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Usefulness of vitrification device having vitrification solution absorber for cryopreservation of mouse embryos at the blastocyst stage

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Abstract

Vitrification is widely used for cryopreservation of embryos and oocytes in applications of assisted reproductive technology globally. In this study, we evaluated the efficacy of the Kitasato Vitrification System (KVS) as a device for the cryopreservation of mouse embryos and made comparisons with the widely used Cryotop[®] vitrification device. In Experiment 1, the blastocyst survival rates were significantly higher after vitrification using the KVS than after using Cryotop[®] devices. In Experiment 2, the cooling and warming rates of Cryotop[®] increased with decreasing vitrification solution volumes around the embryo and were 336,000°C/min and 320,000°C/min at best, respectively. However, the cooling and warming rates of the KVS were 683,000°C/min and 612,000°C/min, respectively, exceeding those of Cryotop[®].

Key Words: Vitrification, KVS, Embryos, Cryotop, Cryopreservation

Vitrification is a widely used method for the cryopreservation of embryos and oocytes and has replaced the slow-freezing method that was used in assisted reproductive technology (ART). Several vitrification methods based on ultrarapid cooling have been developed for the cryopreservation of animal embryos^{2-4,6,7,10,12,13)}. In order to optimize these methods, minimizing the volumes of extracellular vitrification solutions is central to achieving faster cooling rates. Recently, we developed the Kitasato Vitrification System (KVS) as a novel open vitrification system, which is composed of polyethylene terephthalate film

and a porous membrane as vitrification solution absorber, for the cryopreservation of mouse embryos⁹⁾. In the KVS, absorption of excess vitrification solution around the embryos achieves more rapid cooling and warming. On the other hand, Cryotop[®] (Kitazato BioPharma, Shizuoka, Japan)²⁾ is the most popular vitrification container for human ART in Japan. We have frequently used Cryotop[®] to vitrify human embryos in clinical practices. It is recommended to remove excess vitrification solution for achieving good results of vitrification by using Cryotop[®]; however, the volume remaining around the embryos

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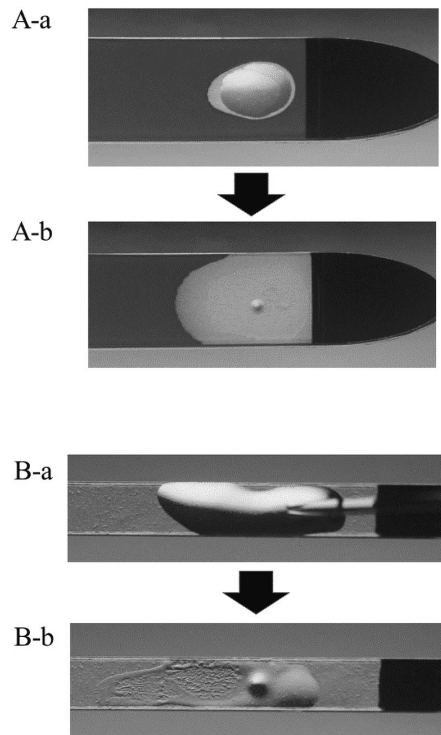


Fig. 1 Embryo vitrification procedures using the Kitasato Vitrification System (KVS) (A) and Cryotop® devices (B). A: Embryos are placed on the vitrification absorber with small volumes ($\leq 0.4 \mu\text{l}$) of vitrification solution added dropwise (a). Subsequently, the vitrification solution surrounding the embryo is absorbed using an absorber within less than 5–6 sec, and the embryo is easily observed with a small remaining volume of vitrification solution (b). B: Embryos are placed on Cryotop® sheets with small volumes ($\leq 1 \mu\text{l}$) of vitrification solution added dropwise (a). Excess solution surrounding the embryo is then removed by pipetting within 10–30 sec (b).

may be different from the practitioners. In our previous report⁹⁾, we set the volume of remaining vitrification solution on the control device as approximately $0.4 \mu\text{l}$. However, the volume of vitrification solution on the Cryotop® device may be less than $0.4 \mu\text{l}$ when a skillful practitioner removes the excess vitrification solution. The objective of this study was to compare the efficacy of the KVS and Cryotop® devices used by different practitioners. We also compared the cooling and warming rates during vitrification procedures between the two vitrification systems by analyzing the thermal transfer using a numerical simulation⁹⁾. These analyses will clarify the relationship between the cooling and

warming rates during vitrification procedures and cryopreservation efficacy of the KVS and Cryotop® devices.

Jcl:ICR mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animals were kept under a 12h light/12h dark cycle (lights on from 07:00 to 19:00) at a constant temperature of $22 \pm 1^\circ\text{C}$ with free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Kitasato University.

