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Preclinical validation of the new vitrification device possessing a feature of absorbing excess vitrification solution for the cryopreservation of human embryos

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Abstract

Aim: The cryopreservation of embryos is essential for assisted reproductive technology field. The aim of the present study is to examine the efficacy and ease of use of a new vitrification device, Kitasato Vitrification System (KVS), in cryopreservation of human embryos.

Methods: Human embryos at the cleavage or blastocyst stage were vitrified and warmed by KVS or Cryotop (control device). The survival of cleavage- and blastocyst-stage embryos and the developmental competence of cleavage-stage embryos were evaluated. Four individuals inexperienced in vitrification and warming embryos tested both KVS and Cryotop. The vitrification time and the detachment time of the embryos were evaluated.

Results: At the cleavage stage, there were no significant differences in the survival rate and the development rate to the blastocyst stage between KVS and Cryotop (100 vs 96.8% and 63.3 vs 61.3%, respectively). At the blastocyst stage, there was no significant difference in the re-expansion rate between KVS and Cryotop (100 vs 88.9%). The vitrification time was shorter for KVS than Cryotop. There was no significant difference in the detachment time between KVS and Cryotop.

Conclusion: Kitasato Vitrification System is easy to operate, even for inexperienced users, and the viability of human embryos vitrified by KVS is comparable to that of Cryotop, a widely used vitrification device.

Key words: assisted reproductive technology, cryopreservation, infertility, Kitasato Vitrification System (KVS), vitrification.

Introduction

Successful embryo cryopreservation is critical for assisted reproductive technology (ART). Patients undergoing *in vitro* fertilization (IVF) can take advantage of vitrification, the process of cryopreserving embryos by placing them in a high concentration of cryoprotectant, followed by ultra-rapid cooling in liquid nitrogen, which prevents ice crystal formation.[1] The ultra-rapid cooling method was introduced in 1996 by Martino *et al*.[2] and led to the development of various cryodevices such as the open pulled straw,[3] Cryoloop[4,5] and Cryotop.[6] Vitrification is superior to slow-freezing as it improves embryo survival and clinical outcomes.[7,8] Furthermore, minimizing the volume of vitrification solution during rapid cooling is critical for embryo viability, since rapid cooling prevents cryodamage. However, it is difficult to control

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the volume of the vitrification solution and to work with small volumes.

Recently, a new vitrification device, Kitasato Vitrification System (KVS) (Mitsubishi Paper Mills Limited), has been developed. The innovative feature of KVS is a vitrification solution absorber consisting of a porous membrane, which absorbs excess vitrification solution surrounding the embryo and helps embryologists minimize the volume of the solution.[9] Momozawa *et al*. reported the efficacy of KVS for vitrification of mouse embryos.[9] The cooling and warming rates of the KVS (683 000 and 612 000°C/min, respectively) exceed those of non-absorbing vitrification devices (26 000 and 25 000°C/min, respectively). In addition, embryos can be observed easily under a stereomicroscope after placement on the porous membrane. Further, the viability and developmental competence of mouse embryos vitrified using the KVS were comparable to or surpassed those of mouse embryos vitrified using control vitrification devices that do not absorb the excess vitrification solution.[9] However, the efficacy of this device for the vitrification of human embryos are still unknown. In this study, we examined the efficacy of KVS in the vitrification of human embryos, compared with a control vitrification device, Cryotop. To determine the ease of handling embryos, we also compared the vitrification and detachment time between KVS and Cryotop.

Methods

Embryos

The study protocol was approved by the review board of the University of Tokyo (approved number, 10830), and signed informed consent was obtained from each patient. Experiments 1 and 2 examined the viability of embryos vitrified-warmed either by KVS or by the control device Cryotop (Kitazato Co.). Embryos were vitrified at our hospital for future transfer between March 2005 and December 2010, but in May 2015, the patients that provided these embryos did not want to store them any longer. Thus, 61 embryos at the cleavage stage and 54 embryos at the blastocyst stage obtained from 45 patients (median age, 35 years; range, 29–42 years) were used in these experiments. Experiments 3 and 4 examined the ease of handling of the devices. Embryos that were degenerated and non-transferable and obtained during ART practice between May 2015 and December 2016 were used.

