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Efficiency of a Closed Vitrification System with Oocytes and Blastocysts

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卵子および胚盤胞におけるクローズドガラス化凍結システムの有効性

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要旨

生殖医療において細胞凍結技術は重要な役割を果たしている．直接液体窒素に接触せずに細胞をガラス化するクローズドシステムは, 二次感染リスクを減少させる. 本研究では, 新開発の CryotopCL を用いてクローズドシステムの有効性をオープンシステムとの比較試験によって検証した.

マウス卵子，胚を用いた基礎実験の後，ヒトの卵子，胚を用いた臨床試験を行い，それぞれの卵子および胚の生存率，凍結融解した卵子の胚発育率を観察した．また臨床試験では凍結融解した胚盤胞の着床率および妊娠率を検証した.

クローズドシステムとオープンシステムの結果は，マウス卵子生存率 96.7%:100%，胚盤胞形成率 75.5%: 75.9%，マウス胚盤胞生存率はともに 100%であった．ヒト卵子生存率 93.9%：97.0%, 胚盤胞形成率 72.0%:66.7%, 胚盤胞生存率 98.4%:98.3%, 着床率 49.1%:48.0%, 継続妊娠率 45.5%:42.0%であり，二つのガラス化技術の同等有効性が確認された．これはクローズドシステムがラボおよび臨床の現場で実践可能な技術であることを示唆している.

キーワード：ガラス化凍結法，クローズドシステム，凍結保存，卵子，胚盤胞

Efficiency of a Closed Vitrification System with Oocytes and Blastocysts

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Abstract

Closed system vitrification may decrease the risk of cross-contamination, because it vitrifies and cryopreserve oocytes and embryos without direct contact with liquid nitrogen. This study evaluated the efficacy of a closed system with the newly developed CryotopCL in comparison with open system Cryotop.

In experiment 1, mouse oocytes were divided into closed and open vitrification groups to compare survival rate. Subsequently, embryo development was evaluated. In experiment 2, mouse blastocysts were compared of their survival rate. In clinical trial 1 and 2, human oocytes and blastocysts were used to compare the two system likewise in experiment 1 and 2. In clinical trial 3, vitrified-warmed blastocysts were transferred to patients to observe implantation and pregnancy rates.

The survival rate with mouse oocytes was 96.7% vs. 100%, blastocyst formation rate was 75.5% vs. 75.9%, and survival rate with mouse embryos were both 100% for the closed and open systems. The survival rate of human oocytes was 93.9% vs. 97.0% and embryo development rate was 72.0% vs. 66.7%. The survival rate of human embryos was 98.4% vs. 98.3%. Clinical outcome showed no significant difference in the implantation rate, 49.1% vs. 48.0% and ongoing pregnancy rate 45.5% vs. 42.0%.

This study indicates that closed system vitrification with CryotopCL is suitable for laboratory and clinical work. It is suggested that the closed system can be put into practical use as an efficient vitrification technique in assisted reproduction technologies.

Keywords: Vitrification, Closed system, Cryopreservation, Oocyte, Blastocyst

CONTENTS

1. INTRODUCTION

Reproductive medicine has greatly facilitated many couples to conceive and to give birth. Cryopreservation is a technology that is used to stop biologic reactions at cryogenic temperatures. This technology allows multiple oocytes retrieved during a single treatment cycle to be preserved, thereby avoiding their being wasted, and providing the opportunity to attempt conception repeatedly [1)]. As a result, this technology has reduced the need to perform Hyperstimulation [2)]. Therefore, cryopreservation plays an important role in fertility treatment.

Slow freezing was the initial method introduced for cryopreservation of cells. The first pregnancy in human with cryotransfer was reported in 1983 [3)]. In slow freeze, the cells are equilibrated gradually with cryoprotectant agent (CPA) and are cooled at a slow cooling rate [4)]. Disadvantage with slow freeze is that it sometimes cause chilling injuries to cells [5)]. Conversely, vitrification which was later introduced as a

new freezing methods, vitrifies cells intra- and extra-cellularly with drastic cooling rate [6] and avoids chilling injuries and ice crystallization in cells [7, 8, 9]. Cryopreservation with vitrification has been conducted as routine work in many laboratories.

