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BRIEF COMMUNICATION

THE VIABILITY OF DEEP-FROZEN AGGREGATED MOUSE EMBRYOS

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Since the first successful aggregation of mouse embryos was reported by TARKOWSKI,[11] many studies on chimera production have been carried out.[5,6,7] Chimeras produced by the aggregation of early embryos have been widely used in experimental investigations of mammalian embryology and developmental genetics.[4,9] Chimeric experimental animals have also been used to study some problems of human genetic diseases. Recently, many mutant mice, some of which were affected by genetic diseases encountered in human beings, have been developed.[3] However, these disease model mice have a relatively lower ability to survive and reproduce. Moreover, their maintenance requires great care and it is economically costly. Freezing of aggregated embryos from normal and disease model mice can be used to alleviate these problems. The purpose of this study is to investigate the viability of frozen-thawed aggregated mouse embryos.

Embryos which were obtained by mating ddY female and ddY or C57/BL male mice were used to investigate the *in vitro* and *in vivo* viability of aggregated embryos. Females were injected with 7.5 IU pregnant mare's serum gonadotropin followed by an injection of 7.5 IU human chorionic gonadotropin (HCG) 48 hours later. Four-, 8- and 12-~16-cell embryos were flushed from the oviduct by using Hanks' solution at 60–76 hours post HCG injection. Recovered embryos were treated with Hanks' solution containing 0.5% pronase at room temperature for 5 to 10 minutes to remove zona pellucida. Then embryos were washed several times and placed in modified Whitten's medium (MWM). Aggregation of embryos was performed by gently pushing the two embryos together in microdrops of MWM containing 0.5% phytohemagglutinin-P (PHA-P). After 10–15 minutes incubation in the MWM containing PHA-P, the aggregates

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were washed twice and placed in MWM. The aggregated embryos were cultured in a CO_2 incubator at 37°C for 48 hours. For freezing, compacted morulae which developed from 8-cell aggregated embryos and single 8-cell embryos with or without zona pellucida after 24 hours' culture were used. Freezing and thawing were carried out as described by URANO et al.[12] Survival of the cultured embryos was assessed by their ability to develop into expanded blastocysts during culture. Diameter of embryos was measured by the photographic technique. Counting of the cell number of embryos was done according to the method of KAMIGUCHI et al.[2] After six hours of *in vitro* culture post thawing, six aggregated embryos were transferred into the uterine horn of a day 3 pseudopregnant ICR mouse (day of vaginal plug=Day 1).

It was observed that embryos in PHA-P medium adhered easily. Over 90% of the aggregated embryos developed into the blastocyst (Table 1). This result was similar with previous results.[8,10] Four-cell stage aggregated embryos did not develop

TABLE 1 Survival rates of control and aggregated mice embryos which developed to blastocysts (BL)

Embryo stage	No. of embryos cultured	No. (%) of embryos developed to		
		Mid BL	Expanded BL	Degeneration
4-cell				
Control				
with ZP	30	12(40.0)	16(53.3)	2(6.7)
without ZP	25	11(44.0)	9(36.0)	5(20.0)
Aggregated	36	24(66.6)	6(18.8)	6(16.7)
8-cell				
Control				
with ZP	33	0(0.0)	31(93.9)	2(6.1)
without ZP	43	3(7.0)	38(88.4)	5(4.7)
Aggregated	97	32(34.0)	58(59.8)	6(7.2)
12-~16-cell				
Control				
with ZP	33	0(0.0)	32(97.0)	1(3.0)
without ZP	22	0(0.0)	22(100.0)	0(0.0)
Aggregated	20	0(0.0)	18(90.0)	2(10.0)

ZP : Zona pellucida

into the blastocyst stage as much as 8-cell and 12-~16-cell embryos. The degeneration rate of 4-cell stage aggregated embryos was similar with that of zona free 4-cell embryos. Zona pellucida removal with pronase may affect the viability of 4-cell embryos.

URANO et al.[12] reported that when ethylene glycol was used as cryoprotectant, the viability of frozen-thawed embryos was not affected by the dilution methods. In the present study, no significant difference was observed between the sucrose and step-wise dilution methods for the aggregated and control embryos (Table 2). The

TABLE 2 Viability of frozen-thawed aggregated embryos 24 hours after *in vitro* culture in relation to the ethylene glycol removal method

Embryos	Ethylene glycol removal methods*	
	Stepwise	Sucrose
Aggregated	62.5% (15/24)[a]	80.0% (36/45)
Control		
with ZP	86.1% (31/36)	89.5% (34/38)
without ZP	79.2% (19/24)	84.2% (32/38)

* : Ethylene glycol was removed in 5 step dilution with PBS or in 0.5 M sucrose solution.
[a] : (No. of blastocysts/No. of frozen-thawed embryos)
ZP : Zona pellucida

sucrose method was recommended as cryoprotectant removal method for the frozen-aggregated embryos because of its convenience.

Diameter and cell number were significantly higher ($P<0.01$) in the aggregated embryos when compared to the control (Table 3). Similar results were reported by

TABLE 3 Means and standard deviations of the diameter and cell numbers of aggregated embryos which developed to blastocysts after *in vitro* culture

Embryo stage	Diameter		Cell number	
	Fresh	Frozen	Fresh	Frozen
4-cell				
	n = 14		n = 17	
Aggregated	110.8 ± 9.3^{a}		114.8 ± 8.3^{d}	
	n = 17		n = 12	
Control	88.3 ± 9.6^{a}		54.3 ± 8.3^{d}	
8-cell				
	n = 57	n = 16	n = 18	n = 20
Aggregated	129.9 ± 12.6^{bg}	114.7 ± 12.7^{cg}	162.5 ± 9.0^{ei}	119.3 ± 14.8^{fi}
	n = 14	n = 14	n = 17	n = 22
Control	99.4 ± 5.1^{bh}	87.7 ± 8.2^{ch}	86.6 ± 8.5^{ej}	60.9 ± 14.7^{fj}

Values with the same superscripts in the column and row are significantly different ($P<0.01$).

other researchers.[1,8] Of the six frozen-thawed aggregated embryos which were transferred to a recipient mouse, only one chimeric young was born. The body weight was 9 g at 14 days after calving and was not different from the other same aged young derived from single embryos. BUEHR & MCLAREN[1] and NAKAGAWA et al.[8] also transferred aggregated embryos to the recipients and produced the same body weight of young as that from single control embryos. It was reported that size regulation of aggregated embryos takes place during development in the uterus.[1]

Diameter and cell number of frozen-thawed embryos were significantly less than those of fresh embryos ($P<0.01$). It was thought to be due to the death of some blastomeres or to developmental delay, as reported by WHITTINGHAM.[13]

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