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Research Article

Production of Chimeric Cattle Embryos by Reaggregation of Blastomeres Obtained from Day 4 Bovine Embryos*

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Abstract: The aim of this study was to investigate the possibility of producing chimeric bovine embryos by reaggregating blastomeres of disaggregated day 4 bovine embryos. Oocytes were obtained from a slaughterhouse. Selected oocytes were matured, fertilized and cultured in vitro. Fertilization day was taken as day 0. Blastomeres of day 4 embryos were disaggregated and then pooled together. Chimeric embryos were randomly constructed by reaggregating blastomeres in empty zonae pellucidae. Non-chimeric handled control embryos were produced by transferring blastomeres from each embryo into their own empty zonae pellucidae. A total of 79 day 4 cattle embryos were used for disaggregation of blastomeres, and 27 chimeric and 12 non-chimeric handled control embryos were obtained from them. The rates of blastocyst formation of chimeric and non-chimeric handled control embryos were 40.7% (11/27) and 50.0% (6/12), respectively, and the difference between them was not statistically important ($P > 0.05$). These results showed the possibility of producing chimeric cattle embryos by reaggregating blastomeres obtained from day 4 embryos.

Key Words: Embryo, in vitro fertilization, chimera, cattle

Dört Günlük Sığır Embriyolarından Elde Edilen Blastomerlerin Yeniden Birleştirilmesiyle Kimerik Sığır Embriyolarının Üretimi

Özet: Bu çalışmanın amacı, 4 günlük sığır embriyolarından ayrıştırılan blastomerlerin (disaggregation) yeniden birleştirilmesiyle (reaggregation) kimerik sığır embriyolarının üretim olanağını incelemektir. Çalışmada kullanılan oositler mezbahadan elde edildi. Seçilen oositler in vitro mature, fertilize ve kültüre edildi. Fertilizasyon günü 0. gün kabul edildi. Çalışmada 4 günlük sığır embriyolarının blastomeleri ayrıştırıldı ve sonrasında birbirleriyle karıştırıldı. Kimerik embriyolar blastomerlerin içi boş zona pellusidalar içerisinde yeniden birleştirilmesiyle oluşturuldu. Kimerik olmayan manipule edilmiş kontrol embriyolar ise, her bir embriyodan gelen blastomerlerin kendi boş zona pellusidaları içerisine transferiyle üretildi. Blastomerlerin ayrıştırılması için toplam 79 adet 4 günlük embriyo kullanıldı ve bunlardan 27 kimerik ve 12 kimerik olmayan manipule edilmiş kontrol embriyo elde edildi. Kimerik ve kimerik olmayan manipule edilmiş kontrol embriyoların blastosist oluşturma oranları sırasıyla % 40,7 (11/27) ve % 50,0 (6/12) olarak saptandı ve aralarındaki fark istatistiksel olarak önemsiz bulundu ($P > 0.05$). Bu sonuçlar kimerik sığır embriyolarının 4 günlük embriyolardan elde edilen blastomerlerin yeniden birleştirilmesiyle üretilebileceğinin olası olduğunu göstermiştir.

Anahtar Sözcükler: Embriyo, in vitro fertilizasyon, kimerik, sığır

Introduction

The term "chimera" is used to describe composite animals containing genetically different cell populations originating from more than one embryo. Primary chimeras can also develop by themselves from double fertilization of an oocyte with 2 maternal nuclei or the spontaneous fusion of zygotes in cases of multiple ovulation. In addition, chimeras may develop as a result of the exchange of cells between fetuses, such as placental circulation of dizygotic twins or freemartinism as in cattle, but in this case chimerism might be limited to an incorporation of blood cells (1).

Two different methods may be used to produce chimeras experimentally. The first is the aggregation method that requires bringing the cell populations from at least 2 different embryos, before tissue differentiation

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and formation of tight junctions, into contact (2-4). The second method is the injection method in which some extra tissue or cells such as the inner cell mass of one embryo, embryonic stem cells or teratocarcinoma cells are injected into developing blastocysts (5-8). When these 2 methods are compared, the aggregation method is easier and faster (3). Chimerism has become one of the most important tools for investigating basic aspects of early embryogenic development, differentiation and sex determination (1,9) and for producing transgenic animals when germ cells or stem cells are injected into blastocysts or aggregated with blastomeres (8,10,11).

