Effect of Cryoprotectants on the Growth Capacity Preservation of Mouse and Cattle Skin

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Abstract: The effect of Dimethylsulphoxide (DMSO), Glycerol (GLY) and Propanediol (PROH) cryoprotectants, each at (10, 20 and 30%) concentration on the preservation of mouse and cattle skin tissue, was investigated. And then, the toxicity test was performed by exposing the skin tissue to DMSO, GLY, PROH, EG without freezing. Tissues were checked by capacity explants. The results indicated that mouse skin tissue frozen in 10% GLY and cattle ear tissue frozen in 20% GLY medium with dry-ice yielded significantly ($p < 0.05$) higher percents (33.3 and 71.4%) of fibroblast than tissue frozen in either concentration of GLY or cryoprotectants. In the toxicity test, mouse skin tissues exposed to GLY and equilibrated at 4°C resulted in higher percent of average explants (41.1%) than that was exposed to PRON and DMSO (average explants 38.3 and 36.5%, respectively), while cattle tissues showed higher toxicity, when exposed to PROH and EG and equilibrated at 4°C (average explants 39.9 and 44.2%, respectively). The present study recommended that 10% GLY with dry-ice freezing was the effective cryoprotectant for the mouse skin tissues, while 20% GLY with dry-ice freezing was effective for cattle skin tissues.

Key words: Cryopreservation, fibroblast, mouse, cattle, skin tissues, China

INTRODUCTION

Nuclear transfer technology assists the improvement of cloning to recover an extinct species as well as to produce transgenic animals from adult animal cells. Nuclear transfer cloning was supported by two cell systems, one being oocyte from ovarian tissues of recipient animals and the other being embryonic or somatic cells from donor animals.

Recently, fetal and adult somatic tissues were biopsied and then the derived fibroblast was efficiently cultured *in vitro* (Rideout *et al.*, 2000; Wakayama and Yanagimachi, 1999).

This was not commonly used, since it was concerned with reproductively active females (Silvstre *et al.*, 2002). Nuclear transfer cloning strategies were helpful to conserve tissue bank of endangered species and genetic diversity of economically important breeds (McLaren, 2000).

Cryopreservation of ovarian tissues and somatic tissues have been used for research and breeding purposes (Villalba *et al.*, 1996). It was effective in different species including human (Aubard *et al.*, 2001), mice (Candy *et al.*, 1997; Newton and Illingworth, 2001), rabbits

(Silvstre *et al.*, 2002), sheep (Gosden *et al.*, 1994), goats (Rodrigues *et al.*, 2004), cattle (Fahrudin *et al.*, 2001; Lucci *et al.*, 2004), pigs (Silvstre *et al.*, 2002; Rendal *et al.*, 2004), marmosets (Candy *et al.*, 1995), cats (Jewgenow *et al.*, 1998) and elephants (Gunaseena *et al.*, 1998).

Generally, Dimethyl Sulphoxide (DMSO), Glycerol (GLY), Ethylene Glycol (EG), Propanediol (PROH), thehalose, methanol and ethanediol were used as cryoprotectants. However, their response was varied greatly among species and from one cell type to another (Lucci *et al.*, 2004; Sommerfeld and Niemann, 1999).

The tolerance and effect of various cryoprotect agents on skin tissue preservation have been performed for human (Villalba *et al.*, 1996), bovine ovarian tissues (Lucci *et al.*, 2004) and bovine fetal skin with DMSO as a cryoprotectant (Fahrudin *et al.*, 2001).

Since, the comparative effect of different cryoprotectants on the preservation of mouse and cattle skins have not been reported yet, the present study aimed to assay the toxicity and effect of cryoprotectants on skin tissues and to explore the possibility of generating somatic cell lines from explants frozen kept for a period of time.

