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Surgical and Non-Surgical Transfer of Mouse Embryos Bisected at Developmental Stage of Morula and Blastocysts

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상실배기 및 포배기에 미세분할한 생쥐 수정란의 외과적 및 비외과적 이식

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초 록

가축의 일란성 쌍태를 생산하기 위한 기술 개발을 확립하고자 상실배 및 포배기에 있는 BALB/c 계통의 생쥐 수정란을 micromanipulator로 분할 수정란을 작출하고 이를 체외배양을 실시하여 발달성적을 조사하였으며, 외과적 및 비외과적 이식을 실시하여 착상율 및 산자생산 성적을 조사한 결과는 다음과 같다.

1. 상실배 및 포배기에 있는 총 811개의 정상적인 수정란을 분할하여 이중에서 666(82.1%)개가 분할시의 불리적인 손상이 없이 분할되었고, 이때 분할 성공율은 발달단계 간에 유의적($P<0.05$)인 차이가 없었다.
2. 분할 수정란중 상실배는 30-36시간, 초기 배반포 및 확장 배반포는 3-6시간 배양을 실시한 결과 분할 수정란중 한쌍이 모두 정상적으로 배양된 것은 각각 70.0%, 80.4% 및 73.1%로써 이들 발달단계 간에 유의적($P<0.05$)인 차이가 없었다.
3. 분할된 상실배와 정상적인 수정란의 이식후 수태율은 각각 63.6% 및 61.3%로써 유의적($P<0.05$)인 차이가 없었다. 그러나 분할된 상실배에 있어서 배양을 하지 않고 이식한 경우에는 전혀 수태되지 않았다 ($P<0.05$).
4. 분할된 포배기 수정란을 체외 배양후 이식한 수태율(55.5%)과 배양과정을 거치지 않고 이식한 성적(43.8 %) 그리고 정상적인 포배기 수정란을 이식한 수태율(55.4%) 간에는 유의적($P<0.05$)인 차이가 없었다.
5. 분할 수정란을 외과적 방법으로 이식한 경우는 52.8%의 수태율을 얻었으나, 비외과적 방법으로 이식한 경우 27.5%로써 외과적 방법으로 이식한 경우보다 수태율이 유의적($P<0.05$)으로 낮았다.

INTRODUCTION

The artifical production of monozygotic twins by embryo bisection has been extensively studied in mammals. Monozygotic twins in sheep(Willadsen, 1979) and cow(Willadsen, 1980) have been produced from totipotent blastomeres isolated microsurgically from 8-cell embryos. For the production of monozygotic twins by dividing early stage of embryos the techniques related to mechanical removal of zonae pellucidae, separation of blastomeres to pair, insertion of the separated blastomeres into evacuated rabbit zonae, embedding in agars and culturing them in ligated rabbit or sheep oviducts were developed and utilized in the early stage of technological development for artifical induction of monozygotic twins.

Relatively simple techniques for bisection of embryos at the stage of compacted morula to hatched blastocyst have been developed in recent, and the pregnancy rates resulted from this improved technique have been reported similar to that

which was obtained after transfer of intact embryos in the sheep (Gatica *et al.*, 1984), cattle (Ozil, 1983), goat(Tsunoda *et al.*, 1985) and mouse(Lee *et al.*, 1989:Kang *et al.*, 1989: Nagashima *et al.*, 1984).

By the application of the technique of embryo bisection not only a significant increase in the number of transferable embryos per donor and collection might be possible but also the genetically identical animals obtained might be very useful for research models in the fields of physiology of nutrition, embryology, genetics and breeding in farm animals.

The present study was conduction to develop a simple technique for the production of monozygotic twins by bisection of morula to blastocyst stage embryos and also to determine the effects of various factors concerning the survival rate of zona-free bisected embryos after surgical or non-surgical transfer in mice.

MATERIALS AND METHODS

1. Animals

Immature virgin female and male BALB/c mice were obtained from Experimental Farm in Gyeongsang National University.

The body weight and age were 15~20 g and 4~6 weeks in females and 25~30 g and 9~10 weeks in males, respectively. They were housed in plastic cages under temperature and light-controlled condition(20-23, 14 hours light: 10 hours dark) with free access of a formulated diet and drinking water.

