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Original Article

New Technique for Human Sperm Cryopreservation Using Emptied Sheep's Ovarian Follicles

Hussein Zwamel, A[1 *], Muhammad-Rashad Fakhrildin, M. B[2], Hadi Hassani, H[3]

1. Radiology Techniques Department, College of Medical Technology, the Islamic University, Najaf, Iraq
2. Department of Medical Physiology, Faculty of Medicine, Jabir Ibn Hayyan Medical University, Najaf, Iraq
3. Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

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Corresponding Author: ahmed.hussein.ali@iunajaf.edu.iq

Abstract

This study aimed to investigate the efficacy of emptied ovarian follicles of sheep as a container for cryopreservation of human spermatozoa to preserve the presence of low concentrations of spermatozoa at the post-thawing stage. This research was performed on 30 semen samples from oligozoospermic patients and 10 samples from normozoospermic males. They were diagnosed according to the standard criteria of the World Health Organization 2010. Semen samples were classified into four groups of G1-G4 according to sperm concentration: 3-5 million/mL, 6-10 million/mL, 11-15 million/mL, and 16-20 million/mL, respectively. Each sample was divided into two equal parts. One part was cryopreserved without cryoprotectant, while the other was diluted 1:1 with 10% glycerol-based cryosolution. The ovarian follicles of sheep were obtained from a local slaughterhouse and prepared by slicing the ovaries and evacuating the follicular fluid and oocyte. The emptied follicles were injected with the prepared semen samples. After cryopreservation and thawing, the semen mixture aspired outside the follicles, and sperm parameters were measured, namely concentration, progressive motility, total motility, and normal morphology. Sperm concentration and progressive and total sperm motility were significantly (P<0.01) decreased in all groups at the post-thawing stage, compared to the pre-freezing stage. The sperm concentration was significantly higher (P<0.01) in samples cryopreserved without cryoprotectant, compared to that in those cryopreserved with glycerol. However, progressive and total motility were significantly (P<0.01) higher in samples cryopreserved with glycerol, compared to that in the samples cryopreserved without cryoprotectant in all groups. Moreover, no significant difference was found between the pre-freezing and post-thawing stages in terms of normal morphology. Emptied ovarian follicles are an appropriate carrier for cryopreservation of human sperms, especially for patients with oligozoospermia. The best sperm survival rate in this technique was observed when using glycerol-based cryosolution.

Keywords: ART, Cryopreservation, Human spermatozoa, Ovarian follicles, Sperm cryopreservation

1. Introduction

Human sperm cryopreservation is a technique used routinely in assisted reproductive technologies (ART) clinics in order to preserve male fertility in case the patient is undergoing a procedure that may affect his fertility, such as chemo- or radiotherapy and vasectomy (1). This technique is also used in the case of sperm donors to prevent the transition of infectious diseases (2). It is approved that the quality of sperm significantly decreases after cryopreservation (post-thawing stage), which is due to the formation of ice crystals and the changes that occur in the sperm plasma membrane (3). The success of sperm cryopreservation is measured by the percentage of the motile sperm recuperated at the post-thawing stage. Based on several studies, about 30-40% of the motility of the sample is lost at the post-thawing stage. This is due to the

structural damage of the plasma membrane, acrosomal contents, and DNA integrity (4-6).

Not only ejaculated sperms can be cryopreserved but also epididymal and testicular sperms can be retrieved and then cryopreserved. The main reason for epididymal or testicular aspiration is obstructive azoospermia; therefore, the amount of semen that is obtained in this technique is relatively few (7).

The conventional sperm cryopreservation techniques are not appropriate for samples from severe oligozoospermic or obstructive azoospermic patients since the sperms are diluted and lost during centrifugation (8, 9). For this reason, several researchers have tried to develop a proper technique to cryopreserve a low number of sperms to replace the conventional sperm cryopreservation techniques in order to preserve the low number of sperms obtained from oligozoospermic or obstructive azoospermic patients (5, 9-13).

This study aimed to preserve the low concentration of cryopreserved sperms at the post-thawing stage by using emptied sheep ovarian follicles as a carrier for sperms during cryopreservation.