The first bovine chimera was obtained in 1983. A Bos taures-Bos indicus chimeric calf was produced by means of blastocyst injection (6). One year later, bovine morula stage embryos were aggregated (12). By using the approach of aggregating inner cell masses with early morula in cattle embryos, aggregation was shown to be affected by the stage of embryonic cells aggregated. It was concluded that bovine embryonic cells can regulate for at least 3 days' difference in development but not for 5 days', even though aggregation is still possible (2). Chimeric calves have been produced by aggregation of in vitro fertilized bovine embryos without zonae pellucidae (13). Using 8-cell stage embryos, 2 chimeric calves were obtained from 5 reaggregated embryos transferred non-surgically to the recipients. Chimeric cattle fetuses were produced by aggregating 8-16-cell stage nuclear transfer (NT) embryos obtained from ES cells with 2 blastomeres from similar stage in vitro fertilized embryos (14).

It was shown that aggregating parthenogenetic bovine embryos could prolong their survival in vitro and in vivo (15). In addition, chimeric calves have been produced from the aggregation of parthenogenetic and in vitro fertilized bovine embryos (4). In that study, male in vitro fertilized embryos and parthenogenetic embryos were used to produce chimeras. Two chimeric calves had XX and XY chromosomes. Therefore, in cattle, parthenogenetic cells can also contribute to normal development of aggregated embryos resulting in chimeric calves. In addition, Rho et al. (16) produced sex chimeric cattle embryos at the blastocyst stage by aggregating male and female blastomeres from 4-cell stage embryos.

The aim of this study was to determine the possibility of the use of day 4 bovine embryos to produce chimeric cattle embryos by reaggregating blastomeres from disaggregated day 4 embryos.

Materials and Methods

In vitro production of cattle embryos

Chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified. Ovaries were obtained from a slaughterhouse and transported to the laboratory in 2 to 4 h after collection in insulated thermoses containing saline (0.9% NaCl) to maintain the temperature between 25 and 32 ºC. They were then washed with tap water at around 30 ºC and placed in a water-bath maintained at 35 ºC. The follicular content was aspirated from small follicules, 2 to 6 mm in diameter, using an 18-gauge needle attached to a pump system, and was collected into 50 ml conical tubes. Then the sediment was diluted with TL-HEPES (17) supplemented with polyvinylpyrrolidone (3 mg/ml PVP-40), Na-pyruvate (0.2 mM) and gentamycin (25 µg/ml) in a 100 mm plate and oocytes were examined under a stereomicroscope in a warm room (30 ± 2 ºC). Oocytes with homogeneously granulated cytoplasm and multiple layers of unexpanded cumulus cells were chosen. Collected oocytes were washed 3 times in TL-HEPES and placed in pre-equilibrated 50 µl drops of maturation medium (10 oocytes per drop) covered with mineral oil. The maturation medium was composed of TCM-199 with Earl's salts (Gibco BRL) supplemented with 10% heat-treated fetal calf serum (FCS; Gibco BRL), 0.2 mM Na-pyruvate, 25 µg/ml of gentamycin, 5 µg/ml of FSH-P and 1 µg/ml of estradiol 17-β. Oocytes were matured for 24 h at 39 ºC, with 5% CO_2 in air and high humidity. These incubation conditions were the same through all phases of the culture (18).

Twenty-four hours after in vitro maturation, oocytes with expanded cumulus were washed twice in TL-HEPES, and then 10 oocytes were transferred into pre-equilibrated 44 µl drops of fertilization medium under mineral oil. Fertilization medium was modified Tyrode's based medium containing 0.2 mM Na-pyruvate, 6 mg/ml of fatty acid free-bovine serum albumin (BSA-FAF) and 25 µg/ml of gentamycin. Oocytes were kept in the incubator until the addition of spermatozoa (19).

Motile spermatozoa were obtained from frozen-thawed bull sperm using density gradient centrifugation (20). A 90:45% Percoll was prepared in a 1.5 ml Eppendorf centrifuge tube, and thawed sperm was layered on the top of 45% Percoll. The sample was centrifuged at 700 x g for 10 min. Then the supernatant

was removed, and the pellet resuspended. Sperm cells in the pellet were counted and diluted with TL-HEPES. Two microliters of sperm suspension was added to each fertilization drop at a final concentration of 1.0 x 10^6 sperm cells/ml; and 2 µl of 2 µg/ml heparin and 2 µl of PHE (20 µM penicillamine, 10 µM hypotaurine and 1 µM epinephrine) were added. Oocytes and sperm were co-cultured for 24 or 48 h (18,21).

