Vitrification Solution의 耐凍劑 組合이 凍結融解後 생쥐 受精卵의 生存率에 미치는 影響

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EFFECTS OF THE COMBINATION OF CRYOPROTECTANTS IN VITRIFICATION SOLUTION ON THE SURVIVAL OF FROZEN - THAWED MOUSE EMBRYOS

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Contents

摘　　要

本 硏究는 vitrification solution의 改善을 目的으로 細胞內 浸透性 耐凍劑의 單用 (glycerol, ethylene glycol and dimethyl sulfoxide(DMSO)) 또는 混用 (glycerol+ ethylene glycol, glycerol+ propylene glycol)에서 氷結晶이 形成되지않는 가장 合理的인 凍結液을 選定하여, 이 凍結液에 acetamide, 非浸透性 耐凍劑(sucrose, Ficoll)의 添加水準과 平衡時間 等이 超急速凍結시킨 mouse morulae의 生存率에 미치는 影響을 究明하기 위하여 實施되었으며 要約된 結果는 다음과 같다.

1. 浸透性 耐凍劑의 濃度를 調査한 實驗에있어서 20, 30, 40%의 單用으로 凍結된 mouse morulae의 FDA-score는 30%(glycerol, ethylene glycol)에서 높았으며, 30% 凍結液중 ethylene glycol(3.6)과 DMSO(1.4)보다 glycerol(4.1)에서 더 높은 生存率을 보여 주었다 ($P<0.05$).

2. m-PBS에 10% sucrose와 20% BSA를 含有한 20%, 30%, 40% 單用耐凍劑에서 凍結時에는 氷結晶이 形成되지 않았으나, 融解時에는 氷結晶이 形成된 反面, 30% 混合 耐凍劑에서는 凍結과 融解時 모두 氷結晶이 形成되지 않았다.

3. 두가지 浸透性 耐凍劑의 組合 實驗에서 20% glycerol과 10% ethylene glycol 混合液(20G10E)이 다른 30% 混合液(10G20E, 15G15E, 20G10P, 15G15P, 10G20P)보다 더 높은 FDA-score(4.5)를 얻었다.

4. 20G10E 溶液에 acetamide 10, 15, 20%를 添加하였을 때, FDA-score는 各各 4.4(對照區), 4.3(10%), 3.6(15, 20%)으로서 生存率에 影響을 주지 못하였다 ($P<0.05$).

5. 20G10E 溶液에 10% sucrose를 添加하여 平衡時間을 檢討한 結果 5分(3.5)과 10分(4.6), 10分(4.6)과 20分(3.2)間에는 有意差($P<0.05$)가 있었다. 20G10E 溶液에 20% sucrose를 添加하였을 때 5分(4.0)과 10分(4.3)間에는 有意差($P>0.05$)가 없었으나, 5分(4.0)과 20分(3.2), 10分(4.3)과 20分(3.2)間에서 有意差($P<0.05$)가 있었다.

6. 20G10E 溶液에 Ficoll(0, 10, 20, 30%)을 添加한 vitrification solution으로 超急速凍結한 mouse morulae의 FDA-score(survival rate)는 各各 4.5(90%), 4.2(84%), 4.4(88%), 4.6(92%)이었으며 添加水準에 관계없이 對照區와 有意差가 없었다 ($P>0.05$).

7. 以上의 結果로부터 mouse morulae의 生存率에 미치는 가장 適切한 凍結液의 單用耐凍劑濃度는 30%였고, 凍結과 融解時 氷結晶이 形成되지 않는 30% 混合凍結液中 20G10E 溶液에 10% sucrose添加가 10分間平衡하여 凍結融解한 mouse morulae에서 가장 良好한

生存率(92%)을 얻었다.

Ⅰ. Introduction

Since the first success in the cryopreservation of mouse embryos was developed by Whittingham *et al.*(1972) and Wilmut (1972) who used the slow freezing (0.2-0.8℃/min) and thawing (4-25℃/min) over an interval 25 to -60℃, further studies have concentrated on the simplification of procedures, improvement of the survival rate of frozen-thawed embryos and application to other species.

Early cryopreservation of mouse embryos depended on the use of cryoprotectants and the partial dehydration of embryos by slow freezing. When sucrose was added to the freezing media however, the sucrose caused the embryos to shrink by losing water (Kasai *et al.*, 1980), so that intracellular ice formation was reduced and the embryos could be frozen rapidly (Nguyen *et al.*, 1984; Renard *et al.*, 1984 ; Taketa *et al.*, 1984; Szell & Shelton, 1986, 1987).

Rapid freezing in LN_2 container required careful control of the quantity of ice formed during freezing and thawing. Intracellular ice formation occurs when the embryos are not sufficiently dehydrated at a given subzero temperature and extracellular ice formation occurs when the concentration of

cryoprotectants is low. Both intracellular and extracellular ice formation is the main cause of an embryo's death during freezing and thawing. Recently, successful ice-free cryopreservation of mouse 8-cell embryos at -196°C by a process called vitrification has been reported by Rall and Fahy (1985).

Vitrification is a process of solidification whereby an aqueous solution does not crystallize during cooling. The original vitrification solution (VS) consisted of 20.5% DMSO, 15.5% acetamide, 10% propylene glycol and 6% polyethylene glycol. Since this Vitrification Solution was highly toxic, however, embryos had to be exposed to this solution at 4℃ and equilibrated in a stepwise manner at different concentrations.

The present studies were carried out to examine the effects of the mixture of cryoprotectants in VS on the survival of vitrified morulae mouse embryos and to develop the proper vitrification solution by simplifying the procedure of vitrification.