The IVF procedure for obtaining embryos was as follows.[10] Each patient received controlled ovarian stimulation with clomiphene citrate (Clomid; Fuji Pharma Co.) or a daily injection of hMG (HMG TEIZO or Gonapure; ASKA Pharmaceutical Co.) and a GnRH agonist (Nasanyl; Pfizer Japan) or antagonist (Ganirest; MSD K.K.). When the leading follicle reached a diameter of 18–20 mm, ovulation was induced with a single injection of hCG (10 000 IU; HCG Mochida; Mochida Pharmaceutical Co.). Oocytes were retrieved at 34 h after hCG administration. After oocyte retrieval, cumulus-enclosed oocytes were cultured in medium (Global; LifeGlobal) containing 10% human serum albumin (InVitroCare Inc.) for insemination. Fertilization was confirmed at 16–18 h after insemination by the presence of two pronuclei. Embryos were cultured in medium under conditions of 6.5% CO_2, 5% O_2, and 88.5% N_2 at 37°C. Embryos were observed on day 3 or 5.

Vitrification device

Kitasato Vitrification System and Cryotop, as a control, were used as vitrification devices in this study (Fig. 1a). The innovative feature of KVS is a vitrification solution absorber consisting of a porous membrane that is placed on the polyethylene terephthalate film.[9] A schema of the embryo vitrification procedures using each vitrification device is shown in Figure 1b. After the embryo was placed on KVS, the excess solution was absorbed spontaneously by the porous membrane. By contrast, the excess solution on KVS was removed by pipetting. The resulting volume of vitrification solution surrounding the embryo was estimated, based on microscopic images, as 1.3 nL for KVS and 30–50 nL for Cryotop, as previously reported.[9]

Vitrification and warming protocol

The vitrification and warming of embryos were conducted utilizing the Vitrification Media Kit (Kitazato Co.) containing hydroxypropyl cellulose as a cryoprotectant. Vitrification and warming protocols were as follows. Briefly, embryos at the cleavage or blastocyst stage were equilibrated in the equilibration solution for 15 min at room temperature, and then transferred to the vitrification solution for 50 s. One embryo was placed on each of the vitrification devices, and the surrounding solution was minimized either by pipetting for Cryotop or by spontaneous absorption for KVS. Then, the device was submerged into liquid nitrogen and covered with a

