FULL PAPER *Theriogenology*

Intrauterine embryo transfer with canine embryos cryopreserved by the slow freezing and the Cryotop method

Tatsuya HORI[1)]*, Hitoshi USHIJIMA[2)], Taku KIMURA[1)], Masanori KOBAYASHI[1)], Eiichi KAWAKAMI[1)] and Toshihiko TSUTSUI[1,3)]

[1)]*Laboratory of Reproduction, Nippon Veterinary and Life Science University, 1–7–1 Kyonan-cho, Musashino-shi, Tokyo 180–8602, Japan*
[2)]*Laboratory of Animal Reproduction, Nippon Veterinary and Life Science University, 1–7–1 Kyonan-cho, Musashino-shi, Tokyo 180–8602, Japan*
[3)]*International Institute of Small Animal Medicine (Bio Plus), AHB Inc., 3–7–11 Kiba, Koutou-ku, Tokyo 135–0042, Japan*

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ABSTRACT. Canine embryos (8-cell to blastocyst stages) frozen-thawed using the slow-freezing method with glycerol (four recipients) or dimethyl sulfoxide (three recipients) as a cryoprotectant and vitrified-warmed using the Cryotop method (five recipients) were surgically transferred into the unilateral uterine horn of recipient bitches. As a result, the morphology of embryos frozen-thawed using the slow-freezing method was judged to be normal, but no conception occurred in any of the recipient bitches. Two of the five bitches that received transferred embryos (morula to early blastocyst stages) vitrified-warmed using the Cryotop method became pregnant and produced normal pups (1/9 embryos, 11.1% and 1/6 embryos, 17.0%). It was concluded that the Cryotop method was more appropriate for canine embryo cryopreservation than the slow-freezing method, which is used for the cryopreservation of embryos of other mammalian species.
KEY WORDS: cryopreservation, Cryotop method, dog, embryo, slow freezing method

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For the reproduction of assistance dogs, including guide dogs, greater importance is attached to the strain. The cryopreservation of canine embryos is necessary to manage and reproduce excellent gametes on a worldwide scale, and the establishment of techniques for this process is needed. Embryo-freezing techniques are also assumed to be useful for the preservation of gametes of rare dog breeds and valuable dog strains that serve as models for human disease as well as studies on the embryo preservation of endangered wild Canidae [3, 6]. However, although many mammalian species, such as cattle [23], pigs [15], cats [5, 7] and mice [22], have been obtained by the transfer of frozen–thawed embryos, studies on frozen canine embryos lag behind these other studies, and no pup had been obtained by frozen canine embryo transfer (ET) until Abe *et al.* reported it in 2011 [2]. Few studies on frozen canine embryos have been reported thereafter, and no cryopreservation techniques for canine embryos have been established.

The slow-freezing method has been used for mammalian embryo cryopreservation, in which embryos penetrated using a preservative solution containing a cryoprotectant (glycerol [Gly] or dimethyl sulfoxide [DMSO]) are packed in straws, slowly frozen in a programmed freezer and stored in liquid nitrogen (LN_2) [17]. The toxicity of cryoprotectants in embryos and the requirement of sufficient time for freezing are problems associated with this method, but the offspring of many animal species have been obtained by ET of frozen-thawed embryos prepared using this method [13, 17, 21]. However, there have been no reports concerning dogs, with the exception of one study in which embryos frozen–thawed using the slow-freezing method with Gly as a cryoprotectant were transferred but pregnancy was not achieved [10]. Since the slow-freezing method using other cryoprotectants has not been investigated, the usefulness of this freezing method for the cryopreservation of canine embryos remains unclear.

