Comparison of Three Different Culture Systems for Establishment and Long-Term Culture of Embryonic Stem-like Cells from *In Vitro*-Produced Bovine Embryos

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ABSTRACT

Although embryonic stem cells (ESCs) or ES-like cells are reported from many mammalian species other than the mouse, the culture system for murine ESCs may not be suitable to the other species. Previously many other research groups have modified either human or mouse ESC culture systems for bovine ESC culture. In this study, we compared three different culture mediums consisting of DMEM, α-MEM or KnockOut™-DMEM (KO), which are modified from human or mouse ESC culture system, for the generation of bovine ESCs. In this study, some pre-requisite events which are important for establishment and long-term propagation of ESCs such as inner cell mass (ICM) attachment on feeder cells, primary colony formation and sustainability after passaging. Once the ICM clumps attached on feeder cells, this was designated as passage 0. In regards to the rate of ICM attachment, α-MEM was superior to the other systems. For primary colony formation, there was no difference between DMEM and α-MEM whereas KO showed lower formation rate than the other groups. For passaging, the colonies were split into 2～4 pieces and passed every 5～6 days. From passage 1 to passage 3, DMEM system seemed to be appropriate for maintaining putative bovine ESCs. On the other hand, α-MEM tended to be more suitable after passage 6. Although α-MEM support to maintain a ES-like cell progenies to passage 15, all three culture systems which are modified from human or mouse ESC culture media failed to retain the propagation and long-term culture of putative bovine ESCs. Our findings imply that more optimized alternative culture system is required for establishing bovine ESC lines.

(Key words : bovine, embryonic stem cells, culture medium, pluripotency)

INTRODUCTION

Embryonic stem cells (ESCs) can be derived from inner cell mass (ICM) of blastocysts and they have the capacity to self-renew and give rise to differentiation of all embryonic germ layers. Since ESCs were isolated and established in the mouse, there have been many reports on derivation of ESCs from various species including domestic animals (Evans and Kaufman, 1981; Notarianni *et al*., 1991; Saito *et al*., 2002; Dattena *et al*., 2006; Huang *et al*., 2007). ESCs from domestic animals such as bovine not only provide commercial benefits in livestock industry but are also a biological model system for studying properties of ESCs. To establish ESCs in animals, most traditional culture media shared several important contents such as leukemia inhibitory factor (LIF) and fetal bovine serum (FBS) (Notarianni *et al*., 1991; Saito *et al*., 1992; Talbot *et al*., 1993; Li *et al*., 2003). However, the culture media may not be suitable to all species because ESCs in some domestic species are still hard to be generated (Saito *et al*., 2003; Yu *et al*., 2008; Kim *et al*., 2010) and the reasons remain largely obscure. Previously many other research groups have used either human or mouse ESC culture systems with some modifications for the generation of bovine ESC and LIF and FBS are essential components in the most culture system. Serum is one of the major factors for inhibiting differentiation of ESCs in culture (Ogawa *et al*., 2004; Tsuji *et al*., 2008). However, most suggested culture conditions are not suitable for the generation of bovine ESCs because the systems are not able to permit their propagation and long-term culture (Yadav *et al*., 2005; Cao *et al*., 2009; Anand *et al*., 2011). To develop

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novel culture system for bovine ESCs, here we used three different culture media for bovine ESC generation and evaluated the capability of ESC establishment with the criteria of ICM attachment to feeder cell layer, formation of primary colonies and ability to maintain stemness after passaging in culture to find appropriate culture system for the generation of bovine ESCs.

