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Viabilities of Biopsied Mouse Embryos after Ultrarapid Refreezing and Thawing

Shin, S. T., J. H. Lim, M. J. Kang*, Y. M. Han* and K. K. Lee*
College of Veterinary Medicine, Chungnam National University, Taejon 305-764, Korea

미세조작된 생쥐수정란의 초급속 재동결융해 후 생존성

신상태 · 임준호 · 강만종* · 한용만* · 이경광*
충남대학교 수의과대학

요 약

본 연구는 미세조작된 수정란의 초급속 재동결 후 발생능을 조사하기 위해서 수행하였다. 먼저 4세포기 생쥐 수정란으로부터 할구세포 한 개를 떼어내고 이들 수정란을 동결액에 넣어 상온에서 2.5분간 평형시킨 다음, 0.25ml straws에 넣어 곧바로 액체질소에 침지시켰다. 4세포기 수정란의 동결액으로는 4.0M ethylene glycol 및 0.25M sucrose가 함유된 dPBS를 사용하였다. 상실배기 수정란의 동결을 위해서는 항동해제로 4.0M ethylene glycol 대신에 5.0M glycerol을 사용하였다. 융해후 biopsied 4세포기 수정란을 M16 배양액에서 상실배기까지 발달시킨 다음 상실배기용 동결액을 이용하여 초급속 재동결을 실시하였다. 재동결융해 후 발달한 biopsied 배반포기 수정란을 대리모에 이식하여 이들 수정란의 생존성을 검토하였다.

제1 차 동결후 biopsied 수정란의 체외발달률은 78%로서 정상적인 수정란의 발달율 (91%) 보다 낮은 성적을 보여주었으나 (P<0.01), 이들 수정란의 이식 후 임신률은 각각 25 및 30%로서 두 실험군간에 차이가 인정되지 않았다. 그리고 biopsied 상실배기 수정란의 초급속 재동결후 체외 발달률 및 임신률은 각각 89 와 27%로서 biopsied 되지 않은 수정란의 성적 (각각 95 및 28%)과 유사하였다. 이러한 결과를 종합하여 볼 때, 본 연구에서 사용된 초급속 재동결 과정이 미세조작된 생쥐수정란의 생존성에 영향을 미치지 않는 것으로 나타났다.

Ⅰ. INTRODUCTION

Embryo refreezing could be assessed as a method for saving valuable frozen embryos which could not be transferred immediately. Embryo biopsy together with assay techniques such as PCR can be used for sexing or diagnosis of genetic deseases at preimplantation stages in mammals (Bradbury et al., 1990; Gomez et al., 1990; Handyside et al., 1990; Han et al., 1993). Preimplantation diagnoses of genetic diseases currently require the biopsy of one or more cells from each embryo, although it may be possible in the near future to diagnose some inherited diseases in early human embryos by non-invasive methods (Edwards & Hollands, 1988). It has been reported that embryo biopsy did not affect the subsequent development of mouse embryos *in vitro* and *in vivo* (Roudebush et al., 1990;

* KIST 생명공학연구소 (Korea Research Institute of Bioscience and Biotechnology, KIST)
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Takeuchi et al., 1992; Han et al., 1994). If a second biopsy and analysis are neede for an accurate diagnosis and to confirm previous results, refreezing of the embryos must be accompanied sometimes. Thus, refreezing may be necessary for efficient utilization of the manipulated embryos. Recently, it was reported that refreezing of biopsied mouse embryos by a conventional slow freezing method did not affect viability of biopsied embryos (Fields et al., 1991; Snabes et al., 1993). However, there have been few reports on refreezing biopsied embryos by an ultrarapid freezing method. In this study, *in vitro* and *in vivo* viabilities of biopsied mouse embryos after ultrarapid refreezing and thawing were investigated.

Ⅱ. MATERIAS AND METHODS

1. Embryos

F1 hybrid (C57BL/6×DBA) female mice were superovulated by intraperitoneal injections of 5 IU pregnant mare's serum gonadotropin (PMS, Serarumon, Japan) followed 48 h later by injections of 5 IU human chorionic gonadotropin (hCG; Sigma Chemical Co., St. Louis, MO)and then mated to hybrid male mice. Four-cell embryos were collected by flushing the oviducts with M2 medium (Quinn et al., 1982) on 54 h agter hCG injection.

2. Micromanipulation

Zona cutting, blunt, and holding pipettes for micromanipulation were pulled from glass capillary tubing (O.D., 1mm; I.D., 0.5mm; length, 130mm; Garner Glass, USA) using a vertical pipette puller Model 720 (David Korf, USA). Cutting and polishing of pipette tips were carried out using the microforge (Alcater, France). The micromanipulations were performed on an inverted Nikon microscope installed DIC (differential interference contrast) system.