Presently, there are two methods of vitrification; closed and open systems. Open system means that the samples are in direct contact with liquid nitrogen during vitrification and storage. There are concerns that the open system has a risk for cross-contamination in two situations: when the sample is vitrified and when it is stored long term. Although cross-contamination with the open system has not been reported, some countries regulate its use. Therefore, there is an increasing demand for a closed system that prevents direct contact with liquid nitrogen.

When vitrification is considered, it is true that the cooling rate is essential to obtaining a high survival rate, but there are also reports indicating that the warming rate has a decisive influence on survival [10]. The efficacy of the traditional Cryotop

open system has been demonstrated in multiple papers [11, 12, 13, 14, 15, 16, 17, 18, 19, 20]. The newly developed CryotopCL closed system retains the benefits of Cryotop and is designed to prevent direct contact with liquid nitrogen, while maintaining the cooling rate. Regarding the warming rate, the basic protocol for the closed system is the same as that for the open system. Theoretically speaking, if a sufficient cooling rate to prevent cooling injuries and ice crystallization is maintained and the same warming rate is realized, an efficient closed system should be possible.

The purpose of this study was to evaluate the efficacy of the closed system with CryotopCL. Mouse oocytes and embryos were used in basic experiments, followed by clinical trials with human oocytes and embryos.

2. MATERIALS AND METHODS

A comparative study of the CryotopCL closed system and the Cryotop open system was conducted. The study was structured in two parts. The first part consisted of experiments on mouse oocytes and the second part consisted of experiments on human oocytes.

2-1-1. Guidelines for animal experiments

In all experiments using mice, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All procedures and treatments were conducted according to the guidelines established in the Animal Research Guidelines of Kitazato BioPharma Co., Ltd. All experiments with mice were performed at this facility.

2-1-2. Collection of mouse oocytes

The mice used in this study were female Crlj:ICR mice for the collection of MII oocytes and male Crlj:BDF1 mice for sperm collection. Both kinds of mice were purchased from Charles River Laboratories Japan (Yokohama, Japan).

Female mice (4–8 weeks of age) were superovulated by intraperitoneal injection of 5 IU equine chorionic gonadotropin (Nippon Zenyaku Kogyo, Tokyo, Japan), followed by a second injection of 5 IU hCG (ASUKA Pharmaceutical, Tokyo, Japan) 48 h later. Cumulus-oocyte complexes at the MII stage were collected in a modified-HTF medium (Kitazato BioPahrama Co, Ltd., Shizuoka, Japan) with 0.1% hyaluronidase (Sigma-Aldrich Corp., St. Louis, MO). At 2–5 min later, the denuded oocytes were washed 3 times with fresh m-HTF medium and used for vitrification.

2-1-3. Fertilization and culture of mouse oocytes

Sperm were collected from the cauda epididymis of male mice, suspended in

TYH medium [21], and covered with mineral oil (Kitazato BioPharma, Shizuoka, Japan). After pre-incubation for 1.5 h at 37°C with 5% CO_2 in air, a small volume of the sperm suspension was added to TYH medium containing oocytes (final sperm concentration: 150 cells/μL).

2-1-4. Clinical Trial

All clinical trial with human from sperm collection, oocyte retrieval, vitrification, insemination and embryo transfer were performed at Life IVF Center (Santa Ana, CA, USA) between April 2013 and April 2014.

2-1-5. Informed consent

Clinical trials with human were approved by the ethics committee of the facility where all the trials were conducted: Life IVF Center. All patients were given a detailed explanation from physicians at the institution about the purpose, design,

expected outcome, and risks of the experiments. Oocytes taken only from patients who had signed informed consent for this clinical trials were used in this study. For the *in vivo* clinical study, the patients received a full explanation of the methodology and provided signed informed consent for their participation.