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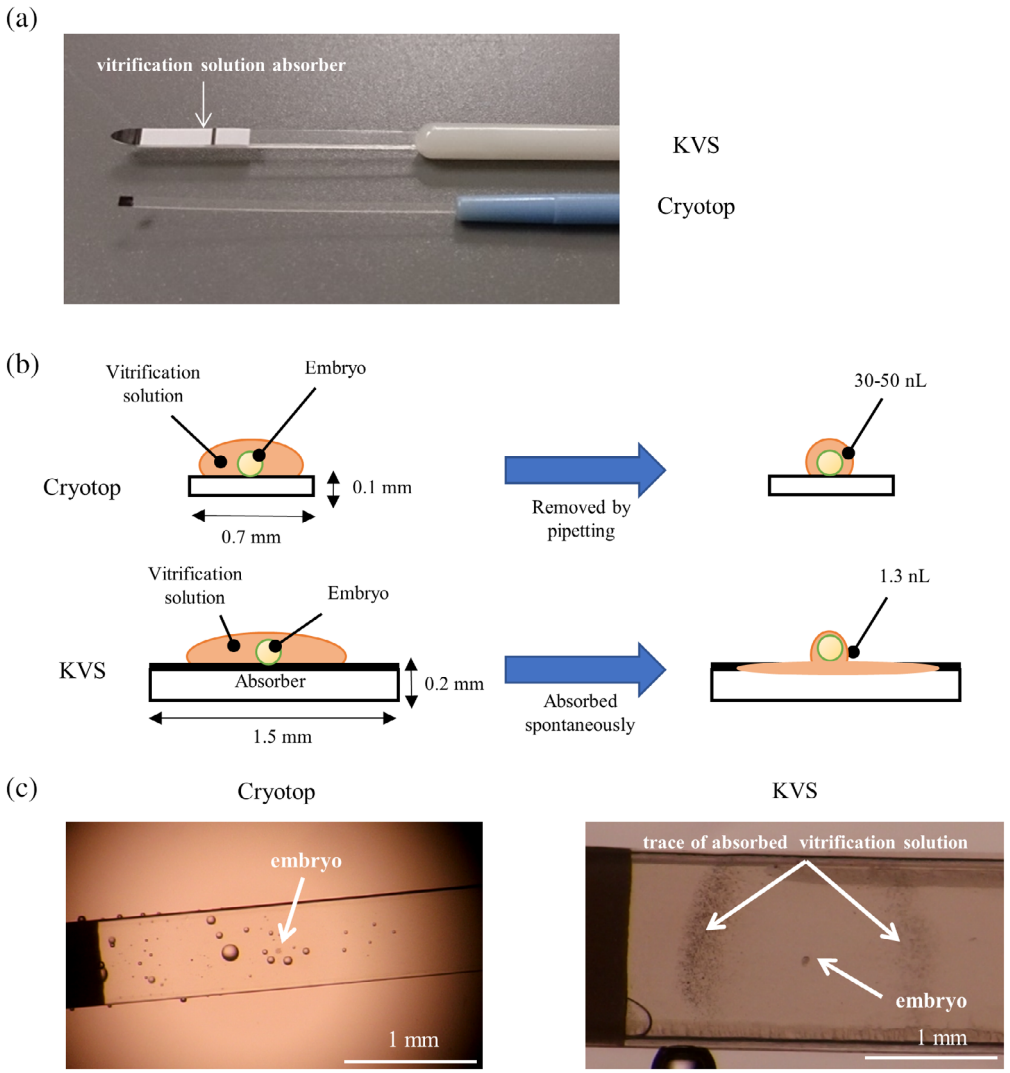

Figure 1 Kitasato Vitrification System (KVS) for embryo vitrification. (a) The device, KVS and Cryotop (Control). KVS is equipped with a vitrification solution absorber, consisting of a porous membrane that is placed on the polyethylene terephthalate film. (b) Embryo vitrification procedures using KVS and Cryotop. The excess solution on Cryotop was removed by pipetting, whereas the excess solution on KVS was absorbed spontaneously by the porous membrane. The resulting volume of vitrification solution surrounding the embryo was estimated using microscopic images as 1.3 nL for KVS and 30–50 nL for Cryotop. (c) KVS and Cryotop with the vitrified embryo during warming. In KVS, a trace of absorbed vitrification solution surrounding the embryo was observed. Scale bars, 1 mm

protective straw-cap. On warming the vitrified embryos, the protective straw-cap was removed from the device, and the device with the vitrified embryo was transferred to the warming solution for 1 min at 37°C. Figure 1c shows vitrified embryos on KVS and Cryotop. Subsequently, the embryos were transferred to the dilution solution for 3 min, rinsed in washing solution for 10 min at room temperature, and cultured in medium for 3 h.

Experimental design

Experiment 1: Survival and development of vitrified-warmed human embryos at the cleavage stage

Embryos vitrified at the cleavage stage were warmed. After 2 h of culture, the viability of embryos was assessed. Embryos displaying greater than or equal to six blastomeres and greater than or equal to Grade III (Veeck criteria) were included in this study. Of

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61 embryos, 30 were vitrified and warmed by KVS, whereas the remaining 31 embryos were vitrified and warmed by Cryotop and served as controls. Embryos from each patient were allocated randomly to each group. After warming, the viability of the embryos was evaluated. Embryos displaying fewer than two degenerated blastomeres were considered viable. After 48 h of culture, the rate of development to blastocysts of Grade ≥ 3 (Gardner criteria) was evaluated. Blastocysts graded with at least one A for the inner cell mass or trophectoderm (Gardner criteria), namely, AA, AB and BA blastocysts, were considered good blastocysts.

