Short Communication

Sex differences in single IVF-derived bovine embryo cultured in chemically defined medium

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\textbf{ABSTRACT}

Single embryo culture is essential for culturing embryos derived from few oocytes obtained from elite cows through ultrasonography guidance. Bovine \textit{in vitro} fertilization (IVF) and individual embryo culture is a challenge as it generally leads to impaired embryo development. In this study, we explored the embryonic development and the sex ratio of IVF-derived bovine embryo cultured individually in chemically defined two-step culture medium. Total 63 cumulus-oocyte complexes were collected, in vitro matured, in vitro fertilized and the resultant fertilized oocytes were randomly cultured individually (4 trials, 15–16 oocytes each) in microdrops of 5 µL of a chemically defined two-step culture medium. Blastocysts were counted in every trial (n = 32, 50.79%) and all of them were used for both genomic DNA and total RNA extraction, cDNA synthesis and PCR using specific primers for \textit{GAPDH}, \textit{GDP6}, \textit{XIST} and \textit{SRY} genes. Results showed significant difference in expression of \textit{XIST} (positive expression in 11 blastocysts) and \textit{SRY} (positive expression in 21 blastocysts) mRNAs, \(P < .05\). This result supports the hypothesis of sexual dimorphism among the pre-implantation embryos produced in vitro production.

1. Introduction

Single bovine embryo \textit{in vitro} culture is a challenge but it is essential for culturing embryos derived from oocytes where obtained from elite cows through ultrasonography guidance. Several researchers reported the beneficial effect of group culture medium for bovine \textit{in vitro} produced embryos [1–5], while the demand for improving single embryo culture is essentially needed for accurate study of the early embryonic development [6,7], and for toxicity screening [8–11]. Therefore, extensive research was performed to improve single embryo culture through co-culturing with somatic [10,12] or embryonic cells [13].

We have previously shown that in vitro embryonic development and the yield of cloned, transgenic embryos and viable calves were increased by using a two-step chemically defined medium for IVF-derived bovine embryos [14–18].

In the current study, we explored the embryonic development and the sex ratio of IVF-derived bovine embryo cultured individually in a chemically defined two-step culture medium using genomic DNA marker and RT-PCR.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals and hormones were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

2.2. Oocyte collection and in vitro maturation (IVM)

Ovaries were collected from a slaughterhouse into NaCl solution 0.9% at 30–33 °C and transported to the laboratory within 2–3 h. Cumulus-oocyte complexes (COCs) from follicles 2–8 mm in diameter were aspirated using an 18-gauge needle attached to a 10 mL disposable syringe. The COCs with evenly-granulated cytoplasm and enclosed by more than three layers of compact cumulus cells were selected, washed three times in HEPES-buffered tissue culture medium-199 (TCM-199; Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS, 2 mM NaHCO3, and 1% penicillin–streptomycin (v/v). For IVM, COCs were cultured in four-wells dishes (30–40 oocytes per well; Falcon, Becton-
2.3. Sperm preparation, in vitro fertilization (IVF) and in vitro culture of embryos (IVC)

Motile spermatozoa were purified and selected using the Percoll gradient method [19]. Briefly, spermatozoa were selected from the thawed semen straws by centrifugation on a Percoll discontinuous gradient (45-90%) for 15 min at 1500 rpm. The 45% Percoll solution was prepared with 1 mL of 90% Percoll (Nutricell, Campinas, SP, Brazil) and 1 mL of capitation-TALP (Nutricell) [20]. The sperm pellet was washed twice with capitation-TALP by centrifugation at 1500 rpm for 5 min. The active motile spermatozoa from the pellet used for insemination of matured oocyte (At 24 h of IVM). Following maturation, COCs were randomly distributed in 30 µL microdrops of IVF-TALP medium (Nutricell) and were then inseminated (day 0) with 1–2×10⁶ spermatozoa/µL for 18 h. Each microdrop contained two COCs and overlaid with mineral oil at 37 °C in a humidified atmosphere of 5% CO₂. In vitro culture (IVC) dishes were labelled and numbered to specifically monitor the individually cultured embryos. Presumptive zygotes were denuded and cultured individually in 5 µL two-step defined culture medium (first 5 days with stage-1 medium then transferred to the later stage medium [16]) overlaid with mineral oil and incubated at 39 °C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂. Cleavage and blastocyst rates were recorded on days 2 and 7, respectively.

2.4. Sex determination with genomic DNA and expression of sex-specific mRNA transcripts in single embryo by RT-PCR

Each blastocyst was washed in TALP medium then transferred into 5 µL of diethylpyrocarbonate (DEPC) treated water (Invitrogen) and stored at −80 °C. Individual embryos were transferred into 100 µL Trizol reagent and mixed very well then were divided (50 µL each) into genomic DNA or total RNA extraction procedures according to specific kits (Qiagen, Valencia, CA, USA) according to the manufacturer’s instruction [14,21]. Specific primers were used to amplify Y-specific segment from the extracted genomic DNA by PCR (Table 1). Different somatic cell lines were used [from transgenic male cow (Tet_M); brain tissue (Invitrogen) in a 20 µL reaction. 10 ng cDNA subjected to reverse transcription-polymerase chain reaction (RT-PCR) using Maxime PCR PreMix kit-i-starTaq (Intron Biotech., Seoul, Republic of Korea) and 1 µL of reverse transcriptase (Invitrogen) using random hexamer and superscript TM III reverse transcriptase-polymerase chain reaction (RT-PCR) using Maxime PCR PreMix kit-i-starTaq (Intron Biotech., Seoul, Republic of Korea) and 1 µL of capitation-TALP (Nutricell) [20]. The sperm pellet was washed twice with capitation-TALP by centrifugation at 1500 rpm for 5 min. The active motile spermatozoa from the pellet used for insemination of matured oocyte (At 24 h of IVM). Following maturation, COCs were randomly distributed in 30 µL microdrops of IVF-TALP medium (Nutricell) and were then inseminated (day 0) with 1–2×10⁶ spermatozoa/µL for 18 h. Each microdrop contained two COCs and overlaid with mineral oil at 37 °C in a humidified atmosphere of 5% CO₂. In vitro culture (IVC) dishes were labelled and numbered to specifically monitor the individually cultured embryos. Presumptive zygotes were denuded and cultured individually in 5 µL two-step defined culture medium (first 5 days with stage-1 medium then transferred to the later stage medium [16]) overlaid with mineral oil and incubated at 39 °C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂. Cleavage and blastocyst rates were recorded on days 2 and 7, respectively.

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Table 1
Primer sequences and primers used for reverse transcription-PCR (RT-PCR) and genomic PCR analysis.

| Gene  | F            | R             | Size bp (°C) | Accession No. |
|-------|--------------|---------------|--------------|---------------|
| RT-PCR | G6PD         | CAAGATGATGAGCAAGAAGG | 195 (55) | NM_001244135.1 |
|       | XIST         | TTGGCTTTTAGATTAAATTTGATGAAAGCAT | 99 (60) | NR_001464.2 |
|       | SRY          | CCGTCAAGGGGACGAGGC | 329 (60) | EU294189.1 |
|       | GAPDH        | TGGCAGACACATCAGTCCGTAG | 267 (60) | NM_001034034.2 |

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* Y-specific: Y chromosome specific STS marker BovY4.
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