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Original Article

Modern human sperm freezing: Effect on DNA, chromatin and acrosome integrity

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ABSTRACT

Objective: Presence of vitrification method in sperm freezing and the introduction of solid surface vitrification beside rapid freezing in vapour, opens an easy and safe way to help infertility centres. While the effects of cryopreservation on motility, morphology and viability of sperm are documented, the question of the probable alteration of sperm DNA, chromatin and acrosome integrity after freezing and thawing procedures in different methods is still controversial.

Materials and methods: Normal sample were collected according to WHO strict criteria. Sperm suspensions were mixed 1:1 with 0.5 M sucrose and divided into four equal aliquots for freezing: fresh, nitrogen direct immersion vitrification (Vit), solid surface vitrification (SSV) and in vapour (Vapour).

Sperm suspensions were transferred into a 0.25 ml sterile plastic. Then straw was inserted inside the 0.5 ml straw. For thawing, the straws were immersed in a 42 °C water bath. Beside the sperm parameters, we assessed the acrosome reaction by double staining, chromatin integrity by toluidine blue (Tb) and chromomycin A3 (CMA3) and DNA integrity by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) respectively.

Results: In progressive motility, the highest rate occurred in Vit (39.9 ± 13.3). Moreover, the lowest rate of immotile sperm was in Vit (32.7 ± 16.3). In normal morphology, the group Vit was similar to the fresh, while SSV and Vapour were significantly different from the fresh. The percentage of acrosome-reacted sperms was more in Vit (81.3 ± 10.2) than the fresh group. TUNEL+ results showed that DNA fragmentation was significantly increased in Vit (p-value = 0.025). While in SSV and Vapour results were comparable to fresh. There was a significant correlation between TUNEL+ and normal morphology, TB, CMA3 and presence of intact acrosome.

Conclusion: Sperm in Vapour was healthier in terms of DNA, chromatin and acrosome integrity. In contrast of higher motility and normal morphology; DNA, chromatin and acrosome integrity were decreased in Vit. However, these findings were more acceptable in SSV or Vapour.

Introduction

Earlier sperm cryopreservation required expensive biological equipment and the process was time-consuming. A fast alternative method like vitrification would provide significant benefits regarding simple equipment and easy procedure in assisted reproductive technology (ART).

Now, vitrification accounts as an acceptable alternative to slow cooling [1]. New vitrification techniques are preferred in all relevant areas of freezing and also in sperm cryopreservation areas [2]. However, the potential risk of disease transmission through contaminated liquid nitrogen during freezing procedure and storage created much concern [3].This issue has been solved with the introduction of closed systems [1].

Vitrification in its evolution provided another version called Solid surface vitrification (SSV) which has been applied successfully to preserve oocytes and ovarian tissue [4]. In this method direct exposure of tissue happens to a precooled metal surface at about −160° C, in which provides enough space for tissue, high cooling rates and avoids producing nitrogen bubbles and

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evaporation around to cause not falling of cooling rate [5]. SSV has been described to vitrify ovine mature oocytes with high rates of survival on the surface of the metal in liquid nitrogen [6]. In another method, direct contact between the straws and the nitrogen vapour for 8–10 min and immersion in liquid nitrogen at −196 °C in rapid freezing have been used in sperm cryopreservation [7].

While the effects of cryopreservation on motility, morphology and viability of sperm are documented, the question of the probable alteration of sperm DNA integrity after different freezing-thawing procedures still exists. There is no clear confirmation in the studies on which cryopreservation techniques induces DNA or chromatin damage. In some studies, authors have reported significant alterations in sperm DNA integrity after cryopreservation and warming [8–10].

Loss of motility and vitality, increased membrane damage, induced acrosome reaction, induction of apoptosis due to oxidative stress are disadvantages of vitrification [2,3]. However, during cryopreservation, sperm is exposed to physical and chemical stress that results in adverse changes in the composition of membrane lipid. All these changes reduce the fertilising ability of human spermatozoa after cryopreservation [8,11–14].

