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Development of Chimeric Embryos Aggregated with Blastomeres from Parthenogenetic and *In vitro* Fertilized Bovine Embryos

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소의 단위 발생란과 체외수정란 유래의 할구 응집에 의한 키메라 수정란(Chimeric Embryo)의 발달

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SUMMARY

발생학에서 키메라(chimera)는 2개 이상의 다른 유전자형의 세포, 또는 다른 종의 세포로부터 만들어진 1개의 생물개체를 뜻하는 말로, 이는 초기 수정란의 발달과 포유류의 분화를 연구하는데 이용되고 있다. 키메라를 만드는 방법에는 할구와 내세포괴를 응집시키는 방법과 배반포 내에 여러 종류의 세포를 주입하는 방법이 있다. 본 실험에서는 서로 다른 두 가지 방법의 활성화 처리법, 즉, ionomycin 처리 후 Cycloheximide (CHX) 또는 6-Dimetylaminopurine (6-DMAP)에 따른 단위 발생란의 분할과 단계적인 발달율을 살펴 보고자 하였으며, 서로 다른 방법에 의해 생산된 단위발생란 유래의 할구와 체외수정란 유래 할구를 응집하여 키메라 수정란(chimeric embryo)를 만든 후 체외수정란과 발달율을 비교해 보았다.

도축장 유래의 난소에서 난자를 채취하여 체외에서 22~24시간 성숙시킨 후 난구세포를 제거하고 metaphase II 단계의 난자를 5 μM ionomycin에 4분간 처리한 후, 10 μg/ml CHX/5 μg/ml cytochalasin B (CCB)에 5시간 또는 1.9 mM 6-DMAP에 4시간 처리하여 분할율과 배반포기 발달율을 비교 조사하였다. 난자 분할율에서는 체외수정란과 6-DMAP처리 단위발생란에서 각각가 83.7 와 85.5% 로 CHX/CCB 처리 단위발생란의 57.9% 보다 유의적으로 높게($P<0.05$) 나타났으며, 배반포기 발달율에 있어서는 체외수정란의 발달율이 27.8%로 6-DMAP처리 활성란 12.3%와 CHX/CCB 처리 활성란 5.3%보다 유의적으로 높게 ($P<0.05$) 나타났다. 키메라 수정란(chimeric embryo)은 서로 다른 두 가지 처리에 의해 생산된 단위발생란의 할구 2개와 체외수정란 유래의 할구 2개를 빈 투명대 내에서 응집시켜 제조하였다. 빈 투명대 내에 키메라 수정란(chimeric embryos)의 8 세포기까지의 발달율은, 체외 수정란

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할구 2개와 CHX/CCB 처리에 의한 할구 2개를 응집한 그룹은 46.1%, 체외 수정란 할구와 6-DMAP 유래 할구 2개를 응집한 그룹은 52.8% 였으며, handled control은 54.7%로 체외 수정란 77.7%에 비해 유의적으로 낮게(P<0.05) 나타났다. 배반포기까지의 발달율은 체외 수정란과 CHX/CCB에 의해 생산된 키메라 수정란(chimeric embryo)은 12.8%, 체외 수정란과 6-DMAP에 의한 키메라 수정란(chimeric embryo)은 18.8%로 handled control의 21.4%에 비해 유의적으로 낮았으며(P<0.05), 이들 키메라 수정란(chimeric embryos)은 체외 수정란의 34.9%에 비해 유의적으로 낮게(P<0.05) 나타났다. 6-DMAP 처리 단위발생이 유기된 수정란 할구 2개와 체외수정란의 할구 2개의 응집에 의한 키메라 수정란(chimeric embryos)의 발달율이 CHX/CCB와 체외수정란의 응집에 의한 키메라 수정란(chimeric embryos)에 비해 다소 높게 나타났으나, 유의적인 차이는 없었다.