MATERIALS AND METHODS

Sample collection and preparation

Mouse: Transgenic mice (oct-4/GFP) at 45 days old and 120 days old were raised in animal-house in Institute of Animal Science, Mariensee, 31535, Nested, Germany. Mouse abdominal skin was incised to expose subcutaneous tissues and then the tissues and bottom layer of skin were collected and placed onto cultural dish. The tissues were rinsed 4-5 times in sterile PBS and cut into pieces of 1 mm³ in size. Randomly, 10-20 fresh pieces from each mouse were cultured as a control, seeded on calcified plasma drops in tissue culture containing DMEM supplement, 10% Fetal Calf Serum (FCS), 2 mm glutamine, 0.1 mm mercaptoethanol, 1% non-essential amino acids, 100 U mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin. Similarly, 3-5 pieces were placed into vial contain cryoprotectants. In a manner similar to skin tissues, mouse whole ears were collected and then rinsed 4-5 times in sterile PBS. After removal of hair and epidermis, the double-sided pieces of tissues were separated into two layers and cut into 1 mm³ pieces in size and then cultured.

Cattle: The cattle ear samples were collected from local slaughterhouse and cowshed in Institute of Animal Science, Mariensee, 31535, Nested, Germany. They were placed in ice and then transported to the laboratory within 0.5-1 h. In the laboratory, ears were disinfected with 75% ethanol and washed with sterile PBS. Then, subdermis tissues were collected and minced into pieces in size of 2 mm³. Randomly, 10-20 fresh pieces, as a control, were cultured in media containing re-calcified micro drops of bovine plasma, DMEM supplement 10% Fetal Calf Serum (FCS), 2 mm glutamine, 0.1 mm mercaptoethanol, 1% non-essential amino acids, 1% vitamin solution, 100 U mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin. Then 3-5 pieces were placed into vial contain cryoprotectants. The viability of tissue was defined as complete culturing after explants by Hoechst staining. The culture condition was adjusted to 37°C with saturated humidity and 5% CO₂.

Freezing and thawing protocol: In the present study, three cryoprotectants including Dimethylsulphoxide (DMSO), Glycerol (GLY) and Propane-1,2-diol (PROH) at three concentrations each were used. 3-5 pieces of tissues were placed into a cryovial and then 0.5 mL freezing media was added. They were divided into four groups. The tissues of the first group were equilibrated for 20-25 min at room temperature, compared with the second group which contains un-equilibrated tissues. While the third and fourth groups were equilibrated for 20-25 min at room temperature.

The third group of tissue samples was cryopreserved by dry-ice vapour. The fourth group of tissue samples was cryopreserved by plunging directly into liquid nitrogen.

For dry-ice freezing, cryovials were held on the surface of dry-ice until the medium was frozen, then put into -80°C freezer. While liquid-nitrogen freezing, the cryovials were plunged into liquid nitrogen directly, then loaded to minus 80°C freezer. After storage for 1-2 weeks, the cryovials were directly thawed in water bath at 37°C for 1.5 min.

The cryoprotectant was diluted by DMEM and 10% FCS. Following thawing and re-hydration, the tissues were pasted to the culture plate by employing re-calcified micro drops of bovine plasma and overlaid with DMEM as mentioned previously and presented in Table 1.

Tolerance test: The tolerance of mouse and cattle skins was tested by exposing the skins to four cryoprotectants (DMSO, GLY, PROH, EG) at three concentrations and 4°C and room temperature.

Statistical analysis: Growth rates were estimated with the following equations: Growth rate = the number of pieces from which can grow fibroblast after being cultured/sum of pieces cultured after frozen-thawed. The data was statistically analyzed by t-test performed by SPSS11.5 software.

Table 1: The compositions of the freezing media

Media	DMSO (%)	Gly cerin (%)	Propanediol (%)	Ethylene glycol (%)	FCS (%)	DMEM (%)
Medium1 (MD1)*	10	-	-	-	10	80
Medium2 (MD2)	20	-	-	-	10	70
Medium3 (MD3)	30	-	-	-	10	60
Medium4 (MG1)	-	10	-	-	10	80
Medium5 (MG2)	-	20	-	-	10	70
Medium6 (MG3)	-	30	-	-	10	60
Medium7 (MP1)	-	-	10	-	10	80
Medium8 (MP2)	-	-	20	-	10	70
Medium9 (MP3)	-	-	30	-	10	60
Medium10 (ME1)	-	-	-	10	10	80
Medium11 (ME2)	-	-	-	20	10	70
Medium12 (ME3)	-	-	-	30	10	60
Control (DMEM)	-	-	-	-	10	90

