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Blastocele fluid from *in vitro*– and *in vivo*–produced equine embryos contains nuclear DNA

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ABSTRACT

Normal mammalian early embryonic development involves apoptosis of blastomeres as a remodeling process during differentiation, starting at the blastocyst stage. Genomic DNA has been recently detected in the blastocele fluid of human embryos and has been amplified by real-time polymerase chain reaction (PCR) to diagnose the sex of *in vitro*–produced human embryos. This new approach varies from conventional preimplantation genetic diagnosis in that no cells are extracted from the embryo and only the blastocele fluid is aspirated and used as a DNA sample for diagnosis. In the present work, we investigated whether the blastocele fluid of equine preimplantation embryos contains nuclear DNA and whether this DNA could be used to diagnose the sex of the embryos by conventional PCR, using specific primers that target the *TSPY* and *AMEL* equine genes. The sex of 11 of 13 *in vivo*–produced embryos and of four of five *in vitro*–produced embryos was successfully diagnosed. The PCR amplification product was analyzed using genetic sequencing reporting that the DNA present in blastocele fluid was genomic. Additionally, after polyacrylamide gel electrophoresis and silver staining, the blastocele fluid from three different embryos produced a ladder pattern characteristic of DNA fragmented during apoptosis. Therefore, the results presented in this work report that blastocele fluid from *in vivo*– and *in vitro*–produced equine embryos contains nuclear DNA which is probably originated by apoptosis of embryonic cells, and this DNA could be used to diagnose the sex of preimlpantation embryos by conventional PCR.

1. Introduction

Apoptosis, a form of cell death that affects isolated cells, is characterized by nucleus and DNA fragmentation, cytoplasm condensation, membrane changes, and cell death without lysis or damage to neighboring cells. It is a normal physiological phenomenon that occurs in multicellular organisms and is genetically determined.

During early development of mammalian embryos, the blastomeres differentiate at the blastocyst stage into two different cell lineages: the trophoblast, which will give rise to extraembryonic tissue, and the inner cell mass, which forms the fetus. In a normal developing embryo, these two cell lineages undergo apoptosis [1], a process that occurs as

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a mechanism to eliminate unwanted or genetically defective cells.

Recently, Palini et al. [2] reported for the first time the presence of genomic DNA in the blastocele fluid of *in vitro*–produced human embryos. These authors reported that this DNA was amplifiable by real-time polymerase chain reaction (PCR) and hypothesized that this DNA could be released into the blastocele cavity from cells undergoing apoptosis. In addition, they were able to determine the sex of 26 of 29 human embryos by amplifying the DNA present in the blastocele fluid from these embryos.

Preimplantation genetic diagnosis (PGD) allows the identification of specific genetic traits of early embryos. Preimplantation genetic diagnosis was first used in humans more than 20 years ago [3] and has also been used in bovine [4], caprine [5], and equine [6] preimplantation embryos. Conventional PGD involves obtaining blastomeres by biopsy from early embryos, which has to be performed carefully to avoid impairing the viability of the embryo.

Blastocele fluid aspiration is a simpler technique to obtain genomic DNA from embryos because only fluid is collected from the blastocysts and therefore, whether it can be used to determine the sex of preimplantation embryos efficiently, gender by PGD could be determined faster. This is important whether PGD is performed to avoid transferring embryos of the undesired sex because the determination needs to be completed on the same day of uterine flush. In some horse breeds, such as Polo Argentino, females are preferred to males because their higher abilities for performance in Polo.

Therefore, the aim of this work was to study whether (1) the sex of *in vivo*– and *in vitro*–produced equine embryos can be determined by amplification of DNA from blastocele fluid, (2) the blastocele fluid of preimplantation equine embryos contains nuclear DNA, and (3) the DNA present in blastocele fluid is originated by apoptosis.

2. Materials and methods

2.1. Animal care and welfare

The protocol (011/2013) for this study was approved by the Committee on the Ethics of Animal Experiments of the Universidad Nacional de San Martín following the recommendations in the Guide for the Care and Use of Animals of the National Institute of Health.

