Simple vitrification for small numbers of human spermatozoa involving clinical outcomes

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Abstract

Conventional freezing procedures are not appropriate for sperm from the testis because of their low number and poor *in-situ* motility. Techniques for the cryopreservation of small numbers of sperm have attempted to use various types of containers, but these are the only currently available options and the lack of an easily implemented technology has remained a major bottleneck. This study was aimed to develop the simple vitrification techniques for a single spermatozoon using Cryotop (Kitazato Biopharma, Japan) and Cell Sleeper (Nipro, Japan) as non-biological containers, which are already commercially available.

1. Single spermatozoon freezing using Cryotop

The first experiment was tried to develop a cryopreservation method for a single spermatozoon using Cryotop, which has a simple structure and is easy to handle. Different parameters influencing the freezing procedure, types of container, sources of sperm, and cryoprotectants were evaluated. The sperm recovery rate after thawing was similar between the sperm frozen using Cryotop or zona pellucida as containers (98.0% vs. 88.0%). Freezing of motile single sperm obtained from ejaculates and testes were evaluated for recovery rate (90.0% vs. 95.0%) and motility rate (44.4% vs. 42.1%), which were not significantly different. The survival rate was significantly higher when sperm were treated with sucrose rather than with glycerol (65.3% vs. 37.3%, $P < 0.01$). These results demonstrated that Cryotop was a highly effective tool for the cryopreservation of a single spermatozoon, and sucrose was determined to be an efficient cryoprotectant.

2. Simple vitrification for small numbers of human spermatozoa

The second experiment was conducted to develop a vitrification method for small numbers of sperm using Cell Sleeper, which is a closed type of cell-freezing container. The container with sperm were cooled in the vapor of liquid nitrogen and then stored in a cryotank. Sperm motility parameters improved significantly ($P < 0.05$) by freezing in oil-free droplets rather than in droplets covered with oil. After vitrified five sperm per container, all sperm were recovered and the viable sperm rate was significantly higher when sperm were vitrified in 3.5 μL of droplet rather than in 0.5 μL (72.0% vs.38.0%, $P < 0.01$). Recovery, motility and viability rates of vitrified-warmed sperm were similar with Cell Sleeper and Cryotop groups as containers. From these results, it was concluded that Cell Sleeper was a highly effective tool for the cryopreservation of small numbers of sperm and limited cells could be vitrified quickly and simply without significant loss.

3. Successful delivery derived from vitrified-warmed spermatozoa from a patient with non-obstructive azoospermia

Finally, it was undertaken to examine the clinical outcomes following intracytoplasmic sperm injection (ICSI) with vitrified sperm from patients with severe male factor infertility. Three patients with severe oligozoospermia or non-obstructive azoospermia (NOA) have undergone ICSI using vitrified-warmed sperm. Limited numbers of sperm were vitrified using Cryotop and Cell Sleeper as non-biological containers. Four cycles underwent ICSI with vitrified sperm. A total of 148 sperm in 18 containers (8.2 sperm per container) were vitrified and 36 of them (5 containers) were warmed. Thirty-three sperm (92%) were retrieved successfully and injected individually into 17 mature

oocytes. Fertilization was observed in 12 (71%) oocytes and all zygotes (100%) cleaved. A couple with NOA achieved a singleton pregnancy and concluded with full-term delivery of a healthy boy (2632 g). A successful delivery was achieved after transfer of a blastocyst derived from vitrified-warmed sperm. This clinical result showed that a small number of vitrified sperm cells were used for ICSI to fertilize oocytes with predictable timing.

In conclusion, results of the current three studies indicated that both Cryotop and Cell Sleeper vitrification were clinically useful and reliable methods for single spermatozoon storage for severe male factor patients. This simple methods can vitrified and warmed only the numbers of sperm cells needed for ICSI without significant loss, and healthy child was born derived from a vitrified-warmed spermatozoon from a patient with NOA.

Declaration

This thesis contains no materials which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, the best of my knowledge and belief, contains no material previously published or written by another person, except for when due reference has been made in the text.

Sign in:

Date:

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Published articles:

1. Single spermatozoon freezing using Cryotop.

   Endo Y, Fujii Y, Shintani K, Seo M, Motoyama H, Funahashi H.

   Journal of Mammalian Ova Reserch, 28, 47-52, 2011.

2. Simple vitrification for small numbers of human spermatozoa.

   Endo Y, Fujii Y, Shintani K, Seo M, Motoyama H, Funahashi H.

   Reproductive BioMedicine Online, 24, 301-7, 2012.

3. Successful delivery derived from vitrified-warmed spermatozoa from a patient with non-obstructive azoospermia.

   Endo Y, Fujii Y, Kurotsuchi S, Motoyama H, Funahashi H.

   Fertility and Sterility, 98, 1423-7. 2012.

Conferences (Oral and Posters presentations):

1. 等張凍結液によるヒト精子凍結法の検討

   遠藤雄史、藤井好孝、本山洋明

   第49回日本不妊学会、旭川大会、2004年9月

2. 極少数精子の回収方法～エンブリオロジストの視点から～

   遠藤雄史、藤井好孝、本山洋明

   第23回日本受精着床学会、大阪大会、2005年8月

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   第 26 回日本受精着床学会、福岡大会、2008 年 8 月

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   遠藤雄史、藤井好孝、本山洋明

   第 53 回日本生殖医学会、神戸大会、2008 年 10 月

5. クライオトップを用いた精子 1 個の凍結保存方法の検討

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6. Sucrose を凍結保護物質として用いた精子 1 個の凍結保存方法の検討

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26th European Society of Human Reproduction and Embryology, Roma, Italy, June 2010.

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第 28 回日本受精着床学会、横浜大会、2010 年 7 月

9. Cryotop を用いたヒト少数精子凍結保存法における生存率の改善

遠藤雄史、藤井好孝、本山洋明

第 55 回日本生殖医学会、徳島大会、2010 年 11 月

10. A successful pregnancy and live birth after intracytoplasmic sperm injection with cryopreserved limited numbers of spermatozoa stored in the practical container.

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12. Single spermatozoon vitrification: Effect on motility, viability, acrosome

status, DNA integrity and sperm binding.

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4th ASPIRE, Osaka, Japan, September 2012.

13. 単一精子ガラス化凍結保存した高度乏精子症患者の臨床報告

遠藤雄史、藤井好孝、本山洋明

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List of abbreviations

°C	Degrees, Celsius
%	Percent(s)
mL	Milliliter(s)
µL	Microliter(s)
cm	Centimeter(s)
mm	Millimeter(s)
µm	Micrometer(s)
nm	Nanometer(s)
min	Minute(s)
sec	Second(s)
M	Molar
mM	Micromolar
ART	Assisted reproductive technology
ICSI	Intracytoplasmic sperm injection
IVF	*In vitro* fertilization
LN_2	Liquid nitrogen
ZP	Zona pellucida
TESE	Testicular sperm extraction
NOA	Non-obstructive azoospermia
SSS	Serum substitute supplement
et al.	et alii (and others)
vs.	versus
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

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Chapter 1

General introduction

1.1 Preface

The history of human semen cryopreservation stretches back about 200 years to the first recorded experiments involving cooling of sperm in snow. Despite this early success, it was not until the fortuitous discovery of glycerol as a cryoprotectant (Polge et al., 1949) and subsequent live birth of a calf (Stewart, 1951) in the early 1950s. Cryopreservation of human semen for assisted reproduction has now became a feasible option. The techniques of human semen storages greatly improved the flexibility of donor insemination treatment, resulting in the first live human births in 1953 (Bunge and Sherman, 1953). Semen cryopreservation as a method for the patients with severe male infertility became a mainstay of fertility treatment, and many couples continue to benefit from the combined use of cryopreservation with assisted fertilization technologies (Al-Hasani et al., 1999). As it is now possible for men with severe male factor infertility to father offspring, many previously intractable cases, such as oncology patients, are now encouraged to store semen prior to sterilizing therapy in the knowledge that fatherhood is a viable prospect.

Cryobiologists have been continued to investigate and unravel the complex mechanics of cryoprotectants and cryopreservation (Gilmore et al., 1997), and with the aid of molecular technologies identify those genetic factors that influence cryoservival (Donnelly et al., 2001). Practitioners and regulatory

authorities of the assisted reproductive techniques (ART) must play a major role in the introduction of peer-reviewed guidelines and evidence-based practice, assuring the safety of recipients and healthy offspring.

1.2 Cryopreservation and storage of testicular sperm

1.2.1 Surgical sperm retrieval

Men with azoospermia, who are previously considered hopelessly infertile, can now potentially initiate a pregnancy with the advent of intracytoplasmic sperm injection (ICSI), if mature sperm can be harvested from their testis.

Percutaneous epididymal sperm aspiration (PESA) carried out on the epididymis and testicular sperm extraction (TESE) carried out on the testes are the most commonly used techniques to obtain sperm from the patients with azoospermia (Tournaya et al., 1998). Testis biopsy may be useful to observe the sperm if they have been produced in the testes. When the patients with obstructive azoospermia with normal spermatogenesis, in almost all cases sperm can extracted by means of PESA or TESE. PESA is carried out for this initially. If PESA is unsuccessful and there are no sperm that can be extracted from the epididymis, TESE is then the next option. However, the patients with nonobstractive azoospermia involve impaired spermatogenesis or testicular failure. In approximately 50% of cases of nonobstractive azoospermia would be extracted the sperm from their testes (Silber, 2000).

In the PESA procedure, a butterfly needle is injected throughout the scrotal

skin and into the epididymis. To accomplish these procedures, the epididymis is located by palpation, fixed and subsequently injected to aspirate the contents. In contrast with a TESE, the scrotal skin is partially or completely opened for the purpose of taking samples of the testes from which sperm can be isolated.

Microdissection testicular sperm extraction (micro-TESE) can improve sperm retrieval for men with nonobstructive azoospermia over those achieved previously with standard testis biopsy techniques (Tsujimura, 2007). This technique requires an operating microscope and it can be visually identified which tubules containing sperm with a brief learning curve after opening the testis. Micro-TESE makes the embryologist easier in finding sperm, since only seminiferous tubules have to be examined. Furthermore, it allows identification of blood vessels with the testicle, minimizing the risk of vascular injury and loss of other areas of the testis.

