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ARTICLE

Simple vitrification for small numbers of human spermatozoa

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Abstract Conventional freezing procedures and containers are not appropriate for spermatozoa from the testis because of their low number and poor in-situ motility, and various types of container have been utilized to freeze small numbers of spermatozoa. This study tried to develop a vitrification method for small numbers of spermatozoa using the Cell Sleeper, which is a closed type of cell-cryopreservation container. The container with spermatozoa were cooled in liquid nitrogen vapour and then stored in a cryotank. Sperm motility parameters improved significantly ($P < 0.05$) by vitrification in oil-free droplets rather than in droplets covered with oil. After vitrification of five spermatozoa per container, all spermatozoa were recovered and the viable sperm rate was significantly higher when spermatozoa were vitrified in a 3.5-μl droplet rather than in 0.5 μl (72.0% versus 38.0%; $P < 0.01$). Recovery, motility and viability rates of vitrified–warmed spermatozoa were similar between the Cell Sleeper and the CryoTop groups. In conclusion, the Cell Sleeper is a highly effective tool for the cryopreservation of small numbers of spermatozoa and limited cells can be vitrified quickly and simply without significant loss.

KEYWORDS: Cell sleeper, cryopreservation, CryoTop, simple vitrification method, single-sperm freezing

Introduction

Cryopreservation of human spermatozoa has now become a routine procedure in assisted reproductive technology. In cases of severe male infertility, intracytoplasmic sperm injection (ICSI) is a treatment option with high success in IVF and oocytes can be fertilized by ICSI even if spermatozoa have poor motility or are frozen (Craft et al., 1993; Palermo et al., 1992). In cases of azoospermia, cryopreservation of testicular spermatozoa can avoid repeated

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testicular biopsy and it is useful for effective treatment and management (Schelgel and Su, 1997). Since the first attempts at human sperm cryopreservation (Bunge and Sherman, 1953), many studies have been devoted to the development of optimal freezing techniques for human spermatozoa. However, traditional freezing techniques are not suitable for spermatozoa from the testis because of their low number and poor in-situ motility (AbdelHafez et al., 2009). The lack of an easily implemented technology has remained a major bottleneck for the cryopreservation of small numbers of spermatozoa.

A previous publication (Endo et al., 2011) reported a successful vitrification method for a single spermatozoon using the CryoTop (Kitazato Biopharma, Japan), which consists of non-biological material and is available commercially. However, the samples are in direct contact with liquid nitrogen (LN_2)m because the CryoTop is an open system, and this may result in microbial contamination (Bielanski and Vajta, 2009).

This study cryopreserved small numbers of spermatozoa using the Cell Sleeper (Nipro, Japan), which is a closed system for cell cryopreservation. The objectives of this study were to establish the optimal vitrification procedure using the Cell Sleeper.

Materials and methods

Semen samples

From February 2009 to September 2011, fresh ejaculated spermatozoa were obtained from 31 patients by masturbation after at least 48 h of sexual abstinence. All patients had normal fertilization ability after insemination by conventional IVF or ICSI procedures. Discarded specimens after conventional IVF or ICSI were utilized for the current experiments. Ejaculated samples were prepared by density gradient separation using Percoll (GE Healthcare, Sweden). After centrifugation at 760 ***g*** for 15 min, the supernatant was removed and then 0.5 ml of P1 medium containing serum substitute supplement (SSS; Irvine Scientific, USA) was pipetted over the pellet for swim up of spermatozoa. The sample was then incubated for 20 min and swim-up sperm with >99% motility were recovered.

All patients signed consent forms permitting use of their gametes for research. The Kurashiki Medical Centre and Ethics Committees approved the project. The project did not require International Review Board approval.

Vitrification containers

The Cell Sleeper (**Figure 1**) is a vial type of cell-cryopreservation container and is equipped with an inner tray. The container is sealed with a screw cap and can be mounted on a cane as a regular cryotube. The CryoTop is usually used as a container for the vitrification of oocytes and embryos (Hiraoka et al., 2004; Kuwayama et al., 2005). It consists of a fine polypropylene strip attached to a plastic handle and is equipped with a cover straw. Both containers consist of non-biological material and are available commercially.

