Single Spermatozoon Freezing Using Cryotop

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Abstract: *Conventional freezing procedures are not appropriate for surgically retrieved spermatozoa from the epididymis or testis because of their low numbers. Techniques for the cryopreservation of small numbers of spermatozoa have not been fully established. We 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 spermatozoa, 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 spermatozoa 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 SpermFreeze (65.3% vs. 37.3%, P < 0.01). Cryotop was a highly effective tool for the cryopreservation of a single spermatozoon, and sucrose was determined to be an efficient cryoprotectant.*
Key words: *Cryopreservation, Cryotop, Zona pellucida, A single spermatozoon, Sucrose*

Introduction

In cases of severe male infertility, intracytoplasmic sperm injection (ICSI) is a treatment option with high success in *in vitro* fertilization (IVF) [1]. Oocytes can be fertilized by ICSI even with spermatozoa from azoospermic patients [2]. In cases of azoospermia, cryopreservation of surgically retrieved spermatozoa from the epididymis and testis is useful for effective treatment and management, and it reduces the requirement for repeated surgery [3, 4]. Since the first attempts at human sperm cryopreservation [5], many studies have been devoted to the development of the optimal freezing techniques for human spermatozoa [6]. Cryopreservation of human spermatozoa has now become a routine procedure. However, conventional freezing procedures may not be appropriate for spermatozoa from the epididymis and testis because of their low number and poor *in-situ* motility. Some authors have reported successful pregnancies using a few spermatozoa stored in empty zona pellucida (ZP) [7, 8]. 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 [9], mini-straws [10, 11], micropipettes [12, 13], cryoloops [14, 15], copper loops [16, 17], volvox globator algae [18], agarose microspheres [19], and alginate beads [20]. Unfortunately, these containers are the only currently available options to cryopreserve very low numbers of spermatozoa and they cannot be used universally [21]. The lack of an easily used technology for handling low numbers of spermatozoa remains a major deterrent to the freezing of single spermatozoon.

In this study we tried to cryopreserve a single spermatozoon using Cryotop (Kitazato Biopharma, Japan), which has a simple structure and is easy to handle. We tested different parameters influencing the freezing procedure, including types of container, sources of spermatozoa, and cryoprotectants.

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Materials and Methods

Spermatozoa 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 spermatozoa 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 spermatozoa. 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*. [22], about 3 μl 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 stereo microscope 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 μl of medium with the specimen was diluted with 100 μl of HFF99 containing 20% SSS and checked using a microscope.

For preparation of each specimen, spermatozoa were collected using ICSI injection pipettes (Kitazato Biopharma, Japan), which had an inner diameter of > 4 μm using an inverted microscope (Olympus IX-71; ×100–200 magnification) equipped with a Relief Contrast system and a 21-inch monitor.

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.

Sperm freezing containers

Two sperm containers, Cryotop and ZP were tested. Cryotop (Fig. 1) is usually used as an efficient container for the vitrification of oocytes and embryos [23]. 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*. [24]. 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 μl droplet of medium in accordance with the method of Cohen *et al.* [24]. 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.

Freezing and thawing techniques using Cryotop

A droplet of freezing medium (1 μl) 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 (Fig. 2a and 2b). Immediately, the Cryotop strip was cooled in the vapor of liquid nitrogen (LN_2) for 2 min prior to submersion (Fig. 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 (Fig. 2d). For thawing, the Cryotop strip was taken out of the LN_2 and immediately placed in a flat droplet of medium (2 μl) which was covered by mineral oil in a Falcon 1006 dish at 37°C (Fig. 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.

Freezing and thawing techniques using zona pellucida

Twenty ZPs, including immature and unfertilized oocytes, were obtained from ten consenting ICSI

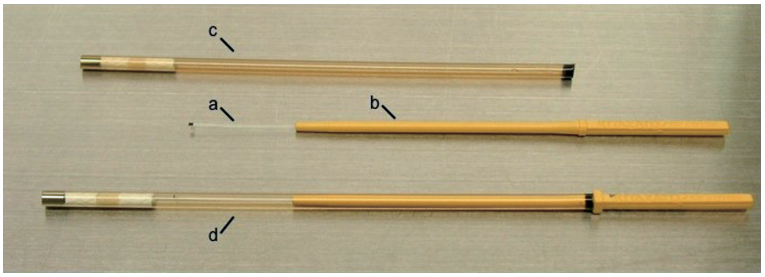

Fig. 1. Cryotop cryopreservation tool. A polypropylene strip (a) is attached to a hard plastic handle (b). After cryopreservation, the hard plastic cover (c) protects from not only physical damage but also virus contamination during storage in LN_2 (d).

