0.385*	0.085
Head damage	0.135	0.563*
Progressive motility	−0.878*	0.017
Discontinuous labelling	−0.768*	0.422*
Vitality	−0.742*	−0.222
Normal morphology	−0.504*	−0.582*
Mid-piece damage/total damage	−0.243	−0.494*
Head damage/total damage	−0.167	0.223
Cytoplasmic droplet	−0.097	−0.224
Mid-piece damage	−0.032	0.255

Significant correlation (*r* Pearson) at $P < 0.01$ (*).

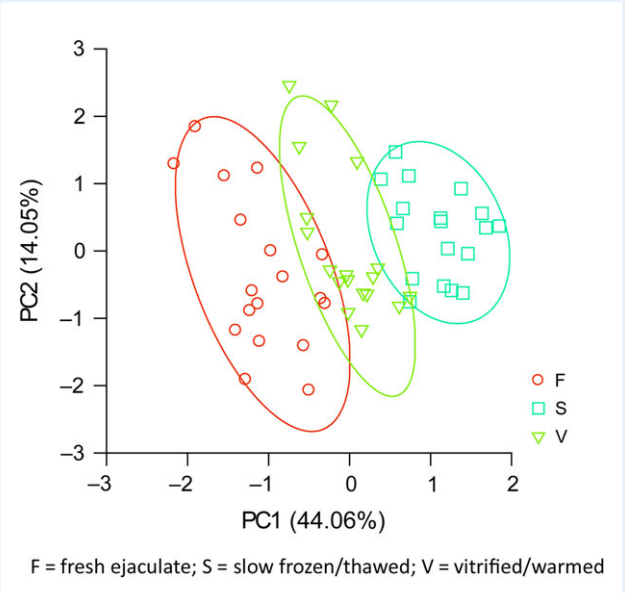

Figure 4 Binary plot of the first two principal components. PC1-2 account for 58.12% of total variance. Note that F vs. V and S vs. V groups exhibit considerable overlap but fresh and slow freezing are clearly separated.

spermatozoa. These alterations may have important consequences on spindle formation and chromosome segregation during mitotic divisions following fertilization.

Adequate DNA integrity maintenance after cryopreservation is crucial for safe transmission of paternal genetic information to the embryo. The presence of DNA breaks in spermatozoa can have a severe negative effect, not only on embryo developmental stages, but also after birth during childhood and adult life (Lewis *et al.*, 2013). It is thus crucial to reduce the DNA damage produced by cryopreservation techniques. A significantly lower SDF was observed after vitrification compared to slow freezing, reducing, on average, the percentage of damaged spermatozoa by about one-third. Differences observed between V and S groups may be due to the usage of glycerol in slow-frozen samples. This substance contributes to the activation of caspases which will ultimately lead to DNA fragmentation (Wündrich *et al.*, 2006). Nevertheless and even though SDF damage on V samples is significantly reduced compared to S samples, V samples damage is still significantly higher than in F samples. Several studies suggest that the main inductor of SDF during cryopreservation is oxidative stress. Elevated reactive oxygen species (ROS) due to an increased production or a defective antioxidant function of semen may have a severe impact on sperm competence (Zribi *et al.*, 2010). Looking at our results, we can hypothesize that ROS production after vitrification is lower than slow freezing.

The global evaluation of structural and functional sperm parameters performed in this study through PCA analysis in pre- and post-cryopreservation groups indicate that the new vitrification protocol evaluated here preserves fresh sperm structural and functional characteristics better than slow freezing. The S group presents parameters that allow their complete differentiation from the F group. The V group presents a set of parameters that allow the separation from the other two groups, although this separation is not so complete. In addition to this, the PCA analysis shows that vitrification preservation of the sperm sample is distinguishable from fresh samples but does not deteriorate the sample as much as slow freezing.

