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Studies on the Efficient Embryo Transfer Methods using Inbred Embryos in Generation of Transgenic Mice*

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Inbred 마우스 수정란을 이용한 형질전환마우스 생산에 있어서의 효과적인 수정란 이식 방법에 관한 연구

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SUMMARY

The objective of this study is to improve the efficiency of embryo transfer in generation of transgenic mice using inbred mouse (C57BL/6J) embryos. The embryos of C57BL/6J and BCF1 mice were superovulated by the standard protocol. One-cell stage of embryos were microinjected and the resulted one- or two-cell were transferred into one- or two-side oviducts of foster mother, respectively. When one-cell stage of embryos were transferred into one-side oviduct of 0.75 d.p.c. foster mother, the results were not ideal because of showing pregnancy ratios of 68.8±7.83% for C57BL/6J and 48.3±14.22% for BCF1, and development ratios of pups vs transferred embryos of 11.9±5.51% for C57BL/6J and 10.5±8.03% for BCF1. However, when two-cell stage of embryos were transferred into two-side oviducts of 0.5 d.p.c. foster mother, we got better results of 94.4±9.64% and 100±0% pregnancy ratio, and 22.1±0.4% and 21.8±0.38% development ratio for C57BL/6J and BCF1, respectively. Therefore, transferring two-cell stage of C57BL/6J embryos into two-side oviducts of 0.5 d.p.c. foster mother may be competitive to the result in BCF1 embryos. Even if there are a lot of other factors affecting these results, we conclude from these experiments that transfer of two-cell embryos into two-side oviducts of 0.5 d.p.c. foster mother is a more efficient and safe method than others in generating transgenic mice using inbred mice embryos.

(Key words: Inbred mouse, Embryo transfer, Superovulation)

Ⅰ. INTRODUCTION

Superovulation and embryo transfer are important procedures in transgenic animal production, *in vitro* fertilization and embryo cryopreservation. The first decision to be made when setting up a colony for the production of transgenic mice is which mouse strain to be used as donor of the embryos

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for microinjection. If genetic background is not critical to the experiment, injections are efficiently performed with F_2 hybrid zygotes generated from mating between F_1 hybrid male and female mice, because of the relatively poor reproductive performance of inbred mice (Brinster et al., 1985). For many experiments, however, it is important to introduce the transgene into mice with a defined genetic background, and in these cases, the genetic advantage of inbred mice will outweigh their embryological disadvantages.

Recently, in many experiments, the inbred C57BL/6J strain mice are widely used for superovulation to obtain the large number of embryos. According to the standard superovulation protocol in "Manipulating The Mouse Embryo" (Hogan et al., 1994), generally one of C57BL/6J strain mouse mated can obtain 20~30 embryos (15~24 injectable fertilized embryos) after superovulation, and the gonadotropin (Sigma G 4527, Sigma C 8544) they used was so expensive (5.89$ for one mouse). When other comparatively cheaper products of gonadotropin were used, the results were unideal (data not shown). It's a problem for the investigators who want to do experiments using inbred mice but with relatively not abundant research funds.

The technique of embryo transfer has been proven useful to a number of biological fields. In the 1890's, Heape's original rabbit experiments addressed what effect a transferred embryo had on the foster mother and on further in vivo development (Heape et al., 1891). These and subsequent studies opened a new venue for exploring facets of genetics, embryology, reproductive physiology, immunology and cancer research (McLaren et al., 1956).

According to efforts of many researchers in this field, it was recently shown that ova can be successfully transferred to the reproductive tracts of recipient mice via a hole made in the oviductal wall rather than by the oviductal ostium (Mountz et al., 1990; Pease et al., 1989). But the oviductal ostium transfer has been used as a main method in transgenic mice production because of the easier manipulation and higher successful incidence. However, so far, no study has provided detailed investigation that showed the relationship between pregnancy ratio and its influencing factors on one- or two-side oviducts embryo transfer methods, one- or two-cell stage of embryo transfer, and transfer time, even though the standard embryo transfer protocol in "Manipulating the Mouse Embryo" had described it superficially.

According to above research backgrounds and the existing problems, the objectives of these experiments were as follows:

1. Adaptation of the superovulation protocol of inbred C57BL/6J strain mouse to an economical way.
2. Initial investigation of relationships between the pregnancy ratio and the affecting factors of one- or two-oviducts embryo transfer methods, one- or two-cell stage embryos transfer, and transfer time for optimizing embryo transfer procedure in mice.

