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Offspring Born From Chimeras Reconstructed From Parthenogenetic and In Vitro Fertilized Bovine Embryos

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ABSTRACT Chimeric embryos were produced by aggregation of parthenogenetic (Japanese Red breed) and in vitro fertilized (Holstein breed) bovine embryos at the Yamaguchi Research Station in Japan and by aggregation of parthenogenetic (Red Angus breed) and in vitro fertilized (Holstein breed) embryos at the St. Gabriel Research Station in Louisiana. After embryo reconstruction, live offspring were produced at each station from transplanting these embryos. The objective of this joint study was to evaluate the developmental capacity of reconstructed parthenogenetic and in vitro fertilized bovine embryos. In experiment I, chimeric embryos were constructed: by aggregation of four 8-cell (demi-embryo) parthenogenetic and four 8-cell stage (demi-embryo) IVF-derived blastomeres (method 1) and by aggregation of a whole parthenogenetic embryo (8-cell stage) and a whole IVF-derived embryo (8-cell stage) (method 2). Similarly in experiment II, chimeric embryos were constructed by aggregating IVF-derived blastomeres with parthenogenetic blsatomeres. In this experiment, three categories of chimeric embryos with different parthenogenetic IVF-derived blastomere ratios (2:6; 4:4, and 6:2) were constructed from 8-cell stage bovine embryos. In experiment III, chimeric embryos composed of four 8-cell parthenogenetic and two 4-cell IVF-derived blastomeres or eight 16-cell parthenogenetic and four 8-cell IVF-derived blastomeres were constructed. Parthenogenetic demi-embryos were aggregated with sexed (male) IVF demi-embryos to produce chimeric blastocysts (experiment IV). In the blastocyst stage, hatching and hatched embryos were karyotyped. In experiment V, chimeric embryos that developed to blastocysts (zona-free) were cryopreserved in ethylene glycol (EG) plus trehalose (T) with different concentrations of polyvinylpyrrolidone (PVP; 5%, 7.5%, and 10%). In experiment I, the aggregation rate of the reconstructed demi-embryos cultured in vitro without agar embedding was significantly lower than with agar embedding (53% for 0% agar, 93% for 1% agar, and 95% for 1.2% agar, respectively). The aggregation was also lower when the aggregation resulted from a whole parthenogenetic and IVF-derived embryos cultured without agar than when cultured with agar (70% for 0% agar, 94% for 1% agar, and 93% for 1.2% agar, respectively). The development rate to blastocysts, however, was not different among the treatments. In experiment II, the developmental rates to the morula and blastocyst stages were 81%, 89%, and 28% for the chimeric embryos with parthenogenetic:IVF blastomere ratios of 2:6, 4:4, and 6:2, respectively. In experiment III, the developmental rate to the morula and blastocyst stages was 60% and 65% for the two 4-cell and four 8-cell chimeric embryos compared with 10% for intact 8-cell parthenogenetic embryos and 15% for intact 16-cell parthenogenetic embryos. To verify participation of parthenogenetic and the cells derived from the male IVF embryos in blastocyst formation, 51 embryos (hatching and hatched) were karyotyped, resulting in 27 embryos having both XX and XY chromosome plates in the same sample, 14 embryos with XY and 10 embryos with XX. The viability and the percentage of zona-free chimeric embryos at 24 hr following cryopreservation in EG plus T with 10% PVP were significantly greater than those cryopreserved without PVP (89% vs. 56%). Pregnancies were diagnosed in both stations after the transfer of chimeric blastocysts. Twin male (stillbirths) and single chimeric calves were delivered at the Yamaguchi station, with each having both XX and XY chromosomes detected. Three pregnancies resulted from the transferred 40 chimeric embryos at the Louisiana station. Two pregnancies were lost prior to 4 months and one phenotypically-chimeric viable male calf was born. We conclude that the IVF-derived blastomeres were able to stimulate the development of bovine parthenogenetic blastomeres and that the chimeric parthenogenetic bovine embryos were developmentally competent. *Mol. Reprod. Dev. 53:159–170, 1999.* © 1999 Wiley-Liss, Inc.

Key Words: chimera; parthenogenetic; in vitro fertilization; cattle; offspring

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INTRODUCTION

Live offspring have occurred naturally in nonmammalian species from developing parthenogenetic embryos (White, 1978), and occasionally parthenogenetic embryos can be experimentally induced in invertebrates, and in some vertebrates (Nagy et al., 1979). Although parthenogenetically activated diploid mammalian embryos can develop in a normal pattern through the pre-implantation stage, these early conceptuses rarely reach the forelimb-bud stage (Kaufman et al., 1977). The diploid gynogenomes produced from fertilized mouse oocytes by suppression of the extrusion of the second polar body (Niemierko, 1975) or by subsequent removal of the male pronucleus (Modlinski, 1980; Borsuk, 1982) develop to the 25-somite stage, as do the genetically similar diploid parthenogenomes (Surani and Barton, 1983). Barra and Renard (1988) have reported constructing diploid mouse embryos by fusing late 2-cell stage haploid parthenogenomes with late 2-cell stage haploid androgenones. These reconstructed embryos, however, developed into normal-appearing offspring after they were transferred to recipient mice.

Pronuclear transfer experiments have shown that the parental genomes are not equivalent in supporting the embryogenesis in mice, and that both paternal and maternal genomes are required to support full term development of mouse embryos (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984). It has been shown that the paternal genome of the mouse was essential for the development of extra embryonic tissues and the maternal genome was essential for the embryogenesis (Barton et al., 1984; Surani et al., 1984). Genome imprinting during gametogenesis is thought to be responsible for the functional difference of parental genomes during embryogenesis (Surani et al., 1986, 1990).

Clear functional differences between the parental genomes of mammals have been revealed by demonstration of lethality in embryos of parthenogenetic, gynogenetic, and androgenetic uniparental genotypes in mice (Solter, 1988). Although mouse parthenogenomes are unable to complete embryogenesis in utero, diploid parthenogenomes are apparently capable of developing to chimeric adults when parthenogenetic cells contribute to embryonic tissues after they were aggregated with in vivo-derived mouse embryos (Stevens et al., 1977; Surani et al., 1977), and have been found to produce viable germ cells of parthenogenetic origin (Stevens, 1978; Anderegg and Markert, 1986). The cellular interaction between parthenogenetic and in vivo-derived blastomeres in chimeric embryos has been shown to be critical to embryo development (Surani et al., 1987a,b; Thomson and Solter, 1988, 1989).

Various methods have been evaluated for the induction of diploid parthenogenesis in bovine ova. The more effective methods include exposure to Ca^{++} ionophore A23187 (Ware et al., 1989), ethanol, electric stimulation or a combination of ethanol and electric stimulation (Yang et al., 1994). The interest in bovine parthenogenetic development has risen during recent years for both scientific and economic purposes. Parthenogenetic activation of bovine oocytes has been induced by a number of methods with relatively high activation rates (Nagai, 1987; Aoyogi et al., 1994; Boediono and Suzuki, 1994; Goto et al., 1994; Presicce and Yang, 1994). In recent years, parthenogenetic bovine embryos have been transferred to recipient females but no offspring have been produced. In one study, estrus did not occur until 48 days after transfer of single parthenogenetic embryos (Fukui et al., 1992). In another study, the return to estrus was delayed until 67 days after transfer of aggregated parthenogenetic embryos, with pregnancy not being maintained after this period (Boediono and Suzuki, 1994), likely do to premature death of the conceptus (Boediono et al., 1995).

