Research Paper E-ISSN NO : 2455-295X | VOLUME : 2 | ISSUE : 11 | NOV 2016

CRYOPRESERVATION OF TESTICULAR HUMAN SPERMATOZOA BY TWO TECHNIQUES: COMPARISONS OF MSOME, DNA FRAGMENTATION AND MITOCHONDRIAL MEMBRANE POTENTIAL

Sahabeh Etebary [1] | Mohammad Ali Khalili* [2] | Nahid Yari [1] | Seyed Mehdi Kalantar [3] | Morteza Anvari [2]

[1] Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

[2] Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

[3] Reproductive and Genetic Unit, Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

ABSTRACT

Purpose To evaluate the efficacy of two testicular spermatozoa cryopreservation techniques in Azoospermic patients with MSOME, DNA fragmentation and mitochondrial assays.

Methods Testicular sperm extraction samples were obtained from 15 men, aged 20-40 years old with obstructive azoospermia. The prepared samples were divided into three groups. Group I was assessed freshly. Group II was cryopreserved with vitrification method, and Group III was cryopreserved with cooling in liquid nitrogen vapor using droplet. After warming, the samples were examined in terms of motility, morphology with MSOME, DNA fragmentation and mitochondrial membrane potential. For morphologic assessment spermatozoa were classified as class 1 (High-quality), class 2 (Medium-quality) and class 3 (Low-quality).

Results There was significant reduction in motility, viability and the mean percentage of class 1 spermatozoa after cryopreservation by both techniques ($p<0.001$). There was no significant difference between groups II and III regarding morphologic class 1, 2 and 3 ($p>0.05$). The DNA fragmentation was significantly increased to 29.48 % and 29.72 % respectively; also the number of sperms with functional mitochondria significantly decreased to 31.81 % and 31.98 % after vitrification and freezing in liquid nitrogen vapor ($p<0.001$). A correlation was found between morphologic class1 and 3 spermatozoa; mitochondrial membrane potential and DNA fragmentation.

Conclusions cryopreservation had adverse effects on spermatozoa parameters. The best method of testicular sperm cryopreservation should yet to be determined, but selecting spermatozoa with minimum abnormality is an important issue in infertility treatment. To reach this goal assessment of frozen-thawed testicular spermatozoa with MSOME will be of particular value.

Keywords: Cryopreservation, Human spermatozoa, Vitrification, DNA fragmentation, Mitochondrial membrane potential, Azoospermia.

Introduction

The technique of testicular sperm extraction (TESE) was first described by Schoysman et al. (1993) to retrieve spermatozoa in azoospermic patients [1]. While, this approach is frequently successful, it has several disadvantages: TESE procedure must be repeated for each intracytoplasmic sperm injection (ICSI) cycle with the probable risk of damage to the testis [2], and inflammatory changes, it may, as well, result in vascular injuries and intratesticular bleeding, which occur during testicular surgery [3-5]. An alternative solution to avoid these difficulties is testicular sperm cryopreservation and using frozen-thawed sperm in ICSI cycles [2]. This approach prevents the need to synchronize testicular biopsy and oocyte retrieval. It also helps to perform surgical procedures in different days for the couples. .

There are still controversies regarding ICSI outcome using frozen-thawed testicular spermatozoa. Some studies showed lower fertilization and/or pregnancy rates with frozen-thawed sperm in comparison with fresh samples [6, 7] while, others claim that cryopreserved sperm can function as well as fresh sperm and maintain post-thaw viability [8, 9]. In a study by Ghanem and colleagues (2005) the outcome of using cryopreserved TESE-extracted spermatozoa in terms of implantation and pregnancy rates per embryo transfer was comparable to fresh TESE samples [10].The post-thaw survival rate and the motility of cryopreserved spermatozoa depend on several factors including: the quality of initial sperm, the cryoprotectant, the reezing and thawing protocol as well as dilution and washing procedures [11]. On the other hand, the cryopreservation of testicular samples is difficult to manage because of low numbers of spermatozoa and the presence of RBCs [12]. Based on these factors, different techniques for sperm cryopreservation have been used, such as slow freezing [13], freezing in liquid nitrogen vapor [14] and vitrification [15]. Rapid freezing is routinely used for the cryopreservation and in comparison to slow freezing provides better post-thaw sperm motility and recovery [16]. Furthermore, vitrification may be more applicable when compared to rapid freezing technique [17]. However, reports about the real efficacy of these techniques in large series are lacking [18]. Previous studies showed that sperm cryopreservation is associated with sperm motility, morphology and viability changes [19].

Recently, many techniques have been achieved for accurate evaluation of sperm parameters regarding sperm motility, viability,

morphology, energy status, damage to plasma membrane and DNA stability [20, 21]. One example of these techniques is motile sperm organelle morphology (MSOME) which was developed by Bartoov and colleagues [22]. MSOME is performed by means of an inverted microscope equipped with high-power differential contrast which helps the real-time assessment of sperm morphological characteristics at high magnification (up to 6600x) [23]. Balaban and coworkers (2011) showed higher rates of implantation, pregnancy and live birth following ICSI of spermatozoa morphologically selected by MSOME [24]. In addition, another method for sperm evaluation is assessment of changes in mitochondrial membrane potential, which was proposed as an early apoptotic process, seen in infertile men [25]. Kasai and colleagues indicated that in vitro fertilization rates were higher in a group of sperm samples with higher mitochondrial potential [26]. In line with previous reports Tompson et al. (2009) reported that the process of cryopreservation resulted in an increase in DNA fragmentation in human spermatozoa [27]. Sperm DNA integrity is one of the other valuable sperm quality assessment parameters proven to be associated with male infertility and correlated with assisted reproductive technology (ART) outcomes [28]. Therefore, the aim of this study was to compare the efficacy of two different cryopreservation techniques on sperm motility, fine morphology, DNA fragmentation and mitochondrial membrane potential in azoospermic men.

