Biopsy and sex determination by PCR of IVF bovine embryos

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Sex of early bovine embryos was determined by polymerase chain reaction (PCR) using a single blastomere removed at the 16–32 cell stage. Embryos were produced *in vitro* and biopsied on the fifth day after *in vitro* fertilization. Biopsied embryos were cultured on a cumulus cell monolayer until embryo transfer. For the PCR, one pair of bovine-specific and one pair of Y-chromosome-specific primers were used. Definite signals following PCR amplification were obtained in 95.4% of cases indicating that one blastomere from a preimplantation bovine embryo is sufficient for sex determination by PCR. Nineteen biopsied embryos of predetermined sex were transferred into synchronized recipient females to examine their developmental potential *in vivo*. Ten of the recipients (52.6%) were found to be pregnant by ultrasonography 25 days after transfer. This result did not differ significantly from that achieved with the use of the control non-manipulated IVF embryos (54.1%; $P > 0.1$).

Introduction

Producing embryos of predetermined sex would be of importance in modern cattle breeding. There have been several attempts to achieve this goal, such as measurement of X-linked enzyme activity by biochemical microassay, using antibodies that recognize a specific antigen (H-Y antigen) in male embryos, cytogenetic analysis and Y-specific DNA probes (van Vliet *et al.*, 1989), but all of these methods have the disadvantage of being inaccurate or extremely time consuming. The recent development of the polymerase chain reaction (PCR) was significant in this field because this technique allows amplification of Y-chromosome-specific repetitive sequences and thus determination of the sex of the embryo in a relatively short time and with high reliability (Herr *et al.*, 1990).

The technique requires a sample from the preimplantation embryo for which a number of methods exist. It is crucial to all techniques that biopsy must not adversely affect developmental potential of the embryos. We reported (Macháty *et al.*, 1992) that removing one cell from the 16–32 cell stage bovine preimplantation embryos does not alter the development of the embryos *in vitro* until the hatched blastocyst stage. In the present study we tested the developmental potential of the biopsied embryos further by transferring them into recipient females. We also investigated whether one cell removed from a 16–32 cell IVF embryo is sufficient for successful sex determination by PCR amplification.

Materials and Methods

Embryo production

Ovaries of slaughtered cows and heifers were collected and transported to the laboratory in Dulbecco's phosphate-buffered

saline (PBS). Follicles, 2–5 mm in diameter, were aspirated, and the cumulus–oocyte complexes were collected and incubated in 400 μ l Medium 199 (Earle's salt) supplemented with oestrous cow serum (20%), FSH (10 μ g ml⁻¹), calcium lactate (2.92 mmol l⁻¹), sodium pyruvate (2 mmol l⁻¹), sodium bicarbonate (33.9 mmol l⁻¹), Hepes (4.43 mmol l⁻¹) and gentamycin (60 mg ml⁻¹) (Sigma Chemical Co., St Louis, MO) in Nunc four-well multidishes (Nunc, Denmark). Maturation was carried out at 39°C in an atmosphere containing 5% CO₂ in air with maximum humidity. After the maturation period (24–26 h) the oocytes were fertilized with swim-up selected frozen–thawed bull spermatozoa (Parrish *et al.*, 1986). The fertilization medium was TALP supplemented with BSA (6 mg ml⁻¹), adrenaline (1 μ mol l⁻¹), hypotaurine (10 μ mol l⁻¹) and heparin (10 μ g ml⁻¹) (Sigma Chemical Co.) as described by Ball *et al.* (1983). After incubation of the gametes, fertilized oocytes with adhering cumulus cells were transferred into dishes containing the maturation medium without FSH. The conditions for fertilization and the following embryo culture were the same as those used for maturation (Berg and Brem, 1989).

