韓國家畜繁殖學會誌 15(1) : 49~57(1991)
Korean J. Animal Reprod.

Effects of Equilibration Time, Precooling and Straw Loading Method on Survival of Mouse Embryos Frozen by Vitrification

Kong, I.Keun., E.B. Lee*, D.J. Kang and C.S. Park
College of Agriculture, Gyeongsang National University

생쥐 수정란의 유리화 동결보존에 있어서 동결전 처리방법에 관한 연구

공일근 · 이은봉* · 강대진 · 박충생
경상대학교 농과대학

적 요

생쥐 수정란의 동결보존법을 개선하고자 유리화 동결과정에서 제1보존액에서의 평형시간, 제2보존액의 전냉각 여부 및 straw loading 방법에 따른 동결보존 후 수정란의 생존율을 조사하고, 또한 유리화 동결보존 과정에서 수정란의 부분적인 손상 여부를 확인하기 위하여 동결보존 후 배양하여 수정란의 할구수를 Hoechst 33342로 염색하여 조사하였던 바 그 결과는 다음과 같다.

1. 동결융해 후 생존율은 Medium-1에서 10분간 평형시간을 준 경우(81.0%)는 5분간(40.0%) 및 15분간(74.1%)의 평형시간을 준 경우보다 유의적($P<0.05$)으로 높았다.
2. Double Medium-2 column 방법으로 얻은 생존율(81.0%)은 single Medium-2 column 방법으로 얻은 생존율(62.8%)보다 유의적($P<0.01$)으로 높았다. 그리고 double Medium-2 column 방법에서는 유리화 용액이 희석액에 오염되지 않아 순수하게 투명한 유리화를 이룰 수 있었다.
3. 전냉각을 않은 경우에 비하여 전냉각한 Medium-2로 동결한 수정란은 생존율에 있어서 다소 높은 성적을 보였으나 유의적인 효과는 없었다.
4. 상실배기의 수정란을 24~28시간동안 배양하여 얻은 후기 배반포를 Hoechst 33342로 염색하여 할구수를 조사하였던 바 신선란(53.3±1.6)보다 동결란(41.4±1.5)에서 유의적($P<0.05$)으로 할구수가 적었다. 이는 동결융해 과정에서의 부분적인 손상에 의하였던 것으로 사료된다.

(Key words : vitrification, equilibration time, precooling, straw loading, embryo cryopreservation.)

I. INTRODUCTION

Considerable progress has been made in improving and simplifying the various steps of embryo freezing procedure since the early reports of successful cryopreservation of mammalian embryos(Whittingham et al., 1972 ; Wilmut, 1972).

The development of simple and inexpensive cryopreservation techniques would certainly enhance the feasibility of on-farm embryo freezing. Moreover, it would have a great impact not only in the field of agriculture, biological science and veterinary medicine, but also in the field of human clinical medicine. Most of the recent research activities have been focused on improvement and simplification in

*순천대학교 농과대학(College of Agriculture, Sunchon National University)

the cooling, thawing and post-thaw dilution procedure of embryo cryopreservation.

Vitrification is a new approach to preservation of embryos as demonstrated by Rall and Fahy(1985) who reported high *in vitro* survival of eight cell mouse embryos by using a rather complex vitrification method. Vitrification is the solidification of the solution brought about not by crystallization but by elevation in viscosity during cooling. At sufficiently low temperature, the solutions became so viscous that they could solidify without the formation of ice.

It has been demonstrated that vitrification is an effective cryopreservation procedure of mouse(Rall, 1987; Rall and Fahy, 1985; Rall et al., 1987; Scheffen et al., 1986), rabbit (Smorag, 1989) and bovine(Massip et al., 1986; Scheffen et al., 1986) embryos. Several factors affect the survival of vitrified embryos such as the composition and concentration of vitrification solution(Rall, 1987; Rall and Fahy, 1985), thawing rate(Rall, 1987; Rall and Fahy, 1985) and developmental stage of embryos used(Massip et al., 1986; Massip et al., 1987).

The objectives of the present study were to examine the effects of equilibration time, precooling and straw loading method on the post-thaw *in vitro* survival rate of mouse embryos cryopreserved by vitrification.

II. MATERIALS AND METHODS

1. Collection of embryos

Immature female mice were superovulated by injection of 5IU PMSG(Peamax, Japan) followed 48hours later by injection of 5IU hCG (Sigma, U.S.A.). After the hCG injection, each female was placed in an individual cage with a male and vaginal plug was identified in the next morning and the day of plug identification was considered as the first day of gestation.