Materials and Methods

Samples

In this cross-sectional experiment, TESE samples were obtained from 15 men aged 20-40 years old with obstructive azoospermia referred to Yazd Research and Clinical Center for Infertility from February to July 2016. Demographic data and medical history were documented from their medical records. For each patient, the presence of at least 1 million spermatozoa in TESE samples was essential. Additionally cases with no motile spermatozoa in TESE sample, as well as those with a history of varicocele or diabetes and tobacco addiction were excluded. Informed written consent was obtained from all participants. The study protocole was approved by ethics committee of Yazd Research and Clinical Center for Infertility.

TESE sample preparation

The collected testicular tissue was rinsed in sperm washing medium (Quinn's Sperm Washing Medium, SAGE IVF Inc., CT, USA) to remove the blood and to be transferred to a sterile petri dish. Using a pair of sterile iris scissors, the tissue was minced and 1 ml of the sperm washing medium was added to minced tissue. The dish was tilted, the medium was aspirated, and the suspension was transferred to a conical tube. The tissue fragments were remained in the dish. 2 ml of the sperm-washing medium was added to the petri dish. A 5-cc syringe was attached to a 21-gauge needle, to gently aspirate the tissue suspension repeatedly. The suspension then was transferred to the same conical tube and let to settle for 5 mins. The supernatant was transferred to a new conical tube. 1-2 ml of the sperm-washing medium was added to the tissue pellet and mixed with a pipette. After 5 mins the tissue fragments were settled and the supernatant was transferred to the conical tube and centrifuged at 400 g for 10 mins. The supernatant was removed gently, leaving the pellet at the bottom. 3-4 ml of RBC lysis buffer was added to the pellet and centrifuged at 400 g for 5 mins. Afterwards the supernatant was aspirated and threw away. The pellet was re-suspended in 3 ml of the sperm-washing medium and centrifuged at 400 g for 10 mins to wash off the RBC lysis buffer [29]. The supernatant was discarded and the pellet was suspended in 0.5 ml of the sperm-washing medium.

The prepared TESE samples were divided into three aliquots. The first part (group I), was assessed regarding the count, motility, viability, morphology, mitochondrial membrane potential and DNA fragmentation. The second part (Group II) was cryopreserved with vitrification method, and the third part (Group III) was cryopreserved with cooling in LN vapor. After a week, warming was done for the last two groups and all the aforementioned parameters were examined.

Vitrification of samples

Vitrification was done according to Isachenko (2008) [15]. The sperm suspension was mixed with sperm freezing solution (Vitrolife, Sweden) briefly in a ratio of 1:1 and left at room tempreture for 10 mins. A basket was plunged into a container filled with liquid nitrogen. By using a micropipette in 45° and 10 cm from liquid nitrogen level, 30 µl drops of sperm suspension were dropped into the liquid nitrogen. The drops formed into a spherical shape in contact with liquid nitrogen and plunged in the basket after a few sec. The solidified droplets were collected and put into cryovials and stored in liquid nitrogen for at least 1 week until warming.

Cooling in liquid nitrogen vapor using droplet

Cooling in LN vapor using droplet was performed as described by Isachenko (2005) [30]. Sperm suspension was mixed with sperm freezing solution in a ratio of 1:1 and was kept at room temperature for 10 mins. Then, aliquots of 30 ml of sperm suspension were located onto sterile aluminum foil previously cooled in LN vapor at distance of 30 cm. During cooling, the droplet of sperm suspension adopted a spherical form. After 5 mins of cooling, these solidified droplets were placed into cryovials, pre-cooled in liquid nitrogen. Then, the vials were plunged into liquid nitrogen for subsequent storage until the time of warming.

Warming

For warming, up to five spheres, one by one, were plunged into 5 ml Ham's F10 supplemented with 5 % HSA which was pre-warmed to 37°C and vortexed for 5–10 s. Finally; the spermatozoa were centrifuged at 400 g for 5 mins and pelleted. The post-warming sperm suspension was maintained at 37° C under 5 % CO_2 for 1 hr. and sperm analysis assays were done as described above.

Motility and viability assessment

10 µl of prepared sample was put on Makler' s chamber and assessed by light microscope under 400x according to WHO 2010 criteria

[31]. Approximately, 100 spermatozoa were assessed for different categories and the result was expressed as percentage.

HOS (Hypo-osmotic swelling test) test was done according to Jeyendran and colleagues (1984) [32]. 100 µl of sample was mixed with 1 ml of HOS solution followed by 30 mins incubation at 37° C. Then 1 drop of sample was assessed under a light microscope to evaluate the results. HOS solution was prepared by mixing 7.35 g sodium citrate (Na3C6H5O7.2H2O) and 13.51 g fructose in 1000 ml distilled water [33]. The osmolality measured (Osmomat 030 Gonotec; Van Hopplynus, Brussels, Belgium) was 155 mosmol/kg. Spermatozoa with intact membranes swelled within 5 mins in HOS medium and all flagellar shapes were stabilized by 30 mins. Finally, spermatozoa with a swollen (alive) and un-swollen (dead) tail were calculated.