Twenty-four or forty-eight hours after insemination, cumulus cells were removed by vortexing in an Eppendorf tube for 3 min. Cumulus free fertilized oocytes (zygotes after 24 h and 2-4-cell embryos after 48 h) were washed 3 times in TL-HEPES and transferred into 50 µl drops of bovine embryo culture medium, CR1aa under mineral oil (25 to 30 fertilized oocytes per drop). CR1aa is a balanced salt solution supplemented with 5 mM hemicalcium lactate, 1 mM L-glutamine, 0.4 mM Na-pyruvate, 25 µg/ml of gentamycin, 3 mg/ml of BSA-FAF, 10 µl/ml of 100xMEM and 20 µl/ml of 50xBME (22,23). The embryos were allowed to develop until day 4 (approximately the 16-cell stage). Fertilization day was taken as day 0.

Disaggregation of day 4 embryos

On day 4, well developing embryos were selected under a stereomicroscope, and each was transferred into a 30 µl drop containing TL-HEPES supplemented with 3 mg/ml of BSA-FAF, 0.2 mM Na-pyruvate, 25 µg/ml of gentamycin and 10% FCS in a 100 mm petri dish containing drops covered with mineral oil. The purpose of using BSA-FAF and FCS was to prevent the blastomeres sticking to each other or to the pipettes during the manipulation of embryos. All manipulations were performed with a Nikon Diaphot microscope equipped with Hoffman optics and a Narishige micromanipulator. Embryos were held with a holding pipette having an outer diameter of approximately 100-120 µm and an inner diameter of approximately 25-30 µm (Figure 1a). A slit on the zona pellucida of each embryo was made by inserting a very fine pulled pipette through the embryo, and the pipette was pushed down until the embryo became free without withdrawing the pipette (Figure 1b). During the procedure, at least 1, but sometimes 2 blastomeres were damaged. The pipette was changed to one with a 45-60 µm outer diameter and 20-30 µm inner diameter for the removal of the blastomeres. Blastomeres from embryos were usually taken out one by one with great care (Figures 2a and b); however, some blastomeres were lost because of fast suction pressure. Blastomeres with good-looking morphology from each embryo were transferred into a 100 µl drop of CR1aa, but empty zonae pellucidae were kept in the same drops for further use.

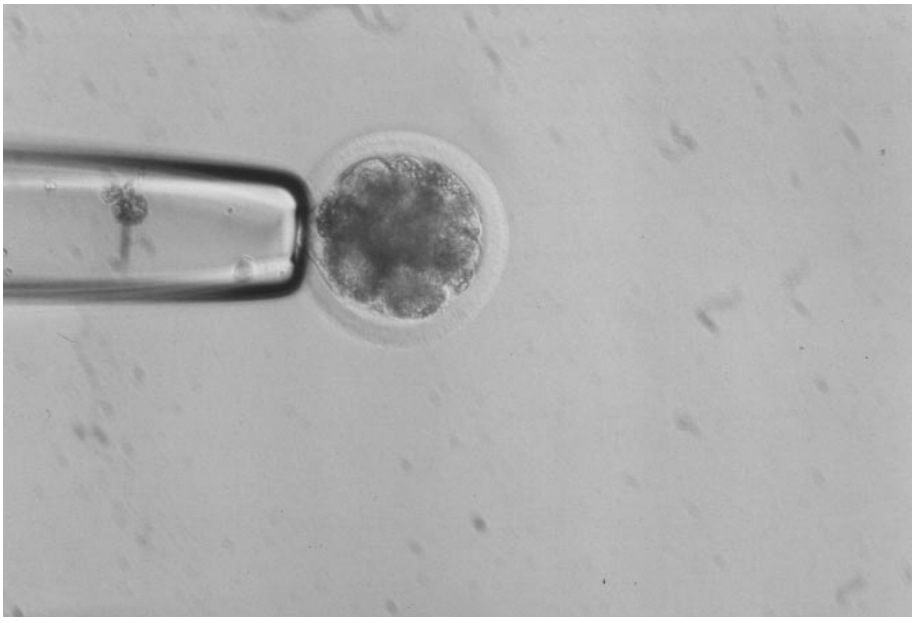

Figure 1a. Holding a day 4 bovine embryo with a holding pipette for manipulation.

