Production of Chimera by Embryos Aggregation Techniques in Bovine - Review -

T. Suzuki

Department of Veterinary Sciences, Yamaguchi University, Yamaguchi 753-8515, Japan

ABSTRACT : A tetraparental chimeric bull was successfully produced by aggregating bovine IVF embryos of F1 (female Holstein× male Japanese Black) and F1(female Japanese Brown x male Limousin) and culturing in vitro without the zona pellucida at Yamaguchi Research Station in Japan. In the microsatellite genotyping, 12% (28/228) microsatellite primer sets ware potentially useful for this parentage analysis in the chimeric bull, 78.6% (22/28) of microsatellite present in the chimeric bull were uniquely contributed from the Japanese Black and 21.4% (6/28) from Limousin. This chimeric bull semen was used in producing IVF embryos. The chromosome preparations were made from peripheral lymphocytes. Based on chromosome analysis the Chimera had apparently normal chromosomes (29 acrocentric pairs, one large sub metacentric X chromosome and one small sub metacentric Y chromosome). The proportion of acrosome reacted spermatozoa after 1 h of incubation was higher ($p<0.01$) with the Chimera than with the Holstein and in Japanese Brown bulls. But did not differ from Japanese Black and Limousin bull sperm. Fertilization rates observed after 5 h of sperm-oocyte incubation with Chimera sperm were higher ($p<0.05$) than with Japanese Brown and ($p<0.01$) than with Holstein sperm, but did not differ from Japanese Black and Limousin sperm. The cleavage rates of IVF oocytes inseminated with Chimera sperm were also higher ($p<0.001$) compared with Holstein, ($p<0.01$) Japanese Brown and ($p<0.05$) Limousin, but did not differ from Japanese Black sperm. The blastocyst rates of IVM oocytes inseminated with sperm were higher ($p<0.05$) than in Limousin, Japanese Brown and Holstein, but did not differ from Japanese Black. Chimeric cattles were produced by aggregation of parthenogenetic (Japanese Brown) and in vitro fertilized (Holstein) bovine embryos at the Yamaguchi Research Station in Japan and by aggregation of parthenogenetic (Red Angus) and in vitro fertilized (Holstein) embryos at the St. Gabriel Research Station in Louisiana. The aggregation rate of the reconstructed demi-embryos cultured in vitro without agar embedding was significantly lower than with agar embedding. The aggregation was also lower when the aggregation resulted from a whole parthenogenetic and IVF-derieved embryos cultured without agar than when cultured with agar. The development rate to blastocysts, however, was not different among the treatment. To verify parthenogenetic and the cells derieved from the male IVF embryos in blastocyst formation, 51 embryos 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 h following cryopreservation in EG plus T with 10% PVP were significantly greater than those cryopreserved without PVP. Pregnancies were diagnosed in both stations after the transfer of chimeric blastocysts. Twin male and single chimeric calves were delivered at the Yamaguchi station, with each having both XX and XY chromosomes detected. Three pregnancies resulted from the transfer of 40 chimeric embryos at the Louisiana station. Two pregnancies were lost prior to 4 months and one phenotypically chimeric viable male born. *(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 8 : 1188-1195)*

Key Words : Chimera, Aggregation, Parthenogenetic. *In vitro* fertilization, Bovine