2.2. Experiment 1: gender determination by amplification of DNA from blastocele fluid from in vivo– and in vitro–produced equine embryos

2.2.1. In vivo–produced equine embryos

Ovarian follicular development in estrous, crossbreed, donor mares was monitored daily using ultrasound scanning. When the dominant follicle(s) reached 35 mm or greater in diameter, the mares were artificially inseminated once with fresh semen from a fertile stallion and administered 1 mg of a GnRH analog (BioRelease deslorelin acetate; BET Pharm, Lexington, KY, USA) intramuscular for ovulation induction. Fixed twice daily (7 AM and 7 PM), ultrasound examinations of the ovaries were continued to diagnose the occurrence of ovulation (Day 0).

Exactly on Day 8 after ovulation detection on each donor mare, they were placed in stocks and embryos were nonsurgically collected as described previously by Losinno et al. [7] using sterile Ringer Lactate as flushing media and in-line embryo filter. The residual medium in the filter was transferred to a sterile plastic Petri dish, and the embryo was searched using a stereomicroscope under a laminar flow. Once the embryo was located, it was morphologically graded using the scale proposed by McKinnon and Squires [8]. Only morphologically normal (grade one) and blastocyst stage embryos were included in the study.

2.2.2. Equine embryos produced in vitro by intracytoplasmic sperm injection

Ovaries were obtained from a pool of mares with unknown reproductive history from an equine slaughterhouse located 50 km from our laboratory. Oocytes were obtained by aspiration of follicles with a 19-ga needle connected to an aspiration pump and matured *in vitro* for 18 to 22 hours in 50-μL microdroplets under mineral oil of TCM 199 supplemented with 1-mM glutamine, 0.19-mM sodium pyruvate, 5 μg/mL FSH (Bioniche), 100 ng/mL epidermal growth factor, 100 ng/mL insulin-like growth factor I, and 10% fetal bovine serum (FBS; Gibco, South America). After *in vitro* maturation, expanded cumulus–oocyte complexes were incubated in a 0.1% hyaluronidase solution for 10 minutes, and their cumulus cells were removed by vigorous pipetting through a fine-bore glass pipette.

All oocytes with a visible polar body and an intact cytoplasm were selected for injection, and intracytoplasmic sperm injection was performed as described previously by Palermo et al. [9]. Briefly, sperm cells were immobilized by disruption of the plasma membrane with the injection pipette. Each oocyte was injected with one stallion sperm while held in place by the holding pipette. All injected oocytes were cultured *in vitro* in synthetic oviductal fluid medium (SOFm) with 19-mM D-glucose with 10% FBS at 38 °C, in 7% O_2 and 5% CO_2 for 48 hours [10]. At this time, oocytes were observed under light microscopy, and all cleaved embryos were cultured for at least 5.5 more days.

2.2.3. Blastocele fluid collection

In vivo– or *in vitro*–produced blastocyst-stage embryos were placed in 50-μL microdroplets of Dulbecco modified PBS without calcium and magnesium (Sigma) supplemented with 10% FBS and 50 μg/mL of gentamicin (working medium) under mineral oil, on an inverted microscope equipped with a Nikon-Narishige micromanipulation system. Embryos were held in place by suction of a holding pipette, and the inner cell mass was placed 90° clockwise away from the holding pipette. Then, the embryo capsule of *in vivo*–produced embryos or the zona pellucida of *in vitro*–produced embryos was punctured with a beveled micropipette (9 μm inner diameter; ORIGIO, Humagen Pipets, USA). All the blastocele fluid was aspirated and, using the same micropipette, discharged on a 1-μL microdroplet of working medium. The microdroplet containing the blastocele fluid was examined under the microscope for the

presence of embryonic cells that could have been accidentally aspirated during the procedure. Only samples that did not contain any cells were included in the study. The blastocele fluid aspiration procedure (Fig. 1) was performed by manual suction, connecting the biopsy micropipette to a disposable syringe. The complete volume of the microdroplet containing the blastocele fluid was transferred to a 0.2-mL DNAse-free tube containing 4 μL of DNAse-free water. The operators performing the blastocele fluid collection wore lab coats, nonpowdered gloves, masks, and caps. The micropipettes used for blastocele fluid collection were replaced after each procedure, so that each set of pipettes was used for only one embryo. The tips used to handle blastocele fluid samples were sterile, disposable, with filter, and used individually for each sample.

