

# Post-biopsy bovine embryo viability and whole genome amplification in preimplantation genetic diagnosis

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**Objective:** To evaluate the effect of the biopsy of 8-cell to 16-cell bovine embryos on their subsequent development and the effect of whole genome amplification (WGA) on removed blastomeres.

**Design:** Randomized study.

**Setting:** Molecular genetics and animal reproduction laboratories.

**Patient(s):** Cow ovaries obtained from slaughterhouses.

**Intervention(s):** The ovaries were punctured, and the oocytes were matured and fertilized in vitro. On the fourth day after fertilization, 8-cell to 16-cell bovine embryos were biopsied, one quarter of each embryo being removed. The blastomeres were submitted to WGA followed by polymerase chain reaction (PCR). The embryos were returned to culture for evaluation of their development.

**Main Outcome Measure(s):** Subsequent rate of blastocyst development, embryo cell number, WGA efficiency, and sex determination.

**Result(s):** A total of 92 embryos were submitted to biopsy. The blastocyst production was 53.3%, with 44.9% of hatching rate. These results were similar to those of the control group (66.0% and 42.6%) of 103 embryos. Overall, no impact was detected on embryo quality in blastocyst cell number between the two groups. Removed blastomeres were submitted to WGA, resulting in 98.2% of efficiency. However, only 59% of the samples were sexed by PCR.

**Conclusion(s):** Biopsy of 8-cell to 16-cell bovine embryos did not affect their subsequent development. WGA was successful in removed blastomeres. (Fertil Steril® 2010;93:783–8. ©2010 by American Society for Reproductive Medicine.)

**Key Words:** PGD, sexing, embryo quality, hatching rate

Despite the advances in assisted reproduction techniques (ART), failures in in vitro fertilization and embryo implantation continue to occur (1), which makes the preselection of embryos before transfer one of the most important tools in ART. Currently, reduced fecundity is markedly associated with chromosomal anomalies in miscarried fetuses (1) and at about 20% to 70% of human embryos are affected by aneuploidy and other chromosome abnormalities (2). Preimplantation genetic diagnosis (PGD) is a method in which the chromosomal constituent of human embryos developing in vitro can be analyzed, and chromosomally abnormal

embryos can be deselected for transfer (3). Preimplantation genetic diagnosis is indicated to reduce the incidence of spontaneous abortions and the birth of babies with sex-linked diseases (2).

In general, one of the prerequisites of PGD is a successful biopsy technique. Moreover, biopsy could be used to obtain embryonic stem cells, which allows to work under ethical limits and creates a new cell line. In addition, this technique is very useful in choosing the desired sex in animal embryos and has a great impact on the production of cloned and transgenic livestock. However, biopsy is an invasive procedure that could cause embryo damage. Its impact on embryo quality is still poorly studied, and there is no single universally practiced protocol for implementing PGD (1, 4). Another point to consider is the small amount of genomic DNA available to perform the genetic study, due to the reduced number of cells obtained from the biopsy (one or two blastomeres). Besides, methodologies using whole genome amplification (WGA) have been developed, and using these methods, it is possible to generate microgram quantities of DNA from little genomic DNA (5).

Our study evaluated the effect of the biopsy of 8-cell to 16-cell bovine embryos on their subsequent development and the

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effect of whole genome amplification (WGA) on removed blastomeres.

## MATERIALS AND METHODS

### Chemicals

The chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

### Oocyte Recovery

The proposal was submitted to the Ethics Committee and Animal Experiments (CEEa) of the Federal University of Juiz de Fora and was approved in February 2007. The experiment was conducted at the Animal Reproduction and Molecular Genetic Laboratories at Embrapa Dairy Cattle Research Center, Juiz de Fora, Minas Gerais, Brazil. Ovaries were obtained from a local slaughterhouse ( $n = 264$ ) and were transported in saline solution (0.9% NaCl) at 37°C supplemented with 0.005% streptomycin sulfate.

