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# FROZEN STORAGE OF EQUINE EMBRYOS

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## Introduction

The introduction of glycerol as a freeze-thaw protective agent for fowl spermatozoa<sup>54)</sup> ushered in a new era of frozen storage of living cells. But compared with the numerous and successful studies of spermatozoa and other tissue cells, the few attempts to freeze embryos with glycerol have been relatively unsuccessful in the rabbit,<sup>10,21,64)</sup> the mouse<sup>62,63)</sup> and the sheep.<sup>3)</sup> Prior to 1971, efforts were directed mainly to unfrozen storage rather than frozen storage,<sup>26)</sup> and the optimum temperature for storage was determined to be 10°C for the rabbit,<sup>13,25)</sup> 5°C for the rat,<sup>67)</sup> 5-10°C for the sheep<sup>3,28,45)</sup> and 5°C for the mouse.<sup>83)</sup> Although some rabbit ova survived storage for up to 14 days,<sup>25)</sup> these temperatures are too high to suppress physical-chemical activity of embryos completely, so that a prolonged storage period could not be expected.

Besides glycerol, dimethylsulfoxide (DMSO)<sup>37)</sup> and polyvinylpyrrolidone (PVP)<sup>52)</sup> have been shown to have cryoprotective activity to erythrocytes and bone marrow, respectively. Many other compounds, including sugars, glycols and polymers, can also protect some cells under certain conditions.<sup>15)</sup> In 1971, WHITTINGHAM<sup>75)</sup> reported 91% development in culture after freezing of early blastocyst stage mouse embryos to -79°C in 7.5% PVP. This was the first report of high survival rates for frozen-thawed mammalian embryos. However, subsequently in 1972, two independent reports described an even more reliable method for freezing embryos to sufficiently low temperatures to allow prolonged storage. WHITTINGHAM *et al.*<sup>81)</sup> used 1.0 M DMSO as the cryoprotectant, a cooling rate of 0.3-2.0°C/min and a warming rate of 4-25°C/min, and 50 to 70% of the frozen mouse early blastocysts developed into blastocysts in culture after storage at -196°C for up to 8 days. Transfer of the surviving embryos resulted in 65% pregnancy, and 43% of the embryos in pregnant mice developed to full term fetuses or live young. Glycerol (1.0 M) was also able to protect 8-cell embryos from freezing damage, but the

survival rate was about half those obtained with 1.0 M DMSO. WILMUT<sup>89)</sup> cooled mouse early blastocysts at 0.22°C/min in 1.5 M DMSO and rewarmed at 12°C/min, and obtained 86% development in culture after storage for 12 hr. to 12 days. These two initial studies led to the first success in the cattle<sup>91)</sup> the rabbit,<sup>4)</sup> the rat,<sup>72,77)</sup> the sheep<sup>88)</sup> and the goat.<sup>6)</sup>

Artificial insemination of frozen bull semen has considerably accelerated the improvement of cattle by increasing the progeny of genetically superior bulls. In contrast, embryo transfer is expected to facilitate extensive use of chosen females. Optimal use of this technique requires the storage of embryos, which can then be transported long distances and kept until the recipient's optimum stage of estrus cycle. Additionally in animal breeding, effective selection depends upon the availability of great genetic variation and it is important that breeds should not be allowed to disappear merely because they are of no current economic value. Frozen storage of embryos would offer an convenient method for conservation of various genetic resources — what we call a “gene bank” — which would contribute to the future breeding of farm animals.

The technique of frozen storage of cattle embryos has steadily progressed since the latter half of 1970's, and the pregnancy rate calculated from the number of transferred embryos has increased to around 50%<sup>7,10,32,33,39,40,61,68)</sup> In the equine, however, there has been only limited work on embryo transfer. The reason for this is that the equine is not a major source of protein for human consumption, that only natural breeding is allowed for most breed registration and that it has been difficult to induce superovulation to date.<sup>10)</sup> Thus, until 1981, there have appeared no reports on the storage of equine embryos at low temperature by both unfrozen and frozen methods. GRIFFIN *et al.*<sup>24)</sup> palpated one pregnancy at 60 days after the transfer of 4 frozen equine embryos, but the recipient was cycling on day 85. OGURI<sup>49)</sup> was also unsuccessful in freezing 10 equine embryos at -196°C.

In the present study, we explored the possibility of frozen storage of equine embryos in liquid nitrogen using a procedure which is substantially the same for mouse embryos.<sup>81,89)</sup> The first experiment attempting frozen storage at -196°C was unsuccessful. Therefore, the experiments in this study also examine the developmental ability of embryos after (1) being cooled to and/or stored at temperatures of 20°C or 0°C without cryoprotectant; (2) being exposed to DMSO at 20°C; (3) being frozen to temperatures between -5°C and -20°C with DMSO. In addition, we discuss the characteristic structure of equine embryo coverings, as they were observed at embryo recovery or during the freeze-thaw treatment.

### Theoretical Aspects of Freezing and Thawing Embryos

MAZUR<sup>40</sup> proposed the hypothesis of two factors responsible for cell injuries during freezing and thawing. One is intracellular ice formation. When cells are subjected to subzero temperatures, they are initially super-cooled. If they are cooled slowly or if their permeability to water is high, they will keep an intra-extracellular equilibrium of vapor pressure by the transfer of intracellular water to the external ice (by dehydration). But if they are cooled rapidly or if their permeability is low, they will equilibrate by intracellular freezing as the result of insufficient dehydration. Extensive intracellular crystals and their growth during warming (recrystallization) are lethal for cells. Thermodynamic and kinetic calculation indicated that "slow enough" for cells the size of mouse embryos is about 1°C/min or less.<sup>80</sup> Another factor responsible for cell injuries is solution effects. As extracellular water freezes with decreasing temperatures, both intra- and extracellular concentration of solutes increases. Hence, the slower the cooling rate is, the longer cells are exposed to a high concentration of solutes. The optimum rate, then, is a rate that is slow enough to prevent production of intracellular ice and yet is rapid enough to minimize the length of time cells are exposed to solution effects.

WHITTINGHAM *et al.*<sup>81</sup> and WILMUT<sup>89</sup> used slow cooling to  $-80^{\circ}\text{C}$  or  $-110^{\circ}\text{C}$  and to  $-70^{\circ}\text{C}$ , respectively, before direct transfer to liquid nitrogen for successful embryo freezing. LEIBO *et al.*<sup>85</sup> clearly showed that slow cooling (0.23°C/min) should be continued to at least  $-50^{\circ}\text{C}$  for high survival of frozen mouse embryos if a slow warming rate (3°C/min) was employed, and suggested that no freezable water exists in cells at  $-50^{\circ}\text{C}$  or below. However, sheep, cow and mouse embryos survived rapid warming ( $>275^{\circ}\text{C}/\text{min}$ ) when they were cooled about 0.3°C/min to temperatures between  $-25^{\circ}\text{C}$  and  $-50^{\circ}\text{C}$  and then transferred directly to liquid nitrogen, whereas a poor survival rate was obtained when warmed slowly.<sup>55,84,85</sup> This freeze-thaw method is generally called the "rapid method". From the good results achieved in both slow and rapid methods, it has been supposed that rapidly cooled cells contain a certain amount of intracellular ice and that the critical factor that determines injury is the amount of intracellular ice formed in relation to the rate of thawing.<sup>20,30,43,84</sup>

The action of cryoprotectant such as glycerol and DMSO, has not yet been well explained. But it is believed to be related to its colligative properties of reducing the increase in the concentration of salts in the unfrozen

portion of the suspending solution<sup>36,37,58)</sup> and increasing the fraction of the extracellular solution that remains unfrozen at a given temperature.<sup>42)</sup>

## Materials and Methods

### Animals

Twenty-seven Hokkaido native pony mares and seven crossbred mares aged 2.5 to 24 years were used as donors and recipients during breeding seasons (from April to October) of 1979-1983. Except for five Hokkaido native pony mares, these animals were kept under rotational grazing day and night at the Hokkaido University Livestock Farm in Shizunai. They were fed hay *ad libitum* in winter. Five Hokkaido native ponies were fed under normal management conditions at the Ishikawa Hokkaido Native Pony Farm in Toya. The mares were gathered from the fields every morning and the ovaries were palpated per rectum once a day from the onset of estrus to the confirmation of ovulation. The teasing was not carried out. All the mares were left for spontaneous ovulation. Hokkaido native pony mares were mated with a fertile stallion of the same breed once, every day or every other day during estrus until ovulation occurred, and crossbred mares were similarly mated with a fertile Thoroughbred stallion. The day, when the sudden disappearance of Graafian follicles resulting in a depressed surface of the ovary into the former follicular cavity was confirmed, was regarded as the day of ovulation (Day-O).

### Non-surgical Recovery of Equine Embryos

Embryos at early blastocyst to expanded blastocyst stage were flushed non-surgically from the uteri of donors 6 days (144-168 hr.), 7 days (168-192 hr.) and 8 days (192-216 hr.) after ovulation by the method described by OGURI and TSUTSUMI.<sup>50)</sup> The three-way system apparatus used for uterine flushing consisted of an external tube made of vinyl resin (12 mm outside diameter and about 65 cm in length) and an internal vinyl tube (5 mm outside diameter) with an opening near the rounded tip. A hollow latex rubber collar, about 6 cm in breadth, was attached near the anterior end of the external tube. The tip of a fine polyethylene tube through which air could be pumped was led into this collar. Donor mares kept standing in the treatment stocks were injected intramuscularly 1 ml of 1% propionylpromazine (Combelen; Bayer)/100 kg body weight for sedation about 20 min. before the uterine flushing. The apparatus was gently inserted through the cervix into the uterine lumen until the latex collar passed through the cervix. The collar was inflated with about 60 ml air to prevent the apparatus from slipping

out and to block leakage of the flushing medium. Sterile physiological saline (0.85%) containing 2% gelatine kept at 35°C was siphoned through the inner tube by a 1 meter head sufficiently to inflate whole uterine lumen. The flushed fluid returned by gravity flow through the opening between the external and the internal tubes into a 2-litre or a 5-litre Erlenmeyer flask.