This is still of great concern, that increased sperm DNA fragmentation could reduce the full term pregnancy rate in ART [15] and increase the risk of miscarriage [16], where the generation of healthy offspring should be considered the goal of ART services [17]. Meanwhile, widespread use of vitrification could still be leading in the maintenance of sperm DNA, chromatin and acrosome integrity?

Material and method

In this experimental study, the normal samples were collected from 20 healthy men after 2–7 days of sexual abstinence. After signing an informed consent for scientific research, they entered the study. This study was approved by our institutional ethics committee.

Semen samples were liquefied in an incubator at 37 °C for 30 min. Semen parameters as sperm concentration, the percentage of motile sperm and normal morphology were analysed.

All samples should contain at least 15 million spermatozoa per ml with a 32% progressive motility rate and >4% normal morphological spermatozoa. Semen analysis was performed according to guidelines of the World Health Organization (2010). Semen analyses were conducted by the same technician, who was blind from other clinical data. For samples, the swim-up technique was performed for 60 min at 37 °C. Then samples were centrifuged and diluted with sperm medium to achieve a concentration of 20×10^6 sperms per ml.

For freezing, the sperm suspensions were mixed 1: 1 with 0.5 M sucrose and incubated for 10 min at room temperature. The mixture was divided into four equal aliquots: fresh, SSV, liquid nitrogen vapour (Vapour) method and vitrification (Vit) which is the direct immersion into liquid nitrogen.

100 μl of sperm suspensions were transferred into a 0.25 mL sterile plastic straw. Then each of them was inserted inside the 0.5 mL straw. In SSV, straws were positioned onto a metal surface on liquid nitrogen for 10 min and then immersed in the liquid nitrogen. In Vapour, the straws were exposed to the liquid nitrogen vapour 4 cm above the level of liquid nitrogen for 10 min and then immersing in the liquid nitrogen. In Vit, straws were immersed directly into liquid nitrogen and all stored.

For thawing, the straws were taken from the liquid nitrogen, immersed in a 42 °C water bath [18] until the ice melted. In all experimental groups; smears for assessing sperm acrosome, DNA and chromatin integrity were prepared immediately after the warming of straws.

Assessment of sperm motility

In fresh and thawed groups, 10 mL aliquots were added to glass slide and sperms were analysed by phase-contrast microscope.

Assessment of sperm viability

Sperm viability in fresh and thawed groups was carried out using eosin–nigrosin staining technique. 10 μl of semen was mixed with 10 μl of eosin–nigrosin stain on a glass slide and assayed using a light microscope to determine the percentage of live sperm. At least 200 spermatozoa were assessed for each case. For analysis, white or unstained sperms were classified as live and pink or red sperms were considered dead.

DNA integrity assessment

In both fresh and thawed semen, DNA integrity was determined using an in situ terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) detection kit (In situ Cell Death Detection Kit, POD; Roche, Mannheim, Germany).

A droplet of the sperm suspension from each sample was smeared onto glass slides and air dried and fixed by immersion in freshly prepared 4% paraformaldehyde in PBS, pH 7.4 for 20 min at room temperature. Next, the slides were incubated with blocking solution (H_2O_2 in 3% methanol) for 20 min at room temperature.

Slides were rinsed in PBS for 5 min, treated with pre-chilled 0.1% Triton X-100 in 0.1% sodium citrate for 5 min on ice. Then the slides staining were performed according to the manufacturer's instructions. Slides were rinsed twice with PBS for a total of 5 min at room temperature.

CMA3 staining

Chromomycin A3 (CMA3) is a guanine–cytosine specific fluorochrome and competes with protamines for binding to the minor groove of sperm DNA and is a useful tool for identifies abnormalities in the sperm chromatin packaging and protamine deficiency.

