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Improvement of Straw Loading Method on Survival of Mouse IVF/IVC Blastocysts Cryopreserved by Vitrification

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체외수정과 체외배양에서 생산된 생쥐 배반포기배의 초자화 동결에 있어서 Straw Loading 방법의 개선

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요 약

본 실험은 체외수정에 의해 생산된 생쥐 배반포기배를 초자화 동결하였을 때 straw 제작방법 및 융해 조건이 배반포기배의 생존성에 미치는 효과를 검토하고자 실시하였다. 융해시 각 straw내 동결보존액이 초자화 상태로 유지되는지를 확인하기 위하여 동결보존액 충전과 봉인방법이 다른 세 종류의 straw I, II, III가 제작되었는데, 이러한 모든 straw는 실온에 1~10초까지 노출된 후 25℃ 수조에 침지되었다. 수정란은 20% ethylene glycol에 5분, EFS 40 용액에 1분간 노출된 후, straw내로 옮긴 다음 straw I과 II의 경우는 straw powder로, straw III는 straw powder와 열처리로 봉인하여 액체질소에 침지하여 다음과 같은 결과를 얻었다; 1) Straw I embryo column은 융해시 3~6초의 실온 노출을 제외한 대부분의 노출시간에서 불투명한 반초자화 상태로 변화되었다. 그러나 Straw II embryo column을 사용하였을 때 다소 개선되었으나 완전히 초자화 상태를 유지하지는 못하였다. 반면, Straw III embryo column의 경우는 융해시 완전한 초자화 상태를 유지할 수 있었다. 2) Straw III loading 방법을 사용하여 초자화 동결, 융해된 난자의 생존율은 Straw I loading 방법을 사용하였을 때에 비해 유의하게 높았으며 ($P<0.05$), 표준오차 범위는 낮게 나타났다. 3) 초자화 동결보존된 난자를 실온에 3, 5, 10초간 노출한 후 25℃ 수조내에 침지하여 융해하였을 때, 배양 24시간째 생존율은 72.7~87.1% 이었고, 배양 48시간째 탈출배반포기배까지의 발달율은 34.0~48.4% 이었으며, 융해시 각 처리간 유의차는 인정되지 않았다. 본 연구 결과, 초자화 동결 융해 후 높은 생존율 및 낮은 오차범위는 Straw III loading 방법과 double 봉인방법 및 적절한 2 단계 융해법을 사용함으로서 얻을 수 있었다.

I. INTRODUCTION

Vitrification of embryos is an effective and inexpensive cryopreservation method since it dose not require costly freezing equipment. Since the first successful report on the vitrification of mouse 8-cell embryos by Rall and Fahy (1985),

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several methods have been proposed for the cryopreservation of mammalian embryos using vitrification. Kim *et al.* (1996) reported that the excellent results with *in vitro* produced mouse blastocysts were obtained by using a EFS 40 solution (40% ethylene glycol, 18% Ficoll and 0.3M sucrose in m-DPBS), which was first described by Kasai *et al.* (1990) for mouse morulae, with some modifications.

Cryopreservation of embryos by vitrification requires a solution which is cryoprotectant, will vitrify on cooling, will remain vitreous on warming and is not toxic to embryos during the period of exposure before vitrification and during warming (Ali and Shelton, 1993). Kong *et al.* (1991) reported that significantly higher survival rate of embryos was obtained by using an embryo column between double vitrification solution column method than single vitrification solution column method and a decrease in the concentration of cryoprotectant in the single column, which may have been caused by contamination with sucrose solution, was considered to have been caused by ice-crystal formation in the straws. Also, Saito *et al.* (1994) showed straw loading method which is put an embryo column between 2 short columns of vitrification solution to avoid contamination of sucrose and influence of heat sealing. On the other hand, Takeda (1987) and Rall and Mayer (1989) reported that thawing procedure is important in determining embryo survival because zona damage is associated with thermally induced fracturing of the suspension during rapid changes of temperature. Shaw *et al.* (1995) has shown that a 10~15sec. slow warming step (in air) followed by rapid warming (in water bath) resulted in higher viability than either shorter (0sec. in air) or longer (30 and 130sec. in air) slow warming steps. Recently, several two step warming methods have been used for the cryopreservation of bovine IVF-blastocysts (Suzuki *et al.*, 1993; Iwasaki *et al.*, 1994; Kuwayama *et al.*, 1994), zona-drilled mouse embryos (Herman *et al.*, 1991) and mouse blastocysts (Shaw and Trounson, 1994; Shaw *et al.*, 1995).