The primary objective of this joint study was to evaluate the developmental capacity of fresh and frozen-thawed reconstructed bovine parthenogenetic embryos with blastomeres of in vitro fertilized bovine embryos. In a series experiments conducted at two research stations, we investigated (1) the effect of agar embedding for protection of aggregated embryos from disaggregation during culture in vitro, (2) the contribution of parthenogenetic cells to the blastocyst formation after aggregation with an IVF-derived embryo, (3) the freezing media for cryopreservation of zona-free chimeric parthenogenetic blastocysts, and (4) the viability of chimeric blastocysts produced by aggregation of diploid parthenogenetic and IVF-derived embryos and their development to term following transfer to the recipient females.

MATERIALS AND METHODS

The Yamaguchi Station

Parthenogenetic activation. The procedures for producing diploid parthenogenetic bovine embryos in experiments I, IV and V were previously reported (Boediono et al., 1995). In brief, oocytes were aspirated from cattle ovaries (Japanese Red breed) obtained from an abattoir and allowed to mature for 32 hr at 38.5°C under 5% CO_2 in air. Maturation medium consisted of Medium-199 (TCM-199; Gibco BRL, NY) supplemented with 5% (v/v) superovulated cow serum (SCS) (Boediono et al., 1994), 0.01 mg/ml follicle stimulating hormone (FSH, Denka, Kawasaki, Japan) and 50 μg/ml gentamicin sulfate (Sigma, St. Louis, MO). To induce parthenogenetic activation, mature oocytes were suspended in culture medium contained 7% ethanol for 10 min and then they were treated with 5 μg/ml cytochalasin-D for 5 hr to suppress polar body extrusion and to produce diploid parthenogenomes. The embryos were then washed and cultured in vitro on feeder layers of bovine cumulus cells (Goto et al., 1988; Zhang et al., 1992) for further development during a 48- to 72-hr interval after activation.

In vitro fertilization. Embryos allotted to experiments I, IV and V were produced by standard in vitro maturation, fertilization and culture procedures

(Boediono et al., 1994). Ovaries from dairy cows (Holstein) were collected from an abattoir one day later than the ovaries from cows where oocytes were to be used for parthenogenetic activation. Follicular oocytes were allowed to mature for 21 hr at 38.5°C under 5% CO_2 in air. Frozen-thawed sperm (Holstein) 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 5×10^6 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 hr. In vitro-matured oocytes were transferred into fertilization droplets for insemination (20–25 oocytes/droplet). After 18 hr of sperm exposure, oocytes were washed and transferred to a polystyrene dish (4-well multidish; Nunclon, Roskilde, Denmark) containing TCM-199 supplemented with 5% SCS, 5 μg/ml insulin (Wako, Osaka, Japan) and 50 μg/ml gentamicin sulfate for further development.

Embryo reconstruction. Aggregation chimeras in experiments I, IV and V were produced by the following methods: (1) aggregation of four 8-cell parthenogenetic blastomeres and four 8-cell IVF-derived blastomeres, and 2) by aggregation of a whole parthenogenetic embryo (8-cell stage) and a whole IVF-derived embryos (8-cell stage). Embryo reconstruction was executed on day 2 following insemination (day 0 = day of insemination). The procedure for opening the zona pellucida and insertion of blastomeres in method 1 was conducted as previously described by Tsunoda et al. (1986), with minor modifications. Embryo micromanipulation procedures were conducted under an inverted Nikon microscope with Narishige micromanipulation unit (Narishige Scientific Instrument, Tokyo, Japan). Briefly, a parthenogenetic 8-cell stage embryo was held by negative pressure with a holding pipette and a microblade was used to make a rent in the zona pellucida. The injection pipette (30 μm in diameter) was inserted through the opening to remove four blastomeres (half of the embryo). The IVF-derived blastomeres (four of the 8-cell stage) were inserted into the parthenogenetic embryo (four of the 8-cell stage) (Fig. 1).

Aggregation of the embryos in method 2 was achieved by moving two zona-free embryos (8-cell stage) together until the blastomere aggregate was sufficiently stable (Fig. 2). Aggregated embryos produced by methods 1 and 2 were then cultured in culture medium (0.5 ml) in a polystyrene 4-well multidish (Nunclon) with a feeder cell layer of bovine cumulus cells covered with mineral oil (1 embryo/well). Aggregated embryos produced by method 1 were cultured in vitro within the zona pellucida and the aggregated embryos produced by method 2 were cultured without the zona pellucida, as previously described (Boediono et al., 1993).

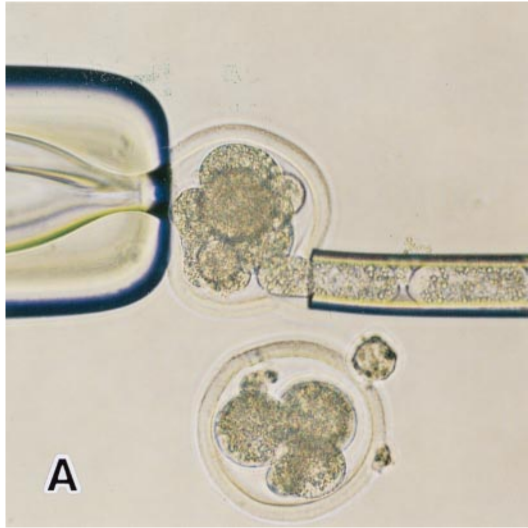
All embryo aggregates were rechecked after 6 hr of incubation to ensure that the cells had not drifted apart. Morphological evaluations were made at 12-hr intervals, with both unaggregated embryos and aggregated embryos with extruded blastomeres removed from the culture system. The chimeric embryos were then maintained in culture until day 9, and the origins of the different embryos that had developed into morphologically normal-appearing blastocysts were recorded.

The Louisiana Station

Parthenogenetic activation. The procedures for producing diploid parthenogenetic embryos in experiments II and III conducted at the St. Gabriel Research Station (Louisiana State University) were developed in this laboratory, and were similar to those reported by Boediono et al. (1994). Red Angus ovaries collected from a local abattoir were maintained in phosphate-buffered saline (PBS) with 5% fetal bovine serum (FBS) and 100 units/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) at room temperature and transferred to the laboratory within 6 hr. Cumulus-oocytes complexes were harvested from ovarian follicles 3–8 mm in diameter, using an 18-gauge needle. The oocytes were washed three times with TCM-199 and then cultured in TCM-199 with 10% FBS at 39°C in 5% CO_2 in an atmosphere of humidified air for 22 hr. Matured bovine oocytes were then treated with 7% ethanol in PBS medium for 10 min, and washed thoroughly with fresh PBS medium immediately after the ethanol treatment. These oocytes were then cultured in TCM-199 with 10% FBS, 10 μg/ml cytochalsin B (Sigma) and 10 μg/ml cyclohexmide (Sigma) at 39°C in 5% CO_2 in an atmosphere of humidified air for 16 hr. The oocytes were thoroughly washed at the end of the culture and then transferred into TCM-199 supplemented with 10% FBS on a granulosa cell monolayer.