3. Biopsy

Single balstomeres were separated from 4-cell mouse embryos by the modification of anaspiration method (Wilton and Trounson, 1989). Briefly, 4-cell embryos were loaded intomicrodrops of embryo biopsy medium (Gordon and Gang, 1990) and then incubated for at least 15 min at 37℃, 5% CO_2 in air prior to biopsy. The embryos were placed in a 5μl droplet of embryo biopsy medium under silicone oil (Sigma) in the well of a cavity microscope slide for micromanipulation.

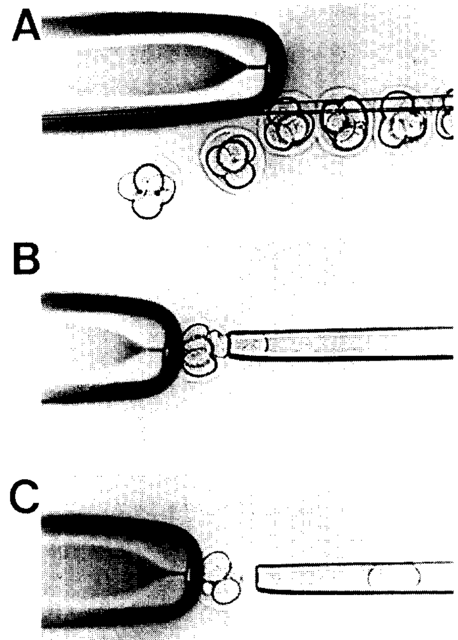

Fig. 1. Biopys of 4-cell mouse embryos.
A. Partial dissection of zona pellucida.
B. Aspiration of a single blastomere using the blunt microneedle.
C. Separation of a single blastomere by sucking into the blunt microneedle.

An embryo was then immobilized at the end of a holding pipette and zona pellucida was pierced with a sharp glass microneedle. The zona pellucida was partially dissected by scraping using both sharp and holding microneedles (Fig. 1a). The embryo with a partially dissected zona pellucida was grasped in the holding pipette and another aspiration pipette with an outer diameter of approximately 20μm was inserted through slit of zona pellucida. A singel blastomere was gently drawn into the aspiration pipette (Fig. 1b), which was removed from the embryo (Fig. 1c). Biopsied embryos were washed twice with M_2 medium and then cultured in M_{16} medium (Whittingham, 1971) under light mineral oil (Sigma) which was equilibrated at 37℃ in 5% CO_2 with humidified air for at least 2 h.

4. Freezing and thawing of biopsied embryos

Biopsied 4-cell embryos were frozen by a modification of ultrarapid freezing method(Trounson et al., 1987). Briefly, the embryos were washed 3 times with modified Dulbecco's phosphate buffered saline (dPBS). They were equilibrated into freezing medium at room temperature for 2.5 min and then were loaded into 40μl of freezing medium in 0.25ml plastic straw(IMV, France) using a finely drawn glass pipette. The straws were sealed with straw powder (FHK, Japan) and then directly immersed into liqiud nitrogen. Freezing medium for 4-cell embryos consisted of 4.0M ethylene glycol (Sigma) and 0.25M sucrose in dPBS supplemented with 6mg/ml BSA (Sigma). Morulae were frozen into freezing medium containing 5.0M glycerol instead of ethylene glycol. The embryos were stored in liqiud nitrogen for 1 to 7d prior to thawing. Thawing was conducted by agitating each straw in 37℃ water for 20 sec. The content of each straw was expelled into 0.5ml of dilution medium which consisted of 0.25M sucrose and 3mg/ml BSA in dPBS. The thawed embryos were rehydrated in dilution medium for 10 min, washed 3 times with dPBS and then cultured in M16 medium at 37℃, 5% CO_2 in air.

5. Embryo culture and transfer

Frozen-thawed embryos were cultrued in 50μl drops of M16 medium supplemented with 0.4% BAS under light mineral oil at 37℃, 5% CO_2 in air. ICR females with spontaneous estrus were mated with vasectomized males. Females with a copulatory plug were used as recipients on the third day. The morning of the plug was designated as Day 0. Approximately 10 blastocysts that developed from biopsied 4-cell embryos after freezing or refreezing were transferred into uteri of pseudopregnant ICR recipient and then developed to full term.

6. Experimental desing

Experiment Ⅰ: Embryos were biopsied at 4-cell stage, incubated at 37℃, 5% CO_2 for 1 h and then frozen by an ultrarapid freezing method. After 48 h of culture, biopsied embryos that developed to blastocysts were transferred to tecipients

Experiment Ⅱ: 4-cell embryos were bipsied, developed to morula stage and then ultrarapidly frozen. After thawing, the embryos were cultured for 24 h and then transfered to recipients.

