Impact of Exposure Period to Liquid Nitrogen Vapor on Criteria of Human Spermatozoa Cryopreserved in New Cryopreservation Technique

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Abstract

Background: The emptied sheep's ovarian follicles recently used as a container for spermatozoa during cryopreservation, it was found a proper carrier to cryopreserving spermatozoa in vapor-dependent cryopreservation. The aim of this study was to evaluate the effect of two periods of exposure to liquid nitrogen (LN_2)vapor on the parameter of spermatozoa during cryopreservation in this technique.

Method: The study was conducted on 30 semen samples from patients with oligozoospermia diagnosed by semen analysis according to the standard criteria of World Health Orgnization (WHO) 2010. Sheep's ovarian follicles obtained from local slaughterhouse and prepared by slicing the ovaries and evacuating the follicular fluid and oocyte. Each semen sample diluted 1:1 with cryosolution (glycerol 10%) and injected within eight emptied sheep's ovarian follicles. The first four follicles represent P1; exposed to LN_2 vapor for 7.5 minutes and the other four follicles represent P2; exposed to LN_2 vapor for 15 minutes before emerged in liquid nitrogen. Sperm progressive motility, total motility, normal morphology and DNA fragmentation index (DFI) were analyzed for all samples pre-freezing and post-thawing.

Results: After two months of cryopreservation, sperm progressive motility and total motility significantly ($P<0.01$) increased post-thawing in P2 as compared with P1, while both of P1 and P2 significantly ($P<0.01$) decreased as compared with pre-freezing. Normal morphology significantly ($P<0.01$) decreased post-thawing in both P1 and P2 as compared with pre-freezing, while no significantly difference foundbetween P1and P2. DFI significantly ($P<0.01$) increased post-thawing in P1 and P2 as compared with pre-freezing, while in P2 DFI was significantly lower than in P1.

Conclusions: The exposure to liquid nitrogen vapor for 15 minutes in emptied ovarian follicles technique gives a better results than exposure to the vapor for 7.5 minutes regarding sperm progressive motility, total motility and DFI.

Keywords: *ART; cryopreservation; human spermatozoa; LN_2 vapor;ovarian follicles; sperm cryopreservation,*

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Introduction

Cryopreservation of human spermatozoa is a wide spread routine work in clinics of assisted reproductive technologies (ART) [(1)]. It is used for preserving male fertility in many cases such as prior to undergoing chemo- or radiotherapy, vasectomy and other activities that may affect male fertility [(2)]. It also can be used in cases of

sperm donors to prevent the distribution of infectious diseases from donor to recipient couples [3]. It is found that cryopreservation cause damage to spermatozoa and reduce its viability and motility and even the fertilization ability[4]. More than 40% of sperm motility found to loss after cryopreservation [2], this may due to the damage of plasma membrane, loss of acrosome function and/or DNA fragmentation [5].Cryopreservation of spermatozoa not only affect sperm motility and viability, but, also the concentration of sperms decreased post-thawing due to the dilutions and washing steps [6]. However, this may not appropriate for low concentrations of spermatozoa because it may loss during washing steps [7,8]. This reason spur many researchers to experimented different techniques for cryopreserving the low concentrations of spermatozoa[7–12].In our recent under-publication study[13], we succeeded in cryopreserving different concentrations of spermatozoa using sheep's emptied ovarian follicles as a vehicle to carry spermatozoa during cryopreservation in order to minimize the loss of cryopreserved spermatozoa. In emptied ovarian follicles technique, spermatozoa cryopreserved by exposing toLiquid Nitrogen(LN_2) vapor before immerged into LN_2. Therefore, this study builds on our prior results, to determine the best time of exposure to LN_2 vaporduring cryopreservation of spermatozoa in this newly invented cryopreservation technique.

Materials and Method

Subjects and samples collection: Thirty semen samples were collected from oligozoospermicpatients (their sperm concentration $\leq 15 \times 10^6$ mL^{-1}) their ages ranged from 22 to 57 years old. They attended to the Fertility Center Clinic at Al-Sadr Medical City, Najaf, Iraq, during the period from February 2019 to November 2019. The samples collected from the subjects by masturbation after 3 days sexual abstinence and analyzed according to the World Health Organization (WHO) 2010standard guidelines using a light microscope (Optica, Italy) to determine the sperm parameters (sperm concentration, total motility, progressive motility, normal morphology). Each sample was analyzed twice by only one experienced biologist to avoid any personal variations.

Ethical approval: This study was ethically approved by the medical ethics committee in Jabir Ibn Hayyan Medical University, Iraq (Approval No: 19-0003). All the patients gave their informed agreement for research before they gave the semen samples.

Experimental design: After seminal fluid analysis (SFA) and DFI assay, each semen sample diluted 1:1 with cryosolutionusing SMART medium [14] with (10%glycerol) and injected within eight emptied sheep's ovarian follicles. The first four follicles represent P1; exposed to LN_2 vapor for 7.5 minutes and the other four follicles represent P2; exposed to LN_2 vapor for 15 minutes before emerged in liquid nitrogen. Sperm progressive motility, total motility, normal morphology and DFI were analyzed for all samples pre-freezing and post-thawing.