Unfortunately, no preoperative parameter, such as a testicular volume and FSH levels, absolutely predicts the chance of sperm retrieval by surgical treatments. Even the histology of a diagnostic biopsy cannot absolutely predict sperm retrieval when the rest of the testis is sampled.

1.2.2 Freezing of testicular sperm

It is a beneficial option to cryopreserve the testicular tissue and sperm prior to initiation of the IVF stimulation cycle for patients who suffer from spermatogenic failure. If the sperm retrieval operation would be timed to

coincide with oocyte aspiration and no mature sperm can be isolated, those patients will have undergone significant physical, emotional and financial burdens without a positive outcome, as there will be no sperm available for ICSI. The fertility treatment is usually only started after sperm have actually been frozen to prevent the premature termination due to the absence of sperm.

It is required to have a method of reliable sperm storage for severe male factor patients. If sperm are not cryopreserved during the initial surgical operation, the patients will require repeated testicular surgical biopsies for sperm extraction. It becomes more challenging to identify healthy testicular parenchyma, and testes are more injured resulting from multiple biopsies. Furthermore, multiple testicular surgeries may cause permanent testicular damage, including partial testicular devascularization, irreversible atrophy, deterioration of spermatogenic development, and even loss of endocrine functions, which would necessitate exogenous testosterone replacement (Schlgel et al., 1997).

Prior to cryopreservation, the harvested testicular tissue needs to be processed. The surgically isolated tissues and seminiferous tubules can be dissected mechanically or dispersed enzymatically (Baukloh 2002). The obtained sperm from the testis can be cryopreserved using several different techniques. One method places testicular tissue homogenate in 1-mL aliquots using cryovials. It is difficult to identify mature sperm in the testicular tissue homogenate, and laborious and time-consuming search under microscope are

often required to find a few mature sperm.

1.2.3 Single spermatozoon freezing

It is advantageous to cryopreserve individual testicular sperm cells, because there is a considerable risk of losing sperm. Conventional sperm freezing procedure would not work with a limited numbers of sperm, and several methods are currently under development to store individual sperm.
In 1997, Cohen et al. first demonstrated the novel method of freezing small numbers of sperm inside empty zona pellucida (ZP). Their technique can provide a storage container for successfully isolating a few testicular sperm and more than >75% of recovery and ICSI fertilization rates have been reported (Cohen et al., 1997). However, the method should not be used because of significant risk of the inadvertent disease transmission from the hamster across species to the human. Utilization of ZP is especially problematic and is should not be employed to freeze sperm (AbdelHafez et al., 2009).

Other methods have been developed that provide effective alternatives to the dilemma about ethical problems. There are some reports that attempting to freeze small numbers of sperm using various types of containers such as droplets on plastic dishes (Sereni et al., 2007), mini-straws (Desai et al., 1998), micropipettes (Gvkharia et al., 2001), cryoloops (Schuster et al., 2003; Desai et al., 2004), copper loops (Isachenko et al., 2004), cryoleefs (Peng et al., 2011), volvox globator algae (Just et al., 2004), agarose microspheres (Isaev et al.,

2007) and alginate beads (Herrler et al., 2006). Unfortunately, these containers are the only currently available options to cryopreserve very low numbers of sperm and they cannot be used universally.

1.2.4 Clinical outcome

Freeze-thawed testicular sperm can fertilize human oocytes in conjunction with IVF-ICSI. Oates et al. (1997) descried that 10 male patients with nonobstructive azoospermia were underwent 19 cycles of ICSI using cryoprerved testicular sperm, and the comparable fertilization rate is obtained between fresh and frozen testis-derived sperm groups. In during last 10 years (2002-11) of our clinical data, 60 patients underwent the PESA, TESE and micro-TESE and 31 patients (52%) could retrieve sperm successfully from their epididymis or testes. Finally, 26 couples (84%) could pregnant and deliver own healthy babies.

For single spermatozoon freezing, in contrast, few pregnancies and deliveries have been reported with the use of post-thaw sperm for ICSI and subsequent ET.

1.2.5 Conclusion

The introduction of ICSI technique has made it possible to realize pregnancy with the use of epididymal or testicular sperm. Sperm retrieval is an effective adjunct for treatment of the patients with azoospermia and the

application of a microdissection technique enhances the chance of sperm retrieval from testis. Using the retrieved sperm from men with obstructive or nonobstructive azoospermia, most couples could deliver own babies when advanced assisted reproduction is applied at Kurashiki Medical Clinic. Retrieved sperm can cryopreserved, stored and used for ICSI at the time of oocyte maturation. Cryopreservation technique is also a beneficial option to reduce the necessity of repeat surgeries for sperm retrieval. Although biological and non-biological carriers have been attempted for preservation of low numbers of sperm, they are the only currently available options. The lack of an easily used technology for handling low numbers of sperm remains a major deterrent to the freezing of single spermatozoon.

1.2.6 Aim of study

Novel cryopreservation technology needs to be further explored, especially designing to treat small numbers and quantities of sperm obtained from the men with azoospermia.

The aims of the present studies were to;

1. Develop the method of a single spermatozoon storage using Cryotop (Kitazato Biopharma, Japan), which is an open type system.
2. Develop the method of a single spermatozoon storage using Cell Sleeper (Nipro, Japan), which is a closed type system.
3. Vitrify the limited numbers of sperm involving clinical outcomes.

Chapter 2

Single spermatozoon freezing using Cryotop

(Endo Y, Fujii Y, Shintani K, Seo M, Motoyama H, Funahashi H. Journal of Mammalian Ova Research 2011;28:47-52.)

2.1 Introduction

In cases of severe male infertility, intracytoplasmic sperm injection (ICSI) is a treatment option with high success in *in vitro* fertilization (IVF) (Palermo et al., 1992). Oocytes can be fertilized by ICSI even with sperm from azoospermic patients (Crafl et al., 1993). In cases of azoospermia, cryopreservation of surgically retrieved sperm from the epididymis and testis is useful for effective treatment and management, and it reduces the requirement for repeated surgery (Schelgel et al., 1997; Ron-El et al., 1998). Since the first attempts at human sperm cryopreservation (Bunge and Sherman, 1953), many studies have been devoted to the development of the optimal freezing techniques for human sperm (Royer et al., 1996). Cryopreservation of human sperm has now become a routine procedure. However, conventional freezing procedures may not be appropriate for sperm from the epididymis and testis because of their low number and poor *in-situ* motility. Some authors have reported successful pregnancies using a few sperm stored in empty zona pellucida (ZP) (Walmsley et al., 1998, Fusi et al., 2001). However, the procedure generates many ethical problems and is no longer used. Other authors have attempted to use various types of containers, including droplets on plastic dishes (Sereni et al., 2007),

mini-straws (Desai et al., 1998; Isachenko et al., 2005), micropipettes (Gvkharia et al., 2001; Sohn et al., 2003), cryoloops (Schuster et al., 2003; Desai et al., 2004), 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, these containers are the only currently available options to cryopreserve very low numbers of sperm and they cannot be used universally (AbdelHafez et al., 2009). The lack of an easily used technology for handling low numbers of sperm remains a major deterrent to the freezing of single spermatozoon.

This study was undertaken to cryopreserve a single spermatozoon using Cryotop (Kitazato Biopharma, Japan), which has a simple structure and is easy to handle. It was tested different parameters influencing the freezing procedure, including types of container, sources of sperm, and cryoprotectants.

2.2. Materials and methods

2.2.1 Sperm sources

From February 2009 to June 2010, all patients who presented at our clinic with severe male infertility, including azoospermia, oligozoospermia, and asthenozoospermia, were enrolled in this study. Fresh ejaculated sperm were collected from 21 of 23 infertile men, and testicular samples were obtained from 2 men with obstructive azoospermia. The testicular sperm were frozen by the standard slow freezing method until the day of oocyte retrieval. Discarded

specimens after 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 was pipetted over the pellet for swim-up of sperm. The sample was then incubated for 20 min, and swum-up sperm (> 99 % motility) were recovered. In accordance with the method of Fujii et al. (1997), about 3 mL of the swum-up sperm was carefully placed in a droplet of 8% polyvinylpyrrolidone (Irvine Scientific, USA) in a Falcon 1006 dish (Becton Dickinson, USA) using a glass fine pipette and a stereomicroscope at ×10 magnification. After 3-10 min of culture at 37°C, the sperm sample was checked using an inverted microscope at ×200 magnification.

Testicular sperm were isolated surgically by testicular sperm extraction (TESE). The sperm from seminiferous tubules were placed into Hepes buffered modified HFF99 (Fuso Pharmaceutical Industries, Japan) containing 20% serum substitute supplement (SSS; Irvine Scientific, USA) and dissected using stainless steel blades. After thorough dissection, 100 mL of medium with the specimen was diluted with 100 mL of HFF99 containing 20% SSS and checked using a microscope.

For preparation of each specimen, sperm were collected using ICSI injection pipettes (Kitazato Biopharma, Japan), which had an inner diameter of >4 mm using an inverted microscope (Olympus IX-71; ×100-200 magnification)

equipped with a Relief Contrast system and a 21-inch monitor.

2.2.2 Cryoprotectants

In this study, two different cryoprotectants, sucrose (Sigma, USA) and SpermFreeze (FertiPro, Belguim) were tested. The sucrose-based freezing medium was 0.1 M sucrose in HFF99 containing 20% SSS. SpermFreeze-based freezing medium was a mixture of SpermFreeze (0.7 mL) and HFF99 (1.0 mL) containing 20% SSS.

2.2.3 Sperm freezing containers

Two sperm containers, Cryotop and ZP were tested. Cryotop (Figure 1) is usually used as an efficient container for the vitrification of oocytes and embryos (Kuwayama et al., 2005). It consists of a fine polypropylene strip attached to a plastic handle and is equipped with a cover straw. Cryotop consists of non-biological material and is available commercially.