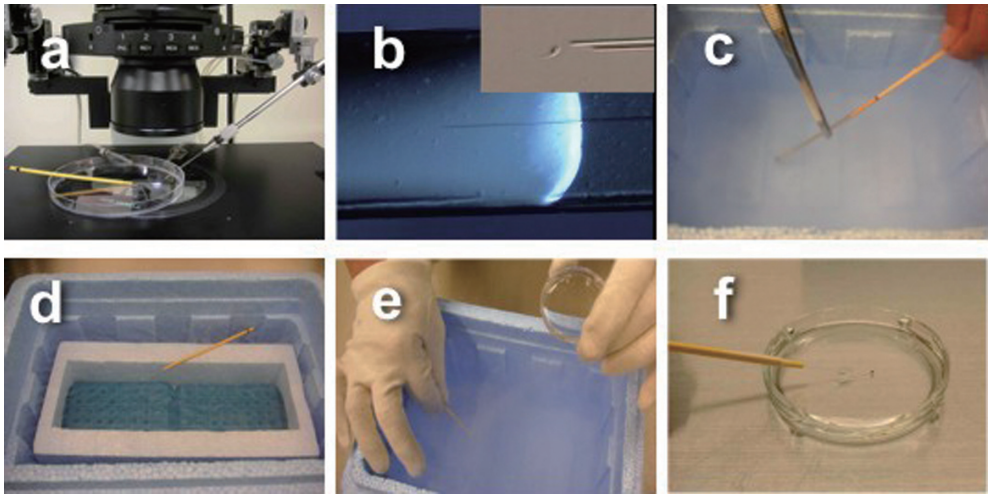

Fig. 2. Description of the freezing and thawing procedures. (a, b) A motile sperm was loaded into freezing medium on a Cryotop strip with the aid of a micromanipulator. (c, d) The Cryotop strip with a single spermatozoon was frozen in LN_2 vapor and then submerged in LN_2. (e, d) For thawing, the Cryotop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

patients. After aspiration of the cytoplasm using an injection micropipette, empty ZP was washed 3 times with HFF99 containing 20% SSS and cultured until sperm insertion. After fixation of the ZP using a holding pipette mounted on a micromanipulator, five motile spermatozoa 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 spermatozoa 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 30ºC 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 μl) which was covered by mineral oil in a Falcon 1006 dish at 37ºC and washed gently with three further droplets.

Table 1. Effect of two different freezing containers on the recovery and motility rates of individually frozen sperm from ejaculates

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. Effect of two different sources of spermatozoa on recovery and motility rates of individually frozen cells using Cryotop

Source of sperm	No. of frozen sperm	No. (%) of sperm recovered after thawing	No. (%) of motile sperm after thawing	Sperm retrieval time (sec)
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 spermatozoa 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 ($P < 0.01$).

Subsequently, the spermatozoa inside the ZP were aspirated using an injection pipette.

Statistical analysis

In this study, thawed spermatozoa 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.

Results

Comparison of sperm freezing containers

The results of sperm freezing using Cryotop and ZP are shown in Table 1. All spermatozoa 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 spermatozoa (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 spermatozoa (12.0%) were lost, 9 (9.0%) of them were not found inside the ZPs after thawing and 3 (3.0%) were trapped in the remains of cytoplasm. The motility rate of the recovered sperm was not significantly different between the Cryotop (30.6%) and ZP (23.9%) groups.

Comparison of spermatozoa sources

Motile spermatozoa 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 sec) in ejaculate, and 286 sec (range 1–1,800 sec) for testicular sperm.

Comparison of cryoprotectants

Two different cryoprotectants, sucrose and SpermFreeze, were tested. Motile spermatozoa 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%).

Discussion

The present study showed that successful freezing of a single spermatozoon was possible using Cryotop. Using the current method, we were able to freeze a single spermatozoon easily, and recover it efficiently and quickly. Cohen *et al*. [24] first demonstrated the practical value of freezing small numbers of spermatozoa 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 [7, 8]. 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 spermatozoa, but no live births have yet been reported [21]. The lack of an easily implemented technology has remained a major bottleneck for the cryopreservation of small numbers of spermatozoa.

In the present study, the sperm recovery and motility rates after thawing were similar between the sperm frozen using Cryotop or ZP as containers. Cryotop is usually used as a vitrification container for oocytes and embryos with excellent results (99% post-thawing survival rate) [23], 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 spermatozoa 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, we consider that Cryotop can be used as a safe container for the cryopreservation of spermatozoa.

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 [25]. In the present study, the testicular spermatozoa 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 spermatozoa.

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 spermatozoa, the best results were always achieved with 0.1 M sucrose (unpublished data). It is well known that human spermatozoa are very sensitive to osmotic stress [16, 17, 26]. 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 [27], our 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 [27]. 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 [26, 27]. 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. Our 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.

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