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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