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Effects of Slow Freezing on Development of Blastomeres Separated from Mouse Preimplantation Embryos

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완만동결이 생쥐 초기배 난할세포의 발달에 미치는 영향

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ABSTRACT

The development of single blastomeres isolated from 2-, 4- and 8-cell mouse embryos and the ability of such blastomeres to survive slow freezing were studied. Of 223, 60 and 188 single blastomeres isolated from 2-, 4- and 8-cell mouse embryos, respectively, 111 blastomeres (49.8%) from 2-cell embryos, 12 blastomeres (20.0%) from 4-cell embryos and blastomeres (16.5) from 8-cell embryos developed into blastocysts after culture for 96 hrs. The recovery rate was 54.2% (65/120), 46.4% (13/28) and 24.3% (17/70) of blastomeres derived from 2-, 4- and 8-cell embryos following freezing and thawing and the survival of frozen-thawed blastomeres was 27.1% (16/59), 36.4% (4/11) and 17.6% (3/17), and respectively. The apparently six normal fetuses were obtained from frozen-thawed blastomere from 2-cell embryos after transferring into the recipients. These results indicate that mouse blastomeres isolated from preimplatation stage embryos can survive storage in liquid nitrogen following slow freezing.

(Key words: Mouse blastomeres, Slow freezing, *In vitro* culture, Embryo transfer)

Ⅰ. INTRODUCTION

Since Nicholas and Hall (1942) proposed that the isolated blastomeres from 2-cell rat embryos were capable of further development, Seidel (1952; 1956) reported birth of live young from 2-cell rabbit embryos in which one of the blastomeres had been destroyed. Also Tarkowski (1959) reported live birth in three mice which had received 2-cell embryos in which one blastomere had been destroyed. Mulnard (1965) observed cytochemically that mouse blastomeres isolated from 2-cell embryos developed to blastocyst stage. Tarkowski and Wroblewska (1967) reported that incidence of blastocyst was about 40%, 30% and 15% among 1/4, 2/8 and 1/8 blastomeres isolated from 4- and 8-cell mouse embryos, respectively. Moore et al. (1968) reported that 30%, 19% and 11% of normal viable young were obtained from single blastomere enclosed in zona pellucida of 2-, 4- and 8-cell rabbit embryos, respectively, but live young was

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not obtained from single blastomere devoid of zona pellucida. Willadsen (1979) produced artificially monozygotic twin lamb by transferring cultured blatomeres to recipients. And Menino Jr. and Wright Jr. (1979) reported successful culture of blastomere isolated from 4- and 8-cell porcine embryos. Subsequently, the method of producing monozygotic twin has been described in cow (Wiiladsen et al., 1981; Ozil et al., 1982).

Since Whittingham et al. (1972) reported that mouse embryos previously frozen to −196℃ developed to the young fetuses after transfer to recipients, deep freezing has been used as a method to store mammalian embryos. Such storage of blastomeres isolated by separation of embryos allowed production of identical sheep (Willadsen, 1980) and cattle (Lehn-Jehsen and Wiiladsen, 1983). These early technique was slow freezing procedure with automatic freezer machine. Vitrification which is rapid freezing without machine has been applied to various embryos and is now a alternative to the slow freezing method (Rall and Fahy, 1985; Rall, 1987). Micromanipulated embryos for DNA microinjection, biopsy or nuclear transfer which were somehow damaged in zona pellucida may be needed for the adjustment of freezing method because blastomeres were directly exposed to cryoprotectants and freezing environments. Even though vitrification method for normal embryos leaded to high survival rate, it may not be a proper method for deep freezing blastomeres. In our laboratory, vitrification for blatomeres resulted in extremely low survival compared with slow freezing method.

The purpose of the present study was to examine the ability of blastomeres isolated from 2-, 4- and 8-cell mouse embryos to survive slow-freezing and the developmental potential to normal fetuses following transfer of such blastomeres to recipients.

Ⅱ. MATERIALS AND METHODS

1. Mouse Embryos

Embryos were collected from ICR mouse after induction of superovulation by the injection of 5 IU Gestyl (Organon Inc., Holland) and 2.5 IU Pregnyl (Organon Inc., Holland) 48 hrs apart on Day 2 for 2-cell embryos and Day 3 for 4- and 8-cell embryos (Day 1 was the day on which a vaginal plug was found). CZB medium was used for recovery, culture and transfer, and PBl (Whittingham, 1971) for freezing and thawing.