In vitro fertilization. In experiment II and III ovaries of dairy cows (Holstein) were collected from a local abattoir were maintained in PBS with 5% FBS and 100 units/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) at room temperature and transferred to the laboratory within 6 hr. Cumulus-oocytes complexes were harvested with an 18-gauge needle from medium-size follicles (3–8 mm in diameter). The oocytes were washed three times with TCM-199 and then cultured in TCM-199 with 10% FBS at 39°C in 5% CO_2 in an atmosphere of humidified air for 22 hr. Frozen sperm cells (from a fertile Holstein bull) were thawed, washed with Brackett-Oliphant medium (B-O medium) supplemented with 5 mM caffeine and then capacitated by the treatment of 0.1 mM calcium ionophore A23187 for 1 min, as previously outlined by Zhang et al. (1992). Briefly, fertilization was conducted for 6 hr in droplets of 100 μl of B-O medium supplemented with 5 mM caffeine and 1% bovine serum albumin (BSA, Fraction-IV, Sigma). The fertilized oocytes were washed twice with TCM-199 and then cultured in TCM-199 supplemented with 10% FBS at 39°C in 5% CO_2 in an atmosphere of humidified air.

Granulosa cell monolayer co-culture. Bovine granulosa cells collected from the ovarian follicles



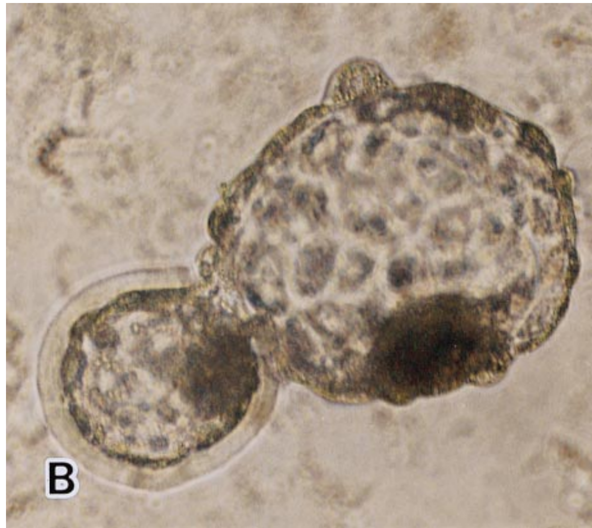
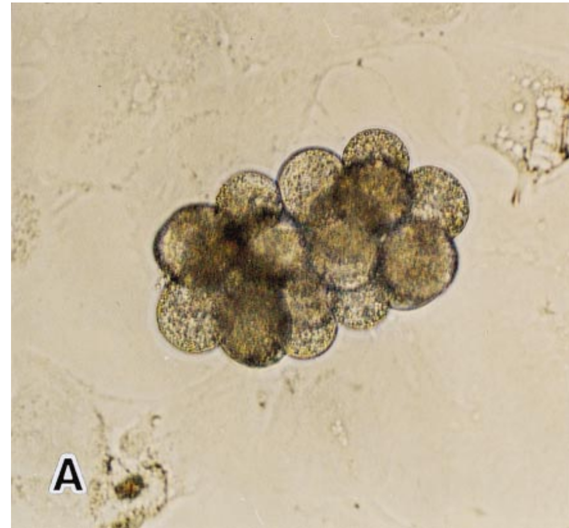


Fig. 1. Reconstructing an embryo produced by aggregation of four 8-cell parthenogenetic blastomeres and four 8-cell IVF-derived blastomeres (**A**). In vitro development of a reconstructed embryo to a hatching blastocyst stage at day 7 post-insemination (**B**).



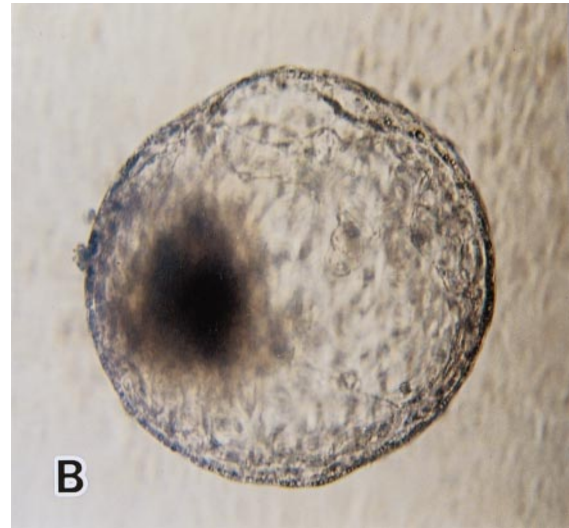


Fig. 2. A reconstructed embryo produced by aggregation of a whole parthenogenetic embryo (8-cell) and a whole IVF-derived embryo (8-cell) (**A**). In vitro development to a hatched blastocyst stage on day 7 post-insemination (**B**).

during oocyte aspiration were washed with TCM-199 supplemented with 10% FBS, and recovered by centrifugation at 500*g* for 5 min (Zhang et al., 1992). The cell pellet was suspended in TCM-199 with 10% FBS and then seeded on a 4-well culture plate (Nunc, France) in droplets of 50 µl TCM-199 supplemented with 10% FBS covered with equilibrated mineral oil. The granulosa cells were cultured at 39°C in 5% CO_2 in an atmosphere of humidified air. The culture medium was changed at 48-hr intervals, and the cells usually formed a confluent monolayer by day 2 of incubation.

Embryo reconstruction. In experiment II and III the zona pellucida of IVF-derived bovine embryos was removed by the treatment of acidic Tyrode's solution and subsequent treatment of 0.25% pronase in PBS. The bovine embryos were then separated into single blastomeres by gently pipetting the zona-free embryos through a fine glass pipette (40 µm in diameter). Micromanipulation was conducted in a microdroplet of PBS with 10% FBS and 10 µg/ml cytochalasin B with a pair of Leitz manipulator units under a Nikon inverted microscope. While a parthenogenetic embryo was held by a holding pipette (80 µm in diameter) via negative pressure, IVF-derived bovine blastomeres were then microinjected into the zona cavity of the parthenogenetic embryo by an injection pipette (30 µm in diameter). Some of the parthenogenetic blastomeres were subsequently removed after the microinjection by using the same injection pipette. The reconstructed embryos were carefully washed and then cultured in TCM-199 supplemented with 10% FBS on a granulosa cell monolayer for 4 days.