본 실험의 결과 서로 다른 방법에 의한 단위 발생란 유래의 할구와 체외 수정란 유래의 할구가 응집에 의한 재조합이 가능하였고 이들을 체외에서 배양하여 배반포기의 수정란까지 발달시켰다.

(Key words : chimeric embryo, parthenogenetic activation, bovine)

INTRODUCTION

Production of chimeras has been an important tool for investigating fundamental aspects of early embryonic development and differentiation in mammals(McLaren, 1976; Wilson and Stern, 1975). Since the first report of chimeric mice by embryo manipulation(Mintz, 1962; Tarkowski, 1961), live chimeric offspring have been produced in sheep (Fehilly *et al.*, 1984; Willadsen and Fehilly, 1983), cattle(Boediono *et al.*, 1999; Picard *et al.*, 1990), pigs(Mueller *et al.*, 1999), and goats(Fehilly and Willadsen, 1986). In addition, chimeric offspring have also been produced between species in *Bos taurus* and *Bos indicus* (Summers *et al.*, 1983), goat and sheep (MeineckeTillman and Meinecke, 1984), and cattle and sheep (S.M. Willadsen, 1988).

Methods used for the production of farm animal chimeras include the aggregation of blastomeres or inner cell mass, and injection of various cell types into the blastocyst cavity(Rho *et al.*, 2001; Boediono *et al.*, 1999; Moens *et al.*, 1996; Picard *et al.*, 1990; Summers *et al.*, 1983). Whichever method is used, however, the proportion of chimeric embryos that continue to develop is relatively low. Simplest explanations for the retardation of development were caused by sex differences, synchrony of cell cycle of blastomeres being aggregated.

In cattle, parthenogenetic activation has been studied extensively during the past decades. However, the efficiency of their development into later embryonic stages is low, causing to mainly by failure of re-accumulation of the maturation promoting factor (MPF) and a decrease in chromosome sets compared with normal fertilized embryos (Rho *et al.*, 1998a). To prevent MPF re-accumulation, combined treatments of calcium elevating agent with an inhibitor of either protein synthesis or protein phosphorylation such as CHX or 6-DMAP (Yang *et al.*, 1994; Neant and Guerrier, 1988) have been widely used. This has been proven effectively enhance the activation stimulus and accelerate the pronuclear formation and parthenogenetic development in the young mouse and bovine oocytes (Rho *et al.*, 1998b; Presicce and Yang, 1994; Susko Parrish *et al.*, 1994). It is interesting to note that one recipient was diagnosed pregnant without a fetal heartbeat on day 35. It and did not return to the estrus cycle until day 48 following the transfer of such a blastocyst(Fukui *et al.*, 1992). In another study, the return to estrus was delayed until day 67 after transfer of aggregated parthenogenetic

embryos. However pregnancy was not maintained after this period(Boediono and Suzuki, 1994). The reasons for which development of parthenogenetic embryos are limited remain unclear but may be related to gene expression causing to lacking male gamete and chromosomal abnormality by haploid and mixoploid(Boediono *et al.*, 1995).

For the purpose of transgenic animal production, in this study, blastomeres produced by parthenogenetic stimuli were combined with those of *in vitro* produced embryos. A few studies have been reported on the production of chimeric embryos aggregated with blastomeres of embryos that produced by *in vitro* and parthenogenetic stimuli (Bedino et al., 1999).

In parthenogenetic embryos, the variable effects of different activation methods on developmental retardation may well constitute limiting factors. Therefore, this study aimed to compare the effects of two different activation methods on cleavage and subsequent development of parthenogenetic embryos. Two different activation methods comprised of combinations of ionomycin, as the calcium ionophore, with either DMAP or CHX. Furthermore, the development of chimeric embryos by aggregation of blastomeres from parthenogenetic and *in vitro* produced embryos was compared with IVF controls.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Company(St. Louis, MO) and media from GIBCO BRL(Canadian Life Technologies, Burlington, ON) unless otherwise specified.