In conclusion, this study shows that the sperm vitrification protocol described in this study allows superior results on all parameters studied (motility, vitality, DNA structure, acrosome, morphology and cytoskeleton) compared to slow freezing and hence, this strategy constitutes an efficient and reliable alternative to conventional freezing methods. Further research requires to be carried out on pathologic samples to determine whether the damage caused by the cryopreservation techniques here are similar and if the application of this methodology in the clinical setting may improve ART outcomes.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Acknowledgements

The authors thank all the patients for donating their samples to this study and IVF-Spain Foundation for economic support of this research.

Authors' roles

J.A. participated in the design of the study and was involved in the manuscript preparation. L.M. participated in the design of the study,

collected and analysed the data and participated in manuscript preparation. M.E. was involved in manuscript preparation and data analysis. J.S. participated in data analysis and figures preparation. A.R. participated in data analysis and figures preparation. M.A.F. supervised sample collection. M.J.G.-T. participated in the design of the study, data analysis and manuscript supervision.

Funding

This study was funded by IVF-Spain Foundation.

Conflict of interest

None declared.

References

Alvarez JG, Lasso JL, Blasco L, Nuñez RC, Heyner S, Caballero PP, Storey BT. Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile lifetime. *Hum Reprod* 1993;**8**:1087–1092.

Bhagwat S, Dalvi V, Chandrasekhar D, Matthew T, Acharya K, Gajbhiye R, Kulkarni V, Sonawane S, Ghosalkar M, Parte P. Acetylated alpha-tubulin is reduced in individuals with poor sperm motility. *Fertil Steril* 2014;**101**: 95–104.

Chaytor JL, Tokarew JM, Wu LK, Leclre M, Tam RY, Capicciotti CJ, Guolla L, von Moos E, Findlay CS, Allan DS *et al*. Inhibiting ice recrystallization and optimization of cell viability after cryopreservation. *Glycobiology* 2012;**22**: 123–133.

Desrosiers P, Legare C, Leclerc P, Sullivan R. Membranous and structural damage that occur during cryopreservation of human sperm may be time-related events. *Fertil Steril* 2006;**85**:1744–1752.

Di Santo M, Tarozzi N, Nadalini M, Borini A. Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. *Adv Urol* 2012;**2012**:1–12.

Doi H, Kawaguchi M, Timasheff SN. Polymerization and calcium binding of the tubulin-colchicine complex in the GDP state. *Biosci Biotechnol Biochem* 2003;**67**:1643–1652.

Fabbri R, Ciotti PM, Di Tommaso B, Magrini O, Notarangelo L, Porcu E, Contro E, Venturoli S, Ostetrica C, Umana R. Tecniche di crioconservazione riproduttiva. *Riv It Ost Gin* 2004;**3**:33–40.

Gomez-Torres MJ, Medrano L, Romero A, Fernandez-Colom PJ, Aizpurua J. Effectiveness of human spermatozoa biomarkers as indicators of structural damage during cryopreservation. *Cryobiology* 2017, http://dx.doi.org/10.1016/j.cryobiol.2017.06.008.

Isachenko E, Isachenko V, Katkov II, Rahimi G, Schöndorf T, Mallmann P, Dessole S, Nawroth F. DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. *Hum Reprod* 2004a;**19**:932–939.

Isachenko V, Isachenko E, Katkov II, Montag M, Dessole S, Nawroth F, van der Ven H. Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. *Biol Reprod* 2004b;**71**:1167–1173.

Isachenko E, Isachenko V, Weiss JM, Kreienberg R, Katkov II, Schulz M, Lulat AG, Risopatrón MJ, Sánchez R. Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose. *Reproduction* 2008;**136**:167–173.

Isachenko V, Isachenko E, Petrunkina AM, Sanchez R. Human spermatozoa vitrified in the absence of permeable cryoprotectants: birth of two healthy babies. *Reprod Fertil Dev* 2012a;**24**:323–326.

Isachenko V, Maettner R, Petrunkina AM, Sterzik K, Mallmann P, Rahimi G, Sanchez R, Risopatron J, Damjanoski I, Isachenko E. Vitrification of human ICSI/IVFspermatozoa without cryoprotectants: new capillary technology. *J Androl* 2012b;**33**:462–468.