2. Splitting Embryos

The zona pellucida was removed with acid tyrode solution. The embryos were kept in tyrode solution until the zona pellicida became disappeared. The embryos devoid of zona washed several times with culture medium and placed in culture medium. The blastomeres were separated by suction several times with pasteur pipettes, allowing the passage of only one or two blastomeres at a time. The suction held the embryos until the blastomeres were completely separated from 2-, 4- and 8-cell embryos.

3. Feezing Embryo

The blastomeres were transferred to PB1 solution for freezing. The blastomeres were dehydrated with 1.5M glycerol by three step procedure (0.5M, 1.0M and 1.5M), allowing ten minutes equilibration period for each step. And then ten to twenty blastomeres were loaded into 0.25mL plastic straw. A straw was separated into three compartments by small air bubbles and embryos were placed in the middle compartment. After that, the loaded straws were placed in freezer (FTS System Inc., USA) with 99% methanol. They were cooled at 1℃/min to −7℃ and ice nucleation (seeding) was induced by touching the surface of the straw with the tip of

a supercooled forcep. After ten minutes, they were cooled at 0.5℃/min to −35℃ and then straws were plunged directly into liquid nirtogen (−196℃).

4. Thawing and Transfer of Embryo

Following storage for 24 hrs to 7 days, the frozen blastomeres were thawed in water at room temperature. After thawing, the blastomeres were rehydrated by adding the following volume of PBI solution every ten minutes ; 0.05, 0.1 and 0.2mL per one straw. The blastomeres washed several times with PBI solution and culture medium. The frozen blastomeres were cultured at 37℃ with a gas atmosphere of 5% CO_2 for 96 hrs. and then the survival was assessed. A portion of those which were judged to survival freezing were transferred to recipient with vaginal plug at that morning from vasectomized male. The recipient was examined on Day 14 of pregnancy for assessment of viability.

Ⅲ. RESULTS

1. Blastomere Development

The development of single blastomeres isolated from two-, four- and eight-cell mouse embryos after culture *in vitro* is shown in Table 1. A total of 223, 60 and 188 single blastomeres were separated from 182 two-, 42 four- and 60 eight-cell embryos, respectively. On average 1.53 blastomeres of a two-cell embryos, 2.43 blastomeres of a four-cell embryo and 3.58 blastomeres of a eight-cell embryo were isolated after blastomere separation. After culture for 96 hrs. 49.8% of blastomeres isloated from two-cell embryos, 20.0% from four-cell embryos and 16.5% blastomeres from eight-cell embryos were developed into morula or blastocysts. The development of blatomeres originated from late preimplatation stage embryos tended to decrease.

Table 1. The development of single blastomeres isolated from 2-, 4- and 8-cell mouse embryos to morula or blastocyst after culture *in vitro*

stage	No. embryo	No. blastomere isolated	No. bastomere developed (%)
2-cell	182	223(1.53)[a]	111(49.8)[b]
4-cell	42	60(2.43)	12(20.0)
8-cell	60	188(3.58)	31(16.5)

[a] Number of blastomeres per one embryo after separation.

[b] Percentages of blastomeres developed to morula and blastocysts.

2. Develpoment of Frozen Blastomeres *In Vitro*

The survival rate of frozen-thawed single blastomeres isolated from two-, four- and eight-cell mouse embryos is shown in Table 2. Recovery rates of blastomeres decreased after thawing as blastomeres were separated as late stage embryos. The survival of frozen-thawed blastomere from two-, four- and eight-cell mouse embryo was 27.1% (16/59), 36.4% (4/11) and 17.6% (3/17), respectively following culture *in vitro*.