Sperm cells were fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) at 48 °C for 10 min. Each slide was then stained with CMA3 solution (0.25 mg/mL in McIlvain buffer; 7 mL citric acid, 0.1 M + 32.9 mL Na_2HPO_4 $7H_2O$ 0.2 M, pH 7.0 containing 10 mM $MgCl_2$) for 20 min in darkness. The slides were washed in buffer, mounted with buffered glycerol (1:1) and analysed by fluorescence microscopy at 390–490 nm.

Bright yellow stained sperm head (abnormal chromatin packaging) were considered as CMA3(+), while yellowish green stained sperms (normal chromatin packaging) were considered as CMA3(−).

Aniline blue staining

The slides were air-dried and then fixed with a solution of 3% buffered glutaraldehyde for 30 min. Then staining was done with 5% aqueous aniline blue solution mixed with 4% acetic acid (pH = 3.5) for 10 min 200 spermatozoa were counted with a light microscope. Spermatozoa with mature nuclei chromatin did not take up the stain and are considered normal while those with immature chromatin and blue stained were considered abnormal.

Toluidine blue (TB) staining and sperm morphology assessment

Dried smears were fixed with freshly made 96% ethanol–acetone (1: 1) at 4 °C for 30 min. Smears were hydrolyzed with 0.1 N HCl at 4 °C for 5 min and washed three times in distilled water

for 2 min. Then staining was done with 0.05% TB (Sigma, St. Louis, MO, USA) for 10 min.

The staining buffer consisted of 50% McIlvain's citrate phosphate buffer (pH 3.5). In a light microscopic study using 100 eyepiece magnification, the chromatin quality of spermatozoa was determined according to metachromatic staining of sperm heads with following scores: score 0 = light blue (good chromatin), score 1 = dark blue (mild abnormal chromatin), score 2 = violet and purple (severe chromatin abnormality). So, the sum of spermatozoa with scores 1 and 2 was considered as TB + or abnormal chromatin, whereas score = 0 as TB- or sperm with normal chromatin [19]. Also, the assessment of sperm morphology was done at the same time [20].

Acrosome reaction (double staining)

According to Kohn et al. (1997) [21] briefly, spermatozoa were fixed in glutaraldehyde (3% PBS, pH 7.4) for at least 30 min at 24 °C. After two washing steps (1000 g, 2 min), the sperm pellet was resuspended and smeared onto a slide. Spermatozoa were stained in Bismarck brown (0.8% in deionized water pH 1.8) for 10 min at 37 °C and washed several times with distilled water, followed by a staining with Rose Bengal (0.8% in 0.1 M Tris buffer, pH 5.3) for 25 min at 24 °C. After the second washing, spermatozoa were dehydrated in 50, 95 and 100% ethanol and rinsed with xylene. Under light microscopy, red or pink staining of the acrosomal region indicated intact spermatozoa, whereas lack of Rose Bengal was interpreted as typical for acrosome-reacted spermatozoa. At least 200 spermatozoa per slide were examined under oil immersion (×1000) using a light microscope.

Statistical analysis

Statistical analysis was performed using the SPSS (SPSS 18.0, Chicago, USA) software. One-way ANOVA test and correlation were used to compare different variables in different groups (Table 2). A p-value of <0.05 was considered significant.

Results

There was a significant decrease in all experimental groups in progressive motion (p-value = 0.001). The highest rate in progressive motility (39.9 ± 13.3), and the lowest rate in immotility (32.7 ± 16.3) happened in Vit. In normal morphology, the higher rate was in Vit and was similar to fresh, while SSV and Vapour were comparable and significantly different from the fresh (p-value = 0.045 and p-value = 0.006 respectively). There was a significant decrease in all experimental groups in viability. Concerning the viability, the highest rate was in Vit (65.5 ± 16.2). While viability in Vapour was significantly lower (49.8 ± 20.1, p-value = 0.045).

