

## Survival of 16-Celled and Morula Stage Rabbit Embryos Frozen to $-196^{\circ}\text{C}$

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Preimplantation stage (16-celled and morula) rabbit embryos were successfully frozen to  $-196^{\circ}\text{C}$ . The cooling rate (from a room temperature to  $0^{\circ}\text{C}$ ), the presence of the mucin layer surrounding embryos, the ice-seeding treatment and the thawing procedure were examined to determine their effects on the survival of the frozen embryos of Japanese white, New Zealand white and Dutch-Belted rabbits. A high proportion (51%; 16-celled, 69%; morula) of Dutch-Belted rabbit embryos developed *in vitro*, when they were frozen to  $-196^{\circ}\text{C}$ , applying the ice-seeding at  $-4^{\circ}\text{C}$  in the presence of 12.5% DMSO, after being cooled to  $0^{\circ}\text{C}$  at the rate of  $7-9^{\circ}\text{C}/\text{min}$ , and were diluted by a stepwise addition of 4 different strength PBS on thawing. The highest rate of *in vitro* development (81%; Japanese white, 75%; New Zealand white, 82%; Dutch Belted embryos) was obtained when the morula stage embryos were frozen to  $-196^{\circ}\text{C}$  applying seeding at  $-4^{\circ}\text{C}$  after being cooled to  $0^{\circ}\text{C}$  at the rate of  $1^{\circ}\text{C}/2.5\text{min}$  and were diluted, on thawing, by stepwise addition of 6, 3 and 1% DMSO solution and a culture medium. No great difference was found in the survival rate between the embryos covered with the mucin layer and those which had not the coat. All the embryos frozen without applying seeding treatment failed to develop *in vitro* after being thawed and diluted. Nine out of 27 does each of which received 6 reimplantations of the embryos frozen-thawed became pregnant and were found to be carrying 37 normal fetuses on the 12th day of pregnancy.

### Introduction

Whittingham, Leibo and Mazur [3] and Wilmut [20] reported independently that they succeeded in obtaining a high proportion of survival in the mouse embryos which were stored at  $-196^{\circ}\text{C}$ . In these studies an administration of Dimethyl Sulfoxide (DMSO) as a cryoprotective agent and concomitant an ice-seeding treatment were employed for freezing. This system of freezing storage has been applied not only for the storage of mouse embryos but also for the embryos of another laboratory animals, and similar survivorship after being frozen and thawed was obtained in the mouse

[6, 8, 9, 12, 13, 14, 16, 18, 19, 22], rat [12, 13] and rabbit [1, 2, 7, 12, 13, 14, 17] embryos. A report [15] of high survival of rat embryos frozen without seeding treatment, however, has not been confirmed by other laboratories. Some successes in the freezing storage of farm animal embryos, applying seeding, have also been achieved with cow embryos [21, 22], but high survival rate has not yet been obtained.

Although the presence of the thick mucin layer surrounding the zona pellucida of the ovum of more than 2-celled stage in the oviduct is a characteristic feature in the rabbit embryos, the embryos seem to have something common with the farm animal embryos in the tolerance to freezing and thawing, because they are conside-

ably larger than the mouse and rat embryos of the equivalent stage.

In the following report we wish to describe a study to obtain an authentic method for the deep freezing storage of rabbit embryos, examining the effects of the cooling rate to 0°C, the removal procedure of DMSO from embryos, the presence of the mucin layer covering embryos and the ice-seeding treatment on the survival of different strain rabbit embryos frozen and thawed.

### Materials and Methods

Japanese white, New Zealand white and Dutch-Belted rabbits were induced to ovulate by the subcutaneous injection of Pregnant Mare Serum gonadotrophin (PMS) 50 i.u. daily for 4 days and the intravenous injection of 50 i.u. Human Chorionic gonadotrophin (HCG) 48 hours after the last injection of PMS. Sixteen-celled and morula stage embryos were collected from the oviducts at 48 and 68 hours respectively following HCG injection and copulation. Some rabbit ova were collected at the mono-celled stage 15 hours after copulation, which were not surrounded by the mucin layer, and were cultured in a medium [11]. Upon reaching the morula stage in the absence of the covering mucin layer, the embryos were subjected to the freezing study.