MATERIALS AND METHODS

1. Chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless indicated in the text.

2. Oocyte Recovery and *In vitro* Maturation (IVM)

Bovine ovaries were collected in pre-warmed 0.9% saline at 30～35℃ and transported from a local slaughterhouse to our laboratory within 2 h. They were washed with 0.9% saline solution on arrival at the laboratory. Follicular fluid containing cumulus–oocyte complexes (COCs) were aspirated using an 18-gauge needle attached to a 10-ml disposable syringe. After washing three times in washing medium which consisted of 10% (v/v) tissue culture medium 199 with Earle's salts and L-glutamine (TCM199, Invitrogen, USA), 2.0 mM $NaHCO_3$, 2.0 mM sodium pyruvate, 25.0 mM HEPES, 1.0 mM L-glutamine, 100 IU/100 mg/ml penicillin/streptomycin plus 30 IU/ml heparin, the oocytes surrounded by two to three layers of cumulus cells were collected and washed in IVM medium. The selected COCs were cultured in 4-well culture dishes (Nunc, Denmark) containing 500 μl of IVM medium under warmed and gas-equilibrated mineral oil for 24 h at 38.5℃ in 5% CO_2. The IVM medium for oocytes is composed of TCM199 supplemented with 10% FBS (Invitrogen), 10 μg/ml FSH, 0.2 mM sodium pyruvate, 1 μg/ml estradiol-17β, and 10 ng/ml EGF.

3. *In vitro* Production (IVP) of Bovine Embryos

The expanded COCs were washed twice in Hepes-buffered Tyrode's solution (hTALP) supplemented with 3 mg/ml fatty acid free-bovine serum albumin (ff-BSA) and placed into 45 μl drops of *in vitro* fertilization (IVF) medium under mineral oil. A frozen semen straw from the HanWoo was thawed in a 37℃ water bath and the semen was deposited on the top of the discontinuous Percoll gradient prepared by depositing 2 ml of 90% Percoll under 2 ml of 45% Percoll in a 15 ml centrifuge tube, and the sample was centrifuged for 20 minutes at 252 ×g. The pellet was removed and resuspended in 300 μl of hTALP and centrifuged at 201 ×g for 10 min. After removal of the supernatant, 5 μl of the sperm suspension (1×10^7 cells/ml) were introduced to IVF drop and this makes final sperm concentration in IVF as 1×10^6 cells/ml. Incubations were carried out at 39℃ in 5% CO_2 for 20～24 h. After insemination, the cumulus cells were removed by repeated aspiration into a pipette and washed 3 times with *in vitro* culture (IVC) medium consisting of CR2 (Rosenkrans and First, 1994) with 0.3% ff-BSA and 1% insulin, transferrin and selenium complex (ITS, Life Technologies, UK). Then denuded fertilized oocytes were transferred to IVC medium consisting of CR2 with 0.3% ff-BSA and 1% ITS for 3 days and then transferred to CR2 medium with 0.15% ff-BSA, 1% ITS and 0.15% FBS for 5 days at 38.5℃ in 5% CO_2, 5% O_2 and 90% N_2. The culture drops were covered by mineral oil and 10 to 15 embryos were placed in each drop.

4. Generation of Bovine ESCs

The zona pellucida (ZP) of blastocysts on Day 7 were removed mechanically by mouth pipetting without chemical treatment such as acid Tyrode's solution or protease and ZP-free blastocysts were slightly torn at the furthermost end of the ICM part of trophectoderm (TE) using a 21 gauge needle to attach the embryos on feeder layer easily and the embryos were placed onto mitomycin-C inactivated STO feeder cell layers and cultured at 38.5℃ in a humidified gas atmosphere of 5% CO_2 with three different culture systems based on the media of Dulbecco's modified Eagle's medium (DMEM, Life Technologies), alpha-minimal essential medium (α-MEM, Life Technologies) or KnockOut^{TM}-DMEM (KO, Life Technologies). DMEM and α-MEM are used with supplementation of 20% FBS, 1% GlutaMAX^{TM} (Life Technologies), 1% non-essential amino acids (NEAA, Life Technologies), 1% ITS, 100 U/ml penicillin, 0.1 mM β-mercaptoethanol, 10 ng/ml human recombinant LIF (hLIF, Merck Millipore, USA) whereas KO replaced FBS with 20% KnockOut^{TM} serum replacement (KSR, Life Technologies). Once the embryonic cell clump attached, this was designated as passage zero (P0). The medium was replaced every other day. After 12 days of culture, the initial outgrowths from the embryos were mechanically dissociated into 2～4 pieces from each clump, and the piece was re- plated individually onto fresh STO feeder layers and

designated as P1. The colonies of the cells were passaged every 5 to 6 days. Only multilayered cells in the central region of the colony were picked up for passaging the presumptive ESCs.

