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Enrichment and culture of spermatogonia from cryopreserved adult bovine testis tissue

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ABSTRACT

Propagation of bovine spermatogonial stem cells (SSCs) from the cryopreserved testicular tissue is essential for the application of SSCs-related techniques. To explore the appropriate conditions for *in vitro* culture of bovine spermatogonia (containing putative SSCs), Sertoli cell monolayer and serum concentration were set as two main control factors. Morphological examination showed that the intactness and structure of adult bovine testicular tissue were well maintained after cryopreservation. The enriched bovine spermatogonia were large round CD_9 and promyelocytic leukemia zinc finger protein (PLZF) positive cells, with high nucleocytoplasmic ratios and multiple types including single, paired-, aligned-cells or grape cluster-like colonies *in vitro*. In Sertoli cell co-culture system, bovine spermatogonia attached quickly and proliferated obviously faster than those in the system without Sertoli cells. Serum-free media was no good for the attachment and proliferation of bovine spermatogonia. When 2.5%, 5% and 10% fetal bovine serum (FBS) was employed in the media, spermatogonia attached easily and divided quickly to form paired-, chained-cells or grape cluster-like colonies with comparable percentages in all groups. However, the contaminated somatic cells proliferated robustly in groups containing 5% and 10% FBS. Together, bovine spermatognia isolated from cryopreserved adult testis tissue express CD_9 and PLZF, can survive and proliferate conspicuously in Sertoli cell co-culture system, and low serum provides an optimal condition for the survival and proliferation of bovine spermatogonia because of avoiding the rapid growth of testis somatic cells.

1. Introduction

Spermatogonial stem cells (SSCs) are undifferentiated spermatogonia showing self-renewal and differentiation characteristics, and are amenable for researches on biology and thremmatology as well as clinical applications. SSCs transplantation (Brinster and Zimmermann, 1994) and testis tissue grafting (Honaramooz et al., 2002; Rathi et al., 2005; Rodriguez-Sosa et al., 2011) provide useful approaches for the studies of spermatogenesis mechanism, therapy of infertility, protection of endangered animals, and especially generation of transgenic large livestock such as pig and cattle. Proper preservation of donor testis tissue and expansion of SSCs for future utilization is especially important for the application of these techniques in large

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domestic animals, taken the difficulties and multiple steps of obtaining their testis into consideration.

Although varied protocols and cryoprotectants have been applied to the cryopreservation of porcine testis tissue (Zeng et al., 2009; Abrishami et al., 2010; Yang et al., 2010; Lee et al., 2014), analogous researches on bovine testis tissue have not been reported. Considering the structural differences in bovine testis (Wrobel, 2000), we examined the effects of several cryoprotectants and serum on the cryopreservation of bovine testis tissue recently (Wu et al., 2011; Wu et al., 2014). In these studies, we initially obtained spermatogonia with a relatively higher recovery rate from cryopreserved bovine testis tissue and explored their characteristics *in vitro*. However, the propagation of bovine male germ cells is still difficult since multiple factors such as serum and temperature (Izadyar et al., 2003), feeder layer (Oatley et al., 2004; Nasiri et al., 2012), extracellular matrices (Kim et al., 2014; Akbarinejad et al., 2015), hormone (Narenji Sani et al., 2013) and growth factors (Aponte et al., 2006; Sahare et al., 2015) etc., are involved.

Serum and feeders are two critical factors involved in the culture of spermatogonia containing SSCs. Studies indicated that higher concentration of serum had adverse effect on the proliferation of SSCs from goat, cattle and piglets (Izadyar et al., 2003; Bahadorani et al., 2012; Zheng et al., 2013). However, its effects on the long-term culture or viability of bovine spermatogonia were different (Izadyar et al., 2003; Xie et al., 2010; Kala et al., 2012). Different feeder layers made of Sertoli cells, bovine embryonic fibroblast (BEF) cells, SIM mouse embryo-derived thioguanine and ouabain resistant (STO) and mouse embryonic fibroblast (MEF) were also tried for the expansion of bovine testicular germ cells containing SSCs (Oatley et al., 2004; Nasiri et al., 2012). In the seminiferous epithelium, SSCs locate in niches composed of Sertoli cells (Hai et al., 2014). Considering the extremely important role of Sertoli cells-based microenvironment for the stabilization of SSCs, Sertoli cells were usually applied for *in vitro* culture of SSCs from domestic animals. Based on our previous work, this study was designed to identify the biological characteristics of bovine spermatogonia (containing SSCs) from cryopreserved adult bovine testis tissue, when cultured in different system containing varied serum concentrations and with or without Sertoli cells.