Another embryo cryopreservation method termed the vitrification method has beem developed. In this method, a smaller amount of a cryoprotectant is used at a higher concentration than that in the abovementioned method, followed by the placement of embryos in LN_2 [9, 17]. This method is useful, because it does not require a specific device, such as a programmed freezer, is less time consuming and has a high survival rate for warmed embryos. Among vitrification methods, the minimum volume cooling (MVC) method is attracting attention [1]. Embryos are sustained in a small volume (<0.1 μl) of vitrifying preservative solution containing a cryoprotectant at a high concentration and are stored in LN_2. A high embryo survival rate can be achieved after warming, because ice crystal formation does not occur in this method, compared with that in the slow-freezing method. The Cryotop method is an MVC method [4, 11]. Cryopreservation using the Cryotop method has been reported to have favorable results for the cryopreservation of immature mammalian oocytes and porcine embryos that are considered slightly difficult to cryopreserve [12, 20], and the only study in which pups were obtained from canine cryopreserved embryos also used the Cryotop method [2]. In this study, the survival rate of frozen canine embryos was high, and a high conception

* CORRESPONDENCE TO: HORI, T., Laboratory of Reproduction, Nippon Veterinary and Life Science University, 1–7–1 Kyonan-cho, Musashino-shi, Tokyo 180–8602, Japan. e-mail: t-hori@nvlu.ac.jp

rate was achieved after thawed embryos were non-surgically transferred into the uterine horn through the cervical canal using a rigid cystoscope. However, although the use of a rigid cystoscope is favorable because it is less invasive for dogs, everybody cannot use it because it is expensive and requires training to learn the technique. Moreover, the application of this method to small-sized dogs is difficult. In contrast, surgical intrauterine ET does not require a specific device and is applicable to all dogs [18]. However, it is invasive for animals, even though it is applied through a small surgical incision within a short period of time. It is necessary to investigate the influence of this method on pregnancy after the transfer of canine cryopreserved embryos, which are damaged compared with fresh embryos. Thus, the investigation of this method may be clinically valuable.

In this study, using the slow-freezing method with Gly or DMSO as a cryoprotectant and the Cryotop method, canine embryos produced by natural mating *in vivo* were cryopreserved. Frozen-thawed embryos were surgically transferred into the uterine horn to investigate the possibility of obtaining pups from these embryos. In our previous study [18], bitches that received a surgical intrauterine transfer of fresh embryos in the 8-cell to blastocyst developmental stages became pregnant. Thus, embryos in these stages were cryopreserved in this study.

MATERIALS AND METHODS

Animals: All animals used in this study were beagles bred at our colony. Fifteen bitches, aged 2–10 years (mean ± S.E.: 5.3 ± 0.4 years), were used as donors, and 12 bitches, aged 2–5 years (mean ± S.E.: 3.2 ± 0.4 years), were used as recipients. Eight of these 12 recipient bitches were parous. For male dogs, nine dogs, aged 2–8 years (mean ± S.E.: 5.1 ± 0.5 years), with normal fertility were used. These experimental dogs were kept in cages measuring 160 × 75 × 65 cm in each two dogs, fed commercial dog food (Health Nutrition, Royal Canin Japon, Inc., Tokyo, Japan) once a day and given drinking water three times daily. Pregnant bitches were kept individually and given food twice from 35 days of gestation. This study was conducted in conformity with the animal study guidelines of Nippon Veterinary and Life Science University.

Estrus and ovulation monitoring: The bitches were observed daily for pudendal enlargement and the presence or absence of vulval bleeding. For hormone measurements, 1 m*l* of blood was collected from the anterior brachiocephalic vein every other day starting from day 6 of vulval bleeding. Blood samples were centrifuged to separate the serum, and the progesterone (P_4) levels were measured by fluorescence enzyme immunoassay using an automated fluorescence immunochemistry analyzer (SPOTCHEM VIDAS SV-5010; ARKRAY, Kyoto, Japan). The day on which the P_4 level initially reached 4–6 *n*g/m*l* or higher was regarded as the ovulation day [8, 14]. Donor bitches were mated once at 3–4 days after the estimated ovulation.