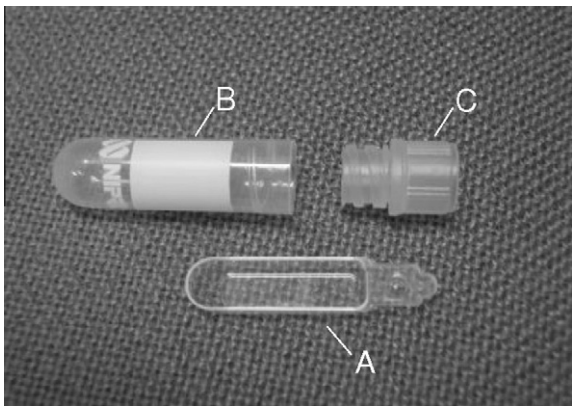

Figure 1 The Cell Sleeper cryopreservation tool. The samples for cryopreservation were placed on a tray (**A**). After placing a tray into a cryotube (**B**), they were sealed with a screw cap (**C**).

Optimal vitrification procedure using the Cell Sleeper

The optimal vitrification volume and procedures were evaluated using six semen samples. Ejaculates were processed through density gradients and swim-up procedures and 100 μl of each sample was diluted with 70 μl SpermFreeze (FertiPro, Belgium) containing 20% SSS, to give a concentration of 5×10^6 cells/ml. After mixing, 0.5, 1.0 and 3.5 μl sample droplets were placed on the trays of the Cell Sleeper. In each tray, droplet was covered with or without 300 μl mineral oil (Fuso Pharmaceutical Industries, Japan). After placing a tray into the cryotube, it was sealed with the screw cap and cooled in LN_2 vapour for 2.5 min prior to submersion. During suspension in the vapour, the Cell Sleeper was placed 0.5 cm above the surface of the LN_2. On warming, the Cell Sleeper was warmed at room temperature for 1 min and the tray was taken out immediately from the cryotube. The tray with oil was incubated at 37°C for 2 min and those with an oil-free droplet were covered immediately with oil and incubated at 37°C for 2 min. Vitrified–warmed sperm motility was analysed using a microscope.

Single-sperm vitrification

Ejaculates from 10 infertile men were processed through density gradients and swim-up procedures. In accordance with the method of Fujii et al. (1997), about 3 μl swim-up spermatozoa was carefully transferred to a droplet of 7% polyvinylpyrrolidone (Irvine Scientific, USA) in a Falcon 1006 dish (Becton Dickinson, USA). After 3–10 min of culture at 37°C, individual motile spermatozoa were picked up using an ICSI pipette equipped with a micromanipulator and transferred to an oil-free droplet of SpermFreeze-based cryopreservation medium (0.5, 1.0 and 3.5 μl) deposited on the tray (**Figure 2A**). Immediately, the tray with the oil-free droplet was put into a cryotube and cooled in LN_2 vapour (**Figure 2B**) as described above. The Cell Sleeper was mounted on a cane (**Figure 2C**) and stored in a cryotank

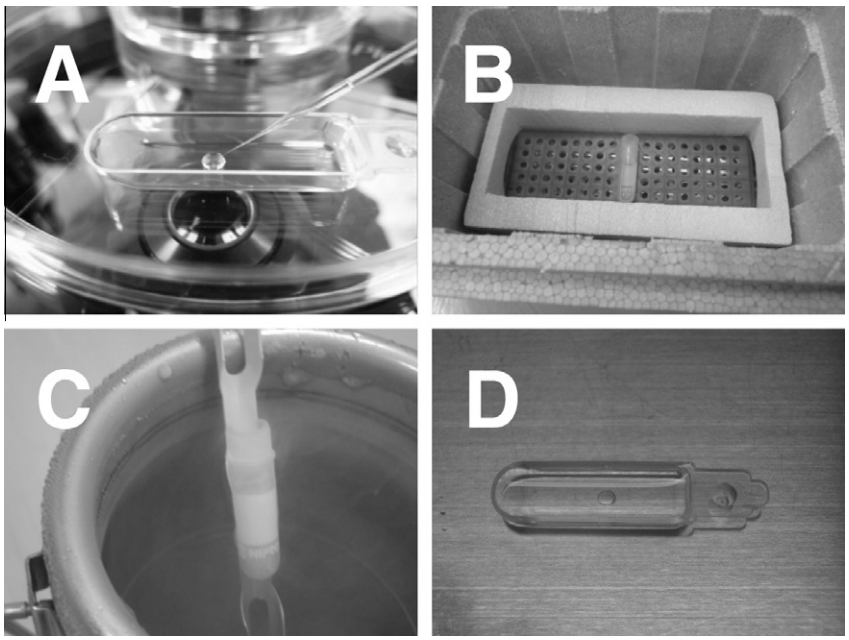

Figure 2 Description of the vitrification and warming procedures using the Cell Sleeper. (**A**) Spermatozoa were loaded into the cryopreservation medium on a tray with the aid of a micromanipulator. (**B**) After placing the tray into a cryotube, the tray was cooled in LN_2 vapour. (**C**) The Cell Sleeper was mounted on a cane and stored in LN_2. (**D**) After warming the Cell Sleeper, the droplet on the tray was covered with oil and observed in order to retrieve the spermatozoa.

at least overnight. After warming, the Cell Sleeper, the tray was taken out immediately from the cryotube and the droplet on the tray was covered with oil (**Figure 2D**). The droplet was cultured at 37°C for 2 min and observed in order to retrieve the spermatozoa.