Ⅱ. Literature review

Since the freezing technique of mouse embryos was developed by Whittingham *et al.*(1972) and by Wilmut (1972), further research has concentrated on the simplification of procedures, application to other species and improvement of viability. Thereafter, as sucrose was added to freezing media, a rapid freezing method has been developed.

Recently, embryo cryopreservation called vitrification or ice-free cryopreservation, was first reported in 8-cell mouse embryos by Rall and Fahy(1985). The vitrification solution used consisted of a mixture of permeable(DMSO, acetamide and propylene glycol) and non-permeable(polyethylene glycol) cryoprotective agentes.

More recently, mouse embryos have been successfully vitrified in a solution of 40% ethylene glycol, 30% ficoll and 20% sucrose (Kasai *et al.*, 1990).

1. Cryoprotectants

Cryoprotectants added to freezing media consisted of permeable(glycerol,dimethyl sulfoxide, ethylene glycol, propylene glycol) and non-permeable cryoprotective agents (polyethylene

glycol, sucrose, rafinose and ficoll, etc.)

In early days, the successful slow freezing of mouse and rat embryos was accomplished in the presence of DMSO as a permeable cryoprotectant (Wilmut, 1972; Leibo *et al.*, 1974; Wittingham, D.G, 1975; Kasai *et al.*, 1980). However, the survival rate of frozen embryos was very low. Recently, it was reported that when glycerol was used as a permeable cryoprotectant, a high survival rate was obtained (Miyamoto & Ishibashi, 1986; Szell & Shelton, 1986ab ,1987; Kim *et al.*, 1988; Kang *et al.*, 1989). Ethylene glycol and propylene glycol were also effective for rapid freezing of mouse embryos (Miyamoto & Ishibashi, 1977,1978,1983; Rall *et al.*, 1984; Renard *et al.*, 1984).

However, a low survival rate was obtained when mouse embryos were frozen in ethylene glycol by a two-step method (Kasai *et al.*, 1980). In 1990, Kasai *et al.* reported that when mouse embryos were vitrified in 40% ethylene glycol solution containing 30% Ficoll + 0.5M sucrose, very high survival rate was obtained. Szell & Shelton(1986) reported that glycerol was more effective than DMSO as the cryoprotectants when embryos were frozen rapidly. In early days, DMSO, propylene glycol and acetamide were used as permeable cryoprotectant in vitrification solution(Rall & Fahy., 1985; Rall *et al.*, 1987; Kono *et al.*, 1988; Nakagata., 1989). Recently the mixture solution of glycerol and propylene

glycol or a single solution of ethylene glycol tend to be used as permeable cryoprotective agents in vitrification solution(Valdez *et al.*, 1989; Kasai *et al.*, 1990).

2. Crystallization

The main causes of cellular damage during freezing and thawing are intracellular and extracellular ice formation (Mazur, 1972). Intracellular ice formation takes place when embryos are not sufficiently dehydrated.The early successful cryopreservation of mouse embryos was found to be due to the partial dehydration of embryos by slow freezing (Wittingham, Leibo *et al.*, 1974; Wilmut, 1972). Intracellular ice formation during freezing can be minimized by dehydration of embryos (Mazur,1977). Mazur (1977) reported that when sucrose was added to freezing media, intracellular ice formation was reduced by removing water from embryos. It was also reported that the embryos exposed freezing media containing sucrose could be frozen rapidly (Renard *et al.*, 1984). Recently, to minimize the injury from ice crystals, a vitrification method was developed by Rall & Fahy (1985). Vitrification is defined as the physical process by which a highly concentrated freezing media solidifies during freezing, without formation of ice crystal (Fahy *et al.*, 1984). However, it can be crystallized during thawing (Rall *et al.*, 1987). To

prevent crystallization during thawing and to facilitate vitrification, Polyethylene glycol has been used as a macromolecule (Rall & Fahy, 1985).

Recently Ficoll was also used as a macromolecule which facilitates non-crystallization by preventing ice crystallization during warming. When 30% Ficoll was added to 40% ethylene glycol solution, it increased the toxicity of the ethylene glycol solution. However, when 30% Ficoll was added to this solution with 0.3M sucrose, it decreased the damage toxicity(Kasai *et al.*, 1990).

3.Equilibration time and sucrose concentration

The early procedure of slow freezing and thawing was the stepwise manner. It took much time to freeze and thaw embryos, for embryos were dehydrated only by permeable cryoprotective agents such as dimethyl sulfoxide or glycerol. However, when sucrose was added to freezing media or dilution media, embryos could be frozen and thawed rapidly by a simple method(Kasai *et al.*, 1980; Nguyen *et al.*, 1984; Renard *et al.*, 1984; Takeda *et al.*, 1984; Leibo ., 1984; Kim *et al.*,1988). The toxic effects of sucrose depend not only on its concentration and duration of exposure, but also on temperature(Kasai *et al.*, 1980; Szell & Shelton, 1986ab; Miyamoto & Ishibashi, 1986).

Equilibration time can be defined as the time required for permeating into cells by permeable agents in the freezing media. When sucrose was added to freezing media, embryos initially lost water and shrank to compensate the extracellular osmolarity caused by freezing media(a combination solution of permeable cryoprotectants and sucrose).

Szell& Shelton(1986a) reported that the highest survival(75%) of rapidly frozen 8-cell mouse embryos was obtained in 10-20% sucrose at 4℃, after 20 minutes equilibration and at 20℃, after 10 minutes equilibration, respectively. Trounson *et al*(1987) also reported that the highest survival of ultrarapid frozen 2-cell mouse embryos was obtained in 10% sucrose after 10 minutes equilibration.