In chromatin integrity, CMA3+ results showed that there was a significant difference between Vit and fresh (p-value = 0.049). This amount was not significant in SSV or Vapour compared to fresh (p-value = 0.99). In TB results, the highest damage was in groups Vit and SSV (153.1 ± 11.1) for both, and the lowest was in the Vapour (145.7 ± 11.6). In DNA integrity, TUNEL+ results showed that DNA fragmentation was significantly increased in Vit (24.6 ± 11.0, p-value = 0.025). While in SSV and Vapour results were comparable to fresh (p-value = 0.950). Acrosome reaction occurred more in Vit (81.3 ± 10.2, p-value = 0.018) than the other groups. The presence of sperm with intact acrosome was higher in Vapour (82.4 ± 7.9, p-value = 0.059) and comparable to fresh (Tables 1 and 2).

TUNEL+ results was significantly correlated with normal morphology (r = −0.356), TB+ (r = 0.013), CMA3+ (r = 0.233) and presence of intact acrosome (r = 0.251).There was a correlation between CMA3+ results with progressive motility (r = −0.282), immotility (r = 0.196), normal morphology (r = −0.318) and viability (r = −0.387) at a significant difference.

Moreover, the rate of presence of intact acrosome was correlated significantly with progressive motility (r = −0.410), immotility (r = 0.470), normal morphology (r = −0.322) and viability (r = −0.421) (Table 3).

Discussion

Various sperm cryopreservation methods induce some alteration and damage in sperm parameters in a variety of ways that may lead to a decrease in sperm survival rate, motility, changes in the plasma membrane, acrosome and DNA integrity [22].

Our results in this study showed that freezing-thawing procedure could induce sperm alteration in all experimental protocols. In all cryopreservation groups; motility, morphology and viability decreased, although these parameters were more acceptable in Vit group. However, DNA and chromatin integrity accompanied to acrosome integrity reduced in this group. We observed that spermatozoa in Vapour and SSV groups could be safer in terms of DNA, chromatin and acrosome integrity.

Perhaps sperm can maintain its DNA, chromatin and acrosome integrity more in the lower cooling rate like Vapour rather than higher one like Vit. The rapid cooling rate in Vit protocol may induce the intracellular crystals which are destructive to the integrity of the cellular membrane. Protamine and DNA complex can be influenced by the physical injury to the chromatin structure that induces DNA damage [23]. This condition induces the uncondensed chromatin as well. It seems, unlike eggs or embryos [24–26], cold shock caused by direct immersion in liquid nitrogen is not suitable for sperm. Acrosome and the chromatin were more vulnerable in this declining temperature rate.

A study used a static vapour phase cooling above the surface of the liquid nitrogen and then plunging the cryovial into liquid nitrogen. After warming, changes in the integrity of the membranes and morphology were apparent. Wrinkling on the plasmalemma or acrosomal change defect was also determined. Moreover, apical head alterations in acrosomal change, lacking continuity, loss of acrosomal content and appearance of vesiculations were detected [27].

Table 1
Sperm parameters, sperm DNA integrity, chromatin integrity and acrosome integrity (Mean ± SD) in different groups.

	Progressive motility	Nonprogressive motility	Immotility	Normal morphology	Viability	CMA3+	TB+	TUNEL+	Intact acrosome
Fresh	66.2 ± 13.7	19.8 ± 9.7	14.0 ± 9.6	7.4 ± 2.7	88.1 ± 10.7	16.9 ± 8.5	132.1 ± 31.0	14.4 ± 9.3	88.9 ± 6.4
Vit	39.9 ± 13.3	27.6 ± 8.1	32.7 ± 16.3	6.1 ± 2.3	65.5 ± 16.2	25.0 ± 10.7	153.1 ± 11.1	24.6 ± 11.0	81.3 ± 10.2
SSV	33.6 ± 11.7	27.0 ± 7.5	39.3 ± 11.0	5.3 ± 2.2	58.3 ± 11.0	23.4 ± 9.2	153.1 ± 11.1	19.8 ± 10.5	81.6 ± 6.5
Vapour	28.8 ± 14.6	24.3 ± 9.7	47.3 ± 18.2	4.8 ± 2.5	49.8 ± 20.1	24.3 ± 10.5	145.7 ± 11.6	21.5 ± 10.0	82.4 ± 7.9
sig	0.001	0.027	0.027	0.008	0.000	0.043	0.010	0.025	0.010

One-way ANOVA test was used. Post hoc test results were shown in Table 2.
p-value < 0.05 was considered significant.