### Fertilization and in Vitro Culture

Cumulus–oocyte complexes (COCs) were aspirated from follicles of 2 to 8 mm in diameter, and those with a homogenous cytoplasm and at least three layers of cumulus cells were used (6). The COCs ( $n = 706$ ) were in vitro matured in 400  $\mu\text{L}$  of TCM 199 medium (GIBCO BRL, Grand Island, NY) with 10% inactivated estrous cow serum and 20  $\mu\text{g}/\text{mL}$  follicle-stimulating hormone (FSH; Pluset, Serono, Italy), for 24 hours, at 38.8°C in an atmosphere containing 5%  $\text{CO}_2$  and at 95% humidity. After maturation, COCs were fertilized with certified bovine semen obtained by the swim-up method (7). In vitro fertilization was performed with 15 to 25 COCs in 100- $\mu\text{L}$  drops of fertilization medium (8) supplemented with 20 mg/mL of heparin and 6 mg/mL of fatty free-acid bovine serum albumin (BSA) fraction V. The COCs were coincubated with  $2 \times 10^6$  spermatozoa/mL for 22 hours in the same conditions of maturation. After fertilization, the probable zygotes ( $n = 706$ ) were partially stripped by mechanical pipetting in a TALP-HEPES medium (9) and then transferred to a cultured CR2aa medium (10) supplemented with 10% fetal calf serum. The embryo culture was performed in the same conditions of fecundation. On the fourth day after fecundation, the cleavage rate was evaluated and up to 50% of the medium was renewed.

After evaluation of the cleavage rate, all the structures were taken out of cultured medium microdrops. Embryos with 8 to 16 cells were rinsed in TALP-HEPES with polyvinyl alcohol (0.003%) added, and they were randomly divided into two groups: control ( $n = 103$ ) and biopsy ( $n = 92$ ).

### Embryo Biopsy

The embryos of the biopsy group were transferred to 5  $\mu\text{L}$  TALP-HEPES microdrops with equilibrated mineral oil. The procedure was performed using micromanipulators (Narishige Co., Tokyo, Japan) attached to an inverted micro-

scope (Carl Zeiss, Feldbach, Switzerland). After embryo immobilization with a holding pipette, a 30  $\mu\text{m}$  microneedle (Transfix, São Paulo, Brazil) was inserted through the zona pellucida at the 3-o'clock position. The number of cells removed was equivalent to a quarter of the embryo (1). After biopsy, embryos were transferred back to in vitro culture to evaluate their capacity for development.

### Evaluation of Embryo Development

The blastocyst rate and stages of the biopsy and control embryos were evaluated on the eighth day after fertilization and the hatching rate on the 10th day. Embryo morphology and quality were evaluated as previously described in the International Embryo Transfer Society manual (11). To evaluate overall quality, embryos were stained on the 10th day of culture, and the blastomeres were counted with the aid of the AxioVision 3.1 imaging software (Carl Zeiss).

### DNA Samples

Male and female bovine DNA samples in six different concentrations (30, 3.0, 0.3, 0.03, 0.003, and 0.0003 ng/ $\mu\text{L}$ ) and embryos at various stages of development (2, 4 to 7, >8 cell, and blastocyst) were used to standardize the polymerase chain reaction (PCR) protocols and set the amplification limits. Embryos were rinsed in Dulbecco's phosphate buffered saline (DPBS) and were transferred to 0.2 mL PCR tubes containing 10  $\mu\text{L}$  of DNA extraction solution (5X PCR buffer and 3 mg/mL proteinase K) and frozen at -20°C until used. Embryos were incubated for 2 hours at 50°C, then for 10 minutes at 95°C to inactivate proteinase K before PCR.

Selected blastomeres were rinsed in distilled water and transferred to 0.2 mL PCR tubes containing 1  $\mu\text{L}$  of distilled water and frozen at -20°C until used. Two  $\mu\text{L}$  of DNA extraction solution (5X PCR buffer and 3 mg/mL proteinase K) were added to each sample to digest the cellular cytoplasm and release the genomic DNA. Blastomere DNA extraction was performed using the same procedure.

