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

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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 nonsurgically 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 calf

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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 blastocyst injection technique (8,11) or aggregation of bisected embryos with the zona pellucida 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 zona pellucidae removed.

In this study, we evaluated the possibility of producing chimeras by the aggregation of zona 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 streptomycin 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 Pharmaceutical Co., Kawasaki, Japan), and 50 µg/ml gentamicin (Sigma Chemical 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% CO₂ in air. The maturation medium (2.5 ml) in a polystyrene culture dish (35 mm diameter, Falcon 1008; Becton Dickinson 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 caffeine 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
and 20 μg/ml Heparin (Shimizu Pharmaceutical Co. Ltd., Shimizu, Japan), to yield a sperm concentration of 5x10^6/ml. A 100-μl 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 μg/ml insulin (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 50 μg/ml gentamicin. 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 μl) 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.

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

Evaluation. 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 diameter) 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.

RESULTS

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 microsurgical 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 blastocysts

<table>
<thead>
<tr>
<th>Method of zonae removal</th>
<th>No. of embryos</th>
<th>Aggregated embryos (pairs)</th>
<th>Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsurgery</td>
<td>74</td>
<td>34 (91.9%)</td>
<td>24&lt;sup&gt;a&lt;/sup&gt; (70.6%)</td>
</tr>
<tr>
<td>0.25% pronase</td>
<td>26</td>
<td>12 (92.3%)</td>
<td>6&lt;sup&gt;b&lt;/sup&gt; (50.0%)</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Within columns, means with different superscripts are significantly different (P<0.05; X<sup>2</sup>-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 control embryos (zonae pellucidae-free) were co-cultured in vitro.

![Figure 1](image-url)
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 pregnancies (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 microsurgical 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 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 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

<table>
<thead>
<tr>
<th>Transferred</th>
<th>Breeds</th>
<th>Pregnant</th>
<th>Calves born</th>
<th>Sex</th>
<th>Chimerism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient</td>
<td>embryo-I</td>
<td>embryo-II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>H x JB + JR x L</td>
<td>+</td>
<td>12.03.93</td>
<td>male</td>
<td>chimera</td>
</tr>
<tr>
<td>B</td>
<td>H x JB + JR x L</td>
<td>+</td>
<td>12.03.93</td>
<td>male&lt;sup&gt;b&lt;/sup&gt;</td>
<td>chimera</td>
</tr>
<tr>
<td>C</td>
<td>H x JB + JR x L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>H x JB + JR x L</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> H = Holstein; JB = Japanese Black; JR = Japanese Brown; L = Limousin.

<sup>b</sup> Calf born dead.
Figure 2. Overtly chimeric male calf produced by aggregating 8 cell-stage, in vitro fertilized bovine embryos (Holstein 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 disjunction. 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 significantly 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 zona pellucidae and transferred nonsurgically to the recipient.

REFERENCES