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ORIGINAL ARTICLE

Tip-microVapour Fast Freezing: A novel easy method for cryopreserving severe oligozoospermic samples

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Abstract

Background: Sperm cryopreservation is an important procedure for oligozoospermic subjects at risk of azoospermia and after surgical recovery of spermatozoa in non-obstructive azoospermic men. Conventional procedures for sperm cryopreservation might be, however, not suitable for samples with a very low sperm number.

Objectives: In this pilot study, we investigated the recoveries of sperm motility and viability in severe oligozoospermic subjects ($n = 39$) after cryopreservation with a tip-microVapour Fast Freezing, a procedure previously developed by our group for men with good semen quality. Sperm DNA fragmentation was also evaluated in a second group of oligozoospermic samples ($n = 16$).

Materials and methods: We used a Vapour Fast Freezing procedure using 10 μL tips as carrier, and Test Yolk Buffer as freezing medium (tip-microVapour Fast Freezing). In a subset of samples ($n = 22$), we compared recovery of motility and viability as obtained with tip-microVapour Fast Freezing and with a Vapour Fast Freezing procedure using 500 μL straws. Sperm DNA fragmentation was evaluated by the sperm chromatin dispersion test.

Results: We found a recovery rate (median [interquartile range]) of 0.29 (0.13–0.41) for progressive motility, 0.30 (0.21–0.52) for total motility and 0.48 (0.29–0.60) for viability. Interestingly, we observed that samples with the poorest motility were apparently less damaged by freezing/thawing. In a subset of samples ($n = 22$), we directly compared values of viability, progressive motility and total motility by freezing/thawing with tip-microVapour Fast Freezing and Vapour Fast Freezing conducted with 500 μL straws. We found much better values of all sperm parameters in samples after freezing/thawing with tip-microVapour Fast Freezing than with Vapour Fast Freezing in 500 μL straws: that is, progressive motility: 7.00 (3.00–8.50)% versus 2.00 (0.00–4.25)%, $p < 0.001$; total motility: 12.00 (8.00–16.25)% versus 6.50 (1.00–9.25)%, $p < 0.001$; viability: 29.75 (23.75–45.25) versus 22.50 (13.75–28.13), $p < 0.001$, respectively. In the second group of oligozoospermic samples, we found that tip-microVapour Fast Freezing produced lower levels of sperm DNA fragmentation than straws (33.00 [19.75–36.00]% vs. 36.00 [22.75–41.87]%, $p < 0.001$).

Discussion and conclusion: Tip-microVapour Fast Freezing appears to be a very promising method to cryopreserve semen samples from severe oligozoospermic patients.

KEYWORDS
carriers, motility, oligozoospermia, sperm cryopreservation, Vapour Fast Freezing, viability

1 | INTRODUCTION

One in every seven couples experiences fertility problems in the western societies, with a male factor responsible for up to 50% of the cases. Men with severe oligozoospermia or azoospermia account for 3.1% and 4.3% of infertile subjects,[1] respectively, and such percentages are expected to increase over time because of the declining trend in sperm count/concentration recently reported.[2,3] The introduction of intracytoplasmic sperm injection (ICSI) gave the chance of parenthood to couples with severe male factor infertility by allowing the treatment of men with severe oligozoospermia and even those with azoospermia. In men with severe oligozoospermia, an extended search of spermatozoa in semen can be pursued, making possible the recovery of a sufficient number of spermatozoa for oocyte injection. However, these patients are at major risk to become azoospermic than men with mild or moderate oligozoospermia.[4] For azoospermic subjects, percutaneous or microsurgical aspiration from epididymis or surgical retrieval from testis of spermatozoa are available,[5] including those mainly used in case of non-obstructive azoospermia (NOA): testicular sperm aspiration (TESA) and testicular sperm extraction (TESE). These approaches have highly variable sperm recovery rates (RRs): in obstructive azoospermia (OA), 45%–97% for epididymal sperm aspiration[6] and near 100% for TESA and TESE[7,8]; in NOA, 36%–64% for TESE and even lower for TESA.[5,6] Such a scenario poses the possibility that no spermatozoon are found on the day of oocyte pick-up. This situation might provoke ICSI cycle cancellation and request for repetition of surgical sperm recovery, increasing the risk of medical complications other than the psychological and economic burden for the couple.