Collection methods of embryos: As previously reported [18], embryos were collected from two donor bitches via laparotomy, using the uterine perfusion method without the excision of the uterus at 10–11 days after ovulation. At 8–11 days after ovulation, embryos were collected from another 13 bitches using oviduct perfusion after conventional ovariohysterectomy (OVHX) [18]. After OVHX, the oviducts and uterine horns were separated; using an 18-G non-beveled needle (Terumo Co., Tokyo, Japan), the oviducts were perfused in the descending direction from the abdominal ostium of the oviduct. The uterus was perfused in the ascending direction from the uterine horn near the corpus uteri. The corpora lutea (number of ovulations) were counted in the excised ovaries or in the ovaries seen through a region of the ovarian bursa without fat. For the perfusion solution, Dulbecco's phosphate-buffered saline (PBS, Gibco®) or TCM199 (Sigma) was used. The perfusate was immediately subjected to the observation of the stage and number of collected embryos under a stereoscopic microscope, immediately followed by embryo cryopreservation. Embryos with abnormal morphology that showed degeneration at this time point were excluded from cryopreservation.

Embryonic Freezing and thawing methods: In the slow-freezing method, 20% fetal calf serum (Gibco®)-containing PBS (m-PBS) was used as the basic preservative solution for freezing. Embryos were equilibrated in stages with m-PBS containing a cryoprotectant at three concentrations. Embryos were first exposed to m-PBS containing 3% Gly or 0.5 M DMSO for 10 min, followed by exposure to m-PBS containing 6% Gly or 1.0 M DMSO for 10 min and then m-PBS containing 10% Gly or 1.5 M DMSO for 30 min. The embryos were subsequently filled in a 0.25-m*l* straw and cooled from room temperature to −4°C at −2°C /min using a programmed freezer (ET-1, Fujihira Industry Co., Ltd., Tokyo, Japan), followed by ice seeding at −4°C. The embryos were then slowly cooled to −34°C at −0.3°C /min, retained at this temperature for 10 min and stored in LN_2. Embryos were thawed by placing the straw in 37°C warm water after keeping them frozen for 4–33 days, exposed to m-PBS containing 6% Gly or 1.0 M DMSO for 10 min at 37°C, followed by exposure to m-PBS containing 3% Gly or 0.5 M DMSO for 10 min and finally placed in m-PBS without cryoprotectants at 37°C.

In the Cryotop method, embryos were placed on the liquid surface of an equilibrium solution (ES) containing 7.5% ethylene glycol (EG) and 7.5% DMSO. The embryos slowly settled to the bottom as they started to shrink due to osmotic pressure differences; they were kept in ES, while they started to swell, and they stopped swelling after 5 min up to a maximum of 15 min. The embryos contained in a minimum volume of ES were then transferred to the liquid surface of a vitrifying preservative solution (VS) containing 15% EG, 15% DMSO and 0.5 M sucrose and washed by pipetting while being moved in VS. The embryos were aspirated, placed with a minimum volume of VS on the tip of the Cryotop sheet under a stereoscopic microscope, rapidly placed in LN_2 1 min after transfer into VS for vitrification and stored. Embryos used for ET were kept vitrified for 150–332 days. To warm the embryos, the Cryotop sheet was taken out of LN_2, and its tip was placed in 1 M sucrose solution (TS) at

