Comparison between mechanical freezer and conventional freezing using liquid nitrogen in normozoospermia

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ABSTRACT

Introduction: This study evaluated the effect of human semen cryopreservation using an ultra-low temperature technique with a mechanical freezer at −85°C as an alternative method to the conventional liquid nitrogen technique at −196°C.

Methods: This was a prospective experimental study conducted in the Medically Assisted Conception unit, Department of Obstetrics and Gynaecology, National University Hospital, Malaysia from January 1, 2006 to April 30, 2007. All normozoospermic semen samples were included in the study. The concentration, motility and percentage of intact DNA of each semen sample were assessed before and after freezing and thawing on Days 7 and 30 post freezing.

Results: Sperm cryopreservation at −85°C was comparable to the conventional liquid nitrogen technique for a period of up to 30 days in a normozoospermic sample. There was no statistical difference in concentration (Day 7 p-value is 0.1, Day 30 p-value is 0.2), motility (Day 7 p-value is 0.9, Day 30 p-value is 0.5) and proportion of intact DNA (Day 7 p-value is 0.1, Day 30 p-value is 0.2) between the ultra-low temperature technique and conventional liquid nitrogen cryopreservation at Days 7 and 30 post thawing.

Conclusion: This study clearly demonstrates that short-term storage of sperm at −85°C could be a viable alternative to conventional liquid nitrogen cryopreservation at −196°C due to their comparable post-thaw results.

Keywords: liquid nitrogen, sperm cryopreservation, ultra-low temperature

Singapore Med J 2011; 52(10): 734-737

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INTRODUCTION

Human sperm cryopreservation is a valuable technique in the field of fertility, especially in the field of oncology. The idea to freeze the human sperm was conceptualised in the 18th century, but it was only in 1953 that the method became a reality.[1] The liquid nitrogen technique was first used in 1963 and had shown promising results.[2] Much later, in 1995, animal studies conducted in male buffaloes and dogs using ultra-low temperature to freeze and store semen showed no significant difference in the semen quality after two months of freezing.[3,4] On the contrary, similar studies in human semen demonstrated mixed results. A previous study in 1945 by Parkes showed that a large proportion of human sperms survive at –196°C or –79°C.[5] A study by Trummer et al in 1998 found that although sperm cryopreservation at –196°C was superior to cryopreservation at –70°C, storage at –70°C for a short period of time (seven days) resulted in a relatively modest loss of motility.[6] In this study, we aimed to evaluate the effect of human semen cryopreservation using the ultra-low temperature technique with a mechanical freezer at –85°C as an alternative method to the conventional liquid nitrogen technique at –196°C.

METHODS

This was a prospective experimental study conducted over a period of 15 months from January 1, 2006 to April 30, 2007 at the Medically Assisted Conception (MAC) unit, Department of Obstetrics and Gynaecology, National University Hospital, Malaysia. All semen samples collected from the MAC laboratory were screened for eligibility. The samples were part of the fertility workup of couples attending the infertility clinic. Samples were collected by masturbation into a clean specimen container after a minimum of 3–5 days of sexual abstinence. The semen was allowed to liquefy at room temperature for 30 minutes, followed by evaluation according to WHO guidelines.[7] Samples with a concentration of > 20 million per ml were identified.

Cryoprotectant (SpermFreeze, FertiPro, Beernem, Belgium) was added to each straw in equal ratio. Each specimen was then divided into four straws. The semen-

cryoprotectant mixture was allowed to equilibrate at 3°C for ten minutes in the lower deck of a refrigerator. Two of the straws were plunged directly and stored in a liquid nitrogen tank (–196°C). The remaining two straws were transferred and stored in an ultra-low temperature (–85°C) freezer, which was equipped with a system to monitor the temperature inside the freezer chamber. The equipment consisted of a microprocessor temperature control that maintained the inner cabinet temperature at –85°C. The temperature could oscillate between –65°C and –100°C (range –83°C ± 11.5°C). The total capacity of the chamber was 128 L.

Thawing was carried out at Day 7 and Day 30 after freezing. The frozen semen was always thawed in the same way, by allowing the straws to warm up in a heating chamber at 37°C for five minutes. This temperature was maintained until the semen was assessed. All samples were assessed before freezing and after thawing. These evaluations were conducted by a trained Andrology laboratory technician who was blinded to the type of freezing method. Sperm concentration was obtained by counting the number of spermatozoa in a column on a Makler counting chamber (× 20) (Irvine Scientific, Santa Ana, CA, USA). The procedure was repeated four times, and the average number of spermatozoa was used to determine the final concentration. Sperm motility was assessed using a Makler chamber (× 400) within 60 minutes of ejaculation. Only sperms belonging to motility class 'a' and 'b' were considered to be motile.