2. Collection of embryos

Immature mice were superovulated by I.P. injection of 5 IU PMSG (PEAMAX, Japan) followed by I.P. injection of 5 IU hCG(Sigma Chem. Co., U.S.A.) 48 to 50 hours later, and 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. Mice were killed by cervical dislocation, and the uterine horns were dissected out and flushed with modified Dulbecco's phosphate buffered saline(D-PBS, Sigma Chem. Co., U.S.A.) by 26 gauge blunt-ended needle attached to a 10 ml syringe. Immediately following the collection, embryos were morphologically evaluated using an inverted microscope(×100). Normally developed embryos were washed twice in modified D-PBS containing 0.1%BSA (Sigma Chem. Co., U.S.A.) and placed in microdrops of the same medium.

3. Bisection of embryos

All micromanipulation was done under liquid paraffin in sterile plastic petri dishs(100 × 15mm: Coster, U.S.A.) at temperature controlled room (30℃). A phase-contrast inverted microscope(×100: Nikon, Japan) with a micromanipulators (Huxley-Goodfellow, England) was used for micromanipulation of embryos. Embryos at the morula stage were held by suction with the holding micropipets(I.D., 25~40㎛) made of capillary tubes(Ests, U.S.A.) and were bisected into halves by lowering the shaft of the microsurgical knife made from breakable special stainless steel blade (WECK, U.S.A.) with 15° to 30° blade angle on a vertical plane. The knife was fixed with glue to a fine glass rod. In bisection of blastocyst, orientation of the blastocyst was always such as to ensure that both the inner call mass and the trophoblast layer were bisected(Fig. 2).

4. Embryo culture *in vitro*

The bisected morula were culture for 32~36 hours in D-PBS supplemented with 0.3%BSA or Ham's F10(Gibco, U.S.A.) supplemented with 20%FCS(Sigma, Chem. Co., U.S.A.) under an

atmosphere of 5% CO_2 in air.

The pH of medium was controlled to 7.2 to 7.4 and then the culture medium was sterilized by filtering though a 0.2 μm disposable filter (Gelman, U.S.A.). The demi-embryos which developed from the bisected morula were classified morphologically by Nagashima *et al.* (1984). Cultured half-embryos were transferred to pseudo-pregnant recipients on the morning of day 4.0 to 4.5. The bisected early or expanded blastocysts were transferred after culture for 3 to 6 hours, but the hatched blastocysts were transferred immediately after bisection. Evidence of survival of half-embryos of morula and blastocyst stages was based on the formation or reformation of a blastocoele cavity and a clear inner cell mass. The formation of trophoblastic vesicles only was not considered as an indication of embryo survival (Fig. 2).

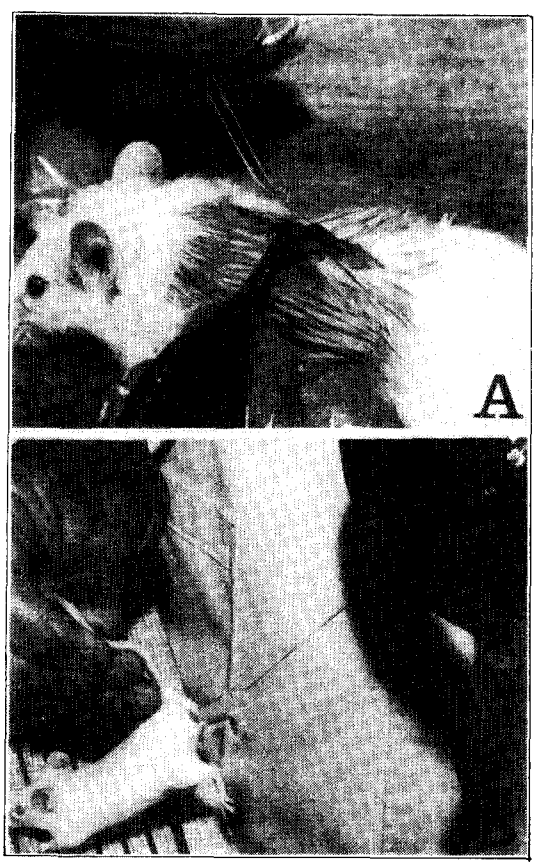

Fig. 1. Methods of transfer for the half mouse embryos at morula or blastocyst stage.
A : Surgical transfer.
B : Non-surgical transfer.