As the temperature of freezing media was higher than room temperature, the equilibration time tended to be short(Szell & Shelton, 1986b).

It was reported that an increase in the intracellular cryoprotectant level was beneficial for the survival of rapidly frozen embryos, but complete permeation was not necessary(Szell & Shelton, 1986ab). This means that dehydration is more important than permeation, and long exposure to freezing media containing sucrose is not necessary. Valdez *et al.*(1989) reported that the highest survival(91%) of vitrified mouse morula embryos was in

10% sucrose at both 4℃ and 20℃ after 10min equilibration. Kang *et al.*(1990) reported that the highest survival of ultrarapid frozen 2-cell mouse embryos was obtained in 10% sucrose after 2.5minutes equilibration. Recently, successful vitrification was accomplished in a solution composed of 40% ethylene glycol, 30% ficoll and 20% sucrose after 2-5minutes equilibration (Kasai *et al.*, 1990).

As reported above, it was suggested that the optimal concentration of sucrose was 10-20% and equilibration time was 2-20minutes generally. The higher the temperature of freezing media was, the shorter the equilibration time. Besides, as sucrose was added to freezing media, equilibration time tended to be short. It is demonstrated that sucrose reduces cryoprotectant permeation and facilitates removal of intracellular cryoprotectants during dilution(Leibo & Mazur, 1978; Szell & Shelton, 1986ab; Kasai *et al.*, 1990).

Ⅰ. Materials and Methods

1. Source and isolation of embryos.

Female ICR mice were housed in plastic cages in a room maintained on a 14 hr light(06:00 to 20:00) and 10 hr dark(20:00 to 06:00) cycle and were fed pelleted food and fresh water ad libitum. Embryos were obtained from 5-8 week old, random-bred, ICR mice. The female mice were induced to superovulate by intraperitoneal injection of 5 I.U. PMSG(Peamex, Sankyozoki, Japan) and 5 I.U. HCG(Puberogen, Sankyozoki, Japan) given 48h apart.

Table 1. Composition of modified Dulbecco's phosphate buffered saline(m-PBS)

Component	Concentration(g/100ml)
NaCl	0.8
KCl	0.02
Na_2HPO_4	0.115
KH_2PO_4	0.02
$CaCl_2$	0.01
$MgCl_2 \cdot 6H_2O$	0.01
Glucose	0.1
Na pyruvate	0.0036
Bovine serum albumin	0.3
Streptomycin	0.005
Penicillin	0.0075
Triple-distilled water	100 ml

After HCG injection, females were paired with ICR and were inspected in the following morning for vaginal plugs. Morulae embryos were flushed from the excised oviducts at times of 72 to 75 after HCG injection using Dulbecco's phosphate buffered saline supplemented with 20% BSA.

After flushing, the embryos were washed in the flesh m-PBS twice and only morphologically normal morulae were used for vitrification.

2. Studies on the toxicity of the permeable cryoprotectants

Toxicity of individual permeable cryoprotectants was studied. Freezing media consisted of three permeable solutes(20, 30, 40% glycerol,ethylene glycol, dimethyl sulfoxide) and 10% sucrose in Dulbecco's modified phophate buffered saline(m-PBS).

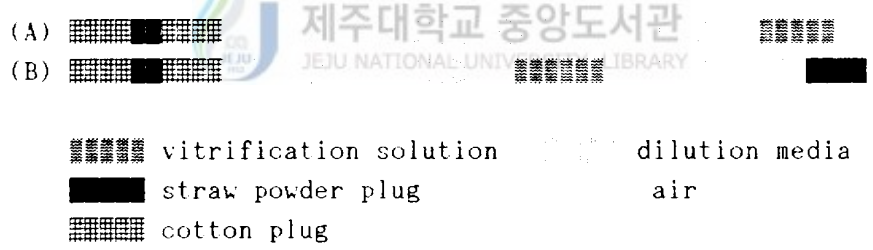

Figure 1.Configuration of vitrification solution, dilution medium and air in 0.25 ml straw, just before loading embryos(A) and after sealing with straw powder(B)

Freezing media were prepared in a 0.25 ml plastic straw by aspiration of PBS + 10% sucrose medium(50μl), air(20μl), PS medium(30μl), air(20μl), with a 1 ml syringe connected to the straw.

Embryos(7-10) were suspended at room temperature in a 50μl drop of freezing medium in a petridish and then were transferred to the other drop(50μl) once. The drops containing embryos, air(20μl), PS(50μl) were aspirated into the prepared straw before being sealed with the straw powder(Figure 1). At 10min of exposure of embryos to the freezing medium straws were immersed directly in liquid nitrogen after being held in liquid nitrogen vapour for 5min.

3. Vitrification test of freezing media

To examine vitrification of the freezing media, glycerol, ethylene glycol, propylene glycol and DMSO were diluted in a concentration of 20, 30 or 40% in m-PBS containing 10% sucrose and 20% BSA.

The mixture solutions of two permeable cryoprotectants also were diluted in m-PBS containing 10% sucrose and 20% BSA.