Katkov II, Isachenko V, Isachenko E, Kim MS, Lulat AG, Mackay AM, Levine F. Low- and high-temperature vitrification as a new approach to biostabilization of reproductive and progenitor cells. *Int J Refrig* 2006;**29**: 346–357.

Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Veeck LL, Morshedi M, Brugo S. New method of evaluating sperm morphology with predictive value for human in vitro fertilization. *Urology* 1987;**30**: 248–251.

Kuznyetsov V, Moskovtsev SI, Crowe M, Lulat AG-M, Librach CL. Vitrification of a small number of spermatozoa in normozoospermic and severely oligozoospermic samples. *Syst Biol Reprod Med* 2015;**61**: 13–17.

Lewis SEM, John Aitken R, Conner SJ, De Iuliis G, Evenson DP, Henkel R, Giwercman A, Gharagozloo P. The impact of sperm DNA damage in assisted conception and beyond: recent advances in diagnosis and treatment. *Reprod Biomed Online* 2013;**27**:325–337.

Lybaert P, Danguy A, Leleux F, Meuris S, Lebrun P. Improved methodology for the detection and quantification of the acrosome reaction in mouse spermatozoa. *Histol Histopathol* 2009;**24**:999–1007.

Merino O, Aguagüiña WE, Esponda P, Risopatrón J, Isachenko E, Isachenko V, Sánchez R. Protective effect of butylated hydroxytoluene on sperm function in human spermatozoa cryopreserved by vitrification technique. *Andrologia* 2015;**47**:186–193.

Mocé E, Fajardo AJ, Graham JK. Human sperm cryopreservation. *EMJ* 2016;**1**:86–91.

Morris GJ. Rapidly cooled human sperm: no evidence of intracellular ice formation. *Hum Reprod* 2006;**21**:2075–2083.

Nawroth F, Isachenko V, Dessole S, Rahimi G, Farina M, Vargiu N, Mallmann P, Dattena M, Capobianco G, Peters D *et al*. Vitrification of human spermatozoa without cryoprotectants. *Cryo Lett* 2002;**23**: 93–102.

O'Connell M, McClure N, Lewis SEM. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. *Hum Reprod* 2002;**17**:704–709.

Ozkavukcu S, Erdemli E, Isik A, Oztuna D, Karahuseyinoglu S. Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. *J Assist Reprod Genet* 2008;**25**:403–411.

Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. *Int J Androl* 2012;**35**:109–124.

Polge C. Low-temperature storage of mammalian spermatozoa. *Proc R Soc Lond B Biol Sci* 1957;**147**:498–508.

Rienzi L, Gracia C, Maggiulli R, Labarbera AR, Kaser DJ, Ubaldi FM, Vanderpoel S, Racowsky C. Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update* 2016;**23**:1–17.

Sanger WG, Olson JH, Sherman JK. Semen cryobanking for men with cancer—criteria change. *Fertil Steril* 1992;**58**:1024–1027.

Silva PFN, Gadella BM. Detection of damage in mammalian sperm cells. *Theriogenology* 2006;**65**:958–978.

Slabbert M, du Plessis SS, Huyser C. Large volume cryoprotectant-free vitrification: an alternative to conventional cryopreservation for human spermatozoa. *Andrologia* 2015;**47**:594–599.

World Health Organization. *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. Geneva: World Health Organization, 2010.

Wündrich K, Paasch U, Leicht M, Glander H-J. Activation of caspases in human spermatozoa during cryopreservationan immunoblot study. *Cell Tissue Bank* 2006;**7**:81–90.

Zou Y, Yin T, Chen S, Yang J, Huang W. On-chip cryopreservation: a novel method for ultra-rapid cryoprotectant-free cryopreservation of small amounts of human spermatozoa. *PLoS One* 2013;**8**:e61593.

Zribi N, Feki Chakroun N, El Euch H, Gargouri J, Bahloul A, Ammar Keskes L. Effects of cryopreservation on human sperm deoxyribonucleic acid integrity. *Fertil Steril* 2010;**93**:159–166.