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Experimental Evaluation of Cross-contamination between Cryotubes Containing Mouse 2-cell Embryos and Murine Pathogens in Liquid Nitrogen Tanks

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Abstract: It has been suspected that embryos stored in liquid nitrogen tanks may become contaminated with murine pathogens, if some pathogens had been introduced to the tanks accidentally. To examine this, we stored tubes containing embryos with tubes containing mouse hepatitis virus (MHV) or *Pasteurella pneumotropica* in liquid nitrogen tanks and examined whether progeny mice derived from the embryos were contaminated with the pathogens or not. After storing for 6 months or 1 year the frozen embryos were thawed and implanted into the oviducts of pseudopregnant female mice, and the mice were bred in vinyl isolators. We could not detect serum antibodies to MHV and isolate *Pasteurella pneumotropica* in the progeny mice, suggesting that cross-contamination between tubes in a liquid nitrogen tank scarcely occurs.

Key words: contamination, embryo, liquid nitrogen tank

The number of transgenic and gene-knockout mice has been increasing, because these genetically modified animals are believed to be not only an effectual tool to figure out the role of a gene of interest *in vivo*, but also beneficial in research on the cause and treatment of human diseases [2]. Breeding of mice is costly and laborious, and needs a lot of space, and in order to save money, labor and space, it is practical to freeze

mouse embryos in liquid nitrogen. Actually, approximately two hundred thousand mouse embryos have been stored in liquid nitrogen tanks in the Center for Animal Resources and Development (CARD), Kumamoto University, a mouse embryo bank in Japan. In addition to CARD, several mouse embryo banks have recently been established and the number of frozen embryos in the world has been increasing.

(Received 14 March 2002 / Accepted 30 August 2002)

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Table 1. Development of cryopreserved 2-cell embryos stored with murine pathogens in liquid nitrogen tanks^{a)}

Tank	Pathogen introduced	Storage time	No. of embryos		No. of mice born (%)
			Frozen	Transferred	
A	MHV	6 mo	60	40	16 (40.0)
		1 yr	60	51	20 (39.2)
B	<i>P. pneumotropica</i>	6 mo	60	48	15 (31.3)
		1 yr	30	26	8 (30.8)

^{a)} C57BL/6J 2-cell embryos were prepared and stored with MHV (tank A) or *P. pneumotropica* (tank B) for 6 months (mo) or 1 year (yr) in liquid nitrogen tanks. Morphologically normal 2-cell embryos were transplanted into pseudopregnant mice and bred in vinyl isolators.

Recent epidemiological evidence suggests the occurrence of accidental hepatitis B virus transmission as a result of contamination of a bone marrow storage liquid nitrogen tank [4, 9]. Another study has claimed that liquid nitrogen is a potential source of microbial contamination of hematopoietic stem cell components [3]. Since liquid nitrogen can penetrate into cryotubes containing mouse embryos in modified vitrification methods [8], there may be a chance of cross-contamination if some samples are contaminated with murine pathogens. In this study we set up experiments to simulate embryo contamination by storing tubes containing mouse 2-cell embryos with tubes containing mouse hepatitis virus (MHV) or *Pasteurella pneumotropica* (*P. pneumotropica*) in liquid nitrogen tanks and evaluated the occurrence of embryo contamination. We chose MHV and *P. pneumotropica* as model pathogens because they are prevalent in contemporary mouse colonies [5, 10, 12] and can be isolated from various tissues including the reproductive organs in mice [1, 7], which may enhance the risk of embryo contamination during processing.

C57BL/6J and MCH:ICR mice were obtained from CLEA, Japan Inc., Japan. C57BL/6J mice were used as oocyte donors for *in vitro* fertilization and MCH:ICR mice were used as pseudopregnant recipients for embryo transfer. Thirty 2-cell embryos derived from *in vitro* fertilization were cryopreserved in a 1 ml cryotube (#366656, Nunc) by the simple vitrification procedure described previously [8]. MHV strain NuU was propagated and titrated on DBT cells as described previously [6]. One milliliter of the supernatant containing 9.8×10^6 plaque-forming units of MHV-NuU was frozen in a

cryotube (2732-002, IWAKI) at -80°C . *P. pneumotropica* was grown in heart infusion broth and titrated on blood agar plates. Bacterial suspension (4.4×10^8 colony-forming units/ml) was mixed with an equal volume of glycerol, aliquoted 1.8 ml in a cryotube (2732-002, IWAKI) and frozen at -80°C . Five tubes of the MHV and ten tubes of the frozen embryos were introduced into a liquid nitrogen tank (SC20/20, MVE, Inc., New Prague, MN) (tank A). Ten tubes of the *P. pneumotropica* and ten tubes of the frozen embryos were put into another liquid nitrogen tank of the same type (tank B). The tanks were placed in an air-conditioned room and topped up with liquid nitrogen every month during the experiments. Since the cryotubes were kept in the liquid phase, they were generally filled with liquid nitrogen.