The composition is as follows:

1) *Single solutions*
   (1) **20G, 30G, 40G** : 20, 30, 40% glycerol
   (2) **20E, 30E, 40E** : 20, 30, 40% ethylene glycol
   (3) **20D, 30D, 40D** : 20, 30, 40% DMSO

2) *Mixture solutions*
   (1) **10G + 20E** : 10% glycerol + 20% ethylene glycol
   (2) **15G + 15E** : 15% glycerol + 15% ethylene glycol
   (3) **20G + 10E** : 20% glycerol + 10% ethylene glycol
   (4) **10G + 20P** : 10% glycerol + 20% propylene glycol
   (5) **15G + 15P** : 15% glycerol + 15% propylene glycol
   (6) **20G + 10P** : 20% glycerol + 10% propylene glycol

The freezing media were aspirated into 0.25 ml plastic straws at room temperature. After being sealed with the powder, the straws were plunged directly into liquid nitrogen. After immersion for 1 minutes, straws were quickly transferred into 38℃ water. Freezing media in all straws which turned opaque were considered crystallized and those which remained transparent were considered uncrystallized. If all the samples turned opaque only once, they were considered crystallized. Samples had 5 replications.

4. Vitrification solution(VS)

In a vitrification test of freezing media, the mixture solutions without ice crystals were selected as vitrification solution. In a freezing test, the highest survival was obtained in the mixture solution of 20% glycerol and 10% ethylene

glycol(20G 10E), which was finally selected as a vitrification solution.

To examine the effects of acetamide, sucrose and Ficoll, they were diluted to vitrification solution(20G 10E) at different concentrations.

In sucrose treatment groups of 7-10 embryos were equilibrated in VS at room temperature(23-25℃) for 5, 10 or 20 minutes.

The composition of vitrification solution is as follows:

1) *Acetamide addition to new vitrification solution*
(1) 20G10E + 10% sucrose + m-PBS with 20% BSA : **Control**
(2) 20G10E + 10% sucrose + **10% acetamide** + m-PBS with 20% BSA
(3) 20G10E + 10% sucrose + **15% acetamide** + m-PBS with 20% BSA
(4) 20G10E + 10% sucrose + **20% acetamide** + m-PBS with 20% BSA

2) *Sucrose addition to new vitrification solution*
(1) 20G10E + 10% sucrose + m-PBS with 20% BSA : **control**
(2) 20G10E + **20% sucrose** + m-PBS with 20% BSA

3) *Ficoll addition to new vitrification solution*
(1) 20G10E + 10% sucrose + m-PBS with 20% BSA : **control**
(2) 20G10E + 10% sucrose + **10% Ficoll** + m-PBS with 20% BSA
(3) 20G10E + 10% sucrose + **20% Ficoll** + m-PBS with 20% BSA
(4) 20G10E + 10% sucrose + **30% Ficoll** + m-PBS with 20% BSA

Each solution was filtered with membrane filter and then stored in a refrigerator(4℃).

5. Vitrification procedures

Vitrification solutions were prepared in 0.25 ml plastic straws by aspiration of dilution medium(PS ; m-PBS + 10%

sucrose)(50μl), air(20μl), dilution medium(30μl), air(20μl), with a 1 ml sylinge connected to the straw. The developmental stage of embryos was the morula. Embryos(7-10) were suspended at room temperature in a 50μl drop of vitrification solution in a petridish and were transferred to the other drop(30μl) once. The drops containing embryos(30μl), air(20μl) and PS(50μl) were aspirated into the prepared straw before being sealed with the straw powder(figure 1). The straws were plunged directly into liquid nitrogen after equilibration for 5, 10 or 20minutes at room temperature and stored in LN_2 container for 2-15 days.

6. Warming

After storage for 1-2 weeks, the straws were thawed rapidly in 38℃ water by shaking slowly for 30seconds. The contents of the straws were expelled into a watching glass and then transferred into a fresh dilution medium(m-PBS + 10% sucrose;PS) at room temperature. At 2 minutes after being transferred into a dilution medium, embryos were washed in fresh PBS twice. The survival rate of frozen thawed embryos was evaluated by FDA-test.

7. FDA - test

A stock solution of 5 mg FDA(Sigma)/ml acetone was added to

m-PBS medium in concentrations of 1:400,000. Embryos were exposed to a drop of FDA solution(50μl) for 1 minutes, and then washed in fresh m-PBS three times. The washed embryos were examined under a reflected-light fluorscence microscope(Nikon).

FDA - scores were classified as follows:

P_5 : 100% fluorscence — Positive
P_4 : Fluorscence over 80%
P_3 : Fluorscence over 60% — Partial
P_2 : Fluorscence over 40%
P_1 : Fluorscence over 20%
P_0 : Non - fluorscence — Negative

Mean FDA-score was calculated by following equation.

$$\text{Mean score} = [(A \cdot 5)+(B \cdot 4)+(C \cdot 3)+(D \cdot 2)+(E \cdot 1)] / N$$

A : No. of P_5 B : No. of P_4
C : No. of P_3 D : No. of P_2
E : No. of P_1
N : Total of P_0 - P_5

8. Statistical analysis

Data were analyzed by the student's t-test using Minitab.

Ⅳ. Results and Discussion

1. Toxicity test of permeable cryoprotectants

Mouse morulae embryos were frozen in 20%, 30% and 40% solutions of glycerol and ethylene glycol, and in 20% and 30% solutions of dimethyl sulfoxide. The results are shown in table 2. The-FDA score of mouse morulae frozen in 20%, 30% and 40% solutions of glycerol and ethylene glycol after 10 minutes equilibration was 3.8, 4.1 and 4.0, and 3.2, 3.7 and 3.2, respectively. The FDA-score was higher in 30% solutions of glycerol and ethylene glycol than in 20% and 40%, and showed significant differences between 30% glycerol and 20%, or 40% ethylene glycol($P<0.05$).

