# CHIMERA PRODUCTION IN BOVINE AND ITS SPERM CAPACITY

Arief Boediono[1], C. Sumantri[2] and T. Suzuki[3]

## ABSTRACT

In vitro produced bovine embryos were used to produce chimeras by combining the 8-cell stage embryos. Aggregated embryos were embedded in either 0, 1.0 and 1.2% agar and cultured in vitro. The aggregation rate of the embryos cultured without agar embedding was lower (P<.05) than with agar embedding (62%, 92% and 94% for 0, 1.0 and 1.2% agar, respectively). Five aggregated embryos were transferred non-surgically, resulting in the birth of 2 chimeric calves. Base on chromosome analysis, the chimera bull had apparently normal chromosomes (29 acrosentric pairs, one large submetacentric X chromosome and one small submetacentric Y chromosome). Capacity of the Chimera (C) sperm were used for IVF to compare with sperm of the Japanese Black (JB), Limousin (L), Japanese Red (JR) and Holstein (H) bulls. Fertilization rates by using Chimera sperm were higher (P<.05) than JR and H sperm, but did not differ from JB and L sperm (81.8%, 80.0%, 69.4%, 44.2% and 18.2% for C, JB, L, JR and H, respectively). The blastocyst rates oocytes inseminated with C sperm were higher (P<.05) than with L, JR and H sperm, but not differ from JB sperm (38.1%, 39%, 25.0%, 23.3% and 17.8% for C, JB, L, JR and H sperm, respectively). These findings suggested that chimera in bovine could be able to produce by aggregation method resulted in the normal calves. The sperm collected from chimeric bull could be used for producing bovine IVF embryos.

Key words: Chimeric bull, Sperm, IVF

## INTRODUCTION

Chimeras have been successfully produced in laboratory animals either by the aggregation of precompaction-stage embryos (Mintz, 1971; Tarkowski and Wroblewska, 1967) or by the microinjection of the inner cell mass (ICM) into the blastocyst (Gardner, 1968; Rorie *et al.*, 1989). Attempts to produce chimeras in domestic animals derived from surgically flushed embryos have been reported in sheep (Fehilly *et al.*, 1984; Polzin *et al.*, 1987; Rorie *et al.*, 1989) and cattle (Brem *et al.*, 1984; Picard *et al.*, 1990; Summers *et al.*, 1983). The blastocyst injection technique (Picard *et al.*, 1990; Summers *et al.*, 1983) or aggregation of bisected embryos with the zone pellucida intact (Brem *et al.*, 1984) was used in cattle to produce chimeric calves.

Sperm from individual bulls differ in their ability to fertilize matured oocytes in vitro and support embryo development to the pre-implantation stage (Iritani *et al.*, 1986; Leibfried-Rutledge *et al.*, 1987). Reproduction of interspecific chimeric between sheep and goat (MacLaren *et al.*, 1993) have been reported. However, the ability of tetraparental bovine chimeric sperm to fertilized matured oocytes and support further embryo development in vitro has not been reported.

The primary objective of this study was to evaluate the developmental capacity of the aggregated embryos. In a series experiments conducted, we investigated (1) the effect of agar embedding for protection of aggregated embryos from disaggregation during culture in vitro, (2) the chromosome composition of chimeric calf (3) the ability of sperm from a tetraparental chimera to fertilize matured oocytes and developmental capacity of in vitro produced embryos.

[1] Fac. of Veterinary Medicine, Bogor Agricultural University, Indonesia.
[2] Fac. of Animal Husbandry, Bogor Agricultural University, Indonesia.
[3] United Graduate School of Veterinary Sciences, Yamaguchi University, Japan.