RESULTS

Culturing mouse and cattle fresh skins and ears: In the present study, fresh pieces of skin ears from mouse and cattle were cultured as a position control. The result showed that adult cattle ear tissues cultured at 11 days yielded more viable cells and proliferated readily and rapidly compared with adult mouse (120 days old) skins and ears, with the explants rates 65.6 and 25.0%, respectively. After tissue frozen, the similar phenomenon happened. However, the 45 days old mouse fresh skin explants rates were 100%. The growth rate of tissues was affected by the capacity and time of explantation. The mouse connective tissues under skin were (85.7%) easier to culture compared with the bottom layer skin (66.7%). Similarly, young adult mouse tissues were (100%) easier to grow than the old adults (25.0%). The growth rate of mouse fresh skin tissues was slightly (57.1%) greater than the growth of ear tissues (25%).

There were individual differences in the capacity and time of fresh tissue growth. This phenomenon was found in process of mouse and cattle tissue culture. In this study, the tissues were explanted from cattle No.167, 148 and 137 after 5 days, from cattle No.1, 2, 3 after 7 days and from cattle No.112, 134 and 177 after 10 days. After being cultured for 11 days, 100% tissues explanted from No.167, 148 and 137 could grow, while 87.6% from No.1, 2, 3 and only 12.3% from No.112, 134 and 177 grew. Meanwhile, some individual tissues from adult mice, did not grow.

Mouse skin and ear freezing

Tolerance test: Mouse skin showed higher growth rate in all cryoprotectant under the 22°C equilibration than that under the 4°C equilibration. It showed that mouse skin had a sensitive reaction to 4°C temperature.

EG observed best growth rate among the other cryoprotectant at 4°C equilibration. Similarly, the best growth rate was observed, when treated with 10%

GLY, 20% PROH and 20%EG at 4°C equilibration. While at 22°C equilibration, the skin growth rate was greater in EG. The results were shown in Table 2.

Mouse skin tissue freezing: The tissues were revived after 2-3 weeks storage and then culture in DMEM. The growth rate of tissues with dry-ice and liquid-nitrogen freezing, with three cryoprotectants at three levels of concentration, was shown in Table 3.

The result showed that the time, when tissues began to grow after being thawed was longer than that of fresh tissues. The growth rate of tissues with dry-ice and liquid nitrogen freezing, was greater in GLY and DMSO medium. Meanwhile, the tissues frozen in 10% GLY medium with dry-ice and liquid nitrogen freezing, yielded significantly higher percentage (33.3, 19.4%, respectively) than that of the control group (0%) and other groups.

Moreover, the result of skin tissues cryopreserved in 30% GLY, 20% PROH, 30% PROH with liquid-nitrogen freezing was similar to that of other groups and no explants were observed. There was significant difference ($p<0.05$) of tissue growth, when treated with GLY DMSO

Table 2: The growth rates of 45-days-old mouse tissue toxicity test

Media	Growth rate (%)	
	Equilibrated for 20-25 min at 22°C	Equilibrated for 20-25 min at 4°C
MD1	81.8 ^{al}	41.7 ^{c2}
MD2	77.8 ^{al}	45.5 ^{b2}
MD3	77.8 ^{al}	22.2 ^{d2}
MG1	60.0 ^{cl}	50.0 ^{a2}
MG2	70.0 ^{bl}	40.0 ^{c2}
MG3	36.4 ^{dl}	33.3 ^{bd}
MP1	37.5 ^{dl}	37.5 ^{cl}
MP2	87.5 ^{al}	50.0 ^{a2}
MP3	55.6 ^{cl}	27.3 ^{bd}
ME1	80.0 ^{al}	38.5 ^{c2}
ME2	75.0 ^{al}	50.0 ^{a2}
ME3	100.0 ^{fl}	35.7 ^{c2}
Con.DMEM	100.0	100.0

^{a-f} $p<0.05$ values within different superscripts in a column are significantly different, ^{1,2} $p<0.05$ values within different numbers in a line are significantly different

Table 3: The growth rates of mouse frozen tissue with dry-ice and liquid nitrogen