Table 1. Results of embryo collected from 15 donor bitches

Cryopreservation method	Donor bitch No.	Age (years)	Body weight (kg)	Male dog No.	Days from ovulation to embryo recovery	No. of CL		Stage of recovered embryo [a] (Number of embryos)	Site of embryo recovery	Recovery rate (%)
						L	R			
Slow Freezing (glycerol)	D1	9.6	7.5	M1	9	5	3 [b]	M (5)	Oviduct	100
	D2	5.3	9.0	M2	9	6 [b]	7	M (6) CM (1)	Oviduct Uterus	100
	D3	4.3	10.0	M3	10	4	3	M (2)	Uterus	28.6 [d]
	D4	5.5	12.0	M3	11	2	5	B (6)	Uterus	85.7 [d]
	D5	4.4	10.0	M3	11	2	6	M (1) B (4)	Oviduct Uterus	62.5
Slow Freezing (DMSO)	D6	4.7	11.5	M4	8	10	– [c]	8-cell (8), Deg (2)	Oviduct	100
	D7	5.5	13.0	M2	8	12	– [c]	8-cell (11), Deg (1)	Oviduct	100
	D8	7.9	12.5	M5	9	– [c]	9	M (8)	Oviduct	88.9
Cryotop	D9	4.6	8.2	M6	8	2	4	8-cell (3), Deg (1)	Oviduct	67.0
	D10	5.8	7.2	M7	9	7	2	8-cell (3), 16-cell (3) Deg (3)	Oviduct	100
	D11	2.1	9.4	M8	9	6	2	M (5), Deg (3)	Oviduct	100
	D12	5.3	9.4	M9	9	6	– [c]	CM (3)	Uterus	50.0
	D13	4.6	9.3	M7	10	4	4	8-cell (7), Deg (1)	Oviduct	88.0
	D14	6.0	7.2	M7	10	2	4	CM (1), EB (5)	Uterus	100
	D15	4.7	9.8	M7	11	5	3	M (8)	Oviduct/Uterus	100
Mean ± S.E.		5.3	9.7							88.7
		0.4	0.5							4.7

a) M: morula, CM: compacted morula, EB: early blastocyst, B: blastocyst, Deg: degenerated ova. b) Not recovery c) Ovary had already been removed d) Uterine flushing by laparotomy.

37°C and slowly moved into TS until the embryos detached from the sheet. The embryos were collected 60 sec after they were transferred into TS, placed on the bottom of a solution containing 0.5 M sucrose (DS) and held for 3 min. The embryos were then transferred to the bottom of a washing solution (WS) and held for 5 min, followed by transfer to the surface of new WS.

After thawing or warming, only embryos estimated to be morphologically normal by macroscopic examination under an inverted microscope were selected and held in each solution (m-PBS or WS) at 37°C until transfer.

Embryo transfer: The transfer day was set so as to adjust the difference between the time to collection of embryos after ovulation in the donor and the time to transfer after ovulation in the recipient to ± 1 day. To shorten the time to ET from thawing, embryo thawing was started corresponding to the state of surgery. As a rule, embryos collected from one donor were transferred to one recipient, but in some cases, embryos collected from multiple bitches with embryos in the corresponding developmental stages were transferred to one recipient bitch. Embryos were transferred using the previously reported surgical intrauterine transfer method [19]. Laparotomy was applied to a recipient bitch, and the uterine horn was exposed. An 18-G injection needle was penetrated into the uterine lumen, a glass capillary containing embryos was inserted with a small volume of solution into this hole, and embryos were slowly infused into the uterine horn. The insertion site was immediately sutured, and the abdomen was closed.

Pregnancy diagnosis: Pregnancy was evaluated at 25–30 days after ovulation in the recipient using an ultrasonographic imaging diagnosis system (ECHOVISOIN SSD-500EV, Aloka Medical, Ltd., Tokyo, Japan). Pregnant bitches were observed every 5 days after pregnancy determination to confirm normal embryonic development until delivery.

DNA test: When pups were obtained by the transfer of frozen embryos derived from multiple donor bitches, a parentage diagnosis was made by DNA testing. The canine oral mucosa to be examined by the DNA test (paternal and maternal dogs of the transferred embryos and pups) was collected using sterile swabs, and the sequences of DNA markers were collated to diagnose parentage by a DNA testing institution, Genetic Technologies. Eleven DNA markers were used including PEZ01, FHC2054, FHC2010, PEZ05, PEZ20, PEZ12, PEZ03, PEZ06, PEZ08, FHC2079 and PEZ16.