The FDA-score of mouse morulae frozen in 20% and 30% solutions of dimethyl sulfoxide was 1.8 and 1.4, respectively. The FDA-score did not show significant differences between 20% and 30% dimethyl sulfoxide($P>0.05$). However, differed significantly between dimethyl sulfoxide and glycerol, or ethylene glycol($P<0.01$).

Table 2. Effect of the concentration of permeable cryoprotectants on the survival of mouse morulae frozen rapidly in liquid nitrogen

Cryoprotectant(%)	No.of embryos frozen	No. of embryos recovered	No. of embryos evaluated by FDA test						Mean FDA score
			P_5	P_4	P_3	P_2	P_1	P_0	
Glycerol									
20	24	19	8	3	0	4	1	3	3.2^{b}
30	60	50	27	5	14	4	0	0	4.1^{bdf}
40	40	33	14	8	7	0	4	0	3.9^{bh}
Ethylene glycol									
20	42	37	10	6	12	3	2	4	3.2^{beg}
30	60	51	20	14	8	2	4	3	3.7^{b}
40	45	38	6	10	11	7	4	0	3.2^{bcg}
Dimethyl sulfoxide									
20	40	34	4	5	3	3	5	14	1.8^{a}
30	50	44	1	2	7	7	15	12	1.4^{a}

Freezing media; 20, 30, 40% glycerol, ethylene glycerol, DMSO + 10% sucrose + 20% BSA + m-PBS
Dilution media; 10% sucrose + m-PBS(PS)
P_5: 100% fluorscence P_4: Fluorscence over 80%
P_3: Fluorscence over 60% P_2: Fluorscence over 40%
P_1: Fluorscence over 20% P_0: Non-fluorscence
Values with different superscripts are significantly different (a-b, c-d, e-f, $P<0.01$; g-h, $P<0.05$).

The FDA-score of frozen-thawed embryos was higher in 30% glycerol(4.1) than in 30% ethylene glycol(3.6) or 20% DMSO(1.8) ($P<0.05$), and the optimal concentration of permeable cryoprotectants in two treatments(glycerol and ethylene glycol) was 30%. It was reported that the high survival of 8-cell mouse

embryos was obtained in 30% glycerol(Szell & Shelton, 1986a) and in 15% glycerol(Miyamoto & Ishibashi, 1986). Miyamoto & Ishibashi also reported that the cryoprotective effect of glycerol was superior to that of DMSO. Kasai *et al.*(1990) reported that when morulae mouse embryos were exposed to a 30% solution of permeable cryoprotectants, a high survival rate was obtained in ethylene glycol(98%) and glycerol(88%).

In experiment for toxicity of permeable cryoprotectants, kind of cryoprotectant was in agreement with the results of Szell and Shelton(1986ab), Miyamoto and Ishibashi(1986) and Kasai *et al.*(1990). The cryoprotective effect of glycerol can be demonstrated by its ability to reduce salt concentration in the unfrozen fraction of a freezing medium(Rall *et al.*, 1987; Szell & Shelton, 1986a).

The concentration of cryoprotectant differed from Miyamoto and Ishibashi's result. It may be due to freezing method, for the freezing in this experiment was carried out in liquid nitrogen while Miyamoto and Ishibashi's freezing was carried out in liquid nitrogen vapour.

2. Vitrification test of freezing media

The results of a vitrification test are shown in table 3. When the single solution of permeable cryoprotectants were

diluted to 20, 30 and 40%, with m-PBS containing 10% sucrose + 20% BSA, all the freezing media remained transparent after freezing.

However, when all the straws were immersed in 38℃ water, they were turned opaque within 3 seconds, which is a sign of ice-crystallization. When 30% mixture solution of two permeable cryoprotectants were diluted to m-PBS containing 10% sucrose + 20% BSA, all the freezing media remained transparent during freezing or thawing. The reason why ice crystallization occurred in a 30% mixture solution of two permeable cryoprotectants rather than in 40% single solution is not clear. It may be due to the character of the mixture solution of two permeable cryoprotectants. Kasai *et al.*(1990) reported that 40% glycerol and ethylene glycol diluted to m-PBS, containing 30% Ficoll or 30% Ficoll + 20% sucrose, did not form ice-crystals. The difference between the results in this work and Kasai *et al.*'s can be explained in that Ficoll, as a non-peremable agent to prevent crystallization was added to 40% freezing media(1990).

Most cellular damage is related to intra and extracellular crystallization during freezing and thawing.

Intracellular crystallization occurs when embryos are not sufficiently dehydrated, which causes embryos to die by alternating the cytoplasm of embryos(Szell & Shelton, 1986ab).

Extracellular crystallization occurs when concentration of permeable cryprotectants in freezing media is low, which causes embryos to die by increasing the salt concentration in the unfrozen section of partly frozen media(Rall *et al.*, 1987).

Table 3. Occurrence of crystallization in freezing media during rapid cooling in liquid nitrogen and warming in 38℃ water

Cryoprotectant	Concentration of permeable cryoprotectant					
	20%		30%		40%	
	Cooling	Warming	Cooling	Warming	Cooling	Warming
Glycerol	-	+	-	+	-	±
Ethylene glycol	-	+	-	+	-	±
Prophylene glycol	-	+	-	+	-	±
Dimethyl sulfoxide	-	+	-	+	-	±
20% G + 10% E			-	-		
15% G + 15% E			-	-		
10% G + 20% E			-	-		
20% G + 10% P			-	-		
15% G + 15% P			-	-		
10% G + 20% P			-	-		

All the transparent samples were considered uncrystallized and were scored (-) and all the opaque samples were considered crystallized and were scored (+).If only all the samples turned opaque once, it was considered crystallized and was scored (±).Each treatment had 5 replications.