### Whole Genome Amplification

The blastomeres were submitted to the GenomiPhi DNA Amplification Kit (GE Healthcare, Munich, Germany) according to the manufacturer's instructions. Briefly, 9  $\mu\text{L}$  of the GenomiPhi sample buffer was added to the PCR tube, and the target DNA was denatured at 95°C for 3 minutes and cooled on ice. Ten  $\mu\text{L}$  of the reaction buffer containing deoxyribonucleotide triphosphates (dNTPs), random hexamers, and Phi29 polymerase were added to each sample. The reaction was incubated at 30°C overnight (18 hours). After amplification, the Phi29 DNA polymerase was heat-inactivated for 10 minutes at 65°C. The amplification product was frozen at -20°C until used.

To evaluate the efficiency of the amplification, 1  $\mu\text{L}$  of the product was electrophoresed on a 1% agarose gel for 2 hours

**TABLE 1**

**Distribution of embryos on the 8th day of culture at different stages of development: early blastocyst (Bi), blastocyst (BI), expanded blastocyst (Bx), and hatching blastocyst (Be).**

Treatment	8th day blastocyst (%)			
	Bi	BI	Bx	Be
Control (68/103)	0 (0/68)	27.9% (19/68)	57.4% (39/68)	14.7% (10/68)
Biopsy (49/92)	2.0% (1/49)	10.2% (5/49)	57.2% (28/49)	30.6% (15/49)

Note: Wilcoxon test ( $P > .05$ ).

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at 120 volts. The gel was stained with 3.0  $\mu\text{g/mL}$  ethidium bromide, and the bands were observed using a ultraviolet transilluminator.

### Polymerase Chain Reaction

The amplification was carried out in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA). The first step was DNA denaturation at 94°C for 10 minutes. The second step consisted of 40 cycles of amplification at the following temperatures: 94°C for 60 seconds for DNA denaturation, 58°C for 30 seconds for primer annealing, and 72°C for 60 seconds for polymerization of DNA, and a final extension step at 72°C for 10 seconds.

The reaction mixture of 20  $\mu\text{L}$  containing 2 mM  $\text{MgCl}_2$ , 5X PCR buffer (Promega, Madison, WI), 0.2 mM of each dNTP (GE Healthcare), 0.05 IU/ $\mu\text{L}$  of GoTaq DNA polymerase (Promega), and 0.25  $\mu\text{M}$  of each primer (Y\_F-5'-AGACCTCGAGAGACCCTCTTCAACACGT-3', Y\_R-5'-GGTCGCGAGATTGCTCGCTAGGTCATGCA-3', Autosomic\_F-5'-CCTCCCCTTGTTCAAACGCCCGGAATCATT-3', Autosomic\_R-5'-TGCTTGACTGCAGGGACCGAGAGGTTTGGG-3') was added to the material to be amplified.

After amplification, the products were submitted to electrophoresis on 8% native polyacrylamide gels for 1 hour at 500 volts. Gels were stained with a silver nitrate procedure. Band scoring and sex determination were performed using 25 bp standard ladder (Promega). Samples from female embryos in the biopsied material showed only the 269 bp whereas the presence of two bands (269 bp autosomic sequence and the Y-specific 213 bp) indicated male samples.

### Statistical Analyses

The chi-square test was used for statistic evaluation of the results of blastocyst and hatching rate between the biopsy group and the control group, to test the efficiency of WGA, and to determine the proportion of each sex of bovine embryos and biopsied samples submitted to PCR. The number of cells per embryo was analyzed by analysis of variance (ANOVA) with SAS software (SAS Institute, Inc., Cary, NC). The proportion of blastocysts was evaluated with the Wilcoxon test.  $P < .05$  was considered statistically significant.

### RESULTS

A total of 264 ovaries were collected, and 706 COCs were aspirated from 2 to 8 mm follicles. The cleavage rate observed was 84.8%, and the proportion of embryos in different stages of development was 2.8% for two-cell, 46.6% for four-cell to seven-cell, and 42.2% for 8-cell to 16-cell embryos. The blastocyst rate for the biopsy group (53.3%, 49 out of 92) was similar ( $P > .05$ ) to the control group (66.0%, 68 out of 103). Furthermore, no difference was noted in the hatching rates between the control group and the biopsied group (42.6% vs. 44.9%, respectively). The proportion of blastocysts in different stages of development on the eighth day did not differ between the groups ( $P > .05$ ) (Table 1). Moreover, no impact of the embryo biopsy was detected on embryo quality (Table 2).

