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Research Article

Comparing the Birth Rates of Microinjected Hybrid Mouse Zygotes Transferred into Outbred and Hybrid Foster Mothers

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Abstract: The purpose of this study was to compare the birth rates after transfers of microinjected hybrid mouse zygotes into hybrid CB6F1 (C57BL/6J X BALB/c) and outbred CD-1 foster mothers. One or two hours after microinjection of a *β-actin-Gfp* gene construct into the male pronucleus, hybrid mouse zygotes were transferred into CB6F1 and CD-1 foster mothers. In addition, non-microinjected hybrid zygotes were transferred into foster mothers of both strains. The overall data demonstrate that outbred CD-1 foster mothers are a better choice for transfer of microinjected hybrid mouse embryos, and there was a statistical difference between the transfers of microinjected zygotes into CB6F1 and CD-1 foster mothers ($P<0.001$). Furthermore, there was also a statistical difference when microinjected and non-microinjected hybrid mouse embryos were transferred into CB6F1 foster mothers ($P<0.05$). However, the same difference was not observed when CD-1 foster mothers were used for transfer. When control groups are compared, data suggest that although there was no difference in terms of birth rate, more pups were obtained from transfer of non-microinjected embryos into CD-1 foster mothers. Thus, we suggest that CD-1 female mice are more suitable foster mothers than CB6F1 female mice,not only for microinjected hybrid mouse embryo transfer but also for non-microinjected hybrid mouse embryos.

Key Words: Microinjection, pronuclear stage, embryo transfer, hybrid, outbred

Mikroenjeksiyon Uygulanmış Hibrid Fare Zigotlarinin Outbred ve Hibrid Alıcılara Transferleri Sonrasında Elde Edilen Doğum Oranlarının Karşılaştırılması

Özet: Bu çalışmanın amacı mikroenjeksiyon uygulanmış hibrid fare zigotlarının, hibrid CB6F1 (C57BL/6J X BALB/C) ve outbred CD-1 alıcı anne farelere transferlerinden sonraki doğum oranlarını karşılaştırmaktır. *β-actin-Gfp* gen konsraktının erkek pronükleusuna mikroenjeksiyonundan 1 ya da 2 saat sonra, bir hücre aşamasındaki hibrid fare embriyoları, doğum oranlarını belirlemek için CB6F1 ve CD-1 alıcı anne farelere transfer edilmiştir. Ayrıca, kontrol olarak her iki grup için, az sayıda mikroenjeksiyon uygulanmamış bir hücre aşamasında hibrid fare embriyoları her iki ırktan alıcı anne farelere transfer edilmiştir. Elde edilen verilerin tümü outbred CD-1 alıcı anne farelerin mikroenjeksiyon uygulanmış hibrid fare embriyolarının transferi için daha iyi bir seçenek olduğunu göstermektedir ve CB6F1 ve CD-1 alıcı fare annelere mikroenjeksiyon uygulanmış embriyolar transfer edildiğinde iki grup arasında istatistiksel fark bulunmuştur ($P<0.001$). Bunun yanında, mikroenjeksiyon uygulanmış ve uygulanmamış hibrid fare embriyoları CB6F1 alıcı anne farelere transfer edildiği zamanda istatistiksel fark bulunmuştur ($P<0.05$). Ancak, aynı fark CD-1 alıcı annelerin transfer için kullanıldığı zaman gözlenmemiştir. Kontrol grupları kendi aralarında karşılaştırıldığında ise, doğum oranı bakımından bir fark olmamasına rağmen bulgular, mikroenjeksiyon uygulanmamış hibrid fare embriyolarının CD-1 alıcı anne farelere transferinden daha çok sayıda yavru elde edildiğini göstermektedir. Bu sebeplerden dolayı, CD-1 alıcı anne fareleri sadece mikroenjeksiyon uygulanmış hibrid fare embriyoları için değil, aynı zamanda mikroenjeksiyon uygulanmamış hibrid fare embriyolarının transferi için CB6F1 alıcı fare annelerden daha uygun alıcı anne fareler olarak önermekteyiz.