Each 6-12 embryos thus collected or cultured *in vitro* were transferred into a pyrex tube (10 mm in diam. 65 mm in length) containing 0.1 ml of a medium [11] at room temperature (20-24°C) and cooled to 0°C at two different cooling rates; the one was at 1°C/2.5 min (slow precooling) and the other at 7-9°C/min (rapid precooling). On cooling, each tube at 2°C received 0.05 ml of 25% DMSO solution (PBS)

previously cooled to 2°C and after 5 minutes received again the same amount of the DMSO solution. The fluid of the tube was then mixed by shaking and maintained at 0°C for 20 minutes. For freezing, 3 different rates of cooling were employed; (1) the tube was cooled to -4°C at the rate of 1°C/2 min and a minute of ice-crystal was then introduced onto the surface of the tube medium to induce crystalline formation. The ice-crystal formation immediately followed and eventually spread towards bottom of the tube, which proceeded to fill the entire medium with a soft crystalline mass within 3 minutes. After 5 minutes keeping at -4°C, the temperature of the tube was lowered to -15°C at the rate of 1°C/3 min then to -80°C at 1°C/min, (2) the tube was cooled at the same rate as in the group (1), but the seeding treatment was not applied and (3) the tube was cooled, without seeding, to -15°C at the rate of 1°C/3 min, from -15 to -40°C within 12-13 minutes and to -80°C at the rate of 1°C/min. When the temperature of the tube reached -80°C, all the tubes frozen were transferred into a gas phase of Liquid Nitrogen (LN) in a LN cabinet and 15 minutes later dipped in LN and stored for 5 to 30 days.

Thawing was produced by placing the tubes in a -120°C alcohol bath (100 ml in a glass flask of 12 cm in diam. 15 cm in depth) and exposure to room temperature. Upon reaching -100°C, the alcohol bath was warmed at the rate of 6°C/min by gradual addition of -20°C alcohol. When the bath reached -4°C, a group of samples was diluted by addition of 0.2 ml of PBS containing 6% DMSO previously kept at 30°C. The tube was immediately transferred into a water bath kept at 35°C and further diluted by stepwise addition of 0.4 and 0.8 ml of PBS containing 3 and 1% DMSO respectively

and 1 mℓ culture medium [11] at 4 minutes intervals. The other group of samples was diluted by the method reported by Bank and Maurer [2] with some exceptions; 0.2 mℓ of hypertonic PBS [4 times single strength(4XSS)] kept at 35°C was introduced into the sample, and the tube was immediately transferred into a 35°C water bath and diluted by adding PBS of 0.2 and 0.4 mℓ (2XSS), 1.0 mℓ (1XSS) and 1.0 mℓ (1/2XSS) at 5 minutes intervals. The tubes were allowed to equilibrate at room temperature for further 15 minutes.

All the samples were transferred to the fresh medium [11] and washed thrice with the renewed medium.

Each samples was examined through phase contrast microscope, and any embryos displaying signs of damage or degeneration (shrinkage and marked indentation of the cell membrane were dominant in these cases) were discarded. The embryos of normal appearance were cultured *in vitro* for development in 3 mℓ of a medium maintained at 37°C for 2 days. A combined synthetic medium (without serum), which was reported by Ogawa et al [11], was used for the freezing, diluting and washing the samples throughout the whole series of this experiment, and for the culture of the embryos was used the medium (with serum) and the closed culture system [10].