2-1-6. Collection of human oocytes

Ovarian stimulation was performed using clomiphene (Clomid; PAR Pharmaceutical, NY, US) supplemented with human menopausal gonadotropin (Synarel; Pfizer, NY, US). Clomiphene administration was initiated on day 3 of the cycle at a dose of 50 mg/day and continued until the “flare” of luteinizing hormone (LH) caused by nasal spraying of 300 μg gonadotropin releasing hormone agonist. Human menopausal gonadotropin was administered on day 8 at a dose of 150 IU and continued every other day until the day before the LH flare. After the oocytes were retrieved, they were cultured in HTF medium (Kitazato BioPharma, Shizuoka, Japan)

for 2 h with 5% CO_2 in air at 37°C and maximum humidity and used for vitrification.

2-1-7. Collection of human Sperm and Preparation

Sperm was liquefied and was prepared with two layer gradient density method with Isolate (Irvine Scientific, LA, USA). Sperm was either used fresh or frozen-thawed. For freezing sperm, freezing medium – TYB with Glycerol & Gentamicine (Irvine Scientific, LA, USA).

2-1-8. Fertilization and culture of human oocytes

After retrieval of the oocytes, they were cultured for 2 h in HTF medium with 5% CO_2 in air at 37°C and maximum humidity. The oocytes that survived vitrification-warming, as determined by morphological examination, were fertilized by intracytoplasmic sperm injection (ICSI). After insemination, they were transferred to 50 µL fresh Global® Total® (LifeGlobal, CT ,US) for culture until the

4-cell stage and transferred to a 100 µL drop of fresh Global® Total® and their development to blastocysts was observed. Blastocysts that were scored at least 3AA without C by Gardner's criteria[22] were defined as good blastocyst.

2-1-9. Vitrification

All oocytes were vitrified using HPC supplemented Vitrification medium VT601 [23] Ca^{2+}-free (Kitazato BioPharma, Shizuoka, Japan) following the manufacturer's instruction. Ca^{2+}-free vitrification media was used to give better fertilization and development after warming [24, 25, 26, 27]. In brief, oocytes and blastocysts were recovered from culture and transferred at 25°C in 3 steps to a solution of 7.4% EG plus 7.4% DMSO prepared in Basic Solution + 20% hydroxypropyl cellulose (HPC) and held for 15 min until they had completely recovered their original isotonic volume, as judged by microscopic observation. The oocytes and blastocysts were then washed in 0.3 mL vitrification solution (14.5% EG

+ 14.5% DMSO + 0.5 M trehalose in Basic Solution supplemented with HPC). The oocytes or blastocysts were then loaded on either CryotopCL or Cryotop. For vitrification of mouse oocyte and blastocyst, 2 or 3 samples were placed on one CryotopCL/ Cryotop. For human oocytes and blastocyst, only one oocyte or blastocyst were cryopreserved on each cryopreservation device.

The CryotopCL is a FDA approved closed system (Fig. 1) device for designed to enable the vitrification without direct contact with liquid nitrogen. The main piece of CryotopCL (105 mm long) consists of a very fine polyethylene terephthalate (PET) strip (2 mm wide × 20 mm long × 0.3 mm diameter at the thinnest part) attached to a plastic handle and has an airtight straw cap (133 mm length). The airtightness of the device is proven by the test performed in accordance with the method for a plastic medicine container of the Japan Pharmacopeia (Japan Food Research Laboratories).

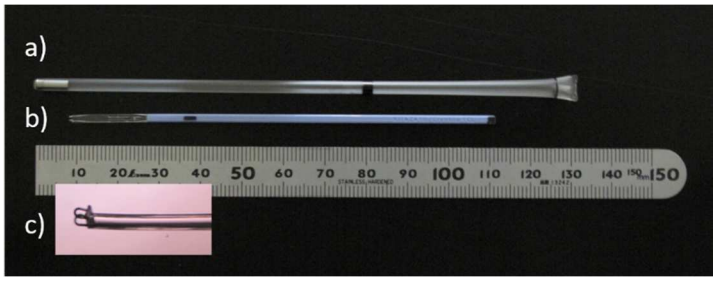

Fig. 1. Picture of CryotopCL (patent pending).

a) Straw cap. The bottom part is sealed and has metal piece to accelerate the cooling rate.

b) CryotopCL main part: PET strip attached to the handle. Metal wires are embedded in both sides of the strip. These wires accelerate the cooling rate.

c) Enlargement of the tip of the PET strip from CryotopCL. The wires are in a loop-shape at the tip to avoid scratching the dish during warming.

