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Developmental competence and cryotolerance of caprine parthenogenetic embryos cultured in chemically defined media

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ABSTRACT

In animal reproduction technologies, the *in vitro* embryo culture system has advanced over the past few decades. However, *in vitro* cultured embryos still have reduced functional and physiological abilities compared with those from *in vivo* conditions, and many factors of oviduct and uterine environments have not yet been revealed. Here, we demonstrated the *in vitro* culture of domestic goat (*Capra hircus*) embryos using two types of culture media, modified synthetic oviductal fluid (mSOF) and a two-step chemically defined medium (DI/II). To obtain parthenogenetic goat embryos, oocytes were matured *in vitro* in tissue culture media-199 supplemented with 10% fetal bovine serum for 22 to 24 hours, and activated with 5 μM, Ca^{2+} ionomycin for 4 minutes, followed by 1.9 mM, 6-dimethylaminopurine treatment for 4 hours. After 2 days of embryo culture in different culture media, there were no significant differences in cleavage rates (96.6% vs. 95.4% in mSOF vs. DI/II, respectively). However, the DI/II group showed improved development competence to blastocysts (64.6% vs. 82.3% in mSOF vs. DI/II, respectively) and the total cell number of blastocysts (144.3 ± 9.2 vs. 264.4 ± 15.2 in mSOF vs. DI/II, respectively) at Day 7. After the cryopreservation of early-stage blastocysts at Day 6 via the conventional slow-freezing procedure, the surviving embryos were analyzed. The re-expansion rate after freezing and thawing was significantly higher in DI/II (39.66% vs. 67.69% in mSOF vs. DI/II, respectively), but there were no statistical differences in total cell numbers (142.3 ± 12.1 vs. 172.1 ± 11.6 in mSOF vs. DI/II, respectively), apoptotic index (4.9 ± 0.8% vs. 3.8 ± 0.7 in mSOF vs. DI/II, respectively), and the gene expression levels (BAX, GLUT1, MnSOD, and OCT4) among the re-expanded blastocysts. Overall, our data reported that the defined *in vitro* culture media for goat embryos were established with high efficiency, which will be very useful for goat embryo production.

1. Introduction

As a key factor of assisted reproductive technologies, *in vitro* culture (IVC) conditions definitely affect the quality of embryos in most mammalian species. A variety of culture

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systems with different formulations have been applied to obtain high quality embryos. According to the main protein source of the additives, they can be classified into three categories as follows: (1) Static co-culture systems with feeder cells; (2) bovine serum albumin (BSA) or serum added, which provides a main nitrogen source from BSA or serum; and (3) Defined media, which are cell-, BSA- and serum-free [1].

A co-culture system with oviductal epithelial cells or cumulus cells was also applied for small ruminants such as goats and sheep in some laboratories [2,3]. Although the *in vitro* development of embryos was successful, some concerns could be raised about how this system's microenvironment could have been contaminated and influenced by unpredictable factors from co-cultured somatic cells. While using the BSA alone or BSA and serum together as the protein source, the *in vitro* environment of the culture media proved effective for goat embryos, and when the BSA was added to the established culture media, they could be categorized as having the semi-defined condition that has been most widely used currently. The positive effects of BSA or serum additives have been evaluated in tissue culture media-199 (TCM-199) [4], B2 media [3], research vitro cleave media, embryo development media, modified Charles Rosenkrans, modified synthetic oviductal fluid (mSOF), and G1/G2 [5,6]. The BSA is in a more purified form and functions as a main protein in *in vivo* conditions, but it still raises some concerns about the potential risks of contamination and disease transmission [7]. Furthermore, embryos exposed to serum before blastocyst formation can show increased incidence of unusual development, accompanied by "large offspring syndrome" and organ defects [8]. There have been intensive efforts to replace serum or serum albumin with defined chemicals for the *in vitro* production of embryos in various species to overcome these unwanted consequences [9–11].

In addition to the replacement of serum or serum-derived materials in embryologic studies, researchers consistently try to reveal *in vivo* conditions and make a culture media that mimics real oviduct and uterine environments. In relation to this, sequential media were formulated to fulfill the energy substrates and physiological differences in accordance with the embryo's developmental stage [12]. Some embryo culture media were designed to change sequentially during the period of preimplantation and were effectively used to culture mammalian embryos, including mouse [13], cattle [14,15], and human embryos [16].

In the present study, we have applied a chemically defined sequential medium, which was successfully optimized for bovine embryo culture, to the IVC of caprine parthenogenetic embryos and compared this with a single-step culture medium containing BSA. In the defined medium, BSA was substituted with polyvinyl alcohol, and the medium composition was optimized specifically for energy sources of pre- and post-compaction [17]. Because no study has performed a development evaluation of goat embryos in a defined sequential media *in vitro*, the goal of this study was to find a more suitable environment for goat embryos *in vitro*; additionally, the effect of the medium was analyzed to determine developmental competence and survival rate after cryopreservation.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma–Aldrich unless otherwise specified.

2.2. Oocyte collection and in vitro maturation

From June to November, goat ovaries were collected at a local abattoir in 25 °C 0.9% saline and transported to the laboratory. Cumulus-oocyte complexes (COCs) from antral follicles (>2 mm in diameter) were aspirated using an 18-gauge sterile syringe. The COCs with a homogenous cytoplasm and dense compact cumulus were selected and washed with HEPES-buffered TCM-199 (Gibco). The selected COCs were cultured in four-well dishes (30–40 COCs per well; SPL) in 500 μL of TCM-199 culture medium supplemented with 10% fetal bovine serum (Gibco), 1% penicillin-streptomycin (v:v, Gibco), 1-μg/mL β-estradiol, 10-ng/mL epidermal growth factor α, 100-μM cysteamine, 0.9-mM sodium pyruvate, and 5-μg/mL follicular-stimulating hormone at 38 °C in 5% CO_2 and humidified air.

2.3. Parthenogenetic activation of oocytes

After 22 to 24 hours of COCs culture, the expanded cumulus cells were removed using 1.2-mg/mL hyaluronidase in HEPES-buffered TCM-199 medium by gentle pipetting. Among the cumulus-free oocytes, only the oocytes with the first polar body were activated by their exposure to 5-μM Ca^{2+} ionomycin in HEPES-buffered TCM-199 medium for 4 minutes, and they were divided into two groups. After washing the oocytes three times in HEPES-buffered TCM-199 medium, they were cultured in the mSOF and the DI/II media, respectively, each containing 1.9-mM 6-dimethylaminopurine, for 4 hours.

2.4. IVC of embryos

The parthenogenetic zygotes were cultured in two different media, mSOF and two-step chemically defined culture medium (DI/II). The mSOF medium was formulated first according to Takahashi et al. [18] and supplemented with 1.5-mM glucose, 8-mg/mL BSA, 1% of MEM non-essential amino acid solution (Gibco), and 2% of MEM amino acid solution (Gibco). The composition of the DI/II medium is presented in the previous study [9], and it is designed to be suitable for the different environments of the early and later stages of the embryo (the DI is for the stage of one-cell to morula and the DII is for the stage of morula to blastocyst). Compared with the previous study, the glucose concentration in the DII medium was altered from 1.5 mM to 2.7 mM and 1 ng/mL of TGF-beta and 10 ng/mL of bFGF were added into either the DI or DII medium. Fifteen to 20 goat zygotes were transferred to 100-μL drops covered with mineral oil. In the DI/II group, the embryos were cultured in the DI medium for the first 4 days and then transferred to the DII medium for the later stage of culturing. The embryos in the mSOF group were also transferred to new medium drops at Day 4. All