Chimera Production by Embryo Aggregation Method and Cultured *In Vitro* Without Zona Pellucida in Mice

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ABSTRACT. **The objective of this research was to study the developmental competence of single and aggregated embryos develop in vitro and in vivo in relation to the phenotypically aspects of the chimeric mice. Chimeric embryos were produced by aggregation of early stage embryos (8-cell stage) collected from mice with different coat coloration (white and brown). Zona pellucida was removed by exposing the embryo into the medium contained 0.25% pronase. The aggregation of zona-free embryo was done by physically micromanipulation with different composition of embryos collected from white and brown mice. The developmental competence of single zona-free or aggregated embryo in in vitro culture was evaluated morphologically and by counting the total of the cell number of the embryos. The treatment of zona removal and aggregation did not affect the developmental competence of embryo to expanded blastocyst after culture in vitro (P>0.05). The development rate of embryo to expanded blastocyst was 86.49%, 75.00%, 72.22%, 81.82% and 76.92% for normal embryo, single zona-free embryo, aggregated two, aggregated three and aggregated four embryos, respectively. The size of aggregated embryos was bigger (P<0.05) when more number of embryos was aggregated than less number of embryos. Similarly, the total cell number of the aggregated embryos developed to blastocyst was significantly different among the treatment (73.85, 120.50, 160.82 and 220.00 cells per embryo for the single zona-free, aggregated two, aggregated three and aggregated four embryos, respectively). The viability of aggregated embryos develop in vivo was examined by transfer of the aggregated embryos to the pseudopregnant female. Twenty four aggregated embryos were transferred and two chimeric mice were born. These results showed that chimeric mice could be produced by aggregation of early stage embryo and cultured in vitro without zona pellucida.**

Key words: chimera, embryo aggregation, in vivo, in vitro

Introduction

Chimera production is one of the various methods have been used on the developmental biology studies. Chimeric animals are those have two or more cell population containing different genetic materials. Chimeras have proved to be valuable in experiments designed to study the cells lineage pathways in developing embryos, sex determination (McLaren, 1975), cells interaction and rescued lethal phenotype (Surani et al, 1977; Stevens, 1978; Boediono et al, 1999).

In addition, chimeras could be a tool to study genetic modification. Essentially three areas of technology exist for genetic modification namely: pronuclear injection, single genotype animal production and chimeric production (Campbell and Wilmut, 1997). Genetic modification is a recent progress that can be applicable for animal breeding.

Chimera could be produced by introducing cell into the early stage of developing embryo (Hogan et al, 1986; Polzin et al, 1987) or by aggregation of the early stage of developing embryos (Piedrahita et al, 1992; Boediono et al, 1993; Boediono et al, 1999). The termination-known part of late stage embryos or fetus derived from different species could be exchanged and form chimeric animals (Gilbert, 1988).

Several experiments indicated that chimeric embryos possessed some advantages over single embryos. Using interactive behaviour between blastomeres, aggregation enhance the viability of parthenogenetic embryos developed both in vitro and in utero resulted the parthenogenetic derived cells contributed in live chimeric animal (Stevens, 1978; Fundele et al, 1991; Boediono et al, 1999). Although the contribution of parthenogenetic cells origin was less than 20% of the total cell population (Surani et al, 1977). Chimera produced by aggregation method possibly reaches the sex ratio (phenotype) and prefers to be male. It has been reported in mice, from the XX-XY chimeras they were 15% developed to female, 6% intersex and

80% developed to male (Mullen and Whitten, 1971; McLaren, 1975). Morphologically, chimeric embryos were larger than single embryos. Boediono et al (1993) reported that aggregated embryo were of significant increment in diameter and formed the giant embryos. It suggested that the embryo viability develops in vitro and in vivo was increased due to the cell number increment.

The aspects of embryo aggregation as a step to produce chimeric animal needs to be studied in order to obtain the clear appraisal on aggregated embryo. This research aims to study the developmental competence and morphometric characteristic of chimeric embryos produced by aggregation method.