## MATERIALS AND METHODS

### In vitro produced (IVP) embryos

Embryos were produced by standard in vitro maturation, fertilization and culture procedures (Boediono *et al.*, 1994). Ovaries from dairy cows were collected from a slaughterhouse. Follicular oocytes were allowed to mature for 21 h at 38.5 °C under 5 % $CO_2$ in air. Frozen-thawed sperm was washed twice with 2.5 mM caffeine in Brackett-Oliphant medium (B-O; Brackett and Oliphant, 1975) without bovine serum albumin. Sperm concentration was adjusted to $5x10^6$ spermatozoa per ml in B-O supplemented with 0.3% bovine serum albumin (BSA, Sigma) and 20 µg/ml heparin (Shimizu, Japan). A 100 µl aliquot of the sperm suspension was pre-incubated for 1 h. In vitro matured oocytes were transferred into fertilization droplets for insemination (20 to 25 oocytes per drop). After 5 h of fertilization, oocytes were washed and transferred to a polystyrene dish (4-well multidish; Nunclon, Denmark) containing TCM-199 supplemented with 5% superovulated cow serum (SCS), 5 µg/ml insulin (Wako, Osaka, Japan) and 50 µg/ml gentamicin sulfate for further development. Two types of frozen-thawed semen were used for in vitro fertilization. Japanese Black breed semen was used for inseminating matured Holstein breed oocytes, and Limousin breed semen was used for inseminating matured Japanese Red breed oocytes.

### Experimental design

*Experiment I: Embryo Reconstruction*

Pronase treatment. The zone pellucida from 8-cell stage embryos was removed by using 0.25% pronase (Pronase E, Sigma) in culture medium for 2 to 3 min and washed with culture medium several times before being aggregated. Aggregation embryos were achieved by moving the two zone-free embryos (8-cell stage) together until the aggregate seemed sufficiently stable. Aggregated embryos were then cultured in culture medium (0.5 ml) in a polystyrene 4-well multidish with a feeder cell layer of bovine cumulus cells covered with mineral oil (one embryos per well). Aggregated embryos were cultured without the zone pellucida as previously described. All aggregates were rechecked after 6 h to ensure that the cells had not drifted apart. Morphological examinations were made 12 h intervals; both un-aggregated embryos and aggregated embryos with extruded blastomeres were removed from the culture dishes. The chimeric embryos were then left in culture until Day-9 and the origins of embryos that had developed into morphologically normal appearing blastocysts were recorded (Figure 1).

Embryo transfer and pregnancy diagnosis. The aggregated embryos that developed into blastocysts were non-surgically transferred to the uteri of synchronized recipients at Day-8 (Day-0 = estrus) of the cycle. Pregnancy diagnosis was done by ultrasound and by rectal palpation per rectum 50 days after transfer.

*Experiment II: Chromosome analysis*

Chromosome preparation was made from peripheral lymphocytes of the tetraparental Chimeric bull. Heparinized blood (1 ml) was cultured for 72 h in 5% $CO_2$ incubator at 38.5 °C in 4 ml TCM-199 medium containing 10% fetal calf serum, 100 ml PHA-M (Difco, USA), and 50 µg/ml gentamicin sulfate (Sigma, USA). Colcemid (0.04 µg/ml; Gibco, USA) was added to the culture 2 h before harvesting. Harvested cells were processed by treating with a hypotonic solution (0.075 M KCl) for 20 min at 38.5 °C and by fixation in Camoy's solution (methanol:acetic acid, 3:1). Two drops of fixed cell suspension were dropped onto a wet glass slides. The air dried chromosome spreads were stained in 5 % Giemsa solution.

*Experiment III: Assessing pronuclear formation and fertilization rates*

Frozen-thawed semen from a 17 month-old tetraparental Chimera were used for in vitro fertilization. Frozen-thawed semen obtained from its sires (Japanese Black and Limousin), and from its maternal grand-sires (Japanese Red and Holstein) were used for comparison. The inseminated ova were stained to observed the fertilization rate. At 16 h after fertilization, the cumulus cells

surrounding the embryos were removed by repeated pipetting in PBS (Gibco, USA) supplemented with 5% SCS, before being fixed in Carnoy's solution for 72 h and stained in 1% aceto-orcein to examine the formation of pronuclei. The fertilization rate was calculated as the percentage of stained ova that had two or more pronuclei.