2. Materials and Methods

2.1. Subjects and Samples Collection

In this study, 30 semen samples were collected from oligozoospermic patients (sperm concentration $\leq 15\times10^6$ mL^{-1}) and 10 samples from non-oligozoospermic males (sperm concentration 16-20×10^6 mL^{-1}) within the age range of 19-54 years old. All semen samples were collected from the subjects by masturbation after 3 days of sexual abstinence. The samples were analyzed according to the guidelines of the World Health Organization 2010 (14). using a light microscope (Optica, Italy) to determine the sperm parameters (sperm concentration, total motility, progressive motility, and normal morphology). Each sample was analyzed twice by only one experienced biologist to avoid personal variations and ensure accurate and precise results.

2.2. Experimental Design

After seminal fluid analysis, 40 samples were equally divided into four groups (G1-G4) according to the sperm concentration (10 samples in each group), and each sample was divided into two equal parts. One part was diluted 1:1 with a simple medium for assisted reproductive technologies (SMART) medium (15) and injected into the emptied ovarian follicles of sheep without the addition of any kind of cryoprotectant. Besides, the other part was diluted 1:1 with cryosolution that contained 10% glycerol and injected inside the emptied ovarian follicles for cryopreservation.

2.3. Preparation of Cryoprotectant

The glycerol-based cryosolution was prepared by adding 1 mL (10%) of glycerol to 9 mL of SMART medium.

2.4. Collection and Preparation of Sheep Ovarian Follicles

In total, 480 ovarian follicles were sliced from the ovaries of 425 sheep in this study. The sheep ovaries were collected from a local slaughterhouse. Both ovaries were collected directly from the ewes after they were slaughtered and kept at 32-35 ºC with normal saline solution (0.9% NaCl) supplemented with two types of antibiotics (100 mg/ml penicillin and 100 mg/ml streptomycin). Afterward, the ovaries were transported to the laboratory within 1 h. In the laboratory, ovaries were washed three times using a normal saline solution (37 ºC) to remove the clotted blood and reduce contamination on the ovarian surface (16).

After washing, the ovaries differentiated according to the size of the ovarian follicles. The ovaries that contained follicles less than 0.3 mm in diameter were excluded and those that contained follicles larger than 0.3 mm in size were sliced to remove the medulla and allow the follicles to fit inside the cryotube. Subsequently, the ovarian pieces that contained the follicles were stored at 4˚C till the semen was prepared.

2.5. Process of Sperm Cryopreservation

The follicular fluid was extracted from the prepared ovarian follicles using a 23-gage sterile hypodermic

needle with a disposable 2 mL syringe. Afterward, each part of the semen samples was injected into four emptied follicles and inserted into 1.8 mL cryotubes (Thermo-Scientific), and covered with cryosolution. The cryotube was then exposed to liquid nitrogen (LN_2) vapor for 15 min (2 cm above the surface of LN_2). Finally, the cryotubes were plunged inside LN_2 and stored at -196 ℃ for 2 months using a cryopreservation LN_2 tank (MVE SC series LN_2 tank of 40 L).

2.6. Thawing Process

After two months of cryopreservation, each cryotube was extracted from the LN_2 and immersed inside a water bath at 35 ℃ for 5 min. Afterward, by using forcipes, the ovarian follicles were transferred from the cryotube to a clean Petri dish and the samples were withdrawn from the follicles using a 23-gage sterile hypodermic needle with a disposable 3 mL syringe. The volume was measured and the samples that were cryopreserved with glycerol were diluted 1:1 volume with the thawing solution (SMART medium plus 0.2 M sucrose) to remove the glycerol and the samples that cryopreserved without cryoprotectant were not diluted. Afterward, the sperm parameters (sperm concentration, motility, and normal morphology) were measured for all samples.

2.7. Statistical Analysis

The statistical analysis system (SAS) software (2012) was used to analyze the data. Parameters in this study were expressed as the mean and standard deviation (mean±SD) and the recovery rate of groups at the post-thawing stage was expressed as a percentage. The least significant difference test and analysis of variance were used to analyze the differences among groups. The *P* values of less than 0.01 were considered statistically significant.