Experimental Design

Experiment I. Aggregated chimeras in experiment I were produced by the following methods: aggregation of

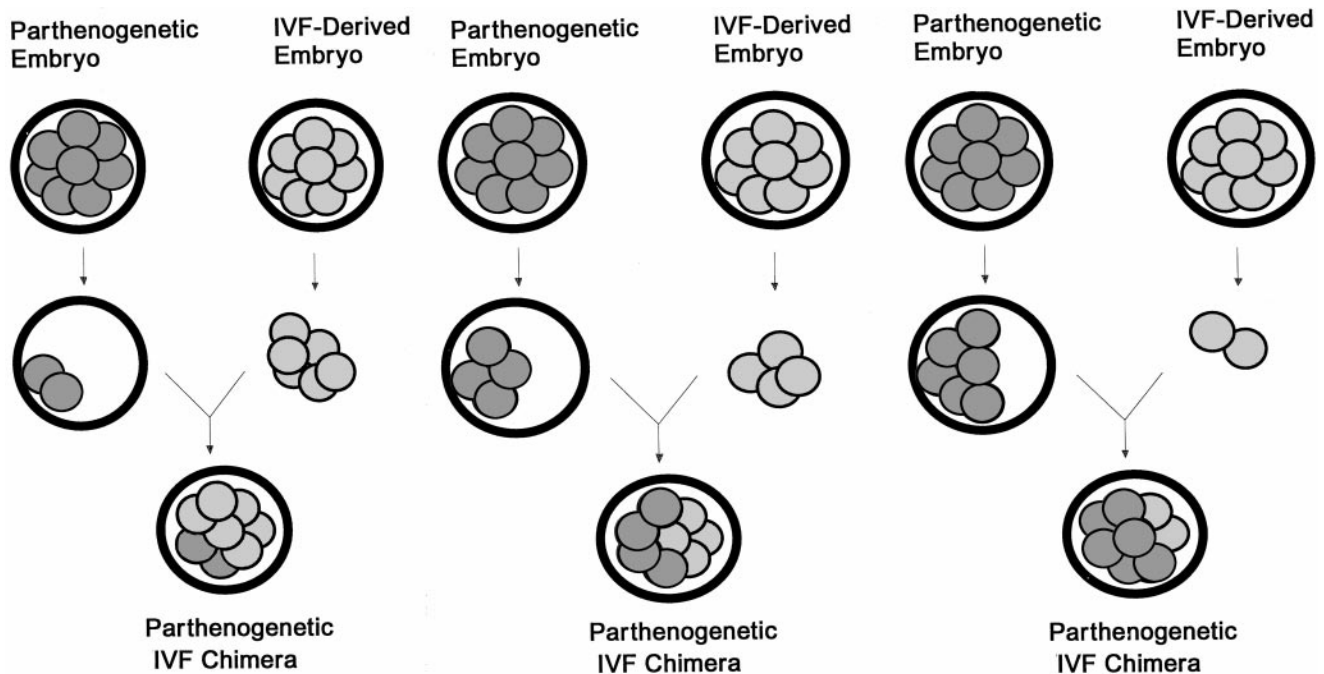


Fig. 3. Reconstruction of parthenogenetic and in vitro–fertilized bovine embryos in experiment II. First, a chimeric embryo reconstructed from two 8-cell parthenogenetic blastomeres and six 8-cell IVF-derived blastomeres (first panel), followed by a chimeric embryo reconstructed from four 8-cell parthenogenetic blastomeres and four 8-cell IVF-derived blastomeres (second panel) and then a chimeric embryo reconstructed from six 8-cell parthenogenetic blastomeres and two 8-cell IVF-derived blastomeres (third panel).

four 8-cell parthenogenetic blastomeres and four 8-cell IVF-derived blastomeres (method 1) and by the aggregation of a whole parthenogenetic embryo (8-cell stage) and a whole IVF-derived embryo (8-cell stage) (method 2). This experiment was designed to evaluate two different methods of embryo reconstruction and three concentrations of agar during embryo embedding (2×3 factorial arrangement) for the protection of aggregated embryos. Solutions of 0% (control), 1%, and 1.2% of agar (Difco) in 0.9% NaCl distilled water were prepared as the treatment groups in this experiment. The basic agar embedding procedure used in this study was that previously described by Willadsen (1979). While the respective agar solution was cooling, the aggregated embryos to be embedded were transferred to medium containing 5% SCS. When the agar had cooled from 39° to 37°C, 2 ml of the solution was poured into a petri dish (Falcon 1008, Becton Dickinson, Oxnard, CA) and then an aggregated embryo was immediately transferred to the agar solution with a fine bore Pasteur pipette. The agar containing the embryo was drawn and held in the tip of the pipette for ~30 sec and then expelled as a solid cylinder into the culture medium. This experiment was composed of five replicates that included 214 aggregated embryos.

Experiment II. To evaluate the effect of blastomeric interaction on the development of bovine parthenogenetic embryos, bovine IVF-derived blastomeres (Holstein breed) were microinjected into diploid bovine parthenogenetic embryos (Red Angus breed) to produce parthenogenetic chimeric embryos. Three categories of parthenogenetic/IVF chimeras were constructed: chimeric embryos with two 8-cell parthenogenetic and six 8-cell IVF-derived blastomeres (treatment B), chimeric embryos with four 8-cell parthenogenetic and four 8-cell IVF-derived blastomeres (treatment C) and chimeric embryos with six 8-cell parthenogenetic and two 8-cell IVF-derived blastomeres (treatment D) (Fig. 3). The intact 8-cell IVF-derived embryos and intact 8-cell parthenogenetic embryos prepared by the same micromanipulation procedure were used as the IVF-derived embryo culture control (treatment A) and parthenogenetic embryo culture control (treatment E). A total of 36 aggregated, parthenogenetic or intact embryos were allotted to respective treatments in three replications. Only viable appearing embryos (morphologically normal) were used in this experiment.

Experiment III. To evaluate if stage of morphological development (number of cell cycles) between the parthenogenetic and the IVF-derived blastomeres was necessary to induce a stimulatory effect of IVF-derived blastomeres on the development of parthenogenetic blastomeres, parthenogenetic and IVF-derived blastomeres at different developmental stages were combined to construct two combinations of aggregated embryos. The two aggregated combinations were chimeras composed of four 8-cell parthenogenetic and two 4-cell IVF-derived blastomeres (treatment B) (n = 40) and chimeras composed of eight 16-cell parthenogenetic and four 8-cell IVF-derived blastomeres (treatment C) (n = 40). Intact 8-cell parthenogenetic embryos (n = 40)

and 16-cell parthenogenetic embryos (n = 40) were used as the culture controls (treatments A and D).