Experiment 2: Survival of vitrified-warmed human embryos at the blastocyst stage

Embryos vitrified at the blastocyst stage were warmed. After 2 h of culture, the viability of embryos was assessed. Embryos displaying greater than or equal to Grade 3 (Gardner criteria) were included in this study. Blastocysts graded with at least one A for the inner cell mass or trophectoderm (Gardner criteria), namely, AA, AB and BA blastocysts, were considered good blastocysts. Of 54 embryos, 27 were vitrified and warmed by KVS, whereas the remaining 27 embryos were vitrified and warmed by Cryotop and served as controls. Embryos from each patient were allocated randomly to each group. After 2 h of warming, the rate of development to the re-expansion stage was evaluated.

Experiment 3: Comparison of the vitrification time between KVS and Cryotop

Degenerated and nontransferable embryos were used for Experiment 3. Four individuals (three medical doctors and one embryologist) inexperienced with embryo vitrification and warming techniques were recruited. These investigators transferred embryos to the device with a pipet, minimized the solution surrounding the embryo, either by pipetting for Cryotop or by spontaneous absorption for KVS, and plunged the device into liquid nitrogen. Subjects performed these steps 20 times by alternating between KVS and Cryotop, and the total time of each trial was recorded.

Experiment 4: Comparison of the detachment time between KVS and Cryotop

Degenerated and nontransferable embryos were used for Experiment 4. Four individuals (three medical doctors and one embryologist) inexperienced with embryo vitrification and warming techniques were recruited. In advance, well-experienced embryologists vitrified embryos on KVS and Cryotop and stored the embryos in liquid nitrogen. The inexperienced investigators transferred the device with the vitrified embryo to the warming solution and kept the device stationary until the embryos detached completely from the device. Although we usually move devices to facilitate the detachment of embryos during daily practice, in the current experiment, the investigators were instructed to keep the device still to exclude the difference between individuals, as well as the difference between trials. Subjects performed these steps 20 times by alternating between KVS and Cryotop, and the detachment time of each trial was recorded.

Statistical analysis

Statistical analysis was performed using JMP Pro 11 Software (SAS Institute Inc.). Fisher's exact test was used to compare parameters between KVS and Cryotop. A *P*-value of less than 0.05 was considered statistically significant, and all reported *P*-values were one-sided.

Results

Experiment 1: Survival and development of vitrified-warmed human embryos at the cleavage stage

As shown in Table 1, there was no significant difference between the Cryotop group and the KVS group in the quality of utilized embryos. Thirty of thirty-one cleavage-stage embryos that were vitrified-warmed by Cryotop survived after warming and recovery (Table 1). One embryo at the 8-cell stage showed two degenerated blastomeres and a broken zona pellucida after warming and recovery. All cleavage-stage embryos (n = 30) that were vitrified-warmed by KVS survived after warming and recovery, and 19 (63.3%) developed to the blastocyst stage with a rate of good-quality blastocysts at 31.6% (6/19) (Table 1). There were no significant differences in the survival rate, the development rate to the blastocyst stage, and the good-blastocyst rate between embryos vitrified-warmed by KVS and Cryotop.

Experiment 2: Survival of vitrified-thawed human embryos at the blastocyst stage

The viability of vitrified-warmed human embryos at the blastocyst stage was examined. As shown in Table 2, there was no significant difference between the KVS group and the Cryotop group in the quality of utilized

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Table 1 Survival and development of vitrified-warmed human embryos at the cleavage stage

	Cryotop (n = 31)	KVS (n = 30)	P-value
Before vitrification			
Stage			
7 cell	3	4	0.8487
8 cell	8	8	
9 cell	4	3	
>10 cell	14	10	
Morula	2	5	
Grade			
1	9	8	0.5268
2	20	17	
3	2	5	
After warming			
Number of embryos survived (%)	30 (96.8)	30 (100)	0.5082
Number of embryos developed to blastocysts with Grade ≥3 (%)	19 (61.3)	19 (63.3)	0.5397
% of good blastocysts (AA, AB or BA)	4/19 (21.0)	6/19 (31.6)	0.3570

Veeck and Gardner criteria were used for grading of cleavage-stage and blastocyst-stage embryos, respectively. and KVS, Kitasato Vitrification System.