5. Embryo transfer

1) Surgical method

For surgical transfer of embryos, recipients were anesthesized with xylazine(Rompun, Bayer Vet. Chem. Co., Korea) 0.05mg and ketamine HCl (Yuhan Coporation, Korea) 0.05mg / g body weight and the ovary and the tubal end of the uterine horn on each side were exteriorized in turn through a dorsolateral incision in the abdominal musculature. The tubal end of the uterine horn was punctured with a 26-gauge hypodermic needle and the half-embryos were deposited in the uterine lumen with a transfer pipet (Fig. 1, A).

2) Non-surgical method

For a simple non-surgical transfer of embryos, 2 to 10 pairs of half embryos were transferred into uterine horns of the recipients using a newly devised transfer instrument. A vaginal speculum was prepared by trimming the end-point of a plastic micropipet tip(O.D., 20 to 30mm). Vaginal speculum was inserted into the opening of the vagina, and a transfer pipet(I.D., 200 μm; medium volume, 1~5 μl) was in fused into uterine lumen via speculum and cervical canal. The half embryos were injected using a hamilton syringe attached to the transfer pipet (Fig. 1, B).

6. Detection of pregnancy

The recipients were autopsied on 5 to 7 days after embryo transfer and the number of young or normal fetuses and resorptions in each uterine horn were recorded.

7. Statistical analysis

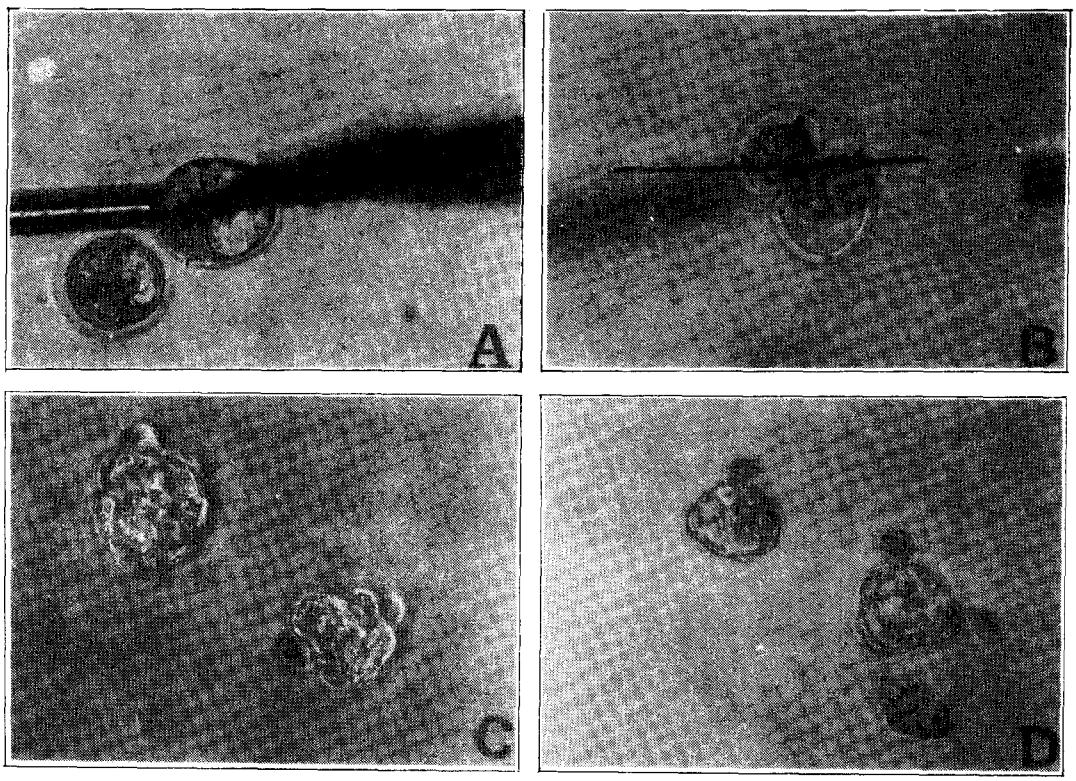

Fig. 2. Bisection of mouse embryos by a WECK microblade and their development ***in vitro***.
A : Blastocyst held to pipet and microblade (×100).
B : A monozygotic pair of half-blastocyst immediately after bisection (×100).
C : A monozygotic pair of half-early blastocyst developed from a bisected morula after 20 hours in culture (×100).
D : A monozygotic pair of half-blastocysts with reformed blastocoeles developed from a bisected blastocyst after 3 hours in culture (×100).