Media	Dry-ice freezing growth rate (%)		Liquid-nitrogen freezing growth rate (%)	
	Equilibrated for 20-25min	Unequilibrated	Equilibrated for 20-25min	Unequilibrated
MD1	11.54 ^l	0	4.17 ²	0
MD2	16.13 ^{al}	0	11.76 ^{bd}	0
MD3	20.59 ^{al}	0	15.39 ^{bd}	0
MG1	33.33 ^{bl}	0	19.36 ^{c2}	0
MG2	17.65 ^c	0	17.65 ^c	0
MG3	19.23 ^c	0	0.00	0
MP1	2.78 ^d	0	3.13 ^d	0
MP2	2.33 ^d	0	0.00	0
MP3	11.43 ^c	0	0.00	0
Con.DMEM	0.00	0	-	0

^{a-f} $p<0.05$ values within different superscripts in a column are significantly different, ^{1,2} $p<0.05$ values within different numbers in a line are significantly different

Fig. 1: Mouse tissue is showed under normal status, no cell explants, x400

Fig. 2: Mouse tissue is showed under fluorescent, cell explants from tissue, x400

Fig. 3: Mouse fibroblast explants from skin tissue, x200

and PROH under the equilibrated or un-equilibrated conditions. The mouse skin fibroblast explanted was shown in Fig. 1-3.

Cattle ear tissue freezing

Results of tolerance test: The control fresh tissues were compared to the tissues treated with four cryoprotectants at three concentration levels. Non-frozen tissues exposed to PROH and EG and equilibrated at 22°C yielded higher

Table 4: The survival and growth rates of cattle toxicity test

Media	Growth rate (%)	
	Equilibrated for 20-25 min at 22°C	Equilibrated for 20-25 min at 4°C
MD1	21.4 ^{ai}	57.9 ^{ba}
MD2	29.6 ^{ai}	75.0 ^{ba}
MD3	24.2 ^{ai}	73.3 ^{ba}
MG1	21.4 ^{ai}	46.7 ^{ca}
MG2	25.0 ^{ai}	64.3 ^{ba}
MG3	25.0 ^{ai}	55.6 ^{ca}
MP1	36.7 ^a	26.7 ^c
MP2	33.3 ^{ai}	42.9 ^c
MP3	30.0 ^{ai}	50.0 ^{ca}
ME1	46.7 ^{bi}	35.7 ^{ai}
ME2	51.7 ^{ai}	28.6 ^{ca}
ME3	41.4 ^{bi}	68.4 ^{ba}
Con. DMEM	100.0	100.0

* $p < 0.05$ values within different superscripts in a column are significantly different, ^a $p < 0.05$ values within different numbers in a line are significantly different

Table 5: The growth rates of cattle frozen-thawed tissue with Dry-ice and Liquid nitrogen

Media	Growth rate (%)	
	Dry-ice freezing equilibrated for 20-25 min	Liquid nitrogen freezing equilibrated for 20-25 min
MD1	0.00	0
MD2	0.00	0
MD3	0.00	0
MG1	28.6 ^a	0
MG2	71.4 ^a	0
MG3	0.00	0
MP1	12.5 ^a	0
MP2	33.3 ^a	0
MP3	0.00	0
ME1	-	-
ME2	-	-
ME3	-	-
Con. MEM	0.00	0

* $p < 0.05$ values within different superscripts in a column are significant

growth rates. Whereas, that exposed to GLY and DMSO was observed higher growth rates when equilibrated at 4°C. Skin growth rate was higher, when equilibrated at 4°C than that equilibrated at 22°C. The results were shown in Table 4.

Results of tissue freezing: The tissues were revived after 1-2 weeks storage and then cultured. The results of three cryoprotectant treatments at three concentration levels, frozen in dry ice and liquid nitrogen and equilibrated at 4 and 22°C were presented in Table 5.

The tissues frozen in 10% GLY, 20% GLY, 10% PROH, 20% PROH media with dry-ice freezing yielded significantly ($p < 0.05$) higher growth rate than the control group and other groups. There were no results observed, when using similar cryoprotectants with liquid-nitrogen freezing. The cattle ear fibroblast explanted was shown in Fig. 4 and 5.

Fig. 4: Bovine tissue is showed under fluorescent, cell explants from tissue, x400

Fig. 5: Bovine fibroblast explants from ear tissue, x800

DISCUSSION

Since, equipment used for tissue cryopreservation was expensive, simple freezing and thawing techniques were recommended. Commercial tissue bank units should be interested in dry-ice or liquid nitrogen freezing for their lower cost compared with specific tissue freezing machines. The present study concluded that dry-ice freezing was better than liquid nitrogen freezing. If the cells or embryos cool slowly, also observed that the larger cell dehydration need longer times.