After processing in the vitrification solution, oocyte and blastocyst samples were loaded on a CryotopCL strip with a small volume of the vitrification solution. A CryotopCL straw cap was pre-cooled in liquid nitrogen and then cut open at its upper part. A CryotopCL containing a sample was inserted into the straw cap. Immediately after introducing CryotopCL into the straw cap, the upper part of the straw was sealed completely with a heat sealer to avoid any entering of liquid nitrogen. 1 - 3 oocytes or embryos were vitrified on each CryotopCL.

The main piece of the Cryotop open system consists of a very fine PET strip (0.7 mm wide × 20 mm long × 0.1 mm diameter at the thinnest part) attached to a plastic handle, and has a straw cap (11.3 cm long). The straw cap of Cryotop is not airtight.

After processing in the vitrification solution, oocyte and blastocyst samples were loaded on a Cryotop strip with a small volume of vitrification solution. The Cryotop was immersed directly into liquid nitrogen and covered with the straw cap.

2-1-10. Warming

With closed CryotopCL, the straw cap was held vertically using an aluminum block placed in the liquid nitrogen. The straw cap was cut open and the samples on CryotopCL were transferred into a thawing solution without direct contact with the liquid nitrogen.

With open Cryotop, the straw cap was removed in liquid nitrogen the samples on Cryotop were in direct contact with liquid nitrogen before they were transferred into a thawing solution.

For the warming procedure, Thawing Medium VT602 (Kitazato BioPharma Co., Ltd., Shizuoka, Japan) was used. Samples either on CryotopCL or Cryotop were immersed directly into a Thawing Solution (4 mL of 1.0 M Trehalose in Basic Solution) at 37°C for 1 min. Then, the samples were recovered from the Thawing Solution and transferred to Diluent Solution (0.3 mL of 0.5 M Trehalose in Basic Solution) for 3 min, washed twice with Washing Solution (0.3 mL of Basic Solution):

for 5 min in the first drop, then 1 min in the second drop, before cultured for 4 h at 37°C.

2-1-11. Embryo transfer, implantation, and pregnancy check

This study was conducted on 105 patients who were aged between 30 and 35 years. Embryo transfer was performed under ultrasound guidance using a Kitazato ET catheter (Kitazato Medical Co., Ltd., Tokyo, Japan). At day 5 or 6, a single blastocyst vitrified and thawed was transferred in the natural cycle. Implantation was determined by the detection of a single intrauterine gestational sac by transvaginal ultrasound at approximately 3 weeks after embryo transfer. Ongoing pregnancy was checked beyond 8 weeks of gestation by ultrasound demonstrating a fetal heart movement.

2-1-12. Measurement and Calculation of Cooling Rate and Warming Rate.

The cooling rate and warming rate were measured using a Midi LOGGER GL900 (Graphtech, Kanagawa, Japan). To calculate the cooling rate and warming rate of CryotopCL, data was put into a graph to compare the temperature change and time passage. Time passage was measured by 4 sec unit in the graph. Slope on regression line was calculated for duration where drastic temperature change was shown

2-2-1. Experiment 1

120 mouse oocytes were collected and cultured to the metaphase II (MII) stage. Oocytes which size was 80μm was used for the experiment. They were divided evenly in the two vitrification group: closed and open and warmed 5 hr ±1 after vitrification on the same day to evaluate their survival rate. After warming, morphologically normal oocytes were fertilized and cultured in vitro to blastocysts

to check their development. An additional 46 fresh mouse oocytes were used as a control to evaluate embryo development.

2-2-2. Experiment 2

120 Mouse oocytes were fertilized and cultured *in vitro* to blastocysts. The blastocysts at Day 5, 160μm were vitrified and warmed 5 hr ±1 after vitrification on the same day to evaluate their survival rate.