Comparison of sperm vitrification containers

Two sperm vitrification containers, the Cell Sleeper and the CryoTop were tested using five additional semen samples, and the spermatozoa were stored for 5 months in a cryotank. Vitrification and warming techniques using the Cell Sleeper were followed as described above. Individual spermatozoa were vitrified in an oil-free 3.5-μl droplet of SpermFreeze-based freezing medium.

Single-sperm vitrification using the CryoTop has been described (Endo et al., 2011). In accordance, the current study deposited a 1-μl droplet of cryopreservation medium on the CryoTop strip at room temperature. Five motile spermatozoa were then transferred to the droplet of cryopreservation medium on the CryoTop strip using an ICSI pipette equipped with a micromanipulator. Immediately, the CryoTop strip was cooled in LN_2 vapour for 2 min prior to submersion. During suspension in the vapour, the CryoTop was placed 4 cm above the surface of the LN_2. For warming, the CryoTop strip was taken out of the LN_2 and placed immediately in a flat 2-μl droplet of medium, which was covered by mineral oil in a Falcon 1006 dish at 37°C. To prevent spermatozoa being left on the CryoTop strip, it was washed with two further droplets. Each droplet was carefully checked in order to retrieve the spermatozoa.

Assessment of acrosome status and DNA fragmentation

Ejaculates from 10 infertile men were processed through density gradients and swim-up procedures. After mixing spermatozoa and SpermFreeze, an oil-free 3.5-μl droplet containing approximately 30,000 motile spermatozoa was vitrified using the Cell Sleeper. Vitrification and warming techniques were followed as described above.

The vitrified–warmed spermatozoa were smeared on a glass slide in a limited area (~4 × 4 mm) and marked with a diamond pen to help find the spermatozoa under the microscope for counting. The percentages of acrosome status and DNA fragmentation after vitrification were normalized to fresh spermatozoa as control samples.

Evaluation of sperm motility and viability

Sperm motility was assessed immediately before and after vitrification of samples using an inverted microscope (×100–200 magnification; Olympus IX-71) equipped with a Relief Contrast system and a 21-inch monitor. For single-sperm vitrification, individual motile or non-motile spermatozoa was transferred to droplets of water using the ICSI pipettes equipped with a micromanipulator to evaluate viability by the hypo-osmotic swelling test (Jeyendran et al., 1984). The spermatozoa with coiled tails were considered as viable. The vitrified–warmed sperm recovery, motility and viability rates were analysed. Time to search for spermatozoa was limited to 30 min per container.

Detection of acrosome status and DNA fragmentation

Sperm acrosome status was assessed using fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (Sigma, USA). The procedures were carried out in accordance with the method of Cross et al. (1986). When more than half of the head of spermatozoa was brightly and uniformly fluorescing, the acrosome was considered intact. Spermatozoa with resting band at the equatorial segment or without fluorescence were considered acrosome-reacted.

Sperm DNA fragmentation was determined by the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay using and In-Situ Cell Death Detection kit with fluorescein (Roche Diagnostics, Germany), following the manufacturer's instructions. A counterstain with propidium iodide was used to visualize the TUNEL-negative nuclei in red. Spermatozoa displaying a spectrum of green fluorescence were considered TUNEL-positive and scored as having DNA fragmentation.

At least 200 spermatozoa per sample were counted with a fluorescence microscope using excitation wavelengths of 450–490 nm and a magnification of ×1000 oil-immersion objective.

Statistical analysis

All semen samples were tested randomly within each experimental group. Statistical analyses of results were used for treatment comparisons and carried out by paired t-test or one-way of variance (ANOVA) using the Stacell 2 program (OMS Publishing, Japan). If the *P*-value was less than 0.05 by ANOVA, the Tukey-Kramer Honestly Significant Difference test was utilized using the same program. The sperm motility, acrosome integrity and DNA fragmentation rates were expressed as means ± SEM. $P < 0.05$ was considered to be statistically significant.