All date were analysed statistically by χ^2-test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

1. Bisection of embryos at various stage

The results of bisection of mouse embryos at morula to hatched blastocyst stages were presented in Table 1.

81.1% of 254 morula, 85.7% of 105 early blastocysts, 81.3% of 342 expanded blastocysts and 83.7% of 110 hatched blastocysts of mouse embryos were successfully bisected without any visible cell damages. There was no difference in the success rate in bisecting embryos of developing stages from morula to blastocysts($P<0.05$).

The present results of embryo bisection show that about 82% of 811 good mouse embryos of morula to hatched blastocyst stages were bisected without visible cell damage, using microsurgical blade attached to a micromanipulator, and the stage of morula to hatched blastocysts did not influence significantly($P<0.05$) in success rate of bisection. However, these results were considered with the results of McEvoy and Sreenan(1987).

Nagashima *et al.* (1984) reported that about 80% of the mouse morulae were bisected success-

Table 1. Production of half-embryos by micromanipulation in mouse embryos at various stages of development

Stage of development	No. of embryos	No. of embryos bisected with damage of half-embryos(%)	No. of embryos bisected without damage(%)
Morulae	254	48(18.9)	206(81.1)
Early blastocyst	105	15(14.3)	90(85.7)
Expanded blastocyst	342	64(18.7)	278(81.3)
Hatched blastocyst	110	18(16.3)	92(83.7)
Total	811	145(17.9)	666(82.1)

There are no significant($P<0.05$) differences in successfully bisected without damage between the cell stages.

fully, but they used pronase for the digestion of zonae pellucidae and a fine glass needle was used to part the blastomeres. They reproted that destruction occurred mainly in the blastomeres in the path of the microneedle. When decompaction of the embryos was in complete it was considered to be difficult to bisect them without injuring the blastomeres.

In the present study the techniques of embryo bisection are considered to be simpler and safe from cell damage. McEvoy and Sreenan(1987) also reported that they employed a simple vertical slicing action with blade for easy bisection of bovine morula and blastocysts including their zonae pellucidae. When bisecting blastocyst orientation of the blastocyst was always such as to ensure that both the inner cell mass the trophoblast layer were bisected. Park *et al.* (1987) also reported that the early to hatched blastocyst in goat embryos were easily bisected, but the embryos of morula stage were very difficult to be bisected microsurgically.

Tsunoda *et al.* (1985) used a micromanipulator system with several hands and a dissecting glass needle, but they also experienced some difficulty in bisection of morula or blastocysts with zonae, compared with the hatched blastocyst. Kim *et al.* (1986) reported that only 39.2% of 255 mouse embryos of morula stage were bisected without visible damage, using a microblade and micromanipulator system.

2. *In vitro* development of half-embryos

In vitro development of the half-embryos from morula to expanded blastocyst stage were presen-

Table 2. *In vitro* development of half-embryos on mouse

Stage of development	No. of embryos	Eu-blastocysts developed from half-embryos(%)		
		Twin	Single	Degenerated
Morulae[1]	137	96(70.1)	25(18.2)	16(11.7)
Early blastocyst[2]	46	37(80.4)	6(13.1)	3(6.5)
Expanded blastocyst[2]	41	30(73.2)	5(12.2)	6(14.6)

There are no significant($P<0.05$) differences in successfully eu-blastocysts twin developed from half-embryos between the cell stages.

[1] Embryos were cultured for 32 to 36 hours after bisection and the half-embryos which had developed to blastocyst stage were identified as survival.

[2] Survival of half-embryos was based on the reformation of a blastocoele cavity and a clear inner cell mass region after culture for 3 to 6 hours.

ted in Table 2.

The half-embryos were cultured *in vitro* for 30 to 36 hours in morula 3 to 6 hours in blastocyst. A total of 224 half-embryos were cultured *in vitro* and 72.8% of them were developed to the expanded or hatched blastocyst stage.

The half-morulae embryos were examined microscopically at 24 hours after culture and morphologically classified into twin eu-blastocyst, single blastocyst and degenerated blastomeres. After culturing for 24 hours the 137 half-morula produced a total of 96 (70.1%) monozygotic pairs of twin eu-blastocyst, 25 (18.2%) pairs single eu-blastocyst and 16 (11.7%) pairs degenerated embryos. The development rates of early blastocyst and expanded blastocyst to expanded blastocyst or hatched blastocyst were 80.4% and 73.2% respectively.