2-2-3. Clinical Trial 1

66 human oocytes were collected after informed consent was given. MII oocytes, 120μm were vitrified and warmed to evaluate their survival rate. After warming, morphologically normal oocytes were fertilized by ICSI and cultured to blastocysts to check embryo development. An additional 30 fresh oocytes were also used as a control for the embryo development rate.

2-2-4. Clinical Trial 2

358 Human blastocysts were fertilized by ICSI and cultured *in vitro* to blastocysts. The blastocysts on Day 5, 200μm were vitrified either with closed system or open system and warmed to check their survival rate.

2-2-5. Clinical Trial 3

A single blastocyst, which was vitrified-warmed, was transferred into each 105 patient. Consequently, clinical pregnancy, implantation rate, and ongoing pregnancy rate up to 10 weeks were observed.

2-3. Data analysis

Data were analysed using contingency tables with Fisher's exact test. P values less than 0.05 were considered to indicate significant differences.

3. RESULTS

There was no significant difference between the closed system with CryotopCL and the open system with Cryotop in all aspects evaluated using mouse oocytes and blastocysts.

Table 1 shows the results from experiment 1 in which the survival rates of MII mouse oocytes in both system were similar: closed system 96.7% vs. open system 100%. Consequently, oocytes that were considered to have survived morphologically were fertilized and cultured to blastocysts. Table 2 shows the fertilization and blastocyst formation rates with fresh mouse oocytes, vitrified mouse oocytes with the closed system, and vitrified mouse oocytes with the open system. The fertilization rates were 93.5% vs. 96.2% vs. 94.4%, respectively. The blastocyst formation rates were 80.4% vs. 75.5% vs. 75.9%, respectively, and the rates of good quality blastocysts among them were 65.2% vs. 60.4% vs. 59.3%, respectively. No

significant difference was found.

Table 1. Survival rates of vitrified-warmed mouse MII oocytes with the closed and open systems.

	No. examined	No. survived	Survival rate
Closed system	60	58	96.7%
Open system	60	60	100%

P value > 0.05 for all measures.

Table 2. Embryo development with control and vitrified-warmed MII oocytes.

	Control (fresh)	Closed system	Open system
No. examined	46	53	54
Fertilized	43 (93.5%)	51 (96.2%)	51 (94.4%)
Blastocyst	37 (80.4%)	40 (75.5%)	41 (75.9%)
Good quality blastocyst	30 (65.2%)	32 (60.4%)	32 (59.3%)

P value > 0.05 for all three measures

In experiment 2, the survival rates of mouse blastocysts vitrified with the closed and open systems were both 100% (Table 3).

Table 3. Survival rates of vitrified-warmed mouse blastocysts with the closed and open systems.

	No. examined	No. survived	Survival rate
Closed system	60	60	100%
Open system	60	60	100%

P value > 0.05 for all three measures

In clinical trial 1 and 2, the study design was similar to the experiments 1 and 2, but human oocytes and embryos were used, while the earlier experiments used mouse oocytes and embryos.

Clinical trial 1 showed that the survival rate for human oocytes with the closed system was 93.9%, while it was 97.0% with the open system (Table 4).

Morphologically surviving human oocytes were fertilized by ICSI and their development was observed. The fertilization rate and embryo development until blastocysts are shown in Table 5.

Table 4. Survival rates of vitrified-warmed human oocytes with the closed and open systems.

	No. examined	No. survived	Survival rate
Closed system	33	31	93.9%
Open system	33	32	97.0%

P value > 0.05 for all measures

Table 5. Embryo development of control and vitrified-warmed human MII oocytes in the open and closed systems.

	Control (fresh)	Closed system	Open system
Survived oocytes	30	31	32
Fertilized	26 (86.7%)	25 (80.6%)	27 (84.4%)
4-cell	25 (96.2%)	24 (96.0%)	24 (88.9%)
8-cell	20 (76.9%)	19 (76.0%)	19 (70.4%)
Blastocyst	19 (73.1%)	18 (72.0%)	18 (66.7%)
Good Quality Blastocyst / Blastocyst	11 (57.9%)	11 (61.1%)	12 (66.7%)

P value > 0.05 for all measures

In clinical trial 2, human blastocysts were vitrified with the closed and open systems and their survival was evaluated morphologically and found to have no significant difference (Table 6).