In this scenario, sperm cryopreservation can be of help for oligozoospermic subjects at risk of azoospermia. In addition, in men undergoing surgical recovery of spermatozoa, it can reduce the number and consequences of repeated surgical interventions and assure that spermatozoa are always available for the ongoing ICSI cycle.

Sperm cryopreservation procedures conventionally consist of adding cryoprotectants, freezing with gradual temperature decrease and finally storing semen samples in liquid nitrogen. These procedures are used for sperm banking in subjects presenting with middle/fine semen quality but appear not suitable for samples with very low sperm number, as they present several drawbacks. First, conventional procedures use relatively large carriers and sample volumes which endanger sperm retrieval after thawing. Second, conventional procedures lead to a high reduction in sperm viability and motility and to sperm loss because of washing step of thawed samples.[9] This issue can be balanced in samples with mild/good quality but represents a serious drawback with samples with very low sperm numbers. Another relevant sperm parameter is the integrity of DNA as it is well known that freezing/thawing provokes an increase in sperm DNA fragmentation (sDF).[10] The latter, in turn, endangers the outcomes of assisted reproductive techniques, including ICSI,[11,12] when frozen samples are used. To overcome some of the drawbacks of conventional cryopreservation procedures, several biological or non-biological carriers have been tested,[6] but the optimal carrier for cryopreservation of a low number of spermatozoa is still sought.

Our group recently tested two procedures of Vapour Fast Freezing (VFF) to cryopreserve spermatozoa in low semen volumes (microVFF) and found that such procedures better maintain sperm viability/motility or DNA quality, depending on the carrier used, with respect to conventional VFF. In particular, using 10 μL tips (tip-microVFF), thawed samples showed percentages of motility and viability similar to a conventional VFF procedure but a lower percentage of sDF.[9] We also found that washing/centrifuging steps after sample thawing provoked a relevant fraction of damage to motile and viable spermatozoa.[9] In this study, we aimed at investigating the recovery of sperm motility and viability in subjects presenting with extremely low sperm number after cryopreservation with a tip-microVFF and skipping the washing/centrifuging steps in thawed samples.

2 | MATERIALS AND METHODS

2.1 | Population and study design

In this pilot study, semen samples were collected from 39 patients with severe oligozoospermia, afferent to the Semen Cryopreservation and Andrology Laboratory of Careggi Hospital, from December 2019 to August 2021, to undergo semen cryopreservation because they were affected by oncological disease (testicular cancer, $n = 7$; lymphoma, $n = 2$; glioma, $n = 1$) or oligozoospermia ($n = 29$). All these subjects were offered sperm banking by a standard procedure used in the laboratory since 1992,[13] that is, a VFF method using 500 μL high security straws (Cryo Bio System) as carrier and Test Yolk Buffer (TYB, containing 20% egg yolk and 12% glycerol as cryoprotectants) (Fujifilm, Irvine Scientific) as freezing medium (from here on indicated as conventional procedure). In all 39 subjects, with remaining semen samples from cryopreservation with conventional procedure, we also used a

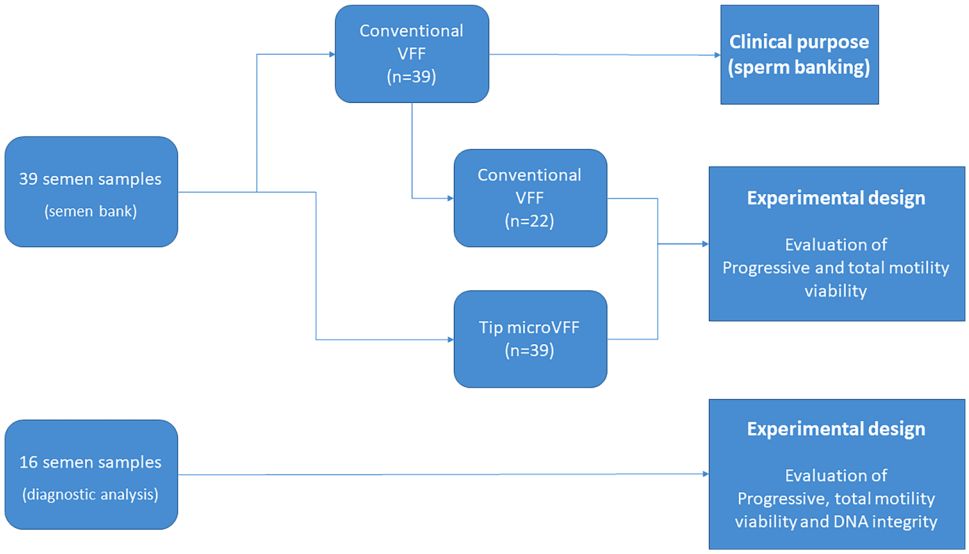

FIGURE 1 Scheme of the study design. VFF, Vapour Fast Freezing.