The final test of viability of the frozen embryos was made by transferring the embryos to the oviduct or uterine horn of the recipient of the same breed. All the embryos used for the transfer study had the mucin layer and were frozen with the seeding treatment after the slow precooling. On thawing, the stepwise dilution by PBS containing DMSO and the culture medium was employed. The morula stage embryos, which showed normal looking morpho-

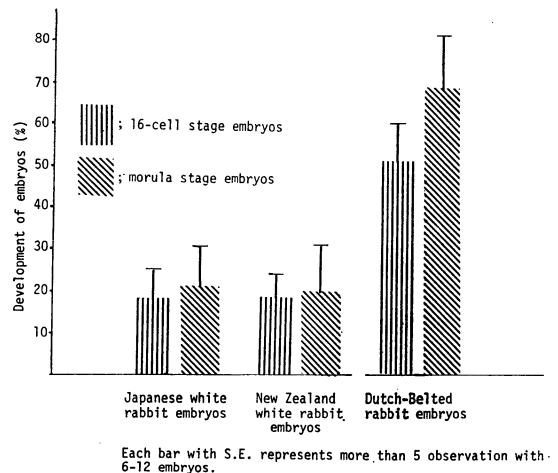
logy on thawing, were transferred immediately after washing to the oviduct of recipients on the 2.5 th day of pseudopregnancy. The blastocysts, obtained by *in vitro* culture after thawing the morula stage embryos, were introduced into the uterine horns, near the uterotubal junction, on the 3.5 th day of pseudopregnancy. Each recipient received 6 embryos. The recipients were rendered pseudopregnant by sterile copulation with the vasectomized male and were inspected by laparotomy on the 12 th day of pregnancy for the presence of fetuses.

Test of significance were performed using  $X^2$  analysis for the rate of *in vitro* development of the embryos frozen and thawed.

## Results

*In vitro* survival of 16-celled and morula stage embryos frozen, thawed and diluted by stepwise addition of hyper- to lower tonic PBS (Fig. 1.).

Fig. 1. Survival of the rabbit embryos which were frozen with seeding after rapid precooling and diluted by stepwise addition of 4 different strength PBS on thawing.

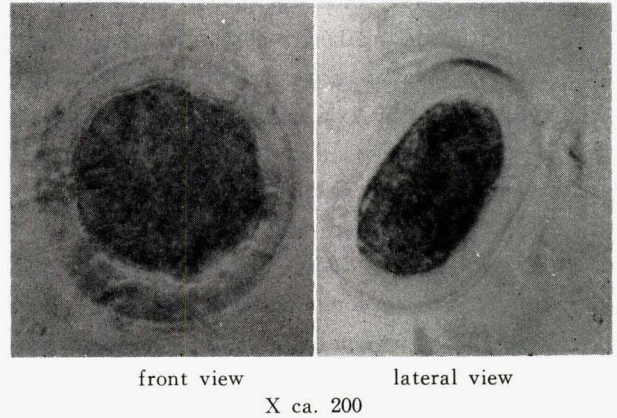


The rapid precooling and the seeding at  $-4^{\circ}\text{C}$  were employed in this experiment. The stepwise addition of 4 different strength PBS upon thawing and dilution to remove DMSO worked well with the embryos from Dutch-Belted rabbits, but poorly with New Zealand and Japanese white rabbits. Out of 45 (16-celled) and 45 (morula) embryos from Dutch-Belted strain, 23 (51%) and 31 (69%) respectively developed *in vitro* into morula and blastocysts. On the contrary, a high proportion of New Zealand and Japanese white strain embryos, although being normal in appearance upon thawing, failed to develop *in vitro*, and out of 95 (16-celled) and 76 (morula) embryos, only 17 (18%) and 16 (21%) respectively cleaved to develop within 24 hours. The difference of survival rate was significant ( $p < 0.005$ ) between Dutch-Belted embryos and the other two strain embryos, but it was not significant ( $p > 0.1$ ) between Japanese and New Zealand strain embryos.

An observation of particular interest was made on the morphology of the rabbit embryos; The embryos recovered at 16-celled stage from the oviduct of Dutch-Belted rabbits could easily be distinguished in shape from those of the other two strain rabbits at equivalent stage, and it was found out that the most of the embryos from Dutch-Belted rabbits were of an elliptical form at their lateral side (Fig.2.). For example, we could observe the shape of lateral side, by shaking the medium in order to rotate the embryos under microscopic field. It is therefore considered that the embryos from Dutch-Belted strain, as a whole, are of a form of thick disc, presenting a striking contrast to spherical form of the other two strain embryos.