Six months later, 60 frozen embryos from liquid nitrogen tank A were thawed and 40 morphologically normal 2-cell embryos were implanted into the oviducts of pseudopregnant female mice as previously described [8] (Table 1). The recipient mice were bred in a vinyl isolator and 16 pups were born. Blood was collected from five randomly selected progeny mice at 8 weeks of age and serum antibody to MHV was examined by means of a commercial enzyme-linked immunosorbent assay kit (Wakamoto Pharmaceutical, Tokyo), according to the manufacturer's instruction. As shown in Table 2, there was no evidence that the progeny mice derived from the frozen embryos stored with MHV in liquid nitrogen were infected with MHV. Similarly, 48 morphologically normal 2-cell embryos from tank B were implanted into the oviducts of other pseudopregnant female mice and 15 pups were born

Table 2. Results of microbiological examination of progeny mice derived from 2-cell embryos stored with murine pathogens in liquid nitrogen tanks

Pathogen	Test method ^{a)}	No. of mice positive / No. of mice examined			
		Tank A		Tank B	
		6 mo ^{b)}	1 yr ^{b)}	6 mo	1 yr
Mouse hepatitis virus	ELISA, IF	0/5	0/5	0/5	0/5
Sendai virus	ELISA, IF	0/5	0/5	0/5	0/5
<i>Citrobacter rodentium</i>	Culture	0/5	0/5	0/5	0/5
<i>Clostridium piliforme</i>	ELISA, IF	0/5	0/5	0/5	0/5
<i>Corynebacterium kutscheri</i>	Culture	0/5	0/5	0/5	0/5
<i>Helicobacter bilis</i>	PCR	0/5	0/5	0/5	0/5
<i>Helicobacter hepaticus</i>	PCR	0/5	0/5	0/5	0/5
<i>Mycoplasma spp.</i>	Culture, ELISA, IF	0/5	0/5	0/5	0/5
<i>Pasteurella pneumotropica</i>	Culture	0/5	0/5	0/5	0/5
<i>Salmonella spp.</i>	Culture	0/5	0/5	0/5	0/5
<i>Aspiculuris tetraptera</i>	Microscopic exam.	0/5	0/5	0/5	0/5
<i>Giardia muris</i>	Microscopic exam.	0/5	0/5	0/5	0/5
<i>Syphacia spp.</i>	Microscopic exam.	0/5	0/5	0/5	0/5
<i>Spironucleus muris</i>	Microscopic exam.	0/5	0/5	0/5	0/5
<i>Trichomonads etc.</i>	Microscopic exam.	0/5	0/5	0/5	0/5
Ectoparasites	Microscopic exam.	0/5	0/5	0/5	0/5

^{a)} ELISA: enzyme-linked immunosorbent assay, IF: immunofluorescence antibody, PCR: polymerase chain reaction, Microscopic exam: microscopic examination. ^{b)} Morphologically normal 2-cell embryos stored with MHV (tank A) or *P. pneumotropica* (tank B) for 6 months (mo) or 1 year (yr) in liquid nitrogen tanks were transplanted into pseudopregnant mice. The 8-week-old progeny mice were examined for infection with the pathogens listed above.

(Table 1). Although we tried to isolate *P. pneumotropica* by culturing swab samples from conjunctiva, trachea, pharyngolarynx, vagina and penis of five progeny mice on 5% horse blood plus heart infusion agar plates, we could not (Table 2). The microbiological status of sentinel mice reared in CARD was monitored every month for the pathogens listed in Table 2. The progeny mice derived from frozen embryos stored with MHV or *P. pneumotropica* for 6 months were checked for infection with other murine pathogens listed in Table 2 as the sentinel mice reared in CARD. There was no evidence that they were infected with those pathogens.

The same experiments were carried out 1 year after the beginning of storage. Briefly, 60 and 30 frozen embryos from tanks A and B, respectively, were thawed and transplanted into the oviducts of pseudopregnant mice (Table 1). The recipient and progeny mice were bred in vinyl isolators and the microbiological status of the progeny mice was examined as described above. There was no evidence that the progeny mice derived from frozen embryos stored with MHV or *P.*

pneumotropica for 1 year were infected with the pathogens listed in Table 2, including MHV and *P. pneumotropica* (Table 2).