TABLE 1 Population characteristics ($n = 39$).

Age (year)	Abstinence (day)	Volume (mL)	pH	Sperm concentration (million/mL)	Sperm count (million/ejaculate)
33.54 ± 9.18	4.00 (3.00–5.00)	3.30 ± 1.99	7.60 (7.40–7.80)	1.20 (0.48–2.10)	2.85 (1.14–8.06)

Note: Values are mean ± SD or median (interquartile range).

new method, that is, a VFF using TYB as the freezing medium but 10 μL polypropylene tips as carrier (tip-microVFF).[9] Finally, when the remaining semen volume was at least 150 μL ($n = 22$), an additional straw was prepared in order to compare recovery of motility and viability of conventional procedure to that of tip-microVFF (Figure 1). The population characteristics are reported in Table 1. To compare tip-microVFF and conventional procedure in terms of sperm DNA integrity after thawing, we recruited further 16 patients with oligozoospermia (sperm concentration <15 million/mL) among men undergoing routine semen analysis, resulting in a group with higher sperm concentration and number than the group with severe oligozoospermia (Tables S1 and 1). Table S1 also reports the values for age, abstinence and the other semen parameters as found in fresh samples in this second group. All the recruited subjects signed a written informed consent form. The study was approved by the Ethical Committee of AOU Careggi (protocol no. 15554_bio).

2.2 | Semen analysis

Semen samples were collected after sexual abstinence for 2–7 days (Table 1). After 30 min for liquefaction, semen analysis was conducted following the WHO 2010 guidelines[14] and consisted of determination of: (i) sperm number and concentration, (ii) progressive and total sperm motility, and (iii) sperm viability. Briefly, sperm concentration was determined by using a Neubauer improved cell counting chamber and examining all nine grids, as suggested by WHO manual 2010, in case of very low sperm number[14]; sperm concentration was then multiplied by semen volume to obtain sperm number/ejaculate. Motility was determined, using light microscopy at 40× magnification on fresh and thawed samples by scoring progressive ($a + b$), non-progressive (c) and immobile spermatozoa (d) in 200 cells, in different fields. Total motility ($a + b + c$) was scored as progressive + non-progressive motility. Sperm viability was evaluated by using eosin test: the sample was mixed with an eosin solution (1:2), spread on a slide and examined by light microscopy, scoring stained and non-stained spermatozoa.

The RR of sperm parameters was calculated by the ratio: post-thaw value/fresh sample value. For RR of sperm concentration, we used the fresh value at freezing time after the addition of freezing medium.

2.3 | Sperm cryopreservation

TYB was slowly added (1:1) to semen samples; hence, the samples were aspirated into the straws, which were subsequently sealed on both sides (conventional procedure). For tip-microVFF, samples were aspired by a micropipette into the tips and then carefully inserted one by one into a cryovial for storage. In both procedures, carriers were first cooled in liquid nitrogen (LN_2) vapour for 8 min by placing them in a floating support at 5 cm above the surface of LN_2 (cooling rate of −15.6°C/min). Hence, the carriers were immersed in LN_2.

2.4 | Thawing

After removal from storage, tips were thawed by carefully opening the cryovial still immersed in LN_2, but attention was given to avoid entry of LN_2 into the vial. Hence, tips were placed vertically on a sterile Petri dish until thawing (few seconds). Samples cryopreserved with conventional procedure were thawed by placing them at 37°C for 15 min. Then, we evaluated motility and viability. In the comparison between tip-microVFF and conventional procedure, in the first 13 samples, we also evaluated post-thawed sperm concentration. All tested parameters were evaluated in duplicate.

