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—Brief Note—

PCR Sexing and Survival Following Embryo Biopsy-Bisection of In Vitro Produced Bovine Embryos

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Abstract: *To obtain identical twins with predicted sex, an efficient and reliable method for manipulation and analysis of bovine embryos has been developed using in vitro produced embryos and PCR. Expanded blastocysts graded as excellent were bisected into halves following biopsy to remove approximately 10% of trophoectoderm. Viability and cell number of these biopsied demi-embryos at 48 h in culture were compared with demi-embryos which have been only bisected. No significant difference was seen in survival rates between the biopsied demi-embryos (80.7%) and demi-embryos (87.0%). The mean cell number of the biopsied demi-embryos (70.00 ± 18.84) was slightly lower than that in the demi-embryos (77.50 ± 20.82). The sex of small sample and one biopsied demi-embryo was determined by PCR. The sex of 48 (96.0%) of the biopsied demi-embryos survived was consistent with the sample-side with 27 males and 21 females. These findings indicate that the sex of biopsied demi-embryos can be determined with a high accuracy, and their in vitro viability is comparable to that of demi-embryos.*

Key words: *Sexing, Biopsy, Bisection, In vitro viability.*

Of new biotechnologies which aim to manipulate bovine embryos before implantation, *in vitro* production (IVP) of embryos, embryo splitting (bisection) and sexing (including embryo manipulation for biopsy) are becoming part of a well organized system for both basic and applied research, and for commercial embryo transfer [1]. Until recently, research in each of these three different technologies was pursued rather independently, except for some attempts to either bisect [2] or sex [3, 4] embryos combined with IVP and/or sex-bisect embryos derived from *in vivo* [5]. If these technologies are merged into a single application, namely, the production of identical twins with predicted sex derived from IVP, the chance for rapidly propagating and tailoring animals will be increased markedly. The main emphasis in this study is on manipulation and analysis of embryos, in taking advantages of both IVP of embryos and polymerase chain reaction (PCR) sexing in our laboratory.

Oocytes from bovine ovaries taken at a slaughterhouse were matured and fertilized by the methods previously reported [4, 6]. At 5 h after insemination, presumptive zygotes together with attached cumulus cells were placed in 400 μl droplets of 25 mM HEPES buffered TCM-199 supplemented with 5% calf serum, 0.5 mM sodium pyruvate and antibiotics under a humidified 5% CO_2 in air atmosphere at 39°C. After 7–8 days of co-culture, only expanded blastocysts graded as excellent (grade 1) were selected and allotted into two groups for either biopsy-bisection or bisection.

Micromanipulation of embryos was performed using the methods of Itagaki *et al.* [4], based on that of Herr and Reed [7], with minor modification. Embryos were transferred individually into 50 μl droplets of BSA-free PB1 medium [8] supplemented with 0.2 M sucrose under mineral oil in a culture dish (60 mm non-treated polystyrene dish, Corning) and fixed to the bottom of the dish charged electrostatically.

To obtain the biopsied demi-embryos, firstly, the embryo was positioned so that the inner cell mass (ICM) was oriented at 12 o'clock using a mouth-operated hand-pulled glass pipette. The culture dish containing the embryo was put under an inverted microscope (TMD-10, Nikon) equipped with a micromanipulator (MO-188, Narishige) with a microblade (Biocut blade: No. 715, Feather Safety Razor Co.). Focusing on the embryo, the edge of the microblade was set to divide approxi-

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mately 10% of trophoectoderm (TE) and a small sample (a small amount of cell mass of TE) was split off by a single descending movement of the blade (Fig. 1-a). As the next step, the culture dish under the microscope was turned 90° and the biopsied embryo was adjusted ICM to a 3 o'clock position. Then the biopsied embryo was bisected symmetrically with a second downward motion of the blade (Fig. 1-b). Following these manipulations (Fig. 1-c), the small sample in 2 μl of medium was placed into an Eppendorf tube preloaded with 8 μl distilled water and stored at −20°C until PCR sexing. A pair of biopsied demi-embryos was washed three times with the culture medium and cultured in 50 μl droplets of the medium with a monolayer of cumulus cells which had been proliferated on the surface of the dish. At 48 h after onset of culture, these demi-embryos were evaluated morphologically and embryos classified as excellent or good were considered to have survived (Fig. 2). When both pairs of biopsied demi-embryos survived, one biopsied demi-embryo was preferentially transferred to an Eppendorf tube (preloaded with 8 μl distilled water) as a sample for PCR assay and the other was fixed onto slides to count the cells [4].

