PRODUCTION OF CHIMERIC CALVES BY AGGREGATION OF
IN VITRO-FERTILIZED BOVINE EMBRYOS WITHOUT ZONAE PELLUCIDAE

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ABSTRACT

Bovine embryos produced by in vitro maturation (IVM), fertilization (IVF) and culture (IVC) were used to produce aggregation chimeras. An aggregated chimera was produced by combining bovine IVF embryos (Holstein x Japanese Black and Japanese Brown x Limousin breeds) which were cultured in vitro without the zonae pellucidae. Forty-eight hours after IVF, embryos at the 8 cell-stage were used to produce aggregation chimeras. In Experiment I, the zonae pellucidae was removed by a microsurgical method using a microblade or by treatment with 0.25% pronase. Holstein x Japanese Black embryos were aggregated with Japanese Brown x Limousin embryos after zonae removal by hand manipulation in culture medium. In Experiment II, the viability of the aggregated embryos developing into blastocysts was examined by measuring the extent of development. The number of aggregated embryos and embryos developed into blastocysts was 34 (91.9%) and 24 (70.6%), respectively, when the zonae pellucidae was removed by the microsurgical method; and 12 (92.3%) and 6 (50.0%), respectively, when the zonae pellucidae was removed using the 0.25% pronase treatment. The size of the aggregated embryos was significantly different from that of the normal embryos when cultured in vitro until Day 10, but not different thereafter. Five aggregated embryos were transferred non-surgically to the recipients, resulting in 1 pregnancy and the birth of 2 chimeric calves. Skin color was used as evidence of chimerism.

Key words: bovine IVF embryo, aggregation chimera, zonae pellucidae, chimeric calve

INTRODUCTION

Chimeras have been successfully produced in laboratory animals either by the aggregation of precompaction-stage embryos (7,13) or by the microinjection of the inner cell mass (ICM) into the blastocyst (3,10). Attempts to produce chimeras in domestic animals derived from surgically flushed embryos have been reported in sheep (2,9,10) and cattle (1,8,11). The blasto-cyst injection technique (8,11) or aggregation of bisected embryos with the zonae pellucidae intact (1) was used in cattle to produce chimeric calves; however, there is yet no report of a bovine chimera produced by aggregating IVF embryos with the zonae pellucidae removed.

In this study, we evaluated the possibility of producing chimeras by the aggregation of zonae pellucidae-free 8 cell-stage bovine embryos (Holstein x Japanese Black and Japanese Brown x Limousin breeds) which had been produced by in vitro maturation, fertilization and culture in vitro.

MATERIALS AND METHODS

IVF Derived from Bovine Embryos

Oocyte maturation. Ovaries were collected from cows at a local slaughterhouse and were brought to the laboratory in Ringer solution supplemented with penicillin-G (100 IU/ml) and streptomicin sulfate (0.2 μg/ml) at 30 to 32°C within 3 h. Oocytes in follicles 2 to 5 mm in diameter were collected by aspiration with an 18-g needle in modified-PBS solution. The oocytes were then washed 3 times using maturation medium (TCM-199, Earle's salt; Gibco, Grand Island, NY USA) supplemented with 5% superovulated cow serum (SCS) collected on Day 7 (5), 0.01 mg/ml follicle stimulating hormone (FSH; Denka Pharmaceuti-cal Co., Kawasaki, Japan), and 50 μg/ml gentamicin (Sigma Che-mical Co., St. Louis MO USA). Oocytes (100 to 200) surrounded by cumulus cells over more than one-third of their surface were introduced into the maturation medium and cultured for 21 h at 38.5°C under 5% CO_2 in air. The maturation medium (2.5 ml) in a polystyrene culture dish (35 mm diameter, Falcon 1008; Becton Dicknson Co. Ltd., Oxnard, CA. USA) was covered with mineral oil (E.R. Squibb & Son, Inc., Princeton, NJ USA).

