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ARE ANY SPERM CRYOPRESERVATION METHODS SUPERIOR OVER OTHERS? COMPARISON OF MOTILITY, VIABILITY, AND MOTILE SPERM ORGANELLE MORPHOLOGY EXAMINATION (MSOME) OF HUMAN SPERMATOZOA

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ABSTRACT

Background: Cryopreservation is an effort to get an optimal number of functional sperms in a clinical setting.

Objective: The present study aimed to evaluate the influence of cooling-warming techniques on human sperm motility, viability and motile sperm organelle morphology examination (MSOME).

Materials and Methods: Semen samples of 28 normozoospermic men prepared by swim-up were divided into four parts. One served as the control group (Group I) and the other three parts cryopreserved using rapid freezing (Group II), cooling with droplet (Group III), and cooling with open-pulled straws (Group IV). For each group, MSOME scoring was used to assess of the sperm head (Normal head score=2), vacuoles (Lack of vacuole score=3), and basis (Normal base score=1) according to Cassuto scoring (Total =6).

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Results: The slightest amount of decrease in motility was noticed in group IV ($P < 0.001$). In group II, the minimum level of viability was achieved. In the morphologic assessment, there were no significant differences between group II and group III in class 1 Cassuto scoring ($P = 0.162$). A significant increase was seen in group IV, though ($P < 0.001$).

Conclusions: Vitrification of human spermatozoa using open-pulled straws method preserved vital parameters of sperm better than other vitrification methodology. The application of MSOME can assist in the selection of the normal quality of spermatozoa in ART programs.

Keywords: Cryopreservation, Human spermatozoa, MSOME, Sperm motility.

Introduction

Cryopreservation is a process by which the cells are cooled to subzero temperatures with immersing in liquid nitrogen for storage. They are then warmed and recovered to renovate their normal function (Leibo and Pool, 2011). Sperm cryopreservation is used in assistant reproductive technology (ART), gamete preservation before cancer treatment, and sperm banking (Talwar, 2011, Meseguer et al., 2006). Leibo and Pool have shown that temperature, the rate of cooling/ warming, and the medium are the most important factors having a principal role in cell survival when the cells were cryopreserved (Leibo and Pool, 2011). Methods of cryopreservation based on high-speed freezing cause liquid to turn into a glassy state without ice crystal formation (Fahy et al., 1984). Although spermatozoa seems to be less sensitive to cryostorage than other cells, changes occur in both sperm structure and function(Watson, 2000). Cryopreservation affects sperm structure, in particular, in the head, the midpiece, and the tail regions (Agha-Rahimi et al., 2014, Hammadeh et al., 1999). Current methods of human sperm cryopreservation are still considered unsatisfactory due to the low recovery rate of sperm motility and viability after thawing(Vutyavanich et al., 2010).

Laboratory methods currently used to detect induced damages by cryopreservation are invasive and sometimes require fixation and staining (Boitrelle et al., 2012). Among semen parameters, the sperm morphology is shown as a prognostic and predictive tool for the predication of human fertility potential. (Van der Merwe et al., 2005). The innovative method proposed by Bartoov and colleagues for the evaluation of sperm morphology utilizes an inverted light microscope equipped with high-power Nomarski optics to achieve a magnification above ×6000 (Bartoov et al., 2002). The fertilization rate, embryo quality, blastocyte formation, implantation, and pregnancy rates are affected by the use of motile sperm organelle morphology examination (MSOME) in sperm selection (Knez et al., 2011, Wilding et al., 2011). Hence, the use of MSOME for sperm selection after thawing may be valuable (Boitrelle et al., 2012). High magnifications selection of spermatozoa is of particular value to screen nuclear abnormalities, vacuoles, and general morphology in ART protocols (Boitrelle et al., 2012). Boitrelle and co-workers (2012) did use MSOME for the assessment of frozen-thawed sperm. They investigated the relationship between cryopreservation and nuclear changes in human sperm(Boitrelle et al., 2012) . There is currently little information in the literature regarding the evaluation and comparison of different methods of- sperm cryopreservation with non-invasive techniques such as MSOME. The main objective of this study was to compare the effects of different methods of cryopreservation on sperm quality (motility, viability, and morphology) and to study the potential value of MSOME for frozen-thaw sperm.