The objective of this study was to examine the effect of straw loading method and thawing protocol on the *in vitro* development of *in vitro* produced mouse blastocysts cryopreserved by vitrification.

Ⅱ. MATERIALS AND METHODS

1. Animals

Four to six weeks old, hybrid F_1 female mice from C57BL/6, ♀×CBA/N, ♂ were superovulated by intraperitoneal (i.p.) injection of 5 i.u. pregnant mare serum gonadotrophin (PMSG ; Sigma), followed by 5 i.u. human chorionic gonadotrophin (hCG ; Sigma) 50h later.

2. Production of blastocysts *in vitro*

In vitro fertilization and blastocysts production were performed as method described by Kim *et al.* (1996). The blastocysts produced *in vitro* were collected middle and hatching blastocysts at day 4 and day 5, individually.

3. Vitrification solutions

The vitrification solution, EFS 40, described by Zhu *et al.* (1993) was used in the present experiments. Ethylene glycol (Sigma) 40% (v/v) in modified Dulbecco's phosphate-buffered saline (mDPBS, Gibco BRL) containing 30% (w/v) Ficoll 70 (average molecular weight 70,000, Sigma) plus 0.5M sucrose (Sigma). 20% ethylene glycol in mDPBS (20% EG) was prepared as an equilibration solution.

4. Vitrification studies

The 0.25ml french straws (I.M.V., L'aigle)

Straw I :

CP	4cm	1cm	0.5 cm	0.5 cm	2cm*	0.5 cm	0.5 cm	1cm	0.5MS	SP

Key (left to right) : Cotton plug, 0.5MS, Air, EFS40, Air, EFS 40 (*Embryo column), Air, EFS 40, Air, 0.5MS, Straw powder

Straw II :

CP	4cm	1.5cm	0.5 cm	0.5 cm	2.5cm*	1cm	0.5MS	SP

Key (left to right) : Cotton plug, 0.5MS, Air, EFS40, Air, EFS 40 (* Embryo column), Air, 0.5MS, Straw powder

Straw III :

CP	4cm	1.5cm	0.5 cm	0.5 cm	0.5 cm	0.5 cm	2cm*	1cm	0.5 MS	SP & HS

Key (left to right) : Cotton plug, 0.5MS, Air, EFS40, Air, EFS 40, Air, EFS 40 (* Embryo column), Air, 0.5MS, Straw powder and Heat sealing

Fig. 1. Configuration of 0.25ml straws loaded vitrification solution before vitrification.

were loaded vitrification solution and 0.5M sucrose solution at room temperature (25℃) by suction with a syringe as three straw loading types described in Fig. 1. The ability of the solution on straw loading methods to remain vitreous during warming was tested by plunging the vitrified straws into a water bath maintained at 25℃. During cooling, vitrification was evidenced by the formation of a transparent glass and during warming, a solution in the 'Embryo column' of each straw that did not devitrify was transformed the solid clear state to the liquid state without evidence of a milky appearance. Devitrification (ice formation) conferred a milky appearance during warming.

Embryos were vitrified by modification of procedures outlined by Kim *et al.* (1996). Embryos to be vitrified were equilibrated to the 20% EG for 5min. and exposed in EFS 40 for 1min. And within this period about ten embryos were loaded into the embryo column of each straw. The plug ends of Straw I and Straw II were sealed with straw powder and Straw III was treated straw powder, followed by heat sealing. For vitrification, the first part of the straw filled with 0.5mol sucrose solution (0.5MS, 4cm) was slowly immersed into liquid nitrogen (LN_2); the remaining part of the straw was then plunged in.

