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Post implantation development reveals that biopsy procedure can segregate "healthy" from "unhealthy" bovine embryos and prevent miscarriages

Clara Slade Oliveira[a,*], Carolina Capobiango Romano Quintão[b], Celio de Freitas[a], Agostinho Jorge dos Reis Camargo[c], Raquel Varella Serapião[c], Luiz Sérgio de Almeida Camargo[b]

[a] Animal Reproduction Laboratory, Santa Monica Experimental Station, Embrapa Dairy Cattle, Fazenda Santa Monica Road, Barao de Juparana, Valença, RJ, 27640-000, Brazil
[b] Animal Reproduction Laboratory, Embrapa Dairy Cattle, 610 Eugenio do Nascimento Ave., Juiz de Fora, MG, 36038-330 Brazil
[c] Agriculture Research Company of the Rio de Janeiro State (PESAGRO RIO), Sao Boa Ventura Ave, 770, Niterói, RJ, 24120-19 Brazil

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ABSTRACT

Embryo biopsy has been performed in bovine in *vivo* produced embryos for the last twenty years, but little could be done with few embryonic cells in the past. Recently, advances in single cell analysis enabled a wide range of applications using embryo biopsy, from morphology to genetics analysis and different omics-techniques, which are promising for in vitro-fertilized (IVF) embryos. The aim of this study was to address if biopsy procedure would affect post implantation development of IVF blastocyts. Here we show that blastocyst stage do not affect re-expansion of biopsied embryos (regular blastocyst: 73.7%; expanded blastocyst: 73.1%), but affects ($p < 0.05$) implantation (regular blastocyst: 37.8%, expanded blastocyst: 61.0%), so ideally biopsy should be performed in expanded blastocysts. No detrimental effect of biopsy procedure was detected for post-implantation development (calving rates, Biopsy: 47.1%, Control: 41.9%), and normal calves were born (Birth weight, Biopsy: 32.10 ± 7.20 kg; Control: 30.95 ± 5.43 kg). Surprisingly, we found interesting results suggesting embryo survival can be increased with aggressive procedures (such as embryo biopsy), and this is highly associated with early pregnancy loss (Biopsy: 0%, Control: 17.4%). This finding also suggests morphological classification of day 7 blastocysts is far from ideal, and supposedly, unhealthy embryos can implant but are bound to miscarriage during the first trimester (non-biopsied embryos). Our results show biopsy procedure is safe for bovine IVF embryos, and shed new light into the importance of conceptus in early pregnancy loss in cattle.

1. Introduction

In vitro embryo technologies in cattle first emerged as alternative tools used for high genetic merit donors that failed to respond to superovulation and *in vivo* embryo production (conventional Embryo Transfer – ET). However, due to its advantages, the use of *in vitro* fertilization (IVF) has increased in the last decade, accounting for over 590,000 embryos produced in 2014 (IETS, 2015).

* Corresponding author.
E-mail addresses: clara.oliveira@embrapa.br (C.S. Oliveira), carolina.quintao@embrapa.br (C.C. Romano Quintão), celio.freitas@embrapa.br (C. de Freitas), agostinhocamargo@yahoo.com.br (A.J. dos Reis Camargo), rserapis@gmail.com (R.V. Serapião), luiz.camargo@embrapa.br (L.S. de Almeida Camargo).

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The emergence of IVF technologies also caused a number of changes in bovine embryo industry. Together with the growing demand for IVF embryos, the features of the modern embryo industry opened new perspectives for selection of high merit embryos based on biological markers present in their genome. Genomic selection allows the prediction of animal genomic breeding value (GEBV), and permits cost and generation interval reduction over the traditional progeny test method (Bouquet and Juga, 2013). By the integration of IVF and genomic selection, embryos instead of animals can be selected based on their GEBV, shortening even more the generation interval and reducing costs with embryos recipients.

To determine the GEBV a group of cells is removed from the embryo by biopsy and then used for genotyping. Embryo biopsies in cattle started being used in the late 80s in embryos produced *in vivo* (ET embryos), and studies reported reasonable success rates of survival after embryo biopsy for IVF embryos (Kirkpatrick and Monson, 1993; Macháty et al., 1993; Bredbacka, 1995) but it was not put into practice in IVF commercial companies.