The growth rate of tissues after freezing was influenced by the original tissues, cryoprotectant and concentration. Two weeks old mouse skin showed quantitatively greater spreading (Oktay *et al.*, 1998). The growth rate of fetal skin tissues was better than that of 20 and 45 days old adult mouse. Ear skin from adult mice was explanted as a source of somatic cells and they showed lower growth rate.

The commonly used cryoprotectant was DMSO, GLY and PROH. The present study indicated that the GLY was better cryoprotectants for cattle ears and mouse skin tissues than the DMSO and PROH with dry-ice freezing.

GLY was usually used for semen storage because it was relatively nontoxic (Stenn and Dvoretzky, 1979). Whereas in the present study, the cattle ear toxicity test with GLY estimated 55.5% and the mouse skin toxicity test with GLY estimated 41.1%, when explanted at 4°C equilibration. This may be due to low permeability of GLY, which delayed the entrance into cells. Thus, after 20-25 min, the amount of GLY inside the tissues was not high enough to be toxic. However, less GLY was observed inside tissues explanted at 22°C equilibration because of dehydration and deleterious somatic stress. The average growth rate of cattle and mouse fibroblast from tissues after cryopreservation in 20% GLY was 71.4 and 23.4%, respectively. It was also shown that the growth rate of cattle ear tissues cryopreserved in GLY was higher than that of mouse skin tissues.

According to the toxicity test, mouse skin showed higher growth rate in all cryoprotectant at the 22°C equilibration than at 4°C equilibration, which showed that mouse skin had a sensitive reaction at 4°C temperature. EG observed highest growth rate among all cryoprotectant at 4°C equilibration. Similarly, highest growth rate was observed, when treated with 10% GLY, 20% PROH and 20% EG at 4°C equilibration. While at 22°C equilibration, the skin growth rate was higher in EG. People have indicated that cryoprotectant and suitable freezing rate were important procedure for improvement of tissue freezing efficiency. Now, cryopreservation of stem cells, embryos and human pancreatic tissues has been successfully performed as well as the livers of mice, rats, dogs, monkeys and human (Cohen *et al.*, 1988; Shengrong *et al.*, 1997; Martignoni *et al.*, 2004).

Freezing preservation of tissues provided a better method to solve the problem of time difference between provisions and requirements. Hypothermal preservation not only was beneficial for accumulating the tissues of donors but also could reduce the immunological rejection obviously.

Several studies reported that the xenogenetic tissues treated at low temperature showed a longer survival time than that of fresh tissues after trans-plantation (Berggren and Lether, 1965; Taylor *et al.*, 1990). Perhaps, the immune antigenicity of skin changed with low temperature treatment, but the specific mechanism was not clear (Taylor *et al.*, 1990).

The results indicated that the tissues in all kinds of cryoprotectants were preserved well if they experienced a process of osmotic equilibrium for 20-25 min before fast or slow freezing preservation. That is to say, tissues should be placed in the cryoprotectant for a period of time before they were frozen and preserved, so that the cryoprotectant could infiltrate them thoroughly.

It was reported that the velocity of lowering temperature had an obvious impact on the effectiveness of tissue freezing (Hullett *et al.*, 1989; Wise *et al.*, 1983), which was confirmed by the present study. With the dry-ice freezing, the tissues in glycerol (10, 20%) experienced osmotic equilibrium for 20-25 min before freeze preservation. The growth rate of *in vitro* culture of mouse and cattle fibroblast was 33.3 and 71.4%, respectively.

CONCLUSION

However, the present study shows that when the condition of freeze preservation changed, the number of fibroblast explanted decreased and some even failed to grow. With the liquid nitrogen freezing, the cattle fibroblast failed to grow, while the mouse fibroblast growth rate showed significant difference: glycerol as a cryoprotectant was better than DMSO and PROH. The results showed that the preservation of tissues with dry-ice freezing was better than with liquid nitrogen freezing. It may be explained, when using liquid nitrogen freezing, water in cytoplasm couldn't permeate out within a short period of time, so plenty of ice crystal formed in cytoplasm, which was harmful to cells.

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