Table 2
Post hoc test results of sperm parameters, sperm DNA integrity, chromatin integrity and acrosome integrity in different groups (p-values are shown in the cells of the table).

		Progressive motility	Nonprogressive motility	Immotility	Normal morphology	Viability	CMA3+	TB+	TUNEL+	Intact acrosome
Fresh	Vit	0.001	0.033	0.000	0.355	0.000	0.049	0.022	0.016	0.018
	SSV	0.000	0.055	0.000	0.045	0.000	0.166	0.015	0.377	0.025
	Vapour	0.000	0.371	0.000	0.006	0.000	0.101	0.232	0.149	0.059
Vit	SSV	0.451	0.997	0.466	0.739	0.430	0.955	1.000	0.826	0.999
	Vapour	0.051	0.653	0.010	0.322	0.008	0.997	0.734	0.988	0.968
SSV	Vapour	0.671	0.770	0.301	0.897	0.294	0.991	0.677	0.950	0.987

Tukey was done as a post hoc test.
A p-value of <0.05 was considered significant.

Table 3
Correlation between different variables.

	Tb+	CMA3+	TUNEL+	Intact acrosome
Progressive motility	r = −0.134 p = 0.243	r = −0.282 p = 0.012	r = 0.251 p = 0.026	r = −0.410 p = 0.000
Nonprogressive motility	r = −0.046 p = 0.243	r = 0.209 p = 0.066	r = −0.017 p = 0.880	r = −0.112 p = 0.323
Immotility	r = 0.172 p = 0.132	r = 0.196 p = 0.086	r = 0.164 p = 0.149	r = 0.470 p = 0.000
Normal morphology	r = −0.218 p = 0.055	r = −0.318 p = 0.005	r = −0.356 p = 0.001	r = −0.322 p = 0.004
Viability	r = −0.245 p = 0.031	r = −0.387 p = 0.000	r = −0.220 p = 0.051	r = −0.421 p = 0.000
TUNEL+	r = 0.013 p = 0.910	r = 0.233 p = 0.042		r = 0.251 p = 0.026

A p-value of <0.05 was considered significant.

Comparing different cooling temperatures in sperm freezing showed that no statistical difference was detected in −86 °C in omitting the use of liquid nitrogen [3] and in −196 °C in the use of liquid nitrogen to sperm progressive motility or DNA fragmentation.

Alike, warmed sperm motility results of samples cryopreserved in liquid nitrogen vapour was comparable to samples cryopreserved with the nitrogen-free freezer [28]. On the other hand, increasing the cooling rate in the freezing of mouse embryos had no advantage for vitrification procedure. Besides, vitrification in double straws with a decline in cooling rate of 400 °C/min was very effective for the cryopreservation of mouse embryos. It was also noted that even lower cooling rate of 120 °C/min was useful for the vitrification of human embryos [1].

Probably cold shock in the lower temperature in Vit can expose sperm to oxidative stress and may be one of the principal causes of increasing sperm DNA fragmentation compared to SSV or Vapour. Cryopreservation may alter the potential of mitochondrial membrane to release produced ROS [29]. Moreover, we examined DNA and chromatin integrity immediately after warming. It was reported that the highest degree of sperm DNA fragmentation occurred during 4 h of incubation after warming samples of fertile donors [30].