Superovulation was induced in female ICR mice (8–16-week old) via intraperitoneal injections of 10 IU of equine chorionic gonadotropin (ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) and 10 IU of human chorionic gonadotropin (hCG) (ASKA Pharmaceutical Co., Ltd.), which were administered 46–48 h apart. Females were paired with males of the same strain immediately after the injection of hCG and were checked for mating the next morning. Embryos were collected from uterine horns at the blastocyst stage (87 h after hCG injection).

Vitrification was performed by several practitioners. The solutions used for vitrification (VT507) and thawing (VT508) were purchased from Kitasato Biopharma. Embryos at the blastocyst stage were equilibrated in an equilibration solution for 10 min at room temperature. The embryos were then transferred to a vitrification solution for 1 min up to less than 2 min and were vitrified using the KVS or a Cryotop® device. Vitrification with the KVS was conducted according to a previous report⁹⁾. Briefly, after equilibration, single embryos with small volumes of vitrification solution (approximately $0.4 \mu\text{l}$) were placed on the KVS (Fig. 1-A-a). Excess vitrification solution covering the embryo was absorbed spontaneously by the absorber on the KVS (Fig. 1-A-b), and the device with the embryo was plunged into liquid nitrogen (LN_2). Thereafter, the device was inserted into a protective straw cap in LN_2 . In vitrification procedures using Cryotop® devices, embryos with small volumes of vitrification solution ($< 1 \mu\text{l}$)

were placed on Cryotop[®] sheets (Fig. 1-B-a) and excess solution around the embryos was promptly removed by pipetting within 10–30 sec (Fig. 1-B-b). Subsequently, the Cryotop[®] sheets were plunged into LN₂. Cryodevices containing single embryos were stored in LN₂ for 0–9 days.

After cryostorage, the KVS with the vitrified embryo was transferred to a thawing solution. After warming at 37°C for 1 min, the embryo was transferred to a diluent solution for 3 min at 37°C and then to a washing solution for 5 min at 37°C. Embryos in Cryotop[®] devices were warmed and diluted using the same procedure. Subsequently, all embryos were washed and cultured in a culture medium (SAGE-1 step medium; Origio, Tokyo, Japan) for 72 h under a humidified atmosphere containing 5% CO₂, 5% O₂, and 90% N₂ at 37°C. Vitrified and warmed embryos were cultured for 24 h, and embryos that formed blastocoels were considered viable. Development to the hatching stage was examined at 24, 48, and 72 h after culture. The percentages of embryo survival, blastocyst development, and blastocyst hatching were compared between the vitrification groups using Chi-square test.

In order to compare the cooling and warming rates between the KVS and Cryotop[®] devices, we simulated thermal transfer as described previously⁹⁾. Briefly, three-dimensional models of embryos were supported by Cryotop[®] sheets of a polypropylene film, which had a width and a thickness of 0.7 and 0.1 mm, respectively¹⁾. Because vitrification solution volumes depend greatly on the handling skill of a practitioner, who minimizes the cooling volumes, we estimated warming and cooling times with vitrification solution volumes around the embryo of 4.2 nl (Model 1), 10 nl (Model 2), or 90 nl (Model 3) based on microscope images. The material properties for numerical simulations are presented in Table 1. As in the previous report⁹⁾, we simulated the cooling and warming rates for Cryotop[®] devices using thermo-fluid analyses with an ANSYS Fluent (Version 14.5; ANSYS Inc., Canonsburg, PA, USA) instrument and made comparisons with

Table 1. Properties of the materials in numerical simulations.

Material	Density	Heat capacity	Thermal conductivity
	(kg/m ³)	(J/kg·K)	(W/m·K)
Polypropylene	905	1,930	0.13
Vitrification solution*	1,255	1,657	0.20
Warming solution*	1,581	1,255	0.28
Embryo**	998	4,182	0.60

* The properties of vitrification and warming solutions were sourced from a report by Tarakanov *et al.*¹¹⁾.

** The thermal properties of embryos matched those of water at 20°C.

previously reported cooling and warming rates for the KVS device.

As presented in Table 2, the survival rates of blastocysts were significantly higher after vitrification using the KVS than after vitrification using Cryotop[®] (100% versus 81.1%, $P<0.01$). Moreover, the embryo-hatching rates after 72 h culture were significantly higher following the use of the KVS compared to after using Cryotop[®] devices (96.7% versus 65.4%, $P<0.01$). In addition, the range of the survival rates of Cryotop[®] for each practitioner was 66.7–100% (A: 91.2% (31/34), B: 66.7% (6/9), C: 70.0% (7/10), and D: 100% (2/2), respectively), although the survival rates of the KVS were 100% in each practitioner.