Materials and methods

Samples:

In this experiment, semen samples of 28 normozoospermic men (20-40 years old) were studied. They were selected among the infertile couples with female factor who referred to Yazd research and clinical center for infertility, Yazd, Iran between February and July 2016. The semen samples were obtained by masturbation after at least 48h of sexual abstinence. Ejaculations were required to contain at least 15 million or more spermatozoa/ml; at least 32% progressive motility, and 4% normal morphology. Semen analysis was performed according to published guidelines of World Health Organization (5th edition, WHO 2010, switzerland). Each sample was swim-up prepared and divided into four equal parts. The first part was evaluated freshly as a control group (Group I) and the remaining three parts were used for cryopreservation using rapid freezing, cooling with droplet and open-pulled straws (Group II, III, IV). For all groups, motility, viability, and morphology with MSOME were assessed. The study protocol was approved by Yazd Research and Clinical Center for Infertility institutional review board. Written informed consent was obtained from all the men on the day of semen sample collection.

Rapid freezing (group II):

Rapid freezing was done according to standard protocol using Sperm Freeze Solution™(Vitrolife, Kungsbacka, Sweden). Briefly, sperm suspension was diluted with Sperm Freeze Solution™ slowly and drop wise in a ratio of 1:1. Then, the suspension was load into cryovials, and the cryovials were placed at 3cm above liquid nitrogen (LN) surface for 30 min. They were then plunged into LN and stored for a minimum 24h until the evaluation. For thawing, vials from LN were removed and placed in 37°C water bath for 10 min. Next, post-thaw sperm suspension was washed with adding 5 ml Hams´F10 medium supplemented with %5 HSA and centrifuged at 300×g for 10 min twice. The resultant pellet was re-suspended in the same medium.

Cooling with droplet (group III):

Cooling with droplet was done according to the method of Isachenko et al, 2008(Isachenko et al., 2008). Briefly, spermatozoa were diluted with Sperm Freeze Solution™ 1:1. Cell suspensions were maintained at 37°C in %5 CO_2 for 5 min before freezing procedure. A metal strainer was plunged in foam box loaded with LN. 30 µl droplets of the spermatozoa suspension were dropped directly into the LN at a distance of 10 cm from LN surface using a micropipette. The formed solid spheres were collected and transferred into cryovials and stored for at least 24h in LN before use. For warming, solid spheres were quickly plunged into pre-warmed Hams´ F10 with %5 HSA one by one, then vortexed gently. The spermatozoa were washed and pelleted as for the rapid freezing method (Isachenko et al., 2008).

Cooling with open-pulled straws (group IV):

Open-pulled straws method was done according to the method of Isachenko and colliques, 2005(Isachenko et al., 2005). Spermatozoa were diluted (1:1) with 0.5mol sucrose at room temperature. In contrast to their method, in the current study 150µl of sperm suspension was drawn inside the 0.25ml sterile straws (Minitube International AG, Germany) which were then placed inside another 0.5ml straws without sealing ball (Minitube International AG, Germany) . The 0.5ml straw was closed using a thermal sealer and immersed into LN. For warming procedure, after expelling out from 0.5 ml straws, 0.25 ml straws were plunged into 5ml pre-warmed HamF10 medium with %5 HSA. Next, the samples were washed and pelleted as for the rapid freezing method (Isachenko et al., 2005).

Sperm motility assessment:

The motility of spermatozoa was assessed immediately after the swim-up procedure (unfrozen) or warming and concentrating the sample by centrifugation as described above (rapid freezing, cooling with droplet and open-pulled straws). The motility was determined in a Makler ´s chamber and under the light microscope using ×400 magnification. Only spermatozoa with forward progression (Categories a and b) were assessed according to WHO guidline (5th edition, switzerland).

The evaluation of sperm viability:

Hypo-osmotic swelling test (HOS) was done according to WHO guidline (5th edition, switzerland), which was as follows: 100µl of a sperm sample and 1 ml of hypo-osmotic solution (0.735g of sodium citrate dehydrate and 1.351g of D-fructose in 100 ml of purified water) were mixed. After 30 minutes of incubation at 37°C, a slide of the mixture was prepared and examined under a phase-contrast microscope at × 400 magnifications. Finally, spermatozoa with swollen tails (live) and unswollen tails (dead) were calculated. At least 200 sperms in each sample were evaluated.