The concept of sperm cryopreservation using empty ZP was first introduced by Cohen et al. (1997). In the current study, ZPs were obtained from human oocytes (germinal vesicles, metaphase I stages, unfertilized metaphase II stages) before and after ICSI procedures. Neither ZP inseminated by conventional IVF procedures nor those obtained from other rodent animals were used. All evacuations of oocytes were performed in HFF99 containing 20% SSS and 0.1% collagenase (Sigma, USA). Micromanipulation was performed in a

Falcon 1006 dish using a 3.5 mL droplet of medium in accordance with the method of Cohen et al. (1997). An injection micropipette was used for evacuation of the oocyte. The injection pipette was moved through the ZP, and the cytoplasm was aspirated until the ZP was empty.

2.2.4 Freezing and thawing techniques using Cryotop

A droplet of freezing medium was deposited on the Cryotop strip at room temperature. A single motile spermatozoon was then transferred to the droplet of freezing medium on the Cryotop strip using an ICSI pipette equipped with a micromanipulator (Figure 2a and 2b). Immediately, the Cryotop strip was cooled in the vapor of liquid nitrogen (LN_2) for 2 min prior to submersion (Figure 2c). During suspension in the vapor, the Cryotop was placed 4 cm above the surface of the LN_2. The Cryotop was soaked in LN_2 for a minimum of 10 min prior to thawing (Figure 2d). For thawing, the Cryotop strip was taken out of the LN_2 and immediately placed in a flat droplet of medium (2 mL), which was covered by mineral oil in a Falcon 1006 dish at 37°C (Figure 2e and 2f). To prevent sperm being left on the Cryotop strip, it was washed with two further droplets. Each droplet was carefully checked using an inverted microscope at ×100 magnification in order to retrieve the sperm.

2.2.5 Freezing and thawing techniques using zona pellucida

Twenty ZPs, including immature and unfertilized oocytes, were obtained

from ten consenting ICSI patients. After aspiration of the cytoplasm using an injection micropipette, empty ZP were washed 3 times with HFF99 containing 20% SSS and cultured until sperm insertion. After fixation of the ZPs using a holding pipette mounted on a micromanipulator, five motile sperm were inserted into the ZP using an ICSI pipette. The sperm were released slowly from the injection pipette to minimize inflation of the ZP. After injection of the sperm, individual sperm cells were counted three times before being cryopreserved. For freezing, the ZP with sperm was placed in freezing medium and kept at room temperature for 3 min. The ZP was then loaded into a 0.25 mL straw (Cryo Bio System, France) between two small air bubbles to indicate their position. One side of the straw was plugged by cotton and the other was sealed with a colored rod plug. The freezing procedure was carried out in accordance with a standard semen cryopreservation protocol, in which the straw was exposed to LN_2 vapor for 5 min followed by plunging into LN_2. It was then kept in LN_2 for at least 1 h. For thawing, the straw containing the ZP was thawed in air for 30 sec, followed by 1 min in a 30°C water bath. Both sides of the straw were cut and the ZP was expelled into HFF99 containing 20% SSS. The ZP was then transferred to a droplet of medium (2 mL), which was covered by mineral oil in a Falcon 1006 dish at 37°C and washed gently with three further droplets. Subsequently, the sperm inside the ZP were aspirated using an injection pipette.

2.2.6 Statistical analysis

In this study, thawed sperm were examined within 30 min. Sperm were recovered individually using a micromanipulator and a microscope. The recovery rate and motility parameters of sperm were assessed. There were no medical utilizations of human oocytes in the current study. The χ^2 test was used to test the significance of differences between the groups.

2.3 Results

2.3.1 Comparison of sperm freezing containers

The results of sperm freezing using Cryotop and ZP are shown in Table 1. All sperm were obtained from ejaculates and treated with SpermFreeze before freezing. There were no lost containers after thawing in either group. A total of 100 sperm were frozen in Cryotops, and 98 of them (98.0%) were recovered after thawing. Two sperm (2.0%) were not found in droplets within 30 min and were lost. A total of 100 sperm were frozen in ZPs, and 88 of them (88.0%) were recovered after thawing. Twelve sperm (12.0%) were lost, 9 (9.0%) of them were not found inside the ZP after thawing and 3 (3.0%) were trapped in the re mains of cytoplasm. The motility rate of the recovered sperm was not significantly different between the Cryotop (30.6%) and ZP (23.9%) groups.

2.3.2 Comparison of sperm sources

Motile sperm were obtained from ejaculates or testes (Table 2). The individual sperm cells were treated with SpermFreeze and frozen using Cryotop.

After thawing, 90.0% of ejaculate and 95.0% of testicular sperm were recovered, respectively. The motility rate was not statistically different between ejaculate (44.4%) and testicular sperm (42.1%). The average time needed per container to find a spermatozoon was 265 sec (range 2 – 1,800) in ejaculate, and 286 sec (range 1 – 1,800) for testicular sperm.

2.3.3 Comparison of cryoprotectants

Two different cryoprotectants, sucrose and SpermFreeze, were tested. Motile sperm were obtained from ejaculates and the individual cells were frozen using Cryotop (Table 3). The recovery rate after thawing was similar between the sucrose (98.0%) and SpermFreeze (95.7%) groups. The survival rate was significantly ($P < 0.01$) higher when sperm was frozen in sucrose (65.3%) rather than in SpermFreeze (37.3%).

2.4 Discussion

The present study showed that successful freezing of a single spermatozoon was possible using Cryotop. Using the current method, it was able to freeze a single spermatozoon easily, and recover it efficiently and quickly. Cohen et al. (1997) first demonstrated the practical value of freezing small numbers of sperm inside empty ZP. The technique may be especially valuable in extreme cases of oligoazoospermia or azoospermia, and successful pregnancies and live births have been reported by others (Walmsley et al., 1998;

Fusi et al., 2001. However, their approach depends on biological materials from human or animal sources, with the implicit danger of disease transmission. Utilization of ZP is especially problematic and is no longer used. Other types of container have been examined for the cryopreservation of small numbers of sperm, but no live births have yet been reported (AbdelHafez et al., 2009). The lack of an easily implemented technology has remained a major bottleneck for the cryopreservation of small numbers of sperm.

In the present study, the sperm recovery and motility rates after thawing were similar between the sperm frozen using Cryotop or ZP as contaners. Cryotop is usually used as a vitrification container for oocytes and embryos with excellent results, 99% post-thawing survival rate (Kuwayama et al., 2005), and it is also considered suitable for the cryopreservation of small numbers of sperm. ZP availability as a container for sperm is very restricted because it is difficult to obtain ZP from a partner unless she decides to undergo oocyte retrieval specifically for sperm cryopreservation. Furthermore, it is impossible to aspirate the cytoplasm completely, even if ZP is available and a little cytoplasm always remains in the ZP. This implies potential contamination of the preserved sperm with infectious or genetic foreign material. New FDA and European Tissue Directive regulations discourage the use of biological carriers for gamete preservation.

Cryotop has been used universally because it consists of non-biological material and is available commercially. The hard plastic cover protects not only

against physical damage, but also against virus contamination during storage in LN_2. Cryotop is now being used in more than 40 countries (700 IVF centers) and has been used in more than 100,000 clinical cases for oocyte and embryo cryopreservation (personal communication from Kitazato Biopharma). Many healthy babies have been born with the aid of the Cryotop method in the last 7 years. Therefore, it is considered that Cryotop can be used as a safe container for the cryopreservation of sperm.

It is well known that sperm collected by TESE has slightly lower motility after cryopreservation than sperm obtained from men with oligoasthenozoospermia or normal sperm quality (Hsieh et al., 2000). In the present study, the testicular sperm motility rate after thawing was not significantly different from that of the ejaculates. This suggests that Cryotop is also an effective tool for the cryopreservation of testicular sperm.

In the present study, the survival rate was significantly higher when sperm were cryopreserved in sucrose rather than SpermFreeze. Although various concentrations of sucrose were tested for cryopreservation of human sperm, the best results were always achieved with 0.1 M sucrose (unpublished data). It is well known that human sperm are very sensitive to osmotic stress (Nawroth et al., 2002; Isachenko et al., 2004). The osmolaritiy of the current freezing medium with 0.1 M sucrose was about 400 mOsm, which appeared to be suitable for sperm. Although a freezing medium with a relatively low osmolarity has been considered to provide little protection for sperm cryopreservation (Hossain and

Osuamkpe, 2007), the present freezing medium with 0.1 M sucrose worked as well as regular freezing media. In addition, the use of only sucrose as a cryoprotectant may provide other advantages. Because sucrose is a non-permeating cryoprotective agent, it does not require processing to remove the cryoprotectant from the cell after thawing (Hossain and Osuamkpe, 2007). As a result, the thawing procedure is very straight forward and sperm loss may be reduced. Glycerol in combination with other additives has been widely and successfully used as a cryoprotectant for human sperm (Nawroth et al., 2002; Hossain and Osuamkpe, 2007). However, the current study showed that SpermFreeze, which includes both glycerol and sucrose, was not as effective for the cryopreservation of a single spermatozoon. DNA integrity of the cryopreserved sperm with sucrose as the sole cryoprotectant requires further exploration.

It may be concluded form the present study that Cryotop is a useful container for the cryopreservation of a single spermatozoon and sucrose is an effective cryoprotectant for the freezing of a single spermatozoon. The current method is a quick, easy, and simple procedure for the cryopreservation of a single spermatozoon. Clinical application of this procedure to extremely poor sperm specimens will be necessary in order to confirm these findings.

Table 1. Recovery and motility rates of ejaculated sperm individually frozen in two different freezing containers

Type of sperm freezing container	No. of frozen sperm	No. (%) of sperm recovered after thawing	No. (%) of motile sperm after thawing
Cryotop	100	98 (98.0)	30 (30.6)
Zona pellucida	100	88 (88.0)	21 (23.9)

Table 2. Recovery and motility rates of ejaculated or testicular sperm individually frozen using Cryotop

Source of sperm	No. of frozen sperm	No. (%) of sperm recovered after thawing	No. (%) of motile sperm after thawing	Sperm retrieval time (seconds)
Ejaculate	20	18 (90.0)	8 (44.4)	265
Testis	20	19 (95.0)	8 (42.1)	286

Table 3. Effect of two different cryoprotectants on the recovery and motility rates of individually frozen sperm using Cryotop

Cryoprotectant	No. of frozen sperm	No. (%) of sperm recovered after thawing	No. (%) of motile sperm after thawing
Sucrose	50	49 (98.0)	32 (65.3)*
SpermFreeze	70	67 (95.7)	25 (37.3)*

*Statistically significant difference ($P < 0.01$).