Materials and Methods

Embryo Collection:

Embryos in the early stage of development (8-cell stage) were collected from the mated mice by the procedures reported previously by Hogan et al. (1986). Briefly, female mice (age 2-3 months) were superovulated by injection i.p. of 5 IU pregnant mare serum gonadotrophin (PMSG, Intervet, Netherland) and 5 IU human chorionic gonadotrophin (hCG, Intervet, Netherland) in 48 h interval. Then, the females were mated with fertile males from the same strain (single mating). The mating females were confirmed by the presence of the vaginal plug in the following morning (Day-1). The embryos were recovered by dissecting the tuba Fallopii at Day-3 in M2 medium supplemented with 0.2% bovine serum albumin (BSA, Gibco, USA). The embryos were washed three times in M2 medium and evaluated for the grade.

Embryo Aggregation:

Only good qualities of 8-cell stage embryos were used for the treatment. The zona pellucida were removed by incubated the selected embryos in M2 medium contained 0.25% pronase (Sigma, USA) for 2 to 4 min. Following zona removal, embryos were gently rinsed in CZB medium supplemented with 0.3% BSA (Chatot et al, 1989). The embryo aggregation method used in this study was similar with reported previously by Boediono et al (1995). The embryos were aggregated by physically pushing the two, three or four of different strain embryos together until the aggregation seemed sufficiently stable. The aggregation were held in 10 µl micro drop CZB medium followed by culture in vitro for further development at 37°C in a humidified atmosphere of 5% CO_2 in air. The aggregated embryos were evaluated after 4 h of incubation; reaggregation was done if they are any embryos disparate.

Evaluation:

The development rate of the aggregated embryos cultured in vitro was evaluated at 24 h interval. In the same time, the morphometric examinations were done by measuring the diameter of the developed embryos. After 48 h of culture in vitro, some embryo that developed to blastocysts was transferred into the female reproductive tract of synchronized recipients (Johnson et al, 1996). The remainder aggregated embryos were prepared for the examination of the total cell number. Total cell number of aggregated embryos was counted by method previously reported by Boediono et al (1995) with some modification. Briefly, aggregated embryos were suspended in a 0.9% sodium citrate as a hypotonic solution for 20 min. Then, they were fixed in a distilled water: acetic acid: methanol: sodium citrate solution (2:4:6:9) for 5 min, followed by distilled water: acetic acid: methanol (1:2:3) for 1 min. The fixed embryos were placed on a free-fatty slide glass. A small amount of acetic acid was dropped onto them to spread the cells. The preparation were stained in 5% Giemsa (Merck, Germany) at pH 6.8 for 20 min and observed under a phase-contrast microscope.

Statistical Analysis:

The data of the development competence of single and aggregated embryos were analysis by Chi-square test. The morphometric data and total cell number were analysis by Student t-test. Means are given as $\pm$ S.E.M. (Steel and Torrie, 1993). Probability of $P<0.05$ were considered to be statistically significant.

Results

Embryo Collection:

Table 1 shown that both strain yielded a great proportion of 8-cell stage embryos. The responsiveness of different strain against superovulation treatment was equal, total embryo recovery was not different ($P>0.05$) between the two strains. However, small portion of collected embryos were 1-cell, 2-cell, 4-cell and degenerated embryos.

Table 1. Stage of embryo collected from superovulated mice on day-3 after fertilization

Development Stage	No. of Collected Embryo (%)	
	Brown	White
1-cell	16 (4.73)	27 (4.96)
2-cell	23 (6.80)	23 (4.23)
4-cell	49 (14.50)	77 (14.15)
8-cell	178 (52.66)	320 (58.82)
Morula	45 (17.37)	62 (11.39)
Degenerated	27 (11.60)	35 (6.43)
Total	338 (100)	544 (100)
Number of mice	14	19
Mean	24.4 ± 7.94	28.63 ± 8.57

Embryo Aggregation:

In this study, the aggregation rate was more than 82% (Table 2). The use of pre-compaction embryos (8-cell stage) gives a greater chance to form aggregated embryo (Figure 1). Only the small number of aggregation failure occurred, it might due by exposure to the medium containing enzyme (pronase) or by mechanical disturbance during handling the embryo.