Embryo biopsy

Five days after fertilization, 90 embryos that reached the 16–32 cell stage were collected and equilibrated for about 30 min in PBS containing 200 mmol sucrose l⁻¹ (Sigma Chemical Co.). Biopsies were carried out in microdrops of the same medium by means of two Narishige micromanipulators (Narishige Co., Ltd, Tokyo) equipped on an Olympus inverted microscope (Olympus Optical Co., Ltd, Tokyo). Embryos were fixed with a flame-polished holding pipette (approximately 120 μ m o.d.) and a bevelled micropipette (approximately 30 μ m o.d.) was pushed through the zona pellucida and one blastomere was removed by gentle suction. Biopsied embryos were transferred separately

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Fig. 1. Gel electrophoresis of bovine DNA samples amplified by PCR. 1: molecular size marker (pBR322 digested with Hae III); 2: female embryo; 3: male embryo; 4: female embryo; 5: female embryo; 6: male embryo; 7: sample without blastomere.

onto a monolayer of cumulus cells in cultivation dishes (prepared previously according to the method of Goto *et al.*, 1988) and cultured *in vitro*. The medium used for cumulus cell coculture was the same as that for embryo culture after fertilization. On the seventh day after fertilization when they reached the transferable stage some embryos were transferred nonsurgically into recipient cows and heifers.

Morula stage embryos produced in the same IVF programmes were also transferred into recipient females as a control group.

Polymerase chain reaction (PCR)

Removed blastomeres in 1–2 µl PBS buffer were transferred into 0.5 ml PCR tubes containing a small amount of Millipore water, and were frozen to release DNA from the cells. After thawing, the components for the amplification were added into the tubes to a final volume of 50 µl. The reaction mixture contained PCR Buffer II (10 mmol Tris-HCl l⁻¹, pH 8.3; 50 mmol KCl l⁻¹), 1.5 mmol MgCl₂ l⁻¹, 0.5 mmol of each dNTP l⁻¹, 2 U of Taq polymerase (Perkin Elmer Cetus, Norwalk) and 0.3 µmol of each primer l⁻¹ overlaid with 50 µl mineral oil (Sigma Chemical Co., St Louis, MO). For the amplification, two pairs of primers, one bovine Y chromosome-specific and one bovine DNA-specific, were used. The sequence of the Y-specific primers were (5' primer) 5'-CCCTCCAGCTGCAGTGTC-3' and (3' primer) 5'-GATCTGTAAGTCAAACCTGGC-3' (Matthews and Reed, 1992). Bovine DNA-specific primers were (5' primer) 5'-TGGAAGCAAAGAACCCCGCT-3' and (3' primer) 5'-TCGTGAGAAACCGCACACTG-3' (Plucienniczak *et al.*, 1982). The length of the Y-chromosome-specific amplification product is 301 base pairs, and that of the bovine DNA-specific amplification product is 216 base pairs. The amplifications were carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk) for 35 cycles each consisting of template denaturation at 95°C for 90 s, primer annealing at 57°C for 90 s and primer extension at 72°C for 180 s. After the last cycle, the samples were incubated at 72°C for 7 min.

After PCR, the amplification products were electrophoresed on a 2% agarose gel (Pharmacia LKB Biotechnology, Uppsala), stained with ethidium bromide and evaluated using ultraviolet

light. If only one band of the bovine-specific product was visible on the gel the blastomere was considered to derive from a female embryo, whereas the presence of two bands referred to a male embryo.

Two additional experiments were performed to test reliability of the sex determination method before calving. First, DNA from male and female bovine liver tissue was used as template in the PCR. DNA was isolated as described by Sambrook *et al.* (1989); samples from each of these lysates (each containing 5 µg of DNA) were amplified under the same conditions used for embryonic DNA amplification. In the second experiment, zonae of five 16–32 cell stage embryos were digested using 0.5% pronase solution; the blastomeres were separated mechanically; and six blastomeres from each embryo were used for PCR. These were placed individually into PCR tubes and sex was determined by the method described above; the results concerning the sex of the same embryo were compared.

Pregnancy rates achieved with the biopsied and control embryos were analysed for significant differences by χ^2 -analysis using contingency tables.

Results

A single blastomere from each of 87 embryos was removed; only three out of 90 embryos were destroyed during manipulation. After biopsy and culture *in vitro*, 49 embryos reached the morula stage. Nineteen morulae were transferred into recipient females on the seventh day after fertilization, and 25 days later ten of the recipients (52.6%) were found to be pregnant by ultrasonography. In the control group 24 embryos were transferred and pregnancy rate was 54.1% (13 of 24).