Ⅱ. MATERIALS AND METHODS

1. Experimental Animals

C57BL/6J (inbred) and BCF1 (C57BL/6J♀ × CBA/J♂) F_1 were used as donor to supply one-cell stage of fertilized embryos for DNA pronuclear microinjection. ICR mice were used as foster mothers. The C57BL/6J and BCF1 were supplied from Genetic Resources Center of Korea Research Institute of Bioscience and Biotechnology at 4~5 week-age, and then were brought up to 8~10 week-age at our SPF mouse house for inducing superovulation to obtain a large number of embryos.

The female ICR mice were purchased from commercial breeding company (Dae Han Biolink Co., Ltd.) at 4～5 week-age. The female mice were brought up to 8～10 week-age (about 28～32g of body weight), and then mated with the vasectomized male ICR mice (12 week-age old) for preparing foster mother. The mice were bred under SPF conditions with regulated light cycle of 12 hours light and 12 hours dark, and light was turned on at 7:00 AM.

2. Inducing Superovulation

For inbred mice C57BL/6J, the Intervet drugs were used. PMSG (pregnant mare's serum gonadotropin) was administered at 4:00 PM and HCG (human chorionic gonadotropin) was administered 46～47 hours later. The dose was 5 IU. After being administered HCG, females were placed in cage of stud male at the ratio of 1:1. On the day following the HCG injection, the females were examined for the presence of vaginal plugs. Females with plugs were removed and sacrificed at about 11:00 AM, the oviducts were excised and the fertilized oocytes were retrieved from the ampullaes.

In order to compare the C57BL/6J strain mouse with the BCF1 strain mouse, the hybrid BCF1 mice were induced superovulation and used the same method as C57BL/6J.

PMSG and HCG were dissolved according to the instruction of products (Intervet).

3. Embryo Transfer

Embryo Transfer (ET) was carried out according to the standard protocol described by Hogan et al., (1994). In our experiments, we initially investigated factors of ET time, development stage of embryos, and one- or two-side oviducts embryo transfer methods that might influence the efficiency of ET. For this purpose, we did four kinds of experiments as follows:

1) One-side oviduct ET with one-cell stage of embryos: One-cell stage of embryos were transferred to foster mother at 5:00～6:00 PM. The number of transferred embryos was 25～30 per foster mother.
2) Two-side oviducts ET with one-cell stage of embryos: One-cell stage of embryos were transferred to foster mother at 5:00～6:00 PM. The number of transferred embryos was 21～30 per foster mother and each oviduct was transferred with 10～15 embryos.
3) One-side oviduct ET with two-cell stage of embryos: Two-cell stage of embryos were transferred to foster mother at 9:00～10:00 AM. The number of transferred embryos was 21～30 per foster mother.
4) Two-side oviducts ET with two-cell stage of embryos: Two-cell stage of embryos were transferred to foster mother at 9:00～10:00 AM. The number of transferred embryos was 21～30 per foster mother and each side oviduct was transferred with 10～15 embryos.

In our experiments, the transferred embryos were all suffered pronuclear microinjection at 1:00 PM～4:00 PM. After ET, the foster mothers were placed in a clean cage and kept for pups birth.

4. Statistical Analysis

The SAS mixed linear model program was used to analysis the data. Treatment means were compared for differences through use of Duncan's Modified Multiple Range test (Duncan, 1955). Differences were considered statistically significant at $P<0.05$.

Ⅲ. RESULTS

1. Inducing Superovulation

The superovulation results were summarized in

Table 1. Surperovulation were induced to total 90 (C57BL/6J, inbred) and 65 mice (BCF1, hybrid) mice, respectively. Among them, 44 (47.2±15.48%) and 48 (72.8±14.34%) mice ovulated ($P<0.05$). The number of total embryos collected were 1,077 and 1,018, respectively. Among them, the number of total injectable embryos obtained were 557 and 825 and the mean numbers of embryos collected form one ovulated mouse were 25.8±5.63 and 21.5±3.14, respectively. However, the collection rate of injectable embryos was higher in BCF1 (17.3±2.66) than in C57BL/6J mice (13.2±1.75).

2. Embryo Transfer for C57BL/6J (inbred) Mice

The embryo transfer results of C57BL/6J mice were summarized in Table 2. When the method of one-side ET with one-cell stage of embryos was used, the lowest ratio of pregnancy (68.8±7.83%) and embryos developed to term (11.9±5.51%) were obtained, The significant difference ($P<0.05$) was showed when comparing the method of one-side ET using one-cell stage embryos with the methods of one- and two-side ET using two-cell stage of embryos. but there was no significant difference comparing with the method of two-side ET using one-cell stage of embryos.

