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Cell death is involved in sexual dimorphism during preimplantation development

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ABSTRACT

In bovine preimplantation development, female embryos progress at lower rates and originate smaller blastocysts than male counterparts. Although sex-specific gene expression patterns are reported, when and how sex dimorphism is established is not clear. Differences among female and male early development can be useful for human assisted reproductive medicine, when X-linked disorders risk is detected, and for genetic breeding programs, especially in dairy cattle, which requires female animals for milk production. The aim of this study was to characterize the development of female and male embryos, attempting to identify sex effects during preimplantation development and the role of cell death in this process. Using sex-sorted semen from three different bulls for fertilization, we compared kinetics of bovine sex-specific embryos in six time points, and cell death was assessed in viable embryos. For kinetics analysis, we detected an increased population of female embryos arrested at 48 and 120 h.p.i., suggesting this time points as delicate stages of development for female embryos that should be considered for testing improvement strategies for assisted reproductive technologies. Assessing viable embryos quality, we found 144 h.p.i. is the first time point when viable embryos are phenotypically distinct: cell number is decreased, and apoptosis and cell fragmentation are increased in female embryos at this stage. These new results lead us to propose that sex dimorphism in viable embryos is established during morula-blastocyst transition, and cell death is involved in this process.

1. Introduction

Sex of preimplantation embryos is usually not considered in reproductive biology studies. However, recent evidence has suggested far from being irrelevant the sex of cell lines, and highlights how X-chromosome inactivation (XCI) and other singularities can be interfering with results in many cell types (Shah et al., 2014). In addition, NIH has recently proposed an initiative aiming the balance in sex of animals in preclinical studies (McCullough et al., 2014), emphasizing the importance of sex at biological responses.

In vitro fertilization (IVF) and embryo culture are extensively used biotechnologies, and are considered valuable tools for human assisted reproduction, developmental science, and for livestock breeding. In addition to notably importance on behalf of livestock, bovine embryonic development also consists in an important model for embryology. Among their similarities to human development are: the closer time of embryonic genome activation, the duration of preimplantation development and the low developmental rates (Niakan and Eggan, 2013). Critical differences between mouse and cattle early embryo development were pointed out in ES cell derivation (Keefer et al., 2007) and trophectoderm differentiation experiments (Berg et al., 2011), highlighting the importance of multi-species studies, and suggesting that mouse might not be the best model for studying embryonic development (Rossant, 2011). In this respect, growing interest in bovine embryology is observed, and knowledge of several basic mechanisms is urged.

The knowledge of embryo sex and its singularities can be useful for human assisted reproductive medicine, specially in cases when X-linked disorders risk is detected. For animal reproduction field, female embryos are especially important, since assisted reproductive technologies are often applied to increase the number of female calves aiming further herd replacement and milk production. Therefore, most commercial bovine systems nowadays apply frozen–thawed sex sorted sperm for oocyte fertilization, and the understanding of female specific requirements is extremely relevant to improve IVP systems. Also, onset of sexual dimorphism characterization could contribute to current knowledge of several phenotypic differences between females and males.

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An intriguing aspect of bovine species is the different behavior between female and male embryos during preimplantation development. Dosage compensation of X-chromosome transcripts occurs in bovine embryos after blastocyst formation (Bermejo-Alvarez et al., 2010, 2011), and sex-specific gene expression patterns are reported for day 6 (Denicol et al., 2015) and day 7 (Bermejo-Alvarez et al., 2010) embryos. However, when and how sex-specific embryos begin to diverge morphologically, and how can we direct culture conditions to mitigate detrimental effects, is not clear.

The aim of this study was to characterize female and male embryos through development, attempting to identify singularities and reveal sex effects in preimplantation embryos. Morphological differences were reported regarding dissemblance in speed of blastocyst formation (Avery et al., 1992) and blastocyst cell numbers (Oliveira et al., 2010; Xu et al., 1992) for day 7 embryos. Also, sensitivity during in vitro culture is reported for female embryos (Edwards et al., 2001). Together, those evidences could suggest female embryos present slower development than male counterparts do, although our personal observations did not support this theory — rates of compaction and cavitation in our system were apparently similar for both genders. Therefore, our hypothesis was that sexual dimorphism is present before day 7 of development, and the main mechanism leading to sex-specific differences reported at blastocyst stage would be cell death rather than delayed development.