Experiment IV. This experiment was designed to further evaluate the contribution of parthenogenetic cells to the blastocyst formation after aggregation with an IVF-derived embryo. Only the sexed (male) IVF embryos were aggregated with parthenogenetic embryos by one of two embryo reconstruction methods (method 1 or method 2, see experiment I) in an effort to produce aggregated chimeric embryos with long term developmental capabilities. The embryo sexing procedure used was that described previously by Itagaki et al. (1993). Briefly, to each of the tubes containing the samples (2–4 blastomeres from IVF-derived embryos), 100 ml of reaction mixture consisting of 10 mM Tris-HCl (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 0.1% sodium cholate, 0.1% Triton X-1000, 50 mM dNTPs, 0.2 mM DNA primer and 2 units of DNA polymerase were added, and then overlaid with 50 ml of mineral oil. The PCR amplification was performed with a DNA thermal cycler (TS-300, Iwaki Glass, Osaka, Japan) by 50 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min) and primer extension (72°C, 1 min). Ten microliter aliquots of amplified products were electrophoresed in 3% NuSieve 3:1 agarose (FMC BioProducts) gel in a Tris-borate-EDTA buffer.

After electrophoresis, the amplified fragments were visualized by ethidium bromide staining and ultraviolet illumination. To verify the participation of both the parthenogenetic and the IVF-derived embryonic cells in formation of the blastocyst, the aggregated embryos (hatching and hatched blastocysts) were karyotyped using the procedure described previously (Boediono et al., 1995). Briefly, the embryos were cultured with 0.04 μg/ml colcemid (Gibco) for 4 hr and suspended in 0.9% sodium citrate as a hypotonic solution for 20 min. Then, the embryos were fixed in a distilled water:acetic acid:methanol:sodium citrate solution (2:4:6:9) for 5 min followed by distilled water:acetic acid:methanol (1:2:3) for 1 to 2 min. The fixed embryos were placed on a glass slide and then a few drops of acetic acid were added. Chromosome preparations were stained in 5% Giemsa solution (Merck, Darmstadt) (pH 6.8) for 20 min and observed under phase contrast microscopy. The total number of cells/embryo were also evaluated using the same staining procedure.

Experiment V. This experiment evaluated several different freezing media for cryopreserving zona-free chimeric blastocysts. Blastocysts derived from aggregation of a whole parthenogenetic and a whole IVF-derived embryos were randomly allotted to one of the following treatment groups: treatment A included 1.8 M ethylene glycol (EG) + 0.05 M trehalose (T), treatment B 1.8 M EG + 0.05 M T + 5.0% PVP, treatment C 1.8 M EG + 0.05 M T + 7.5% PVP and similarly treatment D 1.8 M EG + 0.05 M T but with 10.0% PVP. The freezing procedures used in this experiment were similar to those previously described (Takagi et al., 1993), with minor modifications. To summarize, embryos were exposed to the cryoprotectant for 5 min at room temperature. Following this exposure, 2–5 embryos were loaded into 0.25 ml plastic straws. The loaded straws were then placed into a programmable freezer (ET-1, Fujihira, Japan) maintained at 0°C and held for 2 min. The embryos were cooled from 0° to –7°C at the rate of 1°C/min, seeded at –7°C with supercooled forceps, and then held at this temperature for 10 min. After seeding, the straws were cooled at rate of 0.3°C/min to –30°C, and then immediately plunged into liquid nitrogen. After 7–21 days of storage, embryos were thawed in a 30°C in water bath. The cryoprotectant was removed by a direct method (Suzuki et al., 1993), by washing the embryos several times in culture medium and cultured on feeder layers of bovine cumulus cells. Embryos were then evaluated microscopically at 24 hr post-thaw. A total of 113 zona-free aggregated blastocysts were allotted to respective treatments in four replications of this experiment.

Transfer of Reconstructed Embryos

At the Yamaguchi station, aggregated embryos produced by either aggregation of four 8-cell parthenogenetic and four 8-cell male IVF-derived blastomeres (n = 7) or the aggregation of a whole parthenogenetic with a whole IVF-derived embryos (n = 3) were nonsurgically transferred to the six recipient females (Holstein breed) (1–2 embryos/female) on day 8 (estrus = day 0) of the estrous cycle. One of the recipient females received a single frozen-thawed embryo cryopreserved using the 1.8 M EG plus 0.05 M T and 10% PVP treatment. Correspondingly, at the Louisiana station, a total of 40 co-cultured aggregated embryos from experiment III were nonsurgically transferred to 14 mixed breed beef recipient animals (2–4 embryos/female) either on day 7 or 8 of the estrous cycle. Pregnancy was diagnosed by ultrasonic scanning 40–45 days after embryo transfer and again by rectal palpation and/or ultrasound scanning at 60–65 days post-transfer.

Statistic Analysis

Combined effects of the aggregation methods and/or concentration of the agar embedding were evaluated using a 2×3 factorial arrangement (experiment I). The percentage of the embryo developing to <16 cells, morula and blastocyst stages (experiment II and III) and the post-thaw re-expansion of embryos following cryopreservation (experiment V) were compared by Chi-square analysis. The mean number of cells per embryo (experiment IV) was evaluated across treatment groups using analysis of variance (ANOVA). Differences at a probability value P of 0.05 or less was regarded as significantly different.

RESULTS

Experiment I

The number of agar-embedded and nonembedded aggregated embryos that developed to the blastocyst

TABLE 1. Development of Agar Embedded and Nonembedded Aggregated Bovine Embryos Culture In Vitro (Experiment I)

Aggregation method[b]	n	Embryo composition[a] Partho	IVF	Agar %	No. aggregated[c]	No. of blastocysts
Method 1	36	4/8	4/8	0%	19 (53%)*	16 (84%)
	42	4/8	4/8	1.0%	39 (93%)**	36 (92%)
	38	4/8	4/8	1.2%	36 (95%)**	32 (89%)
Method 2	37	8/8	8/8	0%	26 (70%)*	24 (92%)
	33	8/8	8/8	1.0%	30 (94%)**	32 (97%)
	28	8/8	8/8	1.2%	26 (93%)**	23 (88%)

[a]Partho = pathenogenetic blastomeres; IVF = in vitro-fertilized blastomeres.
[b]Method 1: aggregation of four 8-cell parthenogenetic blastomeres and four 8-cell IVF-derived blastomeres. Method 2: aggregation of a whole parthenogenetic embryo (8-cell stage) with a whole IVF-derived embryo (8-cell stage).
[c]Values within columns with different superscripts are significantly different ($P < 0.05$).

TABLE 2. In Vitro Development of Aggregated Bovine Parthenogenetic/IVF-Derived Embryos After 4 Days in Co-Culture (Experiment II)

Treatment group	n	Embryo composition[a] Partho	IVF	Development (%)[b] <16 cell	Morula/Blastocyst
A	36	—	8/8	8 (22%)*	28 (78%)*
B	36	2/8	6/8	7 (19%)*	29 (81%)*
C	36	4/8	4/8	4 (11%)*	32 (89%)*
D	36	6/8	2/8	26 (72%)**	10 (28%)**
E	36	8/8	—	42 (89%)**	4 (11%)**

[a]Partho = parthenogenetic blastomeres; IVF = in vitro-fertilized blastomeres.
[b]Values within columns with different superscripts are significantly different ($P < 0.05$).