During the return flow, the uterus was massaged by hand per rectum. A total of 1500 ml of media were used for 2 or 3 flushings on each recovery for Hokkaido native pony mares and 3000 ml media for crossbred mares. Day-8 embryos were detected by the naked eye in the flask, and were immediately washed three times in Dulbecco's phosphate buffered saline (Grand Island Biological Co.) containing 100 IU penicillin/ml and 100 µg streptomycin/ml (PBS) at 35°C. For the detection of Day-6 and Day-7 embryos, the flushed media were transferred into bottle-shaped glassware (Text-fig. 1) and kept still until sedimentation of embryo. After 10 to 30 min.,

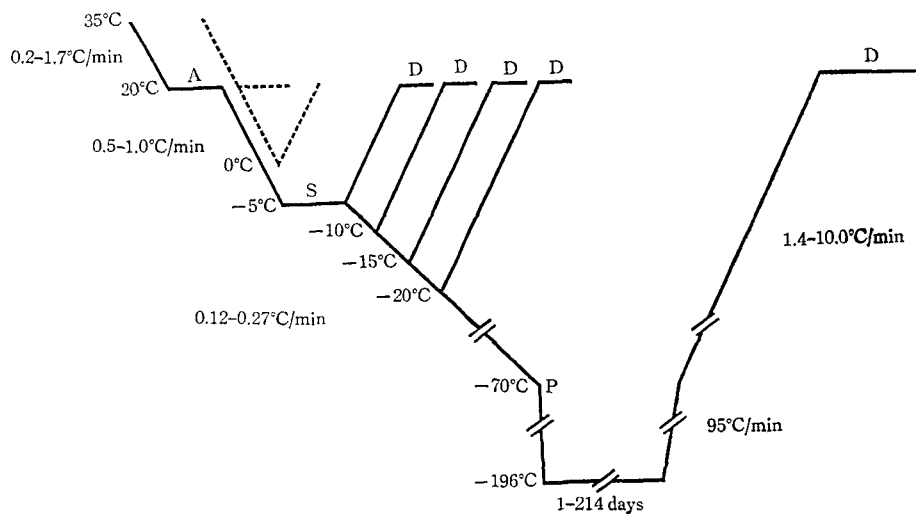
about 13 ml of fluid was pipetted from the bottom of the glassware into a watchglass under a dissecting microscope. Immediately after their discovery, Day-6 and Day-7 embryos were washed similarly in PBS or PBS supplemented with 20% mare serum (PBS+S) at room temperature. The serum was heat-inactivated at 56°C for 30 min. These media were used as the basal medium for all subsequent experiments.

#### Slow Freezing and Thawing of Equine Embryos

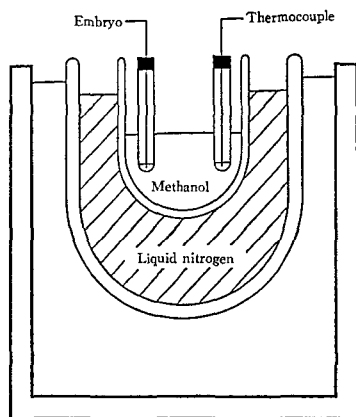
The methods of WHITTINGHAM *et al.*<sup>81)</sup> which are called slow freezing and thawing, were generally followed in the following procedures. The outline of the procedures were diagrammed in Text-fig. 2. All the embryos were cooled at 0.2-1.7°C/min from 35°C to room temperature at which time cryoprotectant was added. Dimethylsulfoxide (DMSO) or glycerol were used as cryoprotectants. DMSO was added by moving the embryos, at 10 min. intervals, through a series of watchglasses containing increasing concentrations of 0.5 M, 1.0 M and 1.5 M-DMSO in PBS or PBS+S. Glycerol, when used, was added similarly in 2 steps (0.5 M and 1.0 M) or 3 steps (0.33 M, 0.66 M and 1.0 M). After 30 min. equilibration in 1.5 M-DMSO or 1.0 M-glycerol at room temperature, the embryos were transferred into Pyrex



**Text-fig. 1.** Diagram of bottle-shaped glassware for the detection of equine Day-6 and Day-7 embryos. It is 10.5 cm at the top and 2.8 cm at the bottom in outside diameter and 41 cm in height. The capacity is about 1500 ml.



**Text-fig. 2.** Procedures and temperature curve in cooling of embryos to various minimum temperatures and in rewarming. A, addition of cryoprotectant; D, dilution of cryoprotectant; S, seeding; P, direct plunge into liquid nitrogen. Broken line represents temperature curve in unfrozen embryos.



**Text-fig. 3.** Diagram of the apparatus for cooling from  $-5^{\circ}\text{C}$  to temperatures between  $-36^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$ . It consists of two evacuated silvered Dewar flasks. The size of inner flask is 10 cm in inside diameter and 13 cm in depth. The inner flask, containing 350 ml methanol, is floated in liquid nitrogen.

test tubes (13 mm × 100 mm) with 0.2–0.3 ml of media. The test tubes, each containing one embryo, were stoppered firmly by screw caps and cooled at 0.5–1.0°C/min from room temperature to –5°C in a Refrigerated Circulating Bath (NESLAB Instrumenta, Inc.). Immediately after the temperature reached –5°C, crystallization of the freezing medium (seeding) was induced by touching the outer wall of the tube with a glass tube precooled in liquid nitrogen. The test tubes were held in the bath at –5°C for 10 min., and then transferred into a deep freezing methanol bath (Text-fig. 3), inducing cooling at rates ranging between 0.12°C/min and 0.27°C/min. The deep freezing bath consisted of an evacuated silvered Dewar flask blank which contained about 350 ml methanol precooled to the seeding temperature, and which was floated in liquid nitrogen. The cooling rate was calculated in three segments of the temperature range between –5°C and –70°C. When the test tubes were cooled to –70°C in this fashion, they were transferred directly into liquid nitrogen where they were stored for 1–214 days.

For thawing, the test tubes were rewarmed at 95°C/min between –196°C and –70°C in air, at 1.4–10.0°C/min to –10°C in 300–500 ml methanol precooled to –70°C in a 1-litre beaker in a 20–60°C water bath, and at about 8°C/min to room temperature in a 20°C water bath. The temperatures of the control media, containing no embryos, in the test tubes adjacent to the sample tubes were monitored and recorded continuously with a copper-constantan thermocouple connected to a Temperature Recorder MODEL EB2P (CHINO WORKS, LTD.) A few minutes after the warming, the embryos were transferred, at 10 min. intervals, to decreasing concentrations of 1.25 M, 1.0 M, 0.75 M, 0.5 M, 0.25 M and 0.0 M-DMSO in PBS or PBS+S to remove DMSO. Glycerol was diluted by the reverse of the procedures used for its addition. After dilution of the cryoprotectant, the embryos were washed once in fresh PBS or PBS+S and were kept at room temperature until they were transferred to the recipient mares.

#### **Rapid Freezing and Rapid Thawing of Equine Embryos**

Day-6 and Day-7 embryos were equilibrated with 1.0 M-glycerol for 30 min. using the 3-step addition method at room temperature, then cooled to –5°C at about 0.9°C/min, held at –5°C after seeding and cooled to temperatures between –36°C and –49°C at 0.12–0.27°C/min. At these temperatures, the samples were plunged directly into liquid nitrogen. After storage for 2–13 days, they were rewarmed rapidly (>200°C/min) to room temperature by agitating them in 20°C water bath. Glycerol was diluted in 3 steps.

**Cooling of Equine Embryos to Various Minimum Temperatures above  $-20^{\circ}\text{C}$** 

In order to detect the reason for failure of initial attempt at frozen storage of equine embryos at  $-196^{\circ}\text{C}$ , the procedure was subsequently interrupted at various steps above  $-20^{\circ}\text{C}$ . In these experiments, the rates of cooling and warming within the corresponding temperature range were the same as those of the deep freezing. The procedures of addition and dilution of cryoprotectant and seeding (Text-fig. 3) were also the same.

1) *Cooling and storage of Day-8 embryos at  $20^{\circ}\text{C}$  or  $0^{\circ}\text{C}$  in cryoprotectant-free media.*

Day-8 embryos were cooled from  $35^{\circ}\text{C}$  to  $20^{\circ}\text{C}$  in 2-3 ml PBS or TCM 199 (Chiba-kessei, containing 100 IU penicillin/ml and  $100\ \mu\text{g}$  streptomycin/ml) in the test tubes, and stored from 8 min. to 550 min. at  $20^{\circ}\text{C}$ . Three of those embryos were transported by rail from Shizunai to Toya (220 km) in the test tubes which were immersed in  $20^{\circ}\text{C}$  water in a Semen Storage Container (Fujihira Industry Co., Ltd.). The other Day-8 embryos were cooled to  $0^{\circ}\text{C}$  and immediately warmed to  $20^{\circ}\text{C}$  or stored at  $0^{\circ}\text{C}$  for 7.7 hr. or 20.3 hr. in PBS.

2) *Examination of the toxicity of DMSO at  $20^{\circ}\text{C}$ .*

Day-8 and Day-6 embryos were only exposed to 1.5 M-DMSO in PBS at  $20^{\circ}\text{C}$  for 30 min.

3) *Freezing of Day-6 embryos to temperatures between  $-5^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$  in the presence of DMSO.*

Day-6 embryos in 1.5 M-DMSO in PBS were cooled to and held at  $-5^{\circ}\text{C}$  for 10 min. after seeding, or were frozen to  $-10^{\circ}\text{C}$ ,  $-15^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$ . All the embryos were rewarmed immediately after each treatment to room temperature, at which time DMSO was diluted.

**Assessment of Viability of Treated Embryos**

After the treatments, the embryos were assessed morphologically and transferred to the recipient mares non-surgically by the technique described by OGURI and TSUTSUMI.<sup>51)</sup> The recipient mares were tranquilized in the stocks as was noted earlier. The vagina was dilated with a vaginal speculum, and a narrow plastic tube, containing an embryo with about 0.2 ml medium positioned some 5 cm from the distal end, was inserted into the external orifice of the uterus. The plastic tube, which had a vinyl resin bulb attached to one end, had an outside diameter of 5 mm, and was 40 cm long for Hokkaido native pony mares and 58 cm long for crossbred mares. Then the speculum was drawn out, and the tip was led through the cervix into the cavity of the left uterine horn or uterine body with the aid of a hand

in the rectum, the embryo with the medium was deposited by pressure on the bulb. The media used for the transfer were the same basal media as used for each treatment. All the transfers were performed by a single operator.

Each recipient received one embryo and its ovulatory cycle was synchronized to within  $-4$  to  $+1$  days of the embryo age at recovery. The degree of synchronization was expressed by the discrepancy between the recipient and the embryo in their post-ovulatory age (days after the most recent ovulation). For example, when the post-ovulatory age of the recipient was one day younger or one day older than the embryo age, the degree of synchronization was defined as  $-1$  day or  $+1$  day, respectively. When the recipient age was the same as the embryo age, the degree of synchronization was defined as 0.