Table 2 Survival of vitrified-warmed human embryos at the blastocyst stage

	Cryotop (n = 27)	KVS (n = 27)	P-value
Before vitrification			
Stage			
3 (blastocyst)	6	9	0.7664
4 (expanded blastocyst)	20	17	
5 (hatching blastocyst)	1	1	
Grade			
AA	1	2	0.5689
AB	5	3	
BA	2	1	
BB	12	18	
BC	3	1	
CB	2	0	
CC	2	2	
% of good blastocysts (AA, AB or BA)	8/27 (29.6)	6/27 (22.2)	0.5444
After warming			
Number of re-expanded blastocysts (%)	24/27 (88.9)	27/27 (100)	0.1179

Gardner criteria was used for grading of blastocyst-stage embryos. and KVS, Kitasato Vitrification System.

blastocysts. All 27 blastocysts vitrified-warmed by KVS re-expanded after warming and recovery (Table 2). Out of 27 blastocysts vitrified-warmed by Cryotop, three blastocysts (4BA, 4BB, 4BC) did not re-expand after warming and recovery (Table 2). However, there was no significant difference in the re-expansion rate between embryos vitrified-warmed by KVS and Cryotop (100 vs 88.9%).

Experiment 3: Comparison of the vitrification time between KVS and Cryotop

To determine the ease of handling embryos, we examined the vitrification time of embryos reported by four inexperienced individuals using KVS or Cryotop as shown in Figure 2a. The vitrification time of embryos reported by all individuals decreased for both devices as the number of trials increased. The vitrification time of embryos vitrified by KVS tended to be shorter than that of embryos vitrified by Cryotop. Furthermore, both the average vitrification time of 16–20 trials and that of 1–20 trials were significantly shorter for KVS than for Cryotop (Fig. 2b).

Experiment 4: Comparison of the detachment time between KVS and Cryotop

We examined whether detachment of the vitrified embryos from KVS during warming is precluded by an efficient absorption of excess vitrification solution during vitrification, which is the innovative feature of KVS. We measured the time for the vitrified embryo to detach from the device. It is recommended to detach embryos in 1 min in daily practice; thus trials were categorized according to detachment time as greater than 1 min and less than 1 min. Furthermore, for trials with a detachment time less than 1 min, the average of detachment time was calculated. As shown in Figure 3a, out of 20 trials, the rate of trials with a detachment time greater than 1 min was 45% for KVS and 56.4% for Cryotop, without significant difference between the groups. Furthermore, there was no significant difference between KVS and Cryotop in the average detachment time among trials with a detachment time less than 1 min (27.3 ± 3.44 vs 20.7 ± 2.87 sec, mean ± SEM) (Fig. 3b).

Discussion

In the present study, we vitrified human embryos using KVS and Cryotop. There was no difference in