To standardize the PCR protocol and to evaluate the power of target DNA detection, a serial dilution of starting DNA from bovines was set up, and successful amplification was observed from 30 to 0.0003 ng of template DNA.

To increase the PCR efficiency, blastomeres were submitted to whole genome amplification before PCR. The technique showed 98.2% efficiency (Fig. 1). The PCR efficiency rate was calculated according to the number successful diagnoses. No difference was detected in the proportion of sexes in embryos at different stages of development ( $P > .05$ ). However, a greater number of female samples was detected in the biopsied material (76%, 25 out of 33) ( $P < .05$ ). The PCR efficiency in blastocysts was statistically significantly greater ( $P < .05$ ) than for embryos in early stages of development and biopsied material (Table 3).

### DISCUSSION

Micromanipulation techniques, such as biopsy and subsequent PGD, could be considered as external aggressions. Rupture of the blastomere and embryo damage caused by biopsy could adversely affect subsequent embryo development (4). As far as we know, only a few studies have emphasized the importance of the biopsy procedure, general conditions during and after micromanipulation, and the quality of biopsied-derived embryos.

**TABLE 2**

**Number of cells in embryo in different stages of development: blastocyst (Bl), expanded blastocyst (Bx), and hatching blastocyst (Be) in the control and biopsy groups.**

Treatment	Average number of cells $\pm$ SD (number of embryos)		
	Bl	Bx	Be
Control	67.1 $\pm$ 3.1 (14)	100.7 $\pm$ 6.9 (12)	189.9 $\pm$ 16.1 (12)
Biopsy	61.1 $\pm$ 5.5 (13)	121.9 $\pm$ 10.6 (14)	187.3 $\pm$ 18.5 (7)

Note: F test ( $P > .05$ ).

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In this study, we evaluated the effect of the biopsy of 8-cell to 16-cell bovine embryos on their subsequent development and the effect of WGA on removed blastomeres. Biopsy of a quarter of each bovine embryo did not affect the development of the embryo to the blastocyst stage. Other reports using the bovine model have shown similar results (12–14). In addition, Jimenez-Macedo et al. (4) found that the biopsy did not affect embryo development in prepubertal 6-cell to 10-cell goat embryos.

The blastocyst hatching rate in the biopsy group did not differ from the control group on the 10th day of development. These findings were also shown by other studies using bovine models (12, 15). Almodin et al. (12) found a hatching rate of 27.5% in the biopsy group on the eighth day. We found a hatching rate of 44.9%, although our results were obtained on the 10th day. In contrast, Park et al. (13) observed a higher hatching rate in biopsied embryos compared with the control group.

The quality of biopsied embryos was not affected during in vitro culture (see Table 2). The results of previous studies coincide with these findings and indicate that biopsy before the formation of gap junctions between blastomeres did not reduce the developmental potential of biopsied embryos (12–14).

Moreover, the experiment conducted by Hardy et al. in 1990 (16) showed that biopsy of human embryos did not affect preimplantation development to the blastocyst stage. Although used as a reference, their work was conducted using mostly good quality and cell-number embryos, which is rarely representative of real-case conditions (1). In our study, we did not make any embryo selection, and the oocytes were obtained from slaughterhouse cows of different genetic composition without any information about their reproductive health. The embryo diversity found in our study is similar to the diversity found in human reproduction patients.