Morphology evaluation by MSOME

5µl drops of MOPS medium (Vitrolife, Sweden) overlaid with mineral oil (Ovoil 100, Vitrolife, Goteborg, Sweden) placed in glass bottom dish (WillCo-Dish, Amsterdam, Netherlands) were incubated for 20 mins. Then, 1 µl of sperm suspension was added to the microdroplets. The dish was put on microscope stage on the objective lens. The inverted microscope (TE300; Nikon, Tokyo, Japan) equipped with high power differential interference contrast optics (Haffman) was used for MSOME (6600×). The image capturing and video recording for further analysis were performed with software (OCTAX Eyeware; Octax). The morphological assessment was done on the monitor and the captured pictures. Observed spermatozoa were classified into 3 classes according to Cassuto scoring system in which head, vacuoles and the base of spermatozoa were evaluated and the suggested formula was (Normal head score : 2) + (Lack of vacuole score: 3) + (Normal base score: 1) = (Total score: 6) for a morphologic ‘‘normal top’’ spermatozoon [34]. At least 100 spermatozoa per sample were evaluated. Then the classifications for the quality of the spermatozoa were calculated as follows:

Class 1 (score of 4 to 6): High-quality spermatozoa

Class 2 (score of 1 to 3): Medium-quality spermatozoa

Class 3 (score of 0): Low-quality spermatozoa

DNA fragmentation assessment

The TUNEL assay was carried out according to the manufacturer’s (Roche Diagnostics GmbH, Manheim, Germany) protocol. PBS/1% BSA was added to the prepared testicular pellet to reach a final concentration of 1×10^6 cells/100 ml of sample. Then on a shaker, a 100µl aliquot of 1% paraformaldehyde was added for 1 h at 15–25° C. After fixation, the cells were washed once with 1ml of PBS/1% BSA. Then, the cells were re-suspended in 100 ml of permeabilization solution (consisting of 0.1% Triton X-100 in 0.1% sodium citrate) for 2 mins on ice. After being washed once with PBS/1% BSA, the labelling reaction was done by incubation with 50ml of labeling solution (supplied with in situ Cell Death Detection Kit, Fluorescin, Roche Diagnostics GmbH, Mannheim, Germany) containing terminal deoxynucleotidyl transferase (TdT) for 1 h at 37° C in the dark. A negative control was prepared by omitting TdT from the reaction mixture for each sample. After labelling, two subsequent washes in PBS/1% BSA were performed and the sample was resuspended in PBS/0.1% BSA to a volume of 500–700ml and counterstained with propidium iodide (0.5ml/ml). Preparation of positive controls for each sample was done as mentioned above, but with further treatment with 2 IU of DNase (Roche Diagnostics GmbH) for 15 mins at 37° C before doing the labelling reaction. Green fluorescence (TUNEL-positive cells) and red fluorescence (propidium iodide-labelled cells) were measured using a 530 ± 30 nm band-pass filter, and a 620 nm long-pass filter, respectively. The two colors were separated by a 560 nm dichroic filter.

Mitochondrial Membrane Potential

Mitochondrial membrane potential was assessed by fluorescent dye JC-1. 5,5',6,6' -tetra-chloro-1 ,1',3,3'-tetraethylben- zimidazolyl-carbocyanine iodide (JC-1, CS0390, Staining Kit, SIGMA ALDRICH, USA). The stock solution was prepared at 1 mg/mL in dimethylsulfoxide (DMSO). Sperm suspention was prepared with the concentration of 50000/ml. 1 ml of sperm suspension was mixed with 1 ml of Staining Solution (25 ml of the 200´ JC-1 Stock Solution in 4 ml of ultrapure water) and incubated for 20 mins at 37 °C in 5% CO2. The cell suspension was centrifuged at 600 *g* for 3–4 mins at 2–8 °C. The supernatant was aspirated and the tube with the cell pellet was placed on ice. The cells were washed with 5 ml of the ice-cold 1´ JC-1 Staining Buffer (10 ml of the 1´ JC-1 staining buffer by diluting 2 ml of the JC-1 staining buffer 5´ with 8 ml of water), and then the cells were re-suspended in 5 ml of the ice-cold 1´ JC-1 staining buffer. The sample was kept on the ice and analyzed within 30 mins after staining. Under fluorescent microscope, the monomeric dye structure emits at 527 nm, while J-aggregates in non-damaged (healthy) mitochondria emit at 590 nm. When mitochondria were intact, the JC-1 reagent aggregate inside the healthy mitochondria and fluoresce red color. If the mitochondria are damaged, the mitochondrial membrane potential was breaking down and the JC-1 reagent was seen dispersed though the entire cells and fluoresce green color [15].

Statistical analysis

Data were analyzed using SPSS version 20 for software (SPSS, Inc., Chicago, IL, USA). The data are given as the mean ± standard deviation (SD). Comparison of means between groups was evaluated by ANOVA test. Spearman's correlations between parametric and Pearson correlations between non-parametric variables of groups were obtained. The level of statistical significance was $p< 0.05$.