5. Thawing and assessment of survival

After a few days of storage in liquid nitrogen, the straws were placed in air for 1~10 sec. and then warmed rapidly in water bath at 25℃. The contents of each straw were expelled into a well of a sterile four-well multi-dish plate containing 0.8ml of 0.3MS and then put into fresh 0.3MS for 5min. The embryos were transferred into a well containing mDPBS medium with 10% FBS and washed two times. After 5min., embryos at each developmental stage of each group were cultured in a 50μl droplet of M16 medium supplemented with 4mg/ml BSA. In all experiments, dilution was performed at room temperature (25℃). Survival rate after warming was defined as the percentage of vitrified embryos that re-expanded at 24h and hatching at 48h of culture.

6. Experiment design

Experiment 1. Effect of straw loading and thawing method

To confirm whether the 'Embryo column' is

vitrified or devitrified according to straw loading types on thawing, the straws frozen were tested separately for 1~10sec. of exposure in air and then plunged into water bath at 25℃. Vitrification conferred that a solution in 'embryo column' of each straw frozen was transformed the solid clear state to the liquid state and devitrification (ice formation) conferred a milky appearance during warming.

Experiment 2. Survival rate and standard error of blastocysts frozen-thawed according to straw loading method

To determine the survival rate and standard error on vitrification of blastocysts according to straw loading type, Straw I and Ⅲ loading methods were used to preserve embryos. After loading of embryos, Straw I was used a single sealing method of straw powder sealing and Straw Ⅲ was used a double sealing method of straw powder and heat sealing. After a few days of storage in LN_2, the straws were warmed rapidly in water bath at 25℃. Survival rate after warming was recorded as the percentage of vitrified embryos that re-expanded at 24h and hatching at 48h of culture.

Experiment 3. Survival of blastocysts vitrified-frozen according to exposure time in air during warming

To examine the influence of thawing method, the straws containing embryos in LN_2 were placed in air for 3, 5 and 10 sec. and then warmed rapidly in water bath at 25℃. The embryos were then cultured and survival rate was recorded as the percentage of vitrified embryos that re-expanded at 24h and hatching at 48h of culture. Straw Ⅲ loading type and double sealing method were used for experiment 3.

7. Statistical analysis

Survival rate of each treatment was compared with that in the control group, with Chi-squire test using SAS Institute software package (SAS Institute Inc., 1985).

Ⅲ. RESULTS AND DISCUSSION

1. Effect of straw loading and thawing method

As shown in Table 1, when three straw loading mentods were tested for vitrification, Straw I embryo column mostly changed from transparent to opaque upon thawing without exposure in air for 3~6 sec. Straw Ⅱ embryo column was improved patially but was not remained completely vitreous during warming. However, when Straw Ⅲ loading method was used, the embryo column was remained vitreous completely.

Vitrification requires that a vitrification solution forms an armophous glass as a result of rapid cooling and remains vitreous in the absence of ice-crystal formation on warming (Ali and Shelton, 1993). But Straw I and Straw Ⅱ loading methods were devitrified, which is considered due to ice-crystal formation, which is thought to damage embryos. The milky appearance, devitrification, in Straw I and Straw Ⅱ embryo columns may have been caused by contamination with the residual 0.5MS, which was first aspirated, adhering to the inner wall of the straws.