2.5 | Sperm chromatin dispersion assay

sDF was detected with Halosperm kit (Halotech DNA) by following manufacturer's instructions with slight modifications. Briefly, 50,000 spermatozoa were added to 1% low melting point agarose and layered on pre-coated agarose slides. Slides were then covered with a coverslip until solidification (4°C). Then, samples were treated with the acid denaturation solution and then with the lysing solution (both provided by the kit). Hence, slides were dehydrated with 70% ethanol and then with 100% ethanol. Staining was conducted with eosin (15 min at RT) and thiazine (15 min at RT) solutions. After drying, slides were scored for halos by bright field microscopy by counting at least 200 spermatozoa. sDF was expressed as the percentage of spermatozoa without or with small halo on total spermatozoa.[9]

2.6 | Statistical analyses

Data were analysed with Statistical Package for the Social Sciences (SPSS 25) for Windows (SPSS, Inc.). The Kolmogorov–Smirnov test was used to check the normal distribution of the variables and data are expressed as the mean ± SD or median (interquartile range), accordingly. We used the Wilcoxon signed rank test to compare: (i) post-thawing values to the fresh ones and (ii) after-thawing sperm parameters obtained with conventional procedure and tip-microVFF. Correlation analysis between the fresh–thawed difference and fresh values was evaluated by calculating the Spearman's correlation coefficient (r). To evaluate the regression to the mean and mathematical coupling effects, we applied the Oldham test.[15] The comparison between conventional procedure and tip-microVFF was sized considering progressive motility as primary endpoint.

2.7 | Sample size calculation

Preliminary experiments of comparison between conventional and tip-microVFF indicated that paired differences showed a mean ± SD of 3.89 ± 4.73 and a normal distribution. Hence, assuming a power of 0.90 and an alpha error of 0.05, the number of subjects to be recruited resulted to be 19, as calculated by a two-sided Wilcoxon signed rank test for quantitative, non-parametric and paired data. Analysis of sample size was computed using PASS software (PASS 2022, v22.0.2, NCSS).

3 | RESULTS

The results of tip-microVFF in the 39 oligozoospermic samples are reported in Table 2. As shown, tip-microVFF recovered nearly all frozen spermatozoa (concentration RR = 0.85), whereas, as expected, both progressive and total motility were highly decreased (RR = 0.29 and 0.30, respectively). Sperm viability was less affected by freezing/thawing, showing an RR of 0.48. Interestingly, when we plotted the difference between fresh and thawed values against fresh values, we observed a sharp direct correlation for progressive ($r = 0.95$, $p < 0.001$, Figure 2A) and total motility ($r = 0.87$, $p < 0.001$, Figure 2B) and viability ($r = 0.43$, $p < 0.01$, Figure S1). Therefore, these results might indicate that the better the value of the aforementioned parameters, in fresh samples, the more severe was their deterioration during cryopreservation. We also investigated whether such relationships were merely because of a regression to the mean and/or mathematical coupling effect, by analysing data with Oldham test.[15] The results showed no evidence of relationship between differences in fresh–thawed and fresh values for sperm vitality (Pearson's correlation coefficient: 0.08; 95% confidence limits: −0.24, 0.39; $p = 0.609$), at variance with both progressive (Pearson's correlation coefficient: −0.88; 95% confidence limits: −0.93, −0.77; $p < 0.001$) and total motility (Pearson's correlation coefficient: −0.69; 95% confidence limits: −0.83, −0.48; $p < 0.001$).

In a subset of 22 semen samples, we compared motility and viability after conventional procedure and tip-microVFF. The results are reported in Figure 3, showing that the new method better recovered both progressive and total motility than conventional procedure (Figure 3A,B). This result is also present when evaluating viability (Figure 3C). Consistently, using tip-microVFF, RR for progressive (RR = 0.29 [0.13–0.41] vs. 0.14 [0.00–0.21], $p < 0.001$) and total motility (RR = 0.30 [0.21–0. 52] vs. 0.19 [0.03–0.29], $p < 0.001$) were higher than those of the conventional procedure. Similar results were found for viability (RR = 0.49 [0.29–0.60] vs. 0.29 [0.18–0.41], $p < 0.001$). Regarding sperm concentration ($n = 13$), the two procedures showed similar recoveries, with a ratio value between after and before cryopreservation of 0.91 [0.68–1.00] for microVFF and 0.87 [0.74–1.00] for conventional procedure ($p = 0.374$). To assess whether baseline semen alteration could affect the comparison between the two cryopreservation methods, we grouped subjects according to baseline semen quality. These analyses, however, confirmed better RRs with tip-microVFF than straws in subjects both below and above the median values of baseline progressive motility, sperm count and viability (data not shown).