The normal embryos of morula stage were obtained from mice at 4.0days after identification of vaginal plug. After collection, morphologically normal embryos were washed in three changes of fresh D-PBS supplemented with 16% fetal calf serum(FCS, Sigma Chem. Co., U.S.A.) then pooled and held at room temperature until used.

2. Preparation of vitrification solutions

The first solution(Medium-1) was prepared by supplement of 10% glycerol(Sigma Chem. Co., U.S.A.) and 20% propylene glycol(Sigma Chem. Co., U.S.A.)(v/v) in basal medium of PBS with 16% fetal calf serum, while the second solution(Medium-2) was prepared by supplement of 25% glycerol and 25% propylene glycol(v/v) in the same basal medium. Precooling of the Medium-2 was allowed to cool inside a refrigerator set at 4℃ for at least 3hours before use.

3. Freezing and thawing procedure

About 10 to 15 compacted morula stage embryos were equilibrated in Medium-1 at room temperature(20℃) for 5, 10 or 15minutes(Fig. 1). After equilibration, they were aspiated with a micropipette and then transferred to 20μl of either precooled Medium-2(4℃) or non-precooled Medium-2(20℃) in a 0.25ml French straw(IMV, L'Aigle, France). The Medium-2 column containing embryos was placed between two Medium-2 column in double Medium-2 column method, but between 1M sucrose column and Medium-2 column in single Medium-2 column method(Fig. 2).

Air bubbles were made between the column of Medium-2 or sucrose solution. Within 30 to 40 sec after embryo loading, the open tips of straw were sealed with straw powder, the prepared straw were held vertically and immersed

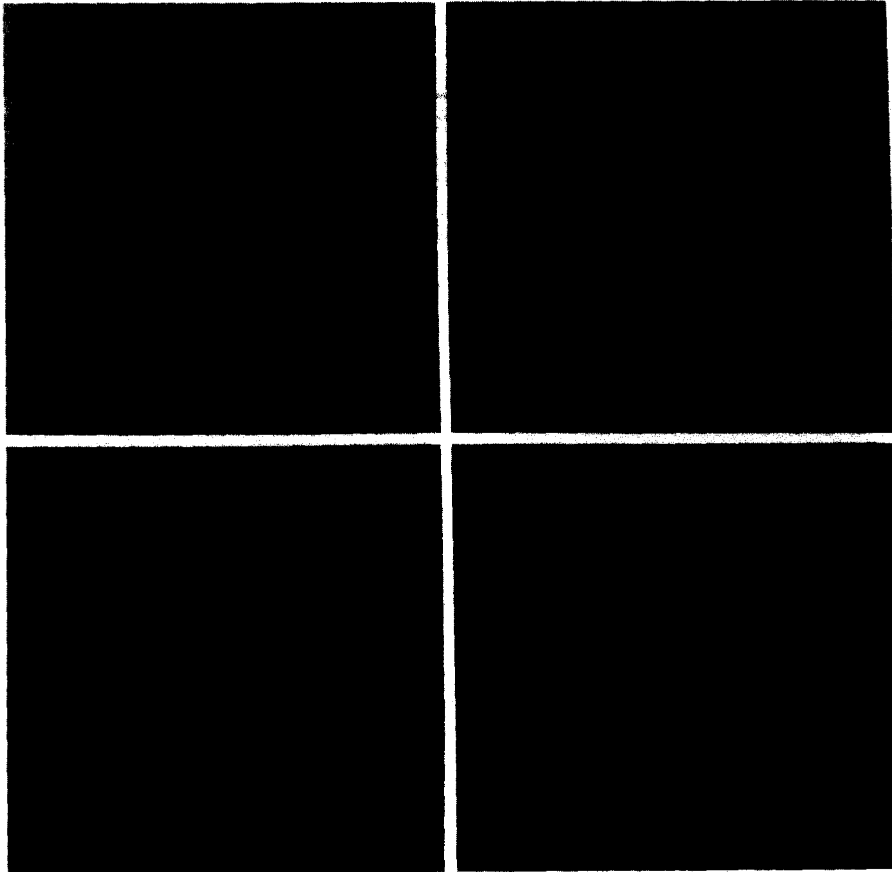
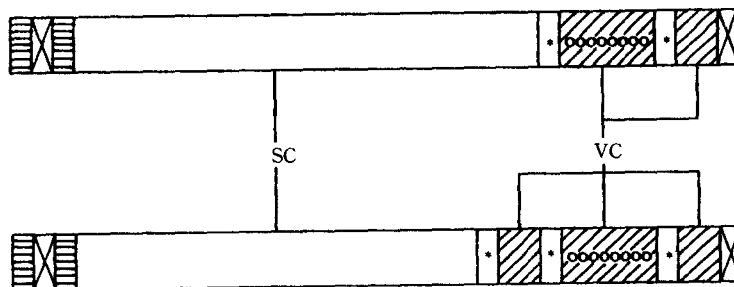