Collection and preparation of sheep ovarian follicles: A total of 240 ovarian follicles were sliced from 186 sheep's ovary used in this study. The sheep ovaries were collected from local slaughterhouse in Najaf city. The ovaries were collected directly from the ewes after slaughtered and kept at 32-35ºC with normal saline solution (0.9%NaCl) supplemented with two types of antibiotics (100mg/ml penicillin and 100mg/ml streptomycin). The ovaries then, transported to the laboratory within 1 hour. In the Laboratory, ovaries washed three times using normal saline solution (37 ºC) to remove the clotted blood and reduce contamination on the ovarian surface [15]. After washing, the ovaries differentiated according to the size of ovarian follicles. The ovaries that contain follicles less than 0.3 mm in diameter were excluded, and those contain follicles larger than 0.3 mm in size sliced to remove the medulla and allows the follicles to be fit inside the cryotube. Then, the ovarian pieces that contain the follicles were stored at 4˚C till the semen prepared.

Process of sperm cryopreservation: The prepared ovarian follicles emptied from the oocyte andfollicular fluidusing 23-gage sterile hypodermic needle with a disposable 2mL syringe. Then, each part of all the semen samples injected in 8 emptied follicles and inserted into two cryotubes (Thermo-scientific 1.8mL) (4 follicles in each cryotube) and covered with cryosolution. One cryotube exposed to LN_2 vapor for 7.5 minutes and the other exposed to LN_2 vapor for 15 minutes (2cm above the surface of LN_2). then, the cryotubes plunged inside LN_2 and stored at -196°C for two months using cryopreservation LN_2 tank (MVE SC series LN_2 tank 40L).

Thawing Process: After two months of cryopreservation, each cryotube was taken out from the LN_2 and immersed inside water bath at 35°C for 5 minutes, then, by using forcipes, the ovarian follicles

transferred from the cryotube to a clean Petri dish and the samples withdrawn from the follicles using 23-gage sterile hypodermic needle with a disposable 3mL syringe. The volume measured and the sample diluted 1:1 with the thawing solution (SMART medium plus 0.25M sucrose) and then utilized for analyzing the sample's parameters (sperm concentration, motility, normal morphology and DFI).

DNA fragmentation assay: Sperm DFI was determined in both fresh and thawed semen using Acridine Orange (AO) fluorescence stain. The AO stain was preparedaccording to Tejada *et al.*(16). The evaluation of DFI done using fluorescent microscope with excitation at 450–490 nm to count sperms in at least 5 fields. The normal spermatozoa with intact double-stranded DNA stained green while spermatozoa with fragmented DNA showed red or orange fluorescence (Figure 1). The DFI was calculated by measuring the percentage of DNA Fragmented spermatozoa[16].

Statistical analysis: The statistical analysis system (SAS) program (2012) was used to analyzed the data. Parameters in this study were expressed as the means and standard deviations (mean ± SD). The least significant difference (LSD) test and analysis of variation (ANOVA) were used to analyze the differences between groups. The p-value of less than (0.01) was considered significantly different.

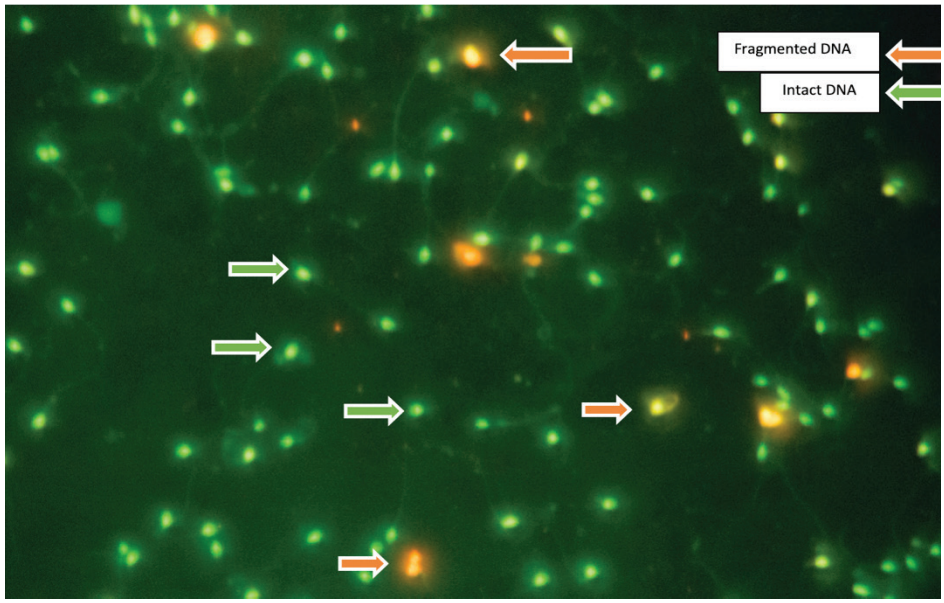

Figure (1): DNA fragmentation assay using Acridine orange stain under fluorescent microscope (magnification power 40X). Green sperms ranked as spermatozoa with intact DNA, orange and yellow sperms ranked as DNA fragmented spermatozoa.