2.2.4. Amplification by PCR of the DNA present in the blastocele fluid

A duplex PCR was performed to amplify the Y-encoded testis-specific protein (*TSPY*) and amelogenin (*AMEL*) genes. The oligodeoxynucleotide primers used to amplify these two sequences and the size of the expected amplification products are shown in Table 1. The total reaction volume was 12.5 mL and contained 6.25-mL MasterMix (Promega, MI, USA), 0.2-μM *TSPY* primers, 0.1-μM *AMEL* primers, and 3.25 μL of the sample. The thermal cycling parameters were 95 °C for 1 minute, 35 cycles of 94 °C for 15 seconds, 58.4 °C for 30 seconds, 72 °C for 30 seconds, and 72 °C for 5 minutes. The PCR was performed twice, using the same primers and cycling protocol. To monitor a possible DNA contamination, one tube was included as a control in the first PCR and contained all the necessary components for the PCR, except 3.25 μL of DNAse-free water placed in the tube instead of the DNA sample. In the second PCR, 3.25 μL of the amplification product from the first PCR was used as DNA template. All the samples and reagents for PCR and the amplification products were handled using sterile disposable tips with filter. The amplification products were electrophoresed on a 2% agarose gel stained with ethidium bromide and visualized under UV light.

2.2.5. Survival of in vivo–produced embryos after blastocele fluid aspiration and in vitro culture

Immediately after blastocele fluid aspiration, seven *in vivo*–produced embryos were placed individually in 50-μL microdroplets of SOFm with 19-mM D-glucose with 10% FBS under mineral oil at 38 °C, in 7% O_2 and 5% CO_2, and observed at 24, 48, and 72 hours. The diameter of embryos was registered immediately before blastocele fluid aspiration and every 24 hours during *in vitro* culture.

2.2.6. Amplification by PCR of the DNA from sampled embryos

After blastocele fluid aspiration or blastocele fluid aspiration and *in vitro* culture for 72 hours, all embryos were sliced into small fragments using a microblade and each fragment was placed in a 0.2-mL DNAse-free tube with 4 μL of DNAse-free water and maintained at −20 °C until analyzed by PCR. The primers and cycling protocol were the same as with the blastocele fluid samples. Results of the amplification of the DNA from sampled embryos were compared with the results of the amplification of the DNA from the blastocele fluid samples from the same embryos.

2.3. Experiment 2: genetic sequence analysis of the amplified DNA from blastocele fluid of in vivo–produced embryos

The band on the agarose gel corresponding to the amplification product of the expected molecular weight of

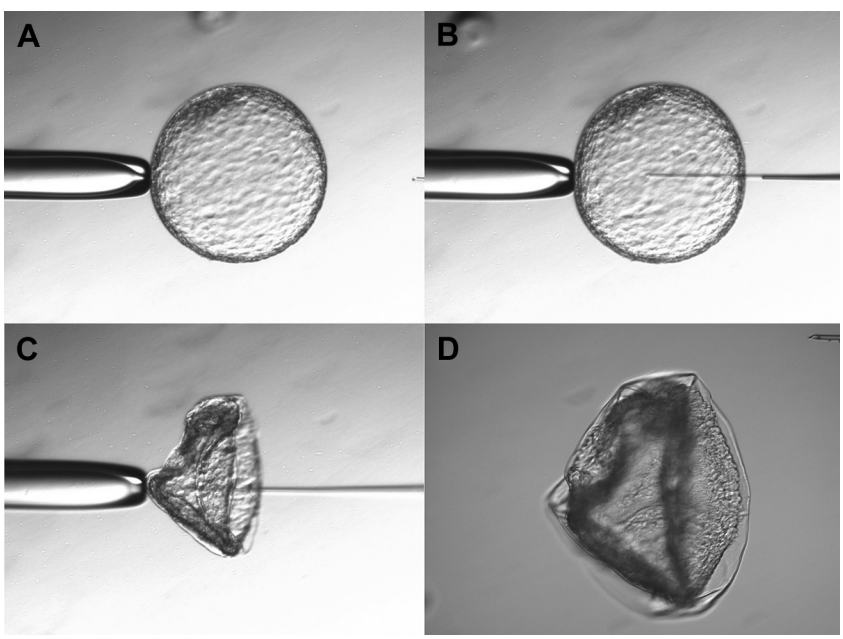

Fig. 1. Blastocele fluid collection from an *in vivo*–produced equine embryo of 500 μm in diameter. (A) *In vivo*–produced equine embryo before blastocele fluid aspiration, (B) the embryo is punctured with the aspiration micropipette, (C) the blastocele fluid is aspirated, and (D) the same embryo as in A after blastocele fluid collection.

