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Frequency of sex chromosomal mosaicism in bovine embryos and its effects on sexing using a single blastomere by PCR

Jong Ho Lee[1], Joong Hoon Park[1], Eun Joo Choi[2], Jong Taek Yoon[2], Chang Sik Park[3], Seong Ho Lee[4], Kyung Soon Im[1] and Dong Il Jin[5]

Seoul National University, Hankyong National University, Chungnam National University, Kongju National University and Sunmoon University, Korea

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Summary

Assessment of nuclear status is important when a biopsied single blastomere is used for embryo sexing. In this study we investigated the nuclear status of blastomeres derived from 8- to 16-cell stage *in vitro* fertilised bovine embryos to determine the representativeness of a single blastomere for embryo sexing. In 24 embryos analysed, the agreement in sex determination between a biopsied single blastomere and a matched blastocyst by polymerase chain reaction (PCR) was 83.3%. To clarify the discrepancies, karyotypes of blastomeres in 8- to 16-cell stage bovine embryos were analysed. We applied vinblastine sulfate at various concentrations and for different exposure times for metaphase plate induction in 8- to 16-cell stage bovine embryos. The 1.0 mg/ml vinblastine sulfate treatment for 15 h was selected as the most effective condition for induction of a metaphase plate (>45%). Among 22 embryos under these conditions, only 8 of 10 that had a normal diploid chromosome complement showed a sex chromosomal composition of XX or XY (36.4%) and 2 diploid embryos showed mosaicism of the opposite sex of XX and XY in blastomeres of the embryo (9.1%). One haploid embryo contained only one X-chromosome (4.5%). Four of another 11 embryos with a mixoploid chromosomal complement contained a haploid blastomere with a wrong sex chromosome (18.2%). In conclusion, assessment of nuclear status of 8- to 16-cell stage bovine embryos revealed that morphologically normal embryos had a considerable proportion of mixoploid blastomeres and sex chromosomal mosaicism; these could be the cause of discrepancies in the sex between biopsied single blastomeres and matched blastocysts by PCR.

Keywords: Bovine embryo, Mosaicism, PCR, Sex chromosome, Sexing

Introduction

Polymerase chain reaction (PCR) combined with a biopsied single blastomere has been used as a tool for embryo sexing in bovine (Machaty *et al*., 1993) and human embryos (Cui *et al*., 1994). Despite the higher efficiency and sensitivity of the PCR method for sexing, more precise assessment of nuclear status was required when using a single blastomere for embryo sexing (Park *et al*., 2001).

Chromosomal analysis of developing embryos has provided the evidence that a considerable proportion of morphologically normal embryos are chromosomally abnormal (Munne *et al*., 1995). Embryonic mixoploidy is a common phenomenon in domestic animal species (Viuff *et al*., 1999). An early and extensive study of bovine embryos developed *in vivo* revealed that

All correspondence to: Dong Il Jin, Department of Applied Biological Science, Sun Moon University, Asan City, Chungnam, 338–708, Korea. Tel: +82 41 302285. Fax: +82 41 5417425. e-mail: dij1@sunmoon.ac.kr

[1]Institute of Animal Science and Technology, College of Agriculture and Life Sciences, Seoul National University, Suwon 441–744, Korea.

[2]Institute of Genetic Engineering, College of Agriculture, Hankyong National University, Ansung, Korea.

[3]Division of Animal Science and Resources, Chungnam National University, Daejeon, Korea.

[4]College of Visual Image & Health, Kongju National University, Kongju City, Korea.

[5]Department of Applied Biological Science, Sunmoon University, Asan City, 338–840, Korea.

41.5% of morphologically normal blastocysts were diploid–tetraploid mosaics (Hare *et al.*, 1980). Moreover, multinucleated or anucleated blastomeres have been found in embryos developing *in vitro* and *in vivo* (Greenlee *et al.*, 1998) and multinucleated blastomeres have been associated with nuclear mortality in degenerating embryos. Sex chromosomal mosaicism has also been reported in bovine embryos fertilised *in vitro* by chromosomal examination and in human preimplantation embryos at the 2- to 8-cell stage (Yoshizawa *et al.*, 1999).