In vitro fertilization. Two types of frozen-thawed semen were used for in vitro fertilization. Japanese Black breed semen was used for matured Holstein breed oocytes, and Limousin breed semen was used for matured Japanese Brown breed oocytes. The Japanese Brown (Bos taurus) is a beef cattle breed from the Kumamoto Prefecture in Japan. Frozen spermatozoa were thawed in a water bath (37°C) and washed 2 times using 2.5 mM caffein in Brackett and Oliphant's medium (Caff-BO) by centrifugation at 500 g for 5 min in each medium. Then the spermatozoa were resuspended in Caff-BO supplemented with 1% bovine serum albumin

(BSA, Sigma) and 20 μ g/ml Heparin (Shimizu Pharmaceutical Co. Ltd., Shimizu, Japan), to yield a sperm concentration of 5×10^{6}/ml. A 100- μ 1 aliquot of the sperm suspension was covered with mineral oil and was preincubated for 1 h under the conditions described above. The in vitro matured oocytes were transferred into sperm microdrops (20 to 25 oocytes/microdrop) for insemination. After 5 h of insemination, the oocytes with cumulus cells were washed and transferred to the culture medium for further development. The culture medium consisted of TCM-199 supplemented with 5% SCS, 5 μ g/ml insulin (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 50 μ g/ml gentamicin. The culture medium (0.5 ml) in a polystyrene dish (4-well multidish; Nunclon, Roskilde, Denmark) was covered with mineral oil (0.5 ml). The cumulus cells surrounding the embryos were removed by pipetting after 48 h of insemination, while the cumulus cell layer (forming a monolayer at this time) attached to the bottom of the culture dish was not removed, and the embryos were cultured on this layer (4). The incubation medium was replaced with new medium every 96 h.

Experiment I

   Microsurgical method. Forty-eight hours after IVF, 8 cell-stage embryos were used to produce aggregation chimeras. Zonae removal was carried out under an inverted Nikon microscope (x200 magnification) with a Narishige micromanipulator unit (Narishige Scientific Instrument Lab., Tokyo, Japan). After stripping the cumulus cells surrounding the embryos by pipetting, the zonae pellucidae was microsurgically removed in a culture medium drop (150 μ 1) into a polystyrene dish (90x20mm, Nissui Pharmaceutical, Tokyo, Japan) covered with mineral oil. Using a microblade, a wide slit was made in the zonae pellucidae, and the blastomeres were removed by pressing the zonae pellucidae using a glass microhook. After complete removal from zonae pellucidae, the blastomeres were then washed with culture medium several times before being aggregated.

   Pronase treatment. The zonae pellucidae from 8 cell-stage embryos was removed 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.

   Embryo Aggregation. Holstein x Japanese Black embryos were aggregated with Japanese Brown x Limousin embryos after zonae removal by hand manipulation in culture medium (0.5 ml) in a polystyrene 4-well multidish which already had a feeder cell layer covered with mineral oil. The aggregated embryos were then cultured in the same dish for further development (2 pairs per well) under the conditions described above. After 5 h of culture, any embryos that had become unaggregated were reaggregated. Culture medium was replaced with new medium every 96 h to support the development of aggregated embryos.

Experiment II

    The possibility of in vitro culture of the aggregated
embryos was examined by means of a cumulus cell co-culture
system. The aggregated embryos were cultured in medium (0.5 ml)
in a 4-well multidish, with 1 aggregated embryo per well. The
multidish already had a feeder cell layer and was covered with
mineral oil (0.5 ml). The normal 8 cell-stage embryos (zonae
pellucidae-free) produced by IVM-IVF were cultured under the
same conditions as the control embryos.

    <u>Embryo transfer and pregnancy diagnosis</u>. The aggregated
embryos that developed into blastocysts were nonsurgically
transferred to the uteri of cows at Day 8 (Day 0=estrus) of the
cycle. Pregnancy diagnosis was done by ultrasound and by rectal
palpation per rectum 50 d after transfer.

    <u>Evaluation</u>. The aggregated embryos were evaluated by
measuring the diameter (longitudinal) every 12 h until 4 d after
aggregation (6 d after IVF). Both the unaggregated embryos and
aggregated embryos with extruded blastomeres (< 180 μm diame-
ter) were removed from the culture dish. The maximum diameter of
single embryos developed in vitro without zonae pellucidae until
6 d after IVF (compacted morulae) was 160 μm. The aggregated
embryos were evaluated every 24 h for further development until
15 d after IVF. The viability of aggregated embryos that had
developed into blastocysts was examined morphologically and by
size.

