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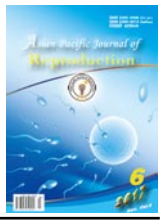

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Cryopreservation enhances vacuolization in human spermatozoa

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ABSTRACT

Objective: To evaluate the impact of freezing–thawing on the human sperm head vacuoles and the potential value of motile sperm organelle morphology examination for selection of frozen-thaw spermatozoa. **Methods:** In 30 sperm samples from infertile men, analysis for conventional sperm parameters (motility, vitality, and normal morphology) and a morphological analysis at high magnification for vacuoles examination were done before cooling and after warming. For description of sperm head vacuoles, two hundred spermatozoa were examined and were classified into three groups according to presence and vacuole areas including no vacuole group (free of any vacuole), small vacuole group (occupy not more than 4% of the nuclear area), and large vacuole group (occupy more than 4% of the normal nuclear area). **Results:** Significant reduction of progressive motility and vitality was observed following cryopreservation (P<0.001). Also, normal morphology decreased significantly after cryopreservation (P<0.05). Spermatozoa with a vacuole-free head had a significant reduction in cryopreservation group (P=0.013). The percentage of spermatozoa with small vacuoles increased slightly, but not significantly after cryopreservation (P=0.296). **Conclusions:** Motile sperm organelle morphology examination is a powerful research tool for investigating spermatozoa abnormalities such as vacuoles that are increased post cryopreservation.

1. Introduction

The nuclear vacuoles in human spermatozoa revealed with the introduction of Nomarski differential interference contrast microscope, which can examine the fine nuclear morphology of motile spermatozoa in real time at a magnification of up to 6 600 ×. Motile sperm organelle morphology examination (MSOME) is a new concept for observing spermatozoa, which enables to examine the fine nuclear morphology of motile spermatozoa[1]. The origin of thevacuoles is subject to controversy. Several studies have found sperm head vacuoles may originate from acrosome origin[2]; whereas, others show nuclear origin for them[3,4].

Cryopreservation plays an important role in reproductive science, in particular for preservation of gametes, embryos, and reproductive tissues[5]. Although, spermatozoa seems to be less sensitive to cryostorage than other cells, cryopreservation is associated with alterations of sperm structure[6]. Cryopreservation affects sperm quality, especially disturbing the motility characteristics[7,8]. The most impairments of sperm morphology after cryopreservation are often described in the literature including damage to sperm membranes[9], coiled tails[10,11] and acrosomal defects[12]. Therefore, less attention has been paid to detailed assessment of sperm head

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structures such as the vacuole.

On the other hand, the presence of nuclear vacuoles in spermatozoa is related to poor assisted reproductive technique (ART) outcomes. Berkovitz *et al*[13] have observed that nuclear vacuoles seem to be a 'pregnancy risk factor' and they should be eliminated by strict selection prior to intracytoplasmic sperm injection (ICSI). The aim of this study was to evaluate the impact of freezing-thawing on the sperm head vacuoles and to assess the potential value of MSOME for selection of frozen-thawed spermatozoa in clinical setting.

2. Materials and methods

2.1. Study design

In this experiment, the subjects were selected between June 2015 and March 2016. Semen samples from 30 men aged 20-40 years who referred to Yazd research and clinical center for infertility, Yazd, Iran. Written informed consent was obtained from all participate in the study. The protocol was approved by ethic committee of the Research and Clinical Center for Infertility (no: IR.SSU.RSI.REC.1394.12). Mean age of men was (33 ± 3) years. Mean sperm concentration was $(63 \pm 11) \times 10^6$/mL.

The samples were collected after 2-7 d of sexual abstinence. After liquefaction, spermatozoa were subjected to sperm count, motility, vitality and morphology analysis and also high-magnification examination of vacuoles. The samples were divided into two parts, control group without any intervention and experimental group includes operations of freezing and warming the samples. After thawing, analyses of sperm motility, vitality, morphology, and vacuoles examination were done.

2.2. Conventional sperm analysis

Semen analysis was performed according to World Health Organization guidelines[14] and sperm morphology was assessed according to Kruger's classification[15]. For the sperm morphology, the air-dried smears were stained with the Diff-Quick Stainkit.

2.3. Cooling

Total of 0.5 mol sucrose was dropwise added to spermatozoa at 1:1 ratio. The diluted spermatozoa were placed in 0.25 mL sterile straws, which were added extra 0.5 mL straw without sealing ball. The sperm straws were then closed using thermal sealer and cooling was carried out by immersed into LN2 container.