To verify whether tip-microVFF protected sperm DNA integrity better than 500 μL straws, we recruited further 16 patients with oligozoospermia among subjects undergoing routine semen analysis. Figure 4 reports the results of this comparison showing the expected increase in sDF after thawing but a lower level of sperm DNA damage

TABLE 2 Recovery of sperm concentration, progressive and total motility and viability after cryopreservation with tip-microVapour Fast Freezing (VFF) ($n = 39$).

	Sperm concentration (million/mL)	Progressive motility (%)	Total motility (%)	Viability (%)
Fresh	0.90 (0.50–2.00)	15.00 (7.00–29.00)	30.00 (17.00–40.00)	75.00 (58.50–80.00)
Thawed	0.80 (0.43–1.50)	4.00 (2.00–8.00)	11.00 (4.00–15.00)	29.5 (20–40.50)
p-Value[a]	<0.001	<0.001	<0.001	<0.001
Recovery	0.85 (0.73–1.00)	0.29 (0.13–0.41)	0.30 (0.22–0.52)	0.48 (0.29–0.60)

Note: Data are median (interquartile range).
[a]Wilcoxon signed rank test.

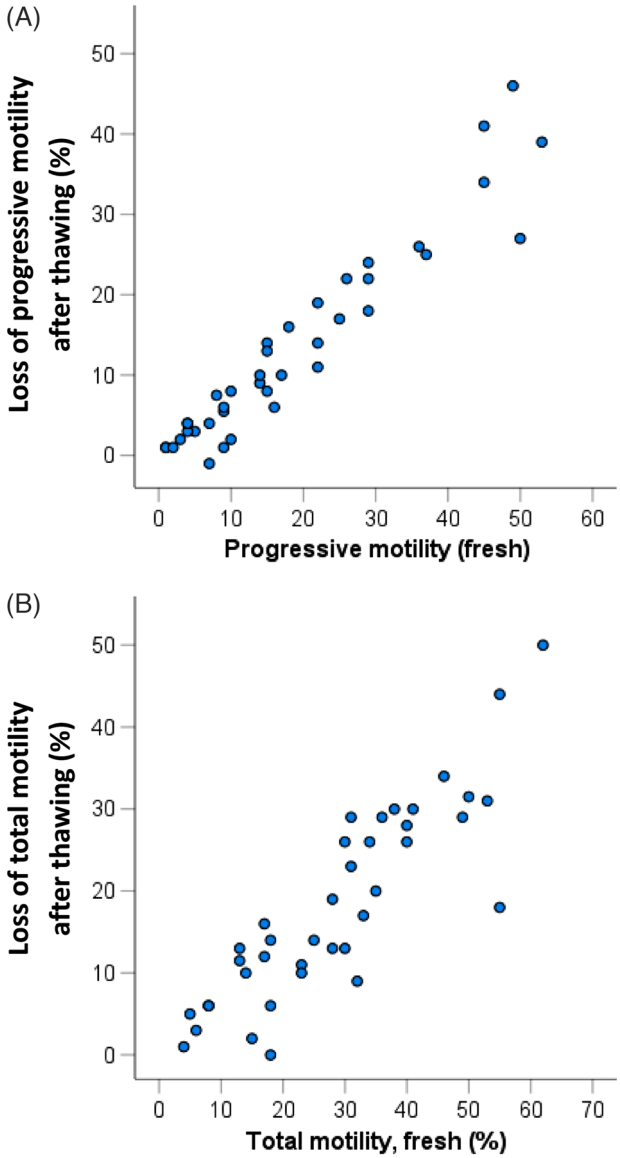

FIGURE 2 Dispersion plots reporting differences in fresh–thawed values (loss) against basal progressive (A) and total motility (B).

in samples cryopreserved with tips than straws. The results of motility and viability in these 16 patients confirmed a better recovery of progressive motility by tips (4.50 [2.00–10.00]%) than straws (4.00 [2.00–8.00]%, $p < 0.05$). A trend towards higher recoveries, albeit not statistically significant, was also observed for total motility (9.00 [3.25–15.75]% vs. 7.50 [3.50–9.75]%, tips vs. straws, respectively) and viability (34.50 [22.50–40.00]% vs. 27.00 [24.00–39.00]%, tips vs. straws, respectively).