    Since no chromosomal markers were available, the skin color
of the calf was used as evidence of chimerism.

Statistical Analysis

    The statistical significance of the differences in
Experiment I was analyzed using the Chi-square test. Data in
Experiment II were analyzed using the Student's t-test, and the
means are given as ± SEM.

<p style="text-align:center">RESULTS</p>

    The aggregated embryos developed into blastocysts (Table
1). A total of 100 Holstein x Japanese Black embryos (8 cell-
stage) and 100 Japanese Brown x Limousin embryos (8 cell-stage)
were used in Experiment I.

    Thirty-four pairs (91.9%) of embryos were completely
aggregated when the zonae pellucidae was removed by the micro-
surgical method. This rate of aggregation was not significantly
different (P<0.05) from that achieved with the 12 pairs (92.3%)
of embryos in which the zonae pellucidae was removed with 0.25%
pronase treatment. However, the viability of aggregated embryos
that developed into blastocysts was significantly different

(P<0.05)   between the embryos with the zonae pellucidae   removed
by   the   microsurgical   method (70.6%) with   the   0.25%   pronase
treatment (50.0%).

Table 1. Viability of aggregated embryos developing into blasto-
          cysts

Method of zonae removal	No. of embryos	Aggregated embryos (pairs)	Blastocysts
Microsurgery	74	34 (91.9%)	24[a] (70.6%)
0.25% pronase	26	12 (92.3%)	6[b] (50.0%)

[a,b] Within   columns,   means   with   different   superscripts   are
significantly different (P<0.05; X^2-test).

     The size of the aggregated embryos cultured in vitro   until
15 d of development after IVF (13 d after aggregation) is   shown
in Figure 1. A total of 8 aggregated embryos and 7 single   con-
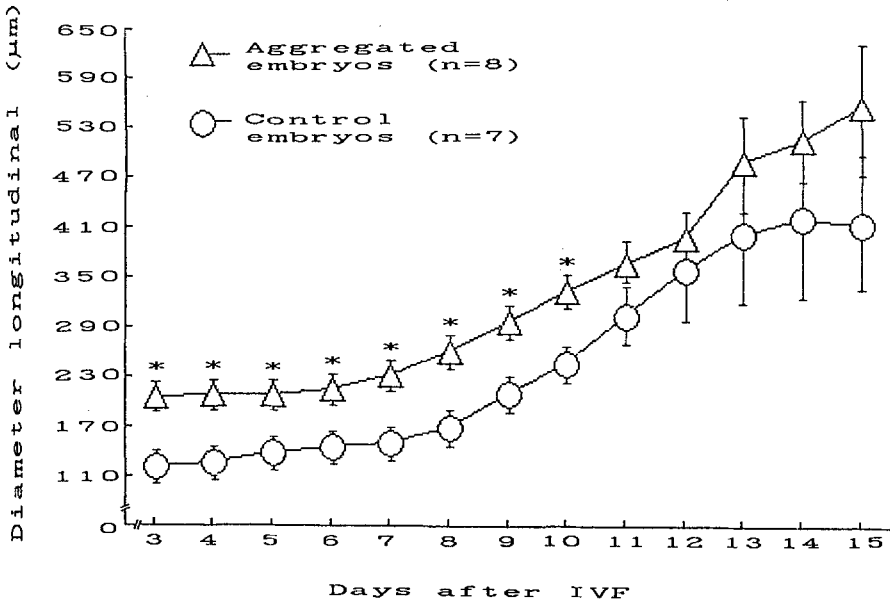trol embryos (zonae   pellucidae-free) were co-cultured in   vitro

Figure 1. The   size   of aggregated embryos   cultured   in   vitro.
          Values   are means ± SEM. Values with an asterisk   (*)
          indicated   significant   difference   (P<0.01)   between
          aggregated embryos and normal embryos in the same day.

with cumulus cells. The size (longitudinal diameter) of the
aggregated embryos was significantly different (P<0.01) from
that of the control embryos until Day 10 after IVF. Their size
after Day 11 of IVF did not differ, but there was a tendency of
control embryos to decrease in size after Day 14 of IVF.