RESULTS

The results of embryo collection from 15 donor bitches are shown in Table 1. The embryo recovery rate was low in the one of two bitches in whom the uterine perfusion method was used (28.6 and 85.7%), but the recovery rate was 50%–100% (mean, 88.7 ± 4.7%) in the 13 bitches in whom the embryos were collected after OVHX. A total of 100 embryos in various developmental stages were collected from 15 donor bitches. Excluding 11 embryos assumed to be degenerated (11.0%), 89 embryos in the 8-cell to blastocyst stages were cryopreserved using the slow-freezing (n=51) or Cryotop method (n=38).

The ET conception results with cryopreserved embryos

are shown in Tables 2 and 3. Four of the 12 recipients received transferred embryos collected from two donors. All of the embryonic morphology was judged to be normal in frozen–thawed embryos prepared using the slow-freezing method with Gly or DMSO as a cryoprotectant, but no conception ocurrred in any of the bitches that received these embryos. On the other hand, vitrified–warmed embryos prepared using the Cryotop method (Fig. 1) were transferred to five bitches, and two of them became pregnant. In one of them, Bitch No. R10, delivery signs and labor started at 65 days after ovulation, but no fetus was delivered. Since the fetal heart rate decreased to about 100 beats/min on echocardiography, cesarean section was immediately performed. All pups were male, no external abnormality was noted, and they developed normally.

One of the two bitches (Bitch No. R10) that became pregnant received embryos collected from two donor bitches. Thus, the donors, pup and the paternal dogs were subjected to DNA testing. As shown in Table 4, D14 and M9 were the parents of the pup.

DISCUSSION

When canine embryos were vitrified using the Cryotop method and surgically transferred into the uterine horn after warming, the conception rate was 40% and normal pups were obtained. This conception rate was slightly lower than that reported in the study by Abe *et al.,* (55.6%; delivery rate, 44.5%) [2], in which embryos were similarly prepared using the Cryotop method and non-surgically transferred into the uterus transcervically using a cystoscope in nine female Labrador retrievers, and five became pregnant. In our study, 35 embryos were transferred, and two pups were born; this rate (5.7%) was slightly lower than that reported by Abe *et al.* (9.1%, 77 embryos were transferred, and seven pups were born) [2]. This difference may have been more likely due to the difference in the transfer method rather than in the dog breed. To reliably transfer embryos, a less invasive nonsurgical method may be appropriate, but transcervical intrauterine transfer using a cystoscope may be more difficult for small-sized dogs than for large-sized dogs. Thus, it is worth investigating surgical intrauterine ET as the transfer method of frozen embryos. Our study clarified that normal pups can be obtained by surgically transferring frozen canine embryos into the uterine horn.

We used embryos in the 8-cell to blastocyst stages for the Cryotop method, but only bitches that received embryos in the morula to early blastocyst stages became pregnant. None of the bitches that received 8- or 16-cell embryos became pregnant. This result is inconsistent with the findings reported by Abe *et al.* [2], in which bitches that received frozen 8- and 16-cell embryos became pregnant but those receiving morulas did not. They subjected frozen embryos to fluorescent staining and observed that the survival rate of embryos transferred after the morula stage was low. It is generally known that developmental embryos are more suitable for freezing and more easily frozen than early stage embryos, and blastocysts are considered most appropriate

Table 2. Conception results of intrauterine transfer of frozen-thawed embryos prepared using the slow freezing method