3. Results

Sperm concentration was significantly (P<0.01) decreased at the post-thawing stage, compared to the pre-freezing stage for all the studied groups. Moreover, in all groups, the sperm concentration significantly (P<0.01) increased in samples that were cryopreserved without cryoprotectant, compared to those that were cryopreserved with glycerol (Table 1). About 50% of the sperm concentration was recovered at the post-thawing stage in samples cryopreserved without cryoprotectant, while about 25% of sperm concentration was recovered in samples that were cryopreserved with glycerol (Figure 1a).

Progressive sperm motility and total motility also significantly (P<0.01) decreased at the post-thawing stage in all the studied groups, compared to the pre-freezing stage. In all groups, the samples that were cryopreserved with glycerol showed a significant (P<0.01) increase in progressive and total motility, compared to those that were cryopreserved without cryoprotectant (Tables 2 and 3). The highest and lowest progressive motility recovery rates were observed in groups four and one (Figure 1b), respectively. Moreover, the highest total motility recovery rate was observed in groups three and four, while the lowest was observed in groups one and two (Figure 1c).

Normal sperm morphology (%) did not differ significantly (P<0.01) neither between pre-and post-thawing stages nor between post-thawing cryoprotectant and cryoprotectant-free treats in all studied groups (Table 4). The best recovery rate of normal morphology was found in samples that were cryopreserved with glycerol in group three (97.58%). However, the lowest recovery rate of normal morphology was observed in samples that were cryopreserved without cryoprotectants in group four (91.99%) (Figure 1d).

Table 1. Sperm concentration (x 10^6 mL^{-1}) in pre-freezing and post-thawing using sheep's ovarian follicles

Group (X 10^6 mL^{-1})	Fresh	CF	Gly 10%
G1: (3-5)	3.98±0.72^a	1.96±0.21^b	0.95±0.11^c
G2: (6-10)	7.52±1.48^a	3.74±1.09^b	1.88±0.05^c
G3: (11-15)	12.65±1.37^a	6.30±0.89^b	2.98±0.18^c
G4: (16-20)	17.77±1.35^a	8.86±1.16^b	4.40±0.32^c

Data are presented as the mean ±SD. Fresh: fresh semen pre-freezing, CF: cryoprotectant-free cryopreserved sperms, Gly: glycerol. Different small letters (a, b, and c) indicate significant differences (P<0.01) in the same row

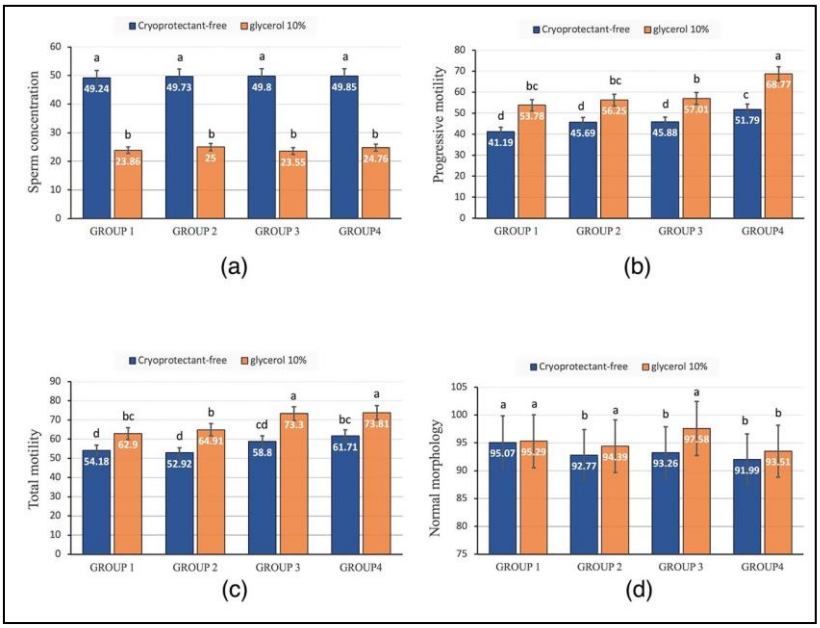

Figure 1. Recovery rates (%) of human sperm parameters for all study groups after freezing-thawing using sheep's ovarian follicles. (**a**) sperm concentration, (**b**) progressive motility, (**c**) total motility, (**d**) normal morphology. Different letters on the percentage bars indicate significant differences (P<0.01)