2.4. Warming

For warming procedure, the sperm straws were plunged into 5 mL pre-warmed HamF10 medium, and the contents were emptied. Washing was carried out by centrifugation at 300 *g* for 5 min. Finally, the pellet was resuspended in 500 μL HamF10 medium supplemented with 5% human serum albumin. Sperm analysis was done according to WHO guideline[14].

2.5. High-magnification morphological examination

For evaluation of the fresh or thawed spermatozoa, an aliquot (3 μL) of sperm suspension was placed in a glass-bottomed dish (WillCo-dish; WillCo Wells BV, The Netherlands) supplemented with 5 μL droplet of polyvinylpyrrolidone (ICSI^{TM}-100, Vitrolife, Goteborg, Sweden). Spermatozoa were observed and examined by Nomarski interference contrast microscopy. The images were captured using a color video camera, which was displayed on a color video monitor. Sperm head vacuoles were analyzed using OCTAX software, which measured sperm head areas and vacuole areas by calculating the diameter of the circle and ellipse (Figure 1).

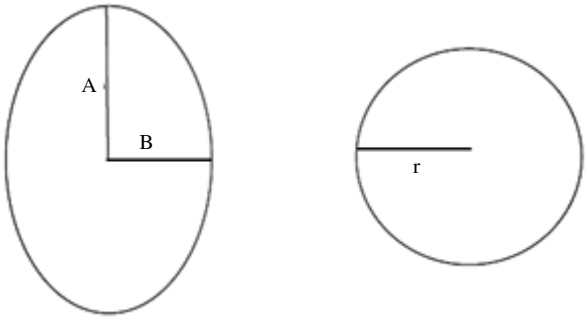

Figure 1. Schematic drawings of the sperm head vacuoles areas measurement.

For description of sperm head vacuoles, they were classified into 3 groups[16]. No vacuole group was considered, if spermatozoa were free of any vacuole. One or more vacuoles were observed that occupying no more than 4% of the nuclear area considered as small vacuole, and occupying more than 4% of the normal nuclear area considered as large vacuole (Figure 1).

Small vacuole: smaller than ½ B≤4% of the nuclear area

Large vacuole: more than ½ B≥4% of the nuclear area[17]

Ellipse area= $\pi \times A \times B$

Circle area= $\pi \times r^2$

2.6. Vitality test using hypo-osmotic swelling

Hypo-osmotic swelling test was carried out according to the original protocol[18]. The sperm samples were diluted with equal volumes of hypo-osmotic solution [equal parts sodium citrate (150 mOsmol) and fructose (150 mOsmol) were mixed]. After incubation at 37 ℃ for 30 min, 10 μL aliquot was transferred to a clean slide and examined under a phase-contrast microscope. Two hundred spermatozoa

were examined under microscope using a 40 × magnification. Spermatozoa with coiled tail indicated positive reaction (vital) and uncoiled tail as the negative response (dead).

2.7. Statistical analysis

Data were analyzed using SPSS version 20.0 software (SPSS, Inc., Chicago, IL, USA). ANOVA test were used for comparison of all parameters. Data were considered significant at $P<0.05$.

3. Results

3.1. Conventional sperm analysis

Significant reduction of progressive motility was observed following cryopreservation (46%±8% *vs.* 26%±5%, $P<0.001$). Also, Vitality decreased significantly after cryopreservation (81%±15% *vs.* 60%±4%, $P<0.001$). Cryopreservation was associated with decreased normal morphology compared with fresh (12.17%±2.35% *vs.* 9.2%±3%, $P<0.05$).

3.2. Presence of vacuoles in sperm head

A total of 6 000 spermatozoa from subjects were individually evaluated at 6 600 × magnification. The percentages of spermatozoa with a vacuole-free head in fresh and cryopreservation groups were 10.23% and 9.07%, respectively. Spermatozoa with a vacuole-free head had a significant reduction in cryopreservation group ($P=0.013$). The percentage of spermatozoa with small vacuole increased slightly, but not significantly after cryopreservation (56.9%±5.4% *vs.* 58.3%±5.5%, $P=0.296$). Similarly, the percentage of spermatozoa with large vacuoles was unchanged after cooling-warming (Table 1).

Table 1
Vacuolar parameters before and after cooling-warming of 30 semen samples (mean±SD).