The evaluation of morphology with MSOME:

For each sample, 5µl of the swim-up sperm fraction or warmed and concentrated sample were transferred to a 5µl droplet of polyvinylpyrrolidone (pvp) (ICSI™-100, Vitrolife, Goteborg, Sweden). These microdroplets were placed in glass-bottom dishes (Willco-dish; Willco Wells BV, Amsterdam, The Netherlands) and covered with mineral oil (Ovoil-100, Vitrolife, Goteborg, Sweden). Motile spermatozoa were observed using an inverted microscope equipped with Nomarski differential interference contrast optics (Eclipse Ti-u, Nikon, Japan). The images were captured using a color video camera, which was displayed on a color video monitor. The morphological evaluation was performed according to Cassuto's scoring system (Cassuto et al., 2009). At least 100 motile spermatozoa per sample were evaluated. In this scoring system, the head, vacuoles and the base of the sperm were evaluated. The criterion for normality of head was the smooth, symmetric and oval shape. The base also must be U-shaped. If one or more vacuoles were observed, nuclear was considered abnormal.

The scored spermatozoa were classified as follows:

Score of spermatozoa= (2×head) + (3 × vacuole) + (base)

Class 1: High-quality spermatozoa with score 4 to 6

Class 2: Medium-quality spermatozoa with score 1 to 3

Class 3: Low-quality spermatozoa with score 0

Statistical analysis:

Data were analyzed using SPSS version 20 software (SPSS, Inc., Chicago, IL, USA). The data was presented as the mean ± standard deviation (S.D). Comparisons between groups were evaluated by ANOVA test. The results were considered significant at $P<0.05$.

Results:

Totally, 28 semen samples were analysed in this study. The mean age of men was 33.71±4.09 years old. The mean of semen parameters, including volume (ml), concentration (10^6/ml), quick motility (%), slow motility (%), viability (%), and normal morphology (%) was 4.14±1.88, 70.11±13.57, 12.32±4.80, 38.50±9.91, 68.99±14.84, and 13.11±5.02 respectively. The motility rate of cryopreserved spermatozoa was diminished after thawing in all groups but this reduction was more noticeable in group II. The effect of the three procedures used for cryopreservation on sperm motility is shown in figure 1.

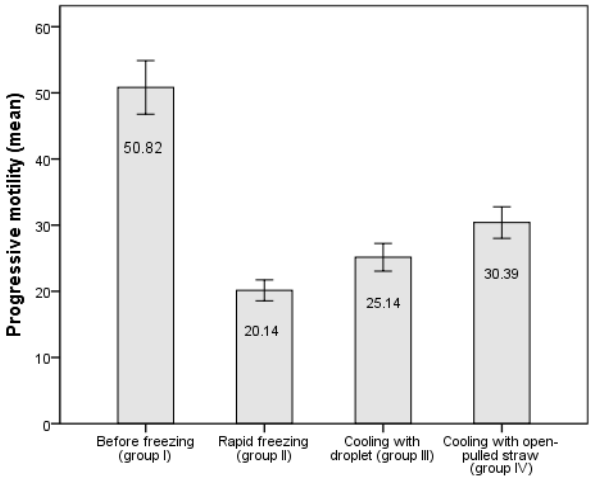

Figure 1. The effects of cryopreservation method on progressive motility. The motility rate reduction was more noticeable in group II. ANOVA test was used.

The mean percentage of live spermatozoa in groups I, II, III, and IV was 92.39%, 54.82%, 59.68%, and 69.07%, respectively. Sperm viability in all cryo group significantly reduced when compared with fresh sample ($P < 0.001$). The effects of cryopreservation on sperm morphology were assessed by MSOME. The effect of different methods of cryopreservation on motile sperm organelle morphology examination is shown in figure 2.