## Results and Discussion

### 1. Structural Characteristics of Embryo Coverings in the Equine Embryos

Intact Day-6 equine blastocysts appeared to be surrounded by a single layer of zona pellucida, as are other species. However, when some of these embryos were exposed to a hyperosmotic solution of cryoprotectants, a thin, transparent, acellular membrane appeared as the embryos contracted away from the zona (Pl. 1, Fig. 4, Pl. 2, Fig. 11). Ultrastructural evidence for the formation of this new layer in Day-6½ equine embryos was clearly demonstrated by FLOOD *et al.*<sup>22)</sup> In agreement with the observation by BETTERIDGE *et al.*,<sup>6)</sup> who called this layer a "capsule", the outer layer of the zona pellucida was dehiscing, peeling or was completely peeled off from the intact inner layer in some Day-7 embryos larger than about 300  $\mu\text{m}$  in diameter (Pl. 4, Fig. 21, Pl. 5, Figs. 25 and 26). The new inner layer was reported to remain until the embryo (Day-21) was at least 34 mm in diameter.<sup>6)</sup> The origin and physiological role of the inner layer are obscure. For expediency, this new inner layer is to be called the "capsule" in this study.

### 2. Attempt at Frozen Storage of Equine Day-8 and Day-6 Embryos at $-196^{\circ}\text{C}$

Day-8 equine embryos (around 1 mm in diameter) are visible to the naked eye, and are readily recovered, manipulated and transferred. Further, the pregnancy rate after transfer of these embryos (about 50%) is relatively high.<sup>5)</sup> For this practical reason, Day-8 embryos were initially chosen to be deep-frozen in liquid nitrogen. However, the results were unsuccessful for both cryoprotectants, DMSO and glycerol (Table 1).

In a hypertonic concentration of DMSO, the trophoblastic layer collapsed

TABLE 1. Results of frozen storage of equine Day-8 and Day-6 embryos at  $-196^{\circ}\text{C}$ 

Age of embryos (days)	Cryoprotectant	Storage period (days)	No. of embryos		No. of pregnancies
			frozen	transferred	
8	DMSO	1-24	5	4	0
8	glycerol	1-30	5	5	0
6	DMSO	3-20	11*	8	0

\* Six of these embryos were frozen in the media containing 20% mare serum.

into blastocoele with the capsule in several places due to osmotic response (dehydration of blastocoele fluid) (Pl. 1, Fig. 1). After thawing, these collapses were further advanced and, additionally, folding was observed in some embryos (Pl. 1, Fig. 2). Most embryos retained these deformations even after the dilution of DMSO. One Day-8 embryo frozen-thawed in 1.5 M-DMSO had dispersed through the broken part of the capsule. Day-8 embryos similarly frozen in 1.0 M-glycerol showed similar collapses to a lesser degree than those treated in 1.5 M-DMSO.

Mouse embryos of all stages, from 1-cell to hatched blastocyst stage, have been successfully frozen.<sup>70)</sup> But in cattle, it is difficult to freeze advanced blastocysts. Transfer of Day-10 or 11 hatched cow embryos frozen to  $-196^{\circ}\text{C}$  resulted in limited success.<sup>91)</sup> TROUNSON *et al.*<sup>68)</sup> also reported poor survival of frozen Day-8 and Day-11 to 13 hatched cow embryos compared with Day-7 to Day-8 zona enclosed embryos, and suggested the effects of the absence of zona and increased size of blastocoele cavity. In an ultrastructural study of frozen-thawed Day-13 cow embryos, the trophoctoderm cells were severely damaged, whereas the embryonic cells remained intact.<sup>44)</sup> Differentiation of cells (embryonic, trophoctoderm and endoderm cells) progressed to some extent in equine expanded blastocysts 8 days after ovulation.<sup>22)</sup> MOHR and TROUNSON<sup>44)</sup> suggested that in cattle, different cell types display different susceptibilities to injuries caused by freezing. This may be one of the factors responsible for zero survival of equine Day-8 embryos after freeze-thawing.

Subsequently, equine embryos in the earlier stages (Day-6 early blastocysts and blastocysts) were similarly frozen in 1.5 M-DMSO, but none of these embryos developed (Table 1). Since they were examined only under low magnification with a dissecting microscope, it was difficult to make a close morphological evaluation, but it was found that five embryos sustained damage to the zona pellucida. Contraction and re-expansion of the Day-6 embryos in DMSO solution are described in detail elsewhere (3. (3)).

### 3. Viability of Equine Embryos Cooled to Various Minimum Temperatures above $-20^{\circ}\text{C}$

#### 1) *Cooling and storage of Day-8 embryos at $20^{\circ}\text{C}$ or $0^{\circ}\text{C}$ in cryoprotectant-free media.*

In the previous experiments, no equine embryos survived storage at  $-196^{\circ}\text{C}$ . The cause of the failure was presumed to be that equine embryos were susceptible to cooling, in the same way that pig embryos are intolerant of cooling even below  $15^{\circ}\text{C}$ .<sup>56)</sup> Then, as the first step to find the deleterious process of the freezing procedure, the viability of embryos after cooling above freezing temperature was examined in the absence of cryoprotectant.

##### a) Cooling to $20^{\circ}\text{C}$ .

Table 2 shows the results of equine embryo transfer after storage at  $20^{\circ}\text{C}$  in PBS or TCM 199. Half of the Day-8 embryos stored at  $20^{\circ}\text{C}$  in PBS developed. Embryos of the mouse,<sup>57)</sup> rat,<sup>14)</sup> rabbit,<sup>26)</sup> sheep,<sup>18)</sup> pig<sup>18)</sup> and cattle<sup>69)</sup> tolerate being held at room temperature for up to an hour. OGURI and TSUTSUMI<sup>50,51)</sup> maintained equine embryos at  $30^{\circ}\text{C}$  during the time from uterine flushing to embryo transfer in flushing medium (physiological saline containing 2% gelatine) and a mixture of mare serum and Ringer's solution, or in flushing medium and TCM 199. They obtained conception rates of

TABLE 2. Viability of equine Day-8 embryos stored at  $20^{\circ}\text{C}$  in PBS or TCM 199

Donor No.	Cooling rate ( $35^{\circ}\text{C}-20^{\circ}\text{C}$ ) ( $^{\circ}\text{C}/\text{min}$ )	Storage medium	Storage time at $20^{\circ}\text{C}$	Recipient No.	Degree of synchronization* (days)	Pregnancy
9	1.7	PBS	79 min	2	+1	-
11	1.0	PBS	34 min	12	-2	+
17	0.6	PBS	11 min	15	-1	-
11	0.6	PBS	9 min	5	-2	-
13	0.6	PBS	8 min	16	-2	+
11	0.2	TCM 199	6.5 hr†	23	-2	-
12	0.2	TCM 199	5.3 hr†	24	-1	-
9	0.2	PBS	9.2 hr†	25	-2	+

\* When the post-ovulatory age (days after the most recent ovulation) of the recipient was 1, 2 or 3 days younger than the age of embryo, the degree of synchronization was defined as -1, -2 or -3, and when 1 day older, it was defined as +1. When the recipient was the same age as the embryo age, it was defined as 0.

† Transportation time included.

Note: All the pregnant recipients foaled.

30-50% from the embryos kept for a total of 45-160 min. ALLEN and ROWSON<sup>28</sup> also successfully kept equine embryos at 30°C in TCM 199 for 18-55 min. In farm animals, most of the embryo transfers are carried out at places poorly equipped or outdoors. Therefore, one of the most important prerequisites for a simple embryo transfer technique is to keep embryos viable at around 20°C for at least the time between embryo recovery and transfer. The conception rate (50%) in this experiment, which is comparable to that of OGURI and TSUTSUMI,<sup>50</sup> indicates that equine embryos favorably retain their viability at room temperature. DOUGLAS<sup>17</sup> recently reported 50% pregnancy rate after transfer of equine embryos maintained at room temperature for up to 3 hr. in PBS supplemented with 20% fetal calf serum.

Some attempts to prolong storage time of mammalian embryos at room temperature have been made. HAFEZ<sup>25</sup> demonstrated a 29% implantation rate after storage of rabbit ova at 20°C for 48 hr. in a mixture of homologous serum and saline. Forty-three percent of pig 4-cell embryos stored at 20°C for 24 hr. developed to fetuses.<sup>53</sup> In cattle, morulae and blastocysts in modified PBS<sup>75</sup> survive storage at room temperature. TROUNSON *et al.*<sup>60</sup> reported a total of 87.5% development in the rabbit oviduct of cattle morulae stored at 18-21°C for 1.5-7.5 hr. Cattle late morulae and blastocysts stored at 20°C for 24 and 48 hr. showed 100 and 64% development in culture, respectively.<sup>60</sup> One of 2 heifers which received morulae stored at 20-25°C in BMOC-3 medium for one day became pregnant.<sup>29</sup>

During storage at 20°C for 9.2 hr. in PBS, one equine Day-8 embryo was transported 220 km by rail without loss of its developmental ability, and prolongation of storage time at room temperature was suggested. However, DOUGLAS<sup>17</sup> who stored equine embryos at room temperature in PBS containing 20% fetal calf serum for 6-24 hr. found no pregnancy at Day-100 after the non-surgical transfer. Since there was only one embryo stored in PBS for several hours in the present experiment, further attempts are required to determine whether equine embryos survive storage at room temperature for one day or so as in the other species mentioned above. Successful storage and long-distance transport of equine embryos in ligated rabbit oviducts was reported by ALLEN *et al.*<sup>13</sup>

b) Cooling to 0°C.

Two of four equine embryos cooled to 0°C and immediately warmed to 20°C developed to foals (Table 3). Sensitivity of embryos to cooling to 0°C is different among the developmental stages within a species and between species. After being held at 0°C for 15 min., few 2- to 4-cell sheep embryos developed normally in the rabbit oviduct, whereas later stage embryos (up to

morula) did well.<sup>89</sup> In cattle, after the same treatment, only one of 19 early morulae (8- to 16-cell) developed to blastocysts in culture, but 7 of 9 late morulae developed to blastocysts.<sup>90</sup> Development of cow Day-7 blastocysts in culture was little affected by storage at 0°C for 30 min.<sup>70</sup> On the contrary, pig embryos at the 8-cell to blastocyst stage were all killed when cooled to 0°C.<sup>86</sup> One reason for these differences was recently explained structurally by MOHR and TROUNSON.<sup>44</sup> They showed evidence that 8- to 16-cell cow embryos have large number of globular vesicles (spherical inclusions variously referred to yolk, globules, lipid droplets and cytoplasmic vesicles) within their blastomeres, whereas blastocysts no longer did. In association with observation related to the cytoplasmic inclusion of these globular vesicles in the sheep,<sup>12</sup> the pig<sup>48</sup> and the mouse,<sup>11</sup> they suggested that a certain amount of globular vesicles within cells has a deleterious effect on the viability of mammalian embryos cooled to low temperatures. Although the number of equine embryos examined here is small, the foaling rate, comparable to that of OGURI and TSUTSUMI<sup>61</sup> with embryos stored at 30°C, suggests a strong cold-resistance of equine Day-8 blastocysts similar to sheep and cattle. Considering that equine expanded blastocysts (>0.5 mm in diameter) have no lipid droplets,<sup>22</sup> the result is consistent with the supposed relationship between the presence of globular vesicles and the viability of cooled embryos.