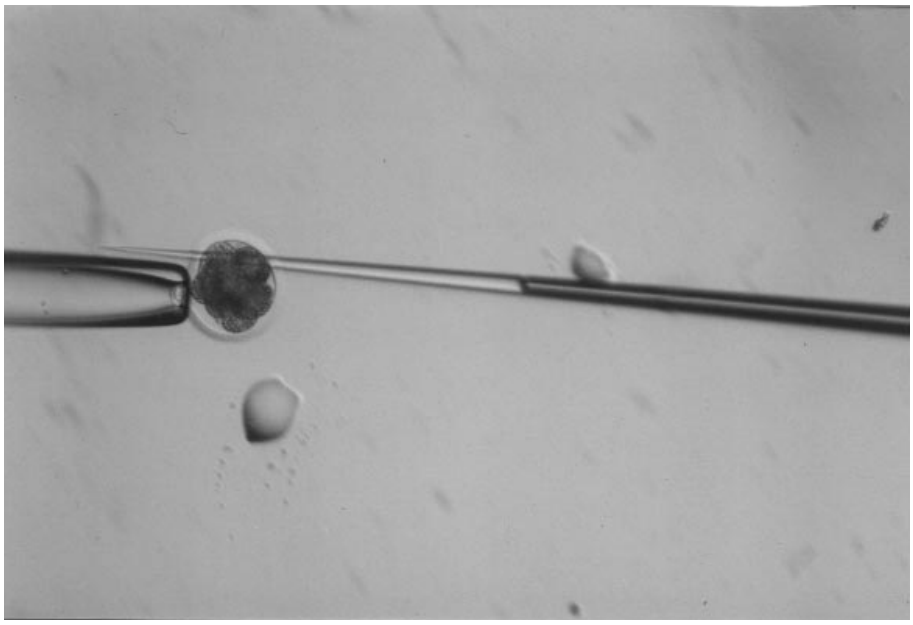

Figure 1b. Making a slit on the zonae pellucidae by means of a very fine pulled pipette.

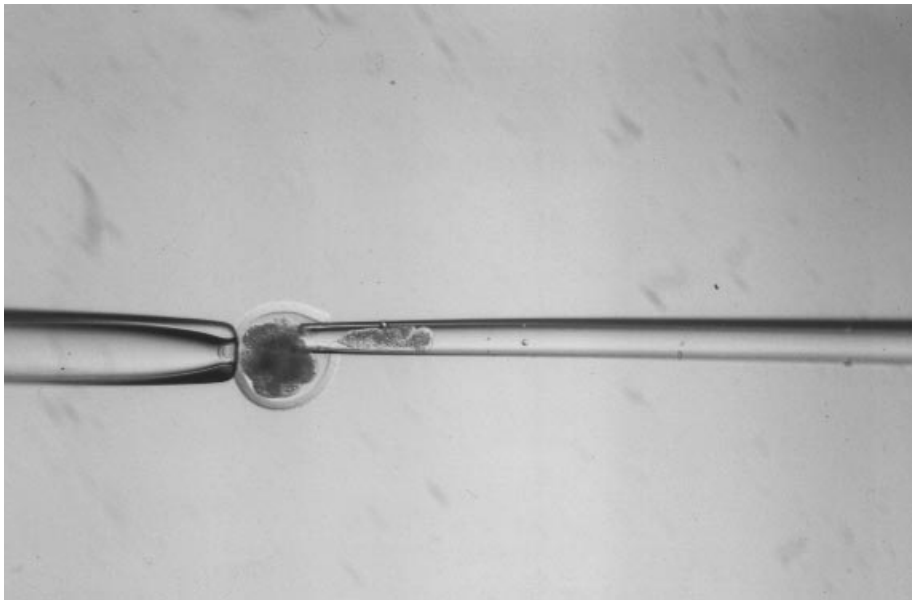

Figure 2a. Taking out blastomeres from an embryo.

