韓國家畜繁殖學會誌 16(3) : 193~198(1992)
Korean J. Animal Reprod.

Pregnancies Following Transfer of Mouse Embryos Preserved by Ultrarapid Freezing

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超急速凍結保存에 依한 생쥐卵의 移植에 따른 受胎率

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적 요

本 實驗은 ICR系統 생쥐의 8細胞胚 및 桑實胚를 Vitrification(VS_3) 凍結保存液을 利用하여 超急速 凍結-融解를 實施하여 胚盤胞胚까지의 體外發生한 胚盤胞胚를 移植 하였을때 受胎率에 미치는 影響을 調査하여 다음과 같은 結果를 얻었다.

1. 8細胞胚의 平衡時間(3分 및 6分)에 따른 超急速 凍結-融解後 胚盤胞胚까지의 體外發生率은 57.6% 및 59.5%였다.
2. 桑實胚의 平衡時間(3分 및 6分)에 따른 超急速 凍結-融解後 胚盤胞胚까지의 體外發生率은 64.4% 및 68.2%였다.
3. 8細胞胚와 桑實胚를 超急速 凍結-融解를 實施하여 胚盤胞胚까지 體外發生한 胚를 僞姙娠되어진 受卵쥐에 19周 및 20周를 利殖하여 7匹(36.8%)과 10匹(50.0%)의 産仔를 얻었다.

(Key words : VS_3, 평형시간, 수정난이식, 생쥐)

I. INTRODUCTION

Most cellular damage occuring freezing and thawing of cells or tissue is related to the formation of intracellular and extracellular ice crystals(Mazur, 1977; Leibo et al., 1978). Recently, successful ice-free preservation of mouse 8-cell embryos at −196℃ by a process termed vitrification has been reported Rall and Fahy (1985). This apporoach relies on the ability of highly concentrated aqueous solutions of cryoprotectants to supercool to very low temperatures. At sufficiently low temperatures, these solutions become so viscous that they solidify into a glass-like state without the formation of ice crystals.

The other main form of cellular death is caused by alterations in the cytoplasm and the surrounding medium. As the liquid water content of these comportments its transformed into ice, the salt concentration increase. These changes were referred to as solution effects by Mazur(1970) who found that the cell died. When the salt concentration in the suspending

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medium reached a critical level during freezing. As a result of damage caused by solution effects, mammalian cells irrespective of the degree of dehydration do not survive freezing below about −20℃ unless the freezing medium contains cryoprotectants.

The role of intracellular vitrification in the cryoprotection of embryos can be studied under conditions which provide various degree of non and permeation before freezing and permit embryos to be cryopreserved by direct transfer into liquid nitrogen(Rall et al, 1985, 1987). Rall and Fahy(1985) described an alternative approach for the cryopreservation of mouse embryos by vitrifiaction. Vitrification is a process of solidification whereby an aqueous solution does not crystallize during cooling; instead, the viscosity of the solution increases with decreasing temperature until an amorphous glass-like solid forms. This is possible because the cells are initially predehydrated at room temperature in hypertonic solution such as sucrose plus non and permeatable cryoprotectant agents. Vitrifiaction is rapid because embryos are plunged directly into liquid nitrogen and thawed by transfer from liquid nitrogen to ice water. This method does not require a biological freezer(Trounson et al, 1987). The toxicity of the cryoprotectants and the need to handle embryos at temperatures around 4℃ during addition and removed of cryoprotectants are disadvantages of vitrification.

We have developed a freezing method that requires a brief, one-step exposure of embryos to the cryoprotectant solution at room temperature followed by plunging the embryos directly into liquid nitrogen(LN_2). Embryos are thawed ultrarapidly in a 37℃ waterbath and the development of this technique and, in classical cryobiology, it would be prodicted that this method would not result in embryo survival. We reported high survival rates on thawing of ultrarapidly frozen 8-cell and morulae mouse embryos and good viability when embryos are cultured *in vitro* or when transferred to foster mothers.

Ⅱ. MARTERIALS AND METHOD

1. Collection and isolation of embryos.

Embryos were obtained from 4~6 week old ICR mice that were induced to superovulate by intraperitoneal injection of 5IU PMSG(Peamax, Japan) and 5IU hCG(Sigma, U.S.A.) given 48h apart. After the hCG injection females were paired with ICR males and inspected copulation plugs at the following morning. Embryos were flushed from the excised oviducts and uterus at 50 to 70h after the hCG injection using a D-PBS(Dulbecco's phosphate buffered saline). The normal 8-cell and morulae embryos were washed in fresh medium D-PBS(3mg BSA/ml).