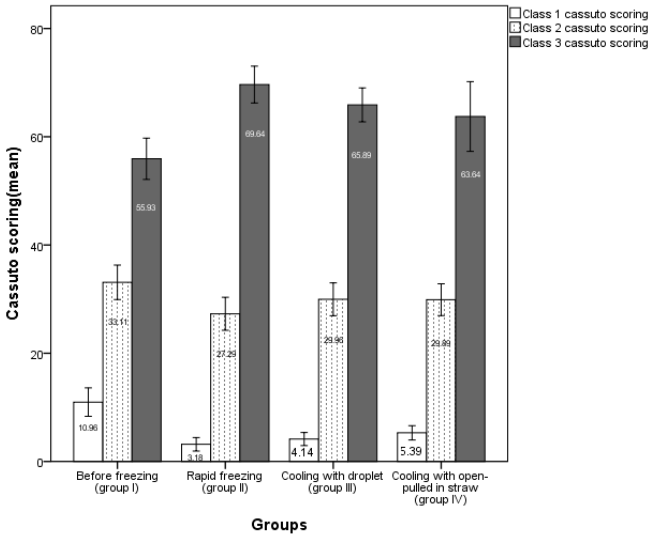

Figure 2. The effects of cryopreservation methods on motile sperm organelle morphology examination and the percentage of different sperm grade.

The percentage of spermatozoa with class 1 significantly reduced from 10.96% (control) to 3.18%, 4.14% and 5.39% (after rapid freezing, cooling with droplet and open-pulled straws, respectively). There were no significant differences regarding class 1 Cassuto scoring between group II and III but a significant difference was seen in group IV. The assessment using MSOME revealed a statically significant decrease in the percentage of class 2 Cassuto scoring after thawing in all groups. The percentage of class 3 Cassuto scoring in the MSOME analysis significantly increased after thawing (Table I).

Table I. The motility, viability and MSOME assessment in fresh and after cryopreservation with rapid freezing, cooling with droplet and open-pulled straws

Parameters	Group I	Group II	Group III	Group IV
Progressive motility (%)	50.82 [a]	20.14 [b]	25.14 [c]	30.39 [d]
Viability (%)	92.39 [a]	54.82 [b]	59.68 [c]	69.07 [d]
Class 1 Cassuto scoring	10.96 [a]	3.18 [b]	4.14 [b]	5.39 [c]
Class 2 Cassuto scoring	33.11 [a]	27.29 [b]	29.96 [c]	29.89 [c]
Class 3 Cassuto scoring	55.93 [a]	69.64 [b]	65.89 [c]	63.64 [c]

Different superscript letters within a row indicate significant differences a,b,c,d ($P < 0.05$).

Class 1: High-quality spermatozoa with score 4 to 6

Class 2: Medium-quality spermatozoa with score 1 to 3

Class 3: Low-quality spermatozoa with score 0

Discussion:

In order to improve sperm recovery and avoiding cryodamage, different technologies for sperm cryopreservation have been developed (Oehninger et al., 2000). Nevertheless, the best method for sperm cryopreservation has yet to be clearly identified (Di Santo et al., 2011). It is well known that conventional slow freezing causes chemical and physical injury to sperm cell membranes, which is related to decrease sperm motility (Alvarez and Storey, 1992). The present study focuses on the relationship between MSOME assessments of human spermatozoa pre and post-cryopreservation using three different methodologies of vitrification/warming.

In our study, the sperm motility decreased significantly in all cryopreservation groups, although it was better in open-pulled straws. This motility reduction after cryopreservation was reported previously (Isachenko et al., 2005, Isachenko et al., 2004, Agha-Rahimi et al., 2014). The sperm motility demands healthy and functional membranes, and the sperm membrane integrity is necessary for sperm motility(VEN et al., 1986). In addition, a decrease in the sperm motility has been correlated with damage to the mitochondrial membrane (O'connell et al., 2002). Oxidative phosphorylation in the inner mitochondrial membrane leads to ATP production which is necessary to drive sperm motility (Zamboni, 1987). Thus, any damage to mitochondrial activity is associated with a reduction in motility. The effects of cryopreservation on the plasma and mitochondrial membranes are well documented (O'connell et al., 2002).