Results

Sperm parameters

The mean age of study group was 32.07 ± 5.06 years (range 24–39) and the duration of infertility was 3.53 ± 2.1 (range 1–8). Table 1 illustrates the comparison of sample parameteres of the three groups: before freezing, after vitrification and cooling in liquid nitrogen vapor. There was a significant reduction in motility and viability after both freezing methods when compared with the fresh sample (p<0.001). Similar results were observed for groups II and III, although. Other parameters (count and morphology by strict criteria) showed no significant differences in three groups (p>0.05).

Morphologic assessment by MSOME:

The comparison between the three groups by MSOME is presented in Fig. 1. The mean percentage of class 1 spermatozoa (normal morphology) significantly decreased after cryopreservation by both techniques (p<0.001). Class 2 and 3 showed no significant difference (p>0.05). There was no significant difference between the vitrification and vapor groups regarding morphologic classes 1, 2 and 3(p>0.05).

DNA fragmentation and mitochondrial membrane potential:

The mean percentage of DNA fragmentation was 11.653 ± 3.10 in the group I, 29.487 ± 6.69 in group II and 29.727 ± 6.28 in group III which significantly increased after vitrification and cooling in liquid nitrogen vapor (p<0.001). The mean percentage of mitochondrial membrane potential was 54.755 ± 4.05 in group 1; 31.814 ± 2.74 in group 2 and 31.981 ± 2.92 in group 3. The number of sperms with functional mitochondria was significantly decreased after cryopreservation by two techniques (p<0.001) (Table 2).

Correlation analysis showed a positive correlation between morphologic class 1 (p=0.0001, Pearson rank correlation r = 0.57) and a negative correlation for class 3 (p=0.009, Pearson rank correlation r = - 0.38) with mitochondrial membrane potential. Also a negative correlation for class 1 (p=0.005, r = - 0.41) and a positive correlation for class 3 (p=0.03, r = 0.31) and DNA fragmentation was seen (Fig. 2).

Table 1: Sample characteristics of three study groups (n = 45)

Variables (mean ± SD)	Group 1 (Before freezing)	Group 2 (After vitrification)	Group 3 After freezing in LN vapor	*P* value
Count × 10^6	4.19 ± 2.32 [a]	3.713 ± 2.01 [a]	3.953 ± 2.0 [a]	0.826
Motility (%)	6.68 ± 2.80 [a]	2.20 ± 1.32 [b]	1.60 ± 1.12 [b]	< 0.001
Morphology (%)	1.4 ± 0.50 [a]	1.333 ± 0.48 [a]	1.20 ± 0.41 [a]	0.502
Viability (%)	61.933 ± 7.9 [a]	27.667 ± 5.75 [b]	19.133 ± 5.33 [c]	< 0.001

* Values are means ± SD; LN: Liquid Nitrogen

Different superscript letters within a row indicate significant differences (ANOVA test)

Fig. **1** The comparison of three groups regarding different sperm classes according to Cassuto – Barak scoring method. Different superscript letters indicate significant differences between respective values of compared groups (p <0.05)

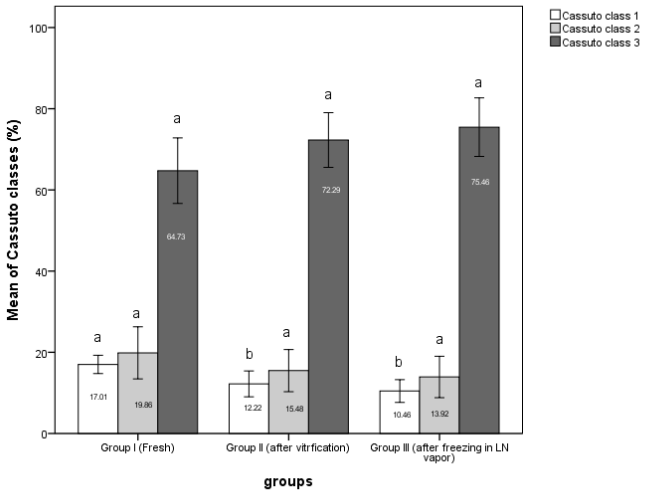
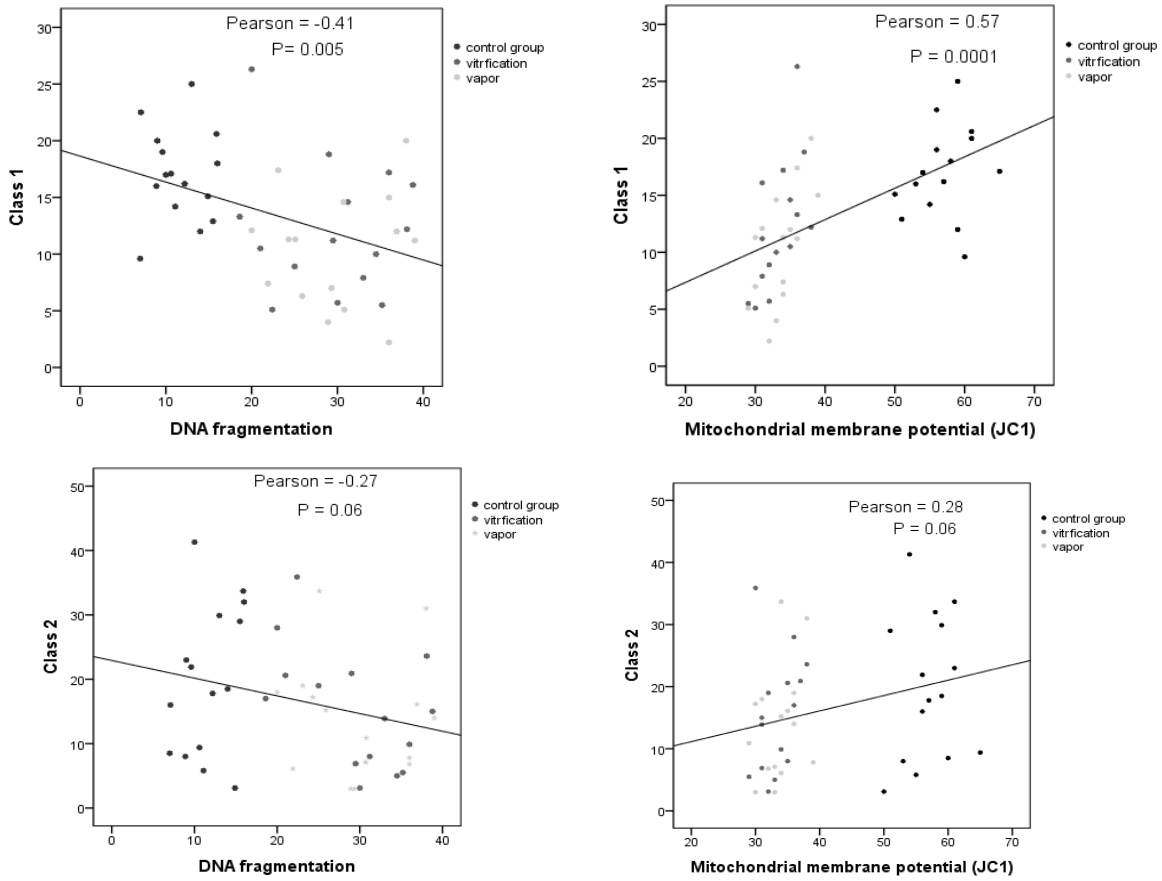