TABLE 3. Viability of equine Day-8 embryos cooled to 0°C in PBS

Donor No.	Cooling rate (35°C-20°C) (°C/min)	Storage time at 0°C (hr)	Recipient No.	Degree of synchronization (days)	Pregnancy
13	0.7	0	5	-3	+
20	0.5	0	2	0	-
11	0.5	0	6	-2	+
10	1.0*	0	11	-1	-
9	0.5	7.7	26	0	-
25	0.5	20.3	11	-1	-

\* Cooling rate from 20°C.

Note: All the pregnant recipients foaled.

CHANG<sup>18</sup> stored rabbit 2-cell embryos at 0°C for 48 hr. in rabbit serum, and 21% of those transferred developed to normal young. Mouse morulae survived storage at 0°C for 48 hr. in modified PBS<sup>70</sup> and modified PBS containing 0.75 M sucrose.<sup>31</sup> Fairly good fetal development (40%) was obtained

by transfer of cow blastocysts stored at 0°C for 48 hr. in PBS supplemented with fetal calf serum.<sup>70)</sup> In contrast, the present attempt to store equine Day-8 embryos at 0°C in PBS for 7.7 and 20.3 hr. met with no success. The transparency of the trophoblast was reduced in the embryo stored for 20.3 hr.

2) *Effect of exposure to DMSO at 20°C.*

Dimethylsulfoxide (DMSO) is widely used as a cryoprotectant for frozen storage of mammalian embryos. However, exposure to DMSO itself, at the concentration and equilibration times required to achieve cryoprotection, caused a number of structural changes in Rhesus kidney cells.<sup>38)</sup> OGURI<sup>49)</sup> examined the viability of Day-8 equine embryos after exposure to 1.5 M-DMSO at -5.5°C, but no development was confirmed. This suggests the harmful effect of DMSO on equine embryos. So, as the next step in investigating deleterious effects of the freezing procedures, the toxicity of DMSO to equine embryos was examined.

Table 4 shows the viability of equine Day-8 and Day-6 embryos after the addition and dilution of 1.5 M-DMSO at 20°C. One of 2 Day-6 embryos developed to a foal, whereas none of six Day-8 embryos developed despite their good, re-expanded morphological appearance after the treatment. A short exposure to DMSO (1.0-2.0 M) at room temperature, 0°C or -6°C to -7°C did not reduce the viability of embryos in the mouse,<sup>89)</sup> rabbit,<sup>80)</sup> sheep or cattle.<sup>68, 88)</sup> By comparison, equine Day-8 expanded blastocysts seemed to be very susceptible to DMSO. The failure in frozen storage of equine Day-8 embryos may be partly due to this deleterious effect of DMSO.

TABLE 4. Effect of exposure to 1.5 M-DMSO at 20°C on the viability of equine Day-8 and Day-6 embryos

Donor No.	Age of embryos (days)	Cooling rate (35°C-20°C) (°C/min)	Recipient No.	Degree of synchronization (days)	Pregnancy
18	8	0.6	2	-2	-
17	8	0.6	4	-2	-
17	8	0.5	7	0	-
1	8	0.2	9	0	-
10	8	0.2	13	-2	-
6	8	0.2	14	-2	-
17	6	0.2	2	-2	+*
9	6	0.2	11	0	-

\* A normal foal was produced.

3) *Freezing of Day-6 embryos to temperatures between  $-5^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ .*

Day-6 embryos were used in this experiment, because the viability after DMSO treatment was confirmed not in Day-8 embryos but in Day-6 embryos in the preceding experiment. As shown in Table 5, the embryos frozen to  $-5^{\circ}\text{C}$  and  $-10^{\circ}\text{C}$  developed, but those frozen to  $-15^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  did not develop. Excellent survival after seeding at  $-5^{\circ}\text{C}$  suggests the innocuous effect of initial phase change in the medium.

TABLE 5. Effects of various minimum frozen temperatures on the viability of equine Day-6 embryos in 1.5 M-DMSO in PBS+S\*

Minimum temperatures ( $^{\circ}\text{C}$ )	Time at minimum temperature (min)	No. of embryos		No. of foals
		treated	transferred	
-5	10†	3	3	3
-10	0	3	3	1
-15	0	1	1	0
-20	0	3	3	0

\* PBS containing 20% mare serum.

† Time after seeding.

When Day-6 embryos were first subjected to 0.5 M-DMSO, they started to contract except for the zona pellucida, and then the blastocoele became smaller and the perivitelline space consequently extended (Pl. 1, Figs. 3 and 4). The contraction continued in 1.0 M-DMSO (Pl. 1, Fig. 5). A few minutes after being pipetted into 1.5 M-DMSO, the size of embryos was reduced to about 80% of the initial diameter (Pl. 1, Fig. 6, Pl. 2, Fig. 11). The outer surface of trophoblast was irregular in the contracted state. During the successive equilibration in 1.5 M-DMSO, the embryos slightly re-expanded, indicating penetration by DMSO, but not fully to the original size (Pl. 2, Figs. 7, 8 and 12). Immediately after thawing to room temperature, most of the embryos retained their good morphological appearance (Pl. 2, Fig. 9, Pl. 3, Fig. 13). The embryos, maintaining the irregular surface of the trophoblast, gradually re-expanded during the dilution process of DMSO, but there still remained a little perivitelline space even after the return into DMSO-free PBS (Pl. 2, Fig. 10, Pl. 3, Fig. 14). Swelling of some trophoblastic cells was observed after the dilution of DMSO (Pl. 3, Fig. 14). There were no detectable differences in morphological quality between the embryos cooled above  $-10^{\circ}\text{C}$  and below  $-15^{\circ}\text{C}$  (Pl. 2, Fig. 10, Pl. 3, Fig. 14).

DMSO is the cryoprotectant in general use for the storage of mouse, rabbit, cow and sheep embryos in liquid nitrogen with the freeze-thawing procedure being used at present. Although no equine embryos frozen below  $-15^{\circ}\text{C}$  in 1.5 M-DMSO developed, it is too early to conclude here that equine embryos were intolerable to freezing below  $-15^{\circ}\text{C}$  in the presence of DMSO.

#### 4. Storage of Equine Day-6 and Day-7 Embryos at $-196^{\circ}\text{C}$ in the Presence of Glycerol

Glycerol has been shown to be an effective cryoprotectant for the embryos of farm animals. The protective capacity of glycerol has been equal to that of DMSO for freezing of cow embryos.<sup>7-9,10</sup> In the goat, glycerol was more effective than DMSO.<sup>6</sup> Simultaneously with the examination of the viability of Day-6 embryos frozen above  $-20^{\circ}\text{C}$  in 1.5 M-DMSO, the other Day-6 embryos were frozen to  $-196^{\circ}\text{C}$  in 1.0 M-glycerol, and later the development of the first deep-frozen embryo was confirmed by rectal palpation of the recipient at Day-30. On this account, glycerol was expected to be a promising cryoprotectant for Day-6 embryos, and it was used in all of the subsequent experiments.

The development of equine Day-6 and Day-7 embryos stored at  $-196^{\circ}\text{C}$  in 1.0 M-glycerol is shown in Table 6. The first foal in the world that was developed from a deep-frozen embryo, was born normally on May 31, 1982 (Pl. 7, Fig. 39). A total of six pregnancies were obtained after the transfer of slowly frozen-thawed embryos; 3 from Day-6 and 3 from Day-7 embryos. Of the eight Day-7 embryos frozen-thawed by the rapid method, one embryo developed. Development was found in all four classified groups; early blastocyst (2/13) (Pl. 3, Figs. 15-18, Pl. 4, Fig. 19), blastocyst (2/13) (Pl. 6, Figs. 35 and 36), early expanded blastocyst (1/6) (Pl. 7, Figs. 37 and 38) and expanded blastocyst (2/17) (Pl. 4, Figs. 21-24).

##### 1) *Morphology of the embryos subjected to the freeze-thawing process.*

During the freeze-thawing process in glycerol, most Day-6 and Day-7 early blastocysts and blastocysts contracted and re-expanded similarly to the embryos frozen in DMSO (Pl. 3, Figs. 15-18, Pl. 4, Fig. 19, Pl. 6, Figs. 35 and 36), and extrusion and swelling of some blastomeres were observed (Pl. 5, Fig. 30, Pl. 6, Fig. 36). Day-7 early expanded and expanded blastocysts, the zona pellucida of which was peeling or completely peeled off, showed various collapses of the trophoblastic layer and the capsule (Pl. 4, Figs. 22-24, Pl. 5, Figs. 27-29). Besides these contraction and collapses, the integrity of the trophoblast was reduced to various extents after thawing. The collapse of the embryos was not necessarily fatal since one severely

TABLE 6. Development of equine embryos stored in 1.0 M-glycerol at  $-196^{\circ}\text{C}$  for 1-214 days

Freeze-thaw method	Age of embryos	Developmental stage of embryos	Medium	No. of embryos		No. of pregnancy
				freeze-thawed	transferred	
Slow	Day-6	EB	S	9	9	2*
		B	S	7	7	1†
	Day-7	B	NS	2	2	1†
		EEXB	S	5	3	1†
			NS	1	1	0
		EXB	S	3	3	1†
	NS		8	6	0	
	Rapid	Day-6	EB	NS	4	4
B			NS	2	2	0
Day-7		B	NS	2	2	0
		EXB	NS	6	6	1†

EB, early blastocyst; B, blastocyst; EEXB, early expanded blastocyst; EXB, expanded blastocyst; S, medium supplemented with 20% mare serum; NS, medium supplemented with no serum.

\* These embryos were aborted at 32-36 days and 43 days after ovulation.

† Recipient foaled.

collapsed Day-7 embryo (Pl. 4, Fig. 24) developed to a foal. In cattle, morphological evaluation of the frozen-thawed embryos was practiced before or after thawing, and the pregnancy rate increased with embryo quality.<sup>19,47,61</sup> In the present study, however, because the inner cell mass could not be well observed, and because the number of embryos examined was so small, it is difficult to establish exact morphological criteria for the viability of frozen-thawed equine embryos.