Experiment Ⅲ: 4-cell embryos were biopsied and ultrarapidly frozen. After thawing, the biopsied embryos were cultured for 24 h, and then ultrarapidly refrozen at morula stage. The refrozenthawed embryos were further cultrued to blastocysts and then transferred to recipients.

Intact 4-cell embryos were subjected to each experiment as a control.

7. Statistical analysis

The statistical significance of the results was analyzed by the Chi-squere test.

Ⅲ. RESULTS

In our preliminary experiment, the highest survival rate of frozen-thawed mouse embryos showed in the groups of 4.0M ethylene glycol and 5.0M glycerol as cryoprotectants at 4-cell and morula stage, respectively (data not shown). Thus, freezing medium containing each cryoprotectant was used for freezing of biopsied embryos at 4-cell and morula stages, respectively. Single blastomeres were biopsied from 4-cell mouse embryos by an aspiration method (Fig. 1). Developmental rate of biopsied 4-cell embryos to blastocysts without freezing was 90% (119/132), which was similar to that (99%, 132/135)of control (intact)embryos.

In Experiments I and Ⅱ, it was examined whether ultrarapid freezing could affect subsequent development of the biopsied 4-cell embryos and morulae (Table 1) 4-cell embryos were biopsied, incubated at 37℃, 5% CO_2 in air for 1 h and then ultrarapidly frozen. After thawing, developmental rate of the biopsied embryos was 78% (101/130), which was lower than that (91%, 114/125) of control ($P<0.01$). However, there was no difference in subsequent development of ultrarapid frozen embryos between biopsied and control embryos after transfer (25 vs 30%, respectively). To test viability of biopsied

Table 1. Viabilities of biopsied embryos ultrarapidly frozen at 4-cell and morula stages

Group	Cell stage frozen	No. of embryos frozen	No. of embryos developed to blastocysts	No. of blastocysts transferred	Pregnant / recipients	No. of pups
Biopsied	4-cell	130	101 (78)[a]	40	2/4	10 (25)
Control		125	114 (91)[b]	40	2/4	12 (30)
Biopsied	Morula	127	115 (91)	40	2/4	10 (25)
Control		133	119 (90)	38	3/4	12 (32)

Experiments were contemporaneously repeated 4 times in each group.
Values in parentheses are percents.
a, b : Values with different superscripts are significantly different ($P<0.01$)

Table 2. Viabilities of biopsied embryos refrozen by an ultrarapid freezing method

Group	[a] No. of embryos		No. of embryos developed to blastocysts	No. of blastocysts transferred	Pregnant /recipients	No. of pups
	frozen	refrozen				
Biopsied	134	118 (88)	105 (89)	48	2/5	13 (27)
Control	140	112 (81)	106 (95)	40	3/4	11 (28)

Experiments were contemporaneously repeated 4 times in each group.
Values in parentheses are percents.
[a] Biopsied embryos at 4-cell stage were frozen-thawed, cultured to morulae and then frozen again.

embryos ultrarapidly frozen at morula stage, 4-cell embryos were biopsied, developed to morula stage and then ultrarapidly frozen. After thawing, biopsied embryos were further cultured for 24 h and transferred to recipients. As shown in Table 1, ultrarapid frozen morulae after 4-cell biopsy showed similar *in vitro* and *in vivo* viabilities compared to intact embryos.

In Experiment Ⅲ, viabilities of biopsied embryos after ultrarapid refreezing were investigated (Table 2). Biopsied 4-cell embryos were frozen-thawed, cultrued for 24 h and then refrozen. *In vitro* developmental rates of biopsied and intact morulae after refreezing and thawing were 89 (105/118) and 95% (106/112), respectively. Following transfer of refrozen embryos, 27 (13/48) and 28% (11/40) of, respectively, biopsied and intact blastocysts yielded normal pups. Thus, *in vitro* and *in vivo* viabilities of biopsied embryos ultrarapidly refrozen were similar to those of intact embryos.

Ⅳ. DISCUSSION

The overall results suggest that refreezing of biopsied mouse embryos by the ultrarapid procedure has no detrimental effect on *in vitro* or *in vivo* viabilities of the embryos.