Anahtar Sözcükler: Mikroenjeksiyon, pronükleer safha, embriyo transferi, hibrid, outbred

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Introduction

Improvements in the production techniques of transgenic animals provide many new opportunities for research in the field of medical, biological and veterinary sciences (1). Transgenic technology also has important impacts on other fields such as agriculture, and animal and human health (1, 2).

The difference between microinjected embryos and non-microinjected embryos is that microinjected embryos carry foreign recombinant DNA after pronuclear microinjection (2, 3, 4). Obtaining a high number of live transgenic mouse pups after transfer depends on the reproductive health of foster mothers (2, 3, 4). Since one of the most important tools in transgenic technology is the embryo transfer procedure, selection of suitable foster mothers is very important (3, 4). It was reported that CD-1 foster mothers have larger ampullae, which makes oviduct transfer easier for less experienced resesarchers, and are mostly good foster mothers. However, some researchers prefer to use CB6F1 foster mothers, which, although their ampullae are smaller, make extraordinarily good foster mothers, rearing litters as small as two pups (2, 4). When comparing microinjected embryos with in vivo developed embryos, microinjected embryos are less advanced than those of in vivo developed embryos in terms of the stage of embryonic development (5). Thus, foster mothers (0.5 day) should be chosen among those which are mated earlier than those donor mice (3). In addition, it was reported that while embryos are transferred into foster mothers from different mouse strains, implantation and birth rates show differences among different mouse strains (4). Hence, it can be concluded from these reports that strain differences in mice have an important impact on implantation and birth rates. In this study, microinjected hybrid mouse embryos were transferred into outbred CD-1 and CB6F1 (C57BL/6 X BALB/c) hybrid foster mothers and the effect of this strain difference on birth rate after transfer was evaluated.

Materials and Methods

Superovulation and embryo recovery

CB6F1 hybrid mice were maintained on a 14:10 hour light:dark cycle. Fifty females (6-7 weeks old) were superovulated by intra-peritoneal administration of 5 IU of pregnant mare serum (PMSG; G-4877; Sigma) followed 48 hours later by 5 IU hCG (Pregnyl; Organon). The females were immediately placed with males for mating and examined the next morning (day 0) for the presence of vaginal plugs (1, 2). In the present study, a total of 42 CB6F1 female mice having vaginal plugs were used for embryo recovery. Mice were sacrificed by cervical dislocation around 17-18 hours after hCG injection and then their oviducts were removed. The ampullae of the oviducts were ripped open with dissecting needles, and a total of 887 embryos with cumulus cells were obtained and placed in drops of M2 medium (Modified Krebs-Ringer solution with partial substitutions of bicarbonate with HEPES) (2).

Pronuclear microinjection

Embryos with two pronuclei were selected under a stereomicroscope and transferred into a 25-30 µl microdrop of M2 medium under mineral oil (M-3516; Sigma) (1, 2). A total of 716 one-cell embryos were used for microinjection. The embryos for manipulation were transferred to the injection chamber. The injection chamber included an inverted microscope equipped with Differential Interference Contrast (DIC) optics (Axiovert 35M, Zeiss), two Leitz micromanipulators (Leitz), and an automatic microinjector (Eppendorf 5242). Holding pipettes and injection needles were prepared in our laboratory as described previously (1, 2, 3, 6, 7). The microinjection of 2-pronuclear stage embryos was performed by using the method described elsewhere (1, 2). Approximately 2 pl of DNA solution (3 ng/pl) was microinjected into the male pronucleus. After microinjection, the embryos were washed three times with M2 medium (2), and then cultured further in 50 µl of CZB medium (8, 9) in a humidified atmosphere of 5% CO_2 at 37oC. Embryos were microinjected with *β-actin-Gfp* (6, 7). About 2 hours after microinjection, 487 of 716 (68%) embryos were determined to have survived by their morphology under a stereo microscope, and all of them were transferred into the oviducts of foster mothers.