Table 2. Aggregation of 8-cell stage embryos with different combination

Combination (Brown:White)	Number of Aggregation	Aggregated Embryos	Aggregation Rate (%)
1 : 1	85	71	85.53
2 : 1	66	58	87.88
3 : 1	29	24	82.76

Embryo Development In Vitro:

The developmental competence of the embryos during in vitro culture among the normal, single zona-free and aggregated embryo was nof different (P>0.05) (Table 3). More than 90% embryos were developed to blastocyst after 48 h of culture.

Table 3 shown that no difference (P>0.05) on developmental competence among single with zona intact, zona-free single, and aggregated embryos. However, embryo with zona intact as control had slightly higher developmental rate than zona-free embryos.

Table 3. Development af aggregated embryos cultured in vitro (48 hours culture in CZB medium)

Combination (Brown:White)	No. of Embryo	Development Stage (%)			
		Compapct Morula	Early Blastocyst	Blastocyst	Expanded Blastocyst
0 : 1 (zona intact)	74	74 (100)	73 (98.65)	72 (97.30)	64 (86.49)
0 : 1 (zona-free)	32	32 (100)	29 (90.65)	27 (84.38)	24 (75.00)
1 : 1	54	54 (100)	49 (90.74)	48 (88.89)	39 (72.22)
2 : 1	44	44 (100)	43 (97.73)	42 (95.45)	36 (81.82)
3 : 1	26	26 (100)	24 (92.31)	22 (84.61)	20 (76.92)

The Morphometry of Developed Embryo

The size of single and aggregate embryo was significantly different (P<0.05) in every stage of development (Table 4). In sight of earlier stage to more advance embryo development, embryo size tends to increase linearly. The size of more number of aggregated embryos was bigger (P<0,05) than less number of aggregated embryos in different stage of development.

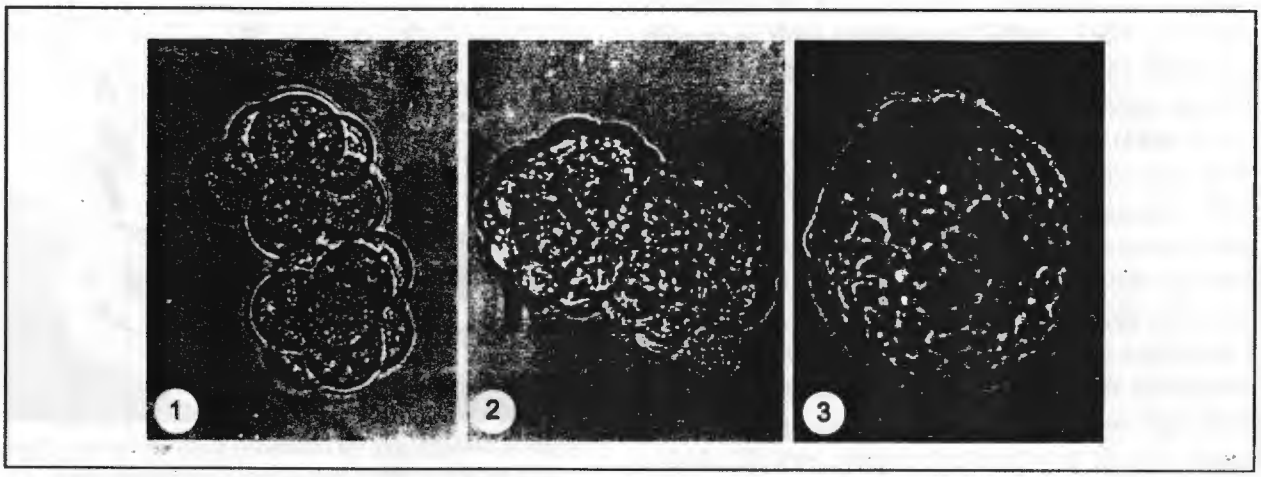

Fig.1. Embryo aggregation for producing chimera mice. (1) aggregation of eight cells stage embryo after zona pellucida removal (two embryo), (2) development of agregated embryo to the compacted morulae. (3) development of aggregated embryo to the expanded blastocyst after cultured in vitro

