Rederivation of Mice by Means of In Vitro Fertilization and Embryo Transfer

Hiroshi SUZUKI¹⁾, Keigo YOROZU, Toshihiko WATANABE¹⁾,
Masao NAKURA¹⁾, and Jiro ADACHI¹⁾

Chugai Pharmaceutical Co., Ltd. and ¹⁾Present address: Laboratory of Molecular Genetics and Embryology, CSK Research Park INC., 1–135 Komakado, Gotemba, Shizuoka 412, Japan

Abstract: In vitro fertilization and embryo transfer were performed for rederivation of four strains of mice harbouring mouse hepatitis virus (MHV) and/or *Pasteurella pneumotropica* (*P. pneumotropica*). Superovulated oocytes were fertilized by preincubated cauda epididymis sperm in vitro. Fertilized eggs at 2-cell stage were transferred into the oviducts of specific pathogen free (SPF) recipients. Microbial examination of sperm and/or oocyte donors verified the presence of *P. pneumotropica* and/or of antibodies to MHV in all strains, but neither in the recipients nor in the offspring antibodies to MHV could they be detected. The results indicate that an in vitro fertilization-embryo transfer (IVF-ET) system is an effective and simple alternative to cesarean operation in infected mice.

Key words: embryo transfer, in vitro fertilization, rederivation

---

Introduction

New strains of SPF mice are generally obtained by cesarean section of conventional or infected pregnant females prior to parturition. After the operation, delivered fetuses are nursed by a foster mother confirmed as SPF. However, since some viral [2, 6] as well as bacterial [4, 5, 9] infections can be transmitted vertically, the cesarean operation-fostering system is not completely reliable.

P. pneumotropica, which is reported to be potential respiratory and/or occasionally suppurative pathogen, was identified in uterus [3, 9] and vagina [11, 20] and has also been isolated from the feces of animals [7]. Fetuses could therefore be infected by this organism

during a cesarean hysterectomy. Flynn *et al.* [3] have reported a high frequency of this infection in mice originally delivered by cesarean operation. Transplacental transmission of MHV to fetuses has also been demonstrated by intravenous inoculation of pregnant mothers [6]. Moreover, Carthew *et al.* [2] have reported that embryos collected from donors experimentally infected with MHV were not infected, but the flushing from the uterine horns was contaminated with the virus. Consequently, many investigators have attempted embryo transfer (ET) to rederive laboratory animals known to harbour pathogens [1, 2, 8–10, 15].

In vitro fertilization (IVF) has been frequently used for treatment of sterility in humans; to improve the

---

(Received 14 April 1995 / Accepted 17 August 1995)

Address corresponding: H. Suzuki, Laboratory of Molecular Genetics and Embryology, CSK Research Park INC., 1–135 Komakado, Gotemba, Shizuoka 412, Japan

quality and increase production in cattle. In laboratory animals, IVF has been used for analysis of fertilization events and to ameliorate defects in the reproductive ability of mutant [13, 14, 21] or aged mice [16]. In this study, we attempted to apply the IVF-ET system for rederivation of mice contaminated with MHV and/or *P. pneumotropica*.

Materials and Methods

Animals: Two inbred strains of mouse (C57BL/KsJ and KK-A^y) and two transgenic mouse strains (Tgm-S and Tgm-A) were introduced from other holders and known to be contaminated with MHV and/or *P. pneumotropica*. These animals were housed in a quarantine unit of our facility before rederivation. SPF KK (Kiwa, Japan) and C57BL/6J (CLEA, Japan) mice were used as oocytes donors for IVF and ICR mice (CLEA,

Japan) were used as pseudopregnant recipients for embryo transfer. These mice were maintained in a barrier unit. Animals in both quarantine and barrier units were kept at $24 \pm 2^\circ\text{C}$ with a relative humidity of 50 to 60% under a lighting regimen of 14L : 10D (lights on 05:00 a.m. to 19:00 p.m.), and air was exchanged 12 to 15 times/hr. Mice were housed in polycarbonate cages (TP-106; $175 \times 245 \times 125$ mm, TP-102; $215 \times 320 \times 140$ mm, TOYO RIKO, Japan) and were allowed access to standard laboratory chow (CA-1; CLEA, Japan) and tap-water.