The objective of this study was to investigate the discrepancy in the PCR sexing in *in vitro* fertilised (IVF) bovine embryos by the analysis of nuclear status of blastomeres derived from 8- to 16-cell stage IVF bovine embryos.

Materials and methods

Embryo production and sex determination by PCR

Bovine embryos were prepared by *in vitro* maturation and fertilisation and cultured for 3 days to develop to the 8- to 16-cell stage, using the method of Park *et al.* (2001). On the third day after insemination, blastomere biopsy was carried out and a single blastomere was obtained for PCR. Development of demi-embryos was observed until the ninth day after insemination, and the hatched blastocysts were collected for PCR analysis.

BOV97M and the bovine 1.715 satellite DNA sequence were selected for amplification of male- and bovine-specific amplification, respectively (Plucienniczak, *et al.*, 1982; Miller & Koopman, 1990). The PCR reaction mixture contained genomic DNA extracted from blastomeres and blastocysts, 10× PCR buffer (100 mM Tris-HCl, pH 8.3, containing 500 mM KCl and 15 mM MgCl2), 0.25 mM of each dNTP, 1 U of *Taq* polymerase (Takara Shuzo, Japan), 1 mM and 0.5 mM of BOV97M and bovine-specific primers, respectively. The sequences of the BOV97M primers were 5′-GAT CAC TAT ACA TAC ACC ACT-3′ and 5′-GGC TAT GCT AAC ACA AAT TCT G-3′, and the bovine-specific primers were 5′-TGG AAG CAA AGA ACC CCG CT-3′ and 5′-TCG TCA GAA ACC GCA CAC TG-3′. The modified multiplex PCR method was used in that the first 10 PCR cycles were done with male-specific primer followed by an additional 23 PCR cycles with bovine-specific primer. DNA was denatured at 95 °C for 30 s, annealed at 55 °C for 30 s and extended at 72 °C for 30 s. After the last cycle, the samples were incubated at 72 °C for 7 min. The PCR reactions were performed in a GeneAmp 2400 (Perkin Elmer, Norwalk, CT) for 33 cycles. The length of the male-specific amplification product is 141 base pairs, and that of the bovine-specific amplification product is 216 base pairs. The accuracy of the procedure was evaluated by comparing the results of biopsied single blastomeres and matched blastocysts.

Metaphase induction and chromosome analysis

Embryos developed to the 8- to 16-cell stage were treated with acidic Tyrode solution (pH 2.3) to remove the zona pellucida (ZP) and then incubated in a small drop of a medium containing 0.1, 1.0, 10.0 mg/ml vinblastine sulfate (Sigma Co.) for 6–24 h. They were immersed separately in 0.4 ml of a hypotonic solution (1% sodium citrate) containing 0.3% bovine serum albumin (BSA) for 10–15 min. A fixative (20 µl) of methanol and acetic acid (1:1) was poured into the hypotonic solution for the fixation of embryos. An embryo was then placed on a glass slide and re-fixed with several drops of the same fixative, and marked on the reverse side. If cytoplasm remained, it was removed by spreading one drop of another fixative, a mixture of methanol and acetic acid (3:1). After they had dried completely, chromosome samples were stained with 10% Giemsa solution for 10 min. Chromosomal normality was determined microscopically.

Results

Sex determination of biopsied single blastomeres and matched blastocysts using PCR

Fig. 1 shows an eletrophoretic representation of the PCR products of a biopsied single blastomere and matched blastocysts. Embryos without a specific male band in PCR analysis of a single blastomere were determined to be female with a matched blastocyst in lanes 3 and 6; similarly for male embryos in lanes 4 and 7. However, in lanes 5 and 8 the sex of the embryo as determined from the biopsied single blastomere and the matched blastocyst did not coincide .

Twenty-four embryos were analysed by PCR. Of these, 20 showed a consistent sex between the biopsied single blastomere and matched blastocyst and 2 embryos did not match for sex (Table 1). Two biopsied single blastomeres did not react by PCR, and thus the sex coincidence between the biopsied single blastomere and matched blastocyst could not be determined. Therefore, the coincident rate of sex determination between biopsied single blastomeres and matched blastocysts by consecutive and multiplex PCR was 83.3%.