Cryoprotectant	Recipient bitch No.	Age (years)	Body weight (kg)	Parity	Days from cryopreservation to transfer	Days from ovulation to transfer	Transfer -red side	Donor bitch No.	Dairy differences of ovulation [a]	Stage of transferred embryo [b] (Number of embryos)	Result
Glycerol	R1	3.8	9.0	parous	13	9	R	D1	0	M (4)	NP [c]
					29			D2	0	M (1)	
	R2	3.2	9.0	parous	4	9	R	D3	−1	M (2)	NP
	R3	5.2	11.0	parous	33	11	R	D4	0	B (4)	NP
	R4	1.9	7.5	nulliparous	14	11	R	D5	0	M (1), B (4)	NP
DMSO	R5	2.6	10.0	nulliparous	24	7	R	D6	−1	8-cell (4)	NP
	R6	1.8	9.0	nulliparous	27	8	R	D7	0	8-cell (5)	NP
	R7	2.2	8.5	nulliparous	9	9	L	D8	0	M (5)	NP
Mean ± S.E.		3.0	9.1							25	
		0.3	0.3								

a) When the ovulation day of the recipient was earlier than that in the donor, the difference was presented as +. b) M: morula, B: blastocyst, c) Not pregnant.

Table 3. Conception results of intrauterine transfer of vitrified-warmed embryos prepared using the Cryotop method

Recipient bitch No.	Age (years)	Body weight (kg)	Parity	Days from cryopreservation to transfer	Days from ovulation to transfer	Transfer -red side	Donor bitch No.	Dairy differences of ovulation[a)]	Stage of transferred embryo[b)] (Number of embryos)	Result (Number of pups)	Days from ovulation to parturition
R8	5.2	7.2	parous	150	9	R	D9	1	8-cell (3)	NP [c)]	
				228			D10	0	16-cell (3)		
R9	2.7	8.4	parous	249	9	R	D10	0	8-cell (3)	NP	
				207			D13	−1	8-cell (6)		
R10	3.4	8.6	parous	238	9	R	D12	0	M (3)	1	65
				213			D11	0	M (6)		
R11	4.0	9.2	parous	332	10	L	D14	0	EB (5), CM (1)	1	64
R12	2.6	7.4	parous	185	11	R	D15	0	M (5)	NP	
Mean ± S.E.	3.6	8.2							35		
	0.3	0.2									

a) When the ovulation day of the recipient was earlier than that in the donor, the difference was presented as +. b) M: morula, CM: compacted morula, EB: early blastocyst, B: blastocyst, c) Not pregnant.

Table 4. DNA analysis of paternal and maternal dogs produced the transferred embryos and puppy (Bitch No. R10)

Dog No.	DNA marker										
	PEZ01	FHC2054	FHC2010	PEZ05	PEZ20	PEZ12	PEZ03	PEZ06	PEZ08	FHC2079	PEZ16
Mother D11	115	160	233	111	181	283	126	194	238	279	308
	119	176	237	115	181	283	129	195	238	279	325
Father M8	115	160	237	111	181	283	126	190	230	275	300
	115	164	241	115	185	283	129	194	238	279	308
Mother D12	115	160	233	111	185	283	126	190	238	291	300
	119	164	237	115	185	283	129	207	238	291	300
Father M9	107	164	233	115	185	269	126	194	238	279	300
	119	176	241	115	185	283	132	195	238	279	325
Puppy	107	164	233	115	185	283	126	194	238	279	300
	115	176	237	115	185	283	126	207	238	291	300

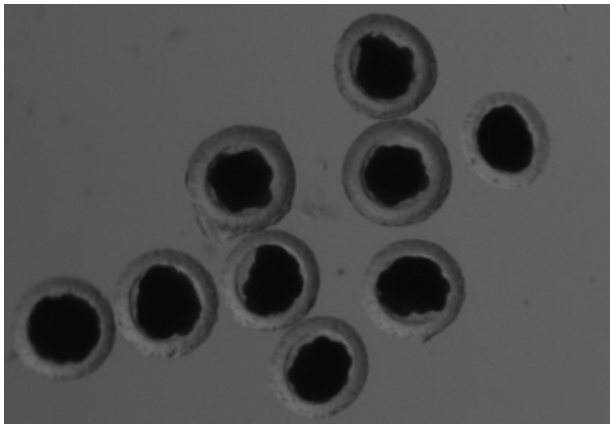

Fig. 1. Embryos of morula stage after vitrified-warmed using the Cryotop method. These embryos were transferred to Bitch No. R10.