Liquid nitrogen is not sterile originally. There is a paper reporting that some liquid nitrogen tanks have been contaminated with bacterial and fungal organisms probably from the environment [3]. In addition, some pathogens might be introduced into a liquid nitrogen tank accidentally, by collecting embryos from infected female mice. It has been suspected that embryos stored in liquid nitrogen tanks may be contaminated with murine pathogens in liquid nitrogen, since liquid nitrogen fills the tubes in the liquid phase of liquid nitrogen tanks.

It is possible that some pathogens have been introduced into a liquid nitrogen tank accidentally as described above. We set this experiment to simulate such a case. In this study, progeny mice derived from frozen embryos in liquid nitrogen tanks where murine pathogens had been experimentally introduced were not infected with the pathogens, even though we used extremely high doses of MHV and *P. pneumotropica*,

which 2-cell embryos are incapable of harboring naturally. Although we demonstrated that only five progeny mice bred in each vinyl isolator were not infected with MHV or *P. pneumotropica*, it is unlikely that other progeny mice were infected with those pathogens because MHV and *P. pneumotropica* are very contagious in mice [7, 10]. Taken together, it is unlikely that cross-contamination between cryotubes occurs in liquid nitrogen tanks, even though some frozen embryos were unintentionally contaminated with some pathogens.

There are some reasons why the cross-contamination did not occur. First, the amounts of the pathogens in the liquid nitrogen might have been insufficient to infect the embryos and recipients. To detect MHV from liquid nitrogen by nested RT-PCR [11], 50 ml of liquid nitrogen was allowed to evaporate, and the detritus was dissolved in water and used as a sample. But, we failed to detect the PCR products of MHV from the samples (data not shown). Secondly, thawed embryos were washed several times with modified Whitten's medium before implantation into the recipient mice. This procedure might also lessen the possibility of infection.

In this study we demonstrated that cross-contamination between cryotubes in liquid nitrogen did not occur. However, we recommend avoiding the storage of mouse embryos with pathogens in the same liquid nitrogen tank, because we could not exclude the possibility that liquid nitrogen could become severely contaminated with pathogens due to an accidental breakage of cryotubes containing pathogens. In addition, a previous study indicated that some liquid nitrogen tanks were

contaminated with bacterial and fungal organisms probably from the environment [3]. Care needs to be taken to avoid microbial contamination in liquid nitrogen tanks.

References

1. Barthold, S.W., Beck, D.S., and Smith, A.L. 1988. *Arch. Virol.* 100: 171–83.
2. Chande, M. 2001. *Lancet* 357: 1684.
3. Fountain, D., Ralston, M., Higgins, N., Gorlin, J.B., Uhl, L., Wheeler, C., Antin, J.H., Churchill, W.H., and Benjamin, R.J. 1997. *Transfusion* 37: 585–591.
4. Hawkins, A.E., Zuckerman, M.A., Briggs, M., Gilson, R.J. C., Goldstone, A.H., Brink, N.S., and Tedder, R.S. 1996. *J. Virol. Method.* 60: 81–88.
5. Itoh, T. 1999. pp. 15–23. *In: Microbial and Phenotypic Definition of Rats and Mice.* Natl. Acad. Press, Washington, DC.
6. Kyuwa, S., Ohsawa, K., Sato, H., and Urano, T. 2001. *Exp. Anim.* 49: 251–257.
7. Mikazuki, K., Hirasawa, T., Chiba, H., Takahashi, K., Sakai, Y., Ohhara, S., and Nenui, H. 1994. *Exp. Anim.* 43: 375–379.
8. Nakao, K., Nakagata, N., and Katsuki, M. 1997. *Exp. Anim.* 46: 231–234.
9. Tedder, R.S., Zuckerman, M.A., Goldstone, A.H., Hawkins, A.E., Fielding, A., Briggs, E.M., Irwin, D., Blair, S., Gorman, A.M., Patterson, K.G., Linch, D.C., Heptonstall, J., and Brink, N.S. 1995. *Lancet* 346: 137–140.
10. Yamada, Y.K., Yabe, M., Kyuwa, S., Nakamura, N., Takimoto, K., and Urano, T. 2001. *Comp. Med.* 51: 319–325.
11. Yamada, Y.K., Yabe, M., Takimoto, K., Nakayama, K., and Saitoh, M. 1998. *Exp. Anim.* 47: 261–264.
12. Zenner, L., and Regnault, J.P. 2000. *Lab. Anim.* 34: 76–83.