Variables	Fresh group	Cryopreservation group	*P* value
No vacuole	10.23±1.79	9.07±1.48	$P=0.01$
Small vacuole	56.90±5.44	58.33±5.57	$P=0.29$
Large vacuole	32.87±5.41	32.60±5.79	$P=0.81$

4. Discussion

A new technique for observing the sperm head vacuoles with Nomarski contrast microscope was developed in 2002[16]. Although MSOME was developed only as a selection criterion, there are few studies about using of this technique after sperm cryopreservation. Conventional semen analysis demonstrates the most important information including the concentration, motility, and morphology of spermatozoa. Sperm morphology has been determined as the best prognostic factor of spontaneous pregnancies[19], or ART outcomes[20,21]. Assessment of human sperm morphology has relied on the method and the optical system used[22].

This study observed cryopreservation of sperm resulted in a decrease of approximately 45% in progressive motility, as already well described in other studies[11,23,24]. Also, a significant reduction in vitality was observed after warming. The percentage of normal morphology was significantly reduced after cryopreservation, which is also reported by others[11,24,25]. The cellular damage related to decrease of motility may be due to some reasons including production of reactive oxygen species, which is related to loss of sperm motility[26], membrane damages created by intracellular or extracellular ice crystals[27], and reduced post-thaw cyclic adenosine monophosphate concentrations, which can influence the energy production required for sperm motility[28].

Since the first description of sperm head vacuoles using real-time observation of motile spermatozoa (MSOME)[1], features defining a classification for vacuole (location, number and size) have not been clearly determined. Large vacuoles have been defined as occupying >4% of the total head area[13,16,29-31]. Also, >50% of total head area[4], and occupying >13% of sperm head area are considered as large sized-vacuoles[32]. A vacuole area occupying >4% of the sperm head was used to describe large vacuoles in this article.

Some studies reported that there is relationship between the presence of sperm head vacuoles and sperm function. Indeed, it was concluded that the vacuole-free spermatozoa had lower rates of DNA fragmentation as compared with vacuolated ones[32-34]. On the other hand, a number of studies demonstrated failure of chromatin condensation at the site of the vacuole and the relationship between nuclear vacuole- like structure and chromatin disorganization[35,36]. Recent studies demonstrated that size and number of sperm head vacuole had negative effects on blastocyst development. Results from these studies showed that incidence of large nuclear vacuoles or abnormal head shape reduced the percentage of good-quality embryos reaching the blastocyst stage[29,37]. Other studies have reported that using of sperm with vacuoles for ICSI tended to result in decreasing pregnancy rates and cause early miscarriage[1,16,38]. Also, recent researches demonstrated that selection of sperms with vacuole-free head and normal shape was positively associated with higher pregnancy and lower abortion rates[29,39,40].

According to Agarwal *et al*[41], vacuoles are associated with impaired chromatin packaging and DNA fragmentation. Therefore, vacuoles had negatively effects on the fertilization, embryo development, pregnancy, miscarriage and healthy babies born. To measure the vacuole area, an objective tool which enabled the

precise calculation of the diameter of the vacuoles and sperm head was used. The area was measured using the formula and vacuoles occupancy rate was calculated. In this study, spermatozoa with large vacuoles represented 32.87% of the samples. This mean value was equivalent to published data varying from 30.00%-40.00%[13,32], but lower than 53.40%[31] and 73.20%[16], and higher than values reported by Falagario *et al*[42] that identified a cut off of 20.00% for sperm nuclear vacuolization on the total sperms in a seminal sample. Finally, the present study found that cryopreservation increased in the percentage of small vacuoles in the spermatozoa, but not significant. Also, a significant reduction in spermatozoa with a vacuole-free head was demonstrated after freezing. Boitrelle *et al*[43] observed that cryopreservation induced sperm nuclear vacuolization, decreased the incidence of grades I+II spermatozoa and the sperm viability rate; while increased incidence of sperm with non-condensed chromatin.

Conversely, Gatimel *et al*[24] demonstrated that the cryopreservation has no effect on human sperm vacuoles. The main difference between the two studies was patient selection. Boitrelle *et al*[43] studied men from infertile couples, while Gatimel *et al*[24] selected fertile men only. According results of this study and this author[43], probably the sperm from infertile men to be more sensitive to cryopreservation damage. Compared with fertile men, a higher susceptibility to cryopreservation was found for parameters such as chromatin condensation[44] and DNA integrity in teratospermic men[45].

In conclusion, cryopreservation procedures caused reduction in spermatozoa with a vacuole-free head. Also, it confirmed that MSOME is a powerful research tool for investigating spermatozoa abnormalities, such as vacuole that may exert a negative effect on ART outcomes.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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