2. Materials and methods

2.1. Reagents

Collagenase IV, hyaluronidase II, deoxyribonuclease (DNase I), trypsin, gentamycin, penicillin–streptomycin and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St., Louis, MO, USA). Minimum essential medium (MEM), fetal bovine serum (FBS), newborn calf serum (NCS), L-glutamine and hepes were supplied by Invitrogen by Life Technology (Carlsbad, CA, USA). Cryovials and Petri dishes were purchased from Nunc (Rochester, NY). Rabbit anti-zinc finger and BTB domain-containing protein 16/promyelocytic leukemia zinc finger protein (ZBTB16/PLZF, sc-22839) was provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit monoclonal CD_9 antibody and DAB were bought from Wuhan Boster Bio-Engineering Limited Company (Wuhan, Hubei, China).

2.2. Testis collection, cryopreservation and thawing

Testes from 2- to 4-year-old healthy Simmental bulls (600–800 kg) were collected from Jilin Province Changchun Haoyue Islamic Meat Co., Ltd. (Changchun, China). All animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University. The testes were treated as we described previously (Wu et al., 2011; Wu et al., 2014). Briefly, the testicular parenchyma were cut into 0.3–0.5 cm^3 fragments and transferred into 5 mL cryovials containing freezing media after decapsulation. The concentrations of DMSO and serum of freezing media were presented as volume percentages (v/v). The freezing media was prepared with a constant 10% DMSO in MEM. The vials were placed in an insulated container at −80 °C for 24 h, then transferred into liquid nitrogen and preserved six months to one year.

For tissue thawing, the cryovials were swiftly transferred in 37 °C water bath and swirled for 3 min. The tissue fragments were moved into a 10 mL tube and diluted slowly with M_1 (MEM supplemented with 10% NCS + 2 mmol glutamine + 100 IU/mL penicillin–streptomycin + 40 μg/mL gentamycin + 15 mmol hepes). Then, the tissue fragments were washed, pooled and centrifuged at 600 *g* for 3 min. After decanting the supernate, the fragments were transferred into Φ35 mm petri dish containing M_1.

2.3. Hematoxylin–Eosin (HE) staining

Regular HE staining of paraffin sections was carried out as described elsewhere. Briefly, after fixation in Bouin's Solution for 24 h, paraffin sections (10 μm thick) of collected bovine testes were made. The sections were then deparaffinized, rehydrated, stained with hematoxylin for 5 min, rinsed in running water for 30 s, differentiated with 1% hydrochloric acid-ethanol for 1 min, washed in running water for 30 s, treated with 1% ammonia for 5 s, and washed with water for 30 s. Next, the sections were stained with 0.5% Eosin for 1 min, dehydrated with gradient ethanol, treated with Xylene twice for 10 min each, and coverslipped for microscopy.

For cultured cells, the samples were rinsed with Phosphate buffer saline (PBS) for 2 times, fixed with 4% paraformaldehyde in PBS for 10 min, rinsing in running water, stained with hematoxylin for 5 min, treated in 1% ammonia for 8 s and water for 10 min. Then the cells were stained with 1% Eosin for 30 s, washed in running water for microscopy.

2.4. Cell culture

Spermatogonia were isolated by a two-step enzymatic procedure as described (Izadyar et al., 2002; Wu et al., 2011, 2014). Briefly, The seminiferous tubules were isolated and subject to digest in digestion mixture (M_1 + 1 mg/mL collagenase IV + 1 mg/mL hyaluronidase II + 1 mg/mL trypsin + 5 μg/mL DNAse I) at 32 °C for 60 min