Results

Optimal vitrification procedure using the Cell Sleeper

Spermatozoa were vitrified in various volumes of droplets covered with or without oil and the results are shown in **Figure 3**. Motility rate was significantly higher when spermatozoa were vitrified in oil-free droplets (37–59%) rather than in droplets covered with oil (9–21%; $P < 0.05$). However, there was no significant relationship between the volume of the droplet and the motility of vitrified–warmed spermatozoa.

Single-sperm vitrification

The results of sperm vitrification using the Cell Sleeper are shown in **Table 1**. After warming, all vitrified spermatozoa were recovered in each group. There was no significant relationship between the volume of the droplet and the sperm motility. However, the viability was significantly higher when spermatozoa were vitrified in a 3.5-μl droplet (72.0%) rather than in 0.5 μl (38.0%; $P < 0.01$).

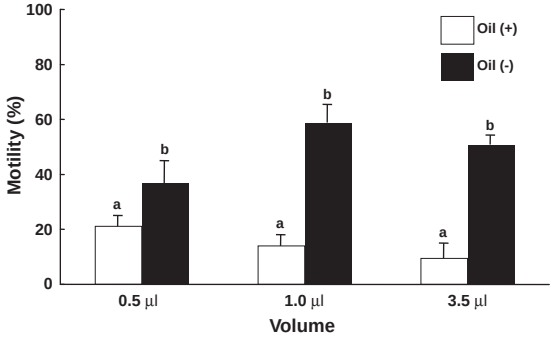

Figure 3 Motility of human spermatozoa after vitrification in 0.5, 1.0 and 3.5 μl droplets covered with or without oil using the Cell Sleeper. Values are mean ± SEM ($n = 6$). Values for the same volume with different letters are significantly different ($P < 0.05$).

Comparison of sperm vitrification containers

Spermatozoa were cryopreserved in the Cell Sleeper and the CryoTop for 5 months and the results are shown in **Tables 2 and 3**. There were no lost containers after warming for either type of container, but LN_2 from the cryotank had leaked in the Cell Sleeper during storage and all of them (10/10) sank in the liquid. After warming, sperm recovery rate was relatively high (≥96%) in both container groups. Motility (29.2% versus 44.0%) and viability (64.6% versus 78.0%) of spermatozoa were similar between the two containers.

Assessment of acrosome status and DNA fragmentation

Sperm acrosome status was significantly ($P < 0.01$) damaged after warming (49.4 ± 3.8%) compared with fresh (4.4 ± 2.3%). However, DNA damage (TUNEL-positive) did not differ between the fresh (2.7 ± 1.5%) and vitrified–warmed (0.4 ± 0.3%) groups, and the rate of sperm DNA fragmentation in spermatozoa incubated for 2 h after warming was also similar (0.8 ± 0.3%).

Discussion

The present study showed that it is possible to cryopreserve small numbers of spermatozoa using the Cell Sleeper. Using the current method, spermatozoa could be vitrified easily and recovered efficiently and quickly. Some authors have reported successful pregnancies using a few spermatozoa stored in empty zona pellucida (Cohen et al., 1997; Walmsley et al., 1998). However, use of zona pellucida generates many ethical problems and is only available on a limited basis, because the procedure depends on biological material from human or animal sources and there is the potential for disease transmission. Furthermore, it is difficult to obtain the zona pellucida from a partner unless she decides to undergo oocyte retrieval specifically for uncertain sperm cryopreservation. Therefore, other authors have attempted to cryopreserve small numbers of spermatozoa using various types of containers such as droplets on plastic dishes (Sereni

Table 1 Effect of different vitrification volume sizes on the recovery, motility and viability rates of individually vitrified spermatozoa using the Cell Sleeper.

Volume (μl)	*Containers (n)*	*Vitrified spermatozoa (n)*	*Spermatozoa after warming (n, %)*		
			Recovered	*Motile*	*Live*
0.5	10	50	50 (100)	8 (16.0)	19 (38.0)[a]
1.0	10	50	50 (100)	15 (30.0)	32 (64.0)[a,b]
3.5	10	50	50 (100)	19 (38.0)	36 (72.0)[b]

[a,b]Values with different superscripts are significantly different ($P < 0.01$).

Table 2 Reliability of containers that had been cryopreserved in a cryotank for five months.

Container	*Type of system*	*Containers (n)*	*Containers (n,%)*	
			Broken after warming	*Soaked inside with LN_2 during storage*
CryoTop	Open	10	0 (0)	–
Cell Sleeper	Close	10	0 (0)	10 (100)

Table 3 Vitrified–warmed recovery, motility and viability of small numbers of spermatozoa vitrified using the CryoTop and the Cell Sleeper.