4 | DISCUSSION

In this pilot study, we show that the use of tip-microVFF to cryopreserve semen samples with very low sperm counts guarantees a better recovery of both motility parameters and viability of spermatozoa than conventional procedure. Interestingly, the new method appears to preserve sperm motility, especially when poor values are present in fresh samples; hence, there is a pressing need for small-volume cryopreservation procedure.

This project stems from a previous study by our group, aimed at comparing the effect of freezing with microVFF and that of the conventional VFF in 500 μL straws in samples of patients with normal sperm count and motility.[9] That study found that microVFF by using 10 μL tips yielded similar recovery of motility and viability but lower sperm DNA damage than conventional VFF in 500 μL straws.[9] The ultimate scope of current research was to develop a new method to cryopreserve very low semen volumes, in order to extend the service of sperm banking to patients with low sperm counts. Because it is well known that recovery of sperm motility and viability highly depends on baseline semen quality,[13,16] in the current study, we challenged tip-microVFF using samples from severely oligozoospermic patients, and in a subset of samples, we compared results with those found using 500 μL straws. Surprisingly, we found that the use of tips yielded very good after-thawing values of both motility and viability, resulting even better than those previously published in 2013 by our group in a similar group of patients (i.e., oligozoospermic ones) using the same procedure but with 500 μL straws.[13] Indeed, Degl'Innocenti et al.[13] showed that the median value of recovery approximated 0% for motility parameters and 20% for viability ($n = 219$), versus 4% and 11% for progressive and total motility, respectively, and 29.5% for viability observed in the 39 recruited men of this study. The better recovery of motility and viability obtained with 10 μL tips than 500 μL straws was also confirmed by the direct comparison done in this study in 22 samples (Figure 3), which also showed that after-thawing motility and viability with straws are similar to those reported in 2013.[13]