Preparation of foster mothers and embryo transfer

Ten to twelve week old foster mothers were mated with vasectomized males one day before microinjection at 3.00 pm. Vasectomized males were kept in individual cages, and two females were usually added to the male cage after hCG injection. For each experimental trial, about 52 CD-1 and 45 CB6F1 females were put into the cages of vasectomized males for mating. The next

morning, females having vaginal plugs were determined and designated as "0.5 day foster mother". Females having vaginal plugs were anesthetized with a mixture of xylazine (16 mg/kg) and ketamine (120 mg/kg) (10). Then, the right ovarium and oviduct were taken out from lateral section opened under the last rib on the back. For embryo transfer, 17-25 microinjected embryos in 1 µl of M2 were loaded into a 150 mm diameter transfer pipette. Under a dissection microscope, the ampullae region of the oviducts was held outside the body cavity with a serrefine clamp attached to the fat pad and a hole in the infindibulum was made by means of a 30-gauge needle. Embryos placed between two air bubbles in the transfer pipette were given into the ampullae from this hole until two air bubbles were seen in the oviduct. In this study, a total of 658 embryos were transferred for both groups with their corresponding control groups.

Statistical Analysis

The data analysis was carried out by using the Graphpad Software Program (Version 2.02, Dr. Granger, LSU Medical Center). Data from the different treatments was compared with Chi squared analysis.

Results

We evaluated pup numbers and birth rates after transfer of microinjected hybrid mouse embryos into CD-1 and CB6F1 foster mothers. In addition, non-microinjected hybrid mouse embryos were transferred as controls for both groups. Two separate experiments were done and each experiment was divided into two groups as shown in Table 1. In the control groups, non-microinjected embryos were used for transfer.

In the first group, a total of 275 microinjected embryos were transferred into 11 foster mothers. In this group, for each foster mother, 25 embryos were used for oviduct transfer. From these transfers, 50 (18%) pups were obtained. In the control group, a total of 125 non-microinjected embryos were transferred into 5 CB6F1 foster mothers and from these transfers, 39 (31%) pups were born alive. The difference between these groups was found to be statistically important ($P<0.05$)

In the second group, a total of 212 microinjected embryos were transferred into 10 foster mothers. In this group, for each foster mother, an average of 21 embryos were used for transfer. From these transfers, 88 (42%) pups were obtained. In the control group, a total of 46 non-microinjected embryos were transferred into 2 CB6F1 foster mothers and from these transfers, 22 (48%) pups were born alive. The difference between these two groups was not significantly important ($P>0.05$).

In addition, the number of pups born from microinjected embryos transferred into CD1 foster mothers was significantly higher ($P<0.001$) than from those transferred into CB6F1.

Table 1. Numbers and percentages of pups born alive after transfer into CB6F1 and CD-1 foster mothers.

	Group 1 (CB6F1 foster mothers)		Group 2 (CD-1 foster mothers)	
Trials	#of transferred zygotes per foster mother / #of used foster mother	#of pup numbers per foster mother / #of transferred total zygotes (% of pups born alive)	#of transferred zygotes per foster mother / #of used foster mother	#of pup numbers per foster mother / #of transferred total zygotes (% of pups born alive)
1.	25/1	2/25 (8%)	17/2	5+10=15/34 (44%)
2.	25/1	3/25 (12%)	25/2	9+12=21/50 (42%)
3.	25/2	6+7=13/50 (26%)	20/2	6+12=18/40 (45%)
4.	25/2	5+9=14/50 (28%)	20/2	12+8=20/40 (50%)
5.	25/5	4+5+3+1+5=18/125 (14%)	24/2	8+6=14/48 (29%)
Total	275/11 (25*)	50/275 (18%)a	212/10 (21*)	88/212 (42%)b
Control**	25/5	8+7+5+9+10=39/125 (31%)b	23/2	10+12=22/46 (48%)b

*Mean number of transferred zygotes per foster mother.