The samples were boiled for 1 min for denaturation. According to the protocol of XY Selector (kit for sexing of bovine embryos: Itoham Foods Inc.), a reaction mixture consisting of 9.9 μl of A solution (2x reaction mixture) and 0.1 μl of B solution (enzyme mixture) was added to each sample and DNA amplified in a Thermal Sequencer (Iwaki Glass Co.). Ten μl of the amplified product was resolved by agarose mini-gel electrophoresis and visualized by ethidium staining and ultraviolet illumination. A gender-neutral sequence of 102 bp was simultaneously amplified, allowing for verification of successful PCR amplification. Samples showing a male-specific sequence of 275 bp were judged to be males (Fig. 2). The PCR assay required approximately 70 min to be

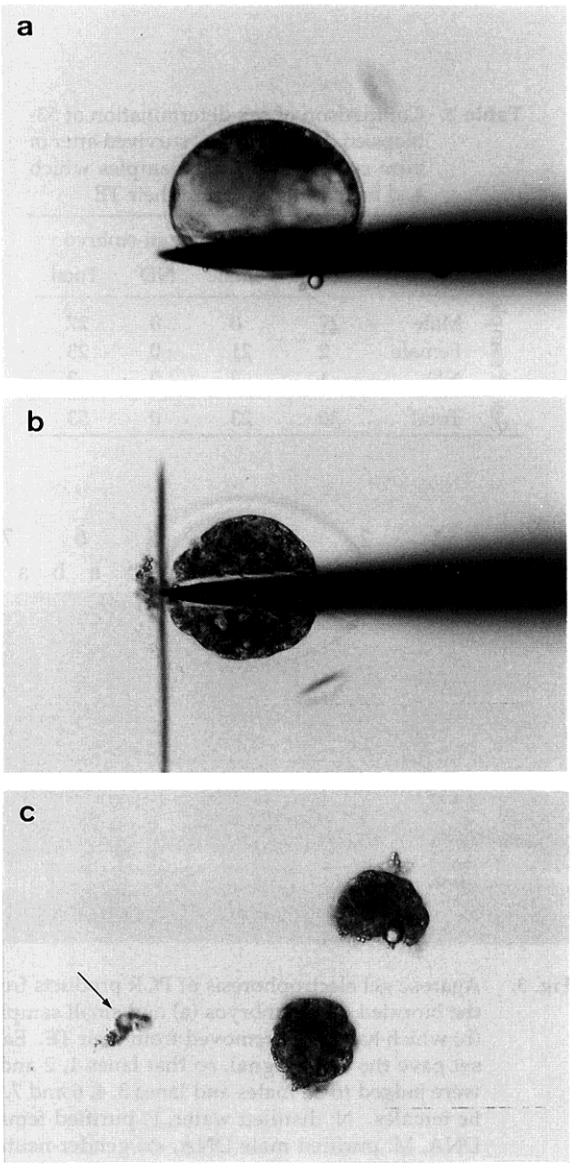

Fig. 1. Micromanipulation of bovine embryo for biopsy-bisection. a) By a single descending movement of the microblade, approximately 10% of the cell mass of TE was removed. b) Following the biopsy, the culture dish was turned 90° so that ICM was oriented towards the blade and the biopsied embryo was bisected symmetrically. c) A pair of the biopsied demi-embryos and a small sample (arrow) removed from TE immediately after biopsy-bisection (× 200).

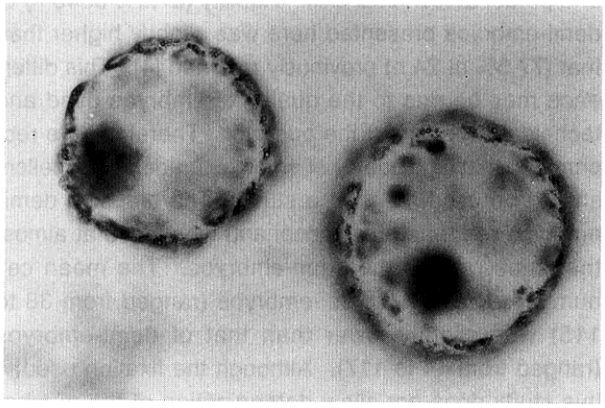

Fig. 2. A pair of the biopsied demi-embryos survived after 48 h *in vitro* culture. Note the presence of clear ICM cells (× 200).

Table 1. Comparison of *in vitro* survival of the biopsied demi-embryos and the demi-embryos at 48 h in culture

Micromanipulation	No. of original embryos (A)	No. of demi-embryos produced (B)	No . (%) of demi-embryos					No. of cells mean ± SD (n=)
			Pair (C)	One	Total (D)	(C/A)	(D/B)	
Biopsy-bisection	57	114	39 × 2	14 × 1	92	68.4	80.7	70.00 ± 18.84 (39)
Bisection	50	100	38 × 2	11 × 1	87	76.0	87.0	77.50 ± 20.82 (38)

completed.