*In vitro survival of morula stage embryos with and without the mucin layer frozen*

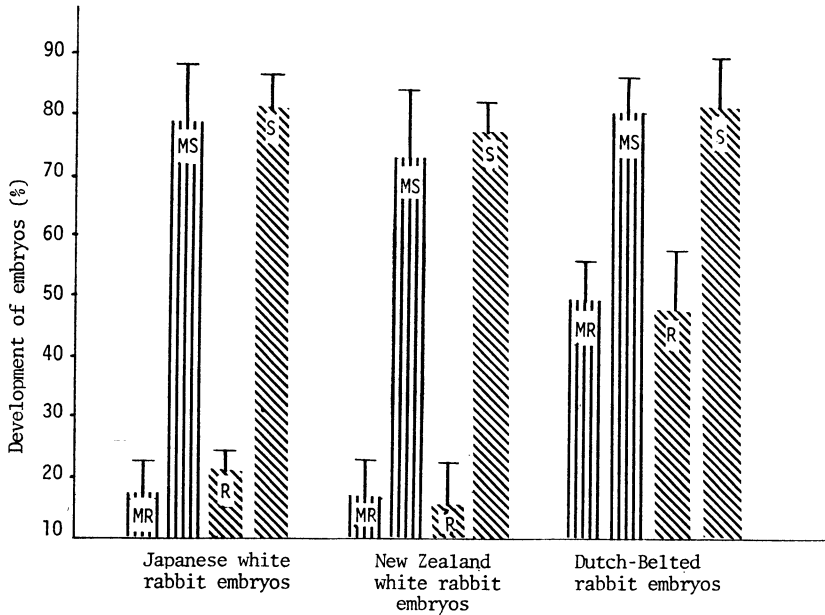
Fig. 2. Morula stage embryo of Dutch-Belted rabbit



*with seeding treatment and thawed in stepwise addition of PBS containing DMSO and culture medium (Fig.3.).*

Extending from 60 to 94% of the embryos were morphologically normal when recovered upon thawing and dilution. Many of the embryos from Japanese and New Zealand white rabbits, which were treated by the rapid precooling before freezing, however, failed to develop *in vitro* after being frozen-thawed. Out of 135 embryos from Japanese white rabbits and 70 from New Zealand white rabbits, 25 (19%) and 11 (16%) respectively developed into the early blastocyst stage. The cultivation of the remaining 169 embryos caused darkening or roughening of the cell membrane and shrinkage of the cell. When the slow precooling was applied, the freezing and thawing procedures resulted in a high rate of survival in these two strain embryos. Out of 119 embryos from Japanese strain and 77 embryos from New Zealand strain, 96(81%) and 58 (75%) respectively developed into the early or expanding blastocyst stage. Although the rapid precooling was applied, a considerably high rate (48%) of Dutch-Belted embryos developed into the blastocyst stage. The rate of development increased in this strain embryos

Fig. 3. Effects of precooling speed and mucin layer surrounding embryos on *in vitro* development of morula stage embryos frozen and thawed.



Each bar with S.D. represents more than 5 observations with 6-12 embryos/tube. Seeding; at  $-4^{\circ}\text{C}$ . Thawing and dilution; stepwise addition of 6, 3 and 1% DMSO solution and a culture medium.

||MR||; embryos, which had the mucin layer, frozen after the rapid precooling.

||MS||; embryos, which had the mucin layer, frozen after the slow precooling.

||R||; embryos, which had not the mucin layer, frozen after the rapid precooling.

||S||; embryos, which had not the mucin layer, frozen after the slow precooling.

when the slow precooling was employed; out of 106 embryos frozen and thawed, 87 (82%) developed into the early or expanding blastocyst.