There was no significant($P<0.05$) difference in *in vitro* developmental rates of cell stages between half-embryos.

These results of *in vitro* development to blastocoele formation of half-embryos in mouse were slightly lower than those from cattle embryos (86%) by McEvoy and Sreenan(1987). Tarkowski and Wroblewska(1967) investigated the *in vitro* development of blastomeres isolated from 4- and 8-cell mouse embryos. They suggested that reduction of blastomere number would results in a blastocyst with a small ICM. Nagashima *et al.* (1984) reported that when half-morulae developed *in vitro* had less than half the normal number of blastomeres because some blasatomeres were destroyed or dissociated by the micromanipulation, regeneration of the ICM precursors might be incomplete.

In present study, it was usually difficult to know how many and what kind of blastomeres had been destroyed or dissociated by the micromanipulation. Tarkowski and Wroblewska(1967) reported that lack of the precursor blastomeres must have resulted in a poorly developed ICM which had no ability to from an entire fetus. However, Moustafa and Hahn(1978) have reported that the half-embryos separated from mouse 8- to 16-cell embryos could be developed normally in culture with or without the zonae.

3. Transfer of intact morula or half-embryos from morulae embryos

The results of surgical transfer of intact, half or cultured half-embryos in morula to pseudo-pregnant recipients were persented in Table 3.

The pregnancy rate of 11 recipients which received intact embryos at morula stage was 63.6% and a similar result(61.5%) were obtained in cultured half-embryos bisected at morula stage. However, there were non-pregnant recipients after transfer of half-morulae without culture. Half-

Table 3. Implantation and development of intact, half or cultured half mouse embryos after morula stage surgical transfer

Embryos transferred	No. of embryos transferred	No. of recipients	No. of pregnant recipients(%)	No. of half-embryos developed to fetuses or youngs(%)
Intact	92	11	7(63.6)[a]	28(30.4)[a]
Half[1]	118	15	0(0.0)[b]	0(0.0)[b]
Cultured half[2]	103	13	8(61.5)[a]	31(30.1)[a]

The percentages with the different superscripts denote significant($p<0.05$) difference between the surgical transfer of intact or half embryos.

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The percentages with the different superscripts denote significant($p<0.05$) difference between the surgical transfer of intact or half embryos.

1) Embryos were transferred immediately after bisection.

2) Embryos were transferred after culture for 32 to 36 hours.

morulae emryos cultured in PBS or Ham's F-10 supplemented with 0.3% BSA or 20% FCS under an atmosphere was transferred immediately after bisection. There was no significant($P<0.05$) difference in pregnancy rates of intact embryos between cultured half-embryos.

In intact morula stage embryos, in present study were in accord with the results of Gosden (1974) and Bronson and Cunnane(1975). However, Seong(1986) reported that the pregnancy rates after transfer of morulae and blastocysts in rats were 50% and 45%, respectively. The pregnancy rate of bisected and cultured morula embryos was 61.5% in this study. This result was higher(53.0%) than Kim *et al.* (1986). However, Tsunoda *et al.* (1985) in goat experiment when five sets of bisected morulae which developed to biastocysts after culture *in vitro* were transferred to five recipients on pregnancies were obtained. However, bisected morulae of without culture transferred half-embryos were not pregnant.

Although the reasons for failure of bisected morulae to survive to term after transfer are not clear in the present study.

4. Transfer of intact blastocysts or half-embryos from blastocysts

The results of surgical transfer of intact, half- or cultured half-embryos in blastocyst stage to pseudopregnant recipients were presented on Table 4.

The pregnancy rates were 55.5%, 43.8% and 55.5% in recipients which were transferred with intact, bisected early to expanded or hatched blastocyst without culture, but some bisected early or expanded blastocyst with culture. There were not significant($P<0.05$) differences in pregnancy rates of recipients transferred with blastocyst embryos between intact and half blastocyst stage and half-embryos between with and without culture following bisection.

In surgical transfer of mouse embryos, the fetuses or youngs to developmental rates were 24.3%, 23.8% and 33.8%, respectively, there were also not significantly($P<0.05$) different in the developmental rates of recipients transferred with blastocyst embryos between intact and half blastocyst stage and half-embryos between with and without culture following bisection. Moreover, there were found no significant differences in conception rates and development after transfer between with and without culture of half-embryos.