Table 2. The survival of single blastomeres isolated from 2-, 4- and 8-cell mouse embryos after freezing and thawing

Embryo separated	No. blastomeres	No. recovered(%)	No. morphologically normal (%)	No. survived(%)
2-cell	120	65(54.2)	59(49.2)	16(27.1)
4-cell	28	13(46.4)	11(39.3)	4(36.4)
8-cell	70	17(24.3)	17(24.3)	3(17.6)

Table 3. The viability of mouse blastomeres isolated from 2-cell embryos

Treatments	No. blastomeres transferred	No. recipient	No. live fetus
Frozen	50	2	6
Unfrozen	60	3	11

3. Viability of Frozen Blatomeres

The final test of viability of the frozen-thawed mouse single blastomeres isolated from 2-cell embryos was made by transfer of morula or blastocysts after culture *in vitro* to the uterus of recipient. And the developed unfrozen blastomere from 2-cell embryos were used as control. The recipient was sacrificed on day 14 of pregnancy to evaluate the viability of fetuses. As shown in Table 3, apparently six normal fetuses at day 14 of pregnancy resulted from the transferred frozen-thawed blastomere.

Ⅳ. DISCUSSION

The development of a single blastomeres from 2-, 4- and 8-cell mouse embryos after culture *in vitro* was low compared with that of the previous reports (Tarkowski, 1959; Mulnard, 1965; Tarkowski and Wroblewska, 1967). However, the trends of development according to blastomeres from different stage embryos were similar to those of Tarkowski and Wroblewska (1967) that reported that incidence of blastocysts decreased from about 40% of 1/4 embryos to about 30% of 2/8 embryos and to about 15% of 1/8 embryos. It was thought that decreasing size of blastomeres resulted to lower development of blastomeres from 4- and 8-cell embryos than that of blastomeres from 2-cell embryos. And the damage originated from improper manipulation might result in low development of the isolated blastomeres. Tarkowski and Wroblewska (1967) reported that there were trophoblastic vesicles which had no inner cell mass, false blastocysts which had false inner cell mass and small blastocyst which was similar to whole blastocyst but small size among the blastocysts developed from blastomeres isolated from the early mouse embryos. These phenomenon could occur in this experiment. Also, the number of isolated blastomeres per embryo was low since theoretically 2 blastomeres from a two-cell embryo, 4 blastomeres from a four-cell embryo and 8 blastomeres from a eight-cell embryo should be obtained. The low isolated blastomere production might result from unskilled manipulation.

Vitrification of mouse blastomeres caused death in our preliminary experiment (data not shown). Mouse blatomeres without zona pellucida may have severe damages in the cytoplasm. This experiment suggests that slow freezing is the another way to store blatomeres at liquid nitrogen. The low recovery of frozen blastomerse may be due to too much rupture or shrinkage of blastomere even during slow freezing. The survival of the frozen-thawed single blastomeres in this experiment was considerably low compared with that of the frozen-thawed normal embryos (Whittingham, 1972). However, even though the development of frozen-thawed blastomeres was low *in vitro*, morula or blastocysts derived from blastomeres of 2-cell embryos can developed to normal fetuses on day 14 of pregnancy in this experiment (Table 3). Although the overall viability was low and no comparison could be made of frozen single blastomere isolated from 4- and 8-cell mouse embryos in this experiment, it is thought that the higher rate of survival will be obtained after improvement of the methods for embryo separation and blastomeres freezing and thawing.

Ⅴ. 요 약

본 연구는 2-, 4- 또는 8-세포기 생쥐 수정란으로부터 분리된 단일 할구의 발달 및 완만동결 후 생존능력을 조사하기 위하여 실시하였다. 2-, 4- 또는 8-세포기 생쥐 수정란에서 각각 223개, 60개, 188개의 할구를 분리하여 96시간 배양하였는데 이 중 2-세포기 수정란으로부터 분리된 할구는 111개(49.8%)가 배반포기까지 발육하였으며, 4-세포기와 8-세포기 수정란으로부터 분리된 할구는 각각 12개(20.0%)와 31개(16.5%)가 배반포기까지 발육하였다. 분리된 할구를 완만 동결한 후 융해하여 배양했을 때 배반포기까지의 발육율은 2-세포기 할구는 27.1%(16/50), 4-세포기 할구는 36.4%(4/11) 그리고 8-세포기 할구는 17.6%(3/17)였으며, 융해 후 할구 회수율은 각각 54.2%(65/120), 46.4%(13/28), 24.3%(17/70)을 나타냈다. 2-세포기 할구를 동결 융해한 후 동기화시킨 생쥐 자궁에 이식하여 정상적으로 발달한 태아를 생산하였다. 본 실험의 결과로 완만동결방법이 생쥐의 할구를 동결보존하는데 이용될 수 있음을 확인하였다.

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