The physiological function of the zona pellucida *in situ* has not yet been well defined. Half of the rabbit embryos<sup>71</sup>) and about 38% of the sheep embryos<sup>88</sup>) sustained damage to the zona after freeze-thawing. Since rabbit embryos denuded of their zona failed to implant after transfer,<sup>59</sup>) damage to the zona after freezing would seem to affect the viability of rabbit embryos. However, in cattle, WILLADSEN *et al.*<sup>87</sup>) and MASSIP *et al.*<sup>40</sup>) reported that about 30% of the frozen-thawed embryos were damaged in the zona pellucida but that this damage did not impair subsequent development.

The zona pellucida and/or the capsule were broken after freezing in three of 22 Day-6 and six of 27 Day-7 equine embryos. None of these embryos with broken zona and/or capsule developed. In four embryos, extrusion or dispersion of the blastomeres through the cleft of the coverings were observed (Pl. 6, Figs. 31-34), but the damage to the coverings and to the embryo proper seemed to occur independently since damage to the embryo coverings was not always associated with poor morphology of the trophoblast. Recently, LEHN-JENSEN and RALL,<sup>34</sup>) after cryomicroscopic observations suggested that the fracture plane formed in extracellular ice during rapid cooling may cause zona damage. In view of the fact that the capsule envelops the spherical embryo until at least Day-21,<sup>5</sup>) damage to or absence of the capsule after freeze-thawing may be detrimental to the equine embryo.

## 2) *Effect of serum.*

Many researchers have supplemented heat inactivated serum to the freezing medium, but no reasons for the use of serum and no evident effect of serum in freezing embryos have been reported. Whether 20% serum was added or not little affected the morphology of the frozen-thawed equine Day-6 and Day-7 embryos except for the embryo coverings. More embryos frozen in PBS+S (10/29) sustained damage to the zona pellucida and/or the capsule than did embryos frozen in PBS (4/31) (combined data with Day-6 embryos frozen in 1.5 M-DMSO), although there was no significant difference ( $P < 0.05$ ,  $\chi^2$ ).

## 3) *Pre-freezing and post-thawing time, and duration of frozen storage.*

Mean time from uterine flushing to the addition of glycerol and from

the dilution of glycerol to the transfer were 88 min. (range: 56–173 min.) and 69 min. (range: 28–168 min.), respectively. Pregnancies were obtained in embryos kept for 45–141 min., and no effect of pre-freezing and post-thawing time *in vitro* was detected. When frozen-thawed cow embryos were kept at room temperature in modified PBS for more than 3 hr. before transfer, fewer produced pregnancies than embryo transferred within 3 hr. of thawing.<sup>61)</sup>

In this experiment, the longest period of successful storage was 214 days. The possibility of prolonged embryo storage cannot be discussed beyond speculation at present. But liquid nitrogen at  $-196^{\circ}\text{C}$  produces a condition in which there is no further biological activity.<sup>79)</sup> Rat embryos stored for 4 years<sup>79)</sup> and mouse embryos stored for 29 months<sup>82)</sup> developed to birth after the transfer.

#### 4) Embryonic loss.

Only one recipient mare showed extended estrus cycle after transfer of Day-7 embryos. Two mares receiving frozen Day-6 embryos aborted spontaneously (Table 6, Pl. 4, Fig. 20). There were no apparent reasons for these two abortions such as accident or disease. OGURI<sup>49)</sup> and DOUGLAS<sup>17)</sup> reported high embryonic loss of 24% and 30%, respectively, in the pregnant recipient mares following the transfer of unfrozen embryos. In the present experiments, it is unknown whether the freezing or the transfer is related to these abortions.

### 5. Degree of Synchronization

An important factor for successful embryo transfer is the relationship of estrus cycle between the donor and the recipient. In cattle<sup>46,60)</sup> and sheep,<sup>60)</sup> the highest pregnancy rate was obtained when the exact synchronization was made. Similar high embryo survival rates resulted from both synchronous and  $\pm 1$  day asynchronous recipients in the pig.<sup>74)</sup> However, in the frozen-thawed rabbit embryos transferred to the oviduct, TSUNODA *et al.*<sup>71)</sup> reported higher survival rate in the recipient ovulating 18 hr. later than the donor, and suggested a delay of frozen-thawed embryos in the resumption of normal embryonic development. The delay was initially found by WHITTINGHAM<sup>78)</sup> who showed that the cell number of frozen-thawed mouse embryos after 24 hr. culture was smaller than that of unfrozen control embryos. But immediate transfer of frozen blastocysts to the uteri of the recipients one day younger than the age of blastocysts did not prevent the delay. The transfer of frozen cow embryos to the recipients one day earlier in an estrus cycle also did not improve the survival rate.<sup>68)</sup>

On the other hand, in unfrozen equine embryo transfer, the pregnancy rate tended to be greater in the recipients that had ovulated 1 to 3 days

after the donors than in the recipients that had ovulated on the same day or 1 to 2 days before the donors.<sup>17,51,65)</sup> Although there is no evidence, a possible explanation for these data is that equine embryos that have experienced the *in vitro* treatment may undergo such a delay as mentioned above even when they were unfrozen. Therefore, in order to overcome the supposed delay caused by both unfrozen and frozen treatment, almost all embryos in this study were transferred to recipients whose post-ovulatory ages were 1 to 3 days younger than the embryo ages, despite there being no effect of choosing a "younger" recipient as reported above. In practice, a good survival rate was obtained from embryos cooled to 0°C and -5°C. However, since no comparison was made of various degrees of synchrony, the relationship between the developmental stage of unfrozen or frozen embryos and uterine environment is unclear in the present study. Further research is needed to determine whether the "delay" in unfrozen embryos occurs and whether additional freeze-thawing affects the optimum degree of

TABLE 7. Birth weight, gestation length and sex of foals in the successful transfer of the embryos cooled to various minimum temperatures

Recipient No.	Minimum temperature (°C)	Birth weight of foal (kg)	Gestation length* (days)	Sex of foal
25	20	43†	316	female
12	20	33	336	female
16	20	25	327	female
5	0	32	326	male
6	0	36	329	female
2	20	27	327	male
15	-5	27	339	male
19	-5	30	342	male
29‡	-5	35	342	female
21	-10	24	333	male
30‡	-196	42	342	male
28‡	-196	36	320	male
14	-196	23	322	male
15	-196	33	319	female
20	-196	29	323	male

\* Length between the day of ovulation and foaling in the recipient.

† Body weight 8 days after the parturition.

‡ Crossbred recipient mares.

synchronization.

#### **6. Gestation Length and Birth Weight of Foals Developed after the Transfer**

These data are listed in Table 7. Mean gestation length was 330 days (range: 316-342 days). This length is well within the range of that generally reported in naturally mated mares.<sup>29)</sup> Birth weight of foals (mean: 31 kg, range: 24-42 kg) was inherent in Hokkaido native ponies. There have been few reports on abnormal gestation periods in the embryos stored at low temperatures. One report<sup>27)</sup> showed gestation periods a full day longer in rabbit embryos stored at 4°C, suggesting the delay in development after storage. In the equine, even though such a delay occurs after low temperature treatment, the viable embryos would have no influence on gestation length and birth weight of the foal.

#### **7. Future Problems in Freezing Equine Embryos**

The results of the present experiments provide the first evidence that equine embryos can survive freezing and long-term storage at liquid nitrogen temperature and develop normally following thawing and transfer to recipients. But the success rate is so limited that the present technique cannot be applied to practical use. At present, optimum conditions of the freeze-thaw procedure are different between species or developmental stages, reflecting differences in cell size, permeability of membrane, and cytoplasmic property of embryos of different species or of different stages. For the establishment of a good embryo freezing method, not only optimization of empirical methods but also study of the structural and physical-chemical property of embryos is needed, in association with basic cryobiology, which is now growing rapidly.

Compared with cow blastocysts at similar stage, equine early blastocysts or blastocysts show a darker appearance, and may include more lipid droplets. If this greater amount affects the viability of cooled embryos as mentioned earlier, these equine embryos may already be less resistant to cooling to around 0°C.

An acellular membrane characteristic of the equine embryo, the capsule, appears six days after ovulation and remains until at least Day-21. The zona pellucida peels off on and after Day-7. The physiological functions of these structures are not known, but if damage to the capsule and/or the zona pellucida after freeze-thawing is fatal for the embryos developing in the uterus of the foster mother, it is necessary to develop a new freeze-thawing method which can keep these coverings intact.

Short-term culture of embryos is a useful method for the evaluation of

the viability of frozen-thawed embryos because embryos developed in culture may have a high potential of development *in vivo*. However, there is little information on the *in vitro* culture of equine embryos, and further research on this subject is necessary.

### Summary

The possibility of frozen storage of equine embryos was examined. A total of 102 embryos at early blastocyst to expanded blastocyst stage were used in this study. The viability of embryos after each treatment was assessed by non-surgical transfer to the recipient mares.

None of five Day-8 and eleven Day-6 embryos in 1.5 M-DMSO and none of five Day-8 embryos in glycerol developed after storage at  $-196^{\circ}\text{C}$ . Three of 6 Day-8 embryos stored at  $20^{\circ}\text{C}$  in PBS for up to 9.2 hr. developed, one of which was transported 220 km by rail. Two of 4 Day-8 embryos cooled to  $0^{\circ}\text{C}$  and immediately warmed in PBS developed, but two Day-8 embryos stored at  $0^{\circ}\text{C}$  for 7.7 and 20.3 hr. and six Day-8 embryos exposed to 1.5 M-DMSO for 30 min. at room temperature resulted in no pregnancy. The results indicate the availability of short-term storage of Day-8 embryos at room temperature and the viability of Day-8 embryos cooled to  $0^{\circ}\text{C}$ , but suggest that Day-8 large expanded blastocysts may be susceptible to DMSO or freezing and thawing. Day-6 embryos survived 30 min. exposure to 1.5 M-DMSO at room temperature (1/2) and subsequent freezing to  $-5^{\circ}\text{C}$  (3/3) and  $-10^{\circ}\text{C}$  (1/2), whereas no development was found after freezing to  $-15^{\circ}\text{C}$  (0/1) and  $-20^{\circ}\text{C}$  (0/3).

In the presence of 1.0 M-glycerol, the viability of equine embryos stored in liquid nitrogen was clearly demonstrated. Three of 16 Day-6 embryos and three of 19 Day-7 embryos slowly frozen-thawed and one of 8 Day-7 embryos rapidly frozen-thawed showed development. Pregnancies were obtained from all four classified groups: early blastocyst, blastocyst, early expanded blastocyst and expanded blastocyst. Twenty-three percent of deep-frozen Day-6 and Day-7 embryos sustained damage to the zona pellucida and/or the capsule, and did not develop after the transfer, suggesting the deleterious effect of damage to embryo coverings on the viability.