These results indicate that the manipulation involved in the bisection and culture of embryos gave no harmful effects or damages to blastocoele reformation and implantation of embryos. The present results in the developmental rates of mouse half-embryos after transfer were superior to those (9.0%) from Kim *et al.* (1986), and similar to

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Embryos transferred	No. of embryos transferred	No. of recipients	No. of pregnant recipients(%)	No. of bisected embryos developed to fetuses or youngs(%)
Intact	210	27	15(55.5)	51(24.3)
Half[1)]	130	16	7(43.8)	31(23.8)
Cultured half[2)]	68	9	5(55.5)	23(33.8)

There are no significant($P<0.05$) differences in embryos developed to fetuses or youngs between intacted or bisected embryos.

[1)] Embryos were transferred immediately after bisection.

[2)] Embryos were transferred after culture for 3 to hours in early or expanded blastocyst.

those(35.7%) from Moustafa and Hahn(1978) and those(41.7%) from Nagashima *et al.* (1984). The differences among the results in this study might be caused by physical and psychic condition of researcher, because the micromanipulations were carried out by hand.

However, mouse embryos can implant and develop successfully without the zona pellucida (Minz, 1962) and the lack of a zona pellucida does not prevent implantation of mouse embryos (Minz, 1962; Tarkowski). Since delay of implantation of the mouse blastocyst is associated with an inhibition of giant cell transformation, it seemed possible that the suppression of trophectoderm activity during this phase was mediated via the ICM(Snow *et al.*, 1976).

However, this experiment, bisected blastocyst embryos were transferred to pseudopregnant recipients on 3.0 to 4.5 days, but Hahn(1984) reported that when mouse embryos were transferred at different developmental stages immediately or shortly after recovery to the uterus of synchronous recipients or recipients asynchronous by -1 day, success rates of 40 to 50% in average can be achieved. after culturing for 3 days culture and subsequent transfer implantation rates of up to 50 % can be obtained using recipients in estrus 2 days after the donor. Even with zona-free mouse embryos, which will only implant if transferred to the uterus, success rates of up to 50% can be achieved. However, these results in the method of treatment of the zona pellucida were different from Willadsen (1979, 1980), Willadsen and Polge (1981), Willadsen *et al.* (1981), Williams *et al.* (1984), Len-Jensen and Willadsen(1983) and Ozil(1983), who removed mechanically the zona pellucida of embryos in mammals.

In this study it was in accord with Ozil(1983), Williams(1984), who reported that the method could be simplified further if the half-blastocysts could survive without the protection of the zona pellucida. Moreover, early and expanding blastocysts resulted in the best pregnancy rate following bisection.

5. Surgical and non-surgical transfer of half-embryos

The results in pregnancy rates after surgical or non-surgical transfer from half-embryos were presented in Table 5.

The pregnancy rates of recipients which were transferred with half-embryos of morula or blastocyst stage were higher in surgical transfer(52.8%) than in non-surgical transfer(27.5%) ($P<0.05$). In both the transfer methods, the pregnancy rate was not significantly($P<0.05$) different between morulae and blastocysts as the stages of

Table 5. Implantation and development of half mouse embryos after surgical or non-surgical transfer

Items	Surgical transfer(%)			Non-surgical transfer(%)		
	Morula	Blastocyst	Total	Morula	Blastocyst	Total
No. of half-embryos transferred	95	199	293	97	121	218
No. of recipients	11	25	36	12	28	40
No. of recipients pregnant(%)	7 (63.6)	12 (48.0)	19 (52.8)[a]	5 (41.7)	6 (21.4)	11[a] (27.5)[a]
No. of fetuses developed(%)	31 (32.6)	54 (27.1)	85 (29.0)[b]	12 (12.4)	14 (11.6)	26 (11.9)[b]

The percentages with different superscripts in the same row are not significantly($P<0.05$) different.

half-embryos.

The survival rate of half-embryos after transfer was also higher in surgical method(29.0%) than in non-surgical method(11.9%), although the difference in the survival rate between both the transfer methods was not significant($P<0.05$). In both the transfer methods, the survival rate of half-embryos after transfer was not significantly ($P<0.05$) different between morula and blastocyst stage(Fig. 4).

To develop a simple methodology for the transfer of half-embryos in mice, a non-surgical transfer technique was devised in the present study. However, the conception rates and the percentage of embryos developed after transfer were much lower in non-surgical transfer method (27.5% and 11.9%), compared with those in surgical method(52.8% and 27.0%).