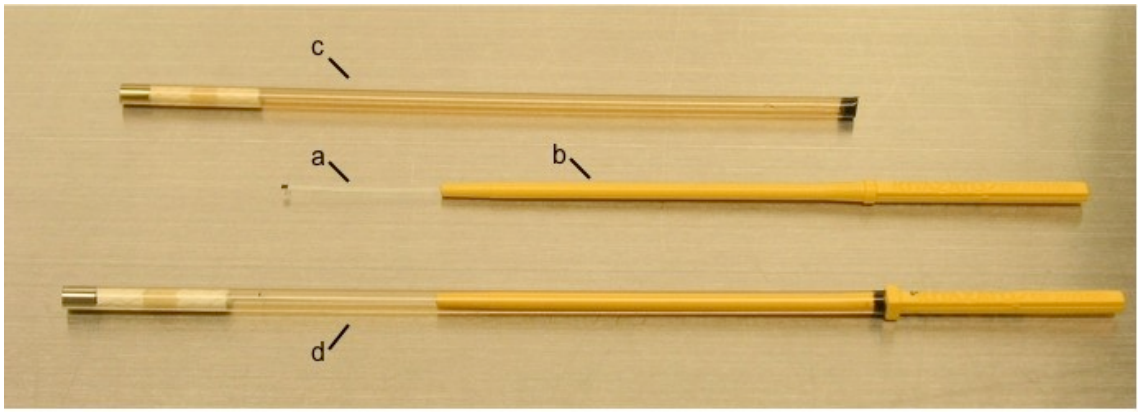

Figure 1. Cryotop cryopreservation tool. A polypropylene strip (a) is attached to a hard plastic handle (b). After cryopreservation, the hard plastic cover (c) protects from not only physical damage but also virus contamination during storage in LN_2 (d).

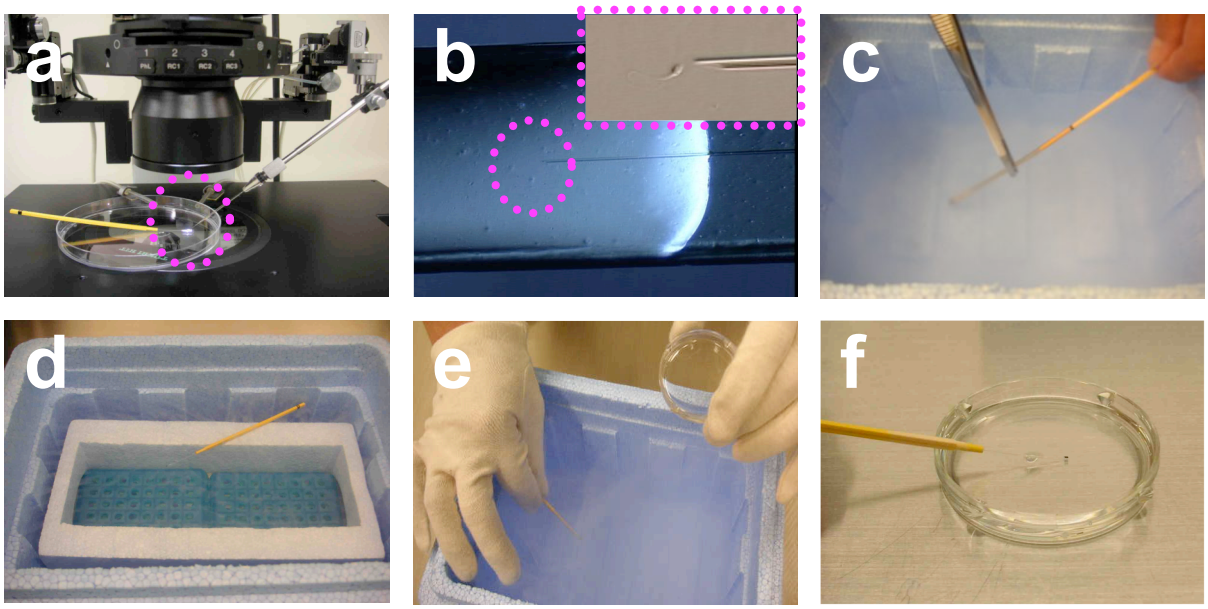

Figure 2. Description of the freezing and thawing procedures. (a, b) A motile sperm was loaded into freezing medium on a Cryotop strip with the aid of a micromanipulator. (c, d) The Cryotop strip with a single spermatozoon was frozen in LN_2 vapor and then submerged in LN_2. (e, d) For thawing, the Cryotop strip was taken out of LN_2 and instantly placed in a droplet of medium which was covered by mineral oil.

Chapter 3

Simple vitrification method for small numbers of human spermatozoa

(Endo Y, Fujii Y, Shintani K, Seo M, Motoyama H, Funahashi H. Reproductive BioMedicine Online 2012;24:301-7)

3.1 Introduction

Cryopreservation of human sperm has now become a routine procedure in assisted reproductive technology (ART). In cases of severe male infertility, intracytoplasmic sperm injection (ICSI) is a treatment option with high success in *in vitro* fertilization (IVF), and oocytes can be fertilized by ICSI even if sperm have poor motility or are frozen (Palermo et al., 1992; Craft et al., 1993). In cases of azoospermia, cryopreservation of testicular sperm can avoid repeated 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 sperm. However, traditional freezing techniques are not suitable for sperm 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 sperm.

In the previous publication (Endo et al., 2011), it has been reported a successful vitrification method for a single spermatozoon using Cryotop (Kitazato Biopharma, Japan), which consists of non-biological material and is

available commercially. However, the samples directly contact with liquid nitrogen (LN_2) because Cryotop is an opened type of cell-freezing system, and this direct contact may result in microbial contamination (Bielanski and Vajta, 2009).

In this study it was conducted to cryopreserve small numbers of sperm using Cell Sleeper (Nipro, Japan), which is a closed type system for cell-freezing. The objectives of this study were to establish the optimal vitrification procedure using Cell Sleeper.

3.2 Materials and methods

3.2.1 Semen samples

From February 2009 to September 2011, fresh ejaculated sperm 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 sperm. 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 Center and Ethics Committees approved the project.

3.2.2 Vitrification containers

The Cell Sleeper (Figure 3) is a vial type of cell-freezing 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. 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.

3.2.3 Establishment of the optimal vitrification procedure using Cell Sleeper

The optimal freezing 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 of 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 Cell Sleeper. In each tray, droplet was covered with or without 300 μL of mineral oil (Fuso Pharmaceutical Industries, Ltd. Japan). After placing a tray into the cryotube, it was sealed with the screw cap and cooled in

the vapor of LN_2 for 2.5 min prior to submersion. During suspension in the vapor, the Cell Sleeper was placed 0.5 cm above the surface of the LN_2. On warming, the Cell Sleeper was thawed 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 analyzed using a microscope.

3.2.4 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 of the swim-up sperm 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 sperm was pick up using an ICSI pipette equipped with a micromanipulator and transferred to an oil-free droplet of SpermFreeze-based freezing medium (0.5, 1.0 and 3.5 µL) deposited on the tray (Figure 4A). Immediately, the tray with the oil-free droplet was put into a cryotube and cooled in vapor of LN_2 (Figure 4B) as described in experiment 1. Cell Sleeper was mounted on a cane (Figure 4C) and stored in a cryotank 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 4D). The droplet was cultured at 37°C for 2 min and

observed in order to retrieve the sperm.

3.2.5 Comparison of sperm vitrification containers

Two sperm vitrification containers, Cell sleeper and Cryotop were tested using 5 additional semen samples, and sperm were stored for five months in a cryotank. Vitrification and warming techniques using Cell Sleeper were followed as described for experiment 1 and 2. Individual sperm were vitrified in an oil-free droplet of SpermFreeze-based freezing medium (3.5 μL).

It has firstly reported the single spermatozoon vitrification using Cryotop (Endo et al., 2011). In accordance with the method, a droplet of freezing medium (1 μL) was deposited on the Cryotop strip at room temperature. Five motile sperm were then transferred to the droplet of freezing medium on the Cryotop strip using an ICSI pipette equipped with a micromanipulator. Immediately, the Cryotop strip was cooled in the vapor of LN_2 for 2 min prior to submersion. During suspension in the vapor, 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 droplet of medium (2 μL), which was covered by mineral oil in a Falcon 1006 dish at 37°C. To prevent sperm being left on the Cryotop strip, it was washed with two further droplets. Each droplet was carefully checked in order to retrieve the sperm.

3.2.6 Assessment of acrosome status and DNA fragmentation

Ejaculates from 10 infertile men were processed through density gradients and swim-up procedures. After mixture of sperm and SpermFreeze, 3.5 µL of oil-free droplet containing approximately 30,000 motile sperm were vitrified using the Cell Sleeper. Vitrification and warming techniques were followed as described for experiment 1 and 2.

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

3.2.7 Evaluation of sperm motility and viability

Sperm motility was assessed immediately before and after vitrification of samples using an inverted microscope (Olympus IX-71; ×100-200 magnification) equipped with a Relief Contrast system and a 21-inch monitor. For single sperm vitrification, individual motile or non-motile sperm 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 sperm with coiled tails were considered as viable. The vitrified-warmed sperm recovery, motility and viability rates were analyzed. Time to search for sperm was limited to 30 min per container.

3.2.8 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 sperm was brightly and uniformly fluorescing, the acrosome was considered intact. Sperm with resting band at the equatorial segment or without fluorescence were considered acrosome-reacted.