Fig. 1. Volume change of compacted morular stage embryos in vitrification solution ; prior to addition of vitrification solution Medium-1(A), and at 5(B), 10(C) and 15(D) minutes after equilibration in Medium-1(×200).

A) Single Medium-2 column method



B) Double Medium-2 column method

Fig. 2. Diagramatic representation of 0.25ml plastic straw loaded with vitrification solution and embryos for vitrification : (SC) 1M sucrose solution column, (VC) vitrification solution Medium-2 column, (*) air bubble column, and (O) embryos in Medium-2 column.

progressively (to prevent irruptive crystallization of sucrose) into liquid nitrogen for freezing.

Thawing was carried out by gently shaking the straw in a 20℃ water bath until the ice had disappeared from the sucrose solution (20sec). In the two-step dilution, all the thawed contents of the straw were poured into dishes, mixed, and left for 10 minutes at room temperature. The embryos were then transferred to 0.25 M sucrose solution, left for 5 minutes at room temperature, and washed in theree changes of fresh D-PBS supplemented with 16% FCS, then pooled and kept at room temperature for 5 minutes.

4. Embryo culture *in vitro*

All recovered embryos were cultured in sterile plastic culture dishes containing microdrops of $NaHCO_3$-BMOC-3 under paraffin oil at 37℃ with 5% CO_2 in air. The pH of medium was controlled between 7.2 to 7.4 and then, although the culture medium was sterilized by filtering through a 0.25μm disposable filter (Gelman, U.S.A.).

The compacted morulae were cultured for 24 to 28 hours and their development to the expanded blastocyst stage was used as the criterion for survival (Fig. 3).

5. Measurement of the number of blastomeres

The umber of blastomeres in non-frozen and frozen embryos after culture for 24 to 28 hours *in vitro* were determined by counting the nuclei of the embryos. The number of blastomeres in non-frozen and frozen embryos at the late blastocyst stage were determined by fluorescent stain without fixation. The embryos were exposed to BMOC-3 containing 5μg/ml Hoechst 33342 (Sigma Chemical Co., U.S.A.) for 10 minutes in 37℃ CO_2 incubator and permounted. Then the number of blastomeres was examined under an epi-fluorescent microscope (Nikon, Japan; Fig. 4).

6. Statistical analysis

All data were analyzed statistically by T-test and chi-square test (Steel and Torrie, 1960). Embryo survival was expressed as the percentage of tested embryos that developed to expanded blastocyst.

III. RESULTS AND DISCUSSION

1. Effect of equilibration time on survival of mouse morulae cryopreserved by vitrification

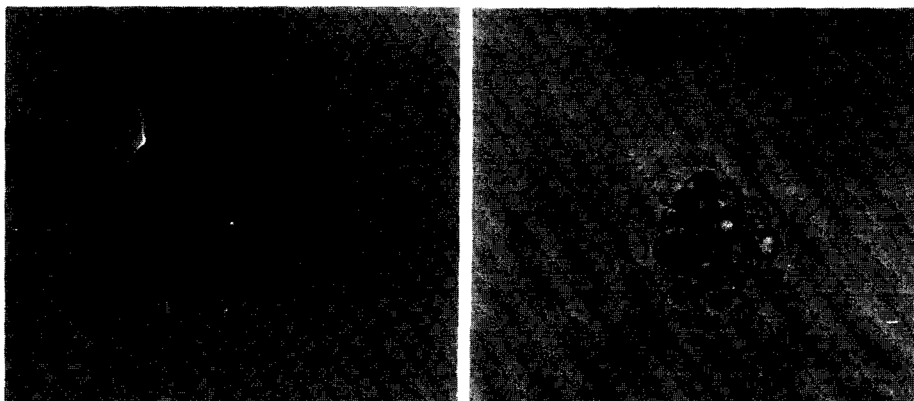


Fig. 3. Expanded blastocyst(A) and degenerated embryos(B) from vitrified-thawed mouse embryos of compacted morular stage after *in vitro* culture for 24 to 28 hours.

