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Effect of charcoal:dextran stripped fetal bovine serum on *in vitro* development of bovine embryos

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ABSTRACT

This study investigated the ability of charcoal:dextran stripped fetal bovine serum (CDS FBS) and heat-inactivated fetal bovine serum (HI FBS) to support *in vitro* development of bovine embryos. The developmental ability and quality of bovine embryos were determined by assessing their cell number, lipid content, mitochondrial activity, gene expression, and cryo-tolerance. The percentage of embryos that formed a blastocyst was significantly ($P < 0.05$) higher in medium containing CDS FBS than in medium containing HI FBS (42.84 ± 0.78% vs. 36.85 ± 0.89%, respectively). Furthermore, the beneficial effects of CDS FBS on embryos were associated with significantly ($P < 0.05$) increased mitochondrial activity, as identified by MitoTracker Green, as well as a reduced intracellular lipid content, as identified by Nile red staining, which increased their cryo-tolerance. Quantitative reverse transcription PCR showed that the mRNA levels of acyl-CoA synthetase long-chain family member 3, acyl-coenzyme A dehydrogenase long-chain, hydroxymethylglutaryl-CoA reductase, and insulin-like growth factor 2 receptor were significantly ($P < 0.05$) increased upon culture with CDS FBS. Moreover, the mRNA levels of sirtuin 1, superoxide dismutase 2, and the anti-apoptotic gene B-cell lymphoma 2 in frozen-thawed blastocysts were significantly ($P < 0.05$) higher in the CDS FBS-supplemented group than in the HI FBS-supplemented group, whereas that of the pro-apoptotic gene BCL2-associated X protein was significantly lower. Taken together, these data suggest that supplementation of medium with CDS FBS improves the *in vitro* developmental competence and cryo-tolerance of bovine embryos.

1. Introduction

In vitro culture (IVC) media play an important role in the development of *in vitro* fertilized embryos and have diverse compositions include defined media composed of simple salt solutions or undefined complex culture media supplemented with serum and with undefined components [1]. Embryo development is frequently better in undefined media than in defined media [2]. Undefined complex culture media are adequate for generating large numbers of embryos for industry [3] because protein supplementation is critical for embryo development [2]. However, a lack of protein supplementation during the morula-to-blastocyst transition improves the developmental competence of bovine embryos [4], indicating that protein supplementation is not necessary for IVC of bovine embryos during the early blastocyst stage. Serum has been widely added to culture media because it contains embryotrophic factors and substances beneficial for embryonic development, such as antioxidants, growth factors, and heavy metal chelators [5], and provides nutrients necessary for cell survival and proliferation [6].

Abbreviations: ACADL, acyl-coenzyme A dehydrogenase long-chain; ACSL3, acyl-CoA synthetase long-chain family member 3; BAX, BCL2-associated X; BCL2, B-cell lymphoma 2; BSA, bovine serum albumin; CDS FBS, charcoal:dextran stripped fetal bovine serum; COC, cumulus-oocyte complex; D-PBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; HI FBS, heat-inactivated fetal bovine serum; HMGCR, hydroxymethylglutaryl-CoA reductase; IGF2R, insulin-like growth factor 2 receptor; ITS, insulin-transferrin-sodium selenite; IVC, *in vitro* culture; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; IVP, *in vitro* produced; LOS, large offspring syndrome; NR, Nile red; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; RT-qPCR, quantitative reverse transcription PCR; SIRT1, sirtuin 1; SOD2, superoxide dismutase 2; SOF-BE1, synthetic oviduct fluid-bovine embryo 1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

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Moreover, serum accelerates *in vitro* embryo development when it is present from the initiation of compaction [7]. The most common serum preparations used for *in vitro* production of bovine embryos are fetal or newborn calf serum [8].

Various types of fetal bovine serum (FBS) are available, including dialyzed FBS, charcoal:dextran stripped fetal bovine serum (CDS FBS), and regular FBS. Dialysis of FBS removes molecules of a small molecular weight, while charcoal:dextran treatment of FBS removes lipophilic chemicals [9]. CDS FBS contains low levels of certain steroid hormones and growth factors, such as estradiol, cortisol, corticosterone, B vitamins T3 and T4, and prostaglandins. Heat inactivation of FBS is routinely performed at 56 °C for 30 min [10]. Such heating has a deleterious effect on the growth-promoting proteins found in FBS [11]. Unlike other types of commercial serum, CDS FBS does not need to be heat-inactivated. To the best of our knowledge, the effect of CDS FBS on bovine embryo development has not been studied.

IVC of embryos is usually performed in 5% CO_2 and ~20% O_2 [12]. This higher O_2 concentration leads to the production of high concentrations of reactive oxygen species (ROS), which can have detrimental effects on embryos [13], and consequently IVC media must be supplemented with antioxidants. Oxidative stress is considered as main limitation in assisted reproductive technology that can induce apoptosis or cell death through peroxidation of lipids and lipoproteins [14]. Therefore, low oxygen in simple medium with no co-culture is a superior culture system for bovine embryos both in terms of development and pregnancy rates. IVC medium is commonly supplemented with insulin-transferrin-sodium selenite (ITS) to counterbalance the effects of ROS [15]. Insulin is important for embryonic growth and metabolism [16]. Transferrin is an iron-transport protein and detoxifies media by removing metals. Selenium protects cells from oxidative damage by reducing free radical production and inhibiting lipid peroxidation [17].