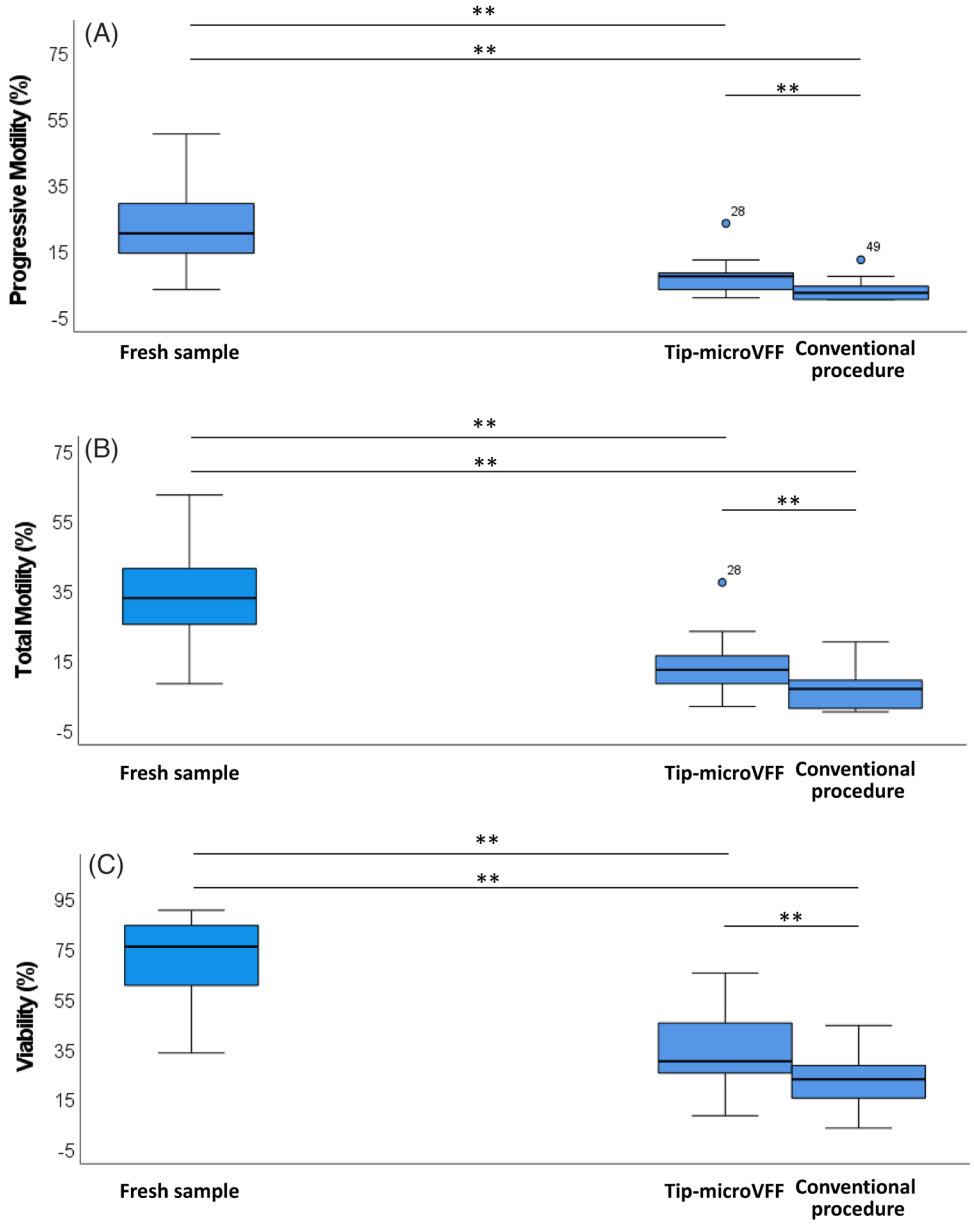

FIGURE 3 Box graphs reporting median values (interquartile range) of progressive (A) and total (B) motility and viability (C), as found before and after cryopreservation with conventional procedure and tip-microVapour Fast Freezing (VFF). $^{**}p < 0.001$; Wilcoxon signed rank test.

As mentioned, tip-microVFF resulted safer than conventional procedure in terms of sperm DNA integrity in semen samples with good quality.[9] To confirm this finding, because of the very low availability of sperm counts in severely oligozoospermic subjects, we recruited a second group of subjects with moderate oligozoospermia, where we compared tip-microVFF and conventional procedure in terms of damage to DNA. We found that straws yielded higher post thawing values of sDF than tips, although the latter remained over the 30% threshold.[17,18] These results further underline the importance of the carrier for protection of semen samples from cryodamage in oligozoospermic subjects, as already indicated by others.[19] As mentioned, when the comparison between tips and straws was conducted in samples with good semen quality, tips and straws yielded very similar results,[9] contrary to what was reported in this study with oligozoospermic samples. One possible explanation for this result might rely on the fact that in current study, we skipped the washing/centrifugation step after thawing samples. The method to thaw samples is very important in terms of induced damage by different cryopreservation procedures. For instance, we recently showed that the additional damage induced by vitrification with respect to VFF was nearly blunted when motility and viability were immediately evaluated after thawing, skipping the centrifugation for sample washing.[9] An alternative intriguing explanation of the different results obtained in oligozoospermic subjects versus normozoospermic ones might rely on intrinsic characteristics of the samples. Indeed, we found a sharp relationship between both progressive and total motility in fresh samples and their loss during cryopreservation, indicating that the lower is the value before and the lower is the damage after thawing. As we excluded that such relationship was