3. Embryo Transfer for BCF1 (hybrid) Mice

The embryo transfer results of BCF1 mice were summarized in Table 3. When the method of one-side ET with one-cell stage of embryos was used, the lowest ratio of pregnancy (48.3±14.22%) and embryos developed to term (10.5±8.03%) were obtained, There was significant difference ($P<0.05$) between the method of one-side ET with one-cell stage of embryos and other methods.

Ⅳ. DISCUSSION

Table 1. Comparison of superovulation ratio between inbred mouse and hybrid mouse

Mouse strains	No. of mice used	No. (%) of mice ovulated	No. (%) of embryos collected	No. (%) of injectable embryos
C57BL/6J	90	44 (47.2±15.48)[1,b]	1,077 (25.8±5.63)[2,a]	557 (13.2±1.75)[3,b]
BCF1	65	48 (72.8±14.34)[1,a]	1,018 (21.5±3.14)[2,a]	825 (17.3±2.66)[3,a]

[1] The mean ratio of plugged mice.

[2] The mean number of embryos collected from one ovulated mouse.

[3] The mean number of injectable embryos collected from one ovulated mouse.

[a,b] Within a column, values with different superscripts are significantly different ($P<0.05$).

Table 2. Comparison of the efficiency of embryo transfer in C57BL/6J mice

ET methods	Developmental stage	No. of embryos transferred	No. (%) of pregnant /foster mother	No. (%) of pups born
One-side oviduct	1-cell	498	12/17 (68.8±7.83)[1,b]	63 (11.9±5.51)[2,b]
	2-cell	651	20/22 (90.8±10.68)[1,a]	136 (20.8±2.29)[2,a]
Two-side oviducts	1-cell	762	23/28 (82.2±5.88)[1,ab]	122 (15.8±3.61)[2,ab]
	2-cell	411	15/16 (94.4±9.64)[1,a]	91 (22.1±0.4)[2,a]

[1] The mean pregnancy ratio.

[2] The mean development ratio of pups vs transferred embryos.

[a,b] Within a column, values with different superscripts are significantly different ($P<0.05$).

Table 3. Comparison of the efficiency of embryo transfer in BCF1 mice

ET methods	Developmental stage	No. of embryos transferred	No. (%) of pregnant /foster mother	No. (%) of pups born
One-side oviduct	1-cell	148	7/15 (48.3±14.22)[1,b]	25 (10.5±8.03)[2,b]
	2-cell	869	39/41 (96.8±6.65)[1,a]	240 (28.4±3.16)[2,a]
Two-side oviducts	1-cell	1,202	42/44 (95.3±4.07)[1,a]	319 (26.4±4.82)[2,a]
	2-cell	526	24/24 (100±0)[1,a]	114 (21.8±0.38)[2,a]

[1] The mean pregnancy ratio.

[2] The mean development ratio of pups vs transferred embryos.

[a,b] Within a column, values with different superscripts are significantly different ($P<0.05$).

Currently, the technique of pronuclear microinjection is the most successful and widely used method for producing transgenic animals. Especially, surperovulation and embryo transfer are the crucial steps for obtaining a large number of fertilized embryos and potential founders from micromanipulated embryos. Embryo transfer efficiency has been remarkably improved, however it is not satisfactory in inbred mice. To improve embryo transfer efficiency using inbred mice in generation of transgenic mice, we investigated several factors affecting superovulation and embryo development.

In our superovulation experiments, when the gonadotropins of Intervet Company (Holland) was used, PMSG was injected at around 4:00 PM, and HCG was injected at 46~47 hours later (about 2:30 PM), the best results were obtained. For one ovulated mouse, 25.8±5.63 embryos were generally produced and among them about 13.2±1.75 embryos had large, clear visible pronuclear that could be used to perform pronuclear microinjection. The gonadotropins (Intervet) were about 140 times cheaper than that (Sigma) used in the standard protocol, while the total yield of embryos was similar to that obtained from the standard protocol.

When comparing with BCF1 (hybrid), the significant different ($P<0.05$) were shown in plug ratio and injectable embryos obtained per plugged mouse, while there was no significant difference in number of collected embryos per plugged mouse. These results are consistent with the knowledge that the inbred mice are less productive than F_1 hybrid mice (Richa, 2001). The undesired results, the lower ratio of injectable embryos against large number of collected embryos for C57BL/6J mice, might be due to the reproductive ability of stud male, strain degeneration or/and the environments of the breeding house, in which the mice were bred. The data of our experiments suggests that the new protocol is highly efficient and economical and can be used widely under normal conditions.