Kong *et al.* (1991) reported that the residual sucrose solution on the inner wall of the straw was removed perfectly when used a double vitrification solution column method. In this experiment, Straw I and Ⅱ loading methods were similar with double vitrification solution column method described by Kong *et al.* (1991) but there were many evidences of devitrification. It may considered to have been the cause of movement

Table 1. Vitrification or devitrification according to straw loading and thawing method

Thawing* time in air (Sec.)	Straw I		Straw II		Straw III**	
	1st	2nd	1st	2nd	1st	2nd
1	DV[1]	DV(1/2)	V(P)	V	V	V
2	DV	DV	DV	V(P)	V	V
3	V[2]	V	V	V	V	V
4	V	V	V	V	V	V
5	V	V	V(P)	V(P)	V	V
6	V	V	V	V(P)	V	V
7	DV	V	V	V(P)	V	V
8	DV(1/2)[3]	DV(1/2)	V(P)	V(P)	V	V
9	V(P)[4]	V(P)	V	V(P)	V	V
10	DV(1/2)	DV(1/2)	V	V	V	V

* Each straw was exposed of various time in air and then plunged into water bath at 25℃ to melt the solutions in straw columns, completely

** The plug end of Straw III was used a double sealing method of straw powder and heat sealing

[1] DV : Devitrification [2] V : Vitrification

[3] DV(1/2) : Devitrified more than half

[4] V(P) : Devitrified partially

of air bubbles in the straw by insufficient sealing using straw powder and then mixing of vitrification solution and the residual sucrose solution on the inner wall.

Straw III method was improved by a triple vitrification column, which can remove perfectly the residual sucrose solution with previously aspirated two vitrification solution columns, and added heat sealing to straw powder sealing to prevent movement of air bubbles inside of the straw. As a result, Straw III embryo column was not contaminated by the sucrose solution and thus maintained an armophous state in all straws independent of exposure time in air through freezing and thawing.

2. Survival rate and standard error of blastocysts frozen-thawed according to straw loading method

The *in vitro* survival rate ± standard error of frozen-thawed mouse blastocysts according to straw loading method and treatment of heat sealing are shown in Table 2. High survival rates and development rates of each groups (middle blastocysts and hatching blastocysts) of vitrified embryos were obtained by using Straw III loading method than Straw I method ($P<0.05$).

When Straw I loading method was used to preserve embryos, milky appearance, ice-crystal formation which is a major cause of cell death (Dobrinsky and Johnson, 1994), came out often times upon thawing. And the survival rates of the embryos in those straws were lower than those of embryos in which transparent of vitrification column was maintained. So, it has been showed that inclusion of an increase of standard error in Straw I method. Also, the embryo column in Straw I was likely to be contaminated with first aspirated sucrose solution column or residual sucrose solution adhering to the inner wall, which was derived from first aspirated sucrose solution column, by movement of air bubble. Such contamination was reponsible for the unstable vitrification, resulting in ice-crystal

Table 2. *In vitro* survival rate and standard error of mouse blastocysts frozen-thawed according to straw loading type and heat sealing

Blastocysts	Treatments	Replic. No.	No. of eggs	Development % ± SE	
				>Bla. (24h)	>Hing (48h)
Middle	Straw I	8	59	57.0±14.0[a]	20.4± 9.8[a]
	Straw Ⅲ*	6	35	78.8± 8.5[b]	32.4± 6.3[a]
Hatching	Straw I	7	41	51.3±21.0[a]	30.7±15.0[a]
	Straw Ⅲ*	6	29	75.8± 7.3[b]	43.7± 4.2[a]

* The plug end of Straw Ⅲ was used a double sealing method of straw powder and heat sealing.

[a, b] : Different superscripts in the same row were significantly different ($p<0.05$)

formation. However, when Straw Ⅲ loading method was used, the embryo column containing the embryos was maintained an armophous state through freezing and thawing. Therefore, the variation according to experimental replication was decreased and, as a result, the high survival rates and low standard errors of the embryos were obtained.

3. Survival of blastocysts vitrified-frozen according to exposure time in air during warming

When the straws containing embryos in LN_2 were placed in air for 3, 5 and 10sec. and then warmed rapidly in water bath st 25℃, the survival rates to re-expanded blastocysts were 72.7~87.1% and the development rates to hatching blastocysts were 34.0~48.4%. There were no significantly differences between the treatment groups.