TABLE 3. In Vitro Development of Aggregated Bovine Parthenogenetic/IVF-Derived Embryos After 4 Days in Co-Culture (Experiment III)

Treatment group	n	Embryo composition[a] Partho	IVF	Development (%)[b] <16 cell	Morula/Blastocyst
A	40	8/8	—	36 (90%)*	4 (10%)*
B	40	4/8	2/4	16 (40%)**	24 (60%)**
C	40	8/16	4/8	14 (35%)**	26 (65%)**
D	40	16/16	—	34 (85%)*	6 (15%)*

[a]Partho = parthenogenetic blastomeres; IVF = in vitro-fertilized blastomeres.
[b]Values within columns with different superscripts are significantly different ($P < 0.05$).

stage is shown in Table 1. The aggregation rate for embryos that were produced by method 1 and cultured in vitro without agar embedding was less ($P < 0.05$) than when similar embryos were agar embedded (19/36, 53% for 0% agar vs. 39/42, 93% for 1% agar, and 36/38, 95% for 1.2% agar, respectively). With reconstructed embryos produced by method 2 and cultured without agar there was less aggregation ($P < 0.05$) than when embryos were cultured with agar (26/37, 70% for 0% agar vs. 30/33, 94% for 1% agar, and 26/28, 93% for 1.2% agar, respectively). In contrast, the developmental rate to the blastocyst stage was not different among the treatments when evaluated using the number of aggregated embryos allotted per group.

Experiment II

After 96 hr of in vitro culture, the reconstructed embryos with more than 4 parthenogeneticly-derived blastomeres (treatments D and E) had significantly more embryos ($P < 0.05$) developing prior to the 16-cell stage (72% and 89%, respectively) than those with 4 or less parthenogeneticly-derived blastomeres (22%, 19%, and 11%, respectively) (treatments A, B, and C) (Table 2).

In contrast, 81% of the parthenogenetic/IVF chimeras composed of two 8-cell parthenogenetic and six 8-cell IVF-derived blastomeres in treatment B developed to morula and blastocyst stages, and 89% of the chimeric parthenogenetic/IVF chimeras composed of four 8-cell parthenogenetic and four 8-cell IVF-derived blastomeres in treatment C developed to morula and blastocyst stages, while only 28% of the parthenogenetic/IVF chimeric embryos composed of six 8-cell parthenogenetic and two 8-cell IVF-derived blastomeres in treatment D the reached morula or blastocyst stage in vitro. Overall, the developmental rates to morula and blastocyst stages for aggregated embryos composed of 4 or more IVF-derived blastomeres (treatments B and C) were similar (78%) to that of the IVF-derived control embryos (treatment A). Correspondingly, a greater ($P < 0.05$) number of aggregated embryos with 4 or more IVF-derived blastomeres (treatments A, B, and C) developed to morulae and blastocysts during culture than did those of aggregated embryos with only 2 IVF-derived blastomeres (treatment D) (28%) and those of the parthenogenetic control group (treatment E) (11%).

Experiment III

Out of 40 of the chimeric embryos composed of four 8-cell parthenogenetic and two 4-cell IVF-derived blastomeres, 60% developed to the morula or blastocyst stage, while only 10% of the intact 8-cell parthenogenetic embryos reached morula and blastocyst stages of development ($P < 0.05$) (Table 3). Of 40 of the chimeric

TABLE 4. Karyotyping of Reconstituted Embryos After Transferring Male IVF-Derived Blastomeres to Parthenogenetic Bovine Embryos (Experiment IV)

Method of reconstruction[a]	No. of embryos	Sex chromosomes			Total cell no./embryo[b]
		XX + XY	XY	XX	
Method 1	28	15 (54%)	8 (29%)	5 (18%)	158 ± 65*
Method 2	23	12 (52%)	6 (26%)	5 (22%)	202 ± 32**
Total	51	27 (53%)	14 (27%)	10 (20%)	

[a]Method 1: aggregation of four 8-cell parthenogenetic blasromeres and four 8-cell IVF-derived blastomeres. Method 2: aggregation of a whole parthenogenetic embryo (8-cell stage) with a whole IVF-derived embryo (8-cell stage).
[b]Values within columns with different superscripts are significantly different ($P < 0.01$).

TABLE 5. Development of Zona-Free Chimeric Blastocysts 24 hr Following Cryopreservation in 1.8 M Ethylene Glycol Supplemented with 0.05 M Trehalose and Different Concentration of Polyvinylpyrrolidone (PVP) (Experiment V)*

Concentration of PVP (%)	No. of embryos/ group	No. of viable embryos at 24 hr (%)
0%	32	18 (56%)[a]
5%	27	18 (67%)[A]
7.5%	27	20 (74%)
10%	27	24 (89%)[bB]

*Values within a column with different superscripts are significantly different ([a,b]$P < 0.01$; [A,B]$P < 0.05$).

embryos composed of eight 16-cell parthenogenetic and four 8-cell IVF-derived blastomeres, 65% developed to morula or blastocyst stage, while only 15% of the intact 16-cell parthenogenetic embryos reached morula or blastocyst stage ($P < 0.05$).

Experiment IV

Chimeric embryos produced either by aggregation of four 8-cell parthenogenetic and four 8-cell male IVF-derived embryos (n = 28) or by aggregation of a whole parthenogenetic and IVF-derived embryos (n = 23) were karyotyped. All embryos used in this experiment produced between four to 20 metaphase plates for evaluation. Karyotyping of aggregated embryos resulted in 27 embryos having XX and XY chromosome plates resulting from the same embryo, 14 embryos with XY and 10 embryos with XX chromosome plates (Table 4). The mean number of cells per blastocyst (± SE) of the aggregated embryos produced by method 1 was less ($P < 0.01$) than those produced by method 2 (158 ± 65 vs. 202 ± 32).

Experiment V

The percentage of zona-free chimeric embryos that re-expanded 24 hr following cryopreservation in ethylene glycol and trehalose with 10% PVP was significantly greater ($P < 0.01$) than those similarly cryopreserved without PVP (89% vs. 56%) (Table 5). Significantly more aggregated embryos cryopreserved in ethylene glycol and trehalose ($P < 0.05$) with 10% PVP re-expanded 24 hr following cryopreservation than those similarly cryopreserved in ethylene glycol and trehalose with 5% PVP (89% vs. 67%).

Transfer of Chimeric Parthenogenesis/IVF-Derived Embryos

At the Yamaguchi station, pregnancies were diagnosed in two recipient dairy females after transfer of the chimeric embryos (one or two embryos/female) produced by aggregation of four 8-cell parthenogenetic and four 8-cell IVF-derived blastomeres (Table 6). After 60 days of gestation, one recipient female (receiving two aggregated day-7 blastocysts) lost her remaining fetus. Twin male calves (stillbirths) were delivered at 234 days of gestation from another recipient female receiving two aggregated day-7 blastocysts. Both XX and XY chromosome plates (originating from white blood cells) were detected from each stillbirth calf (Fig. 4). One of two recipient females was diagnosed pregnant after transferring chimeric embryos (one or two embryos/female) produced by aggregation of a whole parthenogenetic and a whole IVF-derived embryos. A single male calf, with XX and XY chromosome plates (detected from white blood cells), was delivered at 261 days of gestation. Skin pigment color and hair color pattern were both used as phenotypic confirmation of embryo chimerism. The black and white color pattern on the abdomen originated from the Holstein breed (IVF-derived embryo), while the Red color pattern on the distal portion of right hind leg and the scapula region of the left front leg most likely originated from the Japanese Red breed (parthenogenetically-derived embryo) (Fig. 5).