The difference in the rate of development was significant ( $p < 0.001$  in Japanese and New Zealand embryos and  $p = 0.005$  in Dutch-Belted embryos) between the embryos frozen after the slow precooling and those frozen after the rapid precooling, ( $p < 0.001$ ) between Dutch-Belted embryos and the other two strain embryos, which were frozen after the rapid precooling and not significant ( $p < 0.1$ ) among these three strain embryos frozen after the slow precooling. No great difference in the rate of *in vitro* development was found between the embryos

which had the mucin layer and those which had not, and the small difference recorded was not significant ( $p < 0.1$ ). There was no difference ( $p < 0.1$ ) in the viability between the embryos stored for 5 days and those for 30 days.

*In vitro* development of the morula stage embryos frozen with and without applying seeding treatment (Table 1.).

This experiment was conducted with the embryos from Dutch-Belted and Japanese white rabbits. All the embryos were frozen after the slow precooling and the dilution sequence employed was the same as those of serial addition of PBS containing DMSO and the culture medium. In case when seeding was applied for

freezing, a high rate (86%) of the embryos remained normal in morphology upon thawing and dilution. Out of 74 embryos frozen and thawed, 57 (77%) developed *in vitro* into the blastocyst stage in 2 days culture. No difference in the viability was found between these two strain embryos. When frozen without applying

seeding treatment, all the 150 embryos failed in showing any signs of development *in vitro* irrespective of freezing speed, though a few of them were in normal appearance at thawing.

*In vivo development of the frozen and thawed embryos* (Table 2.).

In the transfer studies of the morula stage

Table 1. Effect of ice-seeding on *in vitro* development of morula stage embryos frozen to  $-196^{\circ}\text{C}$

Seeding	Embryos (strain)	No. embryos frozen-thawed	No. embryos in normal shape on thawing	No. which developed <i>in vitro</i> into blastocyst
Applied at $-4^{\circ}\text{C}$	J. W.	34*	29 (85%)	26 (76%)
	D. B.	40*	35 (88%)	31 (78%)
not applied	J. W.	49**	3 (6%)	0
		39***	5 (12%)	0
	D. B.	28**	5 (18%)	0
		34***	0	0

Precooling ; at  $1^{\circ}\text{C}/2.5\text{min}$ .

Thawing and dilution ; by stepwise addition of 6, 3 and 1% DMSO sol. and a culture medium.

J.W. ; Japanese white rabbit, D.B. ; Dutch-Belted rabbit.

\* ; cooled from  $0^{\circ}\text{C}$  to  $-4^{\circ}\text{C}$  at  $1^{\circ}\text{C}/2\text{min}$  (seeded at  $-4^{\circ}\text{C}$ ), from  $-4^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  at  $1^{\circ}\text{C}/3\text{min}$  and from  $-15^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$  at  $1^{\circ}\text{C}/\text{min}$ .

\*\* ; cooled similarly with the group\* but not applied seeding treatment.

\*\*\* ; cooled from  $0^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  at  $1^{\circ}\text{C}/3\text{min}$ , from  $-15^{\circ}\text{C}$  to  $-40^{\circ}\text{C}$  within 12-13 min and to  $-80^{\circ}\text{C}$  at  $1^{\circ}\text{C}/\text{min}$ .

Table 2. Summary of the results of transferring morula and blastocysts.

Embryos (strain)	No. of transferred morula blastocysts	No. of recipients	No. of pregnancies	No. of fetuses	No. of live born
J. W.	42	7	3	14(6, 6, 2)	14
	36*	6	2	6(4, 2)	3
D. B.	48	8	3	11(4, 4, 3)	10
	36*	6	1	6(6)	6

Precooling ; at  $1^{\circ}\text{C}/2.5\text{min}$

Seeding ; at  $-4^{\circ}\text{C}$

Thawing and Dilution ; by stepwise addition of 6, 3 and 1% DMSO sol. and culture medium

\* ; cultured *in vitro* from the morula stage after being frozen and thawed, then subjected to the transfer study

\*\* ; inspected by surgical procedure on the 12th day of pregnancy

J.W. ; Japanese white rabbit. D.B. ; Dutch-Belted rabbit.

embryos, 3 out of 7 Japanese white does (received 42 implants) became pregnant; the 2 does had 6 fetuses respectively and the other one had 2 fetuses. In Dutch-Belted rabbits, 3 out of 8 does (received 48 implants) became pregnant having 4, 4 and 3 fetuses respectively. Therefore, 25 out of 36 implants in these 6 rabbits successfully resulted from the transfer of the embryos frozen and thawed. Seventy two blastocysts were transferred to 12 recipients (6 Japanese and 6 Dutch-Belted rabbits), out of which 3 became pregnant, having 6, 4 and 2 fetuses respectively. therefore 12 out of 18 implants in these 3 rabbits successfully resulted from the transfer of the blastocysts.