In the present study, we supplemented modified synthetic oviduct fluid-bovine embryo 1 (SOF-BE1), a type of IVC medium, with CDS FBS or heat-inactivated fetal bovine serum (HI FBS) and tested their ability to support *in vitro* development of bovine embryos. CDS FBS used as a new type of serum supplement in bovine embryo culture system and compared with HI FBS. We propose that CDS FBS can be used instead of HI FBS for IVC of bovine embryos.

2. Materials and methods

2.1. Media and chemicals

All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted. Experiments were conducted in accordance with the Gyeongsang National University guidelines for the care and use of laboratory animals (approval no. GAR-110502-X0017).

2.2. Oocyte collection and in vitro maturation (IVM)

Ovaries of Korean native cows (Hanwoo) were collected at a local abattoir, placed in physiological saline (0.9% NaCl) at approximately 35 °C, and transported to the laboratory within 2 h after slaughter. Ovaries were washed in fresh Dulbecco's phosphate-buffered saline (D-PBS), and cumulus-oocyte complexes (COCs) were recovered from follicles with a diameter of 2–8 mm using an 18-gauge needle attached to a vacuum pump. Aspirated fluid was expelled into dishes containing TL-HEPES medium (114 mM sodium chloride, 3.2 mM potassium chloride, 2 mM sodium bicarbonate, 0.34 mM sodium biphosphate, 10 mM sodium lactate, 0.5 mM magnesium chloride, 2.0 mM calcium chloride, 10 mM HEPES, 1 μl/ml phenol red, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin) and imaged with a stereomicroscope. Good-quality oocytes with more than three layers of compact cumulus cells and homogenous cytoplasm were selected [18]. COCs were washed three times in TL-HEPES medium and then three times in maturation medium (TCM-199) supplemented with 10% (v/v) FBS (Gibco BRL, Life Technologies, Grand Island, NY, USA), 1 μg/ml estradiol-17ß, 10 μg/ml follicle-stimulating hormone, 10 ng/ml epidermal growth factor, 0.6 mM cysteine, and 0.2 mM sodium pyruvate. Thereafter, groups of up to 50 COCs were transferred to a 4-well dish (Thermo Fisher Scientific, Waltham, MA, USA) containing 500 μl IVM medium and incubated in a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 22–24 h.

2.3. In vitro fertilization (IVF) and IVC

In vitro matured COCs were fertilized with frozen-thawed bovine sperm that had been previously tested for IVF as described by Mesalam et al. [12]. Semen was thawed at 39 °C for 1 min, sperm were washed and pelleted in D-PBS by centrifugation at 750 × *g* for 5 min at room temperature, and motile sperm were recovered. The pellet was re-suspended in 500 μl heparin (20 μg/ml) prepared in IVF medium [Tyrode's lactate solution supplemented with 6 mg/ml bovine serum albumin (BSA), 22 mg/ml sodium pyruvate, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin] and incubated at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 15 min (to facilitate capacitation). Thereafter, sperm were diluted in IVF medium (final density of 1×10^6 sperm/ml). Matured oocytes were transferred to 600 μl IVF medium containing sperm for 18–20 h. After IVF, cumulus cells were removed by pipetting, and groups of up to 50 presumed zygotes were washed and transferred to 4-well dishes containing 500 μl SOF-BE1 medium [19] supplemented with 4 mg/mL fatty acid-free BSA, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite for 3 days. Presumed zygotes were then cultured until day 8 of embryonic development (day 0 = day of IVF) in medium of the same composition (SOF + ITS group) or in which BSA was replaced by 10% (v/v) CDS FBS (Gemini Bio-Products, West Sacramento, CA, USA; SOF + ITS + CDS FBS group) or HI FBS (Gibco BRL; SOF + ITS + HI FBS group). Day 8 expanded and hatched blastocysts (grade 1 and grade 2 [20]) were washed three times in TL-HEPES, transferred to fixative (4% [v/v] paraformaldehyde prepared in 1 M PBS), and stored at 4 °C until used in further experiments (cell counting and assessment of the lipid content and mitochondrial activity).

2.4. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

TUNEL was performed according to the manufacturer's protocol using an In Situ Cell Death Detection Kit (Roche Diagnostics Corp., Indianapolis, IN, USA). Briefly, fixed embryos (n = 30) were washed twice with 0.3% (w/v) polyvinylpyrrolidone (PVP) prepared in 1 M PBS (PVP-PBS) before being permeabilized (0.5% [v/v] Triton X-100 and 0.1% [w/v] sodium citrate) for 30 min at room temperature. After permeabilization, embryos were washed twice with PVP-PBS and incubated in the dark with fluorescently conjugated terminal deoxynucleotide transferase dUTP for 1 h at 37 °C. TUNEL-stained embryos were then washed with PVP-PBS and incubated in PVP-PBS containing 10 μg/ml Hoechst 33342 for 10 min. After washing with PVP-PBS, blastocysts were mounted onto glass slides and their nuclear configuration was analyzed. The number of cells per blastocyst was determined by counting Hoechst-stained cells under an epifluorescence microscope (Olympus IX71, Tokyo, Japan) equipped with a mercury lamp. TUNEL-positive cells were bright red, indicating the occurrence of apoptosis. The apoptotic indexes of blastocysts were evaluated as follows: number of apoptotic blastomeres (TUNEL-positive cells) divided by the total number of blastomeres (positive signals of Hoechst 33342 staining).

2.5. Cytoplasmic lipid content

Nile red (NR), a fluorescent dye specific for intracellular lipids, was