Table 2. Progressive motility (%) in pre-freezing and post-thawing using sheep's ovarian follicles

Group (x 10^6 mL^{-1})	Fresh	CF	Gly 10%
G1: (3-5)	30.25±4.65^{a}	12.46±0.21^{b}	16.27±1.76^{c}
G2: (6-10)	26.22±5.18^{a}	11.98±1.09^{b}	14.75±2.54^{c}
G3: (11-15)	23.10±5.28^{a}	10.60±1.89^{b}	13.17±1.05^{c}
G4: (16-20)	22.90±9.52^{a}	11.86±4.25^{b}	15.75±3.04^{c}

Data are presented as the mean±SD. Fresh: fresh semen pre-freezing, CF: cryoprotectant-free cryopreserved sperms, Gly: glycerol. Different small letters (a, b, and c) indicate significant differences (P<0.01) in the same row

Table 3. Total motility (%) in pre-freezing and post-thawing using sheep's ovarian follicles

Group (x 10^6 mL^{-1})	Fresh	CF	Gly 10%
G1: (3-5)	46.53±9.30^{a}	25.21±4.76^{b}	29.27±3.46^{c}
G2: (6-10)	34.92±6.25^{a}	18.48±4.46^{b}	22.67±3.98^{c}
G3: (11-15)	32.55±6.29^{a}	19.14±3.96^{b}	23.86±2.67^{c}
G4: (16-20)	33.80±7.37^{a}	20.86±4.65^{b}	24.95±4.23^{c}

Data are presented as the mean ±SD. Fresh: fresh semen pre-freezing, CF: cryoprotectant-free cryopreserved sperms, Gly: glycerol. Different small letters (a, b, and c) indicate significant differences (P<0.01) in the same row

Table 4. Normal morphology (%) in pre-freezing and post-thawing using sheep's ovarian follicles

Group (x 10^6 mL^{-1})	Fresh	CF	Gly 10%
G1: (3-5)	40.21±1.14^{Aa}	38.23±1.31 ^{Aa}	38.32±1.37^{Aa}
G2: (6-10)	10.52±5.75^{Ca}	9.76±2.79 ^{Da}	9.93±3.31 ^{Da}
G3: (11-15)	23.16±3.79^{Ba}	21.60±1.05 ^{Ba}	22.60±1.35^{Ba}
G4: (16-20)	20.35±3.01^{Ba}	18.72±1.81 ^{Ca}	19.03±1.41^{Ca}

Data are presented as the mean ±SD. Fresh: fresh semen pre-freezing, CF: cryoprotectant-free cryopreserved sperms, Gly: glycerol. Different small letters (a, b, and c) indicate significant differences (P<0.01) in the same

4. Discussion

In this study, the emptied ovarian follicles of sheep were used as a carrier for human sperm during cryopreservation. It was tried to cryopreserve sperms in this carrier without using cryoprotectant to avoid the loss of sperms through dilutions and washing steps. The results were compared with those of the studies that used glycerol as a cryoprotectant in the same technique. Consequently, different concentrations of sperm were successfully cryopreserved by this technique.

Previously, Cohen, Garrisi (10) described the emptied human and mouse zona pellucida (ZP) as a vehicle to cryopreserve single human spermatozoa. Cohen's technique was upgraded by Hsieh, Tsai (9) to cryopreserve more than one sperm per ZP. In the same concept, Just, Gruber (17) used the spherical algae *Volvox globator* and they succeeded to cryopreserve 15-18 sperms per each spherical *Volvox*. However, these techniques cryopreserved a limited number of sperms and required time to select the sperms by a micromanipulator. In the present study, the sperms were injected with their seminal plasma (hole semen) directly inside the emptied ovarian follicles.

