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Survival of Mouse Morulae and Blastocysts Derived from *In Vitro* Fertilization after Ultrarapid Freezing

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Mouse morulae and blastocysts derived from *in vitro* fertilization were placed in a highly concentrated vitrification solution (DAP 213 : 2 M dimethyl sulfoxide, 1 M acetamide, 3 M propylene glycol in PB 1) in a sampling tube at room temperature, plunged into liquid nitrogen within ten seconds and cryopreserved. Thawing was carried out by directly pouring 37°C 0.3 M sucrose solution into the sampling tube. The ratios of morphologically normal embryos at the morula and blastocyst stages after thawing were 92.0% (149/162) and 13.3% (13/98), respectively. The rate of development from morphologically normal morulae into blastocysts *in vitro* was 83.1% (74/89). Some of the morphologically normal morulae were transferred to pseudopregnant recipients immediately after thawing, and 45.0% (27/60) of the embryos developed into normal young.—KEY WORDS : mouse morula and blastocyst, ultrarapid freezing

Recently, I demonstrated that it is possible to snap-freeze 1-cell [1], 2-cell [2,3], and 4-cell mouse embryos [4] or unfertilized mouse oocytes [5,6] after brief, one-step exposure to a highly concentrated solution at room temperature.

In the present study the viability of mouse morulae and blastocysts was examined.

Animals: All animals were purchased from CLEA JAPAN Inc., Japan and were maintained in a barrier-system sustained animal room with relative humidity at 55 ± 5 , temperature at $23 \pm 1^\circ\text{C}$, and 14 hours light (3:00-17:00).

Source of the embryos: Tubal oocytes recovered from B6C3F₁ mice (2-3 months old) treated with PMSG (Serotropin : Teikoku Zoki Co., Japan) and hCG (Puberogen : San-kyo Zoki Co., Japan) were inseminated *in vitro* with epididymal spermatozoa from ICR strain mice (3 months old) and cultured for 72-96 hours in HTF medium [7] at 37°C under 5% CO₂ in air after insemination.

Freezing and thawing: The embryos devel-

oped into morulae and blastocysts 72 and 96 hours after insemination and were cryopreserved by the ultrarapid freezing described previously [1]. Between 10 and 20 embryos in a small volume of HTF medium ($< 2 \mu\text{l}$) were transferred into 20-30 μl of DAP 213 (2 M dimethyl sulfoxide, 1 M acetamide, and 3 M propylene glycol : Sigma, USA) in PB 1 [1] in sampling tubes (volume 0.5 ml : Cat. No. 72.699, Sarstedt, Germany) at room temperature, and the samples were plunged directly into liquid nitrogen. The duration of oocyte exposure to DAP 213 before freezing was 5-10 seconds. The samples were stored for 2-3 weeks before thawing. They were thawed and diluted by direct addition of 0.3 ml of 37°C 0.3 M sucrose solution to the sampling tubes immediately after removal from liquid nitrogen. The contents of each tube were emptied into a culture dish (35×10 mm, Falcon) and the tubes were rinsed once with 0.3 ml of 0.3 M sucrose solution. The recovered embryos were washed by transfer through four changes of HTF medium, and the number of oocytes and their

Table 1. Survival rates of frozen-thawed mouse embryos at the morula and blastocyst stage after thawing

Embryo stage at the time of freezing	No. of embryos frozen	No. of embryos	
		recovered (%)	morphologically normal (%)
Morula	166	162 (97.6)	149 (92.0)
Blastocyst	102	98 (96.1)	13 (13.3)

Fig. 1. Frozen-thawed mouse embryos at the morula and blastocyst stage after thawing. Most of the frozen-thawed morulae are morphologically normal (a), but there are few morphologically normal blastocysts (b).

Table 2. Development of frozen-thawed morulae into blastocysts *in vitro*

No. of embryos cultured	No. (%) of embryos which developed into blastocysts
89	74 (83.1)

Table 3. Development into live young of frozen-thawed morulae after transfer to pseudopregnant recipients

No. of embryos transferred	No. of recipients used	No. of animals which delivered live young	No. of live young		
			♀	♂	total (%)
60	4	4	11	16	27 (45.0)

morphological appearance were recorded. Morphologically normal cryopreserved morulae were introduced into fresh HTF medium and cultured for 24 hours, and some of the morphologically normal morulae were tran-

sferred to the uterine horns of the recipients on day 3 of pseudopregnancy (day 1 was the day on which the vaginal plug was found). The proportions of morulae and blastocysts which appeared morphologically nor-

mal after freezing and thawing are shown in Table 1. The viability was high for morulae, and 92.0% (149/162) of the recovered embryos were morphologically normal (Fig. 1-a). The viability of blastocysts, however, was very low, and only 13.3% (13/98) of recovered embryos were morphologically normal (Fig. 1-b). Tables 2 and 3 show the *in vitro* and *in vivo* viability assay for frozen-thawed morulae. The proportion of cryopreserved morulae which developed into blastocysts after culture *in vitro* was 83.1% (74/89, Table 2), and the rate of survival to normal young following transfer was 57.5% (46/80, Table 3).

Whittingham reported survival rates for frozen-thawed mouse blastocysts of between 69% and 76% after storage at -196°C , when the slow-freezing and slow-thawing method was used [9]. Schiffen *et al.* also reported that mouse blastocysts can be cryopreserved successfully using the simple technique of vitrification, but the survival rate after warming was low (39%) [8]. In the present study, the viability of blastocysts cryopreserved using the ultrarapid freezing method was lower after thawing than in these reports. Because of this,

it is conceivable that blastocyst sensitivity to freezing may vary markedly depending on the method of freezing. Further study is needed to improve the survival of blastocysts when using the ultrarapid freezing method.

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References

- [1] Nakagata, N. (1989). *Jpn. J. Fert. Steril.*, **34**, 757-760.
- [2] Nakagata, N. (1989). *Jpn. J. Fert. Steril.*, **34**, 470-473.
- [3] Nakagata, N. (1990). *Exp. Anim.*, **39**, 299-301.
- [4] Nakagata, N. (1990). *Exp. Anim.*, **38**, 279-282.
- [5] Nakagata, N. (1989). *J. Reprod. Fert.*, **87**, 479-483.
- [6] Nakagata, N. (1990). *Exp. Anim.*, **39**, 303-305.
- [7] Quinn, P., Kerin, J. F., and Warnes, G. M. (1985). *Fert. Steril.*, **44**, 493-498.
- [8] Scheffen, B., Van Der Zwalman, P., and Massip, A. (1989). *Cryo-letters*, **7**, 260-269.
- [9] Whittingham, D. G. (1974). *J. Reprod. Fert.*, **37**, 159-162.

マウス桑実胚および胚盤胞期胚の超急速凍結保存

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室温にて高濃度の保存液 (DAP 213: 2 M dimethyl sulfoxide, 1 M acetamide, 3 M propylene glycol in PBI) に胚を移し, 10 秒以内に, 直接液体窒素に浸漬することにより, マウス桑実胚および胚盤胞の凍結保存を行った。また, 融解は保存容器内に 37°C の 0.3 M sucrose 溶液を加え, 融解と同時に保存液の希釈を行った。桑実胚における融解時の形態的正常胚の割合

は, 92.0% (149/162) と高値であり, それら胚の体外培養による胚盤胞への発生率および移植による新生児への発生率はそれぞれで 83.1% (74/89) および 45.0% (27/60) であった。しかしながら, 胚盤胞における融解時の形態的正常胚の割合は, わずか 13.3% (13/98) と低値であった。