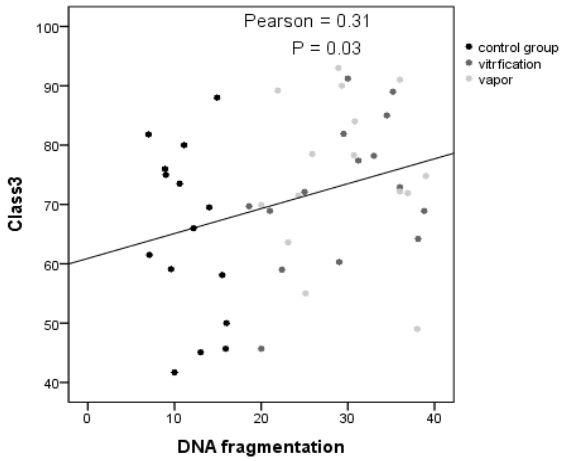
Table 2 The comparison of sperm DNA fragmentation and mitochondrial membrane potential in three groups:

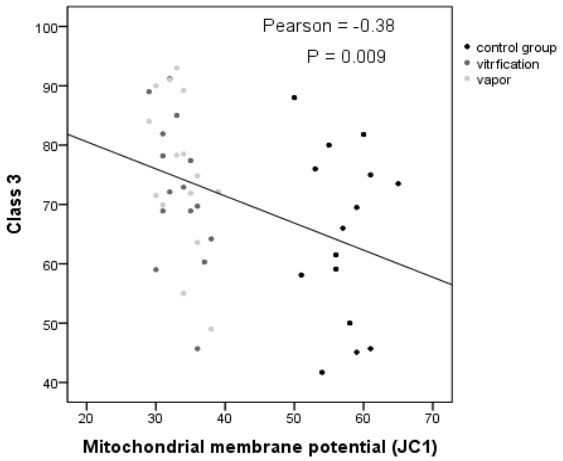
Variable (mean ± SD)	Before freezing (Group 1)	After vitrification (Group 2)	After freezing in LN vapor (Group 3)	*P* value
DNA fragmentation (%)	11.65 ± 3.10 [a]	29.487 ± 6.69 [b]	29.727 ± 6.28 [b]	<0.0001
Mitochondrial membrane potential (%)	54.75 ± 4.05 [a]	31.814 ± 2.74 [b]	31.981 ± 2.92 [b]	<0.0001

Different superscript letters within a row indicate significant differences (ANOVA test)

Fig. **2** The correlation between sperm classes according to Cassuto – Barak scoring method in three groups, mitochondrial membrane potential and DNA fragmentation







Discussion

The main objective was to evaluate the effect of cryopreservation on testicular spermatozoa by MSOME technology. Spermatozoa cryopreservation is a crucial routine procedure for the management of male infertility. There is considerable conflict regarding the effect of cryopreservation on TESE success rates. Some studies demonstrated the efficacy of using frozen and thawed testicular sperm for ICSI by the assessment of sperm parameters after thawing [9]. Although, Kupker et al. showed lower fertilization rate for cryopreservation of spermatozoa in comparison to fresh testicular spermatozoa [35]. Different cryoprotective media have been used for sperm cryopreservation to decrease cryodamage [36].