This technique is an easier and non-time consumer one that also does not require micromanipulation, like previous techniques. The ovarian follicle of sheep is easy to obtain from any slaughterhouse and cost-efficient also its size is suitable for carrying about 0.2-0.5 mL of semen which allows cryopreserving an unlimited number of sperms (depending on the original sperm concentration within semen). The retrieval of the cryopreserved sperms from the follicles at the post-thawing stage is also simple and does not need enzymatic aids. The proteins in the seminal plasma protect the spermatozoa during the attachment between spermatozoa and the granulosa cells of the follicular wall and make the probability of acrosome reaction not anticipated.

In samples that were cryopreserved without cryoprotectant, the semen was diluted 1:1 with SMART medium to reach the volume as that of the semen cryopreserved with glycerol. Meanwhile, samples that were cryopreserved with glycerol were diluted again at the post-thawing stage with the thawing solution to remove the glycerol. Therefore, the recovery of sperm concentration at the post-thawing stage was ~50% for samples cryopreserved without cryoprotectant and ~25% for samples cryopreserved with glycerol in all groups. Cryopreservation of sperms in this technique without using cryoprotectant did not require the dilution of the sample; therefore, the concentration recovery rate at the post-thawing stage will be about 98-100% and this will be a good choice for cases of severe oligozoospermia.

The post-thawing progressive motility and total motility in samples cryopreserved without cryoprotectant were significantly lower, compared to that in samples cryopreserved with glycerol. However, the recovery rate of progressive and total motility in samples cryopreserved without cryoprotectant was hopeful. These promising results reflect the ability of this technique to recover sperm motility at the post-thawing stage without the use of cryoprotectant. Several factors may participate in the protection of sperm viability and support sperm motility, including the presence of granulosa cells, follicular wall, some biochemical components of seminal plasma, and the technique of sperm processing and freezing.

The best recovery rate in sperm motility was obtained by the use of glycerol in all studied groups and this came along with previous publications that mentioned the importance of cryoprotectants and their impact on cryopreservation outcomes (18, 19). It was found that the recovery rate in progressive and total motility increased along with the sperm concentration (the lowest and highest in groups one and four, respectively) and this may refer to an association between sperm concentration and motility recovery at the post-thawing stage. Zhang, Zhou (20) investigated the effect of pre-freezing conditions on the progressive motility recovery rate. They found a significant association

between sperm concentration and progressive motility at the pre-freezing stage with the progressive motility recovery rate, which is in concordance with the results of the present study in this regard.

In the present study, the normal sperm morphology was not significantly affected during cryopreservation with or without cryoprotectant unlike the progressive motility and total motility. It has been declared previously that about 50% of sperms cannot survive cryopreservation even with optimized protocols (21). Le, Nguyen (22) stated that cryopreservation has potential effects on sperm motility, viability, and morphology. The findings of the present research indicated that the harmful effect of freezing in this technique was restricted to the plasma membrane which led to the reduction of the motility more than normal morphology.

In conclusion, ovarian follicles of emptied sheep are an ideal carrier for cryopreservation of different sperm concentrations and it is suitable for cryopreservation of low concentrations of sperms. In this technique, sperms can be successfully cryopreserved without using cryoprotectants; however, the best motility recovery rate can be obtained by using glycerol-based cryosolution. Further studies are required to confirm these results, investigate the outcomes of cryopreserved testicular and epididymal sperms, and evaluate the fertility rate of cryopreserved sperms in this technique.

Authors' Contribution

Study concept and design: A. H. Z.
Acquisition of data: A. H. Z.
Analysis and interpretation of data: M. B. M. R. K.
Drafting of the manuscript: H. H. H.
Critical revision of the manuscript for important intellectual content: A. H. Z.
Statistical analysis: M. B. M. R. K.
Administrative, technical, and material support: H. H. H.

Ethics

Informed consent was obtained from all the participants before the collection of semen samples. Moreover, the study was ethically approved by the local medical Ethics Committee (Approval No: 19-0001).

Conflict of Interest

The authors declare that they have no conflict of interest.

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