For embryo transfer technique, the improved protocol is more efficient than other report that showed the highest pregnancy ratio of 83% (Jonson et al., 1996). It suggests that the two-side ET method may provide an enough space for embryo to develop. When the two-cell stage of embryos were transferred in the morning (0.5 d.p.c. foster mother), we obtained the 90.8±10.68 to 94.4± 9.64% pregnancy ratio for C57BL/6J mice and 96.8±6.65 to 100±0% pregnancy ratio for BCF1. It suggests that 2-cell of embryos can have enough time to adjust themselves to match the condition of the oviduct.

It was concluded that microinjected zygotes were less viable than those that had not been undergone experimental manipulations (Brinster, 1985; Canseco, 1994; Hogan, 1994; Polites, 1994). Typically,

50~80% of the embryos survive against the injection. Among them approximately 10~30% of microinjected embryos transferred into the oviduct will develop to term (Hogan et al., 1994). It was showed that the average number of fetus did not surpass 8.5 per horn and embryonic death after day 9 dependent on uterine crowding (Mclaren and Michie, 1956). It also suggested that the dead embryos may influence the development of other embryos. So, when the one-side ET method was used, crowded environment, influence of dead embryos, competition between transferred embryos and moving to opposite uterine will be the factors to influence the efficient implantation and development of transferred embryos, while the two-side ET method offers more space for the embryo to develop to the date. That may be the reasons that why the two-side ET method was always among the best result groups in our investigation.

According to the common schedule of producing transgenic mice (recovery of superovulated ova at about 11:00 AM and introduction of injection DNA into pronuclear at 1:00~4:00 PM), the embryos were usually transferred to recipients at 0.75 d.p.c. (because the mice were bred in the same light-dark controled SPF mice house). It has been reported that superovulation of female mice and manipulation of embryos *in vitro* delay embryonic and fetal development (Auwera, 2001; Smith, R., 1977). In our investigation, the same worst results were obtained for C57BL/6J and BCF1 mice when the method of one-side ET with one-cell stage of embryos was used. It indicates that the development delayed embryos had some problems for them to match to the environment of the oviducts of 0.75 dpc foster mother. In addition, the crowded environments increase this bad situation.

It has been shown that one- and two-cell embryo transfer have no significant difference in development potence (Hogan et al., 1994). But in our investigation, it has been shown that one-cell embryo transfer using one-side ET method resulted in the lowest ratio of pregnancy and embryos developed to term. These differences may result from the different experiment schedules.

In conclusion, even if there are a lot of other factors affecting this results, two-side ET method, two-cell stage of embryo and 0.5 d.p.c. foster mother are positive factors for ET experiment. It suggests that, for common schedule of producing transgenic mice, transferring two-cell stage of embryos into 0.5 d.p.c. foster mother using two-side ET method may be the most efficient and safe protocol. In addition, our experiments provided an information that economical drugs can be used for superovulation in inbred C57BL/6J mice.

V. 요 약

본 연구의 목적은 inbred 마우스 (C57BL/6J)의 수정란을 이용하여 형질전환마우스를 생산할 때, 수정란이식의 효율성을 증진시키기 위한 것이다. C57BL/6J 및 BCF1마우스로부터 과배란처리 방법에 의해 수정란을 얻고, DNA를 1 세포기 수정란에 미세 주입한 다음, 1세포기 또는 2 세포기의 수정란을 가임신된 마우스의 한쪽 또는 양쪽 난관에 각각 이식하였다. 1세포기의 수정란을 0.75 d.p.c. 가임신된 마우스의 한쪽 난관에 이식했을 때, 임신율이 C57BL/6J는 68.8±7.83%, BCF1은 48.3±14.22% 이었고, 이식한 수정란 당 산자의 발달율은 C57BL/6J가 11.9±5.51%, BCF1은 10.5±8.03%로 성적이 저조하였다. 그러나, 2세포기의 수정란을 0.5 d.p.c. 가임신된 마우스의 양쪽 난관에 이식했을 때, 임신율이 C57BL/6J는 94.4±9.64%, BCF1은 100±0% 이었고, 이식한 수정란 당 산자의 발달율은 C57BL/6J가 22.1±0.4%, BCF1은 21.8±0.38%였다. 따라서 C57BL/6J 마우스의 2세포기 수정란을 0.5 d.p.c. 가임신된 마우스의 양쪽 난관에 이식하는 것이, BCF1마우스와 유사한 성적을 얻어 경쟁력이 있는 것으로 판단되었다. 이러한 결과에 영

향을 미치는 인자가 여러 가지 있을 것으로 판단되지만, C57BL/6J 마우스의 2세포기 수정란을 0.5 d.p.c.가임신된 마우스의 양쪽 난관에 이식하는 방법이 다른 방법보다 형질전환마우스를 생산하는데 효율성이 더 높은 것으로 본 실험에서 확인되었다.

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