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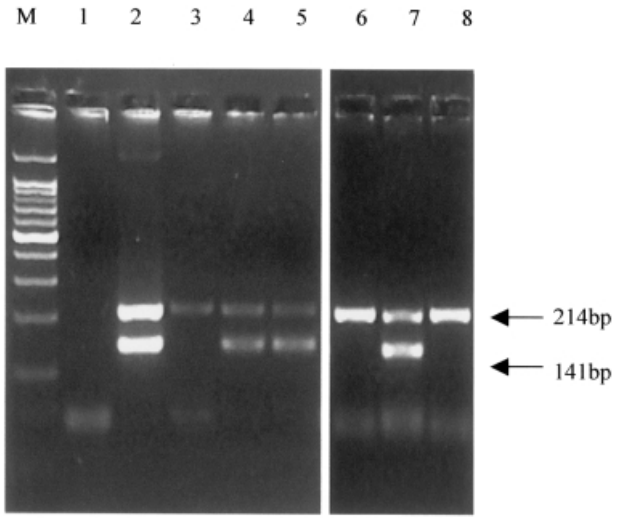

Figure 1 Electrophoretic representation of PCR products of a biopsied single blastomere (lanes 3–5) and matched blastocyst (lanes 6–8). M, 100 bp size marker; lane 1, (–) control; lane 2, (+) control; lanes 3 and 6, matched sex as female in both the single blastomere and matched blastocyst without male-specific band (141 bp); lane s4 and 7, matched sex as male in both the single blastomere and matched blastocyst with a male-specific band; lanes 5 and 8, different sex between the single blastomere and matched blastocyst; 214 bp band, bovine-specific band.

Efficiency of metaphase induction by vinblastine sulfate at various concentrations and exposure times

The optimal condition for metaphase induction in 8- to 16-cell bovine embryos was evaluated by exposing cleaved embryos to various concentrations of vinblastine sulfate for different exposure times. As shown in Table 2, 1.0 and 10.0 mg/ml of vinblastine both effectively induced a metaphase plate (>45%) but the standard deviation of the 1.0 mg/ml concentration was smaller than that of 10 mg/ml vinblastine. Metaphase plates derived from 8- to 16-cell embryos induced in this experiment are shown in Fig. 2. A normal diploid blastomere contained 58 telocentric autosomal chromosomes and 2 metacentric sex chromosomes. A male blastomere contained a large metacentric X-chromosome and a small metacentric Y-chromosome (Fig. 2*D*), while a female blastomere contained two large X-chromosomes (Fig. 2*C*). A haploid blastomere with a single X-chromosome (Fig. 2*E*) and a single Y-chromosome (Fig. 2*F*) was observed, as was a tetraploid blastomere with two X- and two Y-chromosomes (Fig. 2*B*).

Sex chromosomal mosaicism in 8- to 16-cell IVF bovine embryos

Table 3 shows the sex chromosomal compositions of the analysed embryos. Of the 22 embryos analysed, only 8 with a normal diploid chromosome complement showed a normal sex-chromosomal composition of XX or XY (36.4%), and 2 diploid embryos showed mosaicism with the opposite sex of XX and XY in blastomeres (9.1%). One haploid embryo contained only one X-chromosome (4.5%). Four of 11 embryos with a mixoploid chromosomal complement showed sex chromosomal mosaicism (18.2%). Therefore, chromosomal mosaicism was observed in 14 embryos (63.6%), of which 6 (27.3%) showed sex chromosomal mosaicism.

Table 1 Efficiency of sex determination by PCR analysis

No. of embroys analyzed	Consistent	Inconsistent	Not determined	Consistent rate (%)
24	20	2	2	83.3