Rederivation procedure: As shown in the flow chart in Fig. 1, the rederivation procedure in this study was composed of three clean zone phases which utilize four laboratory or animal room units. To avoid contamination, each unit was separately located. IVF, culture of eggs and ET were performed at an embryo manipulation laboratory in a barrier unit. Male and female

Fig. 1. Experimental procedures for rederivation by means of *in vitro* fertilization and embryo transfer.

reproductive organs were excised from infected mice in the quarantine unit and were transferred to the embryo manipulation laboratory. After receiving embryos, recipients were transferred to an animal room and were allowed to deliver at full term. After confirmation of SPF, offsprings were transferred to the animal room for breeding. Microbial examination was conducted in a quarantine unit.

In vitro fertilization and embryo transfer: C57BL/KsJ, KK and C57BL/6J females were superovulated with 5 IU of PMSG (Serotropin; Teikoku Hormone Mfg. Co., Japan) and 5 IU of hCG (Puberogen; Sankyo, Japan) injected 48 hr apart. At 15 to 16 hr after the hCG injection, the females were sacrificed and their reproductive organs were excised in a safety cabinet. To avoid bleeding, each vessel with tissues was ligated in advance. The reproductive organs were exposed in 0.3% benzalkonium chloride solution (Osuban; Nippon Seiyaku, Japan) for 30 sec and rinsed with saline twice. The oviducts were excised on sterilized filter paper and transferred into a dish covered with paraffin oil (Art 7162; Merck, USA) equilibrated overnight at 37°C in 5% CO₂ in air. The ampullar region of oviducts was torn with a pair of needles, and oocytes surrounded by cumulus cells were introduced into 0.4 ml of TYH medium [18] on a dish. Prior to obtaining the oocytes, male mice were sacrificed by cervical dislocation and their reproductive organs were removed. After washing the organs by an aseptic technique in a similar procedure to that used for females, sperm was collected from the cauda epididymis and suspended in 0.4 ml of TYH medium. After preincubation of the sperm for 2 hr, IVF was performed by adding a small volume (3-5 µl) of the sperm suspension to the medium containing eggs (150 cells/µl). At 6 hr after insemination, fertilized eggs were transferred to 0.4 ml of Whitten's medium [19] supplemented with 100 µM EDTA, and cultured at 37°C with 5% CO₂ in air. At 24 hr after insemination, 2-cell stage embryos were transferred into oviducts of ICR recipients on 0.5 day post-coitum as described in a previous report [17].

Microbial examination: To confirm the rederivation of the contaminated animals, microbial examination was performed by detection of serum antibody to MHV and by isolation of *P. pneumotropica*.

Nasal swabs (from male and female donors, recipi-

ents, and their offsprings at 8 weeks old), sperm suspension, homogenized cauda epididymis, and media for IVF and washing were inoculated onto Trypticase soy agar (BBL Microbiological System, Cockeysville, Md., USA) supplemented with 5% of horse blood (Nippon Seibutu Zairyo Center, Japan) and then were cultured at 37°C for 48 hr. Isolated organisms were identified by colonial morphology, motility, staining characteristics, agglutination reaction using anti-serum and appropriate biochemical tests.

The presence of the serum antibody to MHV in male and female donors, recipients and their offsprings at 8 weeks of age was examined by complement fixation (CF) reaction using commercial antigen (MHV-antigen; Labocheck Seiken, Denkaseiken, Japan) and a kit (CF-KIT Seiken, Denkaseiken, Japan). Mouse antibody production (MAP) assay was also performed as described below. 0.5 ml of the homogenized cauda epididymis, sperm suspension and IVF media were inoculated into SPF C57BL/KsJ-dbm mice (bred in our colony) by intraperitoneal injection. Blood samples were recovered from the postcava of the inoculated mice under ether anesthesia at 4 weeks after the injection. Sera were examined by CF reaction as described above.