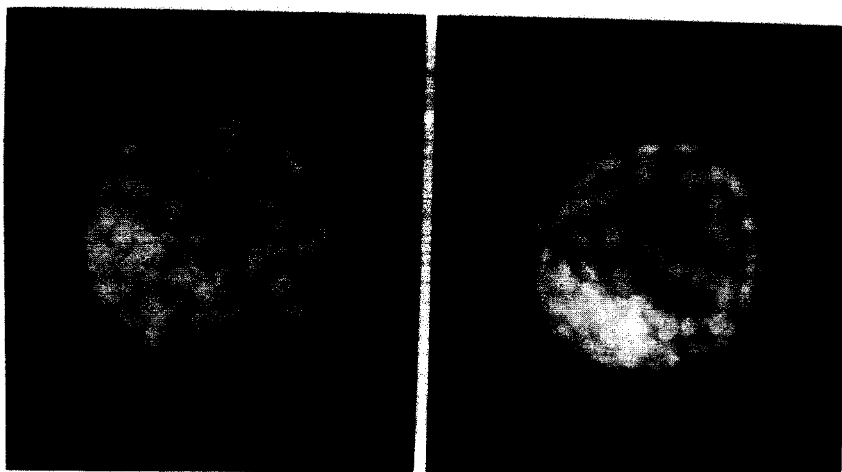


Fig. 4. A frozen-thawed(A) and non-frozen blastocyst(B) stained with Hoechst 33342 following counterstain with trypan blue(×200).

The mouse morulae were frozen by vitrification method after equilibration in Medium-1 for 5, 10 and 15 minutes. The developmental potential of the cryopreserved embryos after thawing and *in vitro* culture was shown in Table 1.

The equilibration in Medium-1 for 5mintes (40.0%) resulted in significantly($P<0.05$) lower survival rates than that for 15minutes(74.1%), and the highest survival from 10minutes equilibration(81.0%), though the difference in survival rate between 10 and 15minutes of equilibration period was not significant.

The survival of mouse embryos cryopreserved by vitrification method could be influenced by several factors(Rall, 1987). The present results showed clearly that the post-thaw survival of vitrified embryos was affected by the length of equilibration time in Medium-1. As shown in Table 1, the present results indicate that exposure to Medium-1 for 5minutes would be insufficient for best equilibration for morula stage embryos.

The survival rate obtained after 5 minutes

Table 1. Effects of equilibration time on survival of mouse morulae cryopreserved by vitrification method with the non-precooled Medium-2 and double Medium-2 column method

Equilibration time(min.)	No. of embryos frozen	No. and(%) of embryos recovered	No. and(%) of embryos developed *in vitro* to		
			Normal blastocyst	False blastocyst	Degenerated
5	92	90(97.8)	36(40.0)[a]	10(11.1)	44(48.9)
10	115	112(97.4)	91(81.0)[c]	8(7.1)	13(11.6)
15	114	112(98.2)	83(74.1)[b]	9(8.0)	20(17.9)

* Values with the different superscript are significant($P<0.05$) difference between the equilibration time.

equilibration(40.0%) was compared to be lower than the result of Valdez et al.(Valdez et al., 1990) who obtained survival rate of 76.3%, but similar to the data of Fukuda et al.(Fukuda et al., 1987) showing the survival rate of 34.0%. According to Rall(Rall, 1987), prolonged exposure of embryos to vitrification solution was not recommended due to problems related to chemical toxicity and osmotic injury.

In the present experiment, it appears that optimum equilibration of mouse embryos at morular stage in Medium-1 could be achieved by exposure of embryos in Medium-1 for 10 minutes in any column method. The highest survival rate of vitrified-thawed morula was reported from equilibration in Medium-1 for 10 mimutes by Valdez et al.(Valdez et al., 1990), but from equilibration for 20mimutes by Scheffen et al.(1986).

Scheffen et al.(1986) reported that the survival rate of mouse embryos of compacted morular stage to blastocysts was very high after 5 and 10 minutes equilibration(>80%), then it decreased with increasing time in the equilibration medium(Medium-1), and advanced stage of development were more sensitive to the duration of the equilibration period.

2. Effect of column method of Medium-2 on survival of mouse morulae cryopreserved by vitrification

In vitro survival rates of embryos frozen with the non-precooled Medium-2 after 10 minutes equilibration was shown in Table 2. As shown in Table 2, the significantly(P<0.01) higher survival rate of embryos was obtained by using double Medium-2 column method than single Medium-2 column method.