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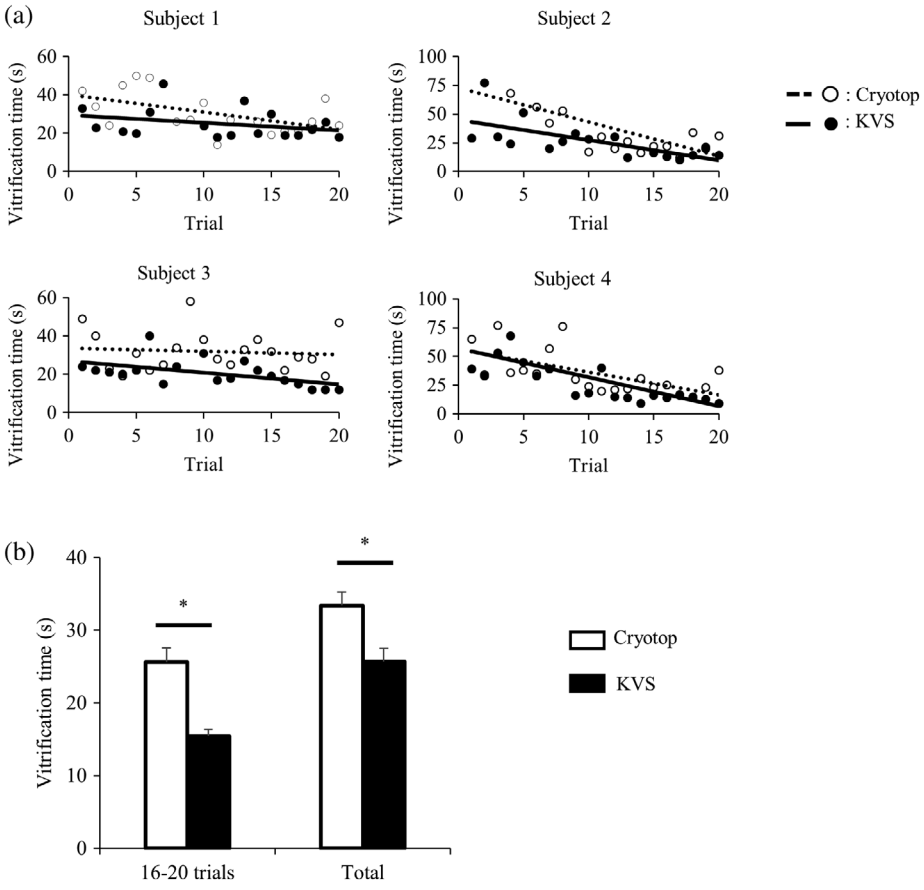

Figure 2 Vitrification time by Kitasato Vitrification System (KVS) and Cryotop as reported by four inexperienced investigators. The investigators transferred embryos to the device with a pipet, minimized the solution surrounding the embryo either by pipetting for Cryotop or by spontaneous absorption for KVS and plunged the device into liquid nitrogen. Subjects performed these steps 20 times by alternating between KVS and Cryotop, and the total time was recorded. (a) The vitrification time of each trial was plotted. (b) The average vitrification time of 16–20 trials and that of 1–20 trials of the four subjects is shown. The values represent mean ± SEM. *, $P < 0.05$.

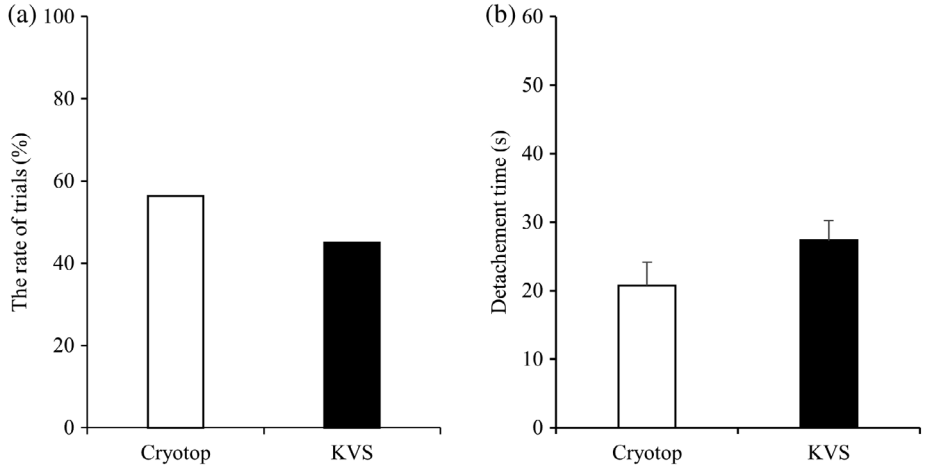

Figure 3 Detachment time by Kitasato Vitrification System (KVS) and Cryotop as reported by four inexperienced investigators. Four inexperienced investigators transferred the device with the vitrified embryo to warming solution and kept the device still until the embryos detached completely from the device. Subjects performed these steps 20 times by alternating between KVS and Cryotop, and the detachment time was recorded. (a) The rate of trials with detachment time greater than 1 min. (b) The average detachment time among trials with detachment time less than 1 min. The values represent mean ± SEM. *, $P < 0.05$

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viability and developmental competence between the cleavage-stage embryos vitrified-warmed by KVS or Cryotop. The survival of blastocyst-stage embryos after vitrification and warming also showed no difference between KVS and Cryotop. Furthermore, regarding the ease of handling embryos, the vitrification time was shorter for KVS than Cryotop, and there was no significant difference in the detachment time between both groups.