Results

Four strains of introduced mice were known to be contaminated with MHV and/or *P. pneumotropica* but not with other pathogens, viz. Sendai virus, *Mycoplasma pulmonis*, Tyzzer's organism, *Corynebacterium kutscheri*, *Bordetella bronchiseptica*, *Escherichia coli* 0-115, *Salmonella* spp., *Streptococcus pneumoniae*, Dermatophytes and *Syphacia* spp.

Results of IVF and ET are shown in Table 1. Fertilization at 6 hr after insemination of C57BL/KsJ, KK-Ay, Tgm-S and Tgm-A occurred at a frequency of 38%, 62%, 84% and 94%, respectively. Subsequent ET at the 2-cell stage of these respective strains developed into newborns with 14%, 16%, 55% and 61% success.

On the course of the IVF, detection of MHV antibody and isolation of *P. pneumotropica* from several specimens of the contaminated donors were carried out. As shown in Table 2, all of the mouse strains tested were demonstrated to be harbouring MHV and/or *P. pneumotropica*, but no pathogens were detected from

Table 1. Results of *in vitro* fertilization and embryo transfer

Strain		<i>In vitro</i> fertilization		Embryo transfer	
Male (N=)	Female (N=)	No. of fertilized/ no. of examined (%)	Mean no. of fertilized eggs	No. of pregnant/ no. of recipients (%)	No. of newborns/ no. of embryos transferred (%)
C57BL/KsJ (1)	C57BL/KsJ (19)	97 / 254 (38)	5.1	5 / 6 (83)	12 / 88 (14)
KK-A ^y (2)	KK (31)	778 / 1249 (62)	25.1	25 / 31 (81)	87 / 558 (16)
Tgm-S (4)	C57BL/6J (53)	1035 / 1231 (84)	19.5	5 / 5 (100)	36 / 55 (55)
Tgm-A (4)	C57BL/6J (54)	1137 / 1211 (94)	21.1	4 / 4 (100)	34 / 56 (61)

Table 2. Microbial examination of donor mice for oocytes and sperm

Microbial exam. specimen	C57BL/KsJ	KK-A ^y	Tgm-S	Tgm-A
MHV antibodies				
Serum	0/5 ¹⁾	4/4	3/4	1/4
Cauda epididymis*	NT ²⁾	0/3	NT	NT
Sperm suspension*	NT	0/3	NT	NT
Fertilization media*	NT	0/4	NT	NT
Isolation of <i>P. pneumotropica</i>				
Nasal swab	10/10	1/2	0/4	0/4
Cauda epididymis	NT	0/3	NT	NT
Sperm suspension	0/1	0/3	0/2	0/2
Fertilization media	0/2	0/2	0/2	0/2
Washing media	0/3	NT	NT	NT

¹⁾ No. of positive/no. of examined. ²⁾ Not tested. *: The specimens were examined by MAP assay. See text.

Table 3. Results of microbial examinations in recipients and offsprings

Specific pathogens	C57BL/KsJ		KK-A ^y	Tgm-S		Tgm-A	
	R ¹⁾	O ²⁾	O	R	O	R	O
MHV-antibodies	0/3 ³⁾	0/6	0/10	0/5	0/25	0/2	0/9
<i>P. pneumotropica</i>	0/3	0/6	0/10	0/5	0/11	0/2	0/9

¹⁾ Recipient. ²⁾ Offsprings. ³⁾ No. of positive/no. of examined.

These animals were also confirmed to be negative for Sendai virus, *Mycoplasma pulmonis*, Tyzzer's organism, *Corynebacterium kutscheri*, *Bordetella bronchiseptica*, *Escherichia coli* 0-115, *Salmonella* spp., *Streptococcus pneumoniae*, Dermatophytes and *Syphacia* spp.

the homogenized cauda epididymides, sperm suspension or fertilization media even in MAP assay. When the homogenized cauda epididymides were cultured on Trypticase soy agar, some gram positive bacteria such as *Staphylococcus* spp., *Bacillus* spp. and *Lactobacillus* spp. were found, but no bacterial growth was observed in the other samples including sperm suspension, in which cauda sperm was suspended in antibiotics-free

TYH medium.