In our study, the evidence is based on the negative correlation between progressive sperm motility and DNA fragmentation after vitrification in normozoospermia. There were reports of a relationship between morphology and DNA fragmentation. It was shown that there is a negative correlation between sperm motility, vitality and sperm DNA damage [31]. It seems that the sperm morphological feature is representative of sperm genomic status. Some authors have been reported direct physical damage to sperm structure or function during sperm freezing related to the ice formation and high osmotic pressure during freezing. Oxidative stress produces free radical cascade leading to lipoperoxidation process [32]. The effects of lipid peroxidation include irreversible loss of motility, leakage of intracellular enzymes, damage to sperm DNA and deficiencies in oocyte penetration and sperm and oocyte fusion [33].

Sperm with the damaged genetic material is still capable of fertilisation and strongly correlated with mutation [34]. Mutations may not be evident and are going to affect divided and developed embryos. Therefore, it should consider ensuring that sperm frozen in these ways could offer the highest protection to the chromatin to prevent possible interference to offspring genome [35]. Regarding advantages or disadvantages of each freezing method, a particular approach should be adopted to achieve the best result of frozen-thawed sperm. Effects of optimising the freezing medium [36–39] or other empirical studies to determine the optimum temperature to maintain membrane or sperm genome integrity are needed. The protective effect on the mitochondrial potential of spermatozoa may help in providing the ATP required for progressive motility or preventing the destabilisation of the plasma membrane of spermatozoa and so maintaining acrosome integrity [37]. It seems that application of our study results would be useful. Our pilot study showed sperm thawing results in single straws were significantly less acceptable than doubles (unpublished data). Genome integrity is necessary to transfer to the next generation, however, cryopreservation might be along with detrimental effects on the genome. Improved warmed sperm quality may, in turn, enhance the processed sperm quality to increase conception rate, embryo development and birth rate in ART.

Conclusion

Sperm in regarding cooling rate temperature could be safer in terms of DNA, chromatin and acrosome integrity. While the motility, morphology and viability may retain high; the DNA, chromatin and acrosome integrity may be deterred in Vit. However, sperm DNA, chromatin and acrosome integrity status were more tolerable in SSV or Vapour rather than in Vit.

Ethics approval

This study was approved by ethics committee of Shahid Sadoughi of Medical Sciences, This study was approved by ethics committee of Shahid Sadoughi of Medical Sciences.

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Authors' contributions

TR, AH, MA, SG and AT participated in Acquisition of data. TR, AH, SG and AT carried out the Analysis and interpretation of data. TR, MA, and AT prepared the manuscript. MA and AT critically revised the manuscript. All authors read and approved the final manuscript version.

Conflicts of interest

All investigators disclose no conflict of interest in this study.