The pregnancy rate of surgical transfer in the present experiment was in accord with Nagashima *et al.* (1984). In bovine embryo transfer, Lambeth *et al.* (1983) was no pregnancy achieved by transfer of bisected blastocyst. However, there are no report in pregnancy rates between surgical and non-surgical transfer in bisected embryos, such low conception and developmental rates of embryos after transfer were considered to be partly due to the technical problems in embryo transfer contamination, and partly due to the errorneous pseudopregnant in recipient mice, which was identified by vaginal plug only. Furthermore, method for non-surgical transfer must be considered. More advanced research is necessary for successful transfer of bisected embryos.

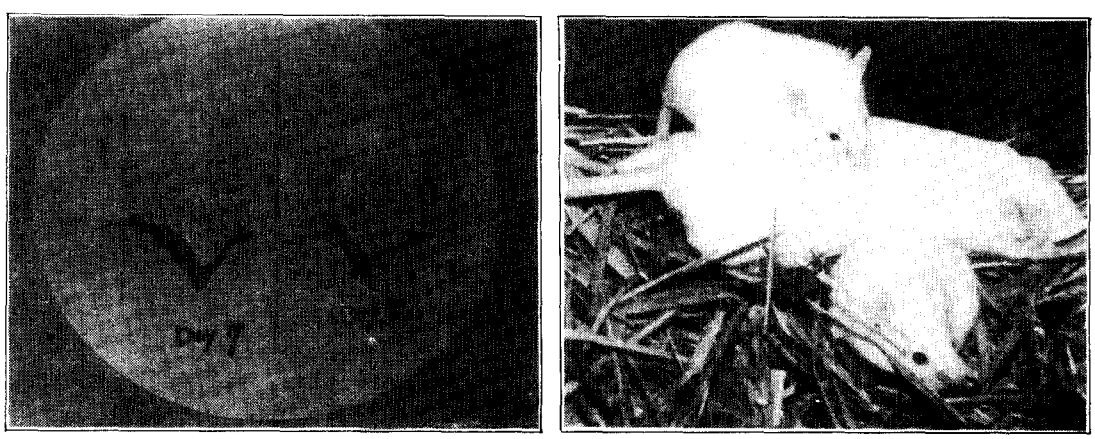

Fig. 3. Detection of pregnancy after transfer of half-embryos.
A : Development assessed by autopsy on 5 to 7 days after embryos transfer.
B : Monozygotic mouse twins obtained from bisection of morula embryo.

SUMMARY

The present study was carried out to develop a simple technique for the production of monozygotic twins by bisection of mouse morula or blastocysts. Random breed BALB/c mice were superovulated by intraperitoneal injection of PMSG following injection of HCG 48 hours later. The embryos of morula to blastocyst stage were bisected vertically using WECK blade attached to micromanipulator. The bisected embryos were cultured for 32 to 36 hours in morula stage and for 3 to 6 hours in blastocyst stage. The cultured half-embryos in the absence of zonae pellucidae were transferred to pseudopregnant recipient mice by surgical or non-surgical methods. The results

optained from this study were summarized as follows:

1. A total of 81.1% of 254 morula, 85.7% of 105 early blastocysts, 81.3% of 342 expanded blastocysts and 83.7% of 110 hatched blastocysts were successfully bisected by a micromanipulation system without any visible damages in cell viability. There were no significant($P<0.05$) differences in the success rate of bisection between stages of embryonic development.

2. When the half mouse embryos were cultured *in vitro* for 30 to 36 hours in morula stage or 3 to 6 hours in blastocyst stage, of 448 half-embryos, 72.8% of them were developed to the expanded or hatched blastocyst stage.

3. When compared with the result of pregnancy rate(63.6%) after surgical transfer of intact morulae, a similar result(61.5%) was obtained with cultured half-embryos bisected at morula stage. However, no one of recipients became pregnant after transfer of half-morulae without culturing ($P<0.05$).

4. When compared with the result of pregnancy rate(55.5%) after surgical transfer of intact blastocysts, similar results were obtained with cultured(55.5%) or non-cultured(43.8%) half-blastocysts($P<0.05$).

5. The pregnancy rate of recipients transferred with half-embryos was higher($p<0.05$), in surgical transfer (52.8%) than in non-surgical transfer (27.5%).

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