*Experiment IV: Assessing cleavage and blastocyst rate*

At 5 h after insemination, the oocytes with cumulus cells were washed and transferred into culture medium for further development. The cleavage (2-, 4-, and 8-cell stages) rates were calculated from the number of the total ova inseminated after 48 h of insemination. The blastocyst rates were calculated from the number of the total cleaved on Days-7, -8, and -9 after insemination.

Statistical analysis

The data were analyzed by Chi-square test. Probabilities of $P<.05$, $P<.01$ and $P<.001$ were considered to be statistically significant. Mean proportions were subjected to least-square analyses of variance with arcsin transformation. Duncan's Multiple Range Test was used for comparison.

## RESULTS

*Experiment I:*

The number of agar-embedded and non-embedded aggregated embryos that developed to the blastocyst stage is shown in Table 1. The aggregation rate for embryos that were produced by aggregation and cultured in vitro without agar embedding was less ($P<.05$) than when similar embryos were agar embedding (45/73, 62% for 0% agar vs. 69/75, 92% for 1.0% agar and 62/66, 94% for 1.2% agar, respectively). However, based on the number of aggregated embryos per group, the developmental rate to blastocysts was not different among the treatments.

The transfer of five aggregated embryos to four recipients (each recipient received one to two aggregated embryos) resulted in two pregnancies (Table 2.). Two calves were born from one of the recipients which received the two aggregated embryos; one calf was born dead while the other was normal and healthy. Both calves had overt characteristics of chimeras of the Holstein x Japanese Black and Japanese Red x Limousin breeds. The calf that is shown in Figure 2, is definitely a chimeric calf. The Red color of its body and tail comes from the Japanese Red x Limousin breed while the black color comes from the Holstein x Japanese Black breed. The white color in the nasal region and on the hoof of the right leg comes from the Holstein breed. Of the remaining three recipients, one was pregnant and the other two failed to become pregnant.

*Experiment II:*

Based on chromosome analysis, this Chimera had apparently normal chromosomes (29 acrocentric pairs, one large sub-metacentric X chromosome and one small sub-metacentric Y chromosome; Figure 3).

Table 1. Development of agar embedded and non-embedded aggregated bovine embryos culture in vitro.

| Agar concentration | n | No. of aggregated | No. of blastocyst |
|---|---|---|---|
| 0% | 73 | 45 (62%) [a] | 40 (89%) |
| 1.0% | 75 | 69 (92%) [b] | 63 (91%) |
| 1.2% | 66 | 62 (94%) [b] | 55 (89%) |

Values within columns with different superscripts are significantly different (a-b, $P<.05$)

Table 3. Pronucleus stages and fertilization rates of IVM oocytes fertilized with Chimera, Japanese Black, Limousin, Japanese Red and Holstein sperm.

| Type of sperm | No. of oocytes | Pronucleus stage (%) | | | Fertilized (%) |
|---|---|---|---|---|---|
| | | 1PN | 2PN | >2PN | |
| Chimera | 44 | 1 (2) | 33 (75)[a] | 3 (7)[a] | 36 (82)[a] |
| Japanese Black | 35 | 1 (3) | 25 (71)[a] | 3 (9)[a] | 28 (80)[a] |
| Limousin | 36 | 1 (3) | 25 (70)[a] | 0 (0)[b] | 25 (69)[a] |
| Japanese Red | 43 | 2 (5) | 17 (40)[b] | 2 (5)[a] | 19 (44)[b] |
| Holstein | 33 | 1 (3) | 5 (15)[c] | 1 (3)[a] | 6 (18)[c] |

Values within columns with different superscripts are significantly different (a-b, P<.05; a-c, P<.01)

*Experiment III:*

A total of 191 inseminated IVM oocytes were stained for observation of pronucleus formation (Table 3). No significant difference was observed between the 1-pronucleus formation of IVM oocytes inseminated with Chimera and the other sperm used. The 2-pronuclei were obtained more frequently in the Chimera groups compared with Japanese Red (P<.05) and Holstein (P<.01) groups, but did not differ with the Japanese Black and Limousin groups. Polyspermy occurred in IVM oocytes inseminated with Chimera, Japanese Black, Japanese Red and Holstein sperm, but did not occur with Limousin sperm. The fertilization rates of IVM oocytes inseminated with Chimera were higher than in Japanese Red (P<.05) and Holstein (P<.01), but did not differ from Japanese Black and Limousin sperm.