Conventional slow freezing can cause mechanical, physical and chemical cell injury by forming intra or extra cellular ice formation, also osmotic damage because of too much cell shrinkage. On the other hand, in this method, lipid-phase transition and lipid peroxidation increases by reactive oxygen production [37, 38]. In contrast to slow freezing vitrification uses high cooling rates (up to 10^4 K/min) [39]. In addition, directly melting the specimens in a warm solution helps to reach high rate of thawing and prevent devitrification (recrystalization) [40]. In this study, for both vitrification and freezing in vapor, equivalent results were achieved regarding count, motility, morphology, DNA fragmentation and mitochondrial membrane potential. For freezing in vapor, we used aluminum foil, to drop specimen on its surface, and to increase thermal conductivity.

In microinjection procedure, selecting spermatozoa with good morphology is of prime importantance. When spermatozoa are immotile or with subtle motility, this selection will become more complicated [41]. By MSOME assessment, the results showed that cryopreservation affected sperm morphology by increasing abnormalities which resulted in boosting classes 2 and 3 spermatozoa by Cassuto-Barak scoring system in comparison to fresh samples. It also, corroborate a significant correlation between sperm morphological classes 1 and 3 and DNA quality. Accordingly, a study by Wilding et al. which demonstrated that morphologic changes assessed by MSOME were related with DNA fragmentation [42]. However, another study, demonstrated no significant correlation between the morphology assessed by strict criteria and DNA integrity [43].

Capability of spermatozoa to move is largely acquired during sperm maturation in the epididymis, so as expected, motility of testicular sperm was low or absent at the extraction time [44]. On the other hand, cryopreservation decreases sperm motility and cause membrane damage because of liquid phase transition and lipid peroxidation [45, 46]. So in this study sperm motility was presented to some degree in almost all specimens. But, motility significantly decreased after freezing, irrespective of the cryopreservation method used.

Studies have shown that cytoplasmic and mitochondrial membranes are very susceptible to cryodamage [47, 19]. Cryopreservation of ejaculated human sperm decreases sperm viability 25 to 50 %. This also hold true for testicular sperm viability and morphology after thawing in comparison to fresh specimens [36, 48]. In our study, 34-42 % reduction in viability was shown after cryopreservation and this reduction was significantly higher in vitrification group. In a study by Prins and associates viability rate after testicular sperm cryopreservation in obstructive azoospermic patients was 62- 64 %. Similar findings was reported by Verheyen and co-workers [9, 6]. Also, Meseguer et al. demonstrated that cryopreservation can disturb mitochondrial activity and viability of spermatozoa [49]. There are different mitochondrial staining techniques to assess mitochondrial membrane potential. The intensity of stain or the percentage of spermatozoa showing the stain exhibits functional mitochondria [25]. It has been previously demonstrated that cryopreservation significantly decreased mitochondrial membrane potential [19]. In agreement, we found that intact mitochondrial membranes decreased 22 % after cryopreservation in comparison to fresh testicular sample.

Sperm DNA damage is related to mutagenic effects which results in future consequences [50]. The percentage of spermatozoa with DNA damage is lower in testis in comparison to ejaculation [51]. By comparing the effects of different cryopreservation techniques on DNA fragmentation of testicular sperm, we found that DNA damage assessed by TUNEL assay increased significantly after cryopreservation but no significant difference was seen between vitrification and freezing in vapor. It was demonstrated that by

worsening spermatozoa morphology, the DNA fragmentation will increase. As previously suggested, non-condensed sperm chromatin was higher in grades II and IV spermatozoa [52]. Indeed, morphologic changes in sperm plasma membrane resulted from intracellular ice formation. This makes sperm DNA sensitive to reactive oxygen species which may result in damaging DNA integrity [53]. This supports data from previous studies which freezing-thawing process caused a reduction in spermatozoa nuclei compactness [54].

Before and after cryopreservation, a correlation was shown between MSOME best and worst grades (class1 and 3) spermatozoa, mitochondrial membrane potential, and DNA fragmentation.The results also confirmed that DNA fragmentation and mitochondrial membrane potential were correlated to morphology assessed by MSOME. It means that human spermatozoa selected by MSOME have lower DNA fragmentation and better mitochondrial status. In this context, Knez et al. showed a higher rate of blastocyst formation with best grade spermatozoa selected by MSOME [55]. Although, this technique is expensive and time-consuming, it is especially useful for cryopreserved testicular samples with impaired morphology. The selection of normal morphology spermatozoa is so important to improve the outcomes. In a study by Hebermann and coworkers it was shown that there was no differences between fresh and frozen-thawed TESE spermatozoa with regards to clinical outcomes [13]. Nonetheless, no study used MSOME and TESE sample cryopreservation at the same time. However, lacking clinical data was the limitation of our study. Also, clinical outcomes of comparative prospective studies with a large number of cases will demonstrate the possible advantages of MSOME/IMSI for testicular sperm cryopreservation. For now, it is obvious that by making use of MSOME, good morphology spermatozoa with high-quality DNA are selected for clinical purposes.

Conclusion

In conclusion, by cryopreservation of testicular spermatozoa, multiple biopsies are prevented and male gametes are available for subsequent ICSI cycles. Although, vitrification and freezing in LN vapor affects sperm parameters, the best method for testicular sperm cryopreservation should yet to be determined; selecting good quality spermatozoa to gain best outcomes is an important issue in ART treatment. To reach this goal, the assessment of frozen-thawed testicular spermatozoa with MSOME will be of particular value.