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References

[1] Vutyavanich T, Sreshthaputra O, Piromlertamorn W, Nunta S. Closed-system solid surface vitrification versus slow programmable freezing of mouse 2-cell embryos. J Assist Reprod Genet 2009;26(5):285–90.
[2] Sanchez R, Risopatron J, Schulz M, Villegas J, Isachenko V, Kreinberg R, et al. Canine sperm vitrification with sucrose: effect on sperm function. Andrologia 2011;43(4):233–41.
[3] Sanchez R, Risopatron J, Schulz M, Villegas JV, Isachenko V, Isachenko E. Vitrified sperm banks: the new aseptic technique for human spermatozoa allows cryopreservation at −86 degrees C. Andrologia 2012;44(6):433–5.
[4] Huang L, Mo Y, Wang W, Li Y, Zhang Q, Yang D. Cryopreservation of human ovarian tissue by solid surface vitrification. Eur J Obstet Gynecol Reprod Biol 2008;139(2):193–8.
[5] Zhang J, Nedambale T, Yang M, Li J. Improved development of ovine matured oocyte following solid surface vitrification (SSV): effect of cumulus cells and cytoskeleton stabiliser. Anim Reprod Sci 2009;110(1):46–55.
[6] Moawad AR, Fisher P, Zhu J, Choi I, Polgar Z, Dinnyes A, et al. In vitro fertilisation of ovine oocytes vitrified by solid surface vitrification at germinal vesicle stage. Cryobiology 2012;65(2):139–44.
[7] 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;2012:854837.
[8] Zribi N, Chakroun NF, Abdallah FB, Elleuch H, Sellami A, Gargouri J, et al. Effect of freezing–thawing process and quercetin on human sperm survival and DNA integrity. Cryobiology 2012;65(3):326–31.
[9] Boitrelle F, Albert M, Theillac C, Ferfouri F, Bergere M, Vialard F, et al. Cryopreservation of human spermatozoa decreases the number of motile normal spermatozoa, induces nuclear vacuolization and chromatin decondensation. J Androl 2012;33(6):1371–8.
[10] Kopeika J, Thornhill A, Khalaf Y. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. Hum Reprod update 2015;21(2):209–27.
[11] Brugnon F, Ouchchane L, Pons-Rejraji H, Artonne C, Farigoule M, Janny L. Density gradient centrifugation prior to cryopreservation and hypotaurine supplementation improve post-thaw quality of sperm from infertile men with oligoasthenoteratozoospermia. Hum Reprod 2013;28(8):2045–57.
[12] Roca J, Martinez-Alborcia MJ, Gil MA, Parrilla I, Martinez EA. Dead spermatozoa in raw semen samples impair in vitro fertilization outcomes of frozen-thawed spermatozoa. Fertil Steril 2013;100(3):875–81.
[13] Valcarce D, Cartón-García F, Herráez MP, Robles V. Effect of cryopreservation on human sperm messenger RNAs crucial for fertilization and early embryo development. Cryobiology 2013;67(1):84–90.
[14] Valcarce D, Cartón-García F, Riesco M, Herráez MP, Robles V. Analysis of DNA damage after human sperm cryopreservation in genes crucial for fertilization and early embryo development. Andrology 2013;1(5):723–30.
[15] Zeke J, Konc J, Kanyo K, Kriston R, Cseh S. Birth and clinical pregnancy from fresh and frozen oocytes fertilized with cryopreserved testicular spermatozoa. Syst Biol Reprod Med 2012;58(3):165–7.
[16] Liow SL, Foong LC, Chen NQ, Yip WY, Khaw CL, Kumar J, et al. Live birth from vitrified–warmed human oocytes fertilized with frozen–thawed testicular spermatozoa. Reprod Biomed Online 2009;19(2):198–201.
[17] Thomson LK, Fleming SD, Barone K, Zieschang J-A, Clark AM. The effect of repeated freezing and thawing on human sperm DNA fragmentation. Fertil Steril 2010;93(4):1147–56.
[18] Mansilla MA, Merino O, Risopatron J, Isachenko V, Isachenko E, Sanchez R. High temperature is essential for preserved human sperm function during the devitrification process. Andrologia 2016;48(1):111–3.
[19] Rosenborg L, Rao K, Björndahl L, Kvist U, Pousette A, Akerlöf E, et al. Changes in human sperm chromatin stability during preparation for in-vitro fertilization. Int J Androl 1990;13(4):287–96.