Sex was determined in the 87 biopsied embryos. In four cases very weak Y-specific signals were obtained which could not be identified unambiguously; in 83 embryos amplification gave results that allowed sex to be determined. The result of a gel electrophoresis after amplification is shown (Fig. 1). In lanes 3 and 6, two bands are visible indicating the presence of both the Y chromosome-specific and the bovine DNA-specific fragments. These are male embryos. Female embryos (lanes 2, 4 and 5) gave a single band referring to the bovine-specific product only.

When DNA samples from liver tissue of animals of known sex were amplified, the sex determined by PCR corresponded to the anatomical sex. In every case the results were as expected, for both male and female.

The sex of five embryos was also determined using six blastomeres from each embryo. The amplification was performed by transferring one blastomere into the PCR tubes. After gel electrophoresis and staining, sex determination gave the same result from all six blastomeres in the case of each embryo: two of them proved to be male, whereas three embryos were female.

Discussion

There are many methods for removal of cells from preimplantation embryos, including zona drilling using acidified solutions (Handyside *et al.*, 1989; Gordon and Gang, 1990), zona thinning (Muggleton-Harris and Findlay, 1991), aspiration using a fine micropipette (Wilton and Trounson, 1989; Wilton *et al.*, 1989) and blastomere displacement (Roudebush *et al.*, 1990). Takeuchi *et al.* (1992) used three methods for biopsy, i.e. enucleation, aspiration and extrusion, and found only slight differences among them regarding their effect on subsequent embryonic development.

A large number of cells biopsied from the embryo are used for sex determination in cows. Bondioli *et al.* (1989) separated 10–20% of the cell mass from uterine-stage embryos collected from superovulated cows and determined the sex of the embryos by DNA hybridization using the biopsied samples. Herr and Reed (1991) and Thibier and Nibart (1992) determined the sex of bovine embryos by PCR after cutting a few cells from morulae or blastocysts using a microblade; others used bisected blastocyst stage embryos for sexing by the PCR (Peura *et al.*, 1991).

Here we demonstrated that DNA content of one single blastomere of an IVF bovine embryo is sufficient for successful PCR amplification. Previously, Morsy *et al.* (1992), using human lymphoblasts and mouse blastomeres, determined that it is possible with one original copy (in a single cell) to amplify reproducibly by the PCR. In this study, the Y-specific signal was insufficiently strong in only 4.6% of the cases. During sexing of bisected blastocysts using two pairs of Y-chromosome-specific primers, Peura *et al.* (1991) obtained a similar number of uncertain results: with one of the two primers used, the Y-specific signal was very weak in one out of twelve embryos.

Since calves have not yet been born after transfers of the sexed embryos, it was not possible to confirm directly the results obtained. Owing to this, DNA isolated from bovine liver tissue was used as a template for the PCR to test the reliability of the sexing method. The sex determined this way from somatic cells was in agreement with the anatomical sex, in every case. Furthermore, when individual blastomeres from the same embryos were analysed, the same bands could be detected after amplification. These indicate that the sexing method used (i.e. PCR from a single separated blastomere of a preimplantation embryo) provides correct results.

The use of bovine-specific primers facilitates the detection of the absence of the blastomere in the reaction mixture and thus excludes the false female results. Owing to the small size of the

blastomeres, it can be extremely useful; however, in this study all samples contained embryonic material as revealed by these primers. Thus, the PCR protocol described above for sexing bovine embryos is very effective, and can be accomplished in about 5 h for a relatively large number of embryos.

The removal of a blastomere caused very little trauma to the embryos. It did not alter developmental potential *in vitro*: after biopsy 56.3% (49 of 87) of the biopsied embryos reached the transferable stage. This rate is similar to that found earlier (Macháty *et al.*, 1992), where 53.3% of the manipulated and 55.1% of the control embryos developed to the blastocyst stage; the difference was not significant ($P > 0.1$). Furthermore, biopsy had no detrimental effect on pregnancy rates achieved using biopsied embryos for transfer. There was no significant difference between pregnancy rates achieved for the biopsied and the control embryos (52.6% and 54.1%, respectively). The method described here is a suitable method for sexing IVF bovine embryos.

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