To prevent intra and extracellular crystallization, a vitrification method was first developed by Rall & Fahy(1985). Vitrification is defined as solidification of freezing media

without crystallization during freezing. The original vitrification solution consisted of 20.5% DMSO, 15.5% acetamide, 10% propyleueglycol and 6% ethylene glycol. Rall & Fahy (1985) reported that high survival(89%) of mouse embryos was obtained by using this solution. Thereafter Valdez *et al.*(1989) reported that high survival(91%) of mouse moralae was obtained from a vitrification solution consisting of 25% glycerol and 20% propylene glycol. Kasai *et al.*(1990) also reported that very high survival(98%) of mouse morulae was obtained from vitrification solution consisting of 40% ethylene glycol, 30% Ficoll and 20% sucrose. In this study, 30% mixture solution of glycerol and ethylene glycol, or glycerol and propylene glycol were selected for a vitrification solution.

3. Effects of the mixture of two permeable cryoprotectants

Table 4. Effects of the mixture of glycerol and propylene glycol on the survival of the vitrified mouse morulae

Cryoprotectant (%)	No.of embryos frozen	No. of embryos recovered	No.of embryos evaluated by FDA test(%)						Mean FDA score
			P_5	P_4	P_3	P_2	P_1	P_0	
10G 20P	30	24	3 (13)	12 (50)	4 (17)	4 (17)	1 (4)	0 (0)	3.5
15G 15P	30	25	6 (24)	5 (21)	8 (33)	3 (13)	0 (0)	3 (13)	3.2
20G 10P	30	29	11 (38)	6 (21)	8 (28)	3 (10)	1 (3)	0 (0)	3.8

10G 20P : 10% glycerol + 20% propylene glycol
15G 15P : 15% glycerol + 15% propylene glycol
20G 10P : 20% glycerol + 10% propylene glycol
Vitrification solution: The 30% mixture solutions of cryoprotectants were diluted in m-PBS containing 10% sucrose
Dilution media : 10% sucrose + m-PBS(PS)

Effects of the mixture of glycerol and ethylene glycol, glycerol and propylene glycol on the survival of vitrified mouse morulae are shown in table 4 and 5.

The FDA-score of mouse morulae frozen in 10G20P, 15G15P and 20G10E was 3.5, 3.2 and 3.8, respectively, but was not significantly different among treatments($P>0.05$). since the proportion(38%) of P_5 in 20G10E was higher than in 10G20E(13%)

and in 15G15E(24%), it seems that 20G10E was more effective for the survival of the frozen mouse morulae.

Table 5. Effects of the mixture of glrcerol and ethylene glycol on the survival of vitrified mouse morulae

Cryopro-tectant (%)	No.of embryos frozen	No. of embryos recovered	No. of embryos evaluated by FDA test(%)						Mean FDA score
			P_5	P_4	P_3	P_2	P_1	P_0	
10G 20E	30	29	13 (45)	7 (24)	1 (3)	3 (10)	3 (10)	2 (7)	3.6^a
15G 15E	30	30	15 (50)	10 (33)	1 (3)	2 (7)	1 (3)	1 (3)	4.1^{ab}
20G 10E	30	25	18 (72)	5 (17)	1 (3)	0 (0)	0 (0)	1 (3)	4.5^b

10G 20E : 10% glycerol + 20% ethylene glycol
15G 15E : 15% glycerol + 15% ethylene glycol
20G 10E : 20% glycerol + 10% ethylene glycol
Vitrification solution : The 30% mixture solutions of cryoprotectants were diluted in m-PB1 containing 10% sucrose
Dilution media : 10% sucrose + m-PBS(PS)
Values with different superscripts are significantly different(a-b, $P<0.05$)

The-FDA score of mouse morulae frozen in 10G20E, 15G15E and 20G10E was 3.6, 4.1 and 4.5, respectively, was significantly different between 10G20E and 20G10E($P<0.05$), however, was not significantly different between 10G20E and 15G15E, 15G15E and 20G10E. As shown in the result that the proportion(72%) of P_5 in

20G10E was higher than in 10G20E(45%) and 15G15E(50%), it seems that 20G10E was more effective for the survival of frozen mouse morulae.

As shown in the above results, a high survival rate was obtained in 20G10E(4.5) and 20G10P(3.8). However, the survival rate was higher in 20G10E than in 20G10P.

The result showed that the optimal glycerol concentration in the new vitrification solution was 20%, and ethylene glycol was more effective than propylene glycol.

It was reported that glycerol, ethylene glycol and propylene glycol were effective for freezing mouse embryos (Miyomoto & Ishibashi, 1977; Szell & Shelton, 1986a). However, Kasai *et al.* (1990) reported that the developing rate of mouse morulae exposed to 30% ethylene glycol was higher in ethylene glycol(98%) and glycerol(88%) than in propylene glycol (16%).

As shown in table 4 and 5, it seems that glycerol and ethylene glycol is more effective for freezing mouse embryos than propylene glycol.

Since the initial vitrification solution were highly toxic, the vitrification procedure had to be carried out in VS1 in a stepwise manner at 2 different temperatures(20℃, 4℃). However, our vitrification procedure in this work was significant in that a simple procedure carried out in one step method at room

temperature.