The vitrification dynamics of two devices were estimated in thermal-transfer-based simulations using numerical modeling (Fig. 2). In three Cryotop[®] models with different vitrification solution volumes, embryo temperatures decreased faster with smaller solution volumes. In comparison, the embryo temperatures dropped much faster on the KVS model, as reported in our previous study⁹⁾, than in all of the above Cryotop[®] simulations. The thermal distribution analyses of Cryotop[®] (Model 2) devices during vitrification are shown in Figs. 2-B and 2-C. These data show that slight thermal energy remained in the embryo on the Cryotop[®] sheet, which was sufficiently cooled by 0.03 sec after LN₂ immersion (Fig. 2-C). The temperature dynamics during the warming procedure are shown in Fig. 3. In the comparisons of the three Cryotop[®] models, embryos with

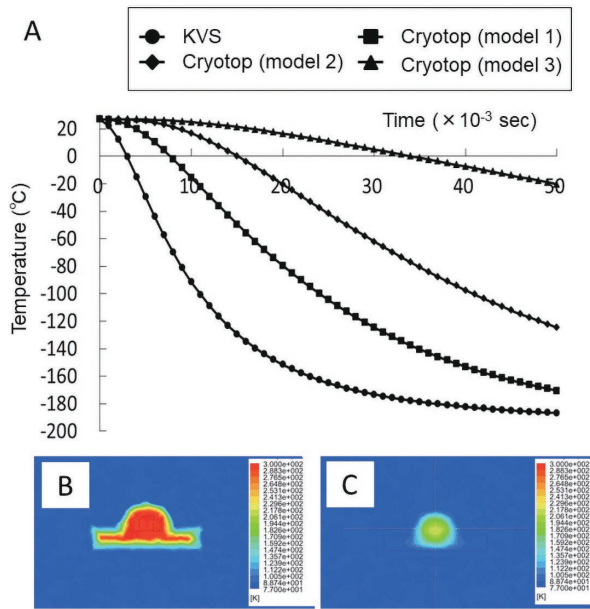


Fig. 2 Vittrification dynamics of the KVS and Cryotop® devices.

A: The embryo temperatures during the vittrification procedures were calculated using thermal-transfer-based simulation. The temperature of the center of the embryo during the vittrification procedure was plotted using the simulations of KVS and three Cryotop® conditions. The data for the KVS are from our previous study⁹⁾. B, C: Thermal distribution of a wide cross section of embryos on the Cryotop® (Model 2) sheet in the vittrification procedure; the thermal distributions immediately after immersion in liquid nitrogen are shown in B and those at 0.03sec after immersion are shown in C.

smaller volumes of vittrification solution warmed faster. In embryos that were vittrified using the KVS model⁹⁾, the temperature rose quickly after immersion in the warming solution (Fig. 3). As shown in Table 3, both cooling and warming rates were more rapid when using the KVS than when using Cryotop® devices.

These results suggest that during vittrification with the KVS, absorption of excess vittrification solution around the embryo achieved more rapid cooling and warming rates⁹⁾, resulting in higher viability and hatching rate. In contrast, Ling *et al.*⁵⁾ reported a 96.8% survival rate of Cryotop® vittrified-warmed mouse blastocysts. In the present study, practitioner A and D showed the highest survival rates (91.2% and 100%, respectively) of Cryotop® and was similar to

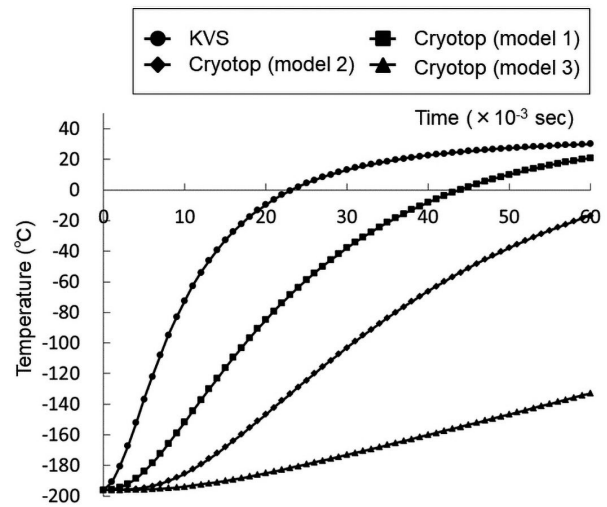


Fig. 3 The embryo temperatures during the warming procedure were calculated using a thermal-transfer-based simulation method.