** In control groups, non-microinjected embryos were transferred into foster mothers in both groups.

Different superscripts in the same rows and columns denote statistical difference ($p<0.05$ for the column and $P<0.001$ for the row).

Discussion

A higher percentage of pups was obtained after transferring microinjected hybrid mouse embryos into CD-1 foster mothers than when transferring the same embryos into CB6F1 foster mothers.

In many reports studied so far, it was reported that hybrid mouse embryos are more efficient not only for pronuclear stage embryo recovery but also for gene transfer studies by pronuclear microinjection (1, 2, 3, 4, 11, 12). In the present study, a total of 50 CB6F1 female hybrid mice were used for superovulation and 42 of them (84%) showed vaginal plugs. The average number of embryos obtained from one donor mouse was 21 and this number was comparable with other studies (2, 3, 4, 12).

The survival rate in zygotes after microinjection can vary according to the preparation, concentration, copy number and pureness of gene constructs, microinjection buffer, volume of DNA solution microinjected into the male pronucleus (about 2 pl) and the skill of the person who applied the microinjection (2, 3). It was reported that 50-85% of microinjected mouse zygotes survive after microinjection (2, 3, 4). In the present study, all parameters mentioned above were taken into consideration and 68% (487/716) of microinjected one-cell embryos survived, which was comparable with some previous results (1, 2, 3, 4, 11).

Microinjected one-cell embryos should be transferred into suitable foster mothers on the same day of microinjection or one day after microinjection when embryos are at the two-cell stage. If the number of foster mothers is not enough for transfer, the remaining zygotes could be cultured until the two-cell stage and then they could be transferred into 0.5-day pseudopregnant females. It was reported that both hybrid and outbred mice could be used as foster mothers but outbred CD-1 mice are especially recommended for successful birth rates (2, 3, 4).

In some studies, after transfer of microinjected one-cell embryos into the oviduct, birth rates were reported to be between 10 and 30% (2, 3, 13, 14, 15). In another study, when microinjected mouse zygotes carrying different gene constructs were transferred into hybrid foster mothers, birth rates for different gene constructs were found to be 29%, 20% and 23% (16, 17).

A total of 275 microinjected embryos were transferred into 11 foster mothers and 50 of them (18%) were born alive in our study. For the control group, a total of 125 non-microinjected embryos were transferred into 5 foster mothers and 39 of them (31%) were born alive. The difference between microinjected and non-microinjected embryo transfer was found to be statistically important ($P<0.05$). Our birth rate for microinjected embryo transfer was lower than a previous study (11). However, the birth rate of the control group was higher than the result of Lin's study with non-microinjected embryos (5).

In the second group, a total of 212 microinjected embryos was transferred into 10 foster mothers and 88 (42%) of them were born alive. In the control group, a total of 46 non-microinjected embryos was transferred into 2 foster mothers and 22 of them (48%) were born alive. The difference between microinjected and non-microinjected embryo transfer was not found to be statistically important ($P>0.05$). In this second group, birth rates for both trial groups and the control group were higher than other studies (2, 4, 5, 18). When the first and second groups' results were compared, birth rates in the first group and second group for microinjected embryo transfer were determined to be 18 % (50/275) and 42% (88/212), respectively and the difference was statistically important ($P<0.001$). In addition, when the control groups were compared with each other, the birth rate was found to be 31% (39/125) for the first group and 48% (22/46) for the second group and the difference was not found to be statistically important ($P>0.05$).

In light of these findings we conclude that outbred CD-1 foster mothers are more suitable than hybrid CB6F1 foster mothers for both microinjected and non-microinjected mouse zygote transfer because of the implantation and birth rates, resistance to anesthesia and convenience for the embryo transfer operation due to the clear appearance of ampullae (2, 4). In addition, this conclusion was supported by the results of transfers of in vitro fertilized or frozen-thawed mouse embryos from different strains when CD-1 foster mothers were used for transfer (unpublished data). The results of transgenic analyses will be published in the future.

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