Toluidine blue (TB) is a basic nuclear dye used for metachromatic and orthochromatic staining of chromatin. It becomes heavily incorporated in damaged dense chromatin. This stain is a sensitive structural probe for DNA structure and packaging. A thin smear of semen was obtained. It was then air-dried and fixed in 96% ethanol-acetone (1:1) and kept in the refrigerator (4°C) for 30 minutes. The smear was then hydrolysed in 0.1N hydrochloric acid for five minutes and rinsed three times in distilled water for two minutes each. It was stained with 0.05% TB (Merck, Poole, Dorset, UK) for ten minutes. The staining buffer consisted of 50% citrate phosphate (McIlvain buffer, pH 3.5). The preparation was embedded in DPX mounting medium. Sperm heads with good chromatin stained light blue and those of diminished integrity stained violet (purple).[2] The results of the TB test were estimated using oil immersion (10 × 100) on a Leica DMLB microscope (Meyer Instruments Inc, Houston, TX, USA). A total of 100 sperms were calculated over four fields, and the average number for those stained light blue and purple was noted.

Table I. Distribution of age group, smoking status and type of subfertility in the sample donors.

Parameter	No. of patients (%)
Age group (yrs)	
25–29	5 (14.3)
30–34	10 (28.6)
35–39	13 (37.1)
40–44	6 (17.1)
45–49	1 (2.9)
Smoker	
Yes	21 (60.0)
No	14 (40.0)
Type of subfertility	
Primary	29 (82.9)
Secondary	6 (17.1)

According to the study by Marcus-Braun et al, there is a reduction of 24.1% from baseline motility following freeze-thaw procedure using liquid nitrogen.[8] Sample size for this study was calculated based on the power of 80% to detect a difference of 50% with an alpha value of 0.05. Thus, a total of 26 samples for each group were required for the study.

Results were presented as mean ± standard error of mean (SEM). All analyses were performed using the Statistical Package for the Social Sciences version 11.0 (SPSS, Chicago, IL, USA). In view of the non-Gaussian distribution of data, the non-parametric Wilcoxon matched pairs test was employed to determine the effects of cryopreservation on semen parameters, including concentration, motility and DNA integrity, that derived from the same ejaculate. A p-value < 0.05 was considered to be statistically significant.

RESULTS

A total of 35 normozoospermic samples were obtained for this study. All the samples were healthy, with no known medical condition. The majority of patients (80%) were < 40 years of age. 60% of the semen donors were smokers (Table I). About 83% of these men had never fathered a child.

A significant reduction in the concentration and motility of post-thaw spermatozoa was observed in both groups compared to the baseline ($p < 0.05$), and the motility was reduced by four-fold. However, no significant reduction in the proportion of intact DNA was noted between the cryopreservation techniques from the baseline. There was no statistical difference in the concentration, motility and proportion of intact DNA between the deep freezing technique and conventional liquid nitrogen cryopreservation (Table II).

The sperm concentrations of the post-thaw samples were reduced to 45% and 40% from the baseline for

Table II. Comparison between conventional liquid nitrogen and deep freezing cryopreservation technique on Day 7 post thawing.

Parameter	Mean ± SD			p-value¶
	Pre-freezing	Deep freezing*	Liquid nitrogen†	
Concentration (× 10^6)	111.3 ± 62.9	65.3 ± 35.1	60.3 ± 34.7	0.1
Motility (%)	45.2 ± 17.1	12.8 ± 12.6	12.9 ± 9.9	0.9
Intact DNA (%)	81.6 ± 14.9	77.6 ± 18.5	79.9 ± 18.1	0.1

*† Comparison of all parameters is made between pre-freezing sample to post-thawed deep freezing and liquid nitrogen cryopreservation technique ($p < 0.05$).
¶ Comparison of all parameters is made between deep freezing and liquid nitrogen cryopreservation technique.
SD: standard deviation

Table III. Comparison between conventional liquid nitrogen and deep freezing cryopreservation technique at Day 30 post thawing.

Parameter	Mean ± SD			p-value¶
	Pre-freezing	Deep freezing*	Liquid nitrogen†	
Concentration (× 10^6)	111.3 ± 62.9	56.2 ± 32.3	50.7 ± 34.0	0.2
Motility (%)	45.2 ± 17.1	12.7 ± 12.5	9.4 ± 10.3	0.5
Intact DNA (%)	81.5 ± 14.8	49.1 ± 25.5	52.3 ± 24.9	0.2

*† Comparison of all parameters is made between pre-freezing sample to post-thawed deep freezing and liquid nitrogen cryopreservation technique ($p < 0.05$).
¶ Comparison of all parameters is made between deep freezing and liquid nitrogen cryopreservation technique.
SD: standard deviation

the deep freezing and liquid nitrogen techniques, respectively. The reduction in motility was greater in the liquid nitrogen group compared to the deep freezing group, although the difference was not significant. At the end of 30 days, the spermatozoa motility was reduced to only 25% of the pre-freezing value in samples that underwent deep freezing and slightly less than 20% for those that underwent liquid nitrogen cryopreservation (Table III). The samples in the liquid nitrogen group had a reduction in the proportion of intact DNA to 64% from the baseline compared to 60% in the deep freezing group. There was no statistically significant difference between the two groups.