Table 6. Survival rates of vitrified-warmed human blastocysts with the closed and open systems.

	No. examined	No. survived	Survival rate
Closed system	182	179	98.4%
Open system	176	173	98.3%

P value > 0.05 for all measures

In clinical trial 3, patients aged between 30 and 35 years received single-embryo transfer treatment, and implantation was checked under ultrasound. The

implantation rate with vitrified blastocysts with the closed system was 49.1% vs. 48.0% with the open system. The ongoing pregnancy rates, confirmed at 10 weeks of gestation, were 45.5% vs. 42.0%, respectively (Table 7). No significant difference was found.

Table 7. Clinical outcome with vitrified-warmed human blastocysts.

	Closed system	Open system
Age (years)	30–35	30–35
No. of patients	55	50
Implantation	27 (49.1%)	24 (48.0%)
Ongoing pregnancy	25 (45.5%)	21 (42.0%)

P value > 0.05 for all measures

Cooling rate and Warming Rate

Temperature change was recorded with Midi LOGGER GL900 every 0.4 sec. In vitrification, predominant temperature drop was recorded between 1.84 sec and 2.72 sec from the time of measuring (Fig.3). Slope of regression line in the range was calculated and it was -51.21, which means cooling rate of -51.21°C/sec. This equals to -3,072.41°C/min, approximately -3,000°C/min. In warming, predominant temperature rise was recorded between 1.08 sec and 1.24 sec (Fig. 4). Slope of regression in the range was 668.29, which means cooling rate of 668.29°C/sec, which equals to 40,097.55°C/min, approximately 40,000°C/min.

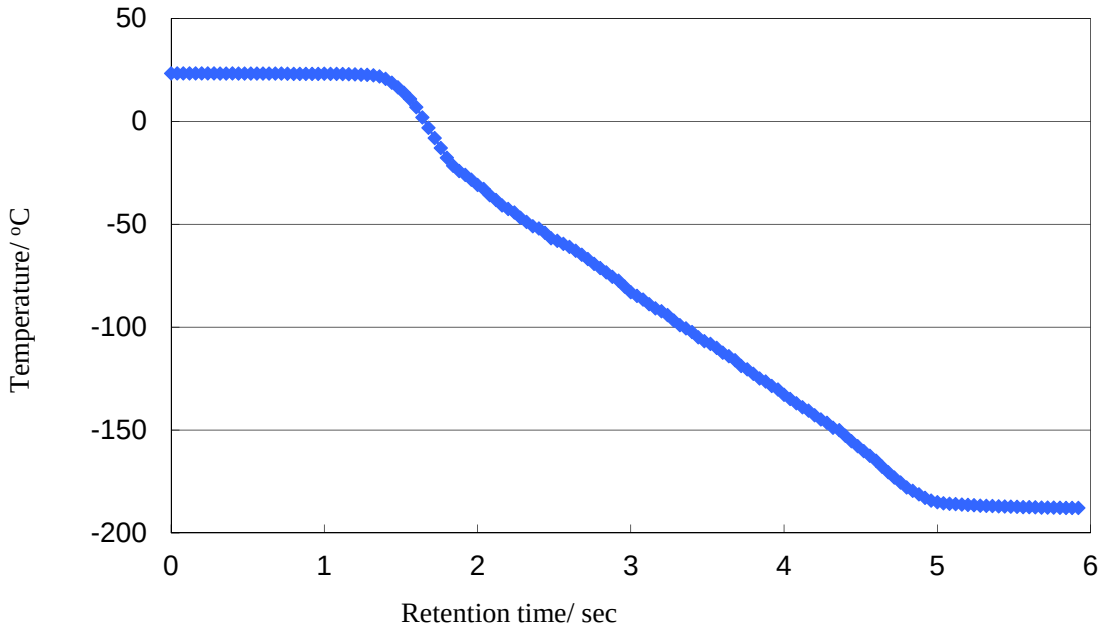

Fig. 3. Temperature change / retention time when CryotopCL is vitrified according to the protocol for the closed vitrification system.

