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Comparision of Cryosurvival Rate of Human Spermatozoa in Cryopreservation Using a Mechanical and Computerized Freezers

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ABSTRACT

Objective To compare the survival rate of human spermatozoa in cryopreservation using two programmed cooling freezers, which have different cooling rates.

Design Semen samples of two groups of men were cooled at different cooling rates using two types of freezers. After thawing sperm motility was assessed for the survival rate and compared.

Setting Andrology Unit, Department of Obstetrics and Gynaecology, Faculty of Medicine, Chulalongkorn University.

Subjects One hundred and forty semen samples were from fertile and infertile men.

Main Outcome Measures The sperm survival rates using two types of freezers were compared. A computer-assisted semen analyser (CASA) was employed to assess sperm motility.

Results The sperm survival rate was significantly higher when using a computerized freezer comparing to the mechanical freezer (paired t-test = 6.95; df = 139; P = 0.05). Using similar method of freezing cryosurvival rate of each group of semen samples showed no significant difference when the results were compared (computer-freezer: t=0.21; P=0.827 with mechanical freezer: t=0.39; P=0.691).

Conclusion Cooling rate used in human sperm freezing is thus an important factor in determining sperm survival in cryopreservation.

Key words: human spermatozoa, cryosurvival rate, cryopreservation

Cryopreservation of human spermatozoa is performed as an assistance to many clinical applications involving the treatment of infertility,[1] such as to improve oliozoospermic semen samples caused by oligoasthenospermia.[2] Cryopreservation is currently a standard procedure used in preventing sexually transmitted diseases, particularly HIV from donating semen.[3] Other reasons are to preserve a reproductive potential after medical, radiological or surgical cancer therapy which might render male sterility and to ensure fertility after vasectomy. However, semen cryopreservation has some problems because there is a great variation in the cryosurvival of spermatozoa from individual among different

methods.[4] Therefore, it is important to understand the etiology of the sperm cells damage during the process of cryopreservation.

There are three phases of mechanism involved in the cells damage during sperm freezing: liquid phase, transition phase and solid phase. The mechanisms of transition from liquid to solid phase are highly destructive causing severe cell damage resulting from the water diffusing out of the cells to maintain a chemical potential equilibrium. It has been reported that a two-factor of cryodamage applied to human spermatozoa and an interaction exists between cooling rate and warming rate.[5-7] These informations obtained from observation of acrosomal outer-membrane shrinkage, coiled tail and swollen mitochondrial midpiece. They are belived causing decrease of postthaw motility and pregnancy rate.[5,7]

Following these problems, it is important to appreciate for the development of human spermatozoa cryopreservation. We have reported the conventional semen analysis and hypoosmotic swelling test are useful in predicting of postthaw motility.[8] This presentation a comparative study of two programmed cooling freezers to find the more efficient method. One hundred and forty specimen were collected form both fertile and infertile men. Each specimen was divided into two aliquots. The first aliquot was loaded into a mechanical freezer (Nicool LM10), which has the liquid nitrogen evaporation rate controlled by a variable speed fan set at different speeds form 1 to 10, while the other aliquot was loaded into a computerized freezer (CryoLogic, CL-863). The survival rates of sperm using different cooling freezers were evaluated using a computer-assisted semen analyzer (CASA).

Materials and Methods

Recruitment of subjects

(a) Fertile men were 70 healthy volunteers with history of having children were recruited. They gave signed informed consents prior to this study.

(b) Infertile men were 70 husbands from couples with history of infertility, coming for routine semen analysis at our laboratory.

Semen samples were produced by masturbation into sterile containers from two groups of subjects and allowed to liquify at 37°C for 30 minutes. All samples were evaluated for sperm parameters such as concentration, motility and morphology using a computer- assisted semen analyzer (CASA) and World Health Organization Laboratory Manual.[9]

Cryopreservation Medium

The cryoprotective medium using in this study was similar to the medium suggested by Matheson et al.[10] One hundred milliliters of the medium contained 15% glycerol, 20% egg yolk (Bacto Egg-Yolk Enrichment, Difco Labs.), 1.15 g sodium citrate, 1.8 g glucose and 1.0 g glycine. The pH was adjusted to 7.2, then activated for 30 minutes at 56°C and kept frozen at-20°C until needed.