Fahy et al.(1984) found that the survival of vitrified frozen liver cells was greatly decreased when the cryostorage solution around the cells was insufficiently amorphous, which resulted eventually in ice-crystal formation in the solution. In the present experiment, when the single Medium-2 column was used to preserve embryos, the Medium-2 column of about half the straw changed from transparent to opaque upon thawing. Moreover, the survival rate of the embryos in those straws were lower than that of embryos in the straws in which the amorphous state(transparent) of the Medium-2 column was maintained.

The opacity of the Medium-2 column in the single Medium-2 column method was considered to be due to ice-crystal formation, which is also thought to damage the embryos. A decrease in the concentration of cryoprotectant in the Medium-2 column, which may have been caused by contamination with 1M sucrose solution, was considered to have been the cause of ice-crystal formation in the straws.

Table 2. Effects of column method of Medium-2 on survival of mouse morulae cryopreserved by vitrification method with equilibration for 10 minutes and non-precooled Medium-2

Medium column	No. of embryos frozen	No. and(%) of embryos recovered	No. and(%) of embryos developed *in vitro* to		
			Normal blastocyst	False blastocyst	Degenerated
Single	115	113(98.3)	71(62.8)[a]	9(8.0)	33(29.2)
Double	115	112(97.4)	91(81.0)[b]	8(7.1)	13(11.6)

* Values with different superscript are significant(P<0.01) difference between the column method.

Using the single Medium-2 column vitrification method, the Medium-2 column in straws containing the embryos was very likely to be contaminated with the residual sucrose solution adhering to the inner wall of the straw, which was derived from the sucrose solution column that was loaded before the Medium-2 column.

The Medium-2 solution used in this experiment consisted of 25% glycerol and 25% propylene glycol in PBS supplemented 16% FCS, and it was considered to be the minimum concentration which was necessary to maintain the vitrified state through freezing and thawing.

The sucrose solution is apt to be mixed with the Medium-2 column containing the embryos when the single Medium-2 column method was used. It was believed that such contamination, occuring even in trace amounts, was responsible for the unstable vitrification of the Medium-2 column, resulting in ice-crystal formation. In contrast, when the double Medium-2 column method was used, 1 M sucrose solution column was first aspirated into the straws, followed by the Medium-2 column without embryos, then by Medium-2 column containing the embryos.

So the residual sucrose solution on the inner wall of the straws was removed perfectly with the previously aspirated Medium-2 column. As a result, the Medium-2 column containing the embryos was not contaminated by the sucrose solution. The amorphous state of the Medium-2 column containing the embryos was thus maintained in all of the straws through freezing and thawing and resulted in a high embryo survival rate.

3. Effect of precooling of Medium-2 on survival of mouse morulae cryopreserved by vitrification

In vitro survival rate of embryos frozen with the precooled and non-precooled Medium-2 was shown in Table 3. The survival rate of morulae was not significantly different($P<0.05$) between precooling and non-precooling of Medium-2.

In the first report on the successful vitrification of mouse eight cell embryos, Rall and Fahy(1985) demonstrated that exposure to highly concentrated vitrification solution produces time dependent injury to the embryos, and one method to reduce or eliminate the harmful effects of exposure to highly concentration solution was to decrease the temperature of exposure to 4℃ or lower(Wood and Farrant, 1980).

4. Effect of cryopreservation by vitrification on number of blastomeres of mouse embryo

As shown in Table 4, the number of blastomeres at late blastocyst stage after

Table 3. Effects of precooling of Medium-2 on the survival of mouse morulae cryopreserved by the vitrification method with equilibration for 10 minutes and double Medium-2 column method

Temperature (℃)	No. of embryos frozen	No. and(%) of embryos recovered	No. and(%) of embryos developed *in vitro* to		
			Normal blastocyst	False blastocyst	Degenerated
4	104	99(95.2)	87(88.0)[a]	55(5.0)	7(7.0)
20	115	112(97.4)	91(81.0)[a]	8(7.1)	3(11.6)

* Values with the same superscript are not significant($P<0.05$) difference between the temperature in Medium-2.

Table 4. Effect of freezing on the number of blastomeres of blastocysts following *in vitro* culture of mouse morulae

Treatment	No. of embryos used	No. of blastomeres (Mean±S.E.M.)
Non-frozen embryos	40	53.3±1.5[b]
Frozen-thawed embryos	54	41.4±1.5[a]

* Values with different superscript are significantly($P<0.05$) different.

culture mouse morula for 24 to 28hours was significantly($P<0.05$) different between the non-frozen and frozen embryos(53.3±1.6 vs 41.4±1.5; $P<0.05$). The present results indicate that reduction of the number of blastomeres in frozen embryos was due to damage during vitrification and freezing procedure and delay of development *in vitro* by handling of embryos *in vitro*.