1. In Vitro Maturation (IVM)

Bovine ovaries collected from slaughterhouse were transported in phosphate buffered saline (PBS) with penicillin-streptomycin(0.06%, Pen-Srep, Gibco BRL) at 25±2℃ within 2 h to the laboratory. The cumulus-oocyte-complexes (COCs) were obtained by aspiration of 2~6 mm antral follicles of ovaries. COCs with dark evenly granulated cytoplasm and more than five layers of cumulus cells were selected for *in vitro* maturation. Selected COCs were washed three times in TL-HEPES containing 0.1% polyvinyl alcohol(PVA).

Fifteen COCs were transferred into a 50 μl drop of tissue culture medium (TCM-199, Sigma, Grand Island, N.Y., USA) supplement with 10% fetal bovine serum (FBS, GIBCO BRL), 25 mM HEPES, 2.5 mM sodium pyruvate, 1 mM L-glutamine, and 0.06% Pen-Strep, 10 ug/ml LH (Sigma), 10 ug/ml FSH (Sigma), that had been previously covered with paraffin oil (Junsei Chemical, Japan) and equilibrated in an atmosphere of 5% CO_2 in air at 39℃. The culture was maintained for 22~24 h at 39℃ in a humidified atmosphere of 5% CO_2.

2. Sperm Preparation and In Vitro Fertilization (IVF)

For the sperm preparation, frozen semen straws were thawed and isolated intact viable spermatozoa using Percoll density gradient method(Rosenkrans et al., 1993). In brief, 100% Percoll(Pharmacia, Uppsala, Sweden) solution was mixed with 10 × salt solution to form 90% Percoll solution. The gradient was loaded 2 ml of 90% Percoll solution into a 15 ml conical tube and then over-layered it with 2 ml of 45% Percoll solution. Frozen-thawed semen were over-layered on the 45% Percoll gradient and centrifuged at 850×g for 15 min. In order to remove the effect of Percoll, sperm pellet from the bottom of the tube was re-suspended 10 ml of TL-HEPES followed by centrifuged at 300 ×g for 10 min. The resulting pellet of sperm was resuspended in 200 μl of IVF-TALP and the concentration of sperm was adjusted to 1×10^5 sperm/μl using a hematocytometer(Superiol, Germany).

For oocytes insemination, 22~24 h matured

oocytes were removed partially its cumulus cells by vortexing them for 10 sec in TL-HEPES, transferred each 15 oocytes into a 49 μl droplet of IVF-TALP containing 10 μg/ml of heparin, and added into the 1 μl of sperm suspension. The final concentration of sperm for insemination was 2 × 10^6 spermatozoa/ml. Oocytes and spermatozoa were co-incubated at 39℃ in a humidified atmosphere of 5% in air for 16~18 h.

3. In Vitro Culture (IVC)

At 18 h post insemination (hpi), cumulus cells and sperm were removed from oocytes by vortexing for 10 sec in TL-HEPES. After being washed three time with TL-HEPES, presumptive zygotes were then first cultured in sets of 15 in 50 μl drops of CR1aa medium supplemented with 0.3% bovine serum albumin (BSA) described previously by Elhassan et al., (1999), for up to day 3, and then transferred further culture into 50 μl of CR1aa medium supplemented with 10% FBS until day 9. At day 5 and day 7, the cultures were fed with 25 μl of fresh CR1aa medium (with 10% FBS) to each drops. The rate of cleavage was assessed at 48 hpi and of development into blastocyst was at 192 hpi.