Ethical approval: "All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

REFERENCES

1. Schoysman R, Vanderzwalmen P, Nijs M, Segal L, Segal-Bertin G, Geerts L et al. Pregnancy after fertilisation with human testicular spermatozoa. The Lancet. 1993;342(8881):1237-.

2. Prince GS, Dolgina R, Studney P, Kaplan B, Ross L, Niederberger C. Quality of cryopreserved testicular sperm in patients with obstructive and nonobstructive azoospermia. The Journal of urology. 1999;161(5):1504-8.

3. Schlegel P, Chang T. The testis, epididymis and ductus deferens. Campbell's urology, 6th ed Philadelphia: Saunders. 1991;190.

4. Schlegel PN, Su L-M. Physiological consequences of testicular sperm extraction. Hum Reprod. 1997;12(8):1688-92.

5. Harrington TG, Schauer D, Gilbert BR. Percutaneous testis biopsy: an alternative to open testicular biopsy in the evaluation of the subfertile man. J Urol. 1996;156(5):1647-51.

6. Verheyen G, Nagy Z, Joris H, De Croo I, Tournaye H, Van Steirteghem A. Quality of frozen-thawed testicular sperm and its preclinical use for intracytoplasmic sperm injection into in vitro-matured germinal-vesicle stage oocytes. Fertil Steril 1997;67(1):74-80.

7. Romero J, Remohi J, Minguez Y, Rubio C, Pellicer A, Gil-Salom M. Fertilization after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Fertil Steril 1996;65(4):877-9.

8. Gil-Salom M, Romero J, Ménguez Y, Rubio C, De los Santos MJ, Remohe J et al. Pregnancies after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Hum Reprod. 1996;11(6):1309-13.

9. Prins GS, Dolgina R, Studney P, Kaplan B, Ross L, NIEDERBERGER C. Quality of cryopreserved testicular sperm in patients with obstructive and nonobstructive azoospermia. J Urol. 1999;161(5):1504-8.

10. Ghanem M, Bakr NI, Elgayaar MA, El Mongy S, Fathy H, IBRAHIM AHA. Comparison of the outcome of intracytoplasmic sperm injection in obstructive and non-obstructive azoospermia in the first cycle: a report of case series and meta-analysis. Int J Androl. 2005;28(1):16-21.

11. Schulze W, Salzbrunn A, Benson D. Pregnancy after intracytoplasmic sperm injection of spermatozoa extracted from frozen-thawed testicular biopsy. Hum Reprod. 1996;11(10):2197-9.

12. Gangrade BK. Cryopreservation of testicular and epididymal sperm: techniques and clinical outcomes of assisted

conception. Clinics. 2013;68:131-40.

13. Habermann H, Seo R, Cieslak J, Niederberger C, Prins GS, Ross L. In vitro fertilization outcomes after intracytoplasmic sperm injection with fresh or frozen-thawed testicular spermatozoa. Fertil Steril 2000;73(5):955-60.

14. Fukunaga N, Haigo K, Kyono K, Araki Y. Efficiency of using frozen-thawed testicular sperm for multiple intracytoplasmic sperm injections. Journal of assisted reproduction and genetics. 2001;18(12):634-7.

15. Isachenko E, Isachenko V, Weiss J, Kreienberg R, Katkov I, Schulz M et al. Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose. Reproduction. 2008;136(2):167-73.

16. Vutyavanich T, Piromlertamorn W, Nunta S. Rapid freezing versus slow programmable freezing of human spermatozoa. Fertil Steril 2010;93(6):1921-8.

17. Slabbert M, Du Plessis S, Huyser C. Large volume cryoprotectant-free vitrification: an alternative to conventional cryopreservation for human spermatozoa. Andrologia. 2015;47(5):594-9.

18. Gil-Salom M, Romero J, Rubio C, Ruiz A, Remohı J, Pellicer A. Intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Mol Cell Endocrinol 2000;169(1):15-9.

19. O'connell M, McClure N, Lewis S. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod. 2002;17(3):704-9.

20. Royere D, Barthelemy C, Hamamah S, Lansac J. Cryopreservation of spermatozoa: a 1996 review. Hum Reprod Update. 1996;2(6):553-9.

21. Hammadeh M, Askari A, Georg T, Rosenbaum P, Schmidt W. Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. Int J Androl. 1999;22(3):155-62.

22. Bartoov B, Berkovitz A, Eltes F, Kogosowski A, Menezo Y, Barak Y. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. J Androl. 2002;23(1):1-8.

23. Bartoov B, Berkovitz A, Eltes F. Selection of spermatozoa with normal nuclei to improve the pregnancy rate with intracytoplasmic sperm injection. N Engl J Med. 2001;345(14):1067-8.

24. Balaban B, Yakin K, Alatas C, Oktem O, Isiklar A, Urman B. Clinical outcome of intracytoplasmic injection of spermatozoa morphologically selected under high magnification: a prospective randomized study. Reproductive biomedicine online. 2011;22(5):472-6.

25. Garner DL, Thomas CA, Joerg HW, Dejarnette JM, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol Reprod 1997;57(6):1401-6.

26. Kasai T, Ogawa K, Mizuno K, Nagai S, Uchida Y, Ohta S et al. Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. Asian journal of andrology. 2002;4(2):97-104.