IV. SUMARY

This study was carried out to investigate the effect of equilibration time, precooling and straw loading method on the post-thaw survival rates of mouse embryos cryopreserved by vitrification method. The effect of the vitrification procedure on the damage of the embryos was also examined by the staining of nuclei using Hoechst 33342. The results obtained were summarized as follows;

1. The equilibration in Medium-1 for 10minutes was considered to be the optimum equilibration time. Embryos equilibrated in Medium-1 for 10minutes(81.0%) showed significantly ($P<0.05$) higher survival rates than those equilibrated for 5minutes(40.0%) or 15 minutes(74.1%).
2. The survival rate of embryos cryopreserved using the double Medium-2 column(81.0%) was significantly($P<0.01$) higher than that using the single Medium-2 column, whish was considered to be due to the double Medium-2 column method being more reliable for preventing contamination of diluent solution of 1M sucrose.
3. The survival rate of compacted morula stage embryos cryopreserved with the precooled and non-precooled Medium-2 was not significantly($P<0.05$) different.
4. The number of blastomeres of embryos at late blastocyst stage after culture of mouse morulae for 24 to 28hours was significantly ($P<0.05$) decreased by freezing embryos using vitrification(53.3±1.6 vs 41.4±1.5), which was considered to be due to the damage of embryos during vitrification and the delay of embryo development by handling *in vitro*.
5. The vitrification procedure is considered to be a very simple and efficient method for cryopreservation of embryos at early developmental stage.

V. REFERENCES

1. Fahy, G. M., D. R. MacFarlane, C. A. Angell and H. T. Merryman. 1984. Vitrification as an approach to cryopreservation. Cryobiol. 21: 407-726.
2. Fukuda, A., Y.Noda, K.Narimoto, H. Matsumoto and T. Mori. 1987. The analysis of vitrification technique compared to two-step freezing for mouse embryos. Jpn. Soc. Implant. 4: 94-98.

3. Massip, A., P.Van Der Zwalmen and B. Scheffen. 1986. Pregnancies following transfer of cattle embryos cryopreserved by vitrification. Cryo-Letters 7 : 270-273.
4. Massip, A., P. Van Der Zwalmen and F. Ectors. 1987. Recent progress in cryopreservation of cattle embryos. Theriogenol. 27 : 69-79.
5. Rall, W.F. 1987. Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiol. 24 : 387-402.
6. Rall, W.F. and G.M. Fahy. 1985. Ice-free cryopreservation of mouse embryos at -196℃ by vitrification. Nature 313 : 573-575.
7. Rall, W. F., M. J. Wood, C. Kirby and D. G. Whittingham. 1987. Development of mouse embryos cryopreserved by vitrification. J. Reprod. Fert. 89 : 499-504.
8. Scheffen, B., P. Van Der Zwalmen and A. Massip. 1986. A simple and efficient procedure for preservation of mouse embryos by vitrification. Cryo-Letters 7 : 260-269.
9. Smorag, Z., B. Cajda, B. Wieczorek and J. Jura. 1989. Stage dependent viability of vitrified rabbit embryos. Theriogenol. 31 : 1227-1231.
10. Steel, R.G.D. and J.H. Torrie. 1960. Principles and procedures of statistics. MaGraw Hill Book Co., New York. U.S.A.
11. Trounson, A., A. Peura, L. Freeman and C.Kirby. 1988. Ultra-rapid freezing of early cleavage-stage embryos and eight-cell mouse embryos. Fertil. Steril. 49 : 822-826.
12. Valdez, C.A., O.Abas Mazni, Y. Takahashi, M. Hishinuma and H. Kanagawa. 1990. Effect of equilibration time, precooling and development stage on the survival of mouse embryos cryopreserved by vitrification. Theriogenol. 33 : 627-636.
13. Whittingham, D.G., S.P. Leibo and P. Mazur. 1972. Survival of mouse embryos frozen to −196℃ and −296℃. Science 178 : 411-414.
14. Wilmut, I. 1972. The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing. Life Sci. 11 : 1071-1079.
15. Wood, M.J. and J. Farrant. 1980. Preservation of mouse embryos by two-step freezing. Cryobiol. 17 : 178-180.