Chimeric reaggregation of blastomeres

Two 50 µl drops containing TL-HEPES supplemented for manipulation as described above were prepared in a 100 mm petri dish and then covered with mineral oil. Pooled disaggregated blastomeres were placed in one drop, and empty zonae pellucidae were transferred into the other drop. Around 14 to 16 blastomeres were transferred with a pipette, similar to the one used for blastomer removal, into an empty zonae on which the slit was in the 2 o'clock position (Figures 3a and b). After finishing the transfer, reaggregated embryos were transferred into CR1aa drops. These CR1aa drops were

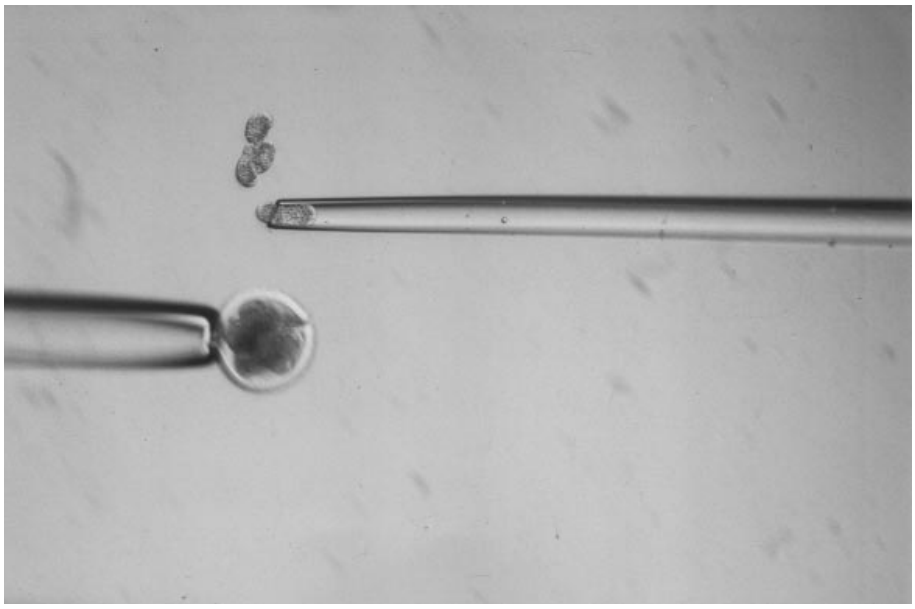

Figure 2b. Leaving blastomeres free.

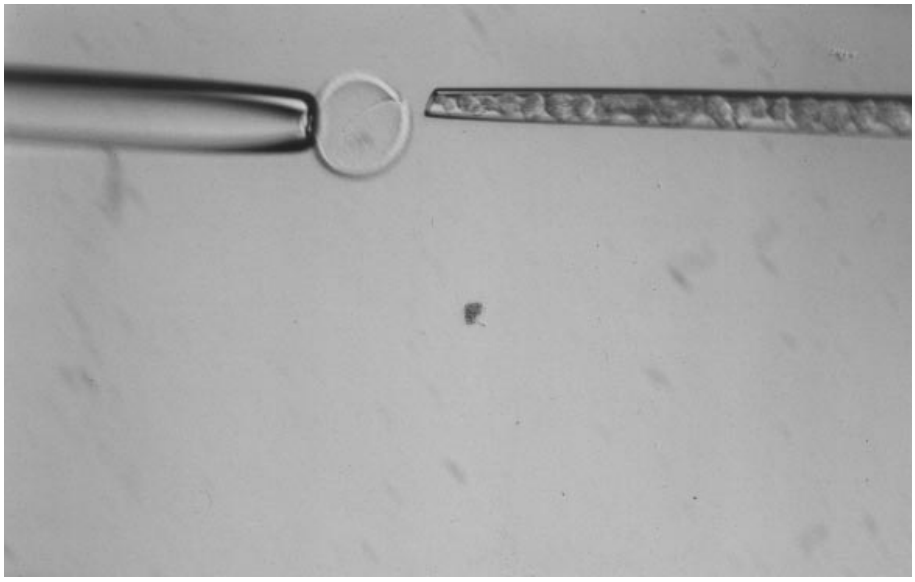

Figure 3a. Positioning the transfer pipette loaded with blastomeres to transfer them into an empty zonae pellucidae.

prepared from the same drops used to culture embryos until manipulation days by taking all the embryos out. Then, the culture plate was put into the incubator. After 12-24 h, 5 µl of heat treated FCS was added into the drops after filtration. Development of the embryos was checked daily.