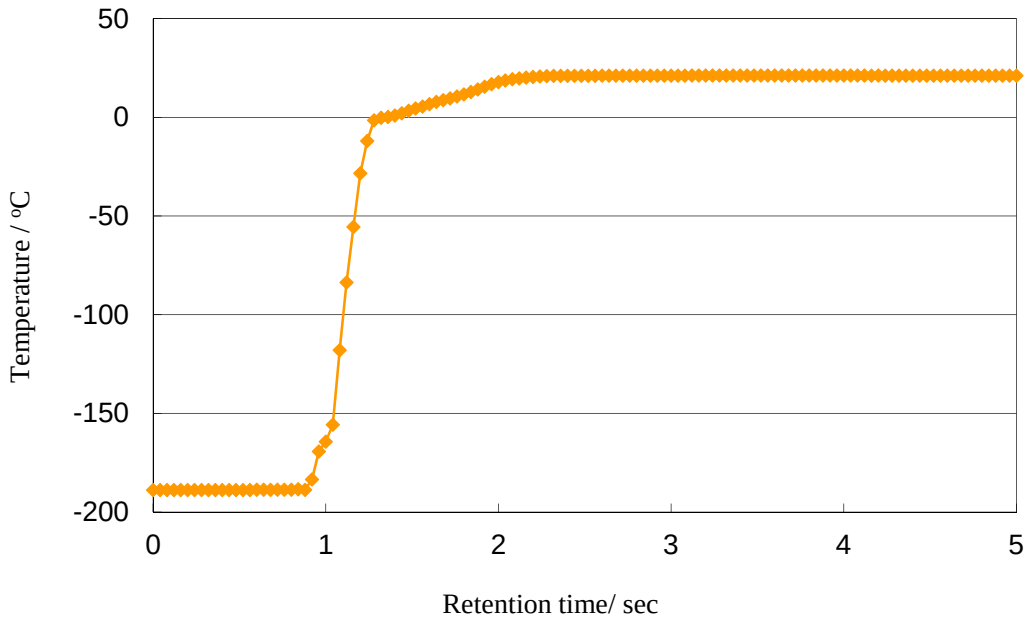

Fig. 4. Temperature change / retention time when CryotopCL is warmed according to the protocol for the closed vitrification system.

4. DISCUSSION

Freezing technology that enabled multiple oocytes and embryos to be vitrified and cryopreserved provided patients with the chance of repeated attempts to conceive without Hyperstimulation. Open system vitrification is likely to be the most well adopted vitrification technique worldwide with higher survival rate compared to slow freezing, and also for more simple protocol compared to closed system.

Conversely, the closed system is a safe vitrification technique with the least risk of cross-contamination. In addition to the requirements of the Food and Drug Administration in the USA, there are some European countries that require the use of a closed system for the cryopreservation of tissues. For this reason, the closed system has become an essential method of cryopreservation.

For a closed system to be practical, it is desirable that the protocol for the system is easy and the equipment for vitrification is reasonable and not too big for a

laboratory. In addition, its efficacy has to be evaluated.

In the present study, the survival, development, implantation, and pregnancy rates were similar between the closed and open systems.

Commercially available systems include CryoTip [28, 29], HSV straw [28, 30], Cryopette [31], and Rapid-i [32]. A report indicated that CryoTip was slightly more vulnerable to technical difficulties during recovery than the other two devices examined [28]. It suggested that any theoretically safe closed system should have an easy protocol that can be performed by any embryologist and provide good stable results.

There are three points in the vitrification procedure where closed CryotopCL and open Cryotop differ. First, the straw cap is cut open in the closed system. Second, CryotopCL with samples is slid into the straw cap from the open-cut part. Third, the CryotopCL straw cap is sealed at its upper part with a sealer. All three procedures are very easy and do not require multiple experience to perform them correctly. For

warming with CryotopCL, the straw cap is cut while partially suspended in liquid nitrogen and there is no contact of the sample with liquid nitrogen, then the CryotopCL in the straw cap is immersed directly into a thawing solution. CryotopCL is placed vertically within an aluminum block holder for ease of handling, so anyone can warm CryotopCL easily without special training.