Table 1
Primers used for sex determination of equine embryos by amplification of DNA in blastocele fluid.

Primer	Sequence	Fragment size (bp)	References
AMEL-F	5′-CCAACCCAAC ACCACCAGCCAA ACCTCCCT-3′	184 (female); 160/200 (male)	Hasegawa et al. [11]
AMEL-R	5′-AGCATAGGGGGCA AGGGCTGCAAGGG GAAT-3′		
TSPY-F	5′-GAA GTC AGG CAC ACC AGT GA-3′	280	Paria et al. [12]
TSPY-R	5′-TAA GGC TGC AGT TGT CAT GC-3′		

the specific PCR using TSPY primers from two different samples obtained from *in vivo*–produced embryos was extracted using the QIAEX DNA extraction kit (Qiagen, CA, USA), according to the manufacturer's recommendation. The purified fragments were cloned into pGEM-T Easy (Promega) and subsequently propagated in *Escherichia coli* TOP10 (Invitrogen, CA, USA). Bacteria were grown in Luria–Bertani medium (10-g Bacto Tryptone, 5-g yeast extract, and 10-g NaCl in 1-L water) overnight at 37 °C with stirring. At 18 hours of culture, a midiprep was performed with 150 mL of medium using the Qiagen Midi Kit (Tip 100, Qiagen), following the manufacturer's recommendations. The plasmids obtained were quantified by spectrophotometry and sequenced with SP6 and T7 primers. Sequencing was performed by an ABI PRISM 3111 sequencer. Sequences were deposited at the National Center for Biotechnology Information and compared using the basic local alignment tool with existing sequences at GenBank. Only sequences with more than 99% of identity were accepted.

2.4. Experiment 3: DNA ladder assay of blastocele fluid from *in vivo*–produced embryos

Blastocele fluid from three different, Day-8, *in vivo*–produced equine embryos was obtained as described in experiment 1. DNA was purified from blastocele fluid samples through Mini Spin Kit Columns (Qiagen), following the manufacturer's protocol. Electrophoresis was performed in 10% polyacrylamide gel in 1X TBE buffer (89-mM Tris, 89-mM boric acid, and 2-mM EDTA) for 2 hours at 100 V.

After electrophoresis, the tray was disassembled and the gel was washed with purified water for 10 seconds. Then, the gel was incubated for 10 minutes in fixing solution (acetic acid in water 7.5% v:v). Once completed, the gel was washed with deionized water for 2 minutes. Additionally, a pretreatment was performed with a solution of formaldehyde (15% in water v:v) for 10 minutes, then incubated for 20 minutes with a silver solution (0.1-g $AgNO_3$ in water), and washed twice with deionized water for 3 seconds. The gel was revealed by incubation in developing solution (3-g Na_2CO_3 and 1-mM sodium thiosulfate in 100 mL of water). The reaction was stopped with stop developer solution (7.5% acetic acid in water).

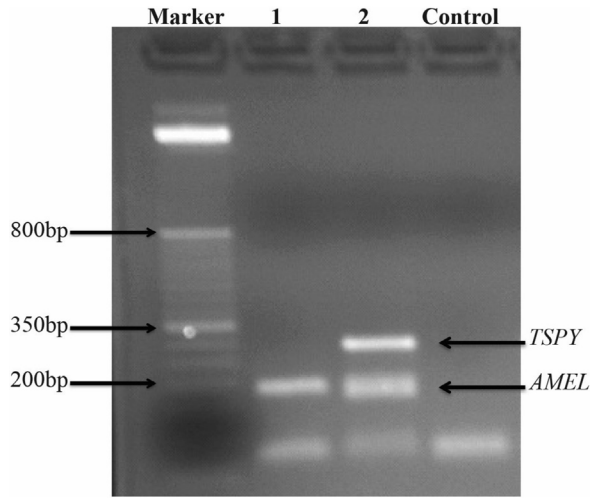

Fig. 2. Electrophoresis in agarose gel of *TSPY* and *AMEL* PCR products. Marker: 50-bp marker; 1, female sample, one *AMEL* band at 184 bp; 2, male sample, one *TSPY* band at 280 bp and two *AMEL* bands at 160 bp and 200 bp; and Control: no DNA control.