The transfer of 5 aggregated embryos to 4 recipients
(1 recipient received 2 aggregated embryos) resulted in 2 preg-
nancies (Table 2). Two calves were born from 1 of the recipients
which received the 2 aggregated embryos; 1 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 Brown x Limousin breeds. The calf which is shown in
Figure 2, is definitely a chimeric calf. The brown color of its
body and tail comes from the Japanese Brown 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
3 recipients, 1 was pregnant and the other 2 failed to become
pregnant.

DISCUSSION

The optimal stage for embryo aggregation in the mouse is
the 8 to 12 cell-stage (7). 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.

The success of embryo aggregation using the microsurgical
method for zonae removal was not significantly different from
results with the 0.25% pronase method. Using the microsurgical
method, some of the blastomeres were injured and the resultant
number of blastomeres for embryo aggregation was lower than for
the group treated with 0.25% pronase. However, the development
of aggregated embryos into blastocysts following the microsur-
gical route was significantly different (P<0.05) from that via
the 0.25% pronase treatment for zonae removal.

Compared with the results of previous studies (1,8,11), the
aggregation technique using whole embryos may prove to be the
more successful means of producing bovine chimeras. The aggrega-
tion 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 fertilized
bovine embryos by aggregation techniques and cultured in vitro

Table 2. Result of transfer of aggregated embryos derived from in vitro fertilized 8 cell-stage bovine embryos

Recipient	Transferred aggregated embryos	Breed[a] embryo-I femalexmale	embryo-II femalexmale	Pregnant	Calves born	Sex	Chimerism
A	2	H x JB +	JR x L	+	12.03.93	male	chimera
		H x JB +	JR x L	+	12.03.93	male[b]	chimera
B	1	H x JB +	JR x L	+	?	?	?
C	1	H x JB +	JR x L	−	−	−	−
D	1	H x JB +	JR x L	−	−	−	−

[a] H = Holstein; JB = Japanese Black; JR = Japanese Brown; L = Limousin.
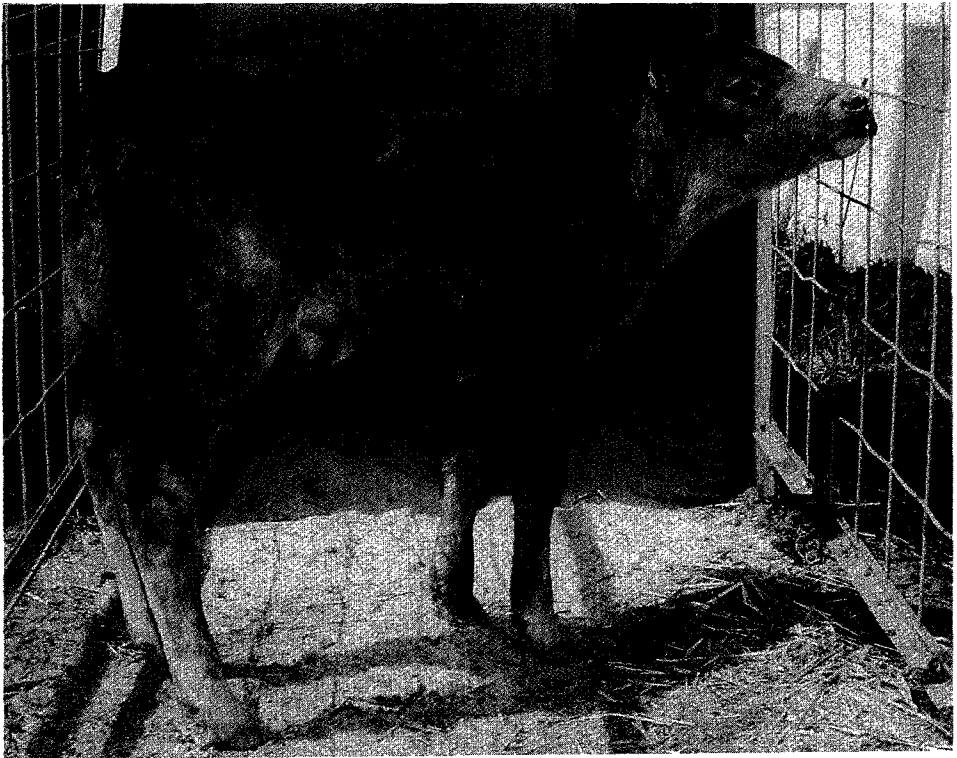[b] Calf born dead.