2. Preparation of vitrification solutions

These solutions were described previously (Rall, 1987). The stock vitrificaion solution is a mixture of permeating solutes(6.5M) glycerol and non-penetrating polymer with 3mg BSA/ml in D-PBS adjusted to pH 8.0.

3. Exposure of embryos to vitrification solutions

The equilibraton procedures were described by Rall and Fahy(1985). Briefly, 8-cell and morulae were equilibrated in VS_3 at 26℃ for 3 or 6 minutes. After a total of 3 or 6 minutes exposure to the vitrification solution, the embryos were either eluted without cooling or vitrified by ultrarapid cooling in liquid nitrogen

(LN_2).

4. Vitrification procedure

The embryo suspensions were vitrified in 0.25 ml plastic insemination straws(FHK Co., Japan) Each vitrification solution [6.5M glycerol+6.0%(w/v) polyethylene glycol(PEG)] was D-PBS and adjusted to pH 8.0.

and these were prepared as follows 1) A 7cm column of appropriate sucrose solution(0.5M) was carefully aspirated into a straw until the cottonplug was wetted 2) A 0.5cm short column of air bubble 3) the embryos were pipetted into the center of this 1cm column and 1.5cm column air bubble and the straws were straw-powder sealed(Fig. 1). After a total of 3 or 6 minutes exposure to the vitrification solution, the straws were held in a vertical position and rapidly immersed in LN_2(~2500℃/min) where they stored for 7days.

5. Rapid thawing

Straws were thawed by gentle agitation in a 37℃ water bath. Embryos not pretreated in VS_3 were removed from the freezing solution in one step by through mixing in the sucrose solution (D-PBS+20% FCS+0.5M sucrose) that was always at the same concentration as in the freezing solution. The embryos were observed by expelling the straw contents into a small petridish and gently mixinng. After 10 minutes, embryos were pipetted directedy into D-PBS+20% FCS, washed three times, and placed into culturedish.

To assess viability, thawed embryos were placed in foetal calf serum under paraffin oil at 37℃ in a humidified atmosphere of air. Embryos viability and morphology were observed at 24 and 48h later. Data are presented as percent survival, the percentage of embryos which developed or redeveloped to normal blastocysts after 48h post-thaw.

6. Embryo transfer

Day 1 of pseudopregnancy was detected by observing vaginal plugs following mating with vasectomized males. Embeyos were surgically transferred into each uterine horn of 3 pseudopregnant recipients that had developed normally to balastocyst. The number of litter after parturition were recorded.

7. Statistical analysis

All data were analyzed by χ^2-test(Minitab INC., 1989, U.S.A.). Embryos survival was expressed as the percentage of embryos that developed to blastocysts *in vitro*.

Ⅲ. RESULTS AND DISCUSSION

1. Effects of equilibration time on survival of mouse 8-cell and morula embryos by ultrarapid freezing

The percentage of developed *in vitro* was considerably elevated after the ultrarapid

Fig. 1. Freezing apparatus with straw loaded as indicated; C : Cotton plug, SD : Sucrose dilution(7.0cm), A : Air bubble(0.5cm), A*: Air bubble(1.5cm), ***: Vitrification solution+embryos(1.0cm), SP : Straw powder(0.5cm)**

Table 1. Effects of equilibration time on survival rate of mouse 8-cell and morula embryos by ultrarapid frozen-thawed

Cell stage	Equilibration time in VS_3(min)	Survival after frozen-thawed(%)	Development to blastocysts(%)
8-cell	3	33/53(62.3)[a]	19/33(57.6)[a]
	6	37/57(64.9)[a]	22/37(59.5)[a]
Morula	3	45/56(80.4)[b]	29/45(64.4)[ab]
	6	44/54(81.5)[b]	30/44(68.2)[b]
Control	–	–	53/67(79.1)[c]

[a, b, c] ($P<0.05$, χ^2)

freezing-thawing, following equilibration time, although the defference was no significant (Table 1).