DISCUSSION

This study explored the option of using ultra-low freezing instead of the conventional liquid nitrogen technique as a means of semen cryopreservation. The post-thaw semen parameters following ultra-low freezing methods appeared to be comparable to that of conventional liquid nitrogen cryopreservation at Day 7 and Day 30. For the past 25 years, liquid nitrogen and cryoprotectants had been widely used for semen cryopreservation. Currently, freezing of semen in liquid nitrogen is the only method used by most *in vitro* fertilisation centres. However, maintaining this facility is costly, as liquid nitrogen is not readily available and requires regular top-up to ensure that the level is adequate. All these factors limit the accessibility of semen cryopreservation to those who require it. The ultra-low freezing method for semen cryopreservation has been used in animals to preserve good stocks as well as to maintain their commercial value. Its application in human semen freezing, however, has yielded mixed results,[2,5,6,9,10] as these studies assessed sperm motility as the main indicator of cryosurvival.

To the best of our knowledge, this is the first study that utilised sperm DNA integrity as the outcome measure besides motility when comparing two different methods of human semen cryopreservation. Although there is global deterioration in the semen parameters following cryopreservation, no significant difference was found in both the motility and DNA integrity between the two techniques. We have taken precautions to avoid type-II bias by performing sample size calculation, as the results failed to reject the null hypothesis. The TB test was chosen to assess the sperm DNA integrity over other more established DNA test, since a TB test is simple to perform and cheaper compared to other available tests. It has been shown to correlate with the results of other tests for sperm DNA and chromatin structure damage.[11] This test can also be performed in the absence of a flow cytometry laboratory. In our study,

calculation of the stained sperm DNA was performed manually using a light microscope. The accuracy could be further enhanced using a microscope with optical density filters, which was not available at the time of the research. The choice and methodology of DNA testing was in consideration of the scope of the study and limited funding.

A larger prospective clinical trial involving normozoospermic semen samples from men who have already fathered a child would be required to show that these two methods are comparable for cryopreservation of the human sperm.

ACKNOWLEDGEMENTS

The TB test was successfully performed with the help of Madam Rahimah, the MAC unit laboratory technologist and aided by Associate Professor CF Leong. The authors also wish to thank Associate Professor SP Ng for contributing to the writing of this article.

REFERENCES

1. Bunge RG, Sherman JK. Fertilizing capacity of frozen human spermatozoa. Nature 1953; 172:767-8.
2. Sherman JK. Improved methods of preservation of human spermatozoa by freezing and freeze-drying. Fertil Steril 1963; 14:49-64.
3. Pironcheva GL, Miteva K, Vaisberg C, Russev G. Influence of freezing at different temperatures on the transcription activity of buffalo sperm chromatin. Cytobios 1995; 83:207-10.
4. Alamo D, Batista M, González F, et al. Cryopreservation of semen in the dog: use of ultra-freezers of −152 degrees C as a viable alternative to liquid nitrogen. Theriogenology 2005; 63:72-82.
5. Parkes AS. Preservation of human spermatozoa at low temperatures. Br Med J 1945; 2:212-3.
6. Trummer H, Tucker K, Young C, Kaula N, Meacham RB. Effect of storage temperature on sperm cryopreservation. Fertil Steril 1998; 70:1162-4.
7. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction, 4th ed. Cambridge:Cambridge University Press, 1999.
8. Marcus-Braun N, Braun G, Potashnik G, Har-Vardi I. Effect of cryopreservation on quality and fertilization capacity of human semen. Eur J Obstet Gynecol Reprod Biol 2004; 116:63-6.
9. Bunge RG, Keettel WC, Sherman JK. Clinical use of frozen semen: report of four cases. Fertil Steril 1954; 5:520-9.
10. Fjällbrant B, Ackerman DR. Cervical mucus penetration in vitro by fresh and frozen-preserved human semen specimen. J Reprod Fertil 1969; 20:515-7.
11. Erenpreiss J, Jepson K, Giwercman A, et al. Toluidine blue cytometry test for sperm DNA conformation: comparison with flow cytometric sperm chromatin structure and TUNEL assays. Hum Reprod 2004; 19:2277-82.