The thawing procedure is known to be important in determining embryo survival (Miyamoto and Ishibashi, 1983; Rall and Meyer, 1989; Takeda, 1987). Rall and Meyer (1989) and Shaw and Trounson (1994) reported that the zona pellucida of frozen embryos is more susceptable to fracture damage when they are thawed in a water bath as compared to those which are thawed in air. But zona fracture damage of vitrified embryos in this experiment was not appeared by thawing first in air for 3~10sec.

Shaw *et al.* (1995) indicated that the viability

Table 3. *In vitro* survival rates of mouse blastocysts according to exposure time in air before plunging into water bath at 25℃ on thawing

Blastocysts	Thawing* time in air (Sec.)	No. of eggs	Development	
			>Bla. (24h)	>Hing (48h)
Middle	3	35	28 (80.0)	12 (34.3)
	5	47	37 (78.7) NS**	16 (34.0) NS
	10	33	25 (75.8)	12 (36.4)
Hatching	3	31	24 (77.4)	13 (41.9)
	5	32	24 (72.7) NS	14 (43.8) NS
	10	31	27 (87.1)	15 (48.4)

** Each straw was exposed of various time in air and then plunged into water bath at 25℃ to melt the solutions in straw columns, completely.

* NS : Not significantly differences

of pronuclear and 4-cell mouse embryos slow cooled was highly dependent on the length of the 'slow warming' step. And the thawing methods are appropriate for the type of cryoprotectant used and embryos which were frozen in ethylene glycol were best thawed by holding the straw in air for a short time 6 to 15 sec. followed by immersion in a water bath. The vitrification solution based on ethylene glycol was used for all this experiments. However, in this experiment, the survival rates between the treatment groups, which was exposed in air for 3, 5 and 10sec. before thawing in water bath at 25℃, were not significantly different. This result suggested that survival of blastocysts vitrified-frozen was not dependent on the exposure time in air for 3 to 10sec. on 'slow warming' step. Show and Trouson (1994) reported that the excellent survival of mouse expanded and hatched blastocysts can be achieved in cryoprotectant solutions, providing that straws are not held in air for longer than 15sec.

Therefore, we conclude that the highly survival of vitrified-frozen mouse blastocysts was obtained by using straw Ⅲ loading, double sealing and appropriate 2 step warming method.

Ⅳ. SUMMARY

This study was carried out to investigate the effect of straw loading method and thawing protocol on the *in vitro* development of *in vitro* produced mouse blastocysts cryopreserved by vitrification. Three loading types of straw Ⅰ, Ⅱ and Ⅲ on loading and sealing method were made for vitrification. The ability of the solution on straw loading methods to remain vitreous during warming was tested by exposed in air for 1 to 10 sec. and then plunged the vitrified straws into water bath at 25℃. Embryos to be vitrified were equilibrated to the 20% EG for 5min. and exposed in EFS 40 for 1min. The plug ends of Straw Ⅰ and Straw Ⅱ were sealed with straw powder and Straw Ⅲ was treated straw powder, followed by heat sealing and then plunged into LN_2. The results obtained in these experiments were summarized as follows: 1) Straw Ⅰ embryo column mostly changed from transparent to opaque upon thawing without exposure in air for 3~6 sec. Straw Ⅱ embryo column was improved partially but was not remained completely vitreous during warming. However, when Straw Ⅲ loading method was used, the embryo column was remained vitreous completely. 2) High survival rates and development rates of each groups (middle blastocysts and hatching blastocysts) of vitrified embryos were obtained by using Straw Ⅲ loading method than Straw Ⅰ method ($P<0.05$). And the range of standard error was low in Straw Ⅲ method. 3) When the embryos vitrified-frozen were placed in air for 3, 5 and 10sec. and then warmed rapidly in water bath at 25℃, the survival rates after 24h of culture were 72.7~87.1% and the development rates to hatching stage after 48h of culture were 34.0~48.4%. There were no significantly differences according to exposure time in air during warming. In conclusion, the present results showed that highly survival and low standard error of vitrified-frozen mouse blastocysts were obtained by using straw Ⅲ loading, double sealing and appropriate 2 step warming method.

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