Cryopreservation of Spermatozoa

Cryopreservation of semen was performed after liquifaction of the sample. Cryoprotective medium was added to the semen in a 1:1 ratio drop by drop and mixed until the solution was homogenous. The mixture was divided into two aliquots, then placed into cryopreservation vials (Nunc, Roskide, Denmark). The first vial was loaded into a mechanical freezer (Nicool LM-10, Compagnie Francaise de Produits Oxygenes, Paris, France), which the evaporation rate was controlled by a variable fan set at a different speed, from 1 to 10. In this study, the speed of fan was set at 10 for 17 minutes as the following protocols: 10°C/min from 20 to 0°C and 5°C/min from 0 to -60°C then plunged into liquid nitorgen. The other vial was loaded into the computeried freezer (Freezer Control CL-863, Cryologic, Australia) using the following protocols: 3° C/min from 32 to 13°C, 4°C/min from 13 to 4°C, 13°C/min from 4 to -5°C, then hold for 5 minutes and continued with 10°C/min at -5 to -60°C. Samples were then plunged into liquid nitrogen, and then kept in storage at -196°C over night.

Thawing Samples

All samples were stored overnight in liquid nitrogen before thawing for assessment. Vials were thawed at 37°C for 20 minutes by placing them directly in a warm water-bath. The post-thawed samples were evaluated for motility within 30 minutes by a computer-assisted semen analyser (CASA).

Statistical Analysis

Statistical evaluation of the results was performed using paired t-test to compared cryosurvival rate of human spermatozoa between two freezers which differ in cooling rates. Probability values <0.05 was considered significant.

Results

One hundred and forty specimen were collected from both fertile volunteers and infertile patients. The characteristics of pre-freezing sperm quality of these specimens are shown in Table 1. Two programmed cooling freezers, which differ in cooling rate were studied. Computeried freezer gradually varies the cooling rates by 3°C, 4°C, 13°C/min while the temperaturs are 32°C to 13°C, 13°C to 4°C, 4°C to -5 °C respectively then hold for 5 minutes and continues with 10°C/min at -5°C to -60°C, while mechanical freezer varies the cooling rate by 10°C and 5°C/min respectively at 20°C to 0°C and 0°C to -60°C. The sperm survival rate by the former method is significantly higher than the later is presented in Table 2 (paired t-test = 6.95; df = 139; P=0.05). Cryosurvival rate is defined as

$$\text{Cryosurvival rate} = \frac{\text{Post thawed motility rate x 100}}{\text{Pre-freezed motility rate}}$$

Analysis comparing the survival rates in each subgroups (fertile and infertile men) using different freezing methods also show significant superiority of computerised freezer (fertile men: paired t-test 5.22; df = 69; P=0.05 while infertile men: t=4.58; df=69; P=0.05) is presented in Table 3. However, using similar methods of freezing cryosurvival rate for each group of samples (fertile group and infertile group) showings no significant difference. The quality of sperm of the two groups are similar except morphology. (Table 4)

Table 1. Pre-freeze semen parameters

Sperm charecteristics	**Fertile men (n=70)**	**Infertile men (n=70)**
Sperm concentration (x10^6/ml)	118.4±76.3	130.0±81.2
Motile sperm (%)	50.4±22.0	49.8±20.4
Progressive motile sperm (%)	28.4±14.6	28.2±14.8
Normal morphology (%)	17.7±9.4	11.8±10.4

Values are mean ±SD

Table 2. Comparing the survival rate between the computerized CL and mechanical freezer in multiple quality samples

All semen samples	Computer-freezer	Mechanical freezer	t	P
Cryosurvival rate (n=140)	52.9±1.85*	40.8±1.57	6.95	0.05*

Values are mean ±SD

*P significantly different from values of Nicool LM 10

Table 3. Comparing the survival rates of semen from fertile and infertile menbetween the computerized and mechanical freezer.