INTRODUCTION

Chimeras have become important in embryological and developmental biology studies (McLaren, 1976). They are produced in mice by aggregating the embryos without zona pellucida (Mintz, 1971). In cattle, chimeras are produced by aggregating bisected embryos within the zona pellucida (Brem et al., 1984), and by micro-injection of inner cell mass into the blastocyst (Picard et al., 1990; Summers et al., 1983). Tetraparental chimeric cattle were successufully produced by aggregating bovine IVF embryos of F1 (Holstein×Japanese Black) and F1 (Japanese Brown× Limousin) followed by culture, in vitro without the zona pellucida (Boediono et al., 1993). Sumantri C et al. (1996) investigated the presence of genetic markers that uniquely exist in the parental genomes, in the chimeric cattle in order to estimate the degree of chimerism in the cattle, and to determine red blood cell types of the cattle. Sperm from individual bulls and rams differ in their ability to fertilize matured oocytes in vitro and support embryo development to the preimplantation stage (Iritani et al., 1986; Fukui et al., 1988; Leibfried-Rutledge et al., 1987). Reproduction of interspecific chimera mice between *M. caroli* and *M. musculus* have been reported (Rossant and Frels, 1980) and between sheep and goat (MacLaren et al., 1993). The ability of tetraparental bovine chimera sperm to fertilize matured oocytes and support further embryo development in vitro has also been reported (Sumantri et al., 1997a, b).

Parthenogenetic development of embryos to yield live offspring occurs naturally, in many non-mammalian species (White, 1978) and it can also be induced experimentally in non-mammalian on vertebrates and invertebrates (Nagy et al., 1979). Although parthenogenetically activated diploid mammalian embryos developed normally through preimplantation stage, they rarely reach the forelimb-bud

* Address reprint request to T. Suzuki, Dept. of Veterinary Sciences, Tel: +81-083-933-5935, Fax: +81-083-933-5935, E-mail: Suzuki @agr.yamaguchi-u.ac.jp.

stage (Kaufman et al., 1977).

There are distinct differences between the paternal and maternal contributions to the embryo development. The paternal genome appears to be more important for the proliferation of the extra-embryonic tissues and the maternal genome plays a key role in preimplantation and early postimplantation development (Barton et al., 1984). Vital functional differences between the parental and maternal genomes of mammals have been revealed by demonstration of lethality to embryos of parthenogenetic, genogenetic and androgenetic uniparental genotypes (Solter, 1988). Cells derived from uniparental embryos can be rescued by integration into normally fertilized embryos, with consequent production of chimeric organism (Stevens et al., 1977; Nagy et al., 1979; Mann and Stewart, 1991; Barton et al., 1991). These phenomena may be relevant to the problem of mammalian parthenogenogenesis.

In the bovine oocytes, there are several effective ways to induce diploid parthenogenesis. They involve, for example, exposure to ionophore A 23187 (Ware et al., 1989), to ethanol, electric stimulation and a combination of ethanol and electric stimulation (Yang et al., 1994). Bovine parthenogenetic embryos have been transferred to recipients. In one such study, estrous did not occur until 48 days after transfer of single embryos (Fukui et al., 1992), while in another study estrous was delayed until 67 days after transfer of aggregated embryos. However pregnancy was not determined after this period (Boediono and Suzuki, 1994), perhaps because of early death of the embryos (Boediono et al., 1995).

The primary objective of our joint study at the Yamaguchi Research Station in Japan and St. Gabriel Research Station in Louisiana USA 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 of experiments conducted at the two research stations, these aspects were 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 balstocyst 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.

Production of chimeric bulls by aggregation of *in vitro*-fertilized bovine embryos without zonae pellucidae

In our study, bovine chimeras were produced by aggregating in vitro-fertilized embryos cultured in vitro without the zonae pellucidae that were then transferred nonsurgically to the recipients (Boediono et al., 1993). The optimal stage for embryo aggregation in the mouse is the 8 to 12 cell-stage (Minz et al., 1971). If bovine embryos of this stage are to be used, they must be collected surgically, because at this stage of development bovine embryos are located in the oviduct. The embryo aggregation technique for producing chimeric bovine offspring was found to be practical since 8 to 12 cell-stage bovine embryos can be produced by the IVF technique.

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 0.25% pronase treatment for zonae removal.

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

The results obtained in the previous study (Boediono et al., 1993) demonstrated that chimeric embryos can be produced using in vitro fertilized bovine embryos by aggregation techniques and cultured in vitro without zonae pellucidae. 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 balstocysts from adhering to the oviduct walls before it reaches the uterine cavity; however, this problem does not arise during in vitro culture.