Table 4. The size of embryo in different stages of development after aggregation

Combination Brown:White	Size of Embryo (μm)			
	Compact Morula	Early Blastocyst	Blastocyst	Expanded Blastocyst
0 : 1	70.39 ± 7.47 [a]	79.51 ± 6.50 [a]	90.00 ± 8.90 [a]	95.70 ± 9.36 [a]
1 : 1	89.30 ± 9.54 [b]	101.20 ± 8.93 [b]	112.09 ± 7.29 [b]	146.18 ± 14.42 [b]
2 : 1	105.46 ± 9.73 [c]	113.89 ± 12.14 [c]	129.38 ± 5.06 [c]	173.94 ± 11.34 [c]
3 : 1	124.71 ± 6.70 [d]	148.24 ± 10.51 [d]	181.25 ± 7.35 [d]	204.38 ± 9.82 [d]

Values within column with different superscripts are significantly different (a-b-c-d, P<0.05)

The embryo size differences seem to be caused by the difference of cell number in the embryos (Table 5). However, the total cell number of blastocyst was not multiplying linearly as reflection of the number of embryos aggregated.

Table 5. Cell number of aggregated embryos obtained from different embryo combination

Embryo Combination	No. of Embryos	Cell Number
Single	20	73.85 ± 5.34 [a]
1 : 1	12	120.50 ± 8.06 [b]
2 : 1	11	160.80 ± 7.36 [c]
3 : 1	14	220.00 ± 21.29 [d]

Values within column with different stage superscripts are significantly different (a-b-c-d, P<0.05)

Embryo Development In Vivo

Twenty four chimeric blastocysts constructed from one brown embryo and one white embryo were transferred to six synchronized females (four blastocysts each). Two chimeric mice were born with phenotypically male and female. Chimerism was observed macroscopically based on coat coloration and external sexual organ (Figure 2).

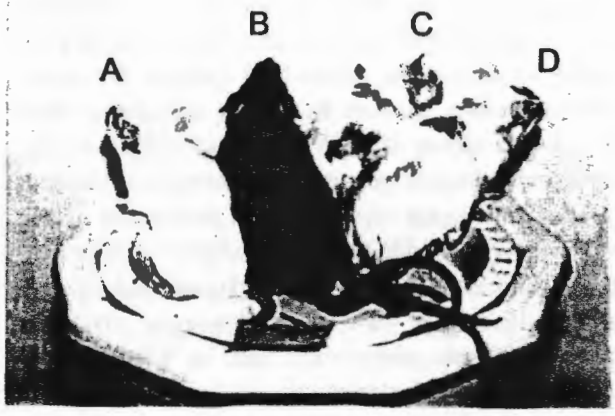

Fig.2. Chimeric mice produced by aggregation of early stage embryos (8-cell stage) collected from mice with different coat coloration (white and brown) and cultured without zona pellucida. A. White mouse, B. Brown mouse, C and D. Chimeric mouse

One chimeric was male having distinct and normal testes, composed from two colors, brown and white (approximately 65:35). The other was female with coat coloration between brown and white (approximately 85:15). After mating with normal mice, both female and male chimeric mice could produce normal offspring.

Discussion

Mullen and Whitten (1971) reported that 8-cell stage embryo collected on Day-3 is a better stage for embryo aggregation. It is due to the embryo compaction was not completed in this stage. The collection time was appropriated with embryo development stage in vivo (Hogan et al, 1986). Table 1 shown that both strain yielded a great proportion of 8-cell stage embryos. Small portion of collected embryos were 1-cell, 2-cell, 4-cell and degenerated embryos. This might due to the quality of the oocytes.