3. Results

3.1. Experiment 1

Thirteen *in vivo*–produced and five *in vitro*–produced equine embryos were used for this experiment. The bands observed after PCR and electrophoresis of the amplified female and male samples are shown in Figure 2. The genetic sex was determined in 11 of the 13 (84.6%) and four of the five (80%) blastocele fluid samples obtained from *in vivo*– and *in vitro*–produced embryos, respectively. Eight of the eleven blastocele fluid samples from the *in vivo*–produced embryos that amplified were diagnosed as males and three were diagnosed as females, whereas two out of the four amplified samples from the *in vitro*–produced embryos were diagnosed as males and two as females. The results obtained by PCR of blastocele fluid were compared with those obtained after PCR of fragments of embryos from which the blastocele fluid was collected. The sex diagnosed from blastocele fluid samples was the same as that diagnosed from the corresponding pieces of embryos in all 15 cases (11 *in vivo*–produced and four *in vitro*–produced embryos, respectively). The two blastocele fluid samples from *in vivo*–produced embryos and the only sample from an *in vitro*–produced embryo that failed to amplify after PCR were obtained from female embryos.

Seven of the 13 *in vivo*–produced embryos were cultured *in vitro* and observed every 24 hours for post–blastocele fluid aspiration survival. Six of seven embryos reexpanded at 24 hours of *in vitro* culture and increased their diameter at 48 and 72 hours. The average percentage increase in diameter at the end of the culture period was 73.7%, from a mean of 633 μm to a mean of 1100 μm. Four of the six reexpanded embryos hatched *in vitro* during the culture period. Figure 3 shows one of the *in vivo*–produced embryos hatching, after 48 hours of *in vitro* culture.

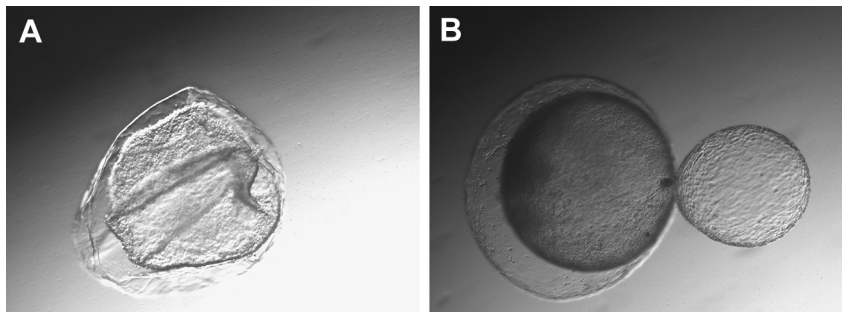

Fig. 3. *In vivo*–produced equine embryo. (A) After blastocele fluid aspiration, (B) after 48 hours of *in vitro* culture.

3.2. Experiment 2

The sequence cloned into pGEM-T Easy and plasmid DNA was isolated and purified by Qiaprep Spin Miniprep Kit (Qiagen), according to the manufacturer's protocol. Then, the plasmid DNA was compared using basic local alignment tool. The fragment obtained after PCR of DNA in the blastocele fluid was 100% identical to a sequence in the Y chromosome of *Equus caballus*, sequence ID, AC215855.2, reporting that the amplified sequence was genomic DNA.

3.3. Experiment 3

This experiment was repeated three times, using blastocele fluid from three different *in vivo*–produced equine embryos. Separation of DNA from blastocele fluid by polyacrylamide gel electrophoresis and silver staining showed a ladder pattern in all the three samples, characteristic of DNA from cells undergoing apoptosis (Fig. 4).

4. Discussion

Preimplantation genetic diagnosis, which allows the detection of genetic traits in early embryos, is a well-established procedure that involves the collection of one or more cells. Recently, Palini et al. [2] found that PGD could also be performed using blastocele fluid of *in vitro*–produced, human, preimplantation embryos, obtained before vitrification.

In the present work, we confirmed the findings of Palini et al. [2] by detecting the sex of *in vitro*–produced equine embryos and showed that this strategy can be applied to *in vivo*–produced equine embryos as well, using conventional PCR.

Although we were able to diagnose the sex of 11 of the 13 *in vivo*–produced embryos and of four of the five *in vitro*–produced embryos, this technique failed to produce a result in three of the samples. The diagnosis rate of this technique was high, but further studies with a larger number of samples will report whether this efficiency can be improved. Using multicopy genes for PCR improves the detection rate in samples with low amounts of DNA, such as those of blastocele fluid. The use of blastocele fluid for PCR is probably limited to multicopy genes and not suitable for the detection of other genes that are in single copy. This was evidenced in experiment 1, in which all three samples that failed to produce an amplification product were from female embryos, reporting that *AMEL*, being a single-copy gene, was not efficiently detected. All male samples were diagnosed successfully, using specific primers that targeted the *TSPY* gene, which is involved in testicular development and is present in multiple copies in the equine genome of male samples [12].