In our study, the aggregated embryos were co-cultured with cumulus cells. As shown by Goto et al. (1988) and Suzuki et al. (1992), 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 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 efficient for the in vitro development of either aggregated or

normal embryos when cultured for more than 2 wk.

Fertility of sperm from a tertraparental chimeric bull

McLaren (1976) reported that Chimeras made by aggregating embryos without regard to the sex, should result in 50% of Chimeras being an XX/XY type. The phenotypic sex of XX/XY individuals will show varying degrees of dominance of the male component with the variation seeming to reflect the relative representation of XY and XX cells in gonadal primordial. Bongso et al. (1981) reported that sterility and infertility in chimerism cattle (60 XX/60 XY) was caused by testicular hypoplasia. However, our Chimera was normal (60 XY); mortility (+++) of frozen-thawed sperm was 30-40%, and 32 to 38% of cleavage embryos could reach balstocyst (Sumantri et al., 1997a, b).

The high fertilization rate after 5 h of sperm-oocyte incubation in the Chimera, Japanese Black and Limousin group were attributed to a high occurrence of acrosome reaction at 1 h after capacitation. The first line of evidence of sperm penetration rate increased until 5 h and the proportion of polyspermy was 4 and 7% at 4 and 5 h after insemination (Saeki et al., 1991). As described by Parrish et al. (1986), differences among bulls in fertilization rates are due to variation in the time for capacitation and the acrosome reaction. The lower fertilization rates observed in Japanese Brown and Holstein may be due to the short sperm-oocyte incubation time of 5 h which may not be sufficient for sperm penetration. The numbers of blastocyst produced on Day 7, 8 and 9 of IVM oocytes inseminated with Chimera were similar to that produced with its sires (Japanese Black and Limousin sperm). These findings indicate there may be a paternal effect in blastocyst production on these days, Shire and Whitten (1980a); Shire and Whitten (1980b) and Goldbard and Warner (1982) reported similar cases in the mouse embryo, where the cleavage rate and speed of development were dependent upon genetic factors, including maternal and paternal effects. In the human embryo, there is a strong paternal effect on preimplantation development and blastocyst formation (Janny and Menezo, 1994).

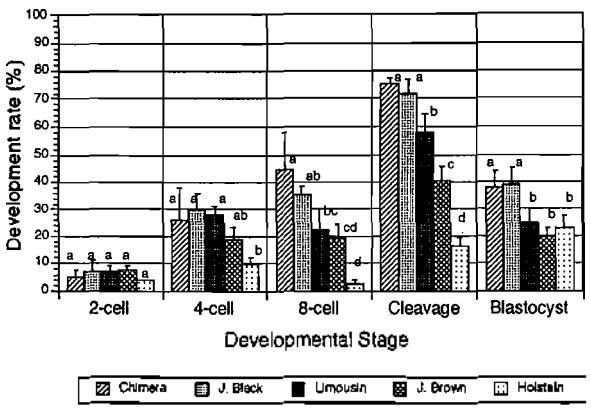

Figure 1. Development of IVF embryos using a tetraparental Chimera (n=239), J. Black (n=171), Limousin (n=186), J. Brown (n=196) and Holstein (n=186), n (No. of oocytes inseminated). Each treatment was replicated 3 times. (ANOVA, Duncan's test, a-b, b-c, c-d; $p<0.05$, a-c, b-d; $p<0.01$ and a-d; $p<0.001$).

The cleavage and blastocyst rates of IVF-zygotes inseminated with the Chimera were similar to its sire (Japanese Black), but differed from another sire (Limousin), and from its maternal grand-sires (Japanese Brown and Holstein). Detailed analysis of mosaicism in interspecific Chimera between *Mus musculus* and *Mus caroli* revealed that cells of the two could coexist and interact normally in all tissues (Rossant and Chapman, 1983), this Chimera originated from aggregated F1 (Holstein×J. Black) and F1 (J. Brown×Limousin) (Boediono et al., 1993). Therefore, the high blastocyst production seen when IVM oocytes were inseminated with Chimera sperm might be due to a heterosis effect. These results suggest that this Chimera sperm can be used for producing bovine IVF embryos.