Sperm DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using In-Situ Cell Death Detection Kit, with fluorescein (Roche Diagnostics GmbH, Germany), following the manufacturer's instructions. A counterstaining with propidium iodide was used to visualize in red TUNEL-negative nuclei. The sperm displaying as spectrum of green fluorescence was considered TUNEL-positive and scored as having DNA fragmentation.

At least 200 sperm per sample were counted with a fluorescence microscope using excitation wavelengths of 450-490 nm and a magnification of × 1,000 oil-immersion objective.

3.2.9 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 HSD 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.

3.3 Results

3.3.1 Establishment of the optimal vitrification procedure using Cell Sleeper

Sperm were vitrified in various volumes of droplets covered with or without oil, and the results are shown in Figure 5. Motility rate was significantly higher when sperm 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 sperm.

3.3.2 Single sperm vitrification

The results of sperm vitrification using Cell Sleeper are shown in Table 4. After warming, all of vitrified sperm were recovered in either group. There was no significant relationship between the volume of the droplet and the sperm motility. However, the viability was significantly higher when sperm were vitrified in 3.5 μL of droplet (72.0%) rather than in 0.5 μL (38.0%; $P < 0.01$).

3.3.3 Comparison of sperm vitrification containers

Sperm were cryopreserved in Cell Sleeper and Cryotop for five months, and the results are shown in Table 5 and Table 6. 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 phase of LN_2. After warming, sperm recovery rate was relatively high (≥96%) in both container groups. Motility (29.2% vs. 44.0%) and viability (64.6% vs. 78.0%) of sperm were similar for Cell Sleeper and Cryotop as containers.

3.3.4 Assessment of acrosome status and DNA fragmentation

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

3.4 Discussion

The present study showed that it is possible to cryopreserve small numbers of sperm using Cell Sleeper. Using the current method, it was able to vitrify sperm easily, and recover them efficiently and quickly. Some authors have reported successful pregnancies using a few sperm 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 procedures depend on biological materials from human or animal sources this implies 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 freeze small numbers of sperm using various types of containers such as droplets on plastic dishes (Sereni et al., 2007), mini-straws (Desai et al., 1998), micropipettes (Gvkharia et al., 2001), cryoloops (Schuster et al., 2003; Desai et al., 2004), 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).

In a previous study (Endo et al., 2011), it was reported a novel vitrification method for a single spermatozoon using Cryotop. The present vitrification and warming techniques were simple and the recovery rate of sperm after warming was relatively high. Furthermore, 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 sperm. Cryotop is an open type of cell-freezing 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 Cryotop realistically make the potential risk of cross-contamination negligible in cases of single sperm freezing.

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

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

It is arguable whether the sperm would be infected with bacterias or pathogens mediated by LN_2, which have leaked into the Cell Sleeper during storage in a cryotank. Because 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 ART. Although there are several papers describing cross-contamination mediated by LN_2 (Charles and Sire, 1971; Piasecka-Serafin, 1972; Tedder et al., 1995; Bielanski et al., 2000), 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) have reported the cryotanks contamination, but the current study failed to detect the contaminants from our laboratoty cryotanks, which have been managed in a clean room at 25-30°C controlled by air conditioner over 20 years of continuous use by our service (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 an important question 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 sperm (Englert et al., 2004; Mencaglia et al., 2005; Kashima et al.,

2009; Sauer et al., 2009; Lutgens et al., 2009). The possibility of infection might be lessened when individual thawed spermatozoon are washed several times with fresh medium before ICSI.

It may be concluded from the present study that Cell Sleeper is a useful container for the cryopreservation of small numbers of sperm and the method is a quick, easy, and simple. 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. It is wished that further clinical studies would be attempted around the world.

Table 4. Effect of different freezing volume sizes on the recovery, motility and viability rates of individually vitrified sperm using Cell Sleeper

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

[a-b]: Values with different superscripts within a column are significantly different ($P < 0.01$).

Table 5. 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 6. Vitrified-warmed recovery, motility and viability of small numbers of sperm vitrified using Cryotop and Cell Sleeper.

Containers	Vitrified sperm (n)	Sperm 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)

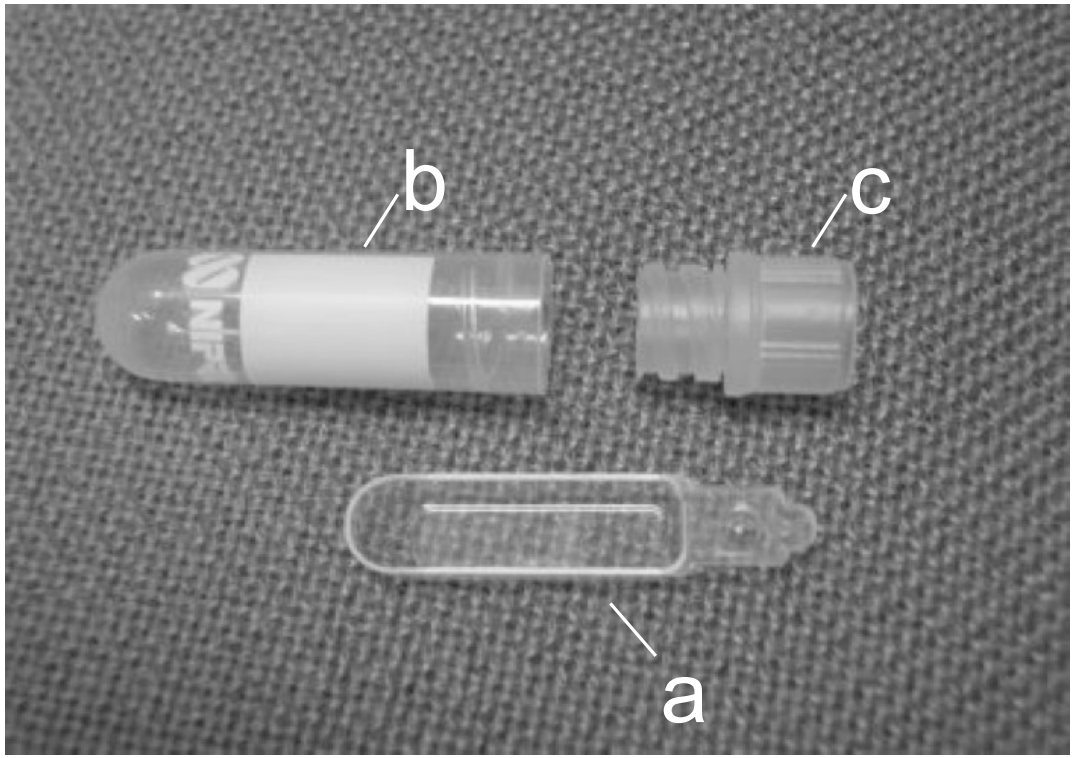

Figure 3. The Cell Sleeper cryopreservation tool. The samples for freezing were placed on a tray (a). After placing a tray into a cryotube (b), they were sealed with a screw cap (c).

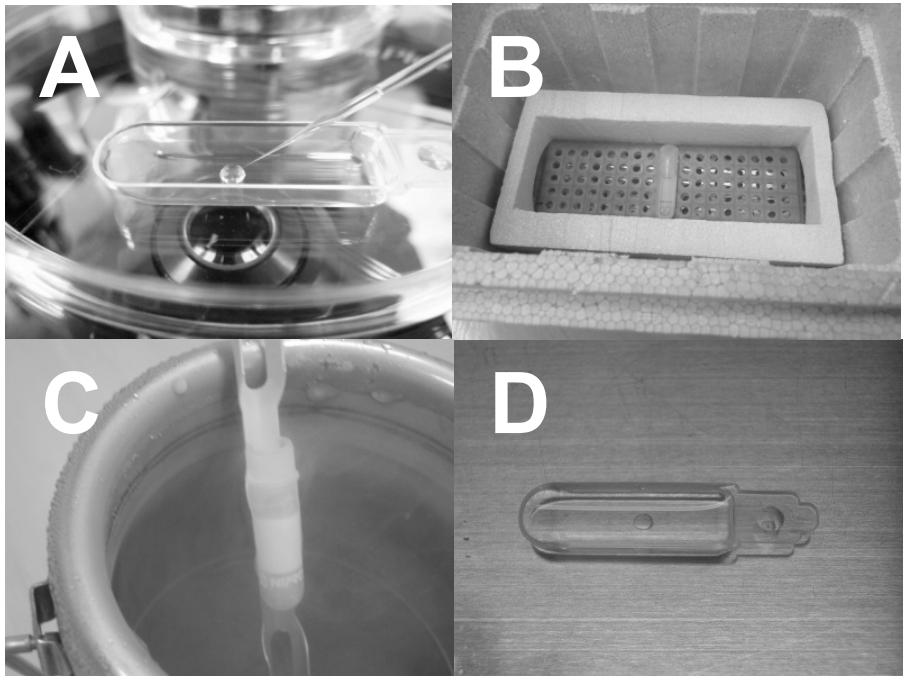

Figure 4. Description of the vitrification and warming procedures using Cell Sleeper. (A) Sperm were loaded into the freezing medium on a tray with the aid of a micromanipulator. (B) After placing the tray into a cryotube, the tray was cooled in vapor of LN_2. (C) Cell Sleeper was mounted on a cane and 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 sperm.