The temperatures at the centers of the embryos during the warming procedures are plotted for the KVS and for three Cryotop® models. The data for the KVS are from our previous study⁹⁾.

the previous report⁵⁾, although all practitioners showed good survival rate (100%) of the KVS. It means that the procedure for the absorption of vittrification solution in the KVS is easy and simple for practitioners and offers stability of outcomes. These advantages will be helpful for embryologists to minimize the volumes of vittrification solution during vittrification.

Although it is difficult to confirm the exact cause of the lower survival rates after Cryotop® vittrification in this study, several factors likely contribute to the differences between the efficacy of KVS and that of Cryotop® devices. First, the different survival rates in the present vittrification system may be related to the different cooling and warming rates, primarily due to the differences in the volume of the vittrification solution. We observed smaller volumes of vittrification solution around the embryo in KVS procedures compared to Cryotop® procedures (Fig. 3). In addition, the cooling and warming rates between these devices differed in thermal-transfer-based simulation analyses using numerical modeling. Specifically, the KVS device achieved a vittrification solution

Table 2. Embryonic development of vitrified–warmed mouse blastocysts after in vitro culture for 72 h.

Type of device	No. of embryos examined	No. of embryos surviving (%)	No. of hatching embryos (%)		
			24 h	48 h	72 h
Cryotop (control)	55	45 (81.1 ^a)	25 (45.5 ^c)	35 (63.6 ^a)	36 (65.4 ^a)
KVS	61	61 (100 ^b)	42 (68.9 ^d)	58 (95.1 ^b)	59 (96.7 ^b)

^{a,b,c,d}Values in the same column with different superscripts are significantly different; $P < 0.01$ for “a” versus “b” and $P < 0.05$ for “c” versus “d”.

KVS: Kitasato Vitrification System.

Table 3. The cooling and warming rates with the KVS and Cryotop® devices were calculated using a thermal-transfer-based simulation method.

Device	Cooling rate (°C/min)	Warming rate (°C/min)
KVS	683,000 ^a	612,000 ^a
Cryotop® (Model 1 [*])	336,000	320,000
Cryotop® (Model 2 ^{**})	215,000	206,000
Cryotop® (Model 3 ^{***})	70,000	68,000

KVS: Kitasato Vitrification System.

^aData from our previous study⁹.

In Models 1, 2, and 3, the volumes of the vitrification solution surrounding the embryo were 4.2^{*}, 10^{**}, and 90^{***} nl respectively.

volume of 1.3 nl and cooling and warming rates of 683,000°C/min and 612,000°C/min, respectively⁹. These rates were extremely high compared with those in all Cryotop® models. In comparison, Mazur and Seki⁸ performed direct thermoelectric coupling measurements of cooling and warming rates for Cryotop® procedures and reported respective rates of 69,000°C/min and 118,000°C/min with a vitrification solution volume of 100 nl. In this study, the cooling and warming rates for Cryotop® procedures (Model 3) with a vitrification solution volume around the embryo of 90 nl were 70,000°C/min and 68,000°C/min, respectively, and were similar to those from direct measurements. In the present study, we estimated that, under optimal Cryotop® conditions (4.2 nl of vitrification solution), cooling and warming rates of 336,000°C/min and 320,000°C/min, respectively, could be achieved. Our simulation analyses also indicated that decreasing the volume of the vitrification solution around the embryo is critical for increasing the cooling and warming rates⁹.

Second, treatments in vitrification solution damage embryonic cells through chemical toxicity of the cryoprotectant, and this damage would be greater after slightly longer treatment times in Cryotop® procedures. In our clinical practice, human embryos were exposed to vitrification solutions for at least 1 min (herein for 1 min to less than 2 min). In addition, in Cryotop® procedures, excess vitrification solution was removed by pipetting, resulting in an additional 10–30 sec handling time depending on practitioner. Longer treatment duration may injure embryos in the present study. The treatment time in vitrification solution was 30 sec in our previous report⁹, shorter than that in this study, and the viability of embryos was 93.4% when we used 0.4 µl of vitrification solution on Cryotop-like device. Further, in a previous study⁵ in which mouse blastocysts were vitrified using Cryotop®, embryos were exposed to a vitrification solution within <60 sec; however, excess vitrification solution after placing onto the sheet was not removed. Therefore, the handling time may be important rather than minimizing solution volume around embryo during the vitrification of mouse blastocysts using Cryotop®, when the volume of vitrification solution is less than 0.4 µl.

In conclusion, this study demonstrated that the KVS minimizes the volume of vitrification solution around the embryo, resulting in ultrarapid cooling and warming regardless of practitioners' skill, and gave a superior outcome for the cryopreservation of mouse embryos. Further clinical examinations of the effectiveness of the KVS with human embryos are warranted.

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