*Experiment IV:*

A total of 978 inseminated IVM oocytes were cultured for observation of development into cleavage and blastocyst (Table 4). The cleavage rate of IVM oocytes inseminated with Chimera sperm did not differ from Japanese Black, but were higher than with Limousin (P<.05), Japanese Red (P<.01) and Holstein (P<.001) sperm. The blastocyst rates of IVM oocytes inseminated with Chimera sperm did not differ with Japanese Black, but were higher (P<.05) than Limousin, Holstein and Japanese Red sperm.

## DISCUSSION

In an effort to protect the developing embryos, agar embedding procedures have been used for freezing of bovine demi-

Table 4. Development of in vitro produced embryos by using Chimera, Japanese Black, Limousin, Japanese Red and Holstein sperm.

| Type of sperm | No. of oocytes | Developed to (%) | |
|---|---|---|---|
| | | Cleavage | Blastocyst |
| Chimera | 239 | 181 (76)[a] | 69 (38)[a] |
| Japanese Black | 171 | 123 (72)[a] | 48 (39)[a] |
| Limousin | 186 | 108 (58)[b] | 27 (25)[b] |
| Japanese Red | 196 | 80 (41)[c] | 16 (20)[b] |
| Holstein | 186 | 30 (16)[d] | 7 (23)[b] |

Values within columns with different superscripts are significantly different (a-b, P<.05; a-c, P<.01; a-d, P<.001)

Table 2. Result of transfer of aggregated in vitro produced bovine embryos

| Recipient | No. of Embryo transfer | Breed[A] embryo-I female x male | Breed[A] embryo-II female x male | Pregnant | Calves born | Sex | Chimerism |
|---|---|---|---|---|---|---|---|
| A | 2 | Hol.x Jap.Black | Jap.Red x Lim | + | 12.03.93 | male | chimera |
| | | Hol.x Jap.Black | Jap.Red x Lim | + | 12.03.93 | male[B] | chimera |
| B | 1 | Hol.x Jap.Black | Jap.Red x Lim | + | Abortion | - | - |
| C | 1 | Hol.x Jap.Black | Jap.Red x Lim | - | - | - | - |
| D | 1 | Hol.x Jap.Black | Jap.Red x Lim | - | - | - | - |

[A]Hol=Hostein; Jap.Black=Japanese Black; Jap.Red=Japanese Red; Lim=Limousin
[B]Calf born dead

embryos (Picard *et al.*, 1988), and bovine nuclear transfer (Wolfe and Kraemer, 1992). Whether agar embedding would be beneficial in developing parthenogenetic embryos remains to be evaluated.

Results from the first experiment (Experiment I) show that agar embedding method was useful for protection of aggregated embryos from disaggregation. However, the development rate was not affected by the presence of agar. Double-layer of agar embedding is usually used for in vivo culture of micromanipulated embryos to protect them from disaggregation and adhering to the walls oviduct of incubator animal (Willadsen, 1979). Our results indicate that using a single layer of agar embedding with low concentration of agar (1.0%) is sufficient for protection of aggregated bovine embryos from disaggregation during culture in vitro.

The optimal stage for embryo aggregation in the mouse is the 8- to 12-cell stage (Mintz, 1971). If bovine embryos of this stage are to be used, they must be collected surgically, because at this stage of development bovine embryos are located in the oviduct. The embryo aggregation technique for producing chimeric bovine offspring was found to be practical since 8- to 12-cell stage bovine embryos can be produced by the IVF technique.