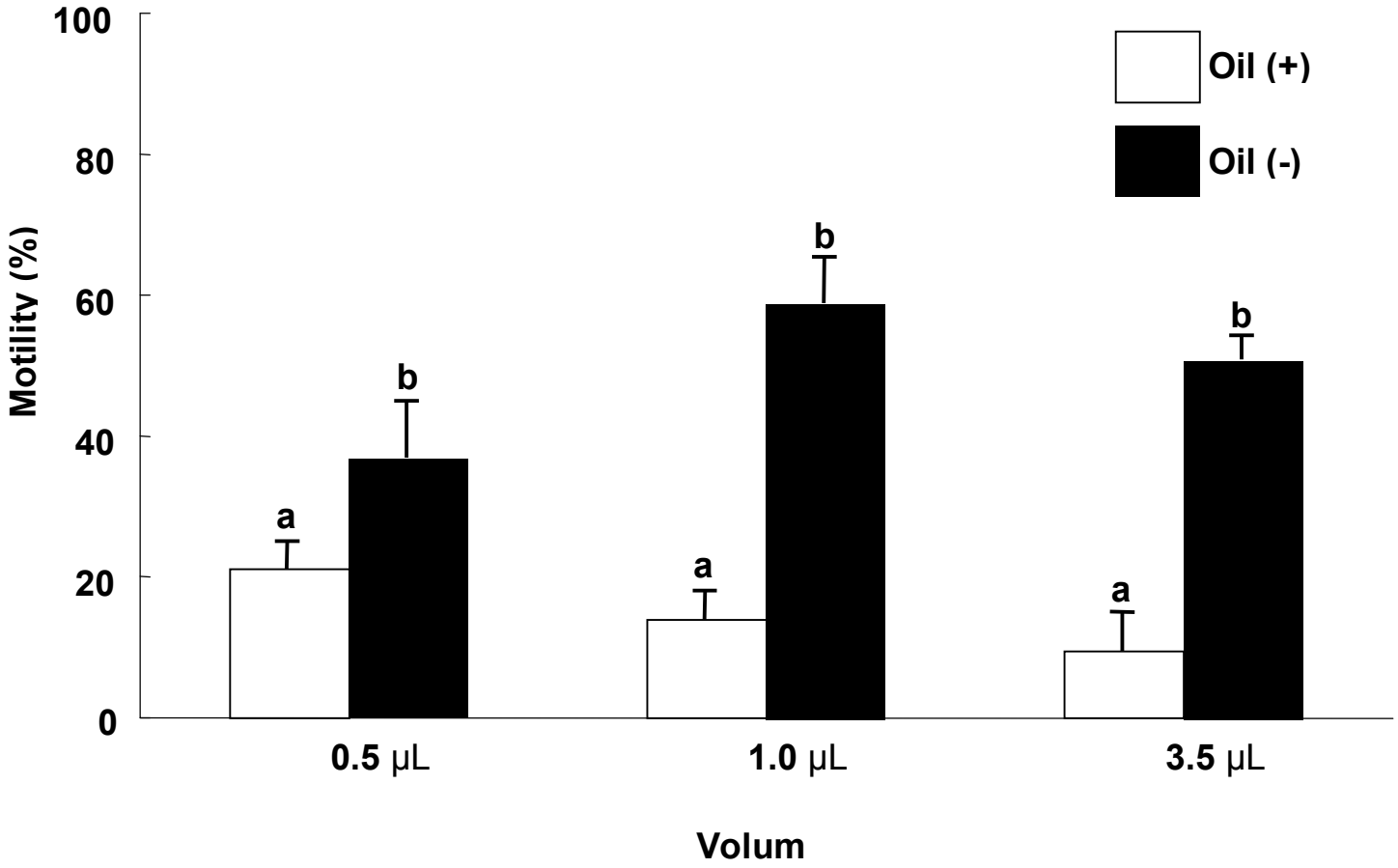

Figure 5. Motility of human sperm (mean ± SEM, n = 6) that were vitrified in 0.5, 1.0 and 3.5 µl of droplets covered with or without oil using Cell Sleeper. [a-b]: Values with different superscripts for the same volume are significantly different ($P < 0.05$).

Chapter 4

Successful delivery derived from vitrified-warmed spermatozoa from a patient with non-obstructive azoospermia

(Endo Y, Fujii Y, Kurotsuchi S, Motoyama H, Funahashi H. Fertility and Sterility 2012;98:1423-7)

4.1 Introduction

Cryopreservation of human sperm has now become an essential technique in the assisted reproductive laboratory. In cases of azoospermia, the use of cryopreserved testis-derived sperm cells for intracytoplasmic sperm injection (ICSI) has a beneficial option of avoiding multiple surgical procedures for sperm extraction (Schelgel and Su, 1997). In the recent studies, small numbers of sperm have been frozen using various types of containers (Cohen et al., 1997; Hsieh et al., 2000; Schuster et al., 2003; Desai et al., 2004; Isachenko et al., 2004; Just et al., 2004; Herrler et al., 2006; Sereni et al., 2007), and successful pregnancies have been reported using a few sperm stored in empty zona pellucida (Fusi et al., 2001; Walmsley et al., 1998). However, these approaches depend on biological materials from human or animal sources and are the only currently available options for freezing a small number of sperm. The lack of an easily implemented technology has remained a major bottleneck for the cryopreservation of limited numbers of sperm (AbdelHafez et al., 2009).

In the previous study (Endo et al., 2011, 2012), it has developed simple, novel

vitrification techniques for a single spermatozoon using Cryotop (Kitazato Biopharma) and Cell Sleeper (Nipro). In this study, it is reported a successful case of delivery after performing ICSI with vitrified sperm.

4.2 Materials and methods

4.2.1 Patients

From December 2007 to October 2011, a total of three couples underwent ICSI using vitrified-warmed sperm. Two couples had been diagnosed with non-obstructive azoospermia (NOA), and testicular sperm were isolated surgically by microdissection testicular sperm extraction (micro-TESE). The other couple had been diagnosed with severe oligozoospermia, and a few motile sperm were obtained from ejaculates (3-10 sperm/ejaculate). All patients gave written consent to be involved in this study protocol, which was approved by a local ethics committee.

4.2.2 Media

As a basic medium, hepes buffered modified HFF99 (Fuso Pharmaceutical Industries) containing 20% serum substitute supplement (Irvine Scientific) was used. Freezing medium was used SpermFreeze (FertiPro), which includes both glycerol and sucrose. Following the manufacturer's instructions, a mixture of SpermFreeze (0.7 mL) and basic medium (1.0 mL) was used for sperm cryopreservation.

4.2.3 Sperm preparation

The seminiferous tubules from patients with NOA were isolated surgically by micro-TESE and placed into 100 μL of basic medium on a glass bottom dish (Wilco Wells BV). Immediately, the tissues were dissected using by stainless steel blades and spread on a dish. Ejaculated sperm from the patient with severe oligozoospermia were washed with basic medium by centrifugation procedures at 760 × g for 15 min. After removing the supernatant, 100 μL of the sperm pellet was spread on a glass bottom dish.

Recovered sperm were diluted with further 100 mL of basic medium containing 7.2 mM pentxifylline (Sigma Chemical) and covered with oil. After 3-10 min of culture at 37°C, the sperm was searched using an inverted microscope (Olympus IX-71; ×100-200 magnification) equipped with a Relief Contrast system and a 21-inch monitor. The found sperm was collected using ICSI injection pipettes (Kitazato Biopharma), which had an inner diameter of >4 mm and equipped with a micromanipulator, and stored in a quite small volume (< 0.1 mL) of basic medium until vitrification procedures.

4.2.4 Cryotop method for a single sperm vitrification

Cryotop consists of a fine polypropylene strip attached to a plastic handle and is equipped with a cover straw (Figure 6A). In accordance with the previously described report (Endo et al., 2011), individual sperm cells were

transferred to a droplet of freezing medium (1 μL) on the Cryotop strip using the ICSI pipettes at room temperature (Figure 6C). Immediately, the Cryotop strip was placed at 4 cm (-120°C) above the surface of the liquid nitrogen (LN_2) for 2 min, and then directly exposed to sterilized LN_2. The Cryotop strip was covered with the hard plastic straw during storage in the cryotank. For warming, the Cryotop strip was placed directly in a flat droplet of basic medium (2 μL), which was covered by oil in a Falcon 1006 dish (Becton Dickinson) at 37°C. To prevent sperm being left on the Cryotop strip, it was washed with two further drops (2 μL). Each droplet was carefully checked and the recovered sperm was transferred to 8% polyvinylpyrrolidone (Irvine Scientific) drop. After washing three times by ICSI injection pipettes, they were stored until ICSI procedures.

4.2.5 Cell Sleeper method for a single sperm vitrification

Cell Sleeper is a type of vial used as cell-cryopreservation container, which is equipped with an inner tray and is sealed with a screw cap (Figure 6B). In accordance with the Cell Sleeper method (Endo et al., 2012), individual sperm were transferred to a freezing medium (3.5 μL) deposited on the tray using the ICSI pipettes (Figure 6D). Immediately, the tray was put into a vial and sealed with a screw cap. The vial was cooled in vapor (-120°C) of LN_2 for 2.5 min and submersed in LN_2. The Cell Sleeper was mounted on a cane and stored in a cryotank. On the day of oocyte retrieval, Cell Sleeper was warmed at room temperature for 1 min and the tray was taken out immediately from the vial. The

sperm on the tray was covered with oil immediately and incubated at 37°C for 2 min. The droplet was observed carefully and the recovered sperm was retrieved individually as described above.

4.2.6 Ovarian stimulation and oocyte collection

The female partners received ovarian stimulation treatment by a combination of recombinant follicle-stimulating hormone (Follistim, Merck) and gonadotrophin-releasing hormone antagonist (Ganirelix, Merck). Vaginal ultrasound-guided follicle puncture was conducted 36 hours after the human chorionic gonadotrophin (Mochida Pharmaceutical) injection. The retrieved oocytes were denudated enzymatically with recombinant human hyaluronidase (Origio) and mechanically by pipetting with narrow glass pipettes. The vitrified-warmed sperm cells were injected into the oocytes in accordance with the previously reported method (Fujii et al., 1997). Oocytes and embryos were cultured in Global medium (LifeGlobal) supplemented with recombinant human albumin (Vitrolife) at 37°C in 6% CO_2, 5% O_2 and 89% N_2. Embryo transfers were performed on day 3 or 5. Clinical pregnancy and implantation were observed by ultrasonic detection of the gestational sac in the uterine cavity at 4-6 weeks after transfer.