RESULTS AND DISCUSSION

Although presumptive bovine ESCs derived from different developmental stage embryos are reported, the cells are still hard to retain stemness during the long-term culture *in vitro* (Stice *et al*., 1996; Iwasaki *et al*., 2000; Mitalipova *et al*., 2001). Once the attached cell clumps form ES-like colonies, long-term maintenance of stemness is focused on the experiment and early important events such as attachment on feeder layer and generation of primary colonies can be easily missed. One of the critical factors to go over early obstacles may be culture medium (Gong *et al*., 2010). To develop optimal culture condition for bovine ESCs, several supplements has been tested such as LIF and bFGF, however stem cell properties are still not able to be maintained with these factors (Cao *et al*., 2009; Gong *et al*., 2010).

In this study, the different culture media for establishing bovine ESCs derived from IVF bovine embryos were compared. First, we evaluated the ability of the attachment of ICM-derived clumps and primary colony formation after seeding of bovine blastocysts. The attachment (63.3%) and primary colony formation (53.3%) rates in α-MEM group was higher than those in the other culture systems (Table 1). Once the clumps attached on the feeder layer, there was no difference of primary colony formation rates between α-MEM and DMEM groups (82.3% and 84.2%, respectively), whereas KO group showed lower colony formation (50.0%). The results above imply that enhancing attachment ability of ICM clumps may be critical as a pre-requisite event for efficient ESCs establishment in bovine species. Serum may be an essential supplement for this event because FBS (DMEM and α-MEM system) and KSR (KO system) are most significantly different supplements among those culture systems. During passaging, the progeny of the cell colonies in all experimental groups was decreased and disappeared before passage 16 (Table 2). More colonies in DMEM group seemed to be maintained than those in other groups until passage 3. However, the number of colonies was quickly disappeared after passage 6. On the other hand, the number of presumptive cell lines in α-MEM tended to decline rapidly during early passages. Interestingly, the colony in α-MEM group was maintained up to passage 15 although the number is very limited. In KO group, no ESC-like colony survived after passage 5. All above results suggest that α-MEM system may be more suitable to retain long-term culture of ESCs in bovine species than other medium tested.

In conclusion, although all three culture systems failed to establish stable line of ESCs, the finding from this study gives some information for the development of optimal culture system to generate ESCs in bovine species.

Table 2. Long-term maintenance capability of presumptive bovine ESCs cultured in three different culture systems

	No. of surviving presumptive ESC lines at				
	Primary colony (Passage 0)*	Passage 3	Passage 5	Passage 11	Passage 15
DMEM	14	14	13	1	0
α-MEM	16	6	6	4	1
KO	4	2	1	0	0

The components of culture media (DMEM, α-MEM and KO) are described in listed in Materials and Methods.
* Each primary colony was derived from different embryos.

Table 1. Comparison of the attachment on feeder layer and primary colony formation of bovine embryonic cells cultured in three different culture systems

	DMEM	α-MEM	KO
Total blastocysts	30	30	30
Number (%) of attachments	17 (56.7)	19 (63.3)	8 (26.7)
Number (%) of primary colonies	14 (46.6)	16 (53.3)	4 (13.3)
Primary colonies / Attachments	82.3%	84.2%	50%

The components of culture media (DMEM, α-MEM and KO) are described in Materials and Methods.

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