Four cell, 8-cell and morula emerged for two reasons. First, the different of ovulation and fertilization time. In mice, ovulation does not occur simultaneously but all mature oocytes were ovulated in 12 to 14 h after hCG injection. The difference of fertilization time leads to a different start of embryo development. Secondly, an intrinsic factor where the different of inherent viability in the oocytes were reflected in embryo development rate (Gordon, 1998).

Mammalian blastomere through 8-cel stage form loose arrangement with plenty of space between them. Following the third cleavage, however, the blastomeres undergo a spectacular change in their behaviour (Gilbert, 1988; Carlson, 1988). Shortening of the microvilli may therefore bring about the flattening of the blastomeres against one another by actin depolymerization (Pratt et al., 1982). Using this phenomenon, embryos would be aggregated physically after zona removal. The blastomeres from different embryos will make surface contact and strong bound together to form compacted morula. Then, the blastomeres of two or more embryos were differentiated to the trophoblast and inner cell mass (ICM) of the chimeric blastocyst.

The aggregation rate reached more than 82% (Table 2). Pre-compaction embryos (8-cell stage) give a greater chance to form aggregated embryo. The aggregation of early development stage embryo after zona removal using pronase was reported

previously in mice (Mintz et al *in* Gilbert, 1988; Mullen and Whitten, 1971), in hamster (Piedrahita et al, 1992) and in ewe (Ruffing et al, 1993). In cattle, the developmental competence of aggregated embryos after zona removal by microsurgery techniques was better than pronase digestion method (Boediono et al, 1993).

There was no difference on developmental competence during in vitro culture among the normal, single zona-free and aggregated embryo. In contrast, Boediono et al (1993) reported that cattle embryos exposure to the medium containing pronase was reduced their developmental competence.

During the preimplantation period, the embryos undergo dynamical change in morphology, physiology and biochemistry (Gardner, 1998). Several hours after 8-cell stage, the embryo transform to morula after compaction. Compacted morula undergo little expanding due to the liquid flow produced by trophectoderm cells to the interior region and formed the blastocoel. In in vivo development, blastocoel formation occurs 94 h after hCG injection. The blastocyst will continue to expand while their cells toward next two cycles. Finally, zona pellucida thinning and blastocyst were hatched, followed by attached to the uterine epithelium and starting implantation.

The dynamics of embryo size linearly associate with embryo morphological changes. During early cleavage stage, embryos size relatively constant until morula. After compaction, embryo begins to expand because of liquid flow to interior region of morula and reach ultimate size while the expanded blastocyst stage (Gilbert, 1988).

The size of single and aggregate embryo was significantly different ($P<0.05$) in every stage of development, reflected cell number difference. The more cell number lead to the increment of liquid flow to interior region of morula, since osmotic action started by Na^+/K^+ATPase pump activity to transport ion across membrane increased. Blastocoel cavity formed in the blastocyst stage was bigger. Interestingly, total cell number of blastocyst was not multiplying linearly as reflection of the number of embryos aggregated. It was apparently as a consequence of the blastomere death and the presence of low quality blastomere brought by pronase exposure so only parts of blastomere running for further development.

The chimeric blastocysts constructed from one brown embryo and one white embryo transferred synchronized female resulted two chimeric mice with phenotypically male and female. Macroscopic observation based on external sexual and coat coloration shown that one chimeric was normal male and the other one was normal female composed from two colors, brown and white approximately 65:35 for male and approximately 85:15 for the female. As female and male chimeric mice could produce normal offspring after mating with normal mice. This finding concluded that female chimeric mice were fertile and produced from two embryos with genotypically XX/XX, and so for male chimeric mice were fertile that produced from two embryos with genotypically XY/XY.

Conclusion

These results concluded that chimeric mice could be produced by aggregation of 8-cell stage embryos and cultured in vitro without zona pellucida followed by transfer to the recipient. The development competence of embryo in vitro was not affected by zona removal and aggregation. Embryo aggregation increased the size and cell number of mice chimeric embryos.

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