Results of microbial examination after rederivation by IVF-ET are shown in Table 3. A total of 10 recipients and 50 offsprings of four mouse strains were consistently negative for MHV antibody and *P. pneumotropica*. These mice were confirmed to be free from the other pathogens described above.

Discussion

When producing SPF animals, the cesarean operation-fostering system is usually applied to prevent pathogens. However, this method is considered unreliable, because some pathogens including MHV and *P. pneumotropica* transmit vertically from the mother to the fetus [3, 6]. If uterine infection occurs, even without infection of the embryo, fetuses could easily become contaminated with infected maternal fluid during surgical delivery.

In the IVF-ET system, contamination in oviducal or uterine fluid with MHV and *P. pneumotropica*, can be eliminated by repeated washing the eggs in fresh medium [2, 12]. Furthermore, as shown in Table 1, the results indicated that the intra-cauda epididymis harboured neither *P. pneumotropica* nor MHV, even in infected animals. It is less likely that the embryos will be infected with pathogens except when pathogens penetrate the zona pellucida surrounding the oocyte and/or plasma membrane. We suggest that our culture conditions facilitated the elimination of pathogens. Hill and Stalley [5] have described that *Mycoplasma pulmonis* adhere to zona pellucida and may possibly penetrate its extra cellular matrix, but *Mycoplasma pulmonis* was inoculated into mouse embryo culture medium M16 and incubated at 37°C, *Mycoplasma pulmonis* was not detected in media after 24 hr. This was probably because of increased acidity in the culture media which potentially causes unfavorable conditions for *Mycoplasma pulmonis* growth [5]. Temporary exposure of zona-intact embryos to MHV had no detrimental effects on further development *in vitro*, whereas MHV-exposed zona-free embryos had degenerated within 24 hr of culture [9]. To avoid transfer of infected embryos, as in the present study, it is recommended that fertilized eggs are cultured for at least 24 hr prior to embryo transfer. In the present study, we used TYH and Whitten's medium containing antibiotics such as penicillin and streptomycin. This is likely to facilitate effective decontamination of sperm and oocytes suspended in culture media as the antibiotics can surround the individual cells in suspension. Furthermore, it has been reported that brief exposure of trypsin (0.25% trypsin, pH 7.6–7.8, for 60–90 sec), is effective in inactivating/removing infectious bovine rhinotracheitis virus (IBRV) and vesicular stomatitis

virus (VSV) from bovine embryos, and pseudorabies virus (PrV), hog cholera virus (HCV) and VSV from porcine embryos [12]. We therefore propose an approach to avoid the vertical transmission of diseases.

Application of IVF to control disease and breeding in animals is advantageous because many eggs can be fertilized from a single male at once and the day of birth can be easily controlled. In fact, as shown in Table 1, only 11 males fertilized 3,047 oocytes from 154 females. Thus the IVF-ET system also has advantages for mass production of mice in less time when compared to natural mating.

Acknowledgments

The authors wish to express their gratitude to Dr P. Kowalski-Saunders for his suggestions concerning the manuscript.