At the Louisiana station, 40 aggregated parthenogenetic/IVF morulae and blastocysts were transferred to 14 naturally synchronized recipient beef females (2–4 embryos/female) at the end of the culture interval. Three of the recipients were pregnant by ultrasonic examination on days 45–60 after transfer. Two of the pregnancies (with one fetus each) had a strong heart beat, and another had an irregular heart beat. Two of these pregnancies were lost after day 60 of gestation. These conceptuses were derived from chimeric embryos composed of four 8-cell parthenogenetic and four 8-cell IVF-derived blastomeres (experiment II). The remaining pregnancy was derived from chimeric embryos with four 8-cell parthenogenetic and two 4-cell IVF-derived blastomeres (experiment III). At 273 days of gestation,

TABLE 6. Summary of Transfers of Reconstructed Embryos Produced by Aggregation Between Parthenogenetic and IVF-Derived Embryos

Embryo recipient[a]	No. of embryos transferred	Morphological stage of the embryo	Diagnosis	Sex of offspring	Chromosome plates
Method 1					
A	2	Blastocyst (day 7)	Pregnant[b]	—	—
		Hatching (day 7)			
B	2	Blastocyst (day 7)	Pregnant	Male	XX + XY
		Hatching (day 7)		Male	XX + XY
C	1	Hatched (day 9)	Not pregnant	—	—
D	2	Hatched (day 8)[c]	Not pregnant	—	—
		Hatched (day 8)[c]			
Method 2					
E	2	Hatching (day 8)	Not pregnant	—	—
		Hatched (day 8)			
F	1	Hatched (day 8)[c]	Pregnant	Male	XX + XY

[a]Method 1: aggregation of an IVF-derived demi-embryo (8-cell stage) with parthenogenetic demi-embryo (8-cell stage). Method 2: aggregation of a whole IVF-derived embryo (8-cell stage) with a whole parthenogenetic embryo (8-cell-stage).
[b]Conceptus no longer present after 60 days.
[c]Frozen-thawed embryo.

this pregnancy produced a live, viable male calf (Fig. 6). Similarly, this calf had a chimeric phenotypic coat color pattern with black and white from the Holstein IVF-derived embryo and red brindle hair coat pattern on the lower shoulder and the abdominal/flank regions indicating parthenogenetic input from the Red Angus breed.

DISCUSSION

In an effort to protect the developing embryos, agar embedding procedures have been used for freezing of bovine demi-embryos (Rorie et al., 1987; Picard et al., 1988) and bovine nuclear transfer embryos (Wolfe and Kraemer, 1992). Whether agar embedding would be beneficial in enhancing pure parthenogenetic embryo development remains to be evaluated.

Results from the first experiment (experiment I) did show that the agar embedding method was useful for protection of aggregated embryos from disaggregation. The overall embryo developmental rate in this study however, was not affected by the presence of agar. A double-layer of agar embedding is usually used for in vivo culture of micromanipulated embryos, to protect them from disaggregation and from adhering to oviductal tissue of the incubator female (Willadsen, 1979). Our results indicate that a single layer embedding method using a 1% concentration of agar would be sufficient for protection of aggregated bovine embryos from disaggregation during in vitro culture.

The in vitro development of pre-implantation parthenogenetic bovine embryos is generally low (Aoyagi et al., 1994; Goto et al., 1994; Presicce and Yang, 1994) and the exact cause of this markedly reduced in vitro development remains unclear. It has been proposed that genomic imprinting during gametogenesis may be responsible for the limited developmental capacity of the parthenogenetic mouse embryos (Barton et al., 1984; Surani et al., 1990). 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; McGrath and Solter, 1984; Surani et al., 1984), and to produce viable germ cells in chimeric adult mice (Stevens, 1978; Anderegg and Markert, 1986).

The results of experiments II and III in this study indicate that the bovine blastomeres of IVF-derived embryos were able to stimulate the development of parthenogenetic blastomeres in aggregated embryos, and this stimulatory effect could take place as early as the 4-cell stage during pre-implantation embryo development. Furthermore, the number of IVF-derived blastomeres in the aggregated embryos appeared to be closely related to the developmental capacity of the reconstructed bovine embryos. Four or more, IVF-derived bovine blastomeres were apparently needed to enhance the in vitro developmental capacity of the parthenogenetic bovine embryos to the level of the IVF-derived embryos. It was also noted that as few as two IVF-derived blastomeres per embryo were capable of partially enhancing the development of the chimeric parthenogenetic embryos. Pregnancies from the transferred chimeric parthenogenetic embryos imply that these embryos were developmentally competent at relatively early stages of in vitro development.

It is presently unknown how the stimulatory effect of the IVF-derived bovine blastomeres is mediated in these reconstructed embryos. The growth factors produced by the IVF-derived bovine blastomeres (Watson et al., 1992) might have played a role in the enhanced development of the aggregated parthenogenetic bovine embryos in the present study. It should not be overlooked that developing intercellular junctions between blastomeres of different origins might play a role in communication and subsequently enhanced development of the chimeric parthenogenetic embryos (Ducibella and Anderson, 1975; Ducibella et al., 1975).

The results of the present study indicate that parthenogenetic bovine embryos were stimulated by the IVF-

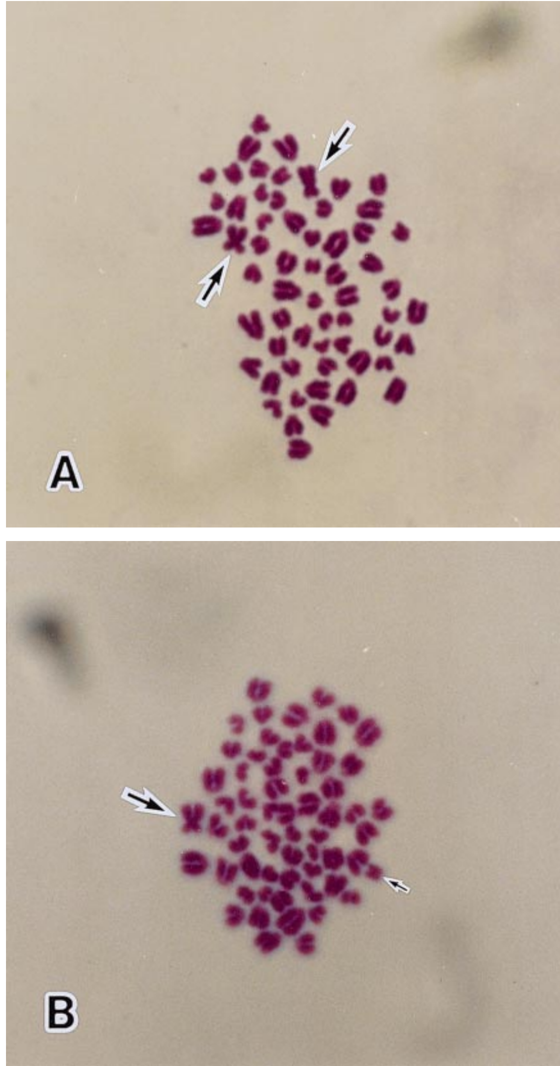


Fig. 4. Metaphase spreads of an embryo transfer chimeric calf (male) produced by aggregation of a whole parthenogenetic embryo (8-cell) and a whole IVF-derived embryo (8-cell): (**A**) 60, XX and (**B**) 60, XY. Long arrow = X chromosome, short arrow = Y chromosome.