To compare the viability and cell number with biopsied demi-embryos, half of the embryos selected were bisected. In the bisection, the embryo was fixed to the bottom of the dish by essentially the same procedure as described above, but ICM was oriented towards the microblade so that the embryo was bisected symmetrically with a single downward motion of the blade. These demi-embryos were cultured and processed as the biopsied demi-embryos were done.

The chi-square test and Student's t-test were used to assess the significance of differences in viability and cell numbers between biopsy-bisection and bisection groups, respectively. A probability of less than 0.05 was considered significant.

Table 1 shows the comparison between the *in vitro* survival of biopsied demi-embryos and that of demi-embryos after culture for 48 h. No significant difference was seen in survival rates (both proportions of original embryos and total demi-embryos produced) between the biopsied demi-embryos (68.4 and 80.7%) and demi-embryos (76.0 and 87.0%). In the data so far reported [9–12], the viability of demi-embryos *in vitro* appears to vary in degrees depending on the procedures for bisection and culture condition, the age or stage of embryos and personal technical skill. Viability (87.0% at 48 h) of demi-embryos presented here was slightly higher than that (77.5% at 24 h) previously reported [4]. This difference may be due to the quality of embryos used and technical expertise of the operator. Therefore, we recommend that high quality of embryos graded as excellent should be used for such a study. The biopsied demi-embryos re-formed blastocoel and developed at almost the same rate as the demi-embryos. The mean cell number of biopsied demi-embryos (ranged from 38 to 115) was slightly lower than that of demi-embryos (ranged from 44 to 117). Although the fixation used in this study does not allow determination of the number of ICM cells, morphologically normal-appearing ICMs were seen in most of both biopsied demi-embryos and demi-embryos (Fig. 2).

Table 2. Comparison of sex determination of 53-biopsied demi-embryos survived after *in vitro* culture and small samples which had been removed from their TE

Sex of sample	Sex of biopsied demi-embryo: Male	Female	ND	Total
Male	27	0	0	27
Female	2	21	0	23
ND	1	2	0	3
Total	30	23	0	53

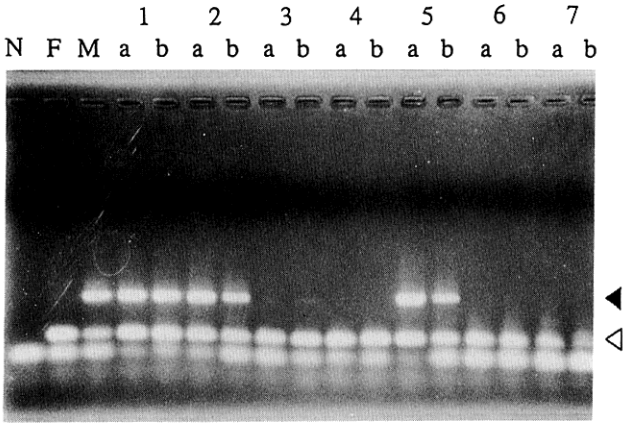

Fig. 3. Agarose gel electrophoresis of PCR products from the biopsied demi-embryos (a) and small samples (b) which have been removed from their TE. Each set gave the same signal, so that lanes 1, 2 and 5 were judged to be males and lanes 3, 4, 6 and 7, to be females. N: distilled water, F: purified female DNA, M: purified male DNA, △: gender-neutral band, ▲: male-specific band.

Table 2 shows the numbers and sex of biopsied demi-embryos together with the results of sample analysis. The 53 biopsied demi-embryos that survived after 48 h of culture were identified as 30 males and 23 females. By contrast, a determination of sex was made on 50 of the same samples which had been removed from TE in

the identical biopsied demi-embryos. Three samples were not sexed due to insufficient DNA or mistake in handling the samples because of the absence of gender-neutral signal. The sex of 48 (96.0%) of biopsied demi-embryos was consistent with the sample-side (27 males and 21 females). The exact nature of discrepancy between assays in two cases remains unclear. There was no skewing of the sex ratio (male/female) for these biopsied (sexed) demi-embryos (1.29 : 1). In addition, all 49 demi-embryos that survived could be sexed and their sex ratio also did not differ between 23 males and 26 females (0.88 : 1). Although further experiments are needed to obtain live identical twins with predicted sex, recent studies [12–14] have shown that the pregnancy rate from bisected embryos was nearly equivalent to that from the whole embryo with the number of newborn nearly doubled.

In conclusion, an efficient and reliable PCR method for sexing has been established using a small cell sample following embryo biopsy-bisection and these sexed demi-embryos are compatible with further development *in vitro* in spite of a small amount of cells lost.

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