The cryopreservation and storage of *in vitro*-developed embryos is critical for ART. Vitrification is the most widely used approach for the cryopreservation of human embryos. To preserve embryos without affecting their viability, embryos should be vitrified in the presence of a high concentration of cryoprotectant, which prevents ice crystal formation.[11,12] Embryos are placed on the device with one of several microtools in a very small volume (0.1–2.0 μL) of vitrification solution.[13] Cryotop, which is the gold standard for the minimal volume vitrification approach, allows rapid vitrification and warming, thus promoting embryo survival.[6,14] Although Cryotop provides excellent clinical outcomes, many find it difficult to work with very small volumes of vitrification solution. KVS device, recently developed by Momozawa *et al.*, is equipped with a porous membrane that absorbs excess vitrification solution, thus facilitating rapid vitrification and warming.[9] The temperature of the embryo in KVS was sufficiently low at approximately 0.05 s after immersion in liquid nitrogen, whereas it took approximately 1 s for the embryo to cool down to the same temperature in Cryotop. It was reported that rapid vitrification and warming is associated with higher survival rate of mouse embryos, with a speculation that either the crystallization of intracellular glass during warming or the growth by recrystallization of small intracellular ice crystals formed during cooling are responsible for the lethality of slow warming.[15] The efficacy of KVS for the cryopreservation of mouse embryos has also been reported.[9] Blastocysts vitrified using KVS showed comparable survival rates to those vitrified using control vitrification devices, such as Cryotop, which do not absorb excess vitrification solution. The rates of embryo re-expansion and hatching were higher for KVS than for the control vitrification devices. There were no differences in the survival rate, blastocyst development rate, and offspring development rate between embryos vitrified with KVS and fresh 2-cell-stage embryos. In the present study, we examined the efficacy of KVS in cryopreserving human embryos. In Experiment 1, we examined the viability of human embryos at the cleavage stage and the development of embryos to blastocysts by KVS. Both of these parameters were comparable to those of Cryotop. In Experiment 2, we evaluated the re-expansion rate of human blastocysts by KVS, which was also comparable to that by Cryotop, although hatching rate was not evaluated in the present study. The present study did not show the superiority of KVS in re-expansion rate, although KVS achieves more rapid cooling and warming rates, with higher re-expansion rate, in mouse blastocysts.[9] One explanation might be a smaller sample size in the present study, 27 blastocysts for each group, compared to the mouse study with 61 for each. Alternatively, it may be attributed to the difference between species, humans and mice. These results indicate that the viability of human embryos vitrified by KVS is not inferior to that of human embryos vitrified by Cryotop. To introduce KVS in ART clinics, further studies are needed to evaluate the implantation and pregnancy rates of embryos vitrified by KVS.

The absorption of excess vitrification solution in KVS not only facilitates rapid cooling, but also simplifies the handling of embryos during vitrification. In this study, we examined the learning curve of inexperienced investigators, as the success of vitrification can vary between embryologists, as well as between ART clinics.[16] All investigators reported that the vitrification time was shorter for KVS than for Cryotop. The key to standardizing the procedure lies in the ability of the investigator to remove the excess solution within seconds for vitrification devices, and this largely depends on the investigator's skill. On the other hand, KVS is equipped with a porous membrane that absorbs the excess volume, thus facilitating the removal of the excess solution and the observation of embryos under a stereomicroscope. The volume control of surrounding solution is easy and stable for KVS, which does not depend on the investigators. In this study, the vitrification time was shorter for KVS than for Cryotop. Furthermore, the average vitrification time of 16–20 trials was shorter for KVS than for Cryotop (15.5 ± 0.94 vs 25.7 ± 1.81 s), indicating that the learning curve of KVS is short. Although we did not examine the vitrification time in experienced investigators, the viability of embryos vitrified by KVS, which was performed by experienced embryologists, was comparable to that of embryos vitrified by Cryotop, as shown in Experiments 1 and 2. Taken together, these results indicate that KVS is easy to operate during vitrification of embryos, both for inexperienced and experienced users.