Because of the small amount of DNA present in blastocele fluid, a double PCR and primers targeting multicopy genes need to be used to achieve amplification. This methodology is highly sensitive to DNA contamination, and therefore, handling of blastocele fluid samples and amplification products needs to be performed with extreme caution. Operators performing the PCR have to wear proper protection, samples and amplification products need to be handled using tips with barriers, and negative controls have to be included on each step of the protocol.

In experiment 2, using specific primers targeting for *TSPY* and genetic sequencing, we were able to confirm that the amplified sequence was from *E caballus* DNA located in the Y chromosome. This result confirms that the DNA in blastocele fluid used as a template for PCR was nuclear DNA.

In experiment 3, we found that the DNA present in the blastocele fluid from *in vivo*–produced embryos is fragmented and shows a characteristic ladder pattern present in cells undergoing apoptosis. To our knowledge, this is the first report presenting evidence that the DNA in blastocele fluid is originated from apoptosis. Tremoleda et al. [13]

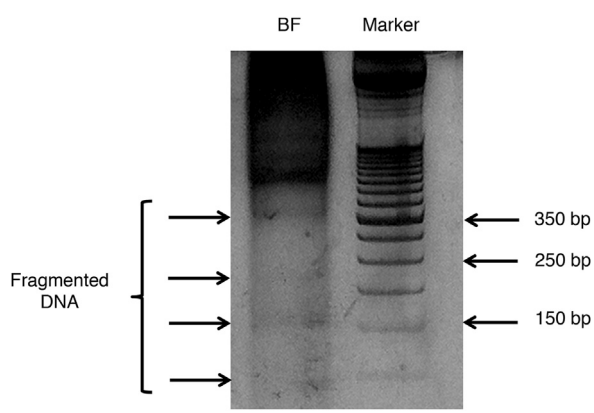

Fig. 4. Polyacrylamide gel electrophoresis and silver staining of blastocele fluid from an *in vivo*–produced equine embryo. BF, blastocele fluid; Marker: 50-bp marker.

showed that equine embryos produced *in vitro* by intracytoplasmic sperm injection present a much greater number of apoptotic cells than *in vivo*–produced embryos, with only 4 out of 10 *in vivo*–produced embryos presenting apoptotic cells. In experiment 1, we were able to amplify the apoptotic DNA present in blastocele fluid by PCR, proving that *in vivo*–produced embryos present enough apoptotic DNA as a template for PCR.

Previous reports have shown that blastomere biopsy of murine embryos produces epigenetic aberrations in the brain tissue of the offspring [14] and that there is an abnormal development and function of neurons of mice generated after blastomere biopsy [15]. Blastocele fluid collection could be less detrimental to the embryo because no embryonic cells are extracted during the procedure, is technically simpler than conventional PGD and is performed in less time. In the present study, we observed that a high proportion of aspirated embryos survived and increased their diameter after *in vitro* culture for 72 hours. Although the pregnancy rates of *in vivo*– or *in vitro*–produced equine embryos transferred after blastocele fluid extraction still need to be studied, the *in vitro* survival of aspirated embryos reports that this approach does not impair their viability. Because it has already been shown that the pregnancy rates of biopsied or intact *in vivo*–produced equine embryos are not significantly different [16,17], similar results are expected using embryos from which only blastocele fluid and no cells are obtained. In a previous report, we aspirated 10 to 30 blastomeres from each biopsied embryo [17] and embryos collapsed completely during the biopsy procedure, similar to that observed after blastocele fluid collection.

4.1. Conclusions

The results presented in this study report that the sex of *in vivo*– and *in vitro*–produced equine embryos can be diagnosed using blastocele fluid as the DNA source for conventional PCR. Our results also confirm that the DNA amplified by PCR is a nuclear DNA and that fragmented DNA, probably originated from apoptosis of embryonic cells, is present in the blastocele fluid of *in vivo*–produced embryos. The large size and consequently the large volume of blastocele fluid present in equine embryos make the horse a suitable animal model to study the presence of apoptotic DNA during early development.

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