No anomaly was apparent in the fetuses, and their postnatal development appeared to be unaffected.

### Discussion

The rate of *in vitro* development was higher in the embryos frozen applying the slow precooling than those treated with the rapid precooling. Among The three strain rabbit embryos, the difference was quite dominant in the embryos from Japanese white and New Zealand white rabbits compared with those from Dutch-Belted rabbits. No great difference ( $p < 0.1$ ) in the proportion of normal looking embryos (on thawing and diluting) were found between the embryos frozen with the slow precooling and those frozen with the rapid precooling. It is therefore considered that the cooling speed before the seeding and freezing eventually affects on the embryonic survival after being frozen and thawed, though it may not induce any noticeable changes in their morphology on thawing.

In the transfer study, the embryos stored

extending from 5 to 30 days proved to develop normally *in vivo*. The rate of development, however, was not so high as in the case of *in vitro*. This might be due to the failure in the transfer procedure or due to the failure in obtaining the fulfilled condition of the recipients to implant. The results obtained accordingly indicate that the 16-celled and morula stage embryos of rabbits can successfully be stored in a frozen state at  $-196^{\circ}\text{C}$ , when DMSO is added at the concentration of 12.5% to the medium and a slow rate of precooling combined with a seeding treatment at early stage of freezing is applied.

Although the rapid precooling was applied, the frozen and thawed embryos from Dutch-Belted rabbits still showed a relatively high rate of survival compared with those from Japanese and New Zealand rabbits. Especially in the case when the stepwise dilution by different strength PBS was employed according to the method of Bank and Maurer [2], 51 to 69% of the embryos developed *in vitro* after thawing. This means that the embryos from Dutch-Belted rabbits might be less damaged by freezing and thawing than the other two strain embryos. The reason why the embryos of this strain can survive in a high rate after being frozen and thawed remains unknown at present. One possible explanation for this phenomenon is that the characteristic morphology of its disc form may afford a benefit for an adaptation to the distortion due to the rapid precooling to  $0^{\circ}\text{C}$ , for a rapid permeation or removal of DMSO, for easy recovery to its original form upon thawing and thus finally less damage are given to this strain embryos on freezing and thawing.

The mucin layer covering the entire surface of embryos may affect the tolerance to the stress of freezing and thawing, acting, for

example, as a partial barrier which prevents rapid permeation and removal of DMSO and rapid osmotic equilibrium between the embryos and the external medium. However, no great difference in the morphology and *in vitro* development were found between the embryos covered with the layer and those which had not the layer after being frozen and thawed.

Utsumi and Yuhara [15] reported that the rat embryos could be stored at  $-79^{\circ}\text{C}$  by freezing without applying seeding treatment. Their report is quite in contrast to our results. When the seeding treatment was not employed at early stage of freezing, the storage resulted in complete failure in obtaining *in vitro* development after freezing and thawing. This result suggests that the seeding treatment is necessary to obtain embryonic survival of rabbit by freezing storage. It is of our opinion that an extrem rapid and hard crystallization followed by a super-cooling, which is eventually produced subsequent to a slow cooling upon freezing, may give a mechanical damage on the entire embryonic mass.