Totals of 10 and 5 foals were produced from the embryos cooled above  $-10^{\circ}\text{C}$  and the embryos stored at  $-196^{\circ}\text{C}$  for up to 214 days, respectively. Gestation length ( $330 \pm 2.4$  days) and birth weight ( $31 \pm 1.5$  kg) of the foals were within the general range of that in naturally mated Hokkaido native pony mares. Successful transfers of the treated embryos resulted in recipients synchronized to  $-1$  to  $-3$  days.

## Appendix

### *In Vitro* Culture of Equine Embryos

*In vitro* culture provides a rapid and useful means of evaluating the viability of frozen-thawed embryos and is particularly efficient in comparison to various other deep-freezing methods. Additionally, the selection of viable frozen-thawed embryos by this means improves the conception rate after the embryo transfer. Presently, little information is available on *in vitro* culture of equine embryos. Then we examined the developmental capacity of fresh equine embryos in varying *in vitro* culture conditions generally used for other mammals.

Early blastocysts were collected on Day-6 and washed with PBS three times at room temperature. The following culture media were used.

1. Ham's F-12 (Gland Island Biological Co.)+1.5% BSA (bovine serum albumine, DAIICH PURE CHEMICALS CO. LTD)
2. TCM-199 (Chiba-kessei)+1.5% BSA
3. PBS+20% heat-treated mare serum
4. MPBS (modified PBS containing 36 mg/l sodium pyruvate, 1 g/l glucose and 4 g/l BSA)+20% heat-treated mare serum
5. BMOC-3 (Gland Island Biological Co.)

100 IU penicillin/ml and 100  $\mu$ g streptomycin/ml were added to all media. After washing with the culture medium, which had been equilibrated overnight with the mixed gas (5% CO<sub>2</sub> and 95% air), the embryos were transferred into Pyrex test tubes containing 1 ml of media, and the mixed gas was blown into the tubes at 300 cc/min for 30 sec. The tubes were immediately sealed firmly with screw caps and were kept in a water bath at 37°C. The embryos were morphologically examined under an inverted microscope at 24 hr. intervals.

TABLE 8. Development of equine Day-6 embryos *in vitro*

Culture medium	No. of embryos cultured	No. of embryos developed to normal expanding blastocyst		
		24 hr.	48hr.	72 hr.
Ham's F-12+1.5% BSA	2	2	2	0
TCM-199+1.5% BSA	2	0	0	0
PBS+20% mare serum	1	0	0	0
MPBS+20% mare serum	1	1	1	0
BMOC-3	2	1	0	0

The results are summarized in Table 8. For the first 24 hr., embryos cultured in Ham's F-12+BSA, MPBS+mare serum or BMOC-3 developed well, and revealed distinct differentiation of inner cell mass and trophoblast cells. But in the TCM-199+BSA and PBS+mare serum cultures, a little development was observed. However, after the first 24 hr., the embryos cultured in Ham's F-12+BSA or MPBS+mare serum developed to normal expanding blastocysts, which, as compared with the same stage of theoretical Day-8 development, were small-sized, indicating a delay of development in embryos cultured for more than 24 hr. One embryo that developed well in BMOC-3 during the initial 24 hr. period shrank after 48 hr. of culture. Poorly-developed embryos in TCM-199+BSA or PBS+mare serum showed signs of degeneration after 48 hr. of culture. Since TCM-199 and PBS media include no sodium pyruvate, this compound may play an important part in the development of equine blastocysts. All embryos were completely degenerated by 96 hr. of culture.

As described in the main paper, equine embryos shed their zonae pellucidae at about 7 days after ovulation. However, in the *in vitro* culture systems employed here, none of the embryos shed their zonae pellucidae.

In conclusion, equine early blastocysts continue morphologically normal development for at least 24 hr. in the media of Ham's F-12+1.5% BSA, MPBS+20% mare serum or BMOC-3. Therefore, it is suggested that these culture methods would apply to the evaluation of the viability of equine embryos.

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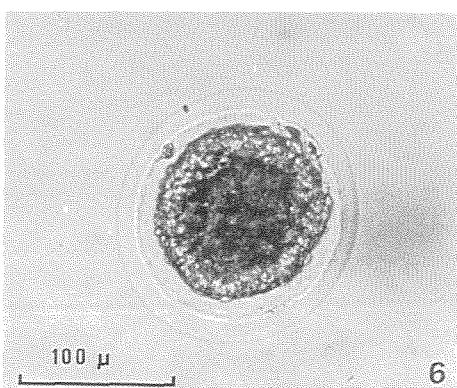
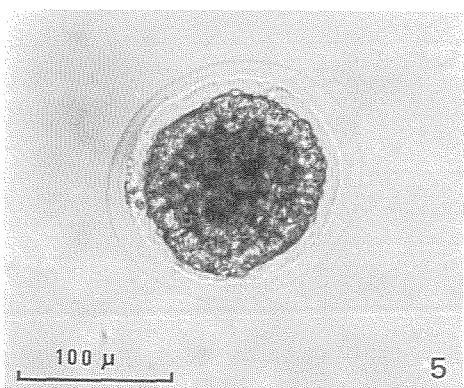
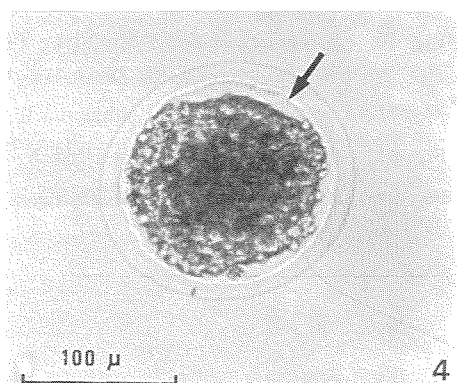
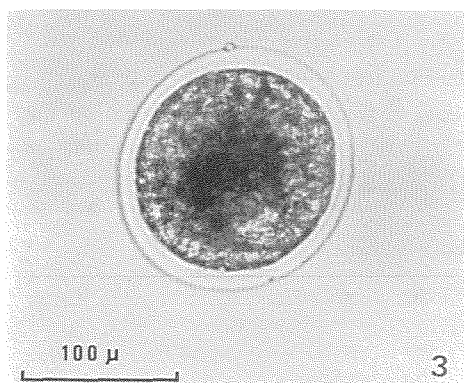
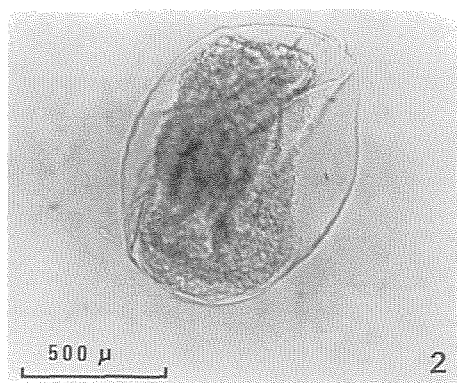
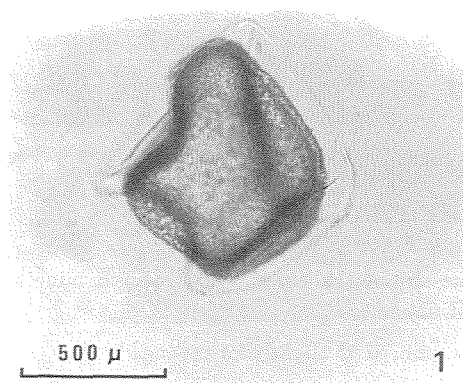
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## LEGEND FOR FIGURES

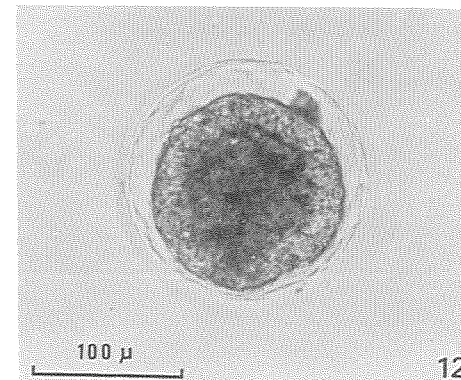
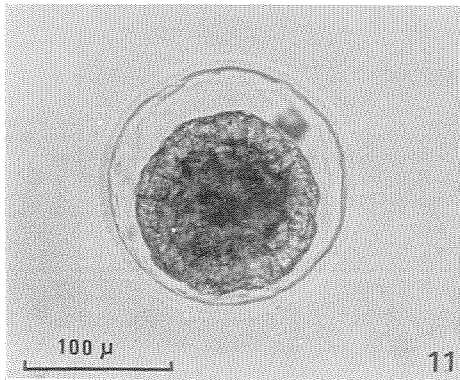
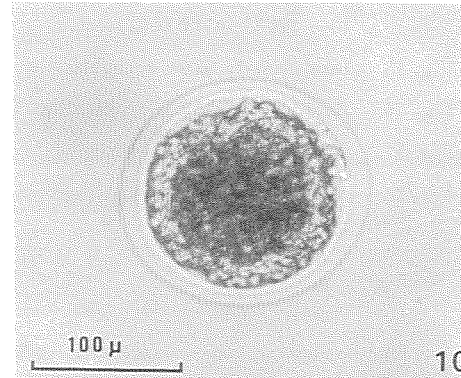
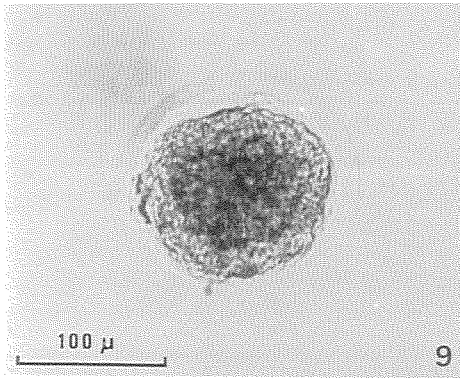
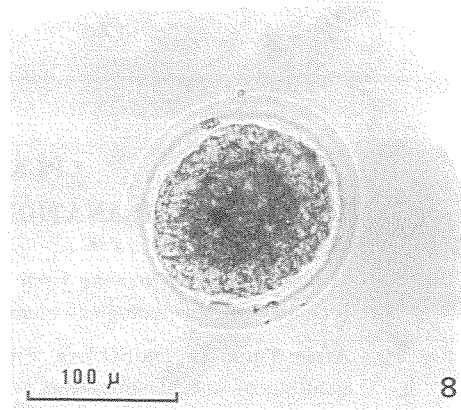
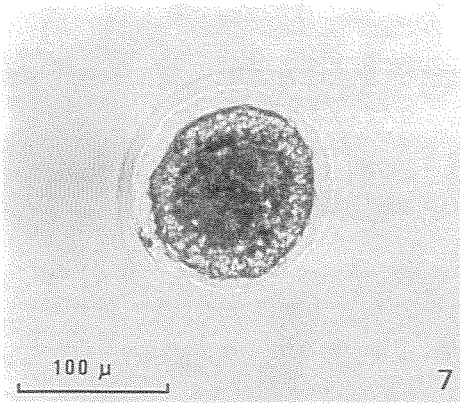
**PLATE 1**  
**EXPLANATION OF FIGURES**

- Fig. 1. Day-8 expanded blastocyst after 25 min. in 1.5 M-DMSO. The embryo collapses at several places.
- Fig. 2. Another Day-8 embryo in 1.5 M-DMSO immediately after thawing from  $-196^{\circ}\text{C}$ . The collapse have advanced and resulted in folding.
- Fig. 3-10. Day-6 early blastocyst subjected to a series of freeze-thaw procedures to  $-10^{\circ}\text{C}$ .
- Fig. 3. Immediately after recovery.
- Fig. 4. After 30 sec. in 0.5 M-DMSO. Contraction of embryo has started and the capsule has become visible and collapsed (arrow). The outer surface of the trophoblast has become irregular.
- Fig. 5. After 9 min. in 1.0 M-DMSO.
- Fig. 6. After 1.3 min. in 1.5 M-DMSO.