Containers	*Vitrified spermatozoa (n)*	*Spermatozoa after warming (n,%)*		
		Recovered	*Motile*	*Live*
CryoTop	50	50 (100)	22 (44.0)	39 (78.0)
Cell Sleeper	50	48 (96.0)	14 (29.2)	31 (64.6)

et al., 2007), mini-straws (Desai et al., 1998), micropipettes (Gvkharia and Adamson, 2001), cryoloops (Desai et al., 2004; Schuster et al., 2003), copper loops (Isachenko et al., 2004), *Volvox globator* algae (Just et al., 2004), agarose microspheres (Isaev et al., 2007) and alginate beads (Herrler et al., 2006). Unfortunately, however, these are the only currently available options and no live births have yet been reported (AbdelHafez et al., 2009).

A previous study (Endo et al., 2011) reported a novel vitrification method for a single spermatozoon using the CryoTop. The vitrification and warming techniques were simple and the recovery rate of spermatozoa after warming was relatively high. Furthermore, the CryoTop can be used universally because it consists of non-biological material and is available commercially, so that it is considered to be suitable for the cryopreservation of small numbers of spermatozoa. The CryoTop is an open type of cell-cryopreservation system and gametes are directly exposed to sterilized LN_2. Furthermore, the gametes are protected from virus contamination during storage in the cryotank because they are covered and sealed with the hard plastic cover in sterilized LN_2. The miniscule volumes of fluid in the CryoTop realistically make the potential risk of cross-contamination negligible in cases of single-sperm cryopreservation.

In the contrast, the Cell Sleeper is a closed type of cell-cryopreservation container. In the present study, spermatozoa with oil had significantly reduced motility rate after vitrification compared with oil-free spermatozoa. On single-sperm vitrification, the best result obtained when spermatozoa were vitrified in an oil-free 3.5-μl droplet and it was the most convenient volume for handling small numbers of spermatozoa using an ICSI pipette equipped with a micromanipulator. The recovery, motility and viability rates of small numbers of vitrified–warmed spermatozoa were similar between the Cell Sleeper and the CryoTop.

In the present study, vitrification procedure might damage the sperm acrosome but DNA integrity was maintained using the Cell Sleeper as a vitrification container. Spermatozoa were processed through density gradients and swim-up procedures to eliminate the non-viable and apoptotic spermatozoa (Younglai et al., 2001). The lack of significant differences among the fresh and vitrified–warmed spermatozoa in terms of apoptotic DNA fragmentation may be because the death of cryo-injured spermatozoa occurs by necrosis rather than by apoptosis (Lachaud et al., 2004).

It is arguable whether the spermatozoa would be infected with bacteria or pathogens mediated by LN_2, which have leaked into the Cell Sleeper during storage in a cryotank. No case of transmission of infectious disease has ever been reported and no report mentions LN_2 as a probable vehicle for disease transmission in assisted reproduction treatment. Although there are several papers describing cross-contamination mediated by LN_2 (Bielanski et al., 2000; Charles and Sire, 1971; Piasecka-Serafin, 1972; Tedder et al., 1995), they are not necessarily applicable to real-life cryostorage in assisted reproduction because using

high concentrations of infective agents may never occur in real situations (Vajta and Reichart, 2011). Bielanski et al. (2003) reported on cryotank contamination, but the current study failed to detect the contaminants in the cryotanks, which have been managed in a clean room at 25–30°C with air conditioning for over 20 years of continuous use (data not shown). Further, Gimenez et al. (2011) have presented the negative evidence of cross-contamination mediated by LN_2 between human pathogens and oocytes/embryos that were clinically vitrified using an open device. Taken together, these results may suggest that the risk of the cross-contamination between gametes and LN_2 is vanishingly small. The detailed cross-contamination mechanism remains unclear (Kyuwa et al., 2003) and further studies and discussions are required.

It is important to consider whether sperm cells act as vectors for vertical transmission of viruses such as human immunodeficiency, hepatitis B, hepatitis C and herpes to oocytes after ICSI. It is known that sperm washing procedures effectively reduce the vertical transmission risk (Kato et al., 2006) and no case of disease transmission to the women or the child has yet been reported after ICSI using washed spermatozoa (Englert et al., 2004; Kashima et al., 2009; Lutgens et al., 2009; Mencaglia et al., 2005; Sauer et al., 2009). The possibility of infection might be lessened when individual thawed spermatozoa are washed several times with fresh medium before ICSI.

It may be concluded from the present study that the Cell Sleeper is a useful container for the cryopreservation of small numbers of spermatozoa and the method is a quick, easy and simple. The Cell Sleeper is commercially available and easy to prepare for use. Clinical application of this procedure to extremely poor sperm specimens will be necessary in order to confirm these findings.

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