Blood typing and microsatellite DNA genotyping of tetraparental chimeric cattle

The purpose of this study was to investigate the presence of genetic markers, which uniquely exist in the parental genomes in the chimeric cattle in order to estimate the degree of chimerism in the cattle, and to determine red blood cell types of the cattle (Sumantri et al., 1996).

Results from blood typing of a tetraparental Chimera bull showed that without differential hemolysis treatment gave 28/66 (42.4%) in partial reaction, after treatment with differential hemolysis test using 5 antisera from different breed, there were no partial reaction found. This might be due to the fact that the Chimera bull, in this case, has more than one blood type mixing in its body. By using this technique, positive reactions in the order of 8-11 antisera from B phenogroup system; 2-4 antisera from C and F phenogroup system; and 1-2 antisera from L, S, Z, and R' were obtained. These antiseras mentioned above can be used for detection of chimerism in the resultant Chimeric bull. However, antisera from A and J phenogroup system could not be detected. This results, contrast to previous report of Summers et al. (1983) who reported that chimerism in *Bos Taurus-Bos indicus* only found in J phenogroup. The differences may be due to the type of chimera, our chimera is a tetraparental from four different breeds. This tetraparental Chimera bull has three types of

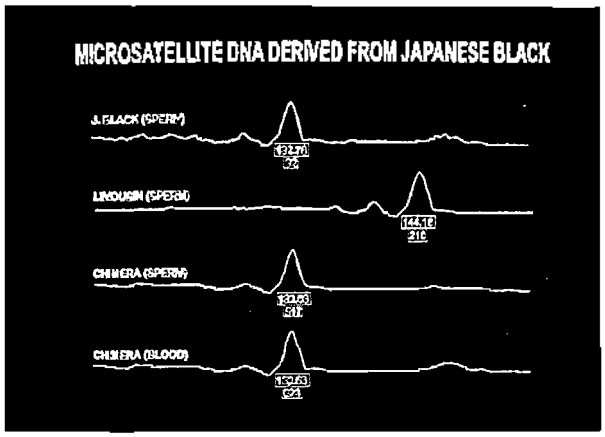

Figure 2. Microsatelite DNA (AG 230 PCR product 132-138 bp) found in tetraparental Chimera derived from its sire Japanese Black

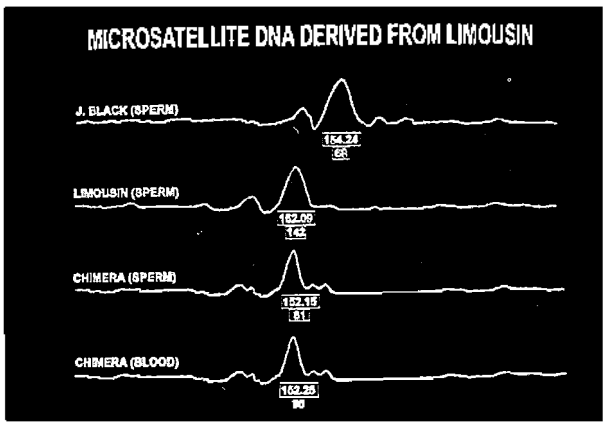

Figure 3. Microsatelite DNA (AG 237 PCR product 150-162 bp) found in tetraparental Chimera derived from its sire Limousin

red blood cells, among these two types are from the chimera and one type is from the co-sib.