Figure 2. Overtly chimeric male calf produced by aggregating 8
cell-stage, in vitro fertilized bovine embryos (Hols-
tein x Japanese Black and Japanese Brown x Limousin
breeds).

without zonae pellucidae (Figure 3). The presence of the zonae
pellucidae is important for development in vivo or in vitro
before compaction, in order to protect the blastomeres from
disjuction. During in vivo development the presence of the zonae
pellucidae 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.

In the present study, the aggregated embryos were co-
cultured with cumulus cells. As shown by Goto et al. (4) and
Suzuki et al. (12) in vitro co-culture with cumulus cells
provides an appropriate environment for embryonic development.
The size of the aggregated embryos cultured in vitro was sig-
nificantly different (P<0.01) from that of the normal embryos
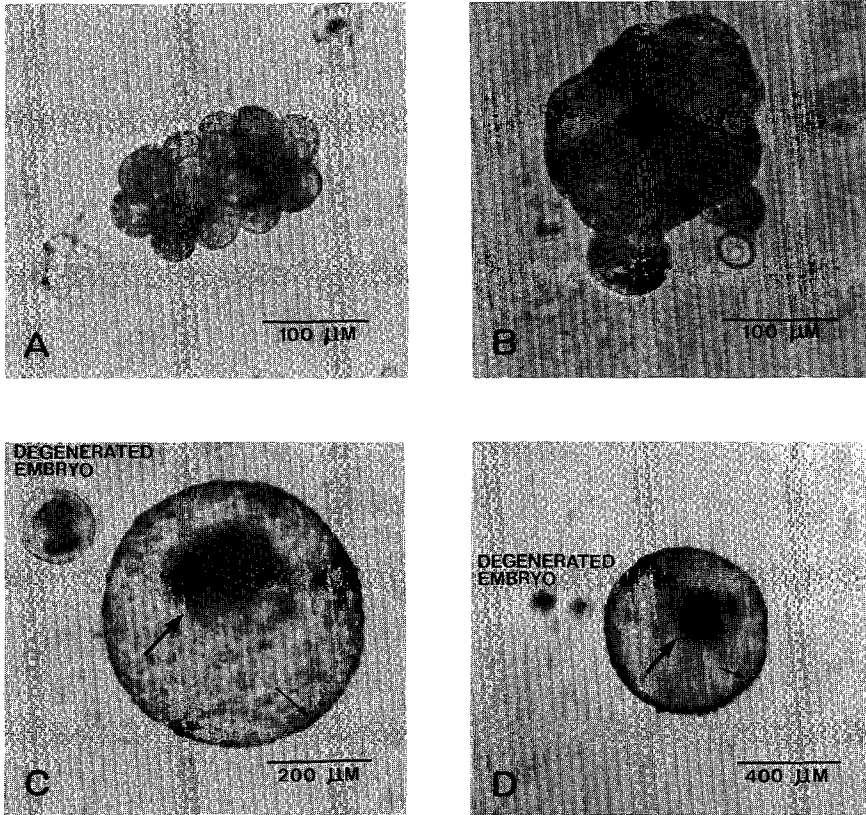Day 10 of IVF, but it was not different thereafter. Normal

Figure 3. Aggregated embryos developed in vitro without a zonae pellucidae. A) Day 1 after aggregation; B) Day 4 after aggregation, when the embryo developed into a compact morulae; C) Day 11 and D) Day 13 after aggregation, when the embryo developed into an expanded blastocyst. Inner cell mass (big arrow); trophectoderm (small arrow).

embryos cultured in vitro have a tendency to decrease in size after Day 14 of IVF. This may be related to the difference in the total cell number of aggregated embryos and normal embryos. Our finding indicates that the culture system used in our study was more efficacious for the in vitro development of either aggregated or normal embryos when cultured for more than 2 wk.

In conclusion, bovine chimeras can be produced by aggregating in vitro fertilized embryos cultured in vitro without the zonae pellucidae and transferred nonsurgically to the recipient.

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