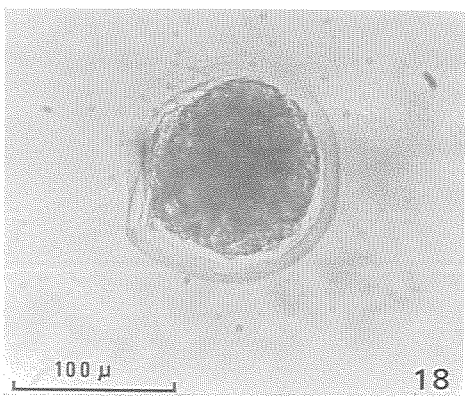
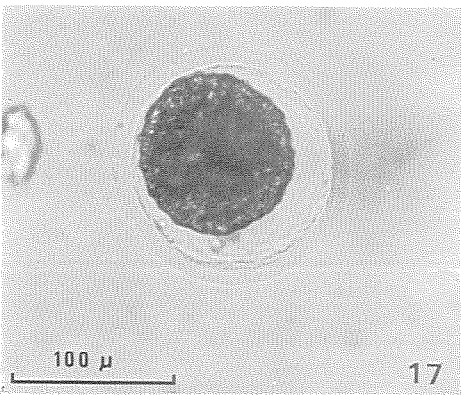
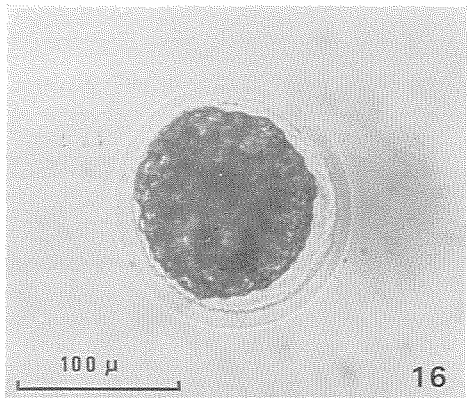
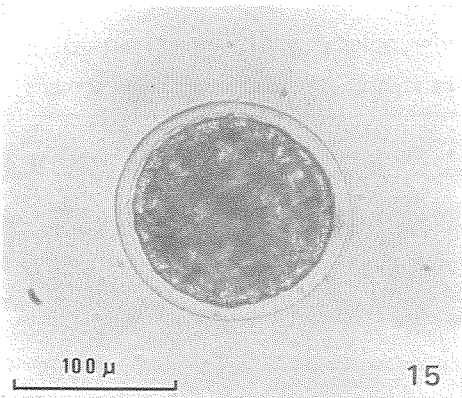
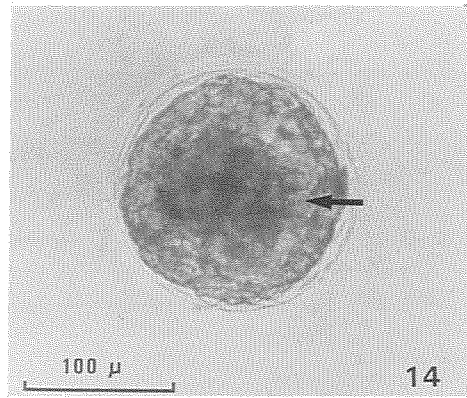
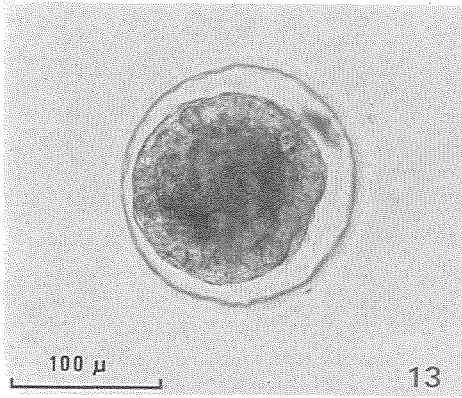
**PLATE 2**  
**EXPLANATION OF FIGURES**

- Fig. 7. After 12 min. in 1.5 M-DMSO. The size of the embryo has reached minimum diameter (about 80% of initial diameter).
- Fig. 8. After 28 min. in 1.5 M-DMSO. The embryo is slightly re-expanded.
- Fig. 9. Immediately after thawing from  $-10^{\circ}\text{C}$  to room temperature in 1.5 M-DMSO.
- Fig. 10. After 10 min. in DMSO-free PBS+S. The embryo has re-expanded, but a little perivitelline space still remains. This embryo developed to a foal.
- Fig. 11-14. Day-6 blastocyst subjected to a series of freeze-thaw procedures to  $-20^{\circ}\text{C}$ .
- Fig. 11. After 2.5 min. in 1.5 M-DMSO.
- Fig. 12. After 28 min. in 1.5 M-DMSO.



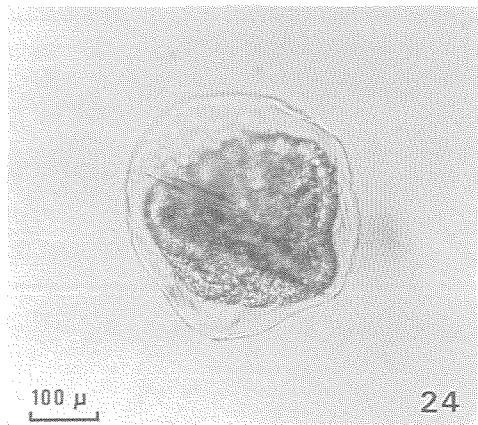
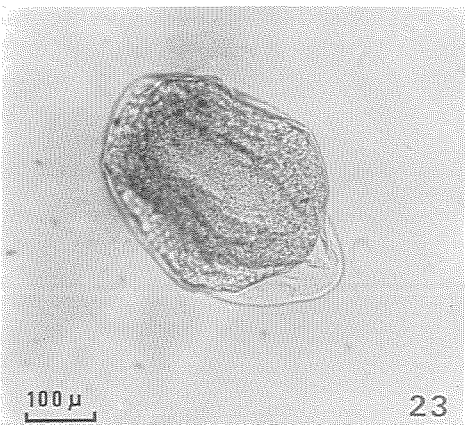
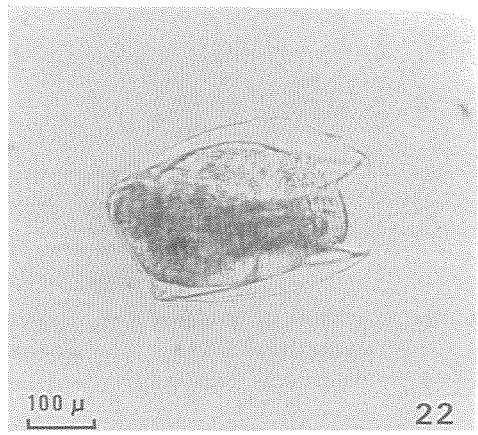
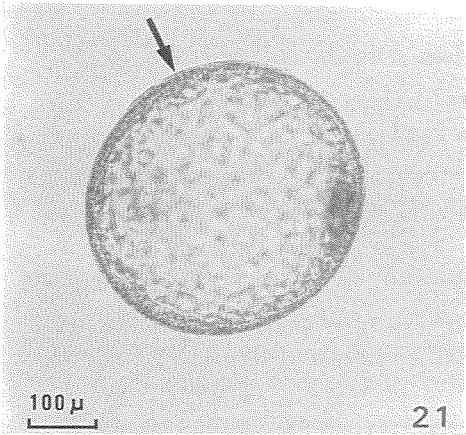
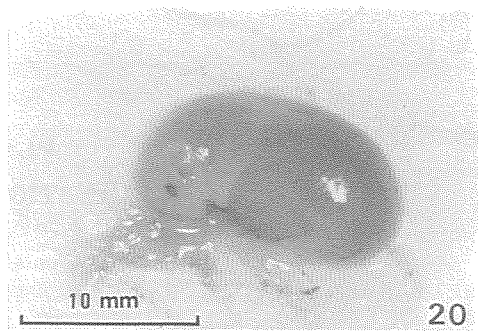
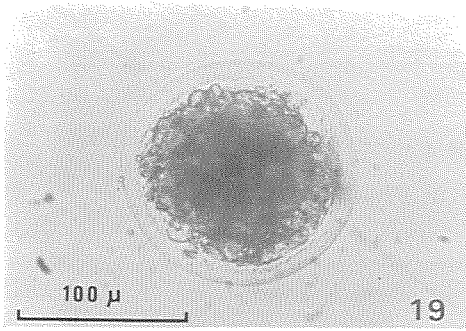
**PLATE 3**  
**EXPLANATION OF FIGURES**

- Fig. 13. *Immediately after thawing from  $-20^{\circ}\text{C}$  to room temperature in 1.5 M-DMSO. It shows no morphological changes as compared with that before freezing.*
- Fig. 14. *After 9 min. in DMSO-free PBS+S. The embryo has re-expanded and swelling of one blastomere is seen (arrow).*
- Fig. 15-19. *Day-6 early blastocyst subjected to a series of slow freeze-thaw procedures to  $-196^{\circ}\text{C}$  in 1.0 M-glycerol.*
- Fig. 15. *Immediately after recovery.*
- Fig. 16. *After 3.5 min 0.5 M-glycerol.*
- Fig. 17. *Ater 2 min. in 1.0 M-glycerol.*
- Fig. 18. *Immediately after thawing from  $-196^{\circ}\text{C}$  to room temperature in 1.0 M-glycerol.*