[20] Zini A, Agarwal A. Sperm chromatin: biological and clinical applications in male infertility and assisted reproduction. Springer; 2011.
[21] Köhn F, Mack S, Schill W, Zaneveld L. Detection of human sperm acrosome reaction: comparison between methods using double staining, *Pisum sativum* agglutinin, concanavalin A and transmission electron microscopy. Hum Reprod 1997;12(4):714–21.
[22] Abdalla H, Hirabayashi M, Hochi S. Demethylation dynamics of the paternal genome in pronuclear-stage bovine zygotes produced by in vitro fertilization and ooplasmic injection of freeze-thawed or freeze-dried spermatozoa. J Reprod Dev 2009;55(4):433–9.
[23] Liu T, Gao J, Zhou N, Mo M, Wang X, Zhang X, et al. The effect of two cryopreservation methods on human sperm DNA damage. Cryobiology 2016;72(3):210–5.
[24] Pope CE, Gomez MC, Kagawa N, Kuwayama M, Leibo SP, Dresser BL. In vivo survival of domestic cat oocytes after vitrification, intracytoplasmic sperm injection and embryo transfer. Theriogenology 2012;77(3):531–8.
[25] Park YS, Kim MK, Lim CK, Lee SH, Park DW, Seo JT, et al. Efficacy of cryopreservation of embryos generated by intracytoplasmic sperm injection with spermatozoa from frozen testicular tissue. J Assist Reprod Genet 2014;31(10):1331–6.
[26] Liang YY, Srirattana K, Phermthai T, Somfai T, Nagai T, Parnpai R. Effects of vitrification cryoprotectant treatment and cooling method on the viability and development of buffalo oocytes after intracytoplasmic sperm injection. Cryobiology 2012;65(2):151–6.
[27] 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(8):403–11.
[28] Creemers E, Nijs M, Vanheusden E, Ombelet W. Cryopreservation of human sperm: efficacy and use of a new nitrogen-free controlled rate freezer versus liquid nitrogen vapour freezing. Andrologia 2011;43(6):392–7.
[29] Gosalvez J, Nunez R, Fernandez JL, Lopez-Fernandez C, Caballero P. Dynamics of sperm DNA damage in fresh versus frozen-thawed and gradient processed ejaculates in human donors. Andrologia 2011;43(6):373–7.
[30] Khalili MA, Adib M, Halvaei I, Nabi A. Vitrification of neat semen alters sperm parameters and DNA integrity. Urol J 2014;11(2):1465–70.
[31] Zhang HB, Lu SM, Ma CY, Wang L, Li X, Chen ZJ. Early apoptotic changes in human spermatozoa and their relationships with conventional semen parameters and sperm DNA fragmentation. Asian J Androl 2008;10(2):227–35.
[32] Gürler H, Malama E, Heppelmann M, Calisici O, Leiding C, Kastelic J, et al. Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species and DNA damage of bovine sperm. Theriogenology 2016;86(2):562–71.
[33] Gadea J, Molla M, Selles E, Marco MA, Garcia-Vazquez FA, Gardon JC. Reduced glutathione content in human sperm is decreased after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders. Cryobiology 2011;62(1):40–6.
[34] Ni W, Xiao S, Qiu X, Jin J, Pan C, Li Y, et al. Effect of sperm DNA fragmentation on clinical outcome of frozen-thawed embryo transfer and on blastocyst formation. PloS One 2014;9(4):e94956.
[35] Donnelly ET, McClure N, Lewis SE. Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. Fertil Steril 2001;76(5):892–900.
[36] Kalthur G, Raj S, Thiyagarajan A, Kumar S, Kumar P, Adiga SK. Vitamin E supplementation in semen-freezing medium improves the motility and protects sperm from freeze-thaw-induced DNA damage. Fertil Steril 2011;95(3):1149–51.
[37] Kotdawala AP, Kumar S, Salian SR, Thankachan P, Govindraj K, Kumar P, et al. Addition of zinc to human ejaculate prior to cryopreservation prevents freeze-thaw-induced DNA damage and preserves sperm function. J Assist Reprod Genet 2012;29(12):1447–53.
[38] Jeyendran RS, Acosta VC, Land S, Coulam CB. Cryopreservation of human sperm in a lecithin-supplemented freezing medium. Fertil Steril 2008;90(4):1263–5.
[39] Jenkins TG, Aston KI, Carrell DT. Supplementation of cryomedium with ascorbic acid-2-glucoside (AA2G) improves human sperm post-thaw motility. Fertil Steril 2011;95(6):2001–4.