During vitrification and cryopreservation, a high cooling rate and high warming rate are necessary for a high survival rate in order to vitrify and thaw without ice crystallization of cells. The cooling rate of for CryotopCL was approximately -3,000°C/min. In comparison, the cooling rate of HSV straw is reportedly -1,200°C/min, while that of Rapid-I is -1,300°C/min [28]. This indicates that CryotopCL has the best cooling rate compared to the other approaches. Conversely, the cooling rate for the open system using Cryotop is -23,000°C/min.

A concern with a closed system is that the cells are not cooled down immediately, but gradually, with a low cooling rate. It is necessary to develop an

environment in which the cells are vitrified immediately. The straw cap of CryotopCL is pre-cooled to keep the temperature inside under -190°C. Two stainless SUS304 wires are embedded in the edges of the CryotopCL strip (Fig. 1). Contact of the strip wires with the metal on the bottom part of the straw cap facilitates efficient heat transfer to the strip. This mechanism of the strip (patent pending) allows CryotopCL to realize a higher cooling rate than other closed system devices. CryotopCL with a sample is transferred from room temperature into the cooled straw cap. The samples are vitrified immediately with a cooling rate of -3,000°C/min, which is a sufficient enough temperature inside the straw to vitrify the cells.

For samples to be vitrified immediately, minimization of the volume of solution in the device is also an important point. The material of the strip used for CryotopCL and Cryotop is the same and has the same features, and the volume of the vitrification solution is minimized with the same procedure. Specifically, samples are vitrified in 0.1 μL vitrification solution. With regard to minimization of medium volume, both

the closed and open systems use the same volume of medium for vitrification.

A slow warming rate results in the development of small intracellular ice crystals by recrystallization, which is lethal to cells. To warm cells with closed system CryotopCL, the straw cap is immersed in liquid nitrogen, but the samples are transferred to a thawing solution at 37°C without direct contact with liquid nitrogen. Whereas with open Cryotop, the samples are in direct contact with liquid nitrogen before being transferred to a thawing solution. The warming rate with closed CryotopCL was 40,000°C/min, while it was 42,000°C/min with open Cryotop. As the similar survival and embryo development rates in both the closed and open systems prove, the samples did not undergo intracellular ice crystallization during the warming process in either system and their viability and embryo development capability were kept.

Clinical comparisons can only be evaluated *in vivo* by transferring vitrified-warmed embryos into the uterus and examining their progress. In the present study,

vitrified-warmed blastocysts were transferred to patients by single-embryo transfer in two groups separated for the closed and open systems. Clinical pregnancy, implantation rate, and ongoing pregnancy rate up to a maximum of 10 weeks were checked, and no significant difference was confirmed. Although a continuous study until live birth will be required, the efficacy of closed system vitrification using CryotopCL was confirmed at the clinical level.

This study indicates that the closed system with CryotopCL can be an alternative to open vitrification systems for both mouse and human oocytes and embryo cryopreservation. There was no significant difference between the open system with Cryotop and the closed system with CryotopCL for both laboratory and clinical outcomes: survival, embryo development, implantation rate, and ongoing pregnancy rate. As a cryopreservation system with a very low risk of cross contamination, the closed system with CryotopCL can be considered to have an important role in the preservation of fertility.

5. ACKNOWLEDGEMENTS

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6. CONFLICTS OF INTEREST

One of the authors of this paper belongs to Kitazato BioPharma Co., Ltd. which is the manufacturer of the two systems, CryotopCL and Cryotop used in the study.

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ETHICAL CONSIDERATIONS

All animal experiments were conducted following Animal Research Guidelines of Kitazato BioPharma Co., Ltd.

Clinical trials at Life IVF Center was performed after approval by ethics committee of this facility. In addition, all donors and patients has received and signed informed consents before their participation in the clinical trial CryotopCL used for this research purpose is FDA approved device which are allowed for human use (USA).