Non-chimeric handled control embryos

As controls, day 4 embryos were first disaggregated as described above and then all non-damaged blastomeres derived from each embryo were transferred into their own empty zonae pellusidae. These embryos were cultured and controlled like chimeric embryos after reaggregation.

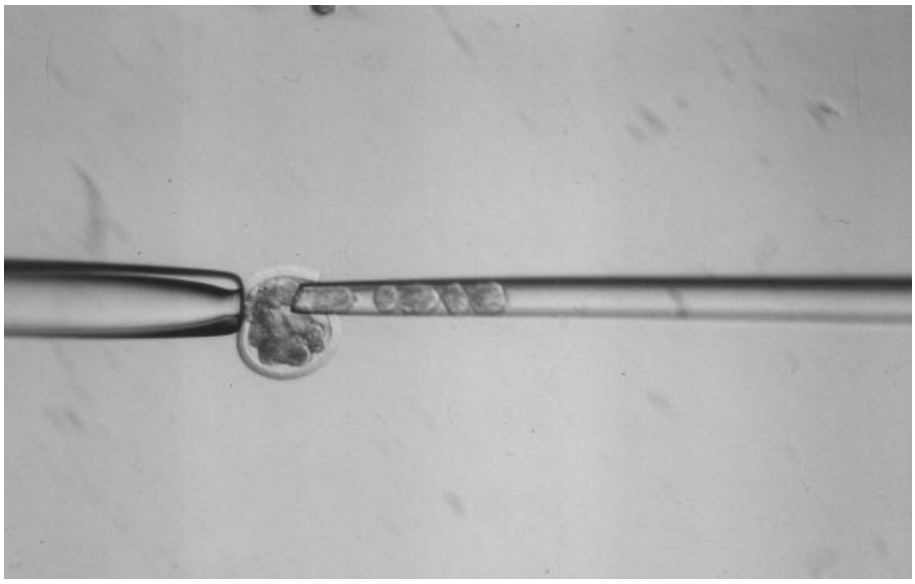

Figure 3b. Placing blastomeres into an empty zonae pellucidae.

Statistical analysis

The difference between the rates of blastocyst formation of chimeric and non-chimeric handled control embryos was analyzed using the Chi-square test of the SSPS Software Program (SSPS 10.0 for Windows). A difference was considered significant when $P < 0.05$.

Results

In this study, 5 replicates were performed and a total of 79 day 4 bovine embryos were used for disaggregation. Sixty-seven out of these were used to construct 27 chimeric embryos by reaggregating the disaggregated good quality blastomeres in empty zonae pellucidae. Of the 27 chimeric embryos, 11 reached the blastocyst stage (40.7%). The remaining 12 disaggregated embryos were used as controls, and 6 of these developed to the blastocyst stage (50.0%). Chimeric blastocyst stage bovine embryos developed from reaggregated embryos are shown in Figure 4, and the Table demonstrates that the rates of blastocyst formation of chimeric and non-chimeric handled control embryos were not significantly different.

Discussion

This study has demonstrated that micromanipulated day 4 bovine embryos, in which blastomeres had been disaggregated, pooled and then randomly reaggregated, formed blastocysts. Normally, the blastomere number of day 4 cattle embryos is approximately 16; however, it is not possible to catch all the embryos at the 16-cell stage at once (24,25). There was no statistical difference ($P > 0.05$) between the chimeric and non-chimeric handled control embryos in this study, although the rate of blastocyst formation of non-chimeric handled control embryos was higher than the rate of blastocyst formation of chimeric embryos. The lower development rate of chimeric embryos could be due to 2 important differences between the chimeric and non-chimeric handled control embryos used in this study. These 2 differences are probably the cell cycle synchronies of the blastomeres and the similarities and dissimilarities of blastomere cell surfaces at the molecular and morphological levels. Therefore, since the characteristics of the blastomere cell surface change considerably as an embryo develops, reaggregating day 4 embryos after disaggregation could have some harmful effects on development (16).