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During warming embryos, all the investigators reported the ease of detecting vitrified embryos for KVS. They found that a brownish trace of absorbed vitrification solution surrounding the embryo, as shown in Figure 1c, helped locate the embryo on the device. This was an unexpected advantage of the absorber on KVS. We wondered whether detachment of the vitrified embryos from KVS during warming may be precluded by an efficient absorption of excess vitrification solution during vitrification. The measurement of the detachment time revealed that the rate of trials with a detachment time greater than 1 min and the average detachment time among trials with a detachment time less than 1 min were similar in KVS and Cryotop. The considerably high rate of trials with a detachment time greater than 1 min for both groups may be due to the protocol in the current study that did not allow investigators to move devices to facilitate the detachment of embryos, as is performed in daily practice. These results indicate that a spontaneous absorption of excess solution by KVS during vitrification does not affect the detachability of vitrified embryos.

In conclusion, KVS is easy to operate, even for inexperienced users, and the viability of human embryos vitrified by KVS is comparable to that of human embryos vitrified by a widely used vitrification device, Cryotop. KVS is applicable as a novel, user-friendly cryodevice for ART clinics, although further studies are needed to evaluate the clinical outcomes, pregnancy and live birth rates, of embryos vitrified by KVS.

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Disclosure

The authors declare that they have no competing interests.

References

1. Practice Committees of American Society for Reproductive Medicine; Society for Assisted Reproductive Technology. Mature oocyte cryopreservation: A guideline. *Fertil Steril* 2013; **99**: 37–43.
2. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biol Reprod* 1996; **54**: 1059–1069.
3. Vajta G, Holm P, Kuwayama M *et al*. Open Pulled Straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev* 1998; **51**: 53–58.
4. Lane M, Schoolcraft WB, Gardner DK. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril* 1999; **72**: 1073–1078.
5. Mukaida T, Nakamura S, Tomiyama T, Wada S, Kasai M, Takahashi K. Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. *Fertil Steril* 2001; **76**: 618–620.
6. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online* 2005; **11**: 300–308.
7. Rienzi L, Gracia C, Maggiulli R *et al*. Oocyte, embryo and blastocyst cryopreservation in ART: Systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update* 2017; **23**: 139–155.
8. Debrock S, Peeraer K, Fernandez Gallardo E, De Neubourg D, Spiessens C, D'Hooghe TM. Vitrification of cleavage stage day 3 embryos results in higher live birth rates than conventional slow freezing: A RCT. *Hum Reprod* 2015; **30**: 1820–1830.
9. Momozawa K, Matsuzawa A, Tokunaga Y *et al*. Efficient vitrification of mouse embryos using the Kitasato Vitrification System as a novel vitrification device. *Reprod Biol Endocrinol* 2017; **15**: 29.
10. Harada M, Takahashi N, Hirata T, Koga K, Fujii T, Osuga Y. Laparoscopic excision of ovarian endometrioma does not exert a qualitative effect on ovarian function: Insights from in vitro fertilization and single embryo transfer cycles. *J Assist Reprod Genet* 2015; **32**: 685–689.
11. Mazur P, Rall WF, Leibo SP. Kinetics of water loss and the likelihood of intracellular freezing in mouse ova. Influence of the method of calculating the temperature dependence of water permeability. *Cell Biophys* 1984; **6**: 197–213.
12. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at −196 degrees C by vitrification. *Nature* 1985; **313**: 573–575.
13. Edgar DH, Gook DA. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. *Hum Reprod Update* 2012; **18**: 536–554.
14. Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method. *Theriogenology* 2007; **67**: 73–80.
15. Mazur P, Seki S. Survival of mouse oocytes after being cooled in a vitrification solution to −196°C at 95° to 70,000°C/min and warmed at 610° to 118,000°C/min: A new paradigm for cryopreservation by vitrification. *Cryobiology* 2011; **62**: 1–7.
16. Gosden R. Cryopreservation: A cold look at technology for fertility preservation. *Fertil Steril* 2011; **96**: 264–268.

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