Compared with the results of previous studies (Brem *et al.*, 1984; Picard *et al.*, 1990; Summers *et al.*, 1983), the aggregation technique using whole embryos may prove to be the more successful means of producing bovine chimeras. The aggregation of demi-embryos or the microinjection of the ICM has the disadvantages of being a complex procedure and of requiring costly equipment.

The results obtained in the present study demonstrate that chimeric embryos can be produced using in vitro produced bovine embryos by aggregation techniques and cultured in vitro without zone pellucida. The presence of the zone pellucida is important for development in vivo or in vitro before compaction, in order to protect the blastomeres from disjunction. During in vivo development the presence of the zone pellucida is important to prevent the blastocysts from adhering to the oviduct walls before it reaches the uterine cavity;

however, this problem does not arise during in vitro culture.

McLaren (1976) reported that Chimeras made by aggregation embryos without regard to the sex, should result in 50% of Chimeras being an XX/XY type. The phenotypic sex of XX/XY individuals will show varying degrees of dominance of the male component with the variation seeming to reflect the relative representation of XY and XX cells in the gonadal primordial. Bongso *et al.* (1981) reported that sterility and infertility in chimerism cattle (60XX/60XY) was caused by testicular hypoplasia. However, our Chimera was normal (60XY) and their sperm could fertilized the matured oocytes and developed to cleavage and blastocyst.

The first line of evidence of sperm penetration rate increased until 5 h and the proportion of polyspermy was 4 and 7% at 4 and 5 h after insemination (Saeki *et al.*, 1991). As described by Parrish *et al.* (1986), differences among bulls in fertilization rates are due to variation in the time for capacitation and the acrosome reaction. The lower fertilization rates observed in Japanese Red and Holstein may be due to the short sperm-oocyte incubation time, 5 h of insemination may not be sufficient for sperm penetration. The cleavage and blastocyst rates of IVM oocytes inseminated with the Chimera were similar to its sire (Japanese Black), but differed from another sire (Limousin), and from its maternal grand-sires (Japanese Red and Holstein). Detailed analysis of mosaicism in interspecific Chimeras in mouse revealed that cells of the two could coexist and interact normally in all tissue (Rossant and Chapman, 1983). Chimera in this study originated from aggregated F1 (Holstein x Japanese Black) and F1 (Japanese Red and Limousin). Therefore, the high blastocyst production seen when IVM oocytes were inseminated with Chimera sperm might be due to a heterosis effect. These results suggested that this Chimera sperm can be used for producing in vitro produced bovine embryos.

The number of blastocyst produced of IVM oocytes inseminated with Chimera was similar to that produced with its sires (Japanese Black and Limousin sperm). These findings indicate there may be a paternal effect in blastocyst production. Shire and Whitten (1980); Goldbard and Warner (1982) reported similar cases in the mouse embryo, where the cleavage rate and speed of development are dependent upon genetic factors, including maternal and paternal effects.