When the equilibration time was supplemented with 3 minutes(8-cell, 19 out of 33, 57.6%; morula, 29 out of 45, 64.4%), the viability of frozen-thawed embryos was lower than 6 minutes(8-cell, 22 out of 37, 59.5%; morula, 30 out of 44, 68.2%), to blastocysts and developing normally after *in vitro* culture.

The present study shows that mouse 8-cell or morula embryos can survive after partial dehydration at room temperature followed by ultrarapid freezing and thawing. Using the 0.5 M sucrose in VS_3 combinating to dehydrate embryos at room temperature, we observed their marked shrinkage. The present results showed clearly that the post-thaw survival of vitrified embryos was affected by the length of equilibration time in VS_3 method. As shown in Table 1, the presetnt results indicate that exposure to VS_3 for 6 minutes would be insufficient for best equilibration for 8-cell or morula embryos.

The survival rate obtained after 6 minutes equilibration(8-cell, 59.5%; morula, 68.2%) were lower than the results of Scheffen et al.(1986) and Reichenbach et al.(1988) who obtained survival rate of 80% and 85%, but similar to the data of Fukuda et al.(1987) showed the survival rate of 34%.

Thus short period of dehydration at room temperature than the one used in the present experiment may not be detrimental to mouse embryos survival. Accordingly, even if a large proportion of embryos developed after ultrarapid freezing and thawing, optimum conditions for dehydration and concentration of non-permeable cryoprotectant showed be found. This would lead to an improvement of the development rate to reach values obtained after rapid freezing.

2. Effects of the development to blastocysts embryos transferred on pregnancy rate of ultrarapid frozen-thawed mouse embryos

The effects of ultrarapid freezing and 20 to 40h *in vitro* culture on the proportion of recipients given with, their mean litter size and the overall survival rate of embryos are summarized in Table 2.

The proportion of ultratrapid frozen-thawed embryos developed to term was less than that of fresh embryos. The pregnancy rate after ultrarapid freezing in VS_3 of 8-cell embryos(36.8%) was approximately 13% higher when compared to the morula embryos(50.0%)

Table 2. Effects of ultrarapid freezing and *in vitro* culture on the development of 8-cell and morula embryos to live young in recipient

Treatment group	No. of Recipient	No. of embryos transferred	No. of recipient pregnant	No. of fetuses(%)
8-cell	2	19	1	7(36.8)[a]
Morula	2	20	2	10(50.0)[b]
Control	3	38	3	23(60.5)[c]

[a, b, c] ($P<0.05$, χ^2)

Pregnancy rate after transferred to blastocysts developed *in vitro* of mouse 8-cell and morula embryos by ultrarapid frozenthawed, although in a limited number of recipient, indicating repaired after rapid freezing(Massip et al., 1986; Trounson et al., 1987). This rapid freezing procedure after partial after dehydration at room temperature in a great interest because it allows several simplifications of the freezing technique the straws can be prepared in advance and kept frozen before embryos recovery.

No manual seeding of the extracelluar medium in necessary during rapid cooling to −30℃. This rapid freezing procedure does not need any equipment, just a refrigerate.

This study shows that mouse embryos may be ultrarapidly frozen after partial dehydrating at room temperature; at can be expected that even more ultrarapid freezing procedure could be efficient for mouse embryos as it has been shown recently(Kobayashi et al., 1990) in laboratory species(rabbit, rat, mouse) that direct plunging into LN_2.

Ⅳ. SUMMARY

This study was carried out in order to investigate effects of vitrification solution(VS_3) and equilibration time on the development and embryos transfer of ultrarapidly frozen 8-cell and morula ICR mouse embryos. Mouse 8-cell and morula embryos, following dehydration by exposure in VS_3 and sucrose, were plunged directly into LN_2 and thawed in 37℃ water bath, survival was defined by embryos transfer and development rate to the blastocysts stage *in vitro* culture in CO_2 incubator.

The results are summarized as follows;

1. The development rate to blastocysts on the following equilibration time in VS_3 of 8-cell embryos were 57.6 and 59.5%, repectively.
2. The development rate to blastocysts on the following equilibration time in VS_3 morula embryos were 64.4 and 68.2%, respectively.
3. The survival *in vitro* of frozen-thawed eight cell and morula embryos from ICR mouse were, cultured to blastocysts and transferred to pseudopregnant recipients, thirty-six percent of the frozen-thawed 8-cell embryos and 50% of the morula embryos into viable fetuses.

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