2. Material and methods

2.1. Experimental design

Matured oocytes were randomly distributed among six groups for fertilization. Semen from three bulls sexed for X- or Y-chromosome spermatozoa were used. In assay 1, kinetics was assessed during development. For that, cleavage of blastomeres was monitored daily for each group, from 24 h.p.i. till 144 h.p.i., and results were compared between bulls and between sexes. In assay 2, quality parameters were assessed in viable embryos. For this analysis, embryos destined for collection and fixation were cultivated in parallel, and embryos with more than 4 cells at 72, 96 and 120 h.p.i.; or more than 8 cells at 144 h.p.i., were selected. Embryos were submitted to caspase 3 immunofluorescence for apoptosis evaluation, and total cell number was assessed by Hoechst 33342 counterstaining. Cell fragmentation, compaction and cavitation were also evaluated in bright field images.

2.2. Supplements

Reagents and culture media were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

2.3. Oocyte recovery

Bovine ovaries were collected at a local slaughterhouse and processed within 3 h after slaughter. The ovaries were washed in saline (37 °C) and follicles measuring 3 to 8 mm in diameter were aspirated with an 18-gauge needle coupled to a 20-mL syringe. Cumulus-oocyte complexes (COCs) presenting at least three layers of cumulus cells and homogenous cytoplasm were selected under a stereomicroscope. The COCs were washed in HEPES-buffered TCM-199 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Cripion, Andradina, Brazil), 16 μg/mL sodium pyruvate and 83.4 μg/mL amikacin (Instituto Biochimico, Rio de Janeiro, Brazil).

2.4. In vitro maturation (IVM)

Groups of 20 COCs were transferred to 100-μL drops of medium containing 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, covered with sterile mineral oil (Dow Corning Co., Midland, MI) and incubated for 24 h at 38.5 °C in an atmosphere of 5% CO_2 in air under saturated humidity.

2.5. In vitro fertilization (IVF)

Groups of 20 matured COCs were washed twice and transferred to 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, and covered with sterile mineral oil. Frozen–thawed straws from three different bulls, containing X-chromosome (female embryo groups) or Y-chromosome (male embryo groups) bearing spermatozoa, sorted by flow cytometry (CRV Lagoa/Sexing Technologies, Sertaozinho, Brazil) were used. For each bull, X- and Y-spermatozoa straws were obtained from the same batch of semen. Flow cytometric sperm sorting based on differences in their DNA content is the best method for separation of X- and Y-chromosome bearing spermatozoa, and its accuracy is about 90% (Seidel, 1999; Hamano, 2007). 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 resuspended in 700 μL TALP-IVF medium and again centrifuged for 5 min at 520 × *g*. After centrifugation, 80 μL of the medium containing the pellet was collected from the bottom of the tube and homogenized in a conic tube. The final suspension was divided among five TALP-IVF drops, in a final concentration of approximately 10^4 spermatozoa for each oocyte. The plates were incubated at 38.5 °C for 20 h in an atmosphere of 5% CO_2 in air under saturated humidity.

2.6. In vitro culture (IVC)

After IVF, presumptive zygotes were partially denuded of cumulus cells by vigorous pipetting and cultured in SOF medium supplemented with 2.5% FBS and 6 mg/mL BSA at 38.5 °C in an atmosphere of 5% CO_2 in air under saturated humidity. Remaining cumulus cells were attached to plastic surface and formed a monolayer of granulosa cells. Groups of 20 presumptive zygotes were cultured in 100-μL drops, and medium was half replaced every 48 h.

2.7. Cleavage assessment

Embryos cultured side by side, female and male, obtained from fertilization with three different bulls were assessed daily (24, 48, 72, 96, 120 and 144 h.p.i.) for developmental progression rates. Presumptive zygotes cleavage was assessed at 48 h.p.i.

2.8. Compaction, cavitation and cell fragmentation analysis

Embryos destined for collection and fixation were cultivated in parallel, in groups of 20 structures per drop, and drops were collected at 72, 96, 120 and 144 h.p.i. Since our goal was to describe viable embryos and detect particularities of female and male development, embryos with less than 5 cells at 72, 96 and 120 h.p.i.; and embryos with less than 9 cells at 144 h.p.i. were considered non-viable and discarded from analysis.

Embryos were fixed in 4% paraformaldehyde for 30 min at 37 °C, and phase contrast images were used to quantify compaction (percentage of embryos exhibiting radial compacted cells), cavitation (percentage of embryos exhibiting fluid filled cavity) and cell fragmentation (estimated number of enucleated cytoplasm fragments inside zona pellucida). The onset of compaction was assessed in this assay, so partially compacted embryos (still presenting non-compacted cells) were considered positive.