In the microsatellite genotyping, 28 out of 228 primer sets gave amplification product in the chimeric cattle's genomic DNA. Of the 28 primer sets, 22 sets gave amplification in genomics DNA from Japanese Black and 6 sets in the Limousin, respectively. The remaining 200 primers sets generated products in all three genomic DNAs, unspecified products, or failed to amplify. These results demonstrated that 12% (28/228) microsatellite primer sets are potentially useful for this parentage analysis in the chimeric cattle, 78.6% (22/28) of microsatellite present in the chimeric cattle were uniquely contributed from the Japanese Black and 21.4% (6/28) from Limousin. Using only limited number of microsatellite markers, this study exposed the powerful approach to the roughly materials derived from Japanese Black than from the Limousin that contributed in the formation of the chimeric cattle.

Parthenogenetic/IVF – derieved embryo ffspring

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 our experiment (Boediono et al., 1999) 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 indicates 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., 1984; 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., 1977), and to produce viable germ cells in chimeric adult mice (Stevens, 1978; Anderegg and Markert, 1986).

The results of our experiment (Boediono et al., 1999) 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 over-looked 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 our study indicate that parthenogenetic bovine embryos were stimulated by the IVF-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 genetically 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 andIVF-derived embryonic cells in blastocyst formation, aggregated embryos (hatching and hatched blastocysts) were karyotyped. The karyotyped embryos in our experiment 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 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 our 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), observed that 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 our experiment, 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_r 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 parhenogenetic cells in the aggregated embryo, inserting

Table 1. Summary of transfers of reconstructed embryos produced by aggregation between parthenogenetic and IVF-deriv ed embryos

Embryo Recipient*	No. of embryos transferred	Morphological stage of the embryo	Diagnosis	Sex of offspring	Chromosome plates
Method 1					
A	2	Blastocyst (day 7)	Pregnant**		
		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)***	Not pregnant	-	-
		Hatched (day 8)***			
Method 2					
E	2	Hatching (day 8)	Not pregnant	-	-
		Hatched (day 8)			
F	1	Hatched (day 8)***	Pregnant	Male	XX+XY

* Method 1: aggregation of an IVF-derieved demi-embryo (8-cell stage) with parthenogenetic demi-embryo (8-cell stage). Method 2: aggregation of a whole IVF-derieved embryo (8-cell stage) with a whole parthenogenetic embryo (8-cell-stage).

** Conceptus no longer present after 60 days.

*** Frozen -thawed embryo.

balstomeres 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.

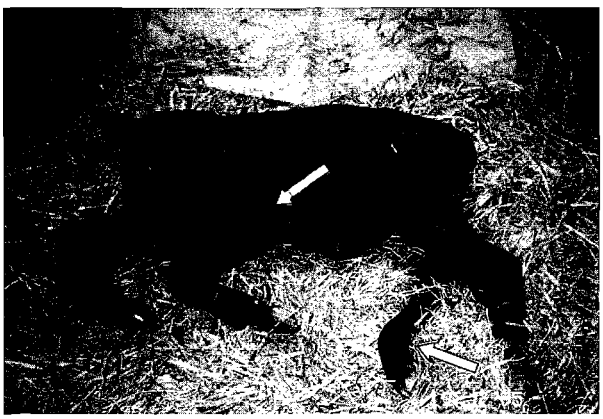

Figure 4. A Chimeric male claf resulting from the transfer of a frozen-thawed embryo originally produced by aggregation of a whole 8-cell parthenogentic embryos (Japanese Red) and whole IVF-derived 8-cell embryos (Holstein). Arrow=a red color pattern originating from a parthenogenetic embryo.

CONCLUSION

Aggregating in vitro fertilized embryos cultured in vitro without the zonae pellucidae and transferred nonsurgically to the recipient could be used as an efficient way to produce bovine chimeras. Sperm from this chimera had the ability to fertilize in vitro matured oocytes and which then developed as blastocysts. Degree of chimerism can obviously be determined by parentage analysis using polymorphic genetic markers. Because microsatellite is abundantly present in the genome and has a high degree of polymorphism, it is the most efficient and powerful genetic marker for this analysis.

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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