4. Parthenogenetic Activation

Two different activation methods by the combinations of ionomycin, as the calcium ionophore, either with 6-dimetylaminopurine (DMAP) or cycloheximide (CHX) and cytochalasin B (CCB) were used for this study as described by Susko- Parrish et al. (1994). Briefly, at 22 h post maturation, COCs were vortexed in order to strip their cumulus cells in 3% sodium citrate solution for 1 min. Denuded oocytes having with the first polar body and dense cytoplasm were selected and then exposed to 5 μM ionomycin in TL-HEPES for 4 min in order to be activated. To stop the activation reaction, the oocytes were incubated in TL-HEPES supplemented with 30 mg/ml BSA for 5 min. After being washed three times in TL-HEPES, the activated oocytes were assigned to two further treatments in order to prevent their re-accumulation of MPF. Oocytes were cultured in CR1aa medium supplemented either with 10 μg/ml CHX and 7.5 μg/ml CCB for 5 h (Treatment A) or 1.9 mM DMAP for 5 h (Treatment B). After being washed three times in CR1aa, the oocytes were cultured in CR1aa medium as the same as IVF embryos. The rates of cleavage and of development into blastocyst were assessed at 48 h post-activation (hpa) and 192 hpa, respectively.

5. Chimeric Embryo Production

Chimeric embryos were produced by slightly modified methods of Rho et al. (2001). On day 2 of culture, embryos developed to 4-cell stage both derived from IVF and parthenogenetic activation were exposed to 0.5% pronase solution (Protease, Type XXV) in D-PBS for 2 min in order to dissolve their zonae pellucidae (ZP). After being washed in TL-HEPES containing 30 mg/ml BSA in order to inactivate pronase, each blastomere were separated in calcium-magnesium-free PBS by pipetting them with a glass pipette. The separated blastomeres were transferred to 20 μl drop of Ham's F-10 medium supplemented with 0.1% polyvinyl alcohol (PVA) under paraffin oil to make aggregation of two set of blastomeres within an emptied ZP by micro-manipulation technique (Fig. 1). For this, an injection pipette inserted blastomeres were injected to an emptied ZP that was immobilized by a holding pipette (an outer diameter: 100 ~120 μm) and expelled them gently. Chimeric embryos were than cultured in a 50 μl drop of CR1aa medium supplemented with 10% FBS for day 6(day 0: the day of injected blastomeres). On day 2 and day 5, the cultures were fed with 25 μl of fresh CR1aa medium(with 10% FBS) to each drop.

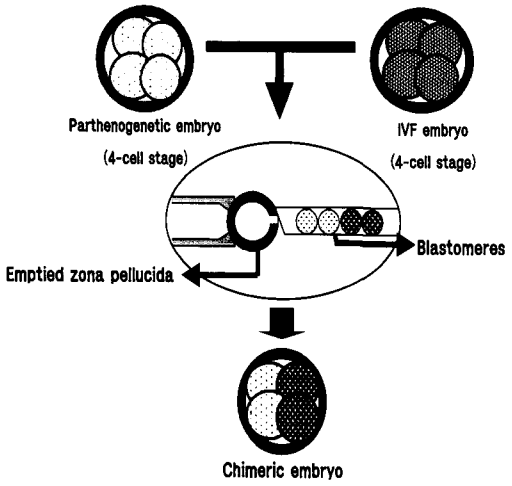

Fig. 1. A diagram for the production of chimeric embryos.

The emptied ZPs were prepared by evacuation with a micromanipulation pipette from presumptive zygotes that had not cleaved. After loading the emptied ZPs in 0.5 ml straws in Ham's F-10, the straws were directly transferred into LN_2 and kept them in until use.

For being categorized embryos produced in this study, 4-cell stage IVF-derived embryos were referred as to IVF controls. Handled controls are non-chimeric embryos that were made by recombining all four blastomeres from a 4-cell stage IVF embryo into an emptied ZP. Chimeric embryos made by aggregation of IVF and parthenogenetic embryos treated with CHX/CCB and DMAP are referred as to Group 1 and 2, respectively.

6. Experimental Design

This study comprised of two experiments. Experiment 1 compared the rates of cleavage and development of parthenogenetic embryos with those of IVF controls. Experiment 2, the rates of cleavage and development of chimeric embryos, which were reconstructed by aggregation with blastomeres of IVF and parthenogenetic embryos were compared with those of handled and IVF controls.