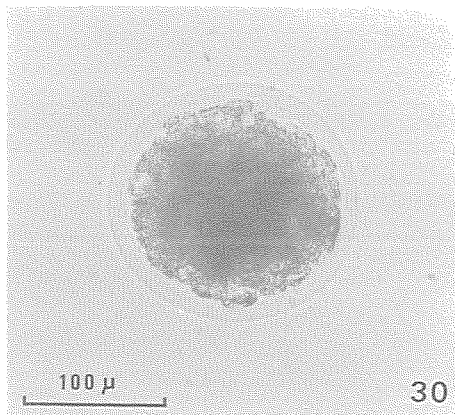
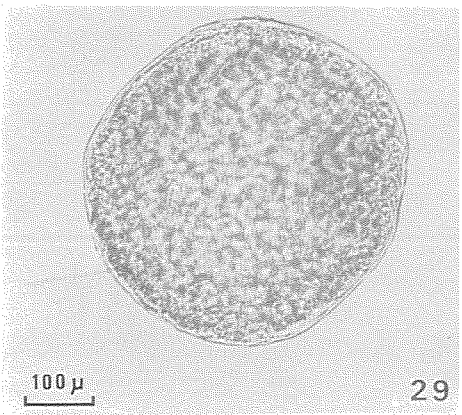
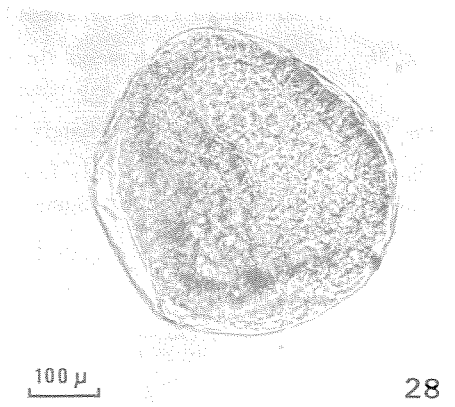
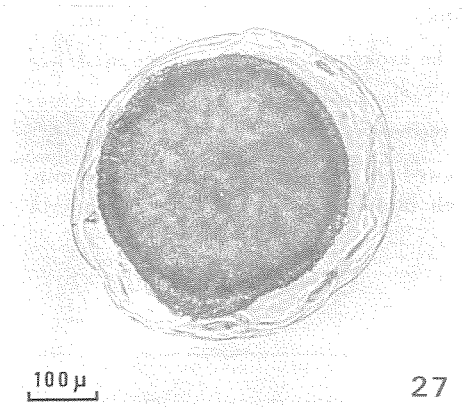
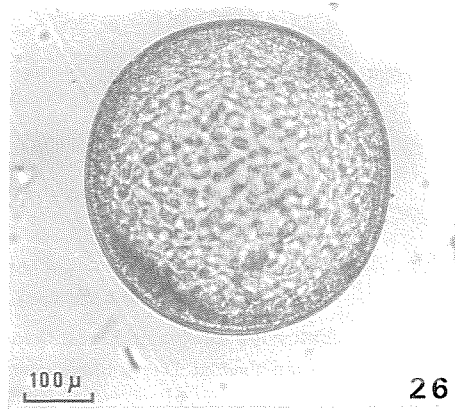
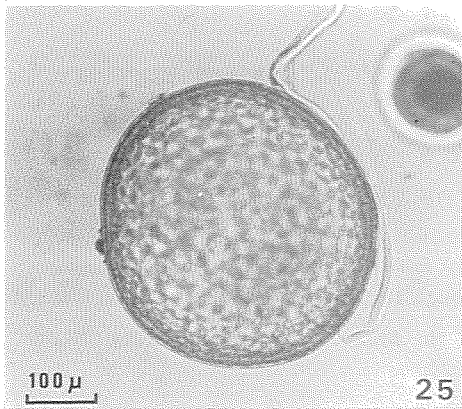
**PLATE 4**  
**EXPLANATION OF FIGURES**

- Fig. 19. After 43 min. in glycerol-free PBS+S.
- Fig. 20. Fetus developed from the same embryo after transfer. It was aborted at Day-43.
- Fig. 21-24. Day-7 expanded blastocyst subjected to a series of slow freeze-thaw procedures to  $-196^{\circ}\text{C}$  in 1.0 M-glycerol.
- Fig. 21. Immediately after recovery. The zona pellucida partly remains (arrow).
- Fig. 22. After 28 min. in 1.0 M-glycerol. The embryo and the capsule have severely collapsed.
- Fig. 23. Immediately after thawing from  $-196^{\circ}\text{C}$  to room temperature in 1.0 M-glycerol.
- Fig. 24. After 25 min. in glycerol-free PBS+S. It remained collapsed until the transfer. This embryo developed to a foal.



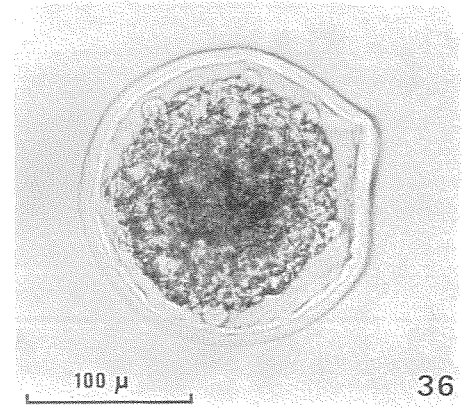
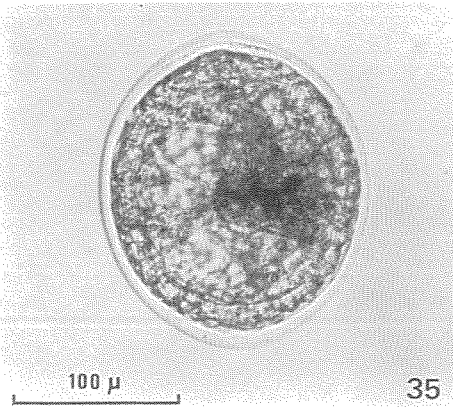
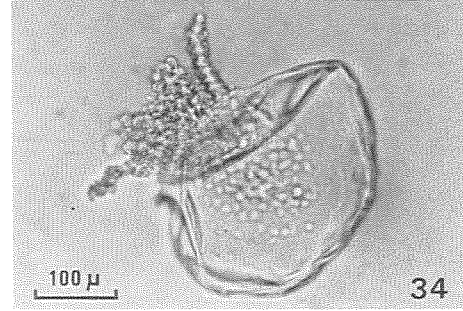
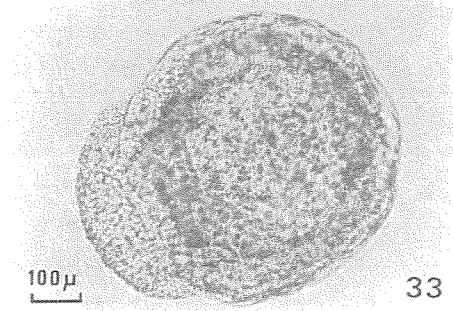
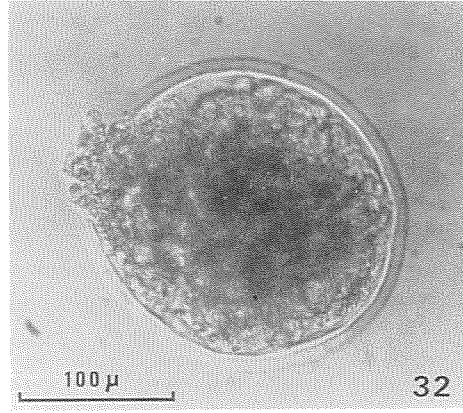
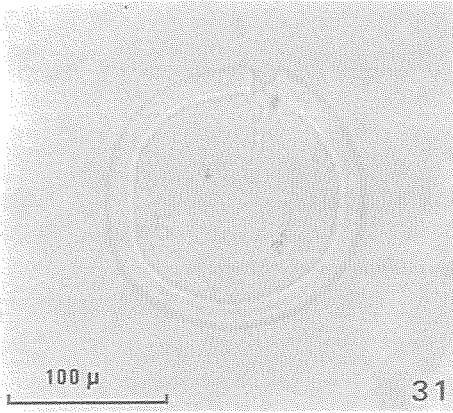
**PLATE 5**  
**EXPLANATION OF FIGURES**

- Fig. 25. Day-7 expanded blastocyst immediately after recovery. The zona pellucida is peeling off.
- Fig. 26-29. Day-7 expanded blastocyst subjected to a series of rapid freeze-thaw procedures to  $-196^{\circ}\text{C}$ .
- Fig. 26. Immediately after recovery. The zona pellucida has completely peeled off.
- Fig. 27. After 25 min. in 1.0 M-glycerol. The embryo has contracted but collapse has not yet occurred except for the capsule.
- Fig. 28. Immediately after thawing from  $-196^{\circ}\text{C}$  to room temperature in 1.0 M-glycerol. The embryo has collapsed but re-expanded as a whole.
- Fig. 29. After 69 min. in glycerol-free PBS. The embryo has fully re-expanded, but did not develop to a foal after transfer.
- Fig. 30. Day-6 early blastocyst in glycerol-free PBS+S after slow freezing to  $-196^{\circ}\text{C}$  and slow thawing. Extrusion of some blastomeres within the zona pellucida is seen. This embryo developed after transfer, but was aborted at Day-32 to 36.



**PLATE 6**  
**EXPLANATION OF FIGURES**

- Fig. 31. Damaged zona pellucida of rapidly frozen-thawed Day-6 early blastocyst. The blastomeres have escaped through the cleft of the zona.
- Fig. 32. Extrusion of blastomeres through the broken part of the zona in slowly frozen-thawed Day-6 blastocyst.
- Fig. 33. Extrusion of blastomeres through the broken part of the capsule in rapidly frozen-thawed Day-7 expanded blastocyst.
- Fig. 34. Dispersion of blastomeres through the broken part of the capsule in slowly frozen-thawed Day-7 expanded blastocyst.
- Fig. 35. Day-7 blastocyst immediately after recovery.
- Fig. 36. The same embryo slowly frozen-thawed in 1.0 M-glycerol and after 30 min. in glycerol-free PBS. Some swollen blastomeres are seen. This embryo developed to foal.



**PLATE 7**  
**EXPLANATION OF FIGURES**

- Fig. 37. Day-7 early expanded blastocyst immediately after recovery.
- Fig. 38. The same embryo slowly frozen-thawed in 1.0M-glycerol and after 47 min. in glycerol-free PBS+S. This embryo developed to a foal.
- Fig. 39. The first foal (male) in the world developed from an embryo stored in liquid nitrogen. The breeds of the foal and the recipient are Hokkaido native pony and crossbred horse, respectively.