for cryopreservation. In addition, we previously reported a study on the surgical intrauterine transfer of fresh canine embryos, in which 16-cell to blastocysts stage embryos were appropriate for transfer [18]. Furthermore, expanded blastocysts are considered slightly difficult to handle *in vitro*, because trophoblasts may shrink away from the zona pellucida in culture medium [16]. Therefore, embryos in the morula to early blastocyst stages may be appropriate for the cryopreservation of canine embryos. To successfully transfer frozen embryos, the timing of transfer may also be important. Studies on fresh ET reported that transfer within a ± 1-day difference between the time to embryo collection after ovulation in the donor and the time to ET after ovulation in the recipient is appropriate [18]. In the study reported by Abe *et al.* [2], the difference in the time after the luteinizing hormone (LH) surge between the donor and recipient bitches was 1–4 days in all bitches and 1–2 days in pregnant bitches, indicating a slight difference in LH levels between the two groups. In our study, the difference in the time to embryo collection after ovulation in the donor and time to ET after ovulation in the recipient was ± 0 days in all bitches, including those that became pregnant, suggesting that the same timing between the donor and recipient bitches was necessary to successfully transfer embryos. However, cryopreserved embryos were damaged to some extent, and they needed some time to adapt to the uterine environment in the recipient bitch. Accordingly, it may be better to set the timing of transfer a little earlier in the recipient bitch, rather than a ± 0-day difference between the donor and recipient bitches, but we could not clarify this because we did not perform the transfer of cryopreserved morulas under this condition.

Parent analysis was performed using 11 DNA markers. The number of DNA markers was less than that used by Abe *et al.* (23 microsatellite markers) [2], but it was sufficient to make a judgment. Since embryos were collected *in vivo*, it is not possible to collect many embryos from one animal. However, the success rate of ET rises as the number of transferred embryos increases. Thus, we transferred embryos collected from different bitches. The maternal and paternal dogs could be identified by parentage analysis using DNA markers, demonstrating that there is no problem with transferred embryos collected from multiple dogs.

Frozen canine embryos prepared by the slow-freezing method [17] with DMSO or Gly as a cryoprotectant, which is used for the embryos of many mammalian species, were similarly surgically transferred into the uterine horn. No conception was obtained, and this result was consistent with the findings reported in the study by Kim *et al.* [10], in which canine embryos frozen–thawed and prepared by the slow-freezing method with Gly were transferred. These findings suggested that the cryopreservation of canine embryos using the slow-freezing method is difficult with any cryoprotectant, although this method is generally used for other animal species. For this reason, the accumulation of phospholipids constituting the cell membrane and neutral fat in egg yolk that serves as an energy source is more abundant in many canine embryos than other mammalian embryos. These lipids are considered to be destroyed by *in vitro* manipulation due to the low-temperature sensitization of embryos, which interferes with embryonic development, and the high ratios of triglycerides and fatty acids constituting fat. These embryonic characteristics may be the reason for the inability to freeze embryos using the slow-freezing method. Regarding this point, Ushijima *et al.* [19] reported that when porcine morulas, known to contain abundant lipids in egg yolk, similar to canine embryos, were isolated by centrifugation and preserved by vitrification after removing the fat in the egg yolk, the embryo survival rate after warming was significantly improved to 95% (35/37) compared with that after the conventional vitrification method (24/42, 57%). A high conception rate was achieved when these embryos were transferred, suggesting that a high conception rate can be obtained in bitches by applying this method to removing the fat from the egg yolk using a micromanipulator and increasing the resistance of embryos to freezing, followed by cryopreservation using the Cryotop method. This remains to be further investigated.

It was concluded that the Cryotop method is more appropriate for canine embryo cryopreservation than the slow-freezing method used for the embryos of other mammalian species. Regarding the embryo developmental stage suitable for cryopreservation, the morula to early blastocyst stages may be appropriate.

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