Our results showed that the motility of spermatozoa in group II displayed the lowest levels of motility as compared to group III and IV. One of the factors affecting the quality of cryopreserved spermatozoa by the rapid freezing method is a slow-rated freezing and thawing (Watson, 1995). During cooling with droplet method, after dropping the droplets into liquid nitrogen, heat transfer from the droplets into the LN leads to the vaporization of LN around the droplet resulting in the production of a nitrogen gas layer which works as an isolator and decreases the cooling rate (Arav et al., 2002). As previously describedwe used sucrose in the vitrification medium in group IV which Isachenko and others have also documented its cryoprotective effect on mitochondrial membrane integrity (Isachenko et al., 2008). It is already known that sperm motility is dependent on mitochondrial action (Kao et al., 1998). The other probable reason for the decrease in motility seems to be in the loss of viability after thawing(Ozkavukcu et al., 2008). Sperm damage may occur during rapid freezing method resulting in slow-rated cooling and warming. In this method, ice crystal formation and osmotic change may occur and can cause cell shrinkage(Gao et al., 1995). Sperm membrane damage may result from increased lipid peroxidation and liquid phase transition changes (Alvarez and Storey, 1992). Ice crystal formation can affect the organelle function and lead to reduced cell survival (Said et al., 2010). Increasing the concentration of solutes in a cell after the flow of water from inner cell space to the external environment due to slow cooling rate can cause cell volume changes, dehydration, and toxicity damage. All these changes can damage sperm plasma membranes(Said et al., 2010).

HOS test is a sperm functional test that evaluates membrane integrity (Jeyendran et al., 1984). In the intact cell membrane, the influx of water leads to expanded cell volume and sperm tail swelling (Drevius and Eriksson, 1966). In this study, as compared to control group

(group I), the spermatozoa viability significantly decreased in all used methods. This reduction was lowest in open-pulled straws method. We observed that the lowest rate of viability was achieved in group II. Our observation implies that the quality of cryopreserved spermatozoa is affected by the rate of cooling which slow rate is a negative factor.

Several reports have shown that freezing can affect sperm morphology including membrane disruption, coiled tails and acrosome damage(Check et al., 1991) . MSOME may be useful for the assessment of frozen-thawed spermatozoa in ART outcomes since the pregnancy rates in intra cytoplasmic sperm injection (ICSI) (Bartoov et al., 2002) and intrauterine insemination (IUI) (Akl et al., 2011), is associated with the ratio of class I spermatozoa. Previous studies showed that grades III and IV spermatozoa have a higher noncondensed chromatin rate (Boitrelle et al., 2011, Franco Jr et al., 2012). In addition, early and late embryo development are associated with chromatin condensation at the time of fertilization (Carrell and Hammoud, 2010). When cryopreserved spermatozoa are used for ICSI, MSOME worthiness in selection of class I motile sperm will be more prominent. The present study showed that cryopreservation is associated with a significant decline in class I sperm morphology. In MSOME assessment, this reduction was not significant between groups II and III, although the group IV showed the best sperm recovery and morphology. In a study by Boitrelle and associate (2012) demonstrated that cryopreservation caused a decrease in class I and II spermatozoa morphology(Boitrelle et al., 2012) . Donnelly et al (2001) also reported a significant decrease in sperm normal morphology after cryopreservation in all groups. They observed increasing in amorphous head, megalo head and midpiece defects (Donnelly et al., 2001). In addition, Agha-Rahimi and coworkers observed defects in sperm midpieces, tails and necks after cryopreservation (Agha-Rahimi et al., 2014).

Sperm mechanical damage is associated with intracellular or extracellular ice formation (GAODY, 1995, Watson, 1995). Membrane rupture causes cell damage during cryopreservation due to intracellular or extracellular ice formation (Mossad et al., 1994). Coiled tails are usually attributed to osmotic changes during freezing and thawing(GAODY, 1995). Donnelly et al showed that fertilization ability is affected by sperm head abnormality. Therefore, normal shaped and condensed nuclei sperm should be injected in ICSI (Donnelly et al., 2001). On the other hand, the base of the sperm head is one of the important factors for sperm quality affecting embryo grading (Mundy et al., 1994). In our study, MSOM morphology evaluations of cryopreserved spermatozoa were Include the head, vacuole and base assessment. We were unable to precisely determine the factors affecting sperm morphology following cryopreservation.

Conclusion: However, we believe that MSOME assessment of frozen-thaw spermatozoa can help to improve ART outcome. Furthermore, straw acts as an insulation which may limit the risk of microbial contamination(Isachenko et al., 2005). In conclusion, the preservation of human spermatozoa with open-pulled straws method preserved vital parameters of sperm better than other vitrification methodologies. It seems that the application of MSOME as non-invasive technology can assist in the selection of the normal quality of spermatozoa in ART programs.

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