Groups of semen samples	Computer-freezer	Mechanical freezer	t	P
Cryosurvival rate of fertile men (n=70)	53.3±2.66*	40.2±2.19	5.22	0.05
Cryosurvival rate of infertile men (n=70)	52.5±2.58*	41.4±2.27	4.58	0.05*

Values are mean ±SE

*P significantly different from values of Nicool LM 10

Table 4. Comparing the survival rates between fertile and infertile semen samples using similar methods

Methods of freezing	Cryosurvival rateof fertile men (n=70)	Cryosurvival rate of infertile men (n=70)	T	P
Computer-freezer	53.3±2.66*	52.5±2.55	0.21	0.857*
Mechanical freezer	40.2±2.19*	41.4±2.27	0.39	0.691*

* P no significant different from infertile men

Discussion

The successful cryopreservation and storage of human spermatozoa with the production of normal offspring using liquid nitrogen vapor freezing and its storage at -96°C was reported by Sherman in 1960.[2] Later, the development of techniques, for cryobanking have practiced serveral freezing protocols using different rates of freezing to achieve the best results. The sperm cryosurvival is depending on several factors such as cryoprotective media, freezing method and the most important the sperm quality. Therefore, more advanced and automated methods of freezing have been advised to improve the success rate.

Previous reports have indicated that cooling rates

exert an important effect on the survival after cryopreservation of many cell types.[6,11-14] One of the most important knowledge is that there are at least two mechanisms of cell death during cryopreservation, cell cooled too slowly and cell cooled too rapidly.

In this study, two programmed cooling freezers, which differed in cooling rates were compared in the outcome of sperm survival rate. Nicool LM-10 is a mechanically assisted vapor freezing machine causing liquid nitrogen vapor to flow around the samples at a controlled rate. Freezing systems, cryotubes are placed above a liquid nitrogen dewar flask, and variable-speed fan disperses liquid nitrogen vapor past the cryotubes at empirically determined rates to achieve the desired cooling curve. For human semen, the fan is set at ç10é for 20 minutes. At this phase, cooled the samples 10°C/min from 20°C to 0°C and then 5°C/min from 0°C to -60°C. Samples were then plunged into liquid nitrogen at temperature of -196°C. While the Freeze Control CL controlled rate freezing by a computer. The cooling rates following: 3°C, 4°C, 13°C/min while the temperatures are 32°C to 13°C, 13°C to 4°C, 4°C to -5°C respectively, then hold for 5 minutes and continues with 10°C/min at -5° C to -60°C. Obviously, there is a difference in cooling rate between a mechanical and a computerized method for the important temperature range resulting in extreme sperm cells damage (0°C to -15°C). At this temperature range, extracellular ice forms causing the supercooled intracellular water, as a result, the water diffuses out of the cells and freezes extracellularly. It has been reported that the cooling rate at 10°C/min is an optimal for this mechanisms and hold at -5°C for 5-10 minutes to equilibrate between the cell and environment before starting range of ice crystal formation.

The sperm characteristics in subgroups of volunteers are similar, especially in motility and progressive sperm and the results of cryosurvival rates are also similar. In spite of the fact that the percentage of normal morphology of fertile men are higher than infertile men (17.7±9.4 >11.8±10.4%), there are no difference in the survival rates of fertile and infertile men using the same freezer, computerized freezer are 53.3±2.66 and 52.5±2.56 respectively and mechanical freezer are 40.2±2.19 and 41.4±2.77 respectively (see Table 3). One drawback of this study is that sperm samples of the infertile group happened to have similar characteristics to the fertile group (infertile: concentration 130.0±81.5; motility: 49.8±20.4; fertile: concentration 118±76.3; motility: 50.4±22) except for some difference in morphology (Perhaps morphology is not a critically important factor for sperm cryosurvival as the motility). Further investigation is necessary to evaluate the efficiency of computerised freezer in cryopreservation of substandard semen of infertile men.

In conclusion, it was clearly demonstrated that the optimal cooling rate of freezer used in human sperm freezing is an important factor in determining the sperm cryosurvival.

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