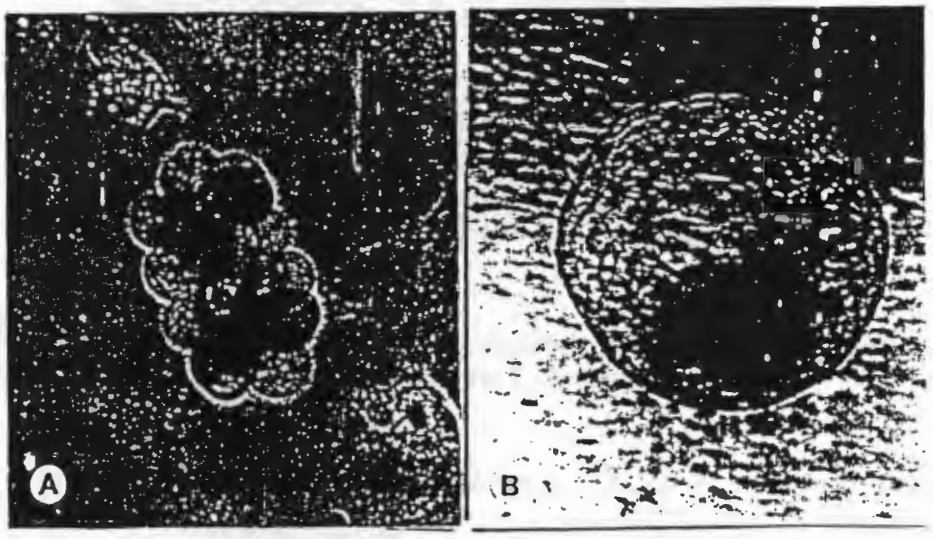


Figure 1. A Chimeric blastocyst produced by aggregation of entire in vitro produced embryos, and when developed in vitro without zone pellucida. (A) Day-4 after insemination, (B) Day-8, developed to expanded blastocyst.
Note: aggregation was done on Day-2 after insemination

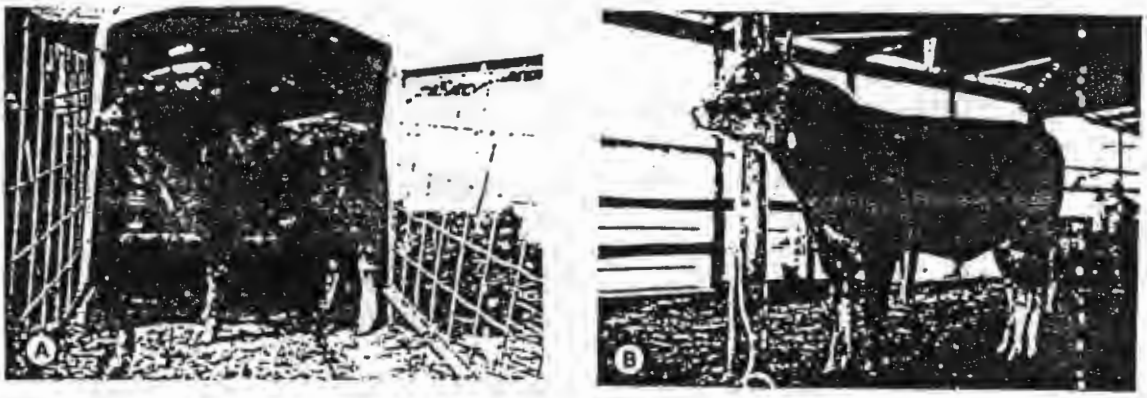


Figure 2. Overtly chimeric bull produced by aggregation of in vitro produced bovine embryos (Holstein x Japanese Black and Japanese Red x Limousin).
(A) One month old, and (B) Seventeen month old.

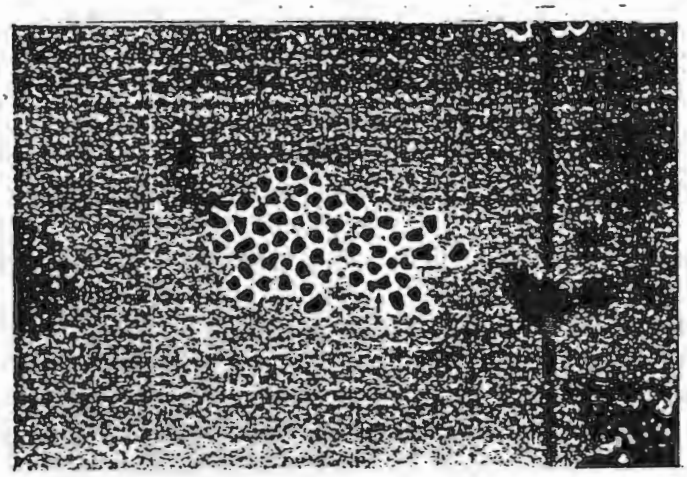

Figure 3. Metaphase spreads of chimeric bull (29 acrocentric pairs, one large sub-metacentric X chromosome and one small sub-metacentric Y chromosome)

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ISSN. 0215-2835

# Hayati

Tahun XII No. 1 - 2, Maret - Juni 1998