7. Statistical Analysis

Differences between treatments were analyzed using one-way ANOVA. A probability of $P < 0.05$ was considered to be statistically significant.

RESULTS

1. Development of Parthenogenetic and IVF Bovine Embryos

As shown in Table 1, the cleavage rates of both IVF controls(83.7%, 274/327) and 6-DMAP treated parthenogenetic embryos(85.5%, 118/138) were significantly higher($P<0.05$) than those of CHX/CCB treated parthenogenetic embryos(57.9%). In the control group, 31.5% of them developed to morula. The developmental rates of parthenogenetic embryos to morula and blastocyst after treating with CHX/CCB was 6.7 and 5.3%, respectively, and the rates of them after treating with 6-DMAP

Table 1. Developmental rate of parthenogenetic and IVF bovine embryos

Treatments	No. of oocytes	Development of embryos (%)		
		2-cell	Morula	Blastocyst
IVF control	327	274 (83.7)*	103 (31.5)*	91 (27.8)*
CHX/CCB	133	77 (57.9)**	9 (6.7)**	7 (5.3)**
6-DMAP	138	118 (85.5)*	21 (15.2)**	17 (12.3)**

** Values with different superscripts within columns are significantly different ($P<0.05$).
'CHX/CCB groups were exposed to 10 μg/ml cycloheximide (CHX) and 7.5 μg/ml cytochalasin B (CCB) for 5 h, 6-DMAP groups were exposed to 1.9 mM 6-dimetylaminopurine (6-DMAP) for 4 h.

was 15.2 and 12.3%, respectively.

These developmental rates were significantly ($P<0.05$) lower than the rates of IVF control embryos.

2. Efficiency of Chimeric Embryo Reconstruction by Aggregation of Each Two Blastomeres of Four-cell Stage Parthenogenetic and IVF Bovine Embryos

Table 2 shows the efficiency of chimeric embryos reconstructed by aggregation of each of the two blastomeres of 4-cell stage parthenogenetic and IVF embryos.

3. Development of Chimeric Embryos by Aggregation of Each Two Blastomeres of Four-cell Stage Parthenogenetic and IVF Bovine Embryos

The development of chimeric embryos aggregated with each two blastomeres of 4-cell stage parthenogenetic and IVF embryos were shown in Table 3. Similarly, the developmental rates of 8-cell stage in the Control group (77.7%) was significantly higher ($P<0.05$) than in Group 1, Group 2 and Handled control (46.1%, 52.8% and 54.7%). Fig. 2 shows the chimeric bovine embryos produced by combining two pairs of 4-cell stage blastomeres (A) and the chimeric embryo developed to morula stage (B).

The rate of blastocyst formation in the control group was significantly higher ($P<0.05$) than Group 1, Group 2 and Handled control (34.9% vs 12.8%, 18.8%, 21.4%). Development into blastocyst among chimeric embryos, in Handled control was

Table 2. The efficiency of chimeric embryos the reconstruction by aggregation of each two 4-cell stage parthenogenetic and IVF bovine embryos

Groups	No. of embryos	No. of (%) chimeric embryos	
		Reconstruction	Degeneration
Group 1	39	19/39 (48.7)	20/39 (51.2)
Group 2	53	30/53 (56.6)	23/53 (43.3)
Handled control	42	25/42 (59.5)	17/42 (40.4)

** Values with different superscripts within columns are significantly different ($P<0.05$).
Group 1: aggregation of IVF and parthenogenetic embryos treated with CHX/CCB and Group 2: aggregation of IVF and parthenogenetic embryos treated with DMAP. Handled control: non-chimeric embryos made by recombined all four blastomeres from a 4-cell stage IVF embryo into an emptied ZP.