27. Thomson L, Fleming S, Aitken R, De Iuliis G, Zieschang J-A, Clark A. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod. 2009;24(9):2061-70.

28. Lopes S, Sun J-G, Jurisicova A, Meriano J, Casper RF. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil Steril 1998;69(3):528-32.

29. Nagy ZP, Verheyen G, Tournaye H, Devroey P, Van Steirteghem A. An improved treatment procedure for testicular biopsy specimens offers more efficient sperm recovery: case series. Fertil Steril 1997;68(2):376-9.

30. Isachenko V, Isachenko E, Montag M, Zaeva V, Krivokharchenko I, Nawroth F et al. Clean technique for cryoprotectant-free vitrification of human spermatozoa. Reproductive biomedicine online. 2005;10(3):350-4.

31. Organization WH. WHO Laboratory Manual for the Examination and Processing of Human Semen. World Health Organization; 2010.

32. Jeyendran R, Van der Ven H, Perez-Pelaez M, Crabo B, Zaneveld L. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 1984;70(1):219-28.

33. Verheyen G, Joris H, Crits K, Nagy Z, Tournaye H, Van Steirteghem A. Comparison of different hypo-osmotic swelling solutions to select viable immotile spermatozoa for potential use in intracytoplasmic sperm injection. Hum Reprod Update.

1997;3(3):195-203.

34. Cassuto NG, Bouret D, Plouchart JM, Jellad S, Vanderzwalmen P, Balet R et al. A new real-time morphology classification for human spermatozoa: a link for fertilization and improved embryo quality. Fertil Steril 2009;92(5):1616-25.

35. Küpker W, Schlegel PN, Al-Hasani S, Fornara P, Johannisson R, Sandmann J et al. Use of frozen-thawed testicular sperm for intracytoplasmic sperm injection. Fertil Steril 2000;73(3):453-8.

36. Weidel L, Prins GS. Cryosurvival of human spermatozoa frozen in eight different buffer systems. J Androl. 1987;8(1):41-7.

37. Gao D, Liu J, Liu C, McGann L, Watson P, Kleinhans F et al. Andrology: Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. Hum Reprod. 1995;10(5):1109-22.

38. Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS, Wu F. Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. J Androl. 1989;10(3):214-20.

39. Karlsson JO. A theoretical model of intracellular devitrification. Cryobiology. 2001;42(3):154-69.

40. Isachenko V, Isachenko E, Katkov II, Montag M, Dessole S, Nawroth F et al. Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. Biol Reprod 2004;71(4):1167-73.

41. Celik-Ozenci C, Jakab A, Kovacs T, Catalanotti J, Demir R, Bray-Ward P et al. Sperm selection for ICSI: shape properties do notpredict the absence or presence of numericalchromosomal aberrations. Hum Reprod. 2004;19(9):2052-9.

42. Wilding M, Coppola G, Di Matteo L, Palagiano A, Fusco E, Dale B. Intracytoplasmic injection of morphologically selected spermatozoa (IMSI) improves outcome after assisted reproduction by deselecting physiologically poor quality spermatozoa. J Assist Reprod Genet. 2011;28(3):253-62.

43. Donnelly ET, Steele EK, McClure N, Lewis SE. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod. 2001;16(6):1191-9.

44. Howards S. The epididymis, sperm maturation, and capacitation. Infertility in the Male. 1983:121-34.

45. Critser JK, Arneson BW, Aaker DV, Huse-Benda AR, Ball GD. Cryopreservation of human spermatozoa. II. Postthaw chronology of motility and of zona-free hamster ova penetration. Fertil Steril 1987;47(6):980-4.

46. Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J Androl 1992;13(3):232-41.

47. Drobnis EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW, Crowe JH. Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. Journal of Experimental Zoology. 1993;265(4):432-7.

48. Sereni E, Bonu M, Fava L, Sciajno R, Serrao L, Preti S et al. Freezing spermatozoa obtained by testicular fine needle aspiration: a new technique. Reproductive biomedicine online. 2008;16(1):89-95.

49. Meseguer M, Garrido N, Martínez-Conejero JA, Simón C, Pellicer A, Remohí J. Role of cholesterol, calcium, and mitochondrial activity in the susceptibility for cryodamage after a cycle of freezing and thawing. Fertil Steril 2004;81(3):588-94.

50. Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proc Natl Acad Sci U S A. 1991;88(24):11003-6.

51. Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S et al. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. Hum Reprod. 2005;20(1):226-30.

52. Franco Jr J, Mauri A, Petersen C, Massaro F, Silva L, Felipe V et al. Large nuclear vacuoles are indicative of abnormal chromatin packaging in human spermatozoa. Int J Androl. 2012;35(1):46-51.

53. Dalzell LH, McVicar CM, McClure N, Lutton D, Lewis SE. Effects of short and long incubations on DNA fragmentation of testicular sperm. Fertil Steril 2004;82(5):1443-5.

54. Hunter J, Bodner A, Hatch F, Balhorn R, Mazrimas J, McQueen A et al. Single-strand nuclease action on heat-denatured spermiogenic chromatin. J Histochem Cytochem. 1976;24(8):901-7.

55. Knez K, Tomazevic T, Zorn B, Vrtacnik-Bokal E, Virant-Klun I. Intracytoplasmic morphologically selected sperm injection improves development and quality of preimplantation embryos in teratozoospermia patients. Reproductive biomedicine online. 2012;25(2):168-79.