Theriogenology 83 (2015) 415–420

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

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

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ARTICLE INFO

Article history:
Received 3 June 2014
Received in revised form 9 September 2014
Accepted 4 October 2014

Keywords:
Preimplantation genetic diagnosis
Equine
Preimplantation embryo
Sex determination
Apoptosis

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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http://dx.doi.org/10.1016/j.theriogenology.2014.10.006

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