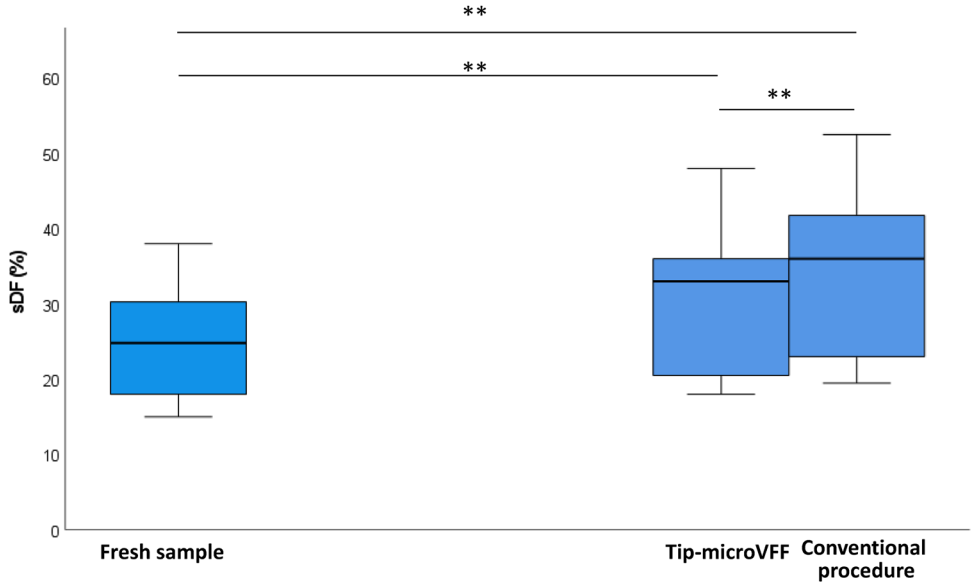

FIGURE 4 Box graphs reporting median values (interquartile range) of sperm DNA fragmentation (sDF), as found before and after cryopreservation with conventional procedure and tip-microVapour Fast Freezing (VFF). $^{**}p < 0.001$; Wilcoxon signed rank test.

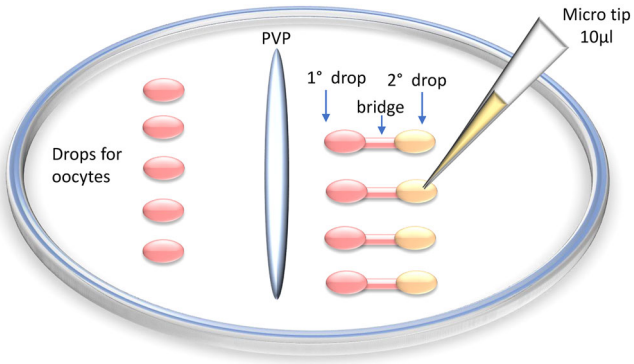

FIGURE 5 A cartoon depicting the suggested use of tip-microVapour Fast Freezing (VFF) thawed samples in the assisted reproductive technique cycle. PVP, polyvinylpyrrolidone.

due to mathematical coupling and/or regression to the mean effects, we can conclude that the worst samples may be somehow more protected by injuries to sperm motility by freezing/thawing with respect to good samples.

As mentioned, in this study, sample washing after thawing was skipped in tip-microVFF, in order to limit sperm loss as already reported with very small semen volumes.[20–22] This aspect guarantees a shortened time for handling samples and avoids noxious effects of centrifugation.[9] On the other hand, skipping washing step might be detrimental for semen samples, as cryoprotectant compounds could be toxic to cells and tissues.[23] The strength point of the study is the idea to overcome this limitation by preparing an ICSI plate with some 20 μL drops of sperm medium, linking them in pair with a thin medium bridge using a pipette (Figure 5) and covering with mineral oil until use. After thawing as described in M&M, tip can be discharged in a drop, allowing spermatozoa to swim across the medium bridge for washing and selection.

Beside the ease of handling sample, the new method is cheap, as it is time-saving and does not require special equipment and material. In addition, it is safe, as tips are stored in closed vials thus preventing viral cross-contamination. Hence, tip-microVFF appears to overcome most drawbacks previously reported with other carriers.[24–28]

This study has two major limitations. Firstly, although better recovery of motility, viability and DNA integrity was observed (Ref.[9] and present study), tips were not tested for biocompatibility at temperature of liquid nitrogen. Secondly, up to now, we have no data on reproductive outcomes with spermatozoa frozen in tips.

In conclusion, this study presents an easy, cheap and simple new method to cryopreserve semen from severely oligozoospermic subjects. In addition, tip-microVFF yields very good values of recovery of both sperm motility and viability. Indeed, such values result much better than those found with 500 μL straws observed in this and in a previous study by our group[13] conducted with similar subjects. Hence, tip-microVFF appears to be very promising new method to cryopreserve semen samples with very low sperm numbers, such as those of severely oligozoospermic subjects.

AUTHOR CONTRIBUTIONS

Sara Dabizzi conceived and designed the study, performed cryopreservation procedures and participated in writing the manuscript. Selene Degl'Innocenti and Costanza Calamai performed cryopreservation procedures. Costanza Calamai also performed sperm DNA fragmentation determination. Luca Boni performed Oldham test. Mario Maggi and Linda Vignozzi prompted the group to challenge the conventional VFF procedure and critically discussed the results. Monica Muratori performed statistical analyses and drafted the manuscript. All authors critically reviewed the manuscript and gave their approval.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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