Initially, interest in embryo biopsy was mainly in regards to sex-determination of ET embryos (Shea, 1999), when sperm sex-sorting was not commercially available. Currently, biopsy of commercial IVF embryos is mainly directed for genomic selection. In this context, several commercial companies produce thousand embryos every day that could be biopsied and selected for desirable traits before being transferred and established a gestation. Biopsy sample is also valuable for experimental purposes. With all recent advances in single cell analysis (Galler et al., 2014), it is now possible to achieve deeper understanding of biological processes throw omics-techniques with those few cells. However, the suboptimal in vitro culture environment can negatively affect metabolism and gene expression of IVF embryos (Lonergan et al., 2003), which differ from ET embryos in several ways (Wrenzycki and Niemann, 2003). Due to such effects of in *vitro* environment, one concern is the post-biopsy viability of IVF embryos.

The aim of this study was to address if biopsied embryos are able to implant and continue pregnancy normally, or if this technique affects any stage of post-implantation development.

2. Methods

2.1. Experimental design

Post-implantation development of biopsied embryos was assessed and compared to non-biopsied (Control) embryos. Girolando embryos were produced in vitro from Gir or F1 (Gir x Holstein crossbred) oocytes. Day 6.5 blastocysts were biopsied and cultured to assess re-expansion. Expanded grade I day 7 blastocysts (biopsied and non-biopsied) were transferred to recipients. Pregnancy diagnosis was performed 30 days after transfer, and gestation was assisted in a mensal basis. Data regarding embryonic loss and delivery was assessed. Procedures followed ethical guidelines for animal experimentation and were approved by local Committee (CEUA–EGL, protocol 29/2013).

2.2. Supplements

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO), culture medium was purchased from Bioklone (Jaboticabal, Brazil) and hormones were purchased from Ourofino (Sertaozinho, Brazil), unless otherwise stated.

2.3. In vitro embryo production

Oocytes from non-lactating Gir and F1 donors were recovered after epidural anesthesia (lidocaine hydrochloride 2%), using a transvaginal device guiding both needle and a 7,5 MHz convex transducer coupled to an ultrasound equipment (Mindray DP2200). Follicles between 2 and 8 mm were punctured with 18 gauge × 50 mm needles and the follicular fluid aspirated through a vacuum system (90 mmHg) wa collected from each donor individually in 50 mL tube containing Dulbeco PBS supplemented with 50UI heparin and 1% fetal calf serum (FCS) solution, at 37 °C. Fluid was filtered and transferred into Petri dishes (100 × 20 mm) for *cumulus* oocyte complexes (COCs) tracking in a stereomicroscope (Olympus SZ40). Viable COCs were matured for 24 h in droplets of sodium bicarbonate-buffered TCM-199 supplemented with 10% FBS, 1.0 μg/mL FSH (Folltropin™, Bioniche Animal Health, Belleville, Canada), 50 μg/mL hCG (Profasi™, Serono, Sao Paulo, Brazil), 1.0 μg/mL estradiol, 16 μg/mL sodium pyruvate and 83.4 μg/mL amikacin. Matured oocytes were submitted to in vitro fertilization for 15–18 h in 30-μL drops of TALP-IVF medium supplemented with 0.6% BSA, 10 μg/mL heparin, 18 μM penicillamine, 10 μM hypotaurine and 1.8 μM epinephrine. Frozen–thawed straws from Holstein bulls, containing X-chromosome bearing spermatozoa, sorted by flow cytometry (CRV Lagoa/Sexing Technologies, Sertaozinho, Brazil) were used. Each straw containing approximately 2 million spermatozoa was centrifuged separately on a discontinuous 45/90 Percoll gradient for 7 min at 3600 × g. The pellet was suspended in 700 μL TALP-IVF medium and again centrifuged for 5 min at 520 × g. After centrifugation, the pellet was collected and divided among five TALP-IVF drops. After IVF, presumptive zygotes were partially denuded of cumulus cells by vigorous pipetting and cultured in SOF-AA medium supplemented with 16 μg/mL sodium pyruvate, 83.4 μg/mL amikacin, 2.8 mM *myo*-inositol, 340 μM tri-sodium citrate dehydrate, 2.5% FBS and 6 mg/mL BSA. Remaining cumulus cells were attached to plastic surface and formed a monolayer of granulosa cells. Medium was half replaced every 48 h. For all steps, structures were cultured in droplets under mineral oil, and cultivated at 38.5 °C in an atmosphere of 5% CO2 in air under saturated humidity. Cleavage was assessed at 72–96 h.p.i. and blastocysts at 155–160 hpi.