4. Effects of acetamide addition to vitrification solution

Table 6. Effect of acetamide addition to new vitrification solution(20G10E) on the survival rate of frozen-thawed mouse morula embryos

Acetamide concentration(%)	No. of embryos frozen	No.of embryos recovered	No. of embryos evaluated by FDA test(%)						Mean FDA score
			P_5	P_4	P_3	P_2	P_1	P_0	
0	50	41	29 (71)	7 (17)	1 (2)	2 (5)	2 (5)	0 (0)	4.4^{b}
10	50	44	29 (66)	9 (21)	2 (4)	2 (4)	2 (4)	0 (0)	4.4^{b}
15	50	40	20 (50)	7 (18)	3 (8)	3 (8)	1 (3)	6 (15)	$3.6^{a,c}$
20	50	50	22 (44)	9 (18)	4 (8)	5 (10)	2 (4)	8 (16)	3.3^{a}

Vitrification solution ; Acetamide(10, 20, 30%) was added to the new vitrification(20G10E)
Control ; a mixture solution of 20% G + 10% E
P_5:100% fluorscence P_4:Fluorscence over 80%
P_3:Fluorscence over 60% P_2:Fluorscence over 40%
P_1:Fluorscence over 20% P_0:Non-fluorscence
Values with different superscripts are significantly different (a-b, $P<0.01$; b-c, $P<0.05$)

Acetamide which had a strong ability to permeate into cells was added to vitrification solution(20G10E) at differant concentrations. The FDA-score of mouse morulae frozen in 20G10E

with 0%, 10%, 15% and 20% acetamide was 4.4, 4.4, 3.6 and 3.3, respectively. The FDA-score was significantly high in 20G10E with 0% and 10% acetamide($P<0.05$), which indicates the reverse effect of acetamide addition since the proportion of the FDA-positive(P_5 and P_4) showed the decreasing of effects by addtion of acetamide. Therefore, it is not recomentable to use acetamide in this work. It seems that an acetamide addition(10, 15, 20%) to vitrification solution had no effect($P<0.05$).

It may be due to the high osmosis. It is thought that as crystallization does not occur in the new vitrification solution(20G10E), acetamide addition is not necessary.

Further studies on the osmotic injury is required. The new vitrification solution might consist of the 30% mixture solution of glycerol and ethlylene glycol, glycerol and propylene glycol in m-PBS containing 10% sucrose and 20% BSA.

5. Effects of equilibration time and sucrose concentration

20% sucrose was added to the new vitrification solution. After 5, 10 and 20 minutes equilibration, embryos were vitrified. The results are shown in table 7.

The survival rate between 5 minutes(3.5) and 10 minutes(4.6), 10 minutes(4.6) and 20 minutes(3.2) of

equilibration in 10% sucrose , and 20 minutes(3.2) and 5 minutes(4.0),or 10 minutes(4.3) in 20% sucrose were significantly different($P<0.05$). When embryos were equilibrated in VS containing 10% sucrose for 10 minutes, the highest survival rate(4.6) could be obtained.

Table 7. Effect of sucrose concentration and equilibration time on the survival of the vitrified mouse morulae

Sucrose conc. (%)	Equili-bration time(min)	No. of embryos recovered	No. of embryos evaluated by FDA test(%)						Mean FDA score
			P_5	P_4	P_3	P_2	P_1	P_0	
10	5	25	8 (32)	8 (32)	2 (8)	3 (12)	3 (12)	1 (4)	3.5^{a}
	10	16	11 (69)	4 (25)	1 (6)	0 (0)	0 (0)	0 (0)	4.6^{bc}
	20	21	3 (14)	6 (29)	9 (43)	1 (5)	0 (0)	2 (10)	3.2^{a}
20	5	16	9 (56)	2 (13)	3 (19)	1 (6)	0 (0)	1 (6)	4.0^{b}
	10	23	14 (61)	3 (13)	5 (22)	1 (4)	0 (0)	0 (0)	4.3^{bc}
	20	20	2 (10)	7 (33)	6 (29)	4 (19)	0 (0)	1 (5)	3.0^{ad}

Vitrification solution;The mixture solution of 20%Glycerol and 10%Ethylene glycol was diluted in m-PBS containing 10% and 20% sucrose.

P_5:100% Fluorscence P_4:Fluorscence over 80%
P_3:Fluorscence over 60% P_2:Fluorscence over 40%
P_1:Fluorscence over 20% P_0:Non-fluorscence

Values with different superscripts are significantly different (a-b, $P<0.05$; a-c, d-e, $P<0.01$)

It was reported that sucrose facilitated removal of

intracellular cryoprotectants during dilution(Leibo & Mazur, 1987: Kasai *et al.*, 1980; Szell & Shelton, 1986a). Rall & Fahy(1985) also reported that full permeation of cryoprotectants was not necessary but harmful for successful embryo preservation.

It was reported that a high survival rate was obtained after 10 minutes equilibration(Rall & Fahy, 1985; Miyamoto & Ishibashi, 1989; Valdez *et al.*,1989), and that there was no differences between 10% and 20% sucrose. Kasai *et al.*(1990) reported that a high survival was obtained after 2 minutes(98%) and 5 minutes(97%) of equilibration in 40% ethylene glycol with m-PBS containing 30% Ficoll + 20% sucrose.

As known in the above results, regardless of concentration of freezing media, the optimal concentration of sucrose and equilibration time was 10% and 10 minutes, respectively and there were no differences between 10% sucrose and 20% in a 10 minutes equilibration.

6. Effect of Ficoll addition to the new vitrification solution

10, 20% and 30% Ficoll as non-permeable cryoprotectants were added to the new vitrification solution(20G10E).

The result, compared with control(0% Ficoll), was shown in table 8.