References

1. Carthew, P., Wood, M. J., and Kirby, C. 1983. Elimination of Sendai (parainfluenza type 1) virus infection from mice by embryo transfer. *J. Reprod. Fert.* 69: 253–257.
2. Carthew, P., Maureen, J. W., and Kirby, C. 1985. Pathogenicity of mouse hepatitis virus for preimplantation mouse embryos. *J. Reprod. Fert.* 73: 207–213.
3. Flynn, R. J., Brennan, P. C., and Fritz, T. E. 1965. Pathogen status of commercially produced laboratory mice. *Lab. Animal Care* 15: 440–447.
4. Heine, W. 1982. Hygienic monitoring and control of breeding colonies of mice and rats. *Zwierzeta. Laboratoryjne.* 19: 2–15.
5. Hill, A. C. and Stalley, G. P. 1991. *Mycoplasma pulmonis* infection with regard to embryo freezing and hysterectomy derivation. *Lab. Anim. Sci.* 41: 563–566.
6. Kitami, K., Taguchi, F., Nakayama, M., Goto, N., and Fujiwara, K. 1978. Vertical transmission of mouse hepatitis virus infection in mice. *Jpn. J. Exp. Med.* 48: 481–490.
7. Moore, T. D., Allen, A. M., and Ganaway, J. R. 1973. Latent *Pasteurella pneumotropica* infection of the gnotobiotic and barrier-held rats. *Lab. Anim. Sci.* 23: 657–661.
8. Okamoto, M., Matsushita, S., and Matsumoto, T. 1990. Cleaning of sendai virus-infected mice by embryo transfer technique. *Exp. Amin.* 39: 601–603.
9. Reetz, I. C., Wullenweber-Schmidt, M., Kraft, V., and Hedrich, J.-H. 1988. Rederivation of inbred strains of mice by means of embryo transfer. *Lab. Anim. Sci.* 38: 696–701.
10. Rouleau, A. M. J., Kovacs, P. R., Kunz, H. W., and Armstrong, D. T. 1993. Decontamination of rat embryos and transfer to specific pathogen-free recipients for the production of a breeding colony. *Lab. Anim. Sci.* 43: 611–

- 615.
11. Saito, M., Kohjima, K., Sano, J., Nakayama, K., and Nakagawa, M. 1981. Carrier state of *Pasteurella pneumotropica* in mice and rats. *Exp. Anim.* 30: 313–316.
  12. Singh, E. L. 1987. The disease control potential of embryos. *Theriogenology* 27: 9–20.
  13. Suzuki, H., Shingai, S., Togashi, M., Miyai, T., and Okamoto, M. 1987. *In vitro* fertilization and the development of eggs following transfer in genetically diabetic obese (db/db) mouse. *Jpn. J. Anim. Reprod.* 33: 96–100.
  14. Suzuki, H., Shingai, S., Togashi, M., Miyai, T., Motohide, Y., and Okamoto, M. 1988. The development of newborn C57BL/KsJ-dbm mice produced from eggs fertilized *in vitro*. *Exp. Anim.* 37: 245–249.
  15. Suzuki, H., Togashi, M., Kumagai, E., Miwa, M., Tatsumi, T., Nakura, M., Nakagawa, T., Tanaka, N., and Adachi, J. 1990. An attempt at embryo transfer as a means of controlling *Bordetella bronchiseptica* infection in the rabbit. *Exp. Anim.* 39: 397–400.
  16. Suzuki, H., Togashi, M., Moriguchi, Y., and Adachi, J. 1994. Relationship between age-related decline in fertility and *in vitro* fertilization rate in IVCS mice. *J. Reprod. Dev.* 40: 107–116.
  17. Suzuki, H., Ueda, O., Kamada, N., Jishage, K., Katoh, M., and Shino, M. 1994. Improved embryo transfer into the oviduct by local application of a vasoconstrictor in mice. *J. Mamm. Ova Res.* 11: 49–53.
  18. Toyoda, Y. and Takasugi, M. 1982. Early development of mouse embryos fertilized *in vitro*. pp. 57–64. *In: Genetic Approaches to Developmental Neurobiology* (Tsukada, O. ed.), Univ. Tokyo Press, Japan.
  19. Whitten, W. K. 1971. Nutrient requirements for the culture of preimplantation embryos *in vitro*. *Advan. Biosci.* 6: 129–141.
  20. Yamada, S., Baba, E., and Arakawa, A. 1983. Proliferation of *Pasteurella pneumotropica* at oestrus in the vagina of rats. *Lab. Anim.* 17: 261–266.
  21. Yokoyama, M., Katsuki, M., and Nomura, T. 1995. The creation of mouse models for human diseases associated with reproductive disturbance by *in vitro* fertilization and embryo transfer. *Exp. Anim.* 44: 139–143.