Fig. 5. A chimeric male calf resulting from the transfer of a frozen-thawed embryo originally produced by aggregation of a whole 8-cell parthenogenetic embryo (Japanese Red) and a whole IVF-derived 8-cell embryos (Holstein). Arrow = a red color pattern originating from a parthenogenetic embryo.

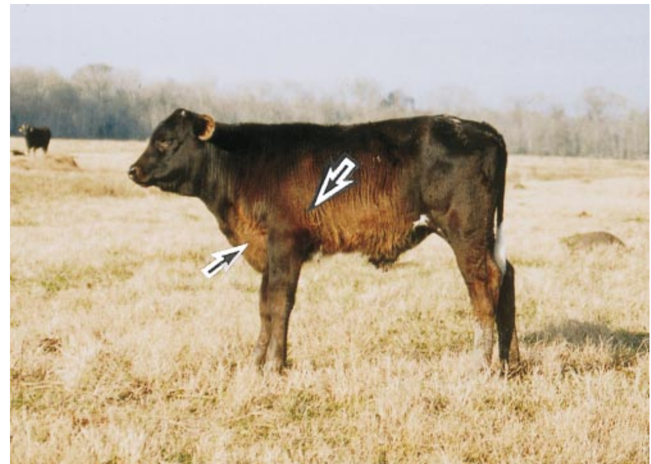


Fig. 6. Chimeric male calf produced by aggregation of four 8-cell parthenogenetic blastomeres (Red Angus) and two 4-cell IVF-derived blastomeres (Holstein) and transferred to a beef recipient female. Arrow = a red color pattern originating from the parthenogenetic embryo.

derived bovine blastomeres over a range of developmental stages in pre-hatched and post-hatched embryos. Indications are that the less advanced IVF-derived bovine blastomeres were capable of enhancing development of the more advanced parthenogenetic bovine blastomeres.

In the mouse, fetal development of parthenogenetic conceptuses is most often restricted by the lack of development beyond the implantation stage (Kaufman, 1983). The life span of parthenogenetically activated cells has been reported to be extended when chimeras were made from parthenogenetic and in vivo-fertilized embryos (Stevens et al., 1977; Stevens, 1978). However, the contribution of parthenogenetic cells to chimeras is generally considered low, representing not more than 20% of the total population of cells in the chimeric mouse (Surani et al., 1977). The survival and integration of parthenogenetic cells, in such chimeras, are probably influenced to a considerable extend by environmental conditions that are mediated by the cells from the IVF-derived embryo. There is evidence for metabolic cooperation between genetically diverse cell types through permeable cell junctions that enable metabolically deficient cells to function in a normal manner (Pitts and Burk, 1976). Similar interactions between parthenogenetic and IVF-derived blastomeres might enhance parthenogenetic cells in the reconstructed embryo.

To verify the participation of both parthenogenetic and IVF-derived embryonic cells in blastocyst formation, aggregated embryos (hatching and hatched blastocysts) were karyotyped. The karyotyped embryos in experiment IV resulted in 53% of the embryos having

XX and XY chromosome plates in the same embryo sample, 27% of the embryos had XY and 20% of the embryos had XX chromosome plates. These findings indicate that the XX chromosomes originated from parthenogenetic blastomeres and the XY chromosomes originated from the IVF-derived blastomeres. These results further indicate that both parthenogenetic and IVF-derived cells can contribute to embryo and conceptus development, although the distribution rate of these different cell types could not be analyzed using this procedure.

In the earliest reports of chimeric mice births, it was evident that the sex ratio was heavily biased in favor of the male, with intersexes relatively uncommon. This may be due to the gene for H-Y antigen production located on the Y chromosome, which could influence the development of the undifferentiated gonad. In sheep, Tucker et al. (1974) reported that two XX-XY chimeras' offspring were male phenotypes at birth. In a subsequent report (Fehilly et al., 1984), 26 of the 36 reconstructed-embryo lambs born had a similar visible male pattern. In the present study, all calves born were phenotypically males with XX-XY chromosome plates. The results further suggest that when multiple-sex are used to make chimeric embryos, the male component exerts a marked effect on sexual differentiation, and consequently most chimeric embryos will develop as phenotypic males.

Embryos frozen in ethylene glycol can be rehydrated directly in a holding medium without step-wise dilution of cryoprotectant (Suzuki et al., 1993). In a subsequent report (Suzuki et al., 1995), a low concentration of trehalose was found to be the most beneficial for the cryopreservation of zona-free blastocysts. The presence of trehalose is thought to reduce osmotic shock while the zona-free blastocysts are suspended in the holding medium for rehydration. Also, Leibo and Oda (1993) have reported that PVP was effective in protecting mouse embryos during the freezing procedure. In experiment V, along with ethylene glycol (cryoprotectant) and trehalose (sugar, as a nonpermeating agent), a macro molecular component (PVP) was evaluated. Since the mechanism of protection of the large polymer, PVP (m.w. = 30,000) is not clear, it is suggested that this substance coats the cells immediately following thawing, giving them physical protection against osmotic stressors. Optimum viability of cryopreservation of zona-free chimeric blastocysts in the present study was obtained when the embryos were cryopreserved in 1.8 M ethylene glycol and 0.05 M trehalose with 10% PVP.

To our knowledge, this is the first report describing parthenogenetic cells derived from cattle developing to term in utero, and their contribution to the production of live chimeric offspring. The results of this study indicate that chimeric blastocysts can be produced by aggregation of blastomeres, demi-embryos or whole embryos obtained from both parthenogenetic and IVF-derived embryos.

In an attempt to increase the participation of parthenogenetic cells in the aggregated embryo, inserting blastomeres obtained from parthenogenetic embryos at an advanced stage (16-cell stage) into IVF-derived embryos at a less advanced stage (4-cell stage) is continuing. The idea is that the more advanced blastomeres (from the parthenogenetic embryo) may contribute to the inner cell mass (ICM) and the less advanced blastomeres (from the IVF-derived embryo) would develop into the trophectoderm (see reviews by Kelly et al., 1978; Godke and Rorie, 1993). As a consequence, this approach could produce live viable transplant calves from parthenogenetic cells.

From the results of the present study, we conclude that the IVF-derived bovine blastomeres were able to enhance the development of the parthenogenetic bovine blastomeres in chimeric embryos, and that the chimeric parthenogenetic bovine embryos can be developmentally competent.

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