4.3 Results

4.3.1 Patient 1

The patient was a 29-year-old woman. Her 30-year-old husband with NOA underwent micro-TESE. A total of two motile sperm and 10 non-motile sperm were retrieved, and all sperm cells were vitrified in two Cell Sleeper vials. On the day of oocyte retrieval, 6 mature oocytes were retrieved and then all sperm were warmed. Ten non-motile sperm were recovered, but two were lost. Sperm were injected individually into oocytes and unused sperm were re-cryopreserved. After ICSI, all injected oocytes were activated by calcium ionophore A23187 (10 IU/mL; Sigma) treatment for 15 min. Normal fertilization was observed in five (83%) oocytes and all zygotes cleaved. A single expanded blastocyst was transferred on day 5, which resulted in a singleton pregnancy. Finally, she concluded with full-term delivery of a healthy boy (2,632 g) at 38 weeks and 3 days, and the infant has had normal physical profiles up to the present.

4.3.2 Patient 2

The patient was a 34-year-old woman, and her 34-year-old husband had been diagnosed with severe oligozoospermia. Total of 55 motile sperm from seven ejaculates were vitrified using Cryotop and Cell Sleeper. In the first cycle, six mature oocytes were retrieved and then seven sperm stored in a Cryotop were warmed. Six non-motile sperm were recovered, but one was lost. After ICSI, normal fertilization was observed in three (50%) oocytes and all zygotes cleaved. A single poor quality morula was transferred into patient’s uterus at day 5 but no pregnancy resulted. In the second cycle, one oocyte was collected and

seven vitrified sperm (7/7; 100%), which were stored in a Cryotop, were recovered. A normal fertilized zygote was obtained after ICSI and an excellent quality embryo (8-cell stage) was transferred on day 3 but failed to implant.

4.3.3 Patient 3

The patient was a 37-year-old woman. Her 37-year-old husband had been diagnosed with NOA and 81 motile sperm collected by micro-TESE were vitrified using ten Cryotops. On the day of oocyte retrieval, four mature oocytes were retrieved and a Cryotop containing ten sperm was warmed. All sperm were recovered, but had lost motility. After ICSI, normal fertilization was observed in three (75%) oocytes and all zygotes cleaved. A single poor quality blastocyst was transferred on day 5 but it did not implant.

All couples refused to perform the chromosome analysis. A total of 148 sperm obtained from three patients were vitrified individually in 18 containers (Table 7) and underwent ICSI using vitrified sperm. On the day of oocyte pick-up, 36 sperm stored in 5 containers were warmed and 33 (92%) were recovered (Table 8). From a total of four clinical results, fertilization was observed in 12 (71%) and all zygotes (100%) cleaved (Table 9). A couple with NOA achieved a singleton pregnancy and delivered a healthy boy.

4.4 Discussion

The current clinical data shows a successful delivery derived from

vitrified-warmed sperm from a patient with NOA. Limited numbers of sperm were vitrified using Cryotop and Cell Sleeper as non-biological containers, which are already commercially available.

The present simple methods can vitrified and warmed only the numbers of sperm cells needed for ICSI without significant loss. Having a method of reliable sperm storage for severe male factor patients may reduce multiple testicular surgical operations and prevent an ICSI failure owing to an unexpected lack of sperm. Another important advantage of the current method is that individually frozen sperm are recovered efficiently without time-consuming searches. Most sperm are recovered within 30 min and used for ICSI with predictable timing. Searching for few sperm from ejaculate or testis on the day of oocyte retrieval is laborious and time-consuming, which may seriously affect the ICSI outcome of the cycle (Walmsley et al., 1998).

In the current clinical cases, comparable sperm recovery rate was seen in both Cryotop (23/24, 96%) and Cell Sleeper (10/12, 83%) groups. The fertilized oocytes were cleaved and developed, regardless whether sperm were stored in Cryotop or Cell Sleeper. Although poor quality sperm lost their motility after warming, viability seemed to be maintained because oocytes were fertilized after ICSI. It is considered that sperm quality is important to maintain their motilities after vitrification. It is well known that sperm collected by micro-TESE has slightly lower motility after cryopreservation than sperm obtained from men with normal sperm quality (Hsieh et al., 2000). In our laboratory studies (Endo et al., 2011,

2012), the healthy ejaculated sperm could be vitrified in Cryotop and Cell Sleeper with similar motility rate (29-44%) after warming.

The only delivery was achieved after oocyte activation with a calcium ionophore. It had been expected the oocyte activation procedure to enhance the fertilization abilities of oocytes and sperm cells. Because all warmed sperm were immobilized by freezing, it was anticipated that the sperm's ability to fuse with the oocyte would be impaired. Fortunately, normal fertilization was observed after oocyte activation and the transfer of one blastocyst stage embryo resulted in singleton pregnancy and live birth. Oocytes activation was not used for the other patients. The oocytes fertilized but no pregnancies were achieved after embryo transfer. the present study is based on a very small sample size and further investigations are required to determine if oocyte activation is required for fertilization after ICSI of sperm vitrified in the Cell Sleeper.

In the current study, Thirty-two vitrified- warmed sperm were recovered and only morphologically normal sperm were selected for ICSI. It was not be attempted to assess the sperm membrane viability by the hypo-osmotic swelling test before ICSI or in sperm not selected for ICSI because the non-selected sperm were morphologically abnormal (i. e. sperm with heads separating from tails, sperm with coiled tails). Most injected sperm were motile just before vitrification and it was expected the sperm would maintain their ability to fertilize oocytes throughout this process. For ICSI procedures, sperm must be immobilized, and they look "dead" in a conventional sense. Along these lines,

Hoshi et al. (1994) have reported the successful delivery after ICSI using freeze-killed sperm. Further, Kusakabe et al. (2008) have reported that both mouse and human sperm killed by freeze-drying techniques maintain a normal karyotype and ICSI of these sperm in the mouse model can lead to development of normal fetuses.

In conclusion, this is a rare case of a successful delivery after transfer of a blastocyst derived from ICSI using limited numbers of sperm stored in novel containers. It is believed that the present simple methods are suitable and clinically useful for the cryopreservation of small numbers of sperm. Further studies of clinical applications with extremely poor sperm specimens will be necessary in order to confirm these findings.

Table 7. Vitrification of small number of sperm using Cell Sleeper and Cryotop.

Patient	Diagnosis	Sperm origin	Container (n)		Vitrified sperm (n)	Sperm per container (mean)
1	NOA	Testis	Cell Sleeper	2	12	6.0
2	Oligo	Ejaculate	Cell Sleeper	6	41	6.8
		Ejaculate	Cryotop	2	14	7.0
3	NOA	Testis	Cryotop	8	81	10.1
Total				18	148	8.2

Note: NOA = non-obstructive azoospermia, Oligo = oligozoospermia

Table 8. Recovery and motility of vitrified-warmed sperm.

Patient	Cycle	Warmed container (n)		Warmed sperm (n)	Collected sperm (n)	
					Motile	Non-motile
		Cell				
1	1	Sleeper	2	12	0	10
2	1	Cryotop	1	7	0	6
	2	Cryotop	1	7	0	7
3	1	Cryotop	1	10	0	10
Total			5	36	0	33

Table 9. Results of embryo development and clinical pregnancy following ICSI using vitrified sperm.

Patient	Cycle	Oocyte injected (n)	2PN fertilization [n, (%)]	Embryo cleaved [n (%)]	Embryo transferred (n)	Outcome
1	1	6	5 (83)	5 (100)	1	Single live birth*
2	1	6	3 (50)	3 (100)	1	No pregnancy
	2	1	1 (100)	1 (100)	1	No pregnancy
3	1	4	3 (75)	3 (100)	1	No pregnancy
Total		17	12 (71)	12 (100)	4	

Note: * Male baby (2632 g)

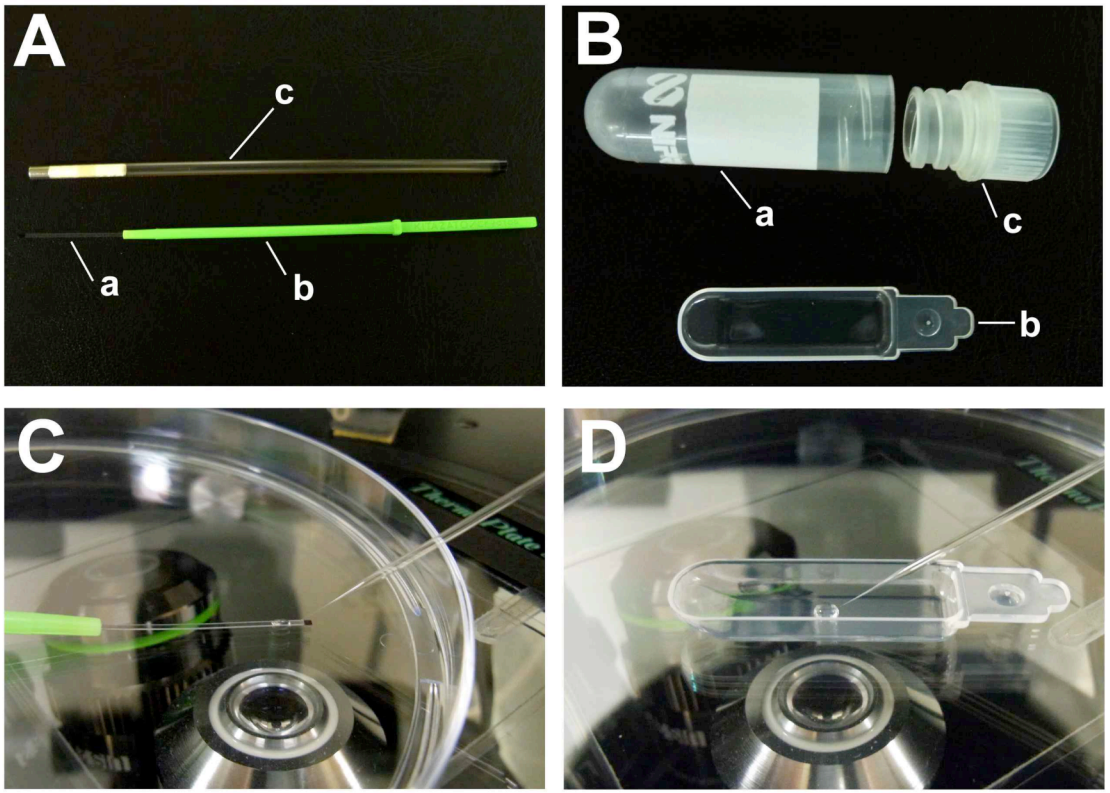

Figure 6. Vitrification procedures of individual sperm. (A) Cryotop consists of a fine polypropylene strip (a), a plastic handle (b) and a cover straw (c). (B) Cell Sleeper vial (a) is equipped with an inner tray (b) and a screw cap (c). Sperm were loaded on a Cryotop strip (C) or a tray of Cell Sleeper (D) using the ICSI pipettes equipped with the aid of a micromanipulator.