Table 3. Development of chimeric embryos by aggregation of each two blastomeres of 4-cell stage parthenote and IVF bovine embryos

Groups	Replicates	No. of embryos	No. of development (%)	
			8-cell	Blastocyst
Control	5	63	49/63 (77.7)*	22/63 (34.9)**
Group 1	6	39	18/39 (46.1)	5/39 (12.8)
Group 2	7	53	28/53 (52.8)	10/53 (18.8)
Handled control	6	42	23/42 (54.7)	9/42 (21.4)*

** Values with different superscripts within columns are significantly different ($P<0.05$).
Group 1: aggregation of IVF and parthenogenetic embryos treated with CHX/CCB and Group 2: aggregation of IVF and parthenogenetic embryos treated with DMAP. Handled control: non-chimeric embryos made by recombined all four blastomeres from a 4-cell stage IVF embryo into an emptied ZP.

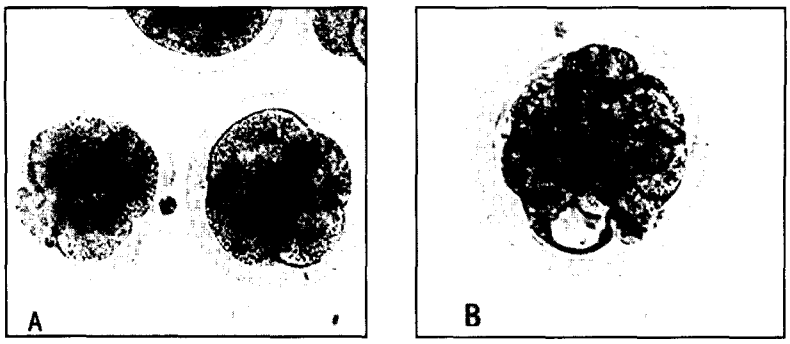

Fig. 2. Chimeric embryos produced by aggregation of each two pairs of blastomeres from 4-cell stage parthenogenetic and IVF bovine embryos. X400.
A: Chimeric bovine embryos reconstructed at the 4-cell stage by combining pairs of blastomeres.
B: A chimeric bovine embryo at morula stage.

significantly higher ($P<0.05$) than Group 1 and Group 2 (21.4% vs 12.8%, 18.8%). Fig. 3 shows the blastocysts developed from chimeric parthenote bovine embryos (A) and hatched IVF blastocysts at day 9 of *in vitro* culture (B).

DISCUSSION

The results of present study show that the developmental rate of chimeric embryos, made by combining pairs of blastomeres from different embryos(parthenogenetic and IVF-derived bovine embryos), was significantly lower than their own four blastomeres(handled control). The result of previous studies of the aggregation technique using whole embryos proved to be more successful means of producing bovine chimeras(Picard et al., 1990; Brem et al., 1984: Summers et al., 1983).

Bovine chimeras were able to be produced by aggregation of *in vitro* fertilized embryos cultured *in vitro* without zona pellucida and transfer of the recipients(Boedino *et al.*, 1993). The optimal stage for embryo aggregation in the mouse was considered to be the 8 to 12 cell stage(Minz *et al.*, 1962). In this study it was shown that the bovine blastomeres of IVF-derived embryos were able to stimulate the development of parthenogenetic blastomeres in aggregated embryos, as similarly repor-

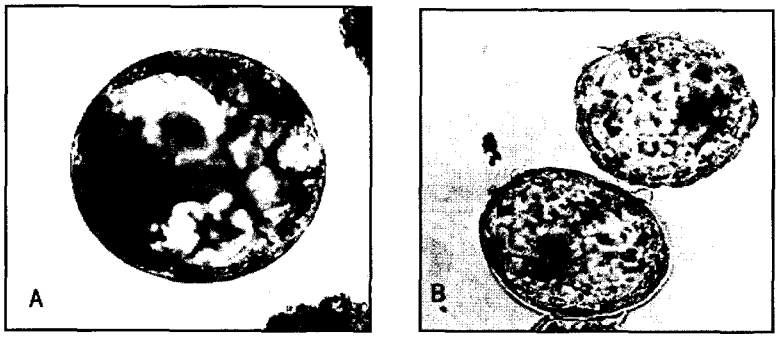

Fig. 3. Blastocysts developed from chimeric bovine embryos produced by aggregation of each two pairs of blastomeres from 4-cell stage parthenogenetic and IVF bovine embryos.
(A) and hatched IVF blastocysts at day 9 of *in vitro* culture. (B). X400