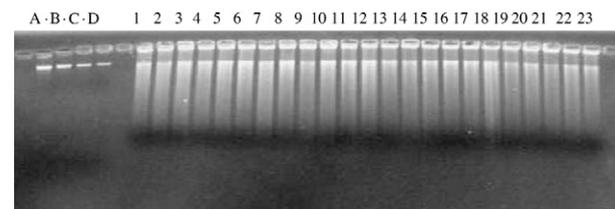
Another limitation to PGD techniques is the requirement of a minimum amount of good quality genomic DNA to perform the analysis of genetic diseases by PCR or fluorescence in situ hybridization (FISH). To increase the efficiency of the PCR diagnosis, we amplified the blastomeres DNA using WGA, a simple isothermic method in which bacteriophage

polymerase is used to generate micrograms of DNA starting from nano or picograms of DNA samples (17). Before WGA, male and female bovine DNA were serially diluted and used to standardize the PCR protocol for sex determination and PCR limit of detection. Thus, it was possible to achieve 98% efficiency in amplifying blastomeres using the WGA kit. Amplified samples showed approximately 400 ng of DNA generated from an estimated initial amount of 12 pg of DNA resulting from two blastomeres per embryo (18). Schneider et al. (19) demonstrated the efficiency of the GenomiPhi DNA Amplification Kit using dilutions of human cell lines. This successful amplification creates a DNA stock sample that could be used for various gene profiling and sex determination analyses of the same embryo, allowing analyses to be rerun to double-check the results, as mentioned by Ballantyne et al. (20). Moreover, the WGA protocol minimizes template loss and excessive sample handling because all steps including DNA isolation, restriction enzyme digestion, primer ligation, and PCR amplification are performed in the same tube (5).

In whole embryos from different stages (see Table 3), we found no differences in the proportion of males and females. Some investigators, however, have described an increase in the proportion of male embryos in the blastocyst stage and have suggested that this could happen due to a possible faster male embryo development when compared with female

**FIGURE 1**

Agarose gel showing blastomere PCR product after whole genome amplification, wells 1 to 23. Lambda DNA: A (400 ng), B (200 ng), C (100 ng), and D (50 ng).



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**TABLE 3**

**Distribution of blastocysts, embryos (2, 4–7, 8–16 cells), and biopsied material according to sex after polymerase chain reaction (PCR).**

DNA sample	Male (n)	Females (n)	Total	% PCR Failure (n)
Blastocyst	12 <sup>a</sup>	13 <sup>a</sup>	27	7 (2) <sup>A</sup>
2 cell	1 <sup>a</sup>	4 <sup>a</sup>	6	17 (1) <sup>B</sup>
4 to 7 cell	1 <sup>a</sup>	1 <sup>a</sup>	5	60 (3) <sup>B</sup>
8 to 16 cell	1 <sup>a</sup>	2 <sup>a</sup>	5	40 (2) <sup>B</sup>
Biopsied material	8 <sup>a</sup>	25 <sup>b</sup>	56	41 (23) <sup>B</sup>

*Note:* The number of male, female, total and PCR failure (%) is indicated. Chi-square test ( $P < .05$ ).

b > a: Sex of DNA sample (rows).

A < B: % PCR failure (column).

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embryos, and greater resistance of male embryos to various stress culture conditions (21–23).

The sex proportion rate in blastomeres found in this study was similar to that found by Almodin et al. (18), who detected an increase in the prevalence of female embryos after PCR (see Table 3). Although no plausible explanation was found for this, our results coincide with findings in the literature indicating a greater proportion of female embryos at early stages of development (21–23).

The efficiency of PCR for sexing whole embryos was greater with blastocysts than with embryos at earlier stages of development and also greater than with biopsied material amplified by WGA (see Table 3). Because PCR sensitivity was very high, detecting as little as 0.0003 ng of DNA, we would expect no differences in PCR efficiency among different DNA samples. These differences could be related to a possible nonextraction of DNA, sample degradation caused by DNA liberation procedures, cytoplasm material present in embryos and biopsied samples, or by sex-chromosomal mosaicism or absence of a nucleus in the biopsied sample (1, 18).

We demonstrated that biopsy of 8-cell to 16-cell bovine embryos did not affect the subsequent development and quality of the embryos. We also described a sensitive PCR protocol for embryos at different stages of development and showed the feasibility of using WGA to amplify biopsied samples before PCR to achieve an efficient PGD.

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