Preimplantation developmental rate of mouse embryos biopsied at 4-cell stage was lower than that of intact embryos after ultrarapid freezing and thawing, although no significant difference was found in the development to full term between biopsied and intact embryos. And *in vitro* and *in vivo* viabilities of morulae that developed from biopsied 4-cell embryos were similar to those of intact embryos (Table 1). Krzyminska et al. (1990) and Roudebush et al. (1990) reported that biopsy of 4-cell mouse embryos resulted in a significant reduction in development rate to the blastocyst stage ($P<0.001$). Therefore they suggested that the 8-cel stage for preimplantation embryo biopsy was more suitable than the 4-cell stage with respect to development of blastocysts and live young. Their results differ from the present study in that they did not freeze the biopsied embryos. Thereafter, it was reported that there was no difference in the development *in vitro* and *in vivo* of the embryos when biopsied 8-cell mouse embryos were frozen by a slow freezing and quick thawing (Krzymiska and O'Neill, 1991). Wilton et al. (1989) showed that ultrarapid freezing of biopsied and control 4-cell embryos had no effect on continued development *in vitro* and *in vivo*. In this study, there was no difference in *in vivo* viability of blastocysts that developed from biopsied 4-cell embryos after ultrarapid freezing. This result indicates that ultrarapid freezing procedure does not affect *in vivo* viability of biopsied mouse embryos.

In our previous report, sex of embryos could be identified from analysis of single blastomeres at 4-cell and morula stages by PCR (Han et al., 1993). Embryo biopsy together with assay techniques such as PCR and probing for specific DNA sequences has been used for sexing or diagnosis of genetic disorders at preimplantation stages in humans and other domestic animals (Handyside et al., 1990 ; Peura et al., 1991 ; Grifo et al., 1992 ; Kirkpatrick & Monson, 1993 ; Horvat et al., 1993 ; AppaRao et al., 1994). Efficient utilization of biopsied embryos requires that they should be cryopreserved. Especially, embryo refreezing could be more profitable for saving valuable frozen embryos which could not be immediately tranfserred due to recipient failures. On this respect, Snabes et al. (1993) reported that 8-cell mouse embryos were frozen-thawed, biopsied and then refrozen by a conventional slow freezing method. They demonstrated that cryopreserved mouse 8-cell embryos could

successfully undergo thawing, biopsy and refreezing. In this study, 4-cell mouse embryos were first biopsied, frozen-thawed, cultured to morulae and then refrozen by an ultrarapid freezing method. As shown in Table 2, there was no significant difference in the rates of *in vitro* and *in vivo* development between refrozen biopsied and intact embryos. Thus, this study resulted in successfully ultrarapid refreezing of biopsied mouse embryos. The results suggest that the procedures including biopsy of 4-cell embryos, ultrarapid freezing and refreezing may be useful for improving utilization of manipulated IVF-derived embryos in humans and domestic animals, although there appears to be some variability between species as to the optional stage of biopsy and the conditions of ultrarapid freezing.

V. SUMMARY

To examine the developmental capacity of manipulated embryos after ultrarapid refreezing and thawing, mouse embryos were biopsied at 4-cell stage, frozen twice at 4-cell and morula stages, respectively, and then transferred to recipients. Single blastomeres were biopsied from 4-cell embryos by a modified aspiration method. Biopsied 4-cell embryos were equilibrated into freezing medium at room temperature for 2.5 min, loaded into 40 μl of freezing medium in 0.25 ml plastic straw and then directly immersed into liqiud nitrogen. Freezing medium for 4-cell embryos consisted of 4.0 M ethylene glycol and 0.25 M sucrose in dPBS supplemented with 6 mg/ml BSA. Morulae were frozen into freezing medium containing 5.0 M glycerol instead of ethylene glycol. Thawing was conducted by agitating each straw in 37℃ water for 20 sec. The content of each straw was expelled into 0.5 ml of dilution medium, which consisted of 0.25 M sucrose and 3 mg/ml BSA in dPBS. The thawed embryos were rehydrated in dilution medium for 10 min, washed 3 times with dPBS and then cultured in M16 medium at 37℃, 5% CO_2 in air. Blastocysts that developed from frozen or refrozne biopsied embryos were transferred to recipients on Day 3 of pseudopregnancy, respectively.

In vitro and *in vivo* developmental rates of the biopsied and intact 4-cell embryos after freezing and thawing were 78 (101/130) and 25% (10/40), and 91 (114/125) and 30% (12/40), respectively. Although the rates of *in vitro* development of biopsied and intact embryos to blastocyst stage were significantly different after freezing and thawing ($P<0.01$), no difference was found between the rates of *in vivo* development of blastocyts produced from biopsied and intact embryos after transfer. Similarly high rates of *in vitro* and *in vivo* development were obtained following the refreezing of morulae from biopsied and intact embryos. Eighty-nine (105/118) and 95% (106/112) of, respectively, biopsied and intact morulae after refreezing developed to blastocysts. Twenty-seven (13/48) and 28% (11/40) of, respectively, biopsied and intact blastocysts yieded normal pups following embryo transfer. Thus, no significant difference was found in the rates of *in vitro* or *in vivo* development of refrozen biopsied and intact embryos. The results indicate that ultrarapid refreezing procedure used in this study has no detrimental effect on viabilities of biopsied mouse embryos.

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