ted by Boedino *et al.*(1999). Boedino *et al.*(1999) indicated that IVF-derived blastomeres per embryos were capable of partially enhancing the development of chimeric parthenogenetic embryos. The growth factors produced by the IVF-derived bovine blastomeres might have played a role in the enhanced development of the aggregated parthenogenetic bovine embryos. And Intercellular junctions between blastomeres of different origins may be in communication and enhanced development of the chimeric parthenogenetic embryos(Ducibella and Anderson, 1975: Ducibella *et al.*, 1975).

The *in vitro* development of parthenogenetic bovine embryos is generally low(Aoyagi *et al.*, 1994; Goto *et al.*, 1994; Presicce and Yang, 1994). Although parthenogenetically activated diploid mammalian embryos developed normally through preimplantation stage, they rarely reach to the forelimb-bud stage. It has been proposed that genomic imprinting during gametogenesis may be responsible for the limited developmental capacity of the parthenogenetic mouse embryos(Surani *et al.*, 1990; Barton *et al.*, 1984). Although some parthenogenetic mouse embryos are unable to develop to term(Kaufman *et al.*, 1977), diploid parthenogenetic mouse embryos have been shown to develop to adults as chimeras(Barton *et al.*, 1984), and to produce viable germ cells in chimeric adult mice(Anderegg and Markert, 1986).

Boedino et al.(1999) did show that the agar embedding method was useful for protection of aggregated embryos from disaggregation. A double-layer of agar embedding was used for *in vivo* culture of micromanipulated embryos, to protect them from disaggregation and from adhering to oviductal tissue of the incubator female(Wlladsen, 1979). T. Suzuki(2001) indicated that a single layer embedding method using a 1% concentration agar would be sufficient for protection of aggregated bovine embryos form disaggregation during *in vitro* culture.

In conclusion, this study shows that blastomeres from parthenotes by two different activation methods of combinations of ionomycin, as the calcium ionophore, either with DMAP or CHX can be reconstituted with IVF-blastomeres by aggregation. Further study still remains to be evaluated chromosome abnormality in chimeric parthenogenetic embryos.

SUMMARY

The objective of this study was to compare the effects of two different activation methods on cleavage and subsequent development of parthenogenetic and chimeric bovine embryos. Furthermore, the development of chimeric embryos by aggregation of each pairs of blastomeres from 4-cell parthenogenetic and IVF embryos was compared with IVF controls.

The cleavage rates of both IVF controls(83.7%, 274/327) and DMAP treated parthenogenetic embryos(85.5%, 118/138) were significantly higher ($P<0.05$) than that of CHX/CCB treated parthenogenetic embryos(57.9%). In control group, 31.5% developed to morula, whereas in 6.7 and 15.2% of oocytes treated with CHX/CCB and 6-DMAP groups developed morula stage, respectively ($P<0.05$). The rate of blastocyst formation in the control group was significantly higher ($P<0.05$) than CHX/CCB and DMAP-treated parthenogenetic embryos (27.8% vs 5.3%, 12.3%).

The developmental rates of chimeric embryos to 8-cell stage, was significantly higher ($P<0.05$) in the control group than Group 1, Group 2 and Handled control (46.1%, 52.8% and 54.7%). Similarly the rate of blastocyst formation in the control group was significantly higher ($P<0.05$) than Group 1, Group 2 and Handled control (34.9% vs 12.8%, 18.8%, 21.4%). On the other hand, the development into blastocyst among chimeric embryos was

significantly higher ($P<0.05$) in Handled control than Group 1 and Group 2 (21.4% vs 12.8%, 18.8%).

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