Chapter 5

General discussions

In the current studies, it was successfully developed the vitrification methods for small numbers of sperm with use of Cryotop or Cell Sleeper. There is no ethical problem because both containers consist of non-biological materials and could be prepared commercially. The simple methods could be retrieved spermatozoon without significant loss and highly maintained their functions without DNA damage. Furthermore, it has suggested that the current reliable methods are clinically useful for single spermatozoon storage for severe male factor patients. The vitrified sperm could be warmed only the numbers of sperm cells needed for ICSI, and subsequent ET resulted in singleton pregnancy and live birth.

5.1 Sperm vitrification

In human or animal oocytes/embryos, vitrification techniques have been widely accepted in many laboratories because the post-warming survival results were much better than those with traditional slow freezing (Kuwayama et al., 2005). Vitrification is a process involving glasslike solidification of living cells without ice crystal formation during cooling, and requires a high concentration of cryoprotective agents (CPAs) and ultra-rapid cooling/warming rates to avoid chilling injury (Cobo et al., 2008). Many healthy babies derived from vitrified

oocytes/embryos have been born and successful human oocyte banks have been established worldwide.

In mammalian sperm, in contrast, conventional vitrification resulted in low or no survival rates and was not adopted as an alternative cryopreservation procedure. This is because a high concentration of CPAs causes the lethal effect of osmotic shock and is not suitable for sensitive mammalian sperm (Isachenko et al., 2003). Moreover, rapid cooling rates lead to osmotic imbalance encountered during thawing and damage the sperm cells, rather than intracellular ice formation (Morris et al., 2006, 2007). Intracellular ice was not formed in the sperm cells and was not a cause of spermatozoon death at cooling rates of up to 3,000°C/min. The mechanisms of sperm vitrification may explain that high intracellular protein content together with the osmotic shrinkage associated with extracellular ice formation leads to intracellular vitrification of sperm during cooling (Morris et al., 2012).

In recent studies, Nawroth et al. (2002) and Isachenko et al. (2004) have developed new sperm vitrification techniques without cryoprotectant and obtained high recovery rates. In these studies, vitrified sperm on loops were warmed very quickly to minimize the cell damage causing by osmotic shock. However, their vitrification method is not suitable for routine cryopreservation of human semen for artificial insemination because only a very small volume of sperm (<20 μL) can be vitrified at one time (Vutyavanich et al., 2010).

The sperm vitrification technique is a beneficial option for small numbers of

sperm. In the current studies, it has been suggested the use of simple novel vitrification techniques for a single spermatozoon using CryoTop and Cell Sleeper. It was able to demonstrate a relatively high recovery rate and normal fertilization was observed after following ICSI using vitrified sperm. Furthermore, successful full-term delivery was achieved after embryo transfer.

5.2 Cryoprotectant

Sucrose is routinely used as a CPA in procedures for freezing and thawing human specimens (Kuwayama et al., 2005). Isachenko et al. (2008) have suggested that statistically higher motility and viability may be achieved for human sperm vitrified in 0.25 M sucrose with half-ionic strength, in a near iso-osmotic condition (420 mOsmol). This may minimize sperm osmotic injury and achieve successful vitrification of sperm. Recent studies showed that sperm motility was completely lost when human sperm were exposed to medium with 600 mOsmol (Rossato et al., 2002), and mouse sperm with 500 mOsmol (Willoughby et al., 1996). Koshimoto et al. (2000) successfully froze mouse sperm in 18% raffinose with quarter-strength phosphate-buffered saline (418 mOsmol), which did not exceed the osmolarity limit of sperm tolerance. Although a freezing medium with a relatively low concentration of CPA has been considered to provide little protection for sperm cryopreservation, sucrose extender with near iso-osmotic condition worked as well as regular freezing media (Endo et al., 2011; Isachenko et al., 2005) and effectively protected the

cells without significant loss of important physiological parameters (Isachenko et al., 2008).

Moreover, the use of only sucrose as a cryoprotectant may provide further advantages. Because sucrose is a non-permeating CPA, it does not require processing to remove the cryoprotectant from the cells after thawing (Hossain and Osuamkpe, 2007). As a result, the thawing procedure is very straightforward and sperm loss may be reduced. When sperm are frozen in glycerol, which is a permeating CPA, centrifugation techniques are required to remove glycerol from the cells after thawing, which causes sperm loss. Another advantage is that the sucrose extender is a chemically defined solution with simple composition and may reduce the possible risk of viral contamination resulting from use of media containing biological macromolecules, such as egg yolk. The results of the experiments described here indicate that sucrose is a suitable, safe and effective CPA to vitrify human sperm.

5.3 Risk of infection

Namely so-called open systems of vitrification require direct contact with the LN_2 during the cryopreservation process. Under such conditions, the hypothetical exposure to pathogenic agents during cooling storage becomes relevant. Some reports have confirmed the presence of with bacteria, fungal organisms and pathogens, which may cause nosocomial infections in LN_2 tanks (Bielanski 2003). Stored samples in cryotanks would be contaminated if once

LN_2 is contaminated. Thus, LN_2 itself can be considered a potential source of pathogen agents during the cryopreservation process and long-term storage. This issue has led to serious concern about the use of specific cryopreservation technology (i.e., vitrification) when using open systems.

It is important to determine the likelihood of detecting pathogens from handling specimens and to evaluate the probability of cross-contamination when using open systems for vitrification. In ART, no case of transmission of infectious disease has ever been reported and no report mentions LN_2 as a probable vehicle for disease transmission (Cobo et al., 2012). Although cross-contamination have been reported under exprimental cnditions in animal cryopreservation research (Bielanski, 2000), 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). Furthermore, sperm washing procedures can effectively reduce the vertical transmission risk of viruses such as human immunodeficiency, hepatitis B, hepatitis C and herpes (Kato et al., 2006). Thus, the possibility of infection might be vanishingly small when individual thawed spermatozoon are washed several times with fresh medium before ICSI.

New FDA and European Tissue Directive regulations discourage the direct contact between samples and LN_2. Therefore, it has been attempted the use of closed system container for sperm vitrification in the current study, but LN_2 have leaked into the container during storage in a cryotank. As a result, sperm were

exposed directly to LN_2 in the cryotanks. Modification of the original technique by enclosing container may require further exploration.

5.4 Conclusion

Vitrification of single human spermatozoon and its use with ICSI is a beneficial option of practical reproductive medicine. Having a method of reliable sperm storage for severe male factor patients can reduce multiple testicular surgical operations and prevent an ICSI failure owing to lack of sperm. It is believed that the current simple methods are suitable and clinically useful for the cryopreservation of small numbers of sperm. Further studies of clinical applications with extremely poor sperm specimens will be necessary in order to confirm these findings.

Chapter 6

Summary

6.1 Single spermatozoon freezing using Cryotop

Conventional freezing procedures are not appropriate for surgically retrieved sperm from the epididymis or testis because of their low numbers. Techniques for the cryopreservation of small numbers of sperm have not been fully established. It was tried to develop a cryopreservation method for a single spermatozoon using Cryotop, which has a simple structure and is easy to handle. Different parameters influencing the freezing procedure, types of container, sources of sperm, and cryoprotectants were evaluated. Comparable sperm recovery rate was seen in both Cryotop and zona pellucida groups as sperm freezing containers. The frozen sperm obtained from ejaculates and testes could be recovered without significant loss and post-thaw motility rate was similar. The survival rate was significantly higher when sperm were treated with sucrose rather than with glycerol. These results demonstrated that Cryotop was a highly effective tool for the cryopreservation of a single spermatozoon, and sucrose was determined to be an efficient cryoprotectant.

6.2 Simple vitrification method for small numbers of human sperm

Conventional freezing procedures are not appropriate for sperm from the testis because of their low number and poor *in-situ* motility. Techniques for the

cryopreservation of small numbers of sperm have attempted to use various types of containers, but these are the only currently available options and the lack of an easily implemented technology has remained a major bottleneck. In the current study, it was successfully established a simple freezing method for small numbers of human sperm using Cell Sleeper, which is vial type of cell-freezing container and is equipped with an inner tray. Cell Sleeper is commercially available and easy to prepare for use. On single sperm freezing, the best result obtained when sperm were frozen in 3.5 μL of oil-free droplet and it was the most convenient volume for handling small numbers of sperm using an ICSI pipette equipped with a micromanipulator. Furthermore, sperm could be recovered efficiently and quickly without significant loss. For these results, it was concluded that Cell Sleeper was a useful container for the cryopreservation of small numbers of sperm.

6.3 Successful delivery derived from vitrified-warmed sperm from a patient with non-obstructive azoospermia

It was examined the clinical outcomes following ICSI with vitrified sperm with the use of Cryotop and Cell Sleeper, which are already commercially available. Three patients with severe oligozoospermia or non-obstructive azoospermia (NOA) have undergone ICSI using vitrified-warmed sperm. Limited numbers of sperm were vitrified using Cryotop and Cell Sleeper as non-biological containers. Four cycles underwent ICSI with vitrified sperm. Most

warmed sperm were recovered successfully and injected individually into mature oocytes. Normally fertilized oocytes were developed and subsequently transferred to patient's uterus. A couple with NOA achieved a singleton pregnancy and concluded with full-term delivery of a healthy boy (2,632 g). A successful delivery was achieved after transfer of a blastocyst derived from vitrified-warmed sperm. The current clinical results showed that a small number of vitrified sperm cells were used for ICSI to fertilize oocytes with predictable timing.

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