Results

Sperm concentration was significantly (P<0.01) decreased in both post-thawing groups when compared to pre-freezing, meanwhile, no significant difference were observed in sperm concentration post-thawing between P1 and P2.

Progressive sperm motility (%) and total motility (%) found to be significantly (P<0.01) higher in samples exposed to LN_2vapor for 15 minutes(11.91±3.56) (22.07±4.36) respectively as compared with samples exposed to LN_2 vapor for 7.5 minutes (8.23±3.05) (14.08±4.73) respectively, while both groups significantly (P<0.01) decreased as compared with pre-freezing (26.22±5.18) (34.92±6.25) respectively.

Normal sperm morphology (%) significantly (P<0.01) decreased in both post-thawing groups when compared with pre-freezing (25.19±3.79), meanwhile, in samples exposed to LN_2 vapor for 15 minutes, normal sperm morphology (23.02±0.35) observed to be slightly but not significantly higher than in those exposed to LN_2 vapor for 7.5 minutes(22.20±0.39).

DFI significantly (P<0.01) increased post-thawing both in P1 (42.91±2.18) and in P2 (39.6±3.01) as compared with pre-freezing (35.75±3.41), while in P2 (samples exposed to LN2 vapor for 15 minutes) DFI significantly decreased as compared with P1(samples exposed to LN2 vapor for 7.5 minutes) (Table 1).

Table 1. Human sperm parameters in pre-freezing and post-thawing using sheep's ovarian follicles.

Parameters	Pre-freezing	Exposure to LN_2 vapor	
		7.5 min	15 min
Sperm concentration (mL $X10^{-6}$)	8.04±3.81[a]	1.95±0.85[b]	1.92±0.49[b]
Progressive motility (%)	26.22±5.18[a]	8.23±3.05[c]	11.91±3.56[b]
Total sperm motility (%)	34.92±6.25[a]	14.08±4.73[c]	22.07±4.36[b]
Normal sperm morphology (%)	25.19±3.79[a]	22.20±0.39[b]	23.02±0.35[b]
DNA Fragmentation Index (%)	35.75±3.41[a]	42.91±2.18[b]	39.60±3.01[c]

Data are presented as the (mean ±SD). Different letters (a, b and c) in the same row indicate significant differences ($p < 0.01$).

Discussion

Human spermatozoa can be cryopreserved by one of three main protocols: slow freezing[17], rapid or vapor-dependent freezing [18] and ultra-rapid freezing (vitrification) [19].In this study, samples were cryopreserved using the direct contact between the cryotube that contain the samples and the LN_2 vapor for two periods of exposure (7.5 and 15 minutes) in view to determine the effect of the two periodson the parameters of cryopreserved spermatozoa. This method is one of the most popular method in human sperm cryopreservation [20]. Esteves *et al.*[6] confirmed that this method preferable than slow graduating cryopreservation for human spermatozoa. Rahiminia *et al.* in 2017[21] concluded that human sperm DNA, chromatin and acrosome integrity status were more tolerable during cryopreservation with LN_2 vapor rather than in vitrification.

Incomparison between the two periods of exposure to LN_2 vapor in this study, the recovery of progressive and total motility increased significantly ($P < 0.01$) in 15 minutes than in 7.5 minutes. This reflect that the extend period of exposure to LN_2 vapor may reduce the plasma membrane damage of the cryopreserved spermatozoa during freezing process, and this in turnincrease the recovery rate of sperm motility post-thawing. For the same reason, normal sperm morphology slightly improved with the exposure to LN_2 vapor for 15 minutes.

In addition to conventional sperm parameters, DFI gives more information on men's reproductive potential. Spermatozoa with fragmented DNA have been reported to be contributed in failure of fertility and loss of pregnancy [22]. Although the negative effect of cryopreservation on sperm motility, morphology, and viability post-thawing has been studied widely [19].The effect of various cryopreservation method on sperm DNA integrity still controversy [23]. Several studies demonstrated that cryopreservation increase sperm DNA fragmentation post-thawing [18,24] and other studies indicated that cryopreservation did not affect sperm chromatin integrity and not increase sperm DNA fragmentation [25,26].

The main causes of DNA fragmentation seemed to be the increase in reactive oxygen species (ROS) during sperm cryopreservation as well as the formation of intracellular ice crystals [27,28]. The results of this study found that DFI significantly decreased with the exposure to LN_2 vapor for 15 minutes when compared with the exposure to LN_2 vapor for 7.5 minutes. This may refer to the positively role of the exposure to LN_2 vapor for 15 minutes in reducing the formation of intracellular ice crystal and reducing the formation of ROSduring freezing process. Exposure to LN_2vapor for 15 minutes previously used in human sperm cryopreservation. In conclusion, the exposure to LN_2 vapor for 15 minutes before emerging the samples directly into LN_2gave better results regarding sperm motility, morphology and DFI than the exposure to LN_2 vapor for 7.5 minutes in this technique of sperm cryopreservation.

Ethical Clearance: The Research Ethical

Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: Non

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