In addition, it was hypothesized that low rates of chimera development could be due to the combining of blastomeres of various sexes, because it is known that male cattle embryos cleave faster than female embryos (25,26). In this study, the best looking and most advanced day 4 embryos were selected. By selecting the most advanced day 4 embryos in terms of development, most embryos used in this study were assumably male. Therefore, we probably obtained a better development

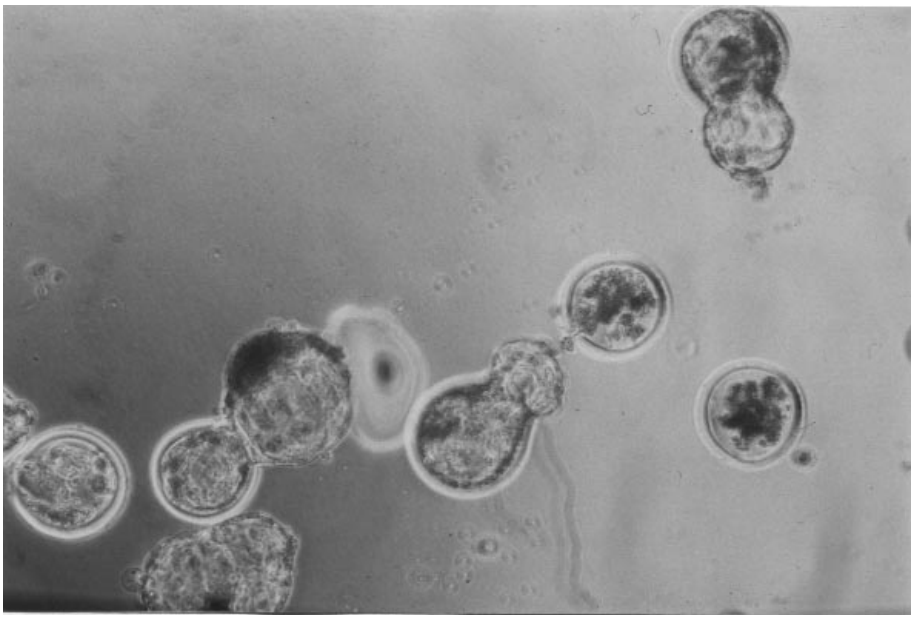

Figure 4. Chimeric blastocyst stage bovine embryos developed after reaggregation of blastomeres from day 4 embryos.

Table. Development of chimeric embryos to blastocyst stage.

Groups	Replicates	n	Blastocyst	%
Chimeras	5	27	11	40.7
Handled controls	5	12	6	50.0

n: numbers of reaggregated embryos

The difference between groups was not significant (P > 0.05)

rate by eliminating the combining of blastomeres of different sexes by choosing the most advanced day 4 embryos. In addition, it was reported that sex differences between the pairs of blastomeres did not affect the development rate of chimeric 4-cell embryos (16).

Blastomeres separated by at least one cleavage stage were successfully used to recombine for the production of live young in sheep (27) and rats (28). In addition, blastocyst stage embryos were obtained easily after recombination of bovine blastomeres separated by one cleavage after in vitro production (29,30). Rho et al. (16) reported that chimeric bovine embryos generated by reaggregating blastomeres from 4-cell stage embryos could develop to the blastocyst stage. They produced 95 chimeric embryos, and 22 of these (23.2%) developed into blastocysts. The blastocyst formation rate in our study (40.7%) was higher than this. One plausible explanation might be the difference between the developmental stages of the embryos used to produce chimeric embryos (24).

Sex chromosome chimeras or XX-XY chimeras can be generated by either method; that is, by reaggregating cleaving embryos of different sexes or by injecting some cells into opposite sex blastocysts. It was hypothesized that if more than 25% of cells in an XX-XY chimera are XY then the embryo develops as a male (31). Rho et al. (16) reported that they produced XX-XY chimeric bovine embryos by combining 2 male and 2 female blastomeres obtained from 4-cell stage embryos. Even with the combination of 1 male and 3 female blastomeres from 4-cell stage embryos, the hypothesis would not be tested because we could not obtain a ratio lower than 25% XY blastomeres. However, with a good development ratio, this study shows that it is possible to test this hypothesis by combining blastomeres of different sexes obtained from day 4 bovine embryos.

In conclusion, the possibility of producing chimeric cattle embryos by reaggregating day 4 embryos has been

demonstrated by this study. By using day 4 bovine embryos to produce chimeric embryos and live young, fundamental aspects of early embryogenic development, differentiation and sex determination could be effectively studied.

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