Table 8. Effect of Ficoll addition to the new vitrification solution(20G 10E) on the survial rate of the frozen - thawed mouse morula

Ficoll concent-ration(%)	No.of embryos frozen	No.of embryos recovered	No. of embryos evaluated by FDA test(%)						Mean FDA score
			P_5	P_4	P_3	P_2	P_1	P_0	
0	20	20	12 (60)	6 (30)	2 (10)	0 (0)	0 (0)	0 (0)	4.5
10	20	17	8 (47)	6 (35)	3 (18)	0 (0)	0 (0)	0 (0)	4.2
20	20	20	12 (60)	4 (20)	3 (15)	1 (5)	0 (0)	0 (0)	4.4
30	20	19	14 (74)	3 (16)	2 (10)	0 (0)	0 (0)	0 (0)	4.6

Vitrificarion solution;Acetamide(10, 20, 30%) was added to the new vitrification(20G 10E)

P_5:100% Fluorscence P_4:Fluorscence over 80%
P_3:Fluorscence over 60% P_2:Fluorscence over 40%
P_1:Fluorscence over 20% P_0:Non-fluorscence

When 0, 10, 20 and 30% Ficoll were added to the new vitrification solution, the FDA-score(survival rate) was 4.5(90%), 4.2(84%), 4.4(88%) and 4.6(92%), respectively. There were no differences among Ficoll addition treatments($P>0.05$). However, according to the results that the proportion of P_5 was higher in 30% Ficoll(74%) than in 0%(60%), 10%(47%) and 20%(60%), it can be considered that 30% Ficoll was more effective for the survival of frozen mouse morulae.

Since polyethylene glycol has been used for a non-permeable

cryoprotectant in vitrification solution, Ficoll was used by Kasai *et al.*(1990) for the first time. They reported that when 30% Ficoll was added to a solution composed of 40% ethylene glycol and 20% sucrose, high survival rate(98%) was obtained.

Ficoll is a non-permeable cryoprotectant to assist vitrification as a macromolecule(molecular weight 70,000), while sucrose, with a low molecular weight, causes embryos to shrink by losing water(Mazur, 1970; Kasai *et al.*,1980,1990).

In vitrification test, ice crystallization did not occur in the new vitrification solution. However, Ficoll was added to the new vitrification solution to examine the effect of a Ficoll addition on protecting extracells. The result from Ficoll addition showed no significant effect($P<0.05$). However, further studies on Ficoll were required since this studies had only a few replications.

In the previous vitrification methods, embryos had to be exposed to several low concentrations of the vitrification solution at 20℃ to prevent toxicity of 100% vitrification solution before equilibration at 4℃(Rall & Fahy, 1985; Rall *et al.*, 1987; Valdez *et al.*, 1989).

In this study, it was observed that after 5 -10 minutes equilibration in the new vitrfication solution(20G 10E) at room temperature, embryos can be frozen in liquid nitrogen by a simple

vitrification method. However, further studies on the non permeable cryoprotectants were required.

Summary

Studies were carried out to select the optimal freezing media which gives no ice crystals in single(glycerol, ethylene glycol, dimethyl sulfoxide(DMSO)) and mixture solutions(glycerol + propylene glycol, glycerol + ethylene glycol) of permeable cryoprotectants. On this freezing media, acetamide and non-permeable cryoprotectants(sucrose, Ficoll) were added to find the proper level and equilibration time in vitrification solution on the survival of vitrified mouse morulae.

The results are summarized as follows:

1. In toxicity test of permeable cryoprotectants, the higher FDA-score(4.1) of mouse morulae frozen in 20, 30 and 40% single solution of cryoprotectants(glycerol, ethylene glycol, DMSO) was obtained in 30% solution. The FDA-score(4.1) of 30% glycerol was higher than 30% ethylene glycol(3.6) and DMSO(1.4) ($P<0.05$).

2. 20, 30 and 40% single solutions of permeable cryoprotectants containing m-PBS with 10% sucrose and 20% BSA did not crystallize during cooling, but crystallized during warming. However, the 30% mixture solution of the two permeable cryoprotectants did not

crystallized both during cooling and warming.

3. When mouse morulae were frozen in 30% mixture solutions of two permeable cryoprotectants(glycerol and propylene glycol, glycerol and ethylene glycol), highest FDA-score(4.5) was obtained in a mixture solution of 20% glycerol and 10% ethylene glycol(20G10E) than other 30% mixture solution(10G20E, 15G15E, 20G10P, 15G15P, 10G20P) and there was significant difference between 20G10E and 10G20E($P<0.05$).

4. When 10, 15 and 20% of acetamide were added to the new vitrification solution(20G 10E), FDA-scores of embryos were 4.4(control), 4.4(10%), 3.6(15, 20%), respectively. The addition of acetamide did not affect the survival of forzen-thawed morulae($P<0.05$).

5. The survival between 5 min(3.5) and 10 min(4.6), 10 min(4.6) and 20 min(3.2) of equilibration in 10% sucrose , and 20 min(3.2) and 5 min(4.0),or 10 min(4.3) in 20% sucrose was significantly different($P<0.05$). The highest survival(4.6) was obtained in mouse morulae equilibrated in VS(20G10E) containing 10% sucrose for 10 minutes.

6. FDA-score of morulae frozen in the new vitrification solution containing 0, 10, 20 and 30% Ficoll was 4.5, 4.2, 4.4 and 4.6, respectively and had no significant effect among concentrations of Ficoll($P>0.05$).

7. The optimal concentration of permeable cryoprotectant in freezing media was 30% and the highest survival of frozen-thawed mouse morulae was obtained after 10 minutes equilibration in 20% glycerol + 10% ethylene glycol + 10% sucrose without crystallization during cooling and warming among 30% mixture solutions.

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