In order to examine the effect of ice-seeding on the characteristics of the freezing medium, an attempt was made to check the concentration of DMSO upon seeding; A small amount of fluid in the tube was removed after seeding from the basal portion of a slush-like medium in the tube with a hypodermic needle (by inserting it to attach the basal wall of the tube) and a tuberculine syringe. The concentration of DMSO in the fluid collected was determined by the colorimetry on the density of the color (dark violet) which was produced [13] by adding an Osmic acid solution to the PBS containing 12.5% DMSO at  $0^{\circ}\text{C}$ . It was found that the concentration of DMSO in the collected fluids at  $-5$ ,  $-7$  and  $-9^{\circ}\text{C}$  was *ca.* 35, 58 and 76%

respectively. It is probable that the increase in DMSO and electrolyte concentration in a slush-like medium during early stage of freezing brings about a condition where the freezing point is lowered as water freezes out of the mixture, and that the ice-seeding combined with a slow cooling may result in an even increase of DMSO concentration and provide the embryos with a special condition whereby the cells are protected from the damage due to freezing. For example, as one of protective effects, this condition might have an effective salt buffer effect, as was suggested by Lovelock [4] and Lovelock and Polge [5] on the protective action of Glycerol, which minimizes electrolyte damage, consequent upon the decrease of intra-cellular ice formation due to the enriched concentration of DMSO. It seems that a part of the toxicity of DMSO may be eliminated not only by its low temperature administration but also by a stepwise dilution on thawing.

Whittingham and Whitten [18] found that 95% of C57BL/6J mouse embryos which had been frozen and thawed developed *in vitro* to the blastocyst stage, whereas only 58% of BALB/cwt embryos similarly treated developed *in vitro* to the blastocyst stage. Similar breed difference was demonstrated by Maurer and Haseman [7] in the morula stage rabbit embryos. They found that *in vivo* survival of the rabbit embryos, after being frozen and thawed using the method of Bank and Maurer [2], was distinctly higher in Dutch-Belted embryos than in New Zealand white embryos. The breed difference was also found in the present study. When the method of Bank and Maurer [2] was employed for freezing and thawing, a higher percentage of Dutch-Belted embryos developed *in vitro* to the blastocyst stage in comparison to Japanese white and New Zealand white

embryos. Therefore, it appears that a freezing-thawing method, which proved to be adequate for the storage of the embryos of a breed of animals, may not always work well for the embryos of other breeds, even though they are from the same species. It is, however, noteworthy that, in the present study, a high rates (75-82%) of frozen-thawed embryos developed *in vitro* irrespective of three different breeds when the factors such as cooling speed, seeding, thawing and dilution procedures are optimized. This results provide encouragement for further research into the methods of embryos preservation.

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## —196°C に凍結された 16 細胞期および桑実期の家兎胚の生存性

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日本白色種, ニューゼーランド白色種, およびダッチ  
 ベルト種家兎の着床前期胚 (16細胞期および桑実期) に  
 ついて凍結 (-196°C) 実験を行なった。特に室温から  
 0°C までの温度下降速度, 胚をとりまくミュンシンの層の  
 存在, 氷晶 Seeding 処理および融解 (凍結からの) 処理  
 の胚の生存におよぼす影響をしらべて凍結保存した結果  
 良好な胚の生存率がえられた。ダッチベルト種胚では  
 7~9°C/min で 0°C まで冷却, 12.5% DMSO 液にて -4  
 °C 氷晶 Seeding を行ない -196°C に凍結し, 融解の際に  
 4種の異なる濃度の PBS を段階的に加えて希釈した結果  
 in vitro で高率で発達した (16細胞期胚で 51%, 桑実期

胚で 71%)。最も高い in vitro 発達例は, 桑実期胚を  
 0°C まで 1°C/2.5min で冷却, -4°C で Seeding 後 -196  
 °C に凍結し, 融解の際には 6, 3 および 1% の DMSO 液,  
 さらに培養液を段階的に加えて希釈した場合にえられた  
 (日本白色種胚で 81%, ニューゼーランド白色種胚で 75  
 %, ダッチベルト種胚では 82%)。ミュンシンの層のある  
 胚とないものとの間には生存率に大差は認められなかつ  
 た。Seeding を行なわず凍結したすべての胚は融解後  
 発達しなかった。凍結融解胚各々 6 個を 27 匹の受容雌に  
 移植した結果 9 匹が妊娠し, 妊娠第 12 日目の検査ではこ  
 れらに 37 の正常胎仔の存在が認められた。