Discussion

Although the PCR method for embryo sexing using a biopsied single blastomere has a high sensitivity, it can not determine the sex of 5–10% of blastomeres probably due to the paucity of the DNA template (Hwang *et al.*, 1995), contamination derived from male cells (Park *et al.*, 2001) or chromosomal aberration in the blastomeres (Navidi & Arnheim, 1991; Machaty *et al.*, 1993; Chen *et al.*, 1999). Of these factors that interfere with the efficiency of PCR on biopsied single blastomeres, chromosomal abnormality is suspected to be the major cause of reducing the representativeness of a single blastomere for the sex of the whole embryo. In this study, the sex discrepancy between biopsied single blastomeres and matched blastocysts was about 10% (Table 1). In cytogenetic studies, sex chromosomal mosaicism has also been reported to occur in approximately 15% of 5- to 10-cell bovine embryos (Yoshizawa *et al.*, 1999) and 2- to 8-cell human embryos (Iwasaki & Nakahara, 1990). Therefore, it is considered that the higher rate of the sex discrepancy could be induced by chromosomal aberrations such as sex chromosomal mosaicism.

Previous studies on chromosomal abnormalities in bovine embryos have been carried out by karyotyping, in which the whole embryo is incubated overnight with colchicine to arrest dividing blastomeres at metaphase, followed by attempts to obtain metaphase plates by HCl/Tween 20 fixative. In our experience, embryos arrested in metaphase with colchicine and fixed using 0.1% Tween 20 in 0.01 N HCl resulted in morphologically poor and excessively condensed chromosomes (data not shown). In this experiment vinblastine sulfate was used instead of colchicine and,

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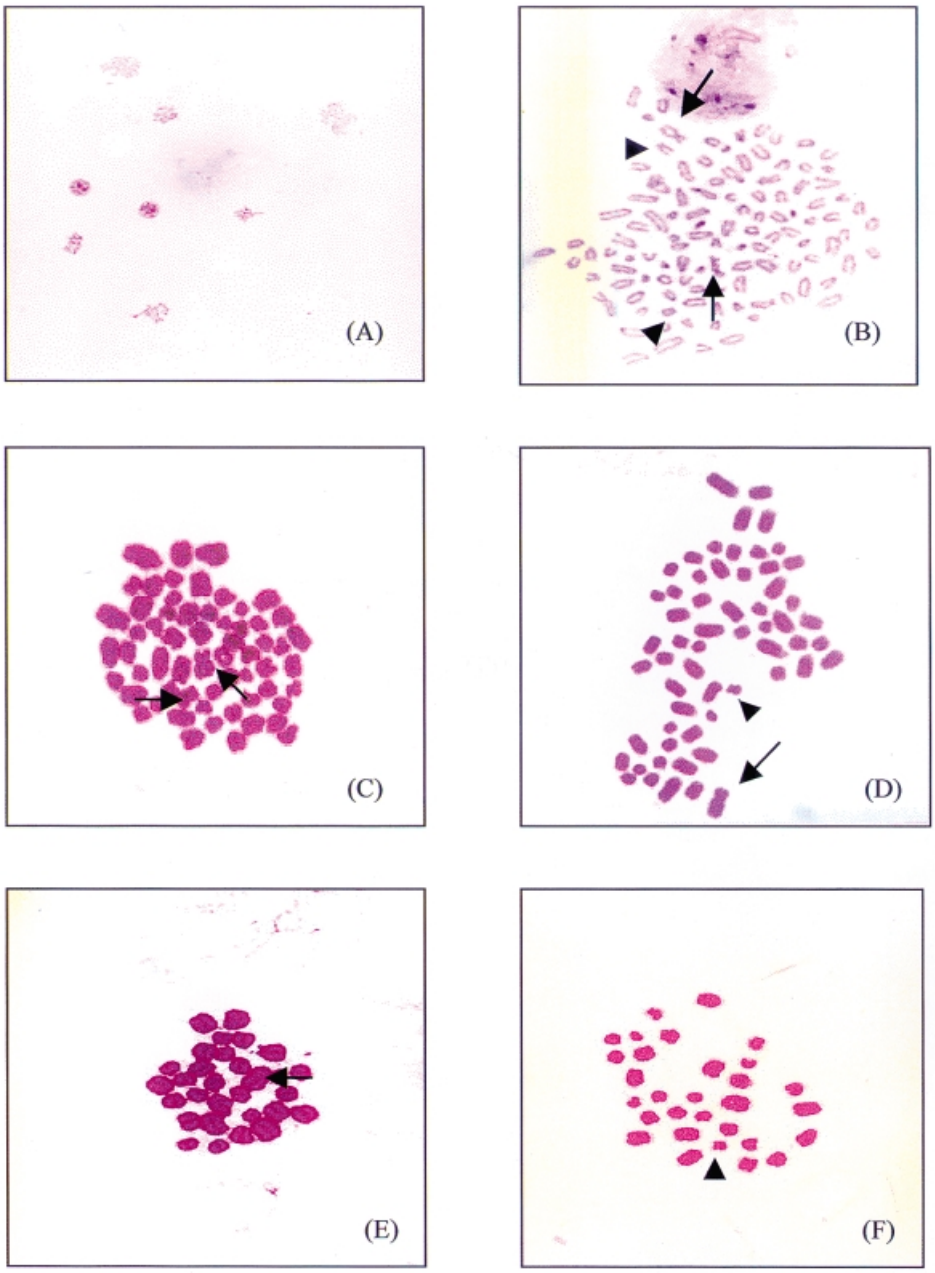

Figure 2 Different chromosomal compositions induced by vinblastine. (*A*) Metaphase induced in 8-cell IVF bovine embryo (×200); (*B*) tetraploid blastomere derived from an 8- to 16-cell IVF bovine embryo; (*C*) normal female diploid blastomere derived from an 8- to 16-cell IVF bovine embryo; (*D*) normal male diploid blastomere derived from an 8-to 16-cell stage IVF embryo; (*E*), (*F*) haploid blastomere with single sex chromosomal composition derived from an 8- to 16-cell IVF embryo. Arrow indicates the X-chromosome and the arrowhead indicates the Y-chromosome (×1000).

prior to fixation of the embryos, hypotonic treatment (1% sodium citrate) was applied. Also, a 1:1 mixture of methanol and acetic acid was used instead of HCl (Yoshizawa *et al.*, 1999). These modifications were sufficiently effective to induce morphologically clear chromosomes compared with HCl/Tween 20 treatment. As shown in Table 2, both 1.0 and 10.0 mg/ml vinblastine effectively induced a metaphase plate (>45%), but the standard deviation of 1.0 mg/ml vinblastine was smaller than that of the 10 mg/ml treatment. The incidence of blastomeres with a metaphase plate in the present study was not lower than the values of mitotic indexes (5–37.5%) reported by other investigators (Iwasaki *et al.*, 1990; Yoshizawa *et al.*, 1999). However, the concentration of vinblastine used was higher and the exposure time longer in this experiment than reported previously (Yoshizawa *et al.*, 1999).

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Table 2 Effect of different concentrations of vinblastine sulfate and exposure time on efficacy of metaphase induction from 8- to 16-cell IVF bovine embryos

Conc. of vinblastine (μg/ml)	Exposure time (h)	No. of ZP-free embryos	Avg. no. of fixed blastomeres	No. of blastomeres with metaphase per embryo ± SD	
				Total (%)	Analysable (%)
0	–	5	8.0	0.4 ± 0.5 (5.0)	0.4 ± 0.5 (5.0)
0.1	9	3	7.3	3.3 ± 1.5 (45.2)	2.3 ± 2.5 (31.5)
	12	3	8.0	0.8 ± 1.0 (10.0)	0.5 ± 0.6 (6.3)
	15	3	7.3	2.3 ± 1.2 (31.5)	1.7 ± 1.2 (23.3)
	24	3	3.3	0.3 ± 0.6 (9.1)	0.0 ± 0.0 (0.0)
1.0	9	6	10.2	2.8 ± 2.7 (27.5)	2.5 ± 2.6 (24.5)
	12	6	6.2	3.2 ± 1.7 (51.6)	2.8 ± 1.6 (45.2)
	15	6	7.3	3.5 ± 2.0 (47.9)	3.3 ± 1.8 (45.2)
	24	6	7.2	3.7 ± 2.4 (51.4)	3.0 ± 2.0 (41.7)
10	9	6	8.2	0.2 ± 0.4 (2.4)	0.0 ± 0.0 (0.0)
	12	6	14.8	3.0 ± 1.4 (20.3)	3.0 ± 1.4 (20.3)
	15	6	8.7	4.2 ± 3.1 (48.3)	3.8 ± 3.0 (43.7)
	24	6	8.2	3.8 ± 4.4 (46.3)	3.5 ± 4.0 (42.7)

ZP, zona pellucida.

Table 3 Sex-chromosomal composition of 8- to 16-cell IVF bovine embryos exposed to 1.0 mg/ml vinblastine for 15 h

Ploidy	No. of embryo	Chromosomal complement	No. of analyzed blastomeres	Sex chromosomal composition
Haploid	1	n	6	X, X, X, X, X, X
Diploid	2	2n	5	XY, XY, XY, XY, XY
	3	2n	5	XY, XY, XY, XY, XY
	4	2n	2	XX, XX
	5	2n	2	XX, XX
	6	2n	6	XY, XY, XY, XY, XY, XX[a]
	7	2n	4	XY, XY, XY, XY
	8	2n	3	XX, XX, XY[a]
	9	2n	4	XY, XY, XY, XY, XY
	10	2n	3	XY, XY, XY
	11	2n	3	XY, XY, XY
Mixoploid	12	n, n, 2n, 2n	4	X, Y, XY, XY[a]
	13	n, 2n, 2n, 2n	4	X, XY, XY, NN[a]
	14	2n, 3n, 3n	3	NN, XYY, XYY
	15	n, 2n, 2n	3	X, XY, XY[a]
	16	n, 2n, 2n	3	X, XY, XY[a]
	17	2n, 2n, 2n, 2n, 2n, 3n	6	XY, XY, XY, XN, XY, NNN
	18	n, 2n	2	Y, XY
	19	2n, 4n	2	XY, XXYY
	20	n, 2n, 2n	3	X, XX, XX
	21	n, 2n, 2n, 2n, 2n	5	X, XX, XX, XX, NN
	22	n, 2n	2	X, XX
Sex-chromosomal mosaicism (%)		6/22 (27)		

N, sex chromosome not determined.
[a]Mosaic embryos with opposite-sex chromosome.

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Yoshizawa *et al*. (1999) and Viuff *et al*. (1999, 2000) reported that 70–80% of day 3 or 4 post-inseminated *in vitro* fertilised bovine embryos were mixoploid. In this study, the proportion of mixoploid embryos was about 50%. The mosaicism of these mixoploid embryos might have resulted from polyspermic fertilisation and non-disjunction of chromosomes in mitotic division (Long & William, 1980; Murray *et al*., 1985). The sperm concentration used in this study was approximately 10 times higher than the convenient sperm concentration for IVF. These high sperm concentrations could be efficient at reducing the proportion of parthenogenetic embryos but increase the production of tri- or tetrapronuclear eggs (Yoshizawa *et al*., 1999).

The early embryonic cell cycles that occur in mammalian embryos immediately after fertilisation serve to subdivide a giant egg cell into many smaller cells as quickly as possible (Bruce *et al*., 1994). In these cycles, no growth occurs and the G1 and G2 phases are dramatically curtailed. However, these gap phases become prolonged after genomic activation. The 8- to 16-cell stage bovine embryos used in this study may not initiate genomic activation completely (Ian, 1994). Therefore, it is possible that incomplete subdivision of early stage bovine embryos induces nondisjunction of mitotic chromosomes, which resulted in mixoploidy. Sex chromosomal mosaicism in bovine and human embryos fertilised *in vitro* has also been found by chromosomal analysis and fluorescence *in situ* hybridisation (FISH) with the explanation of a possible mechanism (Munne *et al*., 1994; Yoshizawa *et al*., 1999; Miller & Therman, 2001). In the present study, 2 of the 10 diploid embryos showed sex chromosomal mosaicism (20%), and of 11 mixoploid embryos, 4 contained an opposite-sex chromosomal composition (374%). These results confirm that a sizable proportion of IVF bovine embryos have sex chromosomal mosaicism in embryonic blastomeres.

In conclusion, assessment of the nuclear status of 8- to 16-cell bovine embryos revealed that morphologically normal embryos contained a sizable proportion of mixoploid blastomeres and sex chromosomal mosaicism. These